From Ecological Epitome to Medical Model:

An investigation into Applications for the use of *Daphnia* in Heart Science.

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300 Word Abstract

"*The Babylon Project was our last, best hope for peace. It failed. But, in the year of the Shadow War, it became something greater: Our last, best hope… for victory.*"

- Babylon 5, season 3 intro.

The primary aim of this research was to determine whether *Daphnia* might become a model for cardiovascular concentration-response trials. This would provide a high throughput means of testing cardiac therapeutics without resort to small mammal trials. We found *Daphnia* are inappropriate in this context due to high population variance and sensitivity to small, subtle, environmental changes. A new aim was developed to determine whether beat-to-beat variation could be correlated with an individual's response to toxic insult. Further, to develop more accurate and efficient means of gathering heart rhythm data by recording heart movement from whole live *Daphnia*. This opens the way to individualising cardio therapeutics; by correlating the stability of individual hearts with response to cardiac insult, regression analysis provides a means of finding a prediction tool. *Daphnia* are a convenient example here, but successful scoring systems might also be applied to the human heart via analysis of ECG readouts. Collecting signals from whole live *Daphnia* did not fulfil the goal of gathering heart data as this instead recorded limb movement. However, this provides a means of improving toxicology testing in aquatic ecology. This thesis offers three contributions to knowledge:

1. *Daphnia* are an inappropriate model for cardiovascular therapeutic dose-response trials due to extreme environmental sensitivities.

2. Baseline heart rhythm can be correlated with paired response to cardiac insult, with significance at the 0.01 alpha level, using an adjusted version of the Lyapnov equation; Finite Time Growth (Wessel, 2010). However, this is only if population variation is adequate. It is better applied to a natural *in situ* population than a homegenic lab population.

3. A novel technique for measuring *Daphnia* electromechanical movement records feeding limbs rather than the heart. This offers a novel and more efficient technique for aquatic ecotoxicology, where visual observation or films of the same are currently used.

From Ecological Epitome to Medical Model:

An investigation into Applications for the use of *Daphnia* in Heart Science.

To my wife, Jamie A. S. Angus-Whiteoak,

For your support and your sense of humour,

which gave this thesis its spirit, and its chaos.

i. Objectives and Chapter Guide

This work began with a minimal approach to equipment availability which evolved over the research period. Gradual acquisition of critical equipment steered the timing of various objectives. Work began by using a Barska light field stereomicroscope (230 volts, 40X zoom), later additions included buying a dark field adapter and building a dark field tent. This explains our choice of the use of light field in chapters 4 and 5; dark field was acquired in chapter 6. Access to ECG equipment granted in the final months of study allowed us to explore new objectives in chapter 7.

Chapter 1: Invertebrate Response to Cardio active Drugs

This chapter will review invertebrate cardiac response to cardiovascular therapeutics according to category i.e. anti-arrhythmic, lipid lowering agents, anticoagulants, antiplatelet agents, cardio tonics, diuretics, fibrinolytics, thrombolytics and glycoprotein inhibitors, vasodilators, vasopressors and herbal remedies. We take a closer look at cholinergic modulators, reviewing invertebrate learning and memory, innate defence response, motility, development, insecticidal effect, toxicity treatment as well as cardiac response to this category. All known *Daphnia* LD50s for cardiac drugs are listed.

Chapter 2: *Daphnia* as an experimental model

The initial objective was to sustainably culture *Daphnia* and their food resources. This chapter will describe the trial animal, and why it was chosen, and the advantages of model systems. We investigate optimum conditions for *Daphnia* culture, by examining the literature for *Daphnia* response to temperature and light, varying oxygen levels, types of food, predator presence and environmental nutrients. We look at physical parameters which may affect lab trials such as reproductive status, age, body size and infection. We conclude with lab specific considerations of how best to negotiate these factors in the lab.

Chapter 3: General Methods

This chapter allows the thesis to 'flow' more easily by placing commonly used and mathematical methods in one place. It covers choice of camera equipment, and the comparative merits of light field and dark field microscopy. It covers experimental procedure for cardiovascular drug lab trials, as well as randomisation, tests for outliers, normalisation and parabolic peak interpolation, and dysrhythmia classification for *Daphnia*.

More importantly it describes programs we specifically developed for image capture in dark field and spectrum analysis for whole *Daphnia* electromechanograms.

Chapter 4: Method Development and Early Insights

The objective of this brief chapter was to perform explorative trials to guide method choice. These revealed insights that influenced project planning and direction. Our first objective was to set up an appropriate *Daphnia* culture. We explore the appropriateness of *in-situ* vs *ex-situ* cultured animals, and whether culture conditions affect trial outcomes. We also experiment with lab protocol including choice of medium for *Daphnia* immobilisation; and give early insights into the application of chaos theory to cardiac dysrhythmia.

Chapter 5: Are *Daphnia* a Model for Cardio therapeutic Trials?

The primary objective of this research is to develop and apply methods to test cardiovascular drug response in *Daphnia*. In this first full trial we measure *Daphnia* response to a selection of the drugs reviewed in chapter 2 using methods described in chapter 3, including development of a semi-automated image capture method. We note difficulties in the measurement of bradycardic responses; where maintaining life support over the time needed before an effect is seen produces confounding factors. We measure response to three tachycardic drugs and give dose response curves for each. Further objectives developed from observations of complex drug responses using phenylephrine, leading to investigation of appropriate buffer choice. We asked whether *Daphnia* alter the pH of their microenvironment, and, whether micro-level changes in pH alter *Daphnia* heartrate. The chapter concludes that *Daphnia*'s extreme sensitivity to micro-level changes in environmental pH (changes subtly below buffer capacity for accurate pH stability) mean that *Daphnia* are not an appropriate model species for medical dose-response trials. This steered the PhD objectives in a new direction in subsequent chapters.

Chapter 6. Heart Variation Scoring Systems

This chapter reviews peer-reviewed literature regarding scoring systems which differentiate dysrhythmias. Our objective is to test whether such systems can be used instead to correlate and individual's baseline heart stability with paired response to cardiac insult. We produce a paired data set, apply each system to the data, then give statistical analyses of the success of each. Our main objective is to compare scoring systems and

deduce which might be best applied to further work. We also discuss methodological limitations and show how the work could be improved to produce more reliable correlations that could then be used to predict cardiac response.

Chapter 7. Towards a More Efficient Data Collection Method

Following on from the work in chapter 6 our final objective was to find a method of measuring *Daphnia* beat-to-beat heart variation in a more efficient manner. Objectives were to build appropriate equipment and related software, and to measure electrical signals from whole *Daphnia*. In this chapter we develop the Whole *Daphnia* Electro Mechanical Movement (EMM) sensing device. It was called this rather than an ECG device as it targeted whole animal movement and not the heart in isolation. We also built attachments to the equipment for better reducing radio frequency interference (RFI) and developed a MatLab based program for interpreting the data. However, we found at the start that these methods did not in fact record the heart, but the feeding limbs. This opened a new door to development of measurement methods for ecotoxicology, where *Daphnia* are established model animals. We examine the effects of neonicotinoids, a group contextualised in their current importance to ecotoxicology, using this new method. We end by discussing the improvements needed to further to this work.

ii. List of Figures and Tables

Ode to a *Daphnia* (performed at Salford Postgraduate Annual Research Conference 2017)

Daphnia, planktonic invertebrate
My muse, you're more to me than mere fish bait
Through your carapace clear I penetrate
With a microscope's gaze, I watch your fate

Your heart like humming bird's wing, fast, does beat
On high speed film to then repeat, and beat
Again, on replay, frame by frame, I seat
On long hours, view, immortalized repeat

Next film; your heart then bradycardic slow
Doxorubicin dosed - I dealt the blow
My camera ready, dark room death, but know
T'was not for naught, your sacrifice will throw

New light on rhythmic patterning, and all
Frames of your myogenic heart I trawl
To analyze a non-linear crawl
Of your heart's variation. I recall

Numbers, which, for this recurrent event
Through simple Lyapunov Exponent
A chaos equation that can be lent
To see if it's stable or divergent

Is lower beat-to-beat variation
In baseline rhythm your damnation?
Behold! Drug response magnification
Tested with Spearman's rank correlation

1. Invertebrate Response to Cardioactive Drugs

This chapter is intended as a review of *Daphnia* response to cardioactive toxins in the environment to show the breadth and limits of *Daphnia* as a model animal in this area. The chapter is divided into sections by drug class in line with the way cardioactive drugs are referred to in environmental science literature. We decided against structuring the chapter according to mechanism of action in humans, as many aspects of *Daphnia* physiology find no analogue in humans. *Daphnia* do not have a vasculature, so vasopressors, antiplatelet agents and anticoagulants are not expected to have the intended effect made for humans. Invertebrates do not synthesise cholesterol and rely on dietary regulation for their sterol intake requirement (Douglass et al 1981) so would not be good candidates for lipid lowering drug trials. In many cases we can only examine how *Daphnia* respond to drugs as environmental toxins. *Daphnia* do have a visible beating heart; thus, it is possible that cardio accelerative or cardio decelerative drugs, or even drugs in the anti-arrhythmic classes, β-blockers or K^+ and Ca^{2+} channel blockers, may have corresponding effects between *Daphnia* and humans.

Cardioactive drugs are one of the most environmentally toxic substances found in waste water effluent (Jones et al 2002), second only to antibiotics (Bona et al 2015). Drugs from anti-depressants, cytostatic, and analgesics to anti-epileptics and x-ray contrast media have been tested against the *Daphnia* acute toxicity LD50 model (Luna et al 2005 and Gomez-Olivan et al 2015). However, beyond life-vs-death acute testing, *Daphnias*' wide-ranging physiological responses to micro molar levels of pharmaceuticals makes them far more applicable to environmental screening than other models (Lilius et al 1995). Therefore, assays have been developed to monitor pollutants via a diverse range of means that examine non-acute effects. For example, feeding rate: McWilliam and Baird (2001) found that post-exposure feeding depression formed a reliable, sensitive endpoint which unified response of *Daphnia* to toxin exposure at geographically disperse sites. Other measures are clustering tendencies of colonies moving away from areas high in toxins (Rosa et al 2008) and changes in swimming speed (Ren et al 2007). The "Dynamic *Daphnia* Toxicity Test," based on monitoring *Daphnia* swimming activity was developed by the German government in 1982, and is widely used across many countries including Austria, the Netherlands, Belgium and South Korea to give constant real time feedback on water conditions (Gunatilaka et al 2001).

For most studies involving cardio active drugs in the environment, only acute fatal effects are observed. A standard has been derived for comparison of these known as the LD50, or Lethal Dose 50%, the dose at which 50% of animals are killed by the drug in question. Toxic LD50s for *Daphnia* and are summed table 1a. As shown in table 1a, Hernando et al (2004) tested a number of drugs found *Daphnia* survival far better in culture medium than waste water. There are many differences between the two aquatic mediums, however until Hernado's study advice on upper-limits for waste water effluent was based on culture medium based lab studies. The study showed that in the presence of mixed toxins, the toxicity of these drugs becomes additive. The table refers to concentrations much higher than pharmacologists are used to discussing, which is unsurprising given these are acute toxic levels. LD50s are time dependent and set to either 24 or 48 hours in order to present comparable results across the literature. This measure ignores subtle physiological changes prior to death, which is of more interest to this research and published more rarely. In this chapter we present studies that look at physiological changes to offer insight into therapeutic rather than toxic effects.

The search strategy for this review included every drug class in turn, then systematically, the names of every drug in each of these drug classes in turn as listed on the webpage *Guide to pharmacology*. This was repeated across databases listed as URLs in the references [9.0]. Where drug effect was non-existent for *Daphnia* the search was widened by phylogenetic distance to their family order, class then phylum, if no data was found then any invertebrate was included. Extrapolation to closely-related species when *Daphnia* data are not available does not imply that we believe drug response will be identical between the two species. Even closely related crustacea are known to have neurogenic hearts while *Daphnia* is thought to be myogenic [2.1]. Using closely related species merely implies that genetically similar species might express similar phenotypes, and given the paucity of any other physiological data, any data at all is a useful starting point. Most literature found for this review comes from environmental science-based sources and may also offer further insight in this area by providing a comprehensive review of cardio active drug effects found in the environment. The chapter is not meant as a review of cardio therapeutic drug action in humans. It is an ecological, not pharmacological review, and does not it imply that any drug class given may have the same action intended for humans in invertebrates.

Table 1a. *Daphnia magna* fatal LD50s shown in μM for 48 hour or 24-hour time frames

β-1 Selective Blocker μM	48hr	Reference

Atenolol in waste water	304		Hernando et al (2004)
Atenolol in culture medium (CM)	751		Hernando et al (2004)
Atenolol in nutrient rich CM	1175		Cleuvers (2005)
Betaxolol in waste water	260		Hernando et al (2004)
Betaxolol in culture medium	976		Hernando et al (2004)
Metoprolol in untreated waste	10		Czech et al (2014)
Metoprolol after photo catalysis	94		Czech et al (2014)
Metoprolol	239		Huggett et al (2002)
Metoprolol in freshwater streams	284		Richard et al (2014)
Metoprolol in waste water	374		Hernando et al (2004)
Metoprolol	497		Moermond and Smit (2015)
Metoprolol in lab hard water	654		Shakya (2011)
Metoprolol in culture medium (CM)	748		Hernando et al (2004)
Metoprolol in nutrient rich CM	1638		Cleuvers (2005)
Non Selective β-Blocker μM	48hr		Reference
Propranolol in pond water	5		Huggett et al (2002)
Propranolol in lab hard water	13		Shakya (2011)
Propranolol in rich culture medium	26		Cleuvers (2005)
Nadolol	246		Lilius et al (1995)
	323		Huggett et al (2002)
Sotalol in waste water	367		Hernando et al (2004)
Sotalol in culture medium	734		Hernando et al (2004)
Ca^{2+} Channel Blocker μM	24hr	48hr	Reference
Diltiazem		68	Kim et al (2007)
Verapamil	121	15	Villegas-Navarro et al (2003)
Lithium	2836		Villegas-Navarro et al (2003)
Anticholesteremic μM	48hr		Reference
Clofibrinic acid	494		Henschel et al (1997)
Anticoagulant μM	48hr		Reference
Bromadiolone	94		Gómez-Canela et al (2014)
Chlorophacinone	1.5		Gómez-Canela et al (2014)
Warfarin	515		Gómez-Canela et al (2014)
Antiplatelet μM	24hr	48hr	Reference
Aspirin		1721	Bang et al (2015)
	5551		Russo (1995)
	8150		Guilhermino et al (2000)
	8150		Lilius et al (1995)
Aspirin components μM	24hr	48hr	Reference
Gentisic acid		2612	Henschel et al (1997)

Salicyclic acid	854	8309	Henschel et al (1997)
O-hydroxyhipponic acid		9223	Henschel et al (1997)
Inotropic Agent μM	24hr	48hr	Reference
Digoxin	31		Lilius et al (1995)
Ouabain		3	Villegas-Navarro et al (2003)
Vasopressor μM	48hr		Reference
Noradrenaline	46		Overturf et al (2015)
Uncatagorized μM	48hr		Reference
Glycophosphate	4613		Bengtsson et al (2004)
p'p'-DDE	0.04		Bengtsson et al (2004)
Cholinergic Modulators μM	24hr	48hr	Reference
Atropine (targets muscarinic receptors)	870		Lilius et al (1995)
	1223	359	Carvalho et al (2003)
Imidacloprid (Neonicotinic Insecticide)	332	250	Pavlaki et al (2014)
		378	Pestana et al (2010)
Methomyl (Carbamate Insecticide)		0.1490	Pereira and Goncalves (2007)
Tebuconazole (Triazole fungicide)		130.27	Sancho et al (2009)
Deltamethrin (This and all below are Organophosphate Insecticides)		0.009	Lopes et al (2006)
		0.031	Barata et al (2006)
Diazinon	0.003		FernadezCasalderrey et al (1994)
Dimethoate	6.98	4.80	Andersen et al (2006)
Dipterex	0.0017	0.0008	Renn et al (2007)
Endosulfan	1.5		FernadezCasalderrey et al (1994)
Gammacyhalothrin		0.0009	Barata et al (2006)
Malathion	0.0115	0.0027	Ren et al (2007)
Nitrofen		0.2464	Ren et al (2007)
Paroxon	2.36	1.89	Carvalho et al (2003)
		0.0007	Guilhermino et al (1996)
Parathion	0.0043	0.0013	Ren et al (2007)
		0.0082	Guilhermino et al (1996)
Propanil		16.28	Pereira et al (2006)
Tetradifon		25.0	Villarroel et al (1998)

1.1 Anti-Arrhythmic Drugs (Using Vaughan-Williams Classifications)

1.1.i. Class 1A

The combined sodium-potassium channel blockade is sparsely studied in invertebrates, no studies have been performed on the invertebrate heart. And, no invertebrate studies were found for **disopyramide** and **procainamide**. **Quinidine** has been used as a 'bitter taste substance' when the training the leopard slug, *Limax maximus* (Culligan and Gelperen, 1983 and Sakura et al 2004) and the tree slug, *Limax marginatus* (Kimura et al 1998 and Kasai et al 2006) to become food averse.

1.1.ii. Class 1B

The weak sodium channel blockade class are used in the removal of unwanted bodily invertebrate parasites by blocking nerve impulses via direct action on voltage gated sodium channels. The medicinal leech, *Hirudo medicinalis*, can be paralysed by 4% **lidocaine** injection, allowing it to be detached from human orifices (Tseng and Ho 2005). **Lidocaine** solution spray completely paralyses peristaltic movement and eliminates suction in the fish-trematodae, *Clinostonum complanatum* (Kitagawa et al 2003). **Lignocaine** injections remove the larval bot fly, *Dermatobia hominis*, which burrow into patient's flesh (Moulton and Adams 1997). However, this class is not universally successful in this role: Lee et al (1994) found that neither subcutaneous injection of **lidocaine** nor **chloroprocaine** were sufficient to remove the American dog tick, *Dermacentor variabilis*, attached to a rabbit. Leffler et al (1993) found that an undescribed immersion oil for microscopy was better at immobilising, and faster at killing American cockroaches, *Periplaneta americana*, which had burrowed into a patient's ear, than any solution of **lidocaine** tested.

Other invertebrate studies focus on neural and genetic effects of this class 1B. In 3rd stage larvae of the fruit fly, *Drosophila*, **prilonest** causes exclusively homologous recombination in somatic cells (Schneider et al 2009), and high levels of **lidocaine** permanently suppress axonal growth in presynaptic neurons (Onozuka et al 2011 and 2012). In the Galician crayfish, *Astacus leptodactylus*, 5000-10,000μM **lidocaine** causes spontaneous activity in slow-adapting neurons, while above 10,000μM it blocks the same neural activity and impulse responses to stimulation (Keceli and Purali 2007). In the freshwater snail, *Lymnaea stagnalis*, 5000μM **lidocaine** destroys neural cell membrane and induces necrosis and apoptosis (Kasaba et al 2006). In the western honey bee, *Apis mellifera*, **procaine** causes reversible, short term, olfactory memory deficits (Muller et al 2003). These effects on

invertebrate neurology made this class a target for the design of insecticides such as **indoxacarb** and **metaflumizone**. They are considered 'safe' as receptor sites for these drugs are significantly different to those on human sodium ion channels (Silver et al 2009). While in invertebrates such as the German cockroach, *Blatella germanica*, **indoxycarb** blocks sodium channels in the nervous system leading to paralysis and death (Djemaoun et al 2015).

Lab based physiological studies largely focus on Class 1B's anaesthetic effect on smooth muscle fibres. Smooth muscle forms supporting tissue for blood vessels and hollow internal organs e.g. stomach, intestine, and bladder. It is composed of myocytes which can tense and relax to help in organ functions such as urination, defecation or arterial pumping. In the olive-brown swimming crab, *Callinectes danae*, 1mM **procaine** or 10mM **lidocaine** inhibit muscle contractions, causing Na^+ retention by activating voltage dependent Na^+ channels, preventing the release of calcium ions from voltage gated channels in the sarcoplasmic reticulum (Madeira and Suarez-Kurtz 1983). In the pond snail, *Lymnaea stagnalis*, **lidocaine** suppresses slow potassium currents through voltage dependent ion channels (Onozuka et al 2005, and Ignatov et al 2005). In the land snail *Euhadra peliomphala*, **lidocaine** suppresses sodium ion currents by phosphorylating cAMP dependent protein kinases (Onozuka et al 1993). A comparative study found that in the common earthworm, *Lumbricus terrestris*, **benzocaine** is 3.25 times more toxic than **lidocaine** in inhibiting motility (Suskevich et al 1966).

Rarer studies look at the sodium channel blockade's bradycardic effect on the invertebrate heart. In the freshwater snail, *Lymnaea stagnalis*, **lignocaine** slows heartrate: It abolishes the inhibitory effects of acetylcholine (Carroll and Cobbin 1969). In the brown-lipped snail, *Cepaeca nemoralis*, **lidocaine** also slows heartrate: It inhibits the cardioexcitatory-peptide, FMRFamide, from producing the signalling molecule nitric oxide (Roszer et al 2006). Lidocaine causes intracellular sodium retention by activating voltage dependent sodium channels, so that intracellular Na+ increases in a dose dependent manner (Onozuka et al 2008). In the American cockroach, *Periplaneta americana*, **lidocaine** slows heartrate by blocking resting state Na+ channels with similar potency to that observed in mammalian Na+ channels (Song et al 2011).

1.1.iii. Class 1C

The strong sodium channel blockade is little studied in invertebrates, with no studies found for **encainide** nor **flecainide**. In the medicinal leech, *Haemopis sanguisuga*, **propafenone** produces a cardiac-like action potential, characterised by rapid depolarization followed by a sustained plateau, terminated by a second rapid depolarization (Dekleva and Beleslin 1995).

1.1.iv. Class 2A

The β–adrenoceptor blockers are more extensively studied in invertebrates due to their importance in environmental waste management. β-blockers are difficult to remove during normal waste water treatment procedures (Murdoch 2015), being very resistant to hydrolysis (Maszkowska et al 2014). They build up as toxins in the environment causing oxidative stress to aquatic organisms (Sun et al 2013). For example, In the rainbow trout, *Oncorhynchus mykiss*, exposure to environmentally relevant concentrations of **atenolol** decreases haemoglobin concentration and increases lactate content in blood plasma suggesting a reduction in the oxygen supply (Steinbach et al 2014). Environmental science studies focus on the outcomes of toxin exposure as opposed to an elucidation of the molecular mechanism by which this occurs.

Metoprolol is the most prevalent β-blocker in freshwater streams, followed in order by **propranolol**, **timolol**, **acebutolol**, and **atenolol**. This closely corresponds to the order of toxicity of drugs in freshwater invertebrates (Zuriaga et al 2014). In Germany, between 55.5 and 111 tonnes **metoprolol**, and 5.2 and 10.5 tonnes **atenolol** are dispensed per year (Cleuvers 2005). 60-65% of **metoprolol** metabolites are excreted in urine (Huschek et al 2004) and concentrations of 5760pM and 8228pM are found in German rivers and waste water effluent respectively (Hirsh et al 1996 and Cleuvers 2003). **Metoprolol** is the 9th most commonly dispensed drug in the US as of 2011 (Shakya 2011). In 2000, **Atenolol** was the 13th most used pharmaceutical by weight in England, 11,554x10^{3} prescriptions were dispensed amounting to 28,968kg (Jones et al 2002).

We found no invertebrate studies for **bezfibrate**, **bisoprolol** nor **esmolol**. However, in *Daphnia magna* **metoprolol** decreased mean body length of new-borns, and reduced offspring numbers at the environmentally relevant concentration of 4488pM (Dietrich et al 2010). **Timolol** is less toxic, inhibiting *Daphnia* growth at a much higher concentration of

100μM (Lang et al 2012). In the free-living ciliate protozoan, *Tetrahymena pyriformis*, **metoprolol** inhibited growth at concentrations of 10μM, and was moderately toxic to the proliferation and migratory behaviour. Even below environmentally relevant levels (0.001pM) **metoprolol** produced a chemorepellant effect on the ciliates (Lang et al 2012). In the freshwater mussel, *Dreissena polymorpha*, 0.02μM **metoprolol** upregulated antioxidant enzymes indicating oxidative stress, and heat-shock proteins indicating protein damage (Contardo-Jara et al 2010).

In *Daphnia magna,* **metoprolol** reduces heartrate at an environmentally relevant concentration of 4488pM (Dietrich et al 2010). The Lowest Observed Effect Concentration, or LOEC, was 12μM which was much lower than those for fecundity (22μM) or growth (45μM). This led Dzialowski et al (2005) to suggest the *Daphnia* heart a useful indicator of the accumulation of biologically harmful toxins in freshwater environments. However, subsequent generations of *Daphnia* became less sensitive to the compound, suggesting an epigenetic effect.

1.1.v. Class 2B

Of the non-selective β-blockers, propranolol is most toxic to aquatic organisms (Cleuvers 2005), due to the strong membrane stabilising properties which other β-blockers lack (Fent et al 2006). In Germany between 1.0 and 2.0 tonnes propranolol per year are dispensed (Cleuvers 2005). Concentrations of 338pM and 1014pM are found in rivers and waste water effluent respectively (Hirsh et al 1996). No invertebrate studies were found for **labetalol**, **oxprenolol** and **pindolol**.

In *Daphnia*, **propranolol** enhances reproduction at low levels (3.38pM) (Rivetti et al 2015) but causes abortion at concentrations above 169nM (Stanley et al 2006). **Propranolol** alters *Daphnia* production of enzymes in manner indicative of oxidative stress. 338pM causes a significant decrease in total glutathione-peroxidase activity occurs, while at 33806pM an increase in glutathione-S-transferase activity is seen (Oliveira et al 2015). Stanley et al (2005) suggested that disruption of membrane integrity, rather than interaction with β-receptors, was responsible for **propranolol** toxicity in *Daphnia*. Their study compared stereoisomers of **propranolol**. R-propranolol was more toxic (LOEC >2.94μM), while S-propranolol, responsible for β-adrenergic blocking, was less toxic (LOEC 1.38μM) in 21-day chronic tests. In the brittle star, *Amphipolis squamata*, R-propranolol at 1μM was found to inhibit

adrenaline dependent bioluminescence, while the less active propranolol S-form had no effect (DuPont et al 2004). In the freshwater ciliate, *Tetrahymena pyriformis*, environmental levels of R-propranolol were highly toxic to growth (LOEC 1μM) (Lang et al 2012). Biodegradation tests for waste water treatment showed that the R-form is far more difficult to degrade than the S-enantiomer (Ribiero et al 2012) making this a high-risk substance even after sewage treatment.

Villegas-Navarro et al (2003) found that **nadolol** caused positive chronotropic effect on *Daphnia* at concentrations less than 0.01μM and negative chronotropy at high concentrations greater than 1000μM. Dzialowski et al (2005) found that **propranolol** significantly reduced *Daphnia* heartrate (LOEC 186nM) at concentrations much lower than its LOEC impact on fecundity (372nM) or growth (1487nM).

1.1.vi. Class 2C

Class 2C comprises the α-blockers, little studied in invertebrates: No invertebrate studies exist for **carvedilol**, a β-blocker with some action at α-receptors. In the burgundy snail, *Helix pomatia*, application of prazosin was found to have no effect on cardiac response (Prokopenko et al 1995). In the American lobster, *Homarus americanus*, **phentolamine** reversibly reduced heartrate at concentrations of 1μM to 10μM, but failed to suppress excitatory activities of noradrenaline, dopamine or 5-hydroxytryptamine (Berlind 2001).

1.1.vii. Class 3

Among potassium channel blockers no studies of **bretylium**, **dofetilide** and **ibutilide** in invertebrates were found. In the cestode parasite, *Echinococcus granulosus*, **amiodarone** increases the expression of p-glycoproteins, allowing the tapeworm to excrete anti-tapeworm drugs via its suckers and tegument, increasing its drug resistance capability (Nicolao et al 2013). **Amiodarone** was developed as a vasodilator in 1961 to treat angina pectoris, however, its pronounced antiarrhythmic effects redirected its use and it is now a class 3 anti-arrhythmic (Van Erven and Schalij 2010). In the mud snail, *Potamopyrgus antipodarum*, 184pM **sotalol** extends the reproductive period so that offspring production is approximately doubled (Feiner et al 2014).

1.1.viii. Class 4

Due their disruption of calcium homeostasis the calcium channel blockers consist of the most highly toxic environmental contaminants known today (Antczak et al 2015). The calcium channel blockers are split into two types (type 1 and type 2). Kim et al (2007) found that, versus nine other pharmaceuticals present in the environment, the type 1 (non-dihydropyridine Ca^{2+} channel Blocker) **diltiazem** was the most acutely toxic. **Diltiazem** was the 20th most used pharmaceutical by weight in England in 2000; 2844x10^{3} prescriptions were dispensed weighing 21,791.50kg (Jones et al 2002).

Data from the blue mussel, *Mytilus edilus*, agrees with Kim et al (2007): the order of potency versus this invertebrate was found to be, first, the type 1 benzodiazepine **diltiazem**; second, the type 1 phenylalkylamine **verapamil**; third and fourth are type 2 dihydropyridines, **nicardipine** then **nifedipine** (Miyahara et al 1993). However, a much different order was found for the parasitic lung fluke, *Paragonimus ohirai*, against which calcium channel blockers were found to stunt development to the sexually mature. In order of potency, equal 10μM concentrations of **nicardipine**, **nimodipine, nitrendipine, verapamil, nifedipine**, and **diltiazem** were used with 100%, 92%, 56%, 33%, 5% and 0% success respectively (Ikeda 2006). No invertebrate studies were found for type 2 calcium channel blockers amlodipine, felodipine and isradipine.

Environmentally relevant concentrations of Ca^{2+} channel blockers have diverse damaging effects on aquatic invertebrate physiology. Ca^{2+} uptake is essential to the metabolic activation of sea urchin eggs. In the black spiny sea urchin, *Heliocidaris crassipina*, exposure to 25μM **verapamil** or **diltiazem** prior to insemination inhibited the acrosome reaction in spermatozoa and caused fertilization failure (Komukai et al 1985). In the pink spiny sea urchin, *Hemicentrotus pulcherrimus*, calcium is critical in the formation protective spicules during gastrulation (Iwata and Nakano 1985). Concentrations as low as 20μM **diltiazem**, 15μM **verapamil** or 50μM **nicarpidine** block Ca^{2+} channels and leave *Hemicentrotus pulcherrimus* neonates stunted and disfigured (Yasumasu et al 1985). Perhaps sea urchins are more susceptible to Ca^{2+} channel blockers than other aquatic invertebrates. For example, for the the medicinal leech, a much higher concentration, 1000μM of either **verapamil**, **gallopamil** or **diltiazem** reduces spermatogenesis by increasing Ca^{2+} in testicular Leydig neurons (Dierkes et al 2004). *Daphnia* are also more resilient than sea

urchins. In a 21-day test Wolfe et al (2015) found up to 24μM diltiazem had no significant effect on *Daphnia* reproductive capacity.

Biphasic effects have caused difficulties in the design of Ca^{2+} channel blockers as insecticides. In the leaf worm, *Mythima loreyi*, production of juvenile hormone is required for development to the sexual stage and is reliant upon extracellular Ca^{2+} (Hseieh et al 2001). 100μM **nifedipine** significantly reduces juvenile hormone production in the first hour of exposure but loses efficacy within 4 hours, while 100μM **verapamil** significantly increases juvenile hormone production in the first hour of exposure losing efficacy within 5 hours (Hseieh et al 2002).

Invertebrate sensory organs and smooth muscle; their sight, smell, communication, locomotion, and food consumption; are all negatively affected by disruption from calcium channel blockers. In the air-breathing sea slug, *Onchidium verruculatum*, 10μM 4-aminopyridine or 30-40μM **diltiazem** reversibly blocked induced Ca^{2+} signals in photoreceptors (Gotow et al 1997). In the American cockroach, *Periplanta americana*, 150μM **verapamil**, 287μM **diltiazem** or 100μM nifedipine reversibly blocked induced Ca^{2+} signals in olfactory interneurons (Husch et al 2008). In the deep sea blue bristleworm, *Tomopteris helgolandicais*, 1mM **verapamil** or 1mM **diltiazem** reversibly blocked induced Ca^{2+} signals used for bioluminescence, a form of intraspecific communication and defence (Gouveneaux and Mallefet 2013). In the blue mussel, *Mytilus edilus*, 10μM **diltiazem** or 10μM **verapamil** reversibly blocked induced Ca^{2+} signals in the smooth muscle reducing locomotion and food consumption (Murakami et al 1984). In the medicinal leech, *Hirudo medicinalis*, 1000μM of either **verapamil**, **gallopamil** or **diltiazem** reversibly blocked induced K^+ and Ca^{2+} signals in motor neurons negatively affecting locomotion of erector muscles in the annuli (Dierkes et al 2004). In the sea cucumber, *Sclerodactyla briareus*, 10μM of either **diltiazem** or **verapamil** reversibly blocked induced Ca^{2+} signals in the smooth muscle (Devlin and Smith 1995). In the red whelk sea snail, *Neptunea antiqua*, 10μM of either **diltiazem**, **nifedipine** or **verapamil** reversibly blocked induced K^+ and Ca^{2+} signals in contracting radula, preventing it from grazing (Alohan 1995).

With regard the invertebrate heart the following studies have been performed. In large predatory sea-whelk, *Busycon canaliculatum*, **verapamil** blocked sarcolemma L-type Ca^{2+} channels accounting for 20% of mobilised Ca^{2+} during excitation-contraction coupling (Devlin

2001). In the edible snail, *Helix pomatia*, L-type Ca^{2+}channels are reversibly inhibited with an EC50 of 79μM for **verapamil** (Prokopenko et al 1995) and 20μM for **diltiazem** (Akaike et al 1981). In the Maine lobster, *Homarus americanus*, 10μM **verapamil** reduced proctolin and FMRF amide-like peptide, F2, induced heart contractions by 10% and 26% respectively. The above two neuropeptide hormones cause contractions via an influx of calcium (Wilkens et al 2005). The effect is different in the sunray clam, *Macrocallista nimbosa*. Neither 10μM of **verapamil**, **nicardipine** or **nifedipine** nor 100μM **diltiazem** could inhibit contractions initiated by 10μM F2, causing researchers to suggest that F2 may work via receptor controlled influx of Ca^{2+} which is less sensitive to Ca^{2+} channel blockers than voltage gated Ca^{2+} channels (Kizawa et al 1990). In the whelk *Busycon canaliculatum*, 0.5μM and 50μM **nifedipine** respectively caused 50% and 100% inhibition of acetylcholine induced contractions, which release Ca^{2+} through a slow-type channel (Alohan 1990). In the *Daphnia magna* heart, **verapamil** has biphasic effect. Concentrations less than 10μM caused positive chronotropy and negative inotropy, while greater than 10μM the opposite occurred (Villegas-Navarro 2003) .

1.1.viii. Class 5

Ca^{2+} channel mediators include serotonin which is more widely known for its Selective Serotonin Reuptake Inhibition (SSRI) properties. In the milky ribbon worm, *Cerebratulus lacteus*, and the pink nemertian worm, *Micrura alaskensis*, serotonin aids in oocyte maturation (Stricker and Smythe 2001). Other class 5 Ca^{2+} channel mediators, such as adenosine, alinidine, cyclopiazonic acid, lithium, nisoldipine and zatebradine are yet to be studied in invertebrates. Serotonin is more widely studied in the invertebrate heart than many Vaugan-Williams anti-arrhythmics presented so far. Human serotonin and molluscan 5-hydroxytryptamine receptors have homologous pharmacological properties in their respective hearts (Murakami et al 1987). Gunnarrsson et al (2008) found 49% homology between human serotonin and *Daphnia* serotonin transporters. In the fruit fly, *Drosophila*, heartrate increases when exposed to serotonin (Vogler and Ocorr 2013). The serotonin reuptake inhibitor fluoxetine increases synaptic concentrations of serotonin in humans. Fluoxitine slows larval *Drosophila* heartrate in a concentration dependent manner, plateauing at 10mM (Majeed et al 2015). In the American hard-shelled clam, *Mercenaria mercenaria*, serotonin also increases heartrate (Walker 1984). And, in the Venus clam, *Katelysia rhytiphora*, serotonin has been shown to increases heartrate at concentrations as low as 0.01μM (Sathanthan and Burnstock 1976). In the large predatory sea-whelk, *Busycon*

canaliculatum, less than 0.0001μM serotonin reversibly stimulates efflux of Ca^{2+} into ventricular muscle primarily from the sarcoplasmic reticulum, with maximal effect at 0.1μM, causing concentration dependent inotropic and chronotropic activity (Devlin 2001).

1.2 Lipid Lowering Drugs

Lipid lowering drugs are split into three types; statins, nicotinic acids and cholesterol absorption inhibitors. The statins include atorvastatin, simvastatin, fluvastatin, mevastatin and rosuvastatin. In humans these act as HMGCo-A reductase inhibitors. As this enzyme is not present in invertebrates they are reviewed here in terms of systemic effects.

Atorvastatin is the most prescribed lipid regulator in Canada (Cavalucci, 2006). It has been detected at concentrations of 40pM to 66pM in waste water treatment effluent and 2pM in surface water (Miao and Metcalfe 2003 a and b). Dussault et al (2008) found 24-hour LD50 values for **atorvastatin** in the non-biting midge larvae, *Chironomus tentans*, and the ghost shrimp, *Hyalella azteca*, at 25.6μM and 2685nM respectively. They concluded that failure to remove **atorvastatin** from sewage treatment would be catastrophic to freshwater life.

Fluvastatin slows oocyte development, significantly reduces oocyte length, fat body vitellogenin and ovarian vitelline which are crucial to metamorphosis and reproduction, due to inhibition of lipid synthesis in invertebrates such as the viviparous cockroach *Diploptera punctata* (Huang et al 2015). In the migratory locust, *Locusta migratoria*, **fluvastatin** was found to prolong instar time in low doses (1μg injections) but not arrest development due to oxidation of the drug, while doses greater than 20μg led to death (Debernard et al 1994). In the Pacific purple sea urchin, *Strongylocentrotus purpuratus*, 2.561μM **mevastatin** decreased capacity to synthesize cholesterols and N-linked glycoproteins. Embryos were rendered incapable of invagination, the stage at which the gut is formed via an indent into the fetal ball of cells. They produce evaginated (externalized) guts or did not gastrulate at all, none survived beyond this (Carson and Lennarz 1979). **Lovastatin** is a red rice yeast extract used to combat bilharzia, or *Schistosomiasis*, infection in rats. 0.124μM **lovastatin** increases membrane permeability in the parasite egg, causing total embryo mortality within 8 days (Araújo et al 2008).

Other lipid lowering drugs such as **ezetimibe**, **cholestyramine**, **gemofibrozil**, and **fenofibrate** have yet to be studied in invertebrates. As an environmental pollutant

cholesterol also has negative impact on invertebrates: Cholesterol derivatives synthesised from the steroid hormone pregnenolone caused ecdysone hormone depression in the migratory locust, *Locusta migratoria*; a hormone crucial to moulting processes and embryonic development (Burger et al 1988).

1.3 Anticoagulants

Anticoagulants are present in the environment as rodenticides. **Warfarin** is most ubiquitous at concentrations up to 145pM in waste water effluent. **Warfarin** is a highly water-soluble compound and activated sludge treatment often fails to remove it, while **bromadiolone** and **chlorophacinone** are rapidly degraded (Gómez-Canela et al 2014). No study of anticoagulant exposure to invertebrates has been found.

1.4 Antiplatelet Agents

Of the antiplatelet agents, only **aspirin** has been studied in invertebrates. **Aspirin** enters the environment via septic tanks (Li 2014) and is extremely resistant to wastewater treatment (Samuel and Teo 2002) making it a major topic of concern for freshwater scientists. It has been found in freshwater streams at concentrations of 1021pM (Spain, 2012) (Lopez-Serna et al 2012) and 1110pM (USA, 2006) (Fent et al 2006). It was the 22nd most prescribed pharmaceutical by weight in England in 2000; 16,769x10^{3} prescriptions dispensed amounted to 18,106kg (Jones et al 2002). In mammals, **aspirin** irreversibly inhibits cyclooxygenase enzymes, which convert arachidonic acid to endoperoxides, decreasing platelet aggregation (Brown and Kozowski 1997). In the free-living ciliate, *Tetrahymena pyriformis*, **aspirin** exposure inhibits growth 10pM, and is chemorepellent at 1pM (Lang et al 2012). These are well below environmentally relevant concentrations. The acorn barnacle, *Balanus amphitrite*, is more resiliiant; no toxic effects were found at concentrations up to 11,000μM (Samuel and Teo 2002).

Aspirin alters protein expression in *Daphnia magna*. Exposure to 172μM **aspirin** over 21 days, significantly downregulated production of vitellogenin (a protein used in egg production), aryl hydrocarbon receptors (transcription factors that regulate gene expression), CYP314 (an oxidizing enzyme found in the gut) and isoenzymes involved in the metabolism of arachidonic acid were significantly downregulated (Bang et al 2015 a and b). *Daphnia* arachidonic acid precursor genes coding for eicosanoids are also downregulated in the presence of **aspirin**. These are local hormones which function as important mediators

in ion transport, which regulate blood pressure (Heckmann et al 2008). The products of metabolic breakdown of aspirin are harmful to *Daphnia*. Gentisic acid exposure inhibits reproduction. Salicyclic and O-hydroxyhipponic acid exposure reduces neonate size and increases mutations and abortion (Marques 2011).

1.5 Cardio Tonic/ Inotropic Agents

Cardio tonic or inotropic agents studied in invertebrates include β-sympathomimetic agents such as dopamine and cardiac glycosides including digoxin and ouabain. In the sea cockroach, *Ligia exotica*, response to dopamine exposure depends on life stage. The crustacean is born with a myogenic heart, which becomes neurogenic during juvenile development. Under 5 days old, 30 second exposures to 50μM dopamine decreased action potential frequency (-17%). Above 25+ days old, the same treatment increased action potential frequency (43%) in the heart. Intermediate ages showed a transitional response (Yamagishi et al 2004).

In *Daphnia*, dopamine upregulates defensive responses to invertebrate predators. It induces growth of neck spines and helmets, increases body size and delays onset of reproduction via interaction with the juvenile hormone pathway (Weiss et al 2015). These help prevent *Daphnia* from being consumed by predator insects by making them too big, or too awkward, to fit through mouth parts. Dopamine agonist exposure slows down *Daphnia*, reducing their speed and time spent swimming (Barrozo et al 2015). The dopamine receptor antagonist, Haloperidol, causes feeding inhibition in *Daphnia magna* (Furuhagen et al 2014). Digitalis exposure causes *Daphnia* heartrate to slow, in contrast to the accelerative human reaction; exposure to 5μM for 80 minutes decreased heartrate by 30% (Bekker and Krijgsman 1950). It is possible this difference may stem from a biphasic dose response. *Daphnia* exposed to 50μM ouabain responded with significant inotropic effect at systole, but heartrate and diastole did not change (Villegas-Navarro et al 2003).

1.6 Vasodilators

There are approximately five types of vasodilator: Angiotensin Converting Enzyme (ACE) Inhibitors, neprilysin inhibitors, angiotensin II receptor antagonists, nitrates and potassium channel openers. No data so far has been found for invertebrate interactions with potassium channel openers. Very little has been observed concerning nitrate interactions with invertebrates. Yost (2004) found that at concentrations greater than 13μM **nitroglycerine** had significant negative effect on the reproduction of *Daphnia magna*. The remaining three (angiotensin related) vasodilator-invertebrate interactions have been studied in much greater depth.

In humans, ACE inhibitors lower blood pressure, are important in treating diseases associated with high circulating components of the renin angiotensin pathway, such as hypertension and congestive heart failure (Vandingenen et al 2002). In the human, ACE is predominantly active in the lungs, and is more elevated in persons with lung problems such as sarcoidosis and asthma (Uçar et al 1997). The somatic isoform regulates blood pressure by cleaving the C-terminal dipeptide from Angiotensin I to form Angiotensin II, a vasoconstrictive peptide. And, by deactivating bradykinin I, a vasodilator. This results in increased blood pressure (Laurent et al 1997). The germinal isoforms of ACE are required for fertility (Lemeire et al 2008 and Riviere et al 2011).

ACE is ubiquitous across the invertebrate kingdom. The enzyme has been isolated in the oyster *Crassostrea gigas* (Riviere et al 2011), the grey flesh fly *Neobellieria bullata* (Vandingenen et al 2002), the Egyptian cottonworm moth, *Spodoptera littoralis*, the silkworm moth, *Bombyx mori* and the buff-tailed bumblebee, *Bombus terrestris* (Vercruysse et al 2005). Invertebrate ACE isolated to date are expressed largely in reproductive tissues, where they are critical to foetal development (Lemeire et al 2008). In the malaria mosquito, *Anopheles stephansi*, ovarian ACE activity increases from an average 0.02nM to 7.33nM following a blood meal (an activity required for egg production in female mosquitos) (Ekbote et al 1999). Invertebrate ACE is also essential during metamorphosis where it becomes highly expressed in cells destined to become adult tissues (Isaac et al 2007). And, invertebrate ACE has also been found to play a role in innate immune response (Lemeire et al 2008). Migratory locusts (*Locusta migratoria*) injected with *E. coli* responded with a tenfold increase of ACE expression in circulating haemocytes (Macours et al 2003).

In the crab, *Carcinus maenas*, ACE was most concentrated in the gills, followed by the brain, muscle and testes, however, none was found in the heart (Chung and Webster 2008). Invertebrate ACE isoforms have a single active region. This germinal-form of ACE has been isolated in the blue mussel, *Mytilus edilus* (Laurent et al 1997) and the blood-sucking leech *Theromyzon tessulum* (Riviere et al 2004). In the fruit fly, *Drosophila*, enalapril, lisinopril and trandolapril, had significantly less potent inhibitory affect than they do on human ACE, which suggested different protein structures for human and invertebrate ACE (Williams et al 1996). *Drosophila* Ance and Acer correspond pharmacologically to the two human somatic ACE binding sites, the N terminal and C terminal, respectively (Coates et al 2000). *Drosophila* Ance is highly potent in cleaving Angiotensin I, while Acer fails to do so (Houard et al 1998). However, In *Theromyzon*, ACE was restricted to the mid-gut, and corresponded closely human germinal ACE, in terms of both gene sequence homology and pharmacological activity (Riviere et al 2004).

ACE inhibition is known to impair invertebrate growth and development (Lamango et al 1997 and Sivitier et al 2002), reduce egg numbers, and prevent cuticle shedding leaving larvae trapped in a carapace too tight to allow the passage of food through the gut, leading to death (Isaac et al 2007). In the malaria mosquito, *Anopheles stephansi*, addition of 1000μM of the ACE inhibitor, captopril, to its blood meal abolished egg production. 1000μM lisinopril did not make a significant difference to the size of the batch (Ekbote et al 2003). In crops pests such as the larval Egyptian cotton leaf worm, *Spodoptera littoralis*, 2.5μM dietary captopril was enough to shut down all ACE activity. At lower concentrations captopril dose dependently down-regulated trypsin activity in the haemolymph, inhibiting growth (Lemeire et al 2007). Topical administration of captopril on *Spodoptera littoralis* larvae prevented a mean 47.5% from reaching adult forms, but egg production was unaffected among survivors. In adult females, 5μl topical application of 46,000μM every two days resulted in an exponential 1.5-fold decrease in egg laying over time. It reduced egg numbers by down regulating ecdysteroid biosynthesis to and upregulating trypsin. Male ecdysteroids and trypsin levels were unaffected (Vercruysse et al 2004 and 2005). 13μM dietary enalapril prevented a significant number of *Spodoptera littoralis* larvae from reaching adulthood. Lisinopril was least potent; only effective when injected into the larvae, not when consumed (Lemeire et al 2008). In the flour moth, *Ephestia kuehniella,* topical application of 23,000μM lisinopril, captopril and enalapril, slowed the onset of

adulthood, completely preventing moths from reaching adulthood by 31%, 43% and 78% respectively compared to controls (Kirane and Soltani 2012).

Very little work has been done to examine Angiotensin II receptor antagonists in invertebrates. In the burrowing crab, *Chasmagnathus granulatus*, Angiotensin II increases learning speed. In a procedure repeated across many works, crabs are habituated to a fear stimulus; a shadow passes overhead which they respond to by freezing, and this is continued until they become accustomed to it and no longer freeze. *Chasmagnathus* injected with 3ng/g 1μM **saralasin** before training failed to habituate (Delorenzi et al 1995), while the learning and memory of those injected one hour after training were unaffected (Delorenzi et al 1996). This suggested that **saralasin** has amnesiac effect on short term but not long-term memories (Delorenzi and Maldonado 1999). This was confirmed by Frenkel et al (2010) who used identical training procedures and found **saralasin** caused memories to be unexpressed in the short term, but not removed from long term memory. The effect of **saralasin** was reversed when the context of the memory was revisited.

1.7 Vasopressors and Inotropes

Vasopressors constrict blood vessels elevating arterial pressure and can be represented by **norepinephrine**. Inotropes increase cardiac contractility and can be represented by **noradrenaline**. Many drugs have both vasopressor and inotropic effects. *Daphnia* heartrate slows when exposed to low concentrations of adrenaline (0.2μM to 2μM), and accelerates at the higher concentration tested, 125μM (Bekker and Krijgsman 1950). 46μM **noradrenaline** delayed *Daphnia magna*'s first moult, from 24 hours after birth to a mean 76.8 hours, reduced the number of moults over the first four days from an average 3.5 to 1, and significantly inhibited reproduction (Overturf et al 2015).

1.8 Herbal Remedies

Shilajit is the guano of the flying squirrel, *Sciurinae*, and is sold as a herbal remedy for male virility. Gaikwad et al (2012) found that *Daphnia* hearts exposed to **Shilajit** produced biphasic response: At low concentrations, 1, 10 and 100ppm heartrate decreased by a mean 7.65%, 15% and 28.45%. At 1000ppm heartrate increased beyond measurement capability then death occurred. No concentration between 100ppm and 1000ppm were tested. Conversion to molar measurement was not possible here as this natural product does not have a known molecular weight.

1.9 Cholinergic Modulators

Cholinergics form the basis of most insecticides and are a hot topic in environmental science. Insecticide leaching into surrounding environments such as streams and land surrounding farmed areas affect crucial ecosystem services such as pollination. More invertebrate studies are published on this category than any other, and form a thorough background to 7.0. Table 1a shows that cholinergic modulators have greater toxic effect at equivalent doses than any other class. In humans, acetylcholine, cholinergic agonists and anticholinesterases increase heartrate, while cholinergic antagonists slow heartrate. Choline and acetly-CoA combine to form acetylcholine in the presynaptic neurons. Acetylcholinesterase breaks down acetylcholine into acetic acid and choline reducing stimulation of the cholinergic receptors. Anticholinesterases inhibit acetylcholine metabolism. This results in free, unbound acetylcholine accumulation at cholinergic nerve endings resulting in potentially fatal continuous stimulation of cholinergic receptors.

There are two classes of cholinergic receptor, muscarinic and nicotinic. Muscarinic (M2) receptors are located on the human heart. Examples of muscarinic agonist are **pilocarpine**, **carbamylcholine** and **carbachol**. Examples of muscarinic antagonists are **atropine**, **hyoscine**, and **scopolamine**. Anticholinesterases include organophosphates and carbamates such as **galantamine** and **physostigmine**. Nicotinic receptors are not present on the human heart but are on intracardiac neurones and also work on skeletal muscle. An example human nicotinic agonist is the competitive acetylcholine inhibitor **tubocurarine**. Studies investigating invertebrate interactions with cholinergic modulators are abundant; however, studies looking at action on the invertebrate heart in particular are relatively rare. This section will examine the variety of actions of this class upon invertebrates.

1.9.i. Learning and Memory

The most abundant research area in this class is learning and memory. The chemical composition of the brain is continually altered or consolidated by new or repeated experiences and memories. Muscarinic receptors are implicated in associative learning. Cholinergic agonists can facilitate memory while cholinergic antagonists impair it by blocking input to the cerebral cortex. In the common flatworm, *Dugesia dorotocephala*, trained to solve maze puzzles, 1000μM **scopolamine** treated worms were 70% poorer at finding their way out than controls. 0.01mM **galantamine** partially reversed this, treated worms then

had 40% poorer memory than controls. Increased doses could not recover mental capacity any further (Ramakrishnan et al 2014). Burrowing crabs, *chasmagnathus granulatus*, were trained to become accustomed to a fear stimulus; a shadow indicating a predator was repeatedly cast over the holding tank. Once accustomed to regular danger, crabs ceased to run away and simply froze until the danger passed. Treatment with 330nM **scopolamine** caused them to revert back to running away. However, when treated animals were retrained, previously trained crabs learned to freeze significantly faster than crabs never trained prior to amnesiac treatment. Higher doses (16.4μM) produced irreversible amnesia (Caffaro et al 2012). Given before training 330nM **scopolamine** had no effect on learning acquisition, and one hour after did not alter the by-then embedded long term memory (Beron-de-Astrada and Maldonado 1998). In the two-spot ocotpus, *Octopus bimaculoides*, injection with 6.6μM **scopolamine** caused short term inability to solve the problem of uncorking jars containing prey. The problem was solved with prolonged effort. Octopi suffered long term memory loss of a previously learned activity involving distinguishing coloured balls to avoid punishment (Fiorito et al 1998). **Scopalamine** affects short term but not long-term memory at lower doses, memory impairment appears to be less reversible with increased concentration. This memory might also be dependent on context; perhaps the immediate presence of a prey animal in a jar was a stronger prompt to memory recall than the more abstract ball test.

The cholinergic effects of insecticides are implicated in the current bee colony collapse crisis with devastating repercussions for farming industries across the world (Brettell and Martin 2017). In the western honeybee, *Apis mellifera*, colony members share odour profiles based on shared genetic patterns of cuticular hydrocarbons. This creates the olfactory memory by which bees distinguish nest mates from invaders. This capability is decreased by **scopolamine**, causing bees to attack nest mates (Ismail et al 2008). Similar outcomes occurred in trials of **scopolamine** which suppressed olfactory neurons in the land slug, *Limax marginatus* (Watanabe et al 2001) and chemo-sensitive antennal neurons of the tobacco hornworm moth, *Manducta sexta* (Torkkeli et al 2004). At two-to-four weeks old bees leave the hive for the first time and begin foraging. During this time the bee brain known as the mushroom body grows by 14%. This is blocked by **scopolamine** causing bees too lose orientation and fail to return to the hive (Weinberger, 2006). Lozano and Gauthier (1997) trained bees to extend their proboscis to a sugar-water source upon being given the scent of vanilla. Bees injected with 0.01M **atropine** before training had no trouble in learning the

response, but bees injected after training suffered significant memory impairment. No studies have been done into *Daphnia* learning and memory.

1.9.ii. Innate Defence Response

The *Daphnia* defensive response is known to be altered by cholinergics (Coors et al 2004). In response to glass worm, *Chaoborus trivittatus*, kairomones, *Daphnia* grow neck-teeth causing them to become less easy to swallow (Weiss et al 2012). 0.001μM **Physostigmine** significantly increases the size of morphological defences, while **atropine** decreases their expression at 5μM and blocks their expression at 50μM (Barry 2001). The Turkish snail, *Helix lucorum*, has cholinergic receptors in the brain and intestine, which communicate via cholinergic fibres in the intestinal nerve. Electric shock causes the snail to close its pneumostoma (breathing orifice) in defence (Gainutdinov et al 1997). The speed and force by which this response occurs is significantly reduced with **atropine** (Palikhova et al 2006). Not all invertebrates have a cholinergically mediated defence response. Lacoste et al (2001) found that, while the Pacific oyster, *Crassostrea gigas*, releases dopamine and noradrenaline into circulation under stress, neither nicotinic nor muscarinic antagonists inhibited this release.

1.9.iii. Motility

Cholinergics have a toxic effect on invertebrate motility (Jemec et al 2007). In the innkeeper worm, *Urechis campo*, acetylcholine increases muscle contraction (Julian et al 2005). In the great pond snail, *Lymnaea stagnalis*, dorsal longitudinal muscle contraction effects of 0.01μM acetylcholine were decreased by 23, 38 and 48% for 0.01, 0.1 and 1μM of **tubocurarine**, and 8, 13 and 37% for the same concentrations of **atropine** (Kononenko and Zhukov 2005). In the neon red crayfish, *Procambarus clarkii*, 100μM **tubocurarine** irreversibly inhibits acetylcholine mediated walking (Bon-Jego et al 2005). In the common flatworm, *Dugesia dorotocephala*, **scopolamine** caused spasms, and movement inhibition (EC50 44μM). Effects of 1000μM **scopolamine** were partially reversed by 10μM **galantamine**. Worms recovered from 80% inhibition to 50%. Increased **galantamine** did not cause further improvement. However, locomotor activity was also reduced 90% in the presence of 100μM **galantamine** alone (Ramakrishnan et al 2014). The spasming effect of **scopalamine** did not increase with increased concentration over 1000μM (Rawis et al 2010). In the migratory locust, *Locusta migratoria*, muscarinic receptors are found on flight initiating neurons. 2% pilocarpine eye drops paralyse the lungworm, *Angiostrongylus*

cantonensis, burrowed in a patient's eye (Mehta et al 2006). Pilocarpine is a cholinergic parasympathomimetic agent with mainly nonselective muscarinic action. Buhl et al (2008) mimicked locust flight initiation with 5000μM **pilocarpine** and reversibly blocked this effect with 10,000μM **atropine** or 10,000μM **scopolamine**. Walking activity in the Indian stick insect, **Carausius morosus**, is also reversibly inhibited by **atropine** (Westmark et al 2009).

1.9.iv. Feeding

Acetylcholine regulates smooth muscle of the GI tract. In the free-living nematode, *Caenorhabditis elegans*, it effects fast pharyngeal pumping via nicotinic receptors (Raizen et al 1995) and muscarinic receptors (Steger and Avery 2004). Receptors are activated during starvation, causing worms to pump food faster. **Arecoline** mimics starvation pumping and is blocked by **atropine** (You et al 2006). In the parasitic hookworm *Ancylostoma caninum*, 5000μM **atropine** abolishes feeding (Hawdon and Datu 2003).

1.9.v. Development

Muscarinic antagonists negatively affect development. In the blowfly, *Chrysomya megacephala*, larval development is negatively impacted by **hyoscine**. Drug-free controls reached optimal pupating weight at 72 hours, while those injected with 720μM never reached optimal weight before pupation at 96 hours (Oliveira et al 2009). In red sea urchin, *Loxechinus albus*, embryos cell movement and communication, crucial to embryonic development, were completely blocked by 200μM **atropine** (Harrison et al 2002).

1.9.vi. The Heart

Acetylcholine slows the *Daphnia* heart; 0.01μM decreases beat frequency by 25% and 0.5μM by 60% (Bekker and Krijgsman 1957). 43μM **melatonin** reversibly slows the *Daphnia* heart. Heartrate decreases linearly with time exposed, reaching a mean 75% of starting rate after two hours (Kaas et al 2009). The anticholinesterase insecticides **tetraethyl pyrophosphate** and **rotenone** cause tachycardia in *Daphnia*, with threshold concentrations of 0.37μM and 250μM, respectively, below which heartrate drops dramatically, and death occurs (Bekker and Krijgsman 1951). Curare has a strong stimulating action on *Daphnia* heart, by reversibly inhibiting the nicotinic receptor. This is in stark contrast to its relatively slight action on the human heart (Carlson 1922). In the heart of the Venus clam, *Katelysia rhyctiphora*, 0.01μM acetylcholine causes contractions to cease completely (Sathanthan and Burnstock 1976). In the heart of bivalve mollusc, *Tapes watlingi*, acetylcholine is a potent

inhibitor of activity (Phillis 1965). In the pupal stage of the fruit fly, *Drosophila* 1000μM acetylcholine decreases heartrate by 60% (Zornik et al 1999). And, in adult *Drosophila*, the decelerative effect of 100μM **carbamylcholine** was blocked by 10μM **atropine** (Yagodin et al 1998). In the common octopus, *Octopus vulagaris*, 6590μM **scopolamine** induced a sharp drop in heartrate and aortic pressure immediately after injection, though recovery was equally sharp (Fiorito et al 1998).

1.9.vii. Insecticides and Nerve Toxins

Invertebrate cholinergic receptors are a popular target for insecticide development (Zhang et al 2000). Nicotinic receptors act on ligand gated ion channels, while muscarinic receptors act on G-proteins. In the insect CNS nicotinic receptors predominate. Neonicotinoids, which target nicotinic receptors, predominated as the early choice for insecticides. Neonicotinoids and their adverse effect on pollinators are discussed in **7.0**. Today the muscarinic agonist insecticides predominate. These include anticholinesterase organophosphates and carbamates such as **pirimicarb**, **methonyl** and **thiodicarb**, which are used for control of pests such as the English grain aphid, *Sitobion avenae* and the bird cherry-oat aphid, *Rhopalosiphum padi* (Lu et al 2013). Much information on the muscarinic agonists is related only to death rather than more subtle physiological changes: Dick et al (1996) found 48-hour LD50s for **ethofenprox** versus rice pests such as the brown plant-hopper, *Nilaparvata lugens* (345nM), and the green leaf-hopper, *Nephotettix cincticeps* (159nM). Dick et al (1996) also found 48-hour LD50s for **propargite** versus the red spider mite, *Tetranychus urticase* (54μM), and the greenfly *Aphis gossypii* (6μM). Iannota et al (2011) estimated **rotenone** and **dimethoate** to be almost equally effective at killing the olive fly, *Bactrocera oleae*, with 76mM and 65mM respectively killing 53% and 45% of flies in 24 hours. LD50s pertaining to *Daphnia* are given in table 2a.

In *Daphnia* 131μM **dimethoate** or 41nM **pirimicarb** caused reversible immobilisation, inhibited reproduction, and reduced average weight and body length of offspring and increased mortality (Andersen et al 2006). Other cholinesterase inhibitors include the organophosphate **parathion**, a weak anticholinesterase, and its metabolite **paraoxon**, which has a potent anticholinesterase effect (Vatanparast et al 2006). In acute *Daphnia magna* toxicity tests, both caused complete immobilisation well before death (Guilhermino et al 1996). And, the pyrethroid **fenvalerate** caused a delay in *Daphnia* offspring production, a decrease in offspring numbers and feeding depression. These had positive linear

correlation with concentration, offspring numbers decreased by 1/7th and feeding rate by 1/3rd at 2381pM (Reynaldi et al 2004). Also, **atrazine**, based on the gram-positive bacteria, *Bacillus thuringiensis*, was found to stimulate haemoglobin accumulation in *Daphnia*. This caused gradual suffocation as fewer haemoglobin particles are available to bind and deliver oxygen. At low levels the onset of the sexual phase and production of males occurred, a common response in *Daphnia* under stress (Rider and LeBlanc 2006).

In freshwater systems, Acetylcholinesterase activity is an established biomarker in toxicity testing for insecticides (Guilhermino et al 2000). **Atropine** interventions are the current treatment for organophosphate toxicity in freshwater systems caused by leaching from treated lands. Pre-treatment with atropine significantly reduced paraoxon toxicity in *Daphnia magna*. Treatment with 38.7μM and 84.0μM atropine improved *D. magna* 24 and 48-hour paraoxon LD50s from 2.36μM and 1.89μM to 8.25μM and 16.90μM respectively (Carvalho et al 2003). In the brine shrimp, *Artemia salina*, 24hr LD50 tests were performed for the insecticides DFP, Fonofos and Phosphamidon (Barahona and Sanchez-Fortun 2007). Pre-treatment with 1000μM 2-pyridine aldoxime methyl chloride (**2-PAM**) achieved almost total inhibition of anticholinesterase activity produced by 98%, 94% and 93% respectively. While pre-treatment with 1000μM atropine achieved only partial inhibition of each by 0%, 21% and 55% respectively. An Atropine-2PAM mixture achieved 100% inhibition of all toxins (Sanchez-Fortun and Barahona 2009). **Atropine** and the acetylcholinesterase reactivator **trimedoxime** are also human antidotes against nerve agent intoxication. Vesela et al (2008) trialled these alone and in combination to treat *Daphnia magna* exposed to the nerve agent tabun, which targets acetylcholinesterase causing irreversible phosphorylation, resulting in cholinergic hyper stimulation. The combination of the two was best at restoring acetylcholinesterase activity, followed by **atropine** alone. **Trimedoxime** alone made no significant improvement.

1.10 Conclusions

In this chapter we searched the literature for all cardiovascular drugs, as well as some cardio active insecticides based on these drugs, when they were observed or tested for a cardiac response in an invertebrate, as well as systemic responses. For all invertebrate cardiac responses found, table 1b was created to compare the responses to mechanism of action in humans. Human molecular targets are also listed. Interestingly, where results pertain to *Daphnia* or the myogenic heart of *Drosophila*, response is largely in line with human

response. For lobsters, known to be neurogenic, the response is opposite, and for other invertebrates the correlation varies, leaning towards the opposite tendencies.

A second objective of this chapter was to bring an ecological perspective to pharmacology; no drug works upon one organ of a whole live animal in isolation, but instead affects a system. Looking at sometimes unexpected secondary effects is a positive way to remove confounding factors in lab studies or understand complex results. This was made very clear in 2.10 where cholinergic modulators, as insecticides and nerve toxins, affect learning and memory, innate defence response, motility, feeding and development as well as the heart. However, in drugs we know as therapeutics, this chapter also found secondary effects that may affect lab data. Some might be expected. The anaesthetic effect of the weak sodium channel blockade class also applies to paralysing invertebrates and is used in parasite removal. And, some might not be expected. β-blockers and ACE inhibitors are detrimental to the reproduction, growth and migratory behaviour of a number of invertebrates. The calcium channel blockers cause mutations in new-born invertebrates, withhold invertebrate maturation, and block sensory abilities. Lipid lowering drugs also caused foetal mutations and delayed maturation. Antiplatelet agents were harmful to egg production. And, some are helpful to invertebrates in unexpected ways. Potassium channel blockers strengthen the tapeworm's ability to reject anti-parasitic drugs and cling to its host, and also strengthen reproductive capacity in snails. Cardio tonic agents strengthened morphological defences in *Daphnia*, however, swim and feeding speeds were reduced.

Our intention was, had we found that *Daphnia* could be used as a model for concentration-response trials, a plethora of drug trials would ensue, and result in a separate chapter comparing *Daphnia* cardiac response to human response. In 5.0, we find that *Daphnia* are not a good model for drug trials. In 6.0 a new research direction is adopted involving the mathematical description of cardiac rhythm which became the keystone of this thesis and not the application of cardiac drugs. This chapter may instead apply to help with further work, for invertebrates more appropriate for drug trials. This might be the fruit fly, *Drosophila*, which is confirmed to have a myogenic heart (Vogler and Ocorr 2013).

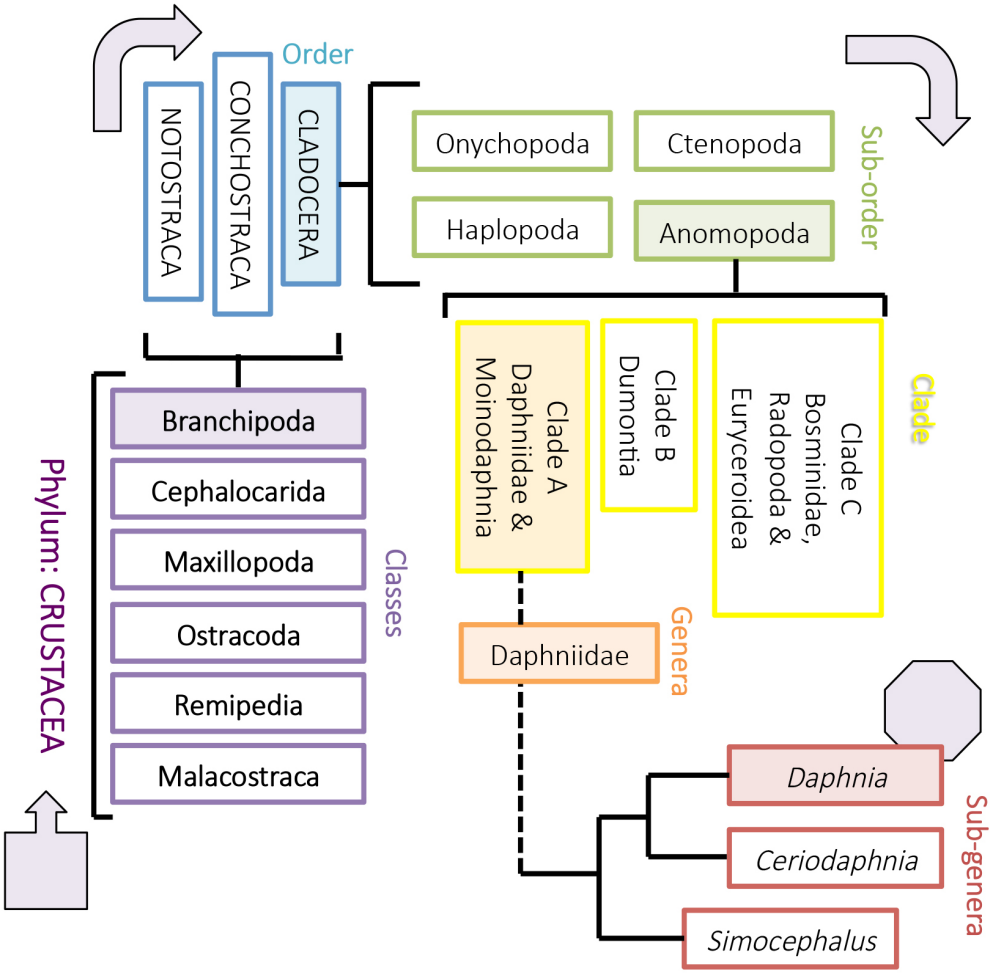
Table 1b. Human mechanisms of action and/or targets for each drug found to be cardio active in invertebrates

Drug	Mechanism of Action in humans from drug bank and guide-to-pharmacology (online, see references)	Invertebrate response similar?
Lidocaine Lignocaine	Increases intracellular Na^+ by blocking the fast voltage gated sodium channels. At high concentrations the membrane of the postsynaptic neuron will not depolarize and will fail to transmit an action potential. This aborts signals, causing heartrate decrease.	Yes, heartrate is reduced in the snail and the cockroach.
Metoprolol Propranolol	These target β-adrenergic receptors in the heart, competing with adrenergic neurotransmitters such as catecholamines at binding sites, inhibiting sympathetic stimulation. The β1 blockade results in decreased heartrate cardiac output and blood pressure to prevent angina.	Yes, heartrate is reduced in *Daphnia* for both drugs.
Phentolamine	Competitively blocks α-adrenergic receptors, leading to smooth muscle relaxation and blood vessel dilation, lowering blood pressure. Blocking is transient and blocking incomplete. The drug is more effective in antagonizing responses to circulating epinephrine or norepinephrine. It also stimulates β-adrenergic receptors and produces a positive inotropic and chronotropic effect on the heart and increases cardiac output.	No, heartrate is reduced in the lobster. However, lobsters are known to have neurogenic hearts. There is no data on *Daphnia*.
Verapamil	Inhibits voltage-dependent L-type calcium channels in the heart causing reduced heartrate and blood pressure. This response is biphasic. It is used to treated cluster headache.	Yes, *Daphnia* response biphasic, heartrate increases at low- and decreases at high- concentrations.
Serotonin	Targets the sodium-dependent serotonin transporter. Produces a complex response; decreased heartrate, vasodilation then longer-lasting lowered blood pressure due to arterial dilation.	No, heartrate is increased in bot flies and whelks. No data on *Daphnia*.
Digitalis Ouabain	Inhibits the sodium/potassium-transporting ATPase membrane pump, resulting in an increase in intracellular sodium. The sodium-calcium exchanger in turn tries to extrude the sodium and in so doing, pumps in more calcium. Increased intracellular concentrations of calcium then promote activation of contractile proteins (actin, myosin), and heartrate is increased.	No, for digitalis, heartrate is reduced but for ouabain, *Daphnia* have significant inotropic effect at systole heartrate is unaffected.
Atropine Scopalamine	Atropine binds to and inhibit muscarinic acetylcholine receptors. Heartrate is increased and atrioventricular conduction improved by blocking parasympathetic influences on the heart.	Yes and no; heartrate is Increased in the fruit fly hearts of *Drosophila*, which are known to be myogenic. It is decreased in the octopus.

2. *Daphnia* as an Experimental Model

The goal of this chapter is to examine the literature for optimal conditions in *Daphnia* culture. There is a wealth of observational information, which broadly agree on the optimal conditions for *Daphnia* growth discussed here. Literature searches were conducted in relation to environmental conditions such as oxygenation, micronutrients, temperature and light; behavioural modifiers such as competition and predator presence; and physical parameters such as genotype, body size, gender, reproduction and infection. And, applied within the databases listed in references. Where *Daphnia* data were not found we widened the search to include closely related species. We conclude with considerations of how me might negotiate these parameters in lab trials. We begin by explaining what a *Daphnia* is, what a model system is, and why we have chosen to investigate this popular ecological model.

Figure 2a. *Daphnia* phylogenetic tree



Legend: Begin at the bottom left, follow the arrows until the octogan at bottom right; purple= class, blue= order, green= sub-order, yellow= clade genera, red= sub-genera.

i. What are *Daphnia*?

Daphnia are a sub-genera of cladoceran crustaceans, comprising over 200 species with worldwide distribution. *Daphnia* eggs are sticky, enabling them to colonise new locations by sticking to the feet of migrating birds. They have differed little morphologically since their split with *Ctenodaphnia* in late Cenozoic (Popova and Kotov 2013). A phylogenetic tree is shown in figure 2a based on data in Wägele et al (2007) and Lourdes (2004). They are microscopic planktonic invertebrates found in still or slow moving freshwater. *D. magna* is widespread in the northern hemisphere and is extensively used as a model species due to its larger size (up to 5mm), though *D. pulex* is also used in lab studies due to prevalence. They live for around 108 days (Hall and Ebert 2012). A *Daphnia* is shown in figure 2b. An anatomical diagram is shown in figure 2e.

Figure 2b. Picture of a *Daphnia magna* giving birth (open source image, Shutterstock)

ii. What is a Model System?

Model systems study non-human species in order to gain an understanding of biological phenomena that may be more widely applicable across other species. Model animals are tested *in vivo* and can be of great advantage in the research of human disease while experimenting on humans is more procedurally difficult to set-up. The notion of making assertions from one species' lab responses to human outcomes is justified by our common genetic heritage, and conservation of gene groupings, developmental, and metabolic

pathways across species. Molecular mechanisms of heart development, and related genes, are conserved from humans to invertebrates (Harvey 1996, Bodmer and Venkatesh 1998, Holland et al 2003, Plageman and Yutzey 2005). A medical model should ideally have some measure that interacts with human physiology or be amenable to interventions humans may need. In 1900, Ernest Warren introduced *Daphnia magna* as a model for studying environmental stress (Warren 1900). Today, *Daphnia* are a well-established model for assessing the impact of toxins on the freshwater systems (Sun and Gy 2005, Martins et al 2007 and Han et al 2010). They are used to estimate the effects of concentrations of pharmacologically active substances released in to the environment by effluent from pharmaceutical companies and hospitals (Villegas-Navarro et al 1997).

This first goal of this study was to answer whether *Daphnia* might be adopted as a pre-clinical model for pharmacological research into the application of cardiovascular therapeutics. Pre-clinical models are studies to test drugs, procedures, or other medical treatment in animals. The rodent is the current preferred model for biomedical research (Ellenbroek and Youn 2016). By studying mouse anatomy, physiology, and metabolism, valuable insight is gained into human function. Rodent models were developed via selective breeding for desired characteristics, in order to simulate human disorders and study their development and possible therapeutic solutions. This has enabled drug development in many key areas including cardiovascular science. Characteristics of the rodent which make it a good model are in list 2c.

List 2c. Characteristics of the rodent model from Iannaccone and Jacob (2009)

1) Their cost effectiveness, as mouse/rat care is cheap and undemanding.
2) Their ability to multiply quickly. Mice can produce litters every three weeks.
3) Their small size, enabling labs to house them in great numbers.
4) Their short generation time, usually around 10 weeks.
5) Their short lifespan, which allows study of aging effects.
6) Their anatomical, physiological and genetic similarity to humans.

Where an invertebrate model can mimic these features, cross applications might be drawn. *Daphnia* are already a well-established as a model species in ecotoxicology, providing a good foundation of published literature. They very clearly meet all but the last criteria, which we shall return to in subheading iii. *Daphnia* may be an ethical alternative to

the mouse. They have a clear carapace, so that in *vivo* viewing of cardiac action is possible. So, unlike with mice, no surgery is necessary to view heart activity. Live animals can metabolise cardiac drugs, whereas isolated hearts do not, leaving the potential effects of metabolites on the larger anatomical system undetected (Valentin et al 2004). As invertebrates, they are not covered by the Animals (Scientific Procedures) Act 1986. Their useful characteristics as a model system are in list 2d.

List 2d. Characteristics of the *Daphnia* model (Stollewerk (2010) and Graham et al (2011))

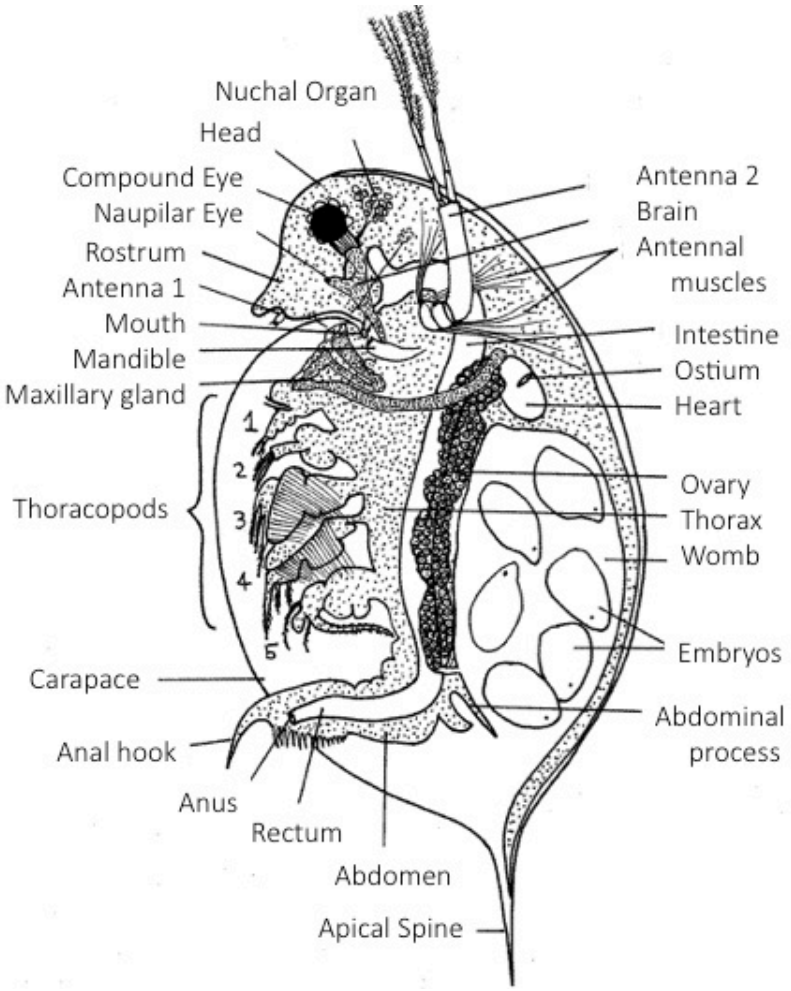
1) Easy to rear, and relatively cheap and easy to maintain.
2) Rapid progenitors with independent new-borns (R-species) as opposed to a large commitment to raising a small number of young (K-species).
3) Short lived with quick life cycles. *Daphnia* have short generation time, producing their first broods at four days old. Time from egg to larval release is three days.
4) Genetically manipulatable and fully sequenced (Colbourne et al 2001).
5) The subject of interest in a wider academic arena; they are economically important in terms of their place in ecotoxicology.

iii. Why Have we Chosen This Particular Model Animal?

The list 2d might well apply to any number of R-species invertebrate. Cardiac viewing through a clear body surface can just as well be achieved with embryonic zebrafish. A physiological similarity to mammals is the key to why *Daphnia* were chosen. Mammals have a myogenic heart, and *Daphnia* are also thought to have a myogenic heart. This is highly unusual for an invertebrate; most crustacea are neurogenic. It is important to address here the reasoning behind the belief that the *Daphnia* have a myogenic heart. Workers have produced pharmacological results which *suggest* this. Some have looked at heart histology and *not found* ganglion. Neurogenic heart beats originate in nerve ganglia while myogenic heart beats originate within the heart muscle itself (Anderson 1973). Prosser (1942) reported a personal communication with Ingle (not referenced) that after a "variety of nerve staining methods" no ganglion was found in the *Daphnia* heart. No further investigation into heart histology has been found other than Stein et al (1965) who mentions no ganglion, but neither does he mention whether it was searched for. No worker has attempted to test whether *Daphnia* hearts beat in isolation as myogenic hearts do. The only evidence for *Daphnia* myogenicity comes from detailed studies over fifty years ago by Bekker and Krijgsman (1951) and Stein et al (1965). A full investigation into

Daphnia heart response since this time is lacking. All work done after 1965, until the final months of this thesis were based on those assumptions and stated them as fact without question. In August 2018, Pirtle et al (2018) published an exciting study which conclusively showed the *Daphnia* heart has distinct ion channels analogous to HCN and T-type channels responsible for automaticity in vertebrate myogenic heart pace-maker cells. This does not change or de-contextualise results in this thesis but instead provides solid confirmation for what had previously been assumed conjecture over the previous half-century. We reserve judgement on Pirtle et al's conclusion that *Daphnia* are therefore a model system for pharmacology until our data is presented and discussed in later chapters.

Figure 2e. Anatomy of a *Daphnia* (adapted from Ebert, 2005) (cited in text on page 35)



Bekker and Krijgsman (1951) suggested the *Daphnia* heart is myogenic as they found that it is not accelerated by acetylcholine, tetraethylpyrophosphate, eserine, digitalin nor pilocarpine but slowed instead. Most arthropods, those which are known to have neurogenic hearts, are accelerated by these. Atropine accelerated *Daphnia* hearts, which is again opposite to the response in known in the neurogenic hearts of other invertebrates. They conclude that a myogenic pacemaker in the *Daphnia* heart is normally under an

inhibitory influence of acetylcholine released from extrinsic nerves. They also found that adrenaline slowed the heart at low concentrations below $2x10^{-7}$M, but increased heartrate thereafter just as is more common in known neurogenic invertebrates. They concluded adrenaline had no physiological function in the heart of *Daphnia*. Stein et al (1965) characterised *Daphnia* heart muscle. Most prominent were large mitochondria thought to provide the energy requirements for rapid heartrate and further suggested myogenicity. Myogenicity relies on energy produced by mitochondrial oxidative phosphorylation, which is positively mitigated my mitochondrial volume (Wagatsuma and Sakuma 2013).

2.1 Optimal Culture Conditions for *Daphnia*

The chapter objective was to research conditions for optimal *Daphnia* culture, to ensure reliable *in vivo* testing. Effects of cardiovascular therapeutics vary dramatically *in vivo* compared to *in vitro*. For example, McGaw and McMahon (1995) found that FMRFamide-related peptides F1 and F2 cause cardiac excitation *in vitro*, and significant inhibition *in vivo* in the crab *Cancer magister*. Isolated hearts are not subject to normal neural and humoral control pathways, as live hearts are. Animal physiology is a product of the system in which they live; environmental, behavioural/ physical parameters. While it is possible to avoid natural variables in the lab such as habitat degradation, suspended solids and mixed invertebrate assemblages (Damasio et al 2008), it is imperative to control and/or monitor all parameters closely. These factors not only influence the reproductive state and size of animals, but for our needs physiological reactions to aquatic toxicants depend on all of these variables (Pieters et al 2006). Reliable and replicable culture conditions might stem the rapid turnover of gene polymorphisms that are known to occur in *Daphnia* in response to changing environments (Schaack et al 2013). By researching conditions for optimal *Daphnia* culture, we hope to ensure trial stages are as reliable and uniform as possible, avoiding confounding responses to bad culture conditions. This section addresses environmental parameters for *Daphnia* culture.

2.1.i Temperature and Light

Temperature is the primary effector in *Daphnia* growth, more critical than food resources (Gerritsen 1982). Changes in temperature affect size, morphology, disease resistance and behaviour. *Daphnia* are sensitive to small temperature changes (0.2-0.5^{o}C) which have dramatic effect on their health; according to Barbosa et al (2014) the bulk of their energy is allocated to thermoregulation, however a quantitative estimate of this is not given.

Seasonal variation causes change in body size known as cyclomorphosis (Brooks 1949). Increased heat causes increased birth rate, smaller offspring, and shorter life spans (Meester et al 2011). Cooling causes a decline in birth rate and an increase in body size among individuals in the population (Shala 2013). Spring clones grow large, while summer clones are small but more abundant (Brzezinski et al 2010). Temperature preference changes with age. Adults are resilient to low temperatures, optimal growth rate occurs at 13.3 to 15.4°C for adults, and 20.2 to 26.2°C for juveniles (Verbitskii and Verbitskaya 2011). Populations peak in spring temperatures of 18.5°C (Straile et al 2012).

Daphnia preferentially graze at night as grazing rate is inversely proportional to light intensity (Jager et al 2007). They migrate upwards at night to take advantage of higher surface temperatures. They have poor vision merely distinguishing changes in light intensity (Schwind 1999). Migration is initiated by decreasing light intensity, continuing after dark due to gradually increasing surface temperature. Longer summer days mean less time spent eating, and lower body size. Increased light intensity in summer also enhances anti-fish morphological defences; armour such as neck spikes and thickened carapaces (Effertz and von Elert 2014). Given large size is preferable for heart observation, a spring-like light: dark cycle was chosen as optimal for lab conditions.

Mimicking the crepuscular cycle with a dusk-dawn simulator, Grover and Miller (1983) found that lab cultures produced a natural response, while an on/off switch caused them to crowd at the bottom of the test tank, or what might be termed the benthic zone in their natural environment. *Daphnia* stay low in evenly distributed lab lighting (mimicking day) and are attracted to point light sources (as in dusk) (Gerritsen 1982). They will swarm in a shaft of light, creating a horizontally circling vortex rotating collectively in a single direction. Circling is the lowest energy state in which moving animals can stay in a group without frequent collisions (Mach and Schweitzer 2007). It is both a cooperative behaviour to allow them to get up high when rising to the surface for food, a means of deterring predators, and an aid to the frequency of sexual encounters during sexual cyclical stages (La et al 2014). In addition to photo tactic orientation, gravitational orientation is important to nightly migrations. Iriji et al (1998) observed *Daphnia* on the MIR space station: In zero gravity they are unable to orient themselves (unlike fish which can) and swim in looping spirals. In the daytime, *Daphnia* sink to the bottom of the water column to avoid fish predation (Rivetti et al 2015), and UV radiation (Hansson and Hylander 2008).

Daphnia also transmit information about their photoperiod to offspring (Jiang et al 2013); mismatches between maternal and offspring light cycles can cause a switch from asexual reproduction to resting-egg production (LaMontague et al 2001). Lari et al (2017) found that *Daphnia magna* heartrate increases linearly with temperature over a range of 5 to 20°C. Thus, a need for consistency in the day/night cycle and temperature exists when culturing and testing *Daphnia* in the lab.

2.1.ii Oxygenation

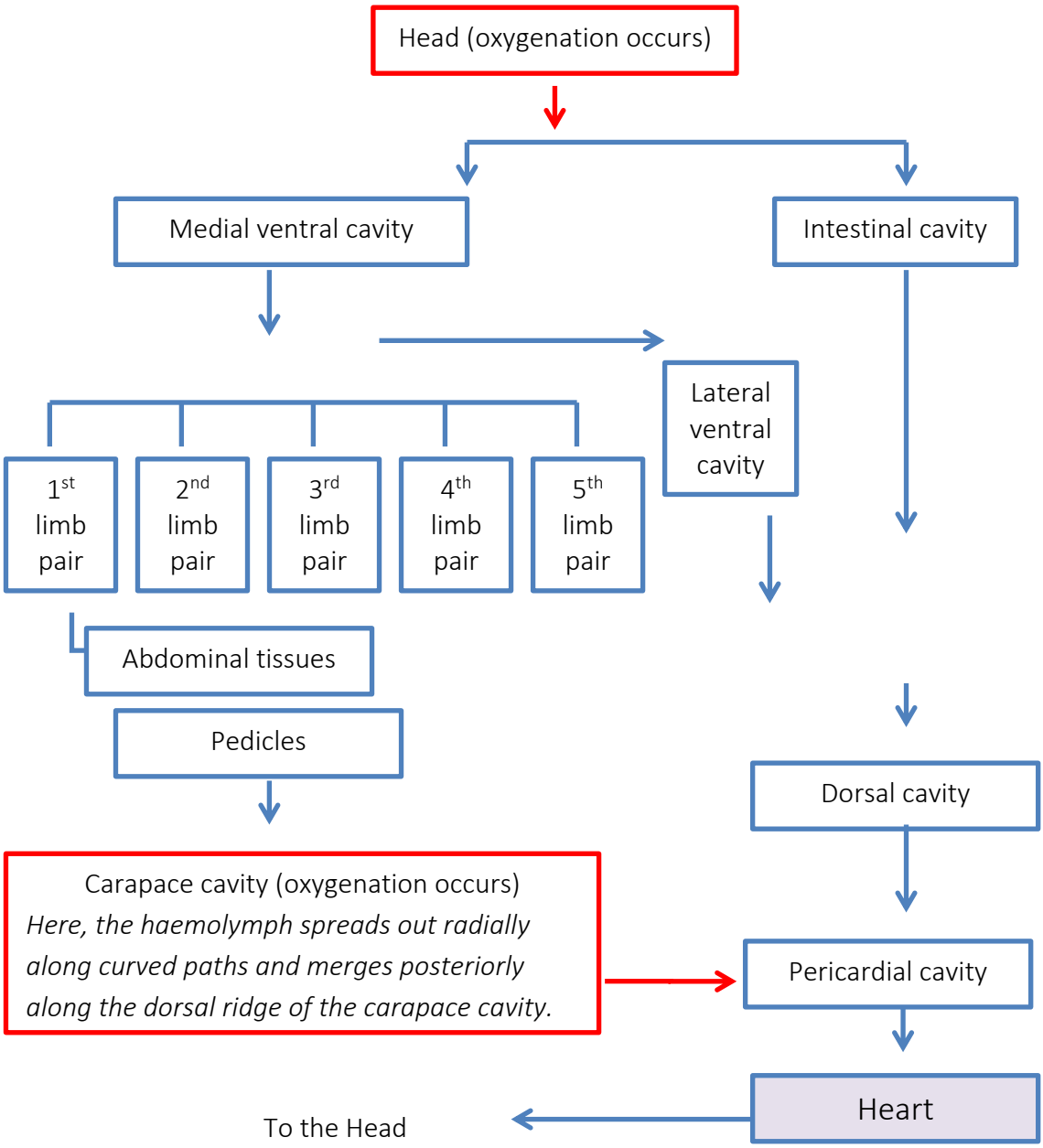
Here we define anoxia as conditions below 0.5 torr (around 0.322% oxygen); hypoxia under 60 torr (around 40% oxygen saturation) and normoxia as 75-100 torr. This is only a general guide based on human measures (Wilkinson 2015) but fits conveniently well with definitions of these three states across environmental science literature described below. A concern in planning lab research was the effect of immobilisation on the *Daphnia*'s ability to oxygenate which would directly affect heart activity. Immobilised *Daphnia* are able to oxygenate freely, without need to move limbs for ventilation: Limb movements stay at a constant rate of 150-200 min^{-1} from normoxia to hypoxia, only slowing in anoxia <0.5 torr (Colmorgen and Paul 1995). In their natural environment, *Daphnia* are exposed to extremes of hypoxia as low as 10-20 torr. *Daphnia* are oxy-regulators; gas exchange occurs via diffusion over the body surface. However, specific sites of medium-to-haemoglobin oxygen exchange occur at the posterior carapace lacuna and rostral region of the head (Pirow et al 2004), these may therefore be potential sites for drug delivery. The heart circulates haemoglobin for internal gas transport at a constant stroke volume, producing compensatory tachycardia at low PO_2 (Paul et al 1997). After one or two hours of anoxia, heartrate switches to a low, stable level. Haemoglobin concentrations are increased. Energy is provided by anaerobic metabolism involving L-lactate accumulation and excretion, prolonging survival for up to 24 hours (Paul et al 1998).

Adamczuk et al (2013) found that dissolved oxygen levels were the critical factor in *Daphnia* distribution in the environment, when compared to relative food availabilities. However, temperature is *still* most important: Stratification of the water column in summer renders surface waters rich in oxygen but too hot. Middle layers are of preferable temperature but with hypoxic oxygen levels (38 torr). The bottom is cool and anoxic. *Daphnia* congregate toward the middle layers, compensating for low oxygen by producing high haemoglobin levels around 0.06mmHg/mg dry weight. The same ponds are more

uniformly oxygenated in winter (87 torr) due to mixing; winter levels sit at around 0.015 mg mmHg/mg dry weight (Wiggins and Frappell 2000 and 2002). Pirow and Buchen (2003) found oxygen levels more important than food concentration when examining changes in *Daphnia* heartrate, which increases with increased food concentration, as more energy is expended pumping thoracic limbs to capture food particles. They then tested heartrate at several partial oxygen pressures under no food and high food conditions and found that difference in heartrate caused by food concentration becomes significantly smaller closer to anoxia. Paul et al (1997) tested well-fed (10^5 cells ml^{-1}) *Daphnia* under three oxygen conditions at a much lower temperature (4°C) and found that decreased temperature lowered heartrate more significantly than oxygen levels. Baumer et al (2002) found that heartrate, stroke volume and had a positive linear relationship with both body size and partial oxygen pressure.

In *Daphnia*, haemoglobin accumulation is regulated by two distinct molecular pathways: an endocrine pathway stimulated by terpinoid hormones, and an oxygen-sensing pathway involving hypoxia inducible factor. Terpinoids are involved in both haemoglobin synthesis and male sex determination (Rider and LeBlanc 2006). Terpinoids include the crustacean juvenile hormone methyl farnesoate, which increases Na+ and K+-ATPase activities and is in turn increased by temperature, anoxia and increased salinity (Purna and Nagaraju 2007). There are two haemoglobin synthesis sites: fat cells (found mainly near the gut) and epipodite epithelial cells. There are at least six haemoglobin genes, four of which are on a single chromosome. The promoter regions contain numerous hypoxia response elements; binding sites for hypoxia-inducible transcription factors (Paul et al 2004). Extracellular fluid comprises 60% *Daphnia* body volume (Kobayashi, 1983). When relaxed the heart aspirates haemolymph from the pericardial cavity, which is expelled into the head via an arterial opening upon contraction. Paul et al (2004) characterised the journey of haemolymph in *Daphnia*'s circulatory system in prose; this journey is described in figure 2f.

Figure 2f. Journey of the haemolymph in *Daphnia*, adapted from Paul et al 2004.



2.1.iii. Food Resources

Choosing and maintaining the correct food is essential for *Daphnia* culture. Increased food levels lead to faster maturation and increased number of eggs per brood. In deprived conditions, young are born larger but fewer in number. This does not infer better health, however, as these neonates born larger will mature later even when well fed (Guinnee et al 2007). Porter et al (1982) found that mandible rates (a proxy for food consumption) increase with food concentration. They do not self-limit but simply eat faster when more food is presented (Furuhagen et al 2014). Feeding rate plateaus at algal concentrations of at $2.5x10^6$ cells cm^3 (Kim et al 2003). Birth rate and adult body size increase with food

availability. Saturation levels for these above which no further increases were seen are found at $5x10^5$ to $6x10^5$ algal cells cm^3, $4x10^5$ to $6x10^5$ algal cells cm^3 (Pavlaki et al 2014) respectively. However, overfeeding is also to be avoided in *Daphnia* cultures. *Daphnia* with high caloric intake are under increased risk of oxidative stress (Furuhagen et al 2014). However, this may also be due to eutrophication: It is unclear how 'overfeeding' can be differentiated from algal overloading of the environment.

Daphnia provide an 'ecosystem service' (Shiny et al 2004 and Pires et al 2005). They consume toxic cyanobacteria arising due to eutrophication (Clement and Zaid 2003) and release the digested toxins into the water for bacterial breakdown (Ekvali et al 2014). Cyanobacteria have defence strategies reliant upon the low nutrient (low phosphorus, high nitrogen) state of their environment (Hall et al 2006). In *Microcystis aeruginosa* low nutrient levels increase toxic micropeptin levels, which inhibit *Daphnia* gut proteases (Schwarzenberger et al 2013 and Rohrlack et al 1999). Low nutrient levels also increase cyanobacterial microviridin-I levels causing lethal moulting (Rohrlack et al 2004 and Rohrlack and Utkilen, 2007), and microcystin levels reducing growth and reproduction (Kurmayer 2011). Toxic inhibition of reproduction varies between species. *Anabaena* and *Oscillatoria* are most harmful at higher temperatures around 23°C, while *Microcystis*, *Nodularia* and *Aphanizomenon* are most harmful around 15°C (Hochmuth and De Schamphelaere 2014).

Volatile organic compounds are produced as a defensive strategy in phytoplankton, produced in external mucilage to deter grazers (Juttner 1999). Further herbaceous defence strategies involve repellent taste upon consumption. *Microcystis* cell rupture activates a rapid carotene oxygenase reaction, producing large amounts of B-cyclocitral, a toxin that causes limb paralysis at high levels (Ferrao-Filho et al 2013). This acts as a repellent and *Daphnia* swim away at speed; ecotoxicological evaluations have been made correlating substance toxicity with speed of their departure (Jeon et al 2008). Trace levels of B-cyclocitral are detected by surrounding *Daphnia* that evacuate the area in response; this protects the larger *Microcystis* colony (Juttner et al 2010). *Daphnia* cannot differentiate between microcystin producing and non-producing cells (Rohrlack et al 2001) so this is does not deter future consumption attempts but merely drive them away from present *Microcystis*. *Daphnia* acquire eventual tolerance to *Microcystis*, and other cyanobacteria such as *Cylindrospermopsis raciborskii*, by remodelling digestive

chemotrypsins (von Elert et al 2012 and Soares et al 2009). *D. magna* is more adaptable than *D. pulex* (Kuster and von Elert 2012). Maternal exposure to *Microcystis* confers offspring resistance; life span and birth rates in subsequent generations are increased compared to that of the parents but are still low compared to unexposed populations (Jiang et al 2013). *Daphnia* acquire this tolerance faster when presented with a variety of food choices. In an environment where only cyanobacteria are available toxic build up slows growth and development (DeMott et al 2009). Lurling et al (2002 and 2003) found that even chemically altered microcystis-free *M. aeruginosa* significantly inhibits growth; they postulated that this was due to their lack of growth enhancing lipids. Yoshida et al (2006) and Nelson et al (2005 and 2006) argue that varied food resources have a diversifying effect on *Daphnia*. However, other workers have found that poor food conditions lead to increased genetic diversity (Weider et al 2008). Hall et al (2011) found that clones raised on digestion resistant *Oocystis* grow faster and were more resistant to infection than clones raised on high quality *Scendesmus*. Consumption of *Oocystis* separated immunologically susceptible and resistant individuals allowing stronger genotypes to dominate.

Daphnia also provide an ecosystem service by removing dangerous human infective bacteria such as *Campylobacter jejuni* (Schallenberg et al 2005), and by consuming waterfowl faeces that would otherwise contribute to eutrophication (Van Geest et al 2007). They are able to consume and breakdown carbon nanotubes (large molecules of pure carbon) making them an efficient and green disposal for medical waste (Roberts et al 2007). The above are facultative survival strategies when all other algal or diatom options are exhausted (Mohamed, 2001). In non-toxic environments *Daphnia* remove small algae, allowing larger oxygen producing macrophytes to dominate and improving pond health in terms of C:P ratio (Jager et al 2007). *Daphnia* strongly prefer nanoplankton such as the diatom *Stephanidiscus parvus*, and cysts of microplankton such as unicellular phytoflagellate *Ceratium hirundinella*. They are less partial to gelatinous algae, which clog their thoracic limbs (Sommer et al 2003). Eskinazi-santana et al (2002) found *Daphnia* were generalists; their gut content is a close matching synopsis of phytoplankton species in their environment. However, Reichwaldt et al (2004) found significant variation in the eating habits of *D. magna* and *D. hyalina* in the same locations, suggesting each species had its own food preferences. Schaltz et al (2007) showed that *Daphnia* of all ages could determine the highest nutrient quality algae when presented with a range of choices.

Spaak et al (2012) found that *D. longispina* prefers eutrophic food but survives facultatively on oligotrophic food, while *D. galeata* fundamentally requires the former.

A vast range of food choices exists for *Daphnia* cultures. Schwarzenberger et al (2013) found that *Daphnia* raised on the green alga *Chlamydomonas klinobasis* have a high growth rate (0.42% a day). Choi et al (2014) compared *Daphnia* cultures fed with *Chlorella vulgaris* or *Stephanodiscus hantzschii*; *Daphnia* grew larger and produced bigger offspring when fed *Chlorella*. Asselman et al (2015) found unicellular alga *Cryptomonas* to be excellent food with respect to *Daphnia* growth and offspring production, while *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* were average. Oliveira et al (2015) found *Daphnia* grew well when fed *Raphidocelis subcapitata*. Martin-Creuzburg et al (2009) found that eukaryotic algae such as *Nannohloropsis limnetica are* rich in both sterols and eicosapentaenoic acid, which are required for maximal growth. Well-fed *Daphnia* are able to withstand sudden drops in temperature due to fatty acid reserves (Schlechtriem et al 2006).

Feeding consistency is important for *Daphnia* cultures. Varying food availability negatively influences *Daphnia* response to toxins (Knillman et al 2012). *Daphnia* that are deprived of food produce slow feeding offspring with large mouths and reduced feeding leg beat rates. Larger mouths maximise feeding efficiency and thus maximise progeny success in hard times (Garbutt and Little 2014). A parallel issue is competition for food caused by crowding (Knillman et al 2012). This leads to physiological stress and increased susceptibility to toxins. Comparisons of population density and *Daphnia* stress responses by Dolciotti et al (2014) showed that populations fared best when under 40 individuals per litre. *Daphnia* are not social and distribute sparsely in their habitat (Schwind 1999), preferring open areas with simple microalgae patches and tending to avoid macrophytes (Adamczuk et al 2013). At the extreme, starvation results in depletion of lipid and glycogen reserves; mitochondrial swelling, reduction of rough endoplasmic reticulum and dictyosomes, and eventual cell size reduction in the most active parts of the digestive system, the anterior mid-gut and caeca (Elendt and Storch 1990).

The algae, *Scendesmus,* have been used as a vector for drug delivery. Bengtsson et al (2004) first exposed *Scendesmus* to the hydrophilic glycophosphate for 4 days, then placed five *Daphnia* in a feeding chamber with a 150μm mesh plankton net through which

Scendesmus could travel in but *Daphnia* could not travel out for 4 more days. ^{14}C activity of the *Daphnia* and the water were measured at the beginning and end of exposure. Exposure to glycophosphate caused a 40% reduction in grazing rate. McGaw (2004) found that invertebrate cardiac beat frequency increases following feeding; in the crab, *Cancer magister*, haemolymph flow rates are increased to facilitate digestive processes.

2.1.iv. Predator Presence

In the presence of predators, sexually active *Daphnia* males attempt to increase the population by further reducing fight time and increasing frequency of sexual contact (La et al 2014). Morphological changes in offspring also ensue in the presence of predators and vary according to the differently sized predators (Beckerman et al 2010). In response to large predators, such as the Grayling fish *Thymallus thymallus* (Fjeld et al 1998), a species of salmon, *Daphnia* keep to a small size and produce many small offspring to increase likelihood of survival (Kramer and Drake 2010). *Daphnia* tend to stay in benthic regions, the lower-most stratum of an aquatic environment (Gliwicz and Maszczyk 2006). This is a necessary strategy as the dark pigmentation in their brood sac makes them more visible to fish than other cladocerans (Adamczuk et al 2013). They reduce pigment levels via upregulation of the arthropod melanin gene dopa-decarboxylase (Scoville and Pfrender 2010). Smaller predators induce growth in individual *Daphnia*. Just such a change can be made with application of predator kairomones (Rietzler et al 2008).

Kairomones are semiochemicals given off by animals which can indicate to predators the location of food prey or indicate to prey animals the location of a predator. This generalised term refers to any combination of chemicals, depending on the animal or plant which emits it. For fish some kairomones derive from gut excreta and are enhanced by having consumed prey animals. Otte et al (2014) kept the three-eyed shrimp, *Triops*, in a net cage, unable to reach *Daphnia* but releasing kairomones. This caused *Daphnia* to grow large to become too big to eat, and produce fewer, but larger, offspring, which tend towards the upper limnetic zone. This response was repeated across several studies involving *Daphnia* and small predators such as *Triops cancriformis* (Hesse et al 2012), the lesser water boatman *Corixa punctata* (Weiss et al 2015) and larvae of the blue-tailed damselfly *Ischnura elegans* (de Block et al 2013). Genes involved in kairomone response include the housekeeping gene cyclophylin, which is upregulated in the presence of fish and down regulated in the presence of invertebrate predators. It increases reproductive

capacity to avoid population decimation by the larger predators (Schwarzenberger et al 2009). Juvenile growth hormones are upregulated in response to the latter. These hormones prevent *Daphnia* from reaching the adult stage and thus growing bigger (Oda et al 2011) and therefore more difficult for invertebrate predators to consume.

Daphnia's extensive chemoreception abilities stem from genes representing 58 orthologues of the insect gustatory receptor family (Penalva-Arana et al 2009). As well as kairomones, *Daphnia* also sense latent pheromones released when other *Daphnia* are eaten and mixed with bacterial enzymes in fish GI tracts (Stabell et al 2003). *Daphnia* are able to sense chemical cues to differentiate hungry and well-fed spiny water fleas, *Bythotrephes,* which are 15x larger and predate on *Daphnia*. *Daphnia* respond by swimming away (Bourdeau et al 2012). The kairomone response can begin before birth. Foetal chemosensitive receptors are able to detect predator presence once the third membrane is shed (LaForsch and Tollrian 2004). At this point foetal *Daphnia culcullata*, *Daphnia pulex* or *Daphnia atkinsoni* develop soft helmets, neck spines or a crown of thorns respectively (Petrusek et al 2008). Embryos are soft and condensed; they expand and harden shortly after birth. Defensive armour grows in successive instars, allowing them a protective advantage over embryos not exposed to kairomones. *Daphnia* with parents that have been exposed to *Triops* produced a more profound response than those with unexposed parents (Otte et al 2015).

Response to *Triops* also includes increased expression of cuticle proteins and chitin modifying enzymes, for defence, at the expense of vitellogenin protein (Otte et al 2014). This creates a 5x harder and 2x thicker cuticle as well as increased diameter of cuticle pillars (Rabus et al 2013). Invertebrate predator kairomones induce toughening of the carapace by 200% (*D. cucullata*) to 350% (*D. pulex*). Pillar height between inner and outer layers is increased to provide fortification with low material expenditure (LaForsch et al 2004). In lab settings this may impede injection of micropipettes. A further issue is that *Daphnia* show significant divergent epigenetic modification in response to small-sized predator cues (Spanier et al 2010, Asselman et al 2015 and Rozenberg et al 2015). Multiple-sized predator presence dulls divergence due to the simple inability to adjust appropriately to all predator types (Dennis et al 2010), and the benefit of enlarged size is lost (Miyakawa et al 2013). In the presence of a mixture of small-sized predators *Daphnia* response is variable. Herzog and LaForsch (2013) found that *Daphnia barbata* exposed to

water boatmen, *Notonecta glauca*, developed larger helmet structures than those exposed to *Triops*. *D. pulex* initiate neck spine growth in the presence of smaller invertebrate predators such as glass worms, the midge larvae of *Chaoborus americanus*, but delay neck spine initiation until body size is larger in the presence of larger invertebrate predators such as the much larger phantom midge larvae, *Chaoborus trivittatus* (Riessen and Trevett-Smith 2009).

Response also varies to differently sized fish. Walsh and Post (2011 and 2012) compared *Daphnia* response to large anadromous vs. smaller landlocked alewife fish *Alosa pseudoharengus*, a species of herring. *Daphnia* predated on by the larger fish matured earlier, produced more offspring and produced a larger ratio of males. These effects were transgenerational and occurring in offspring two generations removed from those predated upon (Walsh et al 2014). Populations undergoing predation over several generations showed increased levels of DNA microsatellites corresponding with an increased plasticity (in the form of negative photo taxis) of response to predators over the generations (Cousyn et al 2001). Ability to grow protective features fails when temperatures vary as they reallocate their energy to thermoregulation (Barbosa et al 2014). Fey and Herren (2014) found *D. lumholtzi* and *D. pulex* varied in tolerance to changing temperatures: *D. lumholtzi* was able to respond defensively to sunfish introduction, while *D. pulex* could not, and so became the more vulnerable species. In a similar study, Adamczuk et al (2013) found that *D. cucullata* proved more successful than *D. longispina* in the presence of the common sunfish, *Lepomis*. In surprising contradiction to the above, Pohnert et al (2007) found that *Daphnia* respond more strongly to the presence of partially eaten or decomposed conspecifics than to predator kairomones.

Daphnia move into vegetation to avoid small predators but prefer open water in the presence of both small and larger predators due to the greater predation risk from fish (Meutter et al 2004). Kairomones induce aggregative behaviour creating swarms of 1000 to 9000 individual per litre (Oien, 2004). This in turn may cause unwanted stress from overcrowding (Garcia et al 2007). *Daphnia* swarms also avoid predators by creating confusion. Feeding leg movement creates a sinusoidal 5Hz frequency, punctuated with occasional 14 to 15Hz blips from antennal swimming movements. However, this also creates a self-selecting system whereby weaker individuals are spun out to the peripheries creating a sub-threshold signal amplified by the noise from the swarm. These signals

benefit the majority who avoid the fish predator and may serve in communication between *Daphnia* to determine inter-neighbour distances within a swarm and to recognize each other versus the predator (Freund et al 2002).

2.1.v Environmental Nutrients

Hooper et al (2008) found optimal pH and calcium values for *Daphnia* population growth under lab conditions and confirmed these by observation of 422 pond sites. Population growth was highest between pH6 and pH8, though *Daphnia* survived at a range of pH 5.75 to pH 9. In higher acidity environments *Daphnia* require more calcium for survival. Survival at pH 5.75 required Ca^{2+} levels above 2495μM. *Daphnia* were most abundant at sites where calcium levels were higher, and pH was around 7.4 to 7.8. Low calcium levels less than 38μM inhibit *Daphnia* growth and weaken the carapace (Riessen et al 2012). The lowermost threshold of Ca^{2+} concentration for growth and survival are 25μM and 12.5μM respectively (Muyssen et al 2009).

Phosphate depletion causes algae to grow detrimentally thicker, more digestion resistant cell walls (Van Donk and Hessen, 1993). A positive nutrient molar ratio for healthy ponds is given by Goldman et al (1979) where C: N: P is 106: 1: 1. *Daphnia* neonates do not adapt well to sudden changes in water type. *Daphnia* acclimatise poorly to salt; losing their spines in an effort to maintain homeostasis; survival is low in brackish water where salt concentration is greater or equal to 0.5% (Warren 1907). Various workers give culture medium recipes, as seen in table 2g. The formula used in our own work, method of production, and suppliers are given at the end of this chapter.

Table 2g. Examples of Artificial Pond Water solutions

CaCl2	KCl	NaCl	NaHCO3	MgS04	Na2SeO3	Reference
1081μM	107μM	-	2286μM	1529μM	-	Shakya (2011)
1300μM	78μM	-	800μM	300μM	0.035μM	Barrozo et al (2015)
600μM	-	6000μM	100μM	-	-	Ramakrishman (2014)
270μM	-	-	55μM	-	0.333μM	Kluttgen et al (1994)

2.2 Physical Parameters

2.2.i. Parthenogenesis vs Sexual Diversity

In the lab setting, *Daphnia* are undesirable when pregnant, due to the strains of sustaining embryonic haemoglobin and fat levels on the animal which may confound results (Paul et al 2004). Many workers choose to experiment on virgin neonates only in the first 4 days of life. It is also important to keep culture conditions optimal because poor conditions will change the way *Daphnia* reproduce. They produce genetically identical clones, ideal for lab studies to reduce variables when parthenogenic. Parthenogenesis forms the bulk of the *Daphnia* reproductive strategy for most of the year. No males are born, and females born from resting eggs which have over-wintered until the spring, simply produce genetically identical clones of themselves until the sexual stage is initiated in response to harsh conditions (Kuwamura et al 2009). The sexual stage causes the production of resting eggs instead of live born clones, which can overwinter until the next spring. Sexual reproduction continues until the last *Daphnia* dies in winter (La et al 2014). Related to this effect of cold on sexual production and diversification, haplotypes of *Daphnia dentifera*, found in cool mountain lakes vary on a lake to lake specific level. Meanwhile, *Daphnia galeata* is found in warm lowland lakes and has identical haplotypes across dispersed geographic regions (Ma et al 2014). Change to the diversifying sexual phase is marked by increased expression of the Doublesex gene (Zhang et al 2014). While sex among *Daphnia* leads to increased genetic variance, it lowers their 'fitness' as defined by their ability to reproduce in the large numbers achievable via parthenogenic reproduction (Allen and Lynch, 2008). Sexual *Daphnia* under predation pressure in summer undergo rapid genetically homogenising selection, while sexual onset in winter is genetically diversifying (Nelson et al 2005).

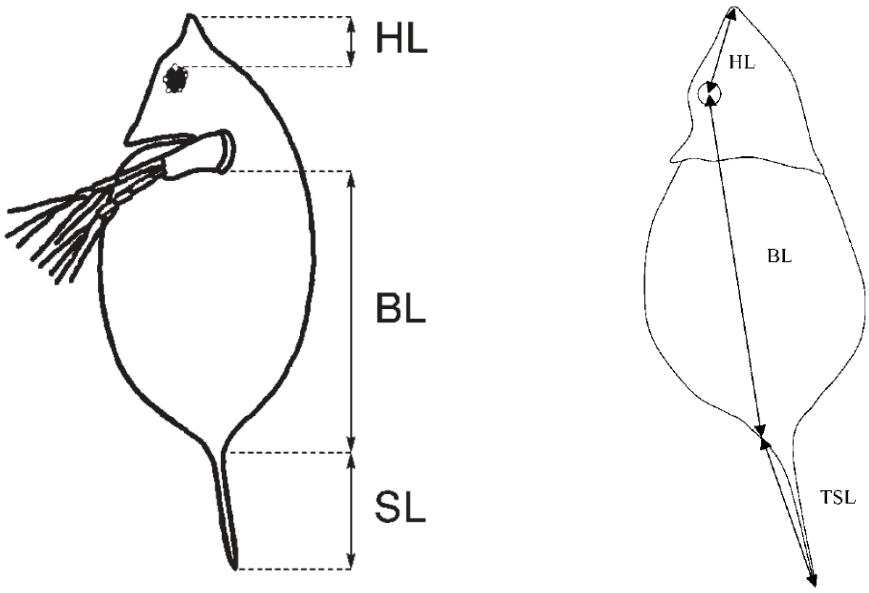
Production of males occurs 1-2 weeks before sexual females to ensure mature males are available for receptive females (Spaak et al 2004). Mating involves ritual fighting between males in the presence of sexual females followed by physical contact between males and females. Sexual contact time is prolonged (up to 25 seconds) while fights are brief (1-3 seconds) (La et al 2014). Males, half the size of the females, chase pheromone tracks then grasp them with hook like ventral antennules before inserting their abdominal claw into the female carapace valve for insemination. Females respond to this by frantically swimming away until released (La et al 2014). *Daphnia* males will mate with females of any genera and are able to form hybrids across genera that go on to reproduce asexually (Hebert and Finston 2000). Toxic response (Guan et al 2005), and population growth

(Weider et al 2003), vary between strains in response to nutrient conditions and food quality. This ability to hybridise can also result in hybrid *Daphnia* parthenogenically producing clones with three sets of chromosomes, this is known as triploidy (Vergilino et al 2009). Triploidy clones are more common at high latitudes, 46°N/ 53°N and high altitudes, such as in the Alps, but it is also possible to find such complex *Daphnia* genetics in the UK. For example, *D. pulex* is a complex of multiple strains that include diploids and triploid hybrid clones (Young, 1979). Likewise, it has been found that *D. obtusa* is composed of two morphologically indistinguishable but genetically separate species (Penton et al 2004). While this may not be ideal in a lab context where homogeny is a goal, hybridisation aids *Daphnia* in the arms race with parasites such as those described in **2.2.iii**. Wolinska et al (2006) observed hybridisation of *D. galeata* and *D. hylalina*, which have low and high resistance respectively, to *Caullerya mesnili,* a protozoan endoparasite of gut epithelium. Hybrid offspring were significantly more resistant than both parental species combined. Genetically diverse *Daphnia* populations are better able to grow in number when encountering new territories, while genetically uniform populations are less able to adapt (Tagg et al 2005).

2.2.ii Body Size

A further reason for selecting neonates for lab study, other than their virginity (the importance of which we discussed in **2.1.i**) is maintaining a homogenous body size. Heartrate increases with increased body length. Baumer et al (2002) found heartrate and body size were positively correlated, with larger *Daphnia* having higher heartrates in normoxic, nutrient free conditions at 20°c. Daphnia were grouped by size. Those around 1.5mm in length had heartrates around 250bpm, those around 3.5mm had heartrates around 300bpm. Neonates are under 1mm in length while adult size is more variant with culture conditions. Standard measurement for *Daphnia* varies somewhat between workers. Stabell et al (2003) measure body length from spine base to base of antennae, while Oda et al (2011) measure from spine base to eye. In many papers, *Daphnia* length is not defined at all and could be any of these or even the entire length from head to spine base. This is seen in figure 2h.

Figure 2h Daphnia measurement, left from Stabell et al (2003), right from Oda et al (2011).



Legend: HL= helmet length, BL= body length and (T)SL= (tail) spine length.

2.2.iii Parasite Status

Daphnia are prone to a range of parasites that affect their interaction with toxins, as well as impede functions such as reproduction, growth, and ability to escape predators. Only decrease in body size – a response to large fish predators – remains unhindered by parasite presence (Hesse et al 2012). *Daphnia*'s most prevalent parasites are the endospore forming bacterium *Pasteuria ramosa* and the single celled fungal parasite *Metschnikowia bicuspidata*. The bacterial parasite *P. ramosa* castrates its host, occupying the *Daphnia* womb for its own use; it causes gigantism allowing it to store as many spores as possible, which are released upon the host's death. It optimally allows *Daphnia* to survive 55-60 days; earlier death prevents the parasite from full exploitation of the host, while late deaths mean a decline in host fitness and therefore parasite resources (Jensen et al 2006). It is more virulent at higher temperatures, with growth reduced below 15°C. *Daphnia* growth is also accelerated at higher temperatures, providing more space and resources for the parasite (Vale et al 2011).

P. ramosa infects the host when spores are consumed and attach to the oesophagus (Duneau et al 2011). It then enters the body via heparin receptors, though some animals are susceptible to this mode of entry while others are resistant. Just as with vertebrates, main purpose of heparin in invertebrates is defence against invading bacteria and other foreign materials (Nader et al 1999). Cavalcante et al (2000) investigated heparin receptor

occurrence in adult tunicates (sea squirts) which were found to line intestinal and pharyngeal epithelium in close contact with the external environment. They speculated this arrangement reflected the role of heparin in invertebrate defence mechanisms. In aquatic invertebrates, such defences involve haemocyte aggregation; hemagglutinating activity is inhibited by heparin which is itself mediated by hemagglutinin that binds heparin. They suggested that heparin release regulates hemagglutinating activity of haemocytes during bacterial invasion (Cavalcante et al 2000). Only *Daphnia* individuals which are able to prevent entry via potential heparin-gated oesophageal barrier survive to produce their lifetime's quota of young (Nader et al 1999). All animals infected via the heparin receptor are sterilised and the varying levels of immune molecules that follow do not improve chance of survival (Graham et al 2011).

Daphnia from well-nourished mothers are more able to resist parasite sterilization in early stages, and thus produce more offspring than other infected animals (Hall and Ebert 2012). The *Daphnia* innate immune system responds to parasite entry by producing a large increase in circulating haemocytes immediately following exposure, and varying levels of proPO, NOS1, NOS2 (nitric oxide synthases 1 and 2) and α2M according to phenotype (Decaestecker et al 2011). Killick et al (2006) found that high environmental temperatures around 25^{o}C outweigh early brood reducing effects caused by *P. ramosa*, causing equally negative effects on this measure of host fitness in both infected and uninfected individuals.

The haemolymph fungus *M. bicuspidata* is also an obligate killing sporulating parasite (Lohr et al 2010); it too awaits host death for spore release and infects when consumed. High-density populations are at higher risk of epidemic as infected animals cast spores via faeces, which uninfected animals meet via filter feeding (Civitello et al 2013). Infected *Daphnia* produce fewer offspring, however far fewer are sterlised in terms of fecundity, by this parasite than by *P. ramosa*, here only they are 20-34% of the population is affected (Hesse et al 2012). *M. bicuspidata* produces fewer spores and causes host death earlier for faster release into the environment; infected animals die within 20 days (Duffy and Hall 2008). Fish hunt *M. bicuspidata* infected *Daphnia* over the uninfected due to their increased opaque visibility (Duffy 2007). Transmission success is aided by invertebrate predators such as *Chaoborus* midge larvae (Hammill and Beckerman 2009), which eat *Daphnia* whole then regurgitate carapace and spores. In competitive situations with *P.*

ramosa, this mode of transmission favours the less productive fungus by releasing spores before *P. ramosa* spores have reached their infective stage (Auld et al 2014).

Less studied *Daphnia* parasites include *Amoebidium parasiticum*, which Pauwels et al (2007) found increased levels of *Daphnia*'s heat shock protein Hsp60 with increased length of time infected. There are sterilizing and sporulating parasites which kill even faster, for example, *Caullerya mesnili* is an intestinal protozoan that produces visible spores 13.8 days post infection (Lohr et al 2010). *C. mesnili* is a driving force behind hybridisation (Wolf and Weider 1991). It wipes out competing *M. bicuspidata* when co-infection occurs (Lohr et al 2010). The bacterium *Spirobacillus cienkowskii* sterilizes around 98% of its hosts and kills within two (25°C) to four (15°C) days (Duffy and Hall 2008). The chytrid fungus *Polycaryum laeve* causes *Daphnia* to become increasingly visible to fish, causing them to become 2-5 x more preyed upon than their non-infected counterparts. This factor is removed in cloudy waters showing that visibility alone rather than chemical cues cause increased predation (Johnson et al 2006).

2.3 Lab Specific Considerations

2.3.i. *Daphnia* Culture Medium

Daphnia culture medium was made as follows, with chemicals purchased from Sigma Aldrich. To prepare 5L, first, 4L distilled water was poured into an appropriately sized vessel. Then the following ingredients were added. Final molarity is given in brackets:

Calcium chloride	980 mg	(1766µM)
Magnesium sulphate heptahydrate	410 mg	(333µM)
Sodium bicarbonate	325 mg	(774µM)
Potassium chloride	390 µL of the stock 1 M solution	(78µM)
Dibasic monosodium phosphate	0.5 ml of the stock 10 mM solution	(1µM)
Monobasic sodium phosphate	0.5 ml of the stock 10 mM solution	(1µM)

Using a magnetic stirrer, the salts were allowed to become fully dissolved in solution for approximately half an hour at room temperature. The vessel was then topped up to 5L with distilled water and mix for approximately 5 minutes.

This chapter has elucidated ideal parameters for lab testing based on observations common across published work. These are summed in table 2i.

Table 2i. Ideal parameters for lab testing

Light: Dark Cycle	12 hours: 12 hours
Temperature	constant 18.5°c
Oxygen	21-kPa
Population Density	40 individuals per litre
Algal Food Density	4x 10^5 to 6x 10^5 cells cm^3
pH	7.4 to 7.8
Calcium	> 1.5mg/L

Monitoring of these parameters is important, as well as regular water changes. *Daphnia* respond negatively to overcrowding. Neonates cultured in water which previously housed another colony, rather than clean water, causes them to change life history strategies. Gust et al (2016) found *Daphnia* cultured in reused water were more susceptible to metal contaminants than animals raised in new water. In light of this, regular water changes, and water changes following population collapses are of importance when culturing *Daphnia*.

2.3.ii. *Daphnia* Suppliers

For the first part of this research [4.0 and 5.0] *Daphnia magna* supplied by Sciento were used. This provided a supposedly genetically identical population, grown *ex situ* in indoor tanks, on which drug trials were performed. The disadvantages of using Sciento were that as a single-staffed company *Daphnia* were often unavailable at random times, and costs were high: £14.50 plus £9.85 shipping for only 100 animals, shipping was by Royal Mail, which was prone to delay. Animals usually arrived in poor condition with empty guts and pinkish staining from lack of oxygenation.

For the second part of this research [6.0 and 7.0] *Daphnia* from Northampton Reptile Centre were used. These provided a genetically diverse population of *D. magna* grown in various outdoor ponds. Using this second company costs were much lower, twelve bags of 200 *Daphnia* cost £12.00, with £3.95 overnight courier delivery. That the animals are not genetically identical is seen as a benefit as this better mimic real life applications. While animals arrived in a healthier condition, these *in situ* bred *Daphnia* arrived along with unwelcome guests due to having been raised in a natural setting.

2.3.iii. Food Supplies

Attempts were made to grow algae from starter supplies. It proved impossible to grow *ex situ* despite attempts to mimic sunlight cycles with grow lights, only attempts *in situ* had any success. Algae was grown from starter batches supplied by Sciento, Manchester. Three algal types were chosen: *Chlamydomonas nivalis*; are small phototrophic motile unicellular phytoflagellates, which in addition to nutritional and oxygenating benefits provide active enhancement activity to *Daphnia* in terms of predator-prey chase situations. *Scenedesmus quadricauda* are a high-quality plant very good for oxygen liberation; they form small colonies of 4 to 8 cells. *Volvox aureus* are motile flagellate colonial cells that form globes with daughter cells contained within. All were grown separately in order to avoid initiating algal defence strategies of mucilage production, and, larger colony aggregation (in the two latter species).

For lab culture of algae, 350g good mulch soil, was dug from St. John's Gardens, Liverpool, and mixed with 5mg $CaCO_3$ in dH_20 (de-ionized water), made up to 1L, in a covered conical flask. This was pasteurised via gradual rising of temperature to approximately 90°C in 15 minutes, then left to cook for a 3-hour duration. The resulting solution was then passed through a tight wire colander to remove large particles then filtered three times to give a dark translucent concentrated soil-water stock solution. A separate 1L algal habitat was made up for each species. 50ml concentrated soil-water was added to 950ml artificial pond water. Ultimately, the culture of algae and *Daphnia* in either the windowless lab using sun-mimic light bulbs or a window ledge in an adjoining lab, which received little sunlight due to being north-west facing, proved unsuccessful despite weekly culture transfers.

For *in situ* algal culture algae was placed in outdoor tanks separate from the tank housing *Daphnia*. This was boosted by the addition of fertilizers (Baby Bio and Dennerle NPK Booster). Green water was moved to animal tanks where algae was able to perpetuate due to natural sunlit conditions. *Daphnia* tanks contained a 2cm thick layer of gravel to trap debris acting as a substrate for further growth. This worked only when strong sunshine occurred over several days. Outside of high summer, algal colonies collapsed with regularity, as seen by a green colour on one day followed by clear water with brown sludge descended to the bottom of the tank the next day. The supplier was unable to provide either regular or reliable supply of algae to meet this shortfall, which in turn led to regular

Daphnia population collapses due to starvation. For the remainder of the study (chapter 3 onwards) a new algal supplier was found that were able to send twice the volume of the previous (1L) for a quarter of the price on a regular three weekly cycle. For the remainder of this research supplies of *Nannochloropsis sp* were provided by Essex Marine Supplies. From there on *Daphnia* populations were maintained with great success. This company was able to provide batches of fresh algae on a regular three-week cycle, which could be set up and paid for in advance as a single batch comprising several months. This allowed for a continuous food supply avoiding delays caused by the normal procurement procedures required for repeat orders. However, the only consistently available algae were provided in highly saline solution which meant that increased water changes were required.

2.3.iv. Dealing with Unwelcome Guests

Unwelcome guests which arrived with *Daphnia* deliveries are discussed here from most common to most rare. Most common was *Chydorus sphaericus,* a cladoceran much smaller than *Cyclops* or *Daphnia*, thought to be herbivorous. These animals proved a threat to the *Daphnia* culture due to both their ability to reproduce in numbers notably greater than *Daphnia*, and to act in large groups when faced with weak or dying animals of other species. *Chydorus* were observed surrounding the larval stage of *Elmidae* en masse; *Elmidae* larvae appeared shredded upon their departure. They were also seen to attack weak swimming *Daphnia*. These appeared on several occasions but were eventually eradicated by first isolating stocks, restarting tank cultures from scratch and careful removal by pipette of any strays seen each day. A *Chydorus* neonate is seen atop a *Daphnia* neonate in figure 2j.

Next most common, *Cyclops bicuspidatus* seen in figure 2k, a copepod smaller than a *Daphnia*, which competes for algal food. These reproduced slowly and presented no threat. Next was larval stage *Elmidae sp.*, a worm-like animal with proto-legs, which become whirligig beetles as adults. All stages survive on algae and detritus; however, their presence was largely curated by *Chydorus* and no larvae survived to adulthood. Next most common was *Dytiscus marginalis*, a predatory diving beetle that preys on *Daphnia*. These are large and relatively easy to spot and remove, though incredibly fast. Finally, *Hydra vulgaris*, which was fortunately the least prevalent but certainly the most unwelcome guest. A Cnidarian, related to jelly fish, which predates on *Daphnia* via searching tendrils, it

paralyses then sucks *Daphnia* into its gut cavity. Within a day of their arrival, an entire colony of *Daphnia* was destroyed. Hydra are impossible to remove, and even to kill, as even when mashed through a fine sieve the cells will regroup into a full animal. Their appearance necessitated the destruction of an entire tank and all its inhabitants and contents including heater (for *ex situ* in winter) and rockery and a fresh start on all aspects of *Daphnia* culture.

Figure 2j. *Chydorus sphaericus* on top of a Daphnia

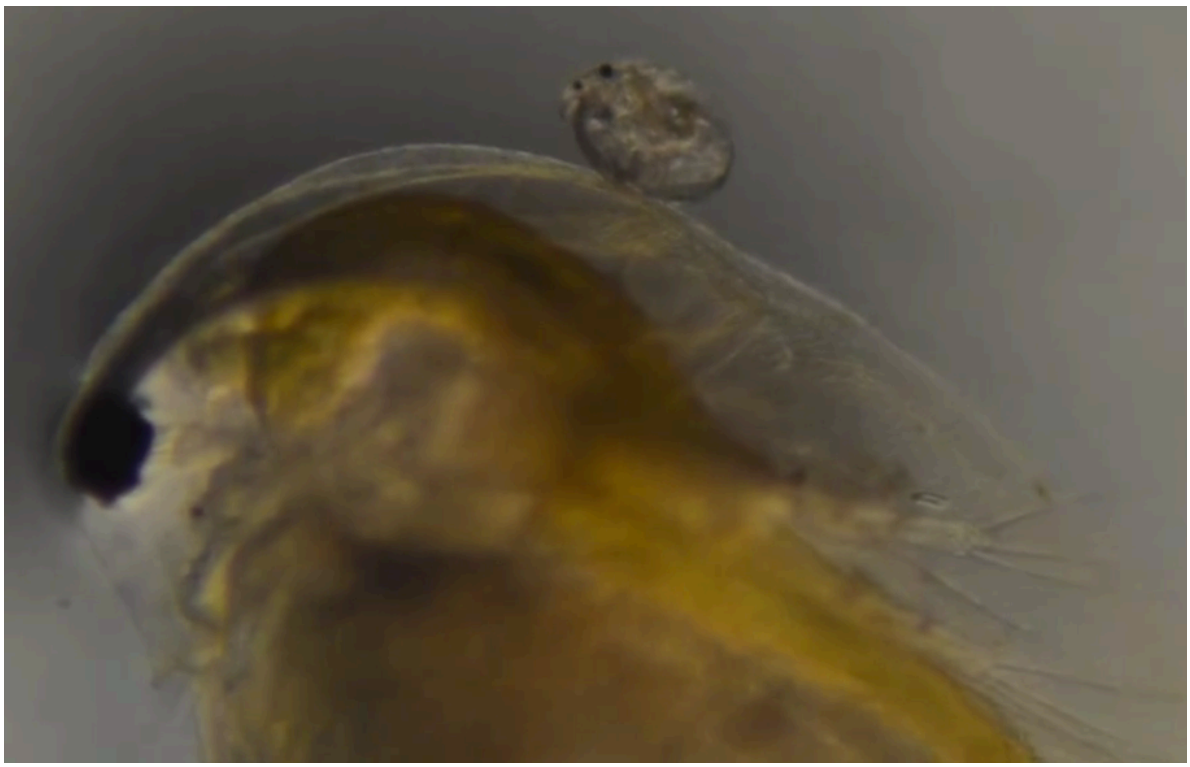
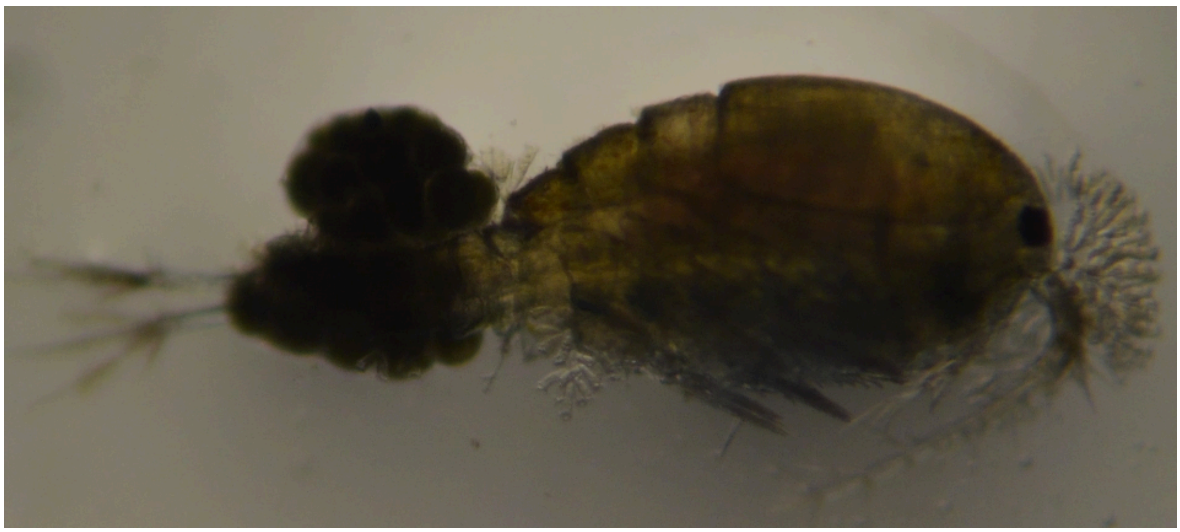


Figure 2k. Cyclops bicuspidatus



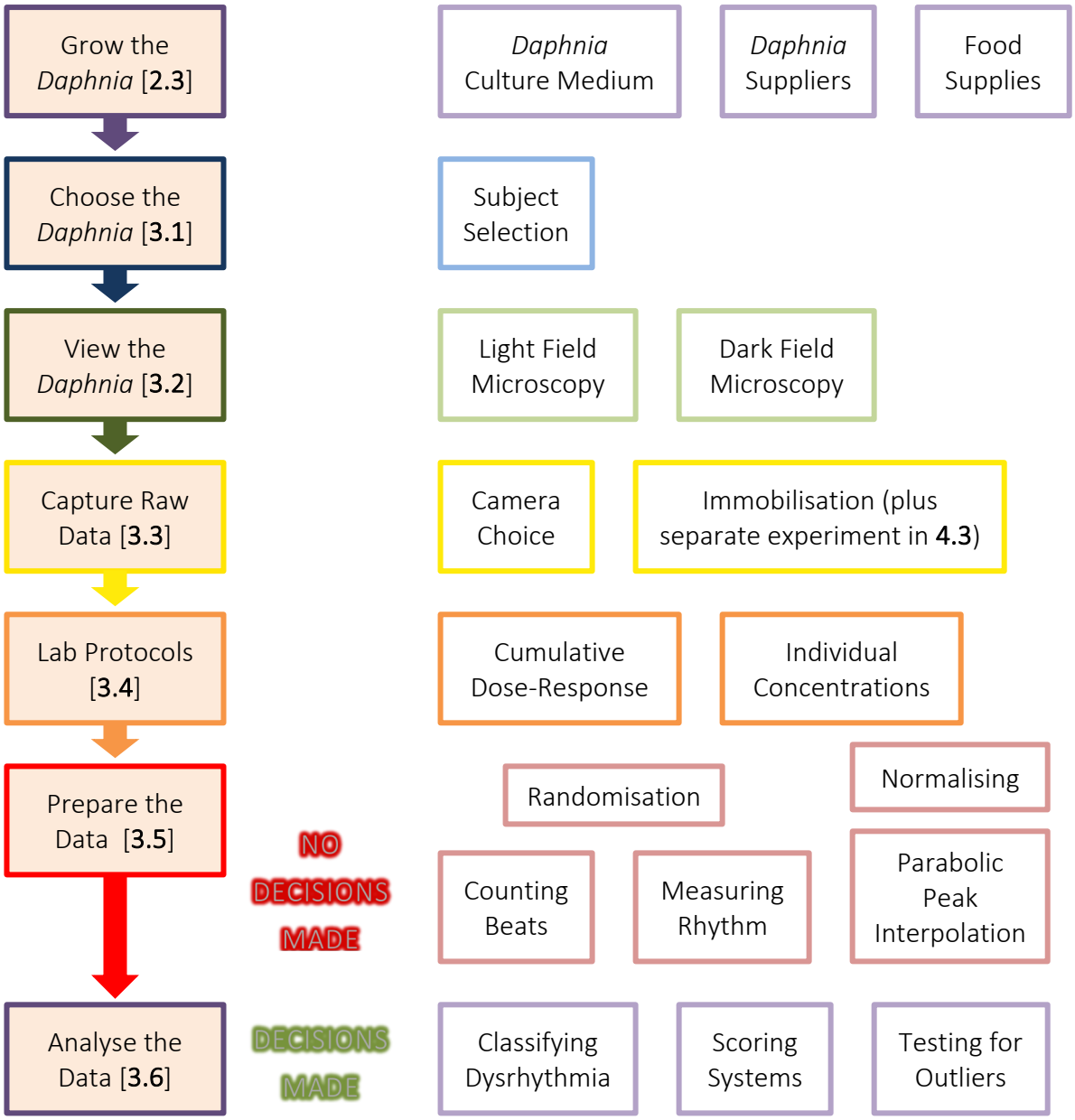
2.3.vii. Summary of Actions Based on this Chapter

Daphnia cultures were maintained for the full research period. During this time the culture tanks were observed daily for the presence of invertebrate predators which were removed as best as possible. Water was changed, by replacing half the tank with fresh water, a minimum of once a week and more often when observation of conditions required further intervention. Temperature was maintained at 18.5°c using a Hidom 75w aquarium heater and observed using a thermometer which remained stuck to the side of the tank. Care was taken to maintain water levels as the Hidom would burn out if not underwater. Oxygenation was maintained using a Hidom 4.0w Aquarium Adjustable Air Pump Twin Valve HD-603. Animals were purchased in groups of 1200 (12 packs of roughly 100 animals per purchase order) and kept in 20L tanks. This would work out to around 60 individuals per litre. However, delivery populations would usually crash before rebuilding over a period of two weeks to around two thirds that density. We assume this is due to stress during packaging and travel. This is in line with the standard *Daphnia* population density of 40 per litre. Algae was added as needed to maintain some green coloration to the tank. Daphnia were observed for green gut content to ensure feeding was occurring. We did not have the means to monitor algal cell level but performed this tacitly by observing how well-fed *Daphnia* were. Overfeeding was clear when the tank remained too green overnight, as *Daphnia* fed at night, and a water change would occur to compensate. pH, calcium, phosphate and nitrate levels were monitored using the API freshwater aquarium master test kit once a week. Unsatisfactory levels were adjusted by pH changes. An aquarium filter pump was not used as it was found to also filter and kill the *Daphnia*. The choice of culturing *Daphnia in situ* rather than *ex situ* is described following brief experimentation in **4.1**, while selection for neonates is described in general methods.

3. General Methods

This chapter presents experimental methods that were used across the thesis. The flow chart in figure 3a gives a logical reasoning behind the choice of these methods and allows for a story arc to guide the reader, where otherwise this chapter would simply be a series of disconnected instructions. In chapter two we reviewed *Daphnia* ecology and used this knowledge to formulate our *Daphnia* culture methods at the end of that chapter. Having prepared a sustainable culture of *Daphnia*, this chapter covers what happened next.

Figure 3a. Flowchart for General Methods used in this thesis



3.i Software referred to in this chapter are Microsoft Excel 14.6.7, MatLab R2007b 7.5, Adobe After Effects CS6 and Adobe Photoshop CS6. Hardware referred to in this chapter are a Nikon D5500 camera, a stereomicroscope model number AY11232 (Barska, 855 Towne Center Drive, Pomona, CA 91767), a dark field condenser with iris diaphragm 2282-410 (Agar Scientific, Unit 7, M11 Business Link, Parsonage Lane, Stansted CM24 8GF) and a DP311 differential amplifier and headstage (Warner Instruments, 1125 Dixwell Avenue, Hamden, CT 06514) attached to a Powerlab 2/26 (AD Instruments Ltd, Oxford OX4 6HD). When referring to equipment in the text we shall refer back to this paragraph as [**3.i**].

3.ii At various places within our results we choose to use either standard deviation or standard error. We will refer to the following paragraph when using either to justify the use of either one so as not to repeat at every turn, this paragraph is defined as [**3.ii**]. The standard deviation is a measure of the average deviation from the estimate of the mean of an individual measurement, for example the mean heart rate of a single *Daphnia*. The standard error is the deviation of the estimated mean from the true population mean, for example the mean heart rate of multiple heart measurements taken from a population of Daphnia. This gives us more general information about how close we are to the true mean given multiple measurements across a population.

3.1 Choose the *Daphnia*: Subject Selection

Daphnia selection for experiments was based on criteria of size, sex and virginity. For consistency neonate *Daphnia* were required; older *Daphnia* may be of any age from mature first brood producing to end-of-life, while age can still be predicted by size for neonates. There is a significant and tested difference between the health of a neonate and that of an ageing *Daphnia*, as described in **2.0**. Neonate size selection was achieved by using a 6ml transfer Pasteur pipette with a 1.5mm opening, which was too small for animals over 5 days old to pass through. Neonates start out life under 1mm in length but quickly double this within roughly 4 days when the carapace is shed, and the first foetuses begin to appear **[2.2]**. The opening restricts by size, not strictly by age. However, we know *a priori* that size and age are related, thus the pipette tip size forms an efficient way to pre-select a study group. After pipette selection, each animal was observed to ensure no pregnancy was underway. Males could be identified by the lack of a womb and were excluded on the basis that their presence only occurs in harsh environmental situations when the sexual stage comes about, at all other times females are parthenogenic. New *Daphnia* bought in from an external

company were allowed to reach their third generation before testing to avoid maternal factors. This is to ensure that parent species are born and raised in a consistently replicable, controlled environment. Further exclusion criteria came at the end of trials. Death was considered to have occurred when no visible heart movement was seen over three separate observations five minutes apart, each for a period of a minute.

3.2 View the *Daphnia*: Microscopy

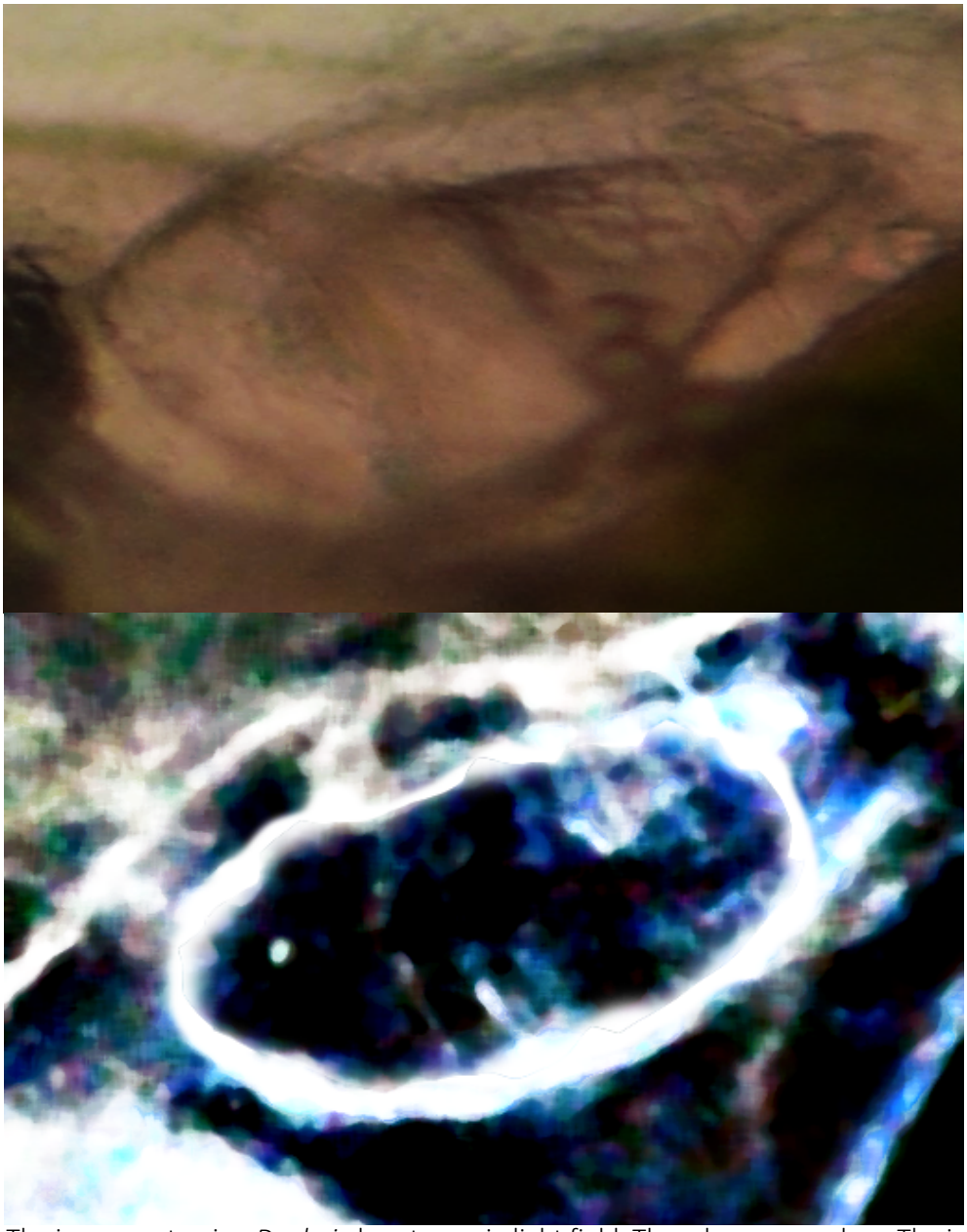
Research in chapters four and five where performed in light field using the available Barska steromicroscope. This equipment was brought in from home and while not ideal for this work, was the only equipment available at the time. Images captured were used for simple beat counting. For chapters six and seven, the microscope was adapted for dark field using the dark field condenser. This allowed for the much more complex measurement of rhythm. Dark field provided significant improvement to image processing. Figure 3b contrasts images in light field vs dark field. The latter allows for accurate edge detection as described in **3.5.iii.** This was not necessary when counting heart rate via slow motion playback of captured film, but crucial for capturing heart rhythm. Edge detection allowed us to develop a semi-automated process, which drastically improved the efficiency and accuracy of data capture.

3.3 Capturing Raw Data

Daphnia heart rate ranges from around 180 to 540 bpm, This has presented difficulties for workers attempting to make an accurate record. Campbell et al (2004) attempt to reduce the problem by slowing the heart by cooling the animals to 12.5°c. They immobilised *Daphnia* on a microscope stage in a small droplet of culture solution. Larger, older animals detach from the surface film while smaller neonates cannot (Gerritsen 1982). However, as we have noted earlier, *Daphnia* consume a great deal of energy in the maintenance of temperature at detriment to other physical factors, so this may confound experimental results. Ideally, *Daphnia* should be tested in the environment which they are normally accustomed to. A less invasive method of monitoring heart rate is via video recording. Until recently, this required the use of specialist high-speed video cameras running at 1000fps, and attendant high-speed computer interfaces. These costly and overpowered tools have little more than the advantage of slow playback for analysis. These include a number of digital image analysing systems including Cell P (Dietrich et al 2010), 2D (Artells et al 2013, Zein et al 2014) and 3D imaging and analytical tools (Ekvall et al 2013, Noss et al 2013) and Fview CTRAX software which runs on MatLab (Branson et al 2009). Workers have attempted

to overcome problems with cheaper, slower cameras by interpolating between lines on frames of interlaced video data (Wagenaar and Kristan 2010). However, the ready availability of progressive cameras today makes this labour intensive and error prone method unnecessary. Today, high speed of video capture is readily available at a reasonable price. With parabolic peak interpolation, a camera running at 60p (progressive scan) provides more than sufficient accuracy when monitoring heart response.

Figure 3b. Comparison of heart in best light field (top) vs best dark field (bottom) images



Legend: The image on top is a *Daphnia* heart seen in light field. The edges are unclear. The image on the right is a Daphnia heart seen in dark field. Heart edges are very clear.

Films were taken with a Nikon D5500 camera attached to the Barska stereomicroscope. Video allowed raw data to be analysed frame by frame, with greater accuracy and efficiency than work done by eye. The microscope-camera combination was focused to 45x at 60fps

in 1920 x 1080 pixel high definition. The Nikon D5500 was chosen due to *Daphnia* heart size and speed. A neonates' heart measures around 100 microns. This camera captures heart images around 270 pixels long. It films a full 60 frames per second, rather than the 60p interlace offered by most cameras at this time. A 60p interlace is created by merging 30p frames either side of an imagined frame; this does not allow for the same degree of precision. The higher sample rate gave more precision, which was essential, especially as questions were refined to look at heart rhythm as well as rate. Parabolic Peak Interpolation [3.5] then gave accuracy. *Daphnia* were immobilised within a drop of water which was pipetted away until individuals were held in place. An experiment which chose this method is described in 4.3.

3.4 Lab Protocols

3.4.i. Cumulative Dose-Response technique for Dose-Response Trials

The cumulative dose-response technique involves measuring the response to successive concentrations one after another using the same individuals, instead of individual concentrations, and is commonly used by pharmacologists to establish EC50 doses. However, at the high concentrations needed to elicit *Daphnia* responses it was discovered to be a very poor predictor of such (see 4.2 and 5.2.ii). This finding agrees with Dunne (1979) which also states that EC50 predictions produced in this manner do not agree with actual results when an individual concentration is used. It is described here only to avoid repetition later. The procedures follow those in part i, however instead of maintaining a single concentration throughout per individual, individuals are instead exposed to a new successively larger concentration every fifteen minutes. Control animals are maintained in drug free artificial pond water (APW) for the duration. A mean starting heart rate is taken before drug exposure and the time of first exposure is taken as zero. After each 15 minute exposure a 12 second film was taken but rather than be placed back in the same solution animals are instead moved to the next higher concentration, and a succession of films is made in this way. For cardio decelerative drugs, this was repeated until animal hearts slowed to a level visibly below 50% of starting rate when seen by eye (this was much lower than actual 50% as the eye cannot judge exacting percentage change as precisely as film enables us to do.) This went on for a minimum four hours and up to seven hours only constrained by lab closing times. For cardio accelerative drugs a heart rate increase peak before a return to normal was always seen in the first two hours. This is discussed further in chapter five.

3.4.ii. Individual Concentration Technique for Dose-Response Trials

Examination of heart rate allows comparison of the relative effectiveness of cardio accelerative and decelerative drugs. Drug concentrations were selected per trial with upper limits set at known *Daphnia* LD50 [1.10]. *Daphnia* were acclimatised to lab temperatures for a half hour prior to the beginning of the experiment. *Daphnia* were trialled in groups of four; one control and three test subjects. Individuals were placed in separate 35mm tissue culture petri dishes, in which they were allowed to swim in approximately 6ml of Artificial Pond Water. Three 12 second high-speed films in light field conditions were made prior to drug administration. This give an approximation for baseline heart rate by taking a mean value of beats over time. *Daphnia* hearts, as seen through the carapace, were filmed in order; control first, followed by subjects one, two, and three, on repeated rotation, as seen in figure 3c. Immediately prior to filming, APW was removed via Pasteur pipette so that the animal was immobilised within a thin film of solution. Following each film, APW was replaced immediately, allowing the animal to swim freely once again. Following the third baseline sample per animal APW was immediately replaced with a solution of whichever drug is being investigated dissolved in water (for all animals other than the control) and the timer started. Only water-soluble drugs were used. The start point is taken as 0 and thereafter measures the time exposed to the drug. The control was kept in drug-free APW. Each animal was filmed on rotation using the test solution and control. Time spent on this was, for cardio-accelerative drugs, the length of time needed for each animal to produce a sharp initial tachycardia followed by a decrease in heart rate back to normal speed. Experiments ended when the decrease in heart rate following this sharp rise could be visually observed (45 minutes to three hours).

The control: trial animal ratio strategy changed along with the move to the individual concentrations method. Instead of testing one control for every one trial animal, one control was used for every three trial animals. As the purpose of controls is simply to know whether a relatively constant heart rate is being maintained during the trial when animals are kept drug free, they offer very little information beyond that. Initial experiments showed that by viewing one animal after another in a cycle, it is possible to view four every five minutes while maintaining the whole lab protocol. Given the problem of low sample numbers compared to processing effort to capture the video data per animal, the cycle in figure 3c was derived to organise animals into groups of 3:1 trials to controls to gain the most information possible from each trial.

Figure 3c. Outline of a cardiovascular drug trial

Thick arrows: *Daphnia* acclimatised to lab temp.

Black rectangles: Individual *Daphnia* is filmed

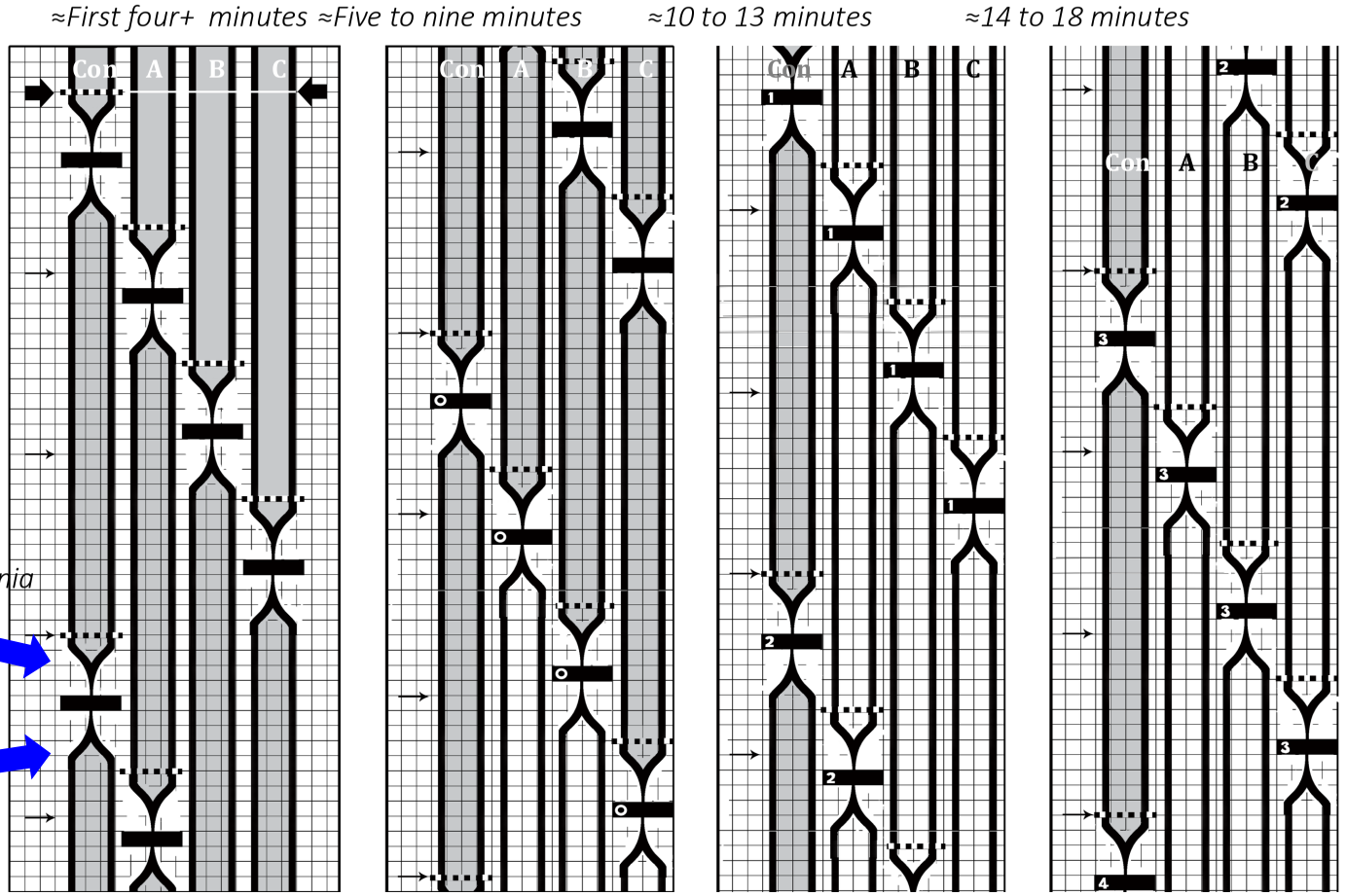
No number in black rectangle: Film made before drug exposure, taken for mean of baseline.

Number in black rectangle: Film made after drug exposure, 0 taken as start time, films numbered thereafter, control done in parallel with no drug.

Thin arrows: Another minute has passed

Narrowing vertical rectangles: Solution is being pipetted out of the bath to immobilise the *Daphnia* for viewing.

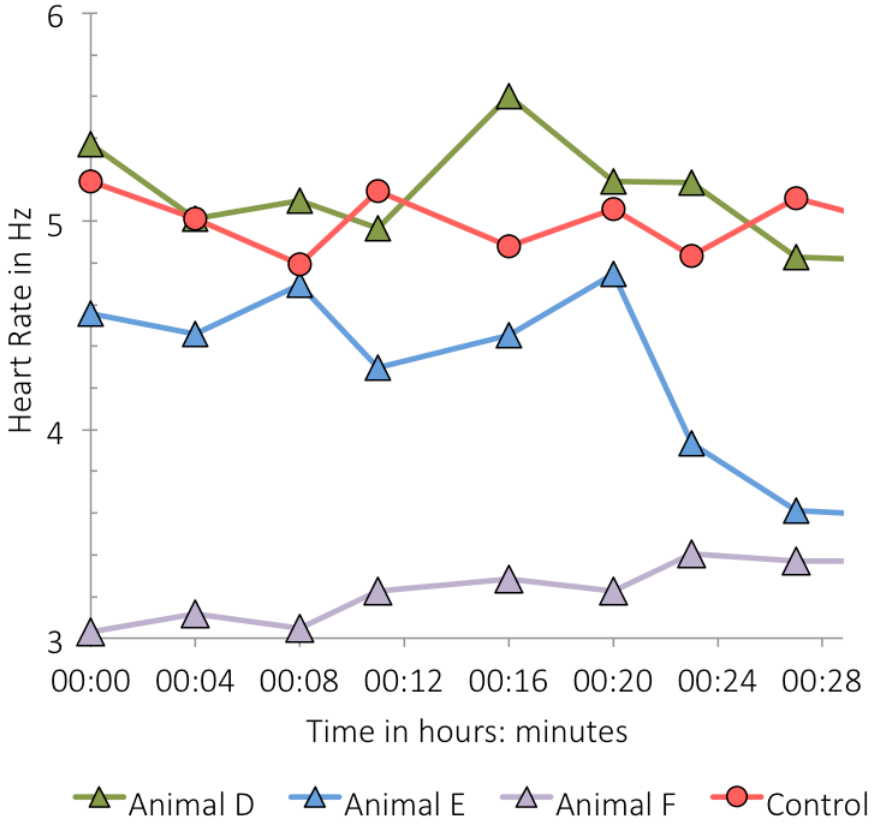
Expanding vertical rectangles: Solution is being pipetted back into the bath so that the *Daphnia* can swim freely.



Legend: The four tracks represent progress through time. Small squares are blocks of 5 seconds along the vertical axis, divided into one minute intervals by thin arrows. Thick arrows indicate the start time. Dotted lines indicate the time at which solution was removed prior to filming that animal. Grey filled vertical rectangles are drug-free controls or the drug-free period prior to drug exposure for trials, Unfilled vertical rectangles indicate where drug is in solution. Three baseline films of each animal were taken to give a mean heart rate prior to drug application. Water was replaced with drug solution for each trial animal except the control, and that time is taken as zero for that animal. Films for each animal were numbered thereafter. Each animal is filmed at roughly equal intervals.

For cardio-decelerative drugs observations continued for five to nine hours, ending when animals died or when the lab closed for the evening. At the end of each trial, drug solution was removed, and animals washed out with three changes of with fresh APW to remove drug residues. Where possible, animals were left to swim freely for period of two to three hours, after which animals were filmed once again to check for recovery. An exemplar graph of heart rate is shown in figure 3d. This is an example of a study not included in this thesis, used here to show what is to be expected from these methods. It shows the first 20+ minutes of raw data was captured.

Figure 3d. Exemplar graph of what is to be expected from the process shown in 3c



Legend: The graph shows that the process shown in 3c resulted in animals being filmed at roughly equal time periods following the first exposure to a drug (here 2560μM atropine, not used elsewhere in this text). The trial continued for a further four hours but is clipped here for the purposes of clear illustration.

Trials with dark field began in the same way as those described above. However, in 6.0 doxorubicin trials, once the trial drug was added animals were left to swim in without disturbance for a full hour before a film was made of the animal's heart response. Recovery was then performed in the same manner as above. Any changes from the above experimental procedure are listed in individual chapters.

3.5 Preparing the Raw Data for Analysis

3.5.i Randomisation

The films were randomised prior to analysis in order to avoid the possibility of user bias. Randomisation avoids this because there is no way of telling the heart rate or beat-to-beat interval of heart rhythm from a consecutive succession of frames once they have been disordered by randomisation. The camera assigns a consecutive number to each film. A matching consecutive number column was made in Excel. In the next column, the function "Rand()" is used so that Excel populates it with non-repeated numbers between 0 and 1, which were then filtered from smallest to largest, to change the order of viewing of the films. Films were then chosen from the camera's memory card and viewed in the order given. When all films for each trial had been observed or processed accordingly the randomized columns were reordered according to film number once more.

3.5.ii Counting Heartrate

Films were loaded into Adobe After Effects CS6 where they could be slowed, and heart beats counted by eye. Each film was checked twice. Heart rate was taken as the number heart beats per film over the length of each film in seconds. And, the time in seconds since exposure to the drug was noted. Each animal's first three baseline results were taken as mean heartrate before drug application and normalised to 100%. Concentration-response curves were produced, and the percentage of animals surviving in each concentration cohort noted. The variance in heartrate over this time was calculated for each animal based on the sample standard deviation, assuming that each heart rate is a discrete sample. F-Tests were performed to find whether each trial animal's heart rate variance at baseline was significantly different from that of the control using, $F = \frac{\Sigma(\,sample\ value - mean)^2}{number\ of\ samples - 1}$. The resulting F-value was compared to an 0.05 F-table. F-values bigger than those in the F table show that the trial animal was significantly different to the control, while numbers below the F-value show no significant difference. This was done to test whether drug exposure had an effect at very low concentrations.

3.5.iii Measuring Heart Rhythm

Each 12 second 60p film was brought into Adobe After Effects and cropped to frame the heart. Films were played through to ensure that Daphnia movement did not cause the heart to move outside of the cropped frame, and cropping was adjusted as necessary if this

occurred. Films were then rendered in lossless TIFF format (with frame blending and motion blur off) to produce 720 corresponding TIFFs corresponding to a single frame each. Images were imported into Adobe Photoshop CS6, in groups of 200 or less. This provided a succession of snapshots of the heart at 1/60th intervals. The program cannot accept more than 200 images open at any one time. Assessment procedure then varied according to whether films were captured in light field or dark field.

3.5.iii (a) Light Field

Light field produces ill-defined image borders, so it was not possible to create an automated process for picking out this area. Instead, for each of the 720 frames in a 12 second film were processed using a sequence comprising an automated action, a manual selection, then another automated action. The three steps are as follows.

Step 1. Press F1. For this, following commands were converted to a single shortcut:

- 'Smart sharpen' (500%; 20px) to attempt to differentiate edges from background;
- 'Colour range inverse' (100%; black) Any pixels lighter than an edge are lassoed;
- 'New layer' added to the image
- 'Reselect layer' and 'delete' - all colour is deleted leaving only the emptied lasso;
- 'Fill' the lasso is infilled in a solid colour e.g. Red (M100; Y100)

Step 2. Manually select the heart.

The heart was selected by drawing a line within spaces left uncoloured in the first step.

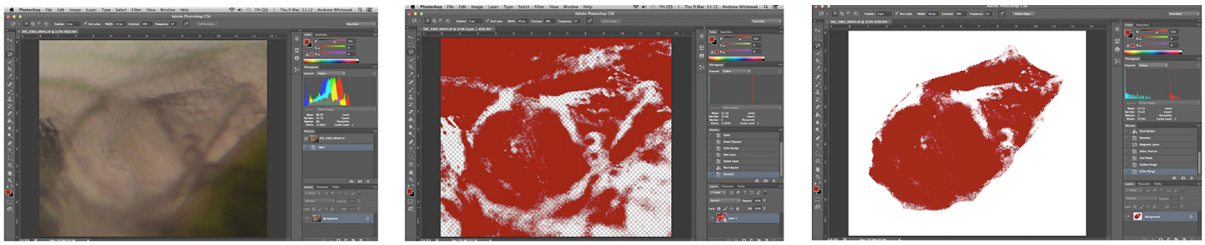
Step 3. Press F2.

- 'Inverse select, delete' the area outside of the manual selection is deleted
- 'Flatten'; leaving the heart in one colour and the remaining image area white, it does not matter how accurate manual selection was as long as it was within a white area.
- 'Select colour'; this selects pixels contained in the heart area.
- 'Histogram function' then shows number of pixels recognised. This gave a 2D area of the heart in pixels. Histogram results were then typed into Excel.

In the final automated function of step 1, "fill", there were pixels external to the heart that were coloured, and pixels within the heart that are not coloured. This is dealt with in the next step. By outlining the heart by hand, a borderline is formed to demark 'in' vs 'out' within the non-coloured space between internal and external those outside will be removed. The problem remains that some pixels within the heart area are not coloured, but filling them in by hand would confound the results by putting the element of human choice into the mix.

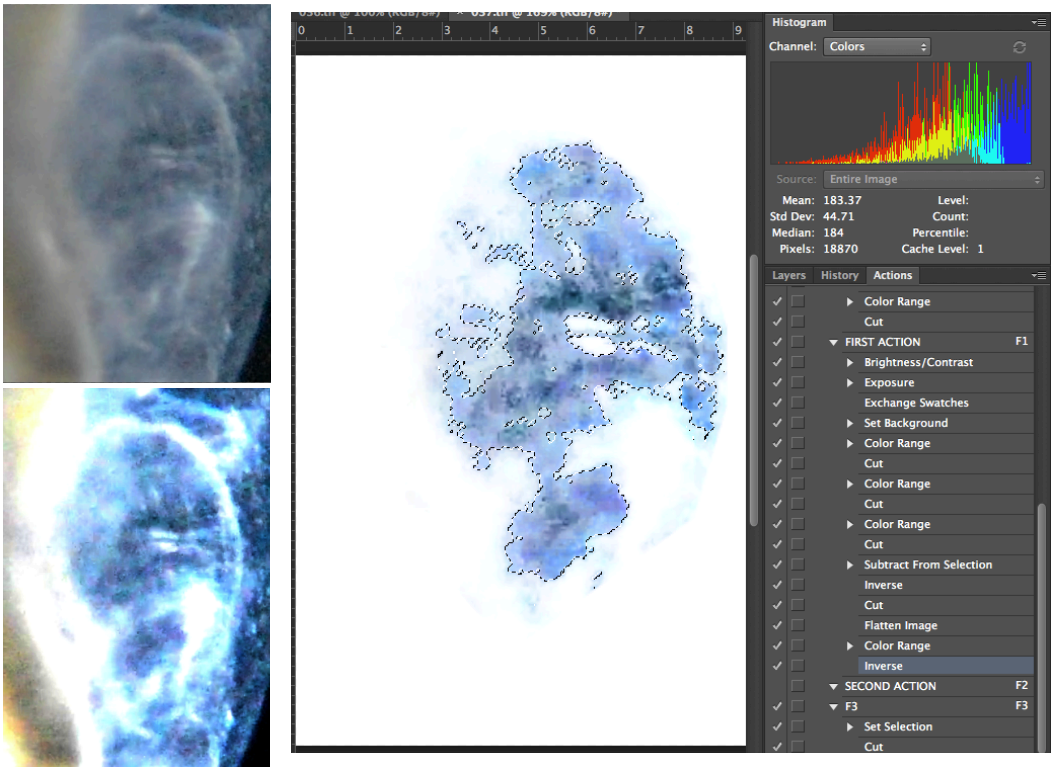
The non-coloured areas have consistency over the film as they represent reflections from folds in the *Daphnia* carapace that do not change over the course of filming, so are left uncounted. The requirement to manually encircle the heart area is open to bias. To avoid this, randomisation was applied to the processing of each frame. Even so, micro changes in lighting over the course of milliseconds can also alter the density of the image leading to error. This was an issue resolved by the use of dark field. The above process is illustrated by a succession of pictures in figure 3e.

Figure 3e. Overview of steps taken in Adobe Photoshop CS6 for one frame of lightfield



Legend: Left to right show the image at the start, after step 1 and then once the heart is selected, after step 3 is complete.

Figure 3f. Overview of the automated process for darkfield in Photoshop CS6



Legend: The above images are screen shots of stages in the automated process, comprising a frame of the original film, then with brightness and exposure adjusted, and finally surrounding pixels removed. This can be done as one automated step for 200 frames at a time, replacing the manual selection per single frame for light field images.

3.5.iii (b) Darkfield

The heart image captured under dark field conditions allows for a clear white line of light to silhouette the heart. After initial set up of the first frame, all remaining frames per film can be captured automatically, at least in the 200 frame batches which Adobe Photoshop CS6 allows the user to open simultaneously. This function is broken into separate parts as a succession of pictures in figure 3f. Once the first set of frames are loaded into the Adobe Photoshop CS6, manual adjustments are made to the automated process itself simply to make it bespoke for each film according to lighting and exposure needs, and to roughly indicate where the heart sits during the film. This done the batch automate function is began and the program allowed to run until completion without further manual intervention. A run of 200 frames took roughly half an hour to complete, so other research and background reading can occur while this takes place. The user simply returns every half an hour to manually type histogram results into Excel then load the next 200 frames.

The single automated step and repeat of Action F3 enacts the following shortcuts.

- Brightness – *this parameter is adjusted at the start of each film process, lighting varies subtly. Manual adjustments are made to best frame the brightly lit heart edges on the dark background. Once brightness level is chosen it is left the same for all 720 frames.*
- Increase contrast to maximum (100%)
- Adjust exposure to bring out white detailing (+1.25, 0.70 gamma correction)
- Create separate layers, so that unwanted data can be easily removed.
- Select interfering colour ranges and remove to reduce interference. Colour ranges removed were yellow (only found in the gut), green (only found in algae) and cyan (a facet of over exposure in some cases)
- Select heart location – *This is performed manually only once as an indicator to the program, once the selection is made it is maintained for all 720 frames*.
- Inverse and cut, to remove the none heart areas of the image.
- Flatten the image and select all none-white pixels to display in the histogram function.

3.5.iv Heart Area Off-set Removal and Normalisation

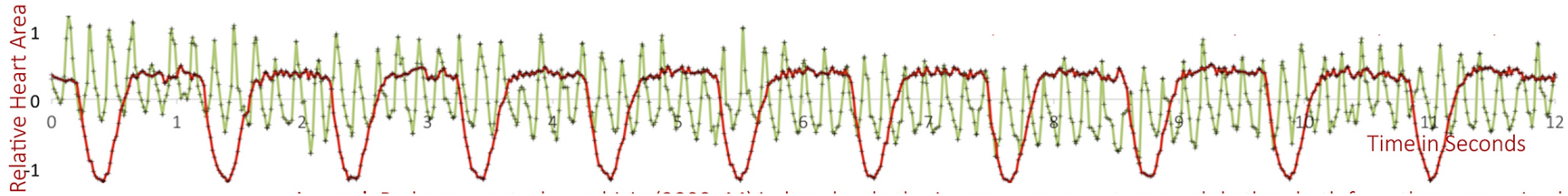
Raw pixel data was normalised by finding the mean heart area pixel value and equating this to zero, while the point of tightest contraction or greatest expansion is equal to -1 or 1 respectively. In this way the normalised pixel value Px_{norm} for each data point is:

$$Px_{norm} = \frac{Px_{current} - Px_{mean}}{Px_{max}}$$

Where $Px_{current}$ = the current pixel value per data point, Px_{mean} = the mean pixel value, and Px_{max} = the maximum pixel value.

This was done so that all heart area results per animal could be directly compared. An exemplar image of heart area results plotted between 0 and 1 is shown in figure 3g, which compares paired data of animal before and after a decelerative drug was applied. Again, this is for example only to show that my normalising the data, heart rates could be compared side by side. This might not otherwise be possible as the area filmed changes for each new film as the *Daphnia* is brought into clear focus by the microscope.

Figure 3g. Exemplar figure showing normalised raw data of heart area over time



Legend: Green represents a *Daphnia* heart before application of a decelerative drug, red is the same heart an hour after drug application.

3.5.v Parabolic Peak Interpolation

Following heart area normalisation, parabolic peak interpolation (PPI), which uses a parabola to determine the true peak of a signal, was performed to find accurate time points for peaks representing the heart in its diastolic state. Diastole was chosen over systole as, when it is plotted in graph form, it forms a sharper and thus more precise point in the analysis of heart movement data. The time between one diastole and the next gave the length of the period between each beat in seconds, from this a more accurate heart rate than counts by eye.

In PPI the peak is replaced with a quadratic polynomial. As the signal is band limited, discrete samples are sufficient to create a reliable interpolation. As the 60p film takes samples at an equidistant timing of 1/60th of a second, the parabola was applied in the time domain so that the three closest consecutive data points to each peak and trough are equidistant. An illustrative example is shown in figure 3h. Here, the time points relevant to each of the three points around the peak can be seen as -1, 0 and 1 due to their equidistance $y(-1) = \alpha \quad y(0) = \beta \quad y(1) = \gamma$

Where α, β and γ are the number of pixels at each time point. The interpolated peak location (p) is found using the equation from (Smith 2011):

$$p = \frac{1}{2}\frac{\alpha - \gamma}{2\alpha - 2\beta + \gamma} \in \left|-\frac{1}{2}, \frac{1}{2}\right|$$

Time points for peak data were found via the equation, $time\ at\ peak = \beta(t) + \left(\frac{p}{60}\right)$ $\beta(t)$ =the time point of the frame labelled β. Then, using the interpolated peak location, the peak magnitude is calculated as,

$$y(p) = \beta - \frac{1}{4}(\alpha - \gamma)p$$

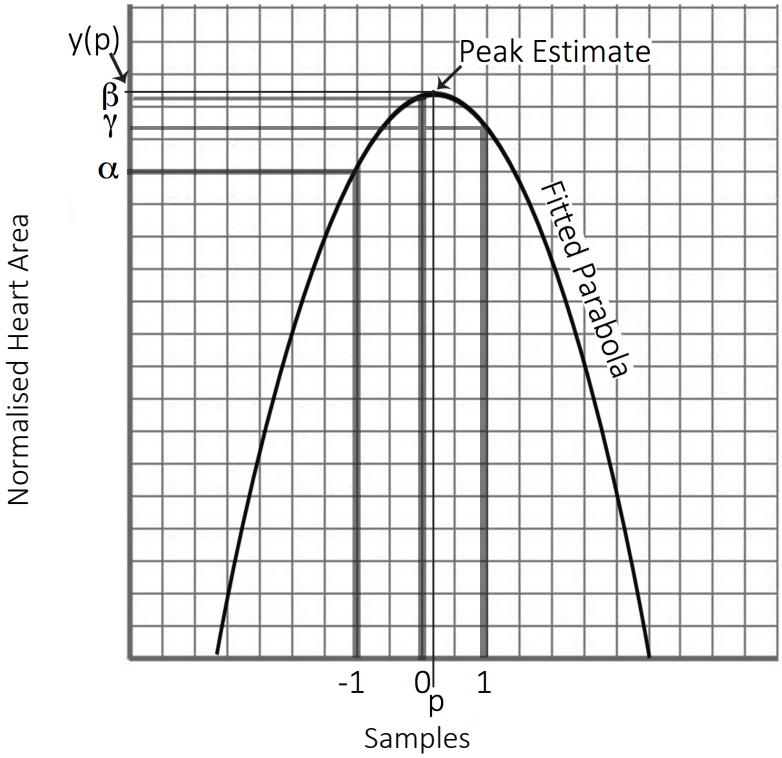
Results gave lists of peak occurrence timings which could then be used to describe heart period (time between successive beats), heart rate, stroke volume and cardiac output as needed. Period was derived by subtracting the time at one systolic trough from the time at the previous. Heart rate = $^1/_{period}$

An *approximation* for stroke volume SV based on 2D heart area was given using,

$$SV = 100 \times \left(1 - \left(\frac{y(p_2)}{y(p_1)}\right)\right)$$

Where $y(p_1)$ is the number of pixels at systole and $y(p_2)$ is the number of pixels at the subsequent systole, and number of pixels is taken as relative heart area.

Figure 3h. Illustrative example of parabolic interpolation



Legend: -1= previous sample, 0= current sample, 1= next sample

To test the precision of parabolic interpolation, a comparison to 1000 fps data was performed via low pass filtering and interpolation of the signal in MatLab. Peaks were then compared via a paired T-test (McDonald, 2009) where the Null Hypothesis argued that mean difference between pairs for comparison will be 0 as there are no differences between interpolation methods. The test statistic, t_s, was calculated using:

$$t_s = \frac{(\mathrm{x} - \mu_0)}{\left(\frac{s}{\sqrt{n}}\right)}$$

Where x is the sample mean, μ is the mean expected under the null hypothesis, *s* is the sample standard deviation and *n* is the sample size. This t value is taken alongside the value for degrees of freedom *n* -1 to obtain a P value. P values merely reveal the existence of an effect but not the size of the effect: the effect size is the main finding of a quantitative study. Effect size describes the magnitude of differences found, whereas a P value tests whether findings are due to chance (Sullivan and Feinn, 2012). Thus, the effect size was calculated using Cohen's d-value. Cohen's d is an effect size used to indicate the standardised difference between two means. For d-values less than 0.2 the difference is trivial even if it is statistically significant. Indeed, results showed that PPI was acceptable as the difference between its use on 60p data and interpolated 1020p data was trivial according to Cohen's

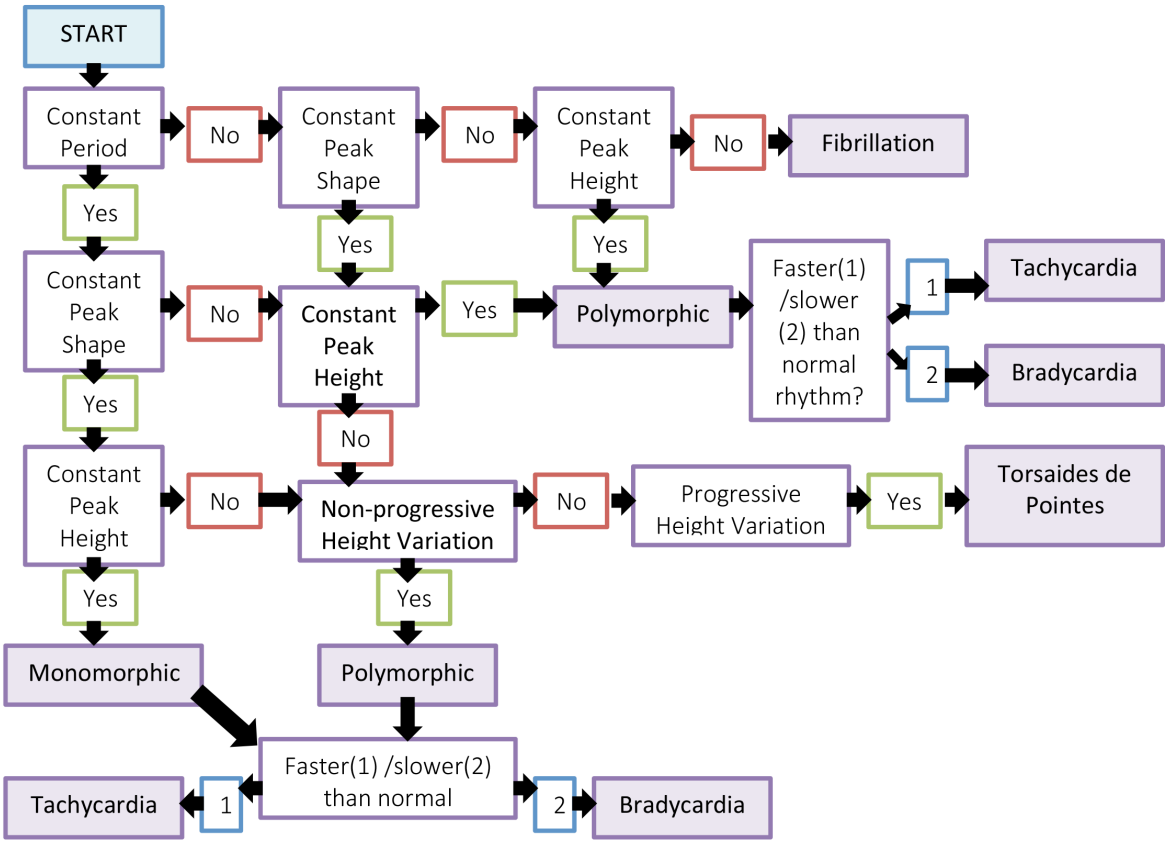
d. This is unsurprising given peak value listings for PPI and 1020fps interpolations were of similar standard deviation and size.

$$Cohen's\ d\ = \frac{(M_2 - M_1)}{\sqrt{\left(\frac{({SD_1}^2 + {SD_2}^2)}{2}\right)}}$$

We know that electrical signals created by the movement of *Daphnia* do not exceed 20Hz (Freund et al 2002). To resolve whether PPI of 60fps data is accurate when compared to higher sample rates, results were compared to interpolation to 1020fps via filtering and up-sampling. As sampling theorem (Nyquist 2002) predicts, a 60 Hz sampling rate produces discrete data samples needed to accurately extrapolate peaks, giving exacting area and time domain data via PPI. While a significant P value (P 0.0066; 0.05) is to be expected for high sample levels (Sullivan 2012) (125+ peaks per film in normal rhythm); PPI and interpolation to 1020fps produced effectively identical results; differences being trivial in paired T-tests (Cohen's *d* < 0.001 SD). PPI on 60 frames per second was used for this thesis.

3.6 Analysing the Data

Figure 3i. Flow chart for classification of *Daphnia* dysrhythmia



3.6.i. Dysrhythmia Classification for *Daphnia*

A method of categorising possible *Daphnia* dysrhythmia was created. The Lambeth Convention guidelines (Curtis et al 2013) state that all cardiac dysrhythmia studies must define the type of dysrhythmia under investigation and state it accordingly. *Daphnia* have only a simple single chamber heart whereby classifications such as 'ventricular' and 'atrial' cannot apply. However, we can borrow many of the terms used in the Lambeth convention to classify *Daphnia* dysrhythmia as accurately as possible, as shown in the flow chart in figure 3i. This figure adapts a tool used to analyse ECGs, in the completely different context of observing heart motion as described by graphs of heart area over time. While it might be clear how simple dysthymias such as tachycardia and bradycardia can be seen in such graphs, more complicated examples such as Torsade's de Pointes are further visualised in 4.5.iii, figure 4L, having been identified using this system of classification.

3.6.ii Assessment of Heart Rate Variability

Scoring systems, given as a literature review in chapter 6, have been used to differentiate dysrhythmias in theoretical (non-lab) based studies of computer-generated dysrhythmias. In our own study we reapply these systems as a means of differentiating heart rate variation at baseline – in normal rhythm – before a drug protocol has been applied. Scoring systems all depend on first creating delay plots. To do this, streams of data in form of beat-to-beat intervals are plotted so that each interval, or period, is plot against the previous period in the same consecutive data stream. Scoring systems then apply various linear and non-linear analyses to these delay plots which result in a spectrum of numbers, or scores, across a population of data that is intended to separate different data streams according to heart rate variability. Papers have had varying success in using this to differentiate types of dysrhythmia, and these are discussed in the review. We performed a brief exploration of all systems in chapter four, then moved on to a selection of the more successful scoring systems in chapter six. Each technique is rarely described in painstaking or easily reproducible detail across papers. So, in this section, we expand on exact mathematical methods from inferences made in the papers and also give related functions in Excel format. These systems will be used to score the stability or variation of beat-to-beat intervals from films of *Daphnia* heart movement in 6.0. As explained in detail in that chapter this will be done to test whether any scoring system differentiates individuals, according to their baseline rhythm, in a way that can be correlated with paired data from the same individual after cardiac insult.

The following is offered in justification for the mathematical analyses and modelling in Chapter 3. These methods are presented because, as will be thoroughly described in experimental chapter five, the initial research question, "Are *Daphnia* an appropriate model for cardiovascular drug testing?" was answered with a resounding "no." And, as shown in chapter six, a new avenue of research was pursued that enabled us to apply films of the Daphnia heart to a different line of questioning: That of the analysis of beat-to-beat patterning in dysrhythmias. The literature behind this are described in more detail there, and the maths involved described in the following passages. However, by way of introduction a discursive, lay description is given here to allow the reader transition between the first question, and the second and most keystone theme of this research, "Can beat-to-beat variation in normal rhythm be correlated with an individual *Daphnia*'s response to toxic insult?" If such a correlation could be found, it was postulated that the new *Daphnia* model could then be applied to human conditions using devices such as the Holter monitor. a Holter monitor is a portable ECG device for monitoring cardiac activity for periods of at least 24 to 48 hours. This too is discussed in more depth in Chapter 6. Various systems are published across the literature that deal with beat-to-beat variation. Here we introduce the maths behind this, and compare their success in differentiating dysrhythmias such as atrial fibrillation (Takahashi et al 1981, Thuraisingham 2006, Park et al 2009, and Mohebbi and Ghassemian 2012) and congestive heart failure (Hayano et al 2012), ventricular tachycardia (Huikuri et al 1996), supraventricular tachycardia (Rydberg et al 2007). However, none of these attempts to use them as predictive assays for potential heart rhythm disturbances on patients in normal rhythm.

These systems are both qualitative and quantitative, with the latter also being divisible into linear and non-linear mathematical systems. Qualitative systems include point density plots and histograms (Park et al 2009, Climent et al 2009, Voss et al 2010). These are not so useful for our analysis. Their interpretation requires in-depth tacit knowledge. Current imaging programs are not able to successfully differentiate dysrhythmias (Nikillus et al 2007). Visual pictures need to be converted into numbers to be useful in algorithmic detection of dysrhythmias. Quantitative linear systems are ellipse fitting, standard deviation of successive difference and line of symmetry analysis. In the first place these are an improbable fit for beat-to-beat variation analysis because beat-to-beat variation is a non-linear process, best described by Chaos Theory. We discuss why this is the case in chapter four, but to explain in brief: The recipe for Chaos is, Chaos = Non-linearity + Memory.

Literature dating back to the re-popularisation of Chaos Theory (Winifred 1981) show the heart's beat-to-beat variation plotted as a Poincare delay plot, where one beat period (the time between one beat and the next) is plotted against the previous beat period on and x and y axis respectively. This tends to produce a coalescence along the line of identity (where x=y) when beat-to-beat variation is quite steady, and a dispersion from that line as variation increases. Non-linear patterns can be formed over time using this plot. Memory applies where, for example, after the heart is stressed by a bout of light exercise and diverges from the line of identity (as well as dispersing more along it due to increased heart rate) a rest period sees the plot return to its steadier convergence with the line of identity. However, as linear analyses are suggested in the literature, they are investigated in chapter four's initial examination of unintentional appearances of dysrhythmia, for the sake of thoroughness.

The ellipse fitting technique (Mohebbi and Ghassemian 2012) is actually quite useful in that it forms the basic idea behind all other equations. It was the first to take an interpretation of the Poincare plot from its visual beginnings into a mathematical analysis, and is a good start point for explaining the maths to a lay audience. This is done later in this chapter. However, it does not analyse the data in great enough depth to form a predictive analysis, while the added dimensions (more than the two used in this system) given by non-linearity do this well. The standard deviation of successive differences (Chua et al 2008, Galland et al 2006, Jovic and Bogunvic 2010, Hindricks et al 2010 and Zarim and Rhaman 2011), is one of the more successful of the linear equations, at least when it comes to differentiating dysrhythmias, and is very simple. It was simply not chosen for chapter 6 due to being linear, and like all linear equations still not as strong as non-linear equations overall.

Finally, line of symmetry analyses (Porta et al 2006 and Guzik et al 2006) were shown to be the poorest equations for forming any kind of discernment between dysrhythmias in chapter four, so were not chosen for predictive analysis in chapter six. Porta (2006) simply counts points either side of the line of identity. Guzik (2006) merely ratios distances from the line of identity, taking no account of the diagonal spread of the data along this line. This author had to adjust the equation given, and the adjusted equation is given and explained in mathematical terms in the next section. In the original equation, rather than simply calculate this as a true 'flight path' distance, gave 'city block distance' instead. In this, to get to any point on the graph, distance could only be taken by running parallel with the x and y axes in steps at right angles to one another. As this adds nothing to the information given by the

analysis, one might only assume that this was taken as an 'easier' way for the authors to come up with an equation that avoided engagement with Pythagorean theorem. All in all, these two types of analyses lose much of the information afforded to us by the formation of a Poincare plot and did not do well at all when analysed in chapter four.

Non-linear analyses techniques were taken forward for the mainstay of this research, in chapter six, where they were applied to beat-to-beat variation in Daphnia in normal rhythm, and results were correlated with the severity of same animal's cardiac response after toxic insult by doxorubicin. These included adaptations of line of symmetry analyses by Karmaker et al (2009), and an original non-linear line of symmetry analysis by Ehler et al (1998). There was also the complex correlation measure (Karmaker et al 2009), multiscale ratio feedback analysis (Huo et al 2014), median stepping increment (Gong et al 2015) and finite time growth (Wessel et al 2010). The non-linear versions of line of symmetry analyses were no more useful nor impressive than their linear counterparts. Cloud adaptations simply added the difference between beat-to-beat intervals into the equation, which allowed results to also show whether hearts were slowing down or speeding up. This did not tell us any more about the heart's beat-to-beat variation.

Next best was multi-scale ratio feedback analysis. What this really does is show us how much the heart is accelerating, decelerating AND the extent of its stability vs instability of rhythm. This is a great concept but doesn't work too well in practice as it looks at all the data in one lump and fails to separate out the detail of what occurs over time; something which finite time growth does do. Finite time growth is not only one of the better systems in discerning one dysrhythmia from another, it also allows for user adaptation. It is the only equation in the literature than can be adjusted along various parameters and so proved the most exciting in terms of the possibility of fine-tuning it to suit the need of the question. In our case we adopted increased dimensions for a broader spectrum of starting scores. Dimensions, as described later (page 157) are simply how many beats in a row are compared to a chain of the same length, repeatedly throughout all chains of that length in the data. Increasing the dimensions makes it more likely that variations, however small, might be found. Another possibility that could be explored in the adaptation of this equation is increasing the number of nearest neighbours compared in the repeated Lyapunov equations which make up the final result for each heart's data. Increasing the range of starting scores gives a stronger foundation from which to make predictions. While increasing the level of

nearest neighbours would also do this in a way that might speak more strongly about the heart's stability over the entire period captured.

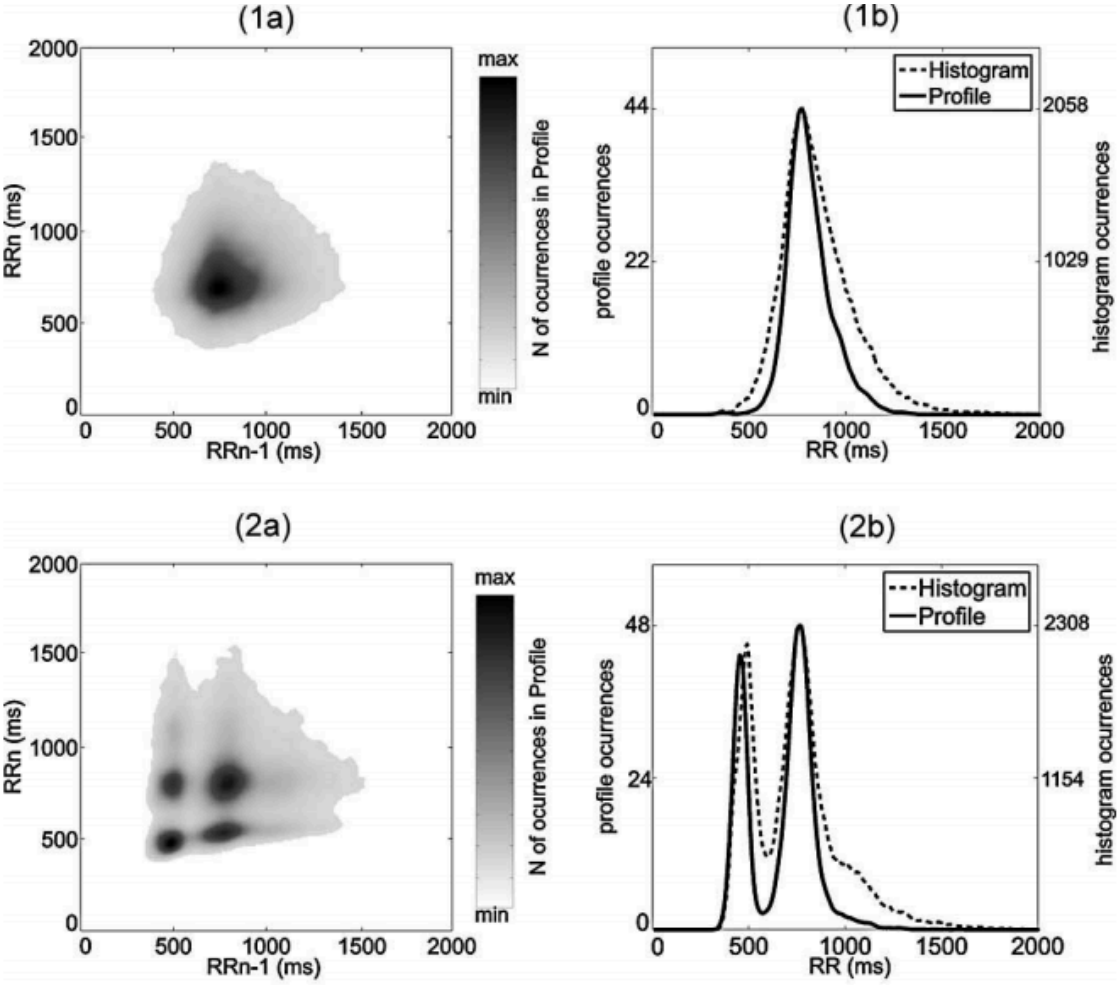
Two equations proved better than Wessel's nine-dimensional finite time growth and both of our own adaptations of this equation, these being complex correlation measure and median stepping increment. The former turned out to be one of the best methods of analysis. I put this down to the use of additional dimensions, and in this case unlike dimensions as described by Wessel et al (2010) being the length of row of beat-to-beat periods, here each new period successively following the next forms part of the analysis. The only drawback is that it is not clear whether it would be possible to increases the number of dimensions simultaneously analysed as chosen by Karmaker et al (2009). If it is, then it is beyond the capabilities of this author. Finally, median stepping increment (Gong et al 2015) proved the best means of discerning dysrhythmias. I adapted this equation for an uneven sampling rate, as the equation assumes samples as if the data were from, say, individual frames in a film are taken, rather than by using continuous sampling or applying parabolic peak interpolation. This method was surprisingly successful but entirely non-adjustable in terms of dimensions or other application to the data. It is still a great contender for its potential predictive value.

This appraisal explores and delineates the attributes, and the strengths and restrictions of the several scoring systems used in the analysis of beat-to-beat signal processing. Further and more thorough explanation of the maths in each system are given in this chapter, and their application and use are shown in chapters four and six.

3.6.ii (a) Point Density Plots (Qualitative Analysis)

Point density histograms were created by listing all periods, sorting them from small to large and using the COUNT function in excel to count periods of them same length to two decimal places. These were then plotted as histogram of period to occurrence. Point density delay plots were similarly created by listing vectors, sorting and counting. Then they were plotted as delay plots and using increasing point size and colour intensity to indicate areas of higher point density. An example figure from Climent et al (2009) is shown as an example in figure 3j, given multi-locus delay plots where not found from *Daphnia* data.

Figure 3j. Example of a Point Density Plot from Climent et al (2009)



Legend: Part one of the plot represents normal rhythm while part 2 represents atrial fibrillation. A and B compare a point density plot to a two-dimensional plot of the same data to show that the point density plot reveals more information about successive periods (4 areas are shown to be dense in points rather than 2 spikes).

3.6.ii (b) The Ellipse Fitting to the Delay Plot (Linear Analysis)

A delay plot of data was made of period data on the x axis to previous period data on the y axis. Figure 3k illustrates this technique. Quantitative application of ellipse fitting comprises is known as the SD1:SD2 ratio and is a simple adaptation of Pythagorean Theorem. The distance Z1 of any point from the line of identity is,

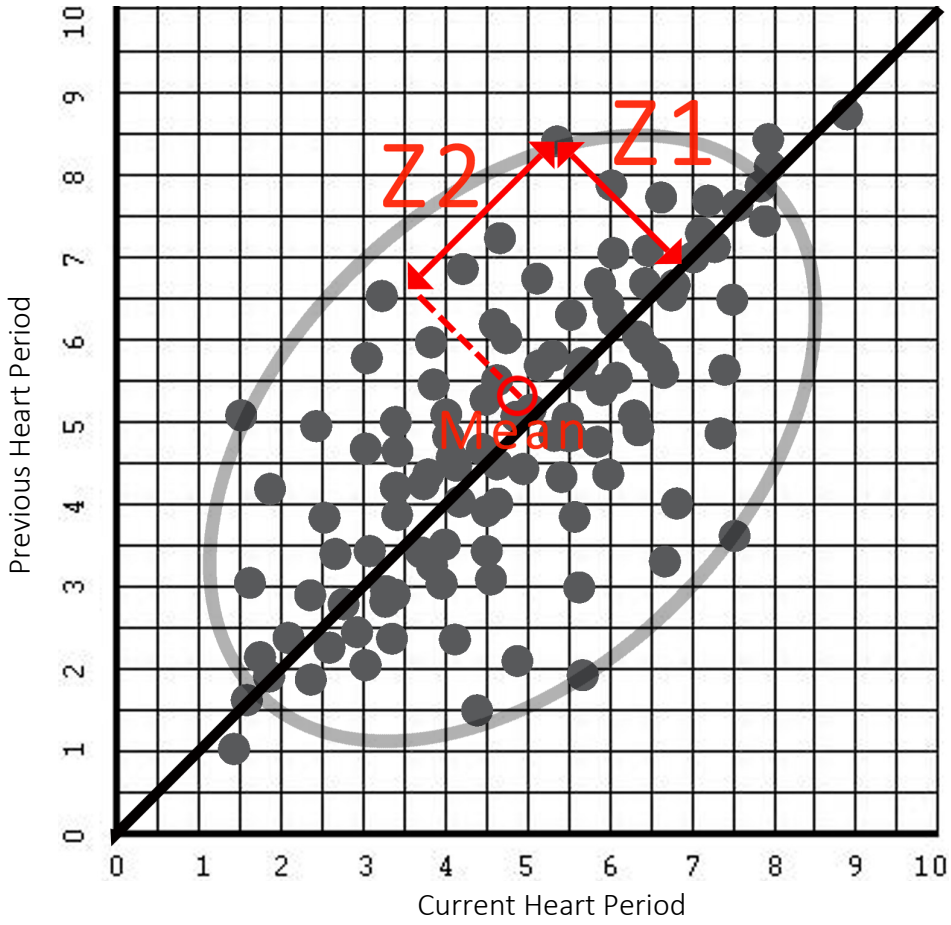
$$z_1 = \frac{\sqrt{(y-x)^2}}{\sqrt{2}}$$

A list of these distances can be made, and a mean and standard deviation derived as SD1. A second line is drawn at 90th to the line of identity, crossing through the mean (x,y) value. This is the line $y = -x + 2RR_m$. The distance Z2 of any point from this line is,

$$z_2 = \frac{\sqrt{2((x+y+2RR_m)^2)}}{2}$$

The standard deviation of Z2 distances is SD2. The ratio of SD1/SD2 is taken so that the higher the resultant ratio the less stable the heart rate variation.

Figure 3k. Illustrative example of the Ellipse Fitting Technique



Legend: This imagined figure shows how a selected point has Z1 distance from the line of identity, and Z2 distance from a line perpendicular to the line pf identity which goes through the mean.

3.6.ii (c) Successive Differences Analyses (Linear)

The Standard Deviation of Successive Differences (SDSD) can be applied to delay plot data using the equation for standard deviation,

$$s_{(x-y)} = \sqrt{\frac{\sum_{i=1}^{n}([x-y]-[\overline{x-y}])^2}{n-1}}$$

where s = SDSD, x and y pertain to period data on the x and y axes and n is sample number.

3.6.ii (d) Line of Identity Symmetry Analyses (Linear)

Porta's Index (**PI**) (Porta et al 2006) involves a simple count of points on the delay plot,

$$PI = 100 \times \left(\frac{b}{a}\right)$$

where b = number of points below the line of identity, a = total number of points. Points were separated according to position above and below the line of identity by the function,

$$if \;\; x - y \; is \; positive \; then \; the \; points \; is \; below \; the \; LOI$$

If x is greater than y the point will be below the line of identity. For Guzik's Index (**GI**) (Guzik et al 2006), the opposite function is used,

$$if \; x - y \; is \; negative \; then \; the \; point \; is \; above \; the \; LOI$$

If x is less than y the point will be above the line of identity; though either choice is arbitrary and makes no difference to results. Then, to find each point's distance from the LOI the following equation is applied,

$$D_i = \frac{|x_i - x_{i+1}|}{\sqrt{2}}$$

where D_i = distance from the line of identity. GI is then,

$$GI = 100 \times \left(\frac{(\Sigma \, D_i \; above \; LOI)^2}{(\Sigma \; all \; D_i)^2}\right)$$

Guzik's equation (Guzik et al 2006) seems both bizarre and incorrect, because It clearly does not give absolute distance as the crow flies but instead "city block distance" as if the journey from point to line were only performed by walking in parallel to the x and y axes. We have already shown a method for calculating the true absolute distance when finding "Z1" during the Ellipse Fitting Technique where

$$z_1 = \frac{\sqrt{(y - x)^2}}{\sqrt{2}}$$

We used this function instead, as we could not justify the use of city block distance.

3.6.ii (e) Line of Identity Symmetry Analyses (Non-Linear)

The application of the function $a = x_i - x_{i-1}$ to all consecutive periods gives a corresponding positive, negative or zero value. For 'cloud' adaptations the function is applied again to the next level of data giving $b = x_{i+x} - x_{i-1} - 2x_i$ to show the tendency of three periods in a row, which may be either increasing (positive values), decreasing (negative values) or neutral (0) (Karmaker et al 2009). The following equations are then applied to the new data:

$$Porta's\ Cloud\ =\ 100\ \times \left(\frac{\Sigma\ \#\ \{\ b\ |<\ 0\ \}}{\Sigma\ \#\ \{b\}}\right)$$

$$Guzik's\ Cloud\ =\ 100\ \times \left(\frac{\left(\Sigma\left\{\frac{|b_i - b_{i+1}|}{\sqrt{2}}\middle|\ >0\right\}\right)^2}{\left(\Sigma\frac{|b_i - b_{i+1}|}{\sqrt{2}}\right)^2}\right)$$

Where $b\ =\ x_{i+x} - x_{i-1}\ - 2x_i$ so that the new distance becomes $D_i = \frac{|b_i - b_{i+1}|}{\sqrt{2}}$, and # requires a count of the contents of the curly brackets {}.

The Ehler's Index (Ehler et al 1998) is given by the equation,

$$Ehler's\ Index\ = \frac{\Sigma\ a^3}{\sqrt{(\Sigma a^2)^3}}$$

Where $a\ =\ x_i - x_{i-1}$.

This was also adapted by Karmaker et al (2009) for increasing cloud adaptation:

$$Ehler's\ Cloud\ =\ \frac{\Sigma\ (\Delta\ I_i)^3\ +\ \Sigma\ (\Delta\ D_i)^3}{\sqrt{(\Sigma(x_i - x_{i+1})^2)^3}}$$

Where if $b\ =\ x_{i+x} - x_{i-1}\ - 2x_i$ is > 0 then $\Delta I_i\ =\ x_{i+1} - x_i$

Or if, $b\ =\ x_{i+x} - x_{i-1}\ - 2x_i$ is < 0 then $\Delta D_i\ =\ x_i - x_{i+1}$.

3.6.ii (f) Complex Correlation Measure (Non-Linear Analysis)

To perform the complex correlation measure, the areas of triangles created by three consecutive vectors in a delay plot are calculated using the determinant equation for a 3x3 matrix where all z values equal 1 as applied to a 2D triangle in linear algebra. An illustration is shown in figure 3L. For the vectors (x_1, y_1), (x_2, y_2) and (x_3, y_3) this area is:

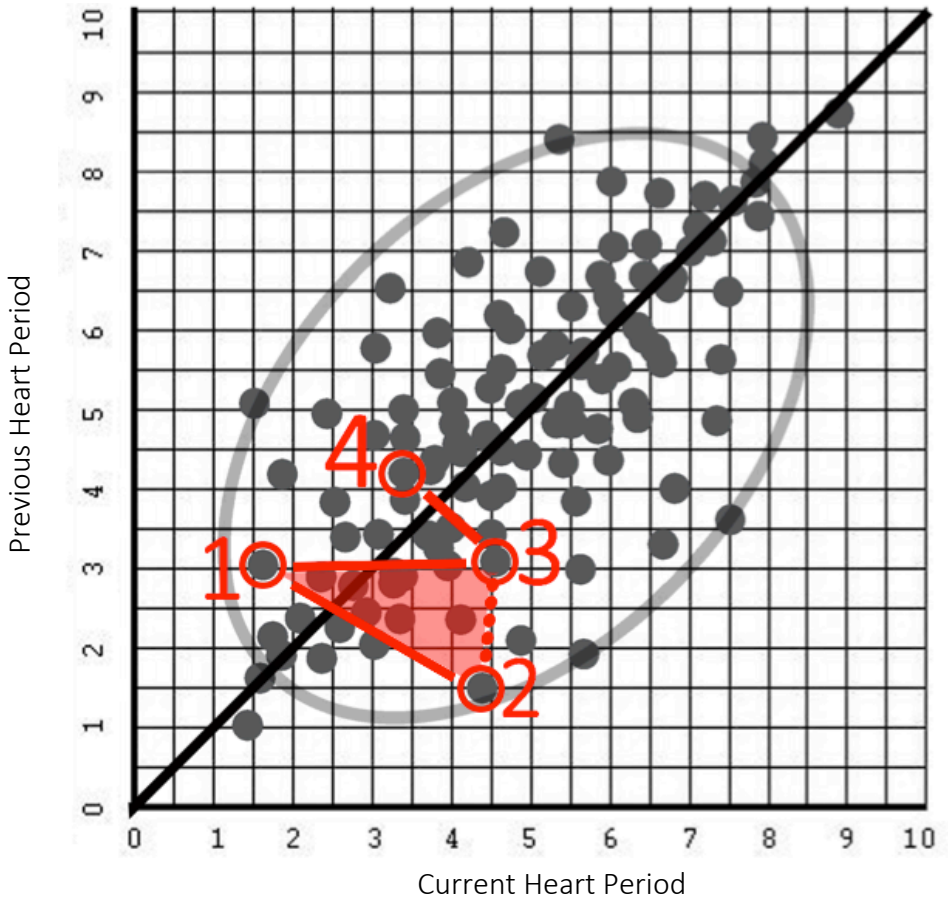
$$A\ =\ \frac{((\ x_2\ -\ x_1)\ \times\ (\ y_3\ -\ y_1))\ -\ ((\ x_3\ -\ x_1)\ \times\ (\ y_2\ -\ y_1))}{2}$$

This formula gives negative and positive values related to clockwise (+) or anticlockwise (-) spin. Given that in delay plots the y axis simply represent the previous x value we could simply say,

$$A\ =\ \frac{((\ x_{i+2}\ -\ x_{i+1})\ \times\ (\ x_{i+2}\ -\ x_i))\ -\ ((\ x_{i+3}\ -\ x_{i+1})\ \times\ (\ x_{i+1}\ -\ x_i))}{2}$$

The standard deviation of the data set is taken for comparison with normal rhythm.

Figure 3L. Illustrative example of the Complex Correlation Measure



Legend: This imagined figure shows how consecutive period data is used to create triangles on the delay plot from which areas are taken for the Complex Correlation Measure.

3.6.ii (g) Multi-scale Ratio Feedback Analysis (MRFA) (Non-Linear Analysis)

To perform Multi-scale Ratio Feedback Analysis, if consecutive periods are taken as,

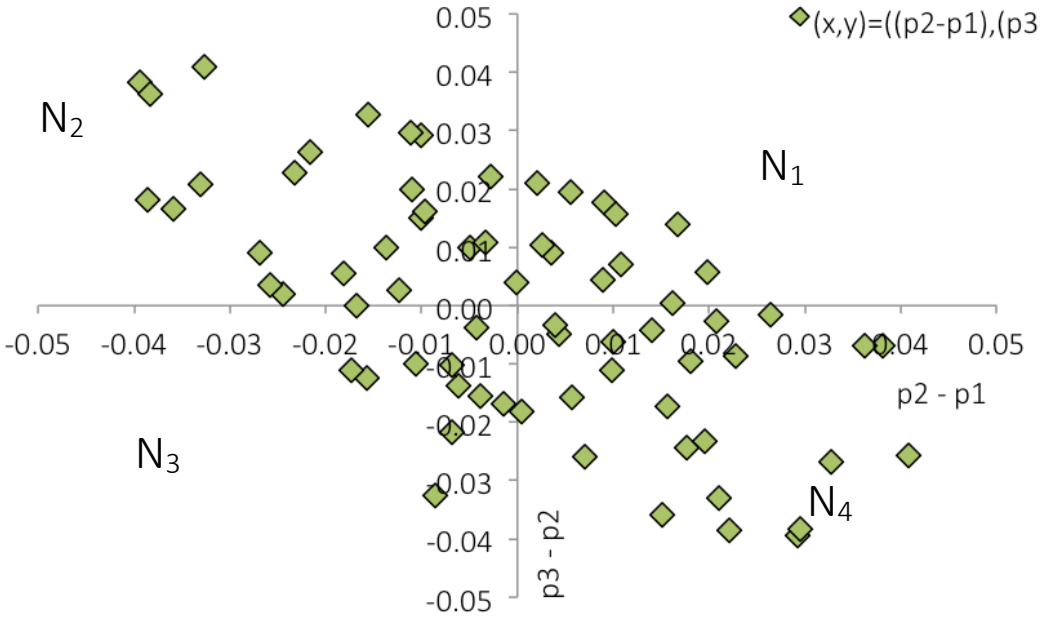
$$p_1,\ p_2, p_3, p_4, p_5, p_6, p_7, p_8 \ldots$$

Then, $(x, y) = ((p_2 - p_1), (p_3 - p_2))$

(x, y) are then separated into quadrants and summed where, $N_1 = \sum(+,+)$, $N_2 = \sum(-,+)$, $N_3 = \sum(-,-)$ and $N_4 = \sum(+,-)$. A real example of this is shown in figure 3m. The MRFA ratio is found using,

$$R_{TF} = \frac{N_1 + N_3}{N_2 + N_4}$$

Figure 3m. MRFA data for *Daphnia P* of the 2800μM doxorubicin cohort in baseline rhythm



Legend: Differences between consecutive period data are shown in four quadrants which indicate either an accelerative change in heart rate (N_1), a decelerative change in heart rate (N_3) or changes in rhythm (N_2 and N_4).

3.6.ii (h) Median Stepping Increment (Non-Linear Analysis)

Gong et al (2015) gave the length of the stepping increment is given as

$$l_i = f_s \times \left(\sqrt{(x_{i+1} - x_i)^2 + (x_{i+2} - x_{i+1})^2}\right)$$

Where, x_i is the magnitude of the i[th] sample and f_s is the sampling rate. We adapted this to account for uneven sampling rates given by parabolic peak interpolation, so that exacting time between samples was used instead. The adapted equation became,

$$l_i = \sqrt{\left(\frac{(x_{i+1} - x_i)}{t_1}\right)^2 + \left(\frac{(x_{i+2} - x_{i+1})}{t_2}\right)^2}$$

t is the time between samples. The median value of the increment is taken as the result.

3.6.ii (i) Finite Time Growth (Non-Linear Analysis)

Wessel et al (2010)'s Finite Time Growth is,

$$\lambda_k^{(n,\tau,T)} = \frac{1}{T} ln \frac{\| \mathrm{X}_k^T - \bar{\mathrm{X}}_k^T \|_2}{\| \mathrm{X}_k - \bar{\mathrm{X}}_k \|_2}, k = 0, \ldots N - (n-1)\,\tau,$$

where, λ = growth rate; T = 1 (the number of steps), N = 9 (The embedding dimension), and τ = 1(the delay used)

This multi-dimensional analysis required more calculation than any of the simpler equations given above. Thus, to understand our workings we would require the reader to examine the Excel program we have written and included in the digital appendix alongside the following reading. The program cannot be printed here as the first spreadsheet alone comprises an array of 144,000 equations to be performed simultaneously. At the smallest print setting possible, with each block ≤1.23cm across the spreadsheet comprises 2168 pages so would be an unreasonable addition to the printed version of this thesis.

We began by taking the first set of nine points as the starting coordinate. These become a_1, b_1, c_1, d_1, e_1, f_1, g_1, h_1 and i_1. Then find the euclidean distance (d) between the nine-dimensional point framed by those coordinates and every other point framed by a set of nine coordinates e.g. a_2, b_2, c_2, d_2, e_2, f_2, g_2, h_1 and i_2, by performing the equation :

$$distance = \sqrt{\begin{matrix}(a_1 - a_2)^2 + (b_1 - b_2)^2 + (c_1 - c_2)^2 + (d_1 - d_2)^2 + (e_1 - e_2)^2 \\ +(f_1 - f_2)^2 + (g_1 - g_2)^2 + (h_1 - h_2)^2 + (i_1 - i_2)^2\end{matrix}}$$

We compare all distances to find, for every nine-dimensional set, which other distances are nearest neighbours, and what are the mean neighbour distances.

The instructions for using the spreadsheet "FTG ARRAY" are as follows:

1. Copy a single column of numbers representing all consecutive period data in question.
2. Go to the first tab entitled "Step 1". This is a 356 column by 121 row grids.
3. Paste the column into box "II2". This is the 243rd column on the second row.
4. Go to the second tab entitled "Auto-arrange" and simply press "Cmd C" to copy the entire highlighted array without changing any element within the tab. A block comprising rows 2 through 121 and columns B through AUI112 is pre-highlighted.
5. Go to the third tab entitled "Step 2" and in node A2 paste values. Make sure to paste values only, not functions. The paste function can take several minutes.
6. Node A1 gives the number of 9 dimensional strings in the array. Take this as "n".

At this point the user can choose to perform the next task the slow way, or the fast way:

a. The slow way

In the first row, observe that an option is given at the top of each set of values to sort the values with a small grey button with an upward pointing arrow. Press this arrow for each of n columns and press "ascending". This takes a while, causes severe eye strain, and saving often is recommended.

b. The fast way

Go to computer system preferences > keyboard > tick “Use F1, F2… as standard function keys”. This will nullify the use of function keys for processes such as adjusting the screen dimness or volume but instead allows for the use of quick functions we have programmed into the spreadsheet. Much of the spreadsheet has been collapsed for its use. Click on the second visible node in the second row of “step 2”. Then press the series of keys “F1” “Enter” “next arrow” n times. As this does not require carefully looking at the screen to press the various ‘sort’, ‘ascending’ and ‘OK’ buttons as they pop up, it means that the “F1” “Enter” “next arrow” sequence can be done with the user’s eyes shut at high speed. We found that the processing speed of the spreadsheet was about 3x slower than the hands could work so, rather than trying to keep a count of how many times the sequence had been types, the user could stop typing the sequence when the program had reached around n=30 (for example for a 90n string) and take a small break (e.g. take a sip of tea) while the spreadsheet caught up with the remaining functions. Going over n series of button presses does not matter as Excel simply does not function when no more n’s exist and instead beeps loudly, at which point the user can press ‘escape’ and move on.

7. Copy rows 2 through 7 onto the clipboard.

8. Go to the fourth tab entitled “Step 3”. Enter into node A1 and paste values. Make sure to paste values only, not functions. While this is still highlighted, copy again. Enter into node A8 and paste transpose. While this is still highlighted, copy again.

9. Go to the fifth and final tab entitled “Step 4”. Enter into node A12 and paste.

10. Return to the first tab “Step 1” and copy the original column of period values.

11. Return again to “Step 4”, enter into node A2 and paste transpose.

12. “Find and Replace” all nonsense “§” symbols with the “=” sign.

13. Near the top of the spreadsheet in columns H and I (the row changes slightly between to between 18, and 22, according to how many dimensions are being calculated) is a value which will read “ J = ”. This value is the final value in the column of results given by the nearest neighbour equations. A slight adjustment of the spreadsheet will be needed by checking the nodes entitled “Mean” and “SD” which will have functions which read, “=SUM(J13:J---)/COUNT(J13:J---)” and “=STDEV(J13:J---)” respectively. For these functions replace “---” with the number given by “J = ”. This will give the mean Finite Time Growth value and standard deviation for the data set in question.

* End of Instructions*

To interrogate the spreadsheet, in “step 1”, the first 122 columns automatically repeat column “ii” in a vertical orientation, and the 123rd to the 242nd columns transpose that same array horizontally. Columns 244 and above call up this horizontal and vertically displayed repeated data, and apply the equation for distance across an 120 x 120 equation array of 144,000 equations. The “Auto-arrange” tab is not a named ‘step’ because absolutely nothing should be inserted into, or adjusted, regarding the content of this tab. It automatically gathers and rearranges answers from “step 1” in a format that can be pasted into “step 2” for further application of the equations. Answers to equations from “Step 1” giving Euclidean distances are interspersed with columns coded with reference to nodes in “step 4” along with the intentionally non-sensical notation §. This nonsense notation was especially chosen as it would not perform a function on the data at this point. “Step 2” is there to arrange all the nearest neighbours for every nine-dimensional array into order. It is interspersed with nonsensical references that will be converted to sense in “step 4.” “Step 3” is simply there as a stop gap in order to make sure values are copy pasted in the correct orientation and free from formulas. Replacing § with = in “step 4” will force the tab to refer to its own columns of 9 dimensional vectors. Functions in this tab automatically take sets of mean Nearest Neighbour values and square the difference between values from each 9-dimensional array and the first nearest neighbour value of each array. For all 9 values the sum of the square root is divided by the sum of the square root of the previous set of Nearest Neighbour values. Then, the natural logarithm LN is applied, a logarithm to the base e, for each n to n+1 comparison.

3.6.ii (j) Adjustment of FTG Spreadsheets to Test Different Dimensions

The FTG Excel was re-written from scratch to incorporate 11 and 13 dimensions. This required entirely new spreadsheets as each increase in dimension required a re-write of the distance equation covering a larger array of numbers. However, instructions for use are no different than those in **3.6.ii(i)**.

3.6.iii Acceptance Criteria

Tests for outliers were performed using a modified Grubb's test (Grubbs, 1969) and visualised in Schmoo plots. It is necessary to exclude outliers to remove false or misleading measures. For example, we cannot know *a priori* whether an animal selected for trial was already sickly or dying before exposure to a drug. We have no control over experimental variables such as this. By applying a blind test for outliers, rather than relying on opinion, we are trying to avoid imposing our own will on the data. The Grubb's test assumes a Gaussian distribution of data, and detects outliers which are so unlikely, that excluding them would not be unreasonable to exclude. A Schmoo plot is a simple scatter plot of input vs output, which visualises starting and response data are plotted for a concentration cohort. It gives a more visual representation of the presence of outliers which are mathematically confirmed by the Grubb's test. An example is given in chapter **5.2**. The centroid (mean of both axes) is also plotted. All data is expected to cluster around the centroid. Possible outliers are points distant from the cluster in both x and y directions. The significance of these outliers was then tested using a generalised Extreme Studentised Deviate (ESD) Test, a modification of Grubbs' Test which handles more than one outlier. Grubb's test handles data in one dimension only, so Schmoo Plot data was converted to vector format as a function of distance from the centroid using Pythagoras,

$$c = \sqrt{a^2 + b^2}$$

a= the absolute difference between individual and population mean starting heart rates, **b**= the absolute difference between individual and population percentage increase in heartrate after drug application and **c** = the vector function of distance from the centroid. A standard deviation for vector data was arrived at using the equation, where **n** = number of individuals tested:

$$sd = \sqrt{\sum c^2/n - 1}$$

The modified Grubb's Test (Zaiontz, 2018) is an iterative process where each possible outlier is tested separately and is indicated as significant outlier – or not – according to an ever-tightening critical value. Vector values are sorted according to length and the largest values, most distant from the centroid, are tested first. For each value in the column a new standard deviation, resulting from removal of the data more distant from itself is calculated. The

largest vector in the data set undergoes the null hypothesis “This value is not an outlier” and the alternative hypothesis “this value is an outlier”. The Grubb’s value (**G**) for this vector is calculated using the equation, $\boldsymbol{G = c/sd}$, where **c**= the individual vector function in question and **sd** = the standard deviation of all vector lengths in the group. The 0.05 alpha level G-crit value, with which to compare individual vectors G values, is found as follows. The significance value (SV) is given as, **SV=α/n,** where α is the significance level desired, here always 0.05, **n**= the number of samples being tested where the vector function under null hypothesis is the largest. Degrees of freedom are given as ***df*** **=n-2**. The critical t value used to calculate G-Crit is found using the Excel function, **T.INV(1−SV,*df*)** where **SV** and ***df*** are values referred to in the spreadsheet from results to equations given above. The critical Grubbs score **G** is given by the equation,

$$GCrit = \frac{t(N-1)}{\sqrt{N(df + t^2)}}$$

Finally, using the suspected range of outliers given by the Schmoo plot, G values and G-crit are compared. If the G value is larger than G-crit, then the value in question is an outlier. The test is repeated with this outlier removed for all suspected outliers +1 until the G value falls lower than the G-crit value.

4. Method Development and Early Insights

In this short pre-experimental chapter, we cover a series of early trials and studies that influenced choices laid out in the General Methodology, or which revealed insights that influenced project planning and direction in the ensuing three chapters. These comprise:

1. An exploration of how culture conditions affect trial outcomes *in-situ* vs *ex-situ*.
2. Trials that led the choice of cardiac therapeutics in chapter **5** based on achievability.
3. An exploration of possible means of immobilising *Daphnia* for microscope viewing.
4. Early insights into the application of Chaos Theory to cardiac dysrhythmia.
5. A brief study which led to chapter **6**, correlating *Daphnia* heart response to toxins with starting heartrate variance.

This chapter describes an exploratory phase that defined the means and course of action for the rest of this research.

4.1 *Daphnia* Culture *In situ* vs *Ex situ*

4.1.i Introduction

This trial compared *Daphnia* cultured *in situ* to those cultured *ex situ*. "*In situ*" refers to *Daphnia* being 'in' the situation which they would be found in nature, for example outdoors, in a freshwater pond or stream. "*Ex situ*" refers to *Daphnia* 'external' to the natural setting, such as those cultured in a lab. To test whether these contrasting initial conditions would also affect lab results, a trial of verapamil was performed according to the Cumulative Dose-Response technique **[3.4.ii]**. This method has been suggested as a means of reaching an EC50 value without the need for multiple trials of individual concentrations. The EC50 is the concentration at which a drug is calculated to give half-maximal response. However, it was discovered this did not produce results nearly as efficiently, nor as accurately as individual concentration trials **[3.4.i]**, which are discussed in chapter **5**. Two separate but related trials were performed. The first used only *in situ Daphnia*, as at the time *ex situ* animals had suffered multiple population collapses. The second trial used both *in situ* and *ex situ Daphnia*.

4.1.ii Methods

Daphnia acquired from the original supplier, Sciento **[2.3.ii]** were divided into two groups at random (simply based on which were poured into their new location without prior examination of individuals) and placed into one of two situations. Either:

1) *In situ*: an open topped outdoor tank filled with oxygenated water drawn from the lock 18 sluice on the Ashton canal (N 53° 28.662 W 002° 09.026), as well as algae for food. Conditions approximated the natural environment, with the tank open to the elements. The tank was supplied with pebbles and décor as algal substrate and to provide crevices and shade options found in natural habitats. Water was changed weekly to prevent nitrogen build-up. Neonates were transported to the lab in a sealed container in a cool bag to avoid overheating. Typical transport time was two hours and a quarter, from placing *Daphnia* in the sealed cool bag to transferring them to a beaker in the lab. On arrival at the lab, *Daphnia* were acclimatised to 18^{o}c lab temperature in an open flask for half an hour before experimentation, with the aim of removing climatic affects during observation.

Or,

2) *Ex situ*: a sealed, indoor tank filled with *Daphnia* Culture Medium **[2.3.i]**, as well as algae for food. Approximations were made to natural conditions while situated in a windowless lab via light bulbs installed in the top of the tank which mimicked sunlight and were timed to turn on and off on a 12:12 cycle. Lab animals were kept at 18^{o}c at all times. Water changed occurred weekly with fresh *Daphnia* Culture Medium. This tank was also stocked with pebbles and décor.

For the first trial, multiple concentrations were trialled in succession using the same individuals **[3.4.ii]**. Concentrations of verapamil made up for the first trial spanned 780μM to 24,960μM. Concentrations tested started with the lowest and changed in increasing increments every fifteen minutes. Control *Daphnia* were maintained in drug free APW for the duration. Following the successive concentrations 780μM, 1560μM, 3120μM, no trial *Daphnia* survived the 3120μM concentration and the trial ended before the next, 6240μM, was added. Ten trial *in situ* trial *Daphnia* and ten *in situ* control *Daphnia* were filmed for a total nine films each. Data were then processed as in **3.5**.

For the second trial, multiple concentrations were again trialled in succession using the same individuals **[3.4.ii]**. Concentrations of verapamil made up for the first trial, all of which were used in the trial, were 750μM, 800μM, 900μM, 1000μM, 1100μM, 1250μM, 1400μM and 1500μM. For this stage, both *ex situ* and *in situ Daphnia* were available and were filmed as separate groups. Concentrations tested started with the lowest and changed in increasing increments every fifteen minutes. Control animals were maintained in drug free APW for the duration. Twelve trial *in situ* control *Daphnia* and twelve *in situ*

trial *Daphnia* were filmed for a total twenty-four films each. No *ex situ Daphnia* survived past the 1250μM concentration so films ended after this concentration for the *ex situ* cohort. Twelve trial *ex situ* control *Daphnia* and twelve *ex situ* trial *Daphnia* were filmed for a total eighteen films each. Data were then processed as in **3.5**.

4.1.iii Results

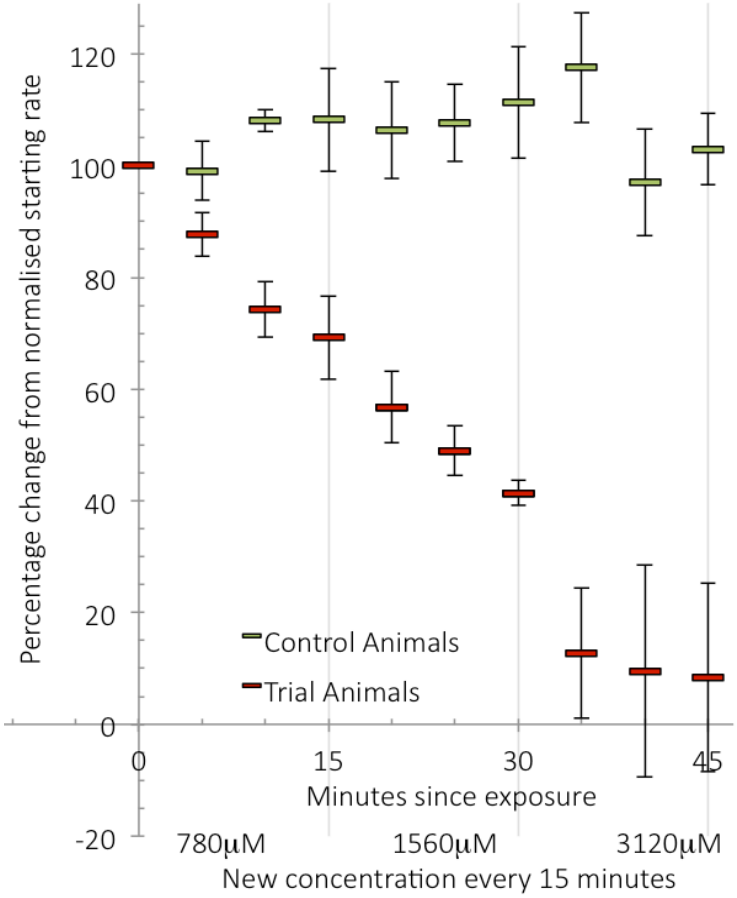
Daphnia response to 780μM, 1560μM and 3120μM verapamil during the first trial are given in figure 4a. Heartrate slowed in response to verapamil exposure. Heartrate falls to fifty percent of the starting rate midway through exposure to 1560μM. However, as no plateau is reached, we might only gather that the EC50 sits at a concentration somewhere below 1560μM. The data is shown as heartrate response over time rather than a dose response curve because it reflects the nature of the trial: We do not assume that responses to concentrations given in succession are equal to *Daphnia* response to individual concentrations. Starting rate was normalised **[3.5.iv]** to 100% so that decelerative response to verapamil is seen decreasing towards 0, where 0 is no heart beats. As concentrations are not trialled separately, we cannot be sure that the lower concentrations would have led to death had the animals been exposed to them for longer.

In the second trial, *in situ* animals survived the entire length of trial while *ex situ* animals as they did not survive past the 1250μM concentration. Paired controls were filmed only while the paired trial animal produced heartbeats. These trials are summed in figure 4b. Both groups show heartrate slowing over time, however, the heartrate of the *ex situ* lab trial group plummets quickly while that of the *in situ* trial group falls more steadily. The *in situ* group appear to reach a plateau by the end of the trial that sits around the 2.5Hz mark for the final three concentrations (1250μM, 1400μM and 1500μM), at a mean 41.34% of the normalised starting HR. For these animals, the figure indicates that EC50 may lie somewhere between 1000μM and 1100μM. However, at these concentrations death (as defined by cessation of heartbeats) had already ended the trial for the *ex situ* group.

4.1.iv. Discussion

In situ animals were exposed to all environmental variables such as sunshine, rain, and changing temperatures. Even invasive invertebrates appeared, competing for space or predating upon them. *Ex situ,* animals were raised in lab, with APW made to mimic natural pond water, artificial lighting and artificial temperature controls free of natural fluctuations

Figure 4a. Trial 1: Successive concentrations of verapamil

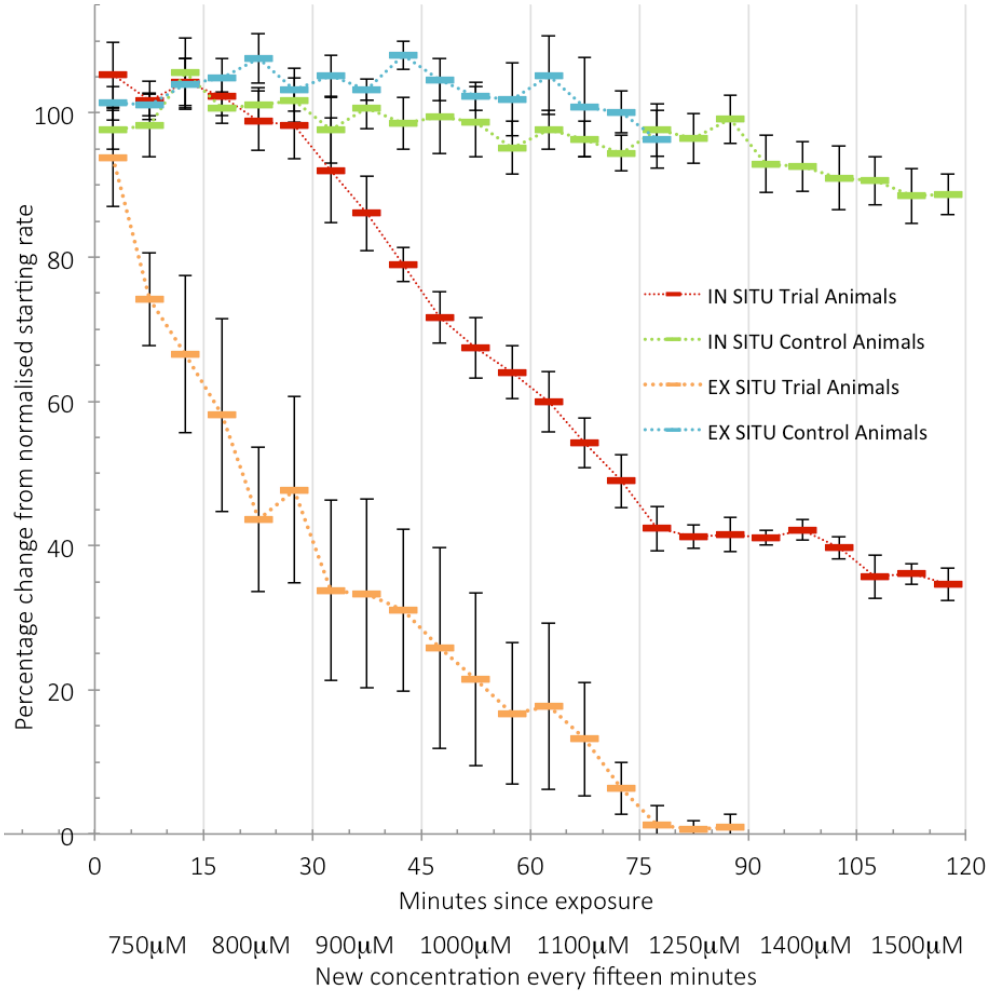


Legend: At time zero (start) no drug has been given. Mean heartrate prior to drug exposure is normalised to 100% for each individual in the population. The x-axis time in minutes. Vertical lines crossing the graph indicate a new concentration was given every 15 minutes. SE error bars on the y-axis indicate how far the sample mean of the data is likely to be from the true population mean. This was deemed more informative than the extraordinarily large SD given by high population variance in response (this subject is challenged later **[5.6 to 5.8]**).

As long as algal supply was maintained and predators kept at bay, *in situ* animals reproduced regularly and did not require restocking. *Ex situ* lab animals struggled from the start with regular population collapses occurring at weekends between lab visits. Despite animals from either group having been sourced from the same supplier, as genetically identical clones, the difference in their living conditions after a small number of successive generations made a notable difference to their survival rate and response to the drug. It may be that while APW is used in lab settings for both drug trials and maintaining *Daphnia* cultures, *in situ* natural freshwater supplies are used. APW may be fine for maintaining a stable pH for lab trials but may not be an ideal medium for *Daphnia* to live and breed within. It may be that animals exposed to *in situ* conditions become either epigenetically,

or by other means adapted to constantly changing conditions. Not everything controlled in the lab can be controlled *in situ*, from lighting, weather, exposure to environmental chemicals such as droppings and detritus from other animals, and even small predators. It is possible that a lifestyle exposed to the more natural conditions better prepares the animals for toxic insult. Lab animals by contrast are given no environmental challenges over the course of their lifetime and thus have no need to either change epigenetically or become hardier individuals. Perhaps lab animals raised in APW are unused to changing conditions, and the stabilising nature of APW prevents them from evolving the natural epigenetic adaptations afforded by living *in situ*.

Figure 4b. Successive concentrations of verapamil comparing two cohorts of *Daphnia*.



Legend: At time zero (start) no drug has been given. Mean heartrate prior to drug exposure is normalised to 100% for each individual in the population. The x-axis time in minutes. Vertical lines crossing the graph indicate a new concentration was given every 15 minutes. SE error bars on the y-axis indicate how far the sample mean of the data is likely to be from the true population mean. This was deemed more informative than the extraordinarily large SD given by high population variance in response.

Even more interesting than the simple conclusion that *in situ* animals may be hardier than *ex situ* animals is the evidence provided by standard error. In figure 4b error-bars are notably wider for *ex situ* animals. Despite the same number of animals having been used in either trial, results for *in situ* animals are less variant and thus more reliable. The wide error bars for *ex situ* animals' would mean we require exponentially more trials to give results deemed as reliable. It was concluded that *in situ* animals should be used for trials going forward. These animals not only survive better, and are more unified in their responses, but also are raised in natural conditions more reflective of the real world.

Possible further work extending from this might be to look at epigenetic differences *in situ* vs *ex situ*; however, this is not within the remit of this study. This early trial provided a dramatic contrast in results using identical methods on supposedly identical animals. This raises the question of how much an individuals' health status at the start of a drug treatment might help us better predict outcomes at the end, a question which, early on, formed the reasoning behind progress toward chapter 6. For further study of verapamil itself, trials using individual rather than successive concentrations of verapamil and *in situ* - only animals are pursued in the next section, and this individual concentration method, with *in situ* animals is taken forward in chapter 5, which looks to answer the original thesis question, "Are *Daphnia* a good model for cardiovascular dose-response trials?"

4.2 Initial Bradycardic Drug Investigations

4.2.i Introduction

This section describes early investigations into bradycardic drug action in *Daphnia,* in an attempt to find the EC50 for verapamil using individual concentrations **[3.4.i.]** as opposed to successive concentrations.

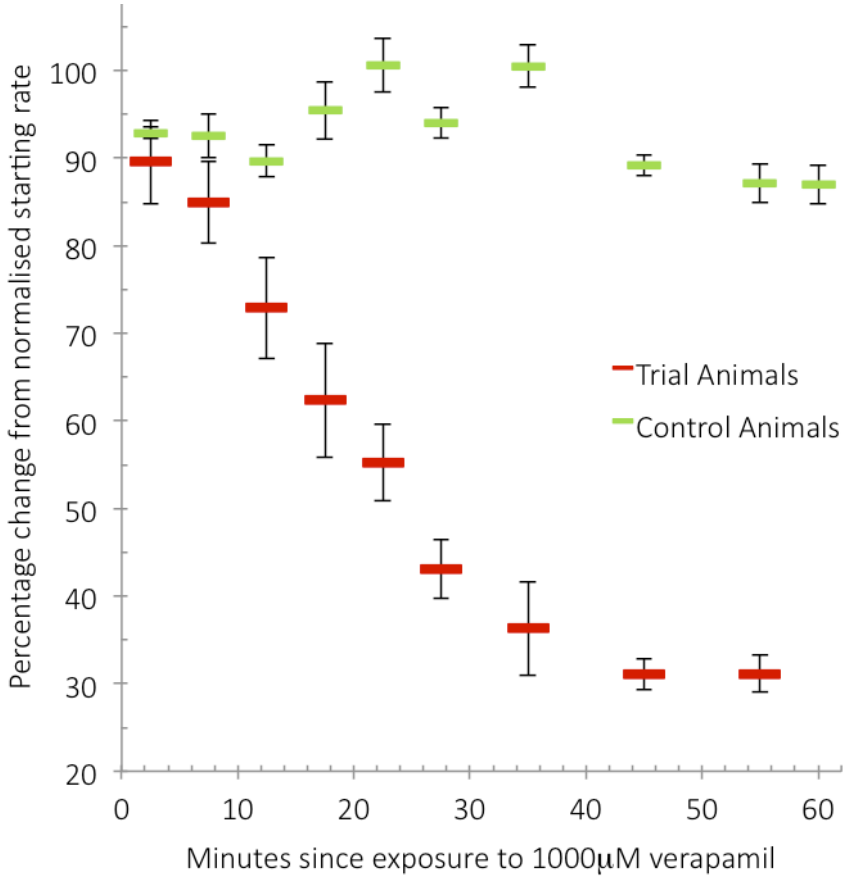
4.2.ii Methods

In **4.1** trials of successive concentrations and suggest that verapamil EC50 may be located close to 1000μM. As it was unclear whether the EC50 would lie above or below 1000μM, animals were first exposed to this concentration alone. From observations made, a series of concentrations were chosen (200μM, 400μM, 600μM, 800μM, and 900μM) to close in on the possible EC50. After the initial foray using 1000μM alone, the remaining concentrations were randomised so that a mix of individual cohorts were trialled per day so as to avoid temporal bias.

4.2.iii Results

Two hundred and twenty-four films of 9 trial and 3 control *Daphnia* were captured at the single concentration of 1000µM. Using 1000µM verapamil, a stable plateau was reached within one-hour exposure. The percent decrease from starting heartrate per animal plateaued at a mean 30.2%±2.5 (Mean+SE) of starting heartrate.

Figure 4c. Trial 1: Single concentration 1000µM verapamil over one hour

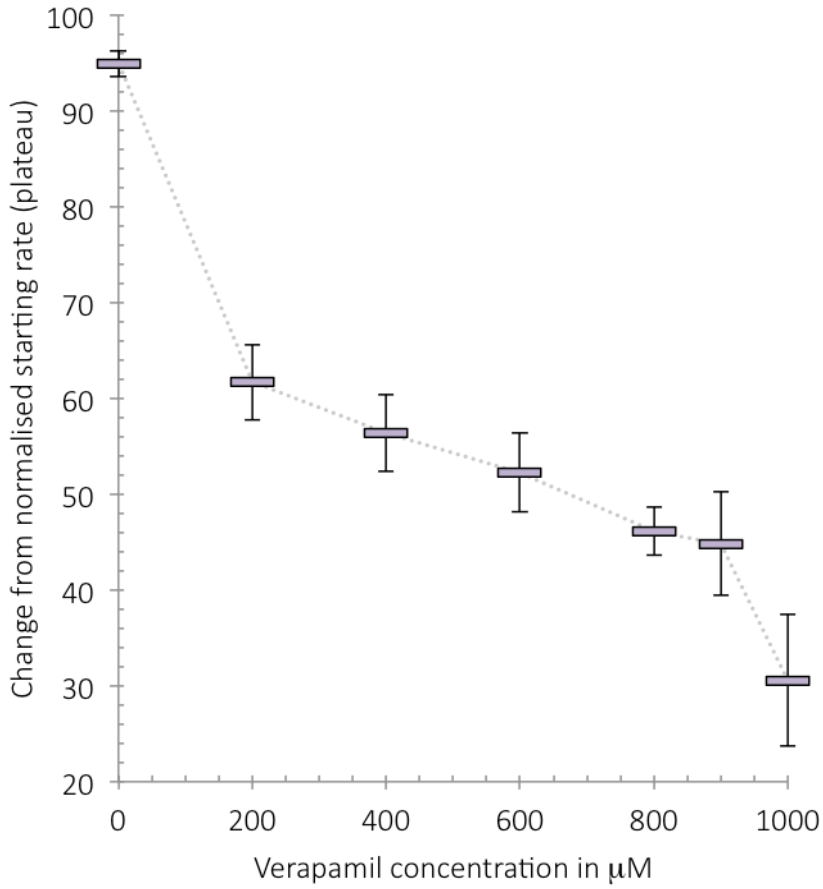


Legend: At time zero (start) no drug has been given. Mean heartrate prior to drug exposure is normalised to 100% for each individual in the population. The x-axis time in minutes. SE error bars on the y-axis indicate how far the sample mean of the data is likely to be from the true population mean. This was deemed more informative than the extraordinarily large SD given by high population variance in response.

A second trial was performed where concentrations of to 900µM, 800µM, 600µM, 400µM and 200µM, as well as a repeat concentration at 1000mM, were exposed to three animals a piece. There were also six drug free controls. Plateaus for the cohorts exposed to 900µM, 800µM, 600µM, 400µM and 200µM were a mean 44.83%, 46.14%, 52.56%, 56.41% and 61.69% of normalised starting heartrate respectively. Results for 1000µM in the second, randomised, trial were a close replicate of the first with a mean plateau at 30.47% of the starting rate. Plateaus were sustained for two hours, after which heartrate

began to decrease once more. Animals showed no recovery three hours after wash-out. It was clear that the one-hour EC50 lay at a lower concentration. Results for brief plateaus sustained between the first and third hours were plotted in the concentration-response curve shown in figure 4d.

Figure 4d. Mean response to each concentration between two hour and three hours



Legend: The mean values from all concentration cohorts are displayed, here the concentration equals zero, these are the results for controls which were exposed to no drug. Plateaus settled upon between two- and three-hours exposure are shown on the y-axis, with the concentration referred to on the x-axis. SE error bars on the y-axis indicate how far the sample mean of the data is likely to be from the true population mean. This was deemed more informative than the extraordinarily large SD given by high population variance in response.

4.2.iv Discussion

Figure 4d suggests that a two-hour EC50 may be found at around ≈ 680μM. A standard EC50 in any concentration curve, for any animal, is customarily set at 24 or 48 hours for cross-comparison across published works. This was not possible here as lab opening times would make it impossible to monitor *Daphnia* response for longer than a nine-hour day, followed by fifteen hours of missing observations before the lab reopens. During this time animals may die, and it would be impossible to discover at when this happened, or whether their deaths were indeed related to the decelerative effect of the drug. However, given the shorter lifespan of *Daphnia* compared to humans (maximum 96 days) a 24hour EC50 may not be sensible as 24 hours of *Daphnia* life may be equivalent to months or years of a human life. And, due to the fast maturation of *Daphnia* a virgin neonate might be pregnant and/or birthing its first clone brood within 24 hours which would then exclude it form the trial.

Another issue was that working with light field microscopy meant image processing could not be automated. Heart edges were rendered as fuzzy and poorly defined. Manual processing of individual frames in each video recording required several hundred hours of labour. The length of time before plateau, which was much longer for other therapeutics, meant an abundant amount of film was required in order to track the response of the heart over time until a plateau was defined. This required large amounts of data processing with a low n-value of trials. Only twelve animals were filmed in trial 1 and only twenty-four in trial 2, however this added up to 224 and 378 films of 720 frames each, so the heart area was circled by hand over 443,440 frames in light field. This problem is later solved by automation once darkfield was acquired. However, while dark field remained unavailable the decision was also made to restrict trials to a smaller number of drugs for quality in results, rather than to aim for less reliable knowledge of a large quantity of drugs. Trials of the heartrate decelerator atropine and phentolamine had also began in tandem with the above, which also made for laborious data requirements with small sample numbers. Atropine and phentolamine trials were dropped in favour of more efficient use of lab time by choosing drugs of possible tachycardic effect going forward. It was also decided that the individual concentrations method would be used given the cumulative concentration technique does not accurately predict EC50 when tested against the single concentration predicted. This is described in chapter 5.

4.3 *Daphnia* Immobilisation

4.3.i Introduction

In an attempt to film multiple *Daphnia* for possible 'high throughput' drug trial, immobilisation methods were researched This would require several *Daphnia* to be immobilised at once, and to be held in place at the same focal point under the microscope. Colmorgen and Paul (1995) immobilised *Daphnia* in the base of small glass Pasteur pipettes, while Penalva-Arana et al (2008) kept them in place by a hair tether using micromanipulators to place them in the field of view. These methods cannot present animals at the same height so the microscopes cannot focus on all simultaneously for filming. Kaas et al (2009) immobilised *Daphnia* on petroleum jelly coated slides, although they did not film multiple *Daphnia* this seemed a means of making this happen. In this experiment we tried out different methods of restraining *Daphnia*.

4.3.ii Methods

Multiple *Daphnia* were placed in various viscous solutions and observed over ten-minute periods for mobility or lack thereof. Solutions included:

- Water chilled for four hours at 4°C
- Methylcellulose, a chemically set gel that is viscous at low temperatures (Ghosal et al 2011) made up according to Sigma Aldrich guidelines.
- Hair gel (Tesco Everyday Value Styling Gel)
- Coconut oil (Coco Loco 100% Organic Aldi's Own)
- Honey (Tesco Everyday Value Clear Honey)

The honey was taken forward for lengthier hour-long observations after proving the most successful at immobilising *Daphnia* without causing cessation of heart beats.

4.3.iii Results

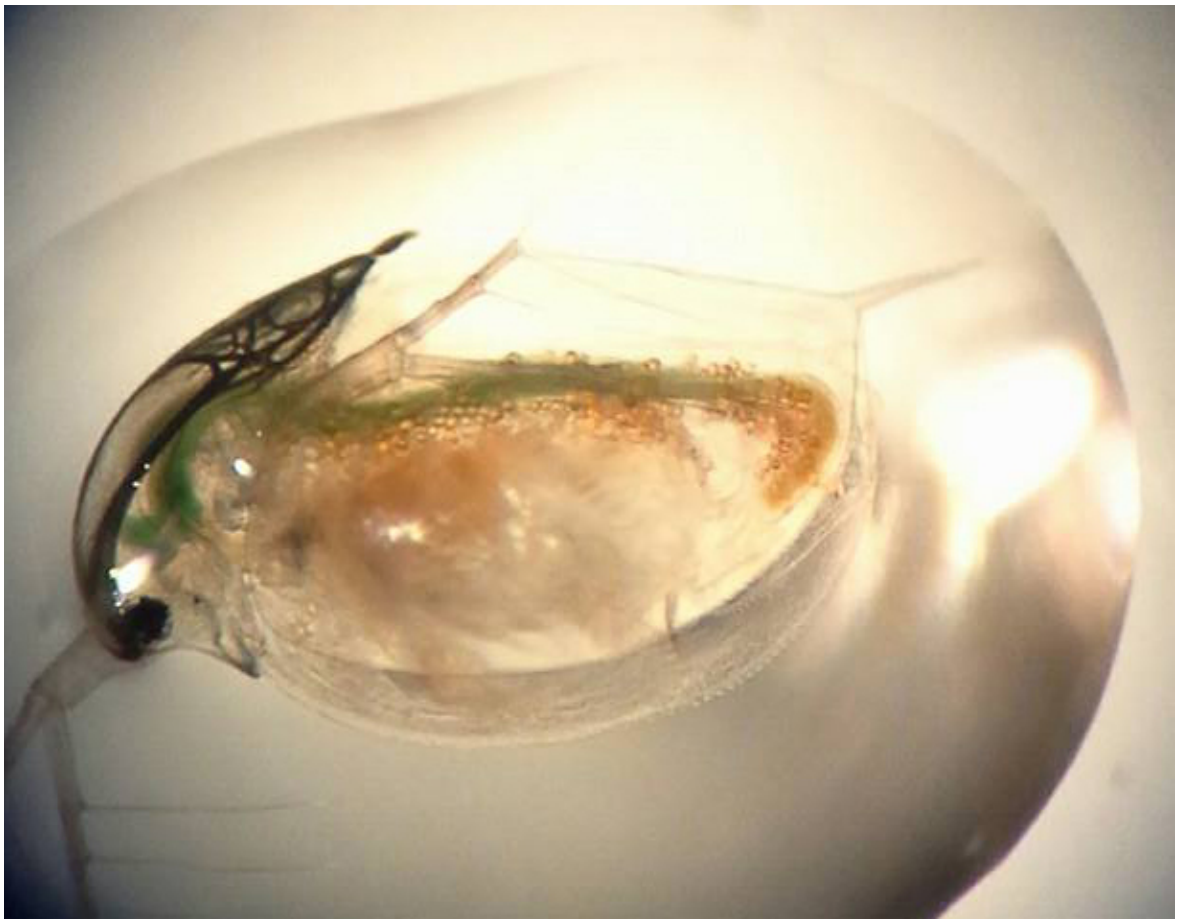
Observations were as follows. Even four hours refrigeration, water larger than a single droplet was not enough to stop multiple *Daphnia* swimming at speed. Methylcellulose took 5 days to stir into solution at 4,000 cP (centipoise) with the heat-stirrer running constantly, yet groups of six, and single, *Daphnia* swam through it easily when cooled. At 8,000 cP it took a fortnight to stir into solution; groups of 6, and individual, *Daphnia*, again, swam through this easily. Hair gel placed over groups of six animals remained lumpy, producing a diffraction effect when viewing *Daphnia*. We could not then see whether *Daphnia* hearts were beating. Coconut oil (20,000cP) killed all 24 animals observed nearly

instantly. The time between placement upon the animals and cessation of heartbeat was too short to measure. Honey (10,000cP) (Bürkle GmbH 2019) settled to a smooth surface film which held animals in place, making cardiac viewing possible. All twentyfour animals survived 10-minute observations and returned to normal swimming activity after washout.

4.3.iv Discussion

Honey appeared a possibility for immobilising multiple *Daphnia*. However, animals did not survive longer periods in honey. It became clear after 38 further Daphnia were observed individually, that no animal survived longer than the first twenty minutes. Also, cardiac dysrhythmia as defined by an irregularity in rhythm, was observed prior to death in all cases. Substances used to immobilise *Daphnia* clearly had potential to cause stress, which might affect the study. Methods of immobilising more than one animal at a time were abandoned in favour of Campbell (2004)'s method of immobilising single animals in a drop of water (or APW or drug laden APW) and pipetting away excess water until an individual remain immobilised, seen in figure 4e.

Figure 4e. *Daphnia* immobilised in a drop of water



4.4 Early Investigations into Cardiac Dysthymia

4.4.i. Introduction

Dysrhythmic hearts were serendipitously captured on film during attempts to immobilise *Daphnia* in honey. These results were presented to the *LJMU School of Pharmacy Seminar Series,* June 2016. The spoken presentation is rewritten here as pre-amble to further investigations **[4.5]** later developed in **[6.0]**.

4.4.ii. Methods

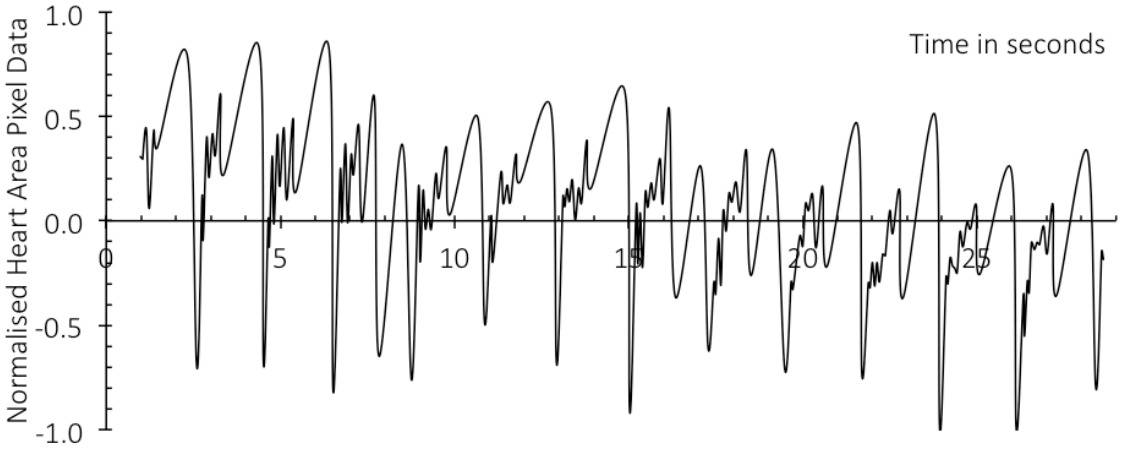
A clear dysrhythmia observed during the honey immobilisation experiment in **4.3**. Heart activity over time was plotted in figure 4f. Data were then tested for a repeat pattern using the autocorrelation coefficient (Chatfield, 1989). Autocorrelation is the correlation of a signal with a delayed copy of itself, giving the similarity between observations as a function of the time lag between them. For this a 7 column Excel spreadsheet was made. Auto covariance is found by dividing the sum of column 6 by the number of periods. Variance is found by dividing the sum of column 7 by the same. Auto covariance is divided by variance to give the first value where lag=zero. For the next "lag" the period values in column 3 are copied and pasted one row down so that correlation is tested between sets of periods one sample apart, and the sums are again repeated for lag=1 and so on.

1. The period observed numbered 1 through N-1, where N is number of heart beats.
2. The period values in seconds (x)
3. The number of pixels at each peak (y)
4. The mean period (x_m)
5. The mean heart area (y_m)
6. $(x-x_m)(y-y_m)$
7. $(x-x_m)^2$

Auto covariance results were plotted as were pixel area change against period.

4.4.iii Results

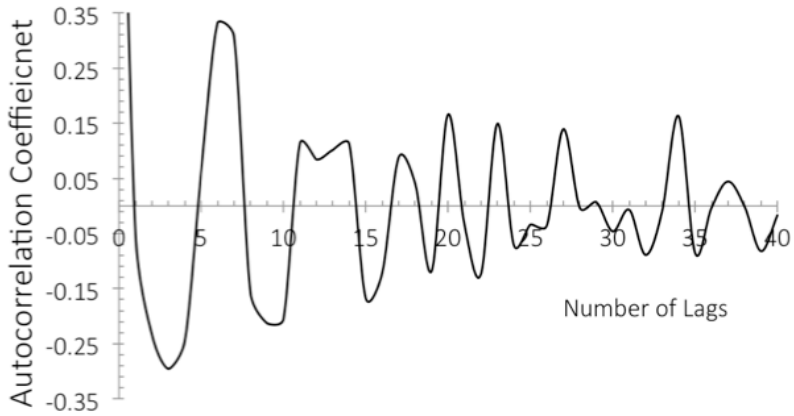
Figure 4f. Approximation of heart activity in an example dysrhythmia over time.



Legend: Heart area over 60fps video was normalised between -1 and 1 and plotted against time to give an approximation of heart activity where points moving towards 1 are heading towards systole and points moving towards -1 are heading towards diastole.

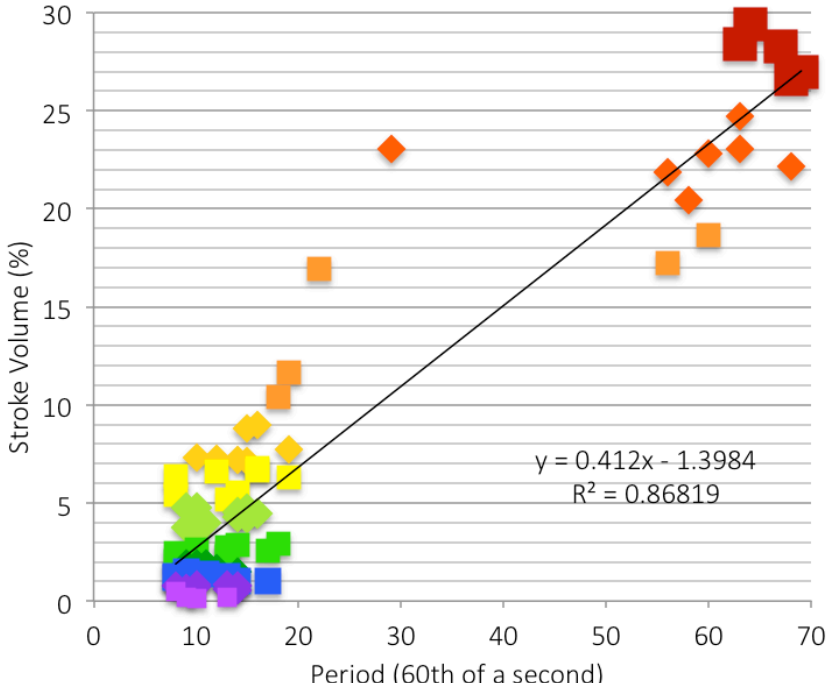
Figure 4f shows a succession of four or more fast heart beats followed by single slower beats, interspersed by seemingly regular systolic intervals, repeating in succession. "Fast" beats are those grouped closely together over time as indicated on the x-axis. Autocorrelation occurred over the 81 heart contractions given in the film where, (N-1)/2=40 lags. Therefore, this was performed for 40 lags. The results were plotted in figure 4g, which shows the period between beats and assesses whether a repeating cycle occurs. The correlation with a value closest to one has a lag of six, therefore where a pattern exists it is most strongly repeated every six beats. However, the numbers are still in the range of randomness, so that the pattern of heart periods within the dysrhythmia is not linear. Each sample fits in two apparent camps of low and high frequency (figure 4h). Large stroke volumes require a longer period to occur, low stroke volumes occur quickly. This pattern sits firmly within two camps.

Figure 4g. Correlation coefficient to test for patterning of heart beats



Legend: On the y-axis, points closer zero have less correlation, while points closer to -1 and +1 have negative and positive correlations. 1 lag = 1/60th of a second.

Figure 4h. Heart period vs. stroke volume in dysrhythmia



Legend: stroke volume % is further catagorised by the following colour scheme:

4.4.iv Discussion

While the dysrhythmia appeared to present a pattern, it was not a linear one. If a pattern can be described over time by a non-linear equation, then it may be 'chaotic'. Dysrhythmias are said to be chaotic, thus may be quantifiable (as discussed in 6.2.iv).

Attempts were made to simulate this waveform using linear methods such as the Fourier series, or by simply adding waveforms together. Taking means from the two camps of low-frequency-high-stroke-volume vs high-frequency-low-stroke-volume seen in figure 4h, and deriving a wave form for each, which were then added together. The following equation was used to approximate waveforms,

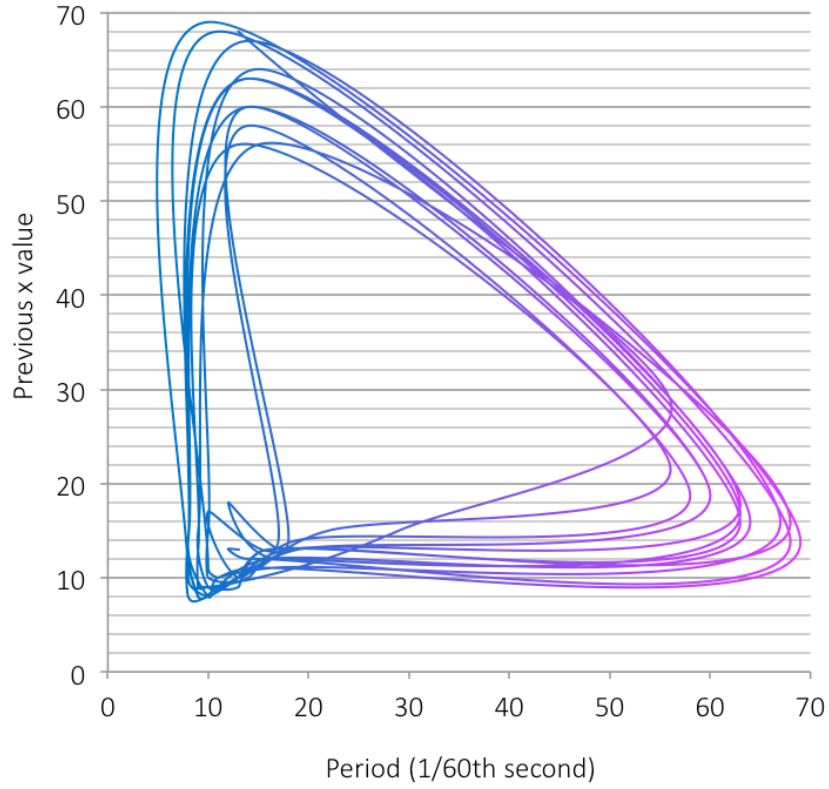
$$y = \left(\frac{magnitude}{2}\right) cos \left(\frac{2\pi}{wavelength} - x\ offset\right) + y\ offset$$

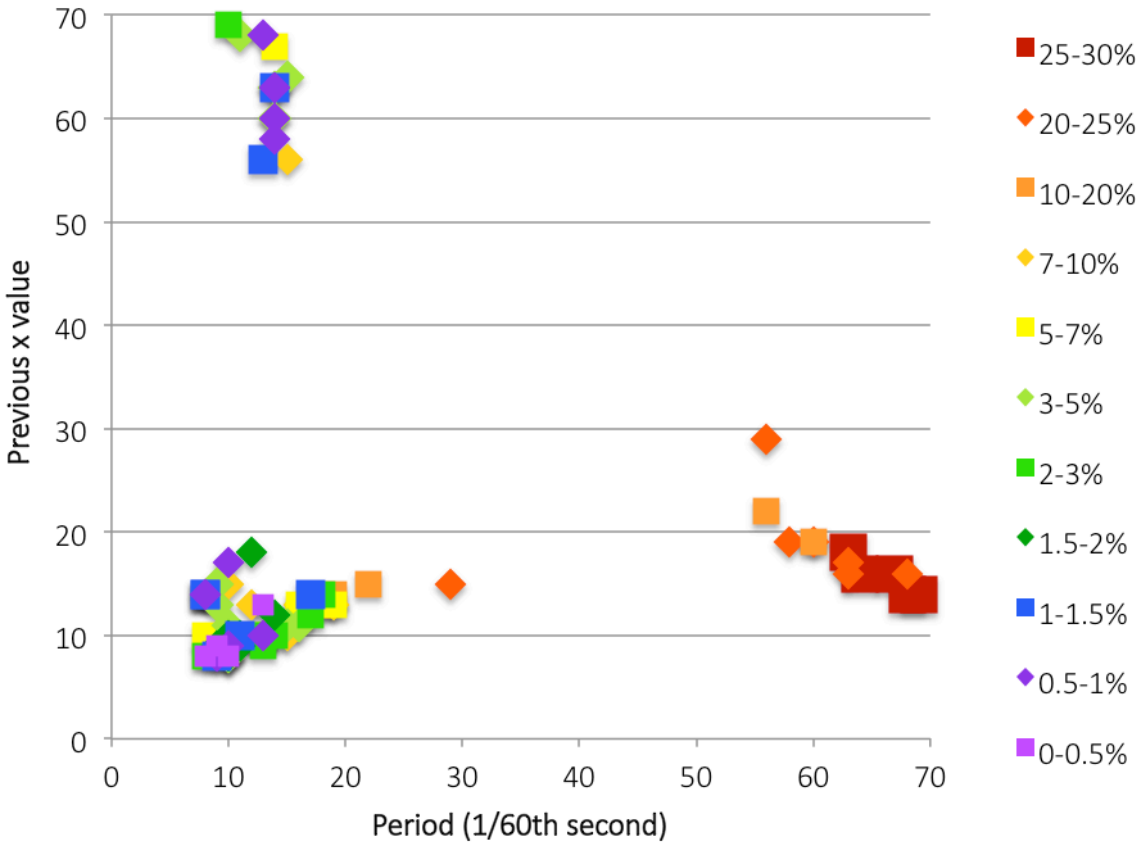
However, we were unable to replicate the wave form in figure 4f using linear methods.

In answer to a similar question, Hsu et al (2012) used delay plots to quantify heart activity in healthy males in their twenties. Heart period were plotted against previous heart period in a series of beats; they used the shape of the graph produced to interpret heart activity. When the subjects were relaxed the plot became oval shaped around the line of identity (LOI). The LOI is where x = y, therefore each beat period equates to the previous beat period. When subjects were exercised the plot became longer and thinner fitting more closely with the LOI.

Daphnia dysrhythmia data were plotted as a delay plot, seen in figure 4i, which is replicated in two different forms. On the left consecutive periods are connected to show how beats are diverging from the line of identity but always return to it in a cyclical manner. This is a non-linear pattern. On the right there is the same graph with the consecutive points unconnected. An attempt is made to indicate stroke volume using a colour scheme, which shows that the largest stroke volumes have a tendency to arrive after groupings of three to four of the smallest stroke volumes during this dysrhythmia. These figures indicate a non-linear repeating pattern.

Figure 4i. Delay Plots of the same dysrhythmia





Legend: Consecutive periods of the dysrhythmia plotted as delay plots. On the top the colour added for aesthetic and has no significance but to enable the eye to realise that period patterns follow a repeating circuit. On the bottom colours are used to identify the largest stroke volumes (red, orange…) in order down to the lowest stroke volumes (… yellow, green, blue, and purple).

This pattern may indicate the heart's homeostatic mechanism attempting to drive heart beat patterns back to a central equilibrium. Lorenz called this a strange attractor, represented in a delay plot by the line of identity. Rosen et al (2015) interpret cardiac memory as a form of stress response; but found it can also emphasise abnormal rhythms overtime. Oestreicher (2007) suggested that these abnormalities of rhythm may be an early predictor of cardiac disease. Delay plots have been suggested as a method of determining differences in the severity of an arrhythmia, where severity is defined by the heart's divergence from normal rhythm. It is known that dysrhythmias can reach a Rubicon, a point of no return, which leads to fibrillation and death. However, there is also sometimes recovery from dysrhythmia. Similarly, *Daphnia* were observed recovering from dysrhythmic states. We postulated that non-linear measurements might be applied to predict individual responses. For example, to predict the result of toxicological insult starting conditions are known, or to differentiate individuals with seemingly healthy hearts to better individualize patient care. We approach this idea in chapter **4.5** and develop it chapter **6.0**.

4.5 Comparison of Dysrhythmia Differentiating Techniques

4.5.i. Introduction

This work was accepted for poster presentation at the Northern Cardiovascular Research Group 2017 conference, seen in figure 4j. Dysrhythmias were observed serendipitously in early concentration-response explorations with tyramine. Exposure to 70 minutes of 200mM lactose was used to induce dysrhythmia in Campbell et al (2004); however, the response here to the same concentration was only a mild tachycardia. All lactose-exposed animals gained pink colouration around the heart over time, indicative of oxygen deprivation. Fibrillation and bradycardia were deliberately induced in animal C after 80 minutes exposure to doxorubicin. This does not form a methodology but merely an exploratory phase – it is films of these explorations which were taken forward for this study, in which systems of quantifying dysrhythmias are researched, applied and compared.

Figure 4j. Northern Cardiovascular Group Conference Poster, June 2017

A new model for quantifying cardiac arrhythmia

LIVERPOOL JOHN MOORES UNIVERSITY

Andrew M. Angus-Whiteoak
Supervisors: Dr. Peter E. Penson,
Dr. Neil Henney and Dr. Gillian Hutcheon

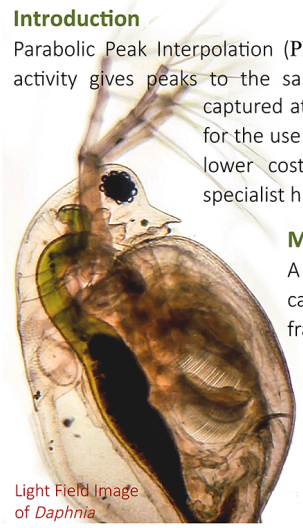
Context

Measuring heart rate variability is a method of risk stratification for serious cardiac arrhythmia and sudden cardiac death. Healthy hearts make continuous adjustments oscillating around mean sinus rhythm to keep within a healthy range. However data interpretation is qualitative and dependent on expert medical opinion. Attempts at quantification largely rely on simulations, while studies which combine quantification with direct observation are rare. By working with a transparent animal *in vivo* we propose to thoroughly test all quantification strategies, and work towards a more reliable predictive model.

Introduction

Parabolic Peak Interpolation (PPI) of 60p HD video of heart activity gives peaks to the same effect size as heart data captured at 1000 Hz. This method allows for the use of standard DSLR cameras with lower cost and higher resolution than specialist high rate cameras.

Daphnia were filmed prior to, and following, induction of cardiac arrhythmia. Data was used to test reliability of quantification methods across the literature. Results point to the superiority of non-linear analyses such as Ehler's Index[1]. On-going work examines the scope for their application in predicting cardiac arrhythmia in real time.

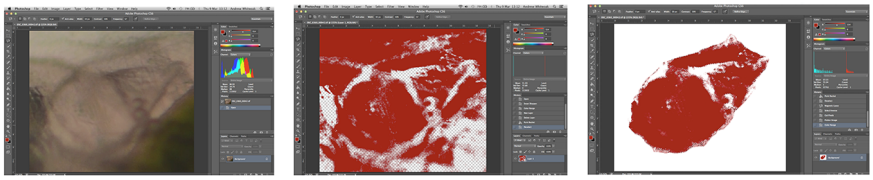


Light Field Image of *Daphnia*

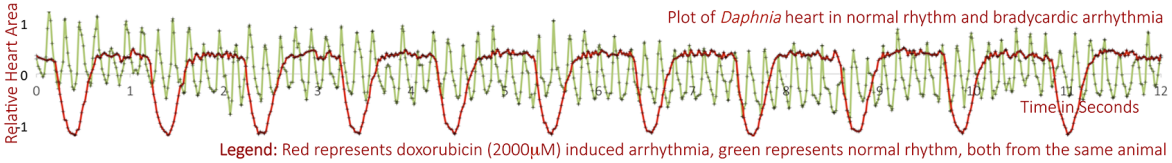
Methods

A novel semi-automatic process captured heart pixel area, each frame was losslessly encoded; edges defined and inverse area filled; heart area is manually selected giving pixel count. True peaks in time domain data and pixel were found by PPI[2], giving heart rate and stroke volume. Normal rhythm and arrhythmia data were analysed in linear terms such as ellipse fitting[3], point density plots, standard deviation of successive differences[4]; and non-linear terms including complex correlation[5], multi-scale ratio feedback analysis[6], median stepping increment[7] and finite time growth[8].

Overview of steps taken in Adobe CS6



Plot of *Daphnia* heart in normal rhythm and bradycardic arrhythmia

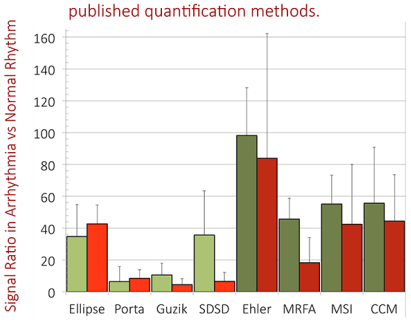


Legend: Red represents doxorubicin (2000μM) induced arrhythmia, green represents normal rhythm, both from the same animal

Results

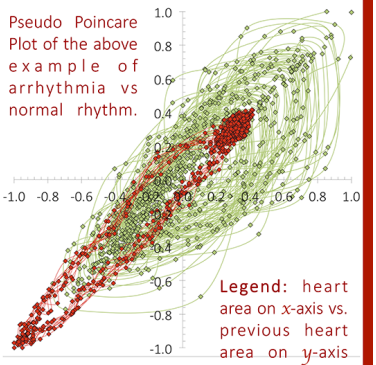
Electrical spectrum from *Daphnia* does not exceed 20Hz[9]. As sampling theorem[10] predicts, a 60 Hz sampling rate produces discrete data samples needed to accurately extrapolate peaks, giving exacting area and time domain data via PPI. While a significant P value (P 0.0066; 0.05) is to be expected for high sample numbers[11] (125+ peaks /film in normal rhythm); PPI and interpolation to 1020fps via filtering and up-sampling produced effectively identical results; differences being trivial in paired T-tests (Cohen's d < 0.001 SD).

Histogram showing relative success of published quantification methods.



Legend: Green = period; red = cardiac output; light = linear techniques; dark = non-linear techniques.

Pseudo Poincare Plot of the above example of arrhythmia vs normal rhythm.



Legend: heart area on x-axis vs. previous heart area on y-axis

Progress

Ongoing work will build a body of data by which quantification methods may be compared for their ability to differentiate healthy vs arrhythmic heart states. Full automation will soon be possible due to recently acquired dark field capability.

1. Ehlers C. L. et al (1998) Low Doses of Ethanol Reduce Evidence for Nonlinear Structure in Brain Activity. J. Neur. 18 (18) 7474-7486.
2. Smith J. O. (2011) Spectral Audio Signal Processing: Sinusoidal peak interpolation. Accessed at https://www.dspreated.com/freebooks/sasp/
3. Mohebbi M. and Ghassemian H. (2012) Prediction of paroxysmal atrial fibrillation based on non-linear analysis and spectrum and bispectrum features of the heart rate variability signal. Comp Meth Prog Biomed 105 (1) 40-9.
4. Galland B. C. et al (2006) Heart rate variability and cardiac reflexes in gestational age infants. J. App .Phys. 100(3) 933-939.
5. Karmakar C. K. et al (2009) Complex Correlation Measure: a novel descriptor for Poincaré plot. BioMed. Eng. 8 (17) 280-305.
6. Huo C. et al (2014) A multi-scale feedback ratio analysis of heartbeat interval series in healthy vs. cardiac patients. Med. Eng. & Phys. 36 (12) 1693–1698.
7. Gong Y. et al (2015) Predict Defibrillation Outcome Using Stepping Increment of Poincare Plot for Out-of-Hospital Ventricular Fibrillation Cardiac Arrest. BioMed. Res. Int. ID 693472 7 pages.
8. Wessel N. et al (2000) Nonlinear analysis of complex phenomena in cardiological data. Herzs. Elek. 11:159–173.
9. Freund J. A. et al (2002) Behavioural Stochastic Resonance: How the Noise from a Daphnia Swarm Enhances Individual Prey Capture by Juvenile Paddlefish. J. theor. Biol. (214) 71.
10. Nyquist H. (2002) Certain topics in telegraph transmission theory. Proc. IEEE 90 (2) 280-305.
11. Sullivan G. M. and Feinn R. (2012) Using Effect Size—or Why the P Value Is Not Enough. Journal of Graduate Medical Education 4 (3) 279-282.

4.5.ii. Methods

Literature searches were performed with search terms such as “Poincare” or “delay plot” or “non-linear analyses” both with and without an ‘AND’ gate which was followed by “arrhythmia” or “dysrhythmia” or “heartrate”, and various alternatives to each term along with various named categories of arrhythmia and named categories of non-linear analysis. Databases are listed in references. All systems of distinguishing dysrhythmias via interpretation of delay plots, (as well as linear and non-linear) from across the literature, are described 3.6, and discussed in chapter 6.0 at length. For this study, dysrhythmias were processed as per 3.4 to 3.5, and categorised as per 3.6.i. Light field was not the ideal method for this work but was the only means available until a dark field adapter was procured. Finally, all published systems were also applied to the data as per 3.6.ii. Application of each scoring system gave a score to baseline data which denoted that system’s own scores, and a paired score to dysrhythmia data. The percentage difference between the baseline score and dysrhythmia score was calculated. Then, this difference was taken as a number between 1 and 0, where scores closer to 1 represent the scoring systems which most differentiated two rhythms.

4.5.iii Results

A strong dysrhythmic response to tyramine came after 104 animals treated in the same manner showed no such response. A mild dysrhythmic response to lactose occurred in one animal. Twenty-two animals exposed to lactose in the same manner showed no such response. It might not be appropriate to attribute the dysrhythmias to tyramine and lactose, as drug exposure may have been only the tipping point in *Daphnia* which could have been less healthy than the population. The very first animal placed in doxorubicin, and all animals, thereafter, suffered severe and rapid onset dysrhythmia, making it a very useful drug for examination of dysrhythmias. These dysrhythmias are summarised in table 4k. Animal A presented with Torsades-de-Pointes-like rhythm when treated with tyramine. Animal A was captured for only 6 seconds in its pre-exposure state as the ‘noise’ seen over light field made heart area at both the beginning and end of the film impossible to elucidate clearly. Animal B produced a mild tachycardic response to lactose. Animal C gave a strong bradycardic response, with mild fibrillation. These are also visualised in figure 4L. Delay plot data for each animal in normal rhythm and dysrhythmia are shown in Figure 4m, Lactose induced dysrhythmia (B) maps close to normal heart activity, while the Torsades-de-Pointes (A) and bradycardia with fibrillation (C) are clearly different.

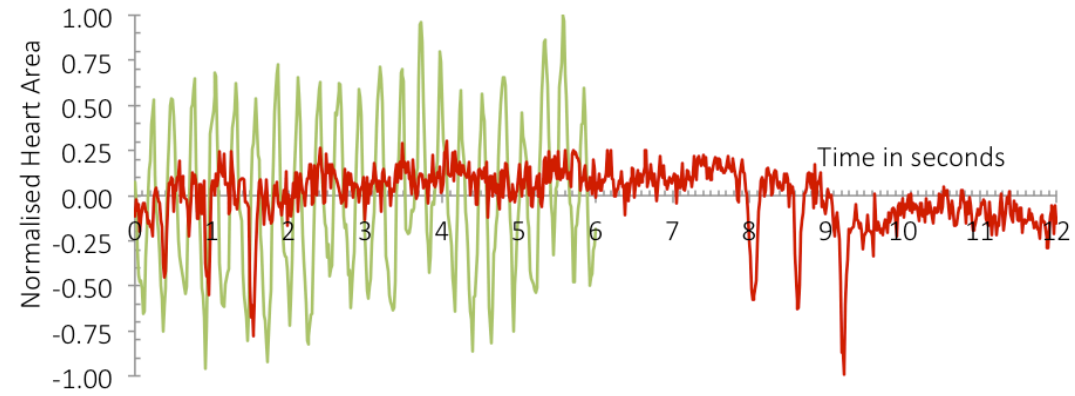
Table 4k. Dysrhythmias filmed under light field microscope

Daphnia	Chemical	Hours:Mins	Heart Action	Recovery	No Effect
A	320µM Tyramine	2:15	Fibrillation with Torsades de Pointes	Dead <1hr	104
B	200mM Lactose	1:10	Mild tachycardia	Recovered <2 hrs	22
C	2000µM Doxorubicin	1:20	Fibrillation and Bradycardia	Dead <2 hrs	0

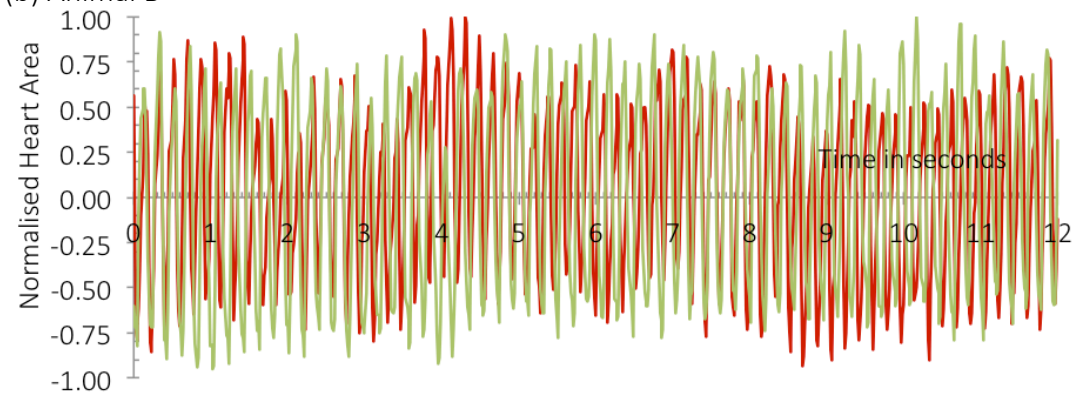
Legend: No Effect= No. animals in the same cohort which bore no dysrhythmic effect. Hours:Mins= Time of exposure before apparent dysrhythmia.

Figure 4L. Three dysrhythmias chosen for exploration of non-linear analysis systems.

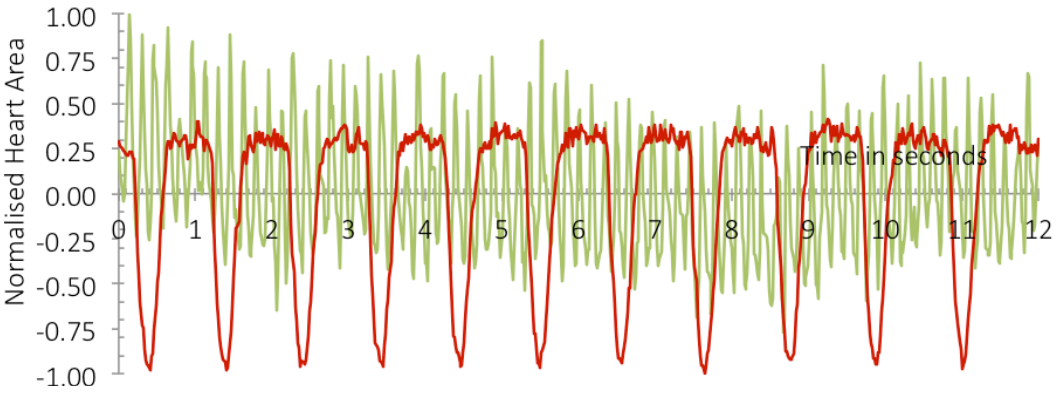
(a) Animal A



(b) Animal B

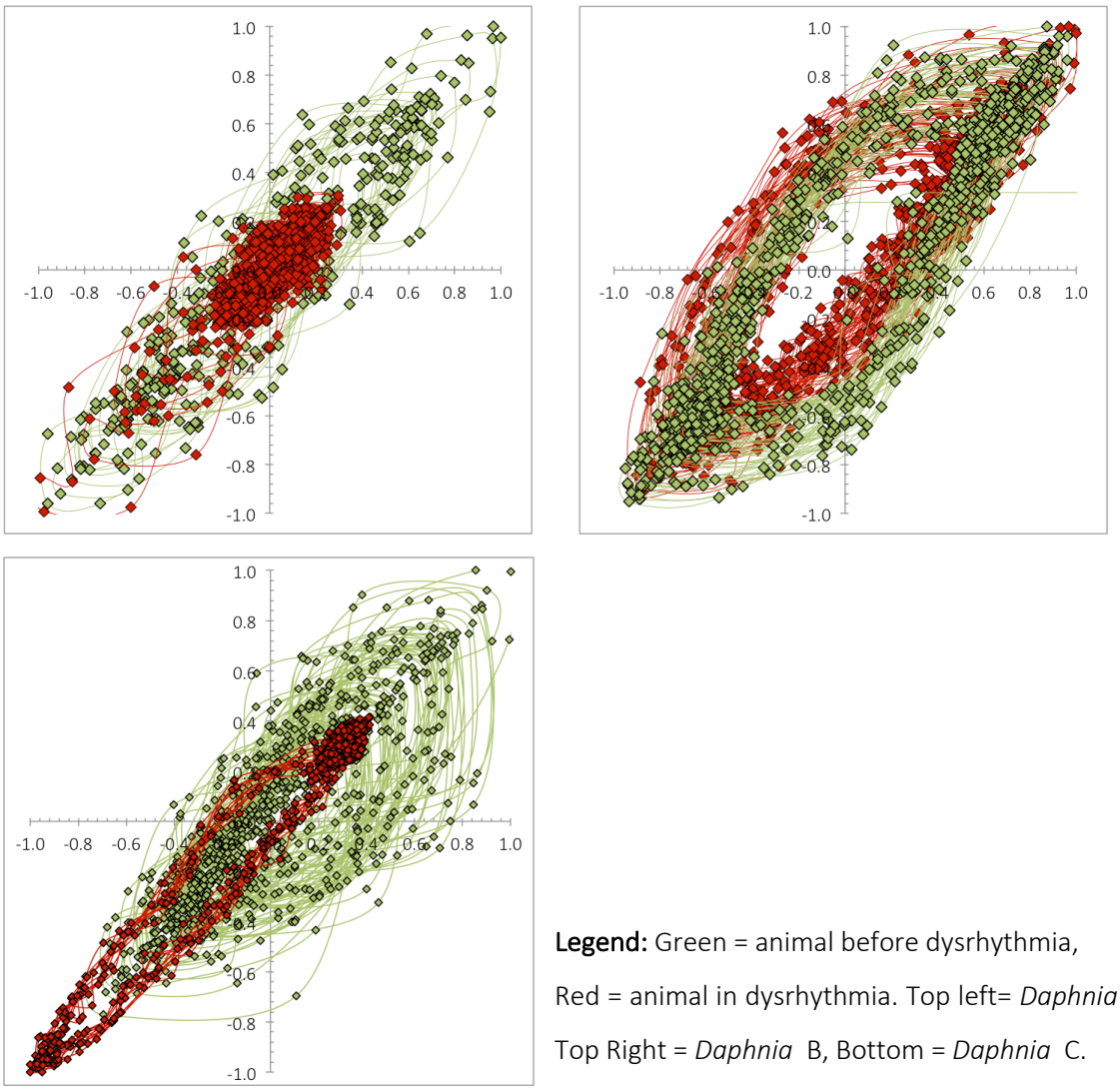


(c) Animal C



Legend: Green= heart before drug exposure, red= drug induced dysrhythmia.

Figure 4m. Delay plots of heart area data in animals A, B and C



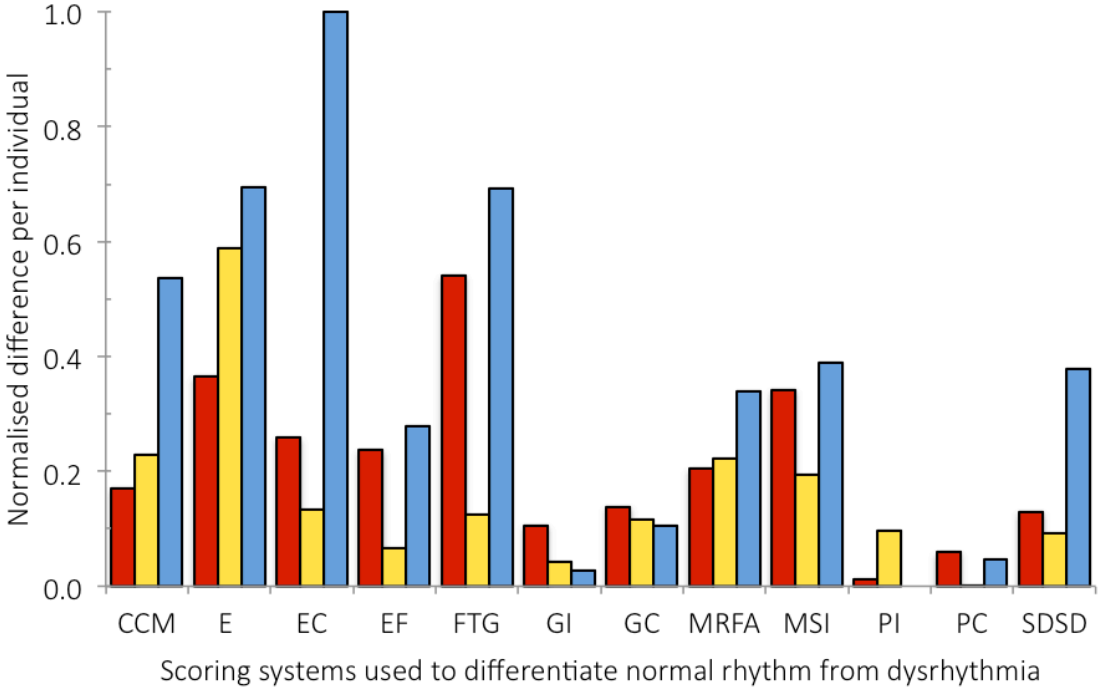
Legend: Green = animal before dysrhythmia, Red = animal in dysrhythmia. Top left= *Daphnia* A, Top Right = *Daphnia* B, Bottom = *Daphnia* C.

4.5.iv Discussion

Scoring systems were compared both for their ability to differentiate paired normal rhythm and dysrhythmia, and to see whether they could differentiate one dysrhythmia from another. For figure 4n and figure 4o scoring systems are abbreviated as follows: CCM= Complex Correlation Measure. E = Ehler's index. EC=Ehler's index Cloud adaptation. EF = Ellipse Fitting. FTG = Finite Time Growth. GI = Guzik's Index. GC = Guzik's index Cloud adaptation. MRFA = Multiscale Ratio Feedback Analysis. MSI = Median Stepping Increment. PI = Porta's Index. PC = Porta's index Cloud adaptation. SDSD = Standard Deviation of Successive Differences. Figure 4n shows the relative success of scoring systems in differentiating dysrhythmias from paired data in normal rhythm. For this we might expect that a useful system would find a greater difference between normal rhythm and high-risk dysrhythmia, than between normal rhythm and low risk dysrhythmia. And, this does indeed occur. *Daphnia* C (shown by the blue bars in the histogram) presented with

fibrillation and did not survive. *Daphnia* C is most differentiated from normal rhythm across almost all systems barring GI, PI and PC. *Daphnia* C was best differentiated by EC, FTG and EI. CCM differentiates the dysrhythmia-baseline pairing by just over 50% while all other systems do not differentiate as well between baseline and fatal fibrillation with bradycardia. The various scoring systems overall do not do so well for *Daphnia* A, which presented with fibrillation with Torsaides de Pointes before death, shown by red bars in the histogram.

Figure 4n. Can scoring systems differentiate normal rhythm from dysrhythmia?

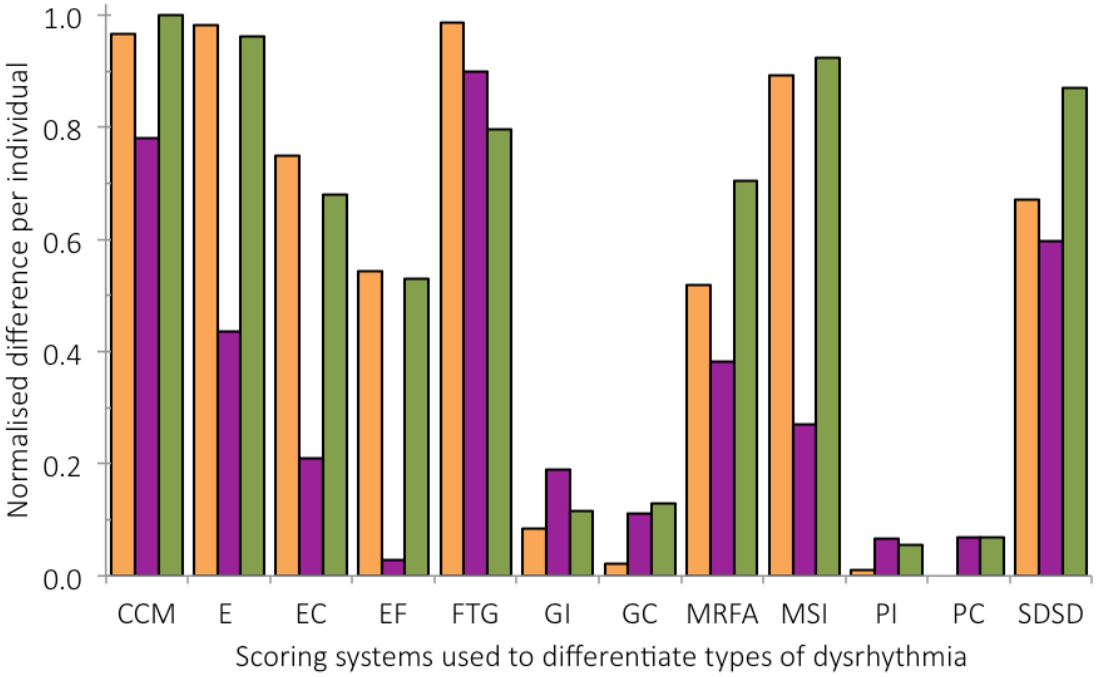


Legend: Scores for baseline heart rhythm and dysrhythmia were found using published scoring systems. Red= Subject A; fibrillation with Torsaides de Pointes; died. Yellow= Subject B; mild tachycardia; survived. Blue= Subject C; fibrillation and bradycardia; died.

For *Daphnia* A, differentiation between heart rhythm and dysrhythmia are best found by FTG which gave a 54% difference between its score at baseline and score for *Daphnia* A in dysrhythmia. No other system had even as much as 40% difference between the two scores given. *Daphnia* B (yellow bars) presented with a mild tachycardia but recovered to normal rhythm and survived. We might be least interested in systems that find a large difference between this and normal rhythm as risk factors were lower in retrospect. Just so, most systems find only weak difference between the paired data for *Daphnia* B. However, EI, CCM and MRFA find a greater difference between the pair for *Daphnia* B than they found for *Daphnia* C, suggesting they may not be appropriate for predicting risk from baseline data. GI, GC, PI, PC, SDSD, MSI and MRFA were unable to achieve even close to a

50% difference in scores for any pairing. From this data it appears that linear methods are far weaker than non-linear methods at differentiating strengths of dysrhythmia from baseline rhythm. This initial data indicates that FTG and EC may be most likely to offer success in establishing as substantive predictive technique in further investigations **[6.0]**.

Figure 4o. Can scoring systems differentiate dysrhythmias?



Legend: Scores for all dysrhythmias were found using published scoring systems. Scoring systems abbreviations are given in figure 4m. Orange= A vs B, Purple= A vs C, Green= B vs C.

Figure O represents each system's success in differentiating dysrhythmias. Scores for Torsades de Pointes are compared to those for mild tachycardia in animals A-vs-B (orange in the figure). Scores for Torsades de Pointes are compared to those for bradycardia with fibrillation in animals A-vs-C (purple in the figure). Scores for mild tachycardia are compared to those for bradycardia with fibrillation in animals B-vs-C (green in the figure). GI, GC, PI and PC perform poorly, and cannot be said to have success in differentiating any dysrhythmia from another. Of the remaining scoring systems, we would expect A-vs-B and B-vs-C to have the highest difference in scores between baseline and dysrhythmia as B is a relatively mild dysrhythmia. This is indeed the case for nearly all other systems (CCM, E, EC, EF, MRFA, MSI and SDSD), but not FTG. While FTG does not follow this pattern, interestingly, it produces the strongest differentiations between dysrhythmias overall.

Scoring systems are clearly stronger at differentiating dysrhythmias themselves than differentiating them from their paired data in normal rhythm. This is interesting as it might indicate that a stronger correlative relationship exists between paired data from the same animal than we might expect when looking at seemingly normal rhythm vs a dysrhythmia. In chapter 6 we explore this correlative relationship and its possibility for use as a predictive tool of cardiac risk. Further early investigations in this regard were presented in a British Cardiovascular Society conference, as seen in figure 4p. Early investigations showed a positive correlation between the normal heart rhythm and drug response using Finite Time Growth.

4.6 Concluding Statement

In this chapter we found that *Daphnia* cultured *in situ* presented with a toxic insult survive at higher concentrations than their *ex situ* counterparts. *Ex situ Daphnia* are more prone to death at lower concentrations. It was decided that the seemingly 'healthier' *in situ Daphnia* would be used in trials going forward. This chapter found that the cumulative dose response method does not accurately predict EC50, if the prediction is tested against individual concentrations over the same time period. This finding agrees with Dunne (1979). It was decided that the individual concentrations technique would be used in ensuing chapters. This chapter also found that *Daphnia* response to decelerative heart therapeutics takes place over several hours, even at high concentrations. With the resources available in the first two years of this research image capture automation could not yet be achieved, so it was decided that accelerative heart therapeutics would be tested next so as to aim for a higher sample number given equal effort. This chapter pursued various means of immobilising *Daphnia* for cardiac viewing and could not find a means to immobilise a group of *Daphnia* so that all might be held within the same focal plane. We found that trapping *Daphnia* within a drop of water was the least harmful way to immobilise them, all other methods caused death or dysrhythmia. Finally, this chapter further investigated the nature of *Daphnia* dysrhythmia in terms of quantifying heartrate variation. This opened up a possibility for further research, investigating heart rhythm as well as heartrate.

Figure 4p. British Cardiovascular Society poster, July 2017

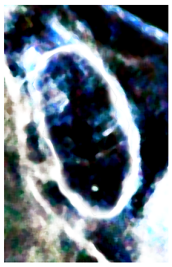
Using Chaos to Predict Personalised Effects of Heart Therapeutics

LIVERPOOL JOHN MOORES UNIVERSITY

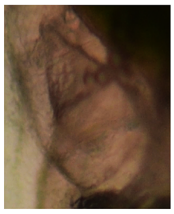
Andrew M. Angus-Whiteoak
Supervisor: Dr. Peter E. Penson

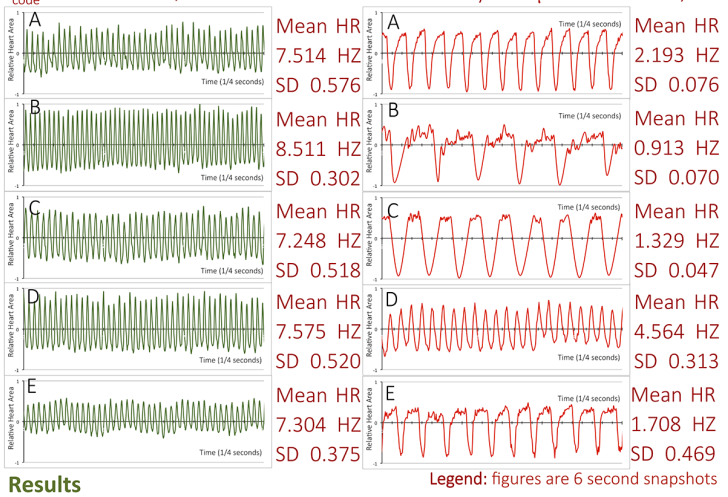
Introduction

Traditional dose-response curves have limited application to personalised medicine as it is difficult to generalise from heterogenous populations. Finite Time Growth[1] (FTG) is a non-linear analyses arising from Chaos theory used to assess heart health. *Daphnia* are aquatic invertebrates <1mm across as neonates. The translucent carapace lends to *in vivo* study of heart response. Our ongoing study links individual fitness with variations in response to therapeutics. By adding this third dimension a better prediction of individual treatment can be made.



Heart in dark field above vs heart in light field, below.





Legend: figures are 6 second snapshots

Results

During work-in progress, so far five *Daphnia* were exposed to 2000µM Doxorubicin. FTG analysis defined all animals as 'healthy' with results close to zero. However, even minute variations in health status were magnified in drug response. Spearman's Rank Correlation show a significant positive relationship between the two with a critical value of 0.900 (significant at the 0.05 level).

Discussion

Results so far indicate that FTG may prove a highly useful method of determining personalised treatment for cardiovascular therapeutics. Further investigation across the dose-response curve is in progress so that a fully three dimensional dose-health-response curve may be established for this drug.

1.Wessel N. et al (2000) Nonlinear analysis of complex phenomena in cardiological data. Herz. Elek. 11:159–173.
2.Angus-Whiteoak A. M., Penson P. E., et al (2017) A New Model for Quantifying Cardiac Arrhythmia. Poster Presentation 25th Northern Cardiovascular Research Group meeting. Available on Researchgate https://www.researchgate.net/publication/316700401
3.Smith J. O. (2011) Spectral Audio Signal Processing; Sinusoidal peak interpolation. https://www.dsprelated.com/freebooks/sasp/

Methods

High speed films of *Daphnia* heart activity were made. Use of dark field resolved variations encountered in previous light field studies[2]. Per animal, 720 frames (≈ to 60+ beats in normal rhythm) made before and after doxorubicin treatment captured heart area over time. Parabolic Peak Interpolation found true peaks[3]. For each film, every 9 consecutive beat periods, were taken as a 9D object in Poincare space. Nearest neighbours (NN) were found for all 9D vectors using a program written by the author in Excel. All peaks were pasted as a column which then cascaded horizontally and vertically in 120x120 fields. The equation,

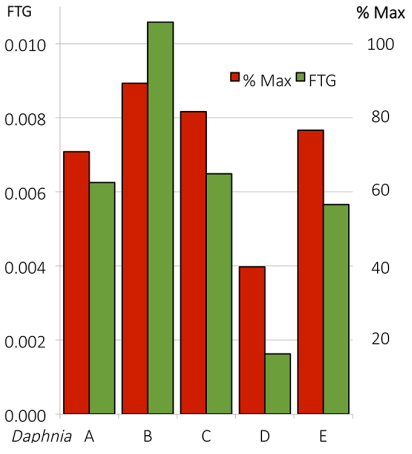
$$d = \sqrt{\begin{matrix}(a_1-a_2)^2+(b_1-b_2)^2+(c_1-c_2)^2+(d_1-d_2)^2+(e_1-e_2)^2\\+(f_1-f_2)^2+(g_1-g_2)^2+(h_1-h_2)^2+(i_1-i_2)^2\end{matrix}}$$

was then applied to each coordinate (a,b,c,d,e,f,g,h,i), returning a value which shows increasing nearness of neighbours approaching zero. Subscripts indicate consecutive vectors. Results were then 'sorted' by lowest NN value, and each consecutive 9D vector compared via the Lyapunov-like FTG equation,

$$\lambda_k^{(n)} = ln\frac{\sqrt{[\Sigma(X_2-\bar{X}_2^{NN})^2]}}{\sqrt{[\Sigma(X_1-\bar{X}_1^{NN})^2]}}, k = 0, \ldots 9-(n-1),$$

λ is growth rate, with an embedding dimension of 9. A Euclidean norm of difference ratios between all vectors was taken as an indication of heart health. Values near 0 show beat-to-beat stability; closer to 1 greater divergence. Results were then linked to drug effectiveness as given by % max response in each animal.

Figure showing Finite Time Growth (FTG) and Drug Response Results for Each Animal



5. Are *Daphnia* a Model for Cardiotherapeutic Response Trials?

It has been proposed that *Daphnia* may be applied as a model for cardiovascular science (Campbell et al 2004). This research was funded based on this proposition, specifically to test whether reliable pharmacological data could be gained from observation of *Daphnia* cardiac response. Initial trials sought to establish whether *Daphnia* might be an appropriate model system for cardiovascular therapy. Water soluble drugs were used to best mimic toxins in the natural *Daphnia* environment, to parallel existing research by ecologists and toxicologists. And, also for ease of drug delivery to the trial *Daphnia*. We know that while some aspects of the *Daphnia* cardiac system are thought to be similar to the mammalian system, many others are not. The intended action of vasopressors **[1.8]** and vasodilators **[1.7]**, for example, would be of little use to investigate in animals which lack blood vessels. Some cardio active drugs are inappropriate, For example anticoagulants **[1.3]**. There are no known platelets in invertebrates; fibrin-like components have been found in the horseshoe crab, *Limulus polyphemus* (Theopold et al 2004) but this is not directly related to cardiac action. Cholinergic modulators **[1.11]** while fascinating in their effects on memory, mobility, development and so forth would also be inappropriate to a cardiac response study. Heartrate change is one of the simplest effects to quantify. Having established the parameters of testing water soluble drugs, with an effect on heartrate, we chose from drugs available in lab, to establish whether the general response of *Daphnia* would follow a comparable response to that of humans.

We initially began by examining drugs with decelerative effect both in initial trials with the verapamil **[4.2]**, and metoprolol; a study performed in parallel to the verapamil trial and discussed in this chapter. Both the verapamil and metoprolol studies produced severely delayed *Daphnia* responses, even at high concentrations. This prevented large sample numbers from being trialled in an efficient time frame, due to the hours of monitoring per *Daphnia* before a response occurred. The length of time before a response may also add a confounding factor to the trials of possible oxygen deprivation due to time spent in a limited volume of solution. Therefore, we moved on to other, water soluble drugs, available in the lab. This time choosing the cardio-accelerants octopamine, phenylephrine and tyramine. All pharmacological agents were chosen from those already available in the lab and originally sourced from Sigma-Aldrich (Sigma-Aldrich Ltd., New Rd, Dorset SP8 4XT) This work produced interesting observations with regard secondary effects in phenylephrine, such as muscle spasms and dysrhythmia. We therefore investigated the

secondary effects themselves. We found that phenylephrine causes abortion, but only at a specific stage of foetal development. We then investigated why secondary effects might occur. This led us to test the appropriateness buffers used in current literature. *Daphnia* may not be appropriate as a medical model, as they respond with extreme sensitivity to microlevel changes in the environment.

5.1 *Daphnia* Response to Metoprolol

5.1.i Introduction

Metoprolol, and its known published effects on the invertebrate heart, were discussed earlier **[1.1]**. To re-cap, it is a high affinity β1 selective beta-blocker and therefore deemed cardio selective as β1 receptors are located in the human heart and mediate heartrate and contractility. β1 blockers/antagonists reduce contractility and slow heartrate. This study was performed in parallel with the initial verapamil study **[4.2]**, and thus also initially uses but later discards the dubious cumulative dose response method **[3.4.i]** which becomes clearly inappropriate for the high concentrations used here. We know from the review in chapter 1, that for *Daphnia* in culture medium have an 48hrLD50 response to metoprolol at around 750μM to 1640 μM. This means that after 48 hours exposure roughly 50% of *Daphnia* are dead at concentrations between these two. Given lab accessibility precludes the possibility of an observable trial for any longer than nine hours, we began with concentrations around these levels in the hope of observing effects within this nine-hour time frame. Three trials were performed, the first two used the cumulative dose response method **[3.4.i]** simply because these trials were performed at the same time as verapamil trials. The third trial used the individual concentrations method **[3.4.ii]** and shows once again that cumulative dose response cannot predict outcomes for individual concentration trials.

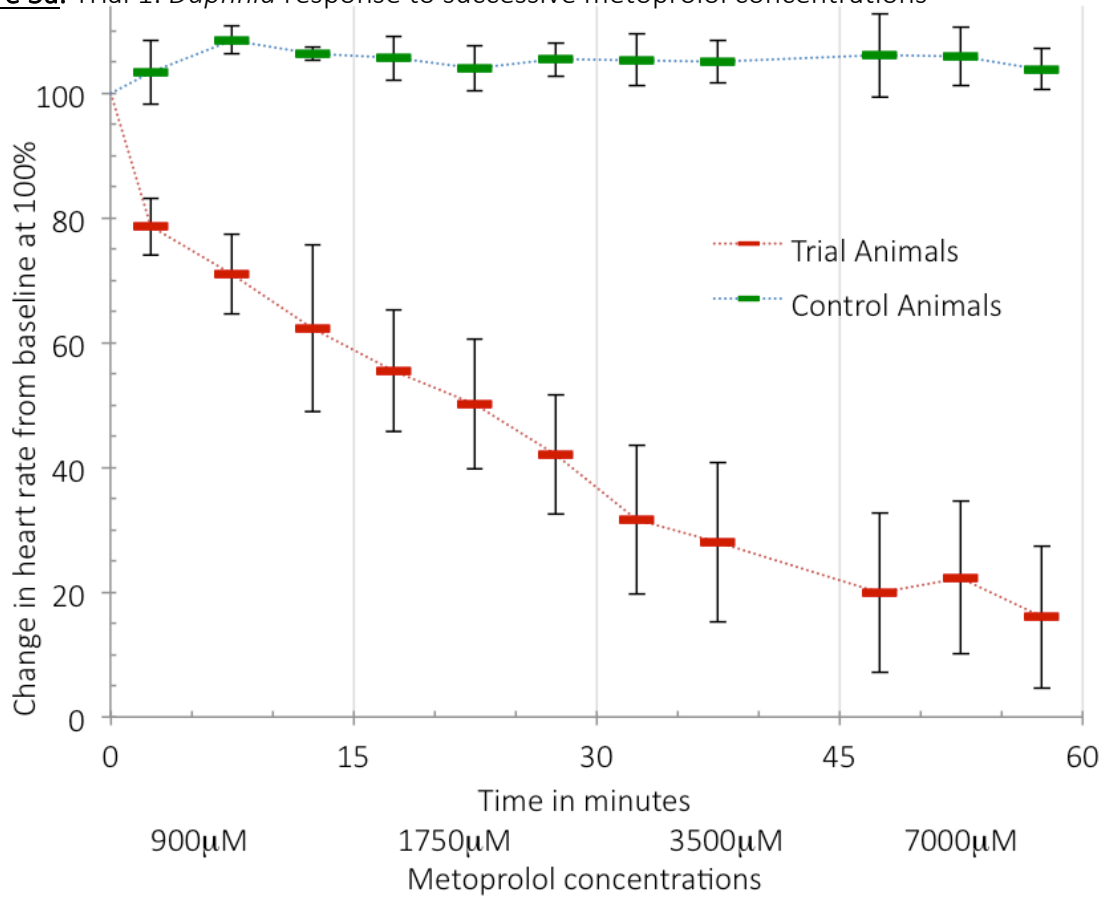
5.1.ii Methods

Metoprolol was dissolved in dH20. In the first trial, successive concentrations of 900μM, 1750μM, 3500μM and 7000μM were tested as discussed in **3.4.i**. Higher concentrations were made up, at 7000μM it was visible even by eye (before video analysis) that heartrate was well below 50% so the trial ended there. In the second trial, the cumulative dose response technique **[3.4.i]** was repeated with a fresh cohort for increasingly narrow concentrations; 700μM, 800μM, 900μM, 1000μM, 1100μM, 1200μM and 1400μM. In the third trial, to test the success of the first two trials in discerning an LD50, a third, fresh

cohort were tested with separate concentrations per individual over time **[3.4.ii]**, using 200µM, 300µM, 350µM, 400µM, 500µM, 550µM, 600µM, 700µM, 750µM, 800µM and 1000µM metoprolol. After drug trials, animals were placed in fresh APW and moved from fresh APW to fresh APW three times to rinse drug residues away as best as possible. Animals were allowed to recover or swim freely for a period of two hours, after which they were observed once again to confirm or deny heart movement. Once lab work had ended, video data was processed and heartrate of each animal over time collected **[3.5]**.

5.1.iii Results

Figure 5a. Trial 1: *Daphnia* response to successive metoprolol concentrations

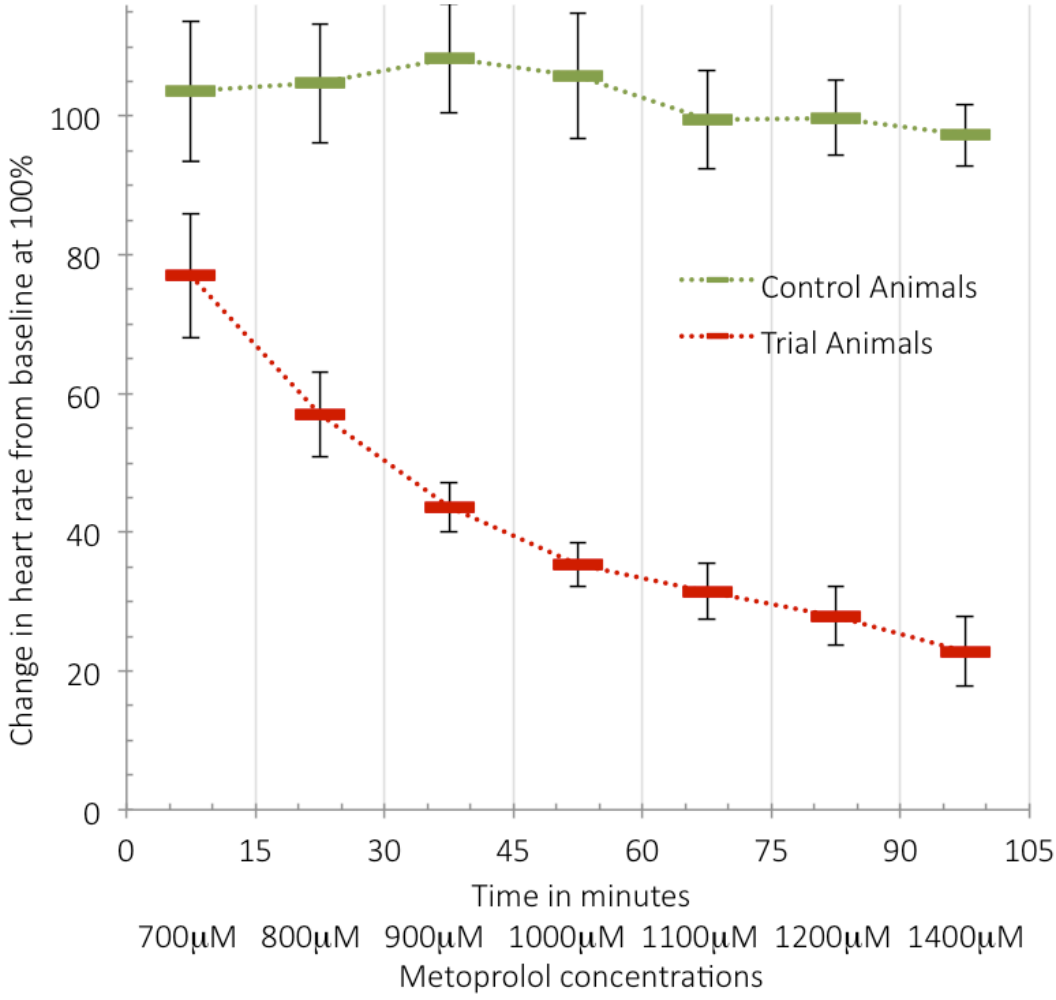


Legend: At time = 0 heartrate was taken as a mean of 3 recordings before exposure. Time begins at moment of first exposure. Solution is changed to next higher concentration every 15 minutes; vertical lines indicate this change. Error bars indicate standard deviation, this was chosen as each cohort comprised only 18 animals.

In the first trial, 18 *Daphnia* were exposed to successive concentrations of metoprolol, and responded with decreased heartrate over time. Concentrations of metoprolol were changed every fifteen minutes. 18 drug free controls maintained relatively constant heartrates. Figure 5a shows the first cohort. Mean trial *Daphnia* heartrate passes below 50% of starting heartrate during exposure to 1750µM metoprolol. Deceleration continued

at successive concentrations. All trial *Daphnia* were found to be dead after the recovery period, all controls survived. In the second trial, twelve *Daphnia* were exposed to successive concentrations spanning 700µM to 1400µM were trialled in an attempt to come closer to an LC50 plateau. There were also 12 drug free controls. All trial *Daphnia* were found to be dead after the recovery period, all controls survived. Trial 2 is shown in Figure 5b and again shows decreasing heartrate over time. A brief plateau occurs around 1000µM, after which heartrate continues to fall. Individuals were trialled with concentrations changing every fifteen minutes, until it was clear when seen by eye that heartrate had fallen below 50% of baseline. From trial 2, we could estimate an LD50 lay somewhere between 800µM and 900µM. However, we believed it necessary to test individual concentrations over time to see whether this was true. This was done in trial 3.

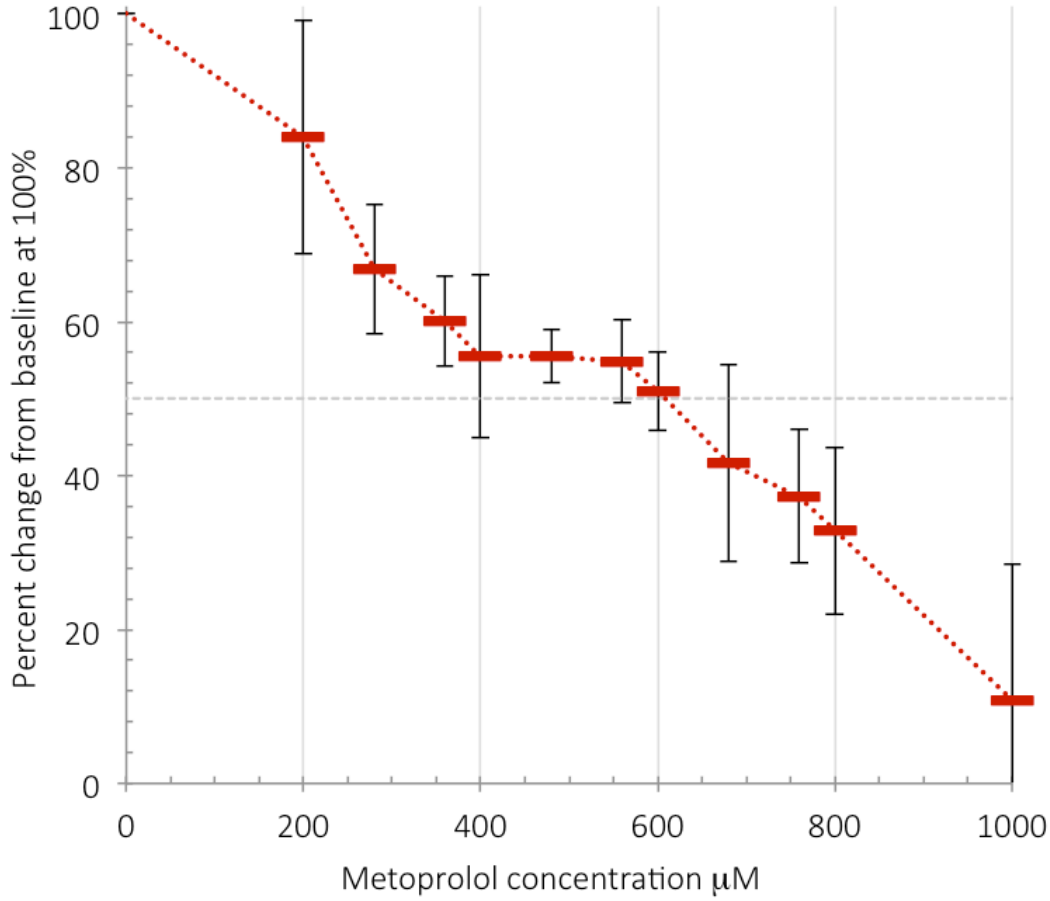
Figure 5b. Trial 2: *Daphnia* response to successive metoprolol concentrations



Legend: At time 0 heartrate was taken as a mean of 3 recordings before exposure. Time begins at moment of first exposure. Solution is changed to next higher concentration every 15 minutes; vertical lines indicate this change. Error bars indicate standard deviation, this was chosen as each cohort comprised only 12 animals.

For the third trial separate concentrations were trialled over time, as opposed to successive concentrations. 408 *Daphnia* were used in this final trial. As discussed in **3.4.ii**, the control: trial animal ratio strategy changed along with the move to the individual-concentrations method. Instead of testing one control for every one trial animal, one control was used for every three trial animals. For the first ten concentrations, 200µM, 280µM, 360µM, 400µM, 480µM, 560µM, 600µM, 760µM and 800µM, 30 trial animals and 10 controls were filmed, until plateaus were achieved. Plateaus were reached between around 3 to 5 hours. However, all animals heartbeats ceased, and no recovery was seen even 3 hours after washout, so they were deemed dead. For the eleventh concentration, 1000µM metoprolol, trials were stopped after 1.5 hours as 50% of animals no longer had heart beats. Only 6 trial animals and 2 controls were done at this concentration as it was obvious that heartrate was well below 50% of baseline and therefore would not be helpful when looking for an EC50. A concentration response curve is shown in figure 5c.

Figure 5c. Metoprolol concentration-response curve (individual concentrations)



Legend: concentration-response curve for bradycardic response plateaus in *Daphnia* during exposure to individual concentrations of metoprolol. Error bars show standard deviation.

During individual concentration trials, visible and lasting plateaus were reached. Aggregate data were not arbitrary but based on the population mean during these plateaus, timing of which was consistent across individuals. The figure shows the decelerative response to metoprolol increasing with concentration and plateauing between 400μM and 550μM and increasing thereafter. 600μM is close to 50% of max response (between the starting heartrate as minimum response and death, or zero heart beats, as maximum response). Heartrate decreases with increasing concentrations. Plateaus are most stable, lasting longer periods of time at 480μM and 560μM. At concentrations 760μM and above the heart decelerates rapidly and plateaus become increasingly brief. At 1000μM heartrate decreases without plateau until death.

5.1.iv Discussion

It is clear from that the presumed LD50 found in successive concentration trials does not represent actual LD50 when testing concentrations on an individual basis. LD50 in ecological papers is a function of time and concentration, rather than successive doses, and the two do not translate. In contrast with the results from the verapamil trial, in metoprolol trials plateaus were reached well within the 9-hour lab-opening time frame, so a concentration response curve could successfully be drawn up. It might be that a nine-hour day was not enough time to see results from verapamil, whereas metoprolol produced plateaus in a timelier manner. This was far from a high throughput way to perform such research. The time required before *Daphnia* reached plateaus equated to an intensive amount of analysis time, not justified by the ensuing low sample number of trials. Three months were spent in lab on a 9 hour per day basis, and yet only one concentration response curve was achieved. As an alternative, accelerative drugs were then chosen for ongoing studies.

5.2 *Daphnia* Response to Octopamine

5.2.i Introduction

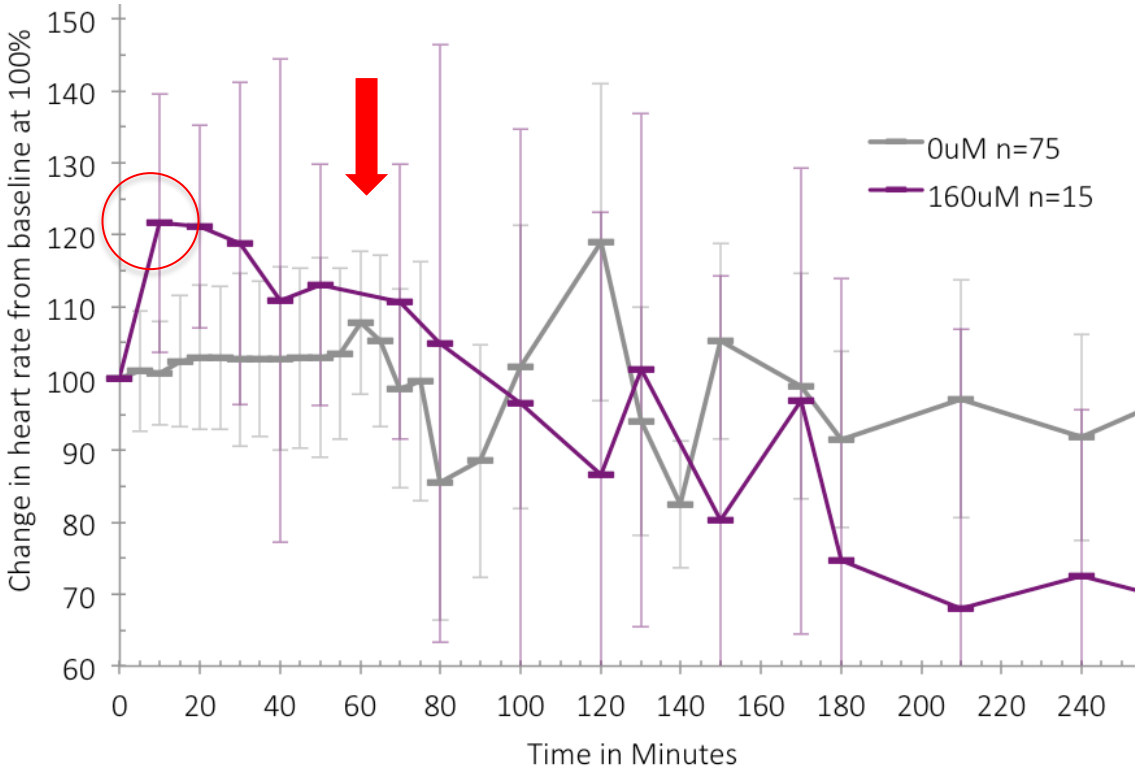
Octopamine's physiological significance is restricted to invertebrates. It is involved in invertebrate modification of muscle performance, fat metabolism, heartrate and respiration. Its action is similar to adrenaline, being mediated via G-protein coupled receptors. Adrenaline is known to increase *Daphnia* heartrate, however, the action of octopamine has not yet been observed in *Daphnia*. Both octopamine-like and tyramine-like receptor gene sequences have been identified in *Daphnia* (McCoole et al 2011).

5.2.ii Methods

Two hundred and ninety-two *Daphnia* were tested, with a 3:1 trial: control ratio. Seventy-five were controls in drug free solution. Seven concentrations of octopamine were separately trialled **[3.4.ii]**; 160µM, 320µM, 640µM, 960µM, 1280µM, 1920µM and 2560µM, for which sample numbers were 15, 41, 32, 29, 29, 38 and 32. A total 2,717 films were observed by eye, and processed as described in **3.5.** Three pre-exposure measurements were taken from each animal to establish baseline.

5.2.iii Results

Figure 5d. Mean data for the 160µM concentration and control over 3 hours

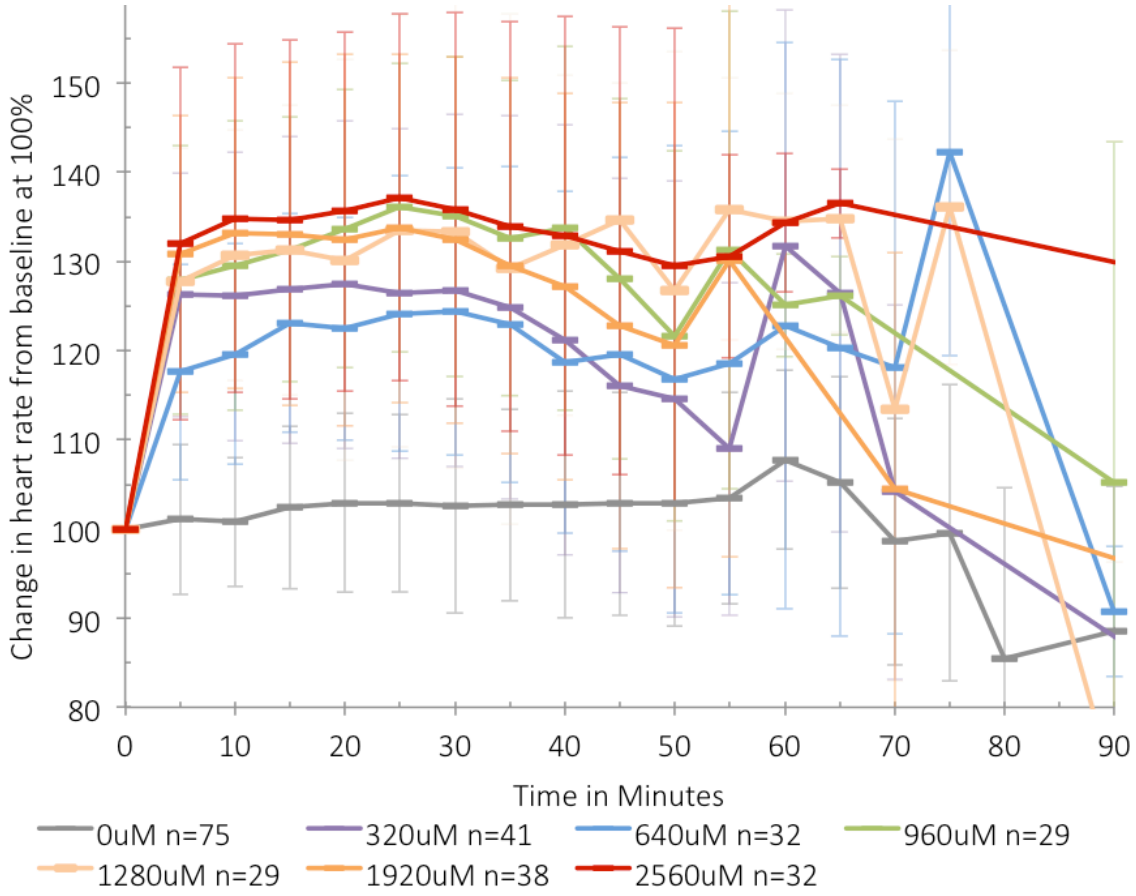


Legend: The arrow points to the one hour after which the control becomes highly variable. The circle shows a typical peak found after exposure to a drug concentration. "uM" is used to mean µM simply because of typesetting restrictions in Excel legends. Error bars show standard deviation.

In the first hour of exposure animals in all concentration cohorts responded with an increase in heartrate which slowly decreased over time thereafter. The change in heartrate from baseline at this peak was taken as maximum response per animal. Animals were exposed for one hour to one hour and half before being allowed to recover in drug free APW. Heartrate was captured after a further hour to three hours (depending on length of recovery allowed by lab opening hours) as determined by the longest period we could allow for recover before the lab closed. This last measure simply was to determine whether heartrate was normalising. Raw data shown in figures 5d and 5e shows that the

controls maintain an even heartrate close to baseline for the first hour. However, after an hour of being placed in and out of solution and filmed every five minutes control heartrate becomes highly variable (figure 5e). This implies that data taken from trial animals after this point is also affected by factors outside of the application of the drug concentrations concerned. When animals are allowed to rest without filming control heartrate returns to an even baseline between the third and fifth hour of observation.

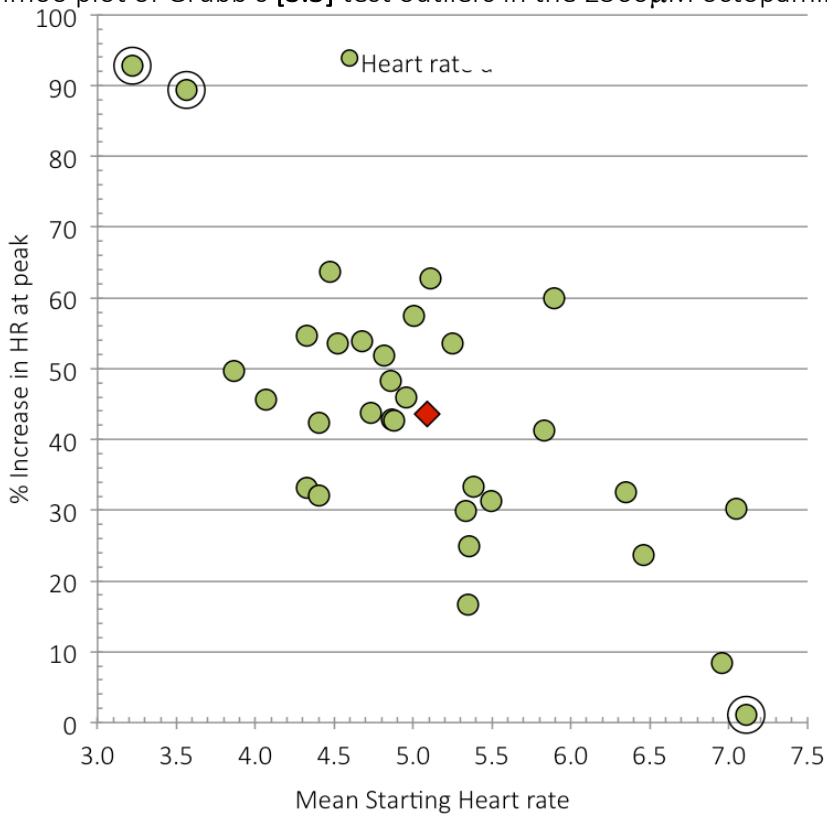
Figure 5e. Mean raw data for all concentrations in the first 1.5 hours



Legend: This figure is presented to indicate the shared trend of a fast heartrate increase followed by a slow decrease, and then increased response variability after one hour. Error bars show standard deviation. "uM" is used to mean μM simply because of typesetting restrictions in Excel legends. Error bars show standard deviation.

Fortunately, as we see in figure 5d, the area of interest in each trial occurs within the first hour after exposure. Heartrate increases immediately or within the first five minute after first exposure to any concentration, and peaks within the first 45 minutes before steadily decreasing as the end of the first hour approaches. The values for each heartrate peak were taken as max response for each concentration. After removal of outliers described below, peaks are then plotted as a concentration-response curve in figure 5h.

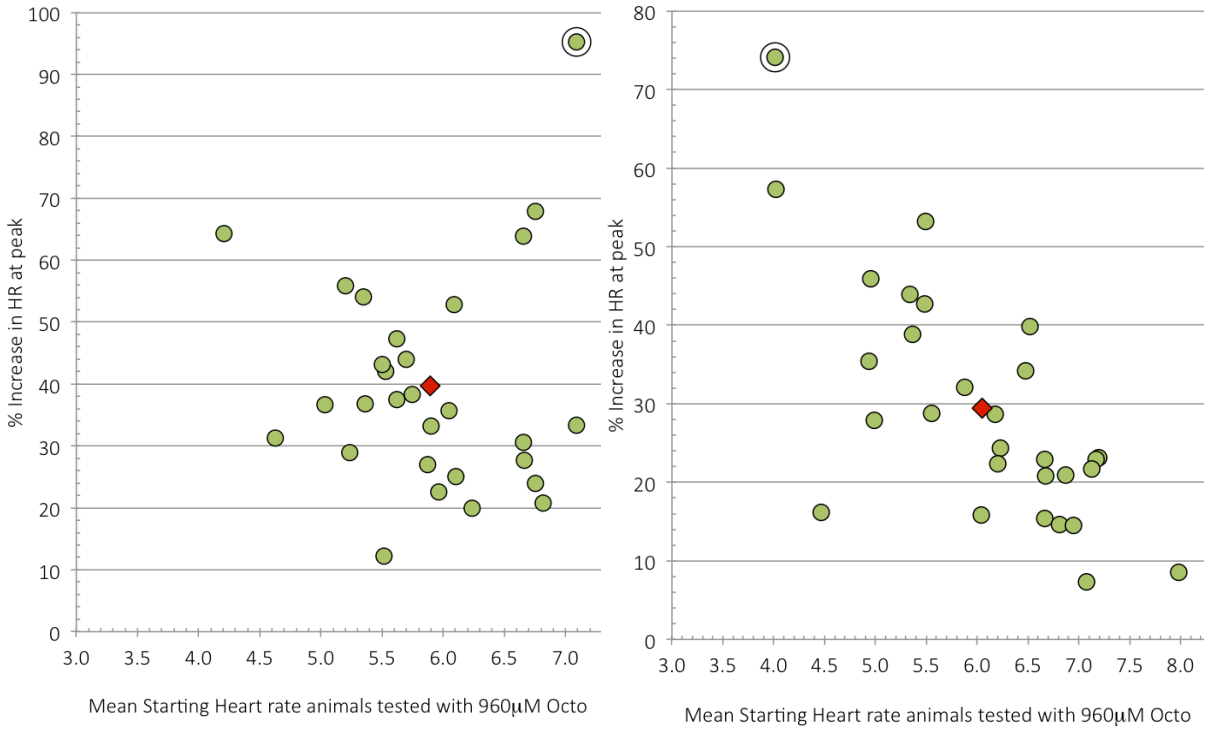
Figure 5f. Schmoo plot of Grubb's [3.5] test outliers in the 2560μM octopamine cohort.



Legend: Diamond = population mean; circles = individual data; encircled points = outliers.

At 2560μM, 65.38% of animals survived. Mean starting heartrate was 4.99Hz (SD 0.93). Mean peak heartrate was +44.99% (SD18.87). Grubbs tests [3.5] found three outliers: Animals M, O2 and K2, this was the largest number of outliers of any cohort. A schmoo plot of outliers is shown in figure 5f. Removing these outliers altered the mean peak heartrate to +41.97% (SD14.00). Interestingly, one animal with the same start rate fell within normal range of the centroid. At 1920μM, 65.38% animals survived. Mean starting heartrate was 5.49Hz (SD1.00). Mean peak +40.22% (SD17.65). At 1280μM, 84.62% animals survived. Mean starting heartrate was 5.92Hz (SD 1.04). Mean peak was +42.18% (SD 14.50). Grubbs testing showed that there were no outliers at either concentration. At 960μM, 88.46% animals survived. Mean starting heartrate was 5.79Hz (SD 0.68). Mean peak was 40.92% (SD 18.28). At 640μM, 84.62% animals survived. Mean starting heartrate was 6.12Hz (SD 0.94). Mean peak was 29.30% (SD 14.60). A schmoo plot showing outliers at 960μM and 640μM is also shown in figure 5g. Removing the 640μM outlier moved mean peak to +32.80% (SD17.85). The animal with this outlier had a low starting heartrate of 4.012Hz. However, another animal with a similar starting rate of 4.022Hz did not have such an extreme response to the drug. Removing the 960μM outlier gave a mean peak of +38.31% (SD14.22). At 320μM 91.30% animals survived, mean starting heartrate was 5.82

Figure 5g. Schmoo plots of Grubb's [3.5] test for outliers in 640μM and 960μM cohorts.



Legend: Diamond = population mean; circles = individual data; encircled points = outliers.

Hz (SD 1.08). Mean peak was 27.88% (SD15.44). Grubbs testing found no outliers. Standard deviation was high, but this is expected at low concentrations. At 160μM all animals survived, and mean peak was +23.06 (SD16.71). Across the 75 control animals involved in all trials above, mean starting heartrate was 5.87Hz (SD 1.25), and mean peak was +9.93% (SD9.80). 100% of control animals survived, and, Grubbs testing showed no outliers. Resulting data can be seen in figure 5j. Heartrate increases with concentration until it plateaus at 1280μM. Perhaps animal receptors are is saturated at this level, as the response does not become much stronger at the two higher concentrations. However, survival decreases dramatically from there on.

5.2.iv Discussion

Control animals in this trial maintained a heartrate very close to baseline for the first hour of being filmed every five minutes. After this heartrates became erratic until a rest period of around two hours allowed them to return to baseline once more. The reason for this change may be that while working in light field, animals were placed in a dish directly over the light source which may have become warm. Efforts had been made to keep the light source cool. An LED bulb was retro-fitted to the equipment in place of the standard halogen to prevent overheating, a blue filter was used to reduce heat transmission, and ice

packs were placed on the equipment for the same reason. However, after an hour, ice packs had thawed. It is possible that heating occurred despite best efforts. A stick-on thermometer attached to the microscope read 18^{o}C for the first hour but was seen to rise to between 19^{o}C and 20^{o}C after two hours had passed, so it is possible a change not recorded by the crude thermometer was already occurring after one hour. This would have depleted oxygen levels in the viewing baths until oxygen exchange from the air could occur during the recovery period. Even within the first hour standard deviation of *Daphnia* responses was high, and peak response within the first 45 minutes had a high standard deviation. This tells us that even though animals were both genetically identical and cultured and treated in the same manner, they still have highly variable responses to treatment.

The octopamine cohort produced a number of outliers. The three outliers in the 2560μM cohort began with extreme low or high starting HRs (animal M: 7.113Hz, animal O2: 3.562Hz and animal K2: 3.221Hz). Grubbs testing was necessary, as any change in heartrate in an animal with a low starting value would create a seemingly higher percent change than those in the middle range. An animal with a high starting value might have seemingly lower heartrate change when given as a percentage. However, it is not such a linear issue. The schmoo plots showed animals with starting HRs similar to outliers that did not produce such extreme responses. A single outlier found at the 960μM test concentration had a starting heartrate of 7.09Hz, however heartrate change was not 'extremely small' but conversely, very high at 95.23%. Another animal of identical starting heartrate responded with a percentage heartrate change of +33.33%, which was very close to the population mean of +38.30%. For 640μM octopamine the outlier had a low starting heartrate of 4.012Hz, however an animal with a similar starting rate of 4.022Hz was within normal range. From this we concluded that animals with extreme starting heartrate should not be arbitrarily excluded from investigation. Grubb's test picks out individual outliers that may not otherwise have been spotted.

5.3 *Daphnia* Response to Phenylephrine

5.3.i. Introduction

Phenylephrine is a post-synaptic α-1 adrenergic agonist, which causes smooth muscle contraction, used as a nasal decongestant and to dilate pupils for ocular examination. Phenylephrine works by acting on alpha receptors on the muscle in the eye that dilates the pupil. This causes this muscle to contract, which makes the pupil open up or dilate (Sandy Harper, personal communication). It is also applied as a cardio tonic agent to increase cardiac output after shock, heart surgery, myocardial infarct or during congestive heart failure. Phenylephrine is chemically related to adrenaline and ephedrine and has potent vasoconstrictor property, causing increased systolic/diastolic pressure and reflex bradycardia. It has not been studied in invertebrate hearts. No information is given in the literature as to *Daphnia* LD50 so the same concentrations as for octopamine were used in order to form comparisons across studies.

5.3.ii. Methods

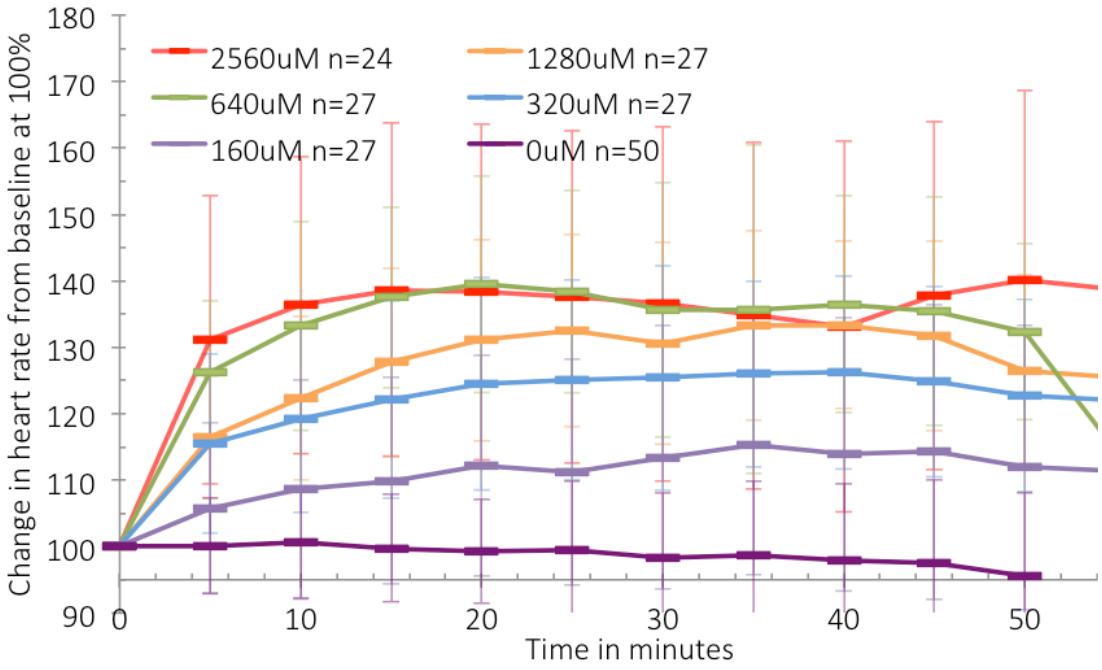
Five cohorts were trialled using separate concentrations [3.4.ii]. These were a drug free control and 160μM, 320μM, 640μM, 1280μM and 2560μM phenylephrine, with sample numbers of 50, 27, 27, 27, 27 and 24 respectively. 2077 videos were made, comprising 424, 408, 416, 421 and 408 for each concentration respectively, and observed by eye. Data processing is described in in 3.6.

5.3.iii. Results

Grubbs testing showed that there were no outliers in any cohort. For 2560μM, 87.5% animals survived, mean starting heartrate was 4.71Hz (SD1.01) and mean peak was +44.92% (SD23.02). In the 1280μM cohort, 100% animals survived, mean starting heartrate was 5.44Hz (SD 0.70), mean peak was +43.80% (SD14.73). At 640μM, 79.17% survived, mean starting heartrate was 5.18Hz (SD0.78) with mean peak at +37.83% (SD14.90). At 320μM, 87.50% survived, mean starting heartrate was 5.46Hz (SD1.12) with mean peak at +30.85% (SD13.27). At 160μM, 100% survived, mean starting heartrate was 6.33Hz (SD1.14), with mean peak at +21.76% (SD15.45). Across all control animals, mean starting heartrate was 5.5Hz (SD1.17) and mean peak was +4.99% (SD8.49). All control animals survived and maintained a constant heartrate throughout that stayed within a mean 1.16% (SD1.47) of the starting rate over one hour. Raw data is presented in figure 5h and shows that for the 50 minutes in which *Daphnia* were exposed to phenylephrine, after

a rapid increase in heartrate, *Daphnia* maintained the new heartrate at a fairly constant level. After this *Daphnia* were allowed to swim freely in drug free APW for a further three hours. At 240 minutes after start time (an hours exposure and three hours recovery) all surviving *Daphnia* were at just below baseline heartrate (mean -10.86%±21.03SD). This is surprising given the extreme secondary effects in the discussion. A concentration-response curve can be seen in figure 5j. It shows an increase of heartrate with concentration, which decreases in gradient for concentrations above 320μM and below 640μM, and taper to a near constant level between 1280μM and 2560μM. This suggests that receptors are saturated at the 1280μM level as the response does not get much higher (there is leap of only +1.12% between 1280μM and 2560μM.)

Figure 5h. *Daphnia* heartrate response to Phenylephrine over 50 minutes



Legend: Error bars show standard deviation.

5.3.iv Discussion

It is surprising that phenylephrine resulted in such steadily maintained increased heartrates given the extreme secondary effects observed during this trial. At 2560μM every animal was affected by muscular spasms, with pronounced contortion of the gut, suggesting that heartrate change may be confounded by a wider toxicological response. Effects on the gastrointestinal tract were also observed at 1280μM. We considered tallying this effect per animal, as in to simply declare yes or no as to whether gut spasms were observed. However, we concluded this decision would be fraught with inaccuracies: Contortions were not always greatly different from an animal cleaning out its feeding legs

with its anal hook. Spasms appeared more vigorous than in control *Daphnia*, but this relied on observer opinion. As an unexpected effect, contortions had not formed part of the experimental protocol therefore significance tests were not performed as consistency in approach could not be pre-scripted. At 640μM, again, gastro-intestinal spasms appeared more common across trial animals than controls. Even at the lowest concentration, animals shook violently or displayed curling of the gut tract.

Further issues were noted during the trial. Three of the animals were trialled immediately after a fresh solution of dissolved drug had been made. Their heartrate changed little compared to the control and we wondered whether the drug had failed to stir into solution thoroughly though no cloudiness could be seen. Another animal appeared to be dying to begin, with blackened and broken tail-spine; it showed an extreme drop in heartrate immediately post drug exposure. However, it could not be excluded because after wash-out, it recovered back to its original heartrate as did all the others.

5.4 *Daphnia* Response to Tyramine

5.4.i Introduction

Tyramine and octopamine are the only biogenic amines whose physiological significance is restricted to invertebrates. They have no known effect on humans but do have known effects on invertebrates. They are placed here as their actions are similar to adrenaline and noradrenaline, being mediated via G-protein coupled receptors, and share pharmacological features with those of the adrenergic system e.g. cardio acceleration. Both are involved in the modification of muscle performance, glycogenolysis, fat metabolism, heartrate and respiration (Roeder 2005). LD50s are unknown so concentrations used across previous trials were again used for comparison.

5.4.ii Methods

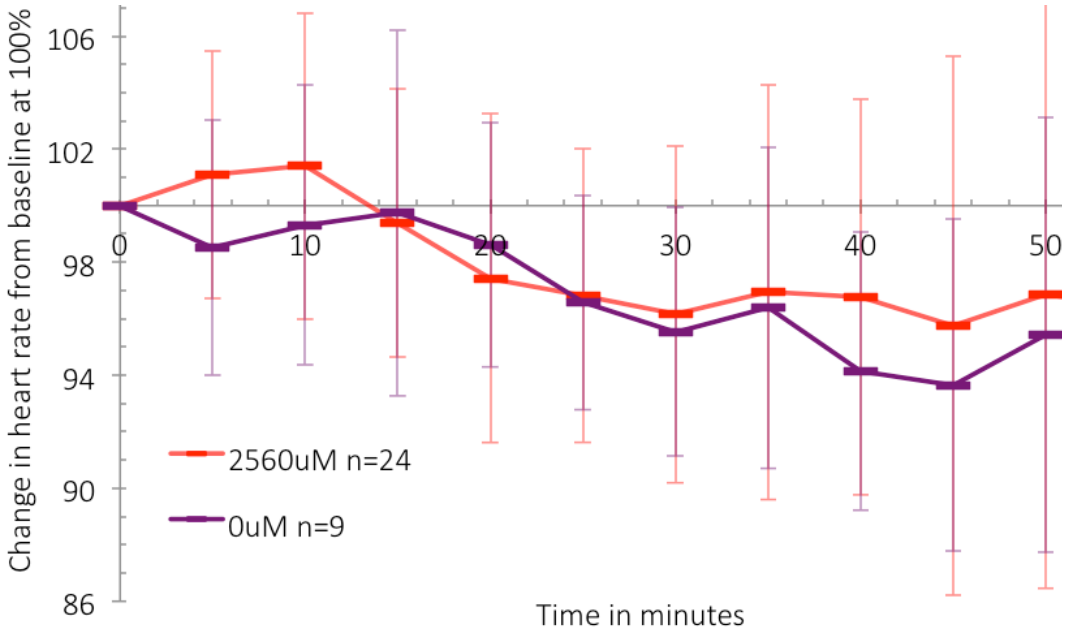
Four cohorts were separately trialled **[3.5]**. These were 320μM, 640μM, 1280μM and 2560μM. Each trial observed 26 trial and 9 control *Daphnia*. A total 1,696 films were made. Data processing is described in in **3.6**.

5.4.iii Results

The 424 films of the largest concentration were observed by eye and became clear that even at this concentration no effects were seen. At 2560μM 100% animals survived. Mean

starting heartrate was 6.68Hz (SD0.70). Heartrates peaked at mean +4.48% (SD5.02). This was not significantly different to heartrate change in controls. Random films across the remaining cohorts were spot checked but no heartrate change was observed. Grubbs testing showed no outliers. Raw data is shown in figure 5i, which shows the control and 2560µM heartrates following very closely mapped paths.

Figure 5i. *Daphnia* heartrate response to 2560µM tyramine over 50 minutes



Legend: Error bars show standard deviation.

5.4.iv Discussion

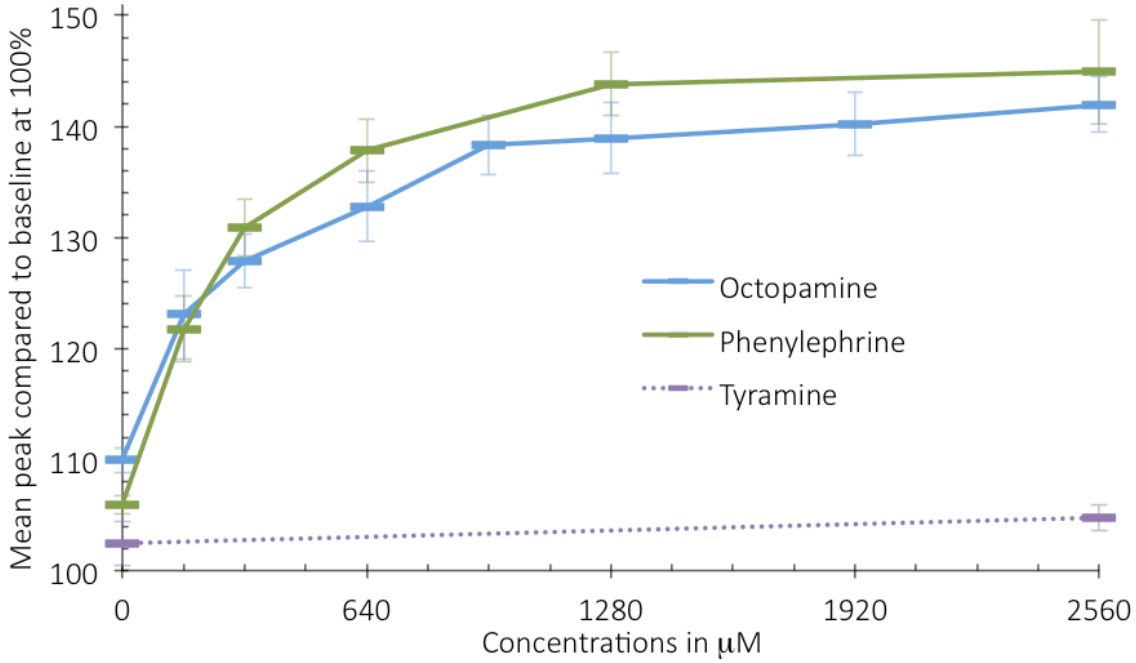
The lab part of the study took 6 weeks and constructively answered the question of how to prioritise ones work load. At the time of this study winter was drawing in, a time in which *Daphnia* produce resting eggs which wait to hatch in the spring while the population dies off. While in previous studies data analysis was performed concurrently with lab work on alternate days so that additional concentrations could be tested should the need arise. For example, if an extreme response was given at a low concentration, lower concentrations would be tested based on ongoing knowledge from data analysis. Instead, we were required to be in the lab every day in order to collect as much data as possible before the *Daphnia* population dwindled. While this may have been a good idea had concentrations been appropriate, this left no time to process data. Therefore, a month of lab work was completed before data analysis begun. Data were observed only after all trials had been performed. It was found that even at the highest concentration *Daphnia* made no heartrate response. 1,272 films were made without interim film observation in an attempt

to maximise lab time in the onset of winter, when *Daphnia* numbers are known to decline rapidly, due to external pressure to increase sample numbers trialled over this chapter. This meant that film analysis could only be performed at evenings and weekends, creating a backlog of data. As a result, rather than choosing higher concentrations according to ongoing results, concentrations were instead maintained on the assumption that the same had worked for the previous two drugs. It became clear that a balance between analysis and lab work must be recognised despite this leading to less time in lab.

5.5 General Discussion of Drug Trials

Phenylephrine appears to produce a slightly faster accelerative heartrate response than octopamine, while tyramine has far less cardio accelerative effect, if any, than octopamine or phenylephrine. This is illustrated in figure 5j. Tyramine may have no effect on *Daphnia*.

Figure 5j. *Daphnia* peak response curves for phenylephrine, octopamine and tyramine



Legend: Error bars now show standard error rather than standard deviation to show how far the given means are from the population mean.

It may have been reasonable to restart tyramine exploration the following spring. However, we instead moved on to an investigation into complex drug effects, to ask why the above studies produced such high individual variation in responses. This was to test for flaws and ensure that our own methods were not responsible for the confounding secondary effects of GI tract spasms seen with phenylephrine, or the high standard deviation in responses seen for all therapeutics.

5.6 Investigation into Lab Protocol Parameters

5.6.i Introduction

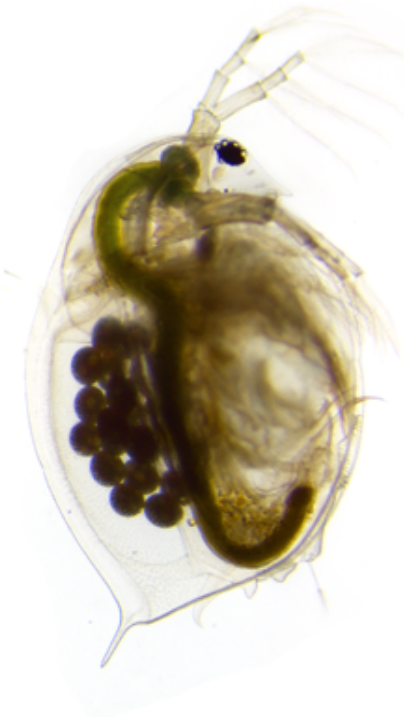
Complex responses were observed during phenylephrine trials. In the 2560μM cohort all neonates responded with physical contortions involving extreme twisting and curling of the gastrointestinal tract. Phenylephrine can become oxidised leading to degradation products such as isoquinolines and aldehydes that may well be the cause of secondary effects. Further observations were made to investigate these secondary effects.

5.6.ii Methods

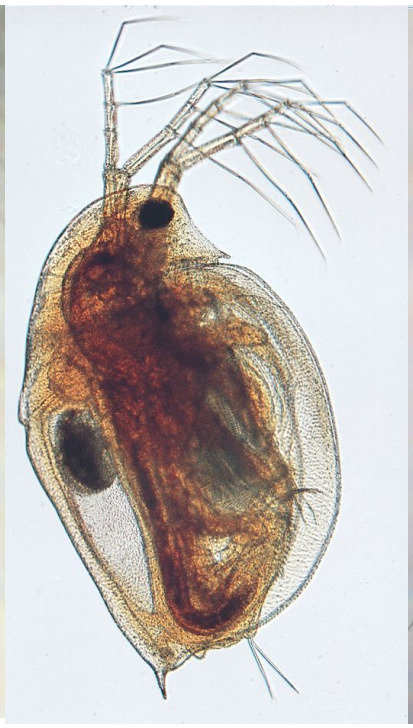
One hundred additional films were made using the individual concentrations protocol [3.5.ii and 3.6] for just 2000μM phenylephrine. One change to the protocol was made: A heterogeneous group of animals across a spectrum of pregnancy statuses were chosen at random rather than selecting for neonates. All remaining available cardio accelerative drugs found in the lab were also tested for secondary effects; tyramine, octopamine, atropine and phentolamine all at 2000μM. Methods beyond this were simply to observe for any secondary effects and make a note of the pregnancy status of each animal. We first categorised the pregnancy statuses we were able to identify by eye, so that any different response by animals in these stages could be noted during observations. Mitmann et al (2014) give a detailed description of each developmental stage in the *Daphnia* embryo, from appearance of the head, antennae then mandibles, through each thoracic segment, the labrum then maxillae and hook shaped abdomen, to the growth of a carapace that partially covers the legs. Stages observable under 40x magnification are seen in figure 5k and are given a Greek-letter code to simplify reference to each stage during observation. Neonates (not pictured) are defined by having empty wombs, at ages above twenty-four hours *Daphnia* are permanently pregnant. Figure 5h shows resting eggs from the sexual stage. These are shed with a carapace layer which has led many to believe the animal dies when producing these eggs. However, from our observation, after the egg is shed along with this layer, the animal will swim away. 5i through 5k represent parthenogenic stages, other than fully developed foetuses seen which were shown in figure 2b. Films of pregnant females were observed carefully a second time to categorise the stage of foetal development which had occurred and to make a note of the presence of abortion or survival among the foetuses.

Figure 5k. Images of *Daphnia* at various stages of pregnancy

Resting eggs (γ) in *Daphnia*, and in shed carapace. Black eggs (β)



Blastulae two photos blastulae, cell division visible Early foetus (δ)



5.6.iii Results

We found that phenylephrine has abortive effect, but only at specific stages of foetal development. Across all drugs one of two responses occurred each time, either:

x = A slight rise and fall in heartrate in the first half hour followed by a return to starting HR, after this heartrate was not significantly different to controls.

Or, y = A slight rise and fall in the first half hour followed by plummeting heartrate ending in dysrhythmia or death.

Of the 49 virgin animals and 51 pregnant animals observed 28.6% and 29.4%, respectively, suffered responses x and y respectively. Table 5L shows the distribution of the two responses and gives ratios for each. Phenylephrine resulted in the least deaths (response y), while notably, tyramine, which produced no significant change in heartrate, killed the highest percentage of animals.

Table 5L. Survival responses across select drugs at 2000μM

Drug response →	x	No. x pregnant	y	No. y pregnant	% y dead	% y of pregnant
Atropine	19	10	4	2	17.4	16.7
Octopamine	18	11	8	6	30.8	35.3
Tyramine	11	4	9	4	45.0	50.0
Phentolamine	9	3	5	1	35.7	25.0
Phenylephrine	15	9	3	1	16.7	10.0
Totals	72	29	29	14	40.3	32.6

For all cases barring phenylephrine, the proportion of pregnant animals killed is in proportion with the total number of animals which died (y), so pregnancy had no effect on death rate. However, with phenylephrine, pregnancy, despite abortions, seems to improve survival. This may be because abortion spares energy to save the mother animal. Abortions did not occur under any treatment other than phenylephrine. When exposed to phenylephrine, blastulae and early stage foetuses were unanimously aborted while eggs were not always aborted. Fully developed foetuses generally survived. Pregnancy status was categorised as one of the following. Either; "α" a virgin, where the womb was seen to be empty and flat, "β" where round black eggs were seen (this status did not occur in randomly chosen subjects for the phenylephrine trials), "χ" referred to blastulae, where eggs appeared to be developed with cell division visible, "δ" for early foetuses, where

divided cells formed a somewhat foetal like shape, “ε” for fully differentiated foetus lying immobile within the womb, “ϕ” for late stage foetuses seen swimming within the womb, and “γ” when resting eggs were seen- these represent the sexual stage as opposed to the parthenogenic stage. Of the pregnant Daphnia seen 1 of 2 with black eggs aborted, 2 of 2 with blastulae aborted, 2 of 2 with differentiated foetuses aborted, though 3 with foetuses moving in the womb and 1 with a resting egg did not abort.

5.6.iv Discussion

Phenylephrine was the drug associated with lowest rate of death among those trialled but caused the greatest level of abortion, and secondary effects were observed with this drug and no other. Table 5m suggests that different stages of foetal development have varying energy demands on the mother *Daphnia*. It could be that the mother has invested so much energy into the young at the late stage that risking her own life is more energy efficient than abortion. It also suggests that the early foetal stage may be when the mother is least able to expend energy on her own health. Though too few animals were observed to be statistically significant. Further animals were not observed, in part because animals were needed for research more relevant to the cardiovascular theme of the work. This finding highlights the importance of a variable in selecting animals for research. Also, while the animals concerned are invertebrates and therefore not covered by the animal rights act, there was no clear justification for further undue suffering. However, observations underlined our original decision to choose only neonate animals for cardiovascular trials. An unregulated mixture of pregnancy statuses which may affect the concentration response curve of any drug trial.

5.7 The Importance of Buffer Choice

5.7.i Introduction

Two issues came about from the drug trials described above. Most obviously, we wondered why it might be that foetuses were aborted or survived at different stages when exposed to phenylephrine. Ascorbic acid (AA) is known to be useful in countering oxidative toxicity (Frank 1985). Background reading suggested that *Daphnia* foetuses may synthesise varying levels of AA according to life stage, leading to age related variation in their ability to withstand oxidative stress. Related to this we were concerned with the overly high levels of variation in responses to drug treatment and ensuing high standard deviations. A more unified response, especially across a population so carefully treated to be cultured in

identical conditions, would be more useful in drug trials. Such variation makes the *Daphnia* model unreliable and necessitates a great deal of repetition. We investigated whether a different buffering system might protect the animals from the oxidative effects which may be the cause of secondary effects or high response variation.

Nespor and Wenig (1939) found that *Daphnia* could be raised on an AA free diet, and, Fisher (1960) concluded that crustacean tissues synthesise their own AA. The strength of this may depend on life stage in the case of foetal development. In the peer-reviewed papers listed in table 5m various concentrations of AA are shown to have different effects on *Daphnia* survival. Wernersson and Dave (1998) bathed *Daphnia* for 18+ hours in about 6μM AA, this was not enough to counteract the lowest oxidant concentration trialled (0.0049μM fluoranthene). Wang et al (2008) found that 10μM AA decreased photo induced oxidative damage of 2μM 1-amino-2,4-dibromoanthraquinone from 96.7% immobility to 40% over a 24-hour period. Bouchnak and Steinberg (2010) found that around 11 μM AA partially countered the oxidising effects of humic substances added to the normal diet of *Daphnia*. Humic substances reduce lifespan and fecundity in female *Daphnia*, while increasing male lifespan and serving as a food substitute beneficial only when compared to fully starved *Daphnia*. The increase in male lifespan may only be due to the need for males during times of stress; under humic conditions eggs laid were ephippia rather than parthenogenic.

Table 5m. Ascorbic acid trials using *Daphnia*

Reference	Antioxidant capability in *D. magna*	Concentration μM
Wernersson and Dave 1998	No effect	5.984
Wang et al 2008	Over 50% toxicity reduction	10.0
Bouchnak and Steinberg 2010	'Partial' toxicity reduction	11.356
Vega and Pizarro 2000	Significant toxicity reduction	567.795
Eaton Technologies 2012	48-hour LD50	2044.060
Olmez-Hanci et al 2014	Significant toxicity reduction	2500.0
Jusadi et al 2008	9-hour LD50	2838.973

Legend: concentrations are reported to the level of precision given in paper – rounding to give identical decimal place levels for aesthetic reasons was decided against in order to maintain accuracy for future researchers.

Vega and Pizarro's (2000) used 568μM AA to counter oxidative effects of UV radiation. This significantly reduced *Daphnia* mortality caused by UV-A but not by UV-B, suggesting that damage in the latter is caused by DNA photo lesion rather than the oxidation caused by the former. Eaton Technologies GMBH (2012) list 2044μM as the *Daphnia* 48-hour LD50 for AA, while Jusadi et al (2008) found a 9-hour LD50 for AA in *Daphnia* at 2839μM. At 2500μM AA Olmez-Hanci et al (2014) found that oxidative effects of equimolar persulphate and equimolar peroxymonosulphate, respectively, were significantly reduced. Addition of this concentration caused a decrease in mobility within half an hour from 85% and 100% to 30% and 25%. These observations also give way to a question of whether the original buffer choice of *Daphnia* Culture Medium (DCM) **[2.3.i]** may not have been the best option for this work. Changes in environmental pH are known to cause toxicological stress which may impact heart response trials. In publications to date, pH of trial and control solutions is are not overtly measured, it appears pH is merely assumed to remain constant when drugs and *Daphnia* alike are buffered within DCM (Campbell et al 2004).

However, small 0.1pH unit variation is known to negatively impact embryo development, and even introduce destructive effects intracellularly (Philips et al 2000). Buffers themselves may induce undesired effects independent of pH. PBS buffer is most commonly used; however, it is known to compromise embryo development and birth rate (Lane et al 1999). According to a systematic review of buffers by Will et al (2011), the Good's buffer MOPs is least toxic and best at buffering pH, with a pKa of 7.15 at 20°c. Will et al (2011) advised that embryo development is supported in the presence of sodium bicarbonate alongside MOPS, but that MOPS alone would be toxic to embryonic development. De Schamphelaere et al (2003) tested the effect of MOPS buffer on *Daphnia magna* reproductive capability and on one of their algal foods *Pseudokirchneriella Subcapitata*. They found that 3.5mM MOPS was the NOEC value with no effect on daphniid or algal survival, or fetal production and survival.

Maintaining a precise stability in environmental pH may be an insurmountable task without more expensive equipment, as *Daphnia* themselves may alter their environment via urination causing the addition of uric acid to solution, or respiration causing CO2 build up. While the culture tank is a mature and stable ecosystem, presumably containing bacteria which deal with the animal's outgoings, protocol requires that animals are moved to a small, clean area such as a viewing plate during trials. Removed from their normal

environment and placed within a small volume of clean buffering solution makes it more possible that the *Daphnia* will alter such an environment in the time allocated for the trial, thus impacting the trial's outcome. Thus, using a range of buffers, it was of interest to ask the following:

- Do *Daphnia* alter the pH of their microenvironment?
- And, do micro-level changes in pH alter *Daphnia* heartrate?

In addition to *Daphnia* Culture Medium, alternative buffering systems were investigated for comparison; MOPS and a simple titration of ascorbic acid and sodium bicarbonate. Sodium bicarbonate undergoes decomposition and production of CO_2 which, if improperly buffered, will increase pH. Meanwhile, ascorbic acid exists as a diprotic acid or zwitterion, with two different ionisable functional groups at pKa 4.2 for the carboxylic acid group and pKa 11.6 for the hydroxyl group (Dollery, 1991). The balanced equation for the neutralization reaction is $NaHCO_3 + C_6H_8O_6 \rightarrow CO_2 + H_2O + C_6H_7O_6\{-\} + Na\{+\}$. This produces a biphasic titration curve in which the pH of AA swings very steeply between pH 7 and 9. A possible alternative to AA might be its stereoisomer, erythorbic acid, a monoprotic acid with a single pKa of 2.1. Erythorbic acid is non-toxic and known not to degrade as easily as AA, and thus is used as a food preservative and reduction agent in photography (Beppu et al 2001). For ease in reading the above questions are separated into two sections representing the two trials used to answer them, where this section forms a natural introduction to both.

5.7(a) Do *Daphnia* Alter the pH of Their Microenvironment?

5.7(a). i Methods

Thirteen trials were performed where two 60ml flasks containing DCM (A and B) and two 60ml flasks containing MOPS buffered solution (C and D) were freshly made at the start of each trial. A pH meter was used; a Mettler Toledo FE20-ATC Kit FiveEasy™ with LE438 pH electrode. pH was measured three times per flask while the solution was stirred lightly by magnetic stirrer, to establish baseline. After this, ten *Daphnia* were placed in solutions B and D respectively. All four solutions were stirred lightly and continuously while the pH of each was taken in turn over the course of half an hour, rinsing the electrode in distilled water between measures. Care was taken when placing the electrode in solutions containing lightly spinning *Daphnia* so as not to damage the specimens. Change in pH per trial was taken as the absolute difference between mean starting pH and the largest range in pH noted once *Daphnia* were placed in solution. Data were compared via Mann-

Whitney U statistical testing; a non-parametric test used for independent samples, less powerful than the unpaired t-test but more reliable in that any significance found is rigorous: it is an exact test in that it makes use of combinatorial probabilities rather than assuming normality. It is recommended for n values between 10 and 20, as it assumes a finite population of data. The n = 10 referred to here are the ten *Daphnia* per trial.

5.7(a). ii Results

The first null hypothesis stated that the presence or absence of *Daphnia* has no effect on the stability of the buffers used. Comparing pH variation in MOPs buffer with versus without *Daphnia* present gives a p-value of 0.429, while comparing pH variation in DCM with versus without *Daphnia* present gives a p-value of 0.360. In both cases we failed to reject the null hypothesis. The results and U scores are given in Table 5n. The second null hypothesis stated that there was no difference in the buffer stability of MOPs versus DCM. When comparing the two buffers with *Daphnia* present in solution, a p-value of 0.119 was found. We failed to reject the null hypothesis, however, note that while not significant at the 0.05 level the p value shows that the two buffers are somewhat different. It is unclear whether these results represent a true 'no difference' or alternatively, a lack of statistical power to detect such a difference. However, it is likely to be the former, because when comparing the two buffers with *Daphnia* absent from solution, a p-value of 0.049 was found. In this case we reject the null hypothesis, the two buffers are significantly different.

5.7(a). iii Discussion

We found that *Daphnia* respiration and excretion does not significantly alter the pH of small volume solutions over the course of half hour observation periods. The two buffers show very different capabilities in their ability to stabilize pH. *Daphnia* Culture Medium shows low stability, changing by a mean 0.46 pH over the half hour period, while MOPS changed by a mean 0.16 pH. In this trial, *Daphnia* made no significant change to the pH of each medium when compared to itself without *Daphnia*. It is interesting an unexpected that the presence of *Daphnia* appears to stabilize the buffering capabilities of DCM when compared to MOPS. In their absence the stability of the two buffers is significantly different. DCM's comparatively unstable pH when seen against MOPS may induce changes in heartrate which might undermine results during trials of cardiovascular therapeutics, bringing previously published methodologies into question.

Table 5n. Absolute change in pH

Trial	MOPs No D	MOPs D	MOPs U No D vs D	CM No D	CM D	CM U No D vs D	No D U MOPS vs CM	D U MOPS vs CM
1	0.16	0.13	6	0.60	0.73	8	1	0
2	0.17	0.21	6.5	0.61	0.55	8	1	4
3	0.15	0.14	6	0.78	0.83	11	1	0
4	0.03	0.36	1.5	0.74	0.85	11	0	7
5	0.04	0.37	2	1.00	0.63	13	0	7
6	0.21	0.33	9.5	0.54	0.66	7	2	7
7	0.10	0.18	2	0.42	0.28	7	0	4
8	0.18	0.01	7.5	0.41	0.28	7	1.5	0
9	0.19	0.03	8	0.11	0.23	0	2	0
10	0.08	0.17	2	0.18	0.19	2	0	1
11	0.27	0.20	10	0.25	0.17	5	3	4
12	0.19	0.14	8	0.28	0.17	6	2	0
13	0.10	0.13	2	0.34	0.19	7	0	0
Mean	0.14	0.18	Score No D	0.48	0.44	Score D	Score MOPs	Score MOPS
SD	0.070	0.112	71	0.260	0.269	77	13.5	34

Legend: No D = No *Daphnia*, D = *Daphnia*, DCM = *Daphnia* Culture Medium, U = U statistic.

A limitation of this trial was that the pH probe required full immersion so that a large amount of solution (60ml) was required rather than the smaller 6ml bath volume used in our trials. In our trials, *Daphnia* are immersed in 10% of this volume, and while we used 10x as many *Daphnia*, this scaling may not translate to real effects. It is possible one *Daphnia* may affect 6ml of APW far more than 10 *Daphnia* effect 60ml. If the effect of increase in water volume were a negative exponential to the effect of the *Daphnia*, then this experiment could not hope to predict the effect of one *Daphnia* in 6ml. A solution would be to add more *Daphnia*, perhaps a hundred, but this itself comes with problems. Even with only 10 *Daphnia* it was difficult to insert the pH probe without squashing an animal, great care was taken not to do so. Squashing a *Daphnia* would result in internal fluids in solution which would bias the experiment. In addition to this, for the brief <30 seconds that the *Daphnia* is immobilised for filming, the animal is held in a thin film of water far smaller than the animal swims in during the experiment, changes may occur during this time that are immeasurable using these methods.

5.7(b) Do micro-level changes in pH alter *Daphnia* heartrate?

5.7(b). i Further Introduction

The first principle of buffer selection is to choose a buffer with a pKa at the pH desired. In this case the pH would be best of it mimicked environmentally neutral pH. MOPs has a pKa of 7.2. While both MOPS and DCM offer this, due to the better stability of MOPS buffered solution, it was decided that MOPS may be a more reliable medium in which to test for change in heartrate, using De Schamphelaere et al (2003)'s recommendation of a maximum final concentration of 3.5mM MOPS.

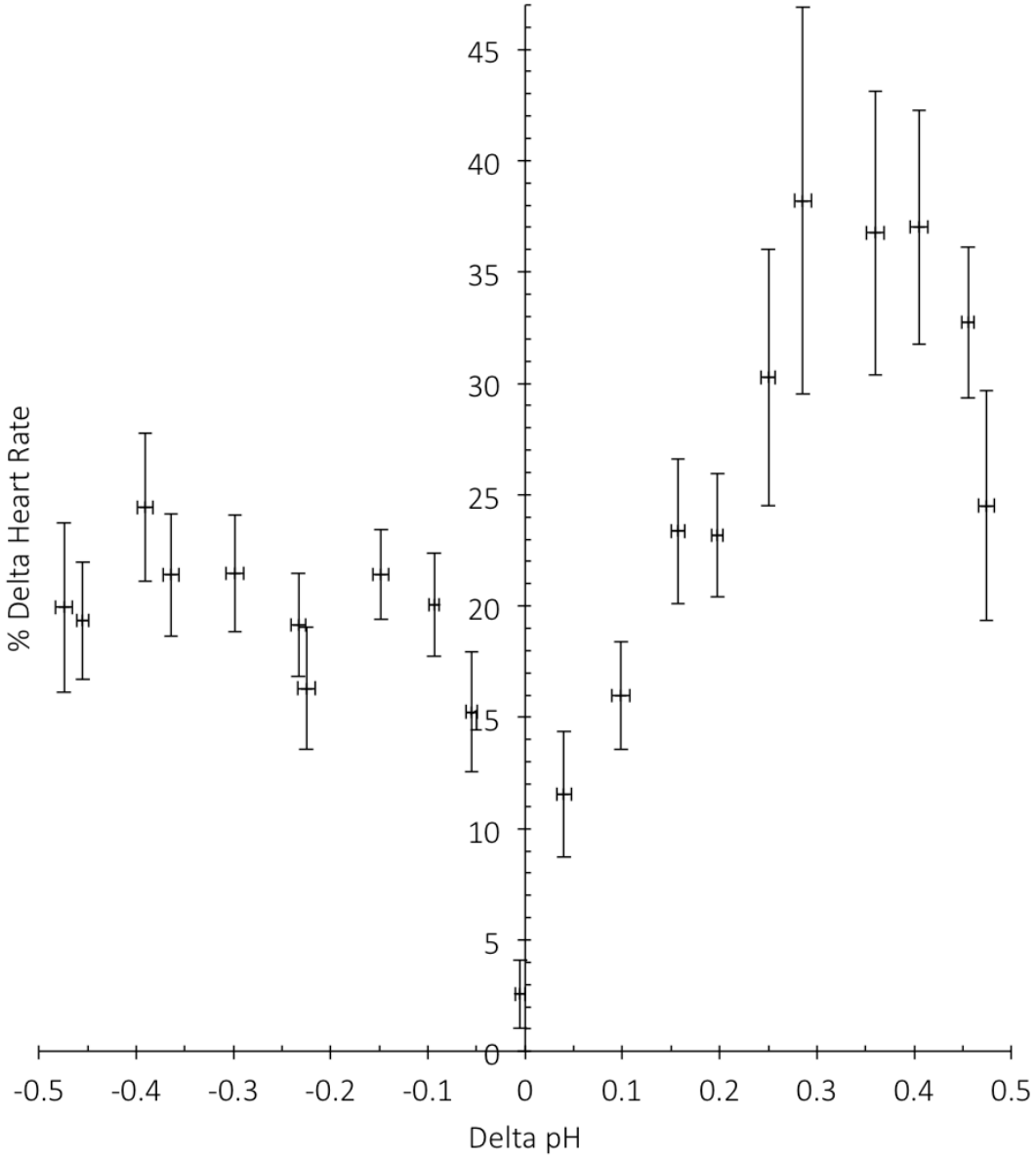
5.7(b). ii Methods

Each trial began by taking a pH measure from a 1000ml beaker containing *Daphnia* and APW. The pH probe was placed in lightly spinning solution for half an hour to establish the starting pH which subjects were accustomed to before removal into test solution. Fresh test solutions were made each day with 3.5mM MOPS adjusted with sodium bicarbonate and ascorbic acid to a final volume of 100ml, in order to span a range of 0.5 pH either side of baseline pH. The animal's environmental pH was recorded prior to each trial in order to calculate delta pH when moving to trial solutions.

Individual *Daphnia* in 6ml pond water were placed in 35mm culture dishes. Pond water was then pipetted away so as to leave the animal immobilized on one side, and high-speed films of the heart seen through the carapace were taken via microscope. 6ml APW were added back into the dish to allow the animal to swim for ten minutes so that it would not become over stressed, while waiting the next test subjects were then treated in the same way. Three initial films were made per animal to establish a mean starting heartrate.

Animals were then moved into a solution of the new pH, in individual 50ml beakers. Animals were allowed to swim for half an hour in the new solution. The process of removing the animals into a 35mm culture dishes, pipetting away the MOPS buffered solution, filming and replacing the animal into solution each time was performed every ten minutes for three new films. The pH of the MOPS solution was recorded when animals were first placed in MOPs buffered solution and after the third and final film to establish any shift in the measured pH range. This record was possible to two decimal places pH using the given pH meter. Films were then processed as in 3.6.

Figure 5o. The effect of change from baseline pH on *Daphnia* heartrate



Legend: Change in pH is given on the x axis, change in heartrate is given on the y axis. A sudden increase in heartrate occurs at a slight shift in pH in either the acid or basic direction. Change toward alkaline produces a greater change in heartrate than that of a change towards acid, suggesting the animals are more tolerant of a change towards the acidic. The dramatic heartrate increase in response to alkali tapers off as the change becomes large, and most likely represents the descent of the animal's health towards its death. Error bars are given as standard error.

5.7(b). iii Results

200 individuals were tested for response to change in pH, between a range -0.59pH to +0.56pH either side of baseline (mean pH 7.16, SD 0.208). This included 20 individuals who were tested at a pH matching the starting value of the tank water itself. Figure 5o shows the mean change in heartrate across this population according to change in pH. These results indicate that *Daphnia* heartrate increases dramatically with micro level changes pH of only 0.05 pH units, regardless of whether this is toward a more acid or baser

environment. When there is zero change in pH the heartrate varies a mean 2.56%. A pH increase of one decimal place leads to an increase in heartrate between mean +15.98% (SD 6.42) while a decrease in pH of a mere 0.15pH leads to an increase in heartrate between mean +21.41% (SD 6.72). This heartrate increase appears to reach a brief plateau in either pH direction before a sudden decrease in heartrate occurs as we near 0.5 of a pH either side of baseline. This decrease is more obvious with a move towards increasing acidity, and may indicate higher animal stress levels.

5.7(b). iv Discussion

It appears that *Daphnia* are very sensitive to their environment. And, while this confirms their use as models for toxicity, it brings into question their use as a model for medical trials unless more rigorous control of drug buffers can be achieved. For some drugs, with phenylephrine as a case in point, simply adding the drug to APW is not enough to stabilise the pH of the solution. pH values close to neutral can be achieved with MOPs buffer solution but increasing amounts of buffer are required to stabilising increasing concentrations of the drug. The pH altering effects of drugs complicate the interpretation of their receptor-mediated effects for a number of reasons. Firstly, we know from this trial that a mere 0.05 change in pH alters heartrate, and that this is beyond buffering capacity. Secondly, we do not know what effect increasing levels of buffer in solution would have on heartrate. A single concentration of MOPs solution was used in this trial, which simply attempted to stabilise dH_20. The effects of increased buffer levels required to stabilise pH of drugs in solutions are unknown, but we assume from this trials that this too would cause further change in heart response.

5.8 Chapter Summary

In this chapter we found that *Daphnia* are an inappropriate model for cardiovascular therapeutic dose-response trials due to extreme environmental sensitivities. Drugs were administered via swamping the *Daphnia* environment. This is in contrast to drug administration in a land-based animal, where drug administration does not alter the entire external environment. It is unclear whether drugs in the environment are consumed and pass through the animal via filter feeding, or whether they enter the animal via osmosis used for respiration. We found that *Daphnia* heartrate produces extreme and sensitive responses to micro level changes in the environment. Intra-population cardiac response is highly variable even in the most controlled environment.

Daphnia did not significantly alter the normal pH variation of either MOPs or DCM buffer solution. MOPs is far more stable a buffer than DCM. However, even the minute pH variation of MOPs is enough to significantly alter *Daphnia* heartrate. It was not possible to maintain a pH stable enough within a micro-range that does not alter *Daphnia* heartrate. This response adds a confounding factor. A solution to the pH problem may be to design a system where drugs are given via superfusion. This would require the *Daphnia* to be held in place by some other means. Perhaps, a tether with multiple light threads, or held between slides made of a plastic with the same refractive index as water so as not to interfere with the microscope's interpretation of the animal. The creation of a superfusion system is a potential extension of this research. However, the means of tethering or pinning the *Daphnia* might add confounding factors by causing stress to the animals.

Daphnia are not a good model for pharmacological bioassays where the measured response is chronotropy. In their natural environment, cardiac responses to environmental toxins might be affected by microenvironment changes from acid rain or calcium leaching, which may account for the paucity of papers using the heart as a signifier of toxicological change. Leg beat rhythm and fatal LD50 assayed in aquatic toxicology papers, while the heart is not so common in such studies. It is less possible to discover why certain stratagems are *not* published while others are, given the difficulty in publishing negative data. Heartrate change with drug concentration was monitored by adding drugs to the animal's environment and analysing film made of the heart under a microscope. Due to the extreme variability of individual-to-individual *Daphnia* heart response, hundreds of animals needed to be trialled per concentration to establish any reliable measure of dose-response. This could improve standard error but not alter the high standard deviation and entrenched unreliability. Standard error is reduced by performing a greater amount of trials, the number of trials needed to do this increases exponentially in relation to a small linear decrease in standard error. The semi-automated method for image capture used for light field saved a great deal of time compared to scanning the data by eye and increased accuracy, but still required intensive user interaction with manual adjustment of each frame captured to ensure the heart area was precisely measured.

6. Heart Variation Scoring Systems

This chapter asks whether we can correlate individual baseline heart variation with cardiac response in individual *Daphnia*. There exist published mathematical scoring systems in the literature which seek to differentiate dysrhythmias with varying success. These systems use beat-to-beat interval data and are discussed in detail below. We evaluate these in terms of a literature review, then apply the most appropriate systems selected from those available towards our own aim of correlating a baseline state with a response to a cardiac drug. Chosen systems are applied directly, or adapted where possible, to differentiate otherwise similar animals according to the stability of their heart rhythm. Animals are considered similar due to culture methods **[2.1]** and life stage **[2.3]**. Baseline rhythm data are collected while animals are still in their natural solution, Artificial Pond Water (APW) so each forms its own "control." Scores differentiate each animal along a numerical spectrum. We then test whether scores might be linked to the individual's response to treatment. We do this by calculating whether there are cross-population correlations between baseline score and response to a select group of concentrations of a chosen drug. Throughout this chapter the following scoring systems will be referred to by the acronyms listed below. This is because repeatedly listing systems becomes so lengthy that sentence structure may otherwise be lost, the methodology for each is also referred to here:

CCM =	Complex Correlation Measure	[3.6.ii(f)]
EI =	Ehler's Index	[3.6.ii(e)]
EC =	Ehler's Index Cloud Adaptation	[3.6.ii(e)]
FTG =	Finite Time Growth	[3.6.ii(i)]
GI =	Guzik's Index	[3.6.ii(d)]
GC =	Guzik's Index Cloud Adaptation	[3.6.ii(e)]
MRFA =	Multiscale Ratio Feedback Analysis	[3.6.ii(g)]
MSI =	Median Stepping Increment	[3.6.ii(h)]
PI =	Porta's Index	[3.6.ii(d)]
PC =	Porta's Index Cloud Adaptation	[3.6.ii(e)]

As we discovered in chapter 5, *Daphnia* are highly sensitive to micro-level environmental change. Despite making every effort to create a homogenous sample population there were still a diversity of responses to identical treatment which made them a bad choice for concentration response trials where a chronotropic response is required **[5.7]**. The primary

objective of this research required a certain level of homogeneity across the population, and *Daphnia* were found to have widely varying responses which were highly sensitive to micro level environmental changes. However, this individual variance could be used for other purposes. Heart rhythm scoring systems allow us to distinguish between individuals. If a link could be made between one scoring system and a resulting response, it would open up the possibility of using these systems as predictive tools. In the best-case scenario predictive tools might then be applied to humans. They might be applied to early stage ECGs to better predict outcomes for patients with early stage heart issues.

6.1 Background

6.1.i. The Drug Chosen

A data set was created based on dose-response to doxorubicin. The actual drug choice is of no real consequence, as the main focus in this trial are the scoring systems and the application of a drug that quickly causes measurable change in heartrate. Given video methods, the clearest change we can record is heartrate. In this case, a drug which slows the heart is more practical as parabolic peak interpolation will produce a more accurate peak given more frames per heart beat **[4.2]**. Doxorubicin is one of the two drugs mentioned in Curtis (2013)'s discussion of dysrhythmia trials in animals in relation to the ARRIVE guidelines (Kilkenny et al 2010) laid out by the Lambeth Convention (Curtis 2013). Doxorubicin has powerful, observable negative chronotropic effect, and has also been used in one trial which relates two published scoring systems (the square root of mean squared differences and the SD1:SD2 method, discussed below) to treatment response by 40 adult Wistar rats. Loncar-Turukalo et al (2015) found that the rat's beat-to-beat variation increased as more Doxorubicin was injected, up to a total of 15mg/kg over 15 days. Rather than working with a cumulative dose, we intend to find heartrate response to a range of individual concentrations and see whether paired starting scores might be correlated with individual *Daphnia* responses to treatment.

Doxorubicin is the prototype compound of the anthracycline class of drugs, and a primary cause of chemotherapy-induced cardiotoxicity in humans (Vejpongsa and Yeh, 2014). It is implicated in early death by congestive heart failure in one third of doxorubicin-treated cancer survivors within a decade of treatment. It induces apoptotic pathways and reduces antioxidant enzyme gene transcription leading to reactive oxygen species production. Concentrations of 740μM, 920μM and 1020μM, have been found to result in fatal

congestive heart failure in 5%, 16% and 26% of cases respectively (Vejpongsa et al 2014). 1100μM can cause acute congestive heart failure within 2-3 days of treatment, or mortality within one year (Chatterjee et al 2010). Postma et al (2002) found that heartrate variation and inotropy were significantly reduced a decade after treatment, suggesting ongoing cardiac damage which reduced the hearts ability to maintain a steady rhythm in the face of cardio accelerative events. Hershman et al (2008) found that a single injection of 'any' concentration of doxorubicin led to a 29% increase in congestive heart failure in a study of 3164 doxorubicin-treated patients over the age of 65 years. A further six injections increased the prevalence of congestive heart failure in the population to 47%.

6.1.ii. Published Studies of the Invertebrate Heart Using Video Methods

There are no heartrate variation quantification studies in invertebrates, nor any invertebrate studies using doxorubicin. A full review of published invertebrate response to cardiovascular drugs is given in chapter 1. In this section we give a synopsis of invertebrate cardiac interventions with drugs not intended for human cardiac use and therefore not included in chapter 1, as they are captured by video methods similar to our own. Bownik et al (2016) filmed *Daphnia* exposed to clove oil (50mM) induced tachycardic dysrhythmia. Heartrate variation increased over exposure time. Ectoine partially reduced these effects. Ectoine is produced by halophilic bacteria and serves as an osmoprotectant to commensal animals (Bernard et al 1993). Prior to this, Campbell et al (2004) used video to capture lactose induced reversible dysrhythmia in *Daphnia* using 100-200mM lactose over 30-60 minutes. Recovery occurred two to three hours after wash-out. Associations were drawn between heart disease and lactose intolerance. Interest in Campbell et al's (2004) work instigated a range of both corroborating and conflicting studies which also used video as a means of data collection. These reversed the effects of 50mM-lactose-induced bradycardia using volatile oils from the leaves of *Cardamomum maton* (Subbulekshmi et al 2016), or from *Artocarpus heterophyllus* (Periyanayagam and Karthikeyan 2013) or from *Trichosanthes cucumerina* (Periyanayagam et al 2015). There are concerns about reliability of methods in these three studies. Animals were mounted on petroleum jelly, which alone can cause *Daphnia* heartrate to decrease **[4.3]** which may form an apparent 'recovery' from dysrhythmic rate increase. Researchers in the above three papers claimed to use only one day old neonates, but their photographs show heavily pregnant *Daphnia* with ten, seven and two eggs respectively. Neonates are, by definition, virgins **[2.2.i]**.

Video was also used to analyse dysrhythmia in excised *Drosophila* hearts (Choma et al 2006), in this study for the development of a program of automated image capture. Santalla et al (2016) used this automated video method to show the *Drosophila* cardiac response to caffeine had an age correlated factor. Older flies were more likely to suffer a more irregular dysrhythmia and death. The image capture program did not fit with our own studies as it is based around an excised *Drosophila* heart stretched as a band and immobilised, and does not cater for live, slightly mobile, *Daphnia* **[4.3]**. Zhang et al (2015) were able to 'strengthen' flies under 4 weeks old by placing them in small exercise wheels for 2.5 hours, making them less susceptible to caffeine. This reduced resting heartrate and reduced the frequency of dysrhythmic episodes.

6.1.iii The Choice of Film and Exposure Timings

We chose to perform videos of 12+ seconds (720+ frames) because this approximates an equivalent to two minutes of human heart beating at 60bpm. In normal rhythm, around 120 *Daphnia* heart beats are produced in 12 seconds. Mohebbi and Ghassemian (2012) found that paroxysmal atrial fibrillation lasts from two minutes to seven days. So, if we were to apply a system as a predictive tool, then to detect episodes at their smallest we must be able to work with two minutes of heart data for a human – approximately 120 beats. For example, Holter monitors record two-minute ECG samples from which diagnoses are made (Gong et al 2015). Ideally our work with *Daphnia* should be able to detect quantifiable differences in heart rhythm using no more data than this. We chose to expose *Daphnia* to single concentrations of doxorubicin for precisely one hour. A baseline video of the heart was taken before exposure, then each *Daphnia* was submerged in a solution of doxorubicin for one hour, then a second video of the heart was taken. This was because both in preparatory set-up work, and throughout ensuing trials, the animals were observed to have a decrease in heartrate over the course of the hour. We knew that heartrate decreased over time as opposed to, for example, maintaining stability followed by a sudden drop in heartrate, as the heart was observed every five minutes full hour in initial observations. We chose an hour because we needed heartrates to drop enough for a comparison across concentrations to be possible, but we were also worried about the viability of animals beyond this point so did not wish to extend the exposure much longer. As we saw in chapter 5, control animals left in drug free APW began to have more variant responses after one hour, while a constant heartrate was maintained for the first hour of observations. Taking any longer than one hour may add confounding factors.

6.1.iv Selection of Scoring Systems from the Literature for this Study

The scoring systems are the means by which we will attempt to link starting heart rhythm with heartrate response to treatment. This section attempts to impart a wider understanding of these systems. Mathematical application of each scoring system is explained in **3.6.ii**. This section will explain what each system does to the data and discuss the studies based around them.

6.1.iv [a] Qualitative Methods of Classifying Cardiac Dysrhythmia

To date, the quantification of heartrate variation is seen purely as a method of risk stratification for serious cardiac dysrhythmia and sudden cardiac death, while the use of scoring systems as tools to predict later cardiac events are not investigated. Healthy hearts make continuous adjustments to heartrate which oscillate around mean sinus rate to keep within a healthy range. Published systems for the most part use simulated data and originate from bioengineering fields. Increased heartrate variation tends to be positively associated with poor outcomes as will be discussed below. However, studies that combine quantification with direct observation are rare. Direct observation uses ECG patient data to predict cardiac events from smaller previously undetected events. For example, Takahashi et al (1981) showed that brief, self-terminating, outbursts of atrial fibrillation occurred prior to 40% of sustained episodes of atrial fibrillation. 18% of patients with paroxysmal atrial fibrillation are diagnosed with permanent atrial fibrillation within four years (Mohebbi and Ghassemian 2012). However, only 13-21% of these episodes are recognised by current algorithms used by pacemakers (Hindricks et al 2010). The authors do not discuss exactly how these episodes were diagnosed. Yan et al (2006) found that Cardiovascular Magnetic Resonance imaging allowed them to significantly predict the possibility of sudden cardiac death in the patient's future: 20% of patients predicted most at risk died within 18 months. Given these examples of smaller events leading to larger events, authors sought means of categorising heartrate variation that may give early warning signs for atrial fibrillation. Grossman and Beek (1990) found that for patients with normal sinus rhythm, heartrate variation is related to breathing (in terms of the normal respiratory sinus arrhythmia that occurs upon breathing in and out) while in hypertensive patients it is less related to respiratory sinus arrhythmia. Farrell et al (1991) found low heartrate variation correlated with lower ability to adapt to physiological insult e.g. dysrhythmic events. From this, Dreifus et al (1993) asserted that heartrate variation is a possible measure of risk stratification for serious cardiac dysrhythmia.

Delay plots have been used as a staple method for examining heartrate variation. They are simple two-dimensional scatter plots, displaying beat period in seconds on the x-axis, and the previous beat period on the y-axis. An example is seen in **3.6.ii(b)** figure 3k. In stable/low heartrate variation, scatter points line up along the line of identity. As heartrate variation increases, points occur further from the line of identity. For Milovanovic et al (2009) tight clustering of delay plot indicated limited heartrate variation in patients with alcoholic liver cirrhosis, while points more dispersed along the line of identity predominated among controls. Similar clustering also occurred during syncope in Holter recordings from boxer dogs, a breed prone to atrial fibrillation, which dispersed around the line of identity when animals were returned to normal sinus rhythm (Moise et al 2010). Hayano et al (2012) likened delay plots to a cone diverging either side of the line of identity. They applied a line of best fit to the cone edge in data from 120 patients and measured the angle of the fitted line. This angle diverged more strongly from the line of identity in 13/120 who died of congestive heart failure during the study, making it a predictor for sudden cardiac death. This method assumes a clear V fan-shape of points extending from the line of identity through which an arbitrary edge can be drawn to divulge an angle, while delay plot data is generally more randomised with decreasing heart health. Nikillus et al (2007) developed a pattern recognition programme based around these relationships with the line of identity and normal sinus rhythm. However, no more is published regarding its success as a predictor of atrial fibrillation (October 2018).

Other qualitative assessments include point density plots, an example is seen in **3.6.ii(a)** figure 3j. Climent et al (2009) created histograms showing the number of occurrences for each period, which revealed multi-peak distributions of periods in atrial fibrillation cases. They compared this to point density plots. Histograms revealed the probability of any length of period occurring, while point density plotting revealed the probability of the occurrence of two consecutive groups of periods following the same pattern. Park et al (2009) applied plot density delay plots to pre-atrial fibrillation event ECG data from Physionet (an open-source web database of physiological signals) and found that plots with more than one cluster signified atrial fibrillation, while those with a single cluster signified normality. This analysis had 91.4% sensitivity and 92.9% specificity when comparing atrial fibrillation cases to known normal sinus rhythm records.

6.1.iv [b] Quantitative Linear Methods of Classifying Cardiac Dysrhythmia

The ellipse fitting technique seen in **3.6.ii(b)** figure 3k, is the most common form of linear quantitative assessment used across the literature. It takes the cluster of points created by plotting heart periods in series against previous heart periods in consecutive succession, and assumes they form an elliptical shape around the line of identity $x = y$. A quantitative result is produced by forming the ratio of SD1:SD2. SD1 is the standard deviation orthogonal to the line of identity $x = y$, and represents short-term variability and fast changes in heartrate. SD2 is the standard deviation along the line of identity $y = -x + 2RR_m$ (where RR_m is the mean of all RR intervals) and represents long-term variability and slower changes in heartrate (Mohebbi and Ghassemian 2012). As points deviate further from the line of identity, heartrate variability increases.

SD1:SD2 differentiates most cardiac abnormalities from normal sinus rhythm. However, it cannot quantify temporal variations in non-linear delay plots so lacks the diagnostic depth given by qualitative assessment of the data. Ellipse fitting relies on there being a single cluster of points, and this hides the complex and dynamic nature of heart activity. It has been used to successfully distinguish between controls with normal sinus rhythm and patients with metabolic syndrome (Kubickova et al 2016); episodes of sustained ventricular tachycardia (Huikuri et al 1996); supraventricular tachycardia (Rydberg et al 2007); paroxysmal atrial fibrillation (Mohebbi and Ghassemian 2012); and sustained atrial fibrillation (Thuraisingham 2006). Using the SD1:SD2 ratio, Chua et al (2008) found that normal rhythm was scored much lower than abnormal conditions (0.3626). However abnormal conditions did not necessarily rank according to severity:

Left branch bundle block	0.5172
Ischaemic cardiomyopathy	0.6767
Ventricular fibrillation	0.6871
Complete heart block	0.7420
Sick sinus syndrome	0.8839
Ectopic beats	1.0016
Atrial fibrillation	1.3960

While we cannot categorically define the relative severity of all of these (for example the degree of complete heart block was not defined so cannot be ranked easily), the most obvious fault with this ranking is that ventricular fibrillation should be ranked with a higher score as it is more severe than atrial fibrillation. In VF the heart cannot pump blood, causing cardiac arrest, while in AF the heart is still beating in an irregular, rapid manner.

SD1:SD2 has been used to test drugs known to cause Torsaides de Pointes. Valentin et al (2004) found higher ratios correlated with higher dysrhythmia risk. However, the technique could not distinguish patients with prior myocardial infarction, which had occurred over a month previously, from healthy controls (Huikuri et al 1996). Catastrophic fatal dysrhythmia is relatively common following myocardial infarction, and therefore we might expect these patients to vary from healthy controls. However, while dysrhythmia risk may be high in the days following immediately following myocardial infarction, it might normalise somewhat over time. In an unusual variation, Hirose et al (1998) used the x-axis itself as SD2 rather than the line of identity, though the reason for this was unstated. A patient had a dysrhythmic reaction to pain management treatment (an alcohol injection) and produced a substantially higher SD1:SD2 ratio compared to 22 unaffected patients.

A second linear technique is Standard Deviation of Successive Differences (**SDSD**) described in **3.6.ii(c)**. This is the standard deviation of the difference in length between each consecutive period. Application of the Successive Differences Technique simply comprised taking heart periods as a single list and finding the difference between one data point and the next by subtraction, then finding the standard deviation of these differences and using the result as a measure of heartrate variation so that a larger SD infers a more varied heartbeat. Galland et al (2006) were able to use SDSD to differentiate heartrate variation in actively sleeping versus quietly sleeping infants, and in infants sleeping prone as opposed to supine. Hindricks et al (2010) applied SDSD to two-minute R-R intervals data from implanted cardiac monitors to diagnose abnormal rhythms and found that while the method was highly sensitive (96%) it had a tendency to produce false positives reflected in a low specificity of only 85%. Thuraisingham, (2006) and, Zarim and Rhaman (2011) found that SDSD could significantly distinguish between atrial fibrillation and normal sinus rhythm using data provided by Physionet and the MIT-BIH Dysrhythmia Data Bank respectively. Chong et al (2015) developed an iPhone application to measure the pulse which interpreted data via SDSD. While their methods could differentiate normal sinus rhythm from abnormal rhythms, they could not differentiate atrial fibrillation from ventricular premature beats or atrial premature beats. Jovic and Bogunovic (2010) found that SDSD was poor at distinguishing between supraventricular dysrhythmia, congestive heart failure or atrial fibrillation and acknowledged this may be due to application of a linear test parameter to non-linear data.

Further linear techniques, so far applied to computer-generated data only, involve comparing points above and below the line of identity. Formulas are given in **3.6.ii(d)**. Porta's Index simply counts the points in a delay plot and assumes that the more equal the number of points above and below the line of identity, the more stable the heart rhythm (Porta et al 2006). Guzik's Index look distances of delay plot points from the line of identity. It is very similar to the SD1:SD2 technique but ignores the tangent to the line of identity formed by crossing through the mean period. It assumes that if points above the line of identity and below it is close to equal in distance from that line, the more stable the heart rhythm (Guzik et al 2006). These two techniques have never been applied to real heart data in publication. Initial tests in chapter 4 found them to be the least able to distinguish any dysrhymia either from normal rhythm or from another dysrhythmia, for this reason we decided not to take these techniques forward in this investigation.

Another linear method is Point Dispersion which is identical to the traditional Schmoo Plot **[3.6.iii]** where distances of all points are calculated from a centroid. Park et al (2009) looked at the dispersion of points from the centroid in comparative atrial fibrillation and normal sinus rhythm cases but could not derive differences between the two. This is perhaps because point dispersion would ignore the direction in which points are dispersed, it would conflate points dispersed along the line of identity representing a simple change in heartrate, with those dispersed tangential to this line which represent an increase in variation in heart rhythm. There is also Segregated Plot analysis, which segregates the normal delay plot using standardised linear frequency bands, then counts points in each band. For Voss et al (2012) these were assigned very low frequency= <0.04 hz, low frequency= 0.04-0.15 hz and high frequency= >0.15Hz. Researchers found that high risk dilated cardiomyopathy patients produced significantly high numbers of points in the low frequency quartile relative to healthy patients. However, as this method relies on frequency bands standardised to the human heart, the establishment of standardised frequency ranges from *Daphnia* would also require a separate study.

6.1.iv[c] Quantitative Non-Linear Methods of Classifying Cardiac Dysrhythmia

Various non-linear analysis methods have been postulated to differentiate dysrhythmia. This discussion is brief as papers focused on mathematical theory but did not apply these equations to real heart data for the large part. Non-linear line of identity symmetry analyses equations is given in **3.6.ii(e)**. Karmaker et al (2009) created non-linear

adaptations of Porta's and Guzik's linear indices, which they called Porta's Cloud (PC) and Guzik's Cloud (GC). The reason for the word 'cloud' is unclear, however, what these equations do is take into account whether three consecutive periods are increasing, decreasing or remaining the same in length. Instead of using two-dimensional data directly from the delay plot, this creates a new delay plot based on the changes between periods. The normal Porta and Guzik's equations are then applied to the new data instead. Ehler's Index (EI) (Ehler et al 1998) is a non-linear analysis also based on comparing symmetries either side of the line of identity. It gives sum of all distances cubed divided by the three halves power of the sum of the square; what this means in real terms is anybody's guess. However, it does make the otherwise two-dimensional delay plot non-linear by adding extra dimensions, and is suggested for period data analysis though it, along with the above, have never been tried on real heart data. However, what we do know from early explorations in chapter 4, is that its application was indeed one of the best at differentiating both dysrhythmias from paired baseline data, and from one another. Karmaker et al (2009) also created an adaptation of this indices using a similar idea of three consecutive periods rather than two. None of these have been tested on real data.

The Complex Correlation Measure (CCM) equation is described in 3.6.ii(f). CCM looks at point-to-point variation across the delay plot rather than a gross description of the entire plot. A moving window of three consecutive points in the 2D delay plot is used to create imaginary triangles, which evolve with time; thus, each triangle is composed of four heart periods. The area of each triangle, as defined by three consecutive coordinates is calculated, and the standard deviation of the data set is taken for comparison with normal rhythm. Karmakar et al (2009) found when delay plots of dysrhythmia, congestive heart failure and normal sinus rhythm were analysed via the CCM differences between them were more significant (having a smaller p-value) than the SD1:SD2 ratio method.

The Multi-scale Ratio Feedback Analysis (MRFA) equation is described 3.6.ii(g). MRFA measures change in heartrate across three successive beats. Coordinates are derived from the differences between successive beats and plotted on a scatter plot. For example, in the consecutive sequence "a, b, c, d, e…" the first coordinate derived from three successive beats would be (x, y) where x = b – a, and y = c – b. In this case, (0 ,0) would indicate no difference in period length across three beats. These new points can be plotted so that the top right quartile (N_1) represents (+,+) coordinates or continued tachycardia, the bottom

left quartile (N_3) represents (-,-) coordinates or continued brady cardia and the top left and bottom right quartiles ($N_2$and N_4 respectively) represent (-,+) and (+,-) coordinates where the heartrate oscillate around a point. The Total Feedback Ratio R_{TF} id then derived from this which gives a ratio of total points in diagonally opposite quadrants, with points representing change in heartrate on top, and points representing change in rhythm on the bottom, much as in the SD1:SD2 ratio but this time with non-linear quantities. Using data from Physionet, Huo et al (2014) found that MRFA differentiated atrial fibrillation patients from those with normal rhythm. Quadrants 2 and 4 were significantly more populated in patients with abnormal rhythms, low scores were significant in atrial fibrillation where wild swings in rhythm indicate fibrillation, and high scores were significant in congestive heart failure, where as healthy subjects had a mid-range score representing relatively smooth heartrate oscillation associated with respiration.

The Median Stepping Increment (MSI) equation is described in 3.6.ii(h). MSI examines Euclidean distance between points and does so via simple adaptation of the Pythagoras equation which accounts for sampling rate. Unlike simple delay plots of period alone, this interpretation takes ECG data into full account and looks at wave magnitude, producing true delay plots of data. However, Gong et al's (2015) equation assumed a constant sampling frequency. Our own sampling frequencies were no longer a constant once parabolic peak interpolation is taken into account. To carry out MSI we adapted the equation (also in 3.6.ii(h)) to use exacting time between samples. This made more sense geometrically, whereas using constant frequency would discard data and therefore obscure results. MSI was used to predict successful defibrillation in ventricular fibrillation patients by providing severity scaling of delay plot data (Gong et al 2015). Results were significantly different in hearts prior to successful versus unsuccessful defibrillation.

The Finite Time Growth (FTG) equation is described in 3.6.ii(i). It is an adaptation of a Lyapunov equation developed by Wessel et al (2010) as a test for how divergent a delay plot might be in nine-dimensional space. This simply means that they go beyond previously discussed explorations which look at distances created by three periods in a row, and instead compare series of nine periods in a row to one another across all consecutive periods. They do this using a nearest neighbour analysis. They chose nine dimensions after testing all dimensions around this number and resolving that the most precise results were given at this level, with higher dimensions not giving a significantly better result.

The equation looks at adjacent samples to determine how the dynamic trajectory evolves one-time step into the future. Every period data point is taken as a coordinate. Nine consecutive coordinates give a position in 9-dimensional space. A series of calculations are performed which compare every 9D vector to every other 9D vector. The five nearest neighbours to each of these 9D vectors in terms of 9D space rather than time are then identified. If, for example, the heart was following a continual circuit, the nearest neighbour of any point will be the next set of points which reach that space at any other point in time. A mean value of each of these five nearest neighbours is then derived. This is compared to the same again applied to the next 9-dimensional vector along, skipping forward one period at a time. A Euclidean norm of the ratio of the difference between these values is found. This is done for all consecutive 9-dimensional vectors and then a mean is taken of all results. This produces a result between -1 and 1. As this result draws closer to 0 there is less variation among nearest neighbours so we can infer that a more conservative plot occurs, as it draws closer to 1 the plot is more divergent indicating a greater degree of heartrate variation. Wessel et al (2010) indicate that while -1 also indicates divergence, they would assume that the negative indicates an animal recovering from divergence, rather than moving towards a greater divergence. However, this has not been trialled on real life data which would differentiate the two states. Wessel et al (2010) applied **9DFTG** to readings from Implanted Cardioverter-Defibrillators of 1000+ beat-to-beat intervals before the onset of fatal dysrhythmias and found significant correspondence between the qualitative strength of the dysrhythmia and the FTG result.

6.1.iv [d] Study Selection for Scoring Systems

Given our earlier study **[4.5]** during the method development stage, we investigated 12 applicable scoring systems. Eight were non-linear and four were linear. It was very clear that non-linear methods held greater potential in correlating starting states with the same animal's response to a cardiac event **[4.5.iv]**. Among these, **FTG**, **EC** and **EI** looked to be the most likely systems in which we might find success in correlating the two. **EI** and **EC** are non-linear methods described in **3.6.ii(e)** but could not be discussed above as they have not been applied to actual heart data and come purely from the field of theoretical mathematics.

For this study we chose to look at the eight published non-linear systems, and not the four linear systems, for the clear and apparent reason that linear systems had failed to perform in earlier work. As discussed in **4.5** each system is based on a comparison of consecutive heart beat periods one against the other, this is essentially a non-linear comparison as it directly compares data streams from different points in time in parallel. It makes sense then that a non-linear system would be best for a non-linear comparison. However, we chose not to narrow our study down to the three 'favourites' as this would not be appropriately rigorous. The remaining non-linear systems have been peer-reviewed and published and no publication exists which denounces their ability to perform. It is not up to us therefore to do so without solid evidence. Therefore, all non-linear systems described in the review are implemented in **3.6.ii** and applied to data gained during the experimental protocol in **6.2.i**.

The goal of this study is to build a regression model to predict the outcome of future observations. The intention is that by testing the scoring systems based on starting rhythm (X), a future unobserved outcome (Y_1) can be predicted based on a paired measured feature (X_1). If the outcome is continuous, then the system ψ is defined as mapping from the incoming feature X to the outgoing feature Y, where $Y= \psi(X)$ denotes the predicted outcome based on the observed X. This is a three-step process: selection of incoming and outgoing features (rhythm variability to and response in terms of changing heartrate), scoring system selection (used to score rhythm variability) and prediction assessment. A difficulty in step two is that there are numerous scoring systems available, so that comparative predictive success becomes hard to determine. It is important to assess a successful system's generalizability when reporting results. In the absence of appropriate independent validation data another approach to estimating predictive accuracy can be achieved by resampling the original data to avoid an overly optimistic assessment of the predictive capability of a system. A further goal is to estimate how well a scoring system, which might be found successful in this trial, might predict the outcome of an observation not included in the original dataset (Molinaro et al 2005).

6.2 Methods

6.2.i Experimental Protocol

This methodology first discusses the experimental protocol, after which scoring systems discussed above and laid out in **3.6.ii** are applied. We follow this section with general results giving dose-responses, then results based on application of each scoring system. Seven concentrations of doxorubicin were made up. Either 1200μM, 1600μM, 2000μM, 2200μM, 2400μM, 2800μM or 3200μM and diluted in APW **[2.3]**. At the start of each trial, four *Daphnia* were removed from their environment via pipette to form a test group. Groups were divided into a ratio of 1 control to 3 trial animals. Each trial animal would be randomly assigned to be exposed to one of the pre-determined concentrations of drug. This may indeed have not always been ideal, as a handful of trials could be performed per day in lab. And, those taken first may be those least able to make a quick escape by swimming from the pipette the fastest. Certainly, it was noted that the introduction of pipette into environment induced a 'swim away' response. All were placed in a shared container, then separated into one individual per dish, so that the order of which of each four was first is not known. These were labelled as a control and three concentrations of those pre-assigned above, never repeating the same concentration twice in the same set.

Data were collected by dark field high-speed film **[3.2** and **3.3]**, so that consecutive beat-to-beat periods are found for each starting film. At the start, each individual's heart was filmed in APW for 12+ seconds **[6.2.iii]**, giving 720 frames from which to derive period data. After the starting film, the animal was placed into the assigned concentration or control solution and allowed to swim freely for one hour. At the end of one hour a second film of 12+ seconds was made. Due to the one hour wait time, multiple test groups could be temporally overlapped, enabling between 12 and 16 animals to be tested per day. Video data was then saved and processed as per **3.6.ii**. Parabolic Peak Interpolation **[3.5.v]** was used for each video to give heartrate before and after drug exposure, and on pre-exposure film data to give accurate beat-to-beat period data. From this a dose-response curve was formulated. Experimental results are presented in **6.4.**

6.2.ii Scoring Systems Analysis

At the end of the experimental phase, we had the following information:

1. Heartrate before exposure
2. Heartrate after exposure
3. Beat-to-beat period data before exposure

This information was used as follows. 1 and 2 were used for concentration-response associations. 3 was input into the scoring methods, which were then correlated with concentration-response.

Scoring methods from across the literature [3.6] were performed on resultant data. As described above, 720 frame sequence films from each *Daphnia* were semi-automatically processed [3.5.iii(b)]. Each item of data (pixel size of heart) typed by hand and double checked before being scored according to all eight published non-linear analysis methods, which comprise 9DFTG, MSI, EC, CCM, GC, MRFA, PC and EI. These scores were then correlated with each individual's heartrate change after one-hour exposure to doxorubicin. Scoring systems were then compared to find which performed the strongest correlation.

In addition to these scoring systems, we attempted to improve the correlative capability of FTG. While other systems are limited, the Lyapunov exponent, also known as the number of dimensions, tested (i.e. how many beat-to-beat intervals in a row we compare and score per *Daphnia* sequence) can be adjusted. Wessel (2010) applied nine-dimensions to this exponent, i.e. nine beats in a row. By altering this number, we sought to test whether improvements could be made in correlations between starting score and response to the drug. While Wessel (2010) 's testing found that increasing dimensions did not improve their differentiation between dysrhythmias which they were attempting to score, we, on the other hand, are not differentiating dysrhythmias from each other. Dysrhythmias are by their nature high in heartrate variation, and so a low dimension FTG were more appropriate. Instead, we are scoring baseline rhythm from individuals in an attempt to correlate it with their response to toxic insult. Thus, higher dimension FTG may offer improved discrimination. The simple adaptations to this equation are described in 3.6.ii. We also applied 11- and 13- dimensional FTG data from all 152 animals.

To decide whether Spearman's or Pearson's rank correlation were more appropriate when assessing the strength of scoring systems, we began by checking for linear relationships via

scatter plots. Looking to the experimental results helped to determine statistical methods. We needed to do this because of the nature of the paired data. One part of the pair is a score of heartrate variation before the drug, based on non-linear scoring of beat-to-beat rhythm. The other is heartrate response to a toxin in a set time, a percentage change function of starting and end heartrate. On one hand we might argue that these are independent variables. One looks at rhythm, the other at rate-based response. Hearts can be fast or slow, and, stable or dysrhythmic; the two do not always relate. This correlation would require Spearman's rank coefficient. However, both are indirectly related as a stressed animal's heart might beat faster. Given that, a regression model such as Pearson's might be required. To this end we needed to test which correlation was more appropriate.

Table 6a. Linear vs non-linear categorisation of scatter plots

	9D FTG	11D FTG	13D FTG	CCM	EI	EIC	GC	PC	MRFA	MSI
1200µM	Not lin	Not lin	Not lin	Amb	Not lin	Not lin	Lin	Not lin	Not lin	Lin
1600µM	Not lin	Lin	Amb	Not lin	Not lin	Amb	Not lin	Not lin	Not lin	Amb
2000µM	Amb	Not lin	Not lin	Not lin	Not lin	Amb	Not lin	Not lin	Not lin	Not lin
2200µM	Not lin	Not lin	Not lin	Not lin	Amb	Not lin	Not lin	Not lin	Not lin	Amb
2400µM	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin
2800µM	Not lin	Amb	Lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Amb
3200µM	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Not lin	Lin

Legend: Lin = the plots resolved into approximately normal bivariate linear distributions; Not lin = the plots were not clearly linear, for example have multiple focal points or form shapes which were clearly not straight lines. Or, where only an irreconcilable congregation of points is seen giving normality but no obvious linearity; Amb = the plots were too ambiguous to tell

Seventy scatter plots were made of score vs response for each of the seven concentrations trialed against each of the ten scoring methods used. For efficiency and as visually presenting these figures is not integral to our findings, but merely a way to choose a statistical system, decisions made by viewing the 70 scatter plots are summarized in table 6a, In this way we attempted to judge whether Pearson's (linear distributions) or Spearman's (non-linear normal distributions) were more appropriate. Most plots gave a normal distribution about a single point with no obvious linearity. No method produced

categorically linear scatter plots across all concentrations, though one or two produced linear scatter plots for a small portion of the concentration cohorts tested.

Spearman's correlation coefficient was chosen to test for correlation between heartrate response and starting scores of beat-to-beat period data. To perform this, animal ID (an alphabetical code), response and score were placed in rows. The latter two categories were ranked from smallest to largest. The following Excel functions were then applied to perform the Spearman's correlations:

Column 1 of ranked numbers indicating Score ↓	Column 2 of ranked numbers indicating response ↓	Rho	=CORREL(column1,colum2)
		P	0
		N	=COUNT(column1)
		Df	= n – 2
		St	=SQRT((1-Rho^2)/df)
		T	=(Rho-p)/St
		alpha	0.05
		t-crit	=T.INV.2T(alpha,df)
		p-value	=T.DIST.2T(ABS(t),df)
		Sig	=IF(p-value<alpha,"yes","no")

Finally, in order to estimate a successful system's generalizability, we performed a leave one out cross-validation, as this test tends toward a small bias with elevated variance. Once a successful system is found, it is fit on a subset of the data called the training set, and the fitted model is used to predict the responses for the observations in the validation set. To do this the data were divided into n parts, n being the sample number. For each sample, the equation of the line, in terms of slope and intercept was found for all samples leaving out the sample in question. The resulting equation was then used to create a predicted $\hat{y}$ value with which to compare the original y value. CV_K is a summation of all the calculations the average Ks that can be derived from n. We can then calculate the standard error of CV_K, which gives the percentage test error. Equations are shown in figure 6b.

Figure 6b. Cross-Validation for K-fold equations

$$MSE_k = \sum_{i \in C_K} \frac{(y_i - \hat{y}_i)^2}{n_k} \qquad CV_{(K)} = \sum_{k=1}^{K} \frac{n_k}{n} MSE_k \qquad \widehat{SD}(CV_K) = \sqrt{\sum_{k=1}^{K} \frac{n_k}{n} MSE_k}$$

Legend: $\hat{y}$=predicted value, y=measured value (cardiac response), n=sample number, K=folds, CV=Mean cross-validation, $\widehat{SE}(CV_K)$ =standard deviation of cross-validation.

6.3 Results

6.3.i Experimental Results.

One hundred and ninety *Daphnia* were exposed to one of the seven concentrations of doxorubicin or a drug free control. Results are tabulated in table 6c.

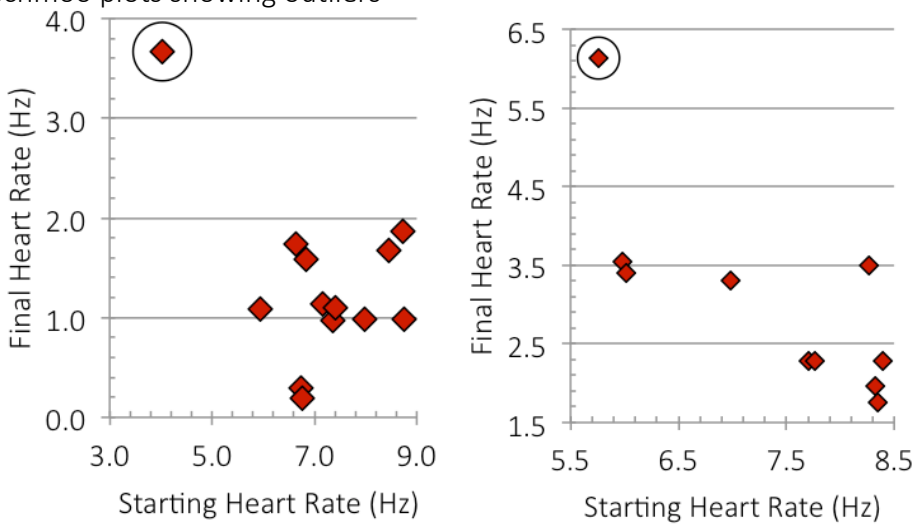
Table 6c. *Daphnia* heartrate response to doxorubicin

Conc	Control	1200µM	1600µM	2000µM	2200µM	2400µM	2800µM	3200µM
N	38	12	26	9	26	26	26	26
Died [ref 3.1]	0	0	2	0	0	13	6	17
HR% change M+SE	+ 0.31 ±1.46%	- 12.29 ±4.67%	-54.38 ±4.21%	-57.06 ±8.88%	-73.44 ±2.72%	-84.71 ±2.05%	-23.90 ±6.09%	-62.64 ±4.80%
Range	-18.89% to +17.41%	-41.61% to +8.76%	-96.35% to -13.04%	-89.27% to -22.93%	-99.03% to -46.34%	-97.23% to -73.75%	-63.30% to +42.06%	-79.0% to -40.7%
Span of range	36.30%	50.37%	83.31%	66.34%	52.69%	23.47%	105.36%	38.30%

Just as in chapter 5, *Daphnia* response is sensitive and varied wildly, producing large standard deviations. Individual variation increased dramatically even between the control and the smallest concentration. Heartrate response becomes more varied with increasing concentrations. For 2400µM and 3200µM cohorts over half of all animals trialled died before the end of the one-hour exposure time. Fewer died in the 2800µM cohort which may suggest that *Daphnia* chosen for these experiments were somehow more able to withstand toxic challenge. However, the range of responses produces by the 2800µM cohort is also the largest. It will be interesting to see whether this greater range of responses produces individual correlations with starting state and response in the next section. The 3200µM cohort responses gave a narrower span of responses than the 2800µM cohort. This may be a facet of there being only 8 surviving individuals.

One animal in the 2400μM cohort was rejected from analysis as Grubbs (1969) testing showed it to be an outlier, illustrated in figure 6d. *Daphnia* I had a Grubbs value higher than G crit. This animal had a very low heartrate to start compared to the population, it was 4.02Hz compared to the population which had a mean starting heartrate of 7.40Hz. The outlier *Daphnia* I had a small decrease in heartrate of only -8.70%. In the 3200μM cohort there were two cases where heart movement was too weak to be captured on film in a way in which image capture processes might reliably isolate the beat pattern, and so these were also excluded from the investigation. *Daphnia* O in the 3200μM cohort was also rejected from analysis, as the Grubbs (1969) test showed it to be an outlier. It was unaffected by treatment instead increasing heartrate by 6.59%, which was within range of control *Daphnia* heartrate change. The right hand Schmoo plot also in figure 6d illustrates this outlier.

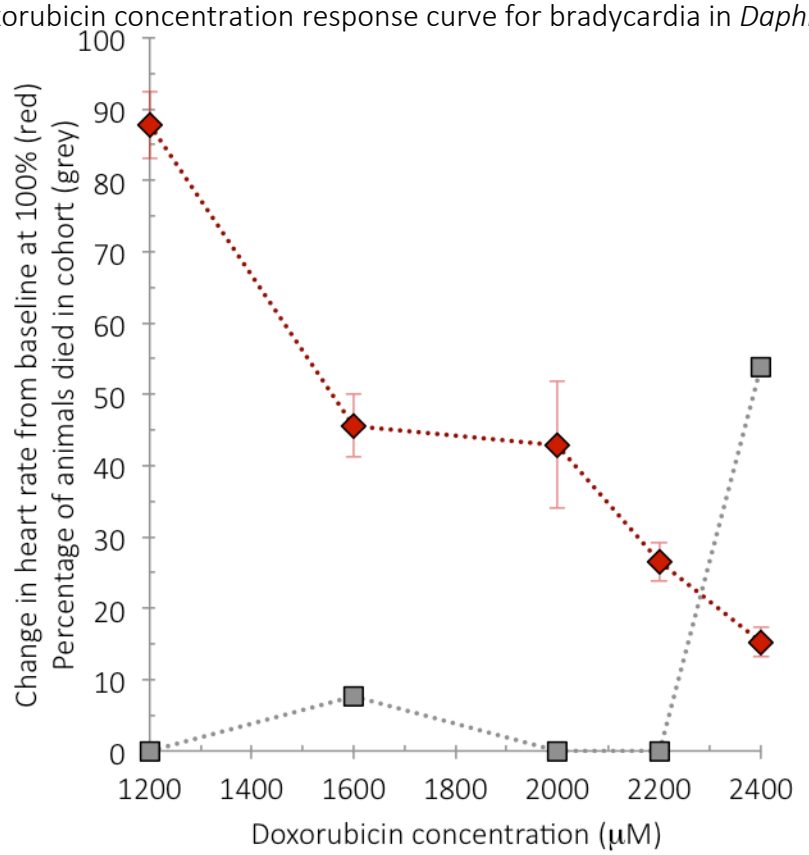
Figure 6d. Schmoo plots showing outliers



Legend: The plots show animals rejected by Grubb's testing clearly circled being visible outliers to others displayed, among 2400μM (left) and 3200μM (right) concentrations

Data were combined to form the dose response curve shown in figure 6e. Bradycardic response to doxorubicin increased concentration-dependently to a plateau between 1600μM to 2000μM in which heartrate sits at just over 50% of its starting rate before exposure. Then, steadily increases once more at 2200μM and 2400μM. After this response becomes erratic due to the toxicity of higher doses and the comparative increase in mortality at these doses.

Figure 6e. Doxorubicin concentration response curve for bradycardia in *Daphnia*



Legend: Diamonds show %mean±SE heartrate decrease after 1-hour exposure to doxorubicin. Each point equates to the mean of a population of animals whom were exposed to a single concentration of doxorubicin for one hour. Squares indicate % animals which died in each cohort during exposure.

6.3.ii Scoring-System Results

Beat-to-beat period data was collected from the 109 *Daphnia* which had paired data and having survived the one-hour exposure (38 died, 4 were excluded **[6.3.i]**), from 78,480 individual frames of video as described in **[3.5.iii(b)]**. Pixel size of heart from each frame was typed by hand and double checked before parabolic peak analysis produced beat-to-beat data **[3.5.vi]**. This was then scored according to the eight published non-linear analysis methods and the two adaptations of FTG described in **6.1.iv(c)**. Resultant scores were compared to drug response in terms of percentage heartrate change, per individual, per dose, via Spearman's rank correlation. Table 6f shows the mean percentage correlations for each scoring system in order to give an overview of each system's general success in correlating baseline rhythm with heartrate response. Spearman's rank found EI, PC, MRFA and GC produced the lowest mean correlations.

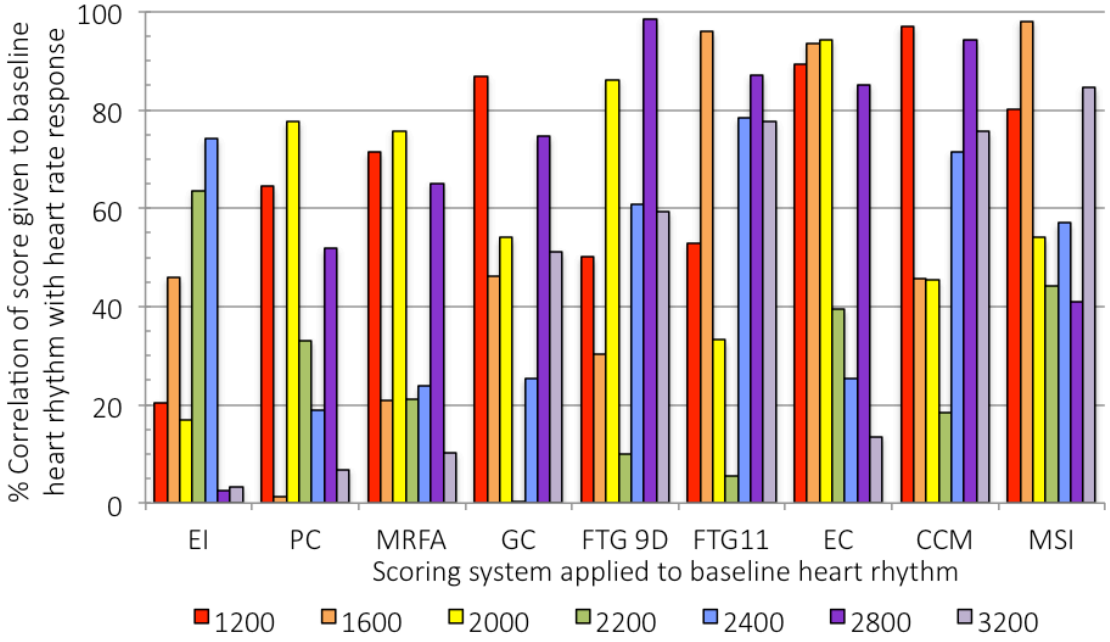
Table 6f. Correlations between starting rhythm score and heartrate response

EI	PC	MRFA	FTG13D	GC	FTG9D	FTG11D	EC	CCM	MSI
32.39	36.27	41.16	48.19	48.39	56.43	61.48	62.95	63.96	65.54
	Lowest correlation			→			Highest correlation		

Legend: numbers show the mean correlation between baseline rhythm and heartrate response across all concentration cohorts for each scoring system

Notably there is variation in how much correlation the different systems had at different concentration. This observation arose repeatedly throughout the analysis and is returned to in the discussion. These are shown in figure 6g. The remaining four published non-linear scoring systems were only incrementally more successful in their correlation. At some concentrations various scoring systems reached 'significant' correlative levels, for example FTG9D, CCM and MSI found significant correlation between their given scores and resultant heartrate response in the 2800μM, 1200μM and 1600μM cohorts, respectively, at the 0.05 alpha level. While EC found significance at the 0.10 alpha level for the 1600μM and 2000μM concentrations, and CCM at the 2800μM concentration. The FTG method was readapted using 11 dimensions, and this was found to improve correlations in the FTG system, however adaptation to 13 dimensions lowered correlations. This may again be due to cohort-to-cohort variance discussed below.

Figure 6g. Correlations for all scoring systems across all cohorts

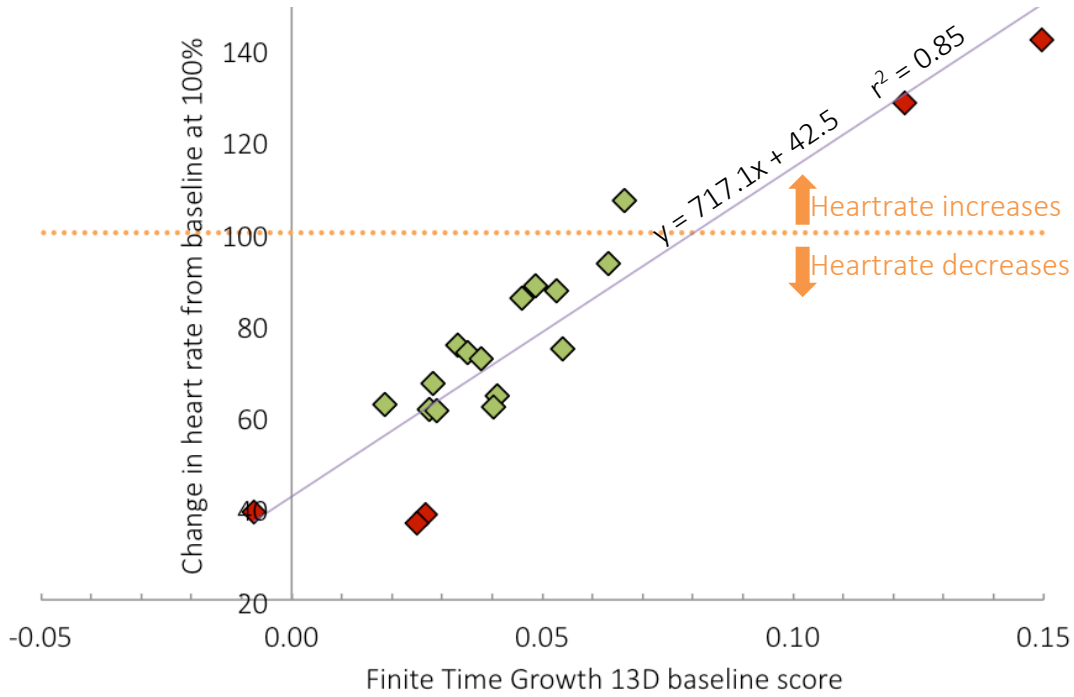


Legend: Concentration cohorts are given in μM. Each histogram bar gives percent correlation between the score given to baseline rhythm for each concentration cohort per scoring system.

For adapted FTG scoring systems, correlations again varied across concentrations. The best correlation (99.999985%) across any concentration was found at 2800µM, using FTG13D. This cohort, as well as the 3200µM cohort, were not included in the concentration-response curve because responses ranged so widely, and because fatalities increased after 2400µM, that we considered the top two concentrations to have toxic effect beyond the primary negative chronotropic action of lower concentrations. Figure 6h suggests a linear relationship may be drawn between baseline FTG13 score and animal response. A line fit to all points gave the equation y = 717.1x + 42.5, with a fit of r^2 = 0.85. Even removing extreme values (red in the figure) did not change this relationship much (y= 855.3x + 40.5, r^2 = 0.72). We understand these equations as rough approximations, but still suggest a relationship could be found if this result could be made consistent for all concentration cohorts, which may provide a useful predictive tool in further studies. The question that remained for discussion were:

- Why do correlations vary so much across cohorts?
- Why are the strongest correlations associated with more toxic concentrations?

Figure 6h. The 2800µM concentration cohort; baseline scores vs heartrate decrease



Legend: Paired data from individual *Daphnia* are shown, with extreme cases highlighted in red for reference in the text.

6.4 Discussion

6.4.i Discussion of Experimental Results.

Daphnia response to doxorubicin was just as varied as expected from work with other drugs in chapter five. Response varied wildly producing large standard deviations. Toxicity was clear at 2400μM where thirteen of the 26 animals trialled were dead before the end of the trial hour. The animal rejected by Grubb's testing (animal I) appeared unaffected by doxorubicin treatment. Its heartrate increased +8.702%, well within the normal control range. The remaining animals produced a bradycardia so strong that it obvious even when seen by the naked eye before being confirmed by video analysis. During this bradycardia the whole heart contracted with observable force. Six of those in the 2800μM cohort were dead before the end of the trial hour. Also, for the 2800μM cohort it became abundantly clear that the chosen concentration was so toxic as to cause aberrations outside of the desired bradycardic affect. Dysrhythmias and complicated beat patterns were clearly visible by eye as well as twitching and signs of stress from those animals who survived the hour. Seventeen of those in the 3200μM cohort were dead before the end of the trial hour. In two individuals the strength of contractions was weakened so that only a small section of the heart wall was seen to tremor lightly, and negative inotropy was generally observed over all animals at this concentration. The Grubb's test rejected animal, O, appeared unaffected by doxorubicin treatment, increasing heartrate by a change of only 6.59%, which is within the range of normal control change. We chose not to show the largest and most toxic concentrations in the concentration-response curve, as results are deeply affected by presumed toxic secondary effects from the 2800μM cohort upwards, underlined by increasing death rates.

6.4.ii Discussion of Scoring-System Results

Given it is doubtful that a linear relationship exists between non-linear scores of baseline heart rhythm and their paired animal response to drugs, we decided that Pearson's correlation was inappropriate, and chose to use Spearman's Rank to compare the success of various scoring systems. It is more probable that non-linear derived scores would also have non-linear correlations when compared to paired data. Notable variation was seen in the success of baseline-to-response correlations between systems at different concentrations. Some systems found significant correlations, but this was not consistent across all concentrations tested.

The FTG system (Wessel 2010) was the only one among those published that allowed for user adaptation. This is done by varying the number of 'dimensions', or more simply, the number of consecutive beats. These are compared to all other same length of consecutive beats across period data. We postulated that increasing dimensions may create a more sensitive way of broadening the range of starting scores. We postulated that a larger dimension would create a greater variance of baseline scores as it would be unlikely that closeness of fit could be maintained across broader spectrums of beats. By widening this baseline range of scores, we hoped that there would be more scope with which to correlate the paired results.

It is not immediately clear whether the adapted FTG equations improved correlations. Overall improvements were only slight with mean overall success increasing by only 5% when comparing FTG 11D to the original and decreasing by just less than 5% using FTG 13D compared to the original. Actual concentration-to-concentration improvements were extremely variable. The 2200μM concentration group produced the poorest correlation between score and response in any FTG-based scoring system, while other groups varied in a non-linear fashion. The best correlation (99.999985%) across any concentration was found at 2800μM, using FTG13D, yet this is a concentration which is in the toxic range in terms of Daphnia death, and also had the widest range of responses.

The variation in success across concentrations for all systems is noticeable. One possible cause for this may be the range of baseline scores in each population. Correlations are better at some concentrations than others, and it seems a wider range of scores in a concentration group allows for greater correlation. The success of each of the eight published scoring methods and two adapted methods, were paired with their baseline score variation across the seven concentrations in paired sample T-Tests. For EI and PC we found that scoring system success and baseline variation of scores were significantly associated at the 0.05 alpha level. These were the bottom two scoring systems. For all other scoring systems baseline-response correlation success and baseline score variation were correlated at the 0.01 alpha level. We can confidently conclude that a broader range of animals to begin, such a natural population or one not selected for age, would have greater success when attempting to correlate starting score with response to a toxin. It may be possible to correlate a starting score with heart response using an adapted FTG but only if animals were selected from a population variant enough to begin.

The 2800μM FTG data set were chosen for leave one out cross-validation, having the most significant correlation between baseline and response, and also meeting our secondary parameter of good variation between starting scores. The measured and predicted results for the 2800μM FTG data set and related calculations are shown in table 6i. Cross-validation calculations found that for 20-folds or leave one out cross-validation of this dataset $MSE_k = 2633.04$, $CV_{(K)} = 131.65$ and $\widehat{SE}(CV_K) = 11.47$. The percentage test error is 11.47%. So overall, the scoring system FTG13 has an 88.53% fit which is very close to the 0.890 Rho value (equivelant to an 89.02% fit) for whole data as given by Spearman's Rank. Indeed, performing root mean squared error over the whole data leaving none out results in 10.36% percentage test error. The cross-validation appear to agree with the predictive accuracy or the original results for this cohort using the FTG13 scoring system.

Table 6i. Measured and predicted results for the 2800μM FTG data set.

Code	x	Y	Line equation of 19 remaining			$\frac{(y-\hat{y})^2}{n_k}$
			A	b	$\hat{y}$	
D	-0.008	60.97	-722.59	57.88	63.32	5.51
Y	0.018	37.38	-726.28	58.31	44.89	56.50
Z	0.025	63.29	-693.02	55.10	37.79	650.55
R	0.027	61.31	-695.65	55.27	36.75	603.12
J	0.027	38.53	-716.50	57.45	37.84	0.48
L	0.028	32.85	-720.98	57.93	37.67	23.22
P	0.029	38.90	-715.27	57.30	36.54	5.59
O	0.033	24.39	-723.06	58.29	34.40	100.24
I	0.035	26.16	-720.34	57.98	32.63	41.81
Q	0.038	27.38	-718.36	57.73	30.63	10.53
N	0.040	37.95	-714.29	56.88	28.10	97.10
E	0.041	35.66	-715.07	57.02	27.72	62.95
H	0.046	14.29	-717.54	58.07	25.15	117.93
X	0.049	11.48	-716.19	58.05	23.23	137.90
U	0.053	12.57	-715.18	57.79	20.07	56.24
S	0.054	25.41	-719.32	57.26	18.30	50.56
T	0.063	6.64	-712.93	57.61	12.53	34.73
F	0.066	-6.90	-701.92	57.69	11.04	321.96
A	0.122	-28.22	-725.96	57.79	-30.95	7.46
G	0.150	-42.06	-787.01	60.00	-57.83	248.67

Legend: x= FTG value, y= response value, a= slope, b= intercept, $\hat{y}$= ax+b.

6.5 Validation Using Data from Physionet

6.5.i. Introduction

The lab-based study suggested a possible application of adapted FTG in the correlation of cardiac response with a score given to baseline of beat-to-beat variation. We attempt to validate the most successful scoring system, 13DFTG using data from Physionet (an open-source web database of physiological signals, in references). We chose a database that would provide paired beat-to-beat interval data both before and during any given cardiac event. Only two Physionet databases met these criteria. These were the CAST RR Interval Sub-Study Database, which recorded data via long-term ECG (Holter) recording, and the Spontaneous Ventricular Tachyarrhythmia Database (SVTD), which recorded data via implanted cardioverter defibrillators. We rejected the former, as it was a pre-selected subset of a larger database which had been reduced to only patients with successful responses to treatment for ventricular premature complexes; a mere 590/3549 of the original database and therefore a biased subset. The bulk of the original patient population did not respond successfully, and this data is lost. It is not possible to go back and recover it, as it was recorded in analogue format and only the pieces relevant to the later study were digitized. Also, analogue recording is open to frequency-domain artefacts which can cause inaccuracies in the data. And, no placebo patient data were digitized for the later study, so no controls exist. Given that all drugs tested in the CAST study were later banned due to fatalities, the placebo group may have been best for our particular study given it is simply the before and after state of patients without confounding intervention. We chose SVTD as it contained paired data of a baseline readout from implanted cardioverter defibrillators, followed by a matched event recording from the same individual, with no confounding drug treatment. Two types of events were recorded, ventricular tachycardia and ventricular fibrillation. The ventricular tachycardia database can be directly compared to our study, as it enables us to correlate starting score with change in heartrate. We will examine this database first. The ventricular fibrillation database is interesting as it allows us to test for correlations between starting score and heart stability in ventricular fibrillation, so we look this secondly. Our validation study also found that score-response correlation was dependent on there being a reasonable range of scores to begin. When a population is more homogenous, correlations are weaker than when a more diverse population is tested. Such diversity is curtailed in the lab setting, which aims to create homogenous populations. It is hoped that a natural patient population might offer more baseline rhythm diversity.

6.5.ii Methods

Beat-to-beat interval data before and during ventricular fibrillation and ventricular tachycardia for each patient were collated in Excel. Over 1000 intervals were provided per record. The final 120 data points of each records were used in FTG analysis, as the recording mechanism records a snap shot of time before and including the cardiac event: The beat data at the end of the recording is therefore the most representative of the event. Pre-ventricular tachycardia data were scored using FTG13, as described in **3.6.ii(i)**. Ventricular tachycardia data were compared against before ventricular tachycardia data to evaluate how much heartrate had increased compared to the baseline reading as percent change from the starting heartrate. For the ventricular fibrillation database both before- and during- data were scored using FTG13. Spearman's rank was applied, and regression analysis performed. To test whether FTG13 might differentiate between an oncoming ventricular tachycardia and a ventricular fibrillation episode, we applied the two sampled t-test to the null hypothesis 'there is no difference between the scores produced by patients prior to ventricular tachycardia and patients prior to a ventricular fibrillation episode'.

6.5.iii Results

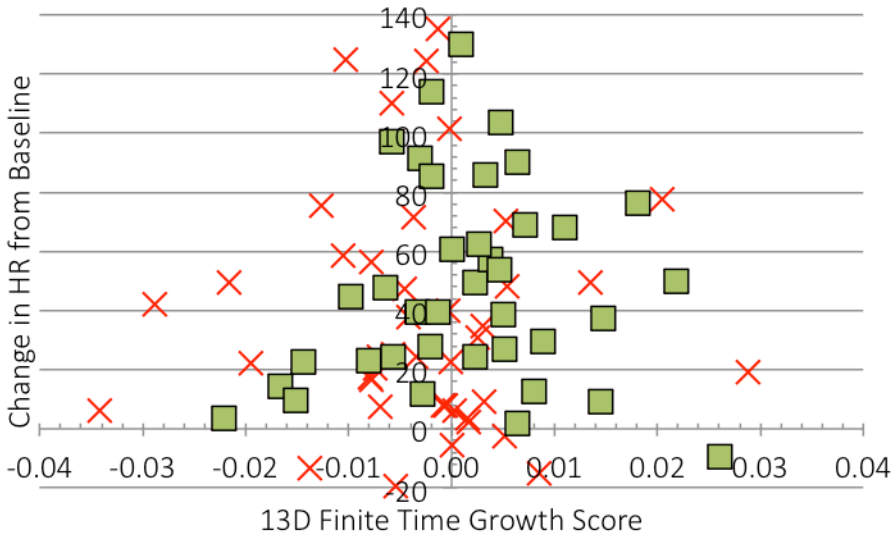
6.5.iii (a) The Ventricular Tachycardia Database

Firstly, in the ventricular tachycardia database, twenty-four of the available 105 paired records were rejected due to false data. Each pair should present a separate baseline reading taken before an event, and the event itself. For patients with multiple ventricular tachycardia events a new baseline each time the cardioverter is reset. This should give unique paired records. However, baselines records were duplicated: baseline records appeared identical. A simple check, deducting one column of data from another found there to be zero difference between records. It is extremely unlikely that any two series of beat-to-beat intervals might be identical in all 1000 beats. Records were rejected as it was unclear which baseline record was the true pair of the ensuing ventricular tachycardia episode each was duplicated against. Further to this, patients 8022 and 8033 bore identical data in their baseline recordings which was also suspect, these records were also rejected.

81 paired records were analysed using 13D FTG, however a further 43 of these were rejected after the work was performed: Errors appeared using the Lyapunov equation when more than one consecutive set of 13 beat-to-beat intervals occurred because the natural logarithm (LN) of zero (a number that occurs if two identical series of periods are compared) is minus infinity. It is unlikely that a heart might have such absolute non-variation in rhythm, and more possible that an error in machine reading has occurred.

These data were not excluded right away as the natural logarithm is the denominator in the Lyapunov equation and simply means that the FTG score will be zero. However, the result flagged these data for closer examination. Each of the 81 datasets were tested for chains of 13+ identical beat intervals. 43 records with more than one consecutive identical sequence of 13+ periods were removed from the data. When Spearman's rank was applied to the original 81 paired data sets, no significant correlation was found. The low Rho of 0.080 was insignificant at alpha 0.48. After rejection of the above, the remaining 38 remaining data pairs still gave a low Rho of 0.096 and an insignificant 0.57 alpha level. Removing repeat beat data worsened the correlation. This may be because spurious, copied, data will be more consistent and have less variation therefore deviation will be much less. Figure 6j shows FTG score for baseline paired data given against change in heartrate from baseline data to ventricular tachycardia data for all 81 records. The secondarily rejected 43 records are shown as crosses while the remaining 38 are shown as squares.

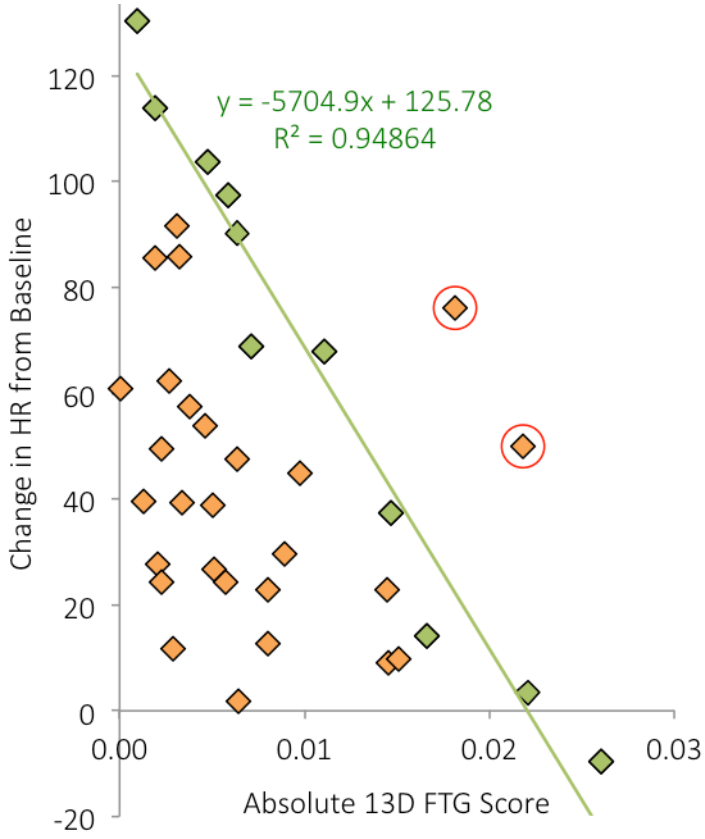
Figure 6j. FTG score vs heartrate change in paired data



Legend: Crosses= rejected data, squares=remaining data.

Nevertheless, figure 6j also offered intuitive insight. The figure has a pyramid shape. If we remember that FTG is based on a scale where zero represents the most stable configuration of beat-to-beat stability, while 1 or -1 represent the most variable then, it is reasonable to select the absolute FTG score as a distance from zero. By turning negative values positive we examine how unstable the heart rhythm is in absolute terms out of 1. If we test this on the first non-select group of 81 patients, correlation does not improve: the critical value of 0.136 corresponds to only a 0.23 alpha level. However! If we use absolute values of the FTG score on the 38 patients non-rejected, we find a critical value of -0.468. This gives a p value of 0.003 and a significance of alpha 0.01. In this case there is *significant negative correlation between the FTG score as a distance from zero and heartrate change from baseline during ventricular tachycardia*. The further from zero the absolute FTG score, then the more the heartrate increases in ventricular tachycardia compared to baseline. A figure showing absolute FTG against change in heartrate from baseline is given in figure 6k. It is interesting that all these patients were considered to be having tachycardia when many of them did not show much change from baseline.

Figure 6k.FTG is negatively correlated with heartrate change from baseline in VF

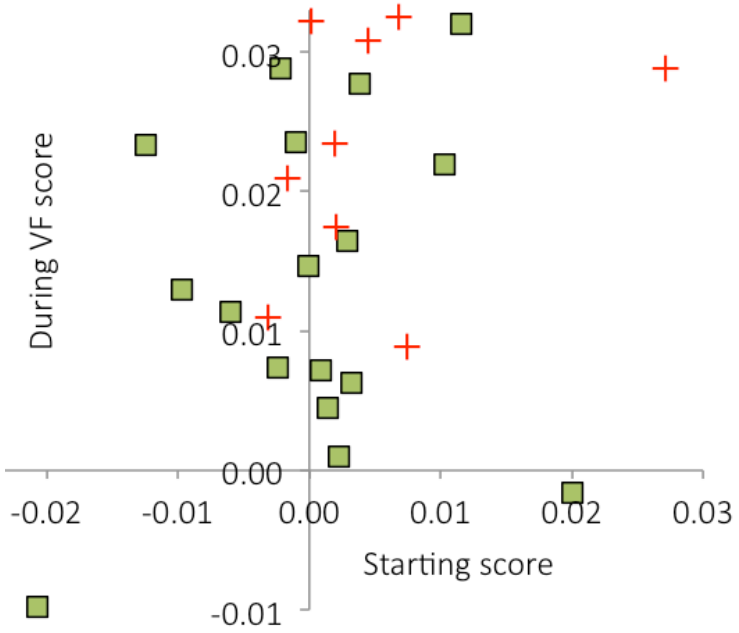


Legend: FTG scores are shown against paired change in heartrate in ventricular tachycardia. The cohort with baseline scores closer to zero has members with higher heartrate increases in ventricular tachycardia than any other cohort. Maximum heartrate increase in any cohort decreases with distance from zero. Fitting a line to all data is not very informative. However, by focusing on maximum response per cohort, we can fit a line to approximate a relationship between score and maximum response. For best fit we ignored the clear outliers, circled in red. The line of best fit in this case was a negative linear one, just as in the *Daphnia* study (y = -5704.9x + 125.78, r^2 = 0.949).

6.5.iii (b) Ventricular Fibrillation Database

The ventricular fibrillation data base contained 29 paired records. Two records were rejected for being duplicated in a similar way to those rejected from the ventricular tachycardia database. One record (patient 269) was rejected as an outlier when paired data were compared via Grubb's testing (Grubb's 5.11; G-crit 2.73). This left 26 data pairs. The low Rho 0.248 was insignificant with alpha 0.23. No correlation was drawn between starting score and end score in ventricular fibrillation. We looked at records for the presence more than one consecutive identical sequence of 13+ periods and found nine instances, these were removed from the data. The remaining 17 data pairs were tested via Spearman's rank, however the low Rho 0.093 remained insignificant with an 0.73 alpha level. Again, removing repeat beat worsened the correlation. Figure 6L shows FTG score for baseline paired data given against FTG score in ventricular fibrillation for all 26 records. The secondarily rejected 9 records are shown as crosses while the remaining 17 are shown as squares. Tests using absolute values of FTG were performed using the above data as before **[6.7.iiia]**. However, this time doing so did not improve any correlation between the two sets giving a critical value of 0.086 and an alpha of 0.75.

Figure 6L. FTG scores of beat-to-beat stability for baseline rhythm and during VF



Legend: Crosses= rejected data, squares=remaining data.

6.5.iv. Discussion

Any results produced by the dataset acquired from Physionet's spontaneous ventricular tachyarrhythmia database are cast into doubt due to the questionable reliability of data therein. Of the 134 paired data units available, 26 had to be rejected as data were duplicated across files. Of the remaining 108 a further pair were rejected as baseline data were so far outside the range of the others than it fell below g-crit in Grubb's testing. Of the remaining 107 paired sets, 53 baseline sequences had more than one set of 13 consecutive identical periods. This in itself, while not impossible, does suggest that some error in data capture occurred. Using the above data, no significant correlations were made between baseline FTG score and either ventricular tachycardia or ventricular fibrillation events that followed, nor between patients of either category. In further analysis, the 81 FTG scores for patients prior to ventricular tachycardia were compared to the 26 FTG scores for patients prior to ventricular fibrillation. This gave a p-value of 0.376. We failed to reject the null hypothesis and found no significant difference between the two data sets. Using only the data were no more than one 13 period consecutive sequence of identical periods were found the p value was 0.488, and again no significant difference was found. FTG was unable to differentiate patients prior to ventricular tachycardia or ventricular fibrillation.

Of the remaining 55 paired datasets 38 belonged to the ventricular tachycardia database and 17 belonged to the ventricular fibrillation database. Using the 17 in the ventricular fibrillation database no significant correlations were made between baseline FTG score and ventricular fibrillation events that followed, nor were significant differences found between these patients and the scores of the 38 in the ventricular tachycardia database. The 38 units of paired patient data in the ventricular tachycardia database, however, showed that, with spurious data rejected, baseline FTG scoring was significantly negatively correlated with change in heartrate from baseline to ventricular tachycardia at the 0.01 alpha level. Patients with scores closer to zero (and thus more stable heart rhythm to begin) would have faster heartrate in ventricular tachycardia than those with scores closer to 1. This suggests that patients with more variable heart rhythm are better able to adapt to ventricular tachycardia events and respond with a less elevated heartrate than those with more stable rhythm. This final conclusion underlines the same one found in the *daphnia* study. However, this result is limited in scope as we found the spontaneous tachycardia database inappropriate as a validation dataset due to duplicated or false data.

6.6 Chapter Summary

The main finding in this chapter was that increased baseline rhythm stability is significantly negatively correlated with the heart's paired response to cardiac intervention. In this chapter, variation in individual-to-individual response formed a basis for the use of *Daphnia* heart rhythm in predictive modelling of paired cardiac response to toxic interventions. We began by linking heartrate variation (HRV) to initial experiments in **4.0** and asked whether scoring systems briefly reviewed there could be applied to HRV in normal *Daphnia*. In chapter 4 we serendipitously observed dysrhythmias which led to a review of scoring systems published for dysrhythmia differentiation. If a scoring system can be used to differentiate one dysrhythmia from another, perhaps it could also differentiate heart rhythm variation in *Daphnia* in their normal environment. This would be akin to differentiating the heart rhythm of otherwise similar or healthy patients. The same *Daphnia* were then given a cardiac intervention, in this case a decelerative drug. This would be akin to a population of patients whom being given a standard dose of a drug which has been decided to be correct for their age/ weight or other parameters based on a dose-response study, but it not individualised for that patient: All trial animals are the same age, from the same culture and get the same treatment for the same amount of time. We then sought to correlate scores of their heart stability to begin with how each animal responded to cardiac intervention. This trial is not intended to tell us anything useful about the nature of the drug itself. Instead, it shows how much otherwise similarly maintained individuals differ in response to the same intervention and attempts to link this to the individual's own heart stability to begin. Scoring systems used to differentiate dysrhythmia were reapplied as methods of quantifying individual heart stability in normal rhythm. Correlations were then performed with paired individual response to cardiac intervention. In this second line of research, *Daphnia* response variability aids rather than hinders discovery.

Animals with more heart rhythm variation to begin were more able to maintain normal heartrates when subjected to a cardio decelerative drug than those with stable rhythm. Doxorubicin was chosen due to its use in inducing dysrhythmia in animal studies. In our trials the highest concentrations caused some visible beat-to-beat variation, while the most common response was bradycardia with no obvious disruption of rhythm. Data resulted in a clear dose-response curve up to 2400μM. This showed a dose dependent decrease in heartrate which plateaued between 1600μM and 2000μM at which

concentrations mean heartrate had fallen to just over 50% of starting rate before slowing once again thereafter. Heartrate becomes erratic at higher doses of 2800μM and 3200μM and mortality increased at this point suggesting toxic effect. We found that baseline heart rhythm can be correlated with paired response to cardiac intervention, with significance at the 0.01 alpha level, using an adjusted version of the Lyapunov equation. However, this is only if population variation is adequate. Our findings contrasted assertions found in the literature search [6.2] which suggested that heart rhythm stability might indicate a less variable heart which could better withstand cardiac intervention. Assertions were based on data simulations with no trial in a real-life clinical setting. We found that increased HRV decreased the tachycardic effect to doxorubicin. Animals with higher HRV to begin were more able to maintain normal heartrates when faced with doxorubicin exposure, while those with a more stable rhythm responded with plummeting heartrates when exposed to the same treatment. This correlation could have implications for personalised heart medicine. HRV scores might indicate risk factors for individuals that would otherwise go unnoticed. HRV scoring might also be used to improve eco-toxicology testing via observation of *Daphnia.*

Few of the scoring systems differentiated starting heart rhythm in a way that could be correlated with paired response. However, by adapting the Finite Time Growth equation to incorporate larger dimensions, we strengthened correlation between score and response. The main limitation identified in this study was the link between the variation in starting scores to begin and the success of correlations. Wider population variation aided correlations, while poor population variation lowered correlations. It is possible that some cohorts were more diverse than others. Individuals were trialled over the span of one year. This included all seasons with their natural effects on algal food supplies as well as other environmental factors including visiting competing species, temperature and day length. All concentration cohorts represented randomly chosen *Daphnia* dispersed over this time period. They should therefore have ranged similarly in their starting score if scores are an absolute reflection their condition at time of trial. This clearly did not happen, the variety of scores for heart-rate-variation per cohort was inconsistent. The 2200μM cohort produced the weakest correlations across scoring systems and had the smallest range of scores to begin. The 2800μM cohort produced the strongest correlations and had the widest range of starting scores to begin. In the 2800μM cohort 6 of the 26 animals trialed died, and clear toxicity indicators were noted. It may be that the more extreme toxic

effects of the higher concentration broadened the range of responses. This may also have made correlations stronger. The toxicity of higher concentrations is a confounding factor as it is unclear whether correlation will only work only at highly toxic levels, or whether it would work at lower concentrations were baseline variability higher.

Maintaining a consistent sample population may have removed the natural variation found in real life situations. In this case variation might only occur in isolated instances rather than across the population as a whole. We meticulously adhered to a systematic feeding timetable. We consistently checked nitrate levels, changed waters, kept the tank at a single temperature, and performed a daily check for invasive predatory or competitive invertebrates from the environment. This was to ensure upkeep of the most consistent population possible over the full year of the trial. Neonate virgins were chosen given their more predictable age. All neonates are under 4 days old and are identified by size and an empty womb. All *Daphnia* between the age of 4 and 96 days old are permanently pregnant. *Daphnia* found in nature rather than maintained in this way might produce a more robust range of starting scores required for good correlations. To increase this range we might ignore the selection for neonates, picking animals of all age groups instead.

A further limitation was the laboriousness of data collection as well as the time involved in applying each scoring system across all data. Up to twelve animals were trialled per lab session. Following each session capture of beat-to-beat data from each 720-frame baseline sequence was performed. However, the method of image capture was greatly improved by acquisition of a dark field filter. An automated method of image capture was written that involved a far more hands-off approach, where before each frame taken in light field required manual adjustment to capture the heart area precisely. Now blocks of 200 frames at a time were processed by the program, and manual intervention was required only per new film rather than per frame in each film. This allowed more data to be processed and allowed the user to step away from the computer workspace and be free for other research processes. However, other processes were added. Each scoring system also had a labour-intensive element. For Finite Time Growth the 4.2MB spreadsheet included a 120 x 120 (14,400 part) net of equations in its first sheet alone to automate the bulk of the Lyapunov equation. It required the full capacity of the available 8GB memory 2.4 GHs processor (MacOS 10.13.1) to perform this per *Daphnia* data set. This gave a low throughput of only 190 *Daphnia*.

7. Towards a More Efficient Heart Data Collection Method

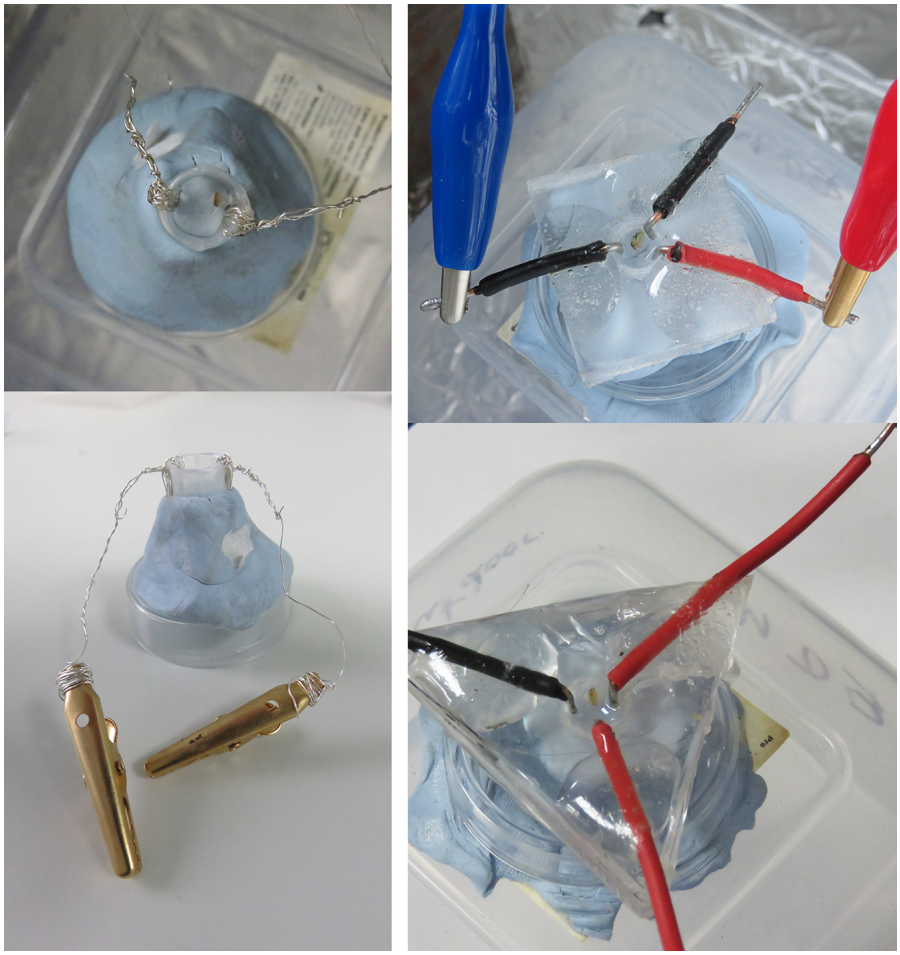
In this chapter we look for a means of vastly increasing the data set. A high throughput method was sought leading to development of way to measure signals from whole live *Daphnia*. So far, we have seen that visual inspection of the heart, even with the dark field microscopy instead of light field, is difficult and time consuming. We asked whether electrical measures from whole, live, *Daphnia* could provide a more efficient and possibly more accurate way of collecting heart data. Collecting data from electrical signals would cut out the data interpretation step added by image capture processes, so may be more accurate. And, removing this step would also allow more time for data capture, making the process more efficient. Forming a direct link between *Daphnia* signal and resulting heart data may be a way to finally achieve a high throughput method of data collection.

The initial aim was to measure an ECG like recording of the *Daphnia* heart. Novel hardware was built and connected to Powerlab via a Warner Instruments DP-311 differential amplifier, to capture signals from live *Daphnia*, which we termed 'Whole *Daphnia* Electro Mechanical Movement' (EMM). To the best of our knowledge, there is no published literature to indicate this has ever been done before. The closest study, Freund et al (2002), measured the electromechanical signal of an entire swarm of *Daphnia*. They recorded leg and antennal movement but not the heart; we therefore knew we could not assume records gained by our method would necessarily represent heart contractions. The first trial aimed to define the signal source. Once the source of the repeating physiological signal was elucidated, the second trial sought to apply our findings to a dose response test using compounds currently used in similar situations in the literature. Having found that the *Daphnia* heart model is too sensitive to small changes in environment to be used in producing reproducible concentration response curves to positive or negative chronotropic agents, we return to the established model in toxicology with a novel method of examining response to water-borne toxins, which forms an exciting basis for further work. This chapter is split into three parts. In 7.1 we describe the development of EMM equipment and software. In 7.2 we investigate what it is the EMM records, and in 7.3 we measure its physiological response to a chosen drug. 7.2 and 7.3 are written in the expected introduction-methods-results-discussion format. 7.1 does not fit this format as it is not an experiment with a set of regulated inbound test measures and measurable outbound results. It is instead a discussion of the main processes developed to perform the studies in 7.2 and 7.3.

7.1 Development of EMM equipment and software

In this chapter we attempt to measure electromechanical movement from live *Daphnia* by putting the animals in a drop of water with two electrodes, one either side of the *Daphnia*. Any muscle action generates electrical signals. Voltages required to detect these signals from the *Daphnia* heart are very small because the *Daphnia* produces a small signal, estimated to be in the order of 50μV. The amplified Powerlab signal is directly proportional to the voltage at the electrodes. 50μV is an educated guess based on the 10K gain used by the amplifier and the typical 0.5V readout displayed in Powerlab.

Figure 7a. Development of the bath structure



Legend: Left; wire and pipette-end bath from early trial, right top; first Plexiglas bath, and, right bottom; later Plexiglas bath, described in detail in the text.

To sense the electric field, it would be best if the water were completely insulating deionized water, because otherwise any ions in the water act like a progressively short circuit to the electric field generated by the *Daphnia*. The more ions the more conductive the water is and the lower the resistance between the two electrodes, which reduces the amplitude of the signal captured. However, in practice this medium stresses *Daphnia* **[1.1.v]** probably due to hypo-osmotic balance, so we use APW **[1g]** which by its nature is

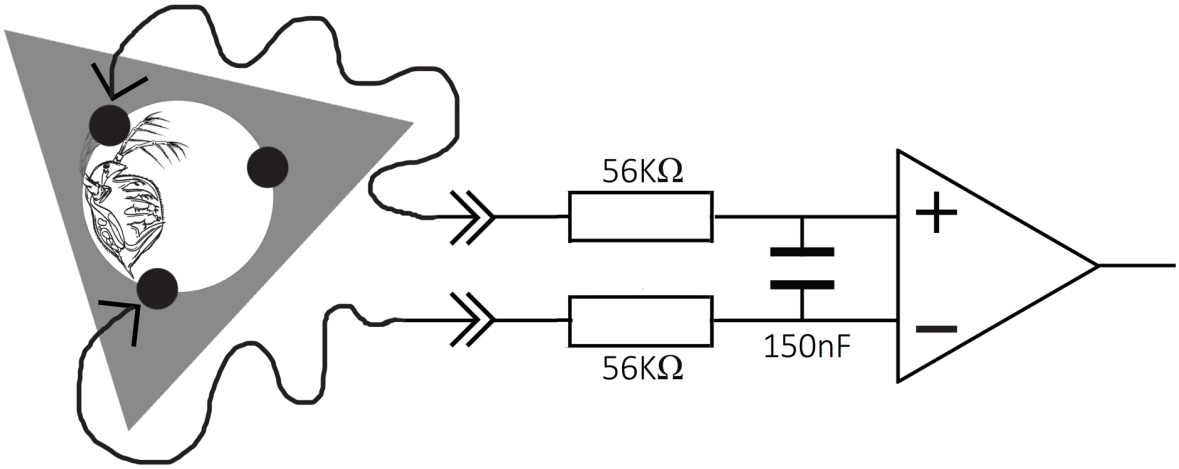
slightly conductive but not overly ionised. The equipment needed to be small enough to hold the *Daphnia* while it straddles two electrodes. The original apparatus, although effective, was also a bit fragile and subject to damage through use, so we built more robust structures (figure 7a).

As the *Daphnia* signal is so weak, we cannot get a reliable measure of frequency from the waveform directly due to underlying noise from normal physiological movement, such as antennal flickers and general wiggling of each individual. Spectrum analysis tools provided in Matlab were used to average a significantly longer record (up to minutes) to infer frequency values in each signal more accurately. We chose ten minutes as *Daphnia* become stressed if they were immobilised for much longer **[4.3]**. This produced some success in picking up the signal, however it became clear that external factors were affecting the ability of the apparatus to work. For narrative flow, these are elaborated upon in greater detail in section **7.1.i**. The problem with these impulsive interference spikes were that they overloaded the sensitive amplification equipment. As a result, all data was being corrupted so that the spectrum averaging process did not work. Therefore, we altered the basic Matlab script to also go through the ten-minute record, identifying the interfering sections and removing them in a way that would minimally effect the remaining data. This is elaborated upon in section **7.1.ii**. The important thing about this software is that it allowed the user to set the thresholds for the interference detection, and the program reported how much data was lost. If around 25% of the data survived there, then a useable record still existed. This corresponded to a mere doubling of the standard error of the parameters for the whole record, which given the interference levels present, was deemed acceptable. Even with all that post processing it became clear that the amplifier was still heavily affected by recorded external interference. We knew, *a priori*, which key frequency ranges we were interested in; at least for the heart which we hoped to capture using this method, key frequencies are within 5 to 7Hz **[5.0]**. So to further reduce interference entering the amplifier a simple passive low pass filter was designed to sit on the amplifier probes. The filter's cut off frequency was set to allow the fundamental frequency range of the *Daphnia* signal to pass through, and to progressively attenuate higher frequency signals so that the latter would get reduced in amplitude.

The component values used in the passive filter were a compromise. Ideally the resistors should have a high value of resistance, to minimise attenuation of the *Daphnia* signal, and

a low enough resistance so as not to generate too much extra noise. Here we must define that interference refers to high frequencies from Radio Frequency Interference (**RFI**), while noise refers to low level background frequencies. Resistors generate low level background noise proportional to the square root of the resistance value. We decided a total resistance of around 100KΩ would suffice, because it would not significantly load the natural shorting impedance of APW and thus would preserve the *Daphnia* signal. Typical impedance of fresh water environments range from around 2.06KΩ to 23.26KΩ (calculated from micro Siemens values given in Talling, 2009, given conductance= 1/resistance). We chose a resistance four times that which we are likely to see in APW, because the resistance of the APW effectively shorts out the *Daphnia*. If the water is very conductive the current will flow around the *Daphnia*, so the voltage we see will be less than the voltage generated by the *Daphnia* muscles. 1/4th current flows through resistor, so total current required from '*Daphnia* battery' will be 1.25 times itself. This will cause a drop in voltage a bit but only by a small amount. A schematic of the circuit is seen in figure 7b. The components chosen were a compromise between calculated desirables and commercially available values. Two balanced resistors at were chosen, as this was the closest resistance value available.

Figure 7b. Circuit diagram of *Daphnia* bath, resistor capacitor and amplifier

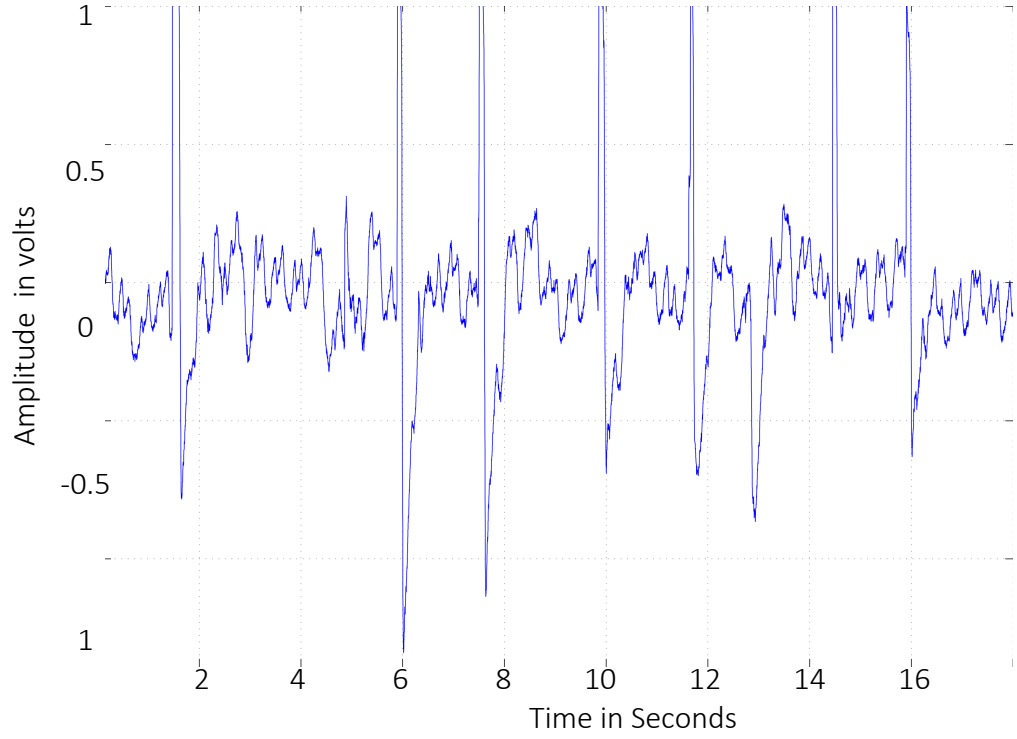


Legend: From left to right, the probes surrounding the *Daphnia* are connected to positive and negative terminals on the amplifier head, via a resistor capacitor which provides passive filtering to remove high frequency noise.

Above 10Hz range input impedance reduces with frequency to a limit of 56K + 56K =112K ohms, below 10Hz the impedance will be the same as that of the Warner DP-311 differential amplifier it is attached to, which is 10^{12} ohms. The capacitor works as an energy storage area, smoothing out the flow of signal coming from the probe to the amplifier. It

acts rather like a dedicated tank in an electric shower, which stores water ready to be released as a tap is turned in a smooth and immediate manner, without which a distant connection to a water tank further away might cause a delay followed by a gush. This metaphor applies to the capacitor as it prevents large bursts of energy, such as high frequency noise from large equipment, from overwhelming the smaller signal from the *Daphnia*, and it also increases the sensitivity of the system to the small, low frequency signals we are interested in. There was a choice between the available sizes of either 220 nanofarads (nF), which would give a cut off frequency of 6.46Hz or 150nF which would give a cut off frequency of 9.47Hz. Higher and lower sizes are available; however, these were chosen to allow the fundamental frequencies through, which we were primarily interested in. Once we decided which resistor to use the capacitor was decided by the cut off frequency. The key decision here was because the higher the cut off frequency, the more *Daphnia* signal goes through unaffected, while the lower it is the more interference rejection will occur. Both affect the harmonics of the *Daphnia*'s frequency, and therefore the shape of the signal coming from the animal. However, we were only interested in the fundamental frequency. The bigger the window the more RFI gets through, so we took this to the 150nF extreme. Further discussion of these components is undertaken in **7.1.iii**.

Figure 7c. Intermittent signal interference by a microwave

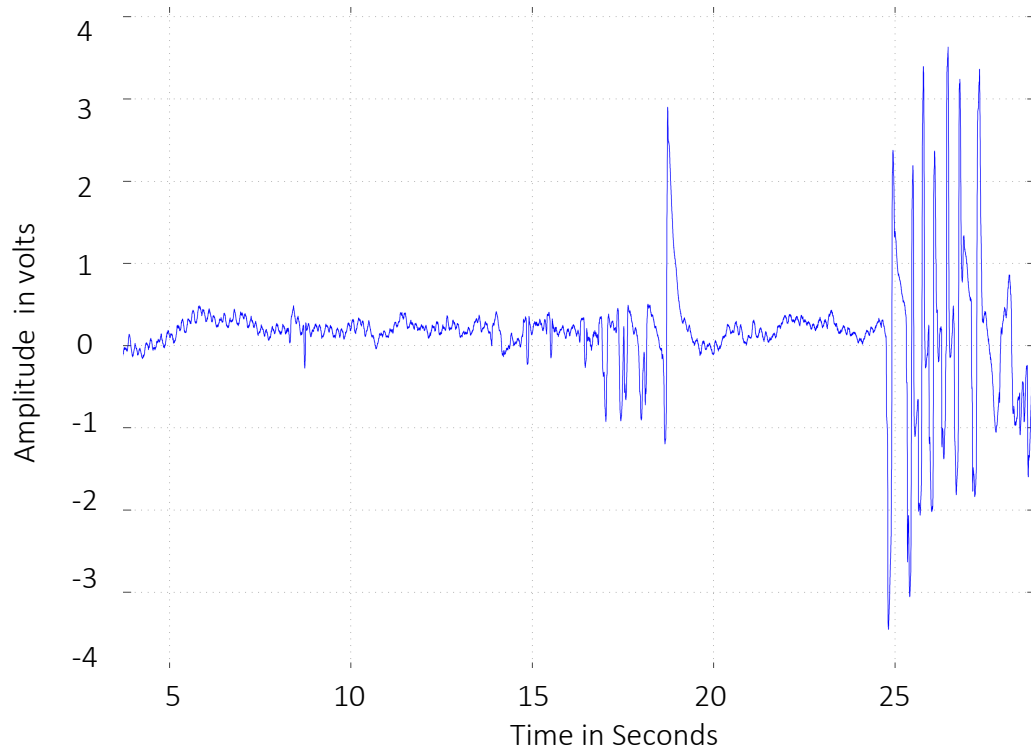


Legend: Leg movement can be seen as smaller waves in between the larger microwave pulses, microwave interference increases depending on power usage.

7.1.i The Problems

Radio Frequency Interference (RFI) providing the greatest problem in setting up and carrying out the studies, and also the greatest opportunity for an array of solutions. This section discusses the problems while the next embarks on solutions. To elaborate on the nature of RFI itself. Mechanically vibrating equipment such as sterilisers and electrically heavy-loading equipment such as the autoclaves produced RFI which could fully obliterate recordings. A neighbouring electrical plant near the second-floor lab caused switching spikes as machinery cycled on and off. Sonic cleaners gave a base line waver to the whole signal when in use. Self-monitoring refrigerators caused sudden and brief spikes in the 60Hz to 200Hz range. Microwaves caused an intermittent disruption, between disruptive spikes *Daphnia* leg movement could still be seen, but this was of course not ideal. Microwave interference is seen in figure 7c. Vibrations from slammed cupboard doors caused clipping in the 100Hz range. However, the most prominent RFI issues were caused by mobile phones, smartwatches, tablets, GPS, Wi-Fi and Bluetooth devices, worn by users in the same and in neighbouring labs. Attempts to resolve this via repeated discussion and educational posters were unsuccessful so alternative solutions were developed.

Figure 7d. Phone based RFI



Legend: *Daphnia* EMM showing cellphone presence (blips), a text (four dashes) then a call (large dashes, right.)

New lab space was used in the second trial. There users were post-doc and technical staff only and responded positively when asked to turn off cell phones in all but one case. 55.17% of data (32/58 records) were lost to personal device RFI in trial 1, in a shared space with around 6-8 phone users at any time. In trial 2, 8.32% of data (80/962 records) were lost to personal device RFI, in a space shared with a single phone user. Taken at face value, data loss appears to be an inverse function of the number of phone users squared. However, the number of records lost in trial 2 (80) represents a mere fraction of data compromised; many more were recovered by careful manual adjustment of the spectrum analysis program where beat frequency could be elucidated in gaps between RFI bursts, but no possibility of beat-to-beat data analysis remained. This meant that development of further work related to chapter 6 was not possible. This occurred in over 50% of remaining cases, suggesting a single cell phone may represent peak saturation for RFI interference when anything more than a baseline frequency is required from the data. Until a method of screening personal devices is achieved, or a means of screening the measurement cell, trials such as this can only be truly efficient if all lab users agree to comply with the non-use of phones. A short sequence of phone RFI disruption, is seen in figure 7d.

7.1.ii The Spectrum Analysis Program

The spectrum analysis program (shown in the appendix) was developed during the preliminary trial, incorporating power averaging software in MATLAB. It does not act as a filter but performs an average power spectrum (APS) of the signal. APS alone cannot fully deal with signals which are corrupted by impulsive noise, as the whole system is driven into non-linear regions. To adapt the program to handle this, a threshold was set to detect and remove impulsive events by blanking them in a way which minimized impact on the APS system: A raised cosine Hann Window formed a fade-out/ fade-in so that noise areas were blanked out effectively without creating facets. This windowing faded the signal in and out in a well-defined way using the Fourier transform. The Hann Window was used for spectral analysis because it minimizes variation with zero scalloping loss (Harris, 1978).

While the program is, in one sense 'adding' a modulation artifact; it is removing the non-linear data which was corrupted already; known *a priori* to be not the data we were looking for. As observed in lab, a clear signal would be interrupted by the appearance of a user with a device causing big clips to appear in the data (figure 7d). Artifacts of removal are very much less than artifacts put into the data by the interference, which is a burst of

broad band noise wiping everything out. The HW merely reduces the amplitude of observed data, making it smaller; it is the lesser of two artifacts. The adapted program allows users to select a narrow band of voltages, to remove noise that would otherwise corrupt the result. It automatically gives a result and produces a power spectrum. By producing a power spectrum, it visualizes the presence of interference at specific Hz measurements, allowing users to better choose a frequency range, or at worse to choose a peak manually, or discard the record.

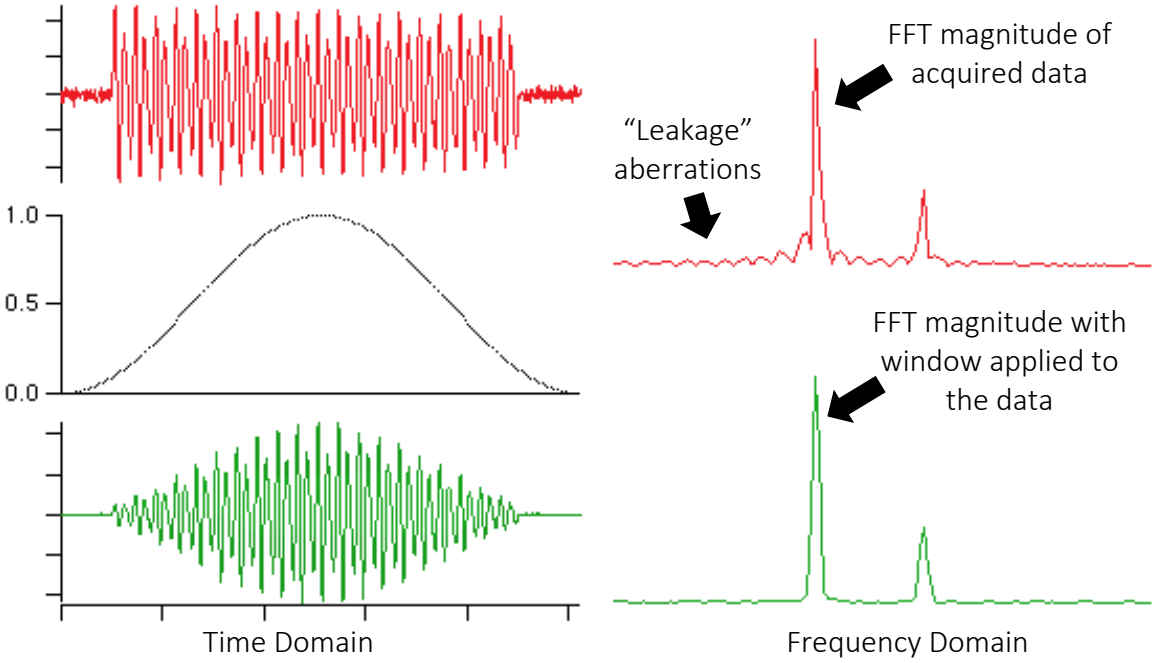
The program then opens the .txt file when the user types in the filename using "*File Name =*". Only the name is required here as the extension has been built in to the program expectations using "if" and "else" functions. The program automatically reads in the sampling time information from the file header and calculates sampling frequency from sampling time. It also captures the date and time of the measurement, as well as the voltage range. It then echoes the extracted header parameters (details which tell us voltage and sample rate) as a double check. It assigns the data to separate named variables "Signal" and "Sample Time," and plots the data in a figure, adds a grid, sets the axes range, labels them and adds date and time of measurement and file name.

On the basis of what is seen in this figure, the user can set a threshold for the interference spikes seen, and interactively repeat for different threshold values until interference is reduced and the *Daphnia* signal is best shown. The program asks whether a new threshold is required via "New Threshold =" and as long as the user types 'Y' new values can be tried. This can be repeated as needed. If not, threshold is inputted by the user the default is to use all of the available voltages. If the user types in a threshold greater than the range of available frequencies (which should not happen) the program will warn the user of their mistake and automatically use the default.

This done, it will plot lines on the power spectrum indicating the thresholds. Using this threshold, it will exclude data that contains interference spikes, using the Hann Window, which has continuity in some of its derivatives. The frequency resolution is given by Sampling frequency (Fs) /Window Size. Fs= 1KHz (chosen in the Power lab software). Window size is 10 seconds long, or 10K samples. This equals a resolution of 0.1Hz, which is how accurately the waveform can be measured. Longer window sizes would give better resolution but less averaging, so that fewer spikes could be removed. Whenever we take a

10 second bit of data out which contains a spike that ten seconds is lost, so a longer window would cause more data to be lost if a spike appears. It uses a periodic version of the $\sin^2$ Hann Window as we are looking for periodic data. The Hann Window also has good side lobe performance, such that frequencies that are far apart are well discriminated, and even those next to one another (0.1Hz apart) only have 1% interference between the two in terms of voltage. The program picks up window sized chunks of data and initializes using the first full window (10K samples). Then, jumps through the input data one half of a window at a time which is the optimum step, as it captures all the data with the smallest amount of overlap. Active windowing throws away data due to the tapering at both ends (see figure 7e) while overlapping windows by 5K samples ensures no data is lost. This is because if $\sin^2$ windows are overlapped together and added up they come out as a constant value only if overlapped by precisely 50%.

Figure 7e. Configuration of a Hann Window



Legend: Red = acquired data, dotted black line = Hann window, green = windowed data. The y-axes in this context refers to normalized heart area data, but in the general context of describing a Hann window this is not relevant as it may refer to any spectrum measured.

The program also keeps a count of how many chunks of data are deleted. More than 50% probably means the data is corrupted by too many RFI spikes and is probably useless. To do this, it keeps a count of the number of chunks in the signal, starting at one because there is always at least one chunk to work with. Finally, the program pre-allocates space for the deleted chunks of signal so that the program runs faster. It sets this at zero so that deleted portions can be 'added' to it. Once set-up is complete, it goes through the signal

one Window Length chunk at a time, in half Window Length steps, checking for spikes and deleting them if present. And, checking whether any of the samples in each chunk are greater than the Spike Threshold. It does this by using the absolute value, which is either the default or user selected value. If it finds any, it adds a windowed version of the signal to the deleted signal array. This produces an array that represents the parts of the signal that needs to be deleted in red, over an array that represents the original signal. In general, a spike will take out two overlapped chunks of data or a Window Length + Window Length /2 sample of data. It calculates the percentage of deleted frames, and outputs to the user.

The program then differentiates the cleaned-up signal to remove the low frequency noise and baseline variation: A Fourier transform of the windowed signal converts time to frequency domain. This is a window that is thin at one end (zero) much, much thicker in the middle (1) and thin at the other end, to which zeros are added. This creates more apparent resolution which is not new information but helps to smooth the curves on the signal. It then calculates the average power spectrum from all the window segments, using Welch's method (https://www.mathworks.com/help/signal/ref/pwelch.html) with 50% overlap between segments. To minimize artefacts the same window was used to clean up the signal. The program then plots the average spectrum, then displays the frequency of the largest peak, which should be the *Daphnia* signal once all higher frequency interference is removed. Finally, the program displays the frequency of the highest peak. This is the heart rate of the *Daphnia*. The graph produces plots the power in decibels (dB) normalized to the highest peak in the spectrum on the x axis. The y-axis is set to cover 0 to 30Hz. It adds a grid, sets axes range, labels them, and adds a title.

The program then checks whether the user is satisfied by asking whether a New Threshold is required. If the largest peak is clear and in the expected *Daphnia* range, we are satisfied and take this as the frequency given by the program. If there is still too much interference for a peak to be seen, we choose a new threshold by typing ‘Y’ and typing in a new number. The default is ‘N’ so by simply pressing enter the program will then save all the results and figures to a pre-assigned folder. If ‘Y’ the program runs through again with the new threshold. Figures are saved in editable (.fig) and readable (.pdf) forms. It names these files by trimming the '.txt' extension off the input file name to form the time domain figure filename and adds the word ‘Spectrum’.

Analogies to the use of the Hann Window in other fields can be drawn. For example, to remove record clicks, the Hann Window technique blanks out the signal for the period of time which subjectively removed the click. Such programs are not freely available, were written for the removal of record clicks, and never intended for measurement systems so do not easily translate into this work.

7.1.iii Summary Discussion of Solutions

Before discussing the larger problem of RFI, we begin by briefly mentioning the improvement which occurred once we built the more substantial plexiglass baths which replaced the pipette-end baths. Using the pipette-end baths seen on the left of figure 7a, the best signals were produced when the animal was close to one or the other electrode and oriented so that the electrodes were perpendicular to the animal's head-tail orientation. However, there was no absolute way to move the animal within the tight space without risk of damage. Animals became distressed at attempts to reposition them leading to an increase in anal hook flicking responses, which were highly disruptive to the signal, creating large spikes in the 1Hz range. The adoption of three electrodes rather than two gave a user option of attaching positive or negative leads in orientations to fit the immobilised position of the *Daphnia*, rather than attempting to move the animal itself.

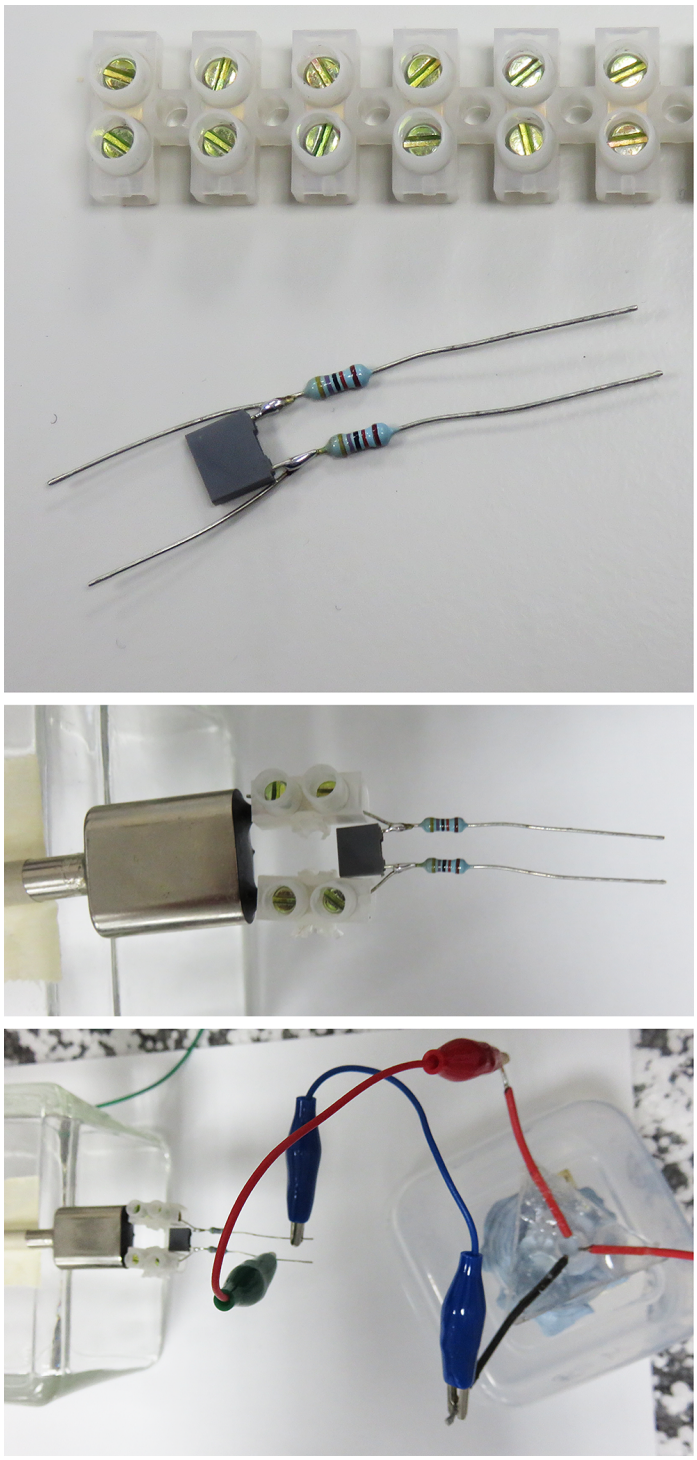
Only three electrodes were practical as they were made from 1.5mm wire and needed space between each wide enough to allow the animal to lay without become trapped between them when water was pipetted away. In some cases where animals were larger than average this would occur, and their trapped situation would only become visible when APW was added and the animal remained immobilised; though it was very obvious from the EMM that animals were stressed by these situations as anal hook flicking signals would peak. In further development of the EMM bath, a printed circuit board idea would bypass this problem as there would be no loose wire for animals to get wedged on.

In order to combat the main issue of RFI, the following solutions were applied, with the solution which had greatest success given first. these are described in further detail below:

- Attaching an external resistor capacitor designed for the specific frequency range.
- A Matlab spectrum analysis which produced a power spectrum giving the strongest frequency and harmonics as visible peaks.
- Agreement from technicians to autoclave down time.

- Increased gain counter intuitively removed some noise.
- Wires shortened, twisted and overlapped.
- Shielding in a copper-wire Faraday cage.
- Gaining cooperation of lab users for mobile device down time.

Figure 7f. Resistor Capacitor and terminal block attached to amplifier probe connection



Legend: top; RC and terminal block, middle; RC attached to the prongs of the regular ECG probe, bottom; full layout of attachment between probe, RC and *Daphnia* bath – note that wires leading to connections on the bath have been crossed to further block interference.

The resistor capacitor (RC) was built by soldering together resistor wire lead, one capacitor and two resistors, and attaching them to the probe head using a terminal block as seen in figure 7f. This removed microwave, large equipment and vibrational interference, but was unable to combat interference emanating from personal devices. The spectrum analysis program [7.2.ii] was of similar importance in removing destructive interference. Together with the RC filter, these formed the most helpful and interesting solutions from a problem-solving perspective.

Third, agreement from technical staff to autoclave down time, during trial 1 and onwards allowed for one to two days per week in which signals were not otherwise completely impossible to capture. Before this agreement EMM measurement occurred with some degree of success before 11 a.m., thereafter no signal at all could be recorded, as EMM equipment was overwhelmed by RFI at all frequencies. The problem would end when autoclaves shut down after 6pm in the evening leaving only one hour before lab closure for further readings to be made. Isolation of the autoclave as a main source of interference was discovered by switching off all large equipment in the lab and by running the EMM and switching each device off and back on. With the help of technical staff, it became clear that the regular use of the autoclave at 11 a.m. daily which continued its cycle until 6pm or thereabouts was the cause of this hiatus. Without the kind allowance for autoclave downtime it is unlikely progression to trial two would have taken place.

Fourth, the system essentially has two amplifiers. An analogue Warner DP-3H differential amplifier on the equipment; and a digital amplifier controlled by software. This creates two levels of noise in the measurement system. Increasing gain on the analogue amplifier counterintuitively removed some noise. When analogue amplifier gain was lowered it was less than optimal; inherent broadband noise tended to increase. More noise was added to the signal, in which the desired animal signal was further hidden. The digital amplifier also picks up RFI, when gain was turned up on this noise also increased further masking the quieter desired signal. We used maximum 10K gain on the analogue amplifier, making the desired signal relative to second stage higher.

Fifth, wires act as antenna for electrostatic and magnetic fields. Wires were crossed over once (figure 7e) to make the area encircled by each loop formed approximately equal, so that any magnetic field going in might cancel itself out. Sixth, metal shielding is believed to

help block RFI, but appeared to do very little in this regard. In trial one, the *Daphnia* bath and amplifier probe were housed in a crude tin foil faraday cage due to available resources. During trial two, access to a lab with copper-wire cage was granted. The new lab did not house large equipment, so it is unknown whether the cage provided any improvement. Unfortunately, personal device interference was not blocked by the copper cage. Finally, mobile device down time is essential for any serious research using EMM, requiring the cooperation of every lab user.

Further work would involve design of an EMM device that could better tolerate RFI interference. Ideas for this include a printed circuit board allowing for removal of intermediate loose wire connections. Also, an amplifier with built in resistor capacitors would improve the current solution of a home-built resistor capacitor attached to the external probe. Data gathered were too noisy for assessment of beat to beat period data or application of any of the rhythm analysis used in chapter 6. The ability to gain RFI free data would be very interesting further work as it would allow us to evaluate patterns of dysrhythmia in feeding leg movement, and whether this has any connection to animal health. EMM potentially forms a quick way to gain beat rate data when compared with the normal method of using film or direct observation.

7.2 Defining What the EMM Signal Measurements

7.2 (a) Brief Pre-Study Trial: Does EMM record heart contractions?

7.2.(a).i. Introduction

EMM from 6 *Daphnia* were compared to films of each individual's heart contractions. The initial intention in this study was to replicate an analogous to ECG on a live animal by measuring the electrical activity of the heart. The null hypothesis stated that the distribution of peak frequency signals on individual *Daphnia* EMMs would not be significantly different to the distribution of heartrates for individual *Daphnia* on film. We aimed to look for differences between two sets of measurements from the same individual, comparing video data and EMM data. Therefore, a paired T-test was required.

7.2.(a).ii. Methods

In this study the earliest version of the Whole D*aphnia* EMM device was used, as seen in the left two images in figure 7a. This was a cut-off pipette, partially sliced on two sides to allow silver wire to be wrapped about it forming a positive and negative electrode area.

Each wire was attached to a crocodile clip which then attached directly to the header from the differential amplifier. It was no more or less successful than the later version simply less robust. Each individual was placed in an observation bath in a small volume of Artificial Pond Water (APW) via Pasteur pipette. Excess APW was sucked away to immobilise the animal. Heart movement was filmed for 12+ seconds as per previous trials [3.4.ii]. This was done before EMM rather than in conjunction with EMM. It is impractical to do both simultaneously due to electrical interference from the microscope.

Next, a ten-minute EMM was recorded. This was done by first pipetting Individuals into the EMM bath, and then pipetting away excess APW to immobilise the animal between two probes. Crocodile clips attached to the amplifier header were attached to probes either side of the *Daphnia* and recording was started. This was done as quickly as possible after filming. The analogue amplifier was set at 1.0Hz high pass, 100 Hz low pass and 10K gain, at a sample rate of 1K/s reading anywhere between 2V and 10V depending on baseline waver interference from background electrical noise in the lab on a per day basis. The EMM's progress was observed by eye in an attempt to ensure that the signal was as free as possible from obliterating interference (see general discussion 7.4(a)). The EMM was saved in the form of a .txt file which could be imported to MatLab. This was repeated for each *Daphnia* trialled. This experimental protocol can be summed for clarity as:

1) Place individual in observation bath in APW
2) Pipette off excess APW to immobilise
3) Film heart movement as per GM 3.4.ii [12+ seconds]
4) Add more APW to the bath
5) Pipette individual into EMM chamber
6) Pipette off excess APW to immobilise
7) Connect probes either side of the *Daphnia* to the amplifier header
8) Record [ten minutes]
9) Observe EMM progress on screen in case of obliterating interference
10) Save in the form of a .txt file
11) Repeat for each individual

Video data were then processed [3.6], while EMM data was processed using an adapted Matlab spectrum analysis made for this purpose. The spectrum analysis program was written in an attempt to remove impulsive electrical interference and produces a power

spectrum giving the strongest frequency and its harmonics as visible peaks. Both give frequency rates for resultant recordings in Hz.

7.2.(a)iii. Results

Heartrate in Hz defined by video methods (GM3.4-3.5) for this cohort was 5.53±1.34 [Mean±SD] (n= 6) while the peak frequency captured on EMM was 8.80±1.84 [mean±SD]. A dependent two-tailed student T-test for paired samples was performed on the data. When comparing paired heart and EMM data, a p value of 0.006 was found. The probability that the observed results are due to random chance is small. The null hypothesis is rejected. The sample distribution of beat data recorded via EMM and that of heart rate captured on film are not associated within a 95% confidence interval.

7.2.(a)iv. Discussion

Daphnia EMM produced a heart-like rhythm not caused by the heart itself. Visual observation suggested that rhythmic feeding limb movement might synchronise with peak pattern movement on the EMM trace. This became the objective of trial 1.

7.2.(b) Trial 1: Does EMM record feeding limb beats?

7.2.(b)i. Introduction

The intention of this trial was to determine the source of the EMM trace when recording whole live *Daphnia*.

7.2.(b)ii. Methods

A more substantial bath was built by adjoining two sheets of 5mm Plexiglas, one with a 7mm diameter hole, with epoxy resin to form a sealed bath. Three 1.5mm silver wires were bent into the bath with equal spacing apart. The idea was that wherever the *Daphnia* landed, when water was partially pipetted away, crocodile clips could easily be placed on two probes either side of the animal. Two wires were soldered with crocodile clips at either end to attach to the device and to negative and positive electrodes of the header from the differential amplifier. These were made short to reduce electrostatic interference, and just long enough to be allow them to cross over one another when attached in order to reduce electromagnetic interference. The bath was then glued to an upturned 35mm petri dish, as the circular shape of the disk better allowed the whole device to be temporarily blu-tacked into place in the equipment housing or removed easily

as needed during a day of testing. The square bath shown at top right in figure 7a was used for trial 1. Methods were similar those in **7.3(a)ii** except where underlined:

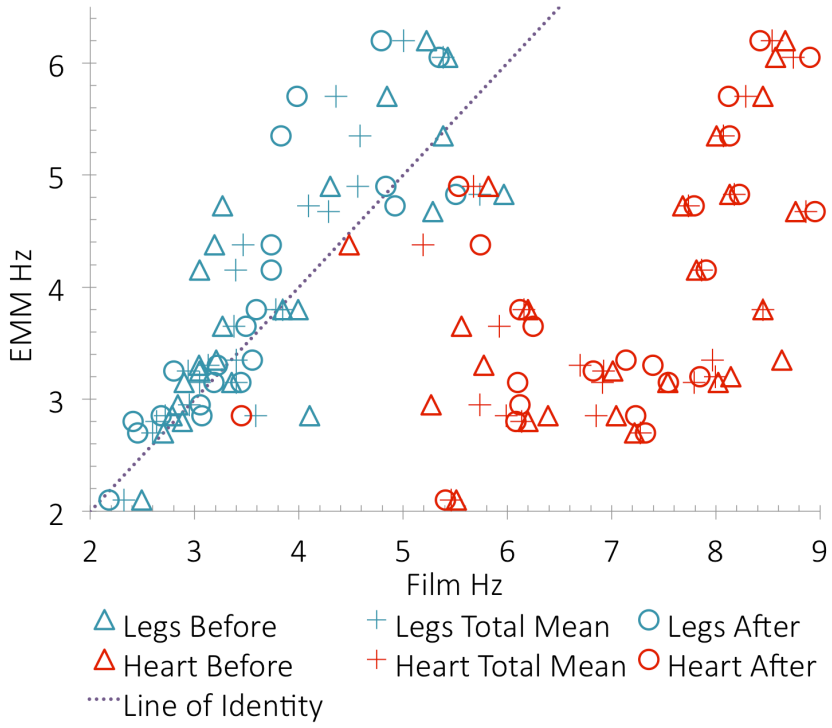
1) Place individual in observation bath in APW
2) Pipette off excess APW to immobilise
3) Film heart movement **[3.4.ii]** [12+ seconds]
4) Also, reposition the animal to focus on limbs and film [12+ seconds]
5) Add more APW to the bath
6) Pipette individual into EMM chamber
7) Pipette off excess APW to immobilise
8) Connect probes either side of the *Daphnia* to the amplifier header
9) Record [ten minutes]
10) Observe EMM progress on screen in case of obliterating interference
11) Save in the form of a .txt file
12) Return individual to observation bath to film heart then legs [12+ seconds each]
13) Repeat for each individual

In this case, films were made both before and after EMM, to combat the possibility of changing beat frequency due to the stresses of testing. Both heart AND legs were captured during the filming stage. Heart video data were then processed as per **3.5-6**, EMM data were processed using the spectrum analysis program. However, limb movement rate was elucidated by eye, looking frame by frame and noting the precise frame at which the limbs would be furthest left or right on screen. These are feathery masses with no particular shape so it is not possible to create a similar automated program as that used for the heart. Time between peaks and troughs were taken as the time between frames showing the limbs at their furthest extent to one side or the other, to an accuracy of $1/60^{th}$ second (due to 60p filming). This does not allow for parabolic peak interpolation as the chosen frame is either a 1 or 0 with regards to whether it is, or is not, the furthest extent of the limb to either side. Mean heart and foot rates were then taken from each data set, both before and after EMM. EMM and video data were then compared via paired distribution T testing as described in **7.2(a)**. Effect size was calculated using Cohen's *d*, an appropriate accompaniment to the above inferential tests where results are considered statistically significant when judged unlikely to have occurred by sampling error alone. It is a standardised mean effect size, that allows for ready comparison with meta-analyses or effect sizes reported in further studies when they occur.

7.2.(b)iii. Results

Fifty-eight EMM records were made of individual *Daphnia* from which twenty-six power spectrums could be obtained. The remaining records were rejected due to entirely destructive RFI that was not resolved even by the spectrum analysis program. For this cohort mean HR on film before and after the EMM record was made were 7.23±0.25Hz [mean±SE] and 7.08±0.26Hz [mean±SE] respectively. Leg movement rate before and after the EMM record was made were 3.69±0.22Hz [mean±SE] and 3.50±0.23Hz [mean±SE] respectively. The EMM record itself recorded a mean 4.018±0.24Hz [mean±SE] across the population. SE is chosen here as we are dealing with a population: SD describes the variance of the data while SE shows how far the population is from the mean and is therefore far more informative in this context. Comparison of EMM record and mean heart and foot rates, before and after EMMs is shown in figure 7g.

Figure 7g. EMM results compared to heart and feeding leg movement rates on film



Legend: 26 *Daphnia* filmed at 60fps before & after EMM. Legs blue; heart red; before EMM △; after EMM O, total mean +. Leg data is close to the LOI, suggesting legs, not heart, were the source of EMM readout. Confidence intervals given in 7.3(b)iii.

To test whether EMM and heartrate data as captured on film were correlated, a null hypothesis assumed no correlation. Comparing paired heart and EMM data via the Student T-test a p value of 1.3919×10^{-9} was found at the 0.05 alpha level. The null hypothesis was rejected. As seen in the preliminary trial, the sample distribution of beat data recorded via

EMM and that of heart rate captured on film are not associated within a 95% confidence interval. The independent paired T-test was then used to compare filmed leg and EMM data. A p-value of 0.0833 was found at the 0.05 alpha level. We 'failed to reject' the null hypothesis. The sample distribution of beat data recorded via EMM and that of limb movement captured on film are correlated within a 95% confidence interval.

Leg and heart data from film were paired to test whether leg activity might correlate with heart activity in a way that allows for a "limb movement proxy" for the heart. A paired T-test gave a p value of 4.52×10^{-10} at the 0.05 alpha level. Limb movement rate data and heart rate also captured on film are not correlated within a 95% confidence interval. Leg movement cannot be used as a proxy for heart activity. Effect sizes were calculated comparing EMM results vs filmed heart rate, EMM results vs filmed limb movement rate, and filmed HR versus filmed limb movement rate, giving d= 4.17, d= 0.51 and d=4.86 respectively. There was no significant difference between EMM and filmed limb movement which indicates they are the same, while significant difference exists between filmed HR and EMM, and between filmed HR and filmed limb movement rate, indicating that they record separate signals.

7.2.(b)iv. Discussion

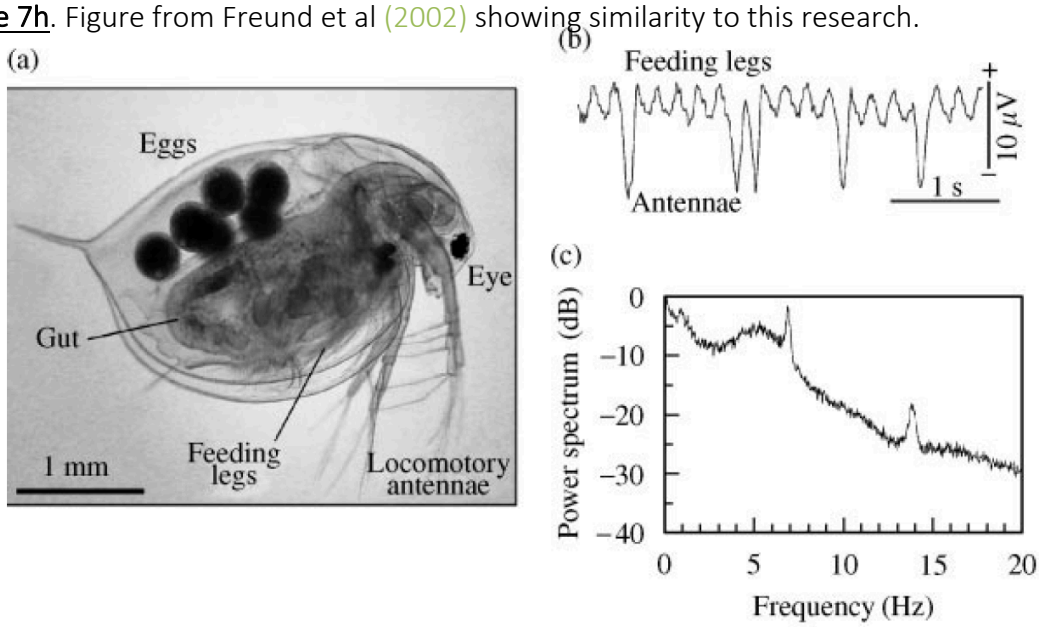
Trial one found that EMM records feeding limb beat frequency and not heart activity. And that limb movement is not a proxy for heart activity. To put this in context, *Daphnia* have four thoracic feeding legs (Shiga et al 2002). Leg movement allows the animal to filter and sweep fine particulate food matter towards the mandibles for consumption (Smirnov, 2013). *Daphnia* forage in a series of hop-pause-turn sequences. Dees et al (2008) found that those capable of the longest hops foraged the most food. They have four thoracic legs called thoracopods, which beat with a frequency around 3.5Hz (D. magna) or 3.85Hz (*D. pulicara*) to create an inflow from which food is filtered, and an abdominal claw to remove unwanted food boluses (Penalva-Arana et al 2007). This frequency agrees with our results.

All instars beat their legs at roughly the same rate. Instars are developmental stages between the neonate and adult, in which carapaces are shed and a softer, larger animal emerges and hardens, until the adult size is reached. There is no difference in leg beat rate between any of these stages, though animal gender does make a difference in leg beat rate. Males beat faster than females due to their higher metabolic demands, as they are in

a constant search for mates, and must maintain high swimming speeds (Penalva-Arana et al 2008). Male presence indicates the onset of the sexual stage and thus poor/ wintering conditions confounding factors, which was a deciding reason in excluding males from trials [3.1]. Leg beat frequency also varies according to culture conditions. During periods of hunger *Daphnia* produce slow feeding offspring with large mouths and slower beat rates maximise feeding efficiency (Garbutt and Little 2014).

EMM may have application within the field of freshwater toxicology. Thoracic limbs are the main site for oxygen uptake; their constant movement creates a water flow facilitating gas exchange (Pirow et al 1999). EMM might be used to observe responses to environmental oxidative state under various conditions. *Daphnia* limb movement is already established as a means of freshwater toxicology testing. Most work in this area has been done via direct observation or film methods, which tend toward low sample numbers or a large expenditure on human resources. In contrast, EMM records thoracic limb motion in real time. And, were we to develop this method as a printed circuit board, the method could be adapted to achieve high-throughput.

Figure 7h. Figure from Freund et al (2002) showing similarity to this research.



Legend: a = a *Daphnia*, b = electrical signal from a tethered *Daphnia*, c = power spectrum

The only publication with methods parallel to this, Freund et al (2002), measured electromechanical signals of an entire swarm, which produced harmonious limb motion at a sinusoidal 5Hz, punctuated with 4-15 Hz blips via antennal swimming movements, suggesting *Daphnia* produce consistent responses when under the same conditions. As a

precursor to their main study Freund et al (2002) tethered a single *Daphnia* and measured electrical signals emanating from it. The signals produced are well within the range of our feeding limb movement results. Figure 7h is taken from Freund et al (2002) and shows the electric potential from a tethered *Daphnia* measured at a distance of 0.5 cm, with oscillations resulting from 5-6Hz motion of the legs and approximately 7 Hz motion of the antennae, as well as a power spectrum of the signal.

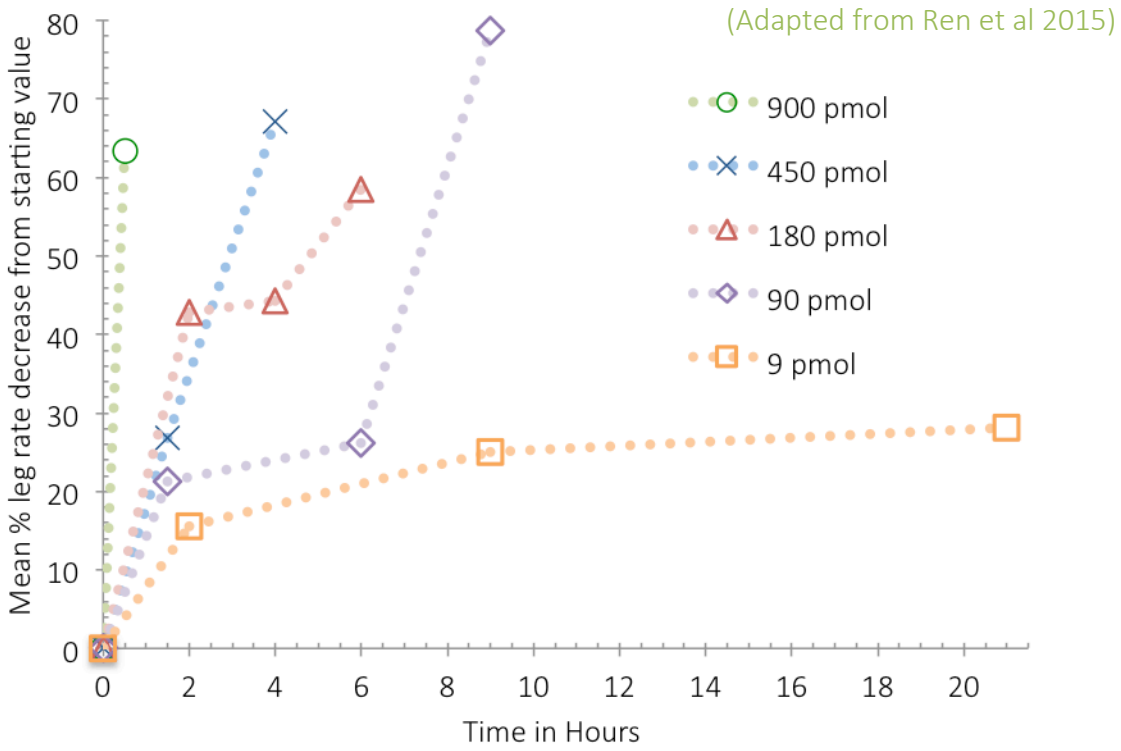
Leg beat frequency has been used in a number of publications. It has been used to show that *Daphnia* feed more quickly as algal food concentration increases (algae unidentified) (Furuhagen et al 2014). A feeding plateau of > 10^4 cells cm^{-3} was found for for *Chlamydomonas reinhardi* (Porter et al 1982). *Escherichia coli* is consumed by *Daphnia* where no other food is presented, with a leg beat plateau at 2.5x 10^6 cells cm^{-3} (Kim et al 2003). Lari et al (2017) found that increasing *Raphidocelis subcapita* concentration had no effect on limb movement, remaining at 7.5Hz. However, only two concentrations were used (5x 10^5 and 5x 10^6 cells/mL), which were perhaps already above maximum saturation for the animal's needs.

The following authors applied limb movement, as seen via slow motion video playback, to the field of toxicology. Lari et al (2017) found that limb movement was reduced in aquatic environments affected by oil-sand bitumen extraction processes. Only twenty animals were used, perhaps due to the laborious nature of acquiring data from film. Bengtsson et al (2004) soaked *Scendesmus* in a glycophosphate solution for 4 days as a food vector for the contaminant; it caused a 40% reduction in limb beat rate.

Ren et al (2015) found increasing concentrations of the insecticide Dichlorvos caused stepwise behavioral changes including hyperactivity, loss of coordination, convulsions and paralysis. An EC50 was given as 0.906pmol. In the current literature (as of September, 2018) only this study uses more than two concentrations, which suggested it as a possible study for comparative work in this thesis. Concentrations were written in non-standard form as 'Toxic Units', based around the dichlorvos LD50 of a rat; we calculated that these refered to 9, 90, 180, 450 and 900pMol; standard units which are more useful for cross-comparison. Ren et al (2015) did not normalise pre-exposure leg beat frequencys, but used mean starting values per trial set. We normalised these values for ease of comparison, making each starting value 100% and adjusting ensuing exposure results accordingly.

Animal response was recorded at inconsistent intervals; not all test groups were observed at the same time. Figure 7i shows our reinterpretation of this work, as no such figures are given in their text. After some experimentation, Dichlorvos was not chosen for comparative study due to the chemical's instability. Batches sublimated shortly after delivery requiring that all studies occur in a brief space of time. This was not practical given the circumstances required trials to be repeated many times due to the presence of RFI.

Figure 7i. Normalised results of *Daphnia* leg movement response to dichlorvos over time



Legend: Data from Ren et al (2015) were interpreted into molar concentrations, normalised for easy comparison between concentration test groups and displayed according to trial time. It was assumed that the 'Toxic Units' given in the paper were made up accurately according to their given LD50 for the rat.

Other workers have used cell counts as a proxy for feeding rate. McWilliam & Baird (2001) placed *Daphnia* in mesh lined chambers at various contaminated sites for 24 hours, then moved them to freshwater containing 5x 10^5 cells/ml *Chlorella vulgaris*, for 4 hours. Cell counts showed consistently lower feeding rates when exposed to the same levels of contamination at geographically disperse sites. Grintzalis et al (2017) used the same method to show that heavy metals ($CdCl_2$ and $NiCl_2$) lowered the feeding rate, and algal concentration increased feeding rate. They also found that older *Daphnia* eat more algae which may be due to their size. They used only two concentrations of each and no controls; too few to produce a reliable dose response curve. Rist et al (2017) also used this

method to investigate the impact of plastic nanoparticles on feeding rate. Due to the similar size of algae to the smaller sized plastic particle (100nM) they were unable to distinguish preference using the cell counter, however they found that the larger size particle (2μM) slowed feeding rates. This may be due to the animal's need to remove obstructive boluses from its mandibular area to facilitate filter feeding.

Toxicological assays have also been performed where swimming (antennal) movement, rather than feeding limb rate are monitored. Swimming is erratic while normal leg movement is sinusoidal and repetitive being required for respiration. Barrozo et al (2015) found that exposure to the dopamine receptor agonist bromocriptine inhibits antennal movement in *Daphnia*, by plotting the course of a swimming *Daphnia* in a petri dish over the course of an hour; movement lessened with increased concentration (EC50 6.6 μM). Bahrndorff et al (2015) used a Locomotor Activity Monitor (LAM) rotated 90° to monitor *Daphnia* up-down swimming activity in 20ml solution, via 9 infrared beams. Using four concentrations, plus a control over 48 hours at 20 ± 1°C, they determined potassium dichromate has an EC50 of 237.95pmol, and 2,4-dichlorophenol has an EC50 of 184.05pmol.

Other organ movements, such as the eye, were not measured in trial one. The *Daphnia* cyclopean compound eye has 242 optic neurons (Lopresti et al 1972), it scans the environment with 4Hz frequency, moving 150° on the sagittal plane and 60° in the horizontal plane. The eye follows moving light sources with such speed, that when made to observe rapidly rotating black and white striped disks, *Daphnia* are quickly sent into nystagmic tremor (Frost 1974). Scan and leg beat frequency are remarkably similar. However, due to the larger size of the legs, they are most likely the dominant frequency measured by these methods.

7.3 Trial 2: *Daphnia* feeding limb response to imidacloprid

7.3.i. Introduction

In this study *Daphnia* limb movement response to imidacloprid is investigated. Imidacloprid is a neonicotinoid, a class of insecticide implicated in honey bee colony collapse, with reverberative effects on both the environment and the economy. Neonicotinoids specifically bind to and activate post synaptic nicotinergic acetylcholine receptors (nACHR) in the central nervous system. They are chemically similar to nicotine

acting antagonistically on these receptors (Tesovnik et al 2017). Binding is nearly irreversible and holds the channel open causing continuous nervous system stimulation, which eventually leads to the death of the neuron (Qi et al 2018). This affinity is equivalent across the neonicotinoids. Permanent effects accumulate with time (Morrissey et al 2015). This is a very good reason for measuring the area under concentration response curves.

The Neonicotinoids are nicotine receptor agonists with biphasic hormesis giving low dose stimulation and high dose inhibition. The three most popular forms are Imidacloprid (IMI), clothianidin (CLO) and thiamethoxam (THIM). More recently developed compounds (as of 2018) are Thiacloprid (THIC) and Acetamiprid (ACE). Less widely used insecticides in this class include guadipyr (GUA) and cycloxaprid (CYC). THIM also interacts with muscarinic receptors (Baines et al 2017). IMI is a partial agonist, while CLO acts as a "super agonist" irreversibly opening nAChR channels (Alkassab and Kirchner 2018).

In the UK, a temporary moratorium on the use of IMI, CLO and THIM has been in place since 2015, restricting their use in summer only, for a minority of crops, rather than a blanket ban (Woodcock et al 2018). Neonicotinoids persist in soil with a half-life of over a thousand days and have been found in commercial honey three years after the ban with CLO in 38.1% of samples (Woodcock et al 2018). Neonicotinoids are primarily used in corn seed coating (Samson-Robert et al 2017), where they form a prophylactic taken up by growing plant tissues to provide lasting toxicity against insect pests (Wood et al 2018). They are highly leachable and prone to water source contamination (Qi et al 2018). Morrissey et al (2015) found neonicotinoids from agricultural run-off in puddles, irrigation channels, rivers, streams and wetlands, all of which exceeded existing water quality guidelines. Lentola et al (2017) investigated neonicotinoid levels in ornamental plants using mass spectrometry; of 29 plants sold as "bee friendly" 23 contained between 2 to 10 different agrochemicals. Most prevalent were the combined presence of neonicotinoids THIM, CLO and IMI in concentrations higher than LD50s for each pesticide alone.

A vast number of publications investigate the effect of neonicotinoids on bee populations. The following have been published in the past year alone. Over 85% of honey bee deaths during corn sewing season are neonicotinoid related: 54% were associated with CLO consumption, and a further 31% deaths with consumption of both CLO and THIM, making colonies in neonicotinoid-coated corn planting areas up to 4x smaller than those outside

the areas (Samson-Robert et al 2017). Also, neonicotinoid exposed colonies produce proportionally less males, and less worker bees, and fewer brood frames compared to controls (Balfour et al 2017). For these, no neonicotinoid concentrations were given. When searching for studies with which to perform a comparison in our own difficulties arose because bee neonicotinoid trials rarely examine more than one concentration. A handful use two concentrations but do not explore the range between them. Where concentrations are given in molar weight, they are compared in table 7j, discussed below.

Table 7j. Comparison of Neonicitinoid effect on bees where concentration is given (μM)

THIM	IMI	THIC	CLO	
0.02	0.02		0.02	No change (Wood et al 2018)
		9.914	0.04	Impaired melanisation (Brandt et al 2017)
0.08	0.08		0.08	Reduced foraging (Wood et al 2018)
			0.2	Sig. impact on mortality (Brandt et al 2017)
Up to 0.429	Up to 0.489			Decreased ability to reheat (Potts et al 2018)
6.410				No change (Baron et al 2017)
8.227				Fewer males born (Stanley and Raine 2017)
	10.170			Reduced foraging (Phelps et al 2018)
14.261			3.845	Queen mate less (Forfert et al 2017)
18.237				Reduced terminal oocyte length (Baron et al 2017)
	39.114			Further reduced foraging (Phelps et al 2018)
34280				Larval immunosuppression (Tesovnik et al 2017)

The following publications test only one concentration against a control. Tesovnik et al (2017) fed honey bees 34280μM THIM loaded protein cakes. In the first of two pupal development stages (white-eyed), THIM deactivated all immune related pathways. At the second, brown-eyed, stage in which melanisation occurs (an immune effector mechanism which also gives eyes their brown colour) 3-4 days later THIM was less harmful; only the immune gene Spaetzle was downregulated. Stanley and Raine (2017) fed honey bees 8227nM THIM loaded protein cakes; proportionally fewer males were produced compared to controls, with larval bees more vulnerable than adults. Forfert et al (2017) fed queens 14261nM THIM or 3845nM CLO loaded protein cakes, both caused queens to become less able to orient and find the scattered drone colonies so that they mated with significantly fewer drones than controls.

The following trials compare only two concentrations of any neonicotinoid. Doses chosen are the minimum and maximum found on farmland; Results unanimously show that the

higher dose causes deleterious effect while the lower has less to no effect. With only two doses no study reveals at what dose toxic impact begins. Baron et al (2017) fed bumble bee queens 6410nM or 18,237nM THIM loaded protein cakes. The higher concentration caused a reduction in the length of terminal oocytes while the lower did not reduce this length. Brandt et al (2017) fed queens 791nM or 9914nM THIC, or, 40nM or 200nM CLO loaded protein cakes. The highest dose of CLO had a significant impact on mortality, the others did not. All concentrations significantly reduced impaired melanisation, which lowered immune response. Wood et al (2018) fed bees 20nM or 80nM of THIM, CLO or IMI loaded protein cakes over twelve weeks. For all three, no effect was seen as 20nM, the 80nM treatment resulted in significant decreases in honey production after 9 weeks due to decreased foraging and navigational ability, CLO had the strongest effect. Phelps et al (2017) fed honeybees 10.170μM or 39.114νM IMI loaded protein cakes. Both doses caused learning deficits compared to controls; with bees less able to associate artificial flower colour to sucrose concentrations. Foraging duration and number of flowers visited was lower for the higher concentration.

The following publications explore more than two concentrations per trial. In the same, dividedly published, study Tosi et al (2017) and Tosi and Nieh (2017) fed honey bees three concentrations of THIM, 1.34ng, 3.48ng and 1.96-2.90ng "/bee" in loaded protein cakes, which initially caused increased movements, until over stimulation lead to long term fatigue and weakened bee ability to fly. The lowest increased flight duration and velocity in the first hour, but significantly decreased flight duration, velocity and thorax temperature after two days of repeated feeding. This impact was stronger at the higher concentration. The higher concentrations impaired locomotion then motor function after 30 minutes, and two days repeat feeding also impaired climbing ability, shortened flight paths and caused photophilia; moving toward light sources which was taken as analogous to leaving the nest. Leaving the nest is a self-sacrifice which prevents contamination of the colony. Alkassab and Kirchner (2018) fed bees 0.1, 0.5, 1.0 and 2.0 ng/bee CLO loaded protein cakes. Compared to controls; the lowest dose had no significant effect; the next two doses caused significant increases in flying distance over 90 minutes, but no difference between one another. The highest dose significantly increased flying distance in the first half hour but caused them to lie on their backs and spasm after 90 minutes. All observations ended at that point. Potts et al (2018) fed bees a range of 9 unspecified concentrations of 313pM- 489nM IMI and 274pM-429nM THIM, which dose dependently decreased bee

ability to heat themselves after chill induced torpor, and decreased the temperature at which bees were able to incubate brood clutches. Baines et al (2017) tested a wide range of concentrations of ACE, CLO, IMI and THIM across three bee species (bumblebee, honey bee and leaf cutter bee) and for resolved LD50 values for all. However, this was done in terms of feeding weight value 'per bee'. And while Samson-Robert et al (2017) give mean bee weight as 0.128g, conversion to a comparable molar weight would not be accurate given the unknown variance of tested bee weight around mean bee weight. For reader reference these values were in order of ACE, IMI, CLO and THIM, for summer honey bees 9.1µg, 32.8ng, 2.6pg and 34.7pg, for winter honey bees 21.5ng, 29.9pg, 0.013pg and 0.02pg, for bumble bees 300µg, 0.23pg, 94.5pg and 4.69pg, and for leafcutter bees 9.3µg, 3.2pg, 0.0006pg and 5pg. All four neonicotinoids caused ataxia in acute stages and shortened lifespans across all species tested. CLO and THIM were most toxic. CLO, IMI and THIM exposure resulted in bi-phasic response curves indicating endocrine disruption. Doses above 25mmol of any overstimulated the nervous system causing acute hyperactivity, trembling, uncontrolled proboscis extension, chronic slowed movements, and ataxia, with no recovery, across all species. Oddly, honey bees lined up in perfect rows prior to death. Younger winter bees were toxically affected at much lower doses of CLO and THIM than in summer, as at this stage bees have a less well developed midgut making neonicotinoids more bioavailable and hastening acute symptoms.

Daphnia are a good choice for such a study as they have the lowest mortality rates compared to many other aquatic invertebrates tested with neonicotinoids, and so were recommended for non-lethal effect studies (Beketov and Liess 2008). Cladocerans are least sensitive to imidacloprid compared to amphibians, crustaceans, fish, insects, molluscs and worms. At doses lethal to these species *Daphnia* grew to significantly smaller body lengths than controls but were unaffected at lower doses (Hayasaka et al 2012). *Daphnia* birth rate remains unaffected until neonicotinoid concentrations increase high enough to cause the population as a whole to undergo mortality (18.54 µM thiamethoxam/14 days). Beketov and Liess (2008) found that while *Daphnia* remain alive in all but abnormally high levels of the neonicotinoid insecticide guadipyr. However, offspring number and body length were very sensitive to low concentrations. Both decreased dose-dependently at concentrations ranging between 0.05 and 1.6mg/L over 21 days (molecular weight unknown), showing response testing is perhaps a more sensitive tool than acute toxicity tests (Qi et al 2014). Tisler et al (2009) found *Daphnia* to be more acutely sensitive to IMI than *Vibrio* bacteria or

Desmodesmus algae though eliciting a detectable non-acute response in these more microscopic species may be difficult. Qi et al (2018) found that animals born under EC50 conditions of imidacloprid had significant abnormality levels occurred including loss of eyes, undeveloped swimming antennae and curved tail spines. EC50 levels for neonicotinoids in *Daphnia* are shown in Table 7k.

Table 7k. LD50 and EC50 μM values for *Daphnia* in the literature

μM	IMI	GUA	CYC	CLO	THIM	ACE
Waterborne	1.25m			0.012m	0.88m	0.17m
Immobilization	64.53q8	50.89q8	57.5q8			
Birth decline	63.36q8	54.80q8	44.20q8			
AchE activity	19.55q8	<3.29q8	<14.04q8			
24hr LD50	172.08m	50.89q4			28.16b	
48hr LD50	169.20h or 221.35t	74.53q4		>1.96d		

Legend: waterborne= mean concentration of neonicotinoid found in freshwater streams; and references, b= Beketov and Liess 2008, d= De Perre et al 2015, h= Hayasaka et al 2012, m= Morrissey et al 2015, t= Tisler et al 2009, q4= Qi et al 2014, q8= Qi et al 2018. Qi 2014 and Qi 2018 assume the unknown molar weight for guadipyr is the same as that of imidacloprid.

Finally, Zein et al (2014) found nicotine could be used as a proxy when testing neonicotinoids : Dose dependent effects of IMI on *Daphnia* cumulative swimming distance, captured on live film, mapped with surprising exactitude to the same action caused by nicotine at four times the molarity : 1, 4, 16, 64 and 256μM nicotine mapped to an equivalent decrease in swimming distance to the use of 4, 16, 64, 256 and 1024μM IMI. Swimming distance increased with the use of 16μM nicotine or 64μM IMI and proceeded to fall once more at higher concentrations of 64 and 256μM nicotine or 256 and 1024μM IMI. Immobilisation occurred at the highest concentrations of both but was recovered from in all cases. As Zein et al (2014)'s study used the widest range of concentrations on Daphnia and produced results from which comparisons might be drawn in our own study – despite the use of the swimming antenna rather than the feeding legs - we chose these concentrations of IMI for our study using Whole *Daphnia* EMM.

For this study we rely entirely on EMM and do not apply video techniques. We know that the adapted spectrum analysis program gives a correct result for leg rate, because in trial one films of heart and leg movement are compared to results from the EMM recording as given by the program. Subsequent statistical correlation showed that the EMM gave leg rate. The program has not changed since then but was simply continued for EMM only

work. Trial one provided an independent check that verified the EMM results were commensurate with the legs.

7.3.ii. Methods

For trial two, five triangular baths were made as shown at the bottom right of figure 7a. One bath was kept contaminant free at all times, used for controls or pre-drug exposure records only, and the others labelled according to drug concentration to be used. This trial used EMM only. Mean starting limb movement rate is derived from the first three EMMs which record beat movement in APW solution, or in cases of RFI interference from EMMs available. Trial animals were next exposed to a concentration of imidacloprid, while a control remained in APW. Animals were trialled three at a time, alternating between a set of three that would be exposed at to 0μM (the control) and either 16μM, 64μM, 256μM or 1024μM imidacloprid, choosing three different concentrations at a time so as not to weight all samples from one cohort into a narrow set of trials. Odd numbered trials have one control, two random concentrations, even numbered trials three random concentrations, performed on consecutive days. In this way one control existed in balance to every five drug-exposed animals.

Figure 7L. Example action chart to ease understanding of lab methods in trial 2

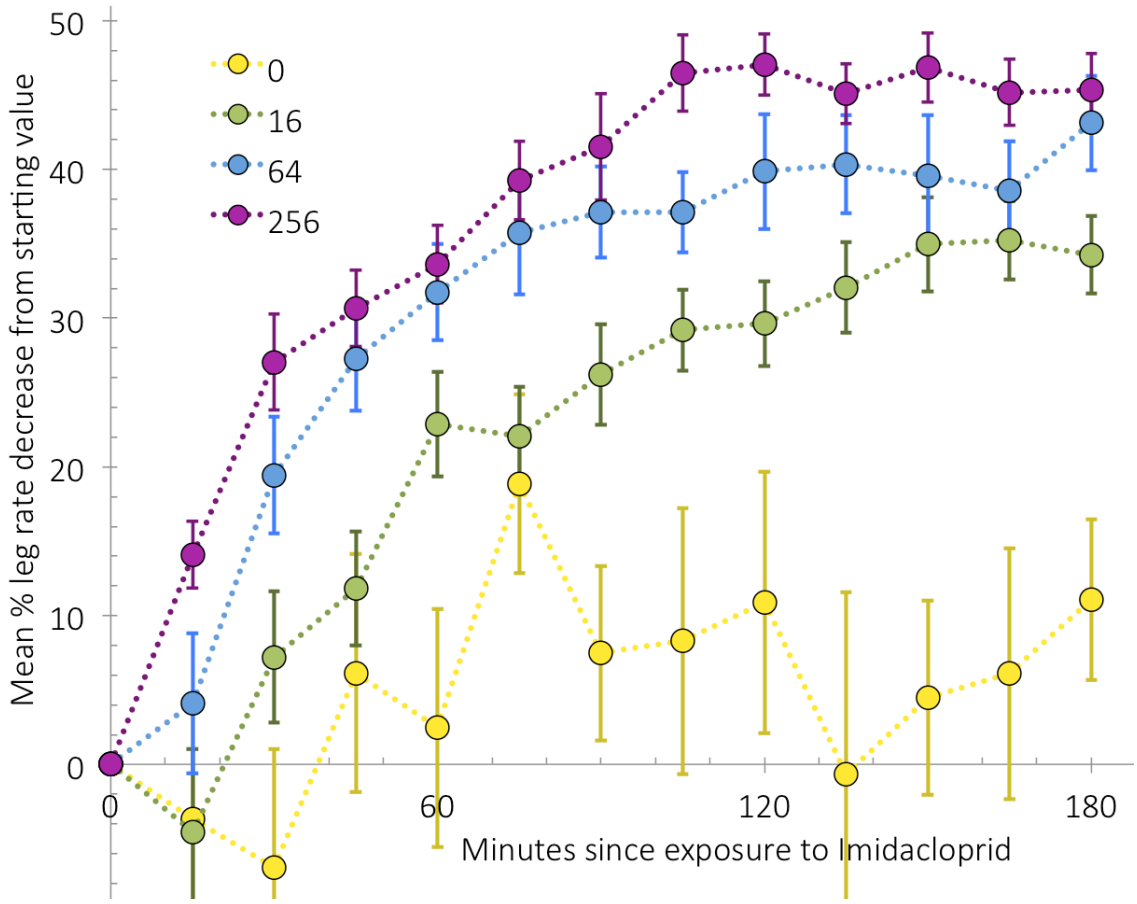
Animal 19		Animal 20		Animal 21
All start by swimmingly freely in APW in individual baths				
1. Record EMM then place back in APW	→	2. Repeat for this animal	→	3. Repeat
4. Repeat	→	5. Repeat	→	6. Repeat
7. Repeat, then place animal in control concentration, 0μM	→	8. Repeat, then place in trial concentration, e.g. 256μM	→	9.Repeat, then place in trial concentration, e.g. 64μM
10. Record EMM then return to solution above	→	11. Repeat for this animal	→	12. Repeat

→ 13. Repeat steps 10-12 for three hours or until no reading is seen.

Animals were recorded one after another in turn, five minutes at a time, for three hours, placing each back in either their appropriate drug or APW solution in between records. A small pool of drug or APW solution immobilised the animal during recording, In between

records the animal was able to swim freely. Three hours was the maximum length of experiment possible owing to constraints imposed by RFI competing equipment and lab opening hours. An example of this process in flowchart form is given in figure 7L for clarity. As before, results were processed using the Matlab spectrum analysis. Standard error was calculated as this is population data, while SD describes variance, SE shows how far the population is from the mean and is therefore far more informative in this context.

Figure 7m. Decreasing leg beat rate response to imidacloprid



Legend: yellow are controls, all concentration values refer to μM, error bars = SE, n = 74.

7.3.iii. Results

Average baseline data in terms of starting HR for each cohort tested were 5.53Hz±0.32 [mean±SE] (controls), 4.71Hz±0.27 [mean±SE] (16μM), 4.07Hz±0.22 [mean±SE] (64μM) and 4.55±0.13 [mean±SE] (256μM). To reiterate, SE is chosen here as we are dealing with a population: SD describes the variance of the data while SE shows how far the population is from the mean and is therefore far more informative in this context. Figure 7m shows *Daphnia* leg response to IMI for 74 animals at 15-minute intervals over 3 hours, plateauing

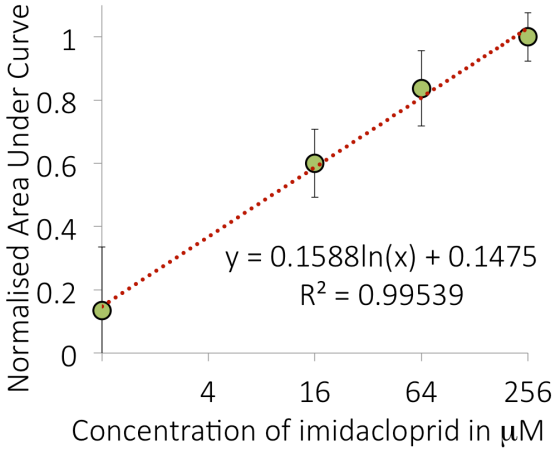
with feeding rate mean decreases of 45.90%, 40.29%, 33.23% and 6.37% for 256µM, 64µM, 16µM and controls respectively.

Related to this Zein et al (2014) found dose dependent effect of IMI on swim distance (antennal movement rather than legs) increased at 16µM and 64µM and fell at 256µM. Immobilisation occurred at 1024µM though recovery occurred in all cases. In our study, leg movement slowed dose-dependently, becoming so weak at 256µM that a second signal is seen. This second, much weaker, signal shows a much faster frequency which also increases over time. We postulate that this may represent the heart due to its greater frequency, though it is not a useful way to measure the heart as it is only visible when leg movement is weak. The observed increasing speed of this second signal over time may indicate cardiac stress as the heart pumps faster to combat oxidative stress.

For the concentration 16µM, 336 separate recordings were made over 24 animals. 32 were rendered unusable by Bluetooth interference despite counter measures. In most *Daphnia* an increase in limb movement occurred in the first 45 minutes after drug exposure, with a mean +4.56%, after this movement rate drops in a linear fashion until the middle of the second hour after which the decline slows and a clear plateau is reached. For this concentration the plateau was reached in the final hour at 66.77% of the starting rate (SE 1.06). The initial increase in limb movement may simply be due to chance rather than a true drug effect. It may be that this lowest concentration required time to affect the system before having an effect. For 64µM, 364 separate recordings were made over 26 animals. Twenty-one were rendered unusable as above. In some but not all animals a small increase in limb movement rate is seen in the first fifteen minutes after drug exposure, with a mean +2.5%, after this movement rate drops in a linear fashion for the first hour after which the decline slows. As the two hour time point is neared the movement rate begins to plateau. For this concentration the plateau was reached in the final hour at 60.71% of the starting rate (SE 0.77). For 256µM, 168 separate recordings were made over the 12 animals. Twenty were rendered unusable as above. Limb movement decreased steadily from the offset becoming so weak in the final two hours that on occasion a second signal could be seen beating at a higher rate. It is possible that only when limb beats became weak, a heart signal, or other signal may have become apparent. These extra signal appearances were too few and far between to perform any reliable analysis, but observation indicated increases in the rate of the second signal over time where it was

seen more than once per individual. If this second signal was indeed the heart then it may indicate the heart under stress due to more toxic conditions at this very high concentration. A plateau was reached in the final hour at 54.10% of the starting rate (SD 0.96, SE 0.43). Fewer animals were selected for exposure to this concentration as it became clear that it caused such weakening of limb movement that signals were very difficult to elucidate, often forcing the user to go back to visual interpretation of the spectrum analysis for confirmation as interference signals out-competed animal signals.

Figure 7n. Normalised area under the curve



Legend: The area under the curve for each dose was normalised between 0 and 1. Error bars show standard deviation. The red dotted line is given by Excel software which gives a linear line of best fit across given points and produces the equation of the line as shown. R^2 gives how well this line fits out of 1: clearly 0.99 is a very good fit. Note that the x-axis is on a logarithmic scale using base 4, as drug concentration increased by base 4 following the imidacloprid trial in Zein et al (2014).

For the control group maintained in drug-free APW, 168 separate recordings were made over 12 animals. Of these six were rendered unusable as above. Animal response was notably more variable than drug-exposed groups. Variance of response grew smaller with increasing concentrations. Perhaps the presence of the compound in solution overwhelmed natural leg beat response that *Daphnia* may have in their normal environment, where limb beating may vary according to any number of factors including micro changes in temperature and light. As its concentration increase it may have become more dominant than these factors.

For the controls, just as with those exposed to 16μM imidacloprid, leg movement increased at the start of the trial, here to a mean +6.07%, however while leg rate decreased overall over the full course of the trial its rate fluctuated up and down and the

standard deviation of this movement was much higher than with any exposed animals. No plateau was reached. In the final hour mean leg rate was 93.63% of starting rate (SD 4.89, SE 2.187), over the entire trial leg rate was a mean 94.63% of starting rate (SD 7.01, SE 2.02). Figure 7n shows the area under the curve for the entire three hour trial period, per dose, was normalised between 0 and 1. This gave a near straight line in log-lin format, so that $y = a \ln(x) + b$, where x = dose and y = response.

7.3.iv. Discussion

In trial two we recorded *Daphnia* leg response to IMI for 74 animals at 15 minute intervals over 3 hours. Responses plateaued with mean decreases of 45.4%, 40.8%, 32.9% and 5.4% for 256µM, 64µM, 16µM and IMI-free controls respectively. Leg movement slowed dose-dependently, becoming so weak at 256µM that a second signal was seen. A faster frequency increasing over time which may be the heart revealed when limb movement weakened. We could postulate the high frequency detected points to increased heart stress but no analysis could be performed as this second frequency was only visible when leg movement was weak.

Thoracopod response to IMI was much more sensitive than antennal response given by Zein et al (2014) for the same concentrations. Leg action increased at 16µM before dropping within half an hour of exposure to this concentration, where as for Zein et al (2014) antennal movement increased with both of 16µM and 64µM IMI dropping only at higher concentrations in the time span tested. In our study, leg rate decreases from the offset using the 64µM and 256µM concentration, causing immobilisation within the first half hour at 1024µM. This is a positive finding as the legs' comparatively deeper sensitivity for detecting lower levels of IMI is important when we consider the destructive effects of low level imidacloprid on pollinators.

Thoracopod response to toxic insult is much more unified and predictable than responses found in cardiac trials. A further interesting observation was that imidacloprid exposures lowers the variability of animals response compared to controls. This is made clear by the lower standard deviation in trials is much lower than among the controls seen in figure 7L. A possible reason for this might be that the drug's toxic effect overwhelms other variant physiological factors, such as a slight age differences (virgin neonates may be anywhere between less than an hour to four days old), food intake variation or individual tiredness

levels passed on the recent swimming activity of each animal. The area under the curve calculations produced a near straight line in log-lin format, so that y = a ln (x) + b, where x = dose and y = response. This outcome suggests that *Daphnia* thoracopod response could be used as a generalised marker of toxicity in freshwater environments and may form an efficient and real time alternative to current methods. More specific chemical analysis could then be used to determine particular toxins.

7.4 Chapter Summary

This chapter found that Whole *Daphnia* EMM may offer new opportunities in ecotoxicology. Specialised equipment was built, and software was written to capture electromechanical movement signals from whole live *Daphnia*. We found that this novel technique records thoracopod movement rather than the heart. We performed a trial using this method nevertheless, as it offers a novel and more efficient technique for aquatic ecotoxicology, where visual observation or films of the same are currently used. EMM has huge advantages over video as a means of gathering data in terms of efficiency, real time application, mechanical accuracy over reliance on human intervention, and the amount of data it can capture in a shorter space of time. Its disadvantages are less to do with the method itself and more to do with external factors in the shape of Radio Frequency Interference (RFI). Solutions to this problem were actively sought and worked upon during the course of method development and the research itself.

Once we found that EMM measures leg beat frequency, we performed a trial of thoracopod response to the neonicotinoid imidacloprid. Neonicotinoids are a current hot topic due to their controversial impact on pollinators, with destructive effect on bee ability to produce and survive or locate and pollinate crops. Along with *Varroa* mites and the effects of monoculture farming, they are responsible for colony collapses worldwide. Bee colony development depends critically on freshwater resources, contamination is linked directly to decline in colony health (Chretien et al 2017). We found that thoracopod response to toxic intervention is much more unified and predictable than responses found in cardiac trials.

In the most similar method to EMM found in the literature, Penalva-Arana et al (2007) recorded of the flow of dopamine via attached microelectrode on a hair-tethered *Daphnia* and found beat with a mean frequency of 3.5 Hz (*D. magna*) or 3.85 Hz (*D. pulicara*). This is

taken as normal leg movement as dopamine concentrations below 10mM have no effect on *Daphnia* leg activity. By using electrochemical traces as a proxy for to limb movement, this method was similar to our own in some ways. EMM works with no drug, while Penalva-Arana et al (2007) require a drug, and the ability to actively measure its concentration. It is possible that limb movement may confound the concentration measured by electrode, as it too is measured by the electrode alongside the drug concentration. In balance it maybe that the electrode used was sufficiently far away for this not to be the case. In EMM, only by placing an electrode directly on either side of an immobilised *Daphnia* are any recordings possible – this underlines the importance of the specific design of the *Daphnia* EMM bath. If the electrodes are placed on the same bath but not either side of the *Daphnia*, even though the bath itself is only of 7mm diameter, little to no signal can be recorded. So sufficient distance from the Daphnia's confounding leg movement may be possible for the electrochemical dopamine trial; however, this would then bring in a second confounding factor – that of dopamine's dispersion in the liquid media before reaching the electrochemical sensors. EMM removes these confounding factors so that we are fairly sure that the only signal emanating from the source is that of limb movement.

EMM has huge advantages over video as a means of gathering data in terms of efficiency, real time application, mechanical accuracy over reliance on human intervention, and the amount of data it can capture in a shorter space of time. Its disadvantages are less to do with the method itself and more to do with external factors in the shape of Radio Frequency Interference. Solutions to this problem were actively sought and worked upon during the course of method development and the research itself.

8. Conclusion

> *"Merely corroborative detail, intended to give artistic verisimilitude to an otherwise bald and unconvincing narrative."* --- Pooh Bah, the Mikado

This research began by investigating the utility of *Daphnia* as a model for cardiac concentration response trials. Secondary effects, such as gastrointestinal spasms and abortion were observed, and population variation in drug response was high. We investigated control of environmental parameters. We found that *Daphnia* physiological activity did not alter environmental pH. However, we found that *Daphnia* are very sensitive to environmental pH, including changes beyond the buffer capacity to stabilise pH in solutions used. Thus, *Daphnia* are an inappropriate model for these trials because of their high sensitivity to micro-level environmental conditions.

The research also found a new direction by using *Daphnia* heart generated data to test various mathematical algorithms. These were scoring systems which were applied to determine stability of individual *Daphnia* heart rhythm in normal environmental conditions. Scores were compared with paired data from the same animal after cardiac intervention in the form of negative chronotropic agent. Heart rate change between baseline and a fixed end point were recorded. Heart rhythm stability scores were compared with paired responses in the form of heart rate change from individual *Daphnia*. We found that hearts more variant to begin were less susceptible to the decelerative effect of the drug, and this may account for high standard deviations or even varying responses to the environment. More variant hearts maintained normal heart rate better than those who had a more stable rhythm. We identified the more successful scoring systems for this analysis, and showed that to achieve significant correlations depended on having an appropriately diverse population to begin. This is something we did not have across all cohorts because of our attempts to maintain a fully homozygous population by selecting neonates, baseline scores were only varied in a handful of cohorts seemingly by chance. We suggested a solution would be to sample a heterogeneous natural population rather than applying the unnatural homogeneity more normal to lab based work, or by simply neglecting to select for size and neonate status. Significant results were found during regression analysis which suggested that measurement of heart rhythm via certain scoring systems may offer potential as a risk indicator for heart patients. A validation of this result was found by application of the most successful scoring system using data from

Physionet. However, any validation using the Physionet dataset are cast into doubt by the sheer volume of duplicated and unreliable data we found in the available databases.

We also attempted to implement a high throughput method of gathering heart data by creating novel instruments and software to record signals from whole live *Daphnia*. We recorded electrical signals from whole, live, *Daphnia*, which we termed whole *Daphnia* Electro Mechanical Movement records, or EMM. We showed that this instead recorded thoracopod movement. Normally, in their current role as a model aquatic ecology, *Daphnia* fatal LD50 is used as a toxicological yardstick. Death is an all or none measure that lacks the subtleties of living physiological response. As the *Daphnia* heart is highly sensitive to micro-level environmental changes, this may also explain why it has not been taken up as a means of observing larger toxicological change in aquatic ecology. However, there are no publications to back this assertion – perhaps because a negative result may not be so easily published. Observation of thoracopod movement in more commonly used in aquatic toxicology, so might be a less sensitive to micro-level changes. Limb beat frequency is known to correspond with environmental toxin levels, and has so far been published via observations by eye or slow motion playback on film. EMM captures data in real time, so is more efficient and accurate than these methods. It offers a novel method of toxicological testing that could be offered back to the field of aquatic ecology from the field of cardiac science.

There is very little published literature with which to contextualise this work. Much of the literature has already been discussed. In this section we will simply summarise, without repeating, connections to the literature discussed in each chapter. And, where possible draw connections between our own work and any related literature. Long reference lists simply seek to legitimise our statement that when we say there is little or no literature with which any one thing may be compared, we do indeed mean it having performed thorough literature searches. As we saw in chapter one, cardiac drugs are a strong focus for ecotoxicologists due to their known toxic levels in the freshwater environment (Jones et al 2002, Bona et al 2015, Luna et al 2005, Gomez-Olivan et al 2015, Lilius et al 1995, McWilliam and Baird 2001, Rosa et al 2008, Ren et al 2007, Gunatilaka et al 2001), especially beta blockers (Murdoch 2015, Maszkowska et al 2014, Sun et al 2013, Steinbach et al 2014, Zuriaga et al 2014, Huschek et al 2004, Hirsh et al 1996, Cleuvers 2003 and 2005, Shakya 2011, Jones et al 2002, Fent et al 2006), but also calcium channel

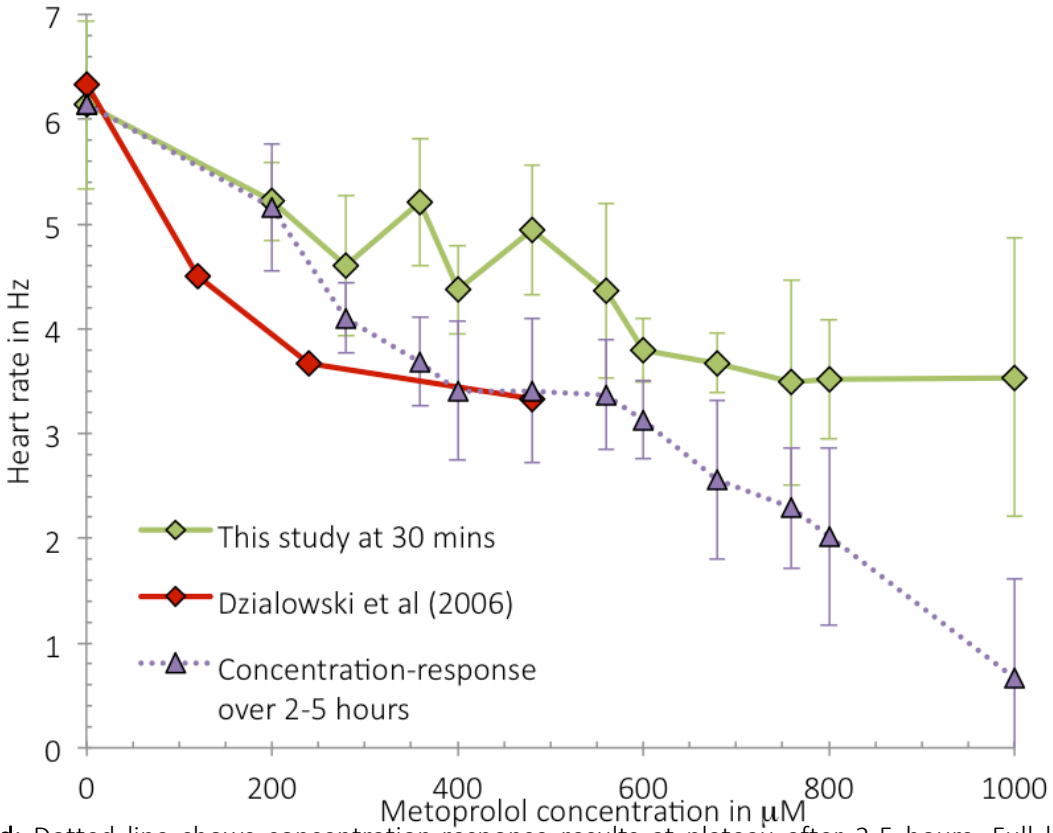
blockers (Antczak et al 2015, Kim et al 2007, Jones et al 2002), lipid lowering drugs (Cavalucci 2006, Miao and Metcalfe 2003 a & b, Dussault et al 2008), anti-platelet agents (Li 2014, Samuel and Teo 2002, Lopez-Serna et al 2012, Fent et al 2006, Jones et al 2002). However, surprisingly, beyond fatal *Daphnia* LD50s (Czech et al 2014, Huggett et al 2002, Richard et al 2014, Moermond & Smit 2015, Shakya 2011, Cleuvers 2005, Huggett et al 2002,Kim et al 2007, Gómez-Canela et al 2014, Bang et al 2015, Russo 1995, Henschel et al 1997, Villegas-Navarro et al 2003, Overturf et al 2015, Bengtsson et al 2004, Lilius et al 1995, Lopes et al 2006, Andersen et al 2006, Fernadez-Casalderrey et al 1994, Pavlaki et al 2014, Pestana et al 2010, Barata et al 2006, Pereira and Goncalves 2007, Carvalho et al 2003, Guilhermino et al 1996, Ren et al 2007, Guilhermino et al 1996 and 2000, Pereira et al 2006, Sancho et al 2009, Villarroel et al 1998) very little is published on *Daphnia* physiological interactions with cardio therapeutics (Dietrich et al 2010, Rivetti et al 2015, Stanley et al 2006, Oliveira et al 2015, Dierkes et al 2004, Wolfe et al 2015, Bang et al 2015 a&b, Marques 2011, Weiss et al 2015, Barrozo et al 2015, Furuhagen et al 2014, Yost 2004, Overturf et al 2015). and still is less regarding *Daphnia* cardiac interactions with the same (Dietrich et al 2010, Villegas-Navarro et al 2003, Dzialowski et al 2005, Bekker and Krijgsman 1950, Gaikwad et al 2012). A little more data can be found on insecticides with effect cardiac receptor mechanisms, but again much of the focus is on *Daphnia* fatal LD50s or physiology outside of cardiac interactions (Coors et al 2004, Weiss et al 2012, Barry 2001, Andersen et al 2006, Guilhermino et al 1996, Reynaldi et al 2004, Rider and LeBlanc 2006, Vesela et al 2008) and still less literature focuses on *Daphnia* heart response to insecticides (Bekker and Krijgsman 1957, Kaas et al 2009, Carlson 1922).

The cardiac drugs this research exposed *Daphnia* to, in chapters four and five, were verapamil, metoprolol, octopamine, phenylephrine and tyramine. We found no papers which discuss *Daphnia* cardiac response to phenylephrine, octopamine nor tyramine. One paper, mentioned in chapter one, looked at *Daphnia* heart interactions with metoprolol. Dzialowski et al (2006) exposed cohorts of 10 *Daphnia* to one of three concentrations of metoprolol or a control, for 30 minutes. After exposure heart rate was filmed using an Nikon 2000 inverted microscope and Matrox video capture card at 30fps. Heart rate was counted by eye by frame-by-frames for 5 seconds of each video. Dzialowski et al's (2006) *Daphnia* were cultured *ex situ*, grown in 300ml beakers of dH20, so we would expect them to have a much stronger response to metoprolol than our own *in situ* subjects

given they would not be accustomed to any natural variations. Also, they were kept at 25°C, and filmed at this temperature, which is incredibly warm for a *Daphnia,* so we would expect starting heart rates to be higher. *Daphnia* were 4 days old and pregnancy status was not checked for, so responses may have been variable, but standard deviations are not clear in the presented figures. Heart rate counts were by eye rather than by image analysis with parabolic peak interpolation, and may not show true variation or may be open to bias. Only 10 *Daphnia* were used per separate concentration, where as our own study used 30 *Daphnia* per concentration.

Exacting heart rate results are not stated in Dzialowski et al (2006) but given as a generalised histogram from which we can only approximate what their results might have been. These appear to be roughly 380bpm (6.33Hz), 270bpm (4.5Hz), 220bpm (3.66Hz) and 200bpm (3.33Hz) for 0μM, 120μM, 240μM and 480μM respectively. It is not possible to tell what the standard deviations are from the figures given. Their results cannot be compared directly to our work in chapter 5 as Dzialowski et al (2006) take only one reading at 30 minutes while we took readings on every individual repeatedly so that each was filmed once every 15 minutes over five hours, we then took plateau results from this to formulate a concentration response curve from plateaus that occurred between two and five hours of exposure, with lower concentrations like those used by Dzialowski et al (2006) taking up to five hours to plateau. However, we can go back to the raw data and show mean data for all our own *Daphnia* at the 30 minute mark for a comparison, this is shown in figure 8a. No starting heart values were taken by Dzialowski et al (2006) for any trial animal nor for any control, but only values after half an hour were taken, their results were compared to the mean population rather than individual change.

Figure 8a. *Daphnia* exposure to Metoprolol for 30 minutes from Dzialowski et al (2006)



Legend: Dotted line shows concentration-response results at plateau after 2-5 hours. Full lines compare our raw data at 30 minutes with that of Dzialowski et al (2006). Error bars show standard deviation. Four data points given in Dzialowski et al (2006) are shown without standard deviations as it was not possible to glean this from figures given.

In figure 8a, at 400µM-500µM our own two hour plateau values and the half hour data point for Dzialowski et al's (2006) 480µM concentration appear to match up in their effect, given both data sets began with roughly the same mean control rate (≈6.8Hz) and both reach ≈3.4Hz at this concentration. However we cannot draw conclusions from this data as it is clear that Dzialowski et al's (2006) single half hour measure was not enough to give a true plateau response across all concentrations. The 80µM concentration we tested is not displayed as it did not reach a plateau even after five hours. It is therefore surprising that Dzialowski et al (2006) are able to claim a drop in heart rate of a full ≈100Hz at the 120µM mark, though this may be entirely possible given the comparatively poor culture conditions described in their paper. Our own raw data at the half hour mark show far less change in heart rate from baseline than in Dzialowski et al (2006) which again may be for that reason.

One paper, also mentioned in chapter one, looked at *Daphnia* interactions with verapamil. As we saw in chapter one, Villegas-Navarro (2003) found that verapamil has biphasic effect in the *Daphnia magna* heart. They sampled four concentrations; 100pM, 1μM, 10μM, 100μM; and a drug free control, with only 6 *Daphnia* per cohort. Heart rate accelerated for the lower three concentrations. No exact data is given in the text but heart rate for 100pM as seen in their histogram sits at around ≈330bpm (5.5Hz), with rates for 1μM and 10μM being incrementally lower but possibly still above ≈320bpm (5.33Hz). Again no starting heart rates were taken. The control animals have a mean ≈265bpm (4.42Hz) heart rate but these animals are different than the trial animals and should have been used only to ensure the trial was not causing variations that might indicate confounding factors, not as a substitute for the trial animal's heart rate at baseline, which was not declared. Animals exposed to 100μM verapamil have a ≈240bpm (4Hz) heart rate which was considered a drop in heart rate when compared to the control given we do not know the starting heart rate of the 100μM cohort *Daphnia* themselves. In Villegas-Navarro's (2003) trial *Daphnia* were kept at warmer temperatures than in their natural environment, this time 23°C, they were selected at 10 days old, which is far beyond neonate age when *Daphnia* are very likely to be pregnant. Pregnancy was not checked for. The time point at which heart rate was checked, though it is clear only one time point was used, is not stated, so we cannot compare their result to an exact time point from our own raw data. It would also not be simple to compare this data as their histogram results are very difficult to define, as well as being closely matched at three concentrations. Our own work found an EC50 plateau at 650μM after three hours, and used no lower concentrations than 200μM.

There ends discussion for all literature with which we can contextualise the area of this work which focuses on drug concentration response. A notable issue in the above papers are poor application of culture conditions. In chapter two we looked at observations in the literature to deduce best culture conditions for *Daphnia*. Papers agreed that temperature is most considered one of the most critical parameters to *Daphnia* culture (Gerritsen 1982, Barbosa et al 2014, Brooks 1949,Meester et al 2011, Shala 2013, Brzezinski et al 2010, Verbitskii and Verbitskaya 2011, Straile et al 2012), and maintenance of a natural crepuscular cycle was also necessary (Jager et al 2007, Schwind 1999, Effertz and von Elert 2014, Gerritsen 1982, Mach and Schweitzer 2007, La et al 2014, Iriji et al 1998, Rivetti et al 2015, Hansson and Hylander 2008, Jiang et al 2013, LaMontague et al 2001), as is

oxygenation (Colmorgen and Paul 1995, Pirow et al 2004, Paul et al 2004, Adamczuk et al 2013, Wiggins and Frappell 2000 and 2002, Rider and LeBlanc 2006, Purna and Nagaraju 2007, Kobayashi 1983) and proper choice and provision of food resources (Guinnee et al 2007, Porter et al 1982, Kim et al 2003, Pavlaki et al 2014, Furuhagen et al 2014, Shiny et al 2004; Pires et al 2005, Clement and Zaid 2003, Ekvali et al 2014, Hall et al 2006, Schwarzenberger et al 2013, Rohrlack et al 1999, Rohrlack et al 2004, Rohrlack and Utkilen 2007, Kurmayer 2011, Hochmuth and De Schamphelaere 2014, Juttner 1999, Ferrao-Filho et al 2013, Jeon et al 2008, Juttner et al 2010, Rohrlack et al 2001, von Elert et al 2012, Soares et al 2009, Kuster and von Elert 2012, Jiang et al 2013, DeMott et al 2009, Lurling et al 2002 and 2003, Yoshida et al 2006, Nelson et al 2005 and 2006, Weider et al 2008, Hall et al 2011, Schallenberg et al 2005, Van Geest et al 2007, Roberts et al 2007, Mohamed 2001, Jager et al 2007, Sommer et al 2003, Eskinazi-santana et al 2002, Reichwaldt et al 2004, Schaltz et al 2007, Spaak et al 2012, Choi et al 2014, Asselman et al 2015, Oliveira et al 2015, Martin-Creuzburg et al 2009, Schlechtriem et al 2006, Knillman et al 2012, Garbutt and Little 2014, Knillman et al 2012, Dolciotti et al 2014, Schwind 1999, Adamczuk et al 2013, Elendt and Storch 1990, Bengtsson et al 2004, McGaw 2004), and also monitoring environmental nutrients, especially nitrogen and phosphorous levels (Hooper et al 2008, Riessen et al 2012, Muyssen et al 2009, Van Donk and Hessen 1993, Goldman et al 1979, Warren 1907). Culture conditions were formulated based on these papers and summed at the end of chapter two. It was also important to review physical parameters, such as body size (Stabell et al 2003, Oda et al 2011) and pregnancy status, for the purposes of subject selection. A review of the effects of pregnancy on Daphnia is given in chapter two, many workers agree that for lab studies a homogenous non-pregnant neonate trial group is best (Kuwamura et al 2009, La et al 2014, Ma et al 2014, Zhang et al 2014, Allen and Lynch 2008, Nelson et al 2005, Spaak et al 2004, La et al 2014, Hebert and Finston 2000, Guan et al 2005, Weider et al 2003, Vergilino et al 2009, Young 1979, Penton et al 2004, Wolinska et al 2006, Tagg et al 2005).

Given early trials in chapter four showed that in situ *Daphnia* are far hardier and better suited to medical trials than ex situ *Daphnia*, features of the natural environments such as predators and parasites also became important to an overview of the *Daphnia* environment. Predator interactions were reviewed in chapter two, (La et al 2014, Beckerman et al 2010, Fjeld et al 1998, Kramer and Drake 2010, Gliwicz and Maszczyk 2006, Adamczuk et al 2013, coville and Pfrender 2010, Rietzler et al 2008, Otte

et al 2014, Hesse et al 2012, Weiss et al 2015, de Block et al 2013, Schwarzenberger et al 2009, Oda et al 2011, Penalva-Arana et al 2009, Stabell et al 2003, Bourdeau et al 2012, LaForsch and Tollrian 2004, Petrusek et al 2008, Otte et al 2014, Rabus et al 2013, LaForsch et al 2004, Spanier et al 2010, Asselman et al 2015, Rozenberg et al 2015, Dennis et al 2010, Miyakawa et al 2013, Herzog and LaForsch 2013, Riessen and Trevett-Smith 2009, Walsh and Post 2011 and 2012, Walsh et al 2014, Cousyn et al 2001, Barbosa et al 2014, Fey and Herren 2014, Adamczuk et al 2013, Pohnert et al 2007, Meutter et al 2004, Oien 2004, Garcia et al 2007, Freund et al 2002), as were the effects of parasites on *Daphnia* (Hesse et al 2012, Jensen et al 2006, Vale et al 2011, Duneau et al 2011, Nader et al 1999, Cavalcante et al 2000, Nader et al 1999, Graham et al 2011, Hall and Ebert 2012, Decaestecker et al 2011, Killick et al 2006, Lohr et al 2010, Civitello et al 2013, Hesse et al 2012, Duffy and Hall 2008, Duffy 2007, Hammill and Beckerman 2009, Auld et al 2014, Pauwels et al 2007, Lohr et al 2010, Wolf and Weider 1991, Lohr et al 2010, Duffy and Hall 2008, Johnson et al 2006). This became especially pertinent when, as we see in chapter four, we found that *in situ* and *ex situ* culture make a big difference to concentration response results. It was decided that all animals would be cultured in situ which made them more robust but meant that predator and parasite monitoring became part of the lab protocol.

In chapter four we also decided on methods for *Daphnia* immobilisation via our own experiments and by looking to the literature for other methods (Colmorgen and Paul 1995, Penalva-Arana et al 2008, Kaas et al 2009, Campbell et al 2004). We decided on the types of cardiovascular drugs that would be tested, and made initial experiments using drugs with negative chronotropic effect. A number of observations came from these initial trials. The first was that the cumulative dose response method does not accurately predict EC50, which agrees with findings by Dunne (1979), and we decided to use the individual concentration method for ongoing work in chapter five. Also, we found that *Daphnia* response to negatively chronotropic drugs was so slow as to require hours of lab observation producing a low n value of *Daphnia* samples, so positive chronotropic drugs were chosen for chapter 5. A serendipitous observation also occurred in this early experimental chapter in which dysrhythmias were observed. This led to further literature searches into how dysrhythmias are quantified (Chatfield 1989, Hsu et al 2012, Rosen et al 2015, Oestreicher 2007) and laid the foundations for the idea behind the chapter six study.

In chapter three general methods are laid out. Here we take the reader through the stages which follow *Daphnia* culture, these being to choose which *Daphnia* to trial, view the *Daphnia*, capture raw data, perform lab protocols, then prepare and analyse the data. Integral in this chapter were works on Parabolic Peak Interpolation (Smith 2011), sampling (Nyquist 2002) , guidance on animal treatment in trials (Curtis et al 2013) and statistical guidance (McDonald 2009, Sullivan and Feinn 2012, Grubbs 1969, Zaiontz 2018). Towards methods for the pivotal study in chapter six, the general methods chapter three also interpreted algorithms for the quantification of heart rate variation for mathematical application in the thesis (Climent et al 2009, Porta et al 2006, Guzik et al 2006, Karmaker et al 2009, Ehler et al 1998, Gong et al 2015, Wessel et al 2010).

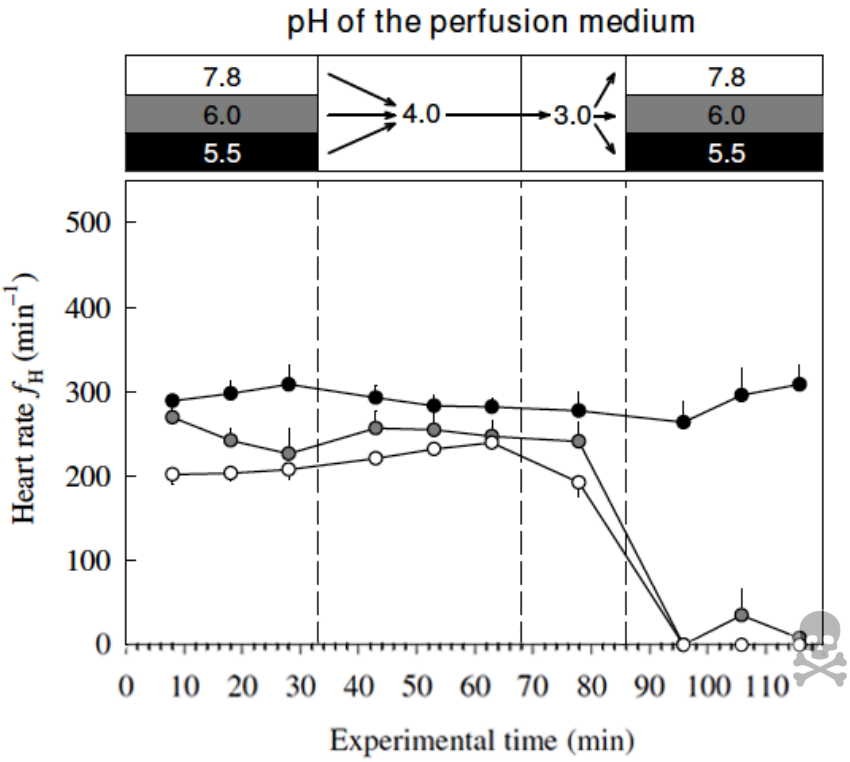
In chapter five, following concentration response trials with high standard deviations and adverse observed secondary effects, we investigated whether lab parameters might confound concentration response results. They key finding was that small changes in pH produced more profound changes in *Daphnia* heart rate. pH changes were outside of the buffer's capacity to stabilise pH. We could not satisfactorily answer whether Daphnia change the pH of the solution they are placed in, as such small amounts are used to immobilise the *Daphnia* that the pH probe would not work. And, scaling the experiment up was open to question as it is unknown whether effects would scale in a linear manner. Various papers suggested that over 3.5mM MOPs buffer itself was toxic to *Daphnia* (Will et al 2011, De Schamphelaere et al 2003). Indeed, many buffers have toxic effects on *Daphnia* at pH stabilisation relevant levels, these include PBS (Lane et al 1999) and ascorbic acid (Eaton Technologies GMBH 2012, Jusadi et al 2008, Olmez-Hanci et al 2014). No trials have been done to test whether the various versions of APW are also of detrimental effect to *Daphnia*. However, concentrations of MOPs below 3.5mM were not enough to stabilise even dH20 to a level which would prevent *Daphnia* from responding with extreme heart rate changes. Additional buffer would be required to stabilise drugs such as phenylephrine. In our trial *Daphnia* heart rate increased ≈15% with only a 0.05pH change from baseline.

We know from the review in chapter two of other environmental factors which have an impact on heart rate. Heart rate increases with increasing temperature (Lari et al 2017), and with decreasing oxygen levels (Paul et al 1997 and 1998, Pirow and Buchen 2003,

Baumer et al 2002) Even body size and heart rate are positively correlated (Baumer et al 2002). However, there are no detailed studies on the effects of small changes in pH on *Daphnia* heart rate.

Weber and Pirow (2009) measured *Daphnia* response to three pH values, pH5.5, 6.0 and 7.8 which gave heart rates as 299 ±12, 246±18 and 205 246±10bpm respectively using a sample size of 3 *Daphnia* per pH. pH values were established using 5mM MES buffer or 5mM citrate and were said to vary by 0.05pH units. The authors state there was high variation in response between individuals, but details are not included – this may be due to the 0.05pH variance. *Daphnia* were starved for 6 hours, then subjected to one of the three trial pH values for 33 minutes, then to pH4 for 35 minutes, pH3 for 18 minutes and then the trial pH once again for 34 minutes before a video was made, and the resulting film processed by counting frames by eye for 5 seconds of 30fps video. The reasoning for this bizarre series of highly specifically times pH values is not given. Therefore, what we have in terms of data are the mean response of three *Daphnia* to three pH values, with additional, highly acidic, values interspersed between them, in the final two of eight hours of seemingly rather stressful environmental exposure (starvation> trial pH> acid> stronger acid> trial pH). We might think there is no wonder responses were highly variant. The authors claim that the three *Daphnia* in the pH7.8 treatment are 'controls' and therefore end pH measurements mean that the lowest pH 5.5 causes an 'increase' in heart rate, while pH 6.0 is similar to the control. No real numbers are given in the text, however, simply looking at the figure from their own text reproduced in figure 8b refutes this. It is clear that Daphnia in the pH 7.8 and pH6.0 cohort either die or come very close to death. This is not a 'change in heart rate', death is an acute toxic effect. What the figure in fact shows us is that the Daphnia in the pH 5.5 cohort maintained a relatively stable heart rate compared to the other two cohorts, possibly because the bizarre interim pH exposures of 4.0 and 3.0 were not as different to 5.5 as they were to 6.0 and certainly to 7.8 where all Daphnia ended the trial at 0bpm, which is death. The only thing that is very surprising in this figure is that animals moved from the test pH to pH4.0 did not respond with a dramatic increase in heart rate, but this may be because no measurements were taken for a full twenty minutes so a lot of possible information is missing, and as we can relate from our own experiment, *Daphnia* heart rate is less responsive to pH moving toward acid than it is when moved towards basic.

Figure 8b.*Daphnia* response to pH change from Weber and Pirow (2009).



Given such extreme individual variation and confounding environmental effects, we took the research in a new direction. Chapter six began with a description of the chosen drug and a background of previous studies which use it (Curtis 2013, Loncar-Turukalo et al 2015, Vejpongsa and Yeh 2014, Vejpongsa et al 2014, Chatterjee et al 2010, Postma et al 2002, Hershman et al 2008). The choice of drug itself is of no great import since the focus of chapter six is the application of mathematical algorithms to heart rate variation, and whether certain algorithms can produce scores which might correlate with individual response to any cardiac insult. It is simply for convenience and reasons discussed in the chapter that doxorubicin was the chosen method of creating that cardiac insult.

Given that chapter six is the first made after darkfield access was available, it meant that the experimental protocol could be improved to the application of semi-automated image capture for detailed examination of frame by frame heart area approximation. To this end we gave background on various published studies of the invertebrate heart using video methods (Bownik et al 2016, Bernard et al 1993, Campbell et al 2004, Ubbulekshmi et al 2016, Periyanayagam and Karthikeyan 2013, Periyanayagam et al 2015, Choma et al 2006, Santalla et al 2016), and gave reasoning behind our choice of film timings based on timings of real world atrial fibrillation diagnosis (Gong et al 2015). After this, we gave background

to the various scoring systems for heart rate variation published in the literature. These included qualitative visual methods of examining delay plots of beat-to-beat variation (Takahashi et al 1981,Mohebbi and Ghassemian 2012, Hindricks et al 2010, Yan et al 2006, Grossman and Beek 1990, Farrell et al 1991, Dreifus et al 1993, Milovanovic et al 2009, Moise et al 2010, Hayano et al 2012, Nikillus et al 2007, Climent et al 2009, Park et al 2009). Linear quantification methods such as the ellipse fitting technique (Mohebbi and Ghassemian 2012, Kubickova et al 2016, Huikuri et al 1996, Rydberg et al 2007, Thuraisingham 2006, Chua et al 2008, Valentin et al 2004, Huikuri et al 1996, Hirose et al 1998), the Standard Deviation of Successive Differences (Galland et al 2006, Hindricks et al 2010, Thuraisingham 2006, Zarim and Rhaman 2011, Chong et al 2015, Jovic and Bogunovic 2010), Guzik's Index (Guzik et al 2006), Porta's Index (Porta et al 2006), point dispersion (Park et al 2009) and segregated plot analysis (Voss et al 2012). And finally, non-linear quantification which included various non-linear adaptation of linear techniques (Karmaker et al 2009), Ehler's Index (Ehler et al 1998), the Complex Correlation Method (Karmaker et al 2009), Multi-scale Ratio Feedback Analysis (Huo et al 2014), Median Stepping Increment (Gong et al 2015) and Finite Time Growth (Wessel et al 2010). All of which are carefully laid out in the 3.6.ii, with figures where relevant, to explain the maths behind them in a clear and plain manner. As well as our own adaptations of Finite Time Growth to additional dimensions as discussed in 3.6.ii(i). and our adapted version of the Median Stepping Increment which accounts for uneven sampling rates given the application of parabolic peak interpolation (Smith 2011) in 3.5.v. As well as our adaptation of Guzik's equation to measure distance geometrically as opposed to their own more bizarre method of summing x and y axes distances in 3.6.ii(d). We then chose to certain of the algorithms for trial based on initial results in chapter 4 (in which work was done in light field) and from assertions made in the literature.

The lab protocol was very similar to previous dose response trials, and produced a dose response curve. After this, films were analysed by the new automated method and data was outputted as strings of heart beat periods. This baseline data was 'scored' by each chosen system. Excel spreadsheets were made up for each system in order to best automate each equation, with the spreadsheets for Finite Time Growth and its extra-dimensional adaptations being the most complex. Scores for each individual's baseline rhythm were correlated with their heart rate response to toxic insult using Spearman's rank correlation. Correlations were highly variable; however it was found that the better

correlations were associated with cohorts that had larger *Daphnia* to *Daphnia* score variation to begin. We discussed that perhaps the homogenising forced of subject selection for neonates and our rigorous control of culture conditions may have created an overly homogenous study population for this purpose. Homogeny is ideal when the goal is a unified response, but not ideal when attempting to differentiate individuals in a population. We found that increased baseline beat-to-beat variation was positively correlated with an individual's ability to withstand toxic insult. *Daphnia* with stable, low variation hearts were more likely to produce a stronger negative chronotropic doxorubicin treatment than those with more variation. Results were validated via leave out out analysis and cross-validation with data from Physionet, which confirmed the same trend. However, physionet data was not considered to be a strong validation as many faults, such a duplicated or 'replaced' data, were detected in the records. Attempts have been made to correlate heart rate variation analyses with various heart conditions, as described in chapter six. However, this is the first time that heart rate variation scoring algorithms have been applied to baseline heart data, and then correlated with a cardiac response to any treatment, in an attempt to find a means of predicting individual response based on individual heart rhythm. There is therefore no literature with which we can contextualise this experiment. However, our study offers an exciting background to further work in which larger population cohorts might be tested with the goal of finding the most appropriate adaptation of the algorithms for prediction of cardiac response from baseline data.

Finally, in chapter seven we looked for a mean of more efficiently capturing heart rate and heart rate variation data. We created novel hardware and wrote complementary software to this end, and we set about resolving issues of radio frequency interference (Talling 2009, Harris 1978). What we instead found was that whole *Daphnia* EMM records the rhythmic beating of the feeding appendages, which we then re-examined in the literature (Shiga et al 2002, Smirnov 2013, Dees et al 2008, Penalva-Arana et al 2007, Garbutt and Little 2014). We went ahead with a dose response test even though we were examining limb response as this has been suggested as a physiological endpoint across literature in aquatic ecotoxicology (Pirow et al 1999, Furuhagen et al 2014, Porter et al 1982, Kim et al 2003, Lari et al 2017, Bengtsson et al 2004, McWilliam & Baird 2001, Grintzalis et al 2017, Rist et al 2017). Ren at al (2015) measured limb beat movement in response to dichlorvos, which we reinterpreted for own comparison given their paper used

their own unique units rather than molar units, but a comparative study could not be drawn up as the powder-form dichlorvos brought into the lab sublimated at cold room temperature (18^{o}C) and we did not have the facilities to work with such a high boiling point compound. We also examined the literature for other *Daphnia* organs which are used in ecotoxicology and these included antennal movement (Barrozo et al 2015, Bahrndorff et al 2015) and eye movement (Lopresti et al 1972, (Frost 1974). We were able to identify antennal movement in our EMM data but this was not as repetitive or rhythmic as leg beating. We were unable to confirm or deny the signal for eye movement in EMM data. Since reports of eye movement frequency are in the leg movement frequency range, either may be being recorded. However, due to the relative size of the eye to the legs, which is perhaps 1/10th, we thought it appropriate to assume that the EMM was more likely to record the legs. EMM data also matched leg beat data given via video microscopy of the same individuals. For our dose response test we chose an insecticide which is a current hot topic in the field of ecology due to its association with honey bee colony collapse. We examined the literature regarding neonicotinoids and colony collapse (Tesovnik et al 2017, Qi et al 2018, Morrissey et al 2015, Baines et al 2017, Alkassab and Kirchner 2018, Woodcock et al 2018, Wood et al 2018, Lentola et al 2017, Samson-Robert et al 2017, Balfour et al 2017, Stanley and Raine 2017, Forfert et al 2017, Baron et al 2017, Brandt et al 2017, Wood et al 2018, Phelps et al 2017, Tosi et al 2017, Tosi and Nieh 2017, Potts et al 2018, Chretien et al 2017) and looked for any papers which gave *Daphnia* response to the neonicotinoids (Beketov and Liess 2008, Hayasaka et al 2012, Tisler et al 2009, Qi et al 2018). Once a dose response curve was found it was compared to work by Zein et al (2014) and found our study concurred with their findings. Limb movement rate decreased dose dependently with imidacloprid concentration. We also compared our methods to those seen in the literature (Penalva-Arana et al 2007) such as it is. Video methods pre-dominate, while the use of electromechanical signals was found in only one paper (Freund et al 2002). We concluded that further study should be done to better isolate *Daphnia* signals from radio frequency interference, and that doing so would offer a more efficient means of collecting limb beat data for use in ecotoxicology.

We summarise with the following brief conclusions pertaining to our three contributions to knowledge:

1. In our first contribution to knowledge, we found that *Daphnia* are an inappropriate model for cardiovascular therapeutic dose-response trials. To do this we:

- Wrote programs in excel for performance of parabolic peak interpolation
- Created a semi-automated method of image capture for light field film
- Performed concentration-response trials for several cardio-therapeutics
- Performed trials to test the effect and stability of pH buffers

We found that:

- *Daphnia* are highly sensitive to micro level changes in their environment. Heart rate varies as much as 45% within 0.5 of a pH change. Their response to microlevel change is so extreme pH cannot be maintained within a narrow enough spectrum to prevent this confounding factor from occurring. This might explain the high standard deviation in response given to all drugs and led us to believe that *Daphnia* are not appropriate for concentration response trials where accurate and reliable results are required for interventions that may well affect human lives.
- *Daphnia* response to all cardio active drugs tested was highly variable with high standard deviations throughout.
- The cumulative concentration technique cannot accurately predict *Daphnia* response to individual concentrations.
- Cardio decelerative drugs verapamil and metoprolol had such a weak effect on *Daphnia*, it took between two to five hours for a plateau to be reached even at high concentrations.
- Phenylephrine produced a mildly stronger positive chronotropic response than octopamine, while tyramine has no negligible effect at matching concentrations.
- Phenylephrine produces deleterious secondary effects, such as gastro intestinal spasms and abortion. Late stage foetuses are less likely to be aborted than blastuae and undifferentiated foetuses, this may reflect energy requirements of the mother.

The initial research question was invalidated by this finding so a new direction was taken in the form of analysis of beat-to-beat variation and its relation to heart rate response in individual *Daphnia*. This removed the problem of population variation and instead focused on why individual *Daphnia* within a population might have differing responses to a drug. We found that *Daphnia* are highly sensitive environmental pH, however, further work might look at other parameters of the *Daphnia* lab environment. We know from other work that Daphnia are very sensitive to temperature, but there is also changes of light to consider, especially when placed under the microscope. Further to this, many recipes

for *Daphnia* Culture Medium are published, but we know of no studies that compare and investigate the optimum recipe. Also, *Daphnia* are trapped in a small volume of water. We know that controls tend to maintain a regular heart rate for the first hour but become variable thereafter, a further study would investigate why this happens.

2. In our second contribution to knowledge, the finding that baseline beat-to-beat rhythm stability is negatively correlated with susceptibility to cardiac intervention via a toxin, we:

- Reviewed all scoring systems for the differentiation of dysrhythmias.
- Worked out how equations are performed in practical terms in 3.6.
- Identified the most useful systems in 4.5 and carried them forward in chapter 6.
- Chose a drug based on earlier experimentation [4.5] as it causes rapid change so that animals could be trialed in a somewhat high throughput fashion and performed a concentration-response trial.
- We were able to use Dark field. This allowed us to develop a semi- automated method of image capture saving a great deal of hands-on time in image processing.
- Wrote fourteen programs in Excel to speed the processes of performing equations for Ehler's, Guxik's and Porta's Indices and the 'cloud' adaptations of the same, and also for multi-scale ratio feedback analysis, complex correlation measure, median stepping increment, ellipse fitting technique, standard deviation of successive differences, and Finite Time Growth for 9 dimensions as well as our own adaptations of this equation in eleven and thirteen dimensions. These fourteen programs were used to score beat-to-beat variation baseline data in all 152 trial animals.
- Correlated baseline scores produced via each system with paired cardiac response to drug exposure for each animal in each concentration cohort.
- Identified the most successful scoring systems in terms of this correlation.
- Identified the limitations and explored what could be done to improve correlations.
- Validated findings using data from Physionet.

We found that:

- The more stable the *Daphnia* heart is to begin the less it is able to withstand toxic effect. A more variant baseline rhythm is correlated with stronger ability to maintain a normal heartrate when challenged with doxorubicin.

- The main limitation is population diversity to begin. Without some natural population variation to begin no scoring system does well in differentiating individual baseline data. We suggested this could be resolved by selecting from all *Daphnia* age groups.
- The least successful scoring systems were Ehler's Index, Porta's and Guzik's Cloud and Multi-scale Ratio Feedback Analysis.
- The most successful systems were Finite Time Growth, Ehler's Cloud, Complex Correlation Measure and Median Stepping Increment. However, even these were less successful in cohorts that displayed less intra-population diversity to begin.
- K-fold validation of the most significant results, where baseline results from adapted Finite Time Growth are strongly correlated with response in the most diverse cohort, agreed with our original finding.
- We validated the main finding using data from Physionet. However, Physionet data validation is given with some hesitation as the veracity of the data was highly questionable due to multiple duplications.

This contribution to knowledge connected a feature of individual *Daphnia* hearts (heart rate variation) with *Daphnia* response. It shows that any research using *Daphnia* in heart science would require multi-dimensional analysis. We cannot simply expose animals to drug and expect a reliable heart rate response to occur. By analysing the heart rate variation in individuals to begin, and correlating this with response, as well as testing multiple concentrations, we may be able to form better predictive correlations.

3. In our third contribution to knowledge, a novel technique for electrically measuring *Daphnia* electromechanical movement which captured thoracopod signals, we:

- Designed and built practical physical adaptations to the Powerlab-connected system which allowed us to EMM record signals from whole, live *Daphnia*.
- Identified and, where possible, resolved real-time RFI issues in lab using hardware solutions such as the external resistor capacitor and shielding, as well as liaising with researchers and staff to seek possible alternatives.
- Developed an adapted Matlab program to not only measure an average power spectrum, but additionally to apply thresholds to detect and remove impulsive events from the data after the recordings were made.

We found that:

- EMM records the movement of the feeding limbs, not the heart, and that heart and limb movement are not correlated.
- Limb movement as recorded by EMM can be applied to the formation of dose-response curves using water-soluble compounds.

Potential exists for a reliable real-time EMM system for toxicological assays using aquatic invertebrates. EMM may well enhance current understanding of emerging contaminants and serve as a novel water quality screening tool.

Further work would seek to further correlate scoring systems with more robust data, perhaps from up-to-date ECG work. Physionet was found not to be an ideal source for this so further work would aim to source or create other bodies of data. We would also seek to automate programs for each algorithm by learning and application of more complex computer languages. Further work might also focus on developing a fully screened EMM device, in which amplifier and probes might be connected as a single unit as opposed to having resistor and capacitor externally attached. Although *Daphnia* have proven unusable as a model for concentration response drug trials, they may in future become a highly useful model for personalised medicine, and also for environmental toxicology, via the use of EMM monitoring.

References

References: Publication Database URLs

Athens open-access	https://www.openathens.net/
EMBL-EBI	https://www.ebi.ac.uk/
IEEE	https://ieeexplore.ieee.org/Xplore/home.jsp
Google Scholar	https://scholar.google.co.uk/
Mendeley	https://www.mendeley.com/research-network/community
NCBI Mesh	https://www.ncbi.nlm.nih.gov/mesh
Research Gate	https://www.researchgate.net/
Science Direct	https://www.sciencedirect.com/
Scopus	https://www.scopus.com/
Springer	https://www.springer.com/
Wiley online library	https://onlinelibrary.wiley.com/

References: Physiological Database URLs

Interbeat (RR) Interval Databases https://www.physionet.org/physiobank/database/#rr

Spontaneous Ventricular Tachyarrythmia Database https://www.physionet.org/physiobank/database/mvtdb/

The CAST RR Interval Sub-Study Database https://www.physionet.org/physiobank/database/crisdb/

References: Guides URLs

Drug Bank	https://www.drugbank.ca/drugs/
Guide to Pharmacology	https://www.guidetopharmacology.org/targets.jsp

References: A

Abramochkin D. V. et al (2010) Bioelectrical activity in the heart of the lugworm *Arenicola marina*. J Comp Physiol 180:5 645-651.

Adamczuk M. and T. Mieczan (2013) Spatial distribution of brood-bearing females of limnetic species of Cladocera. C R Biol 336:9 457-465.

Agatz A. et al (2013) Feeding inhibition explains effects of imidacloprid on the growth maturation reproduction and survival of Daphnia magna. Environ Sci Technol 47:6 2909-2917.

Agnisola C. et al (2003) The Modulatory Effects of Noradrenaline on Vagal Control of Heart Rate in the Dogfish, *Squalus acanthias.* Physiological and Biochemical Zoology 76:3 310-320.

Akaike N. et al (1981) Actions of verapamil, diltiazem and other divalent cations on the calcium-current of Helix neurones. Br. J. Pharmac. 74 87-95.

Allen D. E. and M. Lynch (2008) Both costs and benefits of sex correlate with relative frequency of asexual reproduction in cyclically parthenogenic *D. pulicaria* populations. Genetics 179:3 1497-1502.

Allodi S. et al (2012) Chaoborus and Gasterosteus Anti-Predator Responses in Daphnia pulex Are Mediated by Independent Cholinergic and Gabaergic Neuronal Signals. PLoS One 7:5 e36879.

Alohan F. I. (1991) Dependence upon external calcium for contractile activity in two molluscan proboscis muscles. Camp. Biochem. Physiol. 98:213 369-376.

Andersen T. H. et al (2006) Acute and chronic effects of pulse exposure of Daphnia magna to dimethoate and pirimicarb. Environ Toxicol Chem. 25:5 1187-95.

Anderson M. (1973) Neuromuscular Systems in Neurogenic Arthropod Hearts. American Zoology 13: 291-298.

Aoki K. et al (2008) Monoaminergic modulation of the Na+-activated K+ channel in Kenyon cells isolated from the mushroom body of cricket Gryllus bimaculatus. J Neurophysiol 100:3 1211-1222.

Araújo N. et al (2008) Oxamniquine, praziquantel and lovastatin association in the experimental Schistosomiasis mansoni. Memorias Instute Oswaldo Cruz 103: 450-454.

Artells E. et al (2013) Exposure to cerium dioxide nanoparticles differently affect swimming performance and survival in two daphnid species. PLoS One. 8:8 e71260.

Ashrafi K. (2006) Mapping out starvation responses. Cell Metab 3:4 235-236.

Ask J. A. and Stene-Larsen G. (1984) Functional alpha 1 adrenoceptors in the rat heart during preceptor blockade. Acta Physiol Scand 120 7-13.

Asselman J. et al (2015) Global cytosine methylation in Daphnia magna depends on genotype environment and their interaction. Environ Toxicol Chem 34:5 1056-1061.

Augustine G.J.et al (1985) Calcium entry into voltage-clamped presynaptic terminals of squid. J. Ph. 367 143-162.

Auld S. K. et al (2014) Predators and patterns of within-host growth can mediate both among-host competition and evolution of transmission potential of parasites. Am Nat 184:1 77-90.

Ayalon A. et al (2011) Immediate topical drug delivery by natural submicron injectors. Int J Pharm 419:1 147-153.

References: B

Bahrndorff S. et al (2015) Automated swimming activity monitor for examining temporal patterns of toxicant effects on individual *Daphnia magna*. J. Appl. Toxicol 3212, 7 pages.

Baines R. A. and Bacon J. P. (1994) Pharmacological Analysis of the Cholinergic Input to the Locust VPLI Neuron From an Extraocular Photoreceptor System. Journal of Neurophysiology 72:6 2864-2875.

Bang S. H. et al (2015) Proteomic analysis of Daphnia magna exposed to caffeine, ibuprofen, aspirin and tetracycline. Toxicology and Environmental Health Sciences. 7:2 97-104.

Barahona M. and S. Sanchez-Fortun (2007) Protective effect induced by atropine carbamates and 2-pyridine aldoxime methoiodide Artemia salina larvae exposed to fonofos and phosphamidon. Ecotox Env Saf 66:1 65-73.

Barata C. et al (2006) Toxicity of binary mixtures of metals and pyrethroid insecticides to Daphnia magna Straus. Implications for multi-substance risks assessment. Aquat Toxicol 78:1 1-14.

Barbosa M. et al (2014) Predation life history responses to increased temperature variability. PLoS One. 24:9 9.

Barnes M. and Barnes H. (1982) The total ascorbic acid content and its variation with seasonal and nutritional factors in some common cirripedes. J. Exp. Mar. Biol. Ecol. 60 261-273.

Barnes P. J. (2006) Drugs for asthma. Br J Pharmacol 147 Suppl 1: S297-303.

Baron G. L. (2017) General and species-specific impacts of a neonicotinoid insecticide on the ovary development and feeding of wild bumblebee queens. Proceedings of the Royal Society B 284: 1854.

Barrozo E. R. et al (2015) Exposure to D2-like dopamine receptor agonists inhibits swimming in Daphnia magna. Pharmacol Biochem Behav. 137 101-9.

Barry M. J. (2002) Progress toward Understanding the Neurophysiological Basis of Predator-Induced Morphology in Daphnia pulex. Physiological and Biochemical Zoology 75:2 179-186.

Baumer C. et al (2002) Circulatory oxygen transport in the water flea Daphnia magna. J Com Phys 172:4 275-285.

Bautista-Ortega J. et al (2013) Effects of arginine and antioxidant vitamins on pulmonary artery reactivity to phenylephrine in the broiler chicken. Poult Sci 92:4 1062-1072.

Beckerman A. P. et al (2010) The reaction norm of size and age at maturity under multiple predator risk. J Anim Ecol 79:5 1069-1076.

Bekker J. M. and Krijgsman (1951) Physiological investigations into the heart function of *Daphnia*. J. Physiol 115 249-257.

Bellanger C. et al (2005) In vitro evidence and age-related changes for nicotinic but not muscarinic acetylcholine receptors in the central nervous system of Sepia officinalis. Neurosci Lett 387:3 162-167.

Bengtsson G. et al (2004) Reduced grazing rates in daphnia pulex caused by contaminants: implications for trophic cascades. Environmental Toxicology and Chemistry 23:11 2641-2648.

Bernard T. et al (1993) Ectoine accumulation and osmotic regulation in Brevibacterium linens. Journal of General Microbiology 139 129-136.

Bhatt S. et al (2016) Statistical power analysis of cardiovascular safety pharmacology studies in conscious rats. J Pharmacol Toxicol Methods 81: 128-135.

Bhatt l. K. et al (2005) Experimental animal models to induce cardiac arrhythmias. Indian J Pharm 37:6 348:357.

Bijelic G. et al (2005) Effects of dietary or injected organic cations on larval Drosophila melanogaster: mortality and elimination of tetraethylammonium from the hemolymph. Arch Insect Biochem Physiol 60:2 93-103.

Bollmann A. and Federico L. (2008) Introduction to Atrial Fibrillation: From Mechanisms to Treatment, *from*; Understanding Atrial Fibrillation: The Signal Processing Contribution Synthesis Lectures on Biomed Eng. 30 pages.

Bona M. D. et al (2015) Effects ofenrofloxacin, ciprofloxacin, and trimethoprim on two generations of *Daphnia magna*. Ecotoxicology and Environmental Safety 113 152-158.

Born G. and C. Patrono (2006) Antiplatelet drugs. Br J Pharmacol 147 Suppl 1: S241-251.

Boswell-Smith V. et al (2006) Phosphodiesterase inhibitors. Br J Pharmacol 147 Suppl 1: S252-257.

Botha T. L. et al (2016) Adsorption uptake and distribution of gold nanoparticles in Daphnia magna following long term exposure. Aquat Toxicol 170: 104-111.

Bouchnak R. and C. E. W. Steinberg (2010) Modulation of longevity in D. magna by food quality and simultaneous exposure to dissolved humic substances. Limnologica: Ecology and Management of Inland Waters 40:2 86-91.

Bowery N.G. and T.G. Smart (2006) GABA and glycine as neurotransmitters: a brief history. Br J Ph 147:1 109-119.

Bowman W. C. (2006) Neuromuscular block. Br J Pharmacol 147 Suppl 1: S277-286.

Bownick A. (2016) Protective Effects of Ectoine on Physiological Parameters of *Daphnia Magna* Subjected to Clove Oil-Induced Anaesthesia. Turkish Journal of Fisheries and Aquatic Sciences 16: 691-701.

Bownick A. (2015) Clove essential oil from *Eugenia caryophyllus* Induces Anesthesia, Alters Swimming Performance, Heart functioning and Decreases Survival Rate During Recovery of *Daphnia magna*. Turkish Journal of Fisheries and Aquatic Sciences 15: 157-166.

Brain S. D. and H. M. Cox (2006) Neuropeptides and their receptors: innovative science providing novel therapeutic targets. Br J Pharmacol 147 Suppl 1: S202-211.

Bralet J. et al (1985) Effect of a-adrenoceptor antagonists (phentolamine, nicergoline, prazosin) on reperfusion arrhythmias and noradrenaline release in perfused rat heart. Br. J. Pharmac. 84 9-18.

Brandt A. et al (2017) Immunosuppression in Honeybee Queens by the Neonicotinoids Thiacloprid and Clothianidin. Sci Rep. 7:1 46-73

Brettell L. and Martin S. (2017) Oldest Varroa tolerant honey bee population provides insight into the origins of the global decline of honey bees. Scientific Reports. 7. 45953. 10.1038/srep45953.

Brezden B. L. and Gardner D. R. (1990) Non-voltage-gated calcium channels in snail heart ventricle cells. J Exp. Biol. 150 187-203.

Brezina V. et al (1994) Characterization of the Membrane Ion Currents of a Model Molluscan Muscle the Accessory Radula Closer Muscle of Aplysia californica. III. Depolarization-ActivatedCa Current Journal of Neurophysiology 71:6 2126-2139.

Brezina V. et al (1994) Enhancement of Ca Current in the Accessory Radula Closer Muscle of Aplysia californica by Neuromodulators That Potentiate Its Contractions. The Journal of Neuroscience 14:7 4393-4411.

Broadley K. J. and P. E. Penson (2006) Effects of hypoxia on the vasodilator activity of nifedipine and evidence of secondary pharmacological properties. Eur J Pharmacol 536:3 279-286.

Broadley K. J. (1970) An analysis of the coronary vascular responses to catecholamines, using a modified Langendorff heart preparation. Br. J. Pharmac. 40 617-629.

Brodde O. and Michel M. C. (1999) Adrenergic and Muscarinic Receptors in the Human Heart. The American Society for Pharmacology and Experimental Therapeutics 51:4 651-690.

Brown D. A. (2006) Acetylcholine. Br J Pharmacol 147 Suppl 1: S120-126.

Brown E. R. and S. Piscopo (2011) Ion channels in key marine invertebrates; their diversity and potential for applications in biotechnology. Biotechnol Adv 29:5 457-467.

Bruyere H. and Stith C. E. (1994) Ethyl alcohol reduces cardiac output, stroke volume, and end diastolic volume in the embryonic chick. Teratology 49 104-112.

Brzezinski T. et al (2010) The role of food quality in clonal succession in Daphnia: an experimental test. Oecologia 164:2 379-388.

Buckingham J. C. (2006) Glucocorticoids: exemplars of multi-tasking. Br J Pharmacol 147:1 S258-268.

Buhl E. et al (2008) A muscarinic cholinergic mechanism underlies activation of the central pattern generator for locust flight. J Exp Biol 211:14 2346-2357.

Bürkle GmbH (2019) Viscosity of liquids. https://www.buerkle.de/files_pdf/wissenswertes/viscosity_en.pdf last accessed 31 March 2019

Burnstock G. (2006) Purinergic signalling. Br J Pharmacol 147 Suppl 1: S172-181.

Burger A. et al (1989) Allenic cholesteryl derivatives as inhibitors of ecdysone biosynthesis. Tetrah. 45:1 155:164.

Burykin A. et al (2014) Dynamical density delay maps: simple, new method for visualising the behaviour of complex systems. BMC Medical Informatics and Decision Making 14:6 8 pages.

Butt S. J. and Pitman R. M. (2002) Modulation by 5-hydroxytryptamine of nicotinic acetylcholine responses recorded from an identified cockroach (Periplanta americana) motoneuron. Eur J Neur 15 429-438.

Buttino I. et al (2006) Giant liposomes as delivery system for ecophysiological studies in copepods. The Journal of Experimental Biology 209:5 801-809.

References: C

Caffaro P. A. et al (2012) Dissociation between memory reactivation and its behavioral expression: scopolamine interferes with memory expression without disrupting long-term storage. Neurobiol Learn Mem 98:3 235-245.

Caldwell J. (2006) Drug metabolism and pharmacogenetics: the British contribution to fields of international significance. Br J Pharmacol 147 Suppl 1: S89-99.

Cammarato A. et al (2015) Enhanced assessment of contractile dynamics in Drosophila hearts. Biotec 58:2 77-80.

Campbell A. K. et al (2004) Lactose causes heart arrhythmia in the water flea Daphnia pulex. Comp Biochem Physiol B Biochem Mol Biol 139:2 225-234.

Carroll P. R. and Cobbin L. B. (1969) Cholinergic stimulation in the heart of the mollusc Tapes watlingi. Compedium of Biochemistry and Physiology 28: 1075-1088.

Carson D. D. and Lennarz W. J. (1979) Inhibition of polyisoprenoid and glycoprotein biosynthesis causes abnormal embryonic development. Proc. Natl. Acad. Sci. USA 76:11 5709-5713.

Carvalho F. D. et al (2003) Use of atropine-treated Daphnia magna survival for detection of environmental contamination by acetylcholinesterase inhibitors. Ecotoxicology and Environmental Safety 53: 43-46.

Cavalcante M. C. M. et al (2000) Occurrence of heparin in the invertebrate *Styela plicata* (tunicata) is restricted to cell layers facing the outside environment. The Journal of Biological Chemistry. 275:46 17:36 189-196.

Chatterjee K. et al (2010) Doxorubicin cardiomyopathy. Cardiology 115:2 155-162.

Chatterjee K. and Parmley W. W. (1977) The Role of Vasodilator Therapy in Heart Failure. Progress in Cardiovascular Diseases XIX:4 25 pages.

Chatfield C. (1989) The Analysis of Time Series: An Introduction (Fourth ed.). New York, Chapman Hall.

Chen Y. H. et al (2006) Cocaine elicits action potential bursts in a central snail neuron: the role of delayed rectifying K+ current. Neuroscience 138:1 257-280.

Choi J. Y. et al (2014) Population growth of the Cladoceran Daphnia magna: a quantitative analysis of the effects of different algal food. PLoS One 9:4 e95591.

Choma M. A. et al (2006) In Vivo Imaging of the Adult Drosophila melanogaster Heart With Real-Time Optical Coherence Tomography. Circulation 114 35-36.

Chong J. W. et al (2015) Arrhythmia discrimination using a smart phone. IEEE Biomed Health 19:3 815-824.

Chua K. C. et al (2008) Computer-based analysis of cardiac state using entropies recurrence plots and Poincare geometry. J Med Eng Technol 32:4 263-272.

Chung J. S. and S. G. Webster (2008) Angiotensin-converting enzyme-like activity in crab gills and its putative role in degradation of crustacean hyperglycemic hormone. Arch Insect Biochem Physiol 68:3 171-180.

Civitello D. J. et al (2013) Parasite consumption and host interference can inhibit disease spread in dense populations. Ecol Lett 16:5 626-634.

Clement B. and S. Zaid (2004) A new protocol to measure the effects of toxicants on daphnid-algae interactions. Chemosphere 55:10 1429-1438.

Cleuvers M. (2005) Initial risk assessment for three beta-blockers found in the aquatic environment. Chemosphere 59:2 199-205.

Climent A. M. et al (2009) Poincare surface profiles of RR intervals: a novel noninvasive method for the evaluation of preferential AV nodal conduction during atrial fibrillation. IEEE Biomed Eng 56:2 433-442.

Colbourne J. K. et al (2011) The ecoresponsive genome of Daphnia pulex. Science. 331:6017 555-61.

Collins F. and Epel D. (1977) The role of calcium ions in the acrosome reaction of sea urchin sperm. Experimental Cell Research 106 211-222.

Colmorgen M. and Paul R. J. (1995) Imaging of physiological functions in transparent animals (Agonus cataphractus, Daphnia magna, Pholcus phalangioides) by video microscopy and digital image processing. Comparative Biochemistry and Physiology Part A Physiology 111:4 583-595.

Colquhoun D. (2006) Agonist-activated ion channels. Br J Pharmacol 147 Suppl 1: S17-26.

Coors A. et al (2004) Adaptation to environmental stress in Daphnia magna simultaneously exposed to a xenobiotic. Chemosphere 56:4 395-404.

Corbett A. D. et al (2006) 75 years of opioid research: the exciting but vain quest for the Holy Grail. Br J Pharmacol 147 Suppl 1: S153-162.

Corino V. D. et al (2013) Statistical Modeling of the Atrioventricular Node during Atrial Fibrillation: Data Length and Estimator Performance. Conference Paper: 35th Annual International Conference of the IEEE 2567-2571.

Corino V. D. et al (2011) An atrioventricular node model for analysis of the ventricular response during atrial fibrillation. IEEE Trans Biomed Eng 58:12 3386-3395.

Coronel R. et al (2002) Origin of Ischemia-Induced Phase 1b Ventricular Arrhythmias in Pig Hearts. Journal of the American College of Cardiology 39:1 166-177.

Cousyn C. et al (2001) Rapid local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. Proc Natl Acad Sci U S A 98:11 6256-6260.

Culligan N. and Gelperin A. (1983) One-Trial Associative Learning by an Isolated Molluscan CNS" Use of Different Chemoreceptors for Training and Testing. Brain Research 266 319-327.

Currie M. G. and Newman W. H. (1986) Evidence for alpha-l adrenergic receptor regulation of atriopeptin release from the isolated rat heart. Biochem and Biophys Comm 137:1 94-100.

Curtis M. J. et al (2013) The Lambeth Conventions (II): Guidelines for the study of animal and human ventricular and supraventricular arrhythmias. Pharmacology and Therapeutics 139 213-248.

Curtis T. M. et al (1999) Voltage-activated currents in cardiac myocytes of the blue mussel Mytilus edulis. Comparative Biochemistry and Physiology Part A 124 231-241.

Cuthbert A. W. (2006) A brief history of the British Pharmacological Society. Br J Pharmacol 147:1 2-8.

Czech et al (2014) Ecotoxicological evaluation of selected pharmaceuticals to Vibrio fischeri and Daphnia magna before and after photooxidation process. Ecotoxicology and Environmental Safety 104:1 247-253.

References: D

Damasio J. et al (2008) Combined use of Daphnia magna in situ bioassays biomarkers and biological indices to diagnose and identify environmental pressures on invertebrate communities in two Mediterranean urbanized and industrialized rivers (NE Spain) Aquat Toxicol 87:4 310-320.

David J. A. and Pitman R. M. (1995) Calcium and Potassium Currents in the Fast Coxal Depressor Motor Neuron of the Cockroach Periplaneta americana. Journal of Neurophysiology 74:5 2043-2051.

De Astrade M. B. and H. Maldonado (1999) Two Related Forms of Long-Term Habituation in the Crab Chasmagnathus Are Differentially Affected by Scopolamine. Pharmacology Biochem and Behavior 63:1 109-118.

De Block M. et al (2013) Local genetic adaptation generates latitude-specific effects of warming on predator-prey interactions. Glob Chang Biol 19:3 689-696.

De Coninck D. I. et al (2013) Interactive effects of a bacterial parasite and the insecticide carbaryl to life-history and physiology of two Daphnia magna clones differing in carbaryl sensitivity. Aquat Toxicol 130-131: 149-159.

De Meester L. et al (2011) Thermal genetic adaptation in the water flea Daphnia and its impact: an evolving metacommunity approach. Integr Comp Biol 51:5 703-718.

De Peyer J. E. et al (1982) Ca2+-Activated K+ conductance in internally perfused snail neurons is enhanced by protein phosphorylation. Proc Nat Acad Sci. USA 79 4207-4211.

De Schamphelaere K. A. C. et al (2004) Comparison of the Effect of Different pH Buffering Techniques on the Toxicity of Copper and Zinc to Daphnia Magna and Pseudokirchneriella Subcapitata. Ecotoxicology, 13 697-705.

Dedos S. G. and H. Birkenbeil (2003) Inhibition of cAMP signalling cascade-mediated Ca2+ influx by a prothoracicostatic peptide (Mas-MIP I) via dihydropyridine-sensitive Ca2+ channels in the prothoracic glands of the silkworm Bombyx mori. Insect Biochemistry and Molecular Biology 33:2 219-228.

Dees N. D. et al (2008) Patch exploitation in two dimensions: from Daphnia to simulated foragers. J Theor Biol 252:1 69-76.

Dees N. D. et al (2008) Stochastic resonance and the evolution of Daphnia foraging strategy. Phys Biol 5:4.

Dekin M. S. and Edwards C. (1983) Voltage-dependent drug blockade of L-glutamate activated channels of the crayfish. J. Physiol. 341 127-138.

Dekleva M. N. and B. B. Beleslin (1995) Neurotoxic action of some antiarrhythmic agents: comparative effects of propafenone, lidocaine and amiodarone on leech Retzius nerve cell. Camp. Biochem. Physiol. 110:2 165-169.

Delorenzi A. and H. Maldonado (1999) Memory enhancement by the angiotensinergic system in the crab Chasmagnathus is mediated by endogenous angiotensin II. Neuroscience Letters 266 1-4.

Delorenzi A. et al (1996) Angiotensin II Enhances Long-Term Memory in the Crab Chasmagnathus. Brain Res Bull 41:4 211-220.

Delorenzi A. et al (1995) Acute administration of angiotensin II improves long-term habituation in the crab Chasmagnathus. Neuroscience Letters 196 193-196.

Dembrow N. C. et al (2004) A specific synaptic pathway activates a conditional plateau potential underlying protraction phase in the Aplysia feeding pattern generator. J Neurosci 24:22 5230-5238.

Demott W. R. et al (2010) Ontogeny of digestion in Daphnia: implications for the effectiveness of algal defenses. Ecology 91:2 540–548.

Dennis S. et al (2011) Phenotypic convergence along a gradient of predation risk. Pro Bio Sc 278:1712 1687-1696.

Dermauw W. and T. Van Leeuwen (2014) The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. Insect Biochem Mol Biol 45: 89-110.

Devkota B. P. (2014) Digoxin Level. Medscape. 5 pages.

Devlin C. L. et al (2003) Muscarinic acetylcholine receptor compounds alter net Ca2+ flux and contractility in an invertebrate smooth muscle. Invert Neurosci 5:1 9-17.

Devlin C. L. et al (1999) Pharmacological identification of acetylcholine receptor subtypes in echinoderm smooth muscle (Sclerodactyla briareus) Comparative Biochemistry and Physiology Part C 25: 53-64.

Devlin C. L. and Smith P. J. S. (1995) Acetylcholine-Induced Ca2+ Flux across the Sarcolemma of an Echinoderm Smooth Muscle. Biol. Bull. 189: 207.

Dewael Y. and J. Mellefet (2002) Luminescence in ophiuroids (Echinodermata) does not share a common nervous control in all species. The Journal of Experimental Biology 205: 799–806.

Diamantino T. C. et al (2001) Lactate dehydrogenase activity as an effect criterion in toxicity tests with Daphnia magna strauss. Chemosphere 45 553-560.

Dianat M. et al (2015) Ellagic acid improved arrhythmias induced by CaCL2 in rat stress model. AJP 5:2 120-128.

Dierkes P. W. et al (2004) L-type Ca2+ channel antagonists block voltage-dependent Ca2+ channels in identified leech neurons. Brain Res 1013:2 159-167.

Dietrich S. et al (2010) Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in Daphnia magna--a multigenerational study. Chemosphere. 79:1 60-6.

Diomede L. et al (2013) Oleuropein aglycone protects transgenic C. elegans strains expressing Abeta42 by reducing plaque load and motor deficit. PLoS One 8:3 e58893.

Dionisio Pires L. M. et al (2005) Comparing grazing on lake seston by Dreissena and Daphnia: lessons for biomanipulation. Microb Ecol 50:2 242-252.

Dolciotti I. et al (2014) Competition impedes the recovery of Daphnia magna from repeated insecticide pulses. Aquat Toxicol 147: 26-31.

Dolphin A. C. (2006) A short history of voltage-gated calcium channels. Br J Pharmacol 147:1 56-62.

Dominguez G. A. et al (2015) Effects of charge and surface ligand properties of nanoparticles on oxidative stress and gene expression within the gut of Daphnia magna. Aquatic Toxicology 162: 1-9.

Douglass T. S. et al (1981) The biosynthesis, absorption, and origin of cholesterol and plant sterols in the Florida land crab. Journal of Lipid Research 22 961-971.

Dreifus L. S. et al (1993) Heart Rate Variability for Risk Stratification of Life-Threatening Arrhythmias. JACC 22:3 948-950.

Duffy M. A. (2007) Selective predation parasitism and trophic cascades in a bluegill-Daphnia-parasite system. Oecologia 153:2 453-460.

Duffy M. A. and S. R. Hall (2008) Selective predation and rapid evolution can jointly dampen effects of virulent parasites on Daphnia populations. Am Nat 171:4 499-510.

Dunne A. (1979). Comparison of individual and cumulative dose-response curves [proceedings]. British Journal of Pharmacology 67:3 491-492.

Dupont S. et al (2004) Effect of beta-adrenergic antagonists on bioluminescence control in three species of brittlestars (Echinodermata: Ophiuroidea). Toxicol Pharmacol 138:1 59-66.

Dussault E. B. et al (2008) Toxicity of human pharmaceuticals and personal care products to benthic invertebrates. Environmental Toxicology and Chemistry 27:2 425-432.

References: E

Ebert D. (2005) Ecology, Epidemiology, and Evolution of Parasitism in Daphnia. Bethesda (MD): National Center for Biotechnology Information (US). Available from: https://www.ncbi.nlm.nih.gov/books/NBK2042/

Effertz C. and E. von Elert (2014) Light intensity controls anti-predator defences in Daphnia: the suppression of life-history changes. Proc Biol Sci 281(1782): 20133250.

Ehlers C. L. et al (1998) Low Doses of Ethanol Reduce Evidence for Nonlinear Structure in Brain Activity. The Journal of Neuroscience 18:18 7474-7486.

Ekbote U. et al (2003) ACE inhibitors reduce fecundity in the mosquito Anopheles stephensi. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 134:4 593-598.

Ekbote U. and Isaac R. E. (1999) A mosquito (Anopheles stephensi) angiotensin I-converting enzyme (ACE) is induced by a blood meal and accumulates in the developing ovary. FEBS Letters 455 219-222.

Ekvall M. K. et al (2014) Linking cascading effects of fish predation and zooplankton grazing to reduced cyanobacterial biomass and toxin levels following biomanipulation. PLoS One 9:11 e112956.

Ekvall M. T. et al (2013) Three-dimensional tracking of small aquatic organisms using fluorescent nanoparticles. PLoS One. 8:11 e78498.

Eliot L. S. et al (1993) Imagine terminals of Aplysia sensory neurons demonstrates rols of enhanced Ca2+ influx in presynaptic facilitation. Nature 361: 634-638.

Ellenbroek B. and Youn J. (2016) Rodent models in neuroscience research: is it a rat race?. Disease models and mechanisms. 9:10 1079-1087.

Elwood P. (2008) Lactose and ischaemic heart disease: sweet hypothesis but nothing more! Int J Epi 37 1211-1213.

Eriksson W. A. K. et al (2014) Sucralose induces biochemical responses in Daphnia magna. PLoS One 9:4 e92771.

Ertel E. A. et al (1994) Type III w-Agatoxins: A Family of Probes for Similar Binding Sites on L- and N-Type Calcium Channels. Biochemistry 33 5098-5108.

Eskinazi-Sant A. E. M. et al (2002) On the natural diet of Daphnia laevis in the eutrophic pampulha reservoir (Belo Horizonte Minas Gerais) Brazillian Journal of Biology 62:3 445-452.

Erxleben C. and W. Rathmayer (1997) A Dihydropyridine-sensitive Voltage-dependent Calcium Channel in the Sarcolemmal Membrane of Crustacean Muscle. Journal of General Physiology 109: 313-326.

Evens R. et al (2011) Liposomes as an alternative delivery system for investigating dietary metal toxicity to Daphnia magna. Aquat Toxicol 105(3-4): 661-668.

References: F

Farrell T. G. et al (1991) risk stratification for arrhythmic events in postinfarction patients based on heart rate variability, ambulatory electrocardio variables and the signal averaged electrocardiogram. JACC 18:3 687-697.

Feiner M. et al (2014) Sublethal effects of the beta-blocker sotalol at environmentally relevant concentrations on the New Zealand mudsnail Potamopyrgus antipodarum. Environ Toxicol Chem 33:11 2510-2515.

Feng Y. and Yang Z. (2015) Clinical Application of the Heart Rate Deceleration Capacity Test to Predict Epirubicin-induced Cardiotoxicity. J Cardiovasc Pharmacol 66 371-375.

Fernandezcasalderrey A. et al (1994) Effect of Sublethal Concentrations of Pesticides on the Feeding Behaviour of Daphnia magna. Ecotoxicology and Environmental Safety 27: 82-89.

Ferrao-Filho A. S. et al (2014) Effects of Cylindrospermopsis raciborskii (cyanobacteria) on the swimming behavior of Daphnia (cladocera) Environ Toxicol Chem 33:1 223-229.

Ferreira R. P. and L. C. Salomao (2000) The ionic basis of cardiac activity in the bivalve mollusc Perna perna. Journal of Experimental Marine Biology and Ecology 249: 1-12.

Fink M. et al (2009) A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. Biotechniques 46:1 101-110.

Fiorito G. et al (1998) Scopolamine impairs memory recall in Octopus vulgaris Neuroscience Letters 253: 87-90.

Fischer C. P. and Romero L. M. (2016) The use of α- or β-blockers to ameliorate the chronic stress of captivity in the house sparrow (Passer domesticus). Conserv Physiol 4:1 13 pages.

Fjeld. E. et al (1998) Permanent impairment in the feeding behavior of grayling Thymallus thymallus exposed to methylmercury during embryogenesis. 213 247-254.

Flavahan N. A. and McGrath J. C. (1981) Alpha-adrenoceptors can mediate chronotropic responses in the rat heart. Br. J. Pharmac. 73 586-588.

Florey E. and M. Rathmayer (1978) The effects of octopamine and other amines on the heart and on neuromuscular transmission in decapod crustaceans: further evidence for a role as neurohormone. Biochem Physiol. 61C 229-237.

Flower R. J. (2006) Prostaglandins bioassay and inflammation. Br J Pharmacol 147:1 S182-192.

Foit K. et al (2012) Competition increases toxicant sensitivity and delays the recovery of two interacting populations. Aquat Toxicol 106-107: 25-31.

Forfert N. et a. (2017) Neonicotinoid pesticides can reduce honeybee colony genetic diversity. PLOS ONE 12:10 e0186109

Franks C. J. et al (2002) Ionic Basis of the Resting Membrane Potential and Action Potential in the Pharyngeal Muscle of Caenorhabditis elegans. J Neurophysiol 87 954-961.

Franks N. P. (2006) Molecular targets underlying general anaesthesia. Br J Pharmacol 147:1 72-81.

Fraysse B. and Garric J. (2005) Prediction and experimental validation of acute toxicity of beta-blockers in Ceriodaphnia dubia. Environmental Toxicology and Chemistry 24:10 2470-2476.

Frenkel L. et al (2010) Angiotensin modulates long-term memory expression but not long-term memory storage in the crab Chasmagnathus. Neurobiol Learn Mem 94:4 509-520.

Freund J. A. et al (2002) Behavioral stochastic resonance: how the noise from a Daphnia swarm enhances individual prey capture by juvenile paddlefish. J Theor Biol 214:1 71-83.

Frost B. J. (1975) Eye movements in Daphnia pulex (De Geer). J Exp Biol. 62:1 175-87.

Fryer G. (2008) Jacob Christian Schaffer FRS a versatile eighteenth-century naturalist and his remarkable pioneering researches on microscopic crustaceans. Notes and Records of the Royal Society 62:2 167-185.

Fu B. et al (2011) A reconfigurable real-time compressive-sampling camera for biological applications. PLoS One 6:10 e26306.

Furuhagen S. et al (2014) Feeding activity and xenobiotics modulate oxidative status in Daphnia magna: implications for ecotoxicological testing. Environ Sci Technol 48(21): 12886-12892.

References: G

Gadagkar S. R. and G. B. Call (2015) Computational tools for fitting the Hill equation to dose-response curves. J Pharmacol Toxicol Methods 71 68-76.

Galland B. C. et al (2006) Heart rate variability and cardiac reflexes in small for gestational age infants. J Appl Physiol (1985) 100:3 933-939.

Garbutt J. S. and T. J. Little (2014) Maternal food quantity affects offspring feeding rate in D. magna. Biol Let 10:7

Garcia R. et al (2007) Optimal foraging by zooplankton within patches: the case of Daphnia. Math Biosci 207:2 165-188.

Gerritsen J. (1982) Behavioral response of Daphnia to rate of temperature change: Possible enhancement of vertical migration. Limnol. Oceanogr. 27:2 254-261.

Gho M. and Ganetzky B. (1992) Analysis of repolarization of presynaptic motor terminals in Drosophila larvae using potassiumchannel-blocking drugs and mutations. J. exp. Biol. 170 93-111.

Ghosal K. et al (2011) Hydroxypropyl methylcellulose in drug delivery. Der Pharmacia Sinica 2:2 152-168.

Gielow M. L. et al (1995) Resolution and Pharmacological Analysis of the Voltage-Dependent Calcium Channels of Drosophila Larval Muscles. The Journal of Neuroscience 15 6085-6093.

Gliwicz Z. M. and P. Maszczyk (2007) Daphnia growth is hindered by chemical information on predation risk at high but not at low food levels. Oecologia 150:4 706-715.

Glossmann H. et al (1991) Very high affinity interaction of DPI 201-106 and BDF 8784 enantiomers with the phenylalkylamine-sensitive Ca2+ channel in Drosophila head membranes. British J Pharmacology 102: 446-452.

Gomez-Canela C. et al (2014) Occurrence elimination and risk of anticoagulant rodenticides and drugs during wastewater treatment. Environ Sci Pollut Res Int 21:11 7194-7203.

Gómez-Oliván L. M. et al (2014) DNA damage and oxidative stress induced by acetylsalicylic acid in *Daphnia magna*. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 164 21-26.

Gong Y. et al (2015) Predict Defibrillation Outcome Using Stepping Increment of Poincare Plot for Out-of-Hospital Ventricular Fibrillation Cardiac Arrest. BioMed Research Int 493472 7 pages.

Gotow T. et al. (1997). 4-Aminopyridine and L-cis-diltiazem block the cGMP-activated K+ channels closed by light in the molluscan extra-ocular photoreceptors. Brain Res. 745, 303–308.

Gouveneaux A. and J. Mallefet (2013) Physiological control of bioluminescence in a deep-sea planktonic worm Tomopteris helgolandica. J Exp Biol 216:22 4285-4289.

Grandi E. and M. M. Maleckar (2016) Anti-arrhythmic strategies for atrial fibrillation: The role of computational modeling in discovery development and optimization. Pharmacol Ther 168: 126-142.

Green A. R. (2006) Neuropharmacology of 5-hydroxytryptamine. Br J Pharmacol 147 Suppl 1: S145-152.

Greensmith D. J. et al (2010) The effects of hydrogen peroxide on intracellular calcium handling and contractility in the rat ventricular myocyte. Cell Calcium 48:6 341-351.

Grover P. B. and R. J. Miller (1983) Effects of Crepuscular Photoenvironment on Light-Induced Behavior of Daphnia. Physiology and Behavior 33: 729-732.

Grossman P. et al (1990) A comparison of three quantification methods for estimation of respiratory sinus arrhythmia. Psychophysiology 27:6 702-716.

Grubbs F. E. (1969) Procedures for Detecting Outlying Observations in Samples. Technometrics 11:1 1-21.

Guan R. and W. X. Wang (2006) Comparison between two clones of Daphnia magna: effects of multigenerational cadmium exposure on toxicity individual fitness and biokinetics. Aquat Toxicol 76(3-4): 217-229.

Guevara. M. R. et al (1981) Phase Locking, Period-Doubling Bifurcations, and Irregular Dynamics in Periodically Stimulated Cardiac Cells. Science 214:18 1350-1352.

Guilhermino L. et al (2000) In vitro and in vivo inhibition of Daphnia magna acetylcholinesterase by surfactant agents: possible implications for contamination biomonitoring. Science of the Total Environment 247: 137-141.

Guilhermino L. et al (2000) Acute toxicity test with Daphnia magna: an alternative to mammals in the prescreening of chemical toxicity? Ecotoxicol Environ Saf. 46:3 357-62.

Guilhermino L. et al (1996) Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile Daphnia magna. Chemosphere 32:4 727-738.

Guinnee M. A. et al (2007) The causes and consequences of variation in offspring size: a case study using Daphnia. J Evol Biol 20:2 577-587.

Gunatilaka A. et al (2001) The Evaluation of "Dynamic Daphnia Test" After a Decade of Use. In Biomonitors and Biomarkers as Indicators of Environmental Change 2. Environmental Science Research 56 29-58.

Guzik P. et al (2006) Heart rate asymmetry by Poincare plots of RR intervals Biomed. Tech. 51 272–5

References: H

Haaksma J. et al (2002) The Dimension of 2D and 3D Poincare Plots obtained from 24-hour ECG registrations. Computers in Cardiology 29 453-456.

Hall M. D. and D. Ebert (2012) Disentangling the influence of parasite genotype host genotype and maternal environment on different stages of bacterial infection in Daphnia magna. Proc Biol Sci 279(1741): 3176-3183.

Hall R. S. et al (2006) Inedible Producers in Food Webs: Controls on Stoichiometric Food Quality and Composition of Grazers. The American Naturalist 167:5 628-638.

Hammill E. and A. P. Beckerman (2010) Reciprocity in predator-prey interactions: exposure to defended prey and predation risk affects intermediate predator life history and morphology. Oecologia 163:1 193-202.

Han S. et al (2010) Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aqu Tox 98:3 256-264.

Hanani M. and Shaw C. (1977) A potassium contribution to the response of the barnacle photoreceptor. J. Phys. 270 151-163.

Hanger C. C. et al (1995) Measuring pulmonary microvessel diameters using video analysis. J Phys 79:2 526-532.

Hansson L. A. and S. Hylander (2009) Size-structured risk assessments govern Daphnia migration. Proc Biol Sci 276:1655 331-336.

Hardie R. C. (1991) Voltage-sensitive Potassium Channels in Drosophila Photoreceptors. The Journal of Neuroscience 17:10 3079-3095.

Harris F. J. (1978) On the Use of Windows for Harmonic Analysis with the Discrete Fourier Transform. Proceedings of the IEEE. 66 51-83.

Harrison P. K. et al (2002) Muscarinic signalling affects intracellular calcium concentration during the first cell cycle of sea urchin embryos. Cell Calcium 31:6 289-297.

Hartley P. S. et al (2016) SPARC-Dependent Cardiomyopathy in Drosophila. Circ Cardiovasc Genet.

Hasan K. M. (2015) Comparative Study of Leptin and Phentolamine Effects on Cardiovascular System. Cardiovasc Pharmacol open access 4:5 164-167.

Hawdon J. M. and B. Datu (2003) The second messenger cyclic GMP mediates activation in Ancylostoma caninum infective larvae. International Journal for Parasitology 33:8 787-793.

Hayano J. et al (2009) Circadian rhythm of atrioventricular conduction predicts long-term survival in patients with chronic atrial fibrillation. Chronobiology International 19:3 633-648.

Hebert P. D. N. and Finston T. L. (2000) Macrogeographic patterns of breeding system diversity in the Daphnia pulex group from the United States and Mexico. Heredity 87: 153-161.

Heckman J. J. (2008) Schools, Skills, And Synapses. Economic Inquiry, Western Economic Association International 46:3 289-324, 07.

Heinrich R. et al (2001) A role for muscarinic excitation: control of specific singing behavior by activation of the adenylate cyclase pathway in the brain of grasshoppers. Proc Natl Acad Sci U S A 98:17 9919-9923.

Henschel K. P. et al (1997) Environmental hazard assessment of pharmaceuticals. Regul Tox Pharm. 25:3 220-5.

Hermann A. and Gorman A. L. F. (1984) Action of Quinidine on Ionic Currents of Molluscan Pacemaker Neurons. J. Gen. Phys. 83 919-940.

Hernando M. D. et al (2004) Analysis by liquid chromatography-electrospray ionization tandem mass spectrometry and acute toxicity evaluation for beta-blockers and lipid-regulating agents in wastewater samples. J Chromatogr A. 13:1046 133-140.

Hershman D. L. et al (2008) Doxorubicin, Cardiac Risk Factors, and Cardiac Toxicity in Elderly Patients With Diffuse B-Cell Non-Hodgkin's Lymphoma 26:19 3159-3166.

Herzog Q. and LaForsch C. (2013) Modality matters for the expression of inducible defenses: introducing a concept of predator modality. BMC Biology 11:113.

Hesse O. et al (2012) Fighting parasites and predators: How to deal with multiple threats? Ecology 12:12.

Hill S. J. (2006) G-protein-coupled receptors: past present and future. Br J Pharmacol 147 Suppl 1: S27-37.

Hindricks G. et al (2010) Performance of a New Leadless Implantable Cardiac Monitor in Detecting and Quantifying Atrial Fibrillation Results of the XPECT Trial. Circ Arrhythm Electrophys. 3 141-147.

Hiripi L. et al (1998) An octopaminergic system in the CNS of the snails Lymnaea stagnalis and Helix pomatia. Philosophical Transactions of the Royal Society B: Biological Sciences 353(1375): 1621-1629.

Hochmuth J. D. and K. A. De Schamphelaere (2014) The effect of temperature on the sensitivity of Daphnia magna to cyanobacteria is genus dependent. Environ Toxicol Chem 33:10 2333-2343.

Honda H. et al (2007) Insect Muscarinic Acetylcholine Receptor: Pharmacological and Toxicological Profiles of Antagonists and Agonists. J.Agric.FoodChem. 55: 2276-2281.

Hou P. L. and Burggren W. W. (1995) Cardiac output and peripheral resistance during larval development in the anuran amphibian Xenopus laevis. The American Physiological Society 1126-1133.

Houard et al (1998) The Drosophila melanogaster-related angiotensin-I-converting enzymes Acer and Ance. Eur. J. Biochem. 257 599-606.

Hsu C. et al (2012) Poincaré plot indexes of heart rate variability detect dynamic autonomic modulation during general anesthesia induction. Acta Anaes Taiwanica 50 12:18.

Huang J. et al (2015) Characterization of the juvenile hormone pathway in the viviparous cockroach Diploptera punctata. PLoS One 10:2 e0117291.

Huikuri H. V. et al (1996) Abnormalities in Beat-to-Beat Dynamics of Heart Rate Before the Spontaneous Onset of Life-Threatening Ventricular Tachyarrhythmias in Patients With Prior Myocardial Infarction. Circ93:10 1836-1844.

Huggett D. B. et al (2002) Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch Environ Contam Toxicol. 43:2 229-35.

Huo C. et al (2014) A multi-scale feedback ratio analysis of heartbeat interval series in healthy vs. cardiac patients. Med Eng Phys 36:12 1693-1698.

Husch A. et al (2008) Functional parameters of voltage-activated Ca2+ currents from olfactory interneurons in the antennal lobe of Periplaneta americana. J Neurophysiol 99:1 320-332.

Hsieh Y. et al (2002) Voltage-dependent calcium channels in the corpora allata of the adult male loreyi leafworm, *Mythimna loreyi*. Insect Biochemistry and Molecular Biology 32 547-557.

Hsieh Y. et al (2001) Effects of Calcium Channel Antagonists on the Corpora Allata of Adult Male Loreyi Leafworm Mythimna loreyi. Archives of Insect Biochemistry and Physiology 48 89-99.

References: I

Iannaccone P. M. and Jacob H. J. (2009) Rats! Disease Models and Mechanisms 2:5 206-210.

Ignatov Y. D. et al (2005) Effects of helium-neon laser irradiation and local anesthetics on potassium channels in pond snail neurons. Neuroscience and Behavioral Physiology 35:8 876-881.

Ijiri K. et al (1998) Behavior and Reproduction of Invertebrate Animals During and After A Long-Term Microgravity: Space Experiments Using An Autonomous Biological System. Biological Sci in Space 12:4 377-388.

Ikeda T. (2004) Effects of blockers of Ca2+ channels and other ion channels on in vitro excystment of Paragonimus ohirai metacercariae induced by sodium cholate. Parasitol Res 94:5 329-331.

Ikeda T. (2006) Effects of L-type Ca2+ channel antagonists on in vitro excystment of Paragonimus ohirai metacercariae induced by sodium cholate. Parasitol Res 99:4 336-340.

Inoue S. et al (2014) Ionic channel mechanisms mediating the intrinsic excitability of Kenyon cells in the mushroom body of the cricket brain. J Insect Physiol 68: 44-57.

Ioannou C. C. (2008) The Importance of Predator Behaviour on Risk to Prey The University of Leeds Institute of Integrative and Comparative Biology. Doctor of Philosophy.

Irimia A. et al (2010) Comparison of chaotic biomagnetic field patterns recorded from the arrhythmic heart and stomach. In: Minai A. et al Unifying Themes in Complex Systems. Springer, Berlin.

Isaac R. E. et al (2007) Angiotensin-converting enzyme as a target for the development of novel insect growth regulators. Peptides 28:1 153-162.

Ishida M. and Shinozaki H. (1980) Differential effects of diltiazem on glutamate potentials and excitatory junctional potentials at the crayfish neuromuscular junction. J. Physiol. 298 301-319.

Ismail N. et al (2008) Pilocarpine improves recognition of nestmates in young honey bees. Neuroscience Letters 439:2 178-181.

Ismail N. et al (2006) Stimulation of muscarinic receptors mimics experience-dependent plasticity in the honey bee brain. Proc Natl Acad Sci USA 103:1 207-211.

Iversen L. (2006) Neurotransmitter transporters and their impact on the development of psychopharmacology. Br J Pharmacol 147:1 82-88.

Iwata M. and Nakano E. (1985) Enhancement of spicule formation and calcium uptake by monoclonal antibodies to fibronectin-binding acid polysaccharide in cultured sea urchin embryonic cells. Cell Differentiation 17 57-62.

References: J

Jager C. G. et al (2008) Transient dynamics of pelagic producer–grazer systems in a gradient of nutrients and mixing depths. Ecology 89(5) 1272-1286.

James T. N. et al (1968) Evidence for adrenergic alpha receptor depressant activity in the heart. Am J Phys 215:6.

Jemec A. et al (2007) The applicability of acetylcholinesterase and glutathione S-transferase in Daphnia magna toxicity test. Comp Biochem Physiol C Toxicol Pharmacol 144:4 303-309.

Jenkinson D. H. (2006) Potassium channels--multiplicity and challenges. Br J Pharmacol 147 Suppl 1: S63-71.

Jeon J. et al (2008) Development of a new biomonitoring method to detect the abnormal activity of Daphnia magna using automated Grid Counter device. Sci Total Environ 389(2-3): 545-556.

Jiang X. et al (2013) Maternal effects of inducible tolerance against the toxic cyanobacterium Microcystis aeruginosa in the grazer Daphnia carinata. Environ Pollut 178: 142-146.

Jo H. J. et al (2012) Acute toxicity of Ag and CuO nanoparticle suspensions against Daphnia magna: the importance of their dissolved fraction varying with preparation methods. J Hazard Mater 227-228: 301-308.

Johnson P. T. J. et al (2006) Dining on disease: how interactions between infection and environment affect predation risk. Ecology 87:8 1973-1980.

Jones O. A. et al (2005) Pharmaceuticals: a threat to drinking water? Trends Biotechnol 23:4 163-167.

Jones O. A. H., Voulvoulis N. and Lester J. N. (2002) Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Research 36:20 5013–5022.

Jong W. H. D. and Borm P. J. A. (2008) Drug delivery and nanoparticles: Applications and hazards. International Journal of Nanomedicine 3:2 133-149.

Jordan V. C. (2006) Tamoxifen (ICI46 474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 147 Suppl 1: S269-276.

Jospin M. et al (2002) The L-type voltage-dependent Ca2+ channel EGL-19 controls body wall muscle function in Caenorhabditis elegans. J Cell Biol 159:2 337-348.

Jovic A. and Bogunovic N. (2010) Random Forest-Based Classification of Heart Rate Variability Signals by Using Combinations of Linear and Nonlinear Features. In: Bamidis P.D., Pallikarakis N. (eds) XII Mediterranean Conference on Medical and Biological Engineering and Computing 2010. IFMBE Proceedings 29. Springer, Berlin.

Judge S. and B. Leitch (1999) Modulation of transmitter release from the locust forewing stretch receptor neuron by GABAergic interneurons activated via muscarinic receptors. Journal of Neurobiology: 420-432.

Julian D. et al (2005) Sodium Nitroprusside Potentiates Hydrogen-Sulfide-Induced Contractions in Body Wall Muscle From a Marine Worm. Biol Bull 209 6-10.

Juttner F. et al (2010) beta-cyclocitral a grazer defence signal unique to the cyanobacterium Microcystis. J Chem Ecol 36:12 1387-1397.

References: K

Karagueuzian H. S. et al (2013) Bifurcation theory and cardiac arrhythmias. Am J Cardiovasc Dis 3:1 11-16.

Karim A. H. M. Z. and Rhaman M. (2011) Identification of atrial fibrillation (AFIB) of heart using robust statistical tools and approximate entropy method. Licit 1:2 112-118.

Karmakar C. K. et al (2009) Complex Correlation Measure: a novel descriptor for Poincaré plot. BioMed Eng 8:17.

Karmakar C. K. et al (2009) Defining asymmetry in heart rate variability signals using a Poincare plot. Physiol Meas 30:11 1227-1240.

Kasaba T. et al (2006) Increase in intracellular Ca2+ concentration is not the only cause of lidocaine-induced cell damage in the cultured neurons of Lymnaea stagnalis. J Anesth 20:3 196-201.

Kasai Y. et al (2006) The procerebrum is necessary for odor-aversion learning in the terrestrial slug Limax valentianus. Learn Mem 13:4 482-488.

Kiessling W. (2005) Long-term relationships between ecological stability and biodiversity in Phanerozoic reefs. Nature 433(7024): 410-413.

Kikillus N. et al (2007) algorithm for identifying patients with paroxysmal atrial fibrillation without appearance on the ECG. Proceedings of the 29th Annual International Conference of the IEEE, 275-279.

Kilkenny C. et al (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8:6 412-417.

Killick S. C. et al (2006) Testing the pluralist approach to sex: the influence of environment on synergistic interactions between mutation load and parasitism in Daphnia magna. J Evol Biol 19:5 1603-1611.

Kim J. et al (2010) Molecular cloning of Daphnia magna catalase and its biomarker potential against oxidative stresses. Comp Biochem Physiol C Toxicol Pharmacol 152:3 263-269.

Kim J. H. et al (2003) Rapid bioassay for microcystin toxicity based on feeding activity of Daphnia. Bull Environ Contam Toxicol 70:5 861-867.

Kim Y. et al (2007) Aquatic toxicity of acetaminophen carbamazepine cimetidine diltiazem and six major sulfonamides and their potential ecological risks in Korea. Environ Int 33:3 370-375.

Kimura T. et al (1998) Behavioral Modulation Induced by Food Odor Aversive Conditioning and its Influence on the Olfactory Responses of an Oscillatory grain Network in the Slug Limax marginatus. Learning Mem 4:365-375.

Kirane-Amrani L. and Soltani-Mazouni N. (2012) Comparative activity of three inhibitors of the angiotensin converting enzyme on growth, development and ecdysteroid contents of the mediterranean flour moth, *Ephestia kuehniella* Zeller. African Journal of Biotechnology 11:56 11972-11977.

Kitagawa N. et al (2003) Lidocaine spray used to capture a live clinostomum parasite causing human laryngitis. American Journal of Otolaryngology 24:5 341-343.

Kits K. S. and H. D. Mansvelder (1996) Voltage gated calcium channels in molluscs: classification Ca2+ dependent inactivation modulation and functional roles. Invertebrate Neuroscience 2:1 9-34.

Kizawa Y. et al (1991) Organic Ca2+-antagonist-resistant response to FMRF-NH2 on the molluscan smooth muscle. Gen. Pharraac. 22:5 959-964.

Kjærsgaard A. et al (2015) Plasticity in behavioural responses and resistance to temperature stress in Musca domestica. Anim. Behav. 99: 123–130.

Knillmann S. et al (2012) Intraspecific competition increases toxicant effects in outdoor pond microcosms. Ecotoxicology 21:7 1857-1866.

Komukai M. et al (1985) The Effects of Several Ion Channel Blockers and Calmodulin Antagonists on Fertilization-induced Acid Release and 45Ca 2+ Uptake in Sea Urchin Eggs. Experimental Cell Research 159 463-472.

Kononenko N. L. and V. V. Zhukov (2005) Multiplicity of chemical mechanisms of regulation of muscle contractions in Lymnaea stagnalis L. Journal of Evolutionary Biochemistry and Physiology 41:1 54-62. Kosakai K. et al (2008) Octopaminergic modulation of the single Ca2+ channel currents in Kenyon cells isolated from the mushroom body of the cricket brain. J Insect Physiol 54:12 1479-1486.

Kramer A. M. and J. M. Drake (2010) Experimental demonstration of population extinction due to a predator-driven Allee effect. J Anim Ecol 79:3 633-639. 1.

Kubickova A. et al (2016) Heart rate variability analysed by Poincare plot in patients with metabolic syndrome. J Electrocardiol 49:1 23-28.

Kukkonen J. P. et al (1998) Pseudo-Noncompetitive Antagonism of M1 M3 and M5 Muscarinic Receptor-Mediated Ca2+ Mobilization by Muscarinic Antagonists. Biochem and biophys res communications 243: 41-46.

Kuster C. J. and E. Von Elert (2013) Interspecific differences between D. pulex and D. magna in tolerance to cyanobacteria with protease inhibitors. PLoS One 8:5 e62658.

Kuwamura M. et al (2009) A minimum model of prey-predator system with dormancy of predators and the paradox of enrichment. J Math Biol 58:3 459-479.

Kuwasawa K. And R. B. Hill (1997) Evidence for cholinergic inhibitory and serotonergic excitatory neuromuscular transmission in the heart of the bivalve Mercenaria mercenaria. Journal of Experimental Biology 200: 2123–2135.

References: L

La G. H. et al (2014) Mating behavior of Daphnia: impacts of predation risk food quantity and reproductive phase of females. PLoS One 9:8 e104545.

LaCoste A. et al (2001) Evidence for a form of adrenergic response to stress in the mollusc Crassostrea gigas. The Journal of Experimental Biology 204 1247-1255.

Laforsch C. et al (2004) An acoustic microscopy technique reveals hidden morphological defenses in Daphnia. Proc Natl Acad Sci USA 101(45): 15911-15914.

Laforsch C. and R. Tollrian (2004) Embryological aspects of inducible morphological defenses in Daphnia. J Morphol 262:3 701-707.

Lamango N. S. et al (1996) The endopeptidase activity and the activation by Cl− of angiotensinconverting enzyme is evolutionarily conserved: purification and properties of an angiotensin-converting enzyme from the housefly, Musca domestica. Biochem. J. 314 639-646.

Lammers W. J. E. P. et al (1990) Quantification of spatial inhomogeneity in conduction and initiation of reentrant atrial arrhythmias. The American Physiological Society. Author's pre-print.

Lane M. and Bavister B.D. (1999) Regulation of intracellular pH in bovine oocytes and cleavage stage embryos. Mol Reprod Dev 54:4 396-401.

Lapied B. et al (2001) Indoxacarb, an oxadiazine insecticide, blocks insect neuronal sodium channels. British Journal of Pharmacology 132 587-595.

Lari E. et al (2017a) Oil sands process-affected water impairs feeding by *D. magna*. Chemosphere. 175 465-472.

Lari E. et al (2017b) A novel apparatus for evaluating contaminant effects on feeding activity and heart rate in Daphnia spp. Ecotoxicology and Environmental Safety 135 381–386.

Laurent G. (1991) Evidence for voltage-activated outward currents in the neuropilar membrane of locust nonspiking local interneurons. the Journal of Neuroscience 11:6 1713-1726.

Laurent V. et al (1997) Presence and biochemical properties of a molluscan invertebrate angiotensin-converting enzyme. Regulatory Peptides 69 53-61.

Laurienti P. J. and Blankenship J. E. (1997) Serotonergic Modulation of a Voltage-Gated Calcium Current in Parapodial Swim Muscle From Aplysia brasiliana. The American Physiological Society 0022-3077/97: 1496-1502.

Laurienti P. J. and Blankenship J. E. (1996) Parapodial Swim Muscle in Aplysia Brasiliana. I. Voltage-Gated Membrane Currents in Isolated Muscle Fibers. Journal of Neurophysiology 26:3 1517-1530.

Le Bon-Jego M. et al (2006) State-dependent regulation of sensory-motor transmission: role of muscarinic receptors in sensory-motor integration in the crayfish walking system. Eur J Neurosci 23:5 1283-1300.

LeClerc C. et al (2000) Role of dihydropyridine-sensitive calcium channels in meiosis and fertilization in the bivalve molluscs Ruditapesphilippinarum and Crassostrea gigas. Biology of the Cell 92: 285-299.

Lee M. D. et al (1995) Evaluation of Subcutaneous Injection of Local Anesthetic Agents as a Method of Tick Removal. American Journal of Emergency Medicine 13 Number 1 14-16.

Lee Y. et al (1999) Cloning and Expression of a G Protein-Linked Acetylcholine Receptor from Caenorhabditis elegans. Journal of Neurochemistry 72:1 58-66.

Leffler S., Cheney P. and Tandberg D. (1993) Chemical immobilization and killing of intra-aural roaches: an in vitro comparative study. Ann Emerg Med 22:12 1795-8.

Lemeire E. et al (2008) Effect of ace inhibitors and TMOF on growth development and trypsin activity of larval Spodoptera littoralis. Arch Insect Biochem Physiol 69:4 199-208.

Lemeire E. et al (2007) Presence of angiotensin converting enzyme isoforms in larval lepidoptera (Spodoptera littoralis) Peptides 28:1 119-126.

Li F. and Z. Han (2002) Purification and characterization of acetylcholinesterase from cotton aphid (Aphis gossypii Glover) Arch Insect Biochem Physiol 51:1 37-45.

Lilius H. et al (1995) A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. Environmental Toxicology and Chemistry 14 2085-2088.

Lingle C. (1982) Different types of blockade of crustacean acetylcholine-induced currents. J. Phys. 339 419-437.

Lohr J. N. et al (2010) Prior residency does not always pay off; co-infections in Daphnia. Parasitology doi: 1017/S0031182010000296.

Lončar-Turukalo T. et al (2015) Heart rate dynamics in doxorubicin-induced cardiomyopathy. Physiol Meas 36:4 727-739.

Lopes I. et al (2007) In situ assays with tropical cladocerans to evaluate edge-of-field pesticide runoff toxicity. Chemosphere 67:11 2250-2256.

Lopresti V. et al (1973) Structure and development of neuronal connections in isogenic organisms: cellular interactions in the development of the optic lamina of *Daphnia*. Proc Natl Acad Sci USA. 70:2 433-7.

Lourdes M. A. E-L. (2004) Phylogenetic relationships among families of the order Anomopoda (Crustacea, Brandciopoda, Cladocera). Zootaxa 760 1-26

Lovern S. B. et al (2007) Behavioral and physiological changes in Daphnia magna when exposed to nanoparticle suspensions. Environmental Science and Technology 41:12 4465-4470.

Lozano V. C. et al (2001) Memory impairment introduced by cholinergic antagonists injected into the mushroom bodies of the honeybee. J Comp Phys A 187 249-254.

Lozano V. C. and M. Gauthier (1998) Effects of the Muscarinic Antagonists Atropine and Pirenzepine on Olfactory Conditioning in the Honeybee. Pharmacology Biochemistry and Behavior 59:4 903-907.

Lu Y. H. et al (2012) Comparative studies on acetylcholinesterase characteristics between the aphids, Sitobion avenae and Rhopalosiphum padi. Journal of Insect Science 13:9 1-9.

Lucas S. M. et al (2006) The role of inflammation in CNS injury and disease. Br J Pharmacol 147 Suppl 1: S232-240.

Luna T. O., Plautz S. C. and Salice C. J. (2015) Chronic Effects of 17a-Ethinylestradiol, Fluoxetine, and the Mixture on Individual and Population-Level End Points in *Daphnia magna*. Archives of Environmental Contamination Toxicology 68:4 603-611.

Lundberg J. M. et al (1984) Effects of neuropeptide Y (NPY) on mechanical activity and neurotransmission in the heart, vas deferens and urinary bladder of the guinea-pig. Acta Physiol Scand 121 325-332.

Lurling M. and Grinten E. (2003) Life-history characteristics of daphnia exposed to dissolved microcystin-lr and to the cyanobacterium Microcystis aeruginosa with and without microcystins. Env. Tox. and Chem. 22:6 1281-1287.

References: M

Ma H. et al (2010) Effect of filter-feeding fish silver carp on phytoplankton species and size distribution in surface water: A field study in water works. Journal of Environmental Sciences 22:2 161-167.

Ma H. T. et al (2001) Assessment of the role of the inositol 1 4 5-trisphosphate receptor in the activation of transient receptor potential channels and store-operated Ca2+ entry channels. J Biol Chem 276:22 18888-18896.

Ma L. et al (2010) Arrhythmia Caused by a Drosophila Tropomyosin Mutation Is Revealed Using a Novel Optical Coherence Tomography Instrument. PLoS ONE 5:12 e14348.

Ma X. et al (2014) Diversity of the Daphnia longispina species complex in Chinese lakes: a DNA taxonomy approach. Journal of Plankton Research 37:1 56-65.

Mach R. and F. Schweitzer (2007) Modeling vortex swarming in Daphnia. Bull Math Biol 69:2 539-562.

Machado T. C. et al (2015) Photocatalytic degradation of rosuvastatin: analytical studies and toxicity evaluations. Sci Total Environ 502: 571-577.

Macours N. et al (2003) Molecular evidence for the expression of angiotensin converting enzyme in hemocytes of Locusta migratoria: stimulation by bacterial lipopolysaccharide challenge. J. Insect Physiology 49:8 739-746.

MacPherson M. et al (2001) Model Organisms: New Insights Into Ion Channel and Transporter Function. L-type calcium channels regulate epithelial fluid transport in Drosophila melanogaster. American J Phys 280: 394-407.

Madeira A. C. and Suarez-Jurtz G. (1983) Contractions induced by sodium withdrawal in crab (Callinectes danae) muscle fibres. J. Phys. 338 339-353.

Maestri R. et al (2007) Nonlinear indices of heart rate variability in chronic heart failure patients: redundancy and comparative clinical value. J Cardiovasc Electrophysiol 18:4 425-433.

Majeed Z. R. et al (2013) 5-HT stimulation of heart rate in Drosophila does not act through cAMP as revealed by pharmacogenetics. J Appl Physiol (1985) 115:11 1656-1665.

Malam Y. et al (2009) Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. Trends Pharmacol Sci 30:11 592-599.

Marothu V. K. et al (2014) Photocatalysis of β-blockers – An overview. Arabian Journal of Chemistry. Marsden C. A. (2006) Dopamine: the rewarding years. Br J Pharmacol 147:1 136-144.

Martin-Creuzburg D. et al (2011) Food quality of heterotrophic bacteria for Daphnia magna: evidence for a limitation by sterols. FEMS Microbiol Ecol 76:3 592-601.

Martin-Creuzburg D. et al (2009) Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids. Proc Biol Sci 276:1663 1805-1814.

Martins J., Teles L. O. and Vasconcelos V. (2007) Assays with *Daphnia magna* and *Danio rerio* as alert systems in aquatic toxicology. Environment International 33:3 414-425.

Martinez A. (1995) Nitric oxide synthase in invertebrates. Histochemícal Journal 27 770-776.

Massarsky A. et al (2011) beta-blockers as endocrine disruptors: the potential effects of human beta-blockers on aquatic organisms. J Exp Zool A Ecol Genet Physiol 315:5 251-265.

Matthews S. B. and A. K. Campbell (2000) When sugar is not so sweet. The Lancet 355:9212 1330.

Maxwell L. C. et al (1983) Effects of Ascorbic Acid and EDTA on Vascular Concentration- Response to Catecholamines. Microvascular Research 26 81-88.

McCarthy O. et al (2015) The Effect of social isolation on locomotor activity in the houseflies (Musca Domestica). J. Insect Behav. 28: 288-296.

McDonald J. (2009) Handbook of Biological Statistics. Accessed online www.biostathandbook.com

McGaw I. J. (2005) Does feeding limit cardiovascular modulation in the Dungeness crab Cancer magister during hypoxia? The Journal of Experimental Biology 208 83-91.

McMannus D. D. et al (2016) PULSE-SMART: Pulse-Based Arrhythmia Discrimination Using a Novel Smartphone Application. J Cardiovasc Electrophysiol 27:1 51-57.

McWilliam R. A. and Baird D. J. (2002) Application of postexposure feeding depression bioassays with Daphnia magna for assessment of toxic effluents in rivers. Environmental Toxicology and Chemistry 21:7 1462-8.

Mehta D. K et al (2006), Chemo-paralysis for the removal of a live intraocular worm in ocular angiostrongyliasis. Clinical and Experimental Ophthalmology. 34 493-495.

Milligan G. and E. Kostenis (2006) Heterotrimeric G-proteins: a short history. Br J Pharmacol 147 Suppl 1: S46-55.

Mittmann B. et al (2014) Development and staging of the water flea Daphnia magna (Straus, 1820; Cladocera, Daphniidae) based on morphological landmarks. EvoDevo 5:12.

Miyahara Y. et al (1993) Effects of organic and inorganic Ca2-antagonists on acetylcholine-induced contraction in molluscan (Mytilus edulis) smooth muscle. Gen. Pharmac. 24:6 1419-142.

Miyakawa H. et al (2013) Effect of juvenoids on predator-induced polyphenism in the water flea Daphnia pulex. J Exp Zool A Ecol Genet Physiol 319:8 440-450.

Moermond C. T. A and Smit C. T. (2015) Derivation of Water Quality Standards for carbamazepine, 39 metoprolol and metformin and comparison with monitoring data. Environ Toxicol Chem 40 DOI: 10.1002/etc.3178.

Mohamed Z. A. (2001) Accumulation of cyanobacterial hepatotoxins by Daphnia in some Egyptian irrigation canals. Ecotoxicol Environ Safety 50:1 4-8.

Mohebbi M. and H. Ghassemian (2012) Prediction of paroxysmal atrial fibrillation based on non-linear analysis and spectrum and bispectrum features of the heart rate variability signal. Comp Meth Prog Biomed 105:1 40-49.

Moise N. S. et al (2010) Zone of avoidance : RR interval distribution in tachograms histograms and Poincare plots of a Boxer dog. J Vet Cardiol 12:3 191-196.

Molinaro A. M. et al (2005) Prediction error estimation: a comparison of resampling methods. Bioinformatics 21:15 3301-3307.

Moncada S. and E. Higgs (2006) The discovery of nitric oxide and its role in vascular biology. Br J Ph 147:1 193-201.

Monger D. J. (1985) Insect 3-Hydroxy-3-methylglutaryl-CoA Reductase. Early stages in isoprenoid biosynthesis: Methods in enzymology 110 51-59.

Monger D. J. et al (1982) Compactin inhibits insect HMG-CoA reductase and juvenile hormone biosynthesis. Biochemical and Biophysical Research Communications 105:4 1374-1380.

Morck C. et al (2009) Statins inhibit protein lipidation and induce the unfolded protein response in the non-sterol producing nematode Caenorhabditis elegans. Proc Natl Acad Sci USA 106:43 285-290.

Moriya H. et al (1999) Affinity profiles of various muscarinic antagonists for cloned human muscarinic acetylcholine receptor subtypes and machrs in rat heart and submandibular gland. Life Sci 64:25 2351-2358.

Mosser V. A. et al (2002) Kinetic analysis of M2 muscarinic receptor activation of Gi in Sf9 insect cell membranes. J Biol Chem 277:2 922-931.

Müller D. et al (2003) Procaine impairs learning and memory consolidation in the honeybee. Brain Research 977:1 124-127.

Murakami H. et al (1988) The relaxation induced by indole and nonindole 5-HT agonists in the molluscan smooth muscle. Camp. Biochem. Physiol. 90:1 249-255.

Murakami H. et al (1985) Effects of ca-antagonists and antispasmodic drugs on contraction by ACh in molluscan smooth muscle. Camp. Biochem. Physiol. 80:1 167-173.

Mutoh H. and M. Yoshino (2004) L-type Ca2+ channel and Ca2+-permeable nonselective cation channel as a Ca2+ conducting pathway in myocytes isolated from the cricket lateral oviduct. J Comp Physiol B 174:1 21-28.

Muyssen B. T. et al (2009) Calcium accumulation and regulation in Daphnia magna: Links with feeding growth and reproduction. Comp Biochem Physiol A Mol Integr Physiol 152:1 53-57.

References: N

Na J. et al (2013) A Drosophila Model of High Sugar Diet-Induced Cardiomyopathy. PLoS Genet 9(1): e1003175.

Nagaraju G. P. C. (2007) Is methyl farnesoate a crustacean hormone? Aquaculture 272(1-4): 39-54.

Nash M. P. and Panfilov A. V. (2004) Electromechanical model ofexcitable tissue to study reentrant cardiac arrhythmias. Progress in Biophysics and Molecular Biology 85 501-522.

Nerbonne J. M. and Gurney A. M. (1987) Blockade of Ca+ and K+ Currents in Bag Cell Neurons of Aplysia californica by Dihydropyridine Ca+ Antagonists. The Journal of Neuroscience 7:3 882-893.

Neubig R. R. et al (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on Terms and Symbols in Quantitative Pharmacology. Pharm Rev 55:4 597-607.

Nahorski S. R. (2006) Pharmacology of intracellular signalling pathways. Br J Pharmacol 147 Suppl 1: S38-45.

Neuparth T. et al (2014) Hypocholesterolaemic pharmaceutical simvastatin disrupts reproduction and population growth of the amphipod Gammarus locusta at the ng/L range. Aquat Toxicol 155: 337-347.

Nicolao M. C. et al (2014) P-glycoprotein expression and pharmacological modulation in larval stages of Echinococcus granulosus. Parasitol Int 63:1 1-8.

Nogrady T. and J. Keshmirian (1986) Rotifer neuropharmacology-ii. synergistic effect of acetylcholine on local anesthetic activity in Brachionus calyciflorus (rotifera, aschelminthes). Camp. Biochem. Pharm. 83:2 339-344.

North R. A. (2009) Introduction. British Journal of Pharmacology 147(S1): S1-S1.

Noss C. et al (2013) Three-dimensional analysis of the swimming behavior of Daphnia magna exposed to nanosized titanium dioxide. PLoS One 8(11):e80960.

Nowicka-Zagrajek J. and Wylomanska A. (2007) The autocorrelation and autocovariance functions - helpful tools in the modelling problem. Wroclaw Listopad, 30 pages.

Numata T. and M. Yoshino (2005) Characterization of single L-type Ca2+ channels in myocytes isolated from the cricket lateral oviduct. J Comp Physiol B 175:4 257-263.

Nwoga J. and Bittar E. E. (1990) An Investigation of the Sensitivity of the Ouabain-Insensitive Sodium Efflux in Single Barnacle Muscle Fibers to Pentachlorophenol. Toxicology and Applied Pharmacology 108: 330-341.

Nyquist H. (2002) Certain topics in telegraph transmission theory. Proc. IEEE 90:2 280-305.

References: O

Ocorr K. et al (2009) Semi-automated Optical Heartbeat Analysis of small hearts. J Vis Exp(31)

Oda S. et al (2011) Morphological changes in Daphnia galeata induced by a crustacean terpenoid hormone and its analog. Environ Toxicol Chem 30:1 232-238.

Oestreicher C. (2007) A history of chaos theory. Dialogues Clin Neurosci. 9:3 279-289.

Ohmori H. and Sasaki S.(1977) Development of neuromuscular transmission in larval tunicates. J.Ph. 269 221-254.

Øien A. (2004) Daphnicle dynamics based on kinetic theory: an analogue-modelling of swarming and behaviour of daphnia. Bulletin of Mathematical Biology 66:1 1-46.

Oliveira H. G. et al (2009) The effect of Buscopan on the development of the blow fly Chrysomya megacephala (F.) (Diptera: Calliphoridae) J Forensic Sci 54:1 202-206.

Olmez-Hanci T. et al (2014) Investigation of the toxicity of common oxidants used in advanced oxidation processes and their quenching agents. J Hazard Mater 278: 330-335.

Onozuka S. et al (2004) Lidocaine Increases Intracellular Sodium Concentration through Voltage-dependent Sodium Channels in an Identified Lymnaea Neuron. Anesthesiology 101 110-9.

Onozuka S. et al (2005) Lidocaine excites both pre- and postsynaptic neurons of reconstructed respiratory pattern generator in Lymnaea stagnalis. Anesth Analg 100:1 175-182.

Onozuka S. et al (2008) Lidocaine increases intracellular sodium concentration through a Na+-H+ exchanger in an identified Lymnaea neuron. Anesth Analg 106:5 1465-1472

Onizuka S. et al (2012) Lidocaine treatment during synapse reformation periods permanently inhibits NGF-induced excitation in an identified reconstructed synapse of Lymnaea stagnalis. J Anesth 26:1 45-53.

Onozuka S. et al (2012) Clinical dose of lidocaine destroys the cell membrane and induces both necrosis and apoptosis in an identified Lymnaea neuron. J Anesth 26:1 54-61.

Onozuka M. et al (1993) Lidocaine suppresses the sodium current in Euhadra neurons which is mediated by cAMP-dependent protein phosphorylation. Brain Research 628: 335-339.

Otte K. A. et al (2015) Interclonal proteomic responses to predator exposure in Daphnia magna may depend on predator composition of habitats. Mol Ecol 24:15 3901-3917.

Otte K. A. et al (2014) Proteomic analysis of Daphniamagna hints at molecular pathways involved in defensive plastic responses. BMC Genomics 15:306.

Overturf C. L. et al (2015) Toxicity of noradrenaline, a novel anti-biofouling component, to two non-target zooplankton species, Daphnia magna and Ceriodaphnia dubia. Comp Biochem Physiol C Tox Pharm. 171 49-54.

Ozawa R. et al (1995) Intracellular signal transduction of PBAN action in lepidopteran insects: inhibition of sex pheromone production by compactin, an HMG CoA reductase inhibitor. Regulatory Peptides 57 319-327.

References: P

Palikhova T. A. et al (2006) Cholinergic sensory inputs to command neurons in edible snail. Bulletin of Experimental Biology and Medicine 142:3 275-278.

Pals-Rylaarsdam R. et al (1997) Internalization of the m2 Muscarinic Acetylcholine Receptor. The Journal of Biological Chemistry 272(38): 23682–23689.

Park J. et al (2009) Atrial fibrillation detection by heart rate variability in Poincare plot. Biomed Eng Online 8: 38.

Parsons M. E. and C. R. Ganellin (2006) Histamine and its receptors. Br J Pharmacol 147 Suppl 1: S127-135.

Patel V. et al (2017) Digitalis Toxicity. Medscape. 5 pages.

Paternostro G. et al (2001) Age-Associated Cardiac Dysfunction in D. melanogaster. Circ Res 88 1053-1058.

Pauwels B. et al (2015) The influence of ruthenium on vascular tone. J Pharmacy and Pharmacol67 1263-1271.

Pauwels K. et al (2007) Evolution of heat shock protein expression in a natural population of Daphnia magna. Am Nat 170:5 800-805.

Pavlaki M. D. et al (2014) Changes of chemical chronic toxicity to Daphnia magna under different food regimes. Ecotoxicol Environ Saf 109: 48-55.

Pease J. E. and T. J. Williams (2006) The attraction of chemokines as a target for specific anti-inflammatory therapy. Br J Pharmacol 147 Suppl 1: S212-221.

Pelzer S. et al (1989) Diversity and novel pharmacological properties of Ca2+ channels in Drosophila brain membranes. The EMBO Journal 8:8 2365-2371.

Peñalva-Arana D. C. et al (2007) Studying Daphnia feeding behavior as a black box: a novel electrochemical approach. Hydrobiologia 594:1 153-163.

Peng I. F. and C. F. Wu (2007) Differential contributions of Shaker and Shab K+ currents to neuronal firing patterns in Drosophila. J Neurophysiol 97:1 780-794.

Pennec J. P. et al (2004) Characterization of the voltage-activated currents in cultured atrial myocytes isolated from the heart of the common oyster Crassostrea gigas. J Exp Biol 207(Pt 22): 3935-3944.

Penton E. H. et al (2004) Mitochondrial DNA variation in North American populations of Daphnia obtusa: continentalism or cryptic endemism? Molecular Ecology 13:1 97-107.

Pereira J. L. and F. Goncalves (2007) Effects of food availability on the acute and chronic toxicity of the insecticide methomyl to Daphnia spp. Sci Total Environ 386(1-3): 9-20.

Pereira J. L. et al (2007) Short- and long-term responses of Daphnia spp. to propanil exposures in distinct food supply scenarios. Ecotoxicol Environ Saf 68:3 386-396.

Peretz A. et al (1998) A Ca 21/Calmodulin-Dependent Protein Kinase Modulates Drosophila Photoreceptor K1 Currents: A Role in Shaping the Photoreceptor Potential. The Journal of Neuroscience 18:22 9153-9162.

Periyanayagam K. et al (2015) Cardio protective effect of the leaves of *Trichosanthes cucumerina* L. on *Daphnia magna.* International Journal od Pharmacy and Pharmaceutical Analysis 1:2 1-7.

Periyanayagam K. et al (2013) Cardio protective effect of the leaves of *Artocarpus heterophyllus* L. on *Daphnia magna.* Innovare Journal of Health Sciences 1:3 1-5.

Pertwee R. G. (2006) Cannabinoid pharmacology: the first 66 years. Br J Pharmacol 147 :1 163-171.

Pestana J. L. et al (2010) Pesticide exposure and inducible antipredator responses in the zooplankton grazer Daphnia magna Straus. Chemosphere 78:3 241-248.

Petersen C. I. et al (2004) In vivo identification of genes that modify ether-a-go-go-related gene activity in Caenorhabditis elegans may also affect human cardiac arrhythmia. Proc Natl Acad Sci USA 101:32 11773-11778.

Petrusek A. et al (2009) A crown of thorns is an inducible defense that protects Daphnia against an ancient predator. Proc Natl Acad Sci U S A 106:7 2248-2252.

Phelps J. D. et al (2018) Imidacloprid slows the development of preference for rewarding food sources in bumblebees (Bombus impatiens). Ecotoxicology 27:2 175-187.

Phillips K. P. et al (2000) Intracellular pH regulation in human preimplantation embryos. Hum Repr 15:4 896-904.

Pieters B. J. et al (2006) Modeling responses of Daphnia magna to pesticide pulse exposure under varying food conditions: intrinsic versus apparent sensitivity. Ecotoxicology 15:7 601-608.

Pijanowska J. and M. Kloc (2004) Daphnia response to predation threat involves heat-shock proteins and the actin and tubulin cytoskeleton. Genesis 38:2 81-86.

Pijper A. (1947) Methylcellulose and bacterial motility. J Bacteriol. 53:3 257-269.

Pirow R. (2004) The dichotomous oxyregulatory behaviour of the planktonic crustacean Daphnia magna. Journal of Experimental Biology 207:4 683-696.

Pirow R. et al (1999a) The importance of the feeding current for oxygen uptake in the water flea Daphnia magna. J. Exp. Biol. 202 553-562.

Pirow R. et al (1999b) The sites of respiratory gas exchange in the planktonic crustacean Daphnia magna: an in vivo study employing blood haemoglobin as an internal probe, J. Exp. Biol. 202 3089-3099.

Pirtle T. J. et al (2018) ZD7288 and mibefradil inhibit the myogenic heartbeat in Daphnia magna indicating its dependency on HCN and T-type calcium ion channels. Comp. Biochem. Physiol. Part A 222: 36-42.

Pohnert G. et al (2007) Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. Trends Ecol Evol 22 198-204.

Poirier D. G. et al (1988) toxicity test protocol *Daphnia magna* acute lethality. Ontario ministry of the environment. Aquatic Toxicity Unit: Aquatic Biology Section: Water Resources Branch.

Pope E. C. and Rowley A. F. (2002) The heart of Ciona intestinalis: eicosanoid-generating capacity and the effects of precursor fatty acids and eicosanoids on heart rate. The Journal of Experimental Biology 205 1577-1583.

Popova E. Y. and A. A. Kotov (2013) Latitudinal patterns in the diversity of two subgenera of the genus Daphnia O.F. Muller (Crustacea: Cladocera: Daphniidae) Zootaxa 3736: 159-174.

Porta A. et al 2008 Temporal asymmetries of short-term heart period variability are linked to autonomic regulation Am. J. Physiol. Regul. Integr. Comp. Physiol. 295 550–7.

Portbury A. L. et al (2003) Catecholamines act via alpha adrenergic receptor to maintain fetal heart rate and survival. Am J Physiol Heart Circ Physiol 284 2069-2077.

Porter K. G. et al (1982) The effect of food concentration on swimming patterns, feeding behavior, ingestion, assimilation, and respiration by Daphnia. Limnol. Oceanogr. 27:5 935-949.

Postma A. et al (2002) Cardiac status in bone tumor survivors up to nearly 19 years after treatment with doxorubicin: a longitudinal study. Med Pediatr Oncol 39:2 86-92.

Potts R. et al (2018) The effect of dietary neonicotinoid pesticides on non-flight thermogenesis in worker bumble bees (Bombus terrestris). J Insect Physiol. 104 33-39.

Prokopenko R. A. et al (1995) Effects of Haloperidol and Chlorpromazine on Smooth Muscle Contractility, Platelet Aggregation and Neuronal Calcium Current. Gen. Physiol. Biophys. 14 349-357.

Prosser C. L. (1942) An analysis on the action of acetylcholine on hearts, particularly in arthropods. The Biological Bulletin, Marine Biological Laboratory 83:2 145-164.

Purna G. and Nagaraju C. (2007) Is methyl farnesoate a crustacean hormone? Aquaculture 272 39-54.

References: Q

Qazi S. et al (1996) Characterization of Muscarinic Binding Sites in the Central Nervous System of Larval Manduca sexta. Insect Biochem. Molec. BioL 26:7 721-732.

References: R

Rabus M. et al (2013) Uncovering ultrastructural defences in Daphnia magna--an interdisciplinary approach to assess the predator-induced fortification of the carapace. PLoS One 8:6 e67856.

Ramakrishnan L. et al (2014) Galantamine reverses scopolamine-induced behavioral alterations in Dugesia tigrina. Invert Neurosci 14:2 91-101.

Rang H. P. (2006) The receptor concept: pharmacology's big idea. Br J Pharmacol 147 Suppl 1: S9-16.

Ravet J. L. (2012) Threshold dietary polyunsaturated fatty acid concentrations for Daphnia pulex growth and reproduction. Inland Waters 2:4 199-209.

Ravet J. L. et al (2003) A test of the role of polyunsaturated fatty acids in phytoplankton food quality for Daphnia using liposome supplementation. Limnology Oceanographique 48:5 1938-1947.

Rawls S. M. et al (2010) First evidence that drugs of abuse produce behavioral sensitization and cross sensitization in planarians. Behav Pharmacol 21:4 301-313.

Reichwaldt E. S. et al (2004) The effect of different zooplankton grazing patterns resulting from diel vertical migration on phytoplankton growth and composition: a laboratory experiment. Oecologia 141:3 411-419.

Ren Z. et al (2015) AChE inhibition: one dominant factor for swimming behavior changes of *Daphnia magna* under DDVP exposure. Chemosphere 120 252-7.

Ren Z. et al (2009) Behavioral responses of Daphnia magna to stresses of chemicals with different toxic characteristics. Bull Environ Contam Toxicol 82:3 310-316.

Ren Z. et al (2007) The early warning of aquatic organophosphorus pesticide contamination by on-line monitoring behavioral changes of Daphnia magna. Environ Monit Assess 134(1-3): 373-383.

Reynaldi S. et al (2006) Linking feeding activity and maturation of Daphnia magna following short-term exposure to fenvalerate. Environmental Toxicology and Chemistry 25:7 1826-1830.

Rider C. V. and Leblanc G. (2006) Atrazine Stimulates Hemoglobin Accumulation in Daphnia magna: Is it Hormonal or Hypoxic? Toxicological Sciences 93:2 443-9.

Riessen H. P. et al (2012) Changes in water chemistry can disable plankton prey defenses. Proc Natl Acad Sci USA 109:38 15377-15382.

Rietzler A. C. et al (2008) Laboratory demonstration of morphological alterations in Ceriodaphnia cornuta Sars (1885) fa rigaudi induced by Chaoborus brasiliensis Theobald (1901) Brazillian Journal of Biology 68:2 453-454.

Rinken A. (1996) Formation of the functional complexes of M2 muscarinic acetylcholine receptors with GTP-binding regulatory proteins in solution. Journal of Biochmistry 120: 193-200.

Ritter K. S. (1988) Steinernema feltiae (Neoaplectana carpocapsae) Effect of Sterols and liypolipidemic Agents on Development. Experimental Parasitology 67 257-267.

Riviere G. et al (2011) A crucial role in fertility for the oyster angiotensin-converting enzyme orthologue CgACE. PLoS One 6:12 e27833.

Riviere G. et al (2004) Characterization of the first non-insect invertebrate functional angiotensin-converting enzyme (ACE): leech Tt ACE resembles the N-domain of mammalian ACE. Biochem. J. 382 565-573.

Roberts A. P. et al (2007) In vivo Biomodification of Lipid-Coated Carbon Nanotubes by Daphnia magna. Environmental Science and Technology 41: 3025-3029.

Rocha R. et al (2014) Environmental effects of anticholinesterasic therapeutic drugs on a crustacean species Daphnia magna. Environ Sci Pollut Res Int 21:6 4418-4429.

Rohrlack T. et al (2004) Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in Daphnia pulicaria. Appl Environ Microbiol 70:8 5047-5050.

Rohrlack T. et al (2001) Effects of cell-bound microcystins on survival and feeding of Daphnia spp. Appl Environ Microbiol 67:8 3523-3529.

Rohrlack T. et al (1999) Role of microcystins in poisoning and food ingestion inhibition of daphnia galeata caused by the cyanobacterium Microcystis aeruginosa. Applied and Environmental Microbiology 65:2 737-739.

Romani R. et al (2005) Effects of chlorpyrifos on the catalytic efficiency and expression level of acetylcholinesterases in the bivalve mollusk Scapharca inaequivalvis. Env. Tox. and Chem. 24:11 2879-2886.

Rooij E. et al (2007) The Myriad Roles of microRNAs in Heart Disease. Author's Copy. 575-579

Rosa R. et al (2012) A scaled-up system to evaluate zooplankton spatial avoidance and the population immediate decline concentration. Environ Toxicol Chem 31:6 1301-1305.

Rosa R. et al (2008) Development and sensitivity of a 12-h laboratory test with Daphnia magna Straus based on avoidance of pulp mill effluents. Bull Environ Contam Toxicol 81:5 464-469.

Rosay P. et al (2001) Synchronized Neural Activity in the Drosophila Memory Centers and Its Modulation by amnesiac. Neuron 30 759-770.

Rosen M. R. and L. Bergfeldt (2015) Cardiac memory: The slippery slope twixt normalcy and pathology. Trends Cardiovasc Med 25:8 687-696.

Rosenkranz P. W. (2010) The ecotoxicology of nanoparticles in Daphnia magna. A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University for the degree of Doctor of Philosophy (PhD): 181.

Rosenkranz P. W. et al (2009) A comparison of nanoparticle and fine particle uptake by Daphnia magna. Environmental Toxicology and Chemistry 28:10 2142-2149.

Roszer T. et al (2006) Phe-met-arg-phe (FMRF)-amide is a substrate source of NO synthase in the gastropod nervous system. Cell Tissue Res 325:3 567-575.

Rozenberg A. et al (2015) Transcriptional profiling of predator-induced phenotypic plasticity in Daphnia pulex. Front Zool 12: 18.

Russo R. N. and Parrish S. (1995) Toxicology for Middle School: The Effect of Common Substances on Daphnia. J. Chem. Educ. 72:1 49.

Rydberg A. et al (2008) Can analysis of heart rate variability predict arrhythmia in children with Fontan circulation? Pediatr Cardiol 29:1 50-55.

References: S

Safra T. (2003) Cardiac safety of liposomal anthracyclines. The Oncologist 8:2 17-24.

Sakura M. et al (2004) Impairment of olfactory discrimination by blockade of nitric oxide activity in the terrestrial slug Limax valentianus. Neurosci Lett 370(2-3): 257-261.

Sanchez-Fortun S. and M. V. Barahona (2009) Toxicity and characterization of cholinesterase-inhibition induced by diisopropyl fluorophosphate in Artemia salina larvae. Ecotoxicol Environ Saf 72:3 775-780.

Sanchez-Fortun S. and M. V. Barahona (2001) The use of carbamates, atropine, and 2-pyridine aldoxime methoiodide in the protection of artemia salina against poisoning by carbophenothion. Environmental Toxicology and Chemistry 20:9 2008-2013.

Sancho E. et al (2009) Disturbances in energy metabolism of Daphnia magna after exposure to tebuconazole. Chemosphere 74:9 1171-1178.

Santalla M. et al (2016) Drosophila Melanogaster, an Emerging Animal Model for the Study of Human Cardiac Diseases. Argentine journal of cardiology 84:5 406-412.

Saxena P. R. and Villalón C. M. (1990) Cardiovascular effects of serotonin agonists and antagonists. J Cardiovasc Pharmacol. 7: 17-34.

Schaack S. et al (2013) The effect of spontaneous mutations on competitive ability. J Evol Biol 26:2 451-456.

Schafer S. et al (1994) Ionic Currents of Kenyon Cells from the Mushroom Body of the Honeybee. The Journal of Neuroscience 74:8 4800-4812.

Schallenberg M. et al (2005) Survival of Campylobacter jejuni in water: effect of grazing by the freshwater crustacean Daphnia carinata (Cladocera) Appl Environ Microbiol 71:9 5085-5088.

Schatz G. S. and E. McCauley (2007) Foraging behavior by Daphnia in stoichiometric gradients of food quality. Oecologia 153:4 1021-1030.

Schlechtriem C. et al (2006) Effect of Temperature on the Fatty Acid Composition and Temporal Trajectories of Fatty Acids in Fasting Daphnia pulex (Crustacea Cladocera) Lipids 41:4 397-401.

Schneider L. E. et al (2009) Assessment of genotoxicity of Lidocaine Prilonest and Septanest in the Drosophila wing-spot test. Food Chem Toxicol 47:1 205-208.

Schou T. M. et al (2013) Temperature and population density effects on locomotor activity of Musca domestica (Diptera: Muscidae). Environ. Entomol. 42: 1322–1328.

Schwabedal J. T. C. (2015) Parametric Phase Diffusion Analysis of Irregular Oscillations. Europhysics Letters. Draft copy sent directly by author.

Schwarzenberger A. et al (2009) Target gene approaches: Gene expression in D. magna exposed to predator-borne kairomones or to microcystin-producing and microcystin-free Microcystis aeruginosa. BMC Gen 10: 527.

Schwarzenberger A. et al (2013) Effect of nutrient limitation of cyanobacteria on protease inhibitor production and fitness of Daphnia magna. J Exp Biol 216:19 3649-3655.

Schwerte T. and Pelster B. (2000) Digital motion analysis as a tool for analysing the shape and performance of the circulatory system in transparent animals. The Journal of Experimental Biology 203 1659-1669.

Schwind R. (1999) Daphnia pulex swims towards the most strongly polarized light, a response that leads to 'shore flight'. The Journal of Experimental Biology 202 3631-3635.

Scoville A. G. and M. E. Pfrender (2010) Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. Proc Natl Acad Sci U S A 107:9 4260-4263.

Seifert S. A. (2001) Atropine Use inCentruroidesScorpion Envenomation—Contraindicated or Not? (Commentary) Journal of Toxicology: Clinical Toxicology 39:6 599-599.

Senatore A. et al (2011) Mapping of dihydropyridine binding residues in a less sensitive invertebrate L-type calcium channel (LCa v 1) Channels (Austin) 5:2 173-187.

Shaw J. R. et al (2008) Daphnia as an emerging model for toxicological genomics. 2: 165-328.

Shiga Y. et al (2002) Evolving role of Antennapedia protein in arthropod limb patterning. Dev. 129:15 3555-61.

Shiny K. J. et al (2005) Biotreatment of wastewater using aquatic invertebrates Daphnia magna and Paramecium caudatum. Bioresour Technol 96:1 55-58.

Shinozaki H. (1979) The pharmacology of the excitatory neuromuscular junction in the crayfish. Progress in Neurobiology 14 121-155.

Siciliano A. et al (2015) How Daphnia Assays may be used as Bioindicators of Health Effects? Bio. Endang. Sp. 1:5.

June 2000Journal of Cellular Biochemistry 78(2):297-304

Siddiqui R, Burtschi D. J. and Kovacs R. J. (2000) Phosphatidic acid induces calcium influx in neutrophils via verapamil-sensitive calcium channels. Journal of Cellular Biochemistry 78(2):297-304

Silva-Moraes V. et al (2013) Antischistosomal activity of a calcium channel antagonist on schistosomula and adult Schistosoma mansoni worms. Memórias do Instituto Oswaldo Cruz 108:5 600-604.

Silver K. S. et al (2009) Role of the sixth transmembrane segment of domain IV of the cockroach sodium channel in the action of sodium channel blocker insecticides. Neurotoxicology 30:4 613-621.

Simon T. W. et al (1992) Modulatory effects of FMRF-NH, on outward currents and oscillatory activity in heart interneurons of the medicinal leech. The Journal of Neuroscience 12:2 525-537

Simoes P. M. et al (2013) Phenotypic transformation affects associative learning in the desert locust. Curr Biol 23:23 2407-2412.

Singh S. and Wu C. (1990) Properties of potassium currents and their role in membrane excitability in Drosophila larval muscle fibers. J. exp. Biol. 152 59-76.

Singh S. and Wu C. (1989) Complete Separation of Four Potassium Currents in Drosophila. Neuron 2 1325-1329.

Small D. L. and Morris C. E. (1995) Pharmacology of stretch-activated K channels in Lymnaea neurones. British Journal of Pharmacology 114 180-186.

Smirnov N. (2013) Physiology of the Cladocera, Academic Press, San Diego, CA, USA.

Smith J. O. (2011) Spectral Audio Signal Processing. 'Quadratic Interpolation of Spectral Peaks' in Chapter 4: Spectrum Analysis of Sinusoids. Center for Computer Research in Music and Acoustics.

Soares M. C. et al (2009) Effects of the cyanobacterium Cylindrospermopsis raciborskii on feeding and life-history characteristics of the grazer Daphnia magna. Ecotoxicol Environ Saf 72:4 1183-1189.

Söderhäll I. et al (2003) Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of Pacifastacus leniusculus. Dev. and Comp Immunology 27:8 661-672.

Sommer U. et al (2003) Daphnia versus copepod impact on summer phytoplankton: functional compensation at both trophic levels. Oecologia 135:4 639-647.

Song B. et al (2015) Background-free in-vivo Imaging of Vitamin C using Time-gateable Responsive Probe. Sci Rep 5: 14194.

Song W. et al (2011) Analysis of the action of lidocaine on insect sodium channels. Ins Bioch Mol Bio 41:1 36-41.

Spaak P. (2004) Spatial and temporal patterns of sexual reproduction in a hybrid Daphnia species complex. Journal of Plankton Research 26:6 625-635.

Spaak P. et al (2012) Modes and mechanisms of a Daphnia invasion. Proc Biol Sci 279(1740): 2936-2944.

Spanier K. I. et al (2010) Predator-induced defences in Daphnia pulex: selection and evaluation of internal reference genes for gene expression studies with real-time PCR. BMC Mol Biol 11: 50.

Specter M. J. et al (1975) Studies on Magnesium's Mechanism of Action in Digitalis-induced Arrhythmias. Circulation 52 1001-1007.

Spindler S. R. et al (2012) Statin treatment increases lifespan and improves cardiac health in Drosophila by decreasing specific protein prenylation. PLoS One 7:6 e39581.

Stabell O. B. et al (2003) Inducible Defences in Daphnia Depend on Latent Alarm Signals from Conspecific Prey Activated in Predators. Chem. Senses 28: 141-153.

Stanley D. A. and Raine N. E. (2017) Bumblebee colony development following chronic exposure to field-realistic levels of the neonicotinoid pesticide thiamethoxam under laboratory conditions. Scientific Reports 7: 8005.

Steinbach C. et al (2014) The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (Oncorhynchus mykiss). Science of the Total Environment 497-498; 209-218.

Stollewerk A. (2010) The water flea *Daphnia* - a 'new' model system for ecology and evolution? J. Biology **9**:21

Stow A. et al (2012) Uniform Temperature Dependency in the Phenology of a Keystone Herbivore in Lakes of the Northern Hemisphere. PLoS One 7:10 e45497.

Straile D. et al (2012) Uniform temperature dependency in the phenology of a keystone herbivore in lakes of the Northern Hemisphere. PLoS One 7:10 e45497.

Subbulekshmi N. et al (2016) Antiarrhythmic effect of the leaf volatile oil of *elettaria cardamomum maton*. on the heart of *Daphnia magna.* EJPMR 3:7 282-285.

Sullivan G. M. and Feinn R. (2012) Using effect size; why the p value is not enough. J. Grad. Med. Ed. 4:3 279-282.

Sun H. and Gu X. (2005) Comprehensive toxicity study of nonylphenol and short-chain nonylphenol polyethoxylates on *Daphnia magna*. Bulletin of Environmental Contamination and Toxicology 75:4 677-683.

Suskevich J. et al (1967) A modification of a method for the evaluation of topical anaesthesia in the earthworm. J. Pharm. Pharmac. 19 456-459.

References: T

Tadesse T. et al (2014) Mechanisms underlying odorant-induced and spontaneous calcium signals in olfactory receptor neurons of spiny lobsters Panulirus argus. J Comp Phys A Neuro Sens Neural Behav Physiol 200:1 53-76.

Tagg N. et al (2005) Outcomes of reciprocal invasions between genetically diverse and genetically uniform populations of Daphnia obtusa (Kurz) Oecologia 143:4 527-536.

Taghli-Lamallem O. et al (2016) Drosophila in the Heart of Understanding Cardiac Diseases: Modeling Channelopathies and Cardiomyopathies in the Fruitfly. J. Cardiovasc. Dev. Dis. 3:7 22 pp.

Takaya T. et al (2009) Torsades de Pointes with QT prolongation related to donepezil use. J. Card 54 507-511.

Takekura H. and Franzini-Armstrong C. (2002) The Structure of Ca2+ Release Units in Arthropod Body Muscle Indicates an Indirect Mechanism for Excitation-Contraction Coupling. Biophysical Journal 83: 2742-2753.

Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology (TFESCNASPE) (1996) Heart rate variability: Standards of measurement, physiological interpretation, and clinical use. European Heart Journal 17 354-381.

Tesovnik T. et al (2017) Immune related gene expression in worker honey bee (Apis mellifera carnica) pupae exposed to neonicotinoid thiamethoxam and Varroa mites (Varroa destructor). https://doi.org/10.1371/journal.pone.0187079

Theopold U. et al (2004) Coagulation in arthropods: defence, wound closure and healing. Trends in Immunology 25:6 289-295

Thandroyden F. T. et al (1983) The Effect of Alpha- and Beta-Adrenoceptor Antagonist Agents on Reperfusion Ventricular Fibrillation and Metabolic Status in the Isolated Perfused Rat Heart. J Am Coll Cardiol 1:4 1056-66.

Thuraisingham R. A. (2007) An electrocardiogram marker to detect paroxysmal atrial fibrillation. J Electrocardiol 40:4 344-347.

Thomsen M. B. et al (2006) Beat-to-beat variability of repolarization determines proarrhythmic outcome in dogs susceptible to drug-induced torsades de pointes. Journal of the American College of Cardiology 48:6 1268-1277.

Tissenbaum H. A. et al (2000) A common muscarinic pathway for diapause recovery in the distantly related nematode species Caenorhabditis elegans and Ancylostoma caninum. PNAS 97:1 460–465.

Torkkeli P. H. et al (2005) Expression of muscarinic acetylcholine receptors and choline acetyltransferase enzyme in cultured antennal sensory neurons and non-neural cells of the developing moth Manduca sexta. J Neurobiol 62:3 316-329.

Tosi S. et al (2017) A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. Scientific Reports 7: 1201.

Tricoire H. et al (2014) Methylene blue rescues heart defects in a Drosophila model of Friedreich's ataxia. Hum Molecular Genetics 23:4 968-979.

Turner J. E. et al (1995) Oocyte activation and passage through the metaphase/anaphase transition of the meiotic cell cycle is blocked in clams by inhibitors of HMG-CoA reductase activity. J Cell Biol 128:6 1145-1162

Tseng C. C. and C. Y. Ho (2005) Removal of a nasal leech: a safe and effective method. Otolaryngol Head Neck Surg 132:5 814-815.

References: U

Uçar G1. Et al (1997) Serum angiotensin converting enzyme activity in pulmonary diseases: correlation with lung function parameters. Life Sci. 61:11 1075-82.

Ueda A. and C. F. Wu (2006) Distinct frequency-dependent regulation of nerve terminal excitability and synaptic transmission by IA and IK potassium channels revealed by Drosophila Shaker and Shab mutations. J Neurosci 26(23): 6238-6248.

References: V

Vale P. F. et al (2011) Epidemiological evolutionary and coevolutionary implications of context-dependent parasitism. Am Nat 177:4 510-521.

Valentin J. P. et al (2004) Review of the predictive value of the Langendorff heart model (Screenit system) in assessing the proarrhythmic potential of drugs. J Pharmacol Toxicol Methods 49:3 171-181.

Vallance P. and T. G. Smart (2006) The future of pharmacology. Br J Pharmacol 147 Suppl 1: S304-307.

Van de Meutter F. et al (2005) Spatial avoidance of littoral and pelagic invertebrate predators by Daphnia. Oecologia 142:3 489-499.

Van Den Berg M. P. et al (2004) Clustering of RR intervals predicts effective electrical cardioversion for atrial fibrillation. J Cardiovasc Electrophysiol 15:9 1027-1033.

Van Geest G. J. et al (2007) Goose-mediated nutrient enrichment and planktonic grazer control in Arctic freshwater ponds. Oecologia 153:3 653-662.

Van Erven L and Schalij M. J. (2010) Amiodarone: an effective antiarrhythmic drug with unusual side effects. Heart 96 1593-1600.

Vandingenen A. et al (2001) Captopril a Specific Inhibitor of Angiotensin Converting Enzyme Enhances Both Trypsin and Vitellogenin Titers in the Grey Fleshfly Neobellieria bullata. Arch Insect Biochem Phys 47: 161-167.

Vatanparast J. et al (2006) The functional consequences of paraoxon exposure in central neurones of land snail Caucasotachea atrolabiata are partly mediated through modulation of Ca2+ and Ca2+-activated K+-channels. Comp Biochem Physiol C Toxicol Pharmacol 143:4 464-472.

Vatanparast J. et al (2006) Paraoxon suppresses Ca(2+) spike and afterhyperpolarization in snail neurons: Relevance to the hyperexcitability induction. Brain Res 1083:1 110-117.

Vaynblat M. et al (2002) Simultaneous angiotensin converting enzyme inhibition moderates ventricular dysfunction caused by doxorubicin. The European Journal of Heart Failure 4 583-586.

Vega M. P. and Pizarro R. A. (2000) Oxidative stress and defence mechanisms of the freshwater cladoceran Daphnia longispina exposed to UV radiation. J. Photochem. Photobiol. B: Biol. 54 121-125.

Vehniainen E. R. and J. V. Kukkonen (2015) Multixenobiotic resistance efflux activity in Daphnia magna and Lumbriculus variegatus. Chemosphere 124: 143-149.

Vejpongsa P. and E. T. Yeh (2014) Prevention of anthracycline-induced cardiotoxicity: challenges and opportunities. J Am Coll Cardiol 64:9 938-945.

Verbitskii V. B. and T. I. Verbitskaya (2012) Final thermal preference in parthenogenetic females of Daphnia magna straus (Crustacea: Cladocera) acclimated to various temperatures. Biology Bulletin 38:5 493-499.

Vercruysse L. et al (2004) The angiotensin converting enzyme inhibitor captopril reduces oviposition and ecdysteroid levels in Lepidoptera. Arch Insect Biochem Physiol 57:3 123-132.

Vercruysse L. et al (2005) ACE inhibitor captopril reduces ecdysteroids in moths. An N Ac Sci 1040: 498-500.

Vercruysse L. et al (2005) ACE inhibitory activity in enzymatic hydrolysates of insect protein. J Agric Food Chem 53:13 5207-5211.

Verma P. K. et al (2009) Functional role of cholinergic drugs on spontaneous muscular activity in the amphistome Gastrothylax crumenifer from ruminants. J Helminthol 83:1 77-82.

Vermehren A. and B. A. Trimmer (2005) Expression and function of two nicotinic subunits in insect neurons. J Neurobiol 62:3 289-298.

Vesela S. et al (2008) Daphnia intoxicated by nerve agent tabun can be treated using human antidotes. Environ Toxicol Pharmacol 25:3 329-333.

Villarroel M. J. et al (1999) Daphnia magna Feeding Behavior after Exposure to Tetradifon and Recovery from Intoxication. Ecotoxicology and Environmental Safety 44: 40-46.

Villegas-Navarro A. et al (2003) The heart of *Daphnia magna*: effects of four cardioactive drugs. Comparative Biochemistry and Physiology Part C 136 127–134.

Villegas-Navarro A. et al (1997) Determination of LC50 from Daphnia magna in treated industrial waste waters and non-treated hospital effluents. Environment International 23:4 535-540

Volkov E. M. et al (2001) Effect of cholinergic agonists on resting membrane potential of earthworm body wall muscle cells. Bulletin of Experimental Biology and Medicine 131:4 397-398.

Von Elert E. et al (2012) Inducible tolerance to dietary protease inhibitors in D. magna. J Ex Bi 215:12 2051-2059.

Voss A. et al (2012) Lagged segmented Poincare plot analysis for risk stratification in patients with dilated cardiomyopathy. Med Biol Eng Comput 50:7 727-736.

Vulfius C. A. et al (2001) Nicotinic receptors in Lymnaea stagnalis neurons are blocked by aneurotoxins from cobra venoms. Neuroscience Letters 309 189-192.

References: W

Wagatsuma A. and Sakuma K. (2013) Mitochondria as a Potential Regulator of Myogenesis. The Scientific World Journal. 2013:593267 9 pp.

Wägele J. W. and Mayer C . (2007) Visualizing differences in phylogenetic information content of alignments and distinction of three classes of long-branch effects. BMC Evol. Biol.

Wagenaar D. A. and Kristan W. B. (2010) Automated video analysis of animal movements using Gabor orientation filters. Neuroinformatics. 8:1 33-42.

Walker M. J. (2006) Antiarrhythmic drug research. Br J Pharmacol 147 222-231.

Walsh M. R. et al (2015) Predator-induced phenotypic plasticity within- and across-generations: a challenge for theory? Proc Biol Sci 282(1798): 20142205.

Walsh M. R. and D. M. Post (2011) Interpopulation variation in a fish predator drives evolutionary divergence in prey in lakes. Proc Biol Sci 278(1718): 2628-2637.

Walsh M. R. and D. M. Post (2012) The impact of intraspecific variation in a fish predator on the evolution of phenotypic plasticity and investment in sex in Daphnia ambigua. J Evol Biol 25:1 80-89.

Wang et al (2009) Combined experimental and theoretical study on photoinduced toxicity of an anthraquinone dye intermediate to Daphnia magna. Environmental Toxicology and Chemistry 28:4 846-852.

Warren E. (1900) Memoirs: On the Reaction of Daphnia magna (Straus) to certain Changes in its Environment. Journal of Cell Science 2:43 199-224.

Watanabe G. et al (2011) Potassium-induced cardiac resetting technique for persistent ventricular tachycardia and fibrillation after aortic declamping. Ann Thorac Surg 91 619-20.

Watanabe S. et al (2011) Modulation of oscillatory neural activities by cholinergic activation of interneurons in the olfactory center of a terrestrial slug. Brain Research 896 30-35.

Watkins J. C. and D. E. Jane (2006) The glutamate story. Br J Pharmacol 147 Suppl 1: S100-108.

Watson S. B. et al (2007) *Daphnia* behavioural responses to taste and odour compounds: ecological significance and application as an inline treatment plant monitoring tool. Water Science and Technology 55:5 23-31.

Watson J. A. et al (1985) Isoprenoid synthesis in isolated embryonic Drosophila cells. J Biol Chem 260:26 83-91.

Weber A. K. and Pirow R. (2009) Physiological responses of Daphnia pulex to acid stress. BMC physiology 9:9.

Weckstrom M. et al (1991) Voltage-activated potassium channels in blowfly photoreceptors and their role in light adaptation. Journal of Physiology 440 635-657.

Wegener C. and D. R. Nassel (2000) Peptide-Induced Ca2 Movements in a Tonic Insect Muscle: Effects of Proctolin and Periviscerokinin-2. The American Physiological Society 00022-3077/00: 3056-3067.

Weider L. J. et al (2008) Stoichiometric differences in food quality: impacts on genetic diversity and the coexistence of aquatic herbivores in a Daphnia hybrid complex. Oecologia 158:1 47-55.

Weider L. J. et al (2005) Genotype x environment interactions stoichiometric food quality effects and clonal coexistence in Daphnia pulex. Oecologia 143:4 537-547.

Weiss L. C. et al (2012) Chaoborus and gasterosteus anti-predator responses in Daphnia pulex are mediated by independent cholinergic and gabaergic neuronal signals. PLoS One 7:5 e36879.

Wernersson A. and Dave G. (1998) Effects of different protective agents on the phototoxicity of fluoranthene to *Daphnia magna*. Comparative Biochemistry and Physiology C 120 373-381.

Wessel N. et al (2007) Introduction: Cardiovascular physics. Chaos 015101

Wessel N. et al (2000a) Short-term forecasting of life-threatening cardiac arrhythmias based on symbolic dynamics and finite-time growth rates. Physical Review E 61:1 733-740.

Wessel N. et al (2000b) Nonlinear analysis of complex phenomena in cardiological data. Herzschr Elektrophys 11:159–173.

Westmark S. et al (2009) Pharmacological analysis of tonic activity in motoneurons during stick insect walking. J Neurophysiol 102:2 1049-1061.

Wiggins P. R. and P.B. Frappell (2002) Behavioural thermoregulation in D. carinata from different depths of a natural water body: influence of environmental oxygen levels and temperature. Comp Bioch Phs A 133: 771-780.

Wiggins P. R. and Frappell P. B. (2000) The Influence of Haemoglobin on Behavioural Thermoregulation and Oxygen Consumption in Daphnia carinata. Physiological and Biochemical Zoology 73:2 153-160.

Wiklund A. E. et al (2014) Sucralose Induces Biochemical Responses in Daphnia magna. PLOS ONE 9:4 e92771.

Wilkens J. L. et al (2005) Sites and modes of action of proctolin and the FLP F2 on lobster cardiac muscle. J Exp Biol 208:4 737-747.

Wilkens L. A. (1972) Electrophysiological studies on the heart of the bivalve mollusc, Modiolus demissus. J. Exp. Biol. 56 393-310

Wilkinson J. M. (2015) (expert opinion). Mayo Clinic, Rochester, Minn. Nov. 28, 2015.

Will M. A. et al (2011) Biological pH buffers in IVF: help or hindrance to success. J Ass Reprod Genet. 28:8 711-24.

Williams G. P. (1997) Chaos Theory Tamed. Joseph Henry Press. 520 pages.

Williams T. A. et al (1996) Drosophila melanogaster angiotensin I-converting enzyme expressed in Pichia pastoris resembles the C domain of the mammalian homologue and does not require glycosylation for secretion and enzymic activity. Biochem. J. 318 125-131.

Wood S. C. et al (2018) Comparative chronic toxicity of three neonicotinoids on New Zealand packaged honey bees. PLOS ONE 13(1): e0190517

Woo M. et al (1992) Patterns of beat-to-beat heart rate variability in advanced heart failure. American Heart Journal 123:3 704-711.

Woodcock B. A. et al (2018) Neonicotinoid residues in UK honey despite EU moratorium. PLoS ONE 13(1).

Wooten J. M. (2002) Drug-induced arrhythmias. RN Magazine 1:37 20 pages.

Wright T. J. and H. Huddart (2002) The nature of the acetylcholine and 5-hydroxytryptamine receptors in buccal smooth muscle of the pest slug Deroceras reticulatum. J Comp Physiol B 172:3 237-249.

Wu C. et al (1989) Actions of dendrotoxin on k+ channels and neuromuscular transmission in Drosophila melanogaster, and its effects in synergy with K+ channel-specific drugs and mutations. J. exp. Biol. 147 21-41.

References: X

Xie H. and Bittar E. E. (1989) Nicardipine as a Ca 2+ channel blocker in single barnacle muscle fibers. Biochimica et Biophysica Acta 1014: 207-209.

References: Y

Yabumoto T. et al (2008) Nitric oxide is involved in appetitive but not aversive olfactory learning in the land mollusk Limax valentianus. Learn Mem 15:4 229-232.

Yamagishi H. et al (2004) Developmental changes in dopamine modulation of the heart in the isopod crustacean Ligia exotica: reversal of chronotropic effect. Zoolog Sci 21:9 917-922.

Yan H. L. and Y. M. Hua (2014) Research advances on role of microRNAs in congenital heart diseases. Zhongguo Dang Dai Er Ke Za Zhi 16:10 1070-1074.

Yan A. T. et al (2006) Characterization of the Peri-Infarct Zone by Contrast-Enhanced Cardiac Magnetic Resonance Imaging Is a Powerful Predictor of Post-Myocardial Infarction Mortality. Circulation 114 32-39.

Yano T. et al (2006) A comparison of intracellular lidocaine and bupivacaine concentrations producing nerve conduction block in the giant axon of crayfish in vitro. Anesth Analg 102:6 1734-1738.

Yano T. et al (2013) Dimethylsulfoxide potentiates the nerve conduction-blocking effect of lidocaine without augmentation of the intracellular lidocaine concentration in the giant axon of crayfish in vitro. Fundam Clin Pharmacol 27:4 402-408.

Yasumasu I. et al (1985) Mechanism for Electrosilent Ca 2+ Transport to Cause Calcification of Spicules in Sea Urchin Embryos. Experimental Cell Research 159 80-90.

Yeoman M. S. et al (1999) LVA and HVA Ca2+ Currents in Ventricular Muscle Cells of the Lymnaea Heart. The American Physiological Society: 2428-2441.

Yoshida T. et al (2006) Ecology: mechanisms for consumer diversity. Nature 439(7072)

You Y. J. et al (2006) Starvation activates MAP kinase through the muscarinic acetylcholine pathway in Caenorhabditis elegans pharynx. Cell Metab 3:4 237-245.

Youdim M. B. and Y. S. Bakhle (2006) Monoamine oxidase: isoforms and inhibitors in Parkinson's disease and depressive illness. Br J Pharmacol 147 Suppl 1: S287-296.

Young J. P. W. (1979) Enzyme Polymorphism and Cyclic Parthenogenesis in Daphnia magna Following Sexual Reproduction. Genetics 92: 971-982.

References: Z

Zaiontz C. (2018) Real Statistics Using Excel. www.real-statistics.com

Zapata R. et al (2003) Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase lower fecundity in the German cockroach: correlation between the effects on fecundity in vivo with the inhibition of enzymatic activity in embryo cells. Pest Manag Sci 59:10 1111-1117.

Zein M. A. et al (2015) Toxic effects of combined stressors on Daphnia pulex: Interactions between diazinon 4-nonylphenol and wastewater effluent. Environ Toxicol Chem 34:5 1145-1153.

Zein M. A. et al (2014) Optical bioassay for measuring sublethal toxicity of insecticides in Daphnia pulex. Environ Toxicol Chem. 33:1 144-51.

Zhang A. et al (2000) Insect Nicotinic Acetylcholine Receptor: Conserved Neonicotinoid Specificity of [3H] Imidacloprid Binding Site. Journal of Neurochemistry 75:3 1294-1304.

Zhang L. et al (2015) Automatic recognition of cardiac arrhythmias based on the geometric patterns of Poincare plots. Physiol Meas 36:2 283-301.

Zheng L. et al (2015) Fatiguing exercise initiated later in life reduces incidence of fibrillation and improves sleep quality in Drosophila. Age 37:77 76-88.

Zhang M. et al (2014) Cloning expression and cellular localization of the Doublesex gene in the water flea Daphnia carinata during different developmental stages. Gene 550:2 185-192.

Zhang Y. and Wu C. (1991) Alteration of Four Identified K' Currents in Drosophila Muscle by Mutations in eag. Science Reports 252 1562-1564.

Appendix: MatLab spectrum analysis

MatLab spectrum analysis for removing interference from *Daphnia* EMG results

```
% Close all current graphs and clear all current variables. Then get
the filename and header data
FileName = input('Enter ECG FileName > ','s');
% Enter the name of the ecg file to be processed
if strcmp(FileName(end-3:end),'.txt')
% Do nothing the filename already has an extension
else FileName = strcat(FileName,'.txt');
% Append the required extension
end Header = importdata(FileName,' ',5);
% Get the file header data
SampleTime = sscanf(char(Header(1)),'Interval= %e');
% Get the sampling time information from the file header
Fs = 1/SampleTime;
% Calculate the sampling frequency from the sampling time
DateAndTime = datestr(datenum('30-Dec-1899') +
sscanf(char(Header(2)),'ExcelDateTime= %f'));
%Get the Date and time of the measurement
Range = sscanf(char(Header(5)),'Range= %e');
% Get the Voltage range and echo the extracted Header parameters as a
double check.
display(sprintf('\nMeasurement date and time was
%s',DateAndTime))display(sprintf('Sampling frequency is
%dHz',Fs))display(sprintf('Voltage range is plus or minus
%dv\r',Range))
EcgData = importdata(FileName,'\t',5);
% Then get the ecg data
EcgSignal = EcgData.data(:,2);
% Assign the data to separate named variables Signal and SampleTime
SampleTime= EcgData.data(:,1); figure(1);
% plot the data in a figure, add a grid, set the axes range,
label them, and add a title.
plot(SampleTime,EcgSignal,'b'); grid; axis([0 SampleTime(end) -Range
Range]); ylabel('Amplitude in volts'); xlabel('Time in seconds');
title(['Ecg data from ',FileName(1:end-3),' Measured on ',
DateAndTime]); hold;
% On the basis of what is seen in this figure the user will set a
threshold for the interference spikes and interactively repeat for
different threshold values.
```

```
NewThreshold = 'Y'
% Initialise the need for a threshold to Yes
while NewThreshold == 'Y'
% Repeat until no new threshold is needed
SpikeThreshold = input('Enter interference spike threshold (no input
will set it to ''Range'') > ');
% Enter interference spike threshold
if isempty(SpikeThreshold)
% nothing entered so
SpikeThreshold = Range;
% So use the default
else if SpikeThreshold > Range
%this should not happen!
warning('Input is greater than %dv so Spike threshold is
set to %dv',Range,Range)
% Warn the user
SpikeThreshold = Range;
% And use the default.
end
end
plot([0 SampleTime(end)],[SpikeThreshold  SpikeThreshold],'g')
%Plot lines indicating the thresholds
display(sprintf('\nSpike threshold is plus or minus %dv',
SpikeThreshold));
plot([0 SampleTime(end)],[-SpikeThreshold -SpikeThreshold],'g')
% Using this threshold exclude data that contains interference
spikes, using a window that has continuity in some of its
derivatives.
WindowSize = 10000;
% Set the window size bin with spacing Fs/WindowSize which equals a
resolution of 0.1Hz on the raw data. Longer would
give better resolution but less averaging, however, less spikes could
be removed.
Window = hann(WindowSize,'periodic');
% Use a periodic version of the sin^2(Hann) window as we are looking
for periodic data which has continuous derivatives, and reasonable
sidelobe performance.
Index = 1:WindowSize;
% Pick up Window sized chunks of data and initialise pick up of the
first.
Jump = WindowSize/2;
% Jump through the input data one half of a window at a time which is
```

```
the optimum step.
NumberOfDeletions = 0;
% Keep a count of how many chunks of data are deleted. More than 50%
probably means the data is corrupted by spikes too much and thus
probably usless.
NumberOfChunks = 1;
% To do this keep a count of the number of chunks in the signal.
Start at one because there is always at least one chunk to work with.
DeletedSignal = zeros(size(EcgSignal));
% Finally preallocate space for the deleted chunks of signal so that
the program runs faster. Set it to zero so that we can 'add' the
deleted portions to it. Set up is now complete. Now to go through the
signal one WindowLength chunk at a time, in half WindowLength steps.
Check for spikes and delete if present. In general a spike will take
out two overlapped chunks of data or a WindowLength + WindowLength /2
sample of data.
while Index(end) <= length(EcgSignal)
% While data exists.
if any(abs(EcgSignal(Index)) > SpikeThreshold)
% Are any of the samples in the chunk greater than the
SpikeThreshold? Using the absolute value catches both sides of the
signal. If yes, add a windowed version of the signal to the deleted
signal array.
DeletedSignal(Index) = DeletedSignal(Index) + EcgSignal
(Index).*Window;
% Increment the number of deletions.
NumberOfDeletions = NumberOfDeletions + 1;
end
Index = Index + Jump;
% Move along to the next chunk, by making the index array point to
the next chunk.
NumberOfChunks = NumberOfChunks + 1;
% Count the chunks used
end
% There is now an array that represents the parts of the signal that
needs to be deleted, and an array that represents the original
signal. To delete the spikes we delete the deletions array from the
original signal.
CleanerEcg = EcgSignal - DeletedSignal;
% Calculate the percentage of deleted frames.
PercentageOfDeletedFrames = NumberOfDeletions/NumberOfChunks*100;
% Tell user the bad news:
```

```
display(sprintf('Number of interference corrupted chunks is %d',
NumberOfDeletions));
% How many frames were deleted
display(sprintf('Percentage of signal chunks deleted is
%g%%',round(PercentageOfDeletedFrames*10)/10));
% and what percentage of the whole signal these are. Display the
deleted frames on the signal, in red.
plot(SampleTime,DeletedSignal,'r'); legend('Input Signal','Upper
Threshold','Lower Threshold','DeletedSignal')
title(['Ecg data from ',FileName(1:end-3),' Measured on ',
DateAndTime,' Threshold = ',num2str(SpikeThreshold)]);
DifferentiatedEcg = filter([1 -1],[1],CleanerEcg);
% Differentiate the cleaned up signal to remove the low frequency
noise and baseline variation
FFTsize=4*WindowSize;
% FFT size used in the power spectrum the input is padded with zeros
to interpolate the spectrum by a factor of 4
[AveragePowerSpectrum,Frequency] = pwelch(DifferentiatedEcg,Window,
WindowSize/2,FFTsize,Fs);
% Calcuate the average power spectrum, using Welch's method with 50%
overlap between segments and using the same window which was used to
clean up the signal.
AveragePowerSpectrumdB = 10*log10(AveragePowerSpectrum./max
(AveragePowerSpectrum));
% Convert to dB relative to the peak
display(sprintf('Frequency of the highest spectrum peak is %gHz',
Frequency(AveragePowerSpectrumdB == 0)));
% Display the frequency of the highest peak, which should be the ECG
frequency figure and plot the spectrum that results
plot(Frequency,AveragePowerSpectrumdB)
% Plot the power in decibels (dB) normalized to the highest peak in
the spectrum
axis([0 30 -30 5])
% Set the axes to cover 0 to 30Hz and a typical dB range plot them in
a figure, add a grid, set the axes range, label them, and add a
title.
grid
xlabel('Frequency in Hz')
ylabel('Amplitude in dB relative to the peak')
title(['Ecg spectrum from ',FileName(1:end-3),' Measured on ',
DateAndTime,' Threshold = ',num2str(SpikeThreshold)]);
NewThreshold = input('Do you want a new spike threshold "Y"/"N"? (no
```

```
input will set it to "N") > ','s');
% Go round again if it's yes, stop if it's "No"
if NewThreshold == 'Y'
clf(1) % Clear the old time figure
figure(1) ;
% plot a New time figure, add a grid, set the axes range, label them,
and add a title.
plot(SampleTime,EcgSignal,'b');
grid;
axis([0 SampleTime(end) -Range Range]);
ylabel('Amplitude in volts');
xlabel('Time in seconds');
title(['Ecg data from ',FileName(1:end-3),' Measured on ',
DateAndTime]);
hold;
% Hold that figure so to add more lines to it.
end
end
% Save the figures in editable (.fig) and readable (.pdf)
forms. Save the time domain figure in matlab '.fig' and '.pdf' files.
FigureFileName = FileName(1:end-4);
% Trim the '.txt' extension off the input file name to form the time
domain figure filename.
saveas(1,FigureFileName,'fig');
% Save the figure as a matlab figure
saveas(1,FigureFileName,'pdf');
% Save Spectrum figure in matlab '.fig' and '.pdf' files.
SpectrumFileName = [FileName(1:end-4) 'Spectrum'];
% Trim the '.txt' extension off the input file name and add
'Spectrum' to form the figure filename.
saveas(gcf,SpectrumFileName,'fig');
% Save the figure as a matlab figure
saveas(gcf,SpectrumFileName,'pdf');
% Save the figure as a pdf
```