Genetic Analysis of Tritrophic Interactions between Entompathogenic Nematodes, Symbiotic Bacteria and Blood – Sucking Flies.

A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

Cassandra Edmunds

June 2018

Declaration

I hereby declare that this thesis has been composed by myself and that it has not been accepted in any previous application for a degree. The work of which it is a record was carried out by myself unless otherwise stated. All sources of information have been acknowledged by means of references and all quotations have been distinguished by quotation marks.

Cassandra Edmunds

June 2018

Acknowledgements

I would like to thank my supervisors Dr Robbie Rae, Dr Craig Wilding and Dr Rory Post for imparting their knowledge and expertise throughout this research. Also for their support, patience and unwavering good humour.

I would also like to thank the people whose arms I twisted into helping with soil collection and counting dead arthropods, in particular Natalie Hughes-Crean, David Buckley, Dr Samantha Barlow, Kate Dorman, Peter Andrus, James Waterman and Rupert and Elaine Pearman.

I am also very grateful to Rob Morris for helping to keep my nematode cultures alive while I was away on fieldwork and for always being on "Team EPN". I would also like to thank Professor Michael Clarkson for teaching me about *Ochlerotatus detritus* and their habitat.

I am grateful to BASF for the provision of EPNs and LITE for *Aedes aegypti* mosquito eggs.

Finally I would like to thank my family, especially my husband Paul for their support and patience throughout this research.

Contents

Abstract

Mosquitoes are responsible for millions of deaths a year through the viruses and parasites they vector. Many of these vector species have successfully expanded their range into temperate climates due to a combination of climate change and the easy movement of goods and people around the world. The temperate climate of the U.K. is home to 34 native species, several of which bite humans and are capable of vectoring pathogens more commonly associated with warmer climates, therefore the threat of mosquito-borne illness in the U.K. is a very real possibility. Many vector mosquito species have evolved resistance to traditional chemical insecticides and the search for novel control strategies in endemic areas is a priority in vector control.

Entomopathogenic nematodes (EPNs) are microscopic roundworms, which are obligate parasites of insects from the family Rhabditae. In particular, soil-dwelling nematodes from the genera *Heterorhabditis* and *Steinernema.* Presently EPNs are used in a range of plant-based industries as a chemical-pest control. However, previous laboratory research has shown that EPNs are capable of killing more than 250 species of insect including a selection of vector species and nuisance arthropods. This thesis is concerned with discovering whether commercially available and naturally occurring strains of EPNs from the U.K. could be used as an effective biocontrol agent for mosquito and chironomid species. This study includes a snapshot of the current EPN diversity in the U.K. which found four different *Steinernema* species, including the first molecular confirmation of *Steinernema carpocapsae*. EPNs from both field-collected and commercial sources were capable of killing and parasitizing two native and tropical mosquito species and *Chironomus plumosus*. Commercial strains were more effective at killing both, however, the native field-collected, mosquito species *Ochlerotatus detritus* was susceptible to field-caught EPNs, unlike the tropical, lab-reared *Aedes aegypti*. EPNs were found to be capable of tolerating the extremes of habitat that mosquito species can inhabit in laboratory tests. These studies have shown that with further research including viable field trials that EPNs could be very useful to add to a range of vector and nuisance control measures when used appropriately.

Chapter 1

Introduction

Not just deadly; the problems with nuisance biting, blood-sucking flies.

Outside the field of entomology, positive interpretations of insect-human interactions are scarce. Most people enjoy the sight of charismatic Lepidoptera or Coleoptera but the majority of lay-people consider their encounters with insects to be of the detrimental kind (Kellert, 1993). At one end of the scale, the class Insecta can inflict discomfort and annoyance, whether it be *Vespula vulgaris* attracted to an ice cream, Aphididae spp. destroying a prized plant or the presence of *Musca domestica* pretty much anywhere. At the other end of this scale of encounter is the undisputed death, disease and destruction that insects can bring as vectors of disease and destroyers of whole crops or species. Some examples include Dutch elm disease (*Ophistoma* (*Ceratocystis*) *ulmi*) fungus, vectored by species of *Scolytus* spp. (elm bark beetle) (Strobel and Lanier, 1981); a plague of *Schistocera gregaria* in Africa lasting 4 years (Showler and Potter, 1991) and of course, mosquitoes.

Mosquitoes are frequently described as the most dangerous animal on the planet (Gates, 2014) and they are undisputedly responsible for millions of deaths per year through the parasites and viruses they vector such as malaria, dengue and West Nile fever (Tolle, 2009; WHO, 2016). In between these two experiential extremes lies the phenomena of nuisance or pestiferous insects. The term nuisance denigrates the experience of those who are unfortunate enough to be subject to nuisance populations of insects. The nuisance can come from a range of encounters, in particular when the insects bite. However, non-biting insects such as Chironomidae (non-biting midges) can also become a major nuisance as they swarm upon emergence from the pupa and can cause damage to property and serious allergic issues for those nearby (Stevens et al., 1998; Tabaru et al., 1987). Nuisance biting can come from any haematophagous arthropod, but the predominant families are: Culicidae (mosquitoes), Simuliidae (black fly), Ceratopoginidae (biting midges), Reduviidae (kissing bugs), Tabanidae (horse-fly), Cimicidae (bed bugs) and Ixodidae (ticks). It is notable that the majority of these are known

vectors of pathogenic vertebrate diseases (Adelman et al., 2013; Adler et al., 2010; ECDC, n.d.; Mellor, 1990; Ramsey et al., 2015);

- Simulidae – onchocerciasis;
- Ceratopoginidae – blue tongue virus;
- Reduviidae – *Trypanosoma cruzi*; and
- Ixodidae – Lyme disease.

However, this review will narrow the focus to mosquitoes and non-biting midges and the impact on humans rather than animals or crops. The delineation between vector and nuisance biter is undefined but for these purposes a nuisance biting invertebrate is one that is not currently an established vector of pathogens to vertebrates. However, one study in Galena, Alaska where mosquitoes are described as the "Plague of the Arctic" (Cooke et al., 2006) found a bite rate of 2400 per hour from *Culiseta impatiens* on skin unprotected by insect repellent (Lillie et al., 1988). Whilst *Cu. impatiens* in not a known vector, that level of biting would be intolerable under any circumstances.

The total burden of nuisance biting is not well documented. Clinical data are recorded on those with immuno-reactive bites, that present to a healthcare provider (Anonymous, 2012) whereas the non-health impacts of biting nuisance are not collated. In the case of clinical presentation with bite complications, these data reside with the local Primary Care Trust but there is no single place to report on factors contributing to economic problems and very limited data on work-days lost due to insect bite events, as most people would only seek medical attention for the most troubling reactions to bites or stings. The range of clinically reported insect-bite induced issues is quite extensive. There are the typical weal and flare reaction followed by pruritic papules, allergic reactions such as 'skeeter syndrome' caused by a large local inflammatory reaction and accompanied by fever, bacterial infections like cellulitis and impetigo, loss of sleep, stress and in rare cases anaphylaxis (Carpenter et al., 2013; Simons and Peng, 1999). Misdiagnosis is an issue especially with the more aggressive allergic reactions which results in incorrect and ineffective treatment (Malcolm, 2009; Simons and

Peng, 1999). NHS figures for 2015-2016 show 85,810 attendances at A&E in England for bites and stings and 79,482 attendances for allergic reactions for the same period (allergy data includes other sources of allergic reaction), which is approximately 0.4% of all attendances (NHS Digital, 2017). Data for GP attendance due to insect bites were not available, but as the author of Anonymous (2012) indicates, 5.4 per 100,000 attendances per week are to deal with insect bites. This is figuratively the tip of the iceberg as many insect bite reactions are treated at home, ignored, or dealt with by over the counter treatments and therefore not recorded. This level of activity bears little correlation with the data available from local government authorities. Once a bite becomes infected (acute cellulitis) the typical clinical treatment is the prescription of antibiotics such as flucloxacillin (Eron et al., 2003; National Institute for Health and Care Excellence, 2015). This is increasingly undesirable due to increasing antibiotic resistance (Laxminarayan et al., 2013).

Local authorities (LAs) often undertake mosquito control activity in the U.K., but given increasing pressure on LA budgets, this would often be allocated a very limited budget, if any. In a survey of local authorities in 2009 (Medlock et al., 2012) six LAs returned information on their annual budget allocation, which ranged from £50 to £50,000. There is no central data gathering point to record nuisance biting in the U.K. In 2005 the Government set up Mosquito Watch (Medlock et al., 2012; Public Health England, 2017a), an initiative established to report mosquitos to the Government body tasked with public health in England with the intention of identifying and potentially controlling any invasive mosquitoes found in the U.K. However, there is not a wide public awareness of this scheme and its remit is not to monitor pestiferous biting but to prevent the establishment of non-native vectors (Medlock et al., 2012; Public Health England, 2017a). Medlock et al. (2012) reported that areas with long-standing mosquito problems were among the few proactive with regard control activities. Some places with a localised nuisance-biting problem such as Little Neston, a village that lies next to a saltmarsh on the Dee Estuary in North West England, has a Mosquito Watch system (Fig. 1.1). This offers a clear warning system of when to expect a heavy biting event using data regarding rain-fall,

ambient temperature and high tides. Neither this system nor the similar 'Midge Watch' employed in the Scottish highlands are in receipt of any public funding but are valuable for those visiting or working in these areas to minimise biting risk.

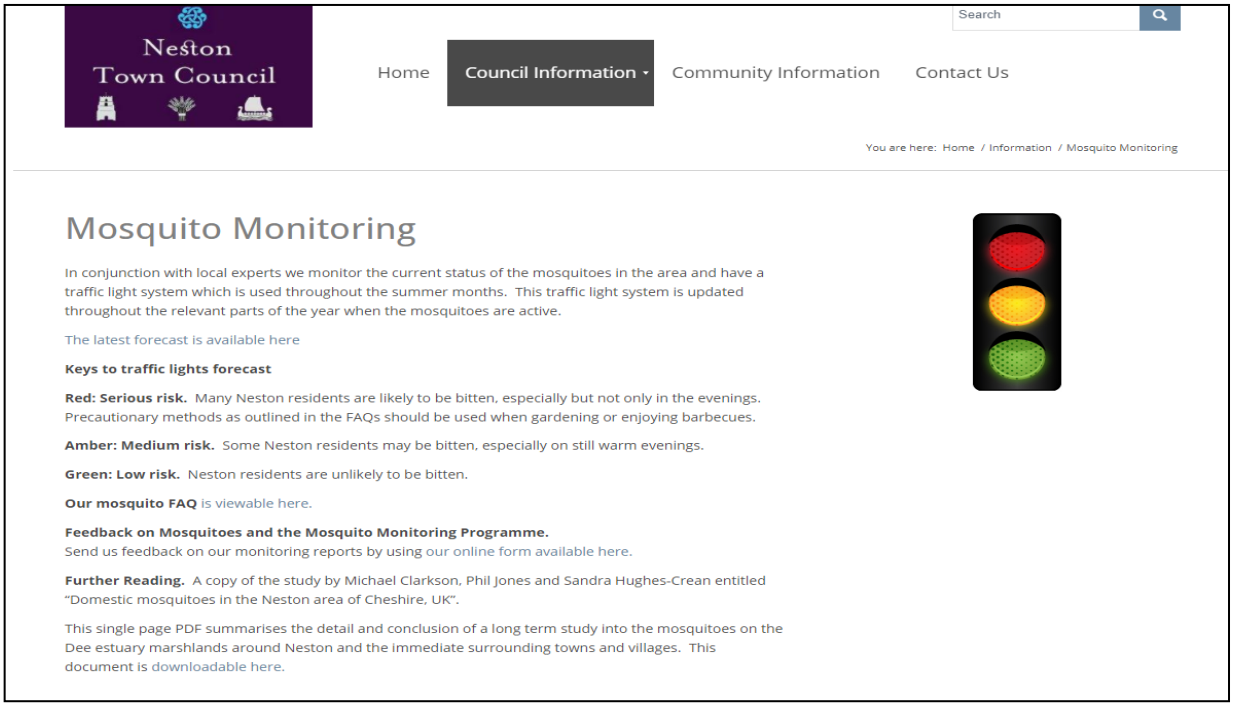


Fig 1.1. Screenshot of Little Neston mosquito monitoring webpages where residents can see the predicted level of biting nuisance caused by *Ochlerotatus detritus*. This is simplified by using a traffic light system.

Today's nuisance, tomorrow's vector?

There are 34 species of native mosquito in the U.K. several of which regularly or preferentially bite humans (Medlock et al., 2012; Medlock et al., 2015). Co-existence with these species has not always been merely a matter of nuisance, as it is currently. Between the 16th and 19th centuries in the U.K. malaria (*Plasmodium vivax*) recorded as ague, tertian or quartian fevers, was thought to be one of the leading causes of human mortality in coastal and marsh areas (Hutchinson, 2004). This was transmitted by the native mosquito *Anopheles atroparvus* (Jetten and Takken, 1994; Lindsay and Thomas, 2001). This started to decline from approximately 1840 onwards due to a number of issues, one of which was drainage of the marshes where the mosquito lived and improvements in housing and sanitation; reducing mosquito breeding and resting sites (Hutchinson, 2004; Lindsay and Birley, 1996). Reducing the opportunities for breeding and resting are still cornerstones of the work to eradicate

mosquito-borne diseases today (Lindsay et al., 2002; Ogoma et al., 2009; WHO, 1982). The issues of concern today are that a range of factors are increasing the likelihood of the establishment of vector species in non-endemic areas and the ability of local mosquitoes to transmit pathogens to the people they bite (Benelli, 2015; Benelli and Mehlhorn, 2016; Medlock and Vaux, 2014, 2011; WHO, 2016).

Human mitigated climate change has created a rise in ambient temperatures globally (Oreskes, 2004). This has enabled tropical vector species of mosquito to extend their range and establish stable populations throughout Europe. For example *Aedes albopictus*, a native of S.E. Asia, has been found as far north as Germany where they have recently been found overwintering (Walther et al., 2017) and is widespread in Europe, putting these communities at a potential increased risk of vector borne disease (Caminade et al., 2014, 2012; Epstein, 2001; Harvell et al., 2002; Met Office, 2017; Purse et al., 2012; Roiz et al., 2011; Townroe and Callaghan, 2014).

The relative ease with which people and goods are now able to traverse huge distances has created a range of problems that was not foreseen by previous generations and is changing the landscape of human/mosquito interactions. Transport of 'Lucky' bamboo (*Dracaena* spp.) has been implicated in facilitating the introduction of *Ae. albopictus* to Europe, as have imports of tyres (Caminade et al., 2012; Gratz, 2004; Hofhuis et al., 2009; Knudsen, 1995; Scholte et al., 2008). Tyres stored outside prior to shipment are perfect oviposition habitats for Aedine mosquitoes when they puddle with rain and provide a relatively sheltered habitat for larvae. A broad selection of other species including Anophelines have been found in tyres imported to Europe (Knudsen, 1995; Schaffner, 2003). The eggs of *Aedes* species are able to diapause and even survive drying events, which enables them to survive relatively long transport times (Becker, 2010; Hofhuis et al., 2009; Kampen et al., 2015). *Ae. albopictus* can tolerate cooler climates (Claeys and Mieulet, 2013; Paupy et al., 2009; Rochlin et al., 2013) which makes it a particular vector threat to temperate climates. *Aedes aegypti*, which is the primary vector of Zika as well as Yellow Fever does not fare so well in the cold but can live its life inside a building (Medlock et al., 2012; Moyes et al., 2017). However, it is particularly well adapted

to living alongside humans and it has been suggested that the 'domesticated' form of *Ae. aegypti* seen today evolved from the tree hole dwelling form *Aedes aegypti formosus* through being transported from sub-saharan Africa via the slave trade, and in doing, adapted from biting non-humans to become an anthropophage that can oviposit in virtually any container of water (Powell and Tabachnick, 2013). The Anopheline mosquitoes that are currently known vectors of malaria (e.g. *An. gambiae)* do not appear to have expanded their range to the same extent as Aedine mosquitoes have, possibly due to a particular sensitivity to temperature and predictions suggest that this will be limited to the African continent (Carvalho et al., 2017; Fuller et al., 2012).

The pathogens that these mosquitoes vector are also coming with them. The European Centres for Disease Control reported that in 2015 (latest available figures) there were 6 cases of locally acquired *P. vivax* infection in Greece (ECDC, 2018a), 122 locally acquired cases of West Nile fever mostly centred around busy urban areas such as Milan or Budapest (ECDC, 2018b) and 6 locally acquired cases of Dengue in France (ECDC, 2018c). Whilst these numbers are low compared to tropical and arid regions, the re-emergence of various mosquito borne diseases in the past 20 years (Hotez, 2016; WHO, 2016) and the fact that these are locally acquired cases is of concern, as this indicates that there is a possible reservoir of disease and a capable vector indicated with each outbreak.

Migration and the increase in exotic travel are aiding the transport of mosquito borne disease. The data above from The European Centre for Disease Control (ECDC, 2018) are just a proportion of the cases of these mosquito borne diseases reported in Europe. The World Malaria Report for 2016 (WHO, 2017) reported that there were 212 million new cases of malaria in 2015 and 6,199 cases reported in continental Europe were acquired outside Europe. Similarly, there were 580 cases of Chikungunya and 2095 Dengue cases acquired outside Europe but reported within (ECDC, 2018c, 2018d). Even the U.K. had 18 cases of travel-related Zika virus in 2017 (Public Health England, 2017b). With the current ease of transport around the globe, you do not even have to go abroad to become infected with malaria. Adult mosquitoes are often found alive in the aeroplane cabin,

passenger luggage or in wheel bays, giving rise to what is known as airport malaria with many contracting the most severe, *P. falciparum* strain (Bruce-Chwatt and de Zulveta, 1980; Danis et al., 1996; Guillet et al., 1998; Isaäcson, 1989). The point of including these slightly alarming statistics is that as the ease of human movement increases, the risk of a human reservoir of a disease such as malaria or Zika being bitten by a vector species in its expanded range also increases. As does the risk that vector transmission may occur from endemic but vector-capable species (Mier-y-Teran-Romero et al., 2017).

Many of the 34 species of mosquito native to the UK have been shown to be vector-competent for various pathogens. Many of the species here are not human biters but several do not discriminate where their blood meal comes from and these are of concern with the potential for zoonotic transmission pathogens such as West Nile virus (Medlock et al., 2005). The mosquitoes that are perpetrators of the worst biting nuisance in the U.K. are *Culesita annulata, Culex pipiens, Anopheles maculipennis, Ochlerotatus cantans* and *Oc. detritus.* An honourable mention goes to *C. pipiens molestus* which has found a particular niche habitat on the London Underground (Medlock and Snow, 2008; Snow, 1998). All of these species have been found to have the potential to vector a range of pathogens including filarial worms such as *Wuchereria bancrofti* (CDC, 2010; Hemingway and Ranson, 2000), West Nile Virus, Tahyna virus, Batai virus and Japanese encephalitis (Mackenzie-Impoinvil et al., 2015; Medlock et al., 2012). Although the complex factors involved in assessing the vectoral capacity of these species makes prediction of the actual risk to humans uncertain, all parties agree that vigilance and monitoring of the mosquito populations is crucial (Hutchinson, 2004; Medlock and Leach, 2015). One of the factors involved in vectorial capacity is the ambient temperature being able to support the pathogenic growth within the vector. For example, *P. falciparum* requires a minimum of 15-16°C to develop within the mosquito mesenteron (Hutchinson, 2004; Kligler and Mer, 1937; Lindsay and Thomas, 2001) and over time, climate change has provided an ever greater range of areas that theoretically support that development (Meterological Office, 2017) see Fig 1.2

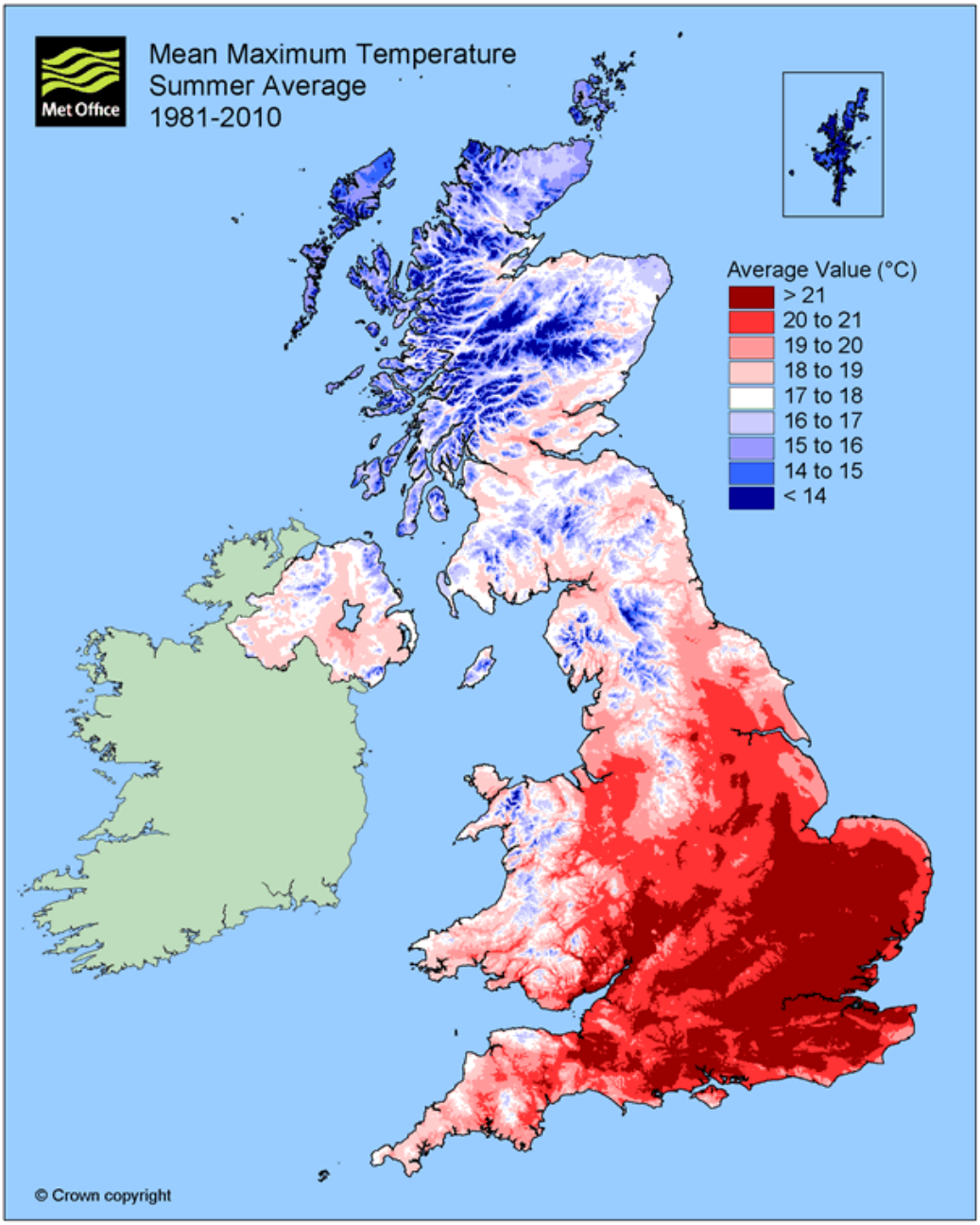


Figure 1.2 U.K. map showing mean maximum summer temperatures over a 29 year period. This illustrates that a large area of the U.K. achieves temperatures in excess of the minimum 15°-16°C required to support development of *Plasmodium falciparum* within the mosquito host (Meterological Office, 2017)

"Either the mosquitoes go, or I go" Aspects of mosquito control

Even before Sir Ronald Ross discovered the link between Anopheline mosquitoes and the malaria parasite in 1894 (Ross, 1911, 1897) there has been a desire to eradicate or control mosquito populations. One of the early pioneers in mosquito control was J.F. Marshall who founded the British Mosquito Control Institute on Hayling Island as a direct result of his refusal to suffer the nuisance

biting experienced by him and his family on moving to the island, and is quoted as saying,

"Either the mosquitoes go or I go, and I refuse to be driven out of my own house."

Although many mosquito researchers have a more altruistic impetus for their research, his findings and that of the institute are still important today (Ross, 1927; Snow and Snow, 2004). One of the issues prevalent in any discussion of vector control is that of evolving resistance. Mosquitoes are short-lived and produce many offspring, which are highly suitable circumstances for resistance to evolve. The organochloride DDT was first used for mosquito control in 1946 and by 1947 two *Aedes* spp. were resistant (Hemingway and Ranson, 2000). Resistance has continued and as a result, in 1976 the World Health Organisation changed the aim of its malaria programme from eradication to control of vectors (Enyati and Hemingway, 2009). Other classes of chemical insecticide have been developed in the intervening time such as organophosphates, carbamates, pyrethroids and include insect growth regulators such as halofenozide which mimic moulting hormones or inhibit chitin production in the larval stages (Bouaziz et al., 2017; Lau et al., 2015). At this point in time, all malaria vector mosquitoes exhibit resistance to all four of these classes (Corbel and N'Guessan, 2013; Sougoufara et al., 2017). The vector control community has particular concerns over mosquito's resistance to pyrethroids as they are used to impregnate bed nets, the use of which has become a key part of the fight against mosquito transmitted tropical disease (Hemingway and Ranson, 2000; WHO, 2017a).

Over time concerns for the environment and the welfare of those coming into contact with these chemical insecticides has driven support for research into non-chemical methods of controlling mosquito species (Bouaziz et al., 2017; Cetin et al., 2005). Biocontrol of insect pests uses a wide range of organisms from parasitoid wasps (Dahlsten et al., 1998) to predatory mites, entomopathogenic nematodes and fungus to bacteria such as *Bacillus thuringiensis* subspecies *israelensis* (*Bt/Bti*) and *Wolbachia pipientis* (Iturbe-Ormaetxe et al., 2011). Although *Bt* and other bacterial control methods including *Wolbachia* make up more than 50% ($218 million) of the biopesticide market this figure only makes up 1-2% of the total crop pest control market (Lacey et al., 2015). With regard to mosquito

and other vector biocontrol, *Bt* and *Lysinibacillus sphaericus* (*Ls*) are popular control methods as there is little evidence of resistance in vector species to *Bt* although some resistance has been reported in crop pests (Huang et al., 2017; Lacey et al., 2015). It combines many of the advantages of chemical pesticides such as conventional application, fast acting and long shelf life along with the advantages sought in biocontrol methods such as selective toxicity and limited environmental damage (Lacey et al. 2015). Some negative effects have been reported; entry of *Bti* into the trophic chain has been implicated in the reduced clutch size and survival of *Delichon urbicum* chicks (Poulin, 2012) and it requires repeated reapplication (Timmermann and Becker, 2017). Entomopathogenic fungi have been of interest to the vector control community since the 1960s when it was found to cause mortality in *An. gambiae* mosquitoes (Benelli et al., 2016). The use of *Beauveria bassiana* is promising as it provides lengthy population suppression in water but the more pathogenic *Metarhizium* spp is not well suited to a water habitat and both genera lack a robust mass production method (Benelli et al., 2016; Huang et al., 2017). Other methods of biocontrol include the introduction of larval predators such as juvenile turtles, copepods or fish. Fish species such as *Gambusia affinis, Poecilia reticulata, Tilapia mossambica* and *Sarotherodon niloticus* have been used as a natural predator of mosquito larvae and were shown to prevent resurgence of the mosquito population when used in combination with *Bt* (Huang et al., 2017). However, the lack of specificity in the fish diet meant that they had the potential to cause damage to the ecosystem and targeted mosquito species were avoiding the water bodies the fish were added to (Angelon and Petranka, 2002; Huang et al., 2017; Van Dam and Walton, 2008).

Releases of genetically modified and sterile mosquitoes have become an important tool in vector control (Alphey, 2014). Sterile insect technique (SIT) releases irradiated/sterilised male mosquitoes into wild populations with the intention of mating with wild females but producing no progeny, and thereby reducing the mosquito population over time (Lees et al., 2015) and has been used in combination with incompatible insect technique (IIT) or cytoplasmic incompatibility, which is induced by infection with *Wolbachia* spp. (Alphey 2014, Lees et al., 2015). However, these methods

are currently quite labour-intensive in terms of rearing the insects, sexing and infecting/ sterilising the mosquitoes and there is also some opposition from the public to the release of any organism considered to be genetically modified, and legislative caution in allowing field trials (Alphey, 2014; Einsiedel, 2005; Subramaniam et al., 2012). There is no current SIT element included in any operational integrated vector management plan (Bourtzis et al., 2016) but large scale open-field trials have been conducted with encouraging results. Oliva et al. (2012) reported a 50% reduction of the fertile mosquito population following a release ratio of 5:1 of sterile to wild male mosquitoes on Réunion Island. Carvalho et al. (2015) reported a 95% reduction following sustained releases of OX513A *Ae. aegypti* in Brazil and an Italian study from 2005 to 2009 showed 18-68% sterility (Bourtzis et al. 2016). RIDL (Release of Insects Carrying a Dominant Lethal) is a newer technique using genetically modified *Ae. aegypti*. The mosquitoes' DNA is modified so that they can only reach adulthood (eclosure) with the addition of the antibiotic tetracycline in their diet (Phuc et al., 2007). The modified mosquitoes are reared to adulthood in the lab with tetracycline and then males are released to mate with wild females. Any offspring from the RIDL males contain the dominant lethal gene and will die at the pupa stage due to the lack of tetracycline in their wild diet. One benefit of this late-stage mortality when compared to traditional SIT is that the offspring will survive the egg and larval stages, thereby competing for resources and space with non-modified conspecifics and but will not achieve adulthood and therefore not able to bite/transmit dengue. (Harris et al., 2012, Phuc et al., 2007). One field study in Brazil reported a 95% reduction in the mosquito population over 1 year (Carvalho et al., 2015). However, the drawbacks are that this level of suppression requires repeated releases of the RIDL mosquitoes and on a far larger scale than other genetic modification techniques (25,000-50,000 males per week/per hectare) (Flores and O'Neill, 2018). So, despite great advances in mosquito control, there does not appear to be any single solution.

Entomopathogenic nematodes past and present use

There are many potential biological control agents that can be used to control mosquitos but one of the most promising are the entomopathogenic nematodes (EPNs). EPNs are insect-parasitic roundworms from the genera *Steinernema* and *Heterorhabditis* (Kaya and Gaugler, 1993) that are widely used in insect pest control (Lacey & Georgis, 2012). They are obligate parasites with one free-living stage, the infective juvenile (IJ) stage or dauer, which persists in the soil waiting for the opportunity to parasitise a new host. EPNs are attracted to host cues including volatile chemical cues but different species have different methods of finding and attacking the insect. These different foraging strategies are found along a continuum from 100% cruiser to 100% ambush with all species strategies found somewhere along it (Gaugler and Campbell, 1993; Lewis et al., 2006). Cruiser strategy (exhibited by *Heterorhabditis bacteriophora*) is when the nematode moves through the soil seeking out a new host. These EPNs respond to volatile chemical cue or faeces that indicate a host is near and respond to a localised search behaviour rather than a ranging movement through the soil (Gaugler, 2002; Grewal et al., 1994). Other species such as *Steinernema carpocapsae* utilise an ambush strategy; waiting until a host passes and attach themselves to it, either through jumping at the insect using muscle contraction to generate a high pressure within the tough EPN cuticle (Morris et al., 2016), or exhibiting nictation; standing on the end of their tail and waving to facilitate uptake by a passing host (Bal et al., 2014; Campbell and Gaugler, 1997; Grewal et al., 1994). *Steinernema feltiae* is described as having an intermediate strategy, which combines elements of both host-seeking methods as it actively moves through the soil and frequently waves the front third of its body, similar to nictation (Campbell and Gaugler, 1997; Gaugler and Campbell, 1993). Once attached, the EPN penetrates to the hemocoel of the insect through the anus, spiracle or mouth. *Heterorhabditis* spp. use a tooth-like structure to pierce the insect cuticle (Bedding and Molyneux, 1982). The attack on a host is not a single event, other dauers will enter the host following the initial penetration (Lewis et al., 2006). The IJ behaviour

towards a host insect it encounters is dependent on a number of factors (Lewis et al., 2006). Some will only enter a recently parasitised host (Lewis et al. 1994), considered to be a strategy to increase the possibility of reproductive success, whereas 10-40% of the nematode population will only enter the host on initial encounter (Glazer, 1997). As the host becomes less able to support a high number of reproducing EPNs, dauers are repelled by chemical cues (3-Methyl-2-buten-1-ol (prenol) and 3-Hydroxy-2-butanone (AMC)) and are reluctant to enter a host in this state, again as a strategy to offer offspring the best survival chances (Baiocchi et al., 2017). Older IJs are less risk-averse and will enter an older or heavily parasitised host as a strategy to have any reproductive success before their demise (Griffin, 2012).

Once inside the hemocoel, IJs expel their symbiotic bacterial gut contents through regurgitation or defecation (*Photorhabdus* spp. for *Heterorhabditis* spp. and *Xenorhabdus* spp. for *Steinernema* spp.) (Ciche and Ensign, 2003; Forst et al., 1997; Thomas and Poinst, 1979; Wouts, 1981). This symbiosis is the cornerstone of the EPNs' success as a killer of insects as the nematode does not show as a high virulence acting alone (Burnell and Stock, 2000; Forst et al., 1997; Gaugler, 2002). Once inside the insect hemocoel the symbiotic bacteria divide and proliferate producing bacterial toxins (Forst et al., 1997) which cause septicaemia and ultimately kill the insect host, usually within 3 days (Ciche and Ensign, 2003; Gaugler and Kaya, 1990). The bacteria evade the insect immune responses by inhibiting the activation of the enzyme phenoloxidase in the case of *Xenorhabdus* spp. (Forst et al 1997), which restricts the phagocytocis and nodule formation that is the usual immune response of the insect to bacterial invasion (Forst et al 1997). Hydrolytic enzymes produced by the bacteria transform the insect cadaver to a biomass that serves as a source of sustenance for the further stages of the EPN reproduction (Ciche and Ensign, 2003). At this stage the IJs mature to adult stages and reproduce, sexually in the case of *Steinernema* and hermaphroditically for *Heterorhabditis*. The new offspring mature and feed on the bacteria produced by the decaying insect carcass and mate producing more offspring. This feeding and reproductive cycle occurs several times (usually three) (Burnell and Stock,

2000) depending on the availability of bacteria to support the process. Larger insects can support much higher numbers of EPN and allow the parasitic stages of the cycle to be completed (Bastidas et al., 2014). As the bacterial resource diminishes a number of processes can happen. The gravid EPN will not oviposit but have an intra-uterine birth and use the parent as a food source, eating their way out and killing it in the process (*endotokia matricida*) (Luc et al., 1979). Low bacterial resource within the host triggers the second stage juveniles to retain a pellet of the bacteria in their gut, retain the second stage cuticle and exit the decaying carcass as the next generation of IJs which go in search of new hosts in the soil (Ehlers and Johnigk, 1999; Taylor et al., 1979).

As well as being lethal insect parasites EPNs were found to be excellent candidates for mass production (Wouts, 1981; Ehlers, 2001; Lacey and Georgis, 2012; Shapiro-Ilan et al., 2012) and are commercially available from a number of producers such as BASF (Nemasys), Koppert, Neudorff, BioLogic and E-nema and sold to farmers and gardeners for insect pest control. In agriculture and floriculture they are utilised for the control of insect pests such as *Otiorhynchus sulcatus* and *Hylobius abietis* to good effect (Georgis et al., 2006; Lacey et al., 2015). The advantage of EPN use over traditional pesticides is that they are considered a 'green alternative', non-chemical pest control and are acceptable for use in organic growing and are non-toxic to vertebrates (Boemare et al., 1996).

.

Under laboratory conditions EPNs are able to parasitize and kill over 250 insects species from the orders Coleoptera, Lepidoptera and Hymenoptera ; Peters, 1996), including the mosquitoes *C. pipiens* and *Ae. aegypti* (Andreadis and Hall, 1976; Dadd, 1971; Poinar and Kaul, 1982; Welch and Bronskill, 1962). These EPNs were able to overcome the melanisation and encapsulation immune reactions of the mosquitoes (Bronskill, 1982). Although interesting and a promising avenue for research in mosquito control, studies using EPNs to control mosquitoes tailed off in the 1990s due to difficulty in replicating results from the lab and in the field (Georgis et al. 2006; Lacey et al. 2015). Despite this, EPNs as vector control has shown a recent revival with several researchers using different

combinations of EPN and mosquito, with some very successful results (Cagnolo and Almirón, 2010; Cardoso et al., 2016a; De Oliveira Cardoso et al., 2015; Pandii et al., 2008; Peschiutta et al., 2014.; Zohdy et al., 2013). Most notably, de Oliveira Cardoso et al. (2015, 2016) found *Heterorhabditis indica* LPP35 induced a high mortality rate (over 85%) in *Ae. aegypti* larvae in both laboratory and field trials. However, these recent assays only go part way to address the issues that exist around how practical using EPNs as a vector biocontrol could be. For example, Zohdy et al. (2013) conducted their pathogenicity assays only in a shallow Petri dish. Whilst this does achieve easily interpretable results, there is little understanding of how these results may translate to a deeper water environment and does not progress the understanding of the potential efficacy beyond that of the early pioneers of EPNs as vector biocontrol. The use of entomopathogenic nematodes for vector biocontrol is an area that has tremendous potential for success and it is important that this is explored thoroughly as an option. EPNs are already in industrial-scale production and there are well-established means of delivery and dispersal, all factors which, make any success in demonstrating their efficacy as a biocontrol for mosquitoes and other nuisance insects a tantalising prospect.

Ochlerotatus detritus; a native nuisance mosquito

Ochlerotatus (Aedes) detritus is a medium sized mosquito typically found in coastal areas throughout the U.K.(Clarkson and Setzkorn, 2011; Mackenzie-Impoinvil *et al.*, 2015; Blagrove *et al.*, 2016). It is highly halo-tolerant and gravid females oviposit in salt-marsh habitats where they lay their eggs on the soil of shallow pools which are subject to regular tidal inundations (Service, 1968; Becker, 2010). Multivoltine and an opportunistic biter of both humans and animals, it is regularly noted as one of the top 3 recorded nuisance biting mosquitoes in the U.K. (Medlock *et al.*, 2012). *Ochlerotatus* spp. mosquitoes can exhibit diapause at the egg/ 1st instar larval stage and are capable of remaining in this state for up to a year to facilitate hatching when environmental parameters are optimal, typically remaining in diapause from autumn until spring (Service, 1968; Becker, 2010; Clarkson and Setzkorn, 2011; Blagrove *et al.*, 2016). This species has several well - studied U.K. populations; Hayling Island,

Brownsea Island and the Dee Estuary. The village of Little Neston on the Dee Estuary (GPS coordinates: 53°16′ 37.2″ N, 3°04′ 06.4″ W) is home to a population of *O.detritus* which has become a severe biting nuisance for local residents and visitors. The Dee Estuary is a silted intertidal landscape with a high tide range of 7m. Over time the previously dredged site filled with silt and became a stable salt marsh habitat with a covering of maritime grasses such as *Juncus maratimus*, *Spartinia anglia* and interspersed with permanent shallow-sided pools which are inundated at high tides but otherwise receive little disturbance. This has created the perfect habitat for *Oc. detritus*. The area is protected from disturbance by legislation (SSSI, SAC, SPA and RAMSAR, due to the importance of the salt-marsh for the wide array of migrating and overwintering birds and the presence of *Salicornia* spp (halotolerant succulent plant). (JNCC, n.d.; JNCC and DEFRA, 2011), Part of the accessible salt-marsh area near Little Neston is owned by RSPB with oversight from Natural England. Cheshire West and Chester unitary local authority do not own the site, but make some attempt to abate the mosquito nuisance as part of their environmental/public health remit (Medlock *et al.* 2012). Since 1986 the local authority has sprayed the marsh with biological insecticide *Bacillus thuringiensis israelensis* (Bti) in response to biting complaints from residents (Clarkson and Setzkorn, 2011). In 2014 ditch digging was undertaken to enable better draining of the saltmarsh was carried out to reduce the oviposition opportunities on shallow sided pools. This was not repeated in subsequent years and accretion has meant that steep-sided drainage ditches are re-filling. Clarkson and Setzkorn (2011) report an improvement in mosquito numbers in years where spraying has taken place, despite spraying not covering the whole area of the mosquito-habitable marsh. However, it is not possible, nor desirable to eradicate this native species from the saltmarsh on the Dee Estuary as it is a valuable part of the food web and local biodiversity and a balance needs to be struck between any potential risk to sensitive ecology and desire to control that population.

Research aims

The central theme of this research is to gain a thorough understanding of whether EPNs are capable of being used to control nuisance insects, in particular Chironomidae and mosquitoes. As part of that, the diversity and distribution of EPNs across the U.K. has been investigated to ascertain whether the species distribution has altered since earlier surveys in 1980s and 1990s (Chapter 3). Any EPNs isolated from this survey were identified through DNA extraction and amplification of the 18SrRNA gene, giving an accurate snapshot of species distribution. Crucially, these isolated EPNs were retained and used in subsequent pathogenicity studies and compared against four available commercial strains of EPN to ascertain whether there was any difference in pathogenicity towards two mosquito species (temperate and tropical) and *Chironomus plumosus* (Chapters 3 and 4). Both commercial and wild strains were challenged with the environmental parameters that they would encounter in the field including pH, salinity and water depth. This will enable an assessment of whether there are any advantages to using wild strains over commercially available ones and whether there are species in the U.K. not currently in mass production that may be beneficial to add. Also, this has the benefit of identifying which EPN species is best to target a particular pestiferous insect. This research has also examined how mosquitoes defend themselves by investigating the molecular mechanisms *Ocherotatus detritus* uses to defend against nematode infection with the objective of understanding the genetic mechanisms underpinning this immune response (Chapter 5). *Ochlerotatus detritus* currently has no reference genome so this involved creating a *de novo* transcriptome from the RNA of this mosquito and using that as a reference to understand what happens when the mosquito is under threat from EPNs. This is the first time this mosquito species has been subject to scrutiny via next-generation sequencing and will provide an important resource for any future study into this temperate climate mosquito.

Chapter 2

The susceptibility of *Chironomus plumosus* to commercially available EPNs; a trial for blood-sucking insects

Introduction

The Chironomidae is a large family of Diptera with a cosmopolitan range including two species found in Antarctica (Lin and Quek, 2011; Oliver, 1971; Usher and Edwards, 1984). Their ubiquitous nature may be in part due to the organism's tolerance of a large range of environmental factors such as water temperature and heavy metal pollution (Pinder, 1986). Chironomids are holometabolous with the vast majority of the life cycle spent in an aquatic larval stage. With few exceptions, the short-lived adult stage of the organism does not feed as the main purpose is to mate and oviposit (Oliver, 1971). To that end, chironomids exhibit adult mass emergence, which is thought to be a strategy evolved to increase the likelihood of finding a suitable mate and provide a sufficient quantity of individuals to allow sexual selection whilst overwhelming potential predators (Oliver, 1971; Lin and Quek, 2011). The ubiquitous presence of chironomids in freshwater systems brings them into contact with human habitations where their mass emergence and aerial swarming can cause a nuisance and some health hazards. The swarming behaviour of the adult stage has been reported as causing a number of issues including allergic reactions (Baur et al., 1982) leading to asthma attacks (Sakai et al., 1993), rhinitis (Cranston et al., 1981), discomfort and distress at adults flying into eyes mouth or nose (Ali, 1980) and even asphyxia (Cranston et al., 1983). Other reported nuisances include causing traffic accidents through adults covering car windshields and lights and dead Chironomidae causing the road and airport runway surfaces to become slippery (Ali, 1980; Armitage et al., 1995; Failla et al., 2015), defacing of paintwork through meconium deposits (Cranston et al., 1983; Sakai et al., 1993) and general restriction of outdoor activity (Ali et al., 2008). The small size of the adults means that they can pass through door and window screens, causing a nuisance inside the house, and larvae have been found to pass through household water systems and contaminate drinking water (Ali, 1980; Failla et al., 2015). The

larvae can also be significant pests of agriculture, causing damage to rice seeds and plants if they enter the irrigated growing environment (Ali, 1980; Failla et al., 2015) where they can cause substantial crop failure through eating the rice endosperm, shoots and causing turbidity in the water (Stevens et al., 1998, 2006). In aquaculture they are equivalently pestiferous, potentially contaminating fish stocks and causing hazards to workers (Failla et al., 2015; Tabaru et al., 1987).

Bacteria associated with egg masses and the gelatinous egg matrix of Chironomidae species, are a cause for potential infection by *Vibrio cholerae*, *Salmonella* spp., and *Aeromonas* spp. (Failla et al., 2015; Figueras et al., 2011) being found in the eggs, some of which can pass over to the larval and adult stages (Failla et al., 2015; Moore et al., 2003). As chironomid larvae form a large part of the diet of many other aquatic species there is concern that these potentially harmful bacteria can find their way through the food web (Failla et al., 2015; Halpern et al., 2008) and may also be transmitted to humans via drinking water systems (Beaz-Hidalgo et al., 2012; Figueras et al., 2011). Similarly, there is concern over the bioaccumulation of metals, which some Chironomidae species have a resistance to and could also be passed up the food chain (Senderovich and Halpern, 2013, 2012).

Current control methods take a number of forms. Most widely used are chemical insecticides and insect growth regulators (Failla et al., 2015; Tabaru et al., 1987). These include the use of organophosphates such as chlorphoxim (obsolete), temephos, fenthion and trichlofon (Ali, 1991; WHO, 2009). Insect growth regulators such as pyriproxyfen and general insecticides such as permethrin, have also been widespread in controlling nuisance populations (Failla et al., 2015; Stevens et al., 2006; Tabaru et al., 1987; WHO, 2009). Other chemical-based methods include shock chloramination (Broza et al., 1998) and adding 35% hydrogen peroxide to water bodies. The use of coagulant polymer Cat-floc LS (Alexander et al., 1997; Tabaru et al., 1987) has also been trialled with varying degrees of success. Physical control methods such as using highly-polarised light in traps and attracting adults with sound (Failla et al., 2015; Hirabayashi and Ogawa, 2000), rotational flooding and drying of breeding areas, increasing depth of water bodies and removal of substrate in concrete

storm drains (Ali, 1980) have also been used. The effectiveness of these treatments is related to the species targeted and the places that they are breeding, with not all species as susceptible as others to particular control methods (Failla et al., 2015). As with the control of any insect species, the use of these different treatments raises concerns as to the effects on non-target species (Failla et al., 2015) and increasing resistance to chemical control is a significant issue for future control programmes for a wide range of pestiferous insects. Biological control methods, using natural enemies of chironomid larvae have included the use of odonate (dragonfly) nymphs (Arena and Calver, 1996) or Cyprinidae (carp) (Bay and Anderson, 1965). The latter was thought to be less valuable as a means of chironomid biocontrol in large lakes as the fish did not augment the existing environmental controls and disturbed the lake environment.

Previous research has found that parasitic nematodes from the family Mermithidae are capable of parasitizing the aquatic larval stages of Chironomidae (de Doucet and Poinar, 1984; Johnson and Kleve, 1996; Poinar, 1964) but there is no research to date that explores the capability of entomopathogenic nematodes (EPNs) from the genera *Steinernema* and *Heterorhabditis* to parasitize Chironomidae. Therefore, one aim of this study was to investigate whether commercially available EPNs can be used to kill *C. plumosus* under lab conditions. However, the primary aim of this chironomid research was to develop and refine methods of testing the control potential of EPNs against native and tropical mosquitoes (see Chapter 4). *Chironomus plumosus* was used for this purpose as they are inexpensive to purchase, easy to acquire from pet supply shops and do not require any specialist handling or rearing in their long larval stage. In contrast, mosquito eggs or larvae have to be acquired from specialist providers such as the Pirbright Institute or the Liverpool Insect Testing Establishment (LITE), which require a significant amount of administrative paperwork and although some providers will supply mosquito eggs free of charge for students, to purchase they can be costly. In addition, mosquito larvae readily pupate and eclose to adulthood in a variety of conditions therefore risk assessment and specialist equipment such as rearing cages and a means of blood-feeding adults

are required. As such, refining testing on a non-biting, non-blood feeding aquatic larvae was deemed to be a sensible step.

Materials and Methods

Sources of invertebrates

Pelagial larvae of *C. plumosus* were purchased from Andy's Aquatics, Wirral, U.K. Aquapets, Liverpool, U.K. and Chico's Pets, Liverpool, U.K. Larvae were immediately transported in a chilled receptacle back to the laboratory where they were stored at 10°C in their original packaging. No food was provided for the *C. plumosus* larvae. Entomopathogenic nematodes (*Steinernema feltiae, Steinernema kraussei, Steinernema carpocapsae* and *Heterorhabditis bacteriophora*) were supplied by BASF Agricultural Specialities in a proprietary storage matrix and kept at 8°C until use, following manufacturer's instructions. All EPNs were used within 28 days of receipt and all Chironomidae experimentation was initiated within a maximum 2 days of receipt of larvae.

Influence of different doses of EPNs on the survival of C. plumosus

EPN challenge was conducted in 200ml disposable plastic drinking cups (70 mm diameter top x 44mm diameter base x 80mm tall), which were sourced from local supermarkets (Tesco plc. and Asda plc.). Following guidelines from the World Health Organisation (2005) for larval testing, twenty-five *C. plumosus* larvae were placed in separate cups containing 100ml of distilled water. To the cups, 0, 1000, 2000, 4000, 8000 or 16000 of each separate EPN species were added. Four replicates were conducted for each dose. These doses were based on testing multiples of the field application rate of 50 nematodes per cm^2 (Campos-Herrera, 2015). The area of the cups (38.46 cm^2) would warrant a dose of 1923 nematodes. The higher doses used had previously been shown to kill a range of insects (Campos-Herrera, 2015; Gaugler, 2002) and would enable any assessment of an ideal effective dosage.

This process was repeated for each of the four tested EPN species. Each cup was covered with netting secured with an elastic band to avoid loss through emergence and were incubated at 10°C to recreate the lower temperature water that *C. plumosus* would ordinarily inhabit (Hilsenhoff, 1966), yet be within the known active range of the EPN species. Larval survival was recorded daily for 21 days. Larvae were adjudged to be dead if they were unresponsive when touched with a seeker. Each condition was tested 12 times. Any *C. plumosus* that died were examined for presence of nematodes as the causal agent of death.

Potential pathogenicity of EPN transport medium on C. plumosus

EPNs are stored in a water dispersible gel carrier (proprietary formulation). To determine if this affected the survival of *C. plumosus*, EPNs were autoclaved at 121ºC for 20 min to kill all EPNs and associated bacteria and allowed to cool to room temperature before being added to the plastic cups containing 25 chironomid larvae. It should be noted that it did not prove possible to separate the EPNs from the gel formulation and therefore it was necessary to kill the nematodes via autoclaving which may have altered the chemical formulation or physical properties of the gel. Each autoclaved EPN species with gel formulation was added to the cups at the same concentrations as the live experiment, which was intended to introduce the same amount of carrier gel at the different dosages as had been present with the live nematodes. The survival of *C. plumosus* was monitored daily for 21 days. Each condition was tested a total of 12 times. Any dead *C. plumosus* were examined for the presence of EPNs.

Survival and distribution of EPNs in a column of water

EPNs from the genera *Steinernema* and *Heterorhabditis* inhabit the water film between soil pores and are considered soil-dwelling organisms rather than aquatic. As such, it was important to discover if their survival would be affected over time in water. Thirty plastic measuring cylinders (1 litre) were filled with 830 ml distilled water (a depth of 30 cm). Each cylinder was marked at 5 cm

intervals. Ten thousand *S. feltiae* were added to each cylinder. *S. feltiae* was used as it exhibited the greatest longevity in preliminary experiments. The cylinders were then covered with fine netting and secured with an elastic band to prevent any external debris contaminating the vessel. Cylinders were kept at room temperature (19-25°C). After 6, 12, 24 and 96 h following nematode application 150 ml was removed from the top 5 cm of the cylinder with a rota-filler and examined for nematodes. This process of removal of 5cm 'slices' of the water in the cylinder continued until the measuring cylinder was empty. The numbers of nematodes were quantified from 3 replicate cylinders. Experiments were done in triplicate resulting in 9 tests of each condition. The rota-filler was used to ensure minimal disturbance to EPN position in the water column and enable the analysis of 'slices' or sections at 5cm intervals throughout the height of the water column

Statistical analyses

The survival of *C. plumosus* exposed to each EPN species and potential pathogenicity of the transport medium were analysed using Log-rank tests in the OASIS software (Yang et al., 2011). OASIS provides a statistical analysis of the complete length of the survival experiment. Full statistical analyses are available at **Appendix 1.**

Results

Survival of C. plumosus to different doses of EPNs

All four EPN species were effective in killing *C. plumosus*. At four days, survival was <20% for treated larvae in comparison to >80% for control larvae unexposed to EPNs (Figures 2.1a to 2.1d). The survival of untreated *C. plumosus* was significantly different from those that were exposed to different doses of *S. kraussei, S. feltiae, H. bacteriophora* and *S. carpocapsae* after 4 days exposure ($P<0.01$) (Figures 2.1a-d).

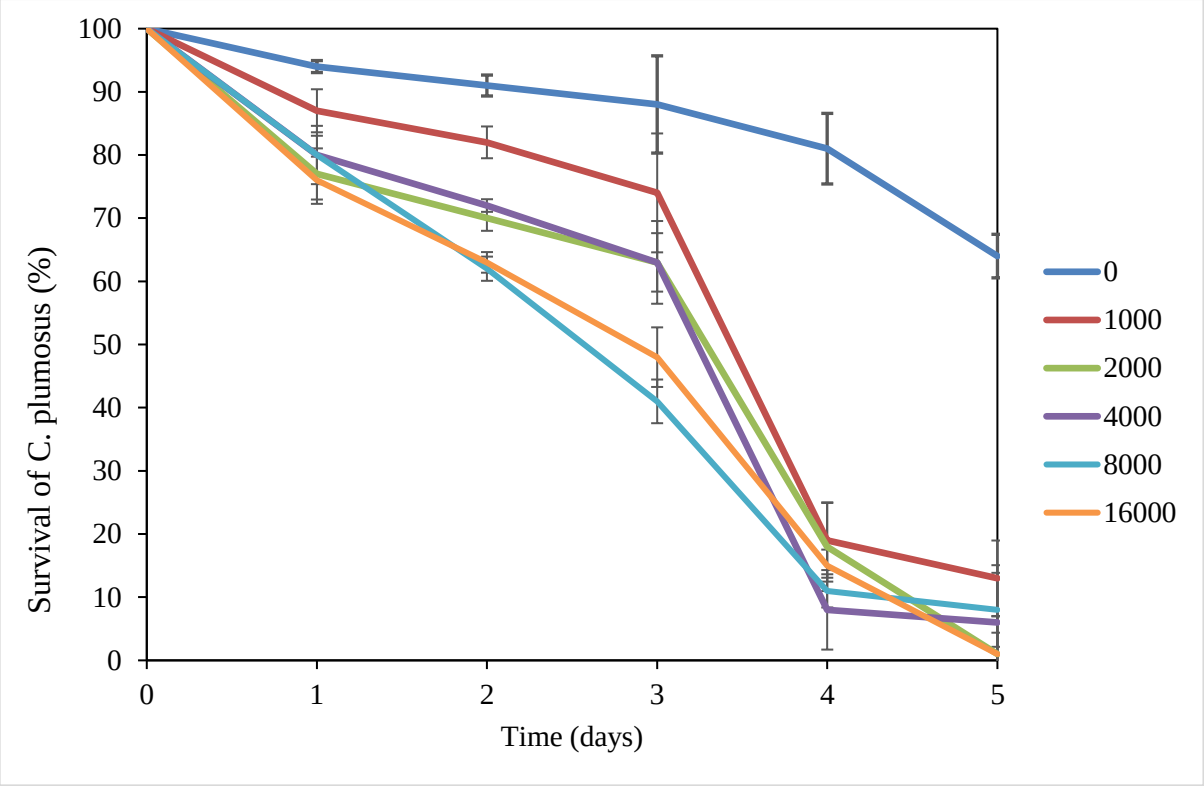


Figure 2.1a Survival of *C. plumosus* (n=25) exposed to *S. feltiae* at different doses. Bars represent ± one standard error of the mean.

All nematodes (*S. feltiae*, *H. bacteriophora*, *S. kraussei* and *S. carpocapsae*) showed no significant dose-response with 1000-16000 being equally effective at killing chironomids (Figures 3.1a to 3.1d) ($P<0.01$) (for complete results of statistical analysis see **Appendix 1**). *H. bacteriophora*, *S. kraussei* and *S. carpocapsae* showed clear larvicidal effects from day two onwards whilst significant effects were not seen until day four for *S. feltiae* ($P<0.01$). *S. kraussei* killed *C. plumosus* fastest with <70% survival after just 2 days ($P<0.001$). When dead, *C. plumosus* were examined for the presence of nematodes after 5 days exposure, infective juveniles were found that had penetrated inside from the initial experimental dose of nematodes and that had developed into adults and reproduced via *endotokia matricida* of the first generation *S. kraussei* females (Figs. 2.2, 2.3).

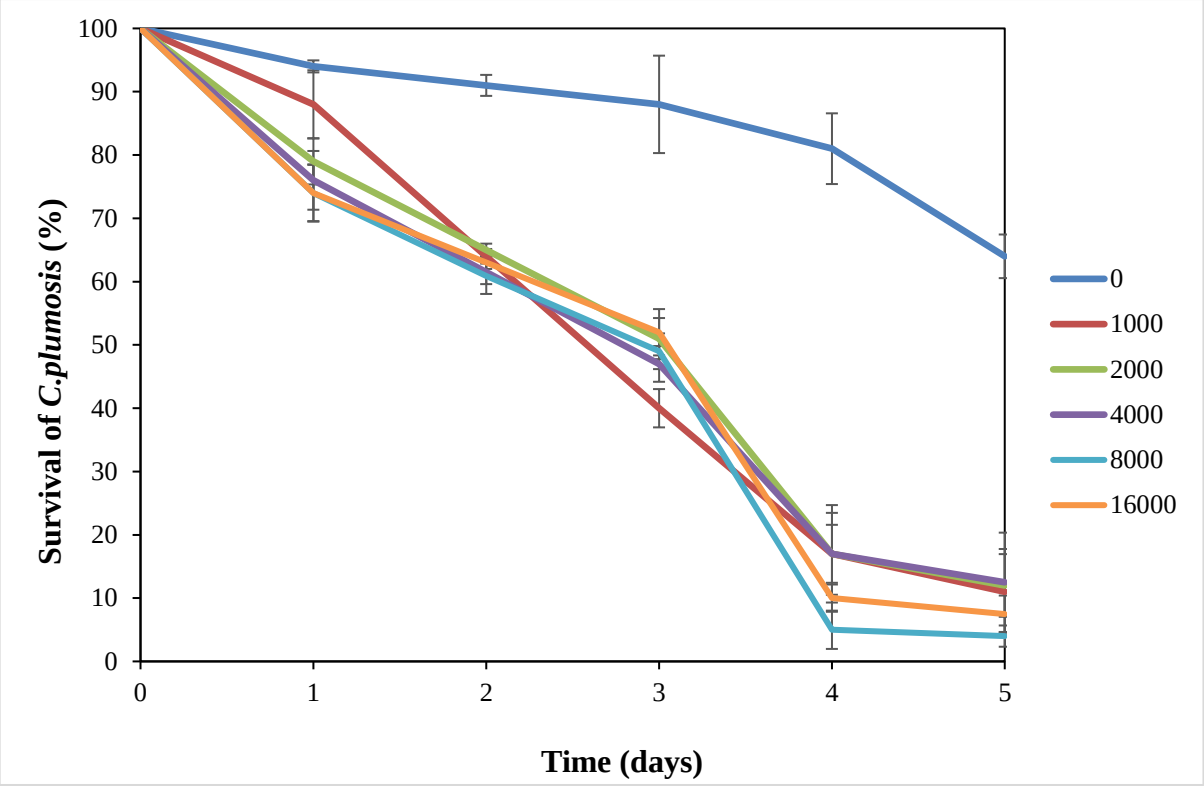


Figure 2.1b Survival of *C. plumosus* (n=25) exposed to *H. bacteriophora* at different doses. Bars represent ± one standard error of the mean.

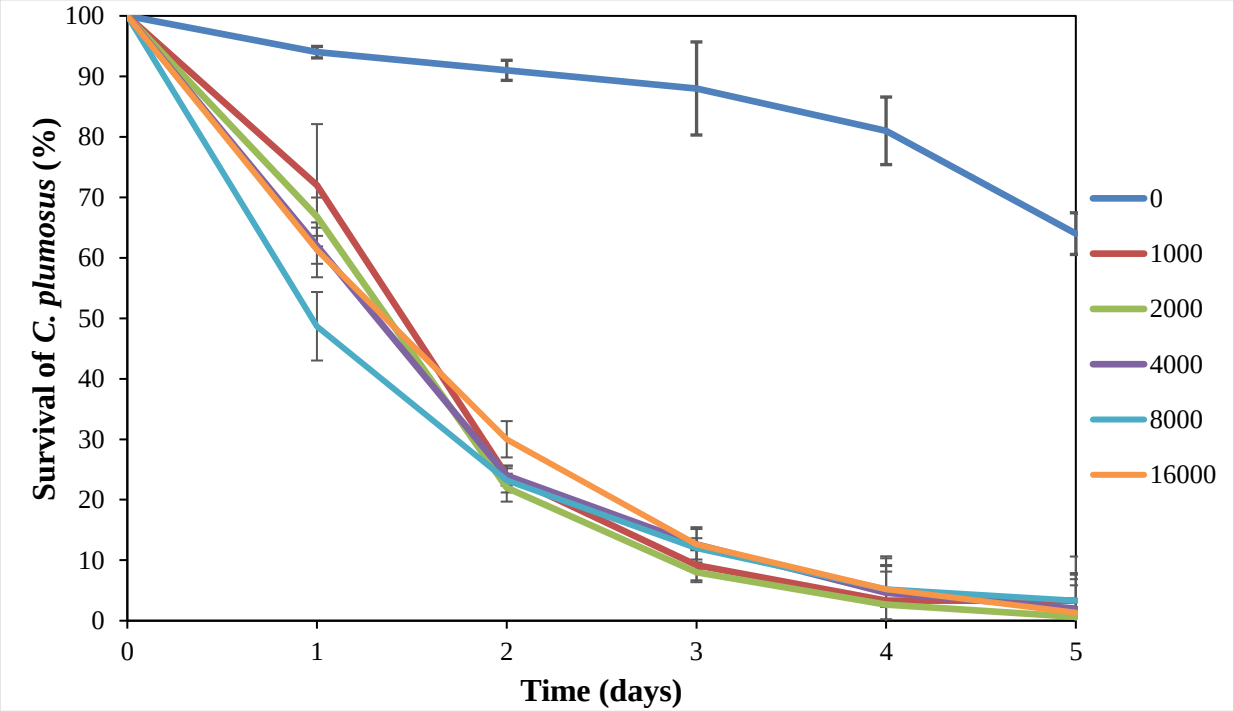


Figure 2.1c Survival of *C. plumosus* (n=25) exposed *S. kraussei* at different doses. Bars represent ± one standard error of the mean.

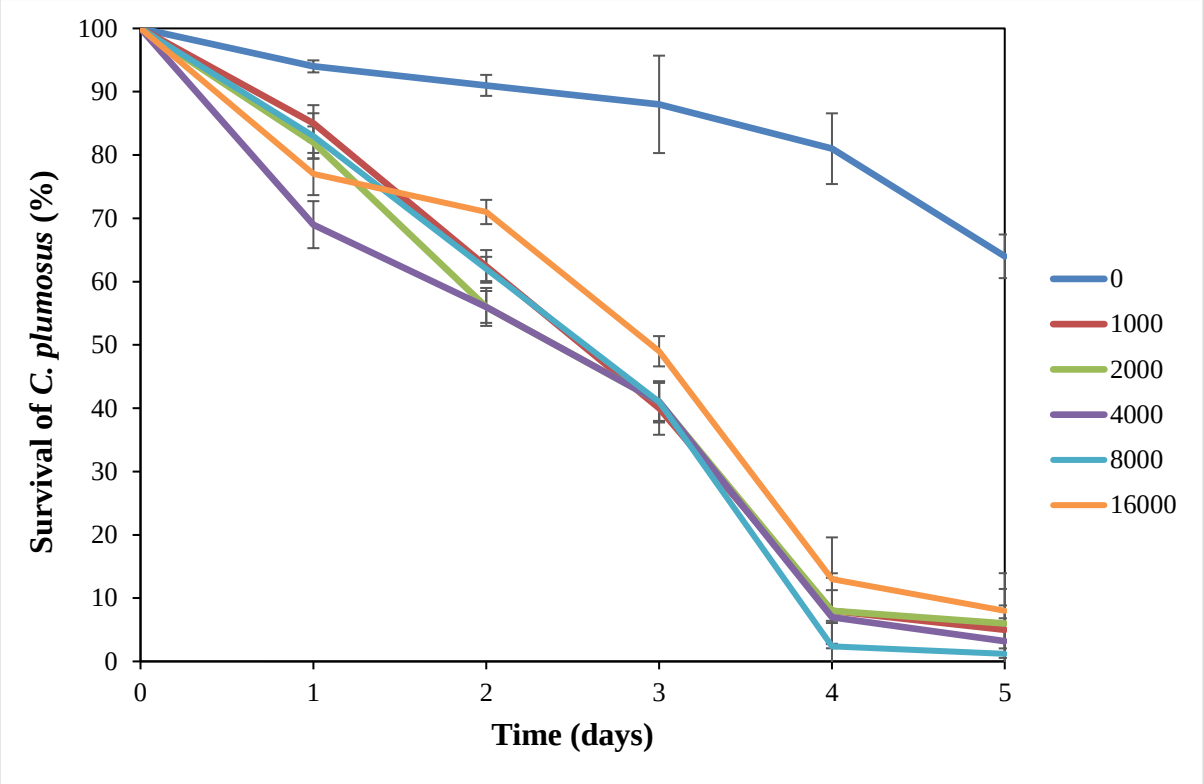


Figure 2.1d Survival of *C. plumosus* (n=25) exposed to *S. carpocapsae* at different doses. Bars represent ± one standard error of the mean

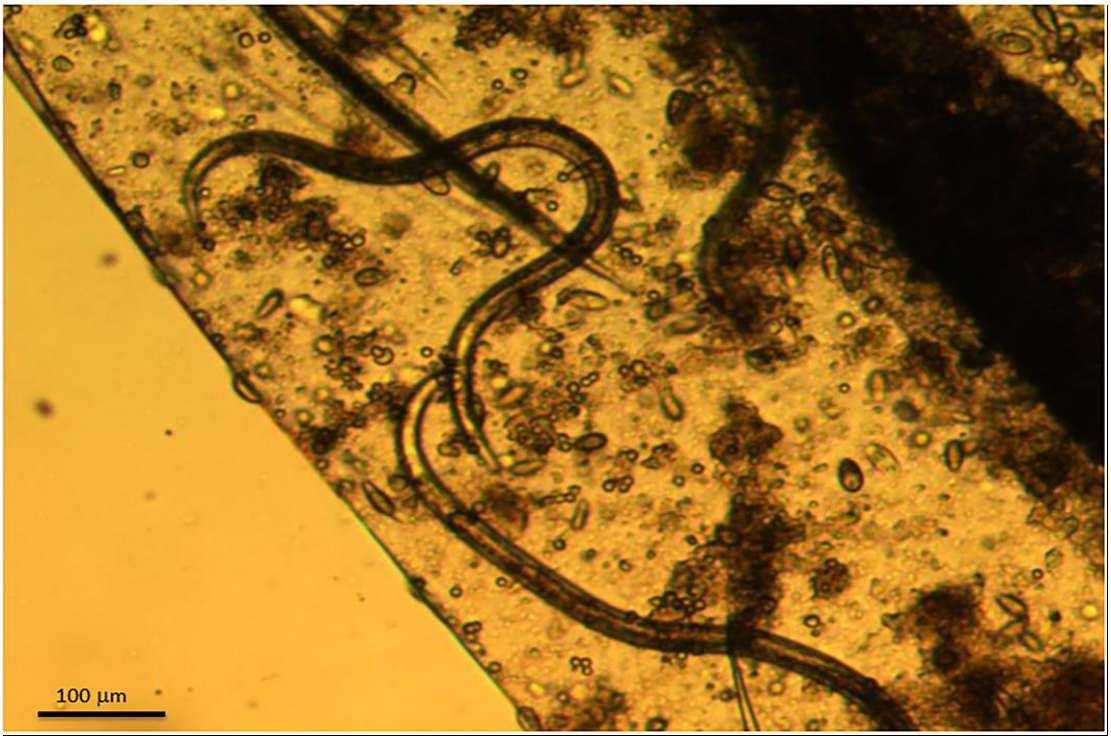


Fig 2.2 Infective juveniles of *S. kraussei* have penetrated the cuticle of the *C. plumosus* larvae.

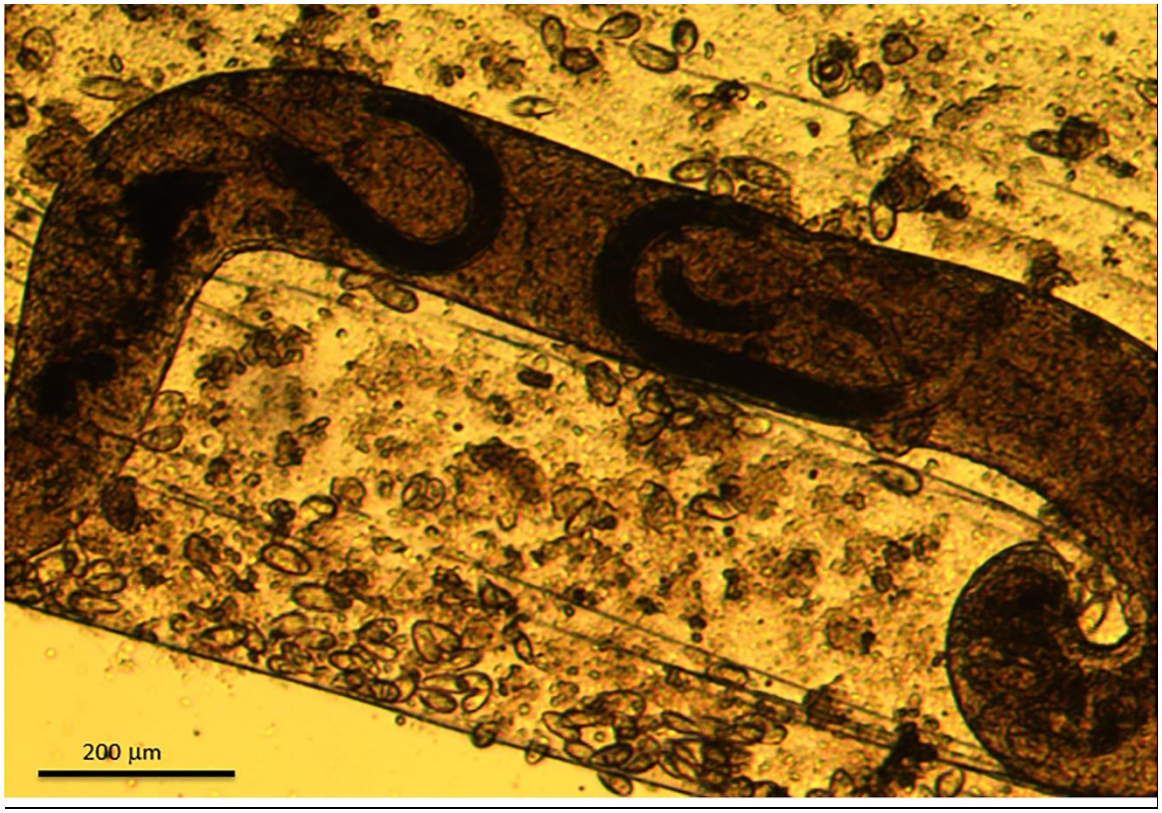


Fig. 2.3 *Endotokia matricida* of adult *S. kraussei* inside *C. plumosus* indicating reproduction has occurred post-penetration of the larvae.

Potential pathogenicity of EPN transport medium

In contrast to the experiments using live EPNs there were no significant differences in the survival of *C. plumosus* exposed to different doses of gel transport medium from *S. kraussei; S. feltiae; H. bacteriophora* or *S. carpocapsae* and the untreated control throughout the 7 days of exposure ($P < 0.05$). The results for *S. feltiae* are shown below as an illustration (Fig 2.4) as all other results were the same.

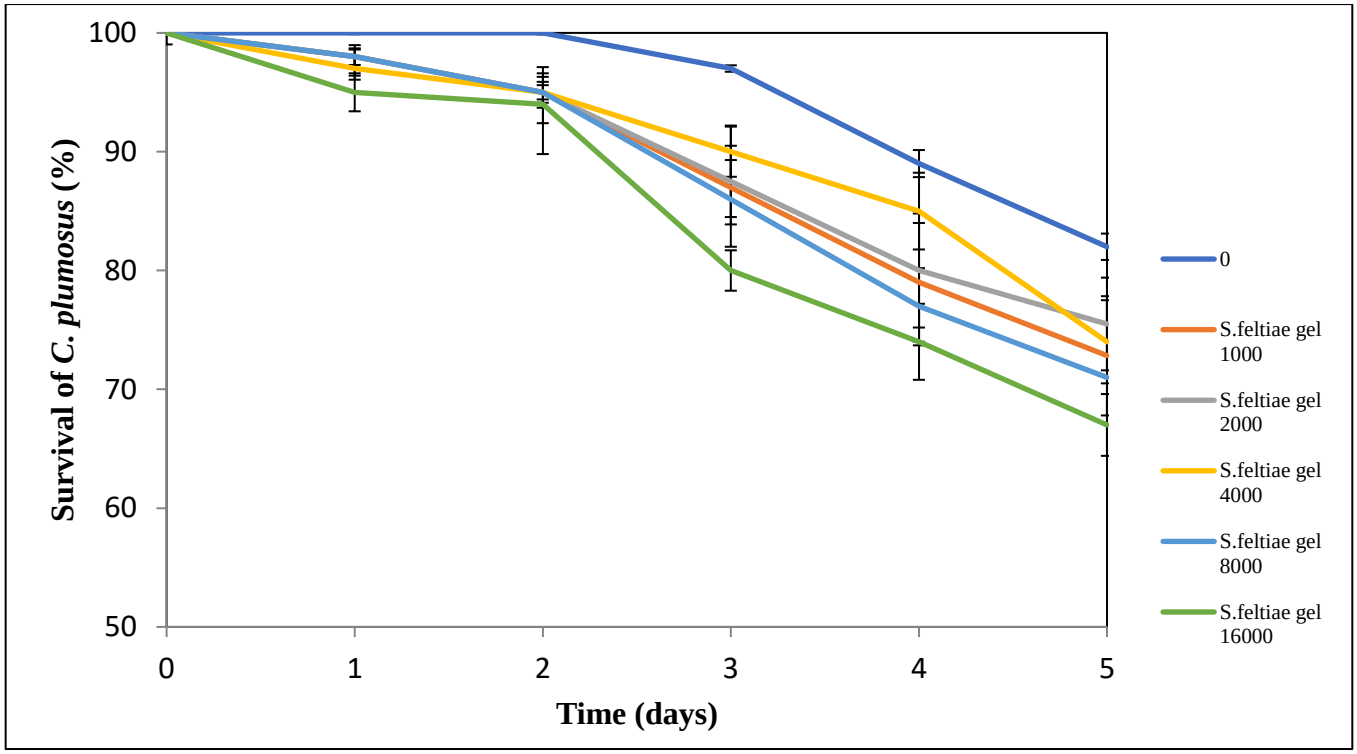


Fig. 2.4 Survival of *C. plumosus* exposed to autoclaved transport medium, at corresponding volumes of the gel/water/EPN solution to that which previously held different doses of live *S. feltiae*. Bars represent ± one standard error of the mean.

Survival of EPNs in water column

Following 6 h in a 30cm water column the majority of nematodes were found at 25-30 cm depth (Table 2.1). Between 6 and 96 h in the water column, > 96% of nematodes were found to be alive at the bottom of the water. Following the first 24 h, all the EPNs found at the top of the water column were dead, suggested they had floated to the top following death.

Table 2.1: Mean number of *S. feltiae* (± 1 SE) found at different water depths in a 30 cm column of water over time following the addition of 10,000 nematodes. Standard error shows high variation in middle depths, likely due to continued sinking of EPNs in water column at point of measurement

Depth (cm)	**Mean number of *Steinernema feltiae* found ± SE**				
	6h	**12h**	**24h**	**48h**	**96h**
0-5	47.11 ± 14.64	36.67 ± 9.99	14.67 ± 3.64	2.00 ± 0.97	5.11 ± 0.73
5-10	59.56 ± 18.61	34.44 ± 9.49	36.67 ± 12.38	2.44 ± 0.99	6.33 ± 1.12
10-15	87.78 ± 22.33	70.33 ± 22.91	83.11 ± 36.92	5.44 ± 1.79	6.33 ± 2.06
15-20	90.67 ± 19.5	87.00 ± 30.26	115.56 ± 54.67	7.44 ± 3.02	8.11 ± 2.04
20-25	119.56 ± 25.65	99.56 ± 30.40	122.56 ± 58.59	27.22 ± 14.91	9.44 ± 2.04
25-30	9596.33± 9.63	9672.00± 7.65	9627.44 ± 8.33	9955.44 ± 8.33	9964.67 ± 6.44

Discussion

This study found that aquatic larval stages of *C. plumosus* were parasitized and killed by four species of EPNs from two genera, with significant mortality observable after 2 days and the clearest mortality across all species visible after 4 days of exposure. *S. kraussei* exhibited the quickest pathogenic effect against the *C. plumosus* larvae. *S. kraussei* also required fewer nematodes (1000 nematodes) to kill faster with only approximately 20% of the chironomid larvae alive after 48 h exposure. *S. kraussei* is known to be a cold tolerant EPN (Dolmans, 1983; Willmott et al., 2002) with the most effective thermal niche breadth of 6-15°C (Mráček et al., 1999; Richardson et al., 2000) although this can be increased through conditioning during storage (Guy et al., 2009). The assays for this experiment took place at 10°C, which is within the infection thermal niche for that species (Willmott et al., 2002). Previous studies have shown that *S. feltiae* has an infective thermal niche

breadth of 8-30°C, *S. carpocapsae* 12-32°C and *H. bacteriophora* 12-32°C (Grewal et al., 1994) so they would not be inactive at the incubation temperature of 10°C but may be more biologically active at warmer temperatures. The successful pathogenic effect of *S. kraussei* at a relatively low temperature, indicates that it may be more suitable for practical application in colder or deeper water bodies and its quicker mortality rate against chironomid larvae may be highly advantageous as a biocontrol agent in a water body.

There was no significant difference between the survival rate of *C. plumosus* exposed to the different concentrations of each EPN species used, indicating that using more nematodes did not increase the mortality rate as might be expected with a traditional chemical insecticide. This is likely to be the result of overcrowding of the EPNs with only 25 potential larval hosts in 100ml water. This result is supported by the findings of Selvan et al. (1993) and Bastidas et al. (2014). Selvan et al. (1993) showed that fewer *S. carpocapsae* were able to successfully penetrate *Galleria mellonella* larvae when the concentration of nematodes increased, with the EPNs themselves dying within the host when applied at higher densities (Selvan et al., 1993). The size of the host organism is likely to be a contributory factor to the results of this experiment. Much standard EPN pathogenicity research is carried out using late instar larvae of *G. mellonella*, which weigh approximately 200-300mg (Selvan et al., 1993; Banville et al 2012) and can produce large numbers of some EPN species when cultured *in vivo*. However, Chironomid species have a wet weight range of 0.89 to 12.82 mg (Edwards 1952) and are therefore much less likely to be able to support as large a number of infective juveniles (Bastidas et al., 2014) as the insect host is a "steadily declining resource," as the more that penetrate, the faster the food resource declines (Selvan et al., 1993). In the highest dosage assays there were 640 EPNs for every *C. plumosus* larvae. The maximum carrying capacity of a *G. mellonella* host was identified as 100 by Selvan et al (1993). If these figures can be extrapolated to other host genera, given the difference in body weight, the maximum carrying capacity of a chironomidae larva (using greatest size range) would be 4.27 EPNs per individual. In a cup containing 25 *C. plumosus*, this would suggest

that the best pathogenic effect might be seen at a dosage of approximately 107 EPNs per cup in this experiment. However, previous studies have showed micro-hosts (< 5mm) to be susceptible to EPN penetration with better rates of infection and host mortality when used as an inundative application (Bastida et al., 2014). Another factor which may contribute to the lack of a correlation between high dose and high mortality is that infective juveniles have been found to be repulsed by olfactory cues (prenol) given off by already parasitized insect hosts. It is theorised that this behaviour is a way of avoiding a dwindling resource and thereby increasing the likelihood of successful parasitic event (Baiocchi et al., 2017), although whether this remains a factor in an aquatic medium remains untested.

The nematodes utilised in this study were supplied by the manufacturer in a water-dispersible gel-based matrix (proprietary formulation). Assays using the autoclaved transport medium were used to determine whether it had any pathogenic effect on *C. plumosus*. The experiments mirrored exactly those with live EPNs so any effect of an increased amount of transport medium could be identified. There was no pathogenic effect identified from the transport medium. It might have been expected that the highest amount of the autoclaved transport gel could have caused mortality in the relatively small volume of the test cup. However, if EPNs were administered in the field using the transport medium, any effect of a large amount of the transport medium may have on target or non-target organisms would not be a relevant drawback to usage, as chironomids (and more pertinently, mosquitoes, which are the ultimate target of this research), typically inhabit much larger bodies of water (Ali, 1980; Ali et al., 2008; Failla et al., 2015; Lin and Quek, 2011).

The larval stages of the Chrionomidae life cycle are benthic (Prat and Rieradevall, 1995). In order to effectively control them, EPNs must be able to reach them at the bottom of the water column and survive for the period required to exhibit mortality effects. The results from the depth assay indicates that the EPNs sink to the bottom of the water body in the first 6-12 h following application and remain at the bottom. The few *S. feltiae* found at the topmost sections of the water column following this time period were dead and those at the lowest two sections (20-30cm) were found to be

alive. This suggests that those higher up had floated to the top following death. This is to be expected as it is understood that EPNs cannot actively swim through the water column due to the lack of friction (Koppenhöfer et al., 1995; Lewis et al., 1998; Wallace, 1958). The pathogenicity assays show that the most effective EPN concentrations were able to penetrate and kill more than 90% of the chironomidae within 4 days. After 96 h the majority of the EPNs in the depth assay were still alive, demonstrating that although the nematode is not considered to be an aquatic invertebrate it would survive long enough to potentially be useful as biocontrol agent for aquatic invertebrates that spend time at the bottom of the water body. When in the water EPNs are presumably able to penetrate *C. plumosus* through the mouth, anus, spiracles or even directly through the cuticle as they do with terrestrial insects (Gaugler, 2002). Penetration of the larvae cuticle was observed in the aquatic larval stage of the mosquito *Culex pipiens*, where *H. bacteriophora* penetrated directly through the anterior portion of the alimentary tract (Poinar and Kaul, 1982).

These results show that EPNs can infect, kill and reproduce in *C. plumosus*, however, it is unknown whether the infective juvenile stage would survive emergence from the host in a deep aquatic environment and this requires further study if they were to be taken further as a potential means of control. In conclusion, field trials of the use of EPNs to combat nuisance populations of Chrionomidae would be an essential step forward in the validity of their use as an effective biocontrol but delivery and survival of EPNs in a deep water environment would likely prove challenging to overcome. However, the results from these studies were highly promising for moving forward to using this technique on mosquito larvae.

Chapter 3

The Diversity and Distribution of Entomopathogenic nematodes in the U.K.

Introduction

 [redacted]

as far apart as Thailand (Thanwisai et al., 2012) to Sweden (Burman et al., 1986) and New Zealand (Ali and Wharton, 2017). Different parts of the U.K. have been surveyed at various times; Scotland by Boag *et al*. (1992), Northern Ireland by Blackshaw (1988), England and Wales by Hominick and Briscoe (1990) and Gwynn and Richardson (1996) covering England, Wales and Scotland. Most recently a survey covered two transects in South West England and Wales (Al-Own, 2013). [redacted] Methods of isolation in these previous surveys differed slightly and may have been a contributory factor to the number of isolates found (Boag et al., 1992).

Hominick and Briscoe (1990) have used up to four successive baitings to achieve such unusually high return rates (Fan & Hominick, 1991). All of the previous U.K. based surveys have used a baiting bioassay to extract EPNs from the soil (see Table 3.1). Hominick (2002) shows that this is actually the lowest efficiency method, however this is the method that best enables establishment of a culture of EPNs. [redacted] Amplification and sequencing of this region is particularly useful when the nematode sample is of unknown origin, as it possible to compare sequences to sequences of known species published in GenBank (Abebe, 2011). It is a rapid and reliable process for identification of EPN species, [redacted]

Figure 3.1 Previous published EPN surveys in the U.K.

Area	Sampling date	Authors	No. of samples	Return rate (%)	Species found	Identification method	Bait technique	Sampling method	Other
Northern Ireland	Nov 1986 to June 1987	Blackshaw (1988)	1093	3.7	*Steinernema bibionis*		1 x *Galleria. mellonella* bait		Loamy soil returned most EPNs
England (excl. S.W) Wales and S.W. Scotland	5 May to 6 Oct 1986 and 29 Apr to 18 Sept 1987	Hominick & Briscoe (1990)	403	48.6	*S. bibionis*, unknown *Steinernema, Heterorhabditis* sp.	Crossbreeding, morphological and RFLP	*G. mellonella* at 2 different temperatures	Randomised drive	Avoided winter sampling
Scotland (mainland)	Apr 1988	Boag, Neilson & Gordon (1992)	1014	2.2	*S. feltiae*, suspected *S. carpocapsae*	Morphological	1 x *G. mellonella* and 1 x *Tenebrio molitor* baits	40 X 40 km grid of whole country, 5 diff. habitats within square	More recovered from arable and forest
East Anglia and South Wales	Jul to Aug 1993	Hominick, Reid & Briscoe (1995)	157	38.2	*S. feltiae*, S. affinis, *Heterorhabditis* (2 single isolates)	RFLP		Randomised drive within 1km of sea	
Warwickshire	Jul to Aug 1993	Chandler, Hay & Reid (1997)	400	4.3	*S. feltiae* and unidentified *Steinernema*	RFLP	1 x *G. mellonella* bait	Research farm and woodland within 20km	More found in hedgerow than arable
England, Scotland and Wales	1996	Gwynne & Richardson	414	11	*S. affinis*, *S. feltiae* and *S. kraussei*	RFLP	*G. mellonella* bait at 3 different temps	Random avoiding clay soils	Sparse coverage of sample area
Swansea and Carmarthenshire	2007	Ansari, Shah & Butt (2008)	95 from 3 sites	7.4	*S. feltiae*, *H. bacteriophora*	*S. feltiae*; morphological, *H. bacteriophora*; sequencing of ITS1-5.S-ITS2 region	1 X *G. mellonella* bait	1.5kg samples from golf course, woodland and potato field	Return rate not directly comparable to other surveys due to heavy sampling of 3 sites
South West England/ Welsh border	2012	Al-Own (2013)	320	5	*S. glaseri*	Sequencing of 18s rDNA gene	1 X *G. mellonella* bait (3 larvae in 50g soil)	Along 2 transects	Survey of soil bacteria, not directly targeting EPNs

A U.K. wide survey was carried out to ascertain what EPN species are currently found in a wide range of habitats and whether these could be utilised to control populations of nuisance Diptera (this is discussed in Chapters 2 and 4). This survey included the first EPN survey of [redacted]. Records indicate that the island has been a human habitation for at least 3000 years with a colourful history of inhabitants and associations including Vikings, pirates and the Knights Templar (The Landmark Trust, 2017). The island itself is designated an SSSI, initially in 1976 and under the Wildlife and Countryside Act (1981). These protections are to maintain the healthy longevity of a wide range of habitats, in particular the granite and slate reef system surrounding the island and intertidal zone. [redacted] Given Lundy's relative isolation from the mainland, it was considered to offer an excellent opportunity to find any previously undescribed species and provide a snapshot of any known EPN species found there.

The aim of this research was to examine the diversity and distribution of EPNs across the U.K. to ascertain whether the species distribution has altered in the intervening years since

the last surveys were conducted, focusing on a range of different habitats and land uses. EPNs that were isolated from this survey were identified through DNA extraction and amplification of the 18SrRNA gene, giving an accurate snapshot of species distribution. These isolated EPNs were retained and used in subsequent pathogenicity studies. Furthermore, several soil parameters that may affect the efficacy of the *Galleria mellonella* baiting method (which is commonly used to isolate EPNs (Bedding and Ackhurst, 1975)) were also examined.

Materials and Methods

Soil collection from around the U.K.

 Urban sampling sites were constrained by finding an area with soil. Within these broad habitat types and land uses, as wide a range of substrate and soil types as possible were targeted including such as granite slack, loam and peat etc. as described by Joint Nature Conservation Committee Handbook for Phase 1 habitat survey (JNCC, 2010) and UKSO Soils map viewer (Cranfield Soil and Agrifood Institute, 2017).

This involved using smaller roads (non-motorway and B classification) to travel between target destinations of different land uses, for example travelling from a rural wooded area to a city and stopping as often as possible to collect soil samples from surrounding area where accessibility and safety permitted. When possible, samples were taken at approximately 16km

intervals along a route. This was dictated in part, whether it was safe to stop the car and exit the vehicle and also whether it was possible to collect a soil sample; for example, where the 16km point coincided with a concrete roadside or requiring entry to a private garden, samples were taken at the nearest viable point. Sampling ceased each day at different times to allow enough time to travel to that night's accommodation during daylight as many accommodation sites were in unfamiliar and remote, unlit areas. Within each sample habitat, the soil was collected from the point that was considered to offer the best opportunity of finding EPNs in the soil such as where the potential host insects may be found. unless the substrate was too hard or too shallow to enable that depth. In those cases, more soil was taken from the surface.

The collected soil was stored immediately in a uniquely numbered plastic bag and sealed to prevent moisture loss or contamination by other soil samples. At the time of collection, GPS coordinates and a photograph were taken. Land use, soil type and any other distinguishing information was also recorded. Collected samples were stored in the boot of the vehicle for the duration of the sampling survey. Isolation of EPNs with *Galleria mellonella* was initiated on the return to the laboratory. Samples were collected between September 2014 and July 2015 (South West England); 1 to 8 November 2014 (Northern Ireland); March 2015 (rest of U.K) 1 to 29 June 2015 (see Figure 2.2 for location of collection sites).

Due to Lundy Island being privately owned and protected by legislation, a request for permission to sample, including a detailed strategy and sampling map was submitted to Natural England. Permission was granted and the sampling of Lundy was conducted from 14 to 19 June 2015. As Lundy is not accessible to motorised transport, the randomised drive method (Hominick and Briscoe, 1990) employed throughout other parts of the U.K. wide survey was not possible, therefore samples were taken using a rough grid system covering the whole of the

island. A total of 46 soil samples were taken, which represents the most intensive area of sampling as part of this U.K. wide survey.

Isolation of EPNs from soil collected from around the U.K.

The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted] obtained from online retailers. *G. mellonella* is a standard bait animal used in EPN studies due to their high susceptibility to EPN attack, therefore, it was not deemed necessary to examine the *G. mellonella* for any signs of pre-infection as discolouration and rapid death are typical. However, the most lively, plump *G. mellonella* were selected for use to ensure initial good health. The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted] (Fig 3.1). Any pupated *G. mellonella* were removed, with live pupae destroyed by freezing. This process was repeated until all the *G. mellonella* were removed from the box. *G. mellonella* survival was monitored for 22 days. Pupated *G. mellonella* were counted as alive, as they had survived their encounter with EPNs as previous studies show that EPNs cannot penetrate the pupal casing (Lewis et al., 1996). Hominick and Briscoe (1990) employed a second round of baiting, which aided a high return rate in their U.K. survey (Table 3.1). Therefore The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted] It was not possible to re-bait all the soil boxes immediately and as a result, some were left dormant for up to 1 month before re-baiting. Where the soil samples remained in a dormant state, the soil was prevented from drying out in that time. The White trapped *G. mellonella* were checked every few days

.

for dauer stage EPNs that had reproduced inside *G. mellonella* and moved into the water.

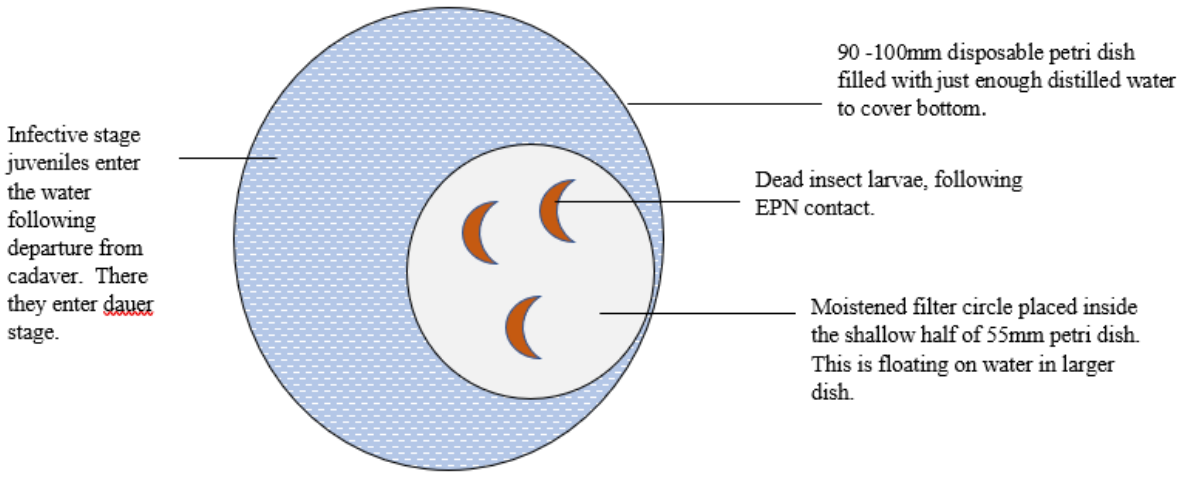


Figure 3.1. Modified White trap. Once the bacterial biomass is depleted in the larval cadaver, the infective juvenile stage of the EPNs leave the host, migrate across the dampened filter paper and enter the surrounding water where they can be harvested or used to repeat the process and grow more EPNs. The large Petri dish is covered with the lid and sealed with Parafilm™ to prevent spillage or desiccation.

Molecular identification of EPNs

The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted] These were centrifuged at 10,000 RPM for 10 minutes and the supernatant removed. This process was repeated until the EPNs had formed a pellet and no more than 20µl supernatant and the pellet remained. The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted]

DNA extraction techniques followed the manufacturer's recommended protocol for tissue samples but with a 3-hour lysis time to enable the breakdown of the EPN cuticle.

Investigating the pathogenic ability of EPNs in soil over time

An experiment was carried out to identify whether there were any differences in the infective persistence of several commercially available EPN species in a standardized soil environment. This could also provide information about whether the time between the first and second baits of the soil collected from the survey affected the number or diversity of EPNs that were found. This study repeated the methods used to isolate EPNs from the collected soil samples. Commercial Peat-free soil (Verve, B&Q), was autoclaved at 120°C and allowed to dry at 80°C for 48 h to remove any excess moisture. Seventy-five grams of soil was placed into 30 separate 1 litre capacity plastic boxes. The capacity of boxes and weight of soil was different to that used in the original isolation assay. This was due to the peat-free soil, once dried, having

a greater volume than the collected soil samples. This combination of box and soil weight ensured the box was sufficiently full for movement of *G. mellonella* without compaction. The soil was remoistened with 30 ml distilled water and allowed to absorb for 24 h before use. Commercial EPNs (*S. feltiae, S. kraussei, S. carpocapsae* and *H. bacteriophora*) were released from their transport medium by adding a small amount into a 50ml centrifuge tube and distilled water was added to 35ml and the water/EPN mix gently agitated. The number of EPNs were calculated by dropping 10µl of the water/EPN mix into a counting dish (5mm Petri dish scored to show a grid pattern). The number of live EPNs were counted under a light microscope and recorded. The dish was cleaned and the process repeated 3 times to obtain a mean count. This figure was used to calculate the required volume of solution containing the requisite number of EPNs. Each species of EPN was tested separately with 8000 of a single EPN species added to 6 boxes. They were added to the surface of the soil as a single inoculation and allowed to disperse. Six boxes received a similar volume of water (no EPNs) and acted as the control. The boxes were then each baited with 10 *G. mellonella* and checked for dead or moribund larvae every 48 h. The experiment ran for 21 days. After this time, 3 of the 6 boxes allocated to each species and control were immediately rebaited with 10 *G. mellonella* and the remaining 3 were left dormant for 30 days before being rebaited with 10 *G. mellonella*. In both of these instances the second bait experiment ran for 21 days. All pupated or dead *G. mellonella* were removed from the boxes. The whole experiment was conducted a total of three times using fresh soil, EPNs and *G. mellonella*. All boxes including those left dormant were opened and remoistened to ensure that conditions were comparable.

Examining the minimum number of EPNs required to cause mortality to G. mellonella

To understand why there was such a low return of EPNs in the soil survey this assay

was devised to examine what the minimum ratio of EPNs to host that were needed to kill *G. mellonella*. This would aid in the understanding of whether EPNs were present in more soil samples but just not in sufficient numbers to be successfully isolated. Seventy-five plastic boxes (16 x 11 x 5 cm) ((4 x EPN species x 3 biological reps x 6 concentrations) + 3 x 0 concentration) - were filled with 75g autoclaved soil and 10 *G. mellonella* were added to each box. Commercial EPNs (*Steinernema kraussei, S. feltiae, S. carpocapsae and H. bacteriophora*) were added to the boxes at the following ratios: 0, 1, 10, 50, 100, 500 and 1000 EPNs per *G. mellonella,* per box. Where the total number of EPNs was low, these were counted individually and transferred using a pipette. Each ratio/EPN species had 3 biological replicates. The boxes were kept at room temperature and checked every 48h for dead or pupated *G. mellonella,* which were removed. The experiment ran for 7 days. The whole experiment was repeated another two times using fresh soil, EPNs and *G. mellonella*.

Statistical analyses

Log-Rank tests were used to analyse survival data using Oasis and Oasis 2 (Yang et al. 2011; Han et al.; 2016). Full statistical results are available in **Appendix 2.**

Results

Isolation and identification of EPNs from soil survey of the U.K.

The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted] (Fig. 3.3 and Table 3.2). The second round of baiting only returned positively identified EPNs from three samples, all of which had EPNs of the same species also identified from the first round of baiting. DNA extraction, PCR and sequence analysis identified four different species of entomopathogenic nematodes from the genera *Steinernema* (Table 3.2) in these 18 samples The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted]. Two separate samples taken from sites in Cornwall yielded isolates of *S. carpocapsae*, which is the first confirmed U.K. record of this species. These were found on a farm under *Ulex europaeus* bushes (gorse) and the second in a deciduous-wooded layby. Seven separate sites yielded *S. glaseri*. These were found in locations covering a broad spread of land uses but were all found in loamy soils (Table 3.2). Isolates of *S. affine* were found at eight separate sites, throughout a broad range of land uses. All of these were found in habitats that had little human interference. Four of the eight sites that yielded *S. affine* were next to roadsides.

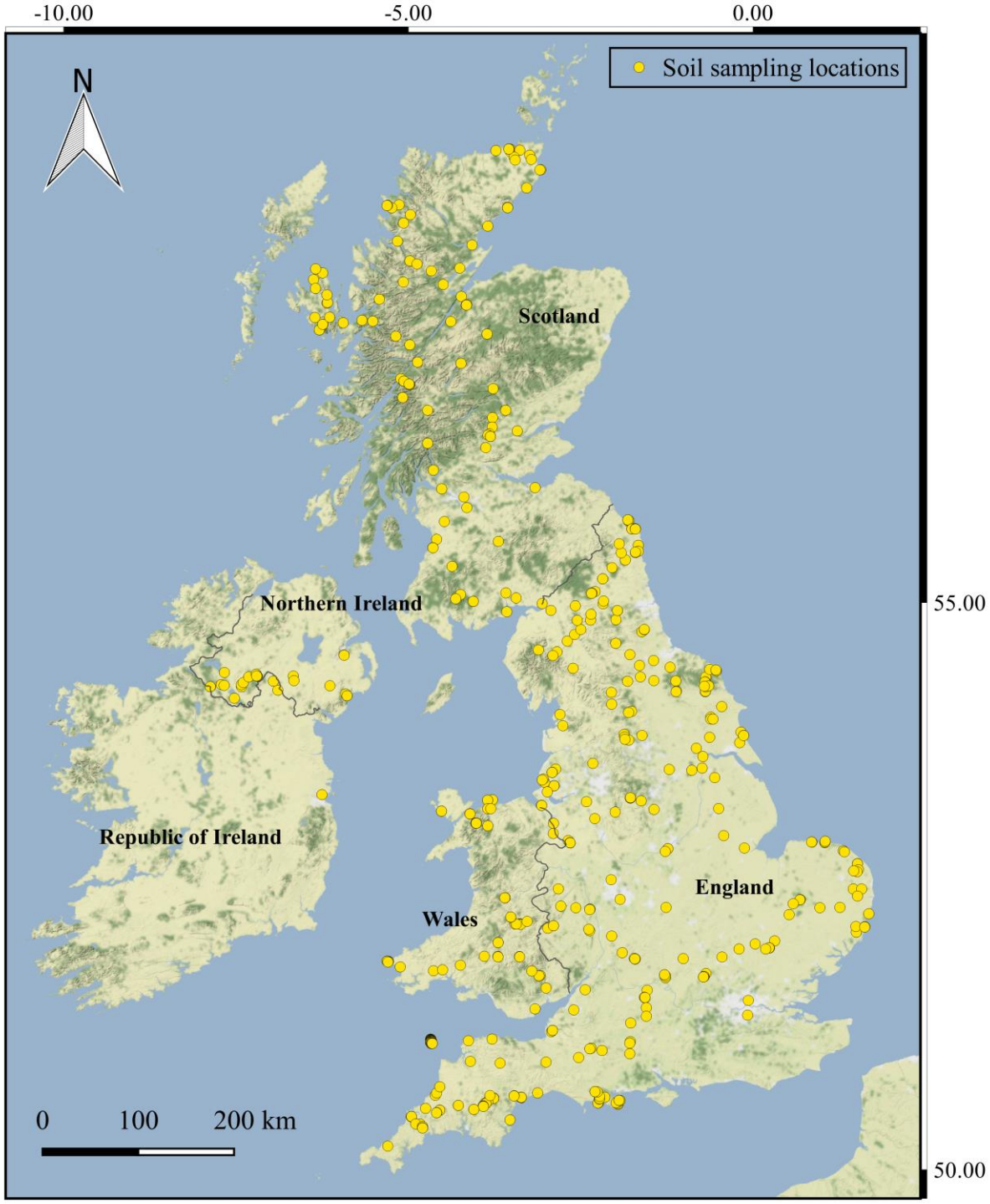


Figure 3.2 Map of the U.K. showing sites where soil samples were collected. The darker shading on Lundy Island represents multiple samples taken from a small area.

Eighteen soil samples had EPNs present, but a much greater number of the soil samples had other nematode species from a variety of trophic groups. Where identification through DNA was successful, nematodes identified to the genus *Acrobeloides* were by far the most numerous with 68

separate isolations from the soil samples. *Cervidellus vexilliger* was the second most abundant with 35 isolations. Nematodes from the genus *Pristionchus* were found in 13 samples, *Rhabditis* spp. were in 9, *Aphelenchus* spp. in 7, *Choriorhabditis cristata* in 2, *Pelodera* spp. in 2, *Oscheius* spp. in 2, *Aphelenchoides* in 2, and single isolates of species from the genera *Synoecnema* and *Phasmarhabditis* were also found. There were several instances of multiple species from one sample, including a soil sample that contained *S. glaseri* and *Oscheius* sp. (identified from separate DNA samples). As these other nematode genera were not being surveyed and are often found on dead *G. mellonella* from these surveys (Mracek, 1980) no further investigation was conducted or information was gathered on non-EPN isolates.

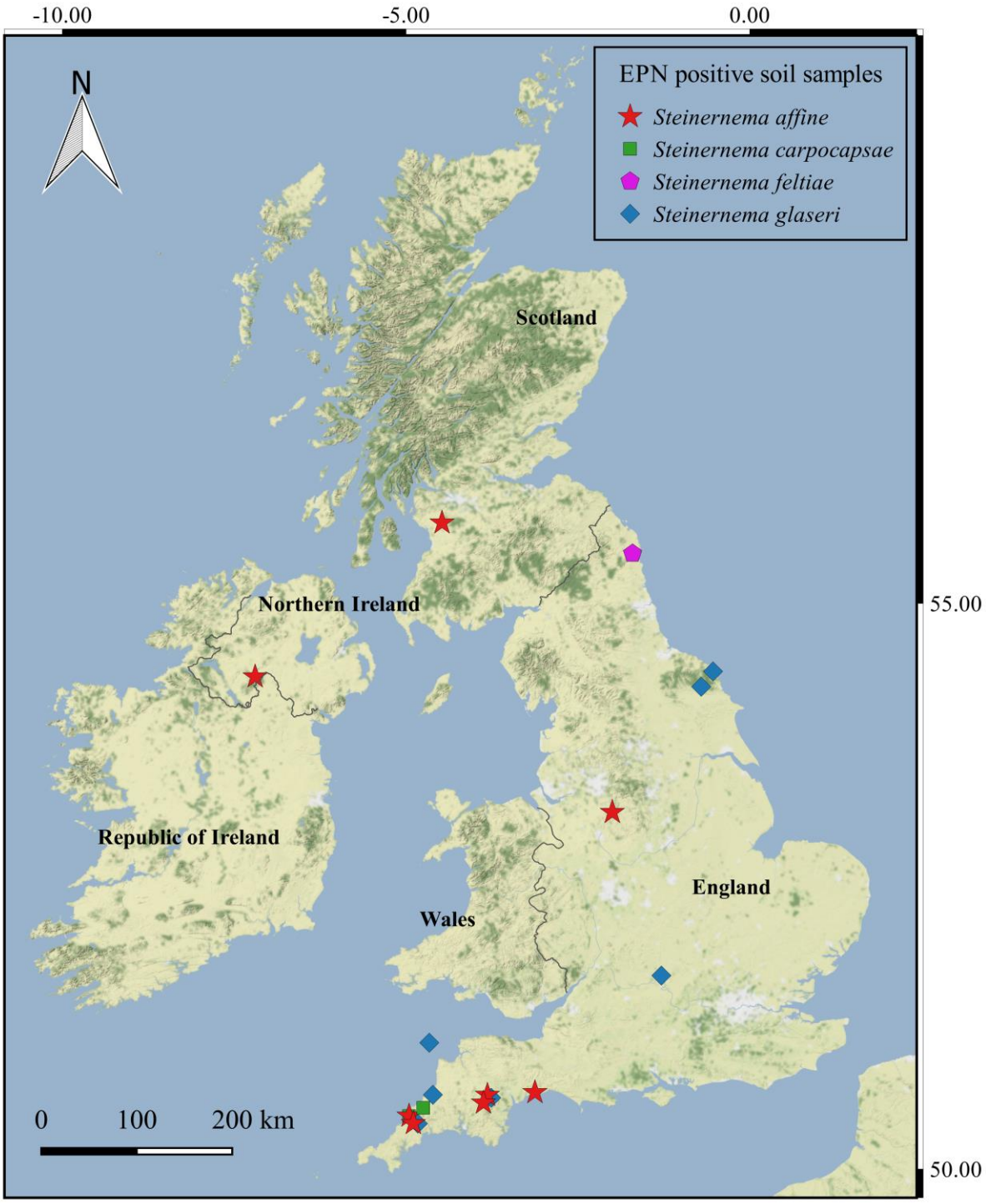


Figure 3.3 Map showing the position of EPN positive soil samples in the U.K.

One isolate of *S. glaseri* was isolated from the samples taken from Lundy Island (Fig. 3.4). This was found in an area of ungrazed pasture close to the farm area and the main settlement. Other nematode species were isolated from Lundy including 10 isolates of *Acrobeloides nanus*, one isolate of *Aplenchoides bicaudatus* and two isolates of *Pangrolamius subelongatus*. There was a

return rate of 2.17% for EPN species on the island, which is lower than that found throughout the rest of the U.K. in this survey.

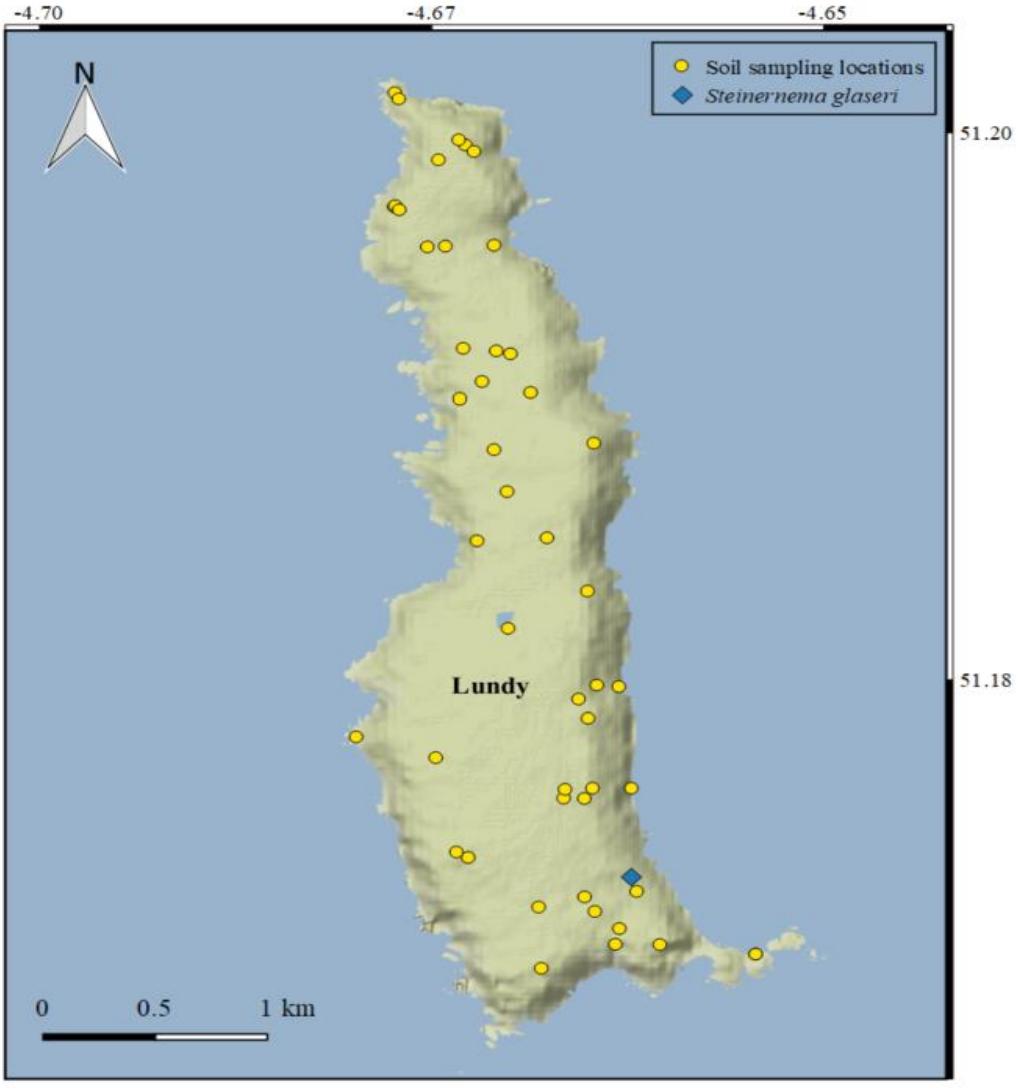


Figure 3.4 Map of Lundy Island showing soil sampling sites (yellow circles) and sample location of *S. glaseri* (blue rhombus).

The table originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions

Minimum numbers of EPNs that can be detected through G. mellonella baiting

There was no significant difference between the survival of *G. mellonella* exposed to a 1:1 ratio per box (regardless of which of the four species was tested) and the untreated soil after 10 days exposure ($P>0.05$) (Figs 3.5a to 3.5d). However, when *G. mellonella* were exposed to any higher ratio – 10, 50, 100, 500: 1 *G. mellonella* - all four species caused a significant reduction in survival ($P<0.001$). Therefore, the minimum ratio required for significant reduction in survival is 10 EPNs to 1 host in all species. However, this is not the most efficient ratio as increasing the ratio of EPNs per host resulted in greater and faster mortality rates.

S. feltiae reduced *G. mellonella* survival to 20% within 4 days at a ratio of 1000 EPNs to 1 *G. mellonella*. Lower *S. feltiae*: host ratios (50, 100 and 500 nematodes per *G. mellonella*) killed 80% of the *G. mellonella* between 5 and 8 days following exposure (Fig 3.5a). *H. bacteriophora* reduced the *G. mellonella* survival to 20% in only two ratios (500 and 1000 nematodes per *G. mellonella*), with 500:1 quicker to reach that point at day 5 compared to 1000:1 by day 7 (Fig 3.5b). *H. bacteriophora* was the only EPN that was faster at killing *G. mellonella* at 500 EPNs per host than 1000. Similar to the other species tested, *S. kraussei* was quicker to induce significant mortality at higher host: parasite ratios with 1000 and 500 EPNs per host reducing *G. mellonella* survival to 20% by day 5 (Fig 3.5c). The other ratios of *S. kraussei* were slower at reaching 20% survival which occurred between days 7 to 10. For *S. carpocapsae* the highest ratios of 500 and 1000 EPNs per host were the quickest acting, causing an 80% reduction in survival by day 5 (Fig 3.5d). By day 7 both 1:50 and 1:100 caused 80% mortality to *G. mellonella*.

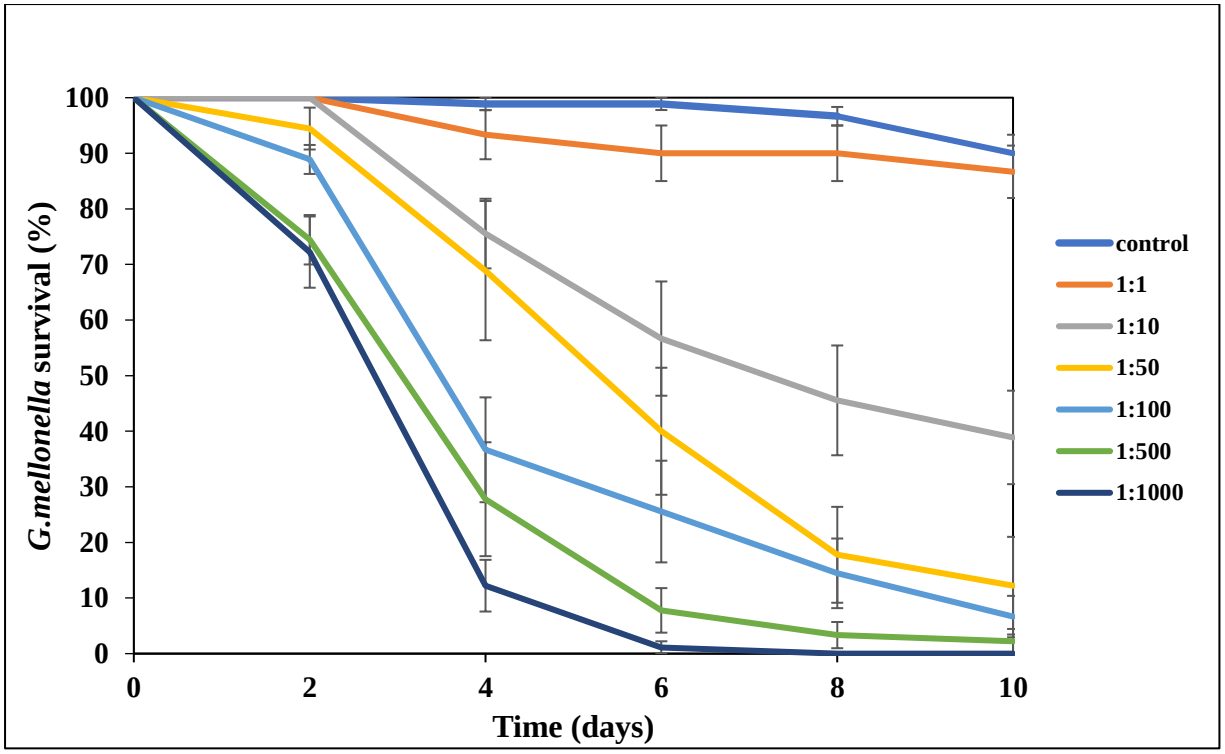


Fig 3.5a. Minimum dose of *Steinernema feltiae* required to induce mortality in *Galleria mellonella* (n=30). Bars represent ± one standard error of the mean.

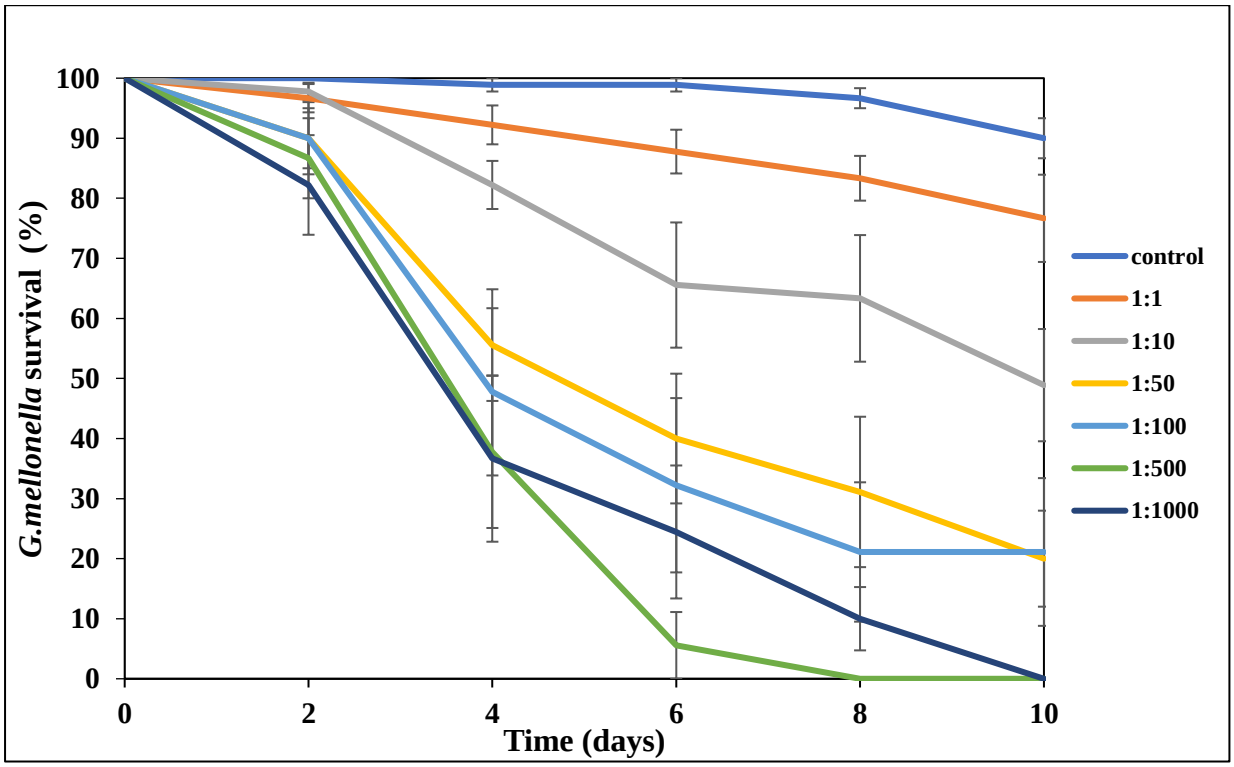


Fig 3.5b. Minimum dose of *Hetorhabditis bacteriophora* required to induce mortality in *G. mellonella* (n=30). Bars represent ± one standard error of the mean.

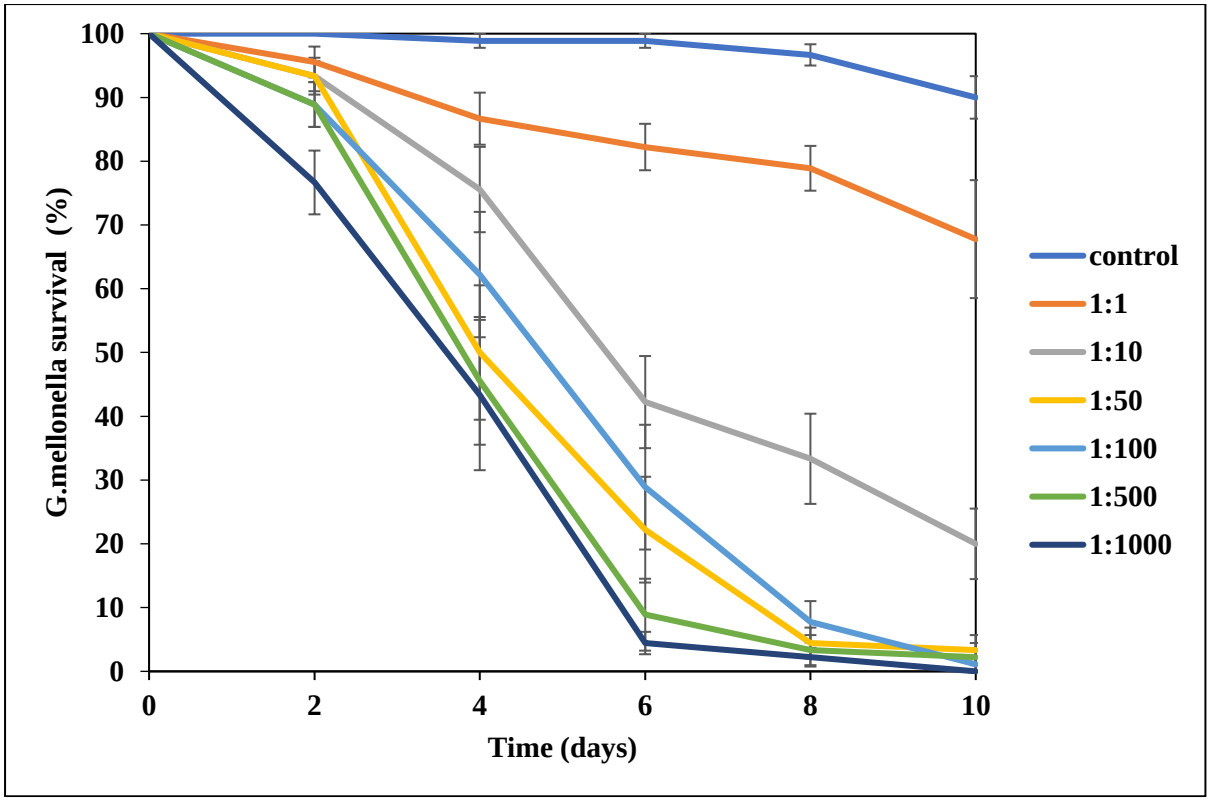


Fig 3.5c Minimum ratio of *S. kraussei* to *G. mellonella* required to induce mortality in *G. mellonella*. (n=30). Bars represent ± one standard error of the mean.

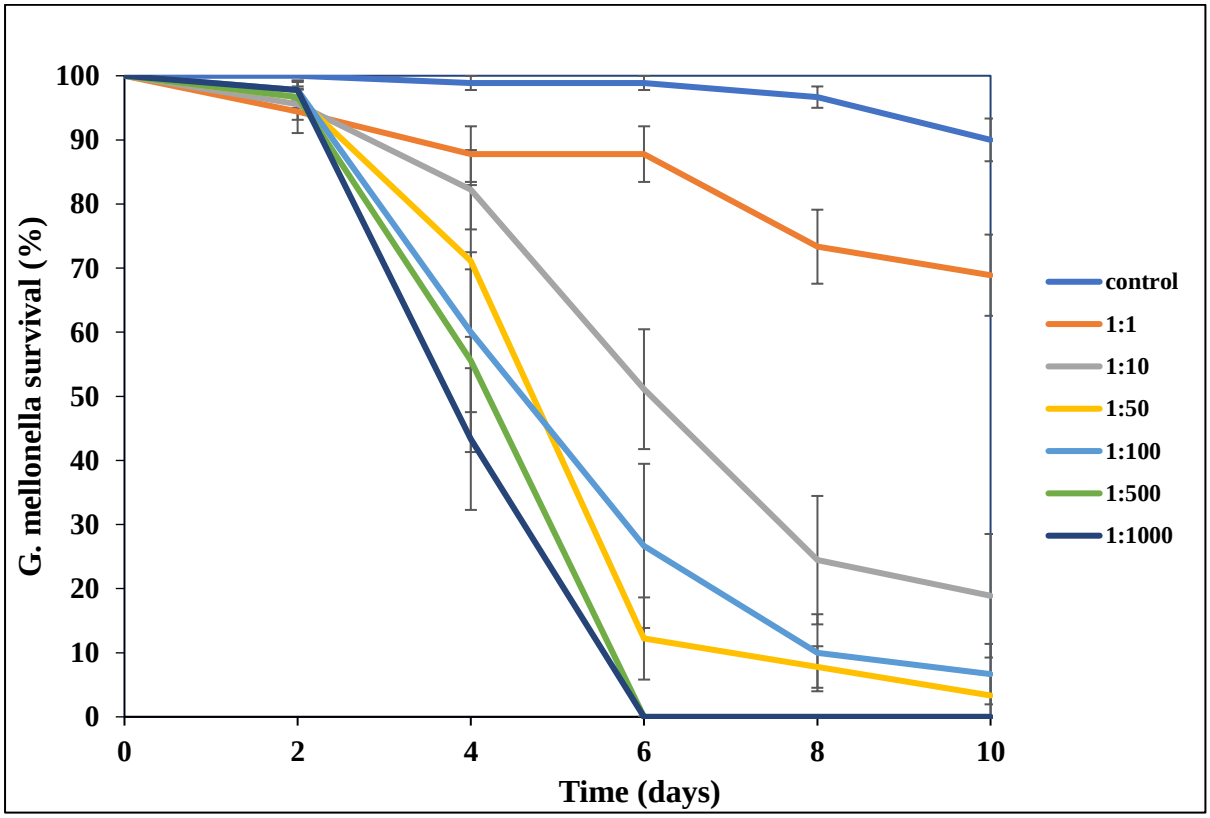


Fig 3.5d Minimum ratio of *S. carpocapsae* to *G. mellonella* required to induce mortality in host. (n=30). Bars represent ± one standard error of the mean.

Persistence of EPNs in soil

An 80% reduction in *G. mellonella* survival has been used as an arbitrary point for clarity to compare and describe these results. Table of full values from the statistical analysis is available at **Appendix 2**.

Survival of G. mellonella exposed to EPNs applied on day 0 (Bait 1)

All four EPN species significantly reduced the survival of *G. mellonella* when applied as the first bait compared to the untreated control ($P<0.05$) (Fig. 2.6a to 2.6d). By day 5-6, all *Steinernema* sp. had killed 80% of the *G. mellonella* and *H. bacteriophora* had killed 80% of the *G. mellonella* by day 7.

Survival of G. mellonella added on day 22 (Bait 2 immediate)

When the soil was immediately baited again on day 22, *Steinernema* sp. killed 80% of the *G. mellonella* by days 3 to 6 ($P<0.05$) (Figs 2.6a, 2.6c and 2.6d). *S. carpocapsae* was 2 days quicker to reach this point than in the 1st bait. By day 20 *H. bacteriophora* had not killed 80% of the *G. mellonella* (Fig 2.62), although this result was significantly different to the corresponding control condition ($P\leq 0.001$).

Survival of G. mellonella added on day 44 (Bait 2 dormant)

Leaving the soil dormant for 1 month slowed the speed at which *S. feltiae* and *H. bacteriophora* killed *G. mellonella* with *S. feltiae* reducing 80% of the population by day 13 ($P\leq 0.001$) and *H. bacteriophora* by day 20 ($P\leq 0.001$). However, there was no significant difference when comparing the second bait of dormant soil against the second bait of the immediately rebaited soil ($P= 1.00$) for *H. bacteriophora*. *S. carpocapsae* and *S. kraussei* were not slowed by the dormant

period in the soil. *S. carpocapsae* killed 80% of the *G. mellonella* by day 5-6 ($P \leq 0.001$) and *S. kraussei* by day 4 ($P \leq 0.001$).

Survival of G. mellonella exposed to 0 EPNs (Control)

The control boxes (without EPNs) did not show 80% reduction under any conditions, with a minimum 37% surviving in those boxes which had been left the longest (Bait 2, dormant). This would indicate that bacterial and fungal pathogens occurring in the soil may have contributed to the mortality rate the longer the soil was left.

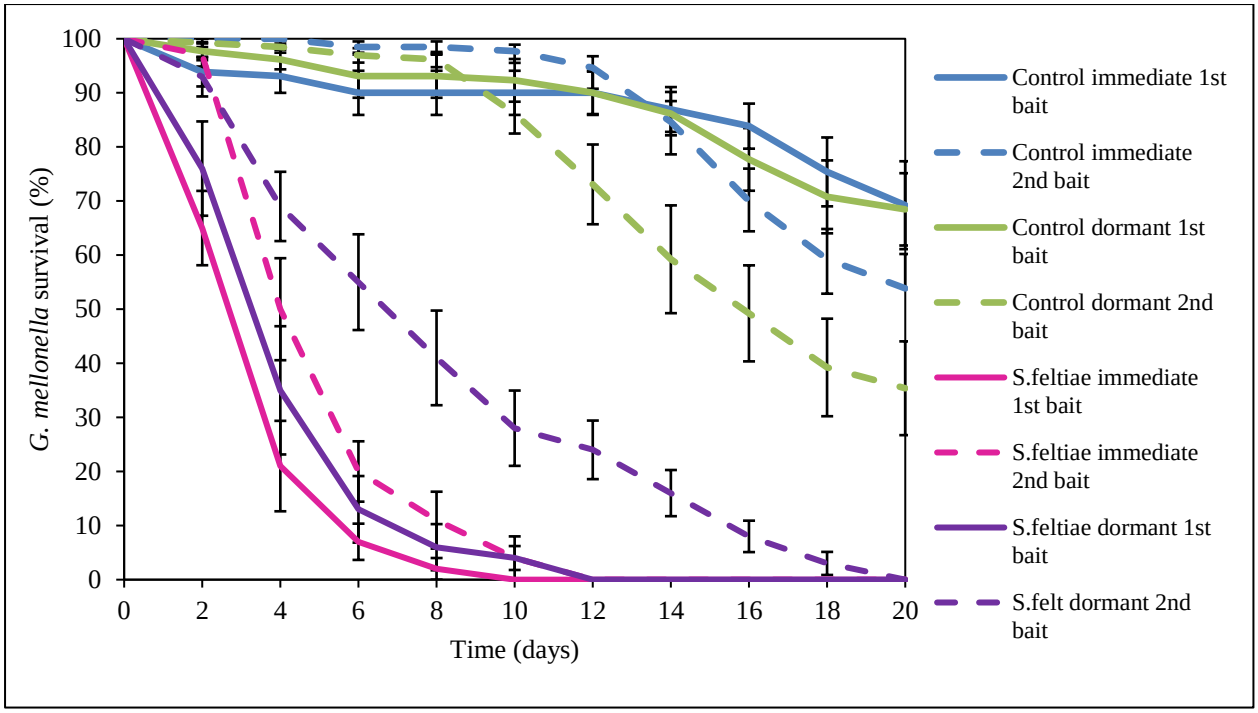


Figure 3.6a. Survival of *Galleria mellonella* used to demonstrate the persistence of *Steinernema feltiae* in soil following successive baits with *G. mellonella* at different times after the introduction of the EPNs. (n=30). Bars represent ± one standard error of the mean.

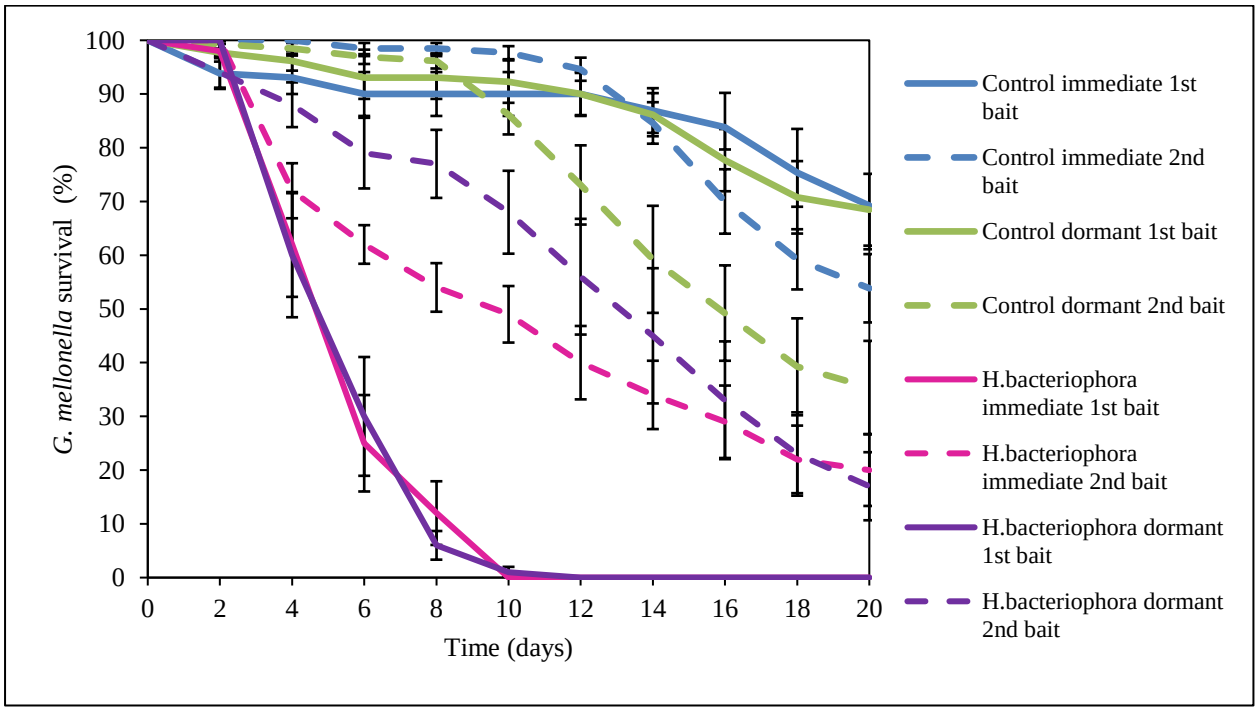


Figure 3.6b Survival of *G. mellonella* used to demonstrate the persistence of *Heterorhabditis bacteriophora* in soil following sucessive baits with *G. mellonella* at different times after the introduction of EPNs. (n=30). Bars represent ± one standard error of the mean.

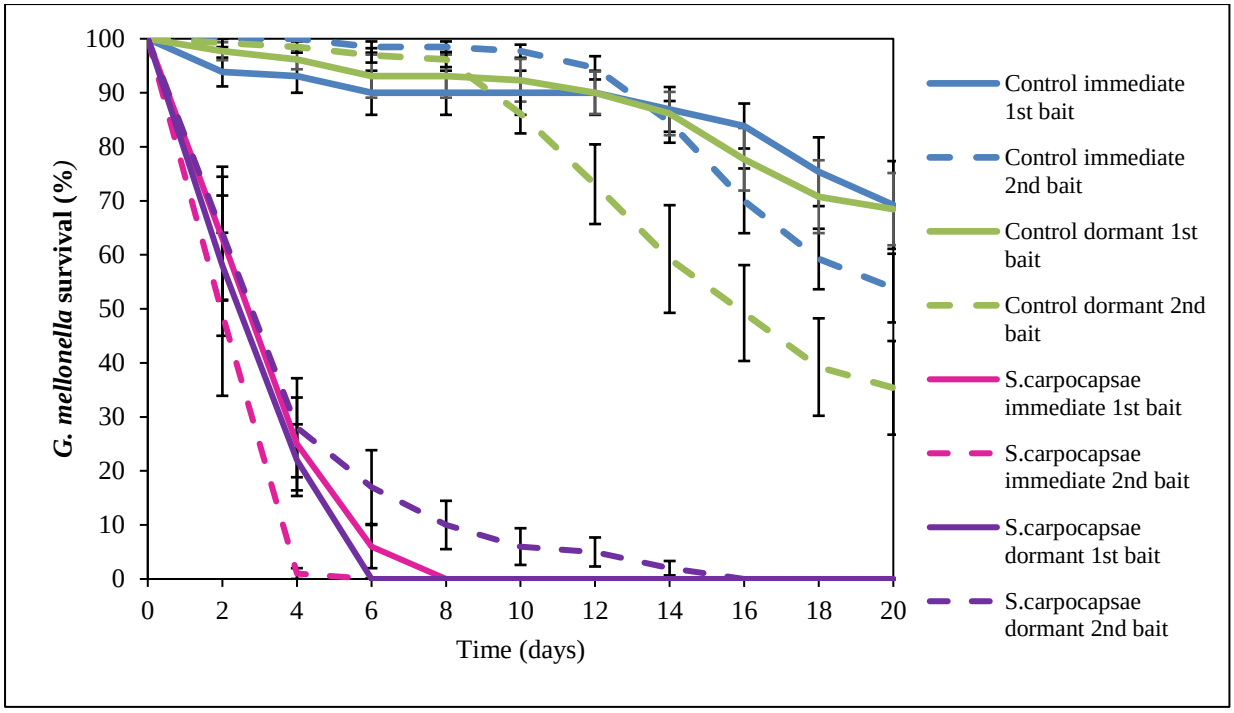


Figure 3.6c. Survival of *G. mellonella* used to demonstrate the persistence of *S. carpocapsae* in soil following successive baits with *G. mellonella* at different times after the introduction of EPNs. (n=30). Bars represent ± one standard error of the mean.

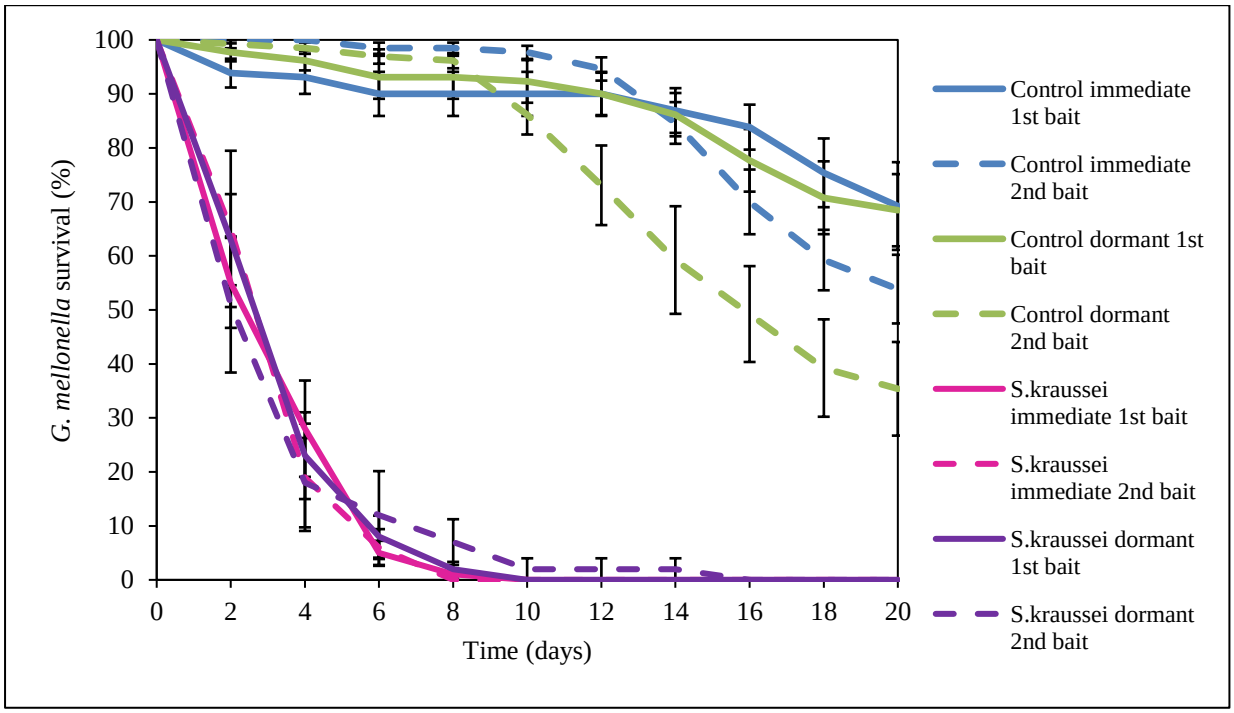


Figure 3.6d. Survival of *G. mellonella* used to demonstrate the persistence of *S. kraussei* in soil following successive baits with *G. mellonella* at different times after the introduction of EPNs. (n=30). Bars represent ± one standard error of the mean.

Discussion

The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted] This is in concurrence with the findings of Fan and Hominick (1991) who found most EPNs were returned from the soil in the first bait. The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted] Two of the previous

U.K. based surveys baited at different temperatures and did produce higher recovery rates (Gwynn and Richardson, 1996; Hominick and Briscoe, 1990). This survey may have produced *S. kraussei* had the soil samples been incubated below 15°C, however without knowing ahead of time what EPNs may have been present, holding the soil samples for any length of time at a low temperature may have prevented the isolation of the EPNs that are active at higher temperatures. The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted]

Steinernema carpocapsae has not been recorded previously in the U.K. The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted]

At the time of these reported isolations of possible *S. carpocapsae*, identification was based purely on morphology. The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted], *S. glaseri* is often associated with turf grasses The text originally presented here cannot be made freely available via LJMU E-Theses Collection because of copyright restrictions [redacted]. This study agreed with the findings of Hominick et al. (1996)

regarding areas of minimal human disturbance. However, it is worth noting that both this study and those of Hominick et al. (1995) and Hominick and Briscoe (1990) employed the randomised drive methodology, which may account for the prevalence of EPNs found at roadsides as more samples were collected from these areas than a systematic sampling such as that of Boag et al. (1992). This study found eight instances of *S. affine* but only one was found within a woodland and that was recovered from a riparian habitat within that woodland. No other samples from any other deciduous or coniferous woodland returned any EPNs despite extensive sampling of these habitats.

Heterorhabditis spp. have not been recovered from U.K. soil surveys with the same abundance or frequency as Steinernematids (Blackshaw, 1988; Boag et al., 1992; Griffin et al., 1994; Gwynn and Richardson, 1996; Hominick et al., 1995).

In this survey, seasonal abundances of EPNs may have been a factor in the relatively high recovery rate from the soil samples collected from South-West England and Northern Ireland (which were collected in November and March, respectively). The rest of the U.K. was sampled in the spring and summer months. Given that host insect activity is diminished in the colder winter months (Puza & Mráček, 2005) it might be assumed that associated parasitic nematodes may also be harder to find. However, this assumption is not borne out by the findings of studies that found autumn and spring peaks in EPN density and declines in the warmer summer months (Akhurst and Bedding, 1986; Griffin et al., 1991; Mráček, 1980). It is thought that this may be an effect of lower soil moisture content in the warmer months (Puza & Mráček, 2005) and insects burrowing further into the soil to avoid hot soil temperatures (Rio

and Cameron, 2000). However, the U.K. has a maritime climate and drought is not as prevalent or pervasive as mainland European countries. Conversely, a lack of seasonal difference was found in studies by Campbell et al. (1995) and Glazer (1996) found no seasonal differences and Mráček (1982) found a peak of EPN activity in June. The presence or absence of EPNs in the soil was described as unpredictable by Hominick & Briscoe (1990) in their repetitive sampling of a single site. Therefore, it is difficult to ascertain whether seasonality did play a part in the recovery of EPNs in this survey. However, soil moisture is an important factor to EPN survival (Hominick, 2002) and whilst some viable EPNs have been returned from desiccated soil (Popiel and Hominick, 1992), the slightly drier conditions found in summer months may prevent a full account of EPNs present in the soil at that time.

One of the EPN isolates (*S. glaseri*) was from Lundy Island. This isolate was found in soil from a livestock farm. How or when EPNs came to inhabit Lundy is unknown but *S. glaseri* has a wide distribution. Future research on EPNs found on Lundy could focus on the relationship they have with the endemic beetle *Psylliodes luridipennis* and weevil *Ceutorhynchus contractus var. pallipes.* This may increase understanding of whether any introductions from the mainland might represent a threat to the endemic invertebrate population and contribute to the understanding of the natural host range for EPNs.

When trying to understand the reasons for a low return rate of the survey conducted here, the changes in the profile of EPNs found in the U.K. since Blackshaw's survey of 1989 may partly be due to the effect that climate and land use change has had on the wider ecosystem. Whilst the presence of *S. carpocapsae* in the U.K. now may be indicative of changes in soil biology, previous studies have found EPNs to be a poor bio-indicator for ecosystem health (Bongers and Ferris, 1999). The opportunistic lifestyles of EPNs make them unpredictable and unreliable for this purpose (Hominick and Briscoe, 1990; Hoy et al., 2008). EPNs are only returned from a comparatively small number of soil samples (Stock et al., 1999), with those

surveys finding 40% or more in the U.K. being the exception. Hoy et al. (2008) suggests that they are most abundant in soils that support their insect hosts. However, previous studies have suggested that the presence of all trophic groups of nematodes is a much more reliable indicator of soil health (Bongers & Ferris, 1999; Hoy et al., 2008). This survey resulted in more bacterivore species than any other, however this information is of little use as this survey's purpose was specifically to find EPNs, and no nematodes were specifically identified that did not reproduce in a White trap. There is increasing concern over the decline in biodiversity and number of insects. This was recently highlighted by a study which recorded a 76% decline in aerial biomass over 27 years (1989 to 2016) in a protected site in Germany (Hallmann et al., 2017; Sorg et al., 2013). This is particularly concerning for associated declines in organisms that rely on these insects as pollinators or food and has been described as "Ecological Armageddon". Declines in other invertebrate taxa have also been recorded over the past few years (Leather, 2018). However, many other taxa are also at risk such as obligate insect parasites, detritivores and scavenger trophic groups that rely on the declining insect biomass for their survival. Given this rate of potential EPN host decline and the incremental warming of temperate climates (Met Office, 2017) the biogeography and abundance of EPNs may also be undergoing radical change. This is all the more reason to regularly survey the same sites for EPNs, not just as a search for novel species to enhance our ability to control pestiferous arthropods but to gain an important understanding of how the invertebrate faunal landscape is changing.

When examining the minimum number of nematodes needed to kill *G. mellonella* it was found that all doses (apart from 1 to 1) killed *G. mellonella*. The time that each EPN species took to kill *G. mellonella* was largely similar with the highest EPN dose (1:1000) resulting in quicker mortality in all tested EPNs apart from *H. bacteriophora*. which had the quickest ratio was that of 1:500. These results suggest that if there were not enough EPNs

present in a particular soil sample, it may not have been possible to isolate the nematodes from the soil using the *G. mellonella* bait method, which is in agreement with the findings of Fan and Hominick (1991) and Molta and Hominick (1989). Although the results from this assay do confirm that in an enclosed space such as the testing boxes that even relatively low doses of EPNs can find and kill hosts.

The persistence of EPNs in soil was also examined. The main aim of this was to understand whether the way soil samples were treated post-collection could have an effect on what EPN species might be isolated. It also shows how long EPNs remain alive and ready to infect a potential host. *H. bacteriophora* was much less effective at killing *G. mellonella* after the first round with the second bait showing almost the same survival as the control in both conditions indicating that they are much less likely to remain infective in the soil for any prolonged period. This suggests that keeping soil samples over any length of time prior to baiting reduces the chances that *H. bacteriophora* will be isolated. However, many of the soil samples collected in this survey that were targeted specifically to find *H. bacteriophora* were baited within 1-2 h of collection and did not return any isolates. Other U.K. surveys found *H. bacteriophora* infrequently (Al-Own, 2013; Ansari et al., 2008; Blackshaw, 1988; Boag et al., 1992; Chandler et al., 1997; Griffin et al., 1994; Hominick et al., 1995; Hominick and Briscoe, 1990). So a combination of its rarity, when compared to the *Steinernema* spp. and short active period in the soil may contribute to why so few surveys have found it in any great abundance in the U.K. *S. feltiae* were also less effective in reducing the survival of *G. mellonella* over time. *S. feltiae* has an intermediate style of host-finding, where it combines a cruiser movement through the soil and also body-waving behaviour to aid attachment to a potential host (Campbell and Gaugler, 1997; Gaugler and Campbell, 1993) or bridge pore-gaps in soil (Kruitbos et al., 2010). This raises the question of whether this is an energetically costly host-finding strategy, as there was only one inoculation of EPNs into the soil and the longer *S. feltiae*

remained in the soil, the less effective they were at reducing *G. mellonella* numbers. *S. kraussei* was almost unaffected by its time in the dormant soil**,** albeit slower to reach 0% survival for host numbers after its 30 day dormancy. However, it was equally quick as the other conditions to reach 20% survival. *S. carpocapsae* was two days faster in the immediately rebaited box at reducing the survival rate to 0% than the other conditions. This may indicate that they were already attracted to the new set of host larvae entering whereas in the first bait they had fewer encounters with the *G. mellonella* at that point. This is in agreement with the findings of Lewis et al. (2006) whereby *S. carpocapsae* required physical contact with a host to then be attracted to its volatiles cues. However, these EPNs have different foraging strategies. *S. kraussei* were found to be the most mobile of the species tested by Jagodič et al. (2017) and may have been well dispersed already in the soil making any avoidance by the host species difficult. With regard to how this relates to this soil survey, it would appear that baiting the soil samples for a second time did not improve the return rate regardless of when that second bait was conducted. Also, that to increase the chances of isolating *H. bacteriophora* it is important to bait the soil as soon as possible.

In conclusion, this soil survey found four species of *Steinernema* and this was the first time *S. carpocapsae* has been confirmed in the U.K. using molecular verification (outside of licenced usage). The virulence of these naturally isolated nematode species and strains was unknown and they were used in Chapters 4 to understand whether they could be used as biological control agents to kill pestiferous mosquito species including *Ochlerotatus detritus* and *Aedes aegypti.*

Chapter 4

Pathogenicity of entomopathogenic nematodes to mosquitoes *Aedes aegypti* and *Ochlerotatus detritus*

Introduction

Mosquito species are widespread globally (Foley et al., 2007) and are one of the world's biggest killers through the pathogens that they vector with more than 1 billion cases and more than 1 million deaths year (WHO, 2017b). The adaptability of mosquitoes to a huge range of different habitats has ensured their success and made them the problem that they are today (Caminade et al., 2012; Medlock et al., 2012; Roche et al., 2015). More recently, climate change has enabled range expansion of vector species such as *Aedes albopictus* towards more temperate climates (Gratz, 2004; Knudsen, 1995; Lambrechts et al., 2010; Rochlin et al., 2013). There is valid concern that, given the right conditions, these vector mosquito species are able to spread serious diseases such as malaria, yellow fever, Japanese encephalitis and Zika beyond the area where these disease are currently endemic (Benedict et al., 2007; Epstein, 2001; Harvell et al., 2002; Lambrechts et al., 2010; Rogers and Randolph, 2000; Roth et al., 2014). Aside from warming ambient temperatures, the increase in global travel and movement of goods has enabled a stochastic range expansion whereby populations of mosquitoes have been found with a random or jumping distribution (not a continuous route). It is well documented that the shipping of used tyres from vector endemic areas introduced *Ae. albopictus* into new territories as mosquitos use the rainwater collecting in the tyres at their point of origin as convenient breeding pools (Hawley et al., 1987; Reiter and Sprenger, 1987). The concerns regarding range expansion are compounded by the ability of native species of mosquito being capable vectors of the life-threatening pathogens mentioned above. *Ochlerotatus detritus*, a saltwater mosquito native to the U.K. (and voracious biter), has been found to be able to transmit Japanese encephalitis under laboratory conditions (Blagrove et al., 2016; Mackenzie-

Impoinvil et al., 2015). This is just one recent example but there are similar studies of many different species and pathogens (Gascon et al., 2010; Otranto et al., 2009; Patz et al., 2000). The relatively recent ease and accessibility of global travel has the potential to exacerbate the issues that range expansion of vector species may bring. Travellers and migrants sometimes journey from warmer climates with cases of malaria or other arboviruses; potentially providing a human reservoir of disease for the range expanding vector species (Gascon et al., 2010; Lobel et al., 1990). Even travellers that have not been to an endemic region have presented with cases of 'runway malaria', likely gained from an infected mosquito entering the cabin of the aeroplane (Hutchinson et al., 2005; Isaäcson, 1989; Rodger et al., 2008; Tatem et al., 2006). It is therefore a genuine (albeit not highly likely) concern that the capable vector species encounter infected travellers.

Much current mosquito research is directed towards understanding and attempting to overcome insecticide resistance. DDT was the insecticide of choice until significant mosquito resistance was recorded. Several different types of insecticide have been used since; organophosphates, carbamates and pyrethroids (Hemingway and Ranson, 2000; Nauen, 2007) with resistance to each of these shown in many classic vector mosquito species (Hemingway and Ranson, 2000; Moyes et al., 2017). Low levels of resistance are also being recorded in mosquito populations treated with the bacterial control agent *Bacillus thuringiensis israelensis* (Bti) despite its multiple toxins (Paris et al., 2011; Paul et al., 2005), however, many studies are underway to monitor and mitigate this (Moyes et al., 2017). Newer techniques such as the sterile insect technique (Alphey et al., 2010) and the introduction of WMel *Wolbachia* infection into mosquito populations (Hoffmann et al., 2011; Iturbe-Ormaetxe et al., 2011) have shown positive results but have not yet had widespread testing (Moyes et al., 2017). Moyes et al. (2017) describe the need for an integrated approach to mosquito control as 'critical', combining the reduction in larval sources with insecticide application. One aspect of many integrated

control programmes is to reduce the mosquito's opportunities for oviposition. As some species have particular oviposition requirements (Becker , 2010) some changes can be achieved simply by individuals reducing the amount of containers or covering collected water in endemic areas (Morales-Pérez et al., 2017). Larger scale strategies are often co-ordinated by local governing authorities such as digging deeper channels in marshy or boggy areas where specific species such as *Ochlerotatus* spp. are known to breed (James-Pirri et al., 2009; Rey et al., 2012).

Entomopathogenic nematodes (EPNs) and mermithid species, which are natural parasites of many vectors of interest, have been tested previously on mosquito species in laboratory studies (Becnel and Johnson, 1998; De Oliveira Cardoso et al., 2015; Poinar and Kaul, 1982; Welch and Bronskill, 1962). Mermithids are able to kill mosquitos and complete their life cycle within the host (Becnel and Johnson, 1998; Petersen, 1985; Petersen and Willis, 1972), but they have not been pursued at a commercial level due to difficulties of mass rearing, despite small-scale success in the laboratory (Creighton and Fassuliotis, 1982; Petersen, 1985).

However, this current research focused on using a range of EPNs that are currently available commercially, and other *Steinernema* species collected from the wild and bred up on a scale large enough to conduct pathogenicity studies (millions of individual EPNs). The advantage of this is that these species have been found in-country and so their potential field use is compliant with the Wildlife and Countryside Act (1981) (JNCC, 2009) which regulates the release of non-native organisms for biocontrol purposes. In the case of the commercially prepared EPNs, they are already produced and packaged on an industrial scale so a novel manufacturing process would not be required should this biocontrol method be adopted. The use of EPNs from the genera *Heterorhabditis* and *Steinernema* as biocontrol for vector species has been researched before (Dadd, 1971; Poinar and Kaul, 1982; Welch and Bronskill, 1962) and showed some promising results with Welch and Bronskill, (1962) showing *Neoplactana* (=Steinernema) DD136 could evade encapsulation by the immune system of *Ae. aegypti* when

invading in large numbers, whereas smaller numbers were more susceptible to the mosquitoes' immune defences. This previous research did not progress beyond laboratory stage because of several factors, one being the lack of consistency of results and methodology of previous pathogenicity assays (De Oliveira Cardoso et al., 2015) another being the relative expense and fragility of EPNs compared to chemical insecticides, which is an issue for all EPN biocontrol functions (Georgis et al., 2006).

The aim of this series of experiments was to ascertain whether EPNs both field-collected (see Chapter 3) and commercial, could be used to kill the larval stages of *Ae. aegypti* and *Oc. detritus* under lab conditions. Experiments were also conducted in order to examine whether EPNs capable of tolerating the environmental stressors found in habitats where mosquitos live including ranges of pH, salinity tolerance and effectiveness in different soil substrates. These questions are posed with the view that this research could be progressed to field study stage.

Pathogenicity methods

Insect sourcing and rearing

Aedes aegypti (New Orleans strain) eggs were obtained from the Liverpool Insect Testing Establishment (LITE) at the Liverpool School of Tropical Medicine. Egg papers were floated in a flat-bottomed tray containing approximately 7cm depth of nutrient-rich medium (cat biscuits in distilled water allowed to stagnate for a minimum of 24h). Hatched larvae were kept at room temperature (19-25°C) until they reached third instar. *Ochlerotatus detritus* larvae were field caught using nets and dippers (plastic cup on long handle) from the saltmarsh at Little Neston (N 53° 16' 40.771" W 3° 4' 6.967"). Once transported back to the laboratory at LJMU they were allowed to develop to third instar, incubated at 15℃ in containers of brackish water (25ppt salinity), collected with the larvae.

Seven different strains of EPN were used in these experiments. Commercial strains were sourced from BASF (*Steinernema feltiae, Steinernema carpocapsae*, *Heterorhabditis bacteriophora* and *Steinernema kraussei*) and others were reared from strains isolated from the EPN distribution survey (see Chapter 3), which included *Steinernema affine* (isolate designated 173) and *Steinernema feltiae* (93, 119 and 367). These field-collected strains were selected to provide a range of different species to test. *Steinernema feltiae* was the most abundant EPN isolated from the distribution survey, and these particular isolates were selected as they had the greatest abundance of individual nematodes, providing sufficient to perform the assays in the same numbers as the commercial strains. With regard to the *S. feltiae* strain (367), this was isolated from Lundy Island and was selected to observe any differences to other strains given the relative biological isolation of the island. *Galleria mellonella* were obtained from a live-food breeder (Internet Reptile). Identified EPNs were grown up to sufficient levels to conduct the pathogenicity assays by infecting *G. mellonella* with IJs which had been previously isolated in White traps. One ml of the water containing the IJ EPNs was pipetted onto a moistened 100mm filter circle in a Petri dish and 10 *G. mellonella* added and the Petri dish sealed. These were checked every 48 h for dead or moribund *G. mellonella*, which were then put into a modified White trap (see fig 2.1). Once the new IJ EPNs had emerged and made their way to the water, the process was repeated until sufficient live IJ stage EPNs were produced.

Pathogenicity assay to test mosquito susceptibility to EPNs

For *Ae. aegypti* assays for each condition a minimum of three 250ml capacity plastic cups were filled with 100ml distilled water, to which approximately 0.025g of crushed cat biscuit was added as food and 25 third instar larvae added to each cup. *Oc. detritus* are much larger larvae than *Ae. aegypti* and inhabit a salt marsh habitat with an average salinity of 25ppt, therefore in assays using that mosquito, only 15 larvae were added to 100ml of 25ppt salinity water. EPNs were added at doses of 0, 2000, 4000 and 8000 to three replicate cups. The amount

of EPNs used was based on using the recommended dose for soil usage as a baseline. Preliminary assays using *Chironomus plumosus* were conducted using a range of EPN doses from 1000 to 16000 (Chapter 2). The results of these experiments suggested that both the values of 1000 and 16000 were not effective and therefore not used in these assays.

EPN numbers were estimated by using a 1000µl micropipette with a trimmed tip to drop a 10µl solution of distilled water and dauer stage EPNs into a counting dish (5mm Petri dish which was scored to show a grid pattern). The numbers of live EPNs were counted under a light microscope and recorded. The dish was cleaned and the process repeated three times to obtain a mean count. This data was used to calculate the required volume of solution containing the requisite number of EPNs. This method of EPN counting was used throughout all assays that required more than 10 EPNs.

The requisite amount of EPNs were added to each cup holding the mosquito larvae using a micropipette with a trimmed tip. The top of each cup was covered with a fine grade netting and secured with an elastic band to prevent possible escape of the eclosed adults. This process was repeated for each different EPN species. The cups containing *Ae. aegypti* were incubated at 20ºC as this was a temperature that was within the known temperature tolerances of the EPN species and one that would also be tolerable for the mosquito species. *Oc. detritus* assays were incubated at 15ºC as they are a U.K. native species and 15ºC was within the tolerances of both EPN and the mosquito. Each experiment ran for 20 days. Each experiment was conducted at three separate time points with a minimum of three replicates per time point. Larvae were inspected every 48h and the number of dead, live, pupated or eclosed individuals were recorded. Any eclosed mosquito larvae were removed from the experiment and destroyed.

Investigating the effect on survival of EPNs when exposed to different water salinities

To investigate the effect of salinity on the survival of EPNs in order to ascertain if they had practical application for the control of brackish water insects, EPN survival was determined across a range of salinities. Using a curved bottomed 96-well plate, 50ul of saline solution (either 0, 10, 20, 30, 40, 50 or 60 parts per thousand (ppt)) was added to 12 wells in a 96 well plate. To each of these wells 1 EPN was added, the plate lid was sealed with Parafilm™ and the plate was incubated at 15ºC. This was repeated for all seven species of EPN. Each plate was checked every 24 h and the number of dead or live EPNs was recorded. The experiment ran for 7 days and was repeated 3 times.

Investigating the effect on survival of EPNs when exposed to different pHs

Fifty microlitres of water adjusted to pH 3, 4, 5, 6, 7, 8, 9 or 10 was added to 8 wells in a 96 well plate. The range of pHs were obtained by adjustment of water using 1M Hydrochloric acid (HCl) or 0.1M Sodium Hydroxide (NaOH). This was confirmed using a portable pH meter (Jenway 3051). Ten EPNs were added to each well. The plate was sealed with Parafilm™ and incubated at 15ºC. This was repeated for all seven species of EPN. The plates were checked daily and the number of dead and alive nematodes were recorded. The experiment ran for 7 days and was repeated 3 times. In this assay the field-collected strains of *S. glaseri* (119) was substituted for *S. glaseri* (93) due to low numbers of *S. glaseri* (93) being available.

Statistical Analyses

Survival of mosquitoes and nematodes was analysed using Log-Ranked tests using Oasis and Oasis 2 statistic software (Yang et al., 2011; Han et al., 2016). Significance levels were taken at $P \leq 0.05$.

Results

Susceptibility of A. aegypti exposed to commercial EPNs

There was a highly significant difference in the survival of *Ae. aegypti* larvae when exposed to all doses (2000, 4000 and 8000 nematodes) of commercially produced *S. feltiae*, *S. carpocapsae*, *H. bacteriophora* and *S. kraussei* (Figures 4.1a-d) when compared to the control. ($p \leq 0.001$). The LT_{50} differed for each EPN species. For example, dosages of 4000 and 8000 *S. feltiae* reached LT_{50} following 6 days exposure (no sig difference between treatments = 0.0845) whereas the dose of 2000 did not reach LT_{50} until 10 days following exposure. For *S. carpocapsae* the 8000 dose reached LT_{50} at 4 days following exposure and resulted in a lower rate of survival overall with less than 20% surviving beyond 8 days. Doses of 2000 and 4000 had killed fewer larvae than the 8000 dosage by the 24 day experimental end. All doses of *H. bacteriophora* reached LT_{50} between 8 and 10 days. The 8000 EPN dose continued to exert a pathogenic effect on the larvae, with, 0% survival after 18 days post exposure. The doses of 2000 and 4000 did not reach that level of mortality. For *S. kraussei* the dose of 8000 LT_{50} was reached at 4.5 days following exposure. LT_{50} was reached at 5 days for the 4000 dose and 2000 at 6 days post exposure. Following this the dosages of 8000 and 4000 followed a similar pathogenic curve and reduced the surviving larvae to 20% by the 8^{th} day following exposure.

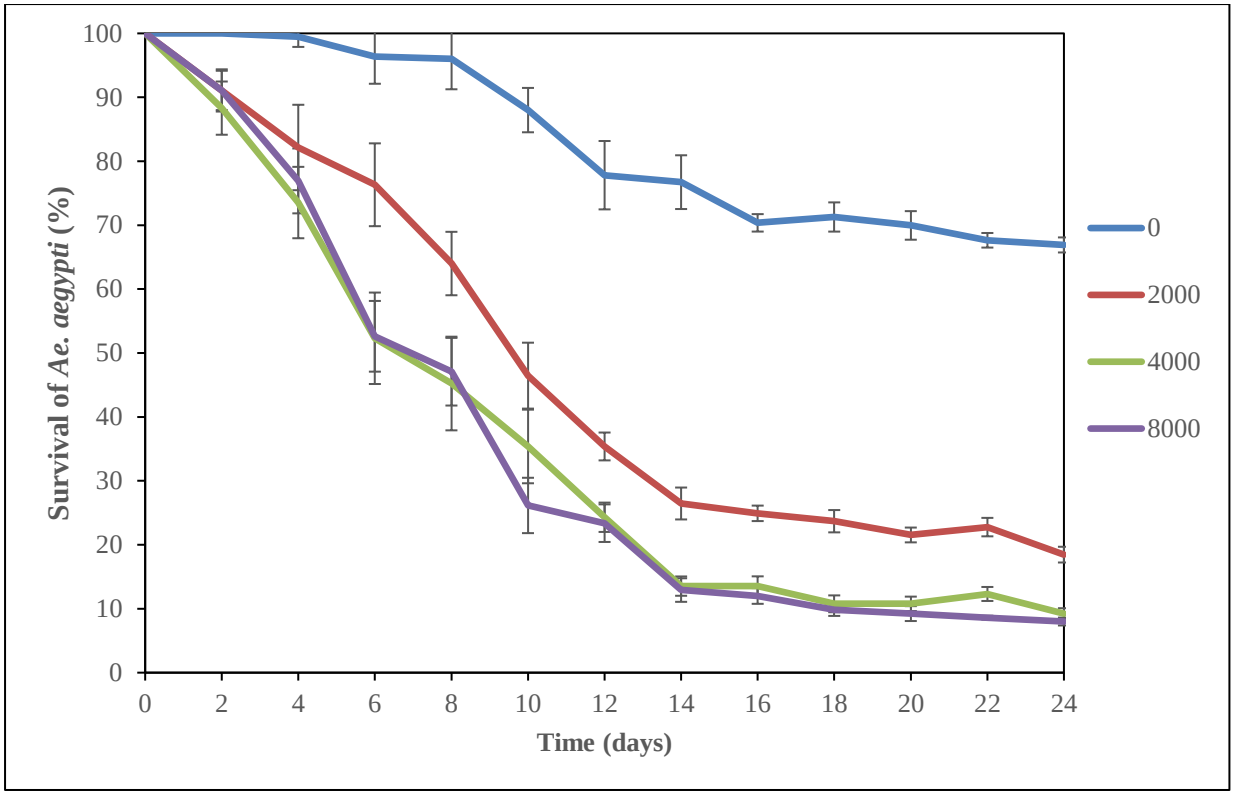


Fig 4.1a Survival of *Ae. aegypti* larvae (n= 25) exposed to the commercial strain of *S. feltiae*. Bars represent ± one standard error.

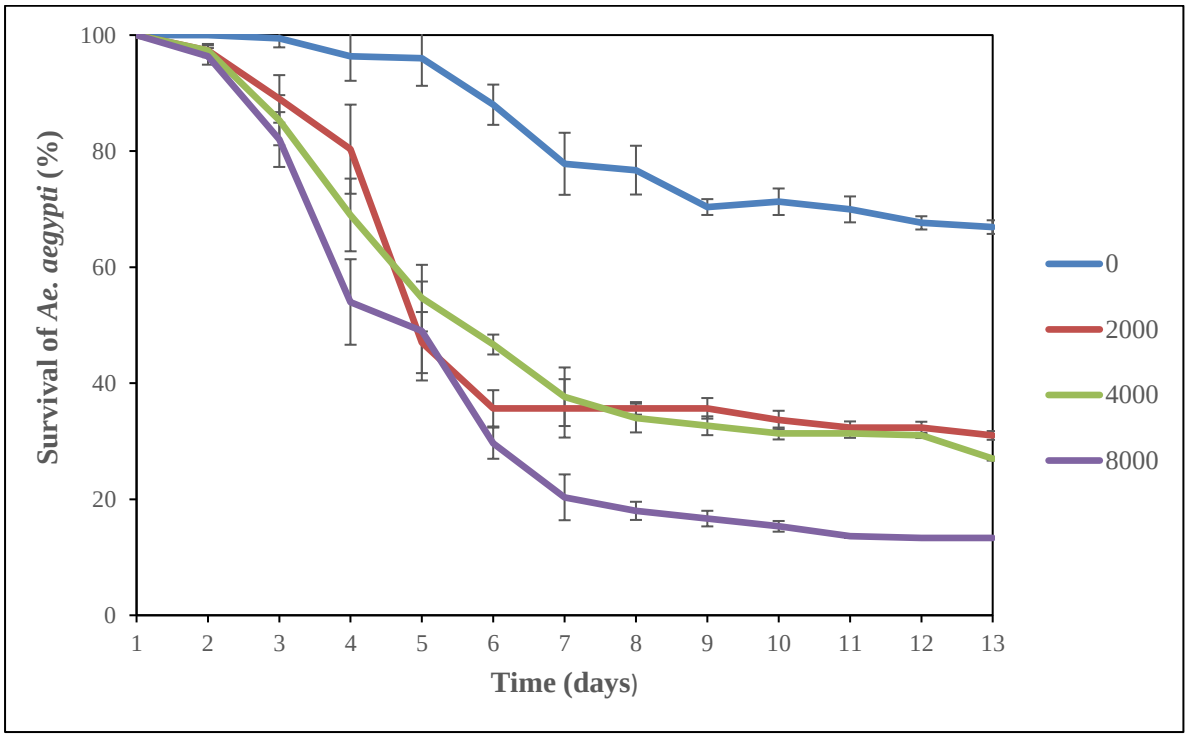


Fig 4.1b Survival of *Ae. aegypti* larvae (n= 25) exposed to the commercial strain of *S. carpocapsae*. Bars represent ± one standard error.

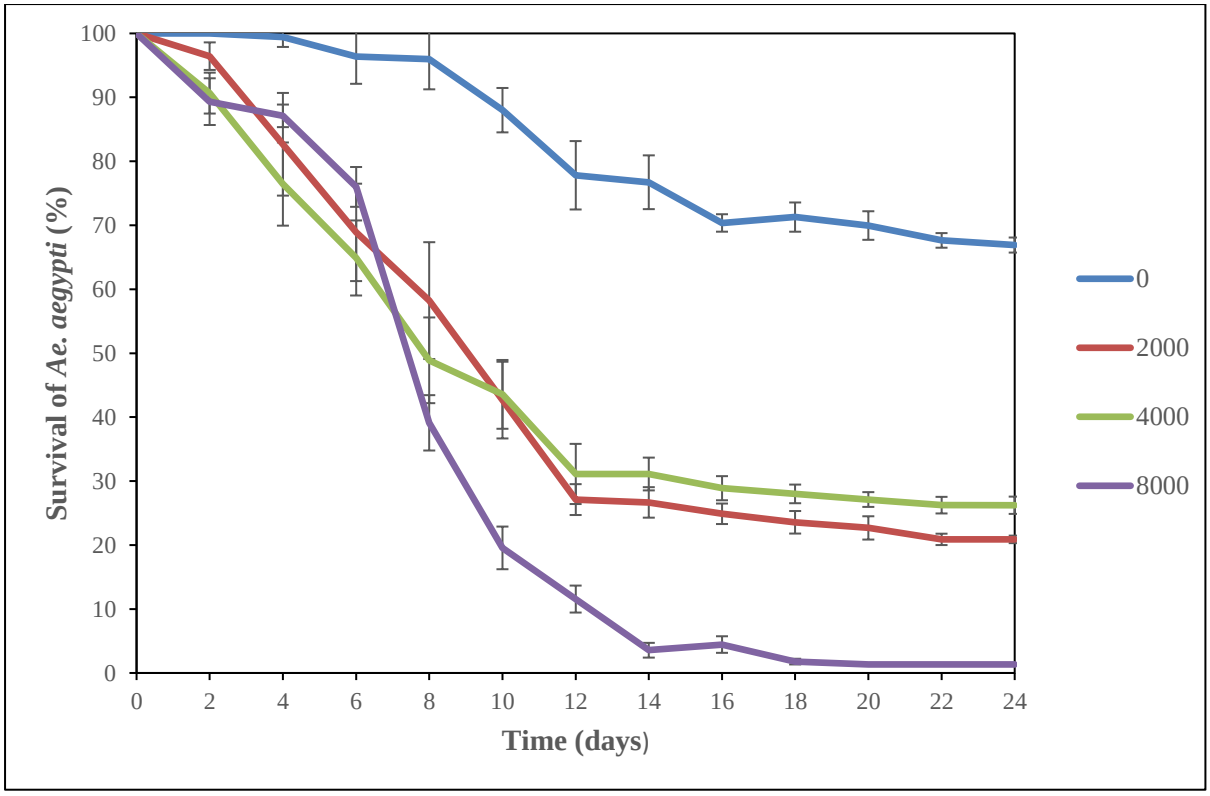


Fig 4.1c Survival of *Ae. aegypti* larvae (n= 25) exposed to the commercial strain of *H. bacteriophora* Bars represent ± one standard error.

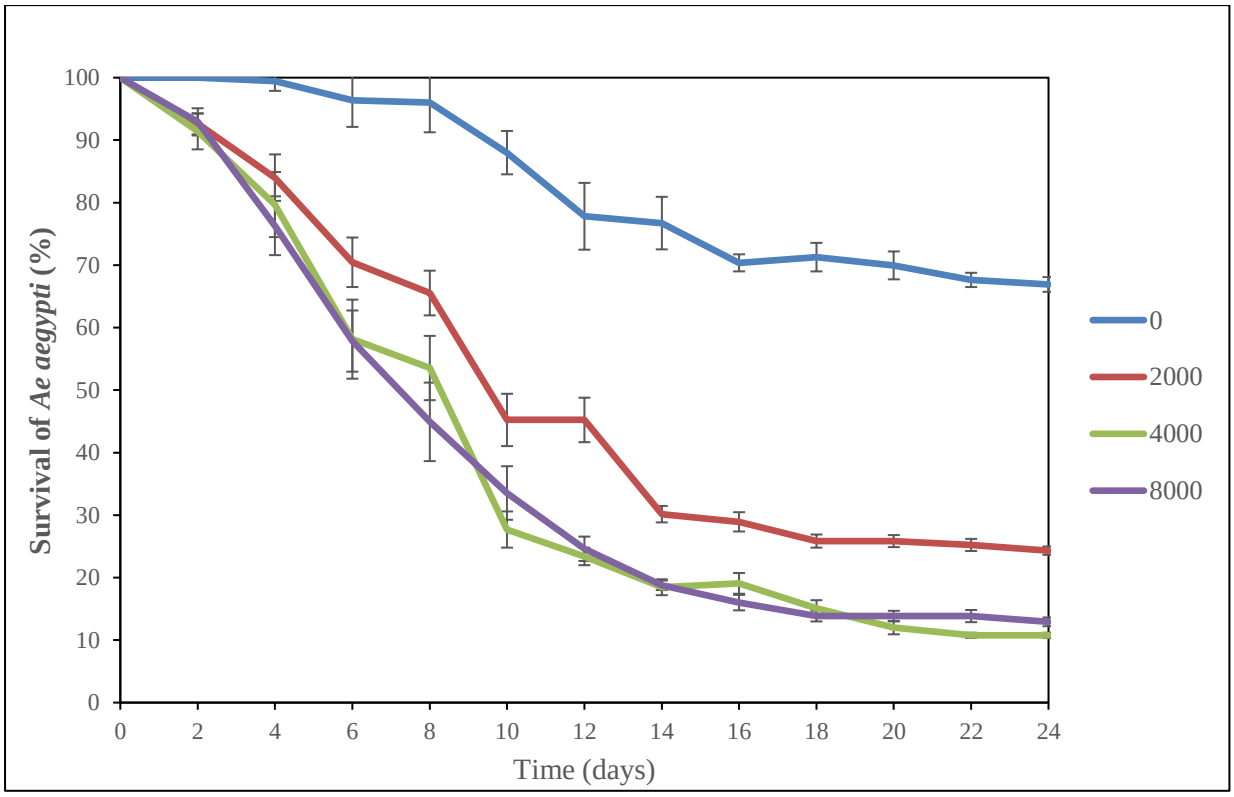


Fig 4.1d Survival of *Ae. aegypti* larvae (n= 25) exposed to the commercial strain of *S. kraussei*. Error bars represent ± one standard error.

Susceptibility of A. aegypti exposed to naturally isolated EPNs

In striking contrast to the commercially available EPNs, naturally isolated *S. glaseri* (strain 93) when applied at does of 2000 (P = 0.0988), 4000 (P = 0.1519) and 8000 (P = 0.1134) nematodes had no significant effect on survival of *Ae. aegypti* although from day 4 onwards no overlap in error bars is seen(Fig. 4.2b). The survival of *Ae. aegypti* was not affected by exposure to *S. glaseri* (367) applied at 2000 (P = 0.4323), 4000 (P = 0.7643) or 8000 EPNs (P = 0.8220) (Fig 4.2a) until the 10th day post exposure. *S. affine* (173) also did not significantly affect the survival of *Ae. aegypti* when exposed to 2000 (P =0.0735), 4000 (P =0.1286) or 8000

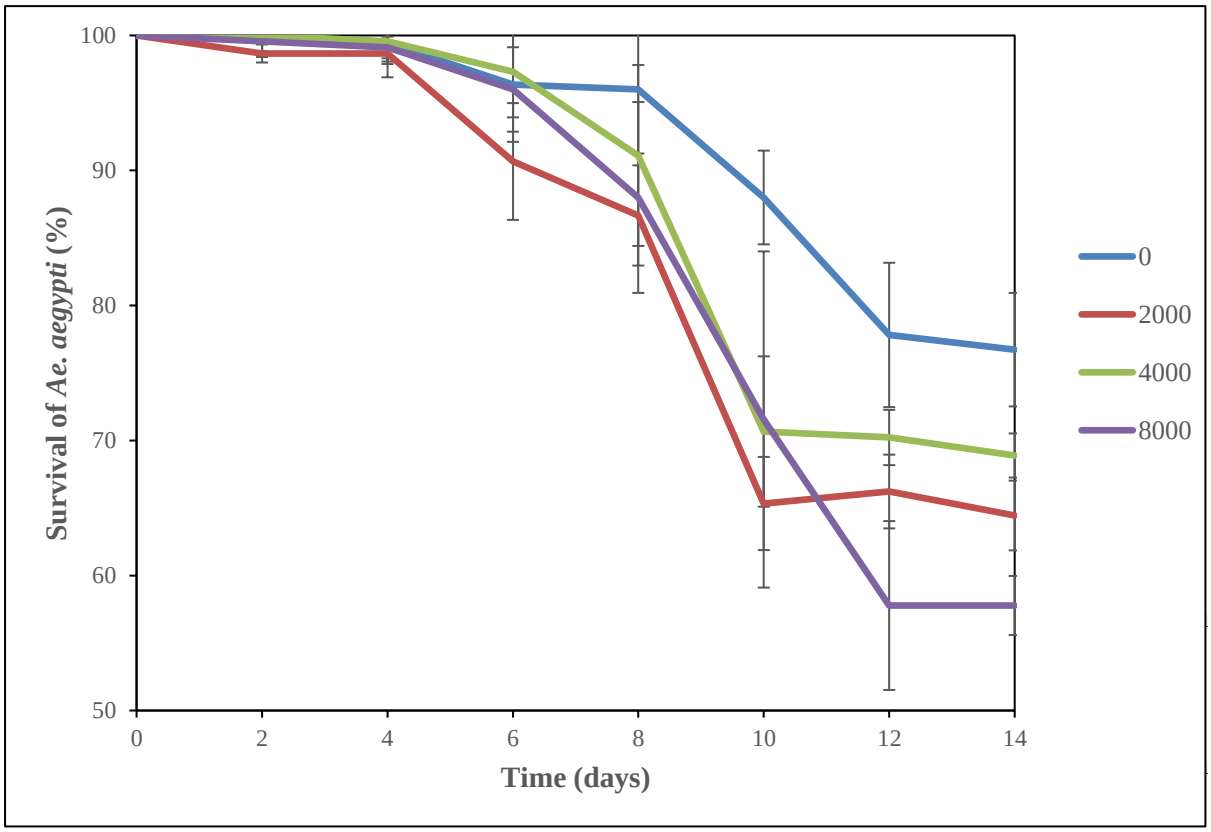


Fig 4.2a Survival of *Ae. aegypti* larvae (n= 25) exposed to the naturally isolated strain of *S. glaseri* (367). Bars represent ± one standard error.

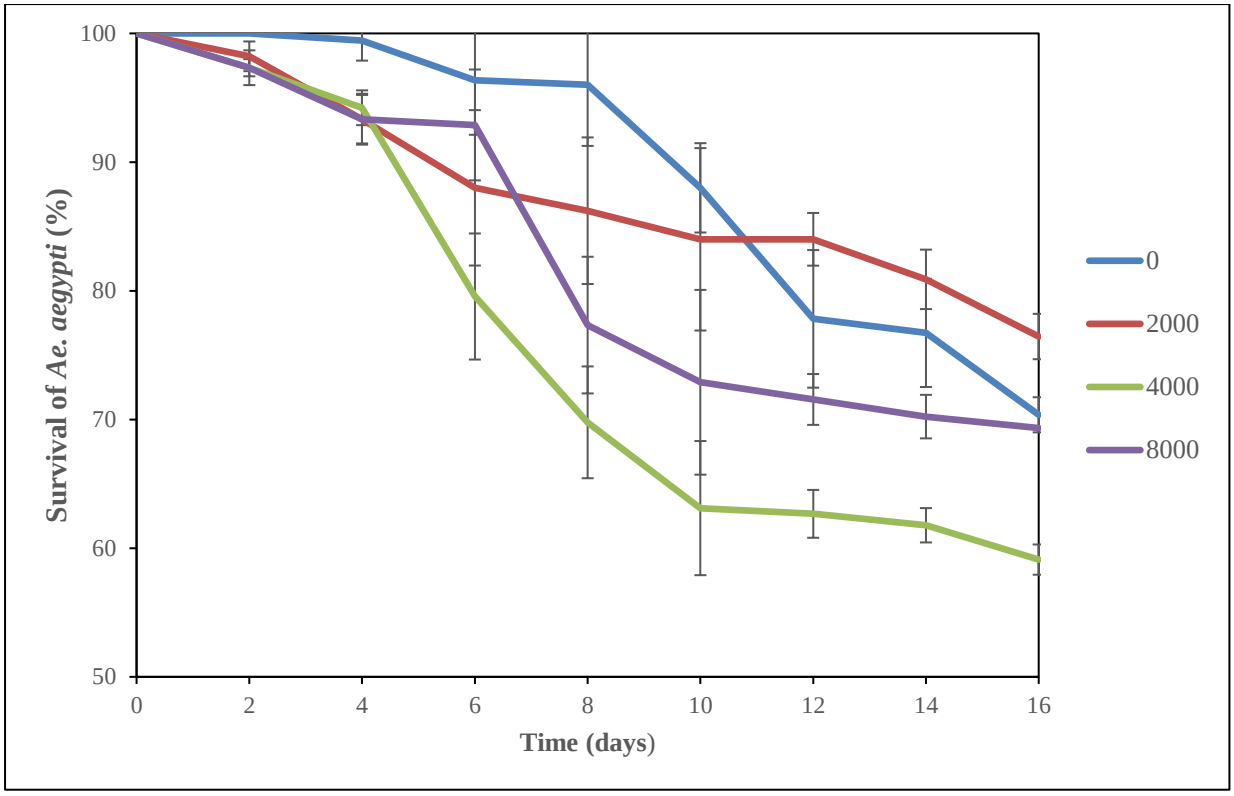


Fig 4.2 b Survival of *Ae. aegypti* larvae (n= 25) exposed to the naturally isolated strain of *S. glaseri* (93). Bars represent ± one standard error.

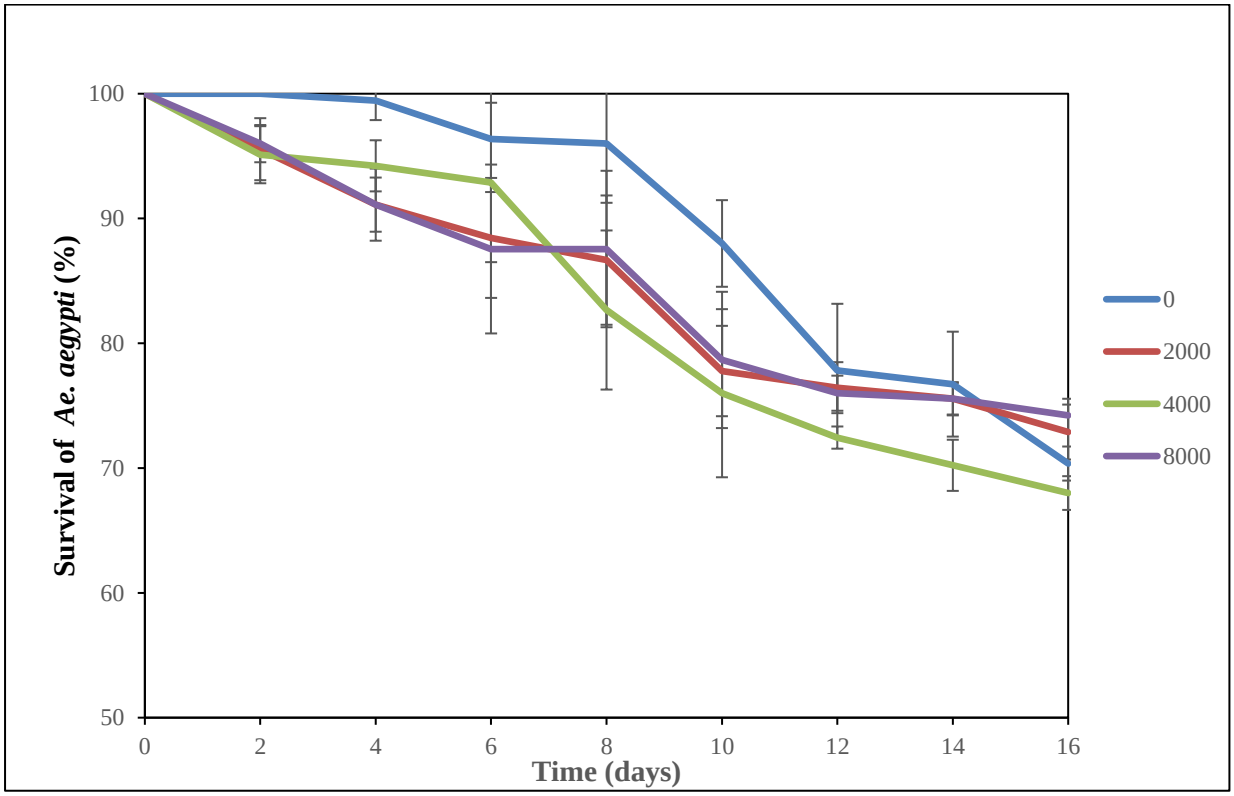


Fig 4.2c Survival of *Ae. aegypti* larvae (n= 25) exposed to the naturally isolated strain of *S. affine* (173). Bars represent ± one standard error.

Pathogenicity of commercial EPNs towards Ochlerotatus detritus

Commercial *S. feltiae*, *H. bacteriophora*, *S. kraussei* and *S. carpocapsae* caused significant mortality to *Oc. detritus* compared to the untreated control when applied at 2000 ($P \leq 0.001$), 4000 ($P \leq 0.001$) and 8000 nematodes ($P \leq 0.001$) (Fig 4.3a-4.3d). However, the LT_{50} differed depending on the nematode species used. For *S. feltiae* the LT_{50} was achieved within 1 day of exposure to *Oc. detritus* using all dosages of EPN. The dose of 8000 *H. bacteriophora* reached LT_{50} at 1.5 days following exposure, 4000 EPNs at 3 days and 3.5 days for the 2000 EPN dose. For *S. kraussei* the quickest pathogenic effect was shown at dosages of 8000 EPNs, with LT_{50} reached at 2.5 days following exposure. There was no significant difference between the survival of *Oc. detritus* exposed to 2000, 4000 and 8000 *S. kraussei* ($P > 0.05$). For *S. carpocapsae* the LT_{50} was reached at 2.5 days following exposure for both dosages of 4000 and 8000 EPNs. The dose of 8000 *S. carpocapsae* was significantly more pathogenic to *Oc. detritus* than 2000 and 4000 nematodes ($P < 0.01$). There were no significant differences between the survival of *Oc. detritus* exposed to 2000 and 4000 *S. carpocapsae* ($P = 0.1573$).

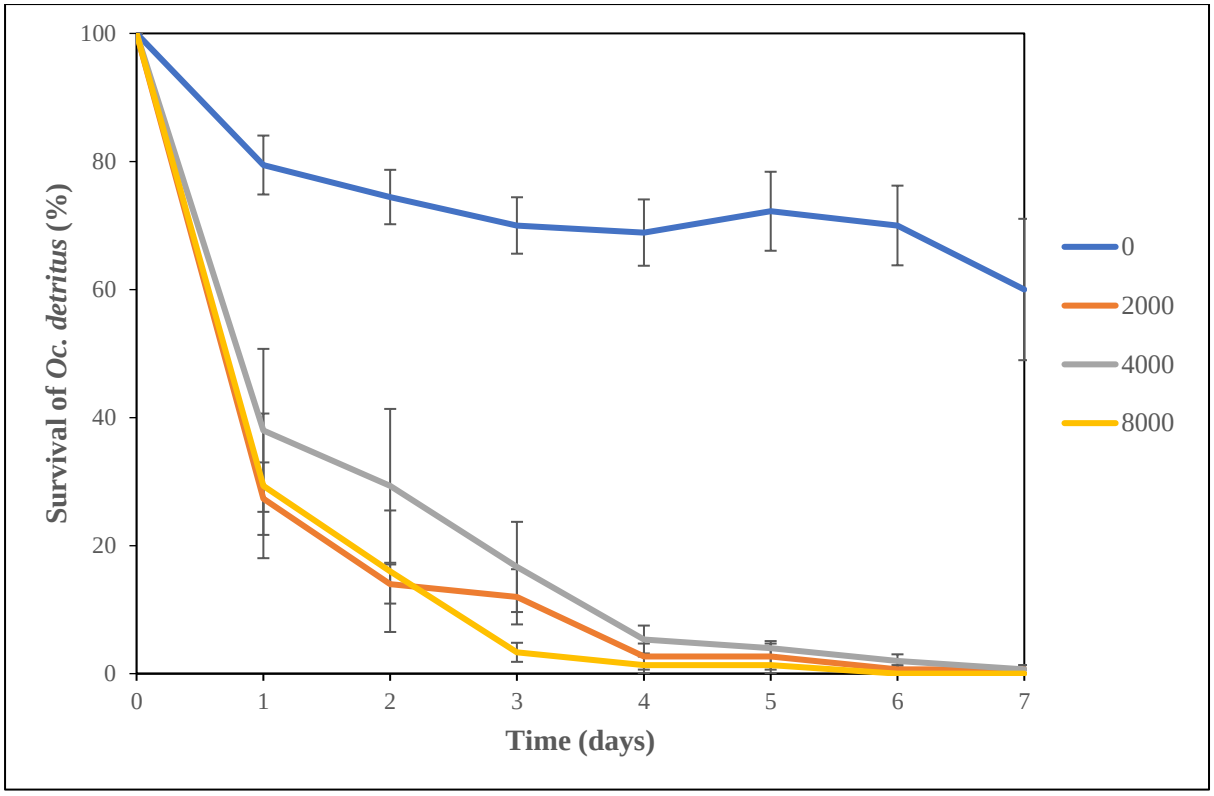


Fig 4.3a Survival of *Oc. detritus* larvae (n = 15) exposed to the commercial strain of *S. feltiae*. Bars represent ± one standard error.

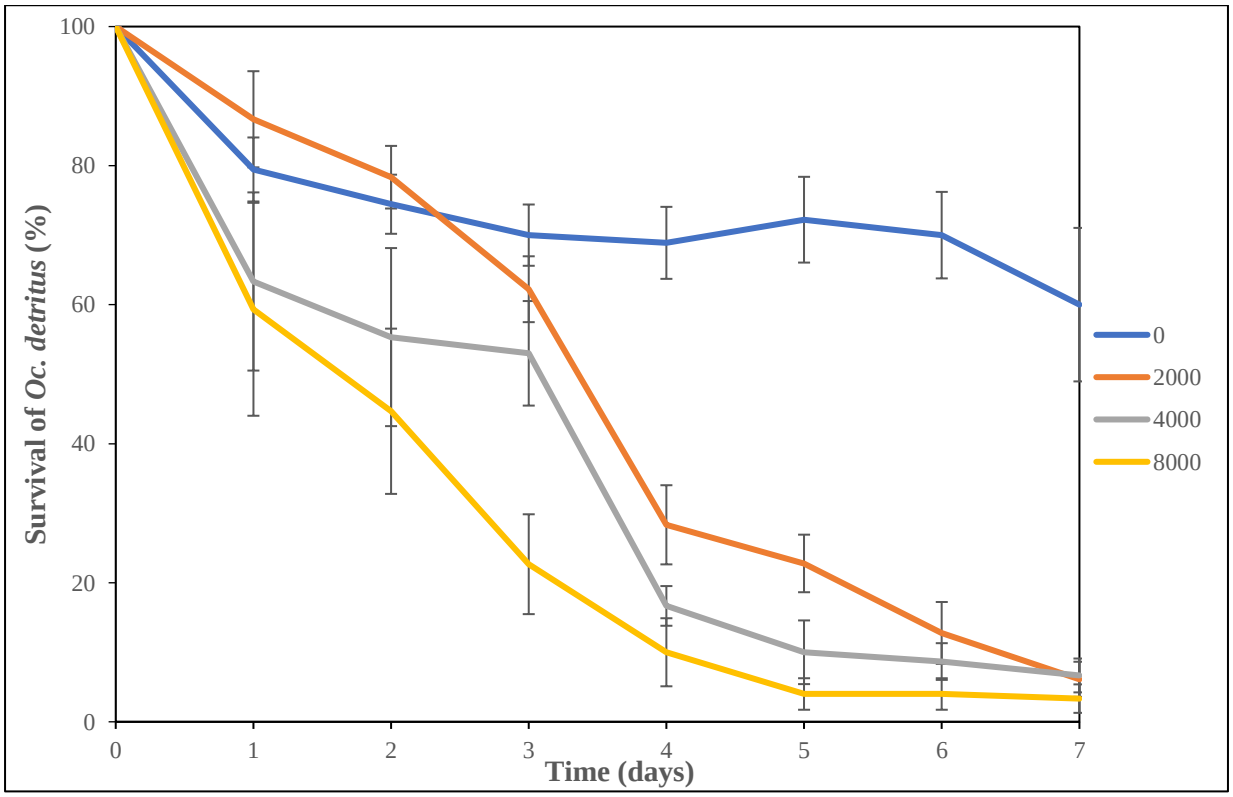


Fig 4.3b Survival of *Oc. detritus* larvae (n = 15) exposed to the commercial strain of *H. bacteriophora*. Bars represent ± one standard error.

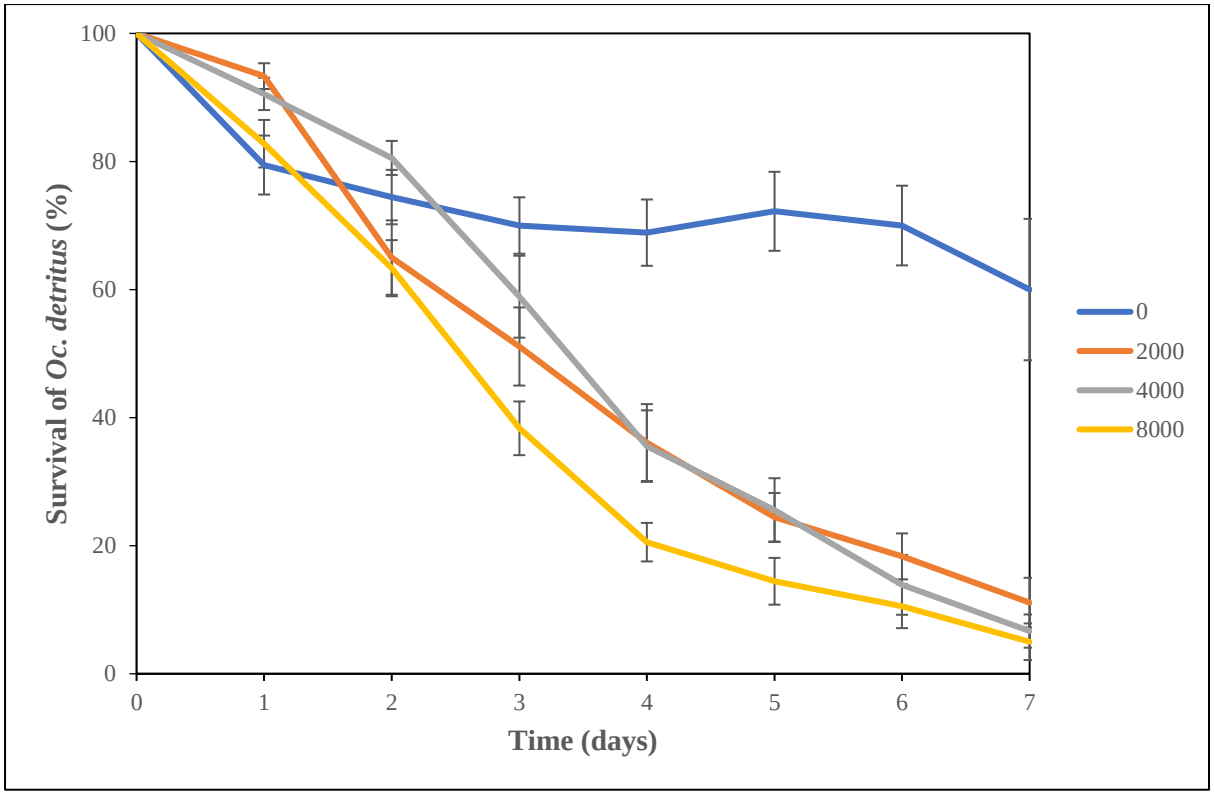


Fig 4.3c Survival of *Oc. detritus* larvae (n = 15) exposed to the commercial strain of *S. kraussei*. Bars represent ± one standard error.

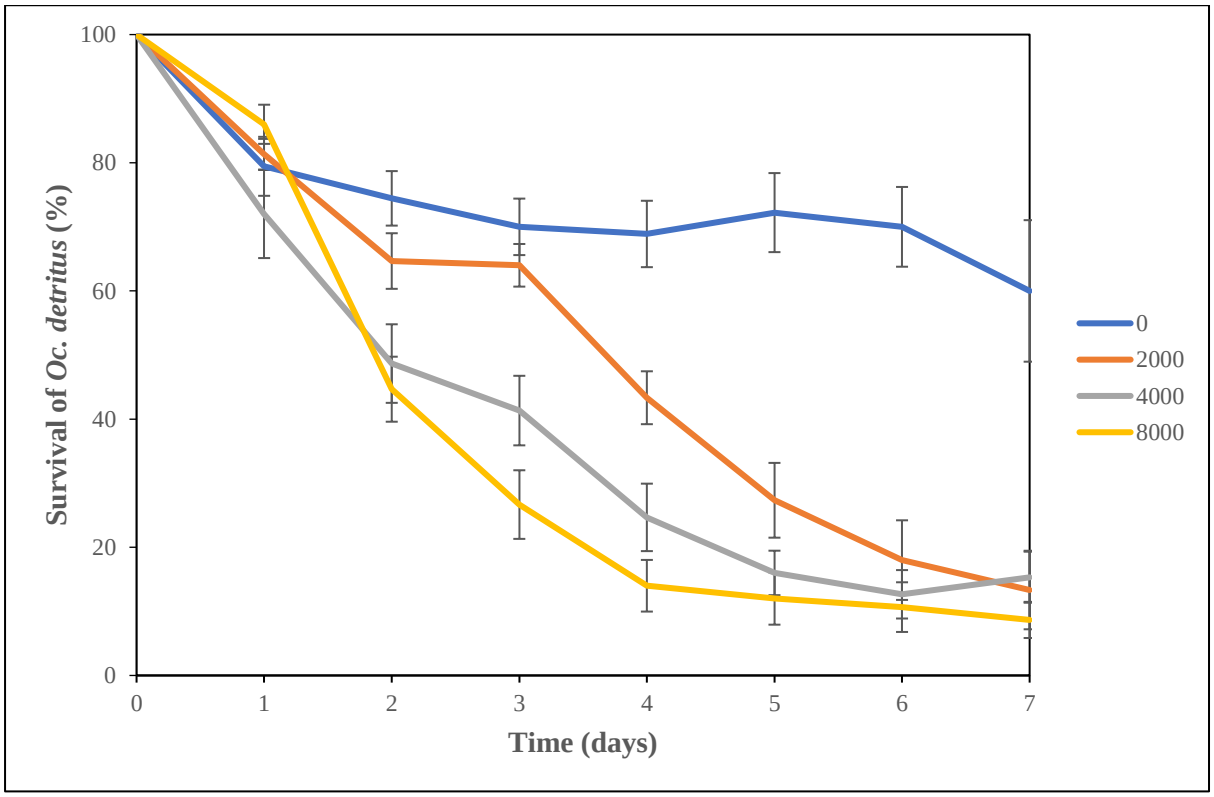


Fig 4.3d. Survival of *Oc. detritus* larvae (n = 15) exposed to the commercial strain of *S. carpocapsae*. Bars represent ± one standard error.

Pathogenicity of naturally isolated EPNs towards Ochlerotatus detritus

Similar to the commercial EPNs, naturally isolated *S. glaseri* (93 and 367) and *S. affine* (strain 173) caused a significant decrease (40-50%) in survival of *Oc. detritus* larvae when exposed to 2000 ($P \leq 0.001$), 4000 ($P \leq 0.001$) and 8000 nematodes ($P \leq 0.001$) compared to the untreated controls (Fig 4.4a – 4.4c). The LT_{50} however differed between nematodes. For example, the dose of 8000 *S. glaseri* (367) caused a LT_{50} of 1.75 days, whereas the dose of 4000 had an LT_{50} of 2.5 days and 2000 had a LT_{50} of 3 days following exposure. There was no significant difference in the survival of *Oc. detritus* exposed to 4000 and 8000 *S. glaseri* (367) ($P > 0.05$) but there was a significant difference between the survival of *O. detritus* exposed to 2000 and 8000 nematodes ($P \leq 0.001$). The doses of 2000 and 8000 *S. glaseri* (93) caused the quickest pathogenic effect to *Oc. detritus* and had an LT_{50} at 24 h. The dose of 8000 *S. glaseri* (93) was significantly more pathogenic towards *Oc. detritus* than 2000 nematodes ($P = 0.2064$) and 4000 nematodes ($P = 0.0629$). Exposure to all doses of naturally isolated *S. affine* (173) resulted in a reduction in survival of *Oc. detritus* to < 20% by day 7. Dosages of 2000 and 4000 EPNs reached a LT50 at 1.5 days and the dose of 2000 EPNs was significantly more pathogenic than 4000 ($P \leq 0.001$) and 8000 nematodes ($P \leq 0.001$). There was no significant difference between the survival of *Oc. detritus* exposed to 4000 and 8000 *S. affine* (strain 173) ($p=0.8096$)(Figs. 4.4a to 4.4c).

Over the course of the experiment, samples of dead *Ae. aegypti* and *Oc. detritus* were examined for any EPNs that had penetrated into the larvae. Fig 4.5 shows 3 *S. kraussei* infective juveniles that had penetrated into the head capsule of the mosquito. This provides visual evidence that EPNs were able to penetrate the larvae and were the cause of pathogenicity in the tested larvae.

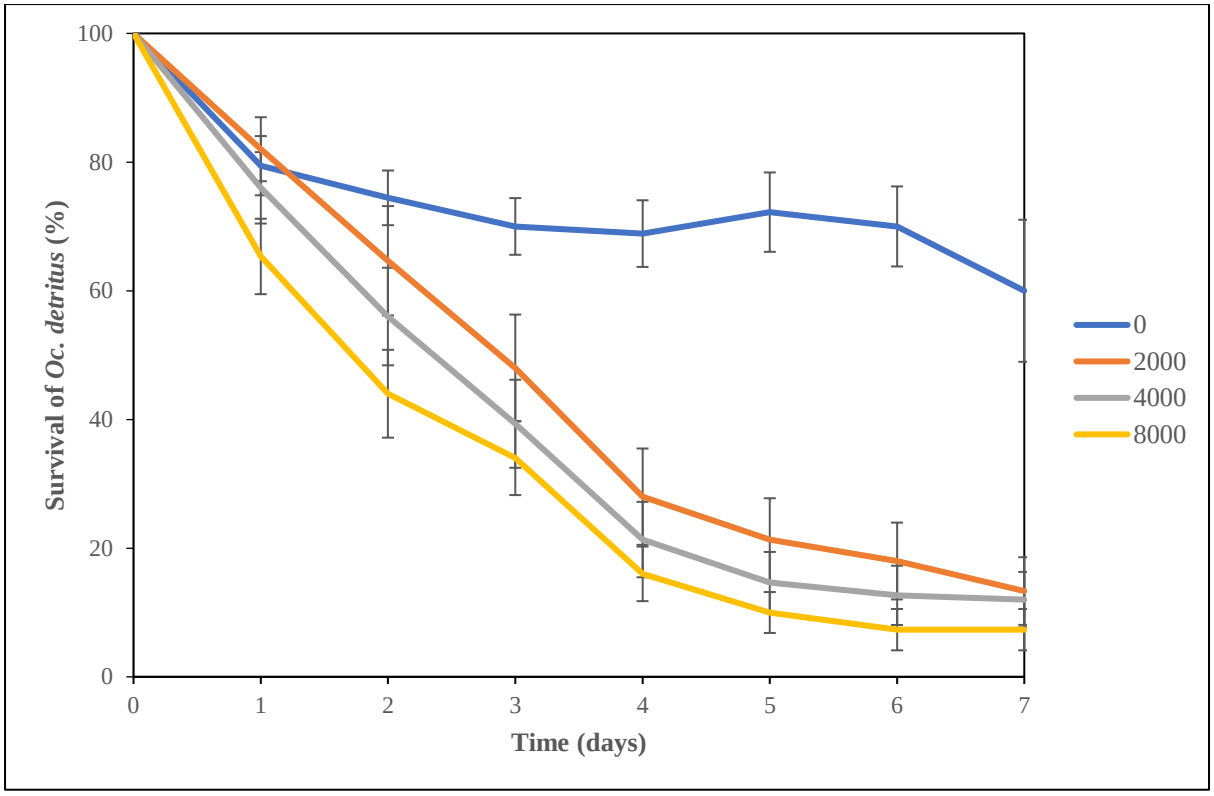


Fig 4.4a Survival of *Oc. detritus* larvae (n = 15) exposed to the naturally isolated *S .glaseri* (strain 367). Bars represent ± one standard error.

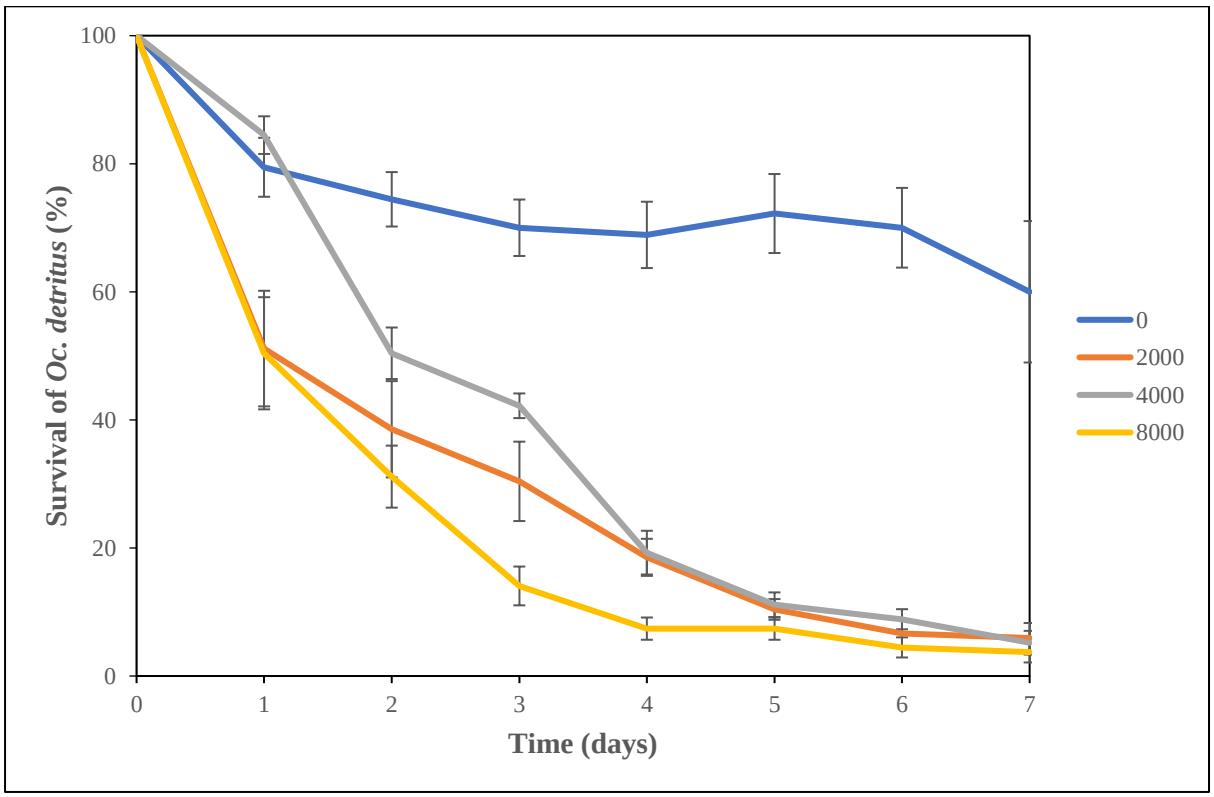


Fig 4.4b Survival of *Oc. detritus* larvae (n = 15) exposed to the naturally isolated *S .glaseri* (strain 93). Bars represent ± one standard error.

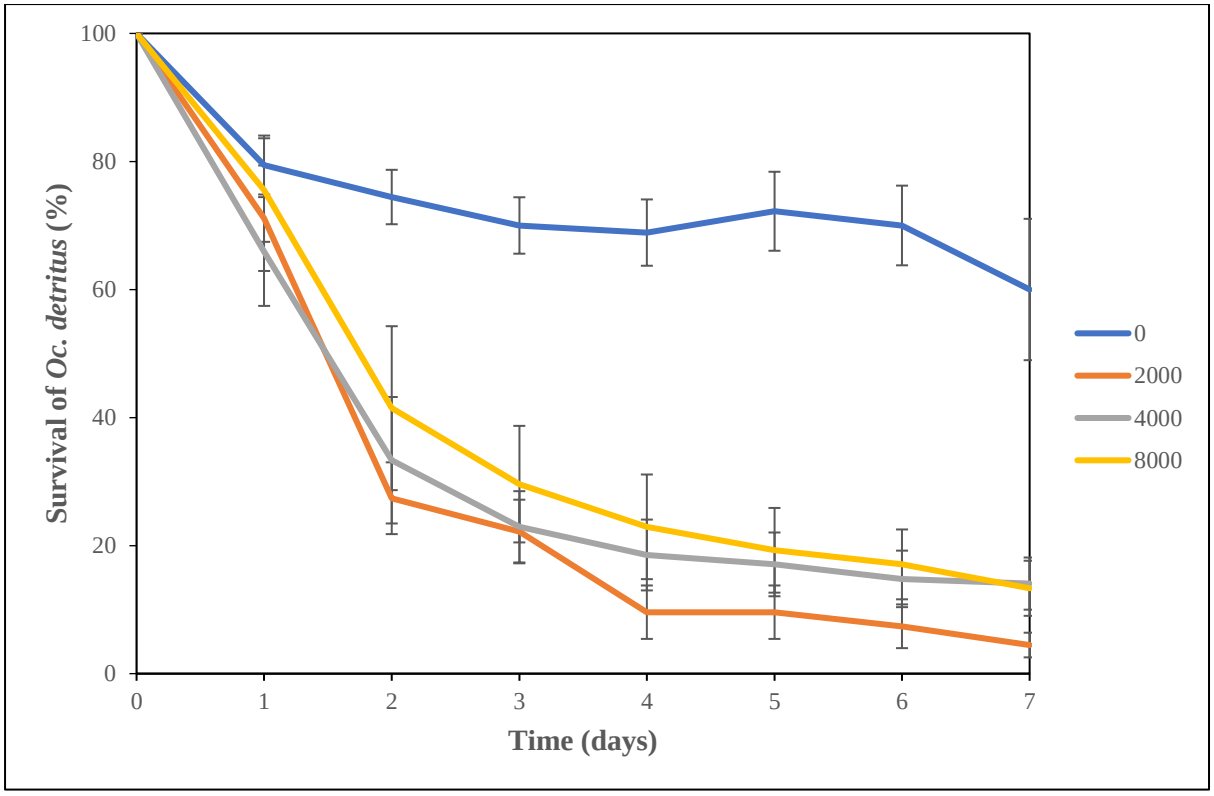


Fig 4.4c Survival of *Oc. detritus* larvae (n = 15) exposed to the naturally isolated *S. affine* (strain 173). Bars represent ± one standard error.

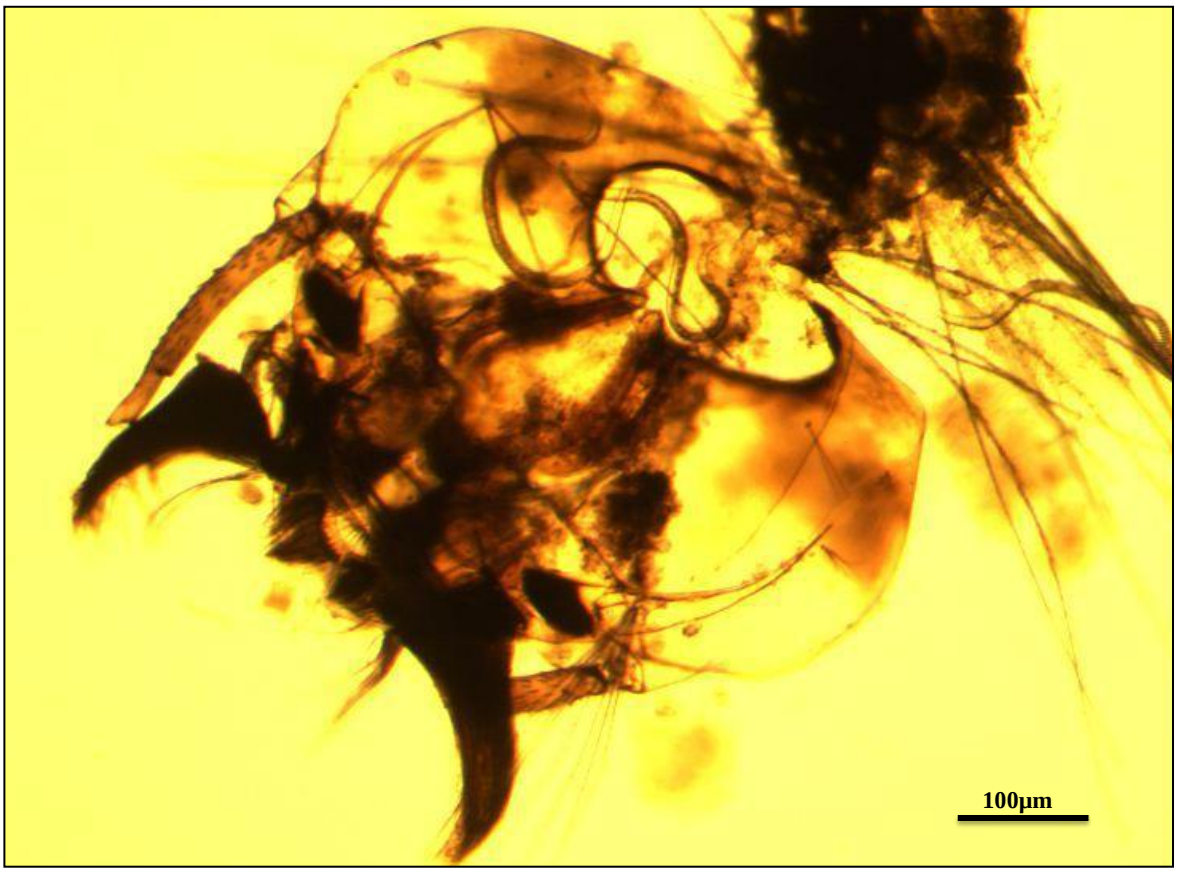


Fig 4.5 *S. kraussei* found inside the head of *Ae. aegypti* after 7 days infection.

Survival of EPNs exposed to different salinities

The survival of both commercial EPNs (*S. feltiae, S. carpocapsae*, *H. bacteriophora* and *S. kraussei*) and naturally isolated *S. affine* (173) and *S. feltiae* (93, 119 and 367) differed in their tolerance to salinities of 0, 10, 20, 30, 40, 50 or 60 ppt over 7 days (Table 4.1). Survival rates in the control condition (0 ppt) were not high overall especially for *S. glaseri* (367). Most EPN strains and species tested showed better survival (compared to the control) at 10 ppt that at greater salinities. From 20 ppt significant decreases in survival were observed in *H. bacteriophora*, *S. carpocapsae* and *S. glaseri* (93). Survival of *S. feltiae* and *S. affine* (173) was significantly decreased from 30 ppt. Interestingly, *S. glaseri* (367), the strain from Lundy Island was observed to have greater survival from 10 ppt to 50 ppt than in the 0 ppt control. Similarly, *S. carpocapsae* survived in greatest numbers from 10 ppt to 40 ppt. All EPN survival was significantly decreased by 60 ppt. Although significantly depleted in comparison to the control, *H. bacteriophora* had the highest survival rate of all EPNs at 60 ppt (11.11 (± 5.73)). Full statistical results are available at **Appendix 3**

Table 4.1 Results of tests of 3 field collected strains of EPN and 4 commercial strains of EPN when exposed to a particular salinity over 7 days and P values for Log Rank statistical analysis when compared to those in 0 ppt salinity.

EPN species	**% live following 7 days exposure (± standard error) P value (bold)**						
	Salinity (parts per thousand)						
	0	10	20	30	40	50	60
***Steinernema feltiae* (commercial)**	57.15 (± 5.27)	40.05 (± 4.48) **0.0546**	41.78 (± 5.82) **0.2034**	30.63 (± 3.52) **0.0015**	37.33 (± 3.52) **0.0491**	18.44 (± 4.20) **0.0086**	2.52 (± 1.31) **≤ 0.001**
***Heterorhabditis bacteriophora* (commercial)**	54.22 (±13.51)	53.11 (±11.69) **0.6143**	33.07 (± 5.09) **0.0030**	16.96 (± 3.60) **≤ 0.001**	18.13 (± 6.90) **0.0001**	22.33 (± 9.79) **≤ 0.001**	11.11 (± 5.73) **≤ 0.001**
***S. carpocapsae* (commercial)**	37.65 (± 5.73)	51.49 (± 6.04) **0.0056**	55.48 (± 4.46) **0.0048**	54.69 (± 4.81) **0.0025**	44.35 (± 7.81) **0.0444**	31.86 (± 5.46) **0.4468**	0.00 (± 0.00) **≤ 0.001**
***S. glaseri* (367)**	18.77 (± 3.65)	40.37 (± 5.58) **0.0167**	41.40 (± 5.9) **0.0905**	45.38 (± 4.93) **0.8249**	31.45 (± 5.76) **0.4442**	27.27 (± 4.71) **0.4785**	1.63 (± 0.871) **≤ 0.001**
***S. glaseri* (93)**	54.08 (± 6.76)	36.33 (3.437) **0.0289**	33.96 (± 7.26) **0.0057**	36.19 (± 4.6) **0.0698**	28.43 (± 6.66) **0.0292**	20.53 (± 5.4) **0.0001**	0.53 (± 0.53) **≤ 0.001**
***S. affine* (173)**	40.43 (± 9.58)	34.9 (± 5.76) **0.737**	29.84 (± 6.16) **0.144**	16.87 (±4.54) **0.0120**	14.10 (± 4.46) **0.0011**	1.25 (± 1.25) **≤ 0.001**	0.00 (± 0.00) **≤ 0.001**

Survival of EPNs exposed to different pHs

There was no significant difference in the survival of the commercial EPNs (*S. feltiae, S. carpocapsae*, *H. bacteriophora* and *S. kraussei*) and naturally isolated *S. affine* (173) and *S. feltiae* (93, 119 and 367) exposed to pH 4, 5, 6, 7, 8, 9 and 10 over 7 days (Table 4.2). Full statistical results available at **Appendix 3**).

Table 4.2 Results of tests of 3 field collected strains of EPN and 4 commercial strains of EPN when exposed to a particular pH over a 7 day period. (n=12) Values are percentage survival (± SE). Significance value (P) Log Rank statistical analysis of survival over 7 day period when compared to pH7 is included.

EPN	**Percentage live following 7 days exposure (± standard error) P value (bold)**						
	pH						
	4	5	6	7	8	9	10
***Steinernema feltiae* (commercial)**	54.17 (± 12.50) **0.688**	68.75 (± 2.08) **0.880**	79.17 (± 7.22) **0.928**	71.67 (± 8.17)	85.43 (± 7.98) **0.743**	79.17 (± 7.98) **0.857**	72.92 (± 13.34) **0.976**
***Heterorhabditis bacteriophora* (commercial)**	66.67 (± 14.83) **0.596**	77.08 (± 14.18) **0.681**	83.33 (± 11.28) **0.872**	91.67 (± 3.73)	83.33 (± 5.89) **0.799**	75.00 (± 15.96) **0.616**	72.92 (± 13.77) **0.481**
***S. kraussei* (commercial)**	83.33 (± 5.89) **0.517**	77.08 (± 9.24) **0.649**	77.08 (± 6.25) **0.686**	72.92 (± 8.89)	81.25 (± 3.99) **0.627**	62.50 (± 20.82) **0.922**	62.50 (± 20.11) **0.895**
***S. carpocapsae* (commercial)**	77.08 (± 3.99) **0.790**	60.42 (± 21.61) **0.625**	58.33 (± 25.00) **0.486**	87.50 (± 4.17)	62.50 (± 21.65) **0.590**	60.42 (± 19.06) **0.565**	54.17 (± 26.68) **0.378**
***S. glaseri* (367)**	31.25 (± 10.96) **0.661**	35.42 (± 9.24) **0.965**	45.83 (± 4.17) **0.797**	40.00 (±3.12)	41.67 (± 11.28) **0.834**	50.00 (± 12.50) **0.643**	47.91 (± 12.14) **0.744**
***S. glaseri* (119)**	64.58 (± 11.47) **0.985**	56.25 (± 10.42) **0.728**	54.17 (± 8.67) **0.820**	55.00 (± 5.00)	64.58 (± 8.67) **0.964**	54.17 (± 8.67) **0.939**	64.58 (± 12.44) **0.96**
***S. affine* (173)**	37.50 (± 12.50) **0.349**	39.58 (± 11.47) **0.440**	43.75 (± 8.59)	70.00 (± 8.12)	35.42 (± 7.12) **0.329**	54.17 (± 7.12) **0.808**	41.67 (± 12.87) **0.496**

Discussion

The results of the pathogenicity assays show that EPNs are not uniformly pathogenic against *Ae. Aegypti* and *Oc. detritus*. EPN strains tested against *Ae. aegypti* were less successful in terms of overall mortality and time to LT_{50} than when tested against *Oc. detritus*. The field-collected strains showed a very poor pathogenic effect towards *Ae. aegypti*, with no significant difference for most strains compared to the control against this mosquito larvae. However, the field-collected strains were much more effective when tested against *Oc. detritus*, with 2 field-collected strains reaching LT_{50} almost as soon as the most pathogenic EPN; the commercially available *S. feltiae.* There are a number of possible reasons for this difference in performance compared to the commercial strains, some of which will be addressed in turn:

- Commercial strains are selectively bred to be more pathogenic;
- The energetic costs of wild living are offset by reduced pathogenicity;
- Differences in EPN thermal tolerance;
- Wild EPNs are highly host-specific; and
- Lab-rearing successive generations reduces pathogenicity, which commercial strains manage to avoid.

The commercial provider of EPNs for this research (BASF), would not reveal the identification of any EPN strain below species level (variant or sub species). Whilst this information may have been obtainable through genetic sequencing, it was not appropriate to do so. Therefore, in this instance it was not possible to understand how far the commercial EPNs in this research differ genetically to the field caught EPNs of the same species. However, it is an established practice in any biocontrol field to enhance desired traits through selective breeding (Hopper et al., 1993; Lacey and Georgis, 2012) so it might be assumed that the better pathogenicity of commercial strains may be a result of this.

The pathogenicity of field collected strains of EPN are infrequently compared to commercial preparations. However, McGraw and Koppenhöfer, (2008) found that naturally occurring EPNs (*S. feltiae* and *S. carpocapsae*) were no less effective than their counterpart commercial strains against the *Listronotus maculicollis* (Coleoptera: Curculionidae) larvae. It is a basic biological principal that any organism's primary aim is to reproduce and in this respect EPNs are no different and pathogenicity to a host could be considered a by-product of this reproductive drive. The efficacy or reproductive success of EPNs was explored by Griffin (2012) who suggests that as pathogenicity and the attendant levels of bacterial symbiont is not an immutable state that exists within the IJ; that there is an energetic cost to carrying the symbiotic bacteria that are responsible for the IJ's ability to kill the host organism (Emelianoff et al., 2008; Mitani et al., 2004) resulting in an ability to survive longer with a lighter bacterial load. This suggests a trade-off between longevity and reproduction. This effect may have been seen during testing for these assays, whereby field collected strains were observed to be surviving much longer in a dauer stage than those from commercial sources, although this was not recorded as it was an incidental observation during the course of preparation for other assays. However, the lack of pathogenic success of the field-caught strains compared to the commercially prepared ones could fit with this hypothesis.

The field collected EPNs from these assays had been isolated from soil, and successive generations reared through using *G. mellonella* as host. From isolation from the soil sample to use in these assays, there were 3 to 4 (depending on strain) passes using *G. mellonella* as the host insect. Several studies have looked at the effect of laboratory rearing on the quality of the EPNs including the host insect where *in vivo* rearing occurs. Inbreeding depression, founder effect and a lack of genetic diversity are all reported as having a negative effect on infectivity and reproductive potential (Abu Hatab et al., 1998; Bilgrami et al., 2006; Shapiro-Ilan et al., 2002; Stuart and Gaugler, 1996). Rearing successive generations on *G. mellonella* is thought

to have a detrimental effect on EPN quality through the parasite's adaptation to the rearing host rather than the target host for field application or the EPNs natural host, which would provide optimal feeding conditions for the EPN (Abu Hatab et al., 1998; Shapiro-Ilan et al., 2002). However, these studies relate to mass rearing, so aside from the potential for a limited genetic diversity in the original EPN population, 3 or 4 passes on a relatively small scale would have been unlikely to demonstrate such effects (Bilgrami et al., 2006), although Wang and Grewal (2002) saw deterioration in *H. bacteriophora* quality after 3 passes through *G. mellonella.* Given the scale of production between this study and the commercial preparation at BASF, it would be unlikely that the commercial strains managed to avoid these effects whereas the field-collected strains did not.

Whilst these are all factors to be considered in the performance of the field-collected strains compared to the commercial ones against *Ae. aegypti*, the field-collected strains did demonstrate a significant pathogenic effect towards *Oc. detritus*, the difference is likely to lie with the host organism rather than the EPN. *Ae. aegypti* New Orleans strain eggs were obtained from The Liverpool School of Tropical Medicine. This strain has been laboratory reared for many years, therefore genetic diversity is likely to be reduced. The *Oc. detritus* larvae that were used in these experiments was collected from a naturally occurring population from Little Neston saltmarsh. The continued survival of this population of mosquitoes has benefited from the stringent environmental legislative protections on the area designed to protect other flora and fauna (see Chapter 1). This has meant that any chemical use is strictly controlled and physical oviposition deterrents such as channel digging has not been as extensive or repeated as necessary to effectively control the mosquito population (Clarkson and Setzkorn, 2011; Clarkson pers comm). No EPNs were found at the Little Neston site following *G. mellonella* baiting of several soil samples, likely due to unsuitability of a heavy clay substrate as a habitat (Kung et al., 1990). However, insect immunity is innate, and no acquired immunity is present

as in vertebrates, the absence or presence of a potential pathogen in the mosquito's natural habitat should not effect its susceptibility. However, the immune system of the invertebrate can provide continuing protection to the invertebrate after the first pathogenic challenge, should it survive (Moret, 2006), which could partly account for the levelling-off of pathogenicity seen in the assays of both mosquito species. Lowenberger (2000) suggests that the diversity of levels of particular immune peptides in mosquitoes reflect an evolutionary exposure to pathogens, so if no EPNs had been encountered by that discrete population of *Oc. detritus* the innate immune response may not have been appropriate to nematode invasion. Without further study it is not possible to draw a conclusion as to why *Oc.* detritus were highly susceptible to the EPNs and may be a combination of captive stress and the factors mentioned above.

The *Ae. aegypti* New Orleans strain is susceptible to insecticides (LITE, 2014) but was more resilient to the pathogenic effects of the EPNs than *Oc. detritus*. *Ae. aegypti* strains used were laboratory bred and reared over many generations and may be more resistant to EPNs when under laboratory stress, whereas *Oc. detritus* was field collected and possible more susceptible as a result. Experiments that have induced stress in mosquitoes have shown a variety of outcomes on the fitness and overall survival of the insect. Stress from overcrowding can induce a range of outcomes on *Ae. aegypti* larvae, including reduced adult weight and overall survival (Barbosa et al. 1972). The larvae in these assays were not overcrowded to the same extent as those in the tests by Barbosa et al., (1972). Whilst 25 larvae in 100ml solution may not be an optimum developmental environment for *Ae. aegypti*, the number of *Oc. detritus* larvae was reduced to 15 per 100ml as the larvae are much bigger. However, as field collected larvae, any change from the comparatively large pools they inhabited may be enough to induce a stress reaction. Studies of other insects show that acute stress reactions move molecular resources away from immune responses, reducing disease resistance but during stress events octopamine (neurotransmitter related to norephinedrine) increases phagocytosis, which is

responsible for encapsulation of invading parasites thereby mitigating the immune deterioration in stressful events (Adamo, 2017, 2014). From this, it could be inferred that stress on *Oc. detritus* may have been somewhat responsible for their mortality rate. However, the control condition for *O. detritus* showed that there was a significant difference in mortality when nematodes were present, indicating that whether the whole of the pathogenic effect observed was direct penetration by the EPNs or that some pathogenicity was stress induced, the EPNs were responsible for the death of the larger mosquito.

Different strains of EPN induced different mortality rates against the two mosquito species but in most cases there was little difference in mortality between the different dosages of particular strains. *S. feltiae* was the most effective EPN towards both mosquitoes, but the most effective concentration was 4000 per 100 ml for *Ae. aegypti* and 8000 per 100 ml for *Oc. detritus*. This is likely to be the effect of the difference in larvae size between the two mosquito species as EPNs are understood to avoid overcrowding within a host, as a strategy for a more favourable chance at reproduction and their own survival (Selvan et al., 1993). At the highest concentration of EPNs for *Ae. aegypti* this would be the equivalent of 320 EPNs targeting one host and for *Oc. detritus* 533 EPNs. Higher concentrations of EPN in a host species can result in reduced pathogenicity as EPNs can be engaged in intraspecific competition strategies (Bashey et al., 2016; Koppenhöfer and Kaya, 1995; Zenner et al., 2014).This effect was also encountered in assays involving *Chironomus plumosus* (see Chapter 2). There is little data on the wet weight of either species of mosquito larvae, which may have given a clearer understanding of the size difference between the two species and therefore why the great difference in most effective concentration. Caution should be exercised when attempting to make a simple assessment of larval size vs. EPN penetration as many other factors could be considered. Infective juvenile EPNs are not a uniform size, one of the largest of the EPNs was *S. feltiae* (750-850µm) which exhibited the most effective pathogenicity whereas the smaller

of the tested species*; H. bacteriophora* (520-600 µm) (Poinar, 1979) was almost as effective as *S. feltiae* in killing *Ae. aegypti*. The difference in LT_{50} for the two mosquitoes was interesting, with *Oc. detritus* assays reaching it at approx. 1-3 days across all tested EPN species, whereas for *Ae. aegypti* it ranged between 6-8 days. The classic hunting style of the EPNs (cruiser/ambush/intermediate) (Campbell et al., 2003; Grewal et al., 1994) is unlikely to be relevant in an aquatic medium. However, it would be interesting to observe EPN movement responding to host cues in an aquatic medium to ascertain whether their terrestrial host-finding strategies afforded them any advantages. Cardoso *et al.*, (2015) tested various EPNs against *Ae. aegypti* larvae and found *H. indica* to be the most pathogenic to *Ae. aegypti* and *S. carpocapsae* ineffective which did not reflect the results of this study. However, Welch and Bronskill (1962) found *Neoplactana* strain DD136 did cause mortality to *Ae. aegypti* although their study was to study encapsulation of the EPN rather than to induce mortality in the mosquito.

As the pathogenicity assays for this study were conducted under conditions most favourable to the host (other than temperatures exceed the EPN active range) the *Oc. detritus* were tested in water that had a salinity of 25 ppt. This makes it difficult to draw any direct comparisons between the two mosquito assays as they were different in terms of temperature, host and salinity. Salinity and pH were tested to ascertain whether the strains of tested EPNs were capable of tolerating the habitats that mosquitoes are found in (Tables 4.1 and 4.2). In the case of the field collected *Oc. detritus* are found in salt-marshes with brackish water measured at 25 ppt. The salinity tolerance test showed that EPNs from all species were able to tolerate a wide range of salinities. *H. bacteriophora* is well established as a halotolerant EPN (Griffin et al., 1994) and showed 11.11% survival after 7 days exposure, which was greater than the other species tested. Thurston et al. (1994) found *H. bacteriophora* virulence remained high (74% ± 5.1) even after storage in sodium chloride (16 d/Sm) for 20 days. This was not in agreement

with the findings of the pathogenicity studies here, as *H. bacteriophora* did not demonstrate a more effective virulence at that salinity in the pathogenicity tests with *Oc. detritus* which were conducted at 25 ppt saline solution although the host difference between *Oc. detritus* and *Ae. aegypti* cannot be ruled out as an important factor. *S. feltiae* showed > 30% survival between 0-30 ppt, although the lower salinities showed the highest survival rate.

S. carpocapsae showed better survival at 20-40 ppt and field collected *S. glaseri* 367 had the highest rates of survival between 10 and 30 ppt. Thurston et al. (1994) show their tested S. glaseri to be the most resilient to a range of salts in terms of survival. The 367 strain of *S. glaseri* was the only EPN isolated from an extensive survey of Lundy Island. This is interesting when compared to the other field-collected *S. glaseri* strain 93, which showed better tolerance of distilled water (0ppt) than the Lundy Island strain 367. It could be theorised that this isolate may have adapted to the coastal conditions that prevail across such a small island.

The UK soil landscape is broad; from a pH of 9.2 basic end of the scale to acidic peats with a pH of 3.1 at the other end of the scale (Cranfield Soil and Agrifood Institute, n.d.; National Soil Resources Institute, 2002). As a soil-swelling parasite the need for an understanding of EPN pH tolerance in soils in clear. However, mosquito larvae are also tolerant of a broad range of acid and basic aquatic conditions (Clark, 2004). Studies on the effect of soil pH on EPNs have shown that they can survive and parasitise within a broad range but high alkaline content of soils acts as a nematocide (Kung et al., 1990) and low pH significantly restricted infection by *S. kraussei* (Barbercheck, 1992). All EPNs survived well when exposed to the range of pHs. It had been expected that the field collected strains would fare better in these assays. However, no significant differences in survival were observed.

In conclusion, *S. feltiae* is the best ‘all-rounder’ in terms of tolerance to environmental extremes and pathogenicity to both mosquito species. The field-collected strains were much less effective than the commercial strains as a potential biocontrol and in terms of

environmental tolerance. As happens in agricultural use of EPNs, the behaviours and lifestyle of a mosquito could dictate what EPN was optimal for biocontrol purposes. For example, *S. feltiae* has an intermediate host seeking style and therefore may be best targeted at mosquitoes that spend some time in a changing aquatic habitat such as *Oc. detritus* that lay their eggs on the soil rather than in the water. This research shows that EPNs might be a viable biocontrol for mosquito larvae, and highlights that this area would benefit from field trials. However, as Cardoso et al, (2015) point out that standardisation of testing for EPN pathogenicity to mosquitoes is crucial to gaining a thorough understanding of the potential for mosquito control as the differences in method of current tests and the dynamic between field caught and commercial or lab-reared strains of both mosquito and EPN make appropriate insect/EPN matching difficult.

Chapter 5

RNA-Seq analysis of gene upregulation in *Ochlerotatus detritus* when challenged with *Steinernema carpocapsae*

Introduction

The immune response of insects has a crucial role in the efficacy of EPNs (Li et al., 2007). Insect immune responses are activated by pattern recognition proteins (PRPs) and activate a range of cellular processes such as phagocytosis, nodulation and encapsulation (An et al., 2017; Hultmark et al., 2005) as they lack an adaptive immune system to recognise pathogen-specific receptors (Aliota et al., 2007). Thus, EPNs need to evade or supress the immune system of their host insect to succeed at parasitizing them. As shown in previous chapters, there are important differences in the ability of different EPNs to kill different hosts, e.g. *Oc. detritus* and *Ae. aegypti* showed very different levels of susceptibility to EPN attack which could limit the usefulness of EPNs as biocontrol agents for certain species.

A complex set of genetic and environmental factors is thought to be at play in the defence against attack from any pathogen (Lazzaro and Schneider, 2014). Understanding why one mosquito species was able to defend itself more effectively against EPNs than the other is to understand why one insect's immune system is able to recognise an invader and switch on its immune responses more effectively than the other: This may provide insight into how certain pestiferous species combat nematode infection at the molecular level. This ability is likely to be partially under genetic control and a range of investigations have produced valuable information on the genetic modulation of the immune system (Gillespie et al., 1997; Hultmark et al., 1980; Steiner et al., 1981) and the advances made in genetic analysis have enabled a much greater understanding of the signalling processes and pathways involved (Adamo, 2017; Stronach et al., 2014).

When dealing with a host/parasite complex it has been found that the parasite can alter the gene expression activity of the host. This is especially interesting in tritrophic arrangements such as the symbiotic bacteria/nematode/insect that occurs with entomopathogenic nematodes. An et al. (2017) found PRPs were upregulated when *Drosophila melanogaster* encountered *H. bacteriophora*, but after a period of time that there was a significant suppression of immune activity that was thought to be the result of the release of the symbiotic bacteria *P. luminescens* which supressed the detrimental immune response in the host. *S. carpocapsae* has also been found to trigger genes in *D. melanogaster* involved in developmental processes, immune function genes (as might be expected), and as yet unidentified genes which are not shared with any other infection models (Yadav et al., 2017).

In order to identify important changes in gene expression involved in phenotypes such as the immune reaction triggered by pathogenic attack, a number of methodologies can be used dependent on whether specific candidate genes are to be studied, or whether the whole transcriptomic response is to be screened. Differential gene expression has typically been studied using either quantitative PCR approaches (when small numbers of genes are studied), or gene-expression microarrays when the relative expression levels of large numbers (hundreds to thousands) of genes are studied. To enable microarray design and construction, information must be available concerning gene sequences from which to design probes to populate the array. When a reference genome is available, transcript information can be obtained from this resource. In the absence of a suitable reference genome, sequencing of cDNA (e.g. EST libraries) is a first step to enable microarray design.

More recently, the use of microarrays in differential expression analysis has been replaced by the introduction of RNA-Seq (RNA sequencing) approaches, which do not necessarily require any prior sequencing information, making them ideal for use on non-model organisms. Wang et al. (2009) described RNA-Seq as a 'revolutionary tool' for transcriptome profiling. Since

then, this technique has become a common method to measure relative gene expression levels. The process involves fractionated or total RNA being converted to cDNA libraries with adaptors attached. High-throughput sequencing is performed on each molecule to obtain short sequences or reads, typically 30-400bp (Wang et al. 2009). Any high throughput sequencing technology can be used for this sequencing process and whilst a number of well-respected analysers have previously entered the market (e.g. Roche-454, Heliscope, Ion Torrent and ABI) Illumina technologies now dominate in this field (Reuter et al 2015) with an increasing presence of PacBio (Sharon et al., 2013). Where one exists, the reads that are produced are then mapped to a reference genome (Figure 5.1). The number of reads mapping to individual contigs (overlapping DNA sequences) are quantified to indicate at what level a particular gene is expressed (Kaplanoglu, 2016; Wang et al., 2009). When using a reference genome, these data are then processed using bioinformatics pipelines to identify genes that are up or downregulated under the different experimental conditions (Kaplanoglu, 2016).

Where there is no reference genome to map reads to, a *de novo* (from new) transcriptome assembly can be performed using software such as Trinity (Grabherr et al., 2011). When one *de novo* transcriptome assembly has been performed, the data from the other experimental conditions can be resequenced using that as its reference genome (Grabherr et al., 2011; Thunders et al., 2017).

Since genomes have thousands of genes (e.g. *C. elegans* has 20,222 genes annotated (Ensembl, 2018)) multiple genes are expected to be up- or down-regulated in RNA-Seq experiments. Pathway analysis using Gene Ontology terms or KEGG terminology allows an understanding to be gained of the types of genes with differential expression.

Pathogenicity experiments (detailed in Chapter 4) showed the wild strain of the mosquito *Oc. detritus* to be particularly susceptible to EPN attack, more so than *Aedes aegypti* (lab reared). Here, mosquitoes from an EPN naïve population were studied to establish if they exhibited any

immune response when challenged with this new threat. Understanding whether the EPN caused any immune response in *Oc. detritus* is important in assessing the viability of EPNs as a form of biocontrol for this mosquito.

Ochlerotatus detritus, (a species with no existing reference genome) was investigated through RNA-Seq for the identification of any differential gene expression when the larvae were challenged with both water containing solute of volatiles [1] of the EPN *Steinernema carpocapsae*, or when exposed to the EPN over a period of time falling short of morbidity (approximate LT_{25}).

Three conditions were therefore examined:

- Baseline expression (control)
- EPN volatile challenge
- Physical proximity of EPN

[1] The term volatile has been used to describe allelochemicals that are involved in EPN interspecific interactions. Although the term volatiles is not technically correct in the current context as that describes a chemical that readily evaporates and these were dissolved in distilled water, this was used as a term of convenience and was the name attached to the set of samples sent to BGI and appears in the figures and analysis they provided. Therefore, the term volatile will continue to be used for simplicity.

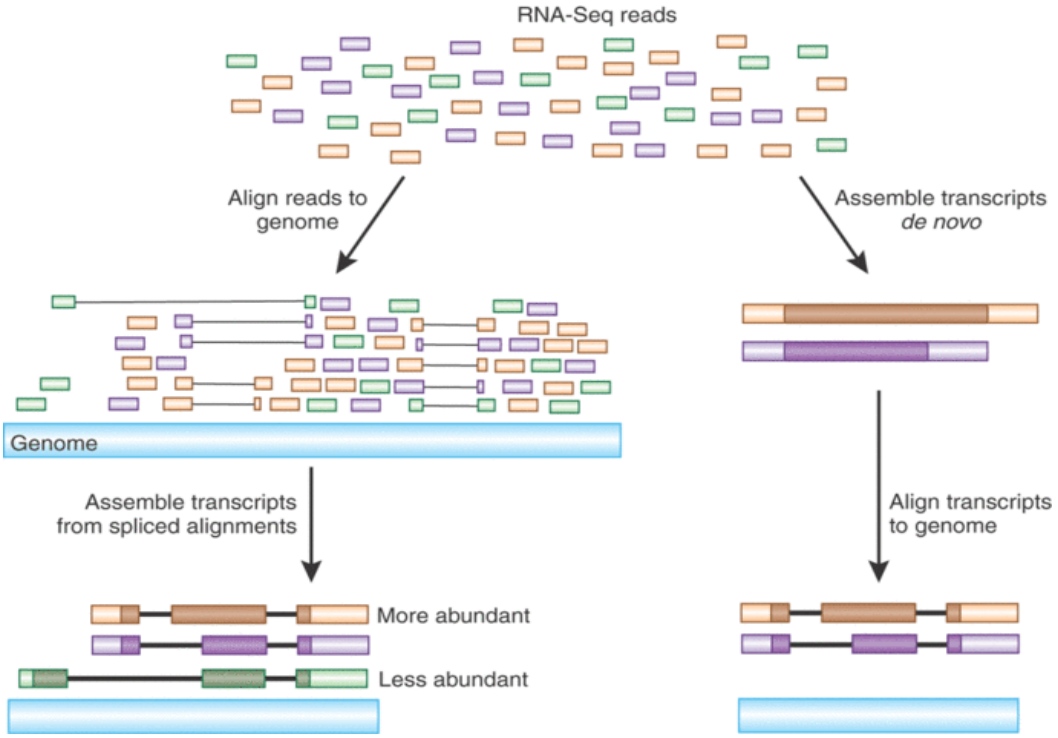


Figure 5. 1. RNA-seq *de novo* transcriptome assembly (Haas and Zody, 2010). The left hand side shows transcript assembly/quantification when using a reference genome, the right hand side shows the *de novo* approach where transcript sequences are assembled without a known reference genome, then transcripts are aligned to the created *de novo* assembly.

Methods.

Oc. detritus larvae were collected from Little Neston on 16 August 2017. All instars were collected and allowed to develop in the saline water (25ppt) found in their natural habitat. The mosquito larvae were kept in a natural dark/light cycle at room temperature (19-23ºC). Only third and fourth instar larvae were used to extract RNA as previous studies showed a much greater success for parasitisation for larger and later instar larvae as EPNs can avoid larvae that are too small for reproduction (Biaochhi et al., 2017). Five individual larvae were used for each RNA extraction as through a previous trial, this number was found to provide sufficient biological material for a high enough concentration of RNA for downstream processes.

The EPN *S. carpocapsae* from BASF was used. EPNs were released from the gel transport medium by agitating in 35ml distilled water in a centrifuge tube. A representative sample was examined under a light microscope to ensure that they were viable.

Volatile exposure

Using the method described previously to approximate abundance (see Chapter 4), approximately 8000 EPNs were pipetted into a microcentrifuge tube containing 1ml distilled water. These were allowed to rest in the tube for 24 h. Following this, the microcentrifuge tube was spun at 14,000 RPM for 10-15 minutes, long enough to gather the EPNs into a pellet at the bottom of the microcentrifuge tube. The supernatant was then removed using a pipette and added to a plastic cup containing 15 late instar larvae and incubated at room temperature (19-23ºC) for 24 h. The EPN pellet was discarded. Five biological replicates were conducted.

EPN exposure

Approximately 8000 *S. carpocapsae* were pipetted into cups containing 15 late instar larvae and incubated at room temperature for a time long enough for EPNs to come into contact with the larvae without causing significant mortality as moribund mosquito larvae were not desirable for this assay. Using data collected in previous experiments detailed in Chapter 4, the LT_{25} was calculated as 1.25 days for *Oc. detritus* when exposed to *S. carpocapsae* and so EPN challenge experiments ran for 32 h. The control samples were late instar larvae incubated in plastic cups for the same amount of time as the other two conditions but without the EPNs or 'volatile' water.

After 24-32 h exposure, larvae that were still alive were immediately washed with PBS before being frozen at -80ºC. Total RNA was extracted from these pellets using the Ambion RNAqueous extraction kit (AM1912) following the manufacturer's instructions. Pellets were ground using plastic pellets (soaked in RNAZap for 15 minutes prior to extraction then rinsed

in distilled water). RNA was eluted from the column using the provided elution buffer, aliquoted, and stored at -80ºC.

RNA Quality

High quality RNA is crucial to obtain the best results from RNA-Seq. Beijing Genomics Institute, or BGI, (the sequencing company used for this study) set the required quality standards for RNA purity and concentration. For insect total RNA for *de novo* transcriptome assembly the following standards were requested to ensure that the sequencing process is effective:

- Volume – 15-100µl
- Concentration > 20mg/ µl
- Electropherogram – baseline smooth and 5S peak is normal.

To discover whether the RNA extracted from the experimental samples was of sufficiently high quality, volume and concentration, aliquots of each sample were kept separately from the main RNA sample and the aliquots were tested using Thermo-Fisher Nano-Drop spectrophotometer and Agilent Tape Station. The Nano-Drop measures the absorbance of a small quantity (1µl) of extracted RNA (or DNA) and calculates the absorbencies at 230nm, 260nm and 280nm. An A260 reading of 1.0 is then equivalent to ~40 µg/ml RNA. The ratio of the three absorbance measurements is also used as a measure of purity for the RNA. As RNA is particularly fragile and prone to quick degradation, the integrity of the extracted RNA was additionally assessed through the use of an Agilent Tapestation. This is an automated electrophoresis machine which gives valuable information on quality for subsequent downstream experiments. It generates a virtual gel image to identify quality and potential contamination of the RNA sample. Only five biological replicates from each condition were required, so those with the highest concentration values and the required volume of total RNA

were used for sequencing. These tests indicate that the majority of extracted RNA samples were of sufficient quality to proceed with a successful RNA-seq analysis. BGI performed their own validation analysis on the samples sent to them, which confirmed these results.

RNA-seq and bioinformatics

Following analyses of RNA quantity and quality, RNA samples were sent to BGI (Shenzen, China) who performed sequencing and performed all bioinformatics analyses following the pipeline in Fig 5.2 and returned both analysed data and raw data for further analysis where necessary. These sequence read data have been deposited in the Short Read Archive (SRA) database with reference number SRP130756.

Library preparation (100PE, 100bp paired end reads length) were carried out by BGI for high-throughput sequencing on the Illumina HISEQ4000 platform, with a minimum of 30 million reads for each sample of the pooled mosquito material guaranteed (15 samples). As part of the agreement with BGI, they performed a *de novo* transcriptome assembly using the sequence data from all sequenced samples, as there is no existing reference genome for *Oc. detritus*, and transcriptome resequencing analysis for the others using this *de novo* transcriptome as reference (Fig 5.3). For the purposes of this investigation, the differentially expressed gene detection (DEG), clustering analysis and GO (Gene Ontology) functional annotation are the most relevant aspects and will provide the information regarding different genes expressed in the three tested conditions.

In this workflow, the 15 RNA extractions that were sent to BGI were reverse transcribed to create 15 cDNA libraries. Each library had an individual bar-coded adaptor and the pooled libraries were sequenced across two lanes of an Illumina Hi-Seq 4000.

Following sequencing, data were processed to remove adaptor sequences and then the assembly stage uses algorithms to assemble the individual reads into transcripts and using Trinity

software generates an assembled *de novo* transcriptome. Sample reads (de-barcoded) are mapped back against this and relative gene expression calculated versus the control sequences using FKPM values (Fragments Per Kilobase of transcript per Million mapped reads). From these data differently expressed genes can be detected and function analysed through GO (gene ontology). From the assembled transcriptome, gene function was also annotated using seven of the ten functional databases, as shown in Fig. 5.2. which are the nucleic acid and protein databases respectively from the National Centre of Biotechnology Information (NCBI) in U.S.A. KOG database is the Eukaryotic Orthologous Groups, a tool for identifying ortholog and paralog proteins. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) is a collection of databases providing bioinformatics information on genomic, chemical and systemic function. The Gene Ontology (GO) database is used to classify genomic functions into molecular, cellular and biological processes. SwissProt is a database that scours other resources to provide all known information about particular proteins. Interpro is a database of protein families that can be used to match identifiable features of known proteins to characterise their functionality. Using this wide range of bioinformatics resources provides the best possible understanding of gene function.

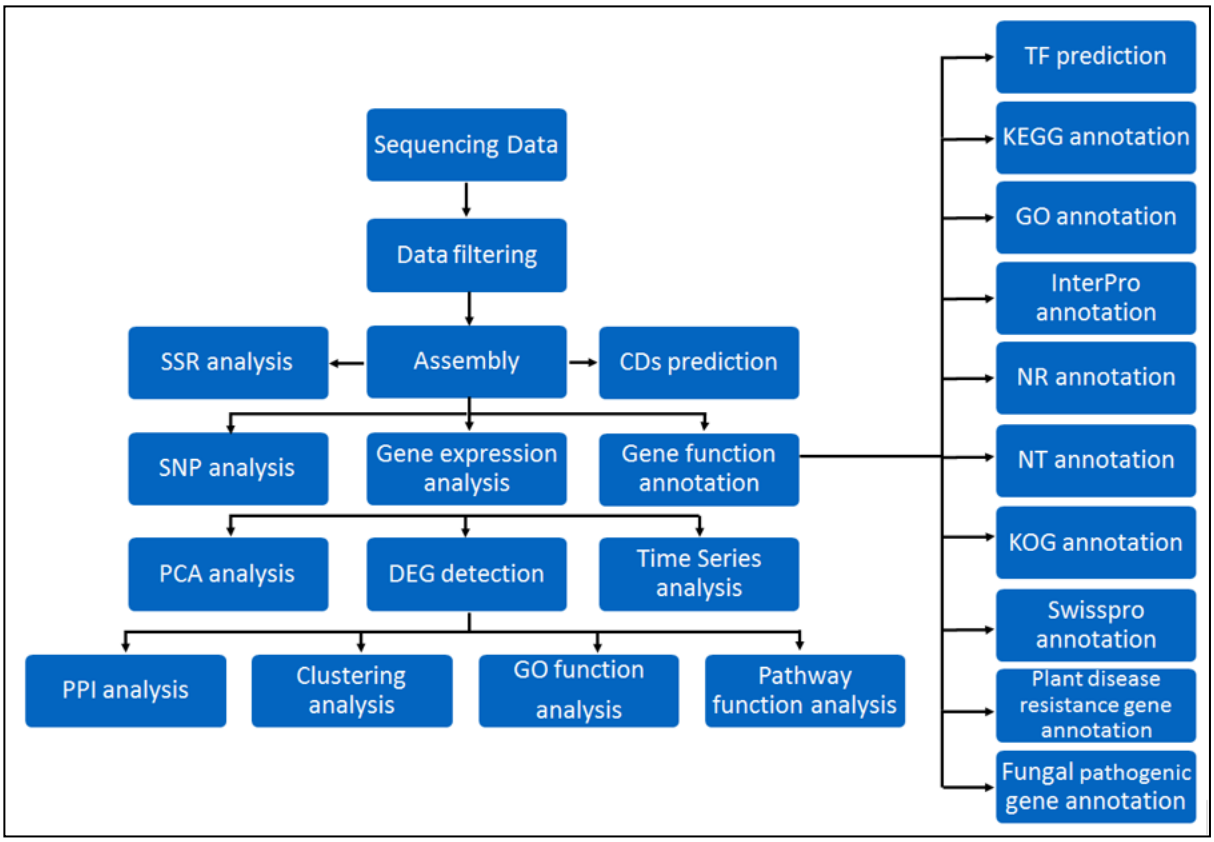


Fig 5.2. Flow chart from BGI showing process of RNA-seq analysis of *Oc. detritus.*

Results

RNA quality

In total, 18 samples were extracted (including a spare of each). All samples demonstrated high concentrations and purity (Table 5.1). Whilst NanoDrop readings showed high concentration and purity, it was necessary to determine quality of RNA. Electropherograms from the Agilent TapeStation output showed sharp bands (indicative of undegraded rDNA species) rather than smears which would suggest poor quality, degraded RNA (Fig. 5.3a and b).

Table 5.1. Results obtained from RNA screening on Nanodrop spectrophotometer, showing 15 samples used for RNA-seq analysis and 3 spares. Ideally, 260/280 absorption ratio (showing potential DNA contamination) should be ~2.0 for the best RNA-Seq results.

Sample ID	**Nucleic Acid Conc. (ng/ul)**	**A260**	**A280**	**260/280**	**260/230**
Control 1	477.75	11.94	5.54	2.16	2.97
Control 2	666.80	16.67	7.69	2.17	2.49
Control 3	516.43	12.91	6.00	2.15	0.97
Control 4	380.63	9.52	4.38	2.18	1.81
Control 5	807.07	20.18	9.27	2.17	2.81
Control 6 spare	317.00	7.92	3.61	2.20	2.30
Volatile 1	447.97	11.20	5.20	2.16	2.22
Volatile 2	642.07	16.05	7.30	2.20	2.45
Volatile 3	479.00	11.98	5.55	2.16	2.15
Volatile 4	545.93	13.65	6.29	2.17	2.42
Volatile 5	500.60	12.52	5.81	2.16	2.17
Volatile 6 spare	302.77	7.57	3.48	2.18	2.42
Exposed 1	783.20	19.58	9.02	2.17	2.39
Exposed 2	655.50	16.39	7.65	2.14	1.60
Exposed 3	744.37	18.61	8.65	2.15	2.45
Exposed 4	694.00	17.35	8.07	2.15	2.44
Exposed 5	651.35	16.28	7.60	2.14	2.48
Exposed 6 spare	325.30	8.13	3.72	2.19	2.84

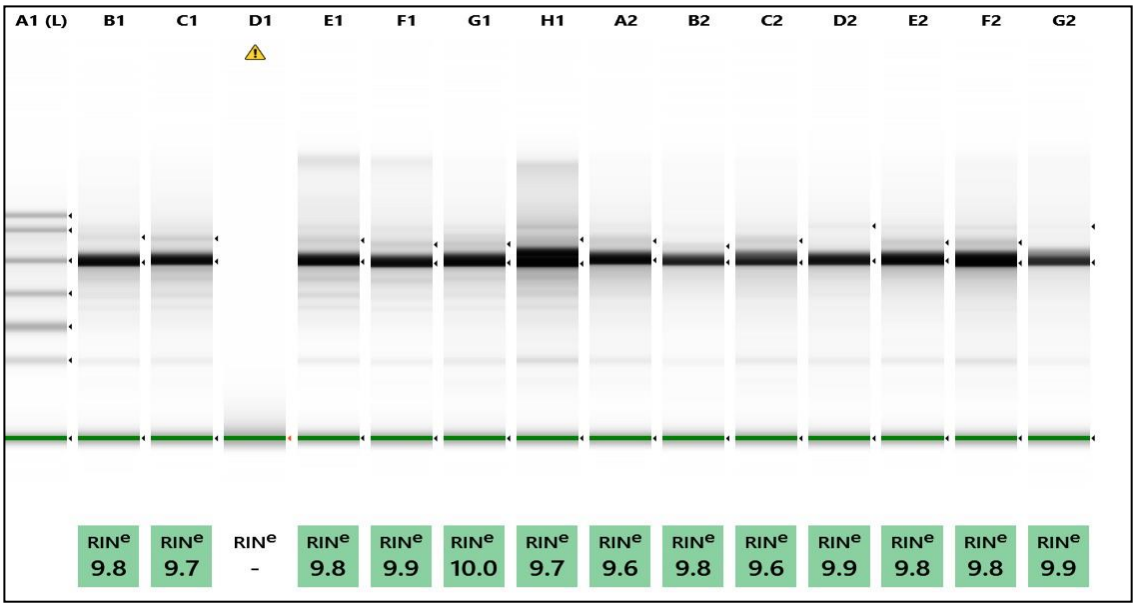


Figure 5.3a

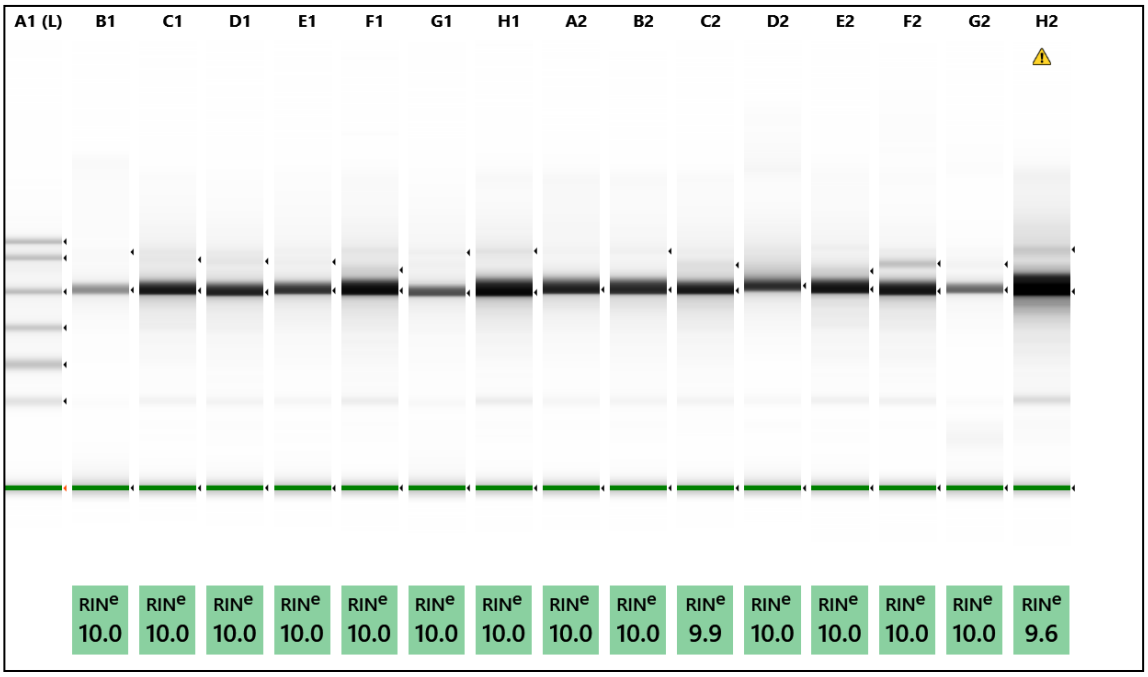


Figure 5.3b

Figures 5.3a and 5.3b Simulated gel image generated by Agilent Tapestation showing extracted RNA (darker bands indicate more RNA) and quality for all the pooled mosquito samples. Image is Scaled to view larger Molecular Weight range. Yellow triangles on the image indicate where RNA concentration is too high or low.

The predominant RNA species in any RNA extractions are the 18S and 28S rRNAs. However, the 28S RNA of insects is prone to splitting on heat denaturation (Winnebeck et al., 2010) and therefore does not appear when samples are run on gels. Agilent Tapestation output provided

further evidence of sample quality in their densitometric output (Figure 5.4) which shows peaks for 18S and relatively undegraded RNA across the sample.

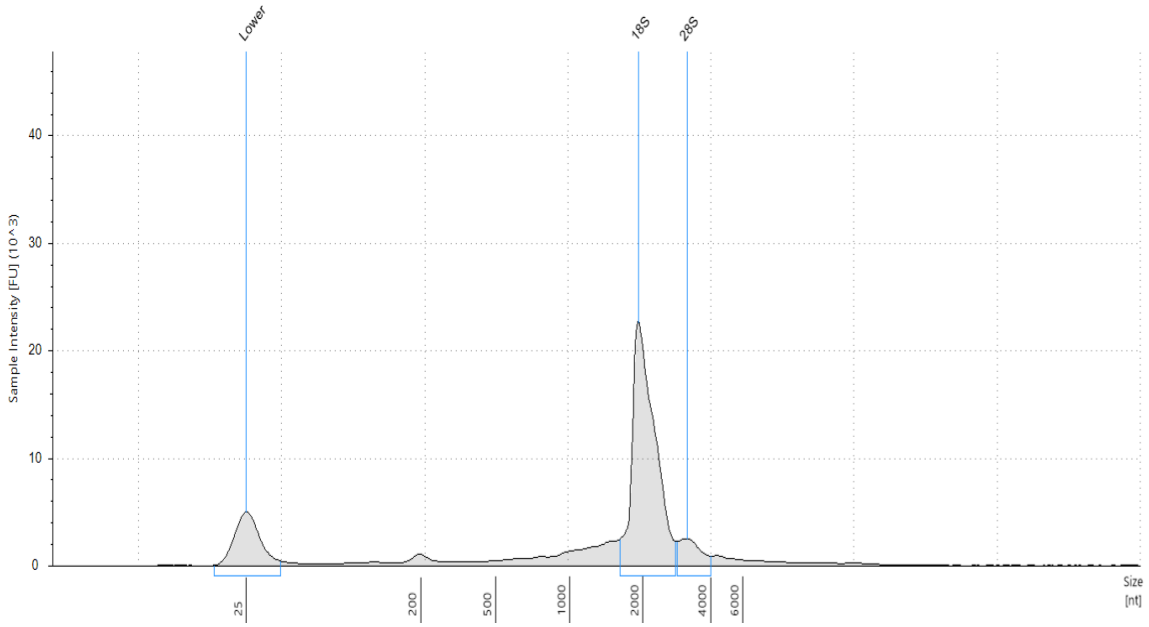


Figure 5.4. Example of expression profile generated for an individual RNA extraction by Agilent Tapestation to determine quality and size of RNA through using S28 and S18 species.

Read quantity and quality

Once the high throughput sequencing had been conducted, it was necessary to check the quality of the reads generated and to clean them, and partition samples by barcode, to enable subsequent bioinformatics programmes to run efficiently and achieve the best results. Contaminants, low quality reads and adaptors which can pollute subsequent downstream analysis are filtered out. Figure 5.5 shows an example of the raw data filter composition chart (Volatile 2). After filtering, the number of clean reads or usable bases were quantified and qualified (Table 5.2). This indicated the amount of high quality reads that could be used to assemble the *de novo* transcriptome.

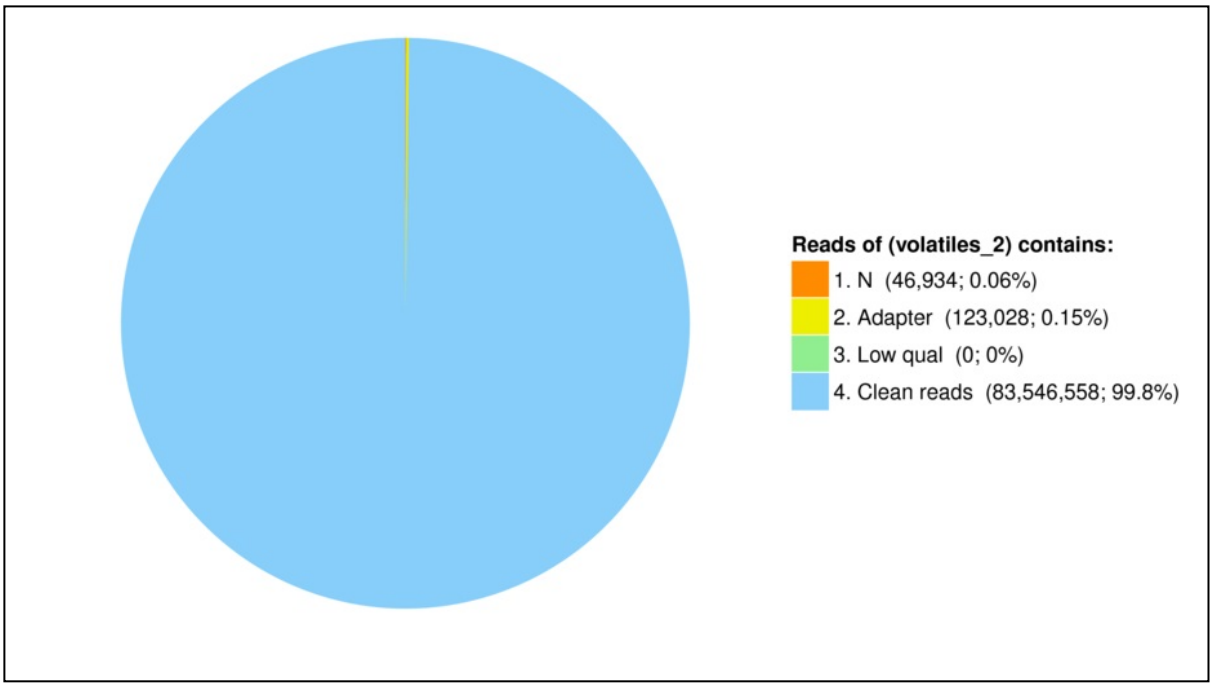


Figure 5.5. Classification of raw reads for the sample named Volatile 2. N is the number of reads which contain more than 5% of a sequence that is not known and may be incorrect. The N, adapter and low quality reads are sequences that are removed before downstream analyses. The majority of reads in this sample are clean.

Table 5.2 Clean reads quality metrics. This shows the number of bases that remained after filtering and the number, which are of a high quality. Mb is a Megabase = 1 million reads, Gb is Gigabase = 1 billion reads.

Sample	Total Raw Reads (Mb)	Total Clean Reads (Mb)	Total Clean Bases (Gb)	Clean Reads Q20 (%)	Clean Reads Q30 (%)	Clean Reads Ratio (%)
Control 1	83.72	83.42	8.34	98.25	95.28	99.64
Control 2	83.72	83.53	8.35	98.39	95.66	99.78
Control 3	83.72	83.53	8.35	98.37	95.62	99.78
Control 4	83.71	83.52	8.35	98.41	95.72	99.77
Control 5	83.72	83.54	8.35	98.45	95.80	99.79
Exposed 1	83.72	83.51	8.35	98.51	95.91	99.75
Exposed 2	83.72	83.49	8.35	98.29	95.50	99.74
Exposed 3	83.71	83.54	8.35	98.40	95.70	99.79
Exposed 4	83.72	83.51	8.35	98.24	95.24	99.75
Exposed 5	83.71	83.52	8.35	98.41	95.67	99.76
Volatile 1	83.72	83.54	8.35	98.41	95.73	99.79
Volatile 2	83.72	83.55	8.35	98.40	95.67	99.80
Volatile 3	83.72	83.39	8.34	98.36	95.62	99.61
Volatile 4	83.72	83.56	8.36	98.46	95.80	99.81
Volatile 5	83.72	83.57	8.36	98.12	95.23	99.82

De novo transcriptome

Ochlerotatus detritus has only been of minor interest to researchers as it is a temperate mosquito with no confirmed vector status, although some recent studies have indicated it may be a potential vector for Japanese encephalitis (Blagrove et al., 2014, Mackenzie-Impoinvil et al., 2015). As such, it has not received the same level of attention at a genomic level as other mosquitoes such as the malaria vector *Anopheles gambiae*. Therefore, there is no existing genome against which to map reads. The *de novo* assembly addresses this issue by using the clean reads from the high throughput sequencing process as input to the Trinity software which assembles the clean reads into collections or clusters of similar length which are described as contigs. Trinity further processes the contigs to form longer sequences. These sequences are described as 'Unigenes' (Shu et al., 2013) as they locate to the same position on the *de novo* transcriptome and appear gene-like but not enough is understood about them to describe them as a gene. The quality metrics of the reads from *Oc. detritus* are shown in Table 5.3.

Table 5.3 Assembly quality metrics of transcripts following Trinity *de novo* assembly. N50, N70 and N90 are weighted median statistics that indicate that 50, 70 or 90% of the total length is contained in Unigenes. The higher the value the better as this indicates that there is less repetition which can introduce mapping errors. GC% is the % of guanine and cytosine bases in all transcripts, a mid-range value indicates that there is no contamination that may skew the value towards A/T rich sequences or higher content G/C. Any GC bias can affect an accurate gene assembly. Bold highlighted results are unusually long when compared to other control samples and when compared to all experimental samples. This suggests that there may be erroneous data.

Sample	Total Number	Total Length	Mean Length	N50	N70	N90	GC(%)
Control 1	**106,481**	**80,723,691**	**758**	**1,668**	**715**	**257**	**45.49**
Control 2	51,338	54,134,185	1,054	2,274	1,264	365	45.25
Control 3	47,802	51,135,639	1,069	2,273	1,282	377	45.36
Control 4	**105,466**	**80,331,877**	**761**	**1,591**	**729**	**263**	**46.11**
Control 5	**97,553**	**72,743,366**	**745**	**1,587**	**690**	**257**	**45.95**
Volatile 1	48,768	51,233,181	1,050	2,221	1,257	369	45.42
Volatile 2	46,101	49,680,060	1,077	2,274	1,295	381	45.43
Volatile 3	52,117	56,536,463	1,084	2,355	1,326	377	45.33
Volatile 4	49,271	52,694,801	1,069	2,301	1,292	374	45.42
Volatile 5	46,046	51,148,588	1,110	2,356	1,366	394	45.38
Exposed 1	49,531	53,491,359	1,079	2,331	1,319	373	45.49
Exposed 2	49,751	52,734,539	1,059	2,280	1,281	365	45.07
Exposed 3	49,522	52,931,829	1,068	2,293	1,294	372	45.28
Exposed 4	54,515	58,091,101	1,065	2,284	1,261	373	44.63
Exposed 5	46,927	49,692,489	1,058	2,239	1,264	369	45.23

There may be magnitudes of difference in the most and least abundant sequences within the cluster transcripts generated by the *de novo* sequencing. Transcript length distribution is used to identify the most abundant which are an elevated number of repetitive elements and cause artefacts which can generate false transcripts. These are then removed to leave Unigenes, which are used for subsequent downstream analysis. Where there is more than one sample, the Unigenes of all samples are analysed to get a final Unigene list, called All-Unigene (Figure 5.6). The clustering quality metrics are shown in Table 5.4.

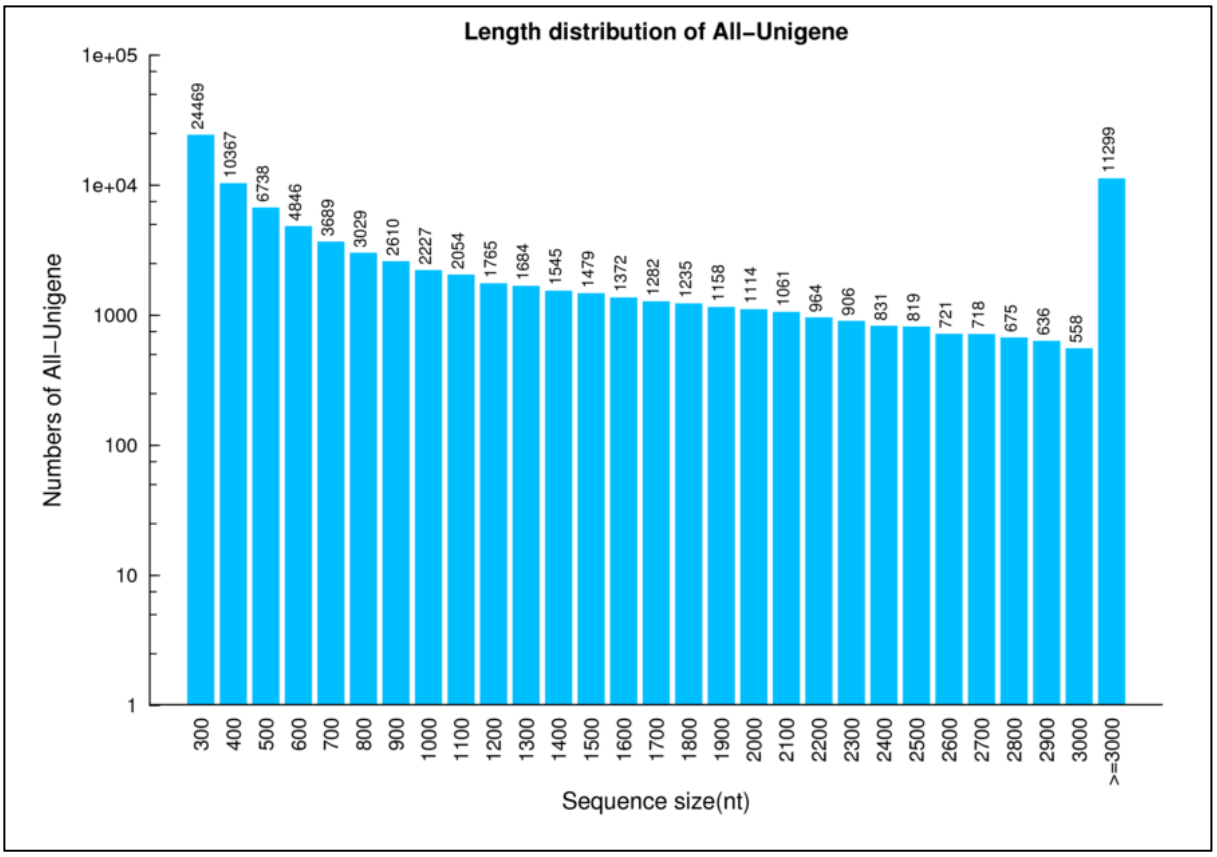


Fig 5.6 Length distribution of All – Unigene. All-Unigene is the final Unigene list for further analysis. TGICL is a gene indices clustering tool which clusters sequences based on pairwise sequence similarity and assembles them to produce more complete sequences, this was used to create All-Unigene when there are more than one sample.

Table 5.4 Clustering quality metrics of Unigenes. Bold highlighted results are abnormally low in length.

Sample	Total no of Unigenes	Read length of Unigenes	Mean length	N50	N70	N90	GC%
Control 1	**58,338**	**52,491,956**	**899**	**1,794**	**881**	**314**	**45.50**
Control 2	32,668	41,800,985	1,279	2,426	1,472	497	45.58
Control 3	31,043	40,351,932	1,299	2,424	1,493	511	45.69
Control 4	**58,162**	**51,607,031**	**887**	**1,689**	**856**	**317**	**45.92**
Control 5	**53,684**	**47,634,643**	**887**	**1,735**	**857**	**313**	**45.88**
Volatile 1	31,492	40,211,106	1,276	2,379	1,463	503	45.74
Volatile 2	30,396	39,283,805	1,295	2,417	1,487	517	45.77
Volatile 3	33,344	43,495,245	1,304	2,482	1,516	507	45.61
Volatile 4	32,237	41,407,319	1,284	2,435	1,492	498	45.75
Volatile 5	29,860	39,841,612	1,334	2,467	1,536	542	45.70
Exposed 1	31,890	40,211,106	1,309	2,482	1,513	518	45.80
Exposed 2	31,299	40,729,712	1,301	2,453	1,502	509	45.67
Exposed 3	31,935	41,139,124	1,288	2,438	1,482	506	45.67
Exposed 4	35,605	45,382,984	1,274	2,419	1,443	494	44.94
Exposed 5	30,778	39,283,805	1,276	2,399	1,468	498	45.61
All-Unigene	91,851	121,344,346	1,321	2,773	1,584	489	44.80

Once assembled into Unigenes, a functional annotation was performed against seven functional databases to discover the identity of these Unigenes when compared to known functional genes recorded in these databases. NT and NR databases are the NCBI official databases for nucleic acids and proteins, respectively. *De novo* identification of the best match hits in these databases also provides useful validatory information to show that no errors have occurred in RNA extraction or during shipping. It is to be expected that the top hit for these Unigenes, when compared to the reference databases, would be from the Culicidae. Fig 5.7 and Table 5.5 indicates that in the *de novo* transcriptome for *Oc. detritus* the majority (87.8%) of Unigenes have top hits against either *Aedes aegypti*, *A. albopictus*, *Culex quinquefasciatus*, *Anopheles gambiae* or *An. darlingi*. The high percentages of genes identified from other mosquito species in both analyses indicates that the data have come from a mosquito and that the genes that were identified in the following annotation were for the correct organism and that there were no substantial amounts of aberrant organisms sequenced.

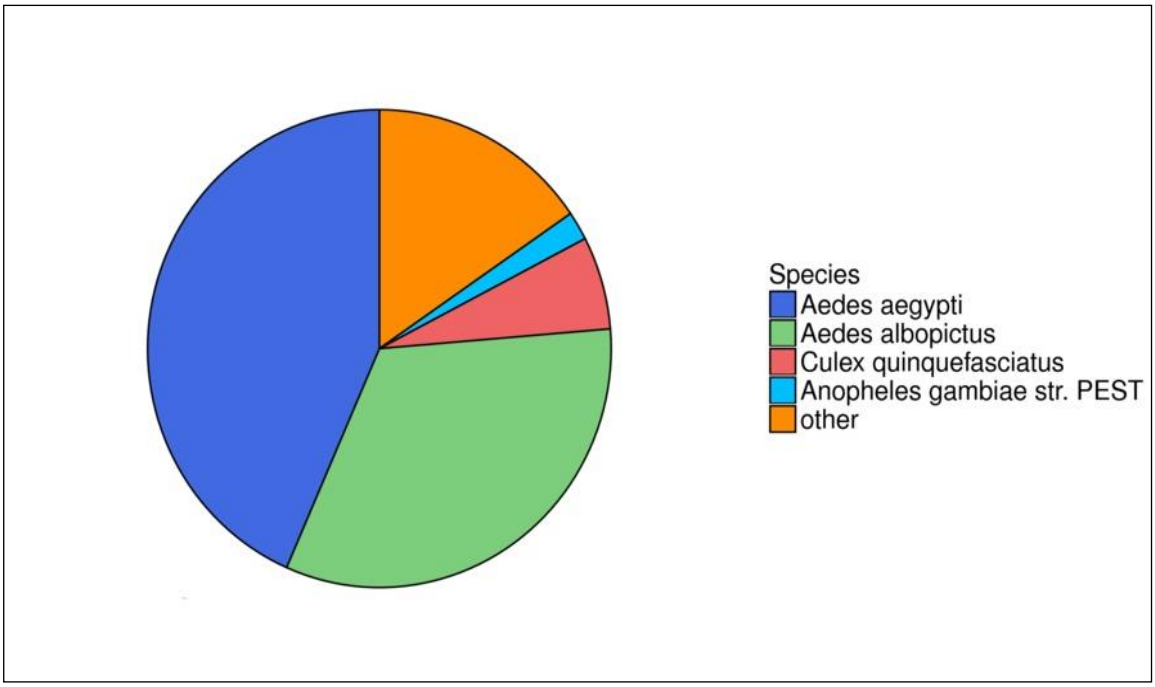


Figure 5.7. Species distribution of Unigenes identified through NR database. This shows that the majority of species identified are mosquitoes, with the largest proportion belonging to Aedine mosquitoes; the group that *Ochlerotatus detritus* belongs to.

Table 5.5 Truncated table of first 20 species identified. These are arranged by percentage (highest first). This corresponds with the NR database findings in Fig 5.7.

Species	Gene numbers	Percentage of total
Aedes aegypti	23958	43.44%
Aedes albopictus	18130	32.88%
Culex quinquefasciatus	3473	6.30%
Anopheles gambiae str. PEST	1074	1.95%
Anopheles darlingi	839	1.52%
Anopheles sinensis	772	1.40%
Papilio xuthus	615	1.12%
Drosophila willistoni	263	0.48%
Eimeria necatrix	248	0.45%
Anopheles gambiae	170	0.31%
Octopus bimaculoides	134	0.24%
Hydra vulgaris	132	0.24%
Lasius niger	125	0.23%
Calliphora vicina	119	0.22%
Dictyostelium discoideum AX4	93	0.17%
Cephus cinctus	90	0.16%
Musca domestica	90	0.16%
Drosophila melanogaster	90	0.16%
Bactrocera cucurbitae	80	0.15%
Monosiga brevicollis MX1	78	0.14%

Gene ontology (GO) annotation categorises the Unigenes into three different categories: molecular function, cellular component and biological processes (Ashburner et al., 2000; GO Consortium, 2017) as seen in Fig. 5.8. For the purposes of this study the biological processes are of the most interest. From the data provided it shows that 3189 genes were identified as those used in response to stimulus and 171 were identified as active in immune response, which

are the genes that would be likely involved in any recognition of, and defence against, invading parasites.

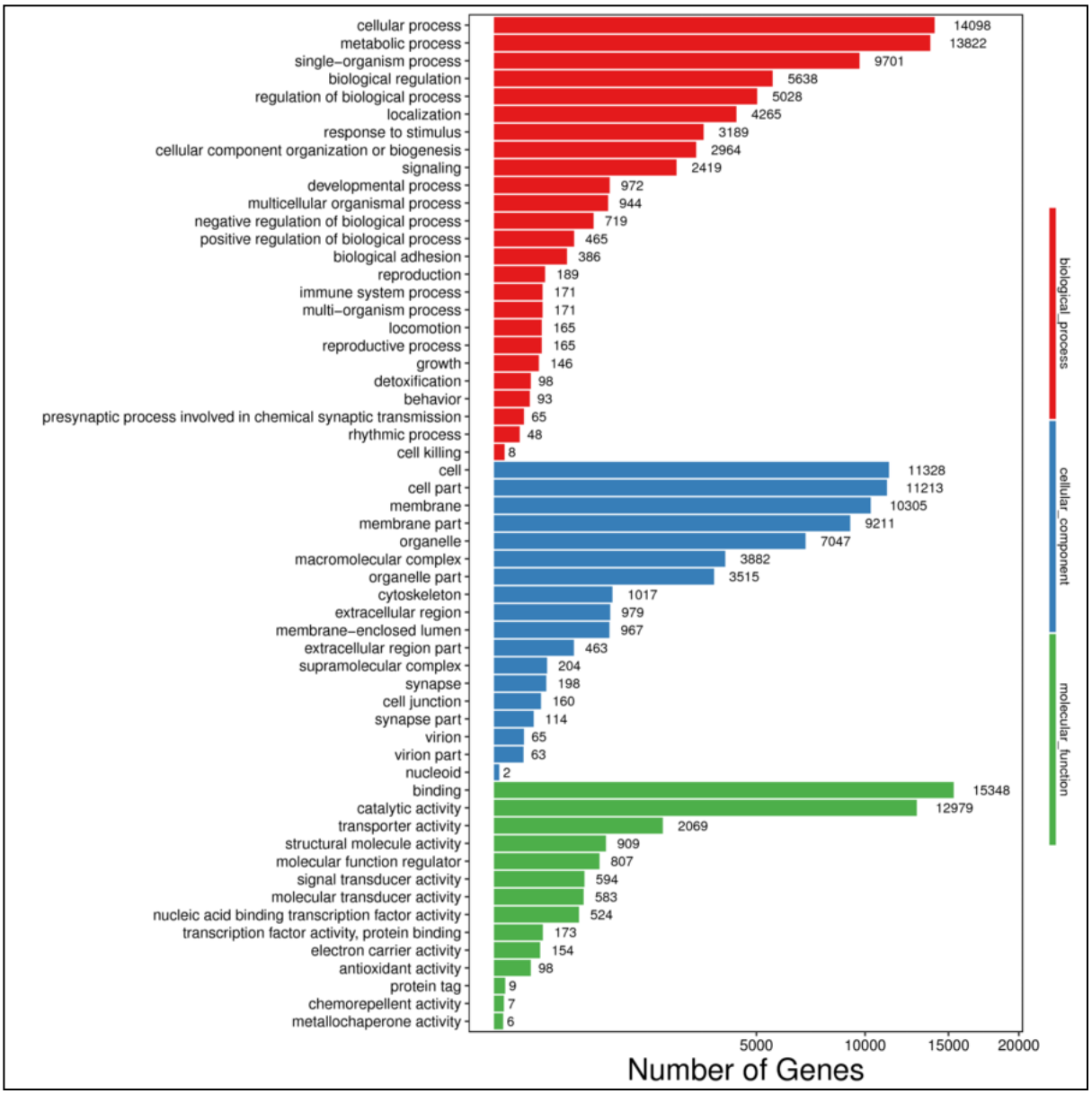


Figure 5.9. Functional distribution of *Oc. detritus* Unigenes aligned to NR database which have been annotated using Blast2GO.

KEGG is a tool used to ascertain high-level biological functions from molecular level information. The resource contains 18 databases split into four broad categories; systems, genomic, chemical and health information. KEGG Pathway falls within the systems category.

The KEGG pathway is a collection of compiled ‘pathway maps’, subdivided into levels which are used to show the current knowledge around molecular interaction, reaction and relation networks to build up a genomic picture of the interaction of these four categories (Kanehisa et al., 2017). KEGG pathway levels 1 and 2 were used to annotate the Unigenes for this project. Level 1 is concerned with metabolism and level 2 genetic information processing. The processes of interest in this instance are KEGG level 1 showing cellular processes and level 2 cell growth and death with 1448 Unigenes annotated as relating to that process. Here, 3833 unigenes were identified as having an immune system function and 1159 were identified that were involved in parasitic disease (Figure 5.10).

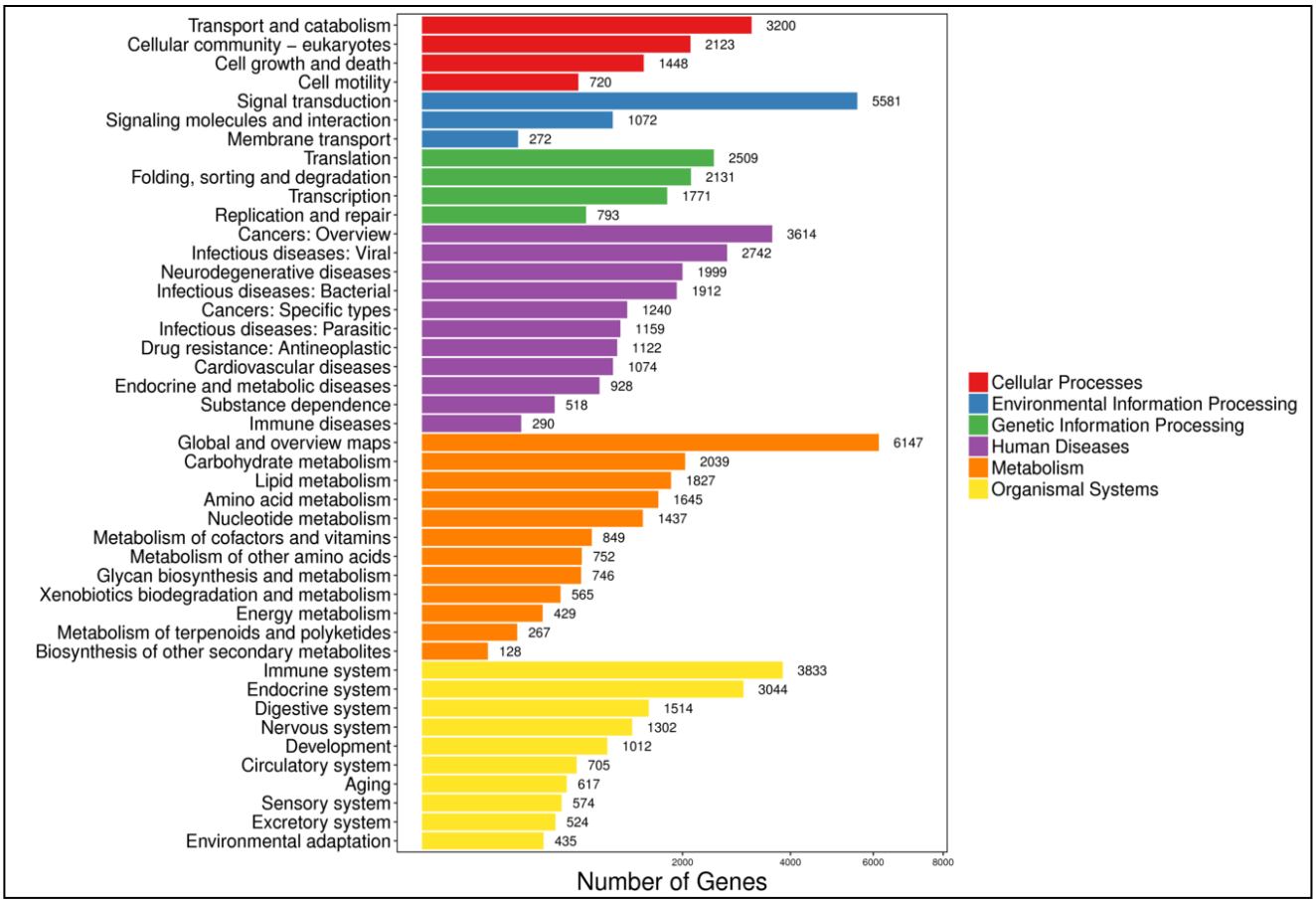


Figure 5.10. Functional distribution of KEGG pathway annotation of All-Unigene from *Ochlerotatus detritus*.

Differential Gene Expression

Once a *de novo* transcriptome had been produced, reads were mapped against this to identify differentially expressed genes. The number of reads mapped (FKPM) varies across all genes (Fig 5.12).

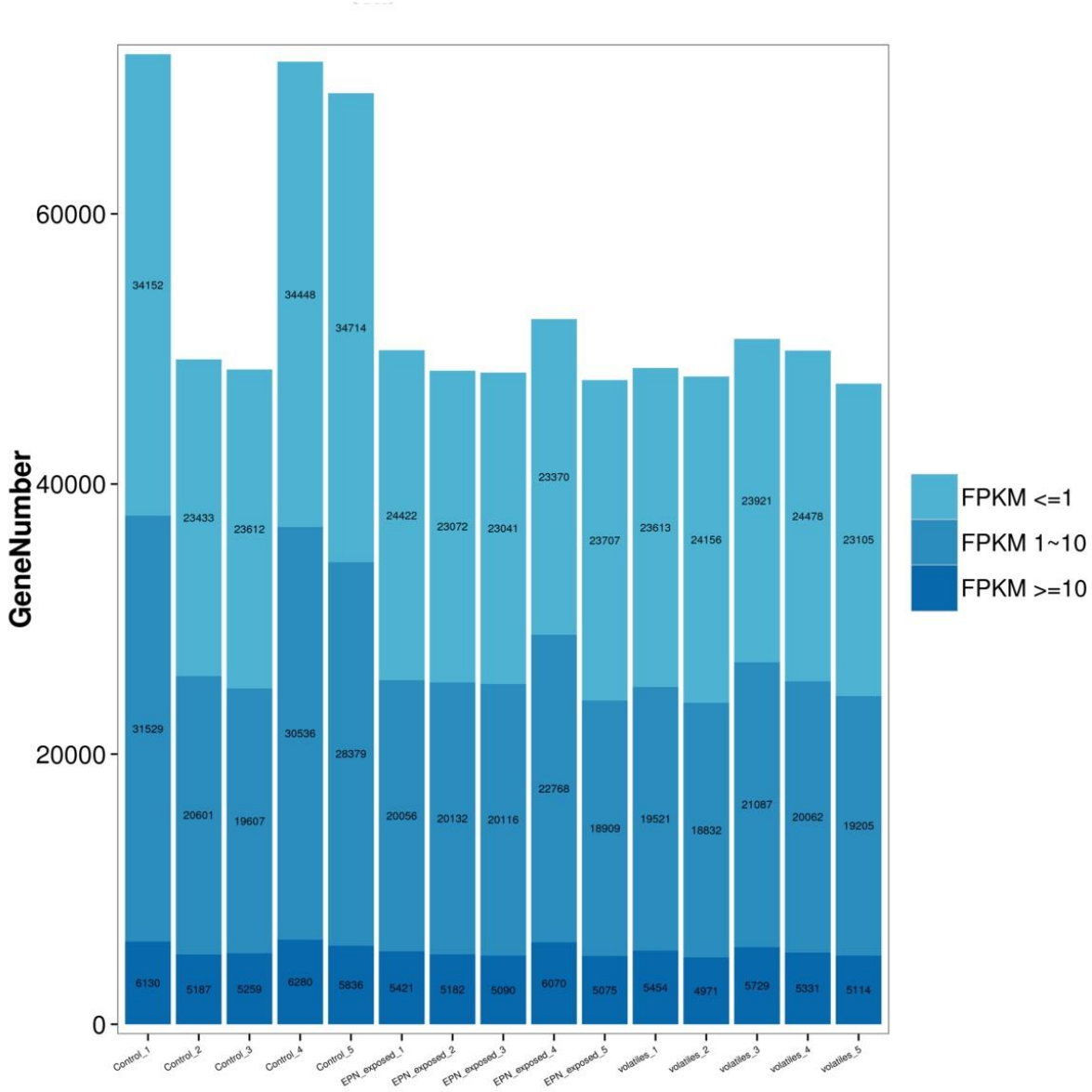
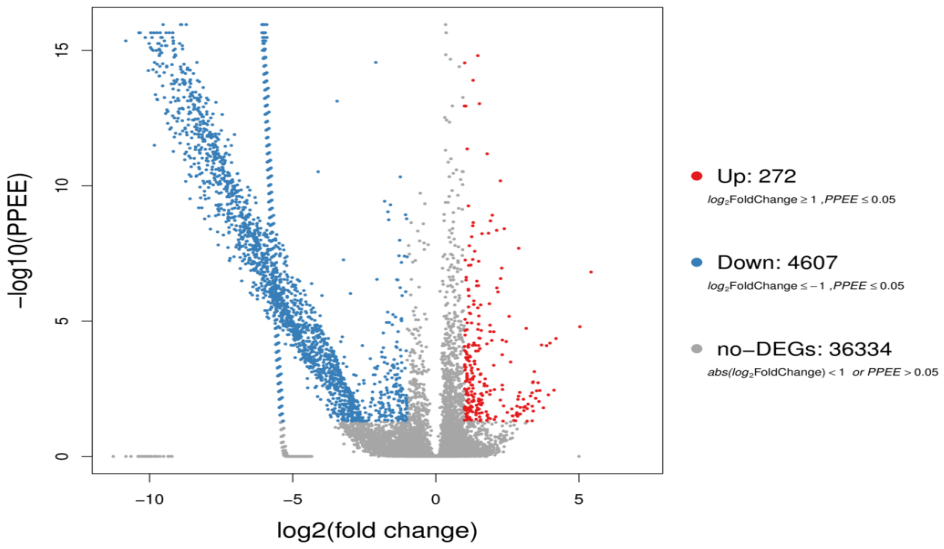


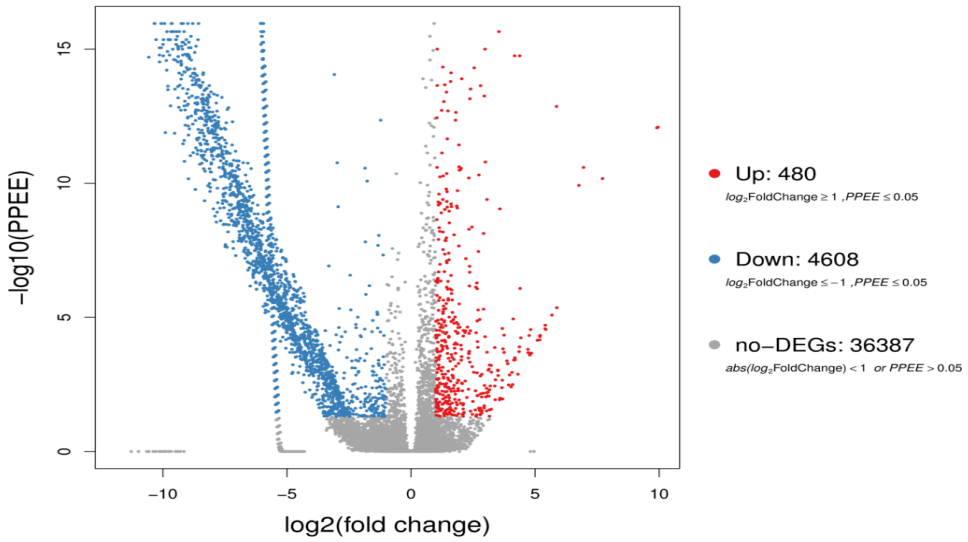
Figure 5.12 Gene expression distribution in each sample. On the Y axis, Gene Number represents the amount of a gene. Fragments per kilobase million or FPKM <= 1 means extremely low expression level, Controls 1, 4 and 5 are expressing a much higher number of genes albeit at a low level.

In order to identify differentially expressed genes volcano plots were produced which plot fold change (experimental condition vs control) against probability of differential expression (calculated from biological replicates). For the two experiments these are shown in figures 5.13a (control vs volatiles) 5.13b and (control vs exposed). Each dot on these figures represents a statistically significant result from the analysis of thousands of replicate data points between the two tested conditions. It can be clearly seen that whilst there are many significantly up-regulated and significantly down-regulated genes there is a larger number of significantly down-regulated genes. The absence of the control conditions in Fig. 5.13c indicates that the would appear to be an issue with the controls, as their absence in testing shows a more regular volcano plot. Figure 5.14 shows the distribution of differently expressed genes, those involving the control again clearly show a large number of downregulated genes. Figure 5.15 shows the gene abundance changing with expression quantity and also further indicates that there are anomalous results.

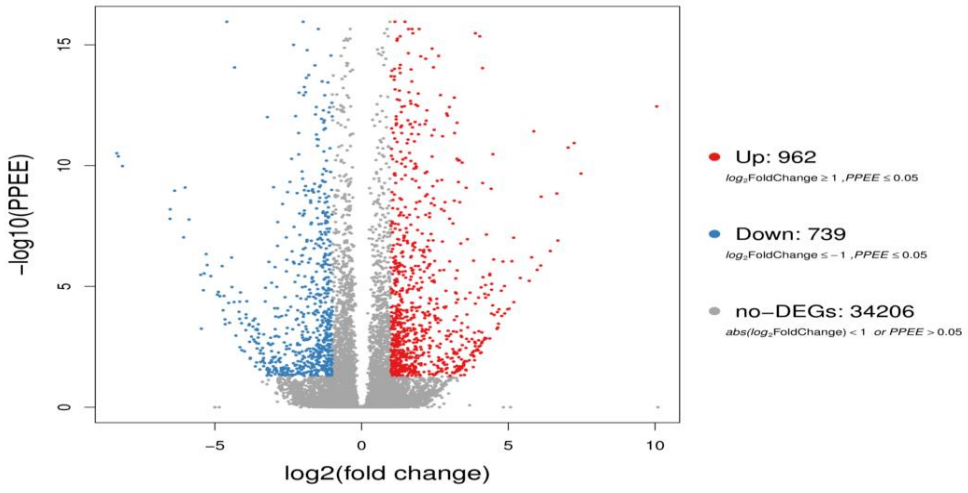
a



b



c



Figures 5.13a - c Volcano plots to show the distribution of DEGS in the compared conditions. Y axis is significance, X is fold-change. 5.13A shows control vs volatile, 5.13B control vs exposed and 5.13C exposed vs volatiles

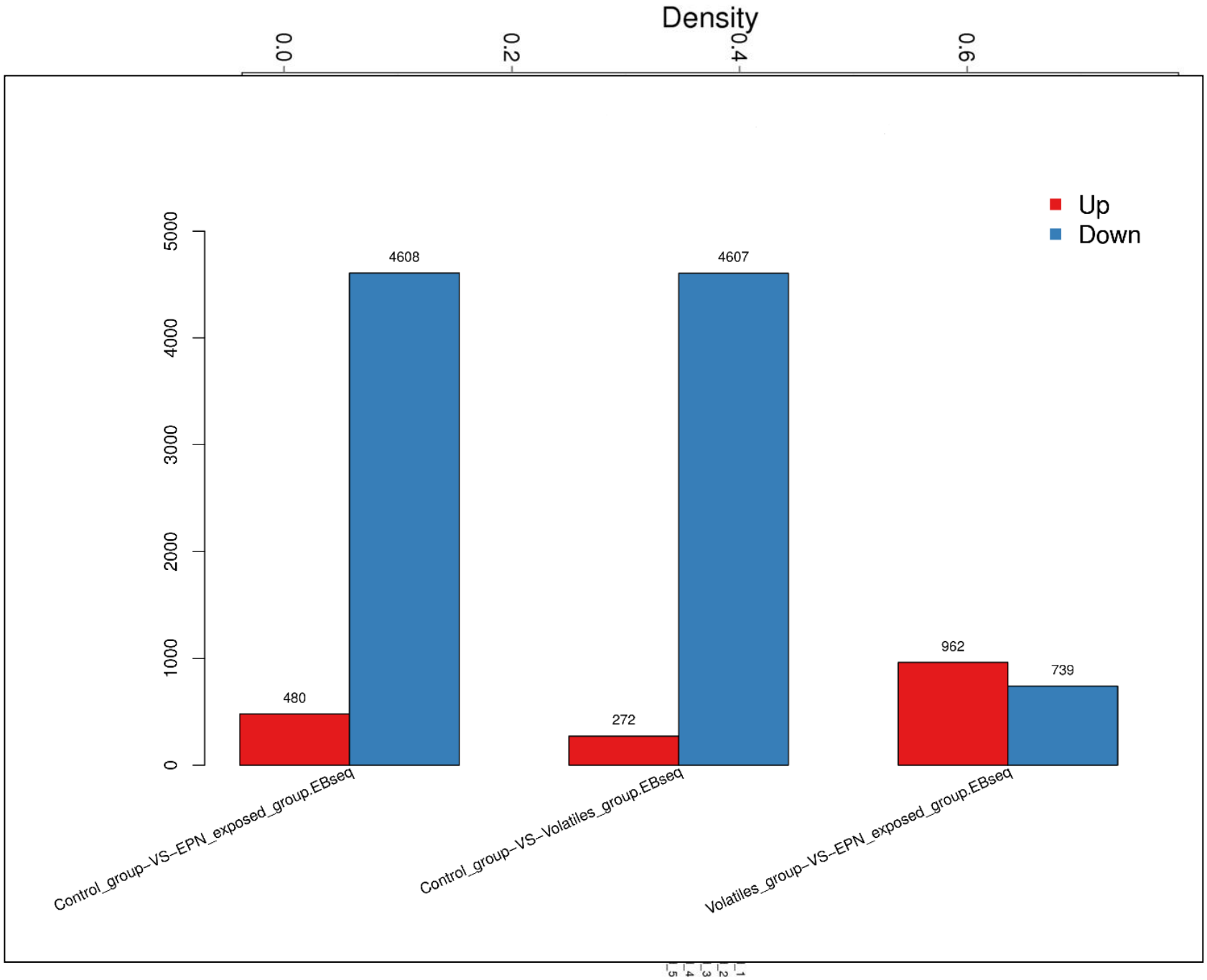


Figure 5.14 Summary of diff)EGs.
Much higher numbers of genes have been downregulated.

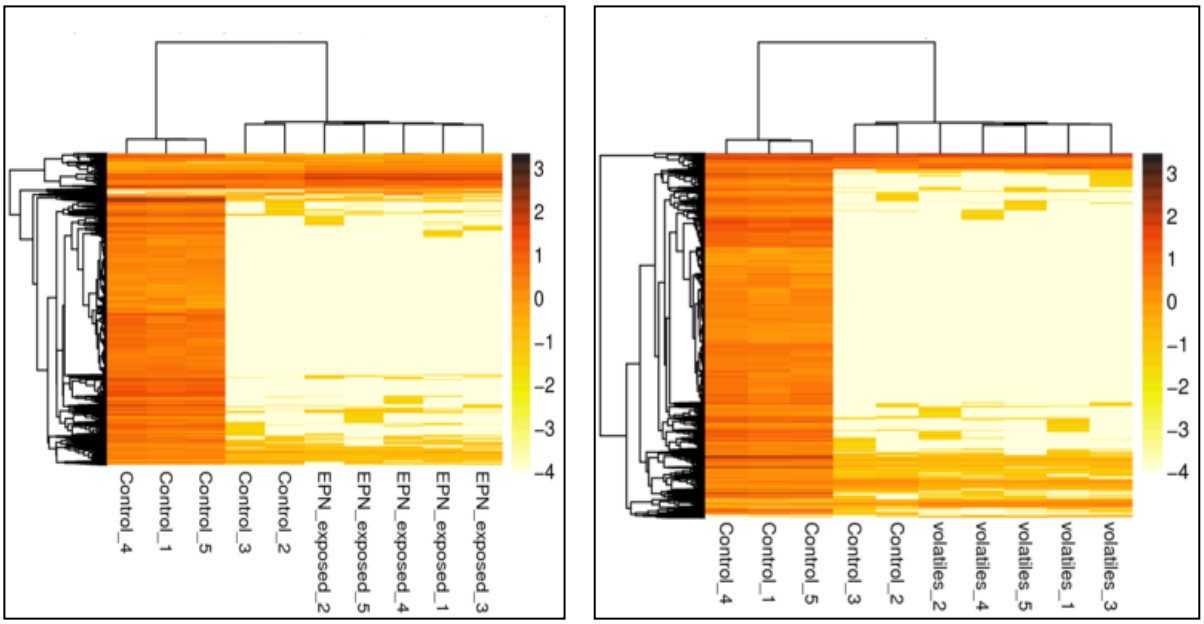
Figure 5.15. The expression density distribution shows the ratio of the gene amount under the specific expression level to total number of expression genes. The top three peaks are indicating that Controls, 1,4 and 5 are showing as having a much higher density compared to the other samples.

In Figures 5.13 a and 5.13b, it is worth noting that there are blue streaks on the left hand side that do not appear to form part of the 'volcano' spread of significant P values. These are also P values, it is not an error in the Figures. The uniform nature of these data points may indicate that this is artefactual, possibly from another biological source as data and plotting errors were excluded through querying with BGI.

Probability heat (Pheat) maps (Figures 5.16a to 5.16c) are a graphical representation of the level of expression of the distribution of differently expressed genes from all the obtained reads. In Figs. 5.16a and 5.16b, both comparing the volatile and exposed conditions to the control, a dark band of colour relating to the control conditions shows that there is a high expression level of genes in the control groups when compared to that of the other conditions it is tested against. When compared against the third set of DEG comparisons of volatiles vs exposed, the difference can be seen markedly.

a

b



c

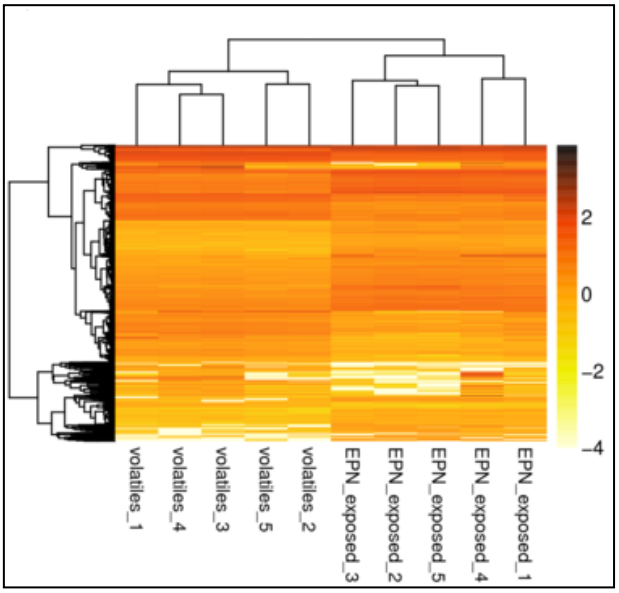


Figure 5.16 a-c Heatmaps of differently expressed genes. Y axis represents DEGs. Dark colour shows high expression levels. a is control vs exposed, b is control vs volatiles, c is exposed vs volatiles. Unusual distribution of highly expressed genes can be observed in heat maps including control samples.

Categories of differentially expressed genes

From the annotated transcriptome the categories of differentially regulated genes can be seen in Figure 5.17 and b. Gene Ontology (GO) analysis places the genes into three structural categories; molecular function, cellular component and biological process. This enables an understanding of the genes that are frequently expressed under the conditions that were being tested for. Molecular function describes the molecular activity of a gene, biological process describes the physiological purpose that a gene carries out and cellular component describes the cell location where the gene performs its function. In Fig 5.17a the biological processes are most highly expressed in cellular and metabolic processes. Binding and catalytic activity are also highly expressed in the molecular function category. In Fig. 5.17b high expression is seen at the same categories, however not to the extent that was found in the exposed samples. This in inkeeping with what would be theorised, that the volatiles exuded by the EPN start to trigger an immune response in the mosquito, whereas the physical proximity of an EPN would induce an increased immune response.

The up and down regulated genes can be seen in Figure 5.18a and b, this gives a more detailed indication of what is happening in the test conditions. However, the high downregulation of genes observed in previous figures prevents a clear assessment.

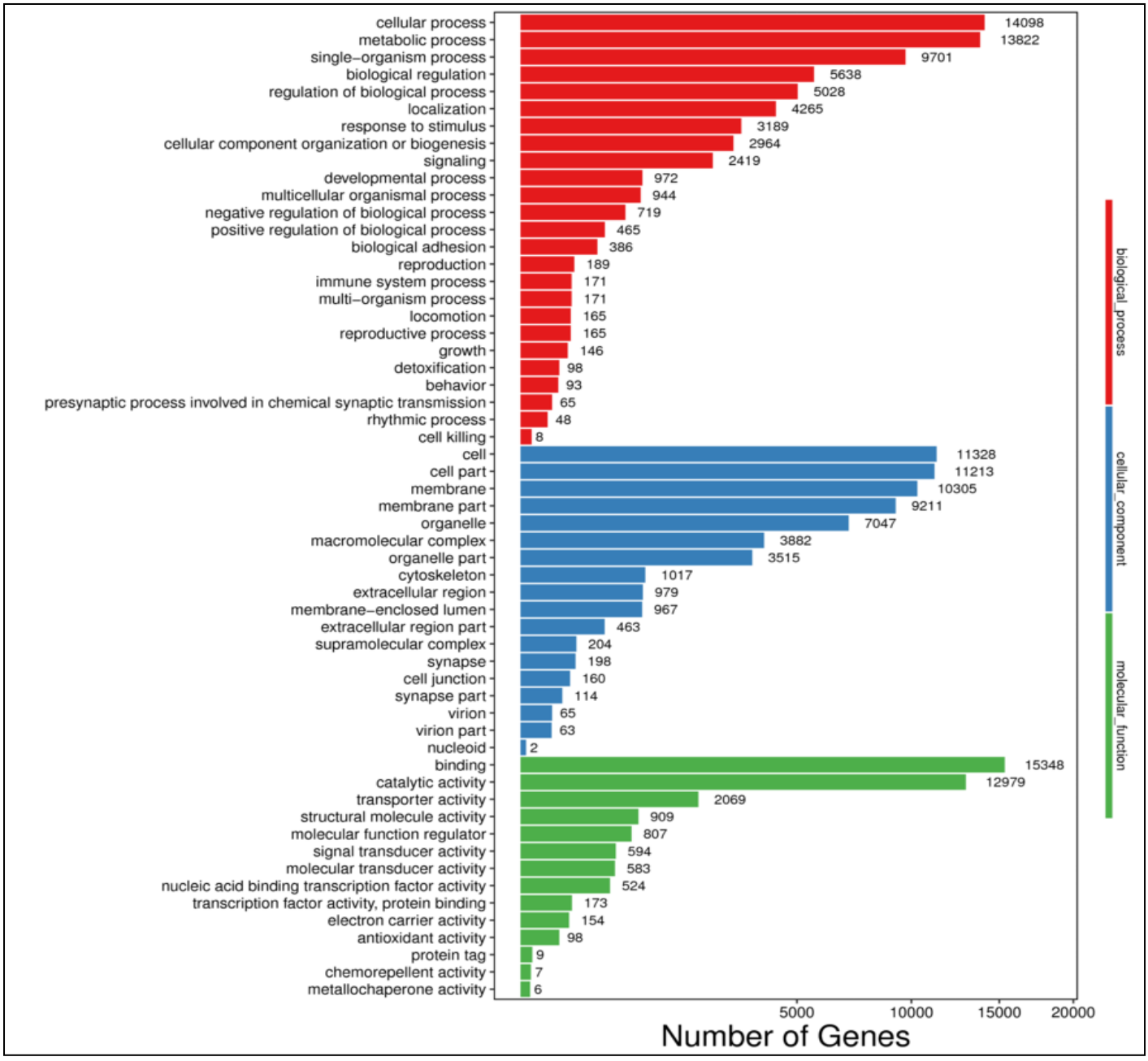


Figure 5.17a. Gene Ontology groupings and expression of differently expressed genes. Control vs exposed.

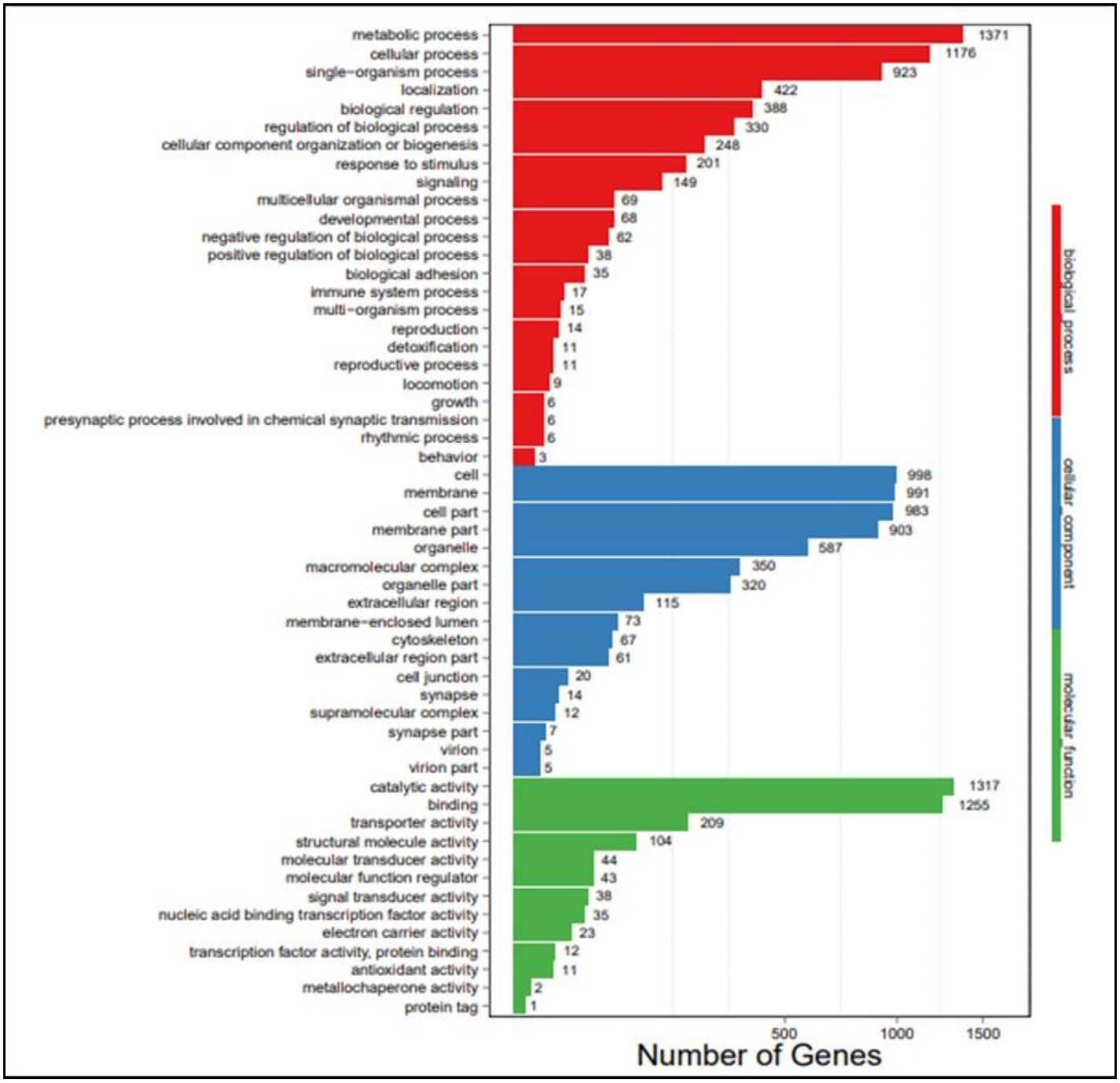


Figure 5.17b Gene Ontology groupings of differentially regulated genes. Contro vs volatile.

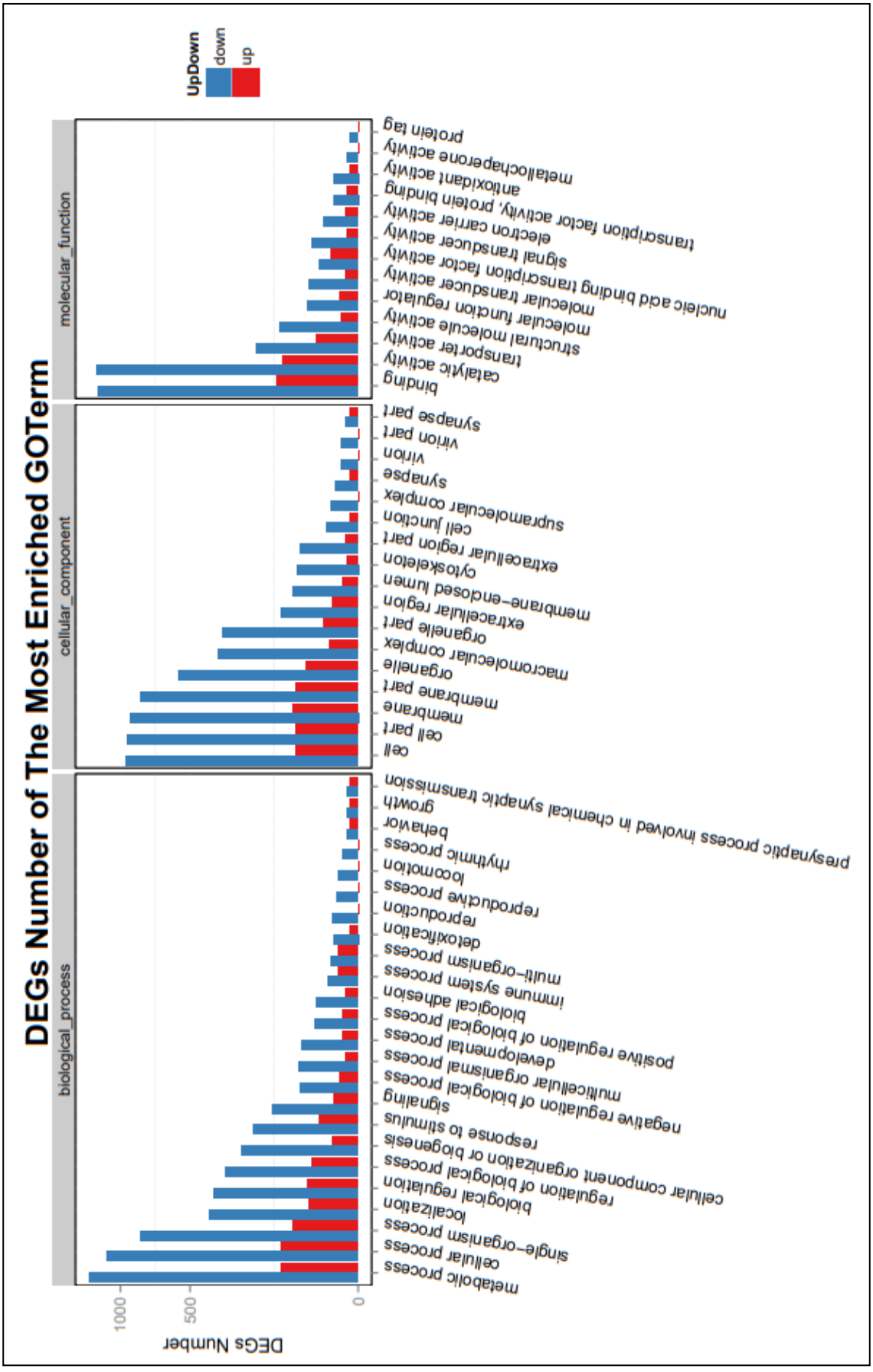


Figure 5.18a Gene Ontology classification of upregulated and downregulated genes. Control vs exposed.

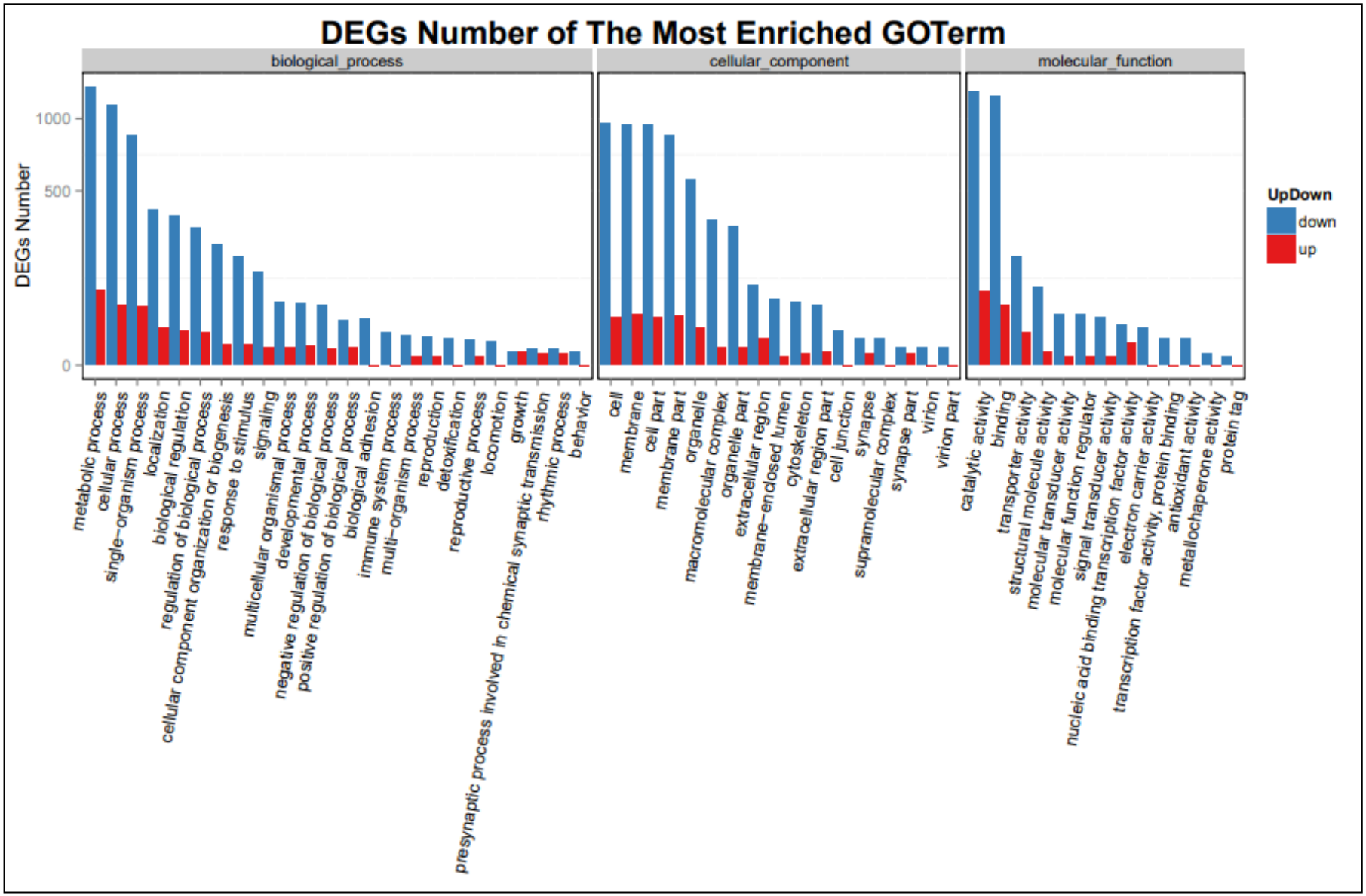


Figure 5.18b. Gene Ontology classification of upregulated and downregulated genes. Cntrol vs volatile

Discussion

This study provides the first transcriptomic dataset for the pestiferous mosquito *Ochlerotatus detritus*. Over 1.17Gb of reads were generated allowing the construction of a larval transcriptome composed of 95,851 Unigenes. Some of these will likely be alternative transcripts and/or fragments of the same gene as not all can be mapped at this point in time. Until a full genome is available for this species, or additional high-quality transcriptome sequencing, perhaps using long range PacBio reads is conducted, then this cannot be known for certain. However, the transcriptome produced is a rich resource for research on this species and for comparative transcriptomic studies.

Differential Gene Expression analysis indicated a large number (272) of transcripts were significantly up-regulated and 4607 were down-regulated when mosquito larvae exposed to water in which EPNs had been ('volatiles') or when mosquitoes were exposed directly to EPNs (but where EPNs had not penetrated) resulting in 480 up and 4608 down regulated. The high number of down-regulated genes, inconsistencies in the heat-maps, and relatively high numbers of Unigenes in controls 1, 4 and 5 suggested that there were issues with these data. Higher numbers of Unigenes in some samples could be caused by contamination. Whilst care was taken to ensure no EPNs penetrated the sample, and there was little evidence of reads mapping to nematode genes (Table 5.5) it is possible that other species of mosquito were accidentally extracted. Whilst *Oc. detritus* is the predominant species at Little Neston it is now apparent that an additional, morphologically similar species, *Oc. caspius*, has been found on one occasion in a mosquito trap near this site (Clarkson, pers comm.). This species is morphologically very similar to *Oc. detritus* with small differences in siphon setae the predominant indicator (Becker, 2010, Clarkson pers. comm.). However, to have three samples contaminated with an infrequently found mosquito would be particularly unfortunate.

Alternatively, there may be differences in strain of *Oc. detritus* present in the pools they were taken from. Including unrecognised additional species in pooled RNA extractions could result in low frequency transcripts, which would then appear differentially expressed if they appear in only one of the conditions. It appears that control samples 1, 4 and 5 probably have suffered from this. Additional analyses of the raw read data indicates that mitochondrial DNA sequences of *Oc. caspius* can be found in these three samples (Gareth Weedall, LJMU, pers. comm.) and it can therefore be concluded that this current iteration of the data analysis cannot be used for its intended purpose and reanalysis is required. Future reanalysis could include either removing these three control samples and using just two samples for controls thereby reducing power, or potentially removing the low frequency transcripts in C1, 4 and 5 which produce the high frequency of down-regulated genes.

Differentially expressed genes

Although these data require reanalysis, numerous genes were up-regulated in the volatile and exposed samples. These should not be as adversely affected by the issue of contamination of the control samples. Some of these (3833) were in immunity families. Immunity gene families are groups of identified genes that have similar functions. Many of the members of these families are found across many metazoan animals such as TEP genes (Christophides et al., 2002) whilst others are species specific (Waterhouse et al., 2007). The most relevant in this study are in three families. Recognition; which, identifies non-self infections or invasions within the mosquito. It contains genes such as PGRP which has diverse functions in activating the insect immune function. In particular melanisation, phagocytosis and gram-negative binding proteins (GNBP), which binds to gram-negative bacteria when upregulated by an incursion to the immune system. This is particularly important in EPN invasion as they release their gram-negative symbiotic bacteria which would be detected by GNBP (Christophides et al., 2002; Steven Forst et al., 1997). Signal modulation phase; which regulates immune

processes following the recognition of an invader (Waterhouse et al., 2007) and finally the signal transduction family of immunity genes. These link the alert of invasion to transcriptional activation (Waterhouse et al. 2007). This group contains the well-studied TOLL signalling pathway which is an antimicrobial response and effector response molecules which also induce melanisation and wound healing (Christophides et al., 2002).

Future work

These data require reanalysis and this will be done in the near future, removing controls 1, 4 and 5. The previous analyses were performed by BGI and time constraints for the completion of this thesis prevent the author performing the analysis. Re-analysing the data with only 2 controls will give less power to the analysis, however, it will remove these erroneous results. Once this is completed this data will provide information that is unique at this point in time about the relationship between EPNs and a mosquito that is becoming increasingly interesting to researchers. It is hoped that this will give an insight into mosquito susceptibility to EPNs when compared to much more resistant species such as *Ae. aegypti*.

General Discussion

Potential for use

One of the unique selling points of using EPNs in pest control is that they are a 'natural' or ecologically friendly method, doing little unintentional damage to non-target insects of the environment. However, the concerns of environmental harm associated with chemical control methods for crop pests do not carry the same gravitas when dealing with vectors of disease because human lives, frequently those of children, are at very obviously at risk (Gates, 2014; Thomas, 2018). Vector-borne disease is at its most devastating in the poorest countries (Worrall et al., 2005) and therefore challenges for all novel control methods are the same: cost, reliability and successful control of mosquitoes that use cryptic habitats and inaccessible breeding sites. New and emergent vector control methodologies have to improve on the use of chemical pesticides in one or more of these ways to achieve take-up in real-world populations (Lacey et al. 2015; Thomas 2018).

In this research, EPNs were not observed to be able to swim, so may not be capable of moving against a flow of water to attack a pelagic dwelling larvae. However, they were able to parasitise and kill larvae that were benthic or spent some of their time at the bottom of the water body as *Ae. aegypti* larvae do. Therefore using EPNs as a control against mosquitoes such as *Culex pipiens,* or *Anopheles* spp., which have been found to spend more time at the surface than other tested species (Merritt et al., 1992; Skiff and Yee, 2014; Yee et al., 2004) would not be as effective. However, Yee et al. (2008) found that mosquito larva adapted their feeding behaviour to a range of physical factors such as the presence of vegetation in the water. The depth of water is an important factor to consider. Some studies show *Aedes* mosquitoes prefer a shallower water body (up to 30cm deep) and *Culex* spp. deeper (greater than 30 cm). The problem of deep water is that if the mosquito larva does not dive to where the EPNs are, they

would not be able to parasitise it, but *Aedes* larvae living in deeper water spend more time feeding on the bottom (Lester and Pike, 2003; Skiff and Yee, 2014), which would be beneficial for EPN control. If the mosquito larvae are inhabiting a broader area of water, they have less chance of coming into contact with the EPNs, who have not shown a capacity for roaming underwater, or the larvae have more room to move away. This is in agreement with the findings of Cardoso et al., (2016) who found that smaller-based containers containing *H. indica* had higher *Ae. aegypti* mortality rates than larger ones. Therefore, using EPNs for control in a restricted area such as a container could be a more effective usage.

Vessels of standing water are a significant problem in vector control (Wong et al., 2011), and by extension the control of nuisance biting insects. Where water is a scarce commodity, communities often collect rain water or grey water (water used for washing dishes or personal ablutions) for use at a later date (Romero et al., 2017). Even in communities where water is not scarce such as the maritime climate of the UK, people collect rain water in water butts for use around the garden or for cleaning (Royal Horticultural Society, 2018). Containers of water are attractive to gravid mosquitoes especially *Ae. aegypti* which preferentially lays its eggs in containers (Day, 2016; Wong et al., 2011) as they are frequently predator free (being an enclosed environment). They are often allowed to stagnate, thereby providing an ideal habitat for the larval stages to feed, grow and eclose with minimal disturbance (Becker, 2010; Bentley and Day, 1989). Integrated vector management research has to balance the need of water-poor or drought affected communities to collect water with the risk of providing a suitable habitat for potential vectors of serious disease (Center for Disease Control, 2016; Trewin et al., 2013).

One issue that has not been given much consideration is whether EPNs could be added to the water container to attempt to address this issue. The success of this would weigh heavily

on the species of mosquito that were problematic in the area and also what the water was being stored for. EPN 'tainted' water for cleaning or watering plants might not be viewed unfavourably. However, the prospect of potentially ingesting water populated with microscopic roundworms is probably an unpalatable thought where it is stored for cooking or consumption even after filtering or boiling. Despite this, EPNs are not thought to pose any threat to human or other vertebrate wellbeing (Boemare et al., 1996) and other biocontrol methods such as the use of turtles or fish to eat larvae in stored water are reasonably well tolerated (Borjas et al., 1993; Romero et al., 2017; Walshe et al., 2017). Human consumption aside, the presence of an EPN population may act as a deterrent to a gravid female in much the same way that they avoid ovipositing in pools that contain predators such as anuran tadpoles through detection of chemical cues (Mokany and Shine, 2003; Saward-Arav et al., 2016) or pools that are overcrowded with competitors (Blaustein and Kotler, 1993). This has not as yet, been tested with regard to EPNs and is something that could be explored further in future research.

When EPNs are used for commercial crops they can be sprayed from a modified delivery system similar to that used for spraying traditional chemical insecticides (Shapiro-Ilan et al., 2012, 2006). For an area such as Little Neston where the whole of the salt marsh is potentially home to millions of *Oc. detritus*, that inhabit shallow pools, spraying in the same manner could produce a good result. However, one of the problems experienced with spraying of *Bti* was that it was done mainly by individuals wearing back-pack style sprayers (Clarkson, pers. comm.), which limited where the bacteria could be sprayed. Data for *Oc. detritus* specifically is limited, but other *Ochlerotatus* floodwater mosquitoes have recorded ranges between 7-11km from their larval and oviposition habitats to seek a blood meal (Bogojević et al., 2011; Elbers et al., 2015). So, for an extensive marshland habitat such as that in Little Neston, a larger area would need to be covered, therefore aerial application may be more

effective, this could be undertaken by a modified drone. A channel digging exercise in 2014 was undertaken to create better run-off from the tide and make favoured oviposition sites less attractive by steepening the sides of the pools. However, in the intervening four years the channels cut have started to re-fill with silt and are creating again more suitable habitats for the nuisance biting mosquito to breed.

The advantages of using EPNs as an effective biocontrol for mosquito species over current control methods is that it could reduce habitat disturbance as channel digging currently does. For a truly effective mosquito management scheme an integrated approach would be best, combining extensive channel digging with aerial application of EPNs. Whilst environmental health matters such as this rest with the local authorites, work of the necessary scale would be unlikely to be undertaken in the current financial and political climate as local authorities are under great financial pressure.

The use of insecticides, whether biological such as *Bti* or chemical based has been the cause of much concern regarding the impact on non-target organisms (Devine and Furlong, 2007). The advantage of using EPNs to target nuisance biting insects is that they are already a part of the environmental landscape, and in the same way they are used in agriculture, this would only apply their usage to a new organism. This use would likely fall within the use permitted by The Convention on Biological Diversity (1992) as they are already used and distributed without licence (with the exception of *S. carpocapsae*). Highly targeted applications of EPNs to a mosquito breeding habitats such as the saltmarsh at Little Neston may show a good result in reducing the biting nuisance.

The potential effect of the use of EPNs on non-target organisms is a concern frequently raised in this field, especially when laboratory studies of more than 250 potential hosts species are cited (Ehlers and Hokkanen, 1996; Lacey and Georgis, 2012a; Laumond et al., 1979). However, as Ehlers and Hokkanen (1996) observed, these conditions are almost never met in

the field and in normal soil applications of EPNs the effect on non-targets has not been noted to be significant due to limited EPN movement, and the ability of non-target hosts to escape or encapsulate the invading nematodes (Bathon, 1996; Ehlers and Hokkanen, 1996). However, their usage in an aquatic environment is little researched and it would be important to carry out further tests to gain an understanding of what happens to EPNs in water and how they interact with fauna found in diverse aquatic habitats. The continuation of the EPN life-cycle in an aquatic environment is not yet fully described but Cardoso et al. (2015) record emergence of IJs from infected *Ae. aegypti* larvae. As EPNs were found not to move independently in the water (Chapter 2) it is unlikely that they would be able to spread freely, infecting new non target hosts, in much the same way that they do not disperse large distances in the soil (Ehlers and Hokkanen, 1996).

Drawbacks to use of EPNs as effective biocontrol for mosquito species.

The problems of using EPNs as a biocontrol for mosquitoes, whether vector or nuisance biter, mirror those issues that affect EPN use in the crop pest control industry: effectiveness, reliability and cost (Georgis et al., 2006; Lacey and Georgis, 2012).

As discussed above, EPNs are, so far, only capable of killing mosquito larvae that go to the bottom of the water body to feed or hide from predators. This issue means that many other species of mosquito including the malaria vector, *Anopheles* spp. would not be affected by larval control with EPNs. Commercially produced EPNs are expensive in comparison to traditional chemical insecticides (Georgis et al., 2006) and unfortunately many of the countries most vulnerable to mosquito-borne disease are the poorest (Tusting et al., 2016; Worrall et al., 2005). Governments and local authorities, which have a dedicated budget and the political will to do so, may be able to afford to use EPNs to control areas of localised biting nuisance.

However, those at serious risk of serious vector-borne illnesses are often reliant on state or charity aid to help with prevention and treatment (Bill and Melinda Gates Foundation, 2018; The Clinton Foundation, 2016). One of the benefits of using EPNs in vector management is that EPNs do not require specialist skill to breed. Potentially, householders could be taught how to rear EPNs once they had access to a source of them and apply dauers to water collection containers or small areas of standing water such as puddles. The powdered format that some companies produce their EPNs in could be used to sprinkle into problematic mosquito breeding areas such as tree holes or rutted tracks. The practice at that scale would be very low-tech and achievable with everyday household items, if not particularly profitable for commercial EPN producers.

EPNs from commercial sources are occasionally not in good condition on receipt, possibly through incorrect storage *en route*. For the average user who would not inspect their EPN order under a microscope, this may just appear to not be an effective control against the target pest and a waste of money. This would be the point where a gardener may resort to traditional chemical pesticides. The same holds true for vector and biting insect control if it is not reliable then it will not continue to be used. Shelf-life and reliability are major issues for commercial EPN production and an issue that is not easily solved (Ansari, pers comm; Georgis et al., 2006). Various techniques have been developed to keep the EPNs hydrated and alive, such as the use of EPN infected insect cadavers (Gumus et al., 2015; Shapiro-Ilan et al., 2012) or a moisture retentive formulations (Menti et al., 2003). EPNs that require refrigeration for effective use and to prolong shelf-life would prove difficult to use in many communities where mosquitoes are problematic due to ambient temperature, remoteness and often a lack of facilities such as fridges or even reliable electricity. For vector control purposes, creating a living bank of locally sourced EPNs that can tolerate local conditions would be more desirable than reliance on commercial preparations.

Conclusion

This research has demonstrated that EPNs currently in commercial production can be used as an effective biocontrol for the larval stages of some mosquito species and pestiferous chironomids. However there are limitations to their usage in an aquatic environment that relies on a thorough understanding of the target host. To progress this research, field trials are critical to thoroughly explore the most effective usages in mosquito control. There is a very real chance that EPN usage could become an important part of integrated vector management in the same way that they are in integrated pest management. This research also begins to further our understanding of the genetic mechanisms underlying the host response to EPN treatment and the resources generated by this work will be invaluable in future studies of this important process.

References

The European Economic Review referencing style has been used throughout. Bibliography generated by Mendeley software.

Abu Hatab, M., Gaugler, R., Ehlers, R.U., 1998. Influence of culture method on *Steinernema glaseri* lipids. *Journal. of Parasitology*. 84, 215–221. https://doi.org/10.2307/3284473

Adamo, S.A., 2017. Stress responses sculpt the insect immune system, optimizing defense in an ever-changing world. *Developmental and Comparative Immunology*. 66, 24–32. https://doi.org/10.1016/j.dci.2016.06.005

Adamo, S.A., 2014. The effects of stress hormones on immune function may be vital for the adaptive reconfiguration of the immune system during fight-or-flight behavior. *Integrative and Comparative Biology*. 419–426. https://doi.org/10.1093/icb/icu005

Adelman, Z.N., Miller, D.M., Myles, K.M., 2013. Bed bugs and infectious disease: a case for the arboviruses. *PLoS Pathogens*. 9, e1003462. https://doi.org/10.1371/journal.ppat.1003462

Adler, P.H., Cheke, R.A., Post, R.J., 2010. Evolution, epidemiology, and population genetics of black flies (Diptera: Simuliidae). *Infection, Genetics and Evolution*. 10, 846–865. https://doi.org/10.1016/J.MEEGID.2010.07.003

Akhurst, R.J., Bedding, R.A., 1986. Natural occurence of insect pathogenic nematodes (*Steinernematidae* and *Heterorhabditidae*) in soil in Australia. *Australian Journal of Entomology*. 25, 241–244. https://doi.org/10.1111/j.1440-6055.1986.tb01110.x

Al-Own, F., 2013. Population structure of insect pathogenic bacteria in UK soil and their associated nematodes. University of Bath. [PhD Thesis]

Ali, F., Wharton, D.A., 2017. A survey of entomopathogenic nematodes from Otago, New Zealand, with the first record of *Steinernema kraussei* (Steiner, 1923) (Rhabditida: Steinernematidae) from the Southern Hemisphere. *New Zealand Journal of Zoology*. 44, 245–255. https://doi.org/10.1080/03014223.2017.1322620

Ali, F., Wharton, D.A., 2013. Cold tolerance abilities of two entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Cryobiology* 66, 24–29. https://doi.org/10.1016/j.cryobiol.2012.10.004

Aliota, M.T., Fuchs, J.F., Mayhew, G.F., Chen, C.-C., Christensen, B.M., 2007. Mosquito transcriptome changes and filarial worm resistance in *Armigeres subalbatus*. *BMC Genomics* 8. https://doi.org/10.1186/1471-2164-8-463

Alphey, L., 2014. Genetic Control of Mosquitoes. *Annual Review of Entomology*. 59, 205–224. https://doi.org/10.1146/annurev-ento-011613-162002

Alphey, L., Benedict, M., Bellini, R., Clark, G.G., Dame, D.A., Service, M.W., Dobson, S.L., 2010. Sterile-Insect Methods for Control. *Vector-Borne Zoonotic Diseaseseases.* 10, 295–311. https://doi.org/10.1089=vbz.2009.0014

An, R., Suri, K.S., Jurat-Fuentes, J.L., Grewal, P.S., 2017. Dynamics of transcriptomic response to infection by the nematode *Heterorhabditis bacteriophora* and its bacterial symbiont *Photorhabdus temperata* in *Heliothis virescens* larvae. *Insect Molecular Biology*. 26, 584–600. https://doi.org/10.1111/imb.12321

Andreadis, T.G., Hall, D.W., 1976. Neoaplectana carpocapsae: Encapsulation in Aedes aegypti and changes in host hemocytes and hemolymph proteins. *Experimental Parasitology*. 39, 252–261. https://doi.org/10.1016/0014-4894(76)90125-9

Angelon, K.A., Petranka, J.W., 2002. Chemicals of predatory mosquitofish (*Gambusia affinis*) influence selection of oviposition site by *Culex* mosquitoes. *Journal of Chemical Ecology*. 28, 797–806.

Anonymous, 2012. Management of simple insect bites: Where's the evidence? *Drug and Theraputics Bulletin*. 50, 45–48. https://doi.org/10.1136/dtb.2012.04.0099

Ansari, M.A., Shash, F., Butt, T., 2008. First report of *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) from UK. *Nematology*. 10, 2, 289-291. https://doi.org/10.1163/156854108783476313

Ansari, M.A., Tirry, L., Moens, M., 2005. Antagonism between entomopathogenic fungi and bacterial symbionts of entomopathogenic nematodes. *BioControl* 50, 465–475. https://doi.org/10.1007/s10526-004-5524-4

Baiocchi, T., Lee, G., Choe, D.-H., Dillman, A.R., 2017. Host seeking parasitic nematodes use specific odors to assess host resources. *Scientific Reports*. 7. https://doi.org/10.1038/s41598-017-06620-2

Bal, H.K., Taylor, R.A.J., Grewal, P.S., 2014. Ambush foraging entomopathogenic nematodes employ 'sprinters' for long-distance dispersal in the absence of hosts. *Journal of Parasitology*. 100. https://doi.org/10.1645/12-165.1

Barbercheck, M.E., 1992. Effect of soil physical factors on biological control agents of soil insect pests. *Florida Entomology*. 75, 539–548. https://doi.org/10.2307/3496136

Barbosa, P., Peters, T.M., Greenough, N.C., 1972. Overcrowding of Mosquito Populations: responses of larval *Aedes aegypti* to stress. *Environmental Entomology*. 1, 89–93. https://doi.org/10.1093/ee/1.1.89

Bashey, F., Sarin, T., Lively, C.M., 2016. Aging alters interspecific competition between two sympatric insect-parasitic nematode species. *Ecology and Evolution*. 6, 3750–3759. https://doi.org/10.1002/ece3.2125

Bastidas, B., Portillo, E., San-Blas, E., 2014. Size does matter: the life cycle of *Steinernema* spp. in micro-insect hosts. *Journal of Invertebrate Pathology*. 121, 46–55. https://doi.org/10.1016/j.jip.2014.06.010

Bathon, H., 1996. Impact of entomopathogenic nematodes on non-target hosts. *Biocontrol Science and Technology*. 6, 421–434. https://doi.org/10.1080/09583159631398

Becker, N., 2010. *Mosquitoes and their control.* Springer. https://doi.org/http://link.springer.com/book/10.1007/978-3-540-92874-4/page/1

Becnel, J.J., Johnson, M.A., 1998. Pathogenicity tests on nine mosquito species and several non- target organisms with *Strelkovimermis spiculatus* (Nemata mermithidae). *Journal of Nematology*. 30, 411–414.

Bedding, R.A., Molyneux, A.S., 1982. Penetration of insect cuticle by infective juveniles of Heterorhabditis spp. (Heterorhabditidae: Nematoda). *Nematologica* 28, 354–359. https://doi.org/10.1163/187529282X00402

Benedict, M.Q., Levine, R.S., Hawley, W.A., Lounibos, L.P., 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. *Vector-Borne Zoonotic Diseaseseases*. 7, 76–85. https://doi.org/10.1089/vbz.2006.0562

Benelli, G., 2015. Research in mosquito control: current challenges for a brighter future. *Parasitology Research*. 114, 2801–2805. https://doi.org/10.1007/s00436-015-4586-9

Benelli, G., Jeffries, C.L., Walker, T., 2016. Biological control of mosquito vectors: past, present, and future. *Insects*. https://doi.org/10.3390/insects7040052

Benelli, G., Mehlhorn, H., 2016. Declining malaria, rising of dengue and Zika virus: insights for mosquito vector control. *Parasitology Research*. 115, 1747–1754. https://doi.org/10.1007/s00436-016-4971-z

Bentley, M.D., Day, J.F., 1989. Chemical ecology and behavioural aspects of mosquito oviposition. *Annual Review of Entomology*. 34, 401–21.

Bilgrami, A.L., Gaugler, R., Shapiro-Ilan, D.I., Adams, B.J., Ilgrami, A.L.B., Augler, R.G., Lan, D.I.S.H., Dams, B.J.A., 2006. Source of trait deterioration in entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* during *in vivo* culture. *Nematology* 8, 397–409. https://doi.org/10.1163/156854106778493394

Bill and Melinda Gates Foundation, 2018. 10 tough questions we get asked | Bill Gates [WWW Document]. www.gatesnotes.com. URL https://www.gatesnotes.com/2018-Annual-Letter?WT.mc_id=02_13_2018_02_AnnualLetter2018_DO-COM_&WT.tsrc=DOCOM (accessed 6.17.18).

Blackshaw, R.P., 1988. A survey of insect parasitic nematodes in Northern Ireland. *Annals of Applied Biology*. 113, 561–565. https://doi.org/10.1111/j.1744-7348.1988.tb03333.x

Blagrove, M.S.C., Sherlock, K., Chapman, G.E., Impoinvil, D.E., McCall, P.J., Medlock, J.M., Lycett, G., Solomon, T., Baylis, M., 2016. Evaluation of the vector competence of a native U.K. mosquito *Ochlerotatus detritus* (*Aedes detritus*) for dengue, chikungunya and West Nile viruses. *Parasites and Vectors*, 9. https://doi.org/10.1186/s13071-016-1739-3

Blaustein, L., Kotler, B.P., 1993. Oviposition habitat selection by the mosquito, *Culiseta longiareolata*: effects of conspecifics, food and green toad tadpoles. Ecological

Entomology, 18, 104–108. https://doi.org/10.1111/j.1365-2311.1993.tb01190.x

Boag, B., Neilson, R., Gordon, S.C., 1992. Distribution and prevalence of the entomopathogenic nematode *Steinernema feltiae* in Scotland. *Annals of Applied Biology,* 121, 355–360. https://doi.org/10.1111/j.1744-7348.1992.tb03448.x

Boemare, N., Laumon, C., Mauleon, H., 1996. The entomopathogenic nematode-bacterium complex: biology, life cycle and vertebrate safety. *Biocontrol Science and Technology*, 6, 333–346. https://doi.org/10.1080/09583159631316

Bogojević, M.S., Merdić, E., Bogdanović, T., 2011. The flight distances of floodwater mosquitoes (*Aedes vexans, Ochlerotatus sticticus and Ochlerotatus caspius*) in Osijek, Eastern Croatia. *Biologia (Bratislava).* 66, 678–683. https://doi.org/10.2478/s11756-011-0073-7

Bongers, T., Ferris, H., 1999. Nematode community structure as a bioindicator in environmental monitoring. *Trends in Ecology and Evolution*, 14, 224–228. https://doi.org/10.1016/S0169-5347(98)01583-3

Borjas, G., Marten, G.G., Fernandez, E., Portillo, H., 1993. Juvenile turtles for mosquito control in water storage tanks. *Journal of Medical Entomology,* 30, 943–946. https://doi.org/10.1093/jmedent/30.5.943

Bouaziz, A., Amira, K., Djeghader, N., Aïssaoui, L., 2017. Impact of an insect growth regulator on the development and the reproduction potency of mosquitoes. *Journal of Entomology and Zoology Studies*, 5, 1662–1667.

Bourtzis, K., Lees, R.S., Hendrichs, J., Vreysen, M.J.B., 2016. More than one rabbit out of the hat: radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. *Acta Tropica*. 157, 115–130. https://doi.org/10.1016/J.Actatropica.2016.01.009

Bruce-Chwatt, L.J. and De Zulueta, J., 1980. *The rise and fall of malaria in Europe: a historico-epidemiological study*. Published for the Regional Office for Europe of the World Health Organization by Oxford University Press, Walton Street, Oxford OX2 6DP.

Burman, M., Abrahamsson, K., Ascard, J., Sjoberg, A., Erikson, B., 1986. Distribution of insect parasitic nematodes in Sweden, in: *Proceedings, 4th International Colloquium of Invertebrate Pathology,* Veldhoven, The Netherlands (Vol 312).

Burnell, A.M., Stock, S.P., 2000. *Heterorhabditis, Steinernema* and their bacterial symbionts - lethal pathogens of insects. *Nematology*, 2, 31–42. https://doi.org/10.1163/156854100508872

Cagnolo, S.R., and Almirón, W.R., 2010. Capacity of the terrestrial entomopathogenic nematode of *Steinernema rarum* (Rhabditida: Steinernematidae) to parasite *Culex apicinus larvae* (Diptera: Culicidae). *Journal of the Entomological Society of Argentina* , 69, 1-2.

Caminade, C., Kovats, S., Rocklov, J., Tompkins, A.M., Morse, A.P., Colón-González, F.J., Stenlund, H., Martens, P., Lloyd, S.J., 2014. Impact of climate change on global malaria distribution. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 3286–91. https://doi.org/10.1073/pnas.1302089111

Caminade, C., Medlock, J.M., Ducheyne, E., McIntyre, K.M., Leach, S., Baylis, M., Morse, A.P., 2012. Suitability of European climate for the Asian tiger mosquito *Aedes albopictus*: recent trends and future scenarios. *Journal of the Royal Society Interface*, 9, 2708–2717. https://doi.org/10.1098/rsif.2012.0138

Campbell, J.F., Gaugler, R.R., 1997. Inter-specific variation in entomopathogenic nematode foraging strategy: dichotomy or variation along a continuum? *Fundamentals of Applied Nematology*, 20, 393–398.

Campbell, J.F., Lewis, E., Yoder, F., Gaugler, R., 1995. Entomopathogenic nematode (Heterorhabditidae and Steinernematidae) seasonal population dynamics and impact on insect populations in turfgrass. *Biological Control*, 5, 598–606. https://doi.org/10.1006/bcon.1995.1071

Campbell, J.F., Lewis, E.E., Stock, S.P., Nadler, S., Kaya, H.K., 2003. Evolution of host search strategies in entomopathogenic nematodes. *Journal of Nematology*, 35, 145–145. https://doi.org/10.1139/z93-101

Cardoso, D. de O., Gomes, V.M., Dolinski, C., Souza, R.M., Idalino, W.S.S., 2016. Efficacy of *Heterorhabditis indica* LPP35 against *Aedes aegypti* larvae in human-generated containers and bromeliads. *Nematoda*, 3, e0172015.

Cardosoa, D. de O., Gomes, V.M., Dolinskia, C., 2016. Efficacy of *Heterorhabditis indica* LPP35 against *Aedes aegypti* larvae in human-generated containers and bromeliads. *Nematoda*, 3, e0172015. https://doi.org/10.4322/nematoda.01715

Carpenter, S., Groschup, M.H., Garros, C., Felippe-Bauer, M.L., Purse, B. V., 2013. *Culicoides* biting midges, arboviruses and public health in Europe. *Antiviral Research*, 100, 102–113. https://doi.org/10.1016/J.Antiviral.2013.07.020

Carvalho, B.M., Rangel, E.F., Vale, M.M., 2017. Evaluation of the impacts of climate change on disease vectors through ecological niche modelling. *Bulletin of Entomological Research*. 107, 419–430. https://doi.org/10.1017/S0007485316001097

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A., Alphey, L., Malavasi, A., Capurro, M.L., 2015. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. *PLoS Neglected Tropical Diseases*. 9, e0003864. https://doi.org/10.1371/journal.pntd.0003864

Center for Disease Control, 2016. Zika, Mosquitoes, and Standing Water, Blogs | CDC [WWW Document]. CDC. URL https://blogs.cdc.gov/publichealthmatters/2016/03/zikaandwater/ (accessed 6.17.18).

Cetin, H., Yanikoglu, A., Cilek, J.E., 2005. Evaluation of the naturally-derived insecticide spinosad against *Culex pipiens* L. (Diptera: Culicidae) larvae in septic tank water in Antalya, Turkey. *Journal of Vector Ecology*, 30, 151–4.

Chandler, D., Hay, D., Reid, a. P., 1997. Sampling and occurrence of entomopathogenic fungi and nematodes in U.K. soils. *Applied Soil Ecology* 5, 133–141. https://doi.org/10.1016/S0929-1393(96)00144-8

Christophides, G.K., Meister, S., Xu, J., 2002. Immunity-related genes and gene families in *Anopheles gambiae*. *Science*, 298, 159–165. https://doi.org/10.1126/science.1077136

Ciche, T.A., Ensign, J.C., 2003. For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? *Applied and Environmental Microbiology* 69, 1890–1897. https://doi.org/10.1128/AEM.69.4.1890-1897.2003

Claeys, C., Mieulet, E., 2013. The spread of Asian tiger mosquitoes and related health risks along the French Riviera: an analysis of reactions and concerns amongst the local population. *International Review of Social Research* 3, 151–173. https://doi.org/10.1515/irsr-2013-0015

Clark, T.M., 2004. pH tolerances and regulatory abilities of freshwater and euryhaline Aedine mosquito larvae. *Journal of Experimental Biology*207, 2297–2304. https://doi.org/10.1242/jeb.01021

Clarkson, M., Setzkorn, C., 2011. The domestic mosquitoes of the Neston area of Cheshire, UK. *European Mosquito Bulletin* 29, 122–128.

Cooke, F.J., Sabin, C.A., Zuckerman, J.N., 2006. Impact of the insect biting nuisance on a British youth expedition to Alaska. *Journal of Travel Medicine* 9, 76–81. https://doi.org/10.2310/7060.2002.2196

Corbel, V., N'Guessan, R., 2013. Distribution, mechanisms, impact and management of insecticide resistance in malaria vectors: a pragmatic review. *Anopheles mosquitoes - New insights into Malaria vectors*.InTech

Cranfield Soil and Agrifood Institute, n.d. Soilscapes soil types viewer - National Soil Resources Institute. Cranfield University [WWW Document]. URL http://www.landis.org.uk/soilscapes/index.cfm# (accessed 11.7.17).

Creighton, C.S., Fassuliotis, G., 1982. Mass rearing a mermithid nematode, *Filipjevimermis leipsandra* (Mermithida: Mermithidae) on the banded cucumber beetle (Coleoptera: Chrysomelidae). *Journal of Economic Entomology* 75, 701–703.

Dadd, R.H., 1971. Size limitations on the infectibility of mosquito larvae by nematodes during filter-feeding. *Journal of Invertebrate Pathology*
18, 246–251. https://doi.org/10.1016/0022-2011(71)90152-2

Dahlsten, D.L., Rowney, D.L., Copper, W.A., Tassan, R.L., Chaney, W.E., Robb, K.L., Tjosvold, S., Bianchi, M., Lane, P., 1998. Parasitoid wasp controls blue gum psyllid. *California Agriculture*, 52, 31–34. https://doi.org/10.3733/ca.v052n01p31

Danis, M., Mouchet, M., Giacomini, T., Guillet, P., Legros, F., Belkaid, M., 1996. Indigenous, introduced and airport malaria in Europe. *Médecine et Maladies Infectieuses* 26, 393–396.

Day, J.F., 2016. Mosquito oviposition behavior and vector control. *Insects*. https://doi.org/10.3390/insects7040065

De Oliveira Cardoso, D., Gomes, V.M., Dolinski, C., Souza, R.M., 2015. Potential of entomopathogenic nematodes as biocontrol agents of immature stages of *Aedes aegypti*. *Nematoda,* 2. https://doi.org/10.4322/nematoda.09015

Devine, G.J., Furlong, M.J., 2007. Insecticide use: contexts and ecological consequences. *Agriculture and Human Values* https://doi.org/10.1007/s10460-007-9067-z

ECDC, 2018a. Malaria: Annual epidemiological report for 2015. Stockholm

ECDC, 2018b. Annual epidemiological report for 2015 - West Nile fever, surveillance Report. Stockholm.

ECDC, 2018c. Annual epidemiological report for 2015 - Dengue fever. Stockholm

ECDC, 2018d. Annual epidemiological report for 2015 - Chikungunya fever. Stockholm.

ECDC, n.d. Key messages about tick-borne encephalitis and tick-borne diseases [WWW Document]. URL https://ecdc.europa.eu/en/tick-borne-encephalitis/facts/key-messages (accessed 4.14.18).

Ehlers, R.-U., Hokkanen, H.M.T., 1996. Insect Biocontrol with non-endemic entomopathogenic nematodes (Steinernema and Heterorhabditis spp.): conclusions and recommendations of a combined OECD and COST workshop on scientific and regulatory policy issues. *Biocontrol Science and Technology* 6, 295–302. https://doi.org/10.1080/09583159631280

Ehlers, R.-U., Johnigk, S.-A., 1999. *Endotokia matricida* in hermaphrodites of *Heterorhabditis* spp. and the effect of the food supply. *Nematology*, 1, 717–726. https://doi.org/10.1163/156854199508748

Ehlers, R.U., 2001. Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology and Biotechnology,* 56, 623-633 https://doi.org/10.1007/s002530100711

Einsiedel, E.F., 2005. Public perceptions of transgenic animals. *Revue Scientifique et Technique*, 24, 149–157.

Elbers, A.R.W., Koenraadt, C.J.M., Meiswinkel, R., 2015. Mosquitoes and *Culicoides* biting midges: vector range and the influence of climate change. *Revue Scientifique et Technique* l'OIE 34, 123–137. https://doi.org/10.20506/rst.34.1.2349

Emelianoff, V., Chapuis, E., Le Brun, N., Chiral, M., Moulia, C., Ferdy, J.B., 2008. A

survival-reproduction trade-off in entomopathogenic nematodes mediated by their bacterial symbionts. *Evolution*, 62, 932–942. https://doi.org/10.1111/j.1558-5646.2008.00319.x

Enyati, A., Hemingway, J., 2009. Malaria management: past, present and future. *Annual Review of Entomology*, 55, 569-591.

Epstein, P.R., 2001. Climate change and emerging infectious diseases. *Microbes and Infections* https://doi.org/10.1016/S1286-4579(01)01429-0

Eron, L.J., Lipsky, B.A., Low, D.E., Nathwani, D., Tice, A.D., Volturo, G.A., 2003. Managing skin and soft tissue infections: expert panel recommendations on key decision points.*Journal of Antimicrobial Chemotherapy*, 52, 711–2.

Fan, X. & Hominick, W.M., 1991. Efficiency of the Galleria (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. *Revue Nématol*, 14, 381-387.

Flores, H. A., & O'Neill, S. L. (2018). Controlling vector-borne diseases by releasing modified mosquitoes. *Nature Reviews Microbiology*, 16, 508–518. doi:10.1038/s41579-018-0025-0

Foley, D.H., Rueda, L.M., Wilkerson, R.C., 2007. Insight into global mosquito biogeography from country species records. *Journal of Medical Entomology*. 44, 554–567. https://doi.org/10.1093/jmedent/44.4.554

Forst, S., Dowds, B., Boemare, N., Stackebrandt, E., 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. *Annual Review of Microbiology*, 51, 47–72. https://doi.org/10.1146/annurev.micro.51.1.47

Fuller, D.O., Parenti, M.S., Hassan, A.N., Beier, J.C., 2012. Linking land cover and species distribution models to project potential ranges of malaria vectors: an example using *Anopheles arabiensis* in Sudan and Upper Egypt. *Malaria Journal*, 11. https://doi.org/10.1186/1475-2875-11-264

Gascon, J., Bern, C., Pinazo, M.J., 2010. Chagas disease in Spain, the United States and other non-endemic countries. *Acta Tropica*. 115, 22–27. https://doi.org/10.1016/j.actatropica.2009.07.019

Gates, B., 2014. The deadliest animal in the world [WWW Document]. Gatesnotes. URL https://www.gatesnotes.com/Health/Most-Lethal-Animal-Mosquito-Week (accessed 1.21.18).

Gaugler, R., 2002. Entomopathogenic nematology. CABI, New York.

Gaugler, R., Campbell, J.F., 1993. Nictation behaviour and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). *Behaviour* 126, 155–169. https://doi.org/10.1163/156853993X00092

Gaugler, R., Kaya, H.K., 1990. Entomopathogenic nematodes in biological control. CRC

Press.

Georgis, R., Hague, N.G.M., 1981. A neoaplectanid nematode in the larch sawfly *Cephalcia lariciphila* (Hymenoptera: Pamphiliidae). *Annals of Applied Biology*. 99, 171–177. https://doi.org/10.1111/j.1744-7348.1981.tb05144.x

Georgis, R., Koppenhöfer, A.M., Lacey, L.A.A., Bélair, G., Duncan, L.W.W., Grewal, P.S.S., Samish, M., Tan, L., Torr, P., van Tol, R.W., 2006. Successes and failures in the use of parasitic nematodes for pest control. *Biological Control* 38, 103–123. https://doi.org/10.1016/j.biocontrol.2005.11.005

Gillespie and, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. *Annual Review of Entomology* 42, 611–643. https://doi.org/10.1146/annurev.ento.42.1.611

Glazer, I., 1996. Survival Mechanisms of Entomopathogenic Nematodes. *Biocontrol Science and Technology* 6, 373–378. https://doi.org/10.1080/09583159631343

Glazer, I., 1997. Effects of infected insects on secondary invasion of steinernematid entomopathogenic nematodes. *Parasitology*, 114, 597-604.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*. 29, 644–652. https://doi.org/10.1038/nbt.1883

Gratz, N.G., 2004. Critical review of the vector status of *Aedes albopictus*. *Medical and Veterinary Entomology*. https://doi.org/10.1111/j.0269-283X.2004.00513.x

Grewal, P.S., Lewis, E.E., Gaugler, R., Campbell, J.F., 1994. Host finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes. *Parasitology* 108, 207. https://doi.org/10.1017/S003118200006830X

Grewal, P.S., Selvan, S., Gaugler, R., 1994. Thermal adaptation of entomopathogenic nematodes: Niche breadth for infection, establishment, and reproduction. *Journal of Thermal Biology*. 19, 245–253. https://doi.org/10.1016/0306-4565(94)90047-7

Griffin, C.T., 2012. Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: traits contributing to nematode fitness and biocontrol efficacy. *Journal of Nematology*. 44, 177–84.

Griffin, C.T., Finnegan, M.M., Downes, M.J., 1994. Environmental tolerances and the dispersal of {IHeterorhabditis}: survival and infectivity of European {IHeterorhabditis} following prolonged immersion in seawater. *Fundamental and Applied Nematology*. 17, 415–421.

Griffin, C.T., Moore, J.F., Downes, M.J., 1991. Occurrence of insect-parasitic nematodes (Steinernematidae, Heterorhabditidae) in the Republic of Ireland. *Nematologica* 37, 92–100. https://doi.org/10.1163/187529291X00097

Guillet, P., Germain, M.C., Giacomini, T., Chandre, F., Akogbeto, M., Faye, O., Kone, A., Manga, L., Mouchet, J., 1998. Origin and prevention of airport malaria in France. *Tropical Medicine and International Health*. 3, 700–705. https://doi.org/10.1046/j.1365-3156.1998.00296.x

Gumus, A., Karagoz, M., Shapiro-Ilan, D., Hazir, S., 2015. A novel approach to biocontrol: Release of live insect hosts pre-infected with entomopathogenic nematodes. *Journal of Invertebrate Pathology*130, 56–60. https://doi.org/10.1016/j.jip.2015.07.002

Guy, A.G., Wright, D.J., Gaffney, M. and Griffin, C.T., 2009. Conditioning of *Steinernema kraussei* and *S. carpocapsae* through storage: enhancing their performance over wide temperature ranges. *IOBC/wprs Bulletin*, 45, 349-352.

Gwynn, R.L., Richardson, P.N., 1996. Incidence of entomopathogenic nematodes in soil samples collected from Scotland, England and Wales. *Fundamental and Applied Nematology*. 19, 427–431.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., De Kroon, H., 2017. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One* 12. https://doi.org/10.1371/journal.pone.0185809

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., Collado, A., Stevenson, J., Scaife, S., Dafa'Alla, T., Fu, G., Phillips, C., Miles, A., Raduan, N., Kelly, N., Beech, C., Donnelly, C.A., Petrie, W.D., Alphey, L., 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology*. https://doi.org/10.1038/nbt.2350

Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., Samuel, M.D., 2002. Climate warming and disease risks for terrestrial and marine biota. *Science* 296, 2158–2162. https://doi.org/10.1126/science.1063699

Hawley, W.A., Reiter, P., Copeland, R.S., Pumpuni, C.B., Craig, G.B., 1987. Aedes albopictus in North America: probable introduction in used tires from northern Asia. *Science*. 236, 1114–1116. https://doi.org/10.1126/science.3576225

Health Organization, W., 2017. World Malaria Report 2017. Geneva.

Hemingway, J., Ranson, H., 2000. Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology* 45, 371–391. https://doi.org/10.1146/annurev.ento.45.1.371

Hoffmann, A.A., Montgomery, B.L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P.H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y.S., Dong, Y., Cook, H., Axford, J., Callahan, A.G., Kenny, N., Omodei, C., McGraw, E.A., Ryan, P.A., Ritchie, S.A., Turelli, M., O'Neill, S.L., 2011. Successful establishment of *Wolbachia* in Aedes populations to suppress dengue transmission. *Nature* 476, 454–457. https://doi.org/10.1038/nature10356

Hofhuis, A., Reimerink, J., Reusken, C., Scholte, E.-J., Boer, A. de, Takken, W., Koopmans, M., 2009. The hidden passenger of lucky bamboo: do imported *Aedes albopictus* mosquitoes cause Dengue virus transmission in the Netherlands? *Vector-Borne Zoonotic Diseases*. 9, 217–220. https://doi.org/10.1089/vbz.2008.0071

Hominick, W.M., Briscoe, B.R., 1990. Survey of 15 sites over 28 months for entomopathogenic nematodes (Rhabditida: Steinernematidae). *Parasitology* 100, 289. https://doi.org/10.1017/S0031182000061291

Hominick, W.M., Reid, A.P., Briscoe, B.R., 1995. Prevalence and habitat specificity of steinernematid and heterorhabditid nematodes isolated during soil surveys of the U.K. and the Netherlands. *Journal of Helminthology*. 69, 27–32. https://doi.org/10.1017/S0022149X00013791

Hominick, W.M., Reid, A.P., Bohan, D.A., Briscoe, B.R., 1996. Entomopathogenic nematodes: biodiversity, geographical distribution and the convention on biological diversity. *Biocontrol Science and Technology* 6, 317–332. https://doi.org/10.1080/09583159631307

Homininck, W.M., Briscoe, B.R., 1990. Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in British soils. *Parasitology* 100, 295. https://doi.org/10.1017/S0031182000061308

Hominick, W. (2002). Biogeography. In: R. Gaugler, ed., *Entomopathogenic Nematology*. Oxon: CABI, pp.115-142.

Hopper, K.R., Roush, R.T., Powell, W., 1993. Management of genetics of biological control introductions. *Annual Review of Entomology* 38, 317–332.

Hotez, P.J., 2016. Southern Europe's coming plagues: vector-borne neglected tropical diseases. *PLoS Neglected Tropical Diseases* 10, e0004243. https://doi.org/10.1371/journal.pntd.0004243

Hoy, C.W., Grewal, P.S., Lawrence, J.L., Jagdale, G., Acosta, N., 2008. Canonical correspondence analysis demonstrates unique soil conditions for entomopathogenic nematode species compared with other free-living nematode species. *Biological Control* 46, 371–379. https://doi.org/10.1016/j.biocontrol.2008.06.001

Huang, Y.-J., Higgs, S., Vanlandingham, D., 2017. Biological control strategies for mosquito vectors of arboviruses. *Insects* 8, 21. https://doi.org/10.3390/insects8010021

Hultmark, D., Steiner, H., Rasmuson, T., Boman, H.G., 1980. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophoracecropia*. *European Journal of Biochemistry* 106, 7–16.

Hultmark, D., Steiner, H., Rasmuson, T., Boman, H.G., 2005. Insect Immunity. Purification and Properties of Three Inducible Bactericidal Proteins from Hemolymph of Immunized Pupae of Hyalophora cecropia. *European Journal of Biochemistry* 106, 7–16. https://doi.org/10.1111/j.1432-1033.1980.tb05991.x

Hutchinson, R.A., 2004. *Mosquito borne diseases in England:: past, present and future risks, with special reference to malaria in the Kent Marshes* (Doctoral dissertation, Durham University).

Hutchinson, R.A., Bayoh, M.N., Lindsay, S.W., 2005. Risk of airport malaria in the UK. *European Mosquito Bulletin* 19, 12–13.

Isaäcson, M., 1989. Airport malaria: a review. *Bulletin of the World Health Organisation* 67, 737–43.

Iturbe-Ormaetxe, I., Walker, T., O' Neill, S.L., 2011. *Wolbachia* and the biological control of mosquito-borne disease. *European Molecular Biology Organisation Reports*. 12, 508–518. https://doi.org/10.1038/embor.2011.84

Jagodič, A., Ipavec, N., Trdan, S., Laznik, Ž., 2017. Attraction behaviors: are synthetic volatiles, typically emitted by insect-damaged *Brassica nigra* roots, navigation signals for entomopathogenic nematodes (Steinernema and Heterorhabditis)? *BioControl* 62, 515–524. https://doi.org/10.1007/s10526-017-9796-x

James-Pirri, M.-J., Ginsberg, H.S., Erwin, R.M., Taylor, J., 2009. Effects of open marsh water management on numbers of larval salt marsh mosquitoes. *Journal of Medical Entomology*. 46, 1392–9. https://doi.org/doi: 10.1603/033.046.0620

Jetten, T.H., Takken, W., 1994. Anophelism without malaria in Europe: a review of the ecology and distribution of the genus *Anopheles* in Europe. Wageningen Agricultural University Papers, Wageningen, Netherlands.

JNCC, 2009. U.K. Wildlife and Countryside Act 1981. *Environmental Conservation*. 9, 42. https://doi.org/10.1017/S0376892900019482

Kampen, H., Medlock, J.M., Vaux, A.G.C., Koenraadt, C.J.M., van Vliet, A.J.H., Bartumeus, F., Oltra, A., Sousa, C.A., Chouin, S., Werner, D., 2015. Approaches to passive mosquito surveillance in the EU. *Parasites and Vectors* 8, 9. https://doi.org/10.1186/s13071-014-0604-5

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. and Morishima, K., 2016. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic acids research* 45, 353-361.

Kaplanoglu, E., 2016. Multi-gene resistance to neonicotinoids in the Colorado potato beetle, *Leptinotarsa decemlineata* (Masters dissertation, Western University).

Kaya, H.K., Gaugler, R., 1993. Entomopathogenic nematodes. *Annual Review of Entomology* 38, 181–206. https://doi.org/10.1146/annurev.en.38.010193.001145

Kellert, S.R., 1993. Values and perceptions of invertebrates. *Society for Conservation Biology* 7, 845–855.

Kligler, I.J., Mer, G., 1937. Studies on the effect of various factors on the infection rate of

Anopheles elutus with different species of *Plasmodium Annals of Tropical Medicine and Parasitology* 31, 71–83. https://doi.org/10.1080/00034983.1937.11684967

Knudsen, A.B., 1995. Global distribution and continuing spread of *Aedes albopictus*. *Parassitologia* 37, 91-97

Koppenhöfer, A.M., Kaya, H.K., 1995. Density-dependent effects on *Steinernema glaseri* (nematoda: steinernematidae) within an insect host. *Journal of Parasitology* 81, 797–799. https://doi.org/10.2307/3283982

Koppenhöfer, A.M., Kaya, H.K., Shanmugam, S., Wood, G.L., 1995. Interspecific competition between steinernematide nemtodes within an insect host. *Journal of Invertebrate Pathology* 154–168.

Kruitbos, L.M., Heritage, S., Hapca, S., Wilson, M.J., 2010. The influence of habitat quality on the foraging strategies of the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis megidis. Parasitology*. 137, 303-309 https://doi.org/10.1017/S0031182009991326

Kung, S.P., Gaugler, R., Kaya, H.K., 1990. Influence of soil pH and oxygen on persistence of *Steinernema* spp. *Journal of Nematology*. 22, 440–5.

Lacey, L.A., Georgis, R., 2012a. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. *Journal of Nematology*. 44, 218–25.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. *Journal of Invertebrate Pathology*132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009

Lambrechts, L., Scott, T.W., Gubler, D.J., 2010. Consequences of the expanding global distribution of *Aedes albopictus* for Dengue virus transmission. *PLoS Neglected Tropical Diseases* 4, e646 https://doi.org/10.1371/journal.pntd.0000646

Lau, K.W., Chen, C.D., Lee, H.L., Norma-rashid, Y., Sofian-azirun, M., 2015. Evaluation of insect growth regulators against field-collected *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) from Malaysia. *Journal of Medical Entomology*. 52, 199–206. https://doi.org/10.1093/jme/tju019

Laumond, C., Mauléon, H., Kermarrec, A., 1979. Données nouvelles sur le spectre d'hôtes et le parasitisme du nématode entomophage *Neoaplectana carpocapsae*. *Entomophaga* 24, 13–27. https://doi.org/10.1007/BF02377505

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K.M., Wertheim, H.F.L., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D., Cars, O., 2013. Antibiotic resistance—the need for global solutions. *The Lancet Infectious Diseases* 13, 1057–1098. https://doi.org/10.1016/S1473-3099(13)70318-9

Lazzaro, B.P., Schneider, D.S., 2014. The genetics of immunity. *Genetics* 197, 467–70. https://doi.org/10.1534/genetics.114.165449

Leather, S.R., 2018. "Ecological Armageddon" - more evidence for the drastic decline in insect numbers. *Annals of Applied Biology*. 172, 1–3. https://doi.org/10.1111/aab.12410

Lees, R.S., Gilles, J.R., Hendrichs, J., Vreysen, M.J., Bourtzis, K., 2015. Back to the future: the sterile insect technique against mosquito disease vectors. *Current Opinion in Insect Science*. 10, 156–162. https://doi.org/10.1016/j.cois.2015.05.011

Lester, P.J., Pike, A.J., 2003. Container surface area and water depth influence the population dynamics of the mosquito *Culex pervigilans* (Diptera: Culicidae) and its associated predators in New Zealand. *Journal of Vector Ecology* 28, 267–74.

Lewis, E.E., Campbell, J., Griffin, C., Kaya, H., Peters, A., 2006. Behavioral ecology of entomopathogenic nematodes. *Biological Control* 38, 66–79. https://doi.org/10.1016/j.biocontrol.2005.11.007

Lewis, E.E., Ricci, M., Gaugler, R., 1996. Host recognition behaviour predicts host suitability in the entomopathogenic nematode *Steinernema carpocapsae* (Rhabditida:Steinernematidae). *Parasitology* 113 (Pt 6, 573–579. https://doi.org/10.1017/S0031182000067627

Li, X.Y., Cowles, R.S., Cowles, E.A., Gaugler, R., Cox-Foster, D.L., 2007. Relationship between the successful infection by entomopathogenic nematodes and the host immune response. *International Journal of Parasitology* 37, 365-374 https://doi.org/10.1016/j.ijpara.2006.08.009

Lillie, T.H., Schreck, C.E., Rahe, A.J., 1988. Effectiveness of personal protection against mosquitoes in Alaska. *Journal of Medical Entomology*. 25, 475–478. https://doi.org/10.1093/jmedent/25.6.475

Lindsay, S.W., Birley, M.H., 1996. Climate change and malaria transmission. *Annals of Tropical Medicine and Parasitology*. 90, 573–588. https://doi.org/10.1080/00034983.1996.11813087

Lindsay, S.W., Emerson, P.M., Charlwood, J.D., 2002. Reducing malaria by mosquito-proofing houses. *Trends in Parasitology*. 18, 510–514. https://doi.org/10.1016/S1471-4922(02)02382-6

Lindsay, S.W., Thomas, C.J., 2001. Global warming and risk of *vivax* malaria in Great Britain. *Global Change and Human Health* 2, 80–84. https://doi.org/10.1023/A:1011995115713

LITE Liverpool Insect Testing Establishment, 2014. Mosquito Colonies [WWW Document]. URL http://www.lite-testing-facility.com/our-services/mosquito-colonies/ (accessed 6.3.18).

Lobel, H.O., Phillips-Howard, P., Brandling-Bennett, D., Steffen, R., Campbell, C.C., Huong, Y., Were, J.B., Moser, R., 1990. Malaria incidence and prevention among European and

North American travellers to Kenya. *Bulletin of the World Health Organisation* 68, 209–215.

Lowenberger, C. (2001) Innate immune response of Aedes aegypti. *Insect biochemistry and molecular biology*, 31, 219-229

Luc, M., Taylor, D.P., Netscher, C., 1979. On *endotokia matricida* and inter-uterine development and hatching in nematodes. *Nemaologica* 25, 268–274. https://doi.org/10.1163/187529279X00299

Mackenzie-Impoinvil, L., Impoinvil, D.E., Galbraith, S.E., Dillon, R.J., Ranson, H., Johnson, N., Fooks, A.R., Solomon, T., Baylis, M., 2015. Evaluation of a temperate climate mosquito, *Ochlerotatus detritus* (*Aedes detritus*), as a potential vector of Japanese encephalitis virus. *Medical and Veterinary Entomology*. 29, 1–9. https://doi.org/10.1111/mve.12083

Malcolm, C.A., 2009. Public health issues posed by mosquitoes An independent report. *Chartered Institute for Environmental Health.* London.

McGraw, B.A., Koppenhöfer, A.M., 2008. Evaluation of two endemic and five commercial entomopathogenic nematode species (Rhabditida: Heterorhabditidae and Steinernematidae) against annual bluegrass weevil (Coleoptera: Curculionidae) larvae and adults. *Biological Control* 46, 467–475. https://doi.org/10.1016/J.BIOCONTROL.2008.03.012

Medlock, J., Hansford, K., Anderson, M., Mayho, R., Snow, K.R., 2012. Mosquito nuisance and control in the U.K. – A questionnaire-based survey of local authorities. *European Mosquito Bulletin* 30, 15–29. https://doi.org/10.1017/CBO9781107415324.004

Medlock, J.M., Hansford, K.M., Schaffner, F., Versteirt, V., Hendrickx, G., Zeller, H., Bortel, W. Van, 2012. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. *Vector-Borne Zoonotic Diseases*. 12, 435–447. https://doi.org/10.1089/vbz.2011.0814

Medlock, J.M., Hansford, K.M., Versteirt, V., Cull, B., Kampen, H., Fontenille, D., Hendrickx, G., Zeller, H., Van Bortel, W., Schaffner, F., 2015. An entomological review of invasive mosquitoes in Europe. *Bulletin of Entomological Research* 105, 637–663. https://doi.org/10.1017/S0007485315000103

Medlock, J.M., Leach, S.A., 2015. Effect of climate change on vector-borne disease risk in the U.K. *The Lancet Infectious Diseases* https://doi.org/10.1016/S1473-3099(15)70091-5

Medlock, J.M., Snow, K.R., 2008. British mosquitoes. *British Wildlife* 19, 338 https://doi.org/10.1016/S0140-6736(00)71108-1

Medlock, J.M., Snow, K.R., Leach, S., 2005. Potential transmission of West Nile virus in the British Isles: An ecological review of candidate mosquito bridge vectors. *Medical and Veterinary Entomology*. 19, 2-21. https://doi.org/10.1111/j.0269-283X.2005.00547.x

Medlock, J.M., Vaux, A.G.C., 2014. Colonization of a newly constructed urban wetland by mosquitoes in England: implications for nuisance and vector species. *Journal of Vector Ecology* 39, 249–60. https://doi.org/10.1111/jvec.12099

Medlock, J.M., Vaux, A.G.C., 2011. Assessing the possible implications of wetland expansion and management on mosquitoes in Britain. *European Mosquito Bulletin* 29, 38–65.

Mellor, P.S., 1990. The replication of bluetongue virus in *Culicoides* vectors. *Current Topics in Microbiology and Immunology*. Springer, Berlin. 143–161. https://doi.org/10.1007/978-3-642-75247-6_6

Menti, H., Patel, M.N., Wright, D.J., Perry, R.N., 2003. Lipid utilisation during storage of the entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis megidis* from Greece and the U.K. *Nematology* 5, 31–37.https://doi.org/10.1163/156854102765216669

Merritt, R.W., Dadd, R.H., Walker, E.D., 1992. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annual Review of Entomology* 37, 349–374. https://doi.org/10.1146/annurev.en.37.010192.002025

Meterological Office, 2017. U.K climate information [WWW Document]. URL https://www.metoffice.gov.uk/public/weather/climate/ (accessed 1.28.18).

Mier-y-Teran-Romero, L., Tatem, A.J., Johansson, M.A., 2017. Mosquitoes on a plane: disinsection will not stop the spread of vector-borne pathogens, a simulation study. *PLoS Neglected Tropical Diseases* 11, e0005683. https://doi.org/10.1371/journal.pntd.0005683

Mitani, D.K., Kaya, H.K., Goodrich-Blair, H., 2004. Comparative study of the entomopathogenic nematode, *Steinernema carpocapsae*, reared on mutant and wild-type *Xenorhabdus nematophila*. *Biological Control* 29, 382–391.
https://doi.org/10.1016/j.biocontrol.2003.07.005

Mokany, A., Shine, R., 2003. Oviposition site selection by mosquitoes is affected by cues from conspecific larvae and anuran tadpoles. *Austral Ecology*28, 33–37. https://doi.org/10.1046/j.1442-9993.2003.01239.x

Molta, N.B. and Hominick, W.M., 1989. Dose-and time-response assessments of *Heterorhabditis heliothidis* and *Steinernema feltiae* [Nem.: Rhabitida] against *Aedes aegypti* larvae. *Entomophaga*, 34, 485-493.

Morales-Pérez, A., Nava-Aguilera, E., Balanzar-Martínez, A., Cortés-Guzmán, A.J., Gasga-Salinas, D., Rodríguez-Ramos, I.E., Meneses-Rentería, A., Paredes-Solís, S., Legorreta-Soberanis, J., Armendariz-Valle, F.G., Ledogar, R.J., Cockcroft, A., Andersson, N., 2017. *Aedes aegypti* breeding ecology in Guerrero: cross-sectional study of mosquito breeding sites from the baseline for the Camino Verde trial in Mexico. *BMC Public Health* 17, 450. https://doi.org/10.1186/s12889-017-4293-9

Morris, R., Wilson, L., Sturrock, M., Warnock, N.D., Carrizo, D., Cox, D., Maule, A.G. and Dalzell, J.J., 2017. A neuropeptide modulates sensory perception in the entomopathogenic nematode *Steinernema carpocapsae*. *PLoS pathogens*, 13, e1006185.

Moyes, C.L., Vontas, J., Martins, A.J., Ng, L.C., Koou, S.Y., Dusfour, I., Raghavendra, K., Pinto, J., Corbel, V., David, J.P., Weetman, D., 2017. Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. *PLoS Neglected Tropical Diseases* https://doi.org/10.1371/journal.pntd.0005625

Mracek, Z., 1980. The use of "Galleria trap" for obtaining nematode parasites of insects in Czechoslovakia (Nematoda, Steinernemadidae). *Acta entomologica bohemoslovaca*, 77, 378-382.

National Institute for Health and Care Excellence, 2015. Cellulitis - Acute [WWW Document]. Clin. Knowl. Summ. URL https://cks.nice.org.uk/cellulitis-acute#!scenariorecommendation:1 (accessed 4.14.18).

National Soil Resources Institute, 2002. Map of soil pH in the UK [WWW Document]. URL http://www.before-n-after.co.uk/soils/ph.html (accessed 11.7.17).

Nauen, R., 2007. Insecticide resistance in disease vectors of public health importance. *Pest Management Science* 63, 628–633. https://doi.org/10.1002/ps.1406

NHS Digital, 2017. Annual A&E statistics published [WWW Document]. URL http://content.digital.nhs.uk/article/7576/Annual-AE-statistics-published (accessed 1.25.18).

Ogoma, S.B., Kannady, K., Sikulu, M., Chaki, P.P., Govella, N.J., Mukabana, W.R., Killeen, G.F., 2009. Window screening, ceilings and closed eaves as sustainable ways to control malaria in Dar es Salaam, Tanzania. *Malaria Journal*. 8, 221. https://doi.org/10.1186/1475-2875-8-221

Oliva, C.F., Jacquet, M., Gilles, J., Lemperiere, G., Maquart, P.-O., Quilici, S., Schooneman, F., Vreysen, M.J.B., Boyer, S., 2012. The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on Reunion Island: mating vigour of sterilized males. *PLoS One* 7, e49414. https://doi.org/10.1371/journal.pone.0049414

Oreskes, N., 2004. The scientific consensus on climate change. *Science*. 306, 1686. https://doi.org/10.1126/science.1103618

Otranto, D., Capelli, G., Genchi, C., 2009. Changing distribution patterns of canine vector borne diseases in Italy: leishmaniosis vs. dirofilariosis. *Parasites and Vectors* 2, S2. https://doi.org/10.1186/1756-3305-2-S1-S2

Pandii, W., Maharmart, S., Boonchuen, S., Silapanuntakul, S., Samsook, V., 2008. Efficacy of entomopathogenic nematodes (Nematoda: Rhabditida) against *Culex gelidus* (Diptera: Culicidae) larvae. *Journal of Vector Borne Diseases*. 5, 24–35.

Paris, M., Tetreau, G., Laurent, F., Lelu, M., Despres, L., David, J.-P.P., 2011. Persistence of *Bacillus thuringiensis israelensis* (Bti) in the environment induces resistance to multiple Bti toxins in mosquitoes. *Pest Management Science* 67, 122–128. https://doi.org/10.1002/ps.2046

Patz, J.A., Graczyk, T.K., Geller, N., Vittor, A.Y., 2000. Effects of environmental change on emerging parasitic diseases. *International Journal of Parasitology* https://doi.org/10.1016/S0020-7519(00)00141-7

Paul, A., Harrington, L.C., Zhang, L., Scott, J.G., 2005. Insecticide resistance in *Culex pipiens* from New York. *Journal of the American Control Association* 21, 305–309. https://doi.org/10.2987/8756-971x(2005)21[305:iricpf]2.0.co;2

Paupy, C., Delatte, H., Bagny, L., Corbel, V., Fontenille, D., 2009. *Aedes albopictus*, an arbovirus vector: from the darkness to the light. *Microbes and Infections* 11, 1177–1185. https://doi.org/10.1016/j.micinf.2009.05.005

Peschiutta, M.L., Cagnolo, S.R. and Almirón, W.R., 2014. Susceptibilidad de larvas de *Aedes aegypti* (Linnaeus)(Diptera: Culicidae) al nematodo entomopatógeno *Heterorhabditis bacteriophora* (Poinar)(Rhabditida: Heterorhabditidae). *Revista de la Sociedad Entomológica Argentina*, 73, 99-108.

Peters, A., 1996. The natural host range of *Steinernema* and *Heterorhabditis* spp. and their impact on insect populations. *Biocontrol Science and Technology* 6, 389–402. https://doi.org/10.1080/09583159631361

Petersen, J.J., 1985. Nematodes as biological control agents: Part I. Mermithidae. *Advances in Parasitology* 24, 307–344. https://doi.org/10.1016/S0065-308X(08)60565-5

Petersen, J.J., Willis, Q.R., 1972. Procedures for the mass rearing of a mermithid parasite of mosquitoes. *Mosquito News* 32, 226–230.

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A. and Coleman, P.G., White-Cooper, H., Alphey, L. 2007. Late-acting dominant lethal genetic systems and mosquito control. *BMC biology*, 5, 11.

Poinar, G.O.. J., 1979. Nematodes for biological control of insects. CRC Press. Boca Raton.

Poinar, G.O., Kaul, H.N., 1982. Parasitism of the mosquito *Culex pipiens* by the nematode *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* 39, 382–387. https://doi.org/10.1016/0022-2011(82)90063-5

Popiel, I. & Hominick, W.M., 1992. Nematodes as biological control agents: Part II. *Advances in parasitology* 31, 381-433. Academic Press.

Poulin, B., 2012. Indirect effects of bioinsecticides on the nontarget fauna: The Camargue experiment calls for future research. *Acta Oecologica* 44, 28–32. https://doi.org/10.1016/j.actao.2011.11.005

Powell, J.R., Tabachnick, W.J., 2013. History of domestication and spread of *Aedes aegypti*; a review. *Memórias do Instituto Oswaldo Cruz* 108, 11-17 https://doi.org/10.1590/0074-0276130395

Public Health England, 2017a. Mosquito: nationwide surveillance - GOV.UK [WWW Document]. URL https://www.gov.uk/government/publications/mosquito-surveillance/mosquito-nationwide-surveillance (accessed 1.25.18).

Public Health England, 2017b. Zika virus: epidemiology and cases diagnosed in the UK - GOV.UK [WWW Document]. Gov.UK. URL https://www.gov.uk/government/publications/zika-virus-epidemiology-and-cases-diagnosed-in-the-uk/zika-virus-epidemiology-and-cases-diagnosed-in-the-uk (accessed 1.30.18).

Purse, B. V., Falconer, D., Sullivan, M.J., Carpenter, S., Mellor, P.S., Piertney, S.B., Mordue Luntz, A.J., Albon, S., Gunn, G.J., Blackwell, A., 2012. Impacts of climate, host and landscape factors on *Culicoides* species in Scotland. *Medical and Veterinary Entomology*. 26, 168–177. https://doi.org/10.1111/j.1365-2915.2011.00991.x

Půža, V., Mráček, Z., 2005. Seasonal dynamics of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* as a response to abiotic factors and abundance of insect hosts. *Journal of Invertebrate Pathology* 89, 116–122. https://doi.org/10.1016/j.jip.2005.04.001

Ramsey, J.M., Peterson, A.T., Carmona-Castro, O., Moo-Llanes, D.A., Nakazawa, Y., Butrick, M., Tun-Ku, E., De La Cruz-Félix, K., Ibarra-Cerdeña, C.N., 2015. Atlas of Mexican Triatominae (Reduviidae: Hemiptera) and vector transmission of Chagas disease. *Memórias do Instituto Oswaldo Cruz* 110, 339–352. https://doi.org/10.1590/0074-02760140404

Reiter, P., Sprenger, D., 1987. The used tire trade: a mechanism for the worldwide dispersal of container breeding mosquitoes.*Journal of the American Mosquito Control Association*. 3, 494–501.

Rey, J.R., Walton, W.E., Wolfe, R.J., Roxanne, C., O'Connell, S.M., Berg, J., Sakolsky-Hoopes, G.E., Laderman, A.D., 2012. North American wetlands and mosquito control. *International Journal of Environmental Research and Public Health* 9, 4537–4605. https://doi.org/10.3390/ijerph9124537

Rio, R. V, Cameron, E.A., 2000. *Heterorhabditis bacteriophora*: seasonal dynamics and distribution in a stand of sugar maple, *Acer saccharum*. *Journal of Invertebrate Pathology* 75, 36–40. https://doi.org/10.1006/jipa.1999.4887

Roche, B., Léger, L., L'Ambert, G., Lacour, G., Foussadier, R., Besnard, G., Barré-Cardi, H., Simard, F., Fontenille, D., 2015. The spread of *Aedes albopictus* in metropolitan France: contribution of environmental drivers and human activities and predictions for a near future. *PLoS One* 10. p.e0125600 https://doi.org/10.1371/journal.pone.0125600

Rochlin, I., Ninivaggi, D. V., Hutchinson, M.L., Farajollahi, A., 2013. Climate change and range expansion of the Asian tiger mosquito (*Aedes albopictus*) in Northeastern U.S.A:

Implications for public health practitioners. PLoS One 8. e60874 https://doi.org/10.1371/journal.pone.0060874

Rodger, A.J., Cooke, G.S., Ord, R., Sutherland, C.J., Pasvol, G., 2008. Cluster of *falciparum* malaria cases in U.K airport. *Emerging Infectious Diseases*. 14, 1284–1286. https://doi.org/10.3201/eid1408.080031

Rogers, D.J., Randolph, S.E., 2000. The global spread of malaria in a future, warmer world. Science 289, 1763–1766. https://doi.org/10.1097/00006454-200101000-00029

Roiz, D., Neteler, M., Castellani, C., Arnoldi, D., Rizzoli, A., 2011. Climatic factors driving invasion of the tiger mosquito (Aedes albopictus) into new areas of Trentino, Northern Italy. PLoS One 6. https://doi.org/10.1371/journal.pone.0014800

Romero, H.B.M., O'Leary, B., Moise, I., Horan, W., 2017. Establishment of an integrated mosquito control program in Monte Verde, Honduras, in: Society of Vector Ecologists, International Congress. Palma de Mallorca. https://doi.org/10.3920/978-90-8686-863-6

Ross, R., 1927. The British Mosquito Control Institute, Hayling Island.*Science Progress in the Twentieth Century (1919-1933)*.121-12

Ross, R., 1911. *The prevention of malaria*. John Murray; London..

Ross, R., 1897. On some peculiar pigmented cells found in two mosquitos fed on malarial blood. *British medical journal*, *2*(1929).1786.

Roth, A., Mercier, A., Lepers, C., Hoy, D., Duituturaga, S., Benyon, E., Guillaumot, L., Souarès, Y., 2014. Concurrent outbreaks of dengue, chikungunya and Zika virus infections – An unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012–2014. *Eurosurveillance* 19. 20929 https://doi.org/10.2807/1560-7917.ES2014.19.41.20929

Royal Horticultural Society, 2018. Using grey water [WWW Document]. RHS website. URL https://www.rhs.org.uk/science/gardening-in-a-changing-world/water-use-in-gardens/using-grey-water (accessed 6.17.18).

Saward-Arav, D., Sadeh, A., Mangel, M., Templeton, A.R., Blaustein, L., 2016. Oviposition responses of two mosquito species to pool size and predator presence: varying trade-offs between desiccation and predation risks. Isr. J. Ecol. Evol. 62, 143–148. https://doi.org/10.1080/15659801.2015.1069113

Schaffner, F., 2003. Mosquitoes in used tyres in Europe: species list and larval key. *European Mosquito Bulletin* 16, 7–12.

Scholte, E.J., Dijkstra, E., Blok, H., De Vries, A., Takken, W., Hofhuis, A., Koopmans, M., De Boer, A., Reusken, C.B., 2008. Accidental importation of the mosquito *Aedes albopictus* into the Netherlands: A survey of mosquito distribution and the presence of dengue virus. *Medical and Veterinary Entomology*. 22, 352–358. https://doi.org/10.1111/j.1365-2915.2008.00763.x

Selvan, S., Campbell, J.F., Gaugler, R., 1993. Density-dependent effects on entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) within an insect host. *Journal of Invertebrate Pathology*62, 278–284. https://doi.org/10.1006/JIPA.1993.1113

Shapiro-Ilan, D.I., Gaugler, R., Tedders, W.L., Brown, I. and Lewis, E.E., 2002. Optimization of inoculation for in vivo production of entomopathogenic nematodes. *Journal of Nematology* 34, 343.

.

Shapiro-Ilan, D., Lewis, E., … Y.S.-J. of I., 2003, undefined, n.d. Superior efficacy observed in entomopathogenic nematodes applied in infected-host cadavers compared with application in aqueous suspension. Acad. Press.

Shapiro-Ilan, D.I., Gaugler, R., Shapiro, D.I., Gaugler, R., 2002. Production technology for entomopathogenic nematodes and their bacterial symbionts. J. Ind. Microbiol. Biotechnol. 28, 137–46. https://doi.org/10.1038/sj.jim.7000230

Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J., Fife, J.P., 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control* 38, 124–133. https://doi.org/10.1016/j.biocontrol.2005.09.005

Shapiro-Ilan, D.I., Lewis, E.E., Son, Y. and Tedders, W.L., 2003. Superior efficacy observed in entomopathogenic nematodes applied in infected-host cadavers compared with application in aqueous suspension. *Journal of Invertebrate Pathology* 83, 270-272.

Shapiro-Ilan, D.I., Han, R., Dolinksi, C., 2012. Entomopathogenic nematode production and application technology. *Journal of Nematology*. 44, 206–17.

Sharon, D., Tilgner, H., Grubert, F., Snyder, M., 2013. A single-molecule long-read survey of the human transcriptome. *Nature Biotechnology*. 31, 1009–1014. https://doi.org/10.1038/nbt.2705

Showler, A.T. and Potter, C.S., 1991. Synopsis of the 1986–1989 desert locust (Orthoptera: Acrididae) plague and the concept of strategic control. *American Entomologist*, 37, 106-110.

Shu, S., Chen, B., Zhou, M., Zhao, X., Xia, H., Wang, M., 2013. De Novo Sequencing and Transcriptome Analysis of Wolfiporia cocos to Reveal Genes Related to Biosynthesis of Triterpenoids. *PLoS One* 8, e71350. https://doi.org/10.1371/journal.pone.0071350

Simons, F.E., Peng, Z., 1999. Skeeter syndrome. *Journal of Allergy and Clinical Immunology* 104, 705–7. https://doi.org/10.1016/S0091-6749(99)70348-9

Skiff, J.J., Yee, D.A., 2014. Behavioral differences among four co-occurring species of container mosquito larvae: effects of depth and resource environments. *Journal of Medical Entomology*. 51, 375–81.

Snow, K., 1998. Distribution of *Anopheles* mosquitoes in the British Isles. *European Mosquito Bulletin* 1, 9–13.

Snow, K.R., Snow, S.E., 2004. John Frederick Marshall and the British mosquitoes *European Mosquito Bulletin* 17, 23–28.

Sorg, M., Schwan, H., Stenmans, W., Müller, A., 2013. Determination of the biomass of flight-active insects in the Orbroicher Bruch nature reserve with malaise traps in 1989 and 2013. *Communications from the Entomological Association Krefeld* 1,1-5.

Sougoufara, S., Doucoure, S., Backe Semebene, P.M., Harry, M., Sokhna, C., 2017. Challenges for malaria vector control in sub-Saharan Africa: resistance and behavioral adaptations in *Anopheles* populations. *Journal of Vector Borne Diseases*. 54, 4–15.

Spiridonov, S.E., Moens, M., 1999. Two previously unreported species of steinernematids from woodlands in Belgium. *Russian Journal of Nematology*. 7, 39–42.

Steiner, H., Hultmark, D., Engström, A., Bennich, H., Boman, H.G., 1981. Sequence and Specificity of Two Antobacterial Proteins Involved in Insect Immunity. *Nature* 292, 246–248.

Stevens, M.., Helliwell, S., Warren, G.., 1998. Fipronil seed treatments for the control of chironomid larvae (Diptera: Chironomidae) in aerially-sown rice crops. *Field Crops Research* 57, 195–207. https://doi.org/10.1016/S0378-4290(97)00146-9

Stock, S.P., Pryor, B.M., Kaya, H.K., 1999. Distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in natural habitats in California, U.S.A. *Biodiversity and Conservation* 8, 535–549. https://doi.org/10.1023/A:1008827422372

Strobel, G.A., Lanier, G.N., 1981. Dutch elm disease. *Scientific American* https://doi.org/10.2307/24964537

Stronach, B., Lennox, A., RA, G., 2014. Domain specificity of MAP3K family members, MLK and Tak1, for JNK signaling in Drosophila. *Genetics* 197, 497–513.

Stuart, R.J., Gaugler, R., 1996. Genetic adaptation and founder effect in laboratory populations of the entomopathogenic nematode *Steinernema glaseri*. *Canadian Journal of Zoology* 74, 164–170. https://doi.org/10.1139/z96-021

Sturhan, D., Liskova, M., 1999. Occurrence and distribution of entomopathogenic nematodes in the Slovak Republic. *Nematology* 1, 273–277. https://doi.org/10.1163/156854199508261

Subramaniam, T.S.S., Lee, H.L., Ahmad, N.W., Murad, S., 2012. Genetically modified mosquito: The Malaysian public engagement experience. *Biotechnology Journal* 7, 1323–1327. https://doi.org/10.1002/biot.201200282

Tabaru, Y., Moriya, K. and Ali, A., 1987. Nuisance midges (Diptera: Chironomidae) and their control in Japan. *Journal of the American Mosquito Control Association* 3, 45-49.

Tatem, A.J., Rogers, D.J., Hay, S.I., 2006. Estimating the malaria risk of African mosquito movement by air travel. *Malaria Journal* 5, 57. https://doi.org/10.1186/1475-2875-5-57

Taylor, D.P., Netscher, C., Luc, M., 1979. On *endotokia matricida* and intra-uterine development and hatching in nematodes. *Nematologica* 25, 268–274. https://doi.org/10.1163/187529279X00299

Thanwisai, A., Tandhavanant, S., Saiprom, N., Waterfield, N.R., Ke Long, P., Bode, H.B., Peacock, S.J., Chantratita, N., 2012. Diversity of *Xenorhabdus* and *Photorhabdus* spp. and their symbiotic entomopathogenic nematodes from Thailand. *PLoS One* 7. e43835 https://doi.org/10.1371/journal.pone.0043835

The Clinton Foundation, 2016. Clinton Health Access Initiative: Malaria [WWW Document]. Clint. Found. website. URL https://www.clintonfoundation.org/our-work/clinton-health-access-initiative/programs/malaria (accessed 6.17.18).

Thomas, G.M., Poinar, G.O., 1979. *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. *International Journal of Systematic Bacteriology* 29, 352–360. https://doi.org/10.1099/00207713-29-4-352

Thomas, M.B., 2018. Biological control of human disease vectors: a perspective on challenges and opportunities. *BioControl* 63, 61–69. https://doi.org/10.1007/s10526-017-9815-y

Thunders, M., Cavanagh, J., Li, Y., 2017. *De novo* transcriptome assembly, functional annotation and differential gene expression analysis of juvenile and adult *E. fetida*, a model oligochaete used in ecotoxicological studies. *Biological Research* 50, 7. https://doi.org/10.1186/s40659-017-0114-y

Timmermann, U., Becker, N., 2017. Impact of routine *Bacillus thuringiensis israelensis* (Bti) treatment on the availability of flying insects as prey for aerial feeding predators. *Bulletin of Entomological Research* 1–10. https://doi.org/10.1017/S0007485317000141

Tolle, M.A., 2009. Mosquito-borne diseases. *Current Problems in Pediatric and Adolescent Health Care* 39, 97-140.

Torr, P., Heritage, S., Wilson, M.J., 2007. *Steinernema kraussei*, an indigenous nematode found in coniferous forests: efficacy and field persistence against *Hylobius abietis*. *Agricultural and Forest Entomology* 9, 181–188. https://doi.org/10.1111/j.1461-9563.2007.00333.x

Townroe, S., Callaghan, A., 2014. British container breeding mosquitoes: The impact of urbanisation and climate change on community composition and phenology. *PLoS One* 9. e95325 https://doi.org/10.1371/journal.pone.0095325

Trewin, B.J., Kay, B.H., Darbro, J.M., Hurst, T.P., 2013. Increased container-breeding mosquito risk owing to drought-induced changes in water harvesting and storage in Brisbane, Australia. *International Health* 5, 251–258. https://doi.org/10.1093/inthealth/iht023

Tusting, L.S., Rek, J., Arinaitwe, E., Staedke, S.G., Kamya, M.R., Cano, J., Bottomley, C., Johnston, D., Dorsey, G., Lindsay, S.W., Lines, J., 2016. Why is malaria associated with

poverty? Findings from a cohort study in rural Uganda. *Infectious Diseases of Poverty* 5, 78. https://doi.org/10.1186/s40249-016-0164-3

Van Dam, A.R., Walton, W.E., 2008. The effect of predatory fish exudates on the ovipostional behaviour of three mosquito species: *Culex quinquefasciatus , Aedes aegypti* and *Culex tarsalis*. *Medical and Veterinary Entomology*. 22, 399–404. https://doi.org/10.1111/j.1365-2915.2008.00764.x

Walshe, D.P., Garner, P., Adeel, A.A., Pyke, G.H., Burkot, T.R., 2017. Larvivorous fish for preventing malaria transmission. *Cochrane Database Systemic Review* https://doi.org/10.1002/14651858.CD008090.pub3

Walther, D., Scheuch, D.E., Kampen, H., 2017. The invasive Asian tiger mosquito *Aedes albopictus* (Diptera: Culicidae) in Germany: local reproduction and overwintering. *Acta Tropica*. 166, 186–192. https://doi.org/10.1016/J.ACTATROPICA.2016.11.024

Wang, X., Grewal, P.S., 2002. Rapid genetic deterioration of environmental tolerance and reproductive potential of an entomopathogenic nematode during laboratory maintenance. *Biological Control* 23, 71–78. https://doi.org/10.1006/bcon.2001.0986

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: A revolutionary tool for transcriptomics. *Nature Review Genetics* 10, 57 https://doi.org/10.1038/nrg2484

Waterhouse, R.M., Kriventseva, E. V., Meister, S., Xi, Z., Alvarez, K.S., Bartholomay, L.C., Barillas-Mury, C., Bian, G., Blandin, S., Christensen, B.M., Dong, Y., Jiang, H., Kanost, M.R., Koutsos, A.C., Levashina, E.A., Li, J., Ligoxygakis, P., MacCallum, R.M., Mayhew, G.F., Mendes, A., Michel, K., Osta, M.A., Paskewitz, S., Sang, W.S., Vlachou, D., Wang, L., Wei, Q., Zheng, L., Zou, Z., Severson, D.W., Raikhel, A.S., Kafatos, F.C., Dimopoulos, G., Zdobnov, E.M., Christophides, G.K., 2007. Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science* 316, 1738–1743. https://doi.org/10.1126/science.1139862

Welch, H.., Bronskill, J.F., 1962. Parasitism of mosquito larvae by the nematode DDD136 (Nematoda: Neoplectanidae). *Canadian Journal of Zoology* 40, 1263–1268. https://doi.org/https://doi.org/10.1139/z62-102

Welch, H.E., Bronskill, J.F., 1962. Parasitism of mosquito larvae by the nematode DD136 (Nematoda: Neoaplectanidae). *Canadian Journal of Zoology* 40, 1263–1268. https://doi.org/10.1139/z62-102

WHO, 2017a. WHO Insecticide resistance [WWW Document]. WHO. URL http://www.who.int/malaria/areas/vector_control/insecticide_resistance/en/ (accessed 2.6.18).

WHO, 2017b. WHO The mosquito. WHO.

WHO, 2016. Mosquito-borne diseases [WWW Document]. WHO. URL http://www.who.int/neglected_diseases/vector_ecology/mosquito-borne-diseases/en/ (accessed 1.21.18).

WHO, 1982. Manual on environmental management for mosquito control, with special emphasis on malaria vectors.

Winnebeck, E.C., Millar, C.D., Warman, G.R., 2010. why does insect RNA look degraded? *Journal of Insect Science* 10, 1–7. https://doi.org/10.1673/031.010.14119

Wong, J., Stoddard, S.T., Astete, H., Morrison, A.C., Scott, T.W., 2011. Oviposition site selection by the dengue vector *Aedes aegypti* and its implications for dengue control. *PLoS Neglected Tropical Diseases* 5, e1015. https://doi.org/10.1371/journal.pntd.0001015

Worrall, E., Basu, S., Hanson, K., 2005. Is malaria a disease of poverty? A review of the literature. *Tropical Medicine and International Health*. 10, 1047–1059. https://doi.org/10.1111/j.1365-3156.2005.01476.x

Wouts, W.M., 1981. Mass production of the entomogenous nematode *Heterorhabditis heliothidis* (Nematoda: Heterorhabditidae) on artificial media. *Journal of Nematology*. 13, 467–469.

Yadav, S., Daugherty, S., Shetty, C., Eleftherianos, I., 2017. RNAseq analysis of the *Drosophila* response to the entomopathogenic nematode *Steinernema*. *G3: Genes Genomes Genetics* 7, 1955–1967.

Yang, J.S., Nam, H.J., Seo, M., Han, S.K., Choi, Y., Nam, H.G., Lee, S.J. and Kim, S., 2011. OASIS: online application for the survival analysis of lifespan assays performed in aging research. *PloS one*, 6, e23525.Centers for Disease Control, 2010. Lymphatic Filariasis - Life Cycle of *Wuchereria bancrofti* [WWW Document]. Parasites - Lymphat. Filariasis. URL https://www.cdc.gov/parasites/lymphaticfilariasis/biology_w_bancrofti.html (accessed 4.14.18).

Yee, D.A., Kesavaraju, B., Juliano, S.A., 2004. Larval feeding behavior of three co-occurring species of container mosquitoes. *Journal of Vector Ecology* 29, 315–322.

Zenner, A., O'Callaghan, K.M., Griffin, C.T., 2014. Lethal fighting in nematodes is dependent on developmental pathway: Male-male fighting in the entomopathogenic nematode Steinernema longicaudum. *PLoS One 9*, e89385. https://doi.org/10.1371/journal.pone.0089385

Zohdy, N., Shamseldean, M., Abd El-Samiee, E., Hamama, H.M., 2013. Efficacy of the Steinernematid and Heterorhabditid nematodes for controlling the mosquito, *Culex quinquefasciatus* Say (Diptera: Culicidae). *Journal of Mosquito Research* 3. https://doi.org/10.5376/jmr.2013.03.0005

Appendices

Appendix 1

Statistics for EPN persistence in soil

Full statistical results for Log Rank test for persistence of EPNs in soil assay

Control

Condition	X2 value	P-value
control immed. v.s. control immed 2nd bait	12.14	<0.001
control immed. v.s. control 1mth	0.70	0.40
control immed. v.s. control 1mth 2nd bait	60.51	<0.001
control 1mth v.s. control immed 2nd bait	6.84	0.01
control 1mth v.s. control 1mth 2nd bait	48.18	<0.001
control immed 2nd bait v.s. control 1mth 2nd bait	20.49	<0.001

Steinernema feltiae

Condition	X2 value	P-value
control 1mth 2nd bait v.s. S. feltiae 1mth 2nd bait	217.40	<0.001
control 1mth 2nd bait v.s. S.feltiae 1 month	778.11	<0.001
control 1mth v.s. S.feltiae 1 mth	994.43	<0.001
control immed 2nd bait v.s. S. feltiae 1mth 2nd bait	346.84	<0.001
control immed 2nd bait v.s. S.feltiae 1mth	961.33	<0.001
control immed 2nd bait v.s. S.feltiae immed. 1st bait	1087.89	<0.001
control immed v.s. S. feltiae 1mth 2nd bait	431.48	<0.001
control immed v.s. S.feltiae 1 month	1014.87	<0.001
S. feltiae 1mth 2nd bait v.s. control 1mth	404.35	<0.001
S. feltiae 1mth 2nd bait v.s. S.feltiae 1 mth	212.30	<0.001
S. feltiae 1mth 2nd bait v.s. S.feltiae immed. 1st bait	284.99	<0.001
S.feltiae 1 mth v.s. S.feltiae immed. 1st bait	11.14	<0.001
S.feltiae immed. 1st bait v.s. control 1 mth 2nd bait	902.28	<0.001
S.feltiae immed. 1st bait v.s. control 1mth	1113.91	<0.001
S.feltiae immed. 1st bait v.s. control immed.	1130.05	<0.001
S.feltiae immed. 1st bait v.s. S.feltiae immed. 2nd bait	38.11	<0.001
S.feltiae immed. 2nd bait v.s. control 1mth	1006.24	<0.001
S.feltiae immed. 2nd bait v.s. control 1mth 2nd bait	770.00	<0.001
S.feltiae immed. 2nd bait v.s. control immed 2nd bait	967.54	<0.001
S.feltiae immed. 2nd bait v.s. control immed.	1030.37	<0.001
S.feltiae immed. 2nd bait v.s. S. feltiae 1 mth 2nd bait	181.90	<0.001
S.feltiae immed. 2nd bait v.s. S.feltiae 1 month	6.57	0.01

Heterorhabdtitis bacteriophora

Condition	X2 value	P-value
control 1mth 2nd bait v.s. H.bacteriophora 1mth	818.87	<0.001
control 1mth 2nd bait v.s. H.bacteriophora 1mth 2nd bait	18.63	<0.001
control 1mth 2nd bait v.s. H.bacteriophora immed	808.49	<0.001
control 1mth 2nd bait v.s. H.bacteriophora immed 2nd bait	20.86	<0.001
control 1mth v.s. H.bacteriophora 1mth	1059.17	<0.001
control 1mth v.s. H.bacteriophora 1mth 2nd	110.65	<0.001
control 1mth v.s. H.bacteriophora immed 2nd bait	102.95	<0.001
control immed 2nd bait v.s. H.bacteriophora 1mth	1022.03	<0.001
control immed 2nd bait v.s. H.bacteriophora 1mth 2nd bait	71.72	<0.001
control immed 2nd bait v.s. H.bacteriophora immed	1011.00	<0.001
control immed. v.s. H.bacteriophora immed	1072.58	<0.001
control immed. v.s. H.bacteriophora immed 2nd bait	116.92	<0.001
control immed. v.s. H.bacteriophora 1mth	1083.03	<0.001
control immed. v.s. H.bacteriophora 1mth 2nd bait	127.29	<0.001
control1mth v.s. H.bacteriophora immed	1048.47	<0.001
H.bacteriophora 1mth 2nd bait v.s. H.bacteriophora immed	544.58	<0.001
H.bacteriophora 1mth 2nd bait v.s. H.bacteriophora immed 2nd bait	0.34	0.56
H.bacteriophora immed 2nd bait v.s. H.bacteriophora 1mth	401.77	<0.001
H.bacteriophora immed 2nd bait v.s. H.bacteriophora immed	396.44	<0.001
H.bacteriophora immed 2nd bait v.s. control immed 2nd bait	68.63	<0.001
H.bacteriophora immed v.s. H.bacteriophora 1mth	0.05	0.81
H. bacteriophora 1mth 2nd bait v.s. H. bacteriophora1mth	551.93	<0.001

Steinernema kraussei

Condition	X2 value	P-value
control 1mth 2nd bait v.s. S. kraussei 1mth	853.19	<0.001
control 1mth 2nd bait v.s. S. kraussei 1mth 2nd bait	614.61	<0.001
control 1mth 2nd bait v.s. S. kraussei immed	886.82	<0.001
control 1mth 2nd bait v.s. S. kraussei immed 2nd bait	971.30	<0.001
control 1mth v.s. S. kraussei immed	1093.81	<0.001
control 1mth v.s. S. kraussei immed 2nd bait	1183.64	<0.001
control immed 2nd bait v.s. S. kraussei immed	1068.42	<0.001
control immed 2nd bait v.s. S. kraussei immed 2nd bait	1160.91	<0.001
control immed 2nd bait v.s. S. kraussei1mth	1031.35	<0.001
control immed v.s. S. kraussei 1mth 2nd bait	828.98	<0.001
control immed v.s. S. kraussei immed	1109.58	<0.001
control immed v.s. S. kraussei immed 2nd bait	1198.19	<0.001
control immed v.s. S. kraussei1mth	1073.68	<0.001
control1mth v.s. S. kraussei1mth	1057.43	<0.001
S. kraussei 1mth 2nd bait v.s. control immed 2nd bait	770.40	<0.001

S. kraussei 1mth 2nd bait v.s. control 1mth	808.71	<0.001
S. kraussei immed 2nd bait v.s. S. kraussei 1mth 2nd bait	16.35	<0.001
S. kraussei immed 2nd bait v.s. S. kraussei1mth	3.03	0.08
S. kraussei immed v.s. S. kraussei 1mth 2nd bait	11.12	<0.001
S. kraussei immed v.s. S. kraussei1mth	0.29	0.59
S. kraussei1mth v.s. S. kraussei 1mth 2nd bait	8.56	<0.001
S. krausseiimmed v.s. S. kraussei immed 2nd bait	1.45	0.23

Steinernema carpocapsae

Condition	X2 value	P-value
control 1mth 2nd bait v.s. S.carpocapsae 1mth 2nd bait	538.51	<0.001
control 1mth 2nd bait v.s. S.carpocapsae immed	938.58	<0.001
control 1mth 2nd bait v.s. S.carpocapsae immed 2nd bait	1070.90	<0.001
control 1mth v.s. S.carpocapsae 1mth	1146.89	<0.001
control 1mth v.s. S.carpocapsae immed	1148.28	<0.001
control 1mth v.s. S.carpocapsae immed 2nd bait	1271.80	<0.001
control 1mth v.s. S.carpocapsae1mth 2nd bait	739.16	<0.001
control immed v.s. S.carpocapsae immed	1163.11	<0.001
control immed 2nd bait v.s. S.carpocapsae 1mth	696.59	<0.001
control immed 2nd bait v.s. S.carpocapsae 1mth	1127.12	<0.001
control immed 2nd bait v.s. S.carpocapsae immed	1124.59	<0.001
control immed 2nd bait v.s. S.carpocapsae immed 2nd bait	1256.44	<0.001
control immed v.s. S.carpocapsae 1mth	1159.32	<0.001
control immed v.s. S.carpocapsae1mth 2nd bait	762.60	<0.001
control immed. v.s. S.carpocapsae immed 2nd bait	1279.56	<0.001
control rebait 2nd bait 2 v.s. S.carpocapsae 1mth	948.59	<0.001
S.carpocapsae immed 2nd bait v.s. S.carpocapsae1mth 2nd bait	104.69	<0.001
S.carpocapsae immed 2nd bait v.s. S.carpocapsae1mth	14.42	<0.001
S.carpocapsae immed v.s. S.carpocapsae 1mth 2nd bait	50.14	<0.001
S.carpocapsae immed v.s. S.carpocapsae immed 2nd bait	28.91	<0.001
S.carpocapsae immed v.s. S.carpocapsae1mth	4.15	0.04
S.carpocapsae1mth v.s. S.carpocapsae1mthrebait	65.57	<0.001

Statistics for minimum amount of EPNs

Steinernema feltiae

Condition	X2 value	P value
1 : 1 v.s. 1 : 10	18.15	0.00002
1 : 1 v.s. 1 : 100	94.19	<0.001
1 : 1 v.s. 1 : 1000	214.72	<0.001
1 : 1 v.s. 1 : 50	71.54	<0.001
1 : 1 v.s. 1 : 500	154.86	<0.001
1 : 10 v.s. 1 : 100	32.69	<0.001
1 : 10 v.s. 1 : 1000	124.52	<0.001

1 : 10 v.s. 1 : 50	17.14	0.000035
1 : 10 v.s. 1 : 500	76.05	<0.001
1 : 100 v.s. 1 : 1000	39.11	<0.001
1 : 100 v.s. 1 : 500	11.53	0.0007
1 : 50 v.s. 1 : 100	3.89	0.0487
1 : 50 v.s. 1 : 1000	72.15	<0.001
1 : 50 v.s. 1 : 500	30.68	3.1e-8
1 : 500 v.s. 1 : 1000	9.88	0.0017
control v.s. 1 : 50	78.18	<0.001
control v.s. 1 : 10	20.79	0.0000051
control v.s. 1 : 1	0.05	0.8203
control v.s. 1 : 100	101.74	<0.001
control v.s. 1 : 1000	228.98	<0.001
control v.s. 1 : 500	165.89	<0.001

Heterorhabditis bacteriophora

Condition	X2 value	P-value
control v.s. 1 to 500	202.50	<0.001
control v.s. 1 to 50	46.86	<0.001
control v.s. 1 to 1000	137.17	<0.001
control v.s. 1 to 100	53.44	<0.001
control v.s. 1 to 10	9.92	<0.001
control v.s. 1 to 1	0.72	0.40
1 to 500 v.s. 1 to 1000	10.52	<0.001
1 to 50 v.s. 1 to 500	56.30	<0.001
1 to 50 v.s. 1 to 1000	24.42	<0.001
1 to 50 v.s. 1 to 100	0.43	0.51
1 to 100 v.s. 1 to 500	41.88	<0.001
1 to 100 v.s. 1 to 1000	16.41	<0.001
1 to 10 v.s. 1 to 500	125.44	<0.001
1 to 10 v.s. 1 to 50	14.33	<0.001
1 to 10 v.s. 1 to 1000	75.77	<0.001
1 to 10 v.s. 1 to 100	18.76	<0.001
1 to 1 v.s. 1 to 500	176.74	<0.001
1 to 1 v.s. 1 to 50	35.68	<0.001
1 to 1 v.s. 1 to 1000	116.86	<0.001
1 to 1 v.s. 1 to 100	41.64	<0.001
1 to 1 v.s. 1 to 10	5.20	0.02

Steinernema kraussei

Condition	X2 value	P-value
control v.s. 1 to 500	165.85	<0.001
control v.s. 1 to 50	141.81	<0.001

control v.s. 1 to 1000	186.92	<0.001
control v.s. 1 to 100	134.71	<0.001
control v.s. 1 to 10	48.50	<0.001
control v.s. 1 to 1	1.96	0.16
1 to 500 v.s. 1 to 1000	1.43	0.23
1 to 50 v.s. 1 to 500	2.43	0.12
1 to 50 v.s. 1 to 1000	7.82	0.01
1 to 50 v.s. 1 to 100	0.70	0.40
1 to 100 v.s. 1 to 500	6.04	0.01
1 to 100 v.s. 1 to 1000	14.18	<0.001
1 to 10 v.s. 1 to 500	42.66	<0.001
1 to 10 v.s. 1 to 50	28.17	<0.001
1 to 10 v.s. 1 to 1000	56.66	<0.001
1 to 10 v.s. 1 to 100	22.91	<0.001
1 to 1 v.s. 1 to 500	130.19	<0.001
1 to 1 v.s. 1 to 50	108.76	<0.001
1 to 1 v.s. 1 to 1000	149.38	<0.001
1 to 1 v.s. 1 to 100	102.21	<0.001
1 to 1 v.s. 1 to 10	30.27	<0.001

Steinernema carpocapsae

Condition	X2 value	P-value
control v.s. 1 to 500	208.32	<0.001
control v.s. 1 to 50	142.65	<0.001
control v.s. 1 to 1000	216.08	<0.001
control v.s. 1 to 100	113.92	<0.001
control v.s. 1 to 10	56.52	<0.001
control v.s. 1 to 1	2.06	0.15
1 to 500 v.s. 1 to 1000	0.82	0.37
1 to 50 v.s. 1 to 500	11.29	<0.001
1 to 50 v.s. 1 to 1000	16.68	<0.001
1 to 50 v.s. 1 to 100	1.57	0.21
1 to 100 v.s. 1 to 500	20.25	<0.001
1 to 100 v.s. 1 to 1000	26.09	<0.001
1 to 10 v.s. 1 to 500	64.68	<0.001
1 to 10 v.s. 1 to 50	24.92	<0.001
1 to 10 v.s. 1 to 1000	72.42	<0.001
1 to 10 v.s. 1 to 100	13.28	<0.001
1 to 1 v.s. 1 to 500	167.97	<0.001
1 to 1 v.s. 1 to 50	109.04	<0.001
1 to 1 v.s. 1 to 1000	175.45	<0.001
1 to 1 v.s. 1 to 100	84.27	<0.001
1 to 1 v.s. 1 to 10	35.97	<0.001

Appendix 2

Log Rank Statistics for *C. plumosus* survival

Steinernema feltiae

Condition	X2	P value
Control *v.s.* S.feltiae1000	90.17	<0.001
Control *v.s.* S.feltiae16000	119.79	<0.001
Control *v.s.* S.feltiae2000	125.75	<0.001
Control *v.s.* S.feltiae4000	147.95	<0.001
Control *v.s.* S.feltiae8000	113.78	<0.001
S.feltiae 1000 *v.s.* S.feltiae 16000	5.84	0.0156
S.feltiae 1000 *v.s.* S.feltiae 2000	5.36	0.0206
S.feltiae 1000 *v.s.* S.feltiae 4000	18.06	2.1e-05
S.feltiae 1000 *v.s.* S.feltiae 8000	5.31	0.0212
S.feltiae 2000 *v.s.* S.feltiae 16000	0.13	0.7145
S.feltiae 2000 *v.s.* S.feltiae 8000	0.05	0.8242
S.feltiae 4000 *v.s.* S.feltiae 16000	3.08	0.0791
S.feltiae 4000 *v.s.* S.feltiae 8000	3.06	0.0804
S.feltiae 8000 *v.s.* S.feltiae 16000	0.00	0.9880
S.feltiae 8000 *v.s.* S.feltiae 4000	3.06	0.0804
S.feltiae2000 *v.s.* S.feltiae 4000	4.80	0.0285

Heterorhabditis bacteriophora

Control	X2	P value
Control *v.s.* H. bacteriophora 1000	87.17	<0.001
Control *v.s.* H. bacteriophora 16000	85.04	<0.001
Control *v.s.* H. bacteriophora 2000	70.32	<0.001
Control *v.s.* H. bacteriophora 4000	69.20	<0.001
Control *v.s.* H. bacteriophora 8000	119.22	<0.001
H. bacteriophora1000 *v.s.* H. bacteriophora 16000	0.01	0.9299
H. bacteriophora1000 *v.s.* H. bacteriophora 2000	0.92	0.3367
H. bacteriophora1000 *v.s.* H. bacteriophora 4000	0.82	0.3656
H. bacteriophora1000 *v.s.* H. bacteriophora 8000	2.13	0.1447
H. bacteriophora2000 *v.s.* H. bacteriophora 16000	0.75	0.3854
H. bacteriophora2000 *v.s.* H. bacteriophora 4000	0.00	0.9639
H. bacteriophora2000 *v.s.* H. bacteriophora 8000	5.91	0.0150
H. bacteriophora4000 *v.s.* H. bacteriophora 16000	0.65	0.4205
H. bacteriophora4000 *v.s.* H. bacteriophora 8000	5.58	0.0181
H. bacteriophora8000 *v.s.* H. bacteriophora 16000	2.26	0.1326

Steinernema kraussei

Condition	X2	P value
Control *v.s.* S. kraussei 1000	191.67	<0.001
Control *v.s.* S. kraussei 16000	181.14	<0.001

Control *v.s.* S. krausse i2000	190.60	<0.001
Control *v.s.* S. kraussei 4000	166.50	<0.001
Control *v.s.* S. kraussei 8000	173.01	<0.001
S. kraussei 1000 *v.s.* S. kraussei 16000	0.25	0.6140
S. kraussei 1000 *v.s.* S. kraussei 2000	0.09	0.7646
S. kraussei 1000 *v.s.* S. kraussei 4000	0.43	0.5129
S. kraussei 1000 *v.s.* S. kraussei 8000	0.00	0.9446
S. kraussei 2000 *v.s.* S. kraussei 16000	0.68	0.4084
S. kraussei 2000 *v.s.* S. kraussei 4000	0.87	0.3521
S. kraussei 2000 *v.s.* S. kraussei 8000	0.17	0.6784
S. kraussei 4000 *v.s.* S. kraussei 16000	0.04	0.8435
S. kraussei 4000 *v.s.* S. kraussei 8000	0.26	0.6120
S. kraussei 8000 *v.s.* S. kraussei 16000	0.10	0.7567

Steinernema carpocapsae

Condition	X2	P value
Control *v.s.* S. carpocapsae1000	150.73	<0.001
Control *v.s.* S. carpocapsae16000	85.04	<0.001
Control *v.s.* S. carpocapsae2000	144.68	<0.001
Control *v.s.* S. carpocapsae4000	147.95	<0.001
Control *v.s.* S. carpocapsae8000	173.88	<0.001
S. carpocapsae1000 *v.s.* S. carpocapsae16000	7.24	0.0071
S. carpocapsae1000 *v.s.* S. carpocapsae2000	0.02	0.8801
S. carpocapsae1000 *v.s.* S. carpocapsae4000	0.05	0.8206
S. carpocapsae1000 *v.s.* S. carpocapsae8000	1.38	0.2398
S. carpocapsae2000 *v.s.* S. carpocapsae16000	6.46	0.0111
S. carpocapsae2000 *v.s.* S. carpocapsae4000	0.15	0.7031
S. carpocapsae2000 *v.s.* S. carpocapsae8000	1.69	0.1929
S. carpocapsae4000 *v.s.* S. carpocapsae16000	7.74	0.0054
S. carpocapsae4000 *v.s.* S. carpocapsae8000	0.95	0.3301
S. carpocapsae8000 *v.s.* S. carpocapsae16000	12.87	0.0003

Appendix 3

Statistics for survival of *Aedes aegypti*

Condition	X2	P-value
control v.s. S. feltiae 2000	86.37	<0.001
control v.s. S. feltiae 4000	175.50	<0.001
control v.s. S. feltiae 8000	212.65	<0.001
S. feltiae 2000 v.s. S. feltiae 4000	10.72	0.0011
S. feltiae 2000 v.s. S. feltiae 8000	15.84	0.0001
S. feltiae 4000 v.s. S. feltiae 8000	0.16	0.6884

Condition	X2	P value
control *v.s.* H. bacteriophora 2000	104.19	<0.001
control *v.s.* H. bacteriophora 4000	114.18	<0.001
control *v.s.* H. bacteriophora 8000	248.39	<0.001
H. bacteriophora 2000 *v.s.* H. bacteriophora 4000	0.53	0.4683
H. bacteriophora 2000 *v.s.* H. bacteriophora 8000	14.54	0.0001
H. bacteriophora 4000 *v.s.* H. bacteriophora 8000	5.17	0.0230

Condition	X2	P value
control *v.s.* S. kraussei 2000	117.05	<0.001
control *v.s.* S. kraussei 4000	206.33	<0.001
control *v.s.* S. kraussei 8000	201.02	<0.001
S. kraussei 4000 *v.s.* S. kraussei 8000	0.79	0.3742
S. kraussei 2000 *v.s.* S. kraussei 4000	8.36	0.0038
S. kraussei 2000 *v.s.* S. kraussei 8000	12.13	0.0005

Condition	X2	P value
S. carpocapsae 2000 *v.s.* S. carpocapsae 4000	0.12	0.7287
S. carpocapsae 2000 *v.s.* S. carpocapsae 8000	2.88	0.0899
S. carpocapsae 4000 *v.s.* S. carpocapsae 8000	3.48	0.0621
control *v.s.* S. carpocapsae 2000	149.23	<0.001
control *v.s.* S. carpocapsae 4000	136.79	<0.001
control *v.s.* S. carpocapsae 8000	257.44	<0.001

Condition	X2	P value
control v.s. S. glaseri 367 2000	42.82	<0.001
control v.s. S. glaseri 367 4000	57.11	<0.001
control v.s. S. glaseri 367 8000	33.54	6.9e-09
S. glaseri 367 2000 v.s. S. glaseri 367 4000	0.65	0.4206
S. glaseri 367 2000 v.s. S. glaseri 367 8000	0.41	0.5244
S. glaseri 367 4000 v.s. S. glaseri 367 8000	2.18	0.1394

Condition	X2	P value
control v.s. S. feltiae 93 2000	76.48	<0.001
control v.s. S. feltiae 93 4000	89.68	<0.001
control v.s. S. feltiae 93 8000	110.64	<0.001

S. feltiae 93 2000 v.s. S. feltiae 93 4000	1.35	0.2451
S. feltiae 93 2000 v.s. S. feltiae 93 8000	4.94	0.0262
S. feltiae 93 4000 v.s. S. feltiae 93 8000	1.35	0.2448

Condition	X2	P value
control *v.s.* S. affine 173 2000	52.65	<0.001
control *v.s.* S. affine 173 4000	43.33	<0.001
control *v.s.* S. affine 173 8000	84.05	<0.001
S. affine 173 2000 *v.s.* S. affine 173 4000	0.49	0.4835
S. affine 173 2000 *v.s.* S. affine 173 8000	0.59	0.4440
S. affine 173 4000 *v.s.* S. affine 173 8000	4.93	0.0264

Comparison at 8000 EPNs

Condition	X2	173 P-value
control *v.s.* felt 8000	319.47	<0.001
control *v.s.* H. bacteriophora 8000	283.45	<0.001
control *v.s.* S. affine 173 8000	84.05	<0.001
control *v.s.* S. carpocapsae 8000	369.47	<0.001
control *v.s.* S. feltiae 367 8000	33.54	6.9e-09
control *v.s.* S. glaseri 93 8000	110.64	<0.001
control *v.s.* S. kraussei 8000	289.98	<0.001
S. feltiae 8000 *v.s.* H. bacteriophora 8000	0.33	0.5628
H. bacteriophora 8000 *v.s.* S. feltiae 367 8000	84.74	<0.001
S. affine 173 8000 *v.s.* S. feltiae 8000	53.28	<0.001
S. affine 173 8000 *v.s.* H. bacteriophora 8000	57.30	<0.001
S. affine 173 8000 *v.s.* S. carpocapsae 8000	78.86	<0.001
S. affine 173 8000 *v.s.* S. feltiae 367 8000	6.94	0.0084
S. affine 173 8000 *v.s.* S. glaseri 93 8000	2.84	0.0917
S. affine 173 8000 *v.s.* S. kraussei 8000	46.86	<0.001
S. carpocapsae 8000 *v.s.* S. feltiae 8000	0.01	0.9059
S. carpocapsae 8000 *v.s.* H. bacteriophora 8000	0.83	0.3619
S. carpocapsae 8000 *v.s.* S. kraussei 8000	0.36	0.5473
S. feltiae 367 8000 *v.s.* S. feltiae 8000	91.77	<0.001
S. feltiae 367 8000 *v.s.* H. bacteriophora 8000	84.74	<0.001
S. feltiae 367 8000 *v.s.* S. carpocapsae 8000	104.70	<0.001
S. feltiae 367 8000 *v.s.* S. kraussei 8000	80.54	<0.001
S. glaseri 93 8000 *v.s.* S. feltiae 8000	30.05	4.2e-08
S. glaseri 93 8000 *v.s.* H. bacteriophora 8000	22.64	2.0e-06
S. glaseri 93 8000 *v.s.* S. carpocapsae 8000	39.92	<0.001
S. glaseri 93 8000 *v.s.* S. feltiae 367 8000	15.78	0.0001
S. glaseri 93 8000 *v.s.* S. kraussei 8000	24.26	8.4e-07
S. kraussei 8000 *v.s.* S. feltiae 8000	0.32	0.5687
S. kraussei 8000 *v.s.* H. bacteriophora 8000	0.03	0.8728

Statistics for survival *Ochlerotatus detritus*

Control

Condition	X2	P value
control v.s. S. affine 173 2000	179.84	<0.001
control v.s. S. affine 173 4000	79.62	<0.001
control v.s. S. affine 173 8000	84.39	<0.001
control v.s. S. glaseri 367 2000	120.18	<0.001
control v.s. S. glaseri 367 4000	99.72	<0.001
control v.s. S. glaseri 367 8000	161.3	<0.001
control v.s. S. glaseri 93 8000	196.26	<0.001
control v.s. S. glaseri 93 2000	142.92	<0.001
control v.s. S. glaseri 93 4000	171.59	<0.001
control v.s. S. feltiae 2000	301.67	<0.001
control v.s. S. feltiae 4000	272.16	<0.001
control v.s. S. feltiae 8000	381.72	<0.001
control v.s. H. bacteriophora 2000	168.9	<0.001
control v.s. H. bacteriophora 4000	159.4	<0.001
control v.s. H. bacteriophora 8000	236.72	<0.001
control v.s. S. carpocapsae 2000	89.76	<0.001
control v.s. S. carpocapsae 4000	96.02	<0.001
control v.s. S. carpocapsae 8000	158.6	<0.001
control v.s. S. kraussei 2000	121.9	<0.001
control v.s. S. kraussei 8000	197.02	<0.001
control v.s. S. kraussei 4000	158.52	<0.001

Interspecific comparisons

S. feltiae 2000 v.s. S. feltiae 4000	4.59	0.0322
S. feltiae 2000 v.s. S. feltiae 8000	5.05	0.0246

S. feltiae 8000 v.s. S. feltiae 4000	21.93	0.0000028
H. bacteriophora 2000 v.s. H. bacteriophora 4000	2.29	0.1303
H. bacteriophora 2000 v.s. H. bacteriophora 8000	33.7	6.4e-9
H. bacteriophora 4000 v.s. H. bacteriophora 8000	15.7	0.0001
S. kraussei 2000 v.s. S. kraussei 4000	1.14	0.2851
S. kraussei 2000 v.s. S. kraussei 8000	12.42	0.0004
S. kraussei 4000 v.s. S. kraussei 8000	7.4	0.0065
S. carpocapsae 4000 v.s. S. carpocapsae 8000	7.1	0.0077
S. carpocapsae 2000 v.s. S. carpocapsae 4000	2	0.1573
S. carpocapsae 2000 v.s. S. carpocapsae 8000	19.29	0.000011
S. affine 173 4000 v.s. S. affine 173 8000	0.06	0.8096
S. affine 173 2000 v.s. S. affine 173 4000	12.27	0.0005
S. affine 173 2000 v.s. S. affine 173 8000	15.92	0.0001
S. glaseri 367 2000 v.s. S. glaseri 367	1.83	0.1758
S. glaseri 367 2000 v.s. S. glaseri 367 8000	11.21	0.0008
S. glaseri 367 8000 v.s. S. glaseri 367 4000	3.79	0.0515
S. glaseri 93 8000 v.s. S. glaseri 93 2000	6.67	0.0098
S. glaseri 93 8000 v.s. S. glaseri 93 4000	8.81	0.003
S. glaseri 93 8000 v.s. S. glaseri 93 4000	8.81	0.003

Comparison between conditions

Condition	X2	P value
S. feltiae 2000 v.s. S. affine 173 2000	24.28	8.3e-7
S. feltiae 2000 v.s. S. affine 173 4000	60.25	<0.001
S. feltiae 2000 v.s. S. affine 173 8000	71.65	<0.001
S. feltiae 2000 v.s. S. glaseri 367 2000	94.65	<0.001
S. feltiae 2000 v.s. S. glaseri 367 4000	69.67	<0.001
S. feltiae 2000 v.s. S. glaseri 367 8000	43.96	<0.001
S. feltiae 2000 v.s. S. glaseri 93 8000	13.44	0.0002
S. feltiae 2000 v.s. S. glaseri 93 2000	39.35	<0.001

S. feltiae 2000 v.s. S. glaseri 93 4000	49.5	<0.001
S. feltiae 2000 v.s. H. bacteriophora 4000	60.58	<0.001
S. feltiae 2000 v.s. S. carpocapsae 2000	119.85	<0.001
S. feltiae 2000 v.s. S. carpocapsae 4000	77.86	<0.001
S. feltiae 2000 v.s. S. carpocapsae 8000	46.13	<0.001
S. feltiae 2000 v.s. S. kraussei 2000	107.83	<0.001
S. feltiae 2000 v.s. S. kraussei 4000	105.2	<0.001
S. feltiae 2000 v.s. S. kraussei 8000	62.31	<0.001
S. feltiae 4000 v.s. S. affine 173 2000	8.94	0.0028
S. feltiae 4000 v.s. S. affine 173 4000	39.43	<0.001
S. feltiae 4000 v.s. S. affine 173 8000	48	<0.001
S. feltiae 4000 v.s. S. glaseri 367 2000	65.43	<0.001
S. feltiae 4000 v.s. S. glaseri 367 4000	44.17	<0.001
S. feltiae 4000 v.s. S. glaseri 367 8000	23.06	0.0000016
S. feltiae 4000 v.s. S. glaseri 93 8000	2.99	0.0839
S. feltiae 4000 v.s. S. glaseri 93 2000	20.95	0.0000047
S. feltiae 4000 v.s. S. glaseri 93 4000	25.95	3.5e-7
S. feltiae 4000 v.s. H. bacteriophora 2000	62.77	<0.001
S. feltiae 4000 v.s. H. bacteriophora 8000	5	0.0253
S. feltiae 4000 v.s. S. carpocapsae 2000	88.42	<0.001
S. feltiae 4000 v.s. S. carpocapsae 4000	52.52	<0.001
S. feltiae 4000 v.s. S. carpocapsae 8000	23.68	0.0000011
S. feltiae 8000 v.s. S. affine 173 2000	51.19	<0.001
S. feltiae 8000 v.s. S. affine 173 4000	93.47	<0.001
S. feltiae 8000 v.s. S. affine 173 8000	110.81	<0.001
S. feltiae 8000 v.s. S. glaseri 367 2000	147.54	<0.001
S. feltiae 8000 v.s. S. glaseri 367 4000	115.29	<0.001
S. feltiae 8000 v.s. S. glaseri 367 8000	81.79	<0.001
S. feltiae 8000 v.s. S. glaseri 93 8000	34.43	4.4e-9

S. feltiae 8000 v.s. S. glaseri 93 2000	73.79	<0.001
S. feltiae 8000 v.s. S. glaseri 93 4000	92.54	<0.001
S. feltiae 8000 v.s. H. bacteriophora 2000	160.35	<0.001
S. feltiae 8000 v.s. H. bacteriophora 4000	109.57	<0.001
S. feltiae 8000 v.s. S. carpocapsae 2000	181.48	<0.001
S. feltiae 8000 v.s. S. carpocapsae 4000	122.94	<0.001
S. feltiae 8000 v.s. S. carpocapsae 8000	82.68	<0.001
S. feltiae 8000 v.s. S. kraussei 8000	111.27	<0.001
H. bacteriophora 2000 v.s. S. affine 173 2000	17.92	0.0000023
H. bacteriophora 2000 v.s. S. affine 173 4000	0.35	0.5564
H. bacteriophora 2000 v.s. S. affine 173 8000	0.91	0.3393
H. bacteriophora 2000 v.s. S. glaseri 367 2000	2.29	0.1299
H. bacteriophora 2000 v.s. S. glaseri 367 4000	0.02	0.9017
H. bacteriophora 2000 v.s. S. glaseri 367 8000	5.86	0.0154
H. bacteriophora 2000 v.s. S. glaseri 93 8000	29.74	4.9e-8
H. bacteriophora 2000 v.s. S. glaseri 93 2000	5.1	0.024
H. bacteriophora 2000 v.s. S. glaseri 93 4000	6.32	0.0119
H. bacteriophora 2000 v.s. S. feltiae 2000	96.6	<0.001
H. bacteriophora 2000 v.s. S. carpocapsae 4000	0.89	0.3466
H. bacteriophora 2000 v.s. S. carpocapsae 8000	5.55	0.0185
H. bacteriophora 2000 v.s. S. kraussei 2000	2.59	0.1075
H. bacteriophora 2000 v.s. S. kraussei 4000	0.35	0.5546
H. bacteriophora 4000 v.s. S. affine 173 2000	7.1	0.0077
H. bacteriophora 4000 v.s. S. affine 173 8000	3.82	0.0507
H. bacteriophora 4000 v.s. S. glaseri 367 2000	6.82	0.009
H. bacteriophora 4000 v.s. S. glaseri 367 4000	1.25	0.2639
H. bacteriophora 4000 v.s. S. glaseri 367 8000	0.85	0.3575
H. bacteriophora 4000 v.s. S. glaseri 93 8000	14.84	0.0001
H. bacteriophora 4000 v.s. S. glaseri 93 2000	0.69	0.4068

H. bacteriophora 4000 v.s. S. glaseri 93 4000	0.86	0.3544
H. bacteriophora 4000 v.s. S. feltiae 4000	35.84	2.2e-9
H. bacteriophora 4000 v.s. S. carpocapsae 2000	15.4	0.0001
H. bacteriophora 4000 v.s. S. carpocapsae 4000	4.18	0.041
H. bacteriophora 4000 v.s. S. carpocapsae 8000	0.82	0.3654
H. bacteriophora 4000 v.s. S. kraussei 2000	8.09	0.0045
H. bacteriophora 4000 v.s. S. kraussei 4000	4.24	0.0395
H. bacteriophora 4000 v.s. S. kraussei 8000	0.27	0.6048
H. bacteriophora 8000 v.s. S. affine 173 2000	0.72	0.3976
H. bacteriophora 8000 v.s. S. affine 173 4000	20.62	0.0000056
H. bacteriophora 8000 v.s. S. affine 173 8000	26.44	2.7e-7
H. bacteriophora 8000 v.s. S. glaseri 367 2000	38.87	<0.001
H. bacteriophora 8000 v.s. S. glaseri 367 4000	22.41	0.0000022
H. bacteriophora 8000 v.s. S. glaseri 367 8000	7.7	0.0055
H. bacteriophora 8000 v.s. S. glaseri 93 8000	0.14	0.7037
H. bacteriophora 8000 v.s. S. glaseri 93 2000	6.95	0.0084
H. bacteriophora 8000 v.s. S. glaseri 93 4000	8.77	0.0031
H. bacteriophora 8000 v.s. S. feltiae 2000	19.11	0.000012
H. bacteriophora 8000 v.s. S. feltiae 8000	48.27	<0.001
H. bacteriophora 8000 v.s. S. carpocapsae 4000	29.75	4.9e-8
H. bacteriophora 8000 v.s. S. carpocapsae 8000	7.68	0.0056
H. bacteriophora 8000 v.s. S. kraussei 4000	39.49	<0.001
H. bacteriophora 8000 v.s. S. kraussei 8000	13.35	0.0003
S. carpocapsae 2000 v.s. H. bacteriophora 2000	8.09	0.0045
S. carpocapsae 2000 v.s. H. bacteriophora 8000	57.75	<0.001
S. carpocapsae 2000 v.s. S. kraussei 2000	1.4	0.2369
S. kraussei 2000 v.s. S. affine 173 2000	26.77	2.3e-7
S. kraussei 2000 v.s. S. affine 173 4000	0.34	0.5572
S. kraussei 2000 v.s. S. affine 173 8000	0.1	0.7518

S. kraussei 2000 v.s. S. glaseri 367 4000	2.00	0.1576
S. kraussei 2000 v.s. S. glaseri 367 8000	12.82	0.0003
S. kraussei 2000 v.s. S. glaseri 93 8000	39.19	<0.001
S. kraussei 2000 v.s. S. glaseri 93 2000	11.56	0.0007
S. kraussei 2000 v.s. S. glaseri 93 4000	13.77	0.0002
S. kraussei 2000 v.s. S. feltiae 4000	74.77	<0.001
S. kraussei 2000 v.s. S. feltiae 8000	166.61	<0.001
S. kraussei 2000 v.s. H. bacteriophora 8000	44.76	<0.001
S. kraussei 2000 v.s. S. carpocapsae 4000	0.15	0.6986
S. kraussei 2000 v.s. S. carpocapsae 8000	11.92	0.0006
S. kraussei 4000 v.s. S. affine 173 2000	21.75	0.0000031
S. kraussei 4000 v.s. S. affine 173 4000	0.04	0.8341
S. kraussei 4000 v.s. S. affine 173 8000	0.3	0.5866
S. kraussei 4000 v.s. S. glaseri 367 2000	1.06	0.3029
S. kraussei 4000 v.s. S. glaseri 367 4000	0.35	0.5516
S. kraussei 4000 v.s. S. glaseri 367 8000	8.44	0.0037
S. kraussei 4000 v.s. S. glaseri 93 8000	34.3	4.7e-9
S. kraussei 4000 v.s. S. glaseri 93 2000	7.39	0.0066
S. kraussei 4000 v.s. S. glaseri 93 4000	9.15	0.0025
S. kraussei 4000 v.s. S. feltiae 4000	70.16	<0.001
S. kraussei 4000 v.s. S. feltiae 8000	170.12	<0.001
S. kraussei 4000 v.s. H. bacteriophora 2000	0.35	0.5546
S. kraussei 4000 v.s. S. carpocapsae 2000	5.32	0.021
S. kraussei 4000 v.s. S. carpocapsae 4000	0.23	0.6313
S. kraussei 4000 v.s. S. carpocapsae 8000	7.87	0.005
S. kraussei 8000 v.s. S. affine 173 2000	5.69	0.017
S. kraussei 8000 v.s. S. affine 173 4000	4.2	0.0404
S. kraussei 8000 v.s. S. affine 173 8000	6.28	0.0122
S. kraussei 8000 v.s. S. glaseri 367 2000	10.87	0.001

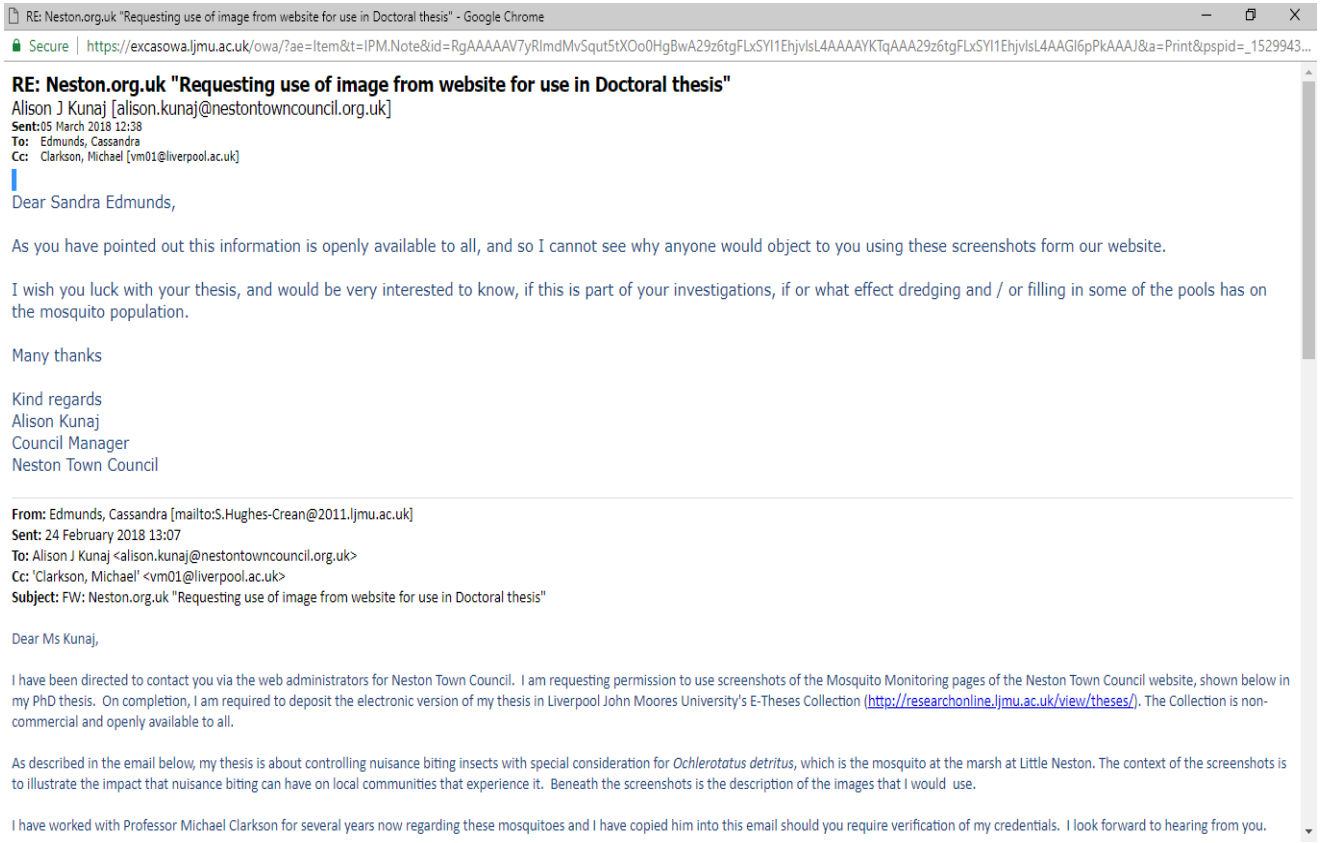
S. kraussei 8000 v.s. S. glaseri 367 4000	2.91	0.0879
S. kraussei 8000 v.s. S. glaseri 367 8000	0.25	0.6149
S. kraussei 8000 v.s. S. glaseri 93 8000	12.97	0.0003
S. kraussei 8000 v.s. S. glaseri 93 2000	0.25	0.62
S. kraussei 8000 v.s. S. glaseri 93 4000	0.26	0.6134
S. kraussei 8000 v.s. S. feltiae 4000	34.45	4.4e-9
S. kraussei 8000 v.s. H. bacteriophora 2000	4.74	0.0295
S. kraussei 8000 v.s. S. carpocapsae 2000	21.28	0.000004
S. kraussei 8000 v.s. S. carpocapsae 4000	6.96	0.0083
S. kraussei 8000 v.s. S. carpocapsae 8000	0.19	0.664

Permissions

Images

Except where specified all images were created by the author. In chapter 5, gel images were generated by Agilent Tapestation and all other figures were provided by BGI as part of RNA-seq analysis.

Mosquito watch website screenshot permitted by Neston Town Council. Please see below.



RE: Neston.org.uk "Requesting use of image from website for use in Doctoral thesis" - Google Chrome

Secure | https://excasowa.ljmu.ac.uk/owa/?ae=Item&t=IPM.Note&id=RgAAAAAV7yRlmdMvSqut5tXOo0HgBwA29z6tgFLxSYl1EhjvlsL4AAAAYKTqAAA29z6tgFLxSYl1EhjvlsL4AAGl6pPkAAAJ&a=Print&pspid=_1529943...

RE: Neston.org.uk "Requesting use of image from website for use in Doctoral thesis"

Alison J Kunaj [alison.kunaj@nestontowncouncil.org.uk]
Sent: 05 March 2018 12:38
To: Edmunds, Cassandra
Cc: Clarkson, Michael [vm01@liverpool.ac.uk]

Dear Sandra Edmunds,

As you have pointed out this information is openly available to all, and so I cannot see why anyone would object to you using these screenshots form our website.

I wish you luck with your thesis, and would be very interested to know, if this is part of your investigations, if or what effect dredging and / or filling in some of the pools has on the mosquito population.

Many thanks

Kind regards
Alison Kunaj
Council Manager
Neston Town Council

From: Edmunds, Cassandra [mailto:S.Hughes-Crean@2011.ljmu.ac.uk]
Sent: 24 February 2018 13:07
To: Alison J Kunaj <alison.kunaj@nestontowncouncil.org.uk>
Cc: 'Clarkson, Michael' <vm01@liverpool.ac.uk>
Subject: FW: Neston.org.uk "Requesting use of image from website for use in Doctoral thesis"

Dear Ms Kunaj,

I have been directed to contact you via the web administrators for Neston Town Council. I am requesting permission to use screenshots of the Mosquito Monitoring pages of the Neston Town Council website, shown below in my PhD thesis. On completion, I am required to deposit the electronic version of my thesis in Liverpool John Moores University's E-Theses Collection (http://researchonline.ljmu.ac.uk/view/theses/). The Collection is non-commercial and openly available to all.

As described in the email below, my thesis is about controlling nuisance biting insects with special consideration for *Ochlerotatus detritus*, which is the mosquito at the marsh at Little Neston. The context of the screenshots is to illustrate the impact that nuisance biting can have on local communities that experience it. Beneath the screenshots is the description of the images that I would use.

I have worked with Professor Michael Clarkson for several years now regarding these mosquitoes and I have copied him into this email should you require verification of my credentials. I look forward to hearing from you.

Published journal articles

Two articles (attached) have been published from the work contained in this thesis so far, these are:

Edmunds, C.V., Wilding, C.S. and Rae, R., 2017. Susceptibility of *Chironomus plumosus* larvae (Diptera: Chironomidae) to entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae): Potential for control. *European Journal of Entomology*, *114*, pp.526-532.

This article is open access

Edmunds, C., Post, R.J., Wilding, C.S. and Rae, R., 2018. A survey investigating the diversity and distribution of entomopathogenic nematodes in the UK and the first confirmed UK record of *Steinernema carpocapsae*. *Nematology*

Permission to use this article has been requested from the publishers.