Optimal foraging behaviour of nectar gathering bumblebees: A doubly labelled water study

by

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To Eddie Saxton

1937-1995

A friend, mentor and coach to many

Abstract

When bumblebees gather nectar, it is generally believed that foragers optimize the energy costs and gains of collecting nectar in a manner which maximizes fitness. The current study investigated the foraging behaviour of nectar gathering bumblebees and whether they maximized one of two currencies:

1. Rate of net energy gain (RNEG), where RNEG = (energy gains - costs) / time.
2. Net energy efficiency (NEE), where NEE = (energy gains - costs) / costs.

Bumblebees were trained to gather nectar from a non-depleting artificial flower patch at which energy gains could be accurately controlled and recorded. Energy gains were combined with time budgets and time / activity / laboratory (TAL) estimates of energy costs, enabling the NEE and RNEG of foraging bees to be estimated. Computer simulations of the optimum behaviour of the same bees when maximizing RNEG and NEE, were then compared to the observed behaviour of the bees. It was found that NEE and RNEG model predictions of behaviour differed significantly to the observed behaviour. It was, therefore, clear that the foragers did not maximize RNEG whilst gathering nectar. However, unlike the RNEG model, NEE model predictions of the bumblebees behaviour required accurate estimates of energy costs. As the data available for TAL estimates of energy costs were limited, based largely on studies of honeybee energetics, it was unclear whether TAL estimated costs were reliable. As a result, it was possible that the variation between the observed foraging behaviour and NEE model predictions was due to errors in TAL estimates of costs.

To provide a more accurate measure of the bumblebees energy expenditure, a protocol was developed to enable the doubly labelled water technique to be applied to bumblebees. This resulted in alterations to the standard DLW analytical and methodological procedures. The developed protocol was validated by simultaneous DLW and infra-red open circuit CO_2 respirometry measures of the energy expenditure of 16 bumblebees during tethered flight. Comparison between DLW and respirometry estimates did not significantly differ from one another. Due to the low variation between DLW and infra-red calorimetry measures of energy expenditure, it was possible to use the DLW technique to measure the energy costs of bumblebees whilst foraging in a field situation.

Results from field DLW measures of the bumblebees energy costs revealed significant errors in TAL estimates. TAL estimates were then removed from the NEE model and replaced by the costs required for the model to predict the observed behaviour of the bees. These costs were significantly different from DLW measured costs, thus showing that the bumblebees were not maximizing NEE whilst foraging for nectar. A significant correlation was, however, observed between the mass of foragers and volume of nectar

collected. It is, therefore possible that bumblebees follow a simple nectar volume threshold rule, possibly in an attempt to maintain a constant water balance.

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a	The incremental increase in flying metabolic rate of a bee with load
a_h	Resting and walking metabolic rates of a bee
a_o	The unloaded metabolic rate of a flying bee
a_τ	Metabolic rate of a bee whilst in the hive
B_H	Background D isotopic enrichment
B_O	Background ^{18}O isotopic enrichment
θ	Foraging energy costs of a bee
C	The weight specific energetic value of nectar
C_1	TAL estimated costs
C_2	Model predicted foraging costs when predicted number of flower visits was altered to equal the same number of flower visits as was observed in the field
C_3	Model predicted foraging costs when metabolic rate of the bee was altered so model predictions of number of flower visits equal observed number of flower visits
C_{DLW}	DLW estimates of a bee's energy expenditure over one foraging cycle
C_p	Total energy expenditure of a bee whilst foraging in the flower patch
C_t	Total energy expenditure of a bee during the flight to the flower patch and back to the hive
DLW	Doubly Labelled Water
EE	Energy expenditure
F_H	Final D isotopic enrichment of a body water pool
F_O	Final ^{18}O isotopic enrichment
G	Energy gained by a bee whilst foraging

GH	Guanidine hydrochloride
gIRMS	gas Isotope Ratio Mass Spectrometry
h	Time taken by a bee to collect nectar in a flower (handling time)
I_H	Initial D isotopic enrichment of body water
I_O	Initial ^{18}O isotopic enrichment of body water
k_H	The apparent fractional turnover rate of the D isotope
k_O	The apparent fractional turnover rate of the ^{18}O isotope
LNVD	Liquid Nitrogen Vacuum Distillation
m	Body mass of *B.terrestris*
MCE	Micro CO_2 Equilibration technique
n	The number of flowers visited by a bee during one foraging cycle
N	Body water pool of an organism
NEE	Net Energy Efficiency
OFT	Optimal Foraging Theory
rCO_2	Rate of CO_2 production
RNEG	Rate of Net Energy Gain
RQ	Respiratory quotient
Rsample	Un-normalized isotopic abundance of a water standard
Rws	Isotopic abundance of a working standard relative to V-SMOW and SLAP
SLAP	Standard Light Arctic Precipitation
t	The time period between the removal of initial and final body water samples during DLW analysis
T	Time taken by a foraging bee to collect nectar and deliver it to the hive

TAL	Time / Activity / Laboratory estimates of energy expenditure
T_0	Time spent by a bee in the hive
τ	Inter-flower flight time of a bee
τ_0	Flight time of a bee from the hive to a flower patch
UHV	Ultra High Vacuum Manifold
V-SMOW	Vienna-Standard Mean Ocean Water
w	The mass of nectar collected on each flower visit by a foraging bee

Chapter 1

Introduction

1.0.0 General Introduction

Organisms evolve towards phenotypes best adapted to survive and reproduce within the ecosystem in which they are situated (Darwin, 1859). Selective pressures should, therefore, result in phenotypes which optimize their foraging behaviour in a manner which maximizes the probability of the individual successfully reproducing (Cody, 1974; Charnov, 1976; Lewontin 1978; Maynard-Smith, 1978; McNeill, 1982; Pyke, 1984; McNamara & Houston, 1986; Stearns, 1986; Krebs & Davies, 1991; Owen-Smith, 1994). In other words:

"Foraging behaviour has been shaped by natural selection, so that foraging strategies which maximize fitness will exist in nature, and these foraging strategies will be optimal with respect to criteria that may be evaluated independently of a knowledge of the fitness of the animals" (Pyke, Pulliam & Charnov, 1977).

The form in which optimal foraging behaviour may express itself is highly variable, depending largely on the life history of the species and the short term energy requirements of the individual or social group (Krebs, Stephen's & Sutherland, 1983). It is, however, possible to describe optimal foraging in terms of three major elements:

1) The time taken to search for, collect and handle the food (time).
2) The energy expended during searching for, and collecting food (energy costs).
3) The energy gained from the food (energy gains).

Optimal foraging theory (OFT) would, therefore, suggest that an organism should employ a strategy in which the combination of time and costs results in the highest net gains to the individual or social group.

This simplistic explanation of optimal foraging theory has, however, been criticized as inadequate (Heindrich, 1983; Hobbs, 1990; Ward, 1992) and even, "a complete waste of time" (Pierce & Ollason, 1987). In particular Pierce and Ollason highlighted 6 main areas[1] in the basic assumptions of OFT they considered invalidated its use.

1. The inability to determine what natural selection maximizes

Pierce and Ollason pointed out that many of the behavioural traits of an individual are inter-connected, with many organisms combining foraging with searching for a mate and predator avoidance *etc.* They argued that as different activities are not independent, natural selection will act on the overall behaviour of the individual, resulting in no single activity being maximized. This was illustrated by Houston & McNamara's (1985) model of prey choice, in which forager's minimized their risk of starvation by selecting prey with minimal quality variation over prey with potentially greater gains, but also greater quality variability. In this case, the organism would forgo maximum gains in order to minimize the chances of starvation. This, however, does not necessarily infer that the organisms are not foraging in an optimal manner. Instead, the risk of starvation could be viewed as another selective pressure acting on OFT, possibly resulting in an animal considering starvation risk as a foraging cost (non-fuel cost), as much as energy expended by foragers when walking or flying (Seeley, 1986, Nonacs & Dill,

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[1] Pierce and Ollason discussed eight areas of perceived inadequacy in OFT, however, the last two points are discussed within points 1 to 6.

1990). Therefore, if foraging costs included the non-fuel cost of starvation risk, collecting a low energy source food with a reduced starvation risk may in fact be optimal, irrespective of whether energy gains are maximized. Thus, although individual activities are not independent, the overall foraging behaviour can still be described as optimal.

2. Variability in environmental conditions reduces the likelihood of optimal foraging behaviour evolving

As environmental conditions are variable, Pierce and Ollason argued that an animal's behavioural foraging traits would have to continually change to remain optimal, making it unlikely that optimal foraging behaviour would evolve. However, many animals are mobile, and actively seek out particular environments (Krebs, 1985). As a result, even in a highly dynamic environment, it is possible for an animal to relocate to a niche which it has optimally evolved to exploit (Pyke, 1984). Also, similar foraging strategies have been reported in animals of unrelated lineage within the same environment (Stearns & Schmid-Hempel, 1987). As similar foraging behaviour has evolved independently, it would tend to indicate that this behaviour is adaptive, if not optimal (Stearns & Schmid-Hempel, 1987)

3. Optimal strategies may not occur in nature

Pierce & Ollason also argued that even if natural selection did tend towards phenotypes which foraged in an optimal manner, optimal behaviour may not be realized due to three factors:

i) Optimal strategies may not yet have evolved.

ii) Animals may have an imperfect knowledge of their foraging environment, thus, making it impossible to forage in an optimal manner.

iii) The genetic variation of the animal may not occur in the direction necessary to permit evolution of optimal foraging behaviour.

Although such arguments highlight reason why optimal foraging may not occur, they do not invalidate the use of OFT to investigate the behavioural traits of an animal. OFT may even be useful in identifying species which do not forage in an optimally manner and provide an explanation for why this is so (Stearns, 1987). A good example of this is the ideal free distribution (IFD) theory (Fretwell & Lucas, 1970). IFD is an optimal foraging model which predicts an equilibrium distribution of foragers among patchy recourses. The model makes two assumptions, i) the forager has perfect knowledge of the gains it will derive from collecting any given unit of food, and ii) foragers can move freely between patches (Harper, 1982; Abrahams; 1986). Providing that these assumptions hold true, the model predicts that at equilibrium, the ratio of the foragers between forage sites should equal the ratio of the resources between those sites, *i.e.*

$$\frac{N_1}{N_2} = \frac{R_1}{R_2} \qquad (1)$$

where N_1 and N_2 equal the numbers of foragers at forage sites 1 and 2 respectively, and R_1 and R_2 equal the amounts or rates of food availability at those sites. This distribution should, therefore, result in the foragers maximizing the food they receive (Pulliam & Carcaco, 1984). However, when this model was tested, large systematic deviations were observed from the predicted (Abrahams, 1986). These deviations were characterized by an

under-use of the more profitable foraging sites and an overuse of the less profitable foraging sites. As a result Gray and Kennedy (1994) investigated errors in the IFD model using a further perception limited model (PLM). Here it was assumed that the IFD model was correct, but the assumption that foragers had perfect knowledge of food gains was false. Using the augmented model, it was subsequently possible to successfully test the predictions of the IFD model on nectar foraging bumblebees (Dreisig, 1995). Therefore, when using an OFT model, it was possible to identify a limitation in an assumption (perfect knowledge), correct it, and use the corrected model to successfully re-test the models assumptions. Although it may not always be possible to correct errors in OFT models, it should be possible to identify these errors and reject the model. Thus, Pierce and Ollason's comments do not invalidate the basis of OFT.

4. The existence of optimal strategies is untestable

Pierce and Ollason asserted that a major limitation of OFT is the inability to identify *a priori* the function of a given foraging strategy by an animal. As a result, an apparently optimal behaviour of an animal may simply be a function of another strategy, for which the animal is not optimally adapted (Pierce & Ollason, 1987). It is possible that this situation may occur, however, the true function of a behavioural trait can be tested through a range of manipulative experiments combined with linear behavioural models (Belovsky, 1994). It should, therefore, normally be possible to determine the function of any given behavioural trait of an organism (Stearns & Schmid-Hempel, 1987).

5. Functional hypotheses are untestable

As OFT models represent a simplification of nature (Cody, 1974), Pierce and Ollason suggested that reality is distorted as it is simplified. It was also argued that assumptions of the types and occurrence of food, and the range of possible behaviour by an animal, must be validated to enable OFT hypothesis to be tested. However, as many food types and behavioural traits do not have defined boundaries (McNeill, 1982), Pierce and Ollason argued that it is not possible to test basic assumptions relating to these elements within optimal foraging models. This could possibly result in OFT models which predict the correct foraging behaviour of an animal whilst the underlying assumptions regarding food types and range of behavioural traits are incorrect (Pierce & Ollason, 1987). If this assumption was true it would be impossible to test the overall validity of any optimal foraging models.

Although it may be reasonable to assume that assumptions and simplifications result in errors in OFT model predictions, the degree of such errors have been testable in previous studies and invalid assumptions rejected or altered (Schmid-Hempel, Kacelnik & Houston, 1985; Stearns & Schmid-Hempel, 1987; Seeley, 1986). However, any study for which basic assumptions, such as energy expenditure, cannot be tested should be viewed critically.

6. Optimal foraging models have not been tested

Pierce and Ollason suggested that many OFT models test for optimal behaviour using experimental conditions which violate the model in question. In particular, Pierce and Ollason pointed to the general assumption that foragers optimize a single behavioural trait, when in fact observed behaviour

is being influenced by multiple parameters (*e.g.* Pulliam, 1974; Charnov, 1976; Oaten, 1977). This may have been true of early OFT studies, however, more recently models have tended to consider numerous variables when attempting to predict foraging behaviour (*e.g.* Schmid-Hempel *et al.*, 1985; Engen & Stenseth, 1986; Ydenberg & Houston, 1986).

Pierce and Ollason also observed that most OFT studies seek agreement between observed foraging behaviour and OFT model predictions. This, they argued, is an incorrect approach, as hypothesis can only be disproved. Although it is reasonable to assume that a hypothesis can be rejected with more certainty than it can be confirmed (Popper, 1934), confirmation can still be of value (Stearns & Schmid-Hempel, 1987). It should also be noted that only after a number of independent confirmations is an OFT hypothesis generally excepted. Confirmation, can in certain circumstances, also be of benefit, as it is also possible to incorrectly reject a hypothesis *e.g.* it is possible that type I statistical errors will falsely reject 1 in 20 studies.

Although OFT has many limitations, and must be applied and interpreted with caution, there is now a large body of literature in which significant relationships have been observed between linear OFT model predictions and observed foraging behaviour (Table 1.0.1). It would, therefore, appear reasonable to assume that despite limitations, OFT is still a valid approach of investigating the foraging rules and strategies employed by animals.

Table 1.0.1. A summary of available linear programming diet model (LP) results. The number of cases refers to the number of independent attempts to validate LP predictions. The r^2 refers to the correlation coefficient reported in the study or computed from the data, where NA refers to an inability to compute the r^2 from the published data. Comments on studies general findings are also presented. (Modified from Belovsky, 1994)

Study	Species	No. of Cases.	r^2	Conclusion
Belovsky 1981	*A. alces*	2	NA	Energy maximizing
Belovsky 1984a	*Microtus pennsylvanicus*, *Tragelaphus strepsiceros*	3	0.95	Energy maximizing
Belovsky 1984b	*Castor canadensis*	1	NA	Energy maximizing
Belovsky 1986a	*Dissosteira carolina, Circottetix undulatus, Melanoplus sanguinipes M. femurrubrum, Microtus pennsylvanicus, Spermophilus columbianus, Sylvilagus nutalli, Marmota flaviventris, Antilocapra americana, Ovis canadensis, Odocoileus virginianus, O. hemionus, Cervus canadensis, Bison bison*	19	0.83	Energy maximizing, except for during the rut when efficiency = time minimizing
Belovsky 1986b	Data on mammalian herbivores from the literature	28	0.86	Energy maximizing
Belovsky 1987a	Human hunter-gathers (data where parameters are available from specific studies) (data combined from numerous studies)	5	0.99	Energy maximizing
		60	0.93	Energy maximizing
Belovsky 1987b	*Microtus pennsylvanicus*	6	0.76	Energy maximizing
Belovsky 1987c	*Odocoileus virginianus*	1	NA	Energy maximizing
Belovsky & Slade 1987	*Equuscaballus, Ovis aries, Bos taurus*	4	0.98	Energy maximizing
Belovsky 1990	*Molothrus ater*	1	NA	Energy maximizing
Belovsky 1991	*Rangifer tarandus*	8	0.96	Energy maximizing
Edwards 1993	*Marmota flaviventris*	12	0.69	Energy maximizing
Doucet & Fryxell 1993	*Castor canadensis*	10	0.57	Energy maximizing
Karasov 1985	*Ammospermophilus leucerus*	1	NA	No result because of missing constraint
Owen-Smith 1993	*Tragelaphus strepsiceros*	3	0.99	Energy maximizing
Ritchie 1988	*Spermophilus columbianus*	132	0.94	Energy maximizing
Ritchie & Belovsky 1990	*S. Columbianus*	20	0.93	Energy maximizing
Schmid-Hempel *et al.*, 1985	*Apis mellifera*	13	NA	Energy efficiency
Schmitz 1990	*Odocoileus virginianus*	6	0.99	Energy maximizing
Spalinger 1980	*O. hemionus*	1	NA	No result because of missing constraint
Vulink & Drost 1991	*Bos taurus*	14	NA	Energy maximizing

One group of organisms for which many of the problems encountered in optimal foraging studies do not apply are the eusocial Apoidea super family, in particular the honeybee's (Apini: *Apis*) and bumblebee's (Bombini: *e.g. Bombus*)[2]. Not only are honey and bumblebee's central place forager's (bound permanently to their hive), but the haploid / diploid nature of their life history (Figure 1.0.1) produces worker caste forager's which are infertile (Heinrich, 1979; Seeley, 1985; Winston, 1991). As the workers are infertile, they do not mate, and restrict their behaviour to hive duties or foraging tasks (Heinrich, 1979, Makela, Rowell, Sames & Wilson, 1993). Temporal polytheism also results in any non-foraging tasks, such as hive duties and colony defense, being performed by non-foraging caste workers (Heinrich, 1979; Seeley, 1985; Winston, 1991). As a result, forager's perform only foraging tasks, making the collection of optimal foraging behaviour data particularly easy compared to data collection on organisms which combine foraging with mating or defensive behaviour (Dukas & Real, 1991).

Testing linear foraging models, using observations of foraging behaviour, is also helped by the relatively simple food the forager's collect; consisting of nectar (sugar and water) for carbohydrates, and pollen for protein (Sladen, 1912; Von Frisch, 1967). As polytheism restricts an individual forager to collecting only nectar or pollen, estimates of foraging gains may be easily observed. Bee's may also be trained to artificial flower patches (Von Frisch, 1967), so that volume and sugar content of nectar collected during a foraging trip (gains) may be controlled (Schmid-Hempel *et al.*, 1985; Seeley, 1986; Wolf & Shmid-Hempel, 1990).

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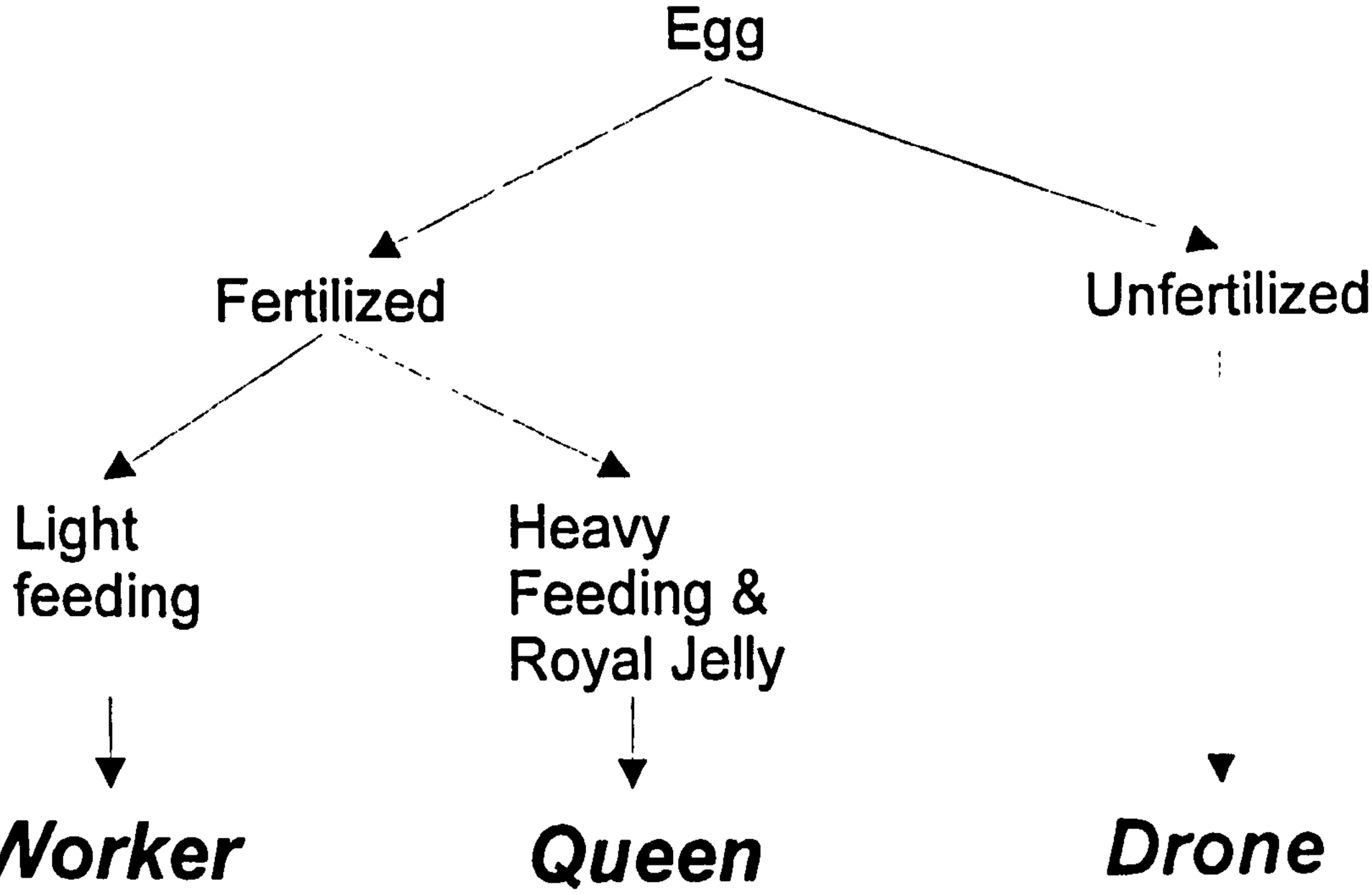
[2] The term bumblebee refers to *Bombus* spp unless otherwise stated. Individual species of *Bombus* will be given were necessary.

Figure 1.0.1 Haploid / Diploid relationship between the production of workers, drones and queens from queen laid eggs (altered from Winston (1991)).



When a queen lays an egg, she can produce offspring with two distinct genotypes: 1) Haploid (unfertilized) offspring with only one set of genes derived from the queen; these offspring will develop into males, otherwise referred to as drones. 2) Diploid (fertilized) offspring, with genes derived from both the queen and one of the drones with which the queen has mated. Diploid offspring are all female and may develop into either worker castes or subordinate queens. The factors determining whether workers or queens are produced is related to the cell size the egg is laid in, and the food the developing larvae are fed on (Winston, 1991).

Due to the relative ease with which the behaviour of honey and bumblebees can be observed and quantified, their optimal foraging strategies have been widely studied (*e.g.* Heinrich, 1976a; Pyke, 1978; Laverty, 1980; Pyke, 1980; Real, 1981; Heinrich, 1983; Willmer, 1983; Hodges, 1985; Schmid-Hempel *et al.*, 1985; Seeley, 1986; Willmer, 1986; Cartar & Dill, 1990a; Cartar & Dill, 1990b; Wolf & Schmid-Hempel, 1990; Cartar, 1991; Greggers & Menzel, 1993; Seeley, 1994; Willmer, Bataw, & Hughes, 1994; Dreisig, 1995).

1.0.1 Intra-patch nectar foraging behaviour of honey and bumblebees

Honey and bumblebees collect nectar from flowering plants. The foraging cycle involves the bee leaving the hive and flying to a nearby flower "patch", usually within a 9 km radius of the hive (Seeley, 1985). Once at the patch, the bee lands on a suitable flower and searches for nectar. When located, the bee draws up the nectar, storing it in an enlarged section of the esophagus called the crop or honey stomach. Once the nectar has been collected, the bee flies to a subsequent flower and collects another "parcel" of nectar. This continues until :

i) The bee's crop is full.
ii) No nectar remains in the patch.
iii) Some form of foraging strategy results in the bee leaving the patch before the crop is full.
iv) The bee is disturbed.

Once the forager has returned to the hive, the bee unloads the collected nectar and typically commences another foraging cycle.

It should, however, be pointed out that sugar content and volume of nectar produced by flowers within the patch are highly variable (Dukas & Real, 1993a), even within plants of the same species located in the same area (Heinrich, 1979a; Pleasants & Zimmerman, 1979; Teuber & Barnes, 1979; Willmer, 1983; Zimmerman, 1981, 1983; Willmer, 1986; Real & Rathcke, 1988; Willmer, 1988; Creswell, 1990; Waser & Mitchel, 1990; Dukas & Real, 1993a). Foraging bees would, therefore, benefit from the ability to identify and forage at nectar rich flowers. It appears that both honey and bumblebees have evolved (or co-evolved with flowering plants) a number of traits which enable them to do just this (*e.g.* Roberts, 1979; Corbet, Kerslake, Brown & Morland, 1984), including:

1) Direct assessment. Where the forager lands on a flower, and uses her glossa to directly determine nectar content by probing the umbel (Pyke, 1978).

2) Visual stimuli. It has also been reported that foragers may be able to visually estimate the nectar content of a flower without the need to land and probe the umbel (Thorp, Briggs, Estes & Ericson, 1975; Kevan, 1976). The visual stimuli used by the forager appears to be the ultraviolet (UV) absorption patterns of some nectars (Thorpe *et al.*, 1975). As honey and bumblebees are capable of visualizing light at UV wavelengths (Weiss, Soraci & McCoy, 1943), it has been argued that a forager can estimate the quantity of nectar contained within a flower from the UV patterns on and within the umbel (Thorpe *et al.*, 1975).

3) Pheromone Marking. There is now growing evidence that bees can, to some degree, mark individual flowers with pheromones, indicating that they are either rewarding (Nunez, 1967; Ferguson & Free, 1979; Cameron 1981;

Marden, 1984; Giurfa & Nunez, 1992), or non rewarding (Frankie & Vinson, 1977; Free & Williams, 1983; Wetherwax, 1986; Schmitt & Bertsch, 1990). As a result it may be possible for bees to avoid landing on non rewarding flowers and concentrate their foraging effort on more rewarding plants.

4) Scent discrimination. It has also been suggested that bees can discriminate between nectar rich and nectar poor flowers by the scent of nectar (Heinrich, 1979a). In particular, odors released from volatile products of yeast metabolism within nectar (Crane, 1975; Williams, Hollands & Tucknott, 1981) may indicate nectar presence or absence.

5) Age selection. Foragers may also be able to distinguish between flowers of different ages *via* traits such as petal quality and colour (Gori, 1983). A good example of age selection was reported by Willmer *et al.* (1994), who observed five species of *Bombus* spp foraging preferentially to younger flowers of the raspberry plant (*Rubus idaeus*).

Although their have been many suggestions as to how honey and bumblebees may select nectar rich flowers, it is unlikely that any one of the above strategies are used in isolation, instead it is more likely that bees use a range cues combined with past experience to determine which flowers will be the most profitable (Corbet *et al.*, 1984).

Although foragers may use some, or all, of the above stimuli to detect nectar rich flowers, bees also have to optimally allocate foraging time and effort within the patch to best maximize fitness. For this reason, bees appear to search for nectar in a systematic manner (Dreisig, 1995) tending to preferentially forage to larger flowers (Wilson & Price, 1977; Thomson, Maddison & Plowright, 1982; Bell, 1985; Andersson, 1988; Schmid-Hempel &

Speiser, 1988) and restrict their inter-flower flight distance and orientation (Dreisig, 1995). The distance which a bee flies, when traveling to a subsequent flower, appears largely dependent on the previous experience of the forager (Dukas & Real, 1993a). It has been observed that bees will decrease their inter-flower flight distance when previously encountered nectar rewards were high (Heinrich, 1979b; Dukas & Real, 1993a). Also when previously encountered nectar rewards were low, foragers generally increase inter-flower distances, "ignoring" many flowers in the process (Dreisig, 1995). It has also been reported that increased inter-flower flight distance is accompanied by an increase in the random probing of previously unvisted flowers by the forager (Heinrich, 1979b). Previous experience also seems to affect the direction in which the inter-flower flights occur. Bees tend to leave flowers in the same direction in which they arrive, if the encountered nectar rewards are low. However, if nectar rewards are high, bees tend to change orientation (Pyke, 1978; Heinrich, 1979b). The combined effect of decreasing inter-flower flight distance and alteration in flight direction results in restricting foraging to a small patch of nectar rich flowers. If the quality of this patch decreases, the resultant increase in inter-flower flight distance and flight direction consistency will result in the forager moving to alternative sites where nectar rewards may be higher (Heinrich, 1979b). As individual flowers in a patch vary greatly in their nectar content (as discussed previously), bees which alter inter-flower flight distances and direction based solely on the experience of the last flower visit would display highly variable and inefficient foraging behaviour (Dukas & Real, 1993a). As a result it would appear that bees are capable of integrating the experience of up to 3 previous flower visits when determining what the subsequent inter-flower flight distance and direction should be (Dukas & Real, 1993b). The ability of bees to integrate past experience from more than 3 flowers appears to be limited due to the inability of the bee to memorize more information (Heinrich, 1976a; Laverty,

1980; Woodward & Laverty, 1992, Greggers & Menzel, 1993; Dukas & Real, 1993b).

Also associated with limited memory is the bees ability to learn and memorize how to efficiently handle nectar once a flower has been located. Ideally, a bee would forage to the flowers within a patch which provided the richest source of nectar, irrespective of the plant's species or morphology. However, this does not appear to be the case, with bees restricting themselves to foraging at between 1 to 3 plant species only (Heinrich, 1976a; Laverty, 1980; Woodward & Laverty, 1992, Greggers & Menzel, 1993). This action appears to be largely due to the limited memory of honey and bumblebees, which restricts the ability of bees to memorize the skills necessary to handle nectar from large numbers of varying flower types efficiently (Dukas & Real, 1991; Dukas & Real, 1993a; Dukas & Real, 1993b; Greggers & Menzel, 1993). It nas also been suggested that the skills learnt when handling one species of flower may "interfere" with handling skills learnt from other species (Woodward & Laverty, 1992; Dukas, 1995) which again would result in honey and bumblebees limiting the number of flower species they could efficiently forage at.

Also, it has been reported that honey and bumblebees often develop foraging "routes", or "traplines", repeatedly visiting the same flowers on every foraging cycle over periods ranging from a few hours to a few days (Corbet *et al.*, 1984; Dreisig, 1995). The foragers also tend to randomly sample previously unvisited flowers whilst flying along a trap line (Corbet *et al.*, 1984). This enables the bee to include newly encountered nectar rich flowers, and exclude previously encountered nectar poor flowers, in subsequent trap line foraging trips (Dreisig, 1995). The viability of trap line foraging does, however, depend on the ability of the flowers to produce sufficient nectar in-

between forager visits to make the foraging trip economically viable to the bee. This not only depends on the innate ability of the plant to synthesize nectar, but also on the ambient air temperature, humidity and age of plant (Willmer, 1986; 1988). Where conditions facilitate rapid synthesis of nectar, trap line foraging may be of benefit to a bee as foragers do not have to spend time searching for nectar rich flowers as these have been previously located.

1.0.2 Differences in the foraging behaviour of honey and bumblebees

Although the within patch foraging behaviour of honey and bumblebees is similar, there are a number of notable differences between the two genera. In particular, it has been reported that bumblebees tend to preferentially forage at flowers providing constant, and thus predictable, nectar rewards (Real, 1981, Wadington, Allen & Heinrich, 1981; Real, Ott & Silverfire, 1982; Real & Carco, 1986; Cartar & Dill, 1990a; Cartar, 1991) whilst honeybees forage at flowers irrespective of variability in nectar contents (Banschbach & Waddington, 1994). The preference, by bumblebees, for flowers which provide a constant supply of nectar has been describe as "risk sensitive" foraging (Cartar & Dill, 1990a; Cartar, 1991) and appears to be a strategy which reduces the probability of energy short fall whilst foraging (Houston & McNamara, 1985). Energy short fall may occur in a patch of nectar variable flowers, even if the total nectar content of all flowers in the patch is high. This is because a forager may only be able to gather nectar from a small percentage of the flowers in the patch and only encounter flowers with low nectar rewards. As a result, the energy obtained by collecting the nectar may be less then the energy expended through flight and handling costs. If, however, the forager could visit all flowers in the patch, variability in nectar content becomes irrelevant, as only total patch nectar content need be considered (Banschbach & Waddington, 1994). As bumblebees have

relatively small colony populations, usually not exceeding 400 individuals (Heinrich, 1979a), the colony's foragers have a significantly lower probability of visiting every flower in a given patch than honeybee foragers, whose colony population may exceed 100,000 workers (Winston, 1991). The relatively large worker population of honeybee colonies, however, is likely to enable foragers to visit all flowers within a patch on a regular basis. As a result, honeybees are unlikely to experience short term energy deficits due to foraging at nectar variable flowers (Banshbach & Waddington, 1994), thus, explaining why bumblebees are risk sensitive foragers whilst honeybees are risk averse.

The relatively large population size of honeybees may also explain other intra-patch differences in the foraging behaviour of honey and bumblebees. As in the case of risk sensitivity, large population sizes tend to reduce any deleterious effects of variability or "mistakes" whilst foraging Houston & MacNamara (1985) argued that the risk of a honeybee colony starving following a mistake by a forager is insignificant when compared to a bumblebee colony. In other words, honeybees do not need to be as efficient as bumblebees when foraging in order to maximize fitness. This may explain some of the difference in foraging behaviour observed by Willmer *et al.* (1994) when studying the behaviour of honey and bumblebees foraging at raspberry plants (*R. idaeus*), variations in behaviour included:

1) Bumblebees made a higher percentage of visits to the flowers (approximately 60% of all visits).

2) Bumblebees were able to more efficiently select and forage at younger flowers than honeybees.

3) Bumblebees visited more flowers per minute than honeybees.

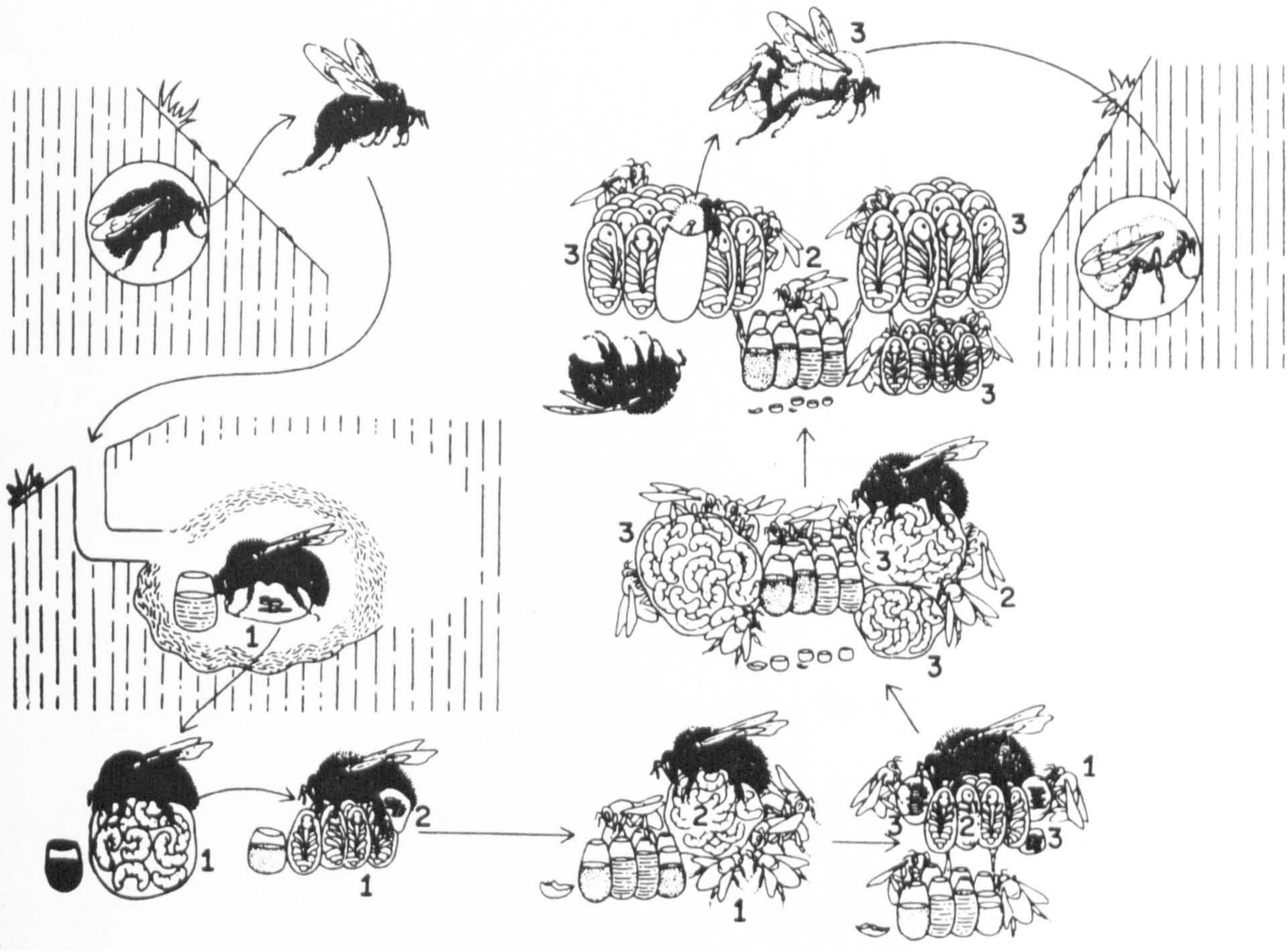
4) Bumblebee foragers were more frequently present in the morning during peak pollen dehiscence.

In general, Willmer *et al.* (1994) found that bumblebees foraged more efficiently than honeybees, possibly reflecting the greater costs experienced by bumblebees of inefficient foraging behaviour.

Another major difference between the two genera is the ability of honeybees to communicate with one another (Von Frisch, 1967). Unlike bumblebees, when honeybees return to the hive, kinetic, audio and trophallaxis communication are used by foragers to indicate the position and quality of potential flower patches (Seeley, 1985; Gould & Gould, 1988; Winston, 1991). This has major implications when considering OFT, as a foraging honeybee may have prior knowledge of the potential costs and gains that may be encountered when collecting nectar from a flower patch it has not yet visited (Seeley, 1994). As a result, foraging honeybees can avoid patches of low quality, and concentrate their foraging effort in patches from which relatively high energy returns can be derived. Bumblebees, however, can only obtain information by visiting the patch (as discussed previously).

There are also differences in the life history of the two genera, which may significantly affect the way they optimize their foraging behaviour (Figure 1.0.2a & 1.0.2b). Unlike bumblebees, honeybees maintain a colony throughout the whole year, provisioning the hive with sufficient nectar and pollen to survive the winter. Bumblebee colonies, however, die at the end of each foraging season, with only fertilized queens surviving the winter through hibernation. As a result, it would appear reasonable to assume that the

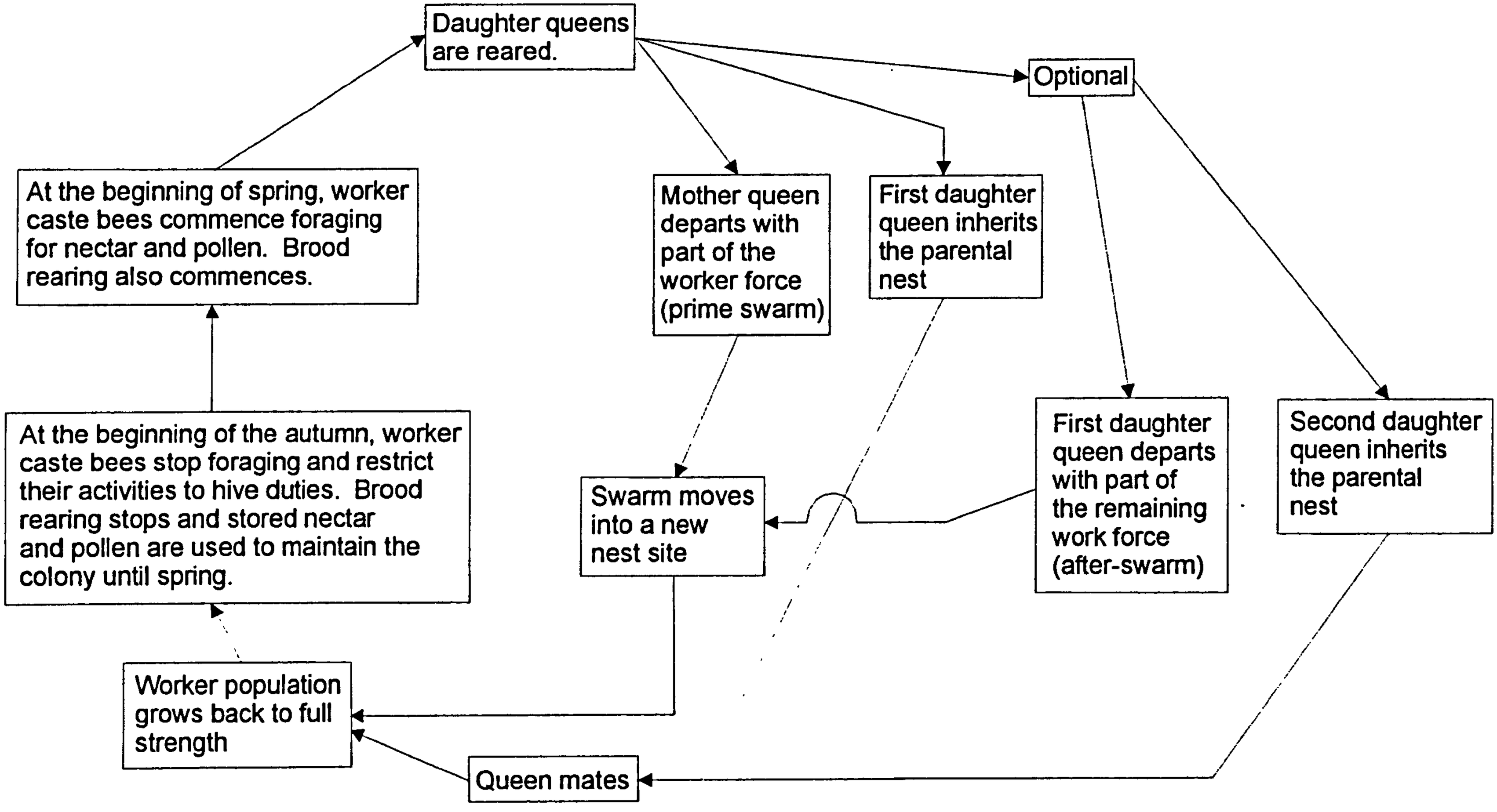
Figure 1.0.2a Annual life cycle of a bumblebee colony



(No. 1-3 indicates the brood generation)

Diagram of a bumblebee colony cycle, from the queen emerging from hibernation (left) to new queens (lightly stippled) emerging from cocoons of the third brood (eggs at lower right), mating, and hibernating (right). Note progression of eggs of specific brood packets to become larvae, pupae, and adults, and the use of empty cocoons for honey or pollen (stippled) storage. The diagram indicates the production of two worker broods and one queen brood, the latter from three separate egg batches. (After Heinrich, 1979).

Figure 1.0.2b. Principle events in the life cycle of honeybee colonies (Modified from Wilson, 1971)



strategy which would confer the greatest fitness to a bumblebee colony would be to produce as many reproductives (drones and queens) as possible, thus maximizing the chances that the genes of the colony will be passed on to the next generation (Heinrich, 1979). Although honeybee colonies also produce reproductives, reproduction also occurs in conjunction with swarming (Seeley, 1985; Winston, 1991). Swarming occurs when a hive worker population reaches a sufficient size for the colony to divide, with one portion of the colony leaving the hive with a subordinate queen, swarming and establishing a new colony. The dominant queen and remainder of the workers stay in the original hive, and begin to build up the colony numbers again. It is, therefore, important that a colony's worker population increases rapidly, allowing the colony to swarm and reproduce as many times in one season as possible. The number of workers in a honeybee colony is also important during over-wintering, as larger colonies are better able to regulate hive temperature (Seeley, 1985). Larger colonies also have a readily available supply of workers, who can commence foraging immediately the weather conditions permit in the spring (Beauchamp, 1992). As a result, it is possible that number of workers is more important to a honeybee colony than is the number of reproductives. This in turn may have considerable implications when considering the optimal foraging strategies which the two genera may employ.

There is one other difference between the two genera which is important when considered energy expenditure, the ability of bumblebees to continue foraging at low temperatures. Generally honeybees do not forage when ambient temperature drops below 15^{o}C (Seeley, 1985, Winston, 1991), although many bumblebee species have been regularly observed foraging at temperatures between 6 and 7^{o}C (large Queen's of *B. vosnesenskii* and *B. edwardsis* have also been reported flying at temperatures nearing 0^{o}C

(Heinrich, 1993)). Bumblebees can fly at low temperatures largely due to the ability of the bees to elevate their thoracic body temperature, *via* shivering thermogenesis, to levels necessary for flight (Heinrich, 1979a; 1993). Shivering thermogenesis is a process were the dorsal longitudinal muscles and dorso-ventral muscles are contracted. As the two sets of opposing muscles are contracted simultaneously, the contractions are tetanic, producing heat but little motion (Ikeda & Boettiger, 1965). The reduction in motion during the contractions results in a number of benefits:

1. Noise levels are reduced, decreasing any interference in communication within the nest.
2. Reducing vibration decreases any wing damage which may occur as a result.
3. Reduction in vibration will also decrease the quantity of pollen lost from the bees body hair during heat production.

It has, however, been demonstrated that honeybees are also capable of shivering thermogenesis (Bastian & Esch, 1970; Esch & Groller, 1991), but are still incapable of flying at temperatures as low as those observed in bumblebees. This would appear to be largely a function of the low surface area / body volume ratio of honeybees relative to the high ratio observed in bumblebees. As the efficiency of shivering thermogenesis to increase thoracic temperatures decreases with decreased surface area / body volume ratio (Heinrich, 1993), the energetic cost of thermoregulation in honeybees are greater than that for the larger bumblebees. As a result, flight (and hence foraging) for honeybees below temperatures of 15^{o}C may simply be energetically too expensive.

1.0.3. Optimal foraging behavior of honey and bumblebees

When studying the optimal foraging behaviour of honey and bumblebees, foragers have been observed to regularly abandon flower patches and return to their hive prior to filling their crops, even when nectar remains in the flower patch (Schmid-Hempel *et al.*, 1985; Seeley, 1986; Carter & Dill, 1990a; Wolf & Schmid-Hempel, 1990). To explain this behaviour, it has been suggested that bees could be maximizing one of two currencies; i) the Rate of Net Energy Gain[3] (RNEG), where foragers maximize the rate of nectar delivery to the hive per unit time, and ii) Net Energy Efficiency (NEE) where foragers maximize their energetic gain per unit of energy expended whilst foraging.

The rationale behind the RNEG currency was based on the belief that fitness of the forager was maximized by delivering the maximum energetic value of nectar to the hive in the shortest possible time (Pyke, 1978; 1980). As a result, bees maximizing RNEG would abandon a patch before filling their crop only if nectar gains decreased with time (Orian & Pearson, 1979). Initially, this approach appeared to fit well with the observed behaviour of foragers in the field (Heinrich, 1983; Pyke, 1984; Cheverton, Kacelink & Krebs, 1985; Harder & Real, 1987). However, as foraging experiments using artificial flowers were used to test RNEG models, discrepancies became apparent between observed behaviour and model predictions. In particular, it was widely reported that honeybees were regularly observed abandoning non-depleting flower patches with only partially filled crops (Schmid-Hempel *et al.*, 1985; Seeley, 1986; Wolf & Schmid-Hempel, 1990). As this phenomenon was counter to predictions from RNEG models, two possible conclusions could be drawn; i) there are constraints on optimal foraging behaviour which

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[3] RNEG is also referred to as the Rate of Net Energy Intake (RNEI).

are not accounted for by energy gains and costs alone, or ii) honeybees do not maximize RNEG.

Honeybee foragers depend heavily on information they can obtain about the cost and gains of foraging from any particular flower patch (Frisch, 1967). Because of this, Nunez (1982) suggested that partial crop loading observed in these species might be a result of a forager's need to acquire information on the potential net gains which could be acquired by foraging in an alternative flower patch. This in turn would permit foragers to concentrate their foraging effort in the most rewarding patches available. As such information can only be acquired by returning to the hive, and communicating with other workers which have foraged at alternative patches, Nunez argued that foragers may benefit from prematurely abandoning a patch in order to acquire this information. As this strategy would result in honeybees collecting partial crop loads, even in non-depleting flower patches, it could explain the discrepancy between the behaviour of the bees observed in the field and RNEG predictions. Honeybees, however, are unique amongst nectar foragers in their ability to communicate the location, costs and quality of a flower patch (Frisch, 1967, Gould & Gould, 1988). If, therefore, Nunez's explanation of partial crop loading in non-depleting patches is correct, other nectivorous organisms, without the ability to communicate similar information, should not display partial crop loading in non-depleting patches. This, however, is not the case, with partial crop loading also being reported by Wolf & Hainsworth (1977) when observing the behaviour of Hummingbirds foraging to artificial flowers containing *ad libitum* nectar. Nunez's information centered hypothesis is not necessarily, therefore, an adequate explanation of the variations between the observed behaviour of foraging honeybees and RNEG predictions.

Another explanation for partial crop loading came from work by DeBenedictis, Gill, Hainsworth, Pyke & Wolf (1978) on hummingbirds. They suggested that time may not be as important a variable in central place foraging as was previously thought. Instead, DeBenedictis *et al.* (1978) suggested that hummingbirds optimized their foraging behaviour by maximizing their Net Energy Efficiency (NEE)). A hummingbird following this strategy not only has to account for the cost of collecting the nectar, but also the added cost of transporting the nectar. As the weight of collected nectar increases, so do the costs of transporting the nectar. The costs of collecting nectar will thus continue to increase with every flower visited, until a point is reached where the gains from collecting a further parcel of nectar would not improve the ratio of gains to costs derived from the foraging trip. When this point is reached, a hummingbird maximizing NEE will abandon the patch. Thus, maximizing NEE provides an alternative explanation for why some nectar foragers abandon non-depleting flower patches with partial crop loads.

As hummingbirds and honeybees both forage for nectar, it was suggested that honeybees might also be maximizing NEE rather than RNEG (Schmid-Hempel *et al.*, 1985). To determine the validity of this hypothesis, Schmid-Hempel *et al.* (1985) produced a mathematical model capable of predicting a honeybees foraging behaviour when maximizing NEE and RNEG. Schmid-Hempel *et al.* (1985) then trained honeybees to an artificial flower patch, where the quantity and energy content of nectar collected was controlled. The observed number of flower visits was then compared to model predictions for the same bee when maximizing NEE and RNEG. It was found that honeybees also appeared to maximize NEE. Subsequent foraging studies on honeybees have also found that NEE predictions of foraging behaviour closely match the behaviour of the bees observed in the field (Seeley, 1986; Wolf & Schmid-Hempel, 1990).

The evolutionary rationale behind honeybee foragers maximizing NEE is linked to two factors i) worker life span, and ii) colony population size.

It has been shown that as energy expenditure in honeybees increases, so their life expectancy decreases (Neukirch, 1982; Schmid-Hempel & Wolf, 1988). Foragers maximizing RNEG and returning to the hive with full crops (when foraging at a non-depleting flower patch), will experience greater flight costs than bees maximizing NEE, which only collect partial crop loads. As increased flight costs reduces life expectancy, a bee maximizing NEE will, on average, be able to complete more foraging cycles before dying than a bee maximizing RNEG. As a result, although a bee maximizing NEE may collect less nectar on each foraging cycle, the total quantity of nectar collected over the life time of the bee may be greater than a bee which maximizes RNEG (Shmid-Hempel *et al.,* 1985). This in turn would convey an increased fitness to the colony, as quantity, rather than rate, of nectar delivery is maximized.

The size of the worker population of a colony is also linked to life expectancy, with increased longevity of workers increasing the population size of the colony (Beauchamp, 1992). As the population size of the colony is important with respect to the ability of the colony to swarm and survive the winter (discussed above), increased worker longevity and the resultant increase in population size would again confer an increased fitness on the colony.

Although there is now strong theoretical and empirical support for the view that honeybees maximize NEE when foraging for nectar, evidence that bumblebees also maximize NEE is lacking. This is largely due to the failure of previous attempts to train bumblebees to forage from artificial flowers (Wolf *pers. com.*). Because of this, it has not been possible to test theoretical predictions of foraging behaviour with the observed behaviour of bumblebees

in the field. This is important, as although it may appear reasonable to assume bumblebees optimize their foraging behaviour in the same manner as honeybees, differences in the behaviour and life histories of the two genera (as discussed previously) may result in their fitness being maximized by using different foraging strategies.

The aim of this study was, therefore, to obtain accurate measurements of energy gains (using artificial flower patches) and costs (using the Doubly Labelled Water technique) of bumblebees foraging for nectar. Comparisons between observed foraging behaviour and NEE / RNEG model predictions could then be used to determine which, if either of the two currencies was maximized by nectar foraging bumblebees.

Chapter 2

Do current optimal foraging models accurately predict the nectar foraging behaviour of bumblebees?

2.0.0 Do bumblebees maximise Net Energy Efficiency, Rate of Net Energy Gain or another currency when foraging for nectar?

Introduction

To explain the foraging behaviour of bumblebees whilst collecting nectar, three factors must first be determined; i) the energy expenditure of the bee whilst foraging (θ), ii) the energy gained by the bee from collected nectar (G), iii) and the time taken to collect the nectar and deliver it to the hive (T). When combined, the variables θ, G and T can be used to predict the optimum number of flower visits a bee should make during a foraging cycle in order to maximise NEE or RNEG. The predicted number of flower visits can then be compared with the observed number of flower visits, and the currency being maximised identified.

To predict the volume of nectar a bee should collect when maximising NEE and RNEG, the maximum value of NEE and RNEG must be determined. This was done using the Schmid-Hempel *et al.* (1985) model (hereafter referred to as the SH model).

2.0.1 Calculating energy gains of a bee whilst foraging

Energy gains of a bee over one foraging cycle can be calculated as:

$$G = nCw \qquad (1)$$

where n = the number of flowers visited, C = weight specific energetic value of nectar (assuming the energy value of sucrose equals 16.7 J mg^{-1}) and w = the mass of nectar collected on each flower visit (mg).

2.0.2 Estimating energy costs incurred by a bee whilst foraging

Initially, the bee must fly from the hive to the flower patch. Assuming the unloaded metabolic rate of a flying bee is a_o (Watt), the cost of flying to the patch in τ_0 seconds can be calculated as $a_o\tau_0$.

The bee then visits n flowers in the patch, collecting w mg of nectar from each flower visit, and taking h seconds to handle the nectar at each flower. Whilst the bee is collecting nectar on the flower, Schmid-Hempel *et al.* (1985) assumed that bees reduce their energy expenditure to resting or walking metabolic rates (a_h (Watt)). After handling the nectar, the bee will take off and fly to the next flower in the patch. As the bee has collected w mg of nectar, her body mass will also have increased by w mg. This increase in

body mass will result in the bee expending more energy whilst flying, thus incrementally increasing the energy cost of every subsequent flower visit. The increased costs of inter-flower flights can be calculated by the incremental increase in flying metabolic rate with load (a Watts), following each flower visit. If, therefore, a bee visits n flowers, with an inter-flower flight time of τ seconds, the total energy expenditure of the bee whilst foraging in the flower patch (C_p) is calculated as:

$$C_p = a_0(n-1)\tau + a\frac{n(n-1)}{2}w\tau + a_h nh \qquad (2)$$

Total energy expenditure during the flight to the flower patch and back to the hive (C_t), can be calculated using equation 3:

$$C_t = a_0\tau_0 + (a_0 + a(Cwn))\tau_0 \qquad (3)$$

Once the bee has returned to the hive, it must deliver the nectar to the honey pots. Thus, if the bee spends T_0 seconds in the hive, with a metabolic rate of a_r (Watts), energy expenditure in the hive will equal $T_0 a_r$.

Total energy expenditure per foraging cycle (C), can, therefore, be estimated as:

$$C = C_p + C_r + T_O a_r \qquad (4)$$

Combining measurements of energy gains, and estimates of energy costs, allows NEE and RNEG to be calculated using:

$$NEE = (G - \theta) / \theta \quad (5)$$

and

$$RNEG = (G - \theta) / T \quad (6)$$

Assuming it is possible to determine the times and distances flown by a bee during a foraging cycle, and estimate the bees metabolic rate, equations (5) and (6) can be used to predict the number of flowers a bee should visit in order to maximise NEE and RNEG.

The aim of this experiment was, therefore, to train bumblebees to forage for nectar at two artificial flowers, recording their behaviour, and to compare the number of flowers the bees visited in these experiments with the number of flowers predicted by the two foraging models.

2.1.0 Materials & Methods

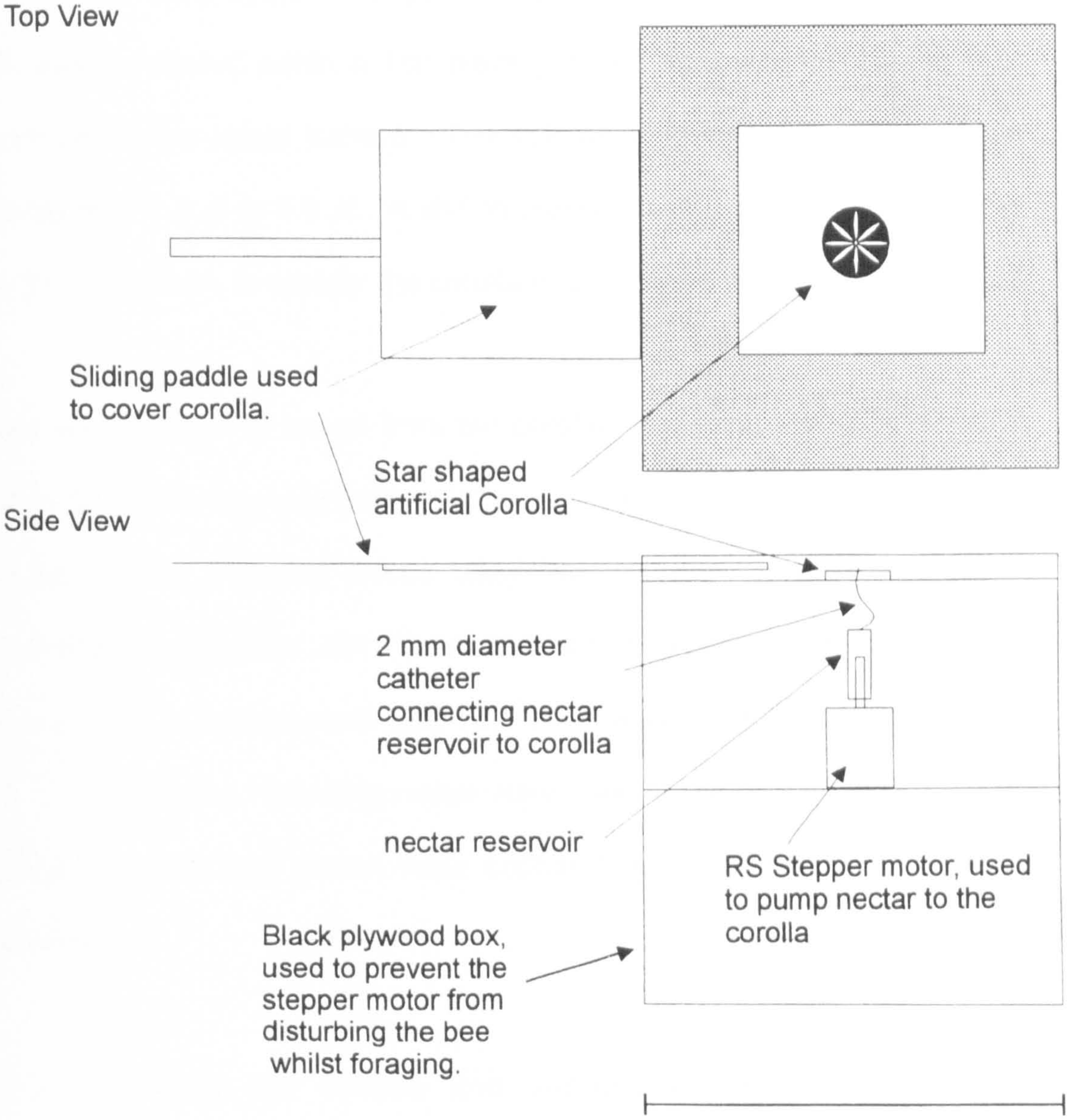
2.1.1 Training bumblebees to artificial flower patches

As the volume and sucrose concentration of nectar in natural flowers cannot be controlled or accurately quantified, foraging bumblebees were trained to artificial flowers (Figure 2.1.1). To provide stable environmental conditions, the artificial flowers were situated in a 150m x 150m Forshaw glasshouse. The internal temperature of the glasshouse was regulated at 22±(sd)1°C (unless otherwise stated, ± will hereafter refer to the standard deviation of a mean), and a relative humidity of 65±2%[1]. The glasshouse contained a mature crop of tomatoes (*Lycopersicum esculentum*), which provided an available source of pollen but no nectar (Wareing & Philips, 1986).

Due to the difficulty in capturing and rearing wild bumblebee colonies, all experiments were performed on bees removed from artificial colonies of *Bombus terrestris*, purchased from Biobest Ltd, Kent. Bumblebee colonies were placed in the glasshouse one week prior to commencing the foraging experiment. This permitted time for the colony to settle following re-location of the hive.

[1]Temperature and humidity within the glasshouse was regulated by a Staern environ regulator system (Staern environ Ltd, Gwent, Wales).

Figure 2.1.1
Design of artificial flower, to which *B.terrestris* were trained (Wolf & Shmid-Hempel, 1990).



The artificial flowers (Figure 2.1.1) were designed following Wolf & Schmid-Hempel (1990), and consisted of an RS™ 19 stepper motor, attached to a nectar reserve. When activated (*via* a remote control box), the stepper motor pumped a known volume of nectar from the nectar reserve to a star shaped blue corolla. To prevent the stepper motor from disturbing foraging bees, the unit was concealed within a $1m^3$ black plywood box, with the corolla fixed externally to the upper surface. The volume of nectar delivered could be altered from 1.2 μl to 0.6 μl. A sliding plastic cover was also attached over the flower surface, to enable the corolla to be covered or exposed.

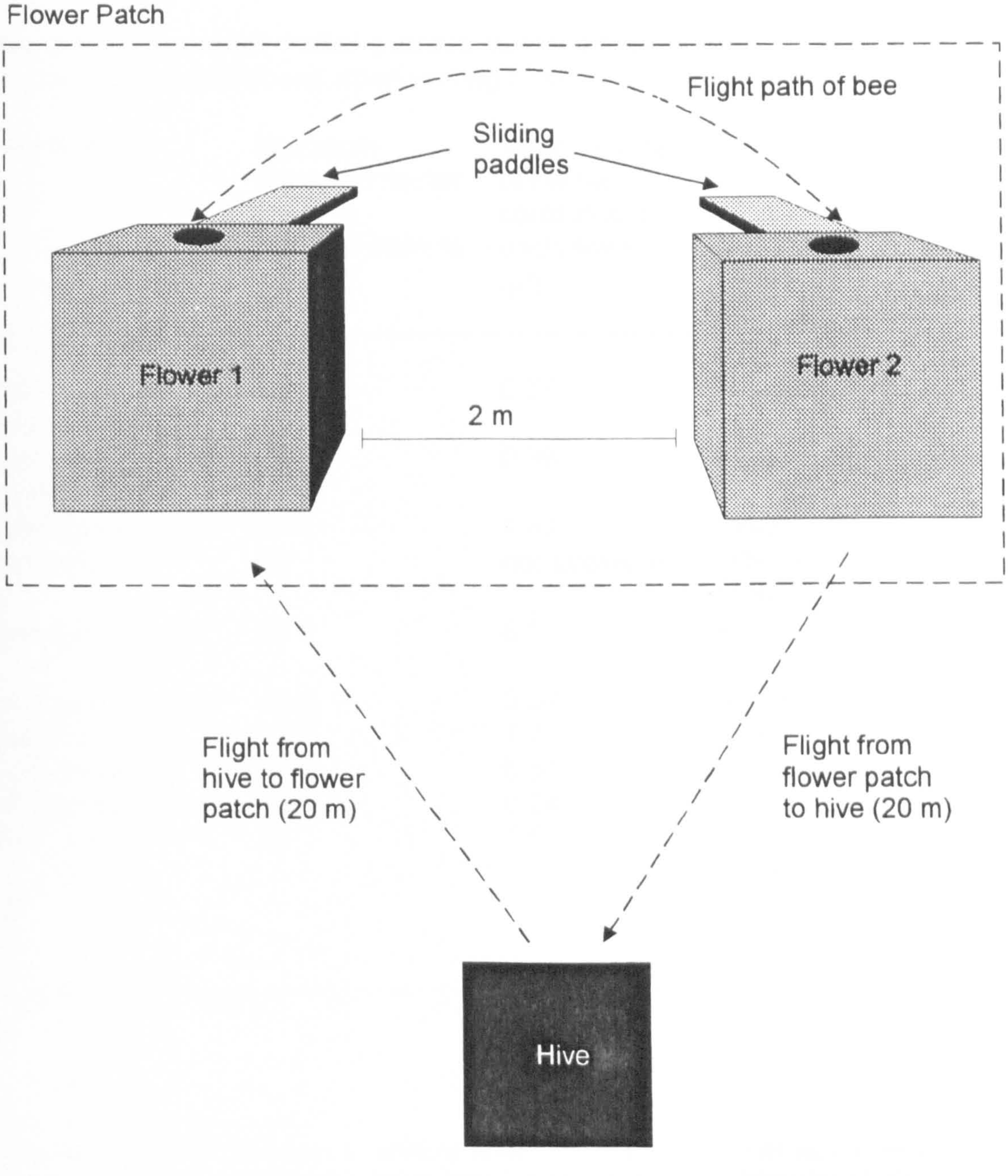
Bees were trained to forage from the corolla by placing the artificial flower within 5m of the hive entrance. Once bees began to feed from the corolla, the flower was gradually moved away from the hive, until the hive-flower patch distance equalled 20m. To simulate a non-depleting flower patch, bees were trained to fly repeatedly between two flowers situated 2m apart (Figure 2.1.2.). Only bees collecting nectar were used in the experiment, any bees collecting nectar and pollen were captured and not used in subsequent experiments.

2.1.2. Changing the volume and sucrose concentration of nectar delivered on each flower visit

Once a bee was trained to the flower patch, the volume of nectar delivered to each flower was set at 1.2 μl with a w/w sucrose concentration of 75% (giving

Figure 2.1.2
Simulation of a non-depleting flower patch using 2 artificial flowers.

To simulate a non-depleting flower patch, bees were trained to fly from the hive to flower 1. Once the bee had collected 1.2 / 0.6 micro-liters of nectar, the sliding paddle was used to cover the corolla. At the same time, the paddle on flower 2 was pulled back to expose the second corolla. The bee was then allowed to fly to flower 2 and collect a second parcel of nectar. This process was repeated until the bee abandoned the patch and returned to the hive.



an energy value of 10.2 J). The sucrose content of the nectar was set at 75%, as it proved difficult to train bees to forage to artificial flowers delivering nectar with lower calorific values (*pers obs*)[2]. It should, however, be noted that natural flowers normally have nectar sucrose contents lower than 75%, although concentrations above this level have been reported (Table 2.1.1).

Table 2.1.1. Reported nectar sucrose concentration and volume of nectar encountered by bumblebees when visiting flowers.

Flower species	Maximum observed nectar sucrose concentration % (w/w)	Mean volume of nectar contained in each flower (µl)	Source
Robinia pseudoacacia	85.6	0.57	Nicolson, 1990
Aconitum columbianum	83.3	0.39	Laverty, 1979
Delphinium barbeyi	64.5	1.83	Laverty, 1979
Rubus idaeus	59	not available	Willmer *et al.*, 1994
Delphinium nelsoni	51.0	6.1	Hodges & Wolf 1981
Podalyria calyptrata	49.6	0.94	Nicolson, 1990
Wisteria sinensis	39.7	1.4	Nicolson, 1990
Mertensia ciliata	39.0	0.61	Laverty, 1979
Oxytropis splendens	36.4	0.24	Laverty, 1979
Agave schottii	29.1	7.5	Schaffer, Jensen, Hobbs, Gurevitch, Todd & Schaffer, 1979

[2] It proved possible to train bees to artificial flowers with nectar contents as low as 20%, however, at low concentrations the flower handling and inter-flower behaviour of foraging bees proved highly variable. For this reason, concentrations of nectar within the artificial flowers were set between 50 and 75%, concentrations at which the variability in foraging behaviour of the bees was minimised (*pers. obs*).

Once trained to the artificial flowers, the bees were marked with queen labelling tags and then allowed to complete a further 6 foraging cycles. This provided the bees with sufficient time to learn the handling skills necessary to collect the nectar from the artificial corollas (Schmid-Hempel *et al.*, 1985; Wolf & Schmid-Hempel, 1990). The assumption that the bees foraged in a stable manner was confirmed by statistically comparing the difference in number of flower visits (G-test), handling and inter-flower times (paired t-test) of the bees in the subsequent seventh and tenth foraging cycles. Any bee whose number of flower visits, handling or inter-flower times significantly differed between the seventh and tenth foraging cycle were rejected.

During foraging cycles 7, 8, 9 and 10 the number of flowers visited, handling and inter-flower times were recorded using an IBM PC and data logger program (written in Microsoft Visual C++ ©)[3]. Four different treatments of nectar sucrose concentration and volume were used in the experiment

1. 1.2 µl of 75% w/w sucrose concentration with a calorific value of 10.2 J (n = 10 bees),
2. 1.2 µl of 50% w/w sucrose concentration with a calorific value of 7.7 J (n = 10 bees),
3. 0.6 µl of 75% w/w sucrose concentration with a calorific value of 5.1 J (n = 6 bees),

[3] The number of flower visits, handling and inter-flower times were recorded by pressing a key on the computer key board, the times were determined *via* the internal clock of the computer.

4. 0.6 µl of 50% w/w sucrose concentration with a calorific value of 3.85 J (n = 6 bees).

The foraging behaviour of 10 bees was recorded when collecting nectar under treatments 1 and 2, whilst the behaviour of 6 of the ten bees were also recorded when collecting nectar under treatments 3 and 4. As individual bee's were recorded foraging under more than one treatment of nectar volume and sucrose concentration, it was necessary to ensure that past experience did not significantly effect the bee's foraging behaviour. As a result, after the bees had foraged at the artificial flower's under treatments 1, 2, 3 and 4, the bees were permitted to completed a further 4 foraging cycles under treatment 1. It was, therefore, possible to compare the behaviour of the bees when foraging under treatment 1 when i) having had no previous foraging experience, and ii) after having previously foraged under treatments 1, 2, 3 and 4. If past experience significantly altered the bees foraging behaviour, a significant difference would be expected in the recorded behaviour of the bees when foraging under treatment 1 with no prior foraging experience, and treatment 1 after having foraged under treatments 1, 2, 3 and 4. For this reason, any bees whose number of flower visits, handling or inter-flower times differed significantly between initial and final nectar treatments were rejected.

2.1.3. Predicting optimum number of flower visits of a bee when maximising RNEG

Although the SH model can be used to predict RNEG, problems are encountered when modelling a bees behaviour in non-depleting flower patches. This is because available gains do not diminish with time spent in the patch, as a result, the SH model will predict an infinite number of flower visits. It was, therefore, assumed that bees maximizing RNEG would visit sufficient flowers to fill their crops prior to returning to the hive. The point at which a bees crop becomes full has been reported by Hiendrich as the mass of nectar equivalent to 90% of the bee's unloaded body mass, as has also been reported in *B.terrestris* by Cooper (1993). One possible source of error in this supposition, is the assumption that the costs of imbibing nectar do not increase as the volume of nectar within the crop increases. If handling costs do increase with crop load, the resultant increase in energy expenditure or time spent handling nectar, may result in the forager abandoning the patch prior collecting a maximum crop load. However, it has been shown that nectar uptake rates, in terms of time, are constant for nectar masses from 0 to 90% of a bumblebee's unloaded body mass (Cooper, 1993). Although this may indicate that time taken to imbibe nectar does not increase with increased nectar crop content, it need not necessarily follow that energy costs of imbibing nectar are also constant. It is possible that as the crop fills, it becomes more difficult for the bee to draw up nectar and pump it into the

crop[4]. However, if a bee is more sensitive to time than energy costs, it may be willing to increase energy expenditure, whilst imbibing nectar, in order to maintain a constant uptake rate. Whether energy costs of handling do increase with increased crop load is unknown, as no work has to date been completed in this area. Also, as little work on crop and gut physiology has been carried out (Willmer, 1986), assumptions of crop load and costs of collecting nectar have to be made on the basis of limited information. As a result, it is possible that a bee maximizing RNEG will not always imbibe nectar with a mass equal to 90% of its unloaded body mass. However, as nectar loading rates in bumblebees remains constant, irrespective of crop load (Cooper, 1993), it was assumed that the maximum number of flower visits a bee could make during one foraging cycle equalled 90% of the bee's body mass divided by the mass of nectar collected on each flower visit.

2.1.4. Prediction of the number of flower visits a bee should make when maximising Net Energy Efficiency

In order to use the SH model to predict the number of flowers a bee should visit when maximising NEE, the unloaded metabolic rate of flying bees, a_0, was estimated using Cooper's (1993) measurements of energy expenditure. In wind tunnel experiments with bumblebees, Cooper found the correlation

[4] Nectar is drawn into the pharynx by the pharyngeal pump, the nectar is subsiquently transported to the crop along the oesophagus by peristaltic movements (Chapman, 1975).

between mass-specific metabolic rate $\dot{V}CO_2$ (ml g^{-1} h^{-1}) and body mass (mg) to be :

$$\dot{V}CO_2 = 0.17.m^{0.83} \quad (6)$$

Although a_O could be estimated directly from equation (6), it was not possible to use Coopers equation to predict a. This was because the Cooper (1993) equation is logarithmic, whilst the SH model is linear. It was, therefore, necessary to alter the C_p (equation 2) and C_t (equation 3) equations in the SH model to account for a logarithmic incremental metabolic rate. This was done by replacing the single value of a, used by Schmid-Hempel *et al.* (1985), with equation (7). The variable a was calculated by subtracting the metabolic rate of the bee before each flower visit from the metabolic rate of the bee following collection of w mg of nectar:

$$a = \frac{\left((0.17m^{0.83}) - \left(0.17(m-w)^{0.83}\right)20.95\right)}{3600} \quad (7)$$

where m = body mass of bee (mg), 20.95 = the caloric equivalent of 1 ml of O_2 (J), and 3600 = constant, converting Jh^{-1} to Watts ($J\,s^{-1}$).

The metabolic rates of the bees whilst handling nectar on the flower (a_h), and in the hive (a_r), were taken from Bastian & Esch's (1970) paper on pre-flight

activity in honeybees and Rothe & Nachtigall's (1989) estimates of the metabolic costs of walking honeybees. These costs were estimated as 12.5% of the metabolic rate of the unloaded flying bee. It should be noted that these values were taken from honeybees and not bumblebees, this was necessary as no equivalent data has yet been published on the energy costs incurred by resting or walking *B.terrestris*.

The values for inter-flower times (t), and the handling times (h) were taken as the mean recorded times of the individual bees. As the experiment was performed by only one person, it was not possible to record the flight times between the flower patch and the hive (t_o) and the hive times (T_o) during individual foraging cycles. Hive times and flight times (between the flower patch and the hive) were, therefore, estimated. Hive times were assumed to be 36 seconds, the mean times observed in 14 bees foraging to the artificial flower patch. Flight times from the flower patch to the hive were estimated as 3.5 seconds, assuming a flight speed of 5.7 ms^{-1} (taken from the mean speed of 6 observed bees flying to the artificial flower patch ($5.7 \pm 1.2\ ms^{-1}$))[5].

The predicted number of flowers was calculated as the number of flowers where NEE is at a maximum (all calculations were performed using an optimal foraging simulation program written in Microsoft visual C++, a copy of which can be found in appendix 1).

[5] The mass of nectar collected by foraging bees has no apparent affect on flight speeds (Cooper 1993).

2.2.0. Results

The number of flower visits, handling and inter-flower times of all bees were found to be highly stable under all nectar treatments, as has been observed in similar honeybee studies (Schmid-Hempel *et al.*, 1985; Seeley 1986; Wolf & Schmid-Hempel, 1990). This was illustrated by the low standard deviation in mean number of flower visits per foraging cycle, which did not exceed ±1.7 (n = 32), for any bee, under any nectar treatment (Table 2.2.1 & 2.2.2). Similarly, the standard deviations of mean handling and inter-flower times were also low, ranging from ±0.0 to ±1.9 (n = 32), and ±0.0 to ±6.9 (n = 32) respectively (Table 2.2.3). When comparing behavioural differences between bees, however, highly significant variations were observed in the mean number of flower visits and handling times under all nectar treatments (Table 2.2.4). Generally, larger bees collected a greater mass of nectar (r_{30} = 29.0, $p<0.001$) (Figure 2.2.1) and took a shorter time to handle the nectar delivered (r_{30} = -0.51, $p<0.05$).

Table 2.2.1. The observed number of flower visits per foraging cycle by *B.terrestris* and the number of flowers and calorific value of nectar predicted by the two models under varying conditions of nectar volume and sucrose content.

		Volume and (w/w) sucrose concentration of delivered nectar									
		1.2 µl of 75%					1.2 µl of 50%				
Bee ID	Body mass (mg)	Mean observed number of flower visits (±sd) (n = 4)	NEE prediction of number of flower visits	Predicted energy value of nectar collected when maximizing NEE (J)	RNEG prediction of number of flower visits	Predicted energy value of nectar collected when maximizing RNEG (J)	Mean observed number of flower visits (±sd) (n = 4)	NEE prediction of number of flower visits	Predicted energy value of nectar collected when maximizing NEE (J)	RNEG prediction of number of flower visits	Predicted energy value of nectar collected when maximizing RNEG (J)
1	172	13.0 ±0.8	6	61	129	1316	13.0 ±0.0	7	54	129	993
2	130	4.5 ±0.6	5	51	98	995	5.0 ±0.0	6	39	98	751
3	127	4.3 ±0.5	4	41	95	972	5.0 ±0.0	5	39	95	731
4	171	19.8 ±1.0	6	61	128	1306	18.5 ±1.7	7	54	128	986
5	135	9.5 ±0.6	6	61	101	1030	10.0 ±0.8	7	54	101	778
6	118	10.8 ±0.5	5	51	89	903	10.5 ±0.6	4	31	89	685
7	125	14.3 ±0.5	6	61	94	949	14.3 ±1.0	7	54	94	724
8	173	18.3 ±1.0	4	41	129	1316	18.3 ±1.0	6	39	129	1001
9	180	18.3 ±0.5	3	31	135	1377	20.3 ±0.5	4	31	135	1039
10	170	15.3 ±0.5	2	20	128	1295	14.5 ±0.6	6	39	128	986

Table 2.2.2. The observed number of flowers visited per foraging cycle by *B.terrestris* and the number of flowers and calorific value of nectar predicted by the two models under varying conditions of nectar volume and sucrose content.

	Volume and (w/w) sugar concentration of delivered nectar										
	0.6 μl of 75% sugar water						0.6 μl of 50% sugar water				
Bee ID	Body mass (mg)	Mean observed number of flower visits (±sd) (n = 4)	NEE prediction of number of flower visits	Predicted energy value of nectar collected when maximizing NEE (J)	RNEG prediction of number of flower visits	Predicted energy value of nectar collected when maximizing RNEG (J)	Mean observed number of flower visits (n = 4)	NEE prediction of number of flower visits	Predicted energy value of nectar collected when maximizing NEE (J)	RNEG prediction of number of flower visits	Predicted energy value of nectar collected when maximizing RNEG (J)
3	127	8.3 ±1.5	7	36	98	500	8.5 ±1.0	13	50	98	377
5	135	21.5 ±1.0	16	82	128	653	20.8 ±1.0	15	58	128	493
6	118	20.5 ±0.6	7	36	101	515	20.3 ±0.5	11	42	101	389
7	125	29.5 ±0.6	6	31	89	454	29.5 ±0.6	10	39	89	343
8	173	34.0 ±0.8	6	31	94	479	35.5 ±0.6	10	39	94	362
9	180	35.5 ±0.6	12	61	129	658	36.5 ±0.6	12	46	129	497

Table 2.2.3. Changes in handling and inter-flower times of *B.terrestris* due to changes in nectar volume and sucrose concentration (n = the number of foraging cycles from which a mean value was calculated).

	Volume and sucrose concentration (w/w) of delivered nectar							
	1.2 µl, 75%				1.2 µl, 50%			
Bee ID	Mean (±sd) handling time (sec)	n	Mean (±sd) inter-flower time (sec)	n	Mean (±sd) handling time (sec)	n	Mean (±sd) inter-flower time (sec)	n
1	9.3 ±0.5	52	3.0 ±0.0	51	5.8 ±1.0	52	3.3 ±1.0	51
2	11.8 ±1.0	18	3.0 ±0.8	17	8.5 ±1.3	20	3.3 ±0.5	19
3	15.5 ±1.9	17	5.0 ±0.0	16	10.3 ±1.3	20	4.0 ±1.1	19
4	5.5 ±0.6	79	2.8 ±1.0	78	4.0 ±0.0	74	3.3 ±1.0	73
5	7.6 ±0.2	38	2.9 ±0.4	37	8.5 ±1.0	40	2.9 ±0.0	39
6	9.3 ±0.7	43	2.7 ±0.8	42	5.4 ±0.4	42	5.3 ±2.6	41
7	6.7 ±0.2	57	2.7 ±0.3	56	6.2 ±0.3	57	2.8 ±0.4	56
8	5.1 ±0.4	73	5.4 ±1.9	72	5.4 ±0.2	73	4.2 ±1.8	72
9	4.7 ±0.3	73	6.3 ±3.1	72	4.8 ±0.5	81	6.5 ±3.9	80
10	5.5 ±0.4	61	9.3 ±6.9	60	5.4 ±0.8	58	4.4 ±1.7	57
mean	8.1 ± 3.5		4.3 ± 2.2		6.4 ± 2.0		4 ± 1.2	
	0.6 µl of 75%				0.6 µl of 50%			
3	9.3 ±0.5	33	5.0 ±2.0	32	5.8 ±1.5	34	2.5 ±0.6	33
5	4.0 ±0.3	86	2.1 ±0.1	85	6.7 ±0.6	83	3.1 ±0.2	82
6	7.6 ±0.3	82	4.8 ±1.8	81	8.0 ±0.7	81	3.5 ±0.5	80
7	4.8 ±0.3	118	6.4 ±0.6	117	5.4 ±1.2	118	3.8 ±1.6	117
8	4.4 ±0.5	136	6.5 ±5.6	135	4.8 ±0.2	142	4.3 ±1.9	141
9	2.6 ±0.1	142	3.4 ±0.2	141	4.8 ±0.1	146	4.4 ±0.9	145
mean	5.45 ± 2.5		4.7 ± 1.7		5.9 ± 1.2		3.6 ± 0.7	

The relationship of body mass and nectar mass and handling times is described by equations 1 and 2 respectively:

$$y = 0.18x - 11.1 \quad (r^2 = 0.49) \qquad (1)$$

where y = mean number of flower visits per foraging cycle, and x = starved body mass of bee (mg)

$$y = 14.9 - 0.06\,x \quad (r^2 = 0.26) \qquad (2)$$

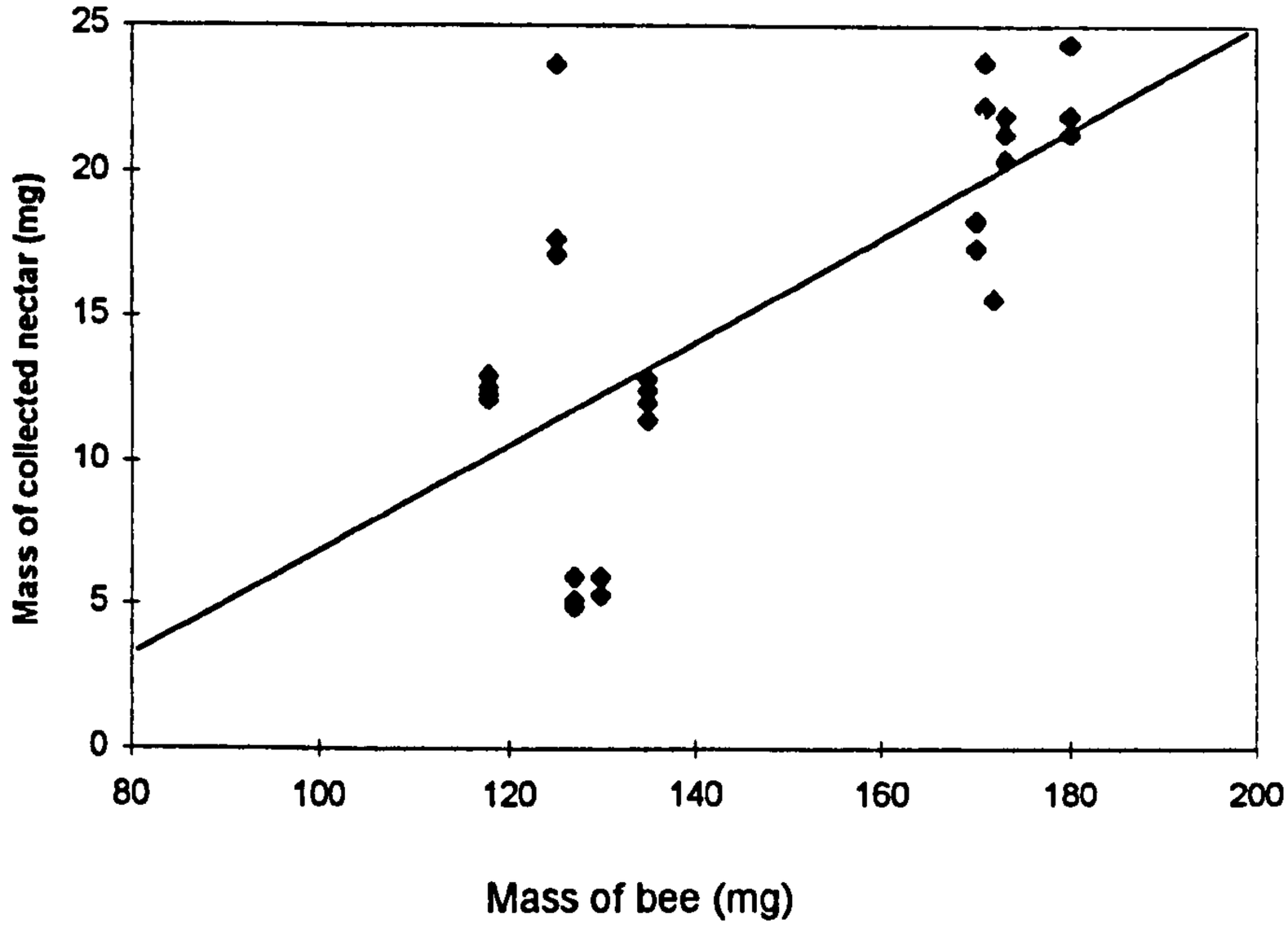
where y = mean handling time per foraging cycle (s).

Despite the significant regression between body mass, mass of nectar collected and handling times, a number of bees of a similar mass displayed a high degree of variation in behaviour, resulting in the low observed r^2. Good examples of this were bees 3 and 6, with bee 6 consistently visiting double the number of flowers visited by bee 3, despite having a body mass 9 mg lighter. Similarly, the handling times of bee 6 were on average 16.4±3.9% (n = 200) lower than bee 3.

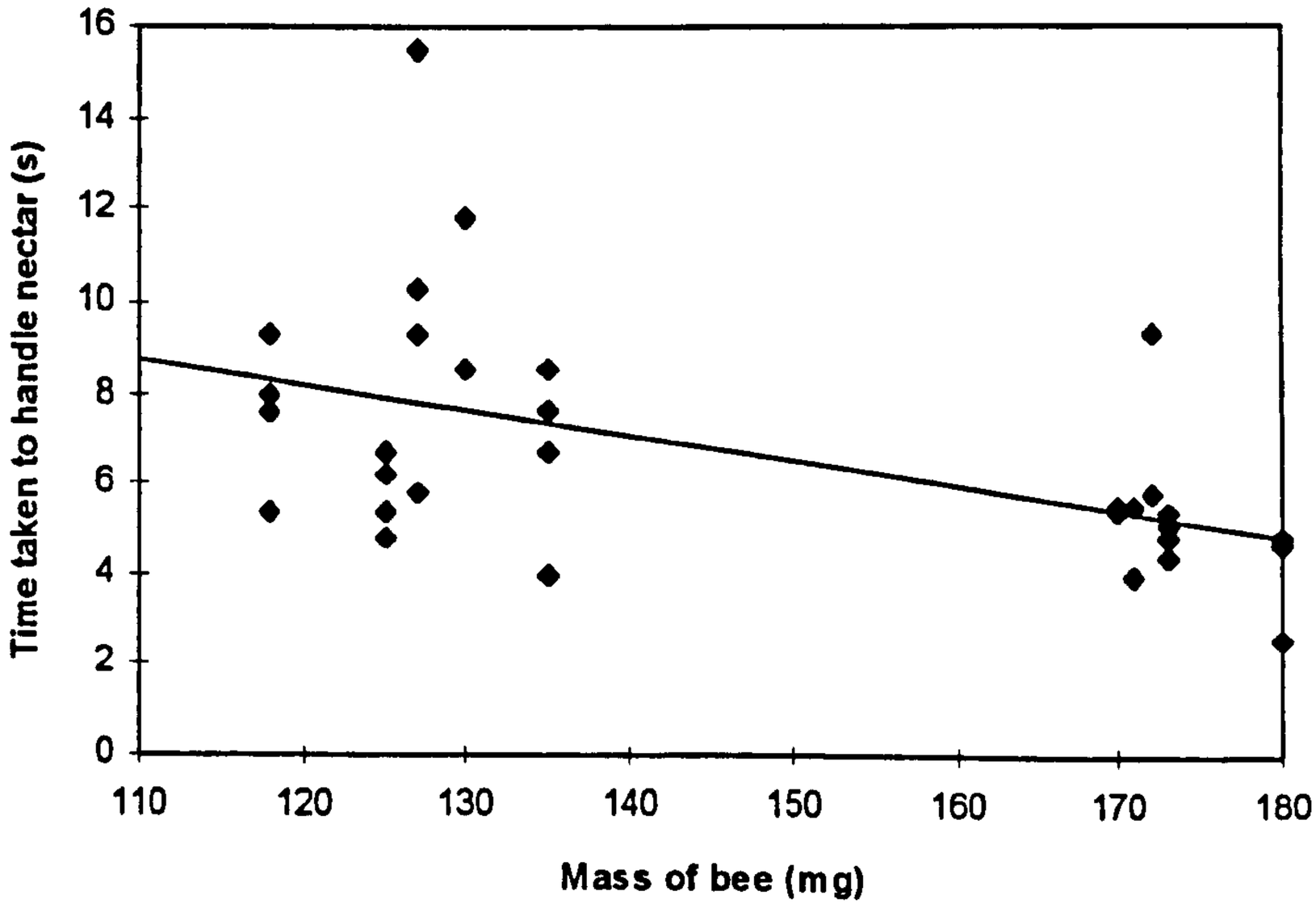
Unlike the number of flower visits and handling time, inter-flower times were insensitive to changes in both nectar volume and sucrose concentration (Table 2.2.4), with mean inter-flower times equalling 4.1±1.6 (n = 32) seconds. Only 3 bees significantly altered inter-flower times when nectar

Figure 2.2.1

a) Relationship between body mass of bee and mass of nectar collected on each foraging cycle



b) Relationship between body mass of bees and time taken to handle nectar within the artificial flowers



concentration was reduced and 2 when nectar volume was halved (Table 2.2.5). Two-way analysis of variance showed no significant difference in inter-flower times with changes in nectar concentration ($F_{1, 123}$ = 2.41, ns) or nectar volume ($F_{1, 123}$ = 0.03, ns).

2.2.1. Changes in foraging behaviour due to changes in nectar volume and sucrose concentration

Altering the sucrose content of the nectar from 75% to 50% had little effect on the number of flower visits performed by each bee. Variation in *n*, following reduction in nectar sucrose content, ranged from -1.3 to +2.0, with a mean of 0.2±0.8 (n = 32). Of the 10 bees observed, only one significantly altered the number of flower visits following reduction in nectar concentration (Table 2.2.6.). When combining the data from all bees, variation in flower visiting behaviour was found to be non-significant (ANOVA; $F_{1, 124}$ = 0.03, ns[♣]). This was also true of model predictions of *n*, with no significant change in NEE predictions ($F_{1, 29}$ = 5.49, ns) and RNEG predictions ($F_{1, 29}$ = 0, ns) following a reduction in nectar sucrose concentrations. When considering the calorific value of the nectar, similarly there was no significant difference between NEE predictions when nectar was changed from 75% to 50% ($F_{1, 29}$ = 0.24, ns), although there was a significant difference between RNEG predictions ($F_{1, 29}$ = 19.04, p<0.001).

♣ As there was a significant regression between body mass and number of flower visits (equation 1), *n* was corrected for body mass of bee to enable inter bee comparisons of foraging behaviour.

Table 2.2.4. Analysis of variance in foraging behaviour between bees.

	Nectar sucrose concentration (w/w) and volume obtained by the bee on each flower visit											
Inter-bee Variable analysed.	1.2 µl, 75%			1.2 µl, 50%			0.6 µl, 75%			0.6 µl, 50%		
	df	F	P	df	F	P	df	F	P	df	F	P
Number of flower visits	9	274.6	p<0.001	9	178.3	p<0.001	5	512.0	p<0.001	5	872.3	p<0.001
Handling time	9	76.9	p<0.001	9	21.4	p<0.001	5	191.4	p<0.001	5	14.3	p<0.001
Inter-flower time	9	3.2	p<0.01	9	1.3	ns	5	1.7	ns	5	1.7	ns

Table 2.2.5. Variation in the inter-flower times of *B.terrestris* when nectar w/w sucrose concentration was reduced form 75% to 50% and volume from 1.2 μl to 0.6 μl.

Bee ID	1.2 μl of 75% *vs* 50% sucrose concentration nectar.			0.6 μl of 75% *vs* 50% sucrose concentration nectar.		
				paired t-test		
	df	F	P	df	F	P
1	5	0.27	ns	-	-	-
2	5	0.27	ns	-	-	-
3	11	8.65	p<0.05*	11	1.59	ns
4	5	0.55	ns	-	-	-
5	11	25.00	p<0.001**	11	9.00	p<0.05*
6	11	0.81	ns	11	<0.001	ns
7	11	7.84	p<0.05*	11	34.94	p<0.001**
8	11	0.2	ns	11	1.11	ns
9	11	0.25	ns	11	5.27	ns
10	5	1.91	ns	-	-	-

* = A significant difference in inter-flower times between different treatments of nectar volume and sucrose concentration.
** = A highly significant difference in inter-flower times between different treatments of nectar volume and sucrose concentration.

When the amount of nectar delivered on each flower visit was halved, the mean number of flower visits approximately doubled, from 12.8±5.2 to 25±9.7 (n = 12), with all bees foraging to significantly more flowers ($F_{1, 124}$ = 83.77, p<0.001♣) (Table 2.2.6). Similarly, model estimates of *n* also increased, with highly significant differences in NEE ($F_{1, 29}$ = 41.01, p<0.001♣) and RNEG ($F_{1, 29}$ = 101.91, p<0.001♣) predictions when nectar volume was changed from 1.2 µl to 0.6 µl. NEE and RNEG model predictions of energy gains also changed significantly with changes in volume of nectar ($F_{1, 29}$ = 93.01, p =<0.001, $F_{1,29}$ = 99.99, p<0.001 respectively).

When foraging at flowers containing 1.2 µl of 75% sucrose concentration nectar, handling times were on average 8.1±3.5 (n = 10) seconds, compared to a mean of 6.4±2.0 (n = 10) seconds following reduction in nectar sucrose content. Of the 10 bees observed, 5 significantly decreased their handling times when sucrose concentration was reduced (Table 2.2.7). When the volume of nectar collected on each flower visit was halved to 0.6 µl, mean handling times were reduced from 7.3±2.9 (n = 20) to 5.7±1.9 (n = 12) s, the change in handling times were significant for 5 of the 6 bees observed (Table 2.2.7). Variations in handling times due to changes in nectar concentration were also reduced, with a mean time of 5.5±2.5 seconds at 75% sucrose concentration and 5.9±1.3 seconds at 50%. Two way analysis of variance showed there was no significant overall difference in handling times due to

Table 2.2.6. Analysis of variation in the number of flower visits made by *B.terrestris* when the sucrose concentration of nectar was altered from 75% to 50% w/w and volume from 1.2 µl to 0.6 µl.

Bee ID	75% *vs* 50% w/w sucrose concentration nectar.			1.2 µl *vs* 0.6 µl of nectar.		
				paired t-test		
	df	F	P	df	F	P
1	5	<0.001	ns	-	-	-
2	5	3.0	ns	-	-	-
3	11	0.4	ns	11	7.5	p<0.05♣
4	5	1.6	ns	-	-	-
5	11	0.1	ns	11	709.8	p<0.001♣♣
6	11	0.9	ns	11	1303.7	p<0.001♣♣
7	11	<0.001	ns	11	2029.6	p<0.001♣♣
8	11	3.2	ns	11	1537.4	p<0.001♣♣
9	11	30.9	p<0.001♣♣	11	3847.7	p<0.001♣♣
10	5	3.9	ns	-	-	-

♣ = A significant difference in the number of flower visits between different treatments of nectar volume and sucrose concentration.
♣♣ = A highly significant difference in number of flower visits between different treatments of nectar volume and sucrose concentration.

Table 2.2.7. Analysis of variation in the handling times of *B.terrestris* when nectar w/w sucrose concentration was reduced form 75% to 50% and volume from 1.2 µl to 0.6 µl.

Bee ID	75% *vs* 50% sucrose concentration nectar.			1.2 µl *vs* 0.6 µl of nectar.		
				paired t-test		
	df	F	P	df	F	P
1	5	42.0	p<0.05♣	-	-	-
2	5	193.4	p<0.001♣♣	-	-	-
3	11	39.5	p<0.001♣♣	11	59.7	p<0.001♣♣
4	5	27.0	p<0.05♣	-	-	-
5	11	26.7	p<0.001♣♣	11	0.2	ns
6	11	0.3	ns	11	56.3	p<0.001♣♣
7	11	3.0	ns	11	8.3	p<0.05♣
8	11	0.3	ns	11	75.0	p<0.001♣♣
9	11	0.3	ns	11,	75.0	p<0.001♣♣
10	5	0.1	ns	-	-	-

♣ = A significant difference in handling times between different treatments of nectar volume and sucrose concentration.
♣♣ = A highly significant difference in handling times between different treatments of nectar volume and sucrose concentration.

changes in nectar concentration ($F_{1, 124}$ = 0.03, ns♠) or volume ($F_{1, 124}$ = 2.41, ns♠).

It should also be noted that inter-flower times were on average 4.1 seconds whilst hive patch times were only 3.5 seconds. As inter-flower distance equalled 2m whilst hive patch distance equalled 20m, it was surprising that inter-flower flight times were longer than hive patch times. This would, however, appear to be due to bees not flying directly between flowers combined with bees hovering over flowers after taking off and prior to landing.

2.2.2. Variation between observed number of flower visits and those predicted by Net Energy Efficiency and Rate of Net Energy Gain models

No bees were observed to fill their crops prior to abandoning the flower patch and returning to the hive, consequently, observed flower visits were consistently lower than those predicted by the RNEG model (Table 2.2.1 & 2.2.2). The difference between observed *n* and RNEG predictions were highly significant for all bees under all nectar treatments (G_{32} = 3933, $p<0.001$)[6] (Table 2.2.8), as were the differences between observed and

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♠ As there was a significant regression between body mass and handling time (equation 2), handling times were corrected for body mass of bee to enable inter bee comparisons of foraging behaviour.

[6] As the degrees of freedom between observed and predicted number of flower visits equalled one, G values had to be used instead of F values in-order to enable statistical analysis.

predicted calorific content of collected nectar (G_{32} = 19596, $p<0.001$) (Table 2.2.9).

Differences between the observed number of flower visits and NEE predictions were lower than those between observed and RNEG predictions. Differences between observed *n* and NEE predictions ranged from +53% to -87%, with mean of -47±3% (n = 32). Except for bees 2 and 3, NEE predictions of number of flower visits underestimated *n* for all bees under all nectar treatments. Only 4 of the 10 bees observed were found to have no significant difference between observed *n* and NEE predictions under all treatments of nectar volume and sucrose concentration (Table 2.2.8). The overall difference in observed *n* and NEE predictions for all bees were highly significant under all treatments of nectar sucrose concentration and volume (G_{32} = 187.2, $p<0.001$).

It was possible that some of the variation between observed behaviour and that predicted by the SH model was due to errors in input parameters. To determine the sensitivity of the SH model to input variable errors, the mean input parameters of the model were incremented by 50% of the mean value observed in the current experiment (Table 2.2.10). The difference between the incremented value and the original observed mean value than provided a relative indication as to the sensitivity of the SH model to errors in each individual input variable. It was evident from this analysis that errors in inter-flower time recordings would produce the largest errors in NEE predicted

Table 2.2.8 Analysis of the difference in number of flower visits observed and those predicted by NEE and RNEG.

	Observed *vs* NEE		Observed *vs* RNEG	
		1.2 µl of 75% w/w sucrose concentration		
	G -stat (df = 1)		G-stat (df = 1)	
bee ID	G	P	G	P
1	2.64	ns	109.92	p<0.001**
2	0.03	ns	104.52	p<0.001**
3	0.01	ns	102.82	p<0.001**
4	7.74	p<0.01*	88.88	p<0.001**
5	0.80	ns	88.71	p<0.001**
6	2.15	ns	69.51	p<0.001**
7	3.46	ns	65.47	p<0.001**
8	9.88	p<0.01*	94.62	p<0.001**
9	12.16	p<0.001**	100.55	p<0.001**
10	11.54	p<0.001**	100.87	p<0.001**
		0.6 µl of 75% w/w sucrose concentration		
3	0.10	ns	206.87	p<0.001**
5	0.81	ns	168.89	p<0.001**
6	6.92	p<0.01*	142.12	p<0.001**
7	6.96	p<0.001**	128.30	p<0.001**
8	21.64	p<0.001**	196.40	p<0.001**
9	12.15	p<0.001**	203.99	p<0.001**
		1.2 µl 50% w/w sucrose concentration		
1	1.83	ns	109.92	p<0.001**
2	0.09	ns	102.14	p<0.001**
3	0.00	ns	99.25	p<0.001**
4	5.38	p<0.01*	92.25	p<0.001**
5	0.53	ns	86.97	p<0.001**
6	3.02	ns	70.28	p<0.001**
7	2.52	ns	65.47	p<0.001**
8	6.48	p<0.01*	94.62	p<0.001**
9	11.90	p<0.001**	94.99	p<0.001**
10	3.63	ns	103.22	p<0.001**
		0.6 µl 50% w/w sucrose concentration		
3	0.95	ns	205.64	p<0.001**
5	0.93	ns	171.39	p<0.001**
6	2.78	ns	142.91	p<0.001**
7	10.06	p<0.01*	128.30	p<0.001**
8	15.15	p<0.001**	192.07	p<0.001**
9	12.97	p<0.001**	55.46	p<0.001**

* = Significant difference between observed and predicted number of flower visits.
** = Highly significant difference between observed and predicted number of flower visits.

Table 2.2.9 Analysis of the difference between observed calorific value of collected nectar and the calorific value of nectar predicted if the bees were to maximize RNEG whilst foraging.

bee ID	Nectar volume and sucrose concentration	observed calorific content of collected nectar (J)	RNEG predicted calorific content of collected nectar (J)	Observed calorific content of nectar *vs* RNEG predictions (G stat (df = 1))	
	1.2 µl 75%			G	P
1		132.6	1316	1121.4	<0.001*
2		45.9	995	1066.7	<0.001*
3		43.9	972	1046.8	<0.001*
4		202.0	1306	902.8	<0.001*
5		96.9	1030	901.5	<0.001*
6		110.2	903	707.8	<0.001*
7		145.9	949	658.4	<0.001*
8		186.7	1316	955.4	<0.001*
9		186.7	1377	1024.1	<0.001*
10		156.1	1295	1020.9	<0.001*
	1.2 µl 50%				
1		100.1	993	846.0	<0.001*
2		38.5	751	786.8	<0.001*
3		38.5	731	761.1	<0.001*
4		142.5	986	708.6	<0.001*
5		77	778	667.7	<0.001*
6		80.9	685	545.3	<0.001*
7		110.1	724	505.4	<0.001*
8		140.9	1001	729.7	<0.001*
9		156.3	1039	729.9	<0.001*
10		111.7	986	799.8	<0.001*
	0.6 µl 75%				
3		42.3	500	454.6	<0.001*
5		109.7	653	429.2	<0.001*
7		104.6	515	296.5	<0.001*
7		150.5	454	159.6	<0.001*
8		173.4	479	148.9	<0.001*
9		181.1	658	288.0	<0.001*
	0.6µl 50%				
3		32.7	377	339.8	<0.001*
5		80.1	493	330.9	<0.001*
6		78.2	389	225.7	<0.001*
7		113.6	343	120.7	<0.001*
8		136.7	362	105.6	<0.001*
9		140.5	497	211.3	<0.001*

* = Highly significant difference between the observed and predicted calorific value of collected nectar.

Table 2.2.10. Sensitivity analysis of the Shmid-Hempel model to errors within input parameters. Errors were calculated by observing the change in predicted NEE and number of flower visits when individual variables within the SH model were incremented by 50%. Variables were varied from the mean input values used in the current experiment.

	Predicted maximum NEE	Predicted maximum NEE after incrementing individual variables by 50%	% difference	Predicted number of flower visits	Predicted number of flower visits after incrementing individual variables by 50%	% difference
Body mass of bee (mg)	32	23	-28.1	19	21	9.5
Unloaded metabolic rate of bee (W) (a_o)	32	23	-28.1	19	21	9.5
Metabolic rate of bee in hive (W) (a_t)	32	32	0.0	19	22	13.6
Metabolic rate of bee on flower (W) (a_h)	32	30	-6.3	19	19	0.0
incremental metabolic rate of bee (W) (a)	32	31	-3.1	19	16	-18.8
Time spent by bee in hive (s) (T_O)	32	32	0.0	19	22	13.6
One way travel time to flower patch (s) (τ_O)	32	31	-3.1	19	23	17.4
Inter-flower flight time of bee (s) (τ)	32	23	-28.1	19	13	-46.2
Time taken by bee to collect nectar at each individual flower (s) (h)	32	32	0.0	19	22	13.6

Volume of nectar collected on each flower visit was set 0.6µl and energetic content of derived nectar 5.1J.

number of flower visits, with a 50% increase resulting in a reduction of a C. 50% in the predicted number of flower visits. Also, errors in estimates of incremental metabolic rates would result in a large reduction in predicted flower visiting behaviour. It should, however, be noted that such sensitivity analysis is limited, as the degree of sensitivity of the model to each input parameter is affected by the value of other variables being used for the same prediction. If, therefore, the experimental design (*e.g.* flight distance and inter-flower distance etc.) were to be altered, the sensitivity of the model to errors in the input parameters may significantly change.

2.3.0. Discussion

Although individual bees foraged in a stable manner, inter-bee variations in handling times and number of flower visits were high. Much of this variation appeared to be due to differences in body mass, with larger bees visiting more flowers per foraging cycle and collecting the available nectar in a shorter period of time. Despite a significant regression between body mass, n and handling time, the size of bee did not explain all inter-bee variation in foraging behaviour. This was particularly true of bees 2 and 3 which displayed a low number of flower visits and high handling times relative to other bees with a similar body mass. The combination of high handling times and low numbers of flower visits may stem from what Heinrich (1976a) described as "worker naiveté", with bees 2 and 3 having little foraging experience and poor handling skills. Heinrich (1976a) noted that bees

foraging to flowers which they had not previously encountered, displayed "errors" when attempting to collect nectar or pollen. Whilst studying *B.vagans* foraging to *Aconitum*, he observed experienced foragers (workers who had previously encountered *Aconitum*) rapidly visiting, probing and extract nectar from up to 150 flowers on each foraging cycle, with few errors. Subsequently, a second patch was exposed to inexperienced foragers, their behaviour was far more erratic, with bees being unable to locate the entrance to the flower, and probing for nectar amongst anthers, were no nectar was present (Figure 2.3.1). The inexperienced bees also abandoned the flower patch having visited fewer flowers than the experienced bees.

During the current study, the only forage plant available to the bees was the pollen providing *Lycopersicum esculentum*, and as a result the bees had no experience in nectar foraging prior to being trained to the artificial flower patches, and could certainly be described as naive nectar foragers. Despite this, all bees were allowed 6 foraging cycles to "learn" how to handle the nectar in the artificial flowers, prior to their behaviour being recorded. As a result, all bees should have been equally efficient at collecting nectar, assuming that all bees had equal learning rates. In a separate experiment, however, Heinrich, Mudge & Deringis (1977) found large variations in the rate at which different bees were capable of learning handling techniques. *B.terricola* were trained to artificial flowers which varied in nectar rewards. To enable the bees to distinguish rewarding and less rewarding flowers, the two nectar treatments were placed in corollas of different colours (blue and

Figure 2.3.1 Foraging behavior of inexperienced and *experienced Bombus vagans* workers at *Aconitum*. ⇑ = entry or attempted entry at a point other than main flower entrance; - = probing for nectar among the anthers, where there is no nectar; P = collecting pollen from the anthers; and N = "correct" entry into the hood of the flower. (Whether or not nectar was collected could not be determined in most cases because the sepals are visually opaque.) (after Heinrich, 1976a).

	Consecutive Flowers Visited																	
Bee ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
The Inexperienced Foragers																		
1	-	-	-	-	-	-	-	-N	-	-N	-	-	-	-	-N	-	-	-N
2	⇑	⇑	⇑	-	-	-	-N											
3	B	-																
4	⇑	⇑	B	B	B	B												
5	-	-N	-N	-	-	-	-	-N	-N	-	-N	-N						
6	-	-	-	-	-	-P	-P	-P	-P	-	-P	-P	-P	-	-	-P	-P	-P
7	-	-	-	B														
8	-N	-	-															
9	-N	-N	-N	-N	-N	-N	-N	-N	-N									
10	-	-P	-P	B	B	-	-P											
:1	-	-	-N	-	-	-	-N	-	-	-N	-	N	-	-	-	-N	N	-N
12	-N	-N	-N	-	-	-N												
13	-																	
14	-	-	-	-	-	-	-	-	-	-	-	-						
15	-	-																
The Experience Foragers																		
A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	→
B	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	→
C	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	PN	→

→ = further flowers were visited without any change in foraging behaviour.

white). The nectar rewards were altered and the subsequent number of flower visits observed. The effects were highly variable between different individuals, with some bees abandoning flowers with the lower nectar rewards in preference for those with higher rewards. Other bees remained in the less rewarding flowers, apparently ignoring the richer and larger nectar content of other flowers. Even when flowers were emptied of all nectar, it took some bees up to 200 flower visits before learning which colour flowers contained nectar.

Such variability in learning rates makes it possible that in the current study, a number of bees may not have been foraging optimally due to naiveté. This appears particularly likely for bees 2 and 3 whose long handling times indicated less efficient nectar collection compared to other bees. As naive bees have also been observed abandon to flower patches prematurely (Heinrich, 1976a), this may also explain why bees 2 and 3 visited fewer flowers than other bees with a similar body mass.

2.3.1 Effects of changes in nectar sucrose concentration and volume on foraging behaviour

With respect to number of flower visits, all bees displayed no changes in behaviour when nectar sucrose concentration was reduced from 75% to 50%. This was also true of NEE and RNEG model predictions of number of flower visits, derived when calculating *n* using the observed flight, handling and hive times of each forager. This might suggest one of two possibilities 1) *B.terrestris* are insensitive to changes in the calorific value of nectar, or, 2) the changes in calorific value of the nectar were insufficient to result in an observable changes in the foraging behaviour of the bees. As model predictions of flower visiting behaviour also indicated insensitivity to changes in nectar sucrose content, it was not possible to determine from the current experiment whether *B.terrestris* were insensitive to changes in calorific content of the nectar.

Changes in nectar volume, however, had a more obvious effect, with bees approximately doubling the number of flower visits performed per foraging cycle. This was similar to the pattern of RNEG estimates, with predictions of *n* doubling upon halving the nectar volume delivered. The number of flower visits of the bees following changes in nectar sucrose concentration and volume do not, therefore, contradict either the RNEG or NEE models.

2.3.2 Do bumblebees optimise Rate of Net Energy Gain whilst foraging

When foraging on the artificial flowers, bees only filled ≈ 10% of their crop with nectar prior to abandoning the flower patch. No significant increase in nectar uptake rate with increased crop load was observed, as has also been reported in previous studies (Cooper, 1993). The relatively low crop content, combined with the constant nectar uptake rate would appear to indicate that the bees will have experienced little increase in handling costs with increased nectar load. Whether nectar uptake rates and handling costs, in the current study, would increase if the mean crop content were to exceed 10% is unclear. It was, however, unlikely that decreased uptake rate could account for a forager, maximizing RNEG, reducing optimum nectar loads by 90%. It should also be noted that when nectar sucrose concentration decreased, thus reducing nectar viscosity and increasing uptake rate, no significant changes in the mass of nectar collected by the bees was observed. Thus indicating that nectar uptake rate was not a limiting factor in the current experiment, and does not explain the differences between observed and RNEG predicted foraging behaviour.

As the RNEG model predicts that bees will always fill their crops in a non-depleting patch, it would appear that none of the observed bees maximized their rate of net energy gain whilst foraging. Schmid-Hempel *et al.* (1985) also found that honeybees trained to artificial patches visited significantly fewer flowers than predicted by the RNEG model (t_{12} = 8.54, $p<0.001$, n =

1-12). Gross differences in observed *n* and RNEG estimates have also been reported elsewhere by Schmid-Hempel (1987) and Wolf & Schmid-Hempel (1990). As discussed previously, Nunez (1982) argued that such observations were a result of the bees prematurely abandoning a patch in order to obtain information about alternative foraging sites. This foraging strategy, however, could not have occurred in this study, primarily because bumblebees are unable to communicate the location of alternative forage sites to one another (Heinrich, 1979a). Even if the bees were able to obtain some form of information by prematurely returning to the hive (*e.g.* from the nectar or pollen reserves within the colony), it is unlikely that this could explain the large differences between observed *n* and RNEG predictions found in all bees under all nectar treatments.

Due to the large differences between observed and predicted foraging behaviour, and similar findings in honeybee studies, it would appear highly unlikely that *B.terrestris* optimise RNEG when foraging for nectar

2.3.3. Do bumblebees optimise Net Energy Efficiency when foraging for nectar

Although NEE predictions of *n* were far closer to the observed behaviour of the bees than RNEG estimates, the observed and predicted behaviours were still significantly different. These findings are contrary to those observed by Schmid-Hempel *et al.* (1985) who, when studying honeybees, found no

significant difference between observed n and n predicted by the efficiency model (pair wise; t_{12} = 0.43, p<0.1, n = 12). Similarly, bees have also been found to conform to the efficiency model by Kacinik, Houston & Schmid-Hempel (1986); Schmid-Hempel (1986); Wolf & Schmid-Hempel (1990) and Cartar & Dill (1990a). Seeley (1986) also concluded that honeybees optimise NEE, despite finding a poor relationship between observed behaviour and behaviour predicted by the efficiency model. Seeley explained the difference between the observed and predicted behaviour by pointing out that many of the variables within the efficiency model were based on estimates rather than empirical measurements. In particular, he noted that current remote calorimetric estimates of energetic costs are inadequate, and do not include non-fuel costs such as predator avoidance, wear and tear on the bee (depreciation costs), and costs of converting nectar to honey. To demonstrate this point, Seeley increased the estimated costs of each foraging trip by adding non-fuel costs of 10 to 15 J[7], and re-calculated the predicted behaviour of the bees when maximising NEE. The results showed that if costs were increased, NEE predictions became closer to the observed behaviour, supporting the theory that costs were underestimated. Although it was not possible from the current experiment to determine whether variables within the SH model underestimated the bees foraging costs, it was, however, possible that a number of non-fuel costs were omitted from the

[7] Seeley does not describe how estimates of non-fuel cost were derived, but simply describes them as "realistic".

model, *i.e.*: i) Costs of predator avoidance, and ii) depreciation cost of decreased life span due to increased foraging effort.

I. Costs of predator avoidance

Overt predator avoidance strategies, such as aggressive posturing, spraying faeces at attackers and stinging, are easily observed and well documented (Cartar, 1991) (a summary of defensive behaviour in *Bombus* spp. can be found in Heinrich (1979a)). As none of these behaviours was observed in the current study, it is clear they did not impinge on the bees energy budgets; however, the effect of more subtle strategies could not be determined.

As bees begin foraging, they expose themselves to greater risks of predation and parasitism than are encountered whilst in the hive (Alford, 1975). It would therefore be beneficial for a risk sensitive bee to increase hive times and decrease patch times. Likewise, a bee exposed on a flower is more likely to be parasitized or predated than a flying bee, thus making it beneficial for a forager to minimize time spent handling nectar. As a result, such strategies would be highly costly to a foraging bee. Whether bumblebees implement any of these strategies is unknown, as no work has been completed in this area, and there have been no reports of bumblebees altering their foraging behaviour due to predation risk. This may be due to the fact that bees depend totally on their overt deterrents

such as their sting and warning colouration. The effectiveness of the bees sting as a defensive measure is such that other species of insect, including many members of the hover fly family (Syrphidae) have evolved to mimic the bumblebees warning colouration (Wickler, 1968; Gilbert & Raven, 1975). However, overt strategies would have little effect on parasitization, occurring largely when foragers visit flowers to collected nectar and pollen. The parasitized bee will than transmit the parasite to the colony when returning to the hive to unload any collected nectar or pollen (Shmid-Hempel, 1994). There is now good evidence to indicate that once parasitized, the behaviour of the bumblebee is significantly affected. In particular, Mueller and Schmid-Hempel (1993) reported that foragers parasitized with canopid flies did not return to the hive in the evening, spending the night in the field. This may have been a strategy to reduce the risk of parasitizing the whole colony, or may also be a result of decreased temperatures, experienced outside the hive, decreasing the reproduction rate of the parasites (Mueller & Schmid-Hempel, 1993). It has also been suggested that bumblebees alter their nectar and pollen foraging behaviour in order to reduce the chance of parasitization (Durrer & Schmid-Hempel, 1994). This may include different bumblebee colonies foraging to different floral types, even when they are located in the same area. This would then reduce the chances of a forager from one colony transmitting a parasite, *via* a flower, to a forager from another colony (Durrer & Schmid-Hempel, 1994). However, empirical evidence for such a strategy has yet to be observed.

It is, therefore, possible that bees do alter their behaviour in response to predator / parasite avoidance, thus incurring a none fuel cost whilst foraging. However, it is not possible from the current experiment to conclude if, and to what degree, the none fuel costs of predator avoidance affects the foraging strategy of bumblebees

II. **Depreciation costs of decreased life span due to increased foraging effort**

When bees begin foraging, they are not only exposed to increased risks of predation and parasitism, but also have to contend with increased wear and tear to their bodies and wings. This is most obvious in older foragers, who often lose their body hair, appearing bald (*pers. obs.*), and displaying substantial fraying to their wings (Cartar, 1992). Although loss of body hair may not immediately appear important, it does result in decreased insulation, possibly resulting in increased costs of maintaining the thoracic temperature required for flight (Cartar, 1992). Of more importance is the loss of wing surface area due to fraying. As estimates of energetic costs of flight are dependent on a constant relationship between body mass and wing area (Cooper 1993), any damage which decreases wing area will result in increases in energetic costs of flight. This will result in underestimates of a_O and a in the SH model. Although small errors in a_O have a limited effect on efficiency model predictions of n, little change is

required in a before predictions of n are significantly altered. For example, if the estimated incremental metabolic rate of a 150 mg bumblebee is increased by only 0.0001 W, the number of flower visits predicted will increase by approximately 10%.

Cartar (1992) also suggested that damage incurred to the wings and exoskeleton resulted in a further cost of decreased life span to the bee. To demonstrate his theory, he clipped the wings of 266 *B. melanopygus* and compared their life span to 266 non-clipped bees. He found that the life spans of clipped bees were significantly shorter than that of non-clipped bees. Cartar suggested that decreased life span was due to increased predation, with decreases in wing area reducing mobility, and thus the ability of the bee to escape predation. Similar studies in honeybees, however, suggest the situation is more complicated, with life span being directly linked to foraging effort (Neukirch, 1982; Schmid-Hempel & Wolf, 1988). Neukirch (1982) suggested that the cause of decreased life span with increased foraging effort is due to a limited ability of bees to replenish glycogen stores in their flight muscles. Neukirch found that young bees were able to synthesise fresh glycogen by utilising excess UDP-glucose (Sacktor, 1970). However, as the work load of the bee increased, the ability of the bee to replenish glycogen decreased. Of particular importance to this study, Neukirch observed that as the ability of the bee to synthesise glycogen decreased, levels of ATP also decreased whilst O_2 consumption increased. This in turn suggests that the rate of energy

consumption of foragers may increase with increased number of foraging cycles. As estimates of a_o and a are made from the body mass of the bee alone, they do not take into consideration increased O_2 consumption due to the decreased ability of the bee to synthesise glycogen. This in turn will result in underestimates of energy consumption and poor predictions of n by the efficiency model.

Not only is it likely that foraging costs have been omitted from the model, it is also possible that estimates of total energy costs (C) were inaccurate. This was due to two factors i) the use of honeybee data to predict the resting metabolic rates of bumblebees, and ii) limitations in Coopers equation relating mass to flying energy expenditure of a bumblebee.

Although honey and bumblebees have a similar physiology, it was unclear whether it was possible to assume that the resting metabolic rate of a bumblebee is equal to that of a honeybee. In particular, when "resting", bumblebees regularly vibrate their thoracic muscles to generate heat in-order to control their body temperature (Heinrich, 1979a, 1993). As heat generation in bumblebees is energetically costly (Heinrich, 1979a), the apparent metabolic resting costs of bumblebees may, therefore, be significantly higher than that of honeybees (Section 1.0.2). This turn would result in an underestimate of C by the SH model.

It was also possible that errors occurred in the SH model due to the use of Coopers (1993) equation, predicting flying metabolic costs of the bumblebees from mass of bee alone. This was due to the fact that mass was not the only variable affecting the bees flight costs, other factors such as aerodynamics and wing area were also likely to have been important. In particular, as bees gathered nectar, their body mass and resultant flight costs will have increased, however the surface area of the bees wings will have remained fixed. As a result flight costs will have increased exponentially with increases in body mass. It was, however, also possible that the low masses of nectar collected by the bees resulted in the increases in metabolic costs occurring on the linear portion of the exponential curve. This in turn would have limited any errors produced by Coopers equation.

Due to the complex relationship between the physiology and behaviour of bumblebees, it was unsurprising that the model acted as a poor predictor of *B.terrestris* foraging behaviour (as was found to be the case in honeybees by Seeley (1986)). Manipulation of the costs incurred whilst foraging do, however, appear to greatly improve the accuracy of the model. It was, therefore, premature to assume that bumblebees did not maximise NEE, as it was not possible accurately to measure the energetic costs of foraging. Hence, to determine whether bumblebees were maximising NEE or another currency, accurate field measures of foraging bumblebee energy metabolism were required. Currently, the only possible way to do this is through the use of the Doubly Labelled Water (DLW) technique (Lifson & McClintock, 1966).

Chapter 3

Development and application of a doubly labelled water protocol for bumblebees

3.0.0 General introduction to the Doubly Labelled Water (DLW) Technique

The Doubly Labelled Water (DLW) method is a non-invasive technique for measuring the rate of CO_2 production (rCO_2), and thus energy expenditure, of free-ranging animals. The technique has been successfully applied to a large number of species, ranging from small insectivorous bats (Speakman & Racey, 1988_a) to large ruminants (Fancey, Blanchard, Holleman, Kokjer & White, 1986). Attempts to use the technique on arthropods have, however, proved less successful, with large errors in estimates of carbon dioxide production (Buscarlet, Proux & Gerster, 1978; King & Hadley, 1979; Cooper, 1983). The reasons why the DLW technique has been less successful in arthropods than vertebrates are unclear, although the relatively small body size is likely to have been an important factor. It is also possible that the relatively high water turn-over rates in certain arthropods may have increased any effect isotopic fractionation may have had on the accuracey of the DLW technique.

3.0.1. Fundamental principles of DLW measurement of CO_2 production

The DLW technique uses heavy isotopes of hydrogen (Deuterium, D) and oxygen (^{18}O) as non-ioxic, non-radioactive labels of water ($D_2{}^{18}O$). When administered to an organism, $D_2{}^{18}O$ behaves metabolically in the same

manner as H_2O, mixing freely with an animals body water pool (N). Given sufficient time, the hydrogen and oxygen isotopes will completely mix (equilibrate) with N, resulting in plasma enriched homogeneously with $D_2{}^{18}O$. Also, due to an equilibrium exchange of oxygen, facilitated by the carbonic anhydrase catalyzed hydration of carbon dioxide, the ^{18}O content of expired CO_2 is in equilibrium with the animals body water (Lifson & McClintock 1966).

As the animal loses water through evaporative water loss (EWL), defecation and respiration, ^{18}O and D isotopes are lost from the body water pool. This occurs in two ways:

1. ^{18}O and D combine in the body water to form[1]; $D_2{}^{18}O$, $D_2{}^{16}O$ or $H_2{}^{18}O$, the isotopes are then lost from the body water pool through EWL, excretion and defecation and respiration.
2. The ^{18}O isotopes also combine with carbon dioxide to form $C^{18}O_2$, and, unlike D, can exit the animal's body during respiration.

As H_2O leaves the animal's body, D and ^{18}O isotopes are lost in the same proportion, therefore, although the overall abundance of D and ^{18}O within the animal decreases, the ratio between the two isotopes in the body water pool remains the same. However, when CO_2 is expired, ^{18}O originating from the bodies water pool is lost without any resultant decrease in D abundance. As a result, the abundance of the ^{18}O isotope will decrease at a greater rate than

[1] Other rare water compounds may also be formed with ^{17}O and ^{19}O.

the D isotopes, which are lost almost solely via H_2O. The difference in the apparent fractional turnover rates between the two isotopes can, therefore, be used to calculate the net rate of CO_2 production (rCO_2) in an animal (Lifson & McClintock, 1966; Tatner & Bryant, 1989; Speakman & Racey, 1988_b).

3.0.2. Measurement and calculation of the rate of CO_2 production in a free ranging animal

Before rCO_2 can be calculated, the turnover rates of D and ^{18}O must be measured. This is normally accomplished by extracting three body water samples from the subject, and analyzing their D and ^{18}O isotopic composition in replicate. The first sample is taken prior to administering the DLW, and measures the background abundance of D and ^{18}O within the animal's body water. Once the administered isotope has equilibrated with the animal's body water, a second sample is taken, which is referred to as the "initial" sample. A third sample is removed after time t, and is referred to as the "final" sample. Once the background abundance has been subtracted from the initial and final abundances, the rate of decrease in D and ^{18}O content between initial and final samples over time t, can be used to calculate the rate of CO_2

production of the organism (rCO_2), using equation (1):

$$rCO_2 = (N/2) \cdot (k_O - k_H) \quad (1)$$

where k_O and k_H = the apparent fractional turnover rates of ^{18}O and D respectively, and 2 = a constant equating two atoms of oxygen in each molecule of CO_2 with one atom in each molecule of water. N = body water content of the organism (moles).

Although the basic DLW method is theoretically applicable to most organisms, the technique depends on a number of basic assumptions, which if violated may result in significant errors in rCO_2 measurement (Nagy, 1980).

3.0.3. Basic assumptions of the doubly labelled water technique

Assumption 1) *Organisms have a constant body water content.*

It is highly unlikely that the body water content of an animal will remain constant over the period of an experiment (Speakman & Racey, 1988_b). The errors created by fluctuations in N are, however, small (Nagy, 1980; Speakman & Racey, 1988_b; Tatner & Bryant, 1989) and are unlikely to be significant unless changes in the body water pool are high (>100%) (Speakman & Racey, 1988_b).

Assumption 2) *Rates of CO_2 and water flux within an organism are constant.*

The technique assumes that the decline in D and ^{18}O (expressed as natural logarithms) is linear. This again is unrealistic, as most animals will have variable activity levels, and hence fluctuating levels of CO_2 and metabolic water production. Changes in environmental conditions will also affect evaporative water loss and energy used in temperature regulation. As a result, the decline in isotope abundance in an animal's body water between initial and final samples will be non-linear (Speakman & Racey, 1988_b). However, using the two sample technique, the change in isotopic abundance between initial and final samples represents the mean CO_2 and water flux over the experimental period (Lifson & McClintock, 1966), creating negligible errors in rCO_2 measurement (Speakman & Racey, 1988_b).

Assumption 3) *Administered Isotopes must label only the body water and CO_2 of the organism.*

It is possible that D and ^{18}O isotopes may become incorporated into non-aqueous molecules by ionic diffusion and anabolic metabolism (Nagy, 1980). This may cause errors in rCO_2 if N is determined by D or ^{18}O isotope dilution space, as incorporation of $D_2{}^{18}O$ into non-aqueous compounds will result in an over estimate of body water content (Nagy, 1980; Nagy & Costa 1980; Schoeller, Ravussin, Schultz, Acheson, Baertshi & Jequier, 1986; Speakman & Racey, 1988_b; Tatner & Bryant, 1989). However, if N is determined independently *e.g.* from body mass, this error will be minimized.

Assumption 4) *D and ^{18}O is lost from the organism's body only as CO_2 and H_2O.*

If D and ^{18}O leave the body in forms other than CO_2 and H_2O, potentially gross errors may occur in rCO_2 measurement. If, however, the isotopes are lost in the ratio of 2 D to 1 ^{18}O, DLW estimates of rCO_2 will not be affected as the ratio between the two isotopes (used to calculate rCO_2) will remain the same (Lifson & McClintock, 1966; Tatner & Bryant, 1989).

The main site of $D_2{}^{18}O$ loss, other than CO_2 and H_2O, is through non-exchangeable H and O components of nitrogenous excretion (Nagy. 1980, Speakman & Racey, 1988_a). In particular, ^{18}O appears to be incorporated into urea through the ornithine - arginine cycle (Speakman & Racey. 1988_a), resulting in over estimates of rCO_2. The degree of error created in final measurements of CO_2 production is not clear, although errors in the DLW technique are lower in animals with low rates of urea synthesis *e g* birds insects and reptiles (Speakman & Racey, 1988_a).

Assumption 5) *The D and ^{18}O isotopic composition of exiting CO_2 and H_2O is in equilibrium with the isotopic abundance of the organism's body water.*

Due to the rapid reaction catalyzed by carbonic anhydrase, CO_2 and H_2O will be in isotopic equilibrium with N prior to exiting the body. However, as the water of the body changes phase from liquid to gas, the lighter isotopes, ^{1}H, ^{16}O and ^{17}O, escape more frequently from the water phase than the heavy isotopes, increasing the relative abundance of D and ^{18}O in the liquid from which the gas has evolved. This process is called fractionation and occurs largely during transcutaneous evaporative water loss and expiration of CO_2 and H_2O. Un-corrected, fractionation may result in significant errors in rCO_2 estimates (Lifson & McClintock, 1966; Haggarty, McGaw & Franklin, 1988; Haggarty, 1991). Lifson & McClintock (1966) attempted to minimize the effect of this error by adding a correction factor to equation (1). The value of the correction factor was determined simply by observing the rate of fractionation of tap water evaporating from a beaker at 25°C. Although attempts have been made to model fractionation in more complex biological systems (Coward, Prentice, Murgatroyd, Davies, Cole, Sawyer, Goldberg, Halliday, & MacNamara, 1985; Schoeller *et al.*, 1986) no subsequent studies have been able significantly to increase the accuracy of Lifson and McClintock's (1966) correction factor (Speakman & Racey, 1988_a).

Assumption 6) *Environmental carbon dioxide and water does not enter the organism through skin or respiratory membranes.*

If CO_2 or H_2O enters an animals body across skin or respiratory membranes, the apparent isotopic turnover rates of D and ^{18}O will increase. Environmental H_2O entering the bee's body should, however, have a limited effect on the accuracy of rCO_2 measurements, as the turnover rates of D and ^{18}O will be altered by the same amount, not effecting the ratio between the two isotopes (Lifson & McClintock, 1966; Nagy, 1980). Environmental CO_2 entering the bee's body will, however, only increase the turnover rates of ^{18}O; thus increasing the rate of ^{18}O fractional turnover relative to D, resulting in over estimates of rCO_2. The degree of error created by exogenous CO_2 is, however, likely to be negligible in most circumstances (Speakman & Racey, 1988_b); although in situations where ambient air humidity is low (≤3.8 mg H_2O/L air) and CO_2 concentrations are high (≥3.4%), gross errors of up +81% in rCO_2 estimates may occur (Nagy, 1980).

3.0.4. Application of the DLW technique to *B.terrestris*

As the DLW technique was originally developed to measure the energy expenditure of vertebrates, it was not possible to transfer the methodology directly to invertebrates without modification. There were 5 main areas of the DLW technique which required alteration before the technique could be applied to *B.terrestris*:

1) *Isotopic analysis of* <u>*B.terrestris*</u> *plasma.*

One of the major problems in applying the DLW technique to bees, and invertebrates in general, is the need to remove relatively large volumes of plasma for isotopic analysis. Analysis of the $D_2{}^{18}O$ abundance within vertebrates normally requires a minimum four 5 µl samples of plasma to be extracted from the animal[2] (Tatner & Bryant, 1989). If this procedure were to be used in *B.terrestris*, 20µl of plasma would have to be removed from the bee. As the removal of 4µl of haemolymph has been found to alter the physiology of honeybees significantly (Maler, Fuchs, Pfeifer & Bounias, 1990), the extraction of 20µl of plasma from *B.terrestris* will almost certainly detrimentally effect the bee's physiology and behaviour. As the mean water content of *B.terrestris* workers is only 105±57 µl (n = 32) (*pers. obs.*), it was necessary to reduce the volumes of plasma required for isotopic analysis, preferably to volumes below 4µl (a mean of C. 4% of a bumblebees total body water volume).

2) *Administering* $D_2{}^{18}O$ *to* <u>*B.terrestris*</u>.

In vertebrates, $D_2{}^{18}O$ is normally administered by intraperitoneal injection, via disposable needles and syringes (Tatner & Bryant, 1989). This is not possible in *B.terrestris* for two reasons; i) bees do not have intraperitoneal

[2] These four samples exclude any extraction of plasma in order to determine background isotopic abundance.

cavities and ii) the wound created during injecting, with disposable needles, would be large relative to the body size of the bee, possibly damaging organs and causing bleeding. It was, therefore, necessary to develop a new protocol for injecting DLW directly into the bee's haemocoel, whilst minimizing the size of injection wound and preventing bleeding and damage to organs.

3) *Removal of plasma from B.terrestris for D and ^{18}O analysis.*

Plasma is normally withdrawn from vertebrates by puncturing a vein with a needle and drawing up the plasma into a syringe or micro-pipette (Tatner & Bryant, 1989). As bees have a low pressure, open circulatory system (Chapman, 1985), haemolymph must be extracted directly from the haemocoel. Although small volumes of plasma can be extracted using micro-pipettes, the tip of the pipettes rapidly become blocked with adipose tissue (*pers. obs.*). This limits the volume of extractable plasma, making it impossible to perform standard D and ^{18}O isotopic analysis. Thus, to permit full analysis of D and ^{18}O abundance, new techniques had to be developed to extract adequate quantities of haemolymph.

4) *Estimation of time taken for administered isotope to equilibrate with B.terrestris body water.*

The time taken for administered isotope to equilibrate with an animal's body water is highly variable, and depends on a range of physiological and behavioural traits such as water conservation strategies and activity levels.

Equilibration is normally determined by the plateau enrichment method (Gales, 1989). This involves plasma samples being extracted sequentially from the organism at regular intervals following isotope administration. As the isotopes equilibrate with the animal's body water, so the abundance of the animal's body plasma will increase. Once the isotope has completely equilibrated, plasma abundances will stop increasing, having reached a plateau. The time at which extracted plasma samples plateau can then be taken as the equilibration time of the organism. As this technique again requires the removal of large volumes of plasma, it was not possible to use it on *B.terrestris*. As a result, the development of a new technique to determine the equilibration time of administered isotopes was required.

5) *Measurement of initial $D_2{}^{18}O$ isotopic composition of <u>B.terrestris</u> plasma.*

Following equilibration of administered isotopes, "initial" plasma samples are normally removed, which involves the extraction and analysis of four 5µl samples of plasma (Tatner & Bryant, 1989). As described in point 1, the removal of such large volumes of plasma is likely to be deleterious to the physiology and behaviour of the bee. Even if it is possible to reduce the volume of haemolymph required for isotopic analysis, the removal of four separate samples would involve repeatedly puncturing the bee's cuticle, greatly increasing the chance of harming the bee. It was, therefore, necessary to develop new techniques for determining initial isotopic plasma abundances of *B.terrestris,* without extracting large volumes of plasma or repeatedly puncturing the exoskeleton.

3.0.5. Validation of the DLW protocol for *B.terrestris*

As the usual DLW protocol had to be altered before it could be used to measure *B.terrestris* energy expenditure, it was also necessary to validate the DLW estimates of rCO_2 with indirect calorimetry measurements. This was carried out to determine the accuracy of the developed DLW protocol, and detect any errors created by physiological or behavioral traits of bumblebees which violate the basic assumptions of the DLW technique.

3.1.0 General methodology for the analysis of ^{18}O and D abundance of water samples

Introduction

Stable Isotope Ratio Analysis (SIRA) by mass spectrometry is now well established as the standard method for determining the abundance of ^{18}O and D isotopes in water samples (Wong & Klein, 1986). SIRA measurements can be performed on water samples in two ways; i) direct analysis of the sample as a liquid, or ii) analysis of the isotopic abundance of gases evolved from the water sample. Direct analysis of the liquid phase of water can be performed using mass spectrometers such as the Isogas Aqua Sira (VG Ltd, Cheshire). This technique is, however, restricted to analysis of liquids with an isotopic abundance near background levels, due to their inability to measure accurately abundances above 1000 delta per mil ($\delta‰$) [1] (Speakman, Nagy, Masman, Mook, Poppitt, Strathearn & Racey 1990). As the DLW technique can require analysis of samples with isotopic abundances in excess of 5000 $\delta‰$ (*pers. obs.*), direct isotopic analysis of enriched water samples was not possible.

To measure accurately high D and ^{18}O abundances, gas Isotope Ratio Mass Spectrometry (gIRMS) is normally used (Speakman *et al.*, 1990, Wong, Klein,

[1] Per mil values refer to parts per 1000.

Parr & Clements, 1993). This involves converting water samples into CO_2, for ^{18}O analysis, and hydrogen gas, for D analysis. There are currently several methods capable of evolving H and CO_2 gas from water (Bigeleisen, Perlman & Prosser, 1952; Speakman *et al.*, 1990), but only four are in common use (Wong & Klein, 1986). These are; the production of CO_2 by the equilibration (Wong, Lee & Klein, 1987a,b) and guanidine hydrochloride conversion techniques (GH) (Boyer, Graves, Suelter & Dempsey, 1961; Dugan, Borthwick., Harmon, Gagnier, Glahn, Kinsel, Macleod & Viglino, 1985; Wong *et al.*, 1987b), and the production of H by the uranium reduction (Bigeleisen *et al.*, 1952; Wong & Klein, 1986) and zinc reduction techniques (Coleman, Shepard, Durnham, Rouse & Moore, 1982; Florkowski, 1985; Kendall & Coplen, 1985; Sudzuki, 1987; Wong, Lee & Klein, 1987a; Tanweer, Hut & Burgman, 1988).

3.1.1. Analysis of ^{18}O abundance in water samples using the equilibration technique

Equilibration is currently the most commonly used gIRMS method of ^{18}O analysis (Crowley, *pers com.*). This technique requires water and CO_2 to be mixed at 25°C in the following reaction:

$$H_2{}^{18}O + C^{16}O_2 \rightarrow H_2{}^{16}O + C^{18}O_2 \qquad (1)$$

After an equilibration period (dependent on the size of sample and quality of analytical equipment) the CO_2 is removed, cryogenically purified and admitted to a mass spectrometer.

The equilibration technique has one advantage over GH conversion; it does not destroy the sample or significantly alter its isotopic composition during analysis (Kishima & Sakai, 1980). It is, therefore, possible to use one sample for multiple analysis, not only determining the ^{18}O isotopic abundance but also the D abundance. This is important when limited volumes of water are available for analysis, as in the case of *B.terrestris*. However, even with the development of the Micro CO_2 Equilibration (MCE) technique (Kishima & Sakai, 1980) it is still not possible to analyse samples of ≤1µl in volume[2]. The technique is also time consuming, with equilibration of small samples taking between 48-72 hours (Speakman *et al.*, 1990). As a result the equilibration technique was not suitable for analysing the *B.terrestris* body water samples.

3.1.2. Analysis of ^{18}O abundance in water samples using the guanidine hydrochloride (GH) conversion technique

Unlike the equilibration technique, small sample analysis (samples of ≤1µl in volume) has been successfully carried out on water samples with the GH

[2] Kishima & Sakai (1980) report being able to analyse sample of between 4-5µl in volume using the MCE technique, they also suggest that it may be possible to analyse samples as small as 1µl in volume but have not validated this assumption. No other published work reports analysing samples below a volume of 10 µl using the equilibration technique.

conversion method. Using the GH method, Boyer *et al.*, 1961, reported reproducible results with samples as small as 0.1µl in volume (when analysing water samples with an isotopic equal to natural abundance).

The repeatability of ^{18}O isotopic analysis is reduced, however, in highly enriched samples and liquids with a high organic content, such as blood or milk, reducing the accuracy of the GH technique (Wong *et al.*, 1987a). As plasma samples taken from *B.terrestris* will contain both enriched levels of ^{18}O and organic matter, the use of the GH technique required further validation before it could be used in the present study. Despite the need for further validation, the GH conversion method was used to analyse the ^{18}O abundance of the bee's body water in preference to CO_2 equilibration, due to the technique's ability to analyse the isotopic abundance of small samples (samples with a volume of ≤1µl).

3.1.3. Analysis of D abundance in water samples using the uranium reduction technique

The most frequently used method for evolving Hydrogen gas from water is the uranium reduction technique (Wong & Klein, 1986). This involves admitting a water sample to an ultra high vacuum (UHV) preparation line; the sample is then vaporised and cryogenically moved through the vacuum system to a uranium furnace. Within the furnace, the water reacts to produce uranium oxide, thus liberating hydrogen gas from the sample. The evolved hydrogen is then collected by a topler pump or adsorbed to the surface of large

volumes of activated charcoal (Wong & Klein, 1986). The gas is then admitted to a mass spectrometer for gIRMS analysis.

Although the uranium reduction technique accurately measures background abundances of D in water samples, there are a number of limitations with this method when analysing small isotopically enriched samples. The major source of error in the uranium reduction technique is caused by isotopic memory within the uranium furnace. This is the effect of heavy isotopes from a water sample adsorbing to the surface of the uranium in the furnace. The trapped isotope is then released when the uranium is oxidised by a subsequent water sample. As a result, the evolved hydrogen from the subsequent samples becomes artificially enriched (Coleman *et al.*, 1982. Wong *et al.*, 1987a). Coleman *et al.*, 1982, reported that errors from the isotope memory effect increased as the volume of water sample decreased. Isotope memory will also increase as the isotopic abundance of the water sample increases (Crowley *pers. com*). This technique could, therefore, produce significant errors if used with small samples of isotopically enriched *B.terrestris* plasma. As a result the uranium reduction technique was not used to analyse the D abundance of *B.terrestris* body water.

3.1.4. Analysis of D abundance in water samples using the zinc reduction technique

The use of zinc for the liberation of hydrogen gas from water samples can be performed in two ways:

1. The zinc furnace technique.

   Zinc may be used in the same manner as uranium, by passing a water sample through a heated furnace containing zinc turnings. The heated zinc reacts with the water to produce zinc oxide, liberating the hydrogen component of the water (Wong & Klien, 1986). This method may, however, also result in analytical error due to isotopic memory within the reactor, making it an inappropriate technique to analyse the D abundance of a bee's body water.

2. The single sample technique.

   µl quantities of water may also be reacted with milligram quantities of zinc. Due to the small quantities of zinc required for the reduction reaction, it is possible to change the zinc and reaction vessel for each individual sample, thus preventing isotopic memory (Coleman *et al.*, 1982; Florkowski, 1985; Kendall & Coplen, 1985; Sudzuki, 1987; Wong *et al.*, 1987a; Tanweer *et al.*, 1988).

As the isotopic memory effect could produce significant errors in small isotopically enriched samples, the zinc single sample technique was used to analyse the D abundance of the *B.terrestris* body water.

3.2.0 Design and construction of ultra high vacuum manifold

To minimise contamination and fractionation of water samples, both ^{18}O and D preparation were performed using a custom built ultra high vacuum manifold.

The design of the UHV manifold had to take two factors into account:

1. The ability of the UHV manifold to attain a pressure sufficiently low to prevent significant contamination and fractionation of the water samples.
2. The ability of the UHV manifold to maintain this pressure during the period of analysis.

The pressures at which ^{18}O and D water preparation were performed in previous isotopic analysis studies varied with the isotope being analysed and the volume of water sample being processed. In large sample water analysis (≥5µl) a pressure of ≤E-2 mb is required for GH preparation (Wong *et al.*, 1987b), whilst zinc reduction analysis of water requires a pressure of ≤E-3 mb (Florkowski, 1985: Tanweer *et al.*, 1988). In small sample analysis (≤5µl), however, contamination and fractionation can result in significant analytical errors at pressures as low as E-3 mb (*pers. obs.*). For this reason the UHV manifold was designed to attain pressures of ≤E-5 mb, thus avoiding any significant contamination of water samples during analysis and preparation.

The general design of the UHV manifold was based on a system currently used at the Scottish Universities Research Reactor Centre (SURRC)[3]. The SURRC system uses two UHV manifolds, one for ^{18}O preparation and one for D preparation. The separate UHV manifolds were necessary due to the use of a uranium furnace attached to the D manifold.

The SURRC UHV manifold was constructed of borosilicate glass, with joints in the glassware connected by greased Quick Fit © sockets. The pumping system comprised a Mercury Vapour pump and an Edwards © Stage 2 Rotary pump. This system could achieve a minimum pressure of 2.6 E-2 mb ±0.2, and could maintain this pressure for a period of 3.4 minutes ±0.5 (*pers. obs.*).

Although these pressures are adequate when analysing samples of ≥5µl in volume, sufficiently accurate analyses were not possible from small samples prepared on this system, due to high levels of contamination from mercury, vacuum grease and room air (*pers. obs.*). The UHV manifold was, therefore, re-designed and built at Liverpool John Moores University to allow the preparation, and subsequent isotopic analysis, of small volumes of plasma extracted from *B.terrestris*.

[3] The design of the SURRC vacuum line is described by Tatner & Bryant (1989)

As in the SURRC line, the main tubing was made from borosilicate glass (Figure 3.2.1 & 3.2.2). All Quickfit © joints were replaced with greaseless 'Cajon™" type "O" ring seals, thus preventing the need for vacuum grease and the contamination which it created in the SURRC line (*pers. obs*). Flexible vacuum tubing was placed at stress points to reduce any cracking within the borosilicate glass.

The vacuum pumping system was also modified (Figure 3.2.2), substituting an Edwards Speedi-Vac © vapour diffusion pump for the mercury diffusion pump used at the SURRC. High grade Santovac 5™ diffusion pump oil was used in order to attain the lowest possible pressures, and to decrease back-flushing[4] within the system. The latter was further reduced by placing a fore-line trap between the diffusion pump and the borosilicate vacuum line. The fore-line trap contained ≈ 200 gm of 23 micron activated aluminium balls. Vaporised diffusion pump oil passing through the fore-line trap was adsorbed on to the surface of the aluminium balls. The fore-line trap could be removed from the UHV preparation line, allowing the activated aluminium to be cleaned[5] or replaced.

For the vapour diffusion pump to operate correctly, a "backing" vacuum was required. The backing vacuum removes any material which is condensed

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[4] "Back-flushing" is the process of vaporised diffusion pump oil being drawn into the UHV manifold due to fluctuations in internal pressure. Back-flushed diffusion pump oil will act as both a sample contaminant and a substance capable of adsorbing isotope, potentially creating fractionation of any water samples.

[5] The aluminium balls where cleaned by heating at 100°c for 48 hours in a desiccating oven, this occurred approximately every 3 months.

Figure 3.2.1. Design of Ultra High Vacuum (UHV) Manifold, used in the preparation of CO_2 and H gas for gIRMS analysis

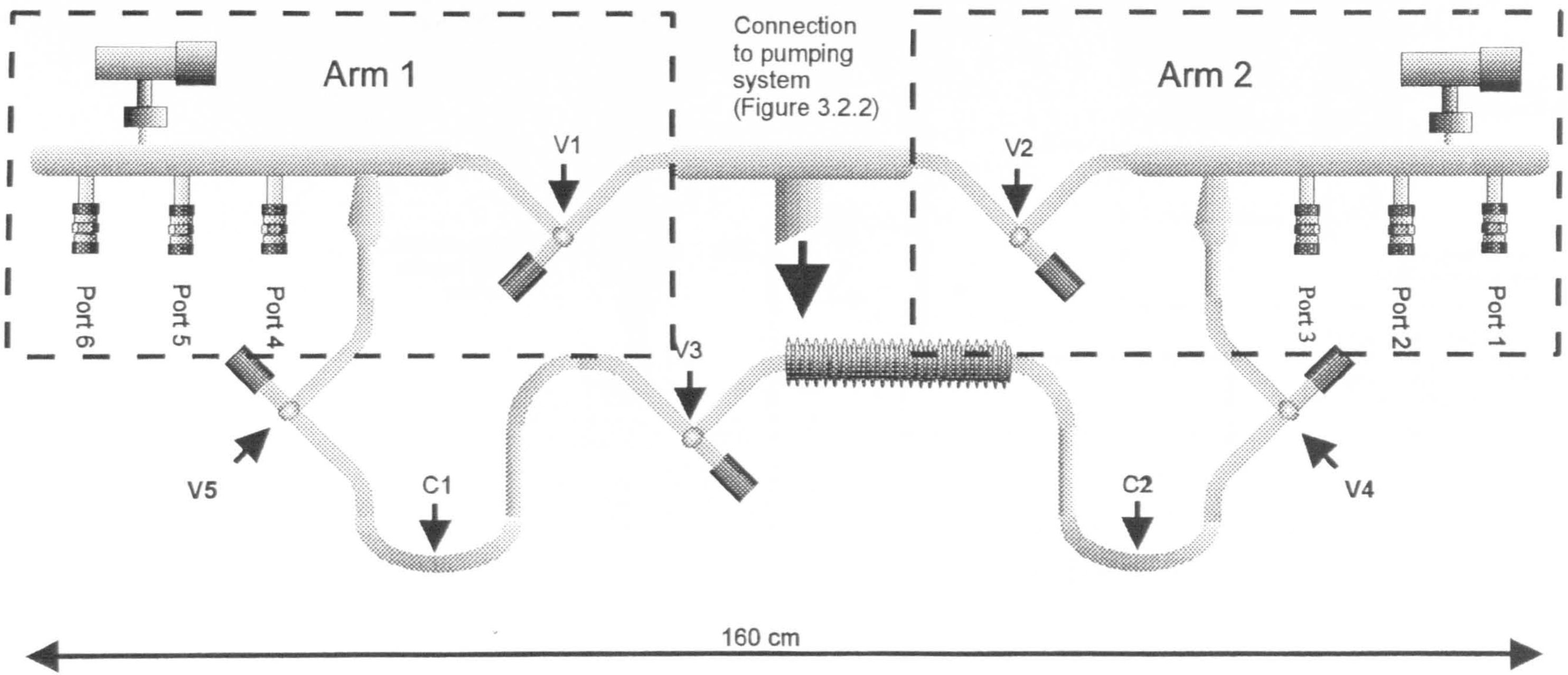
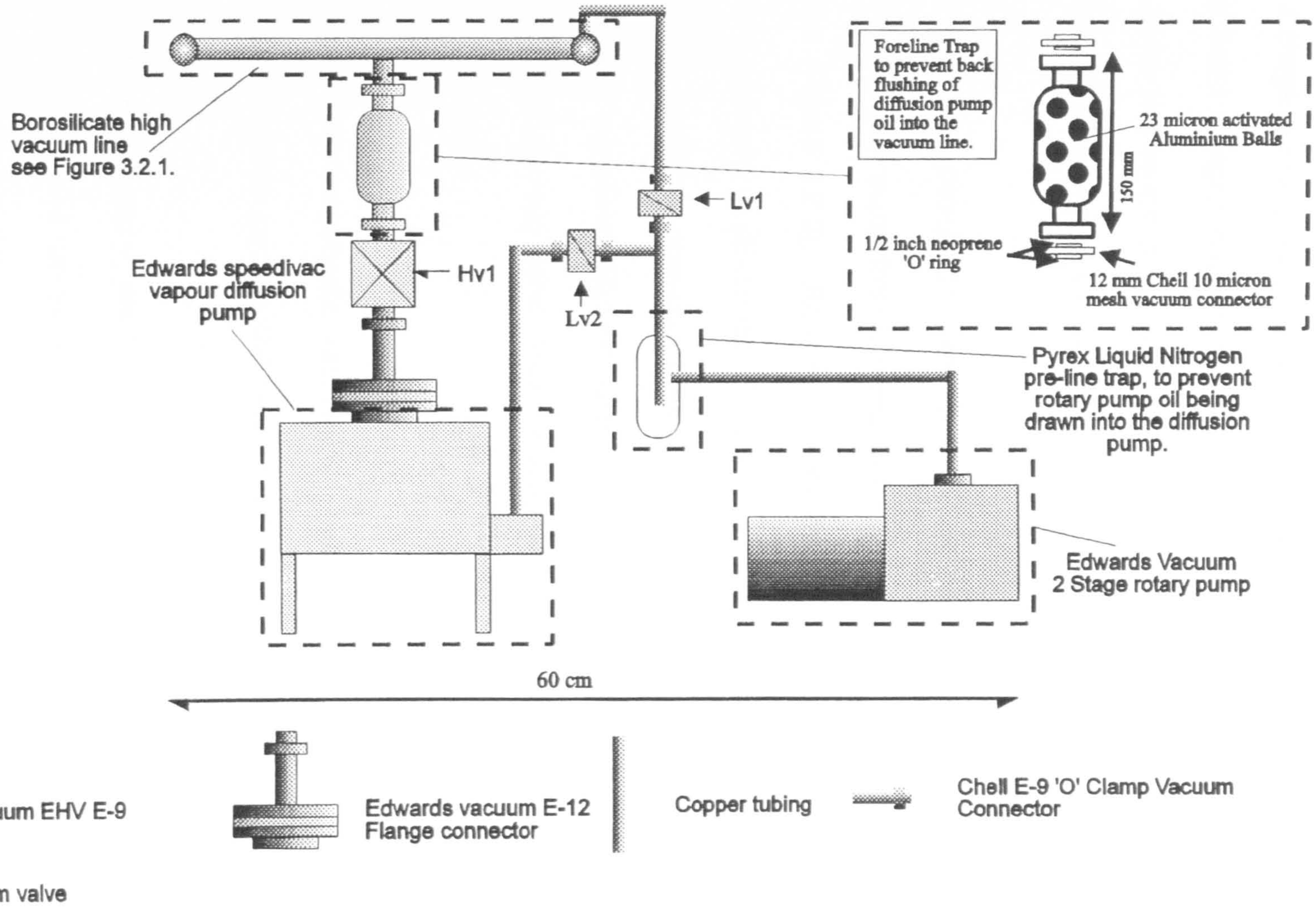


Figure 3.2.2. Ultra high vacuum pumping system.

When evacuating the UHV manifold, it was first necessary to "rough" the line, (evacuate the manifold to a pressure of <E-0.05 mb). This was done by opening Lv1 whilst Hv1 was closed, thus opening the rotary pump to the line. The line could then be opened to the diffusion pump by closing Lv1 and opening Hv1. Lv2 was opened in order to allow the rotary pump to act as a backing pump to the diffusion pump.



within the diffusion pump oil. The backing vacuum was produced by an Edwards 2 Stage © rotary pump, attached to the diffusion pump with copper tubing. A liquid nitrogen pre-line trap was placed between the rotary and diffusion pump to prevent rotary and diffusion pump oil from mixing, thus maintaining optimum pumping conditions.

The rotary pump was also used to perform "roughing" of the UHV manifold, by isolating the diffusion pump from the line and then opening the line to the rotary pump (Figure 3.2.2). Roughing is the process of evacuating the manifold to a pressure of ≤E-0.5 mb. This is necessary as vapour diffusion pumps have a maximum operating pressure of ≤E-0.5 mb. At pressures greater than this, vaporised diffusion pump oil is "sucked" from the diffusion pump by the backing rotary pump, thus reducing the diffusion pump's ability to attain low pressures.

Pressure within the borosilicate vacuum line was monitored using four Edwards E-5 mb Vacuum gauges, connected to an Edwards controller.

With the above modifications, the UHV manifold was capable of attaining a pressure of ≤E-5 mb and maintaining this pressure for a period of C.200 minutes after valve V1 and V2 were sealed (Figure 3.2.1).

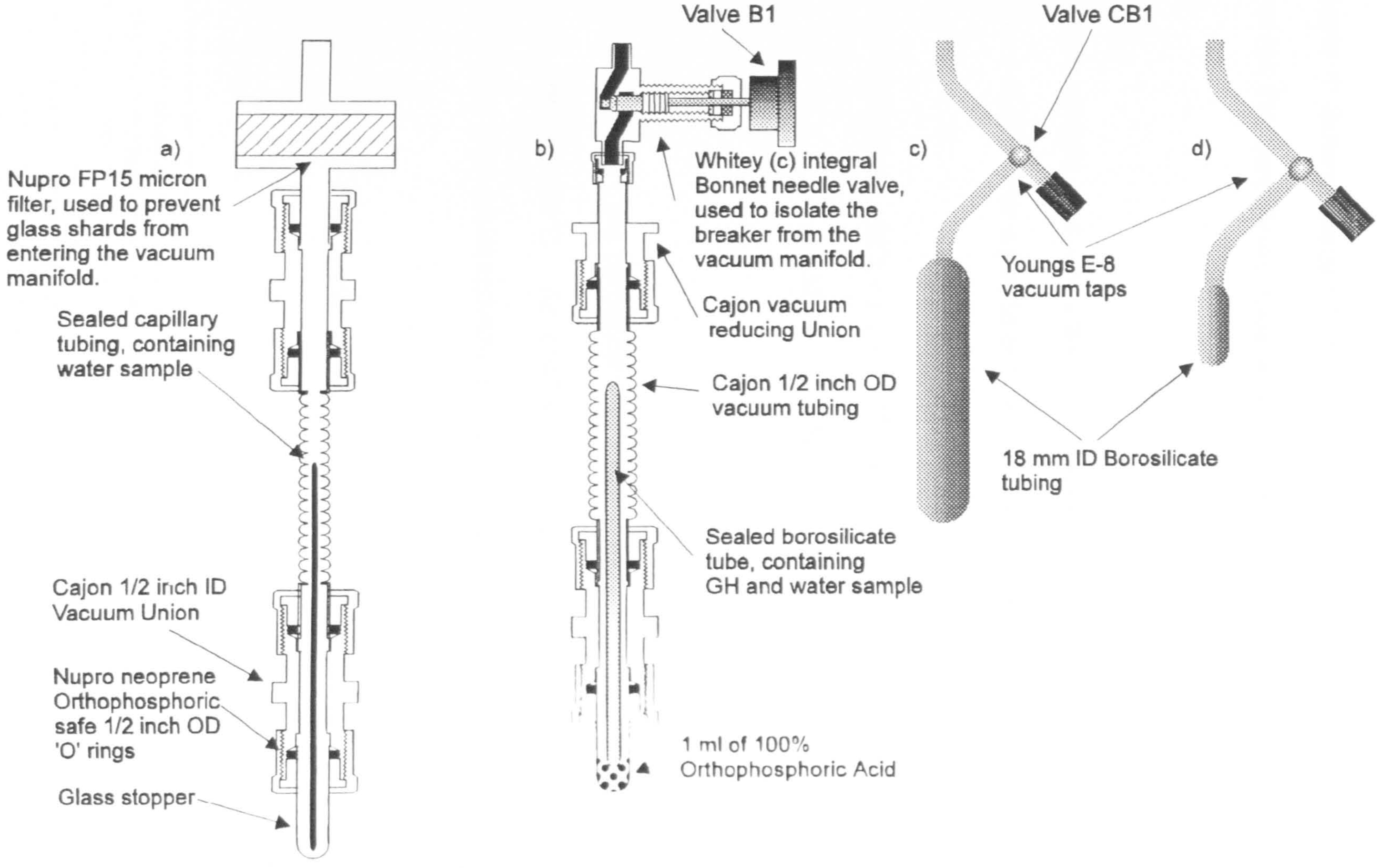
3.2.1. GH preparation and analysis of the ^{18}O isotopic content of water samples

To determine the ^{18}O isotopic composition of *B.terrestris* plasma, samples were converted from H_2O to CO_2 for subsequent gIRMS analysis of δ44 / δ46 ratios. Conversion of the samples was carried out using the GH method (Boyer *et al.*, 1961; Kishima & Sakai, 1980; Dugan *et al.*, 1985; Wong *et al.*, 1987b).

Plasma samples were flame sealed in 5µl Vitrex micro-pipettes © and placed in a stage 1 micro-pipette breaker (Figure 3.2.3). The stage 1 breaker was then attached to the UHV manifold via port 2s Cajon union (Figure 3.2.1). Port 1 was sealed with a glass stopper, whilst a 6mm internal diameter (ID) borosilicate tube containing 100 mg of BDH anhydrous GH was placed on port 3. Valve V2 was then opened and arm 2 of the manifold evacuated to a pressure of ≤E-5 mb. Following evacuation, valve V2 was closed. The micro-pipette containing the plasma sample was then broken by the stage 1 breaker, thus exposing the plasma to the UHV. The plasma sample was then frozen into the borosilicate tube, attached to port 3, by liquid nitrogen distillation[6].

[6] Liquid nitrogen distillation is the process where liquids are evaporated within a vacuum. The liquid is then transported in its gaseous state and condensed back into a liquid by freezing with liquid nitrogen.

Figure 3.2.3.
a) Stage 1 capillary breaker, capillaries were cracked by bending the flexible tubing. **b)** Stage 2 borosilicate tube breaker. The borosilicate tubes were scored and then broken by bending the flexible tubing **c)** Large sample CO_2 collection bulb. d) Small sample CO_2 collection bulb.



When the pressure had stabilised, valve V2 was re-opened to evacuate any non-condensable material[7] from the UHV manifold. The borosilicate tube, containing the frozen plasma sample and GH, was then flame sealed using a Microflame © Butane/Oxygen burner (the dewer of liquid N_2 remained in place whilst the borosilicate tube was sealed). V2 remained open during sealing.

The plasma sample and GH were then heated in a muffle furnace at a temperature of 260°C for a period of 10 hours, thus catalysing the reaction:

$$NH_2C{:}(NH) \bullet NH_2 \bullet HCL + 2H_2O \xrightarrow{>260^{\circ}C} 2NH_3 + CO_2 + NH_4 \bullet CL \qquad (2)$$

Following reaction (2), the borosilicate tube was removed from the oven and allowed to cool to ≤70°C. As cooling occurred, the ammonia and CO_2 combine to form ammonium carbamate by the reaction:

$$2NH_3 + CO_2 + NH_4CL \underset{<70^{\circ}C}{\overset{>70^{\circ}C}{\Leftrightarrow}} NH_4NH_2CO_2 + NH_4CL \qquad (3)$$

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[7] During vacuum preparation, gases are produced and liberated from the plasma samples and the chemical reagents reacted with the plasma. Some of these gases have no liquid phase or have a freezing point below -197°C, thus, they cannot be condensed by freezing with liquid nitrogen. In the process of liquid nitrogen distillation, the presence of these "non-condensable materials" prevents the internal pressure of the UHV manifold from returning to its initial pressure prior to the breaking of the 5μl pipette. As the presence of such material can effect the accuracy of the mass spectrometer, they are removed from the sample by the vacuum pumping system of the UHV manifold.

The CO_2 was then extracted from the ammonium carbamate by reaction with Orthophosphoric Acid. This was achieved by placing the borosilicate tube containing the ammonium carbamate into a Stage 2 breaker (Figure 3.2.3b) containing 1 ml of 100% BDH analar Orthophosphoric acid. Prior to placing the borosilicate tube into the stage 2 breaker, the tube was scored with a glass cutter, thus facilitating the "cracking" of the tube.

The stage 2 breaker was then placed on arm 2 of the UHV manifold via one of the ports Cajon unions. Empty ports were sealed with glass stoppers. Valve V2 was then opened and the UHV manifold and breakers evacuated to a pressure of ≤E-5 mb. Due to the hydrophilic nature of Orthophosphoric acid, significant quantities of water were normally present prior to evacuation[8]. As a result, evacuation took around 12 hours to complete, and was normally performed over night.

Following evacuation of arm 2, valve B1 on the stage 2 breaker was closed. The ammonium carbamate was then exposed to the Orthophosphoric acid by bending the flexible tubing of the stage 2 breaker, thus fracturing the borosilicate tube. The stage 2 breaker was then removed from arm 2 of the UHV manifold and placed in a 90^0C oven for a period of 60 minutes, thus

[8] The Orthophosphoric acid was stored in a desiccating oven.

releasing the CO_2 through the reaction:

$$NH_4 \bullet NH_2 \bullet CO_2 + H_2PO_4 + NH_4 \bullet CL \xrightarrow{90^0 C}$$

$$CO_2 + (NH_4)_2 \bullet PO_4 + NH_4 \bullet CL \qquad (4)$$

The stage 2 breaker was then removed form the 90°C oven and placed on Arm 1 of the UHV manifold, and a CO_2 collection bottle (Figure 3.2.3) placed on arm 2. All empty ports were sealed with glass stoppers. Valves V1 to V5 were then opened, and the line evacuated to a pressure of ≤E-5 mb.

Before transferring the CO_2 sample in the stage 2 breaker into the CO_2 collection bottle, the sample was cryogenically purified. This was done by passing the sample through a series of varying temperature cold traps.

3.2.2. Cryogenic purification of CO_2

After the UHV manifold had been evacuated, Valves V2 and V3 were closed. Valve B1 on the stage 2 breaker was then opened allowing the CO_2 sample into arm 2. A dewer of liquid nitrogen was placed under cold trap C2 of the UHV manifold, thus freezing the CO_2 into C2. Once the gas sample had been frozen into C2, valve V4 was closed. The liquid nitrogen dewer was then replaced by a dewer containing acetone and dry ice, at a temperature of approximately -80°C. This temperature allowed the frozen CO_2 within C2 to

melt and vaporise, but retain any contaminants with a freezing point higher than -80°C.

A dewer containing an acetone/dry ice mix was also placed under cold trap C1, in order to trap any contaminants not frozen in C2. Valve V1 was then closed and a dewer containing liquid nitrogen placed under the CO_2 collection bottle on arm 1. Valve V3 was then opened and the CO_2 frozen into the CO_2 collection bottle. The Valve CB1 on the collection bottle was then closed, allowing the sample to be removed from the UHV manifold and placed on a mass spectrometer inlet manifold for gIRMS analysis.

3.2.3. gIRMS analysis of CO_2 gas for $\delta 44$ / $\delta 46$ ratios

CO_2 gas samples were analysed on a VG SIRA 12 mass spectrometer at the Liverpool University Stable Isotope Laboratory. All samples were analysed following the standard protocol described in the VG users handbook. The results of analysis were expressed in delta (δ) per mil ($^{o}/_{oo}$), defined as:

$$\delta^{18}O,^{o}/_{oo} = \left(\frac{Rsample}{Rws} - 1\right)10^{3} \qquad (5)$$

where Rsample and Rws are the ^{18}O / ^{16}O ratios of the sample and working standard respectively. The Rws was normalised against two international standards, V-SMOW (Vienna-Standard Mean Ocean Water) and SLAP

(Standard Light Arctic Precipitation) according to Gonfiantini (1978), and any mass spectrometer offset corrected for by analysing to internal laboratory standards (Wong *et. al.*, 1993).

3.2.4. Zinc reduction and analysis of the Deuterium abundance of water samples

It has been shown that the ability of zinc to reduce water samples without significantly altering the samples isotopic abundance, is dependent on i) low levels of contaminants within the zinc reagent (Coleman *et al.*, 1982; Florkowski, 1985; Sudzuki, 1987) and ii) the grain size of the zinc shot (Florkowski, 1985; Kendall & Coplen, 1985). At present there are no commercially available sources of zinc of optimal size and contamination. It was, therefore, necessary to use an experimental zinc reagent developed by Indiana University Geological Department. The zinc was optimally contaminated with inter-metallic compounds[9], and supplied as turnings rather than shot.

To prevent contamination of the zinc, the reagent was stored under vacuum conditions at a pressure of ≤2.0 E-2 mb. To react the zinc with water samples, 20 mg of zinc was used per 1 µl of sample water. The zinc was rapidly transferred from the vacuum container to a 6 mm ID Vycor[10] tube ©.

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[9] The exact nature of the inter-metallic compounds within the zinc reagent was not disclosed.

[10] Vycor © tubing was used in preference to borosilicate due to the ability of evolved hydrogen to react with the surface of borosilicate glass (Kendall & Coplen, 1985).

The Vycor © tube was then attached via a Cajon union to port 1 of the UHV manifold (Figure 3.2.1).

The water sample to be analysed was flame sealed into a 5µl Vitrex capillary tube and placed in a stage one capillary breaker (Figure 3.2.3a). The stage one breaker was attached via a Cajon union to port 2 of the UHV manifold. The manifold was then evacuated to a pressure of ≤E-5 mb. Following evacuation, the Vycor tubing containing the zinc was gently heated with a blow torch, thus removing any adventitious water from the surface of the zinc.

Once the Vycor © tube had cooled to room temperature, Valves V2 and V4 were closed, and the capillary in the stage one breaker cracked open. The water sample was liquid nitrogen distilled into the bottom of the Vycor tube. Valve V2 was then opened to remove any non-condensable material from the manifold, following which the Vycor tube was flame sealed.

The reduction process was then catalysed by placing the Vycor tube, containing the zinc reagent and water sample, into a 500°C muffle furnace for a period of 30 minutes.

The Vycor © tube was allowed to cool to room temperature and then placed in a stage 2 borosilicate tube breaker (Figure 3.2.3b) and placed on the inlet manifold of the mass spectrometer. The inlet manifold and stage 2 breaker were evacuated to a pressure of ≤E-5 mb. Once evacuated, valve B1 was

closed and the Vycor tube broken, releasing the gas into the stage 2 breaker. The gas sample could then be admitted to the mass spectrometer for gIRMS analysis. All analyses of hydrogen samples was completed within 30 minutes of the Vycor tube leaving the muffle furnace. This was to minimise any reaction between the surface of the Vycor tubing and the Hydrogen gas.

3.2.5. gIRMS analysis of Hydrogen gas for H/D ratios

The H/D isotopic composition of the gas was analysed using a VG SIRA 602 mass spectrometer at Liverpool John Moores University. The samples were admitted to the mass spectrometer by opening valve B1 on the stage 2 breaker. The samples were then processed according to the VG users manual. The results were recorded using a PC and VG DACC card

All results were expressed in D units defined as:

$$\delta D, ‰ = \left(\frac{Rsample}{Rws} - 1 \right) 10^3 \qquad (6)$$

in which the Rsample and Rws are the D ratios of the sample and laboratory standard respectively. These values were then corrected for H_3^+ contribution to the ion beam of the mass spectrometer using the standard protocol described in the VG SIRA's users handbook. Also, 2 internal laboratory

two standards were run every morning in order to correct for any mass spectrometer offset (Wong *et al.*, 1993)[11].

3.2.6. Calculation of D and ^{18}O PPM abundance from δ ($^{o}/_{oo}$) value

For the calculation of rCO_2 the normalised $\delta(^{o}/_{oo})$ values have to be expressed as parts per million (ppm). The per mil values were converted using equation (8) for ^{18}O and equation (9) for D.

$$^{18}O \text{ PPM} = 2.0052 \times \text{normalised } \delta\ (^{o}/_{oo}) + 2005.2 \qquad (8)$$

$$D \text{ PPM} = 0.15575 \times \text{normalised } \delta\ (^{o}/_{oo}) + 155.75 \qquad (9)$$

where 2005.2 = the ^{18}O abundance of V-SMOW and 155.75 = the D abundance of V-SMOW.

[11] Enrichment of standard 1 equalled 3437 ppm and 4521 ppm for standard 2.

3.3.0 Validation of the analysis of ^{18}O and D abundance in small isotopically enriched water samples

Introduction

The DLW technique requires the measurement of the fractional turnover of $D_2{}^{18}O$ isotopes in an animal's body water pool (Section 3.0.2.). This normally requires the extraction of μl quantities of plasma from the animal for isotopic analysis. However, as discussed previously (Section 3.0.4.), this technique is not applicable to bumblebees due to the relatively small volume of their body water pool. As a result, in order to apply the DLW technique to bees, it was necessary to reduce the volume of plasma required in order to analyze the isotopic composition of the bees body water pool. As no previous attempts have been made to analyze the $D_2{}^{18}O$ abundance of isotopically enriched water samples below 3 μl in volume (Kishima & Sakai, 1980, Dugan *et al.*, 1985, Florkowski, 1985, Kendall & Coplen, 1985, Sudzuki, 1987, Wong *et al.*, 1987a, Tanweer *et al.*, 1988); it was not clear how small sample volumes could be whilst still permitting accurate isotopic analysis. To determine how much plasma needed to be extracted from a bee, it was necessary to determine the minimum

volume of plasma required for accurate D and ^{18}O isotopic analysis. This was achieved using two approaches:

1) The effect of sample volume on precision was investigated by using a series of enriched standards, all with the same isotopic composition, but ranging in volume from 0.1 to 10 μl. If the analytical technique, described in Section 3.2.1 & 3.2.4, was capable of analyzing volumes of plasma ≤3 μl, no significant differences should have been found between the standards of varying volume.

2) Such comparisons will, however, only determine the reproducibility of the analytical procedure, and will not indicate the absolute accuracy of the technique. To determine the absolute accuracy of the analysis, 10μl samples of the same enriched standard were also analyzed by an independent laboratory. Comparisons between the independent analysis of the standard with analysis performed using the technique described in Section 3.2.1 & 3.2.4, therefore, permitted the precision of the procedure to be determined.

3.3.1. Materials and methods

The DLW standard was prepared gravimetrically by diluting 1 ml of 20% atom excess $D_2{}^{18}O$ (Europa Scientific, Crewe, England)[1] with 160 ml of distilled water. The standard was flame sealed into Vitrex © micro-pipettes in volumes of 10, 5, 1, 0.5, and 0.1 µl respectively. The ^{18}O abundance of the standard was analyzed in replicate at Liverpool University using volumes of 10, 5, 1, 0.5, and 0.1µl of standard, and the D abundance was analyzed in replicate at Liverpool JMU, using volumes of 10, 5, 2 and 1 µl of standard. The ^{18}O and D analysis was performed as described in Section 3.2.1 & 3.2.4. Replicates of the standard were also analyzed at Groningen University stable isotope laboratory, Holland, at volumes of 10µl for both ^{18}O and D analysis. The Groningen ^{18}O abundance of the standards were analyzed using the GH reduction method (Section 3.2.1) and the D abundance using the uranium reduction technique (Section 3.1.3).

The results were expressed as δ (‰) and normalized against V-SMOW and SLAP, as described in Sections 3.2.3 & 3.2.6. To enable comparison between previous inter-laboratory studies, the normalized δ (‰) values were then converted to ppm (Section 3.2.3 & 3.2.6.).

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[1] 20% atom excess refers to a water sample which contains a 20% percentage greater ratio of ^{18}O to ^{16}O isotopes and ^{1}H to D isotopes above levels observed in V-SMOW (Gonfiantini, 1978).

3.3.2. Results

Little variation was observed in the isotopic abundances between the standards analyzed at Liverpool University and Liverpool JMU, irrespective of volume of standard analyzed (Table 3.3.1). The variation in abundance between samples of different volumes was found to be non-significant for both the ^{18}O (Anova; $F_{4,20} = 1.008$, $p = 0.426$,) and D ($F_{4,16} = 1.21$, $p = 0.343$) isotopes.

Similarly, there was little variation between the observed mean Liverpool University and Liverpool JMU analyzed abundances of the standards, and the abundances observed by independent analysis at Groningen University. The mean ^{18}O and D abundances observed at Liverpool University and Liverpool JMU were 3110.3±15.5ppm (n = 25) and 506.4±17.3 ppm (n = 20) respectively. These results compared well with the independent analysis of the water standards performed at Groningen University, where an ^{18}O abundance of 3109.0± 34.5 (n= 3) ppm and a D abundance of 505.5± 0.9 (n = 3) ppm was observed (Table 3.3.1). The difference between the Groningen and Liverpool University / Liverpool JMU analysis was non-significant for both ^{18}O (G_1 = <0.001, ns) and D isotopes (G_1 = <0.002, ns).

Table 3.3.1
Repeatability of isotopic analysis of different volumes of enriched water standards.

^{18}O isotopic abundance of water standard (PPM)

Site of analysis	Liverpool University					Groningen
volume of sample	10 µl	5 µl	1 µl	0.5 µl	0.1 µl	10 µl
n	5	5	5	5	5	3
mean	3110	3112	3119	3102	3111	3109
sd	9.5	15.0	15.0	10.9	14.4	34.5
*% error	0.02	0.08	0.32	-0.23	0.07	-

D isotopic abundance of water standard (PPM)

Site of analysis	Liverpool JMU				Groningen
volume of sample	10 µl	5 µl	2 µl	1 µl	10 µl
n	5	5	5	5	3
mean	502	507	504	514	506
sd	17.0	14.4	12.5	29.9	8.8
*% error	-0.70	0.31	-0.33	1.61	-

***% error of the Liverpool analysis was calculated as the % difference in the isotopic composition of the water standard analyzed at Liverpool from the isotopic composition of the water standard analyzed at Groningen.**

3.3.4. Discussion

Comparison of the ^{18}O isotopic abundance of the standard showed no significant variation when analyzed using different volumes of sample. Thus, the analytical technique described in Section 3.2.1 resulted in observations of isotopic abundance which were repeatable in standards as small as 0.1μl in volume. Also, analysis of the ^{18}O isotopic abundance of the standard showed no significant variation between abundances observed at Liverpool University stable isotope laboratory and Groningen. Assuming the ^{18}O abundance reported by the Groningen University was correct, this confirms that the absolute ^{18}O abundance observed in samples as small as 0.1μl in volume were also reliable.

The % error in the ^{18}O analysis, at Liverpool University stable isotope laboratory, compared well to previous inter-laboratory studies, with a mean error in ^{18}O analysis of +0.05±0.4% (n = 25), compared to a mean error of -1.4±0.4%, reported by Speakman *et al.* (1990)[11]. Data regarding analytical error during D analysis from previous inter-laboratory studies were not available

As in the case of the ^{18}O analysis, no significant variation was observed in the D isotopic abundance of the water standards analyzed using different volumes of

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[11] Speakman *et al.* (1990) reported the accuracy of ^{18}O interlaboratory analysis using enriched standards of 3, 5 and 10μl in volume.

sample, again indicating that it was possible to produce repeatable D isotopic analysis on water samples as small as 1μl in volume. The lack of any significant variation between the D abundance of the water standards analyzed at Groningen University and the abundance observed at Liverpool JMU again indicated that the absolute values of observed D abundance were reliable for water samples as small as 1μl in volume.

It was, therefore, clear that it was necessary to withdraw 0.1μl of plasma from a bee for ^{18}O analysis and 1μl of plasma for D analysis. Assuming the analysis was performed in replicate (to reduce analytical error), this would mean a total of only 2.2μl of plasma would have to be extracted from the bee in order to determine the $D_2{}^{18}O$ isotopic abundance of the bees body water pool.

3.4.0 General methodology for Administering Isotope to bumblebees

Before it was possible to use the DLW technique to measure a bumblebee's energy expenditure, it was necessary to administer $D_2{}^{18}O$ isotopes into the body water pool of the bee. Two methods of administration were considered.

1. Mixing isotopes with water and sugar, producing an isotopically enriched "nectar", which could then be fed to the bee. After the bee had consumed the enriched nectar, isotopes within the sugar water could then pass through the crop membrane of the bee as the nectar was metabolized.
2. Direct injection of the isotope into the haemocoel of the bee.

3.4.1. Administering $D_2{}^{18}O$ by feeding isotopically enriched nectar to bumblebees

Although feeding isotopes to a bee may appear the less intrusive of the two techniques, there are a number of problems associated with this approach resulting from a bumblebee's behaviour and physiology. If a bumblebee were to be fed isotopically enriched sugar water, the imbibed nectar would be stored within the crop until it was either metabolized by the bee, or delivered to the hive (Heinrich, 1979). When delivering nectar to the hive, bumblebees contract their

crop and regurgitate the collected nectar into a honey pot, which would result in any isotopically enriched sugar water within the crop being lost from the bee. It was, therefore, not possible accurately to regulate the amount of isotope administered to the bees plasma using this technique. As isotopic enrichment of the bees plasma could not, therefore, be controlled, it would not have been possible to ensure that the bees plasma isotopic abundance were sufficiently high to enable gIRMS analysis.

The speed at which nectar is transported across the crop membrane is variable depending on the energy requirements and water balance of individual bees (Bertsch, 1984). As a result, it was also not possible accurately to determine the time at which imbibed, isotopically enriched nectar had been fully transported from the crop to the bee's body water pool. If DLW measurements of energy consumption were taken before the isotope had fully equilibrated with the bee's body water, significant errors could occur in rCO_2 estimates (Nagy, 1980; Fancey *et al.*, 1986; Speakman & Racey 1988_b). For these reasons, the use of labeled nectar was rejected in favour of direct injection of isotope into the haemocoel.

3.4.2. Administering $D_2{}^{18}O$ to bumblebees by direct injection of isotope into the haemocoel

To enable direct injection of isotopes into bumblebees, the injection procedure had to be capable of:

1. Administering the isotope into the bee without creating an injection wound large enough to significantly affect a bumblebee's physiology and behaviour, and minimizing isotope leakage.
2. Injecting the isotope into the bee without damaging any of the internal organs of the bee, or puncturing the crop[1].

3.4.3. Production of Micro-Needles for injection

To reduce any detrimental effects experienced by the bee following injection, the outside diameter (OD) of the needles used to puncture the bee's body and administer the isotope was minimized. Reduction in the size of injection needle reduced the size of the injection wound, thus minimizing bleeding of haemolymph and isotope leakage from the bee. Commercially available needles were too large for use in micro-injection (Fishel *pers. com.*); however, using

[1] If a bee's crop is punctured during injection, imbibed nectar will leak from the crop into the haemolymph. Not only will this affect the physiology of the bee, but it could also result in significant errors in DLW rCO_2 estimates.

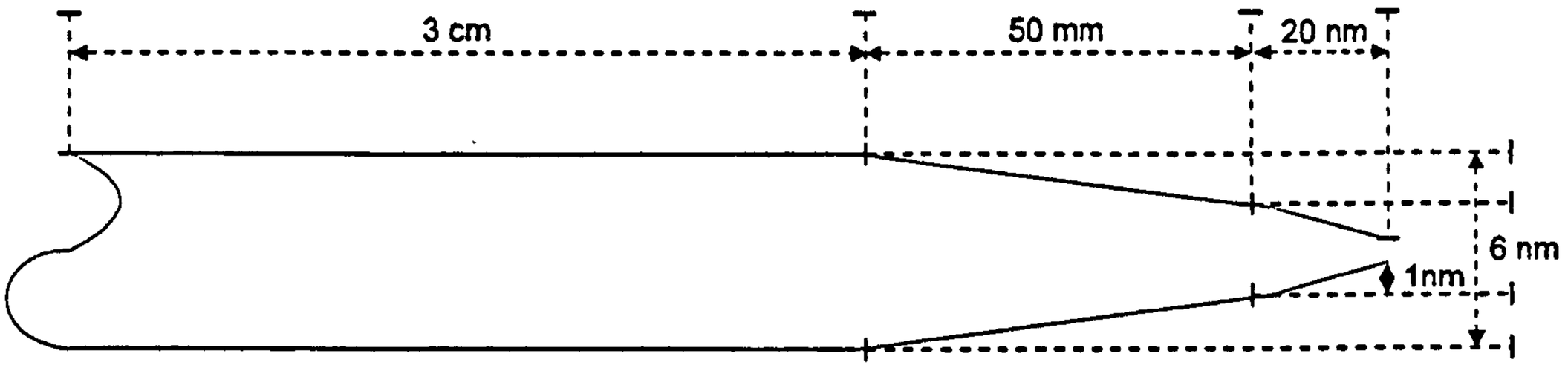
techniques developed for *in-vitro* fertilization (Fishel & Symonds, 1993) it was possible to manufacture needles with an approximate OD of only 4 nm.

Injection needles were produced from borosilicate 5µl Vitrex © micro-pipettes, (any pipette with an OD of ≤ 100 microns can be used (Fishel & Symonds, 1993)). The tip of the needle was formed by heating the central 2 cm portion of the micro-pipette with a platinum filament. Simultaneously, the micro-pipette was stretched, thus thinning the central portion. This process was performed using a Micro-pipette Puller © (Research Instruments Ltd, Cornwall, UK). The heat of the filament and tension force of the pipette puller could be adjusted, enabling the degree of thinning of the micro-pipette to be accurately controlled. This made it possible to produce "pulled" pipettes with an OD of ≥ 6 nm.

The thinned section of the pipette was then cut using a Research Instruments Microforge ©. This produced two needles, the tips of which had an OD equal to that of the thinned section of the pipette prior to cutting (≈ 6 nm). The OD of the micro-needle was further reduced using a Research Instruments Micro-Grinder ©, this enabled a bevel of ≈ 2 nm to be placed on the tip of the micro-injection

needle. The approximate dimensions of the injection needle can be seen in Figure 3.4.1.

Figure 3.4.1.
The typical dimensions of a needle used to inject Isotopically enriched water into *B.terrestris.* (Dimensions are approximated, although electron microscopy has determined the typical standard error to be +/- 0.005 nm (Fishel *pers. com.*)).



Not to scale

3.4.4. Use of micro-needles and micro-manipulator to inject bumblebees with $D_2{}^{18}O$

Although micro-needles enabled control over the size of injection wound, they d'd not provide control over the volume of isotope injected, or the precise positioning of the needle needed during injection to avoid damage to internal organs. This was achieved using micro-manipulation techniques. The volume of $D_2{}^{18}O$ isotope injected into the bee was controlled using a 2µl Hamilton ® micro-syringe and Cheney ™ adapter. The micro-needles were slipped over the needle of the Hamilton syringe, and the connection between the two needles sealed using melted dental wax. Accurate positioning of the needle during

injection was achieved by attaching the Hamilton syringe to a Shufer micro-manipulator ©. Bees were restrained and positioned during injection using a maneuverable injection stage (Figure 3.4.2).

3.4.5 Injection procedure

Before injection, bees were anaesthetized by cooling in a 5°C refrigerator until torpid. Once torpid, the bee was removed from the refrigerator and placed on the rotating cork platform of the injection stage, restrained using entomology pins and positioned for injection. The micro-needle was then pushed dorsally through the inter-segmental membrane of the third and fourth segment of the bee's abdomen. The micro-manipulator was used to position the needle to a depth of 2 to 3 mm within the abdomen (Figure 3.4.3). The Hamilton syringe was then used to inject the isotope into the bee.

The probability of damaging organs in the abdomen was reduced by only injecting bees with empty crops. This prevented a full crop from pushing the organs of the abdomen into the site of the injection. An empty crop was also more difficult to puncture accidentally with the micro-needle.

Following injection the micro-needle was removed from the bee's abdomen and the injection wound sealed with melted dental wax.

Figure 3.4.2.
Injection stage to restrain and manouver *B.terrestris* during injection of DLW

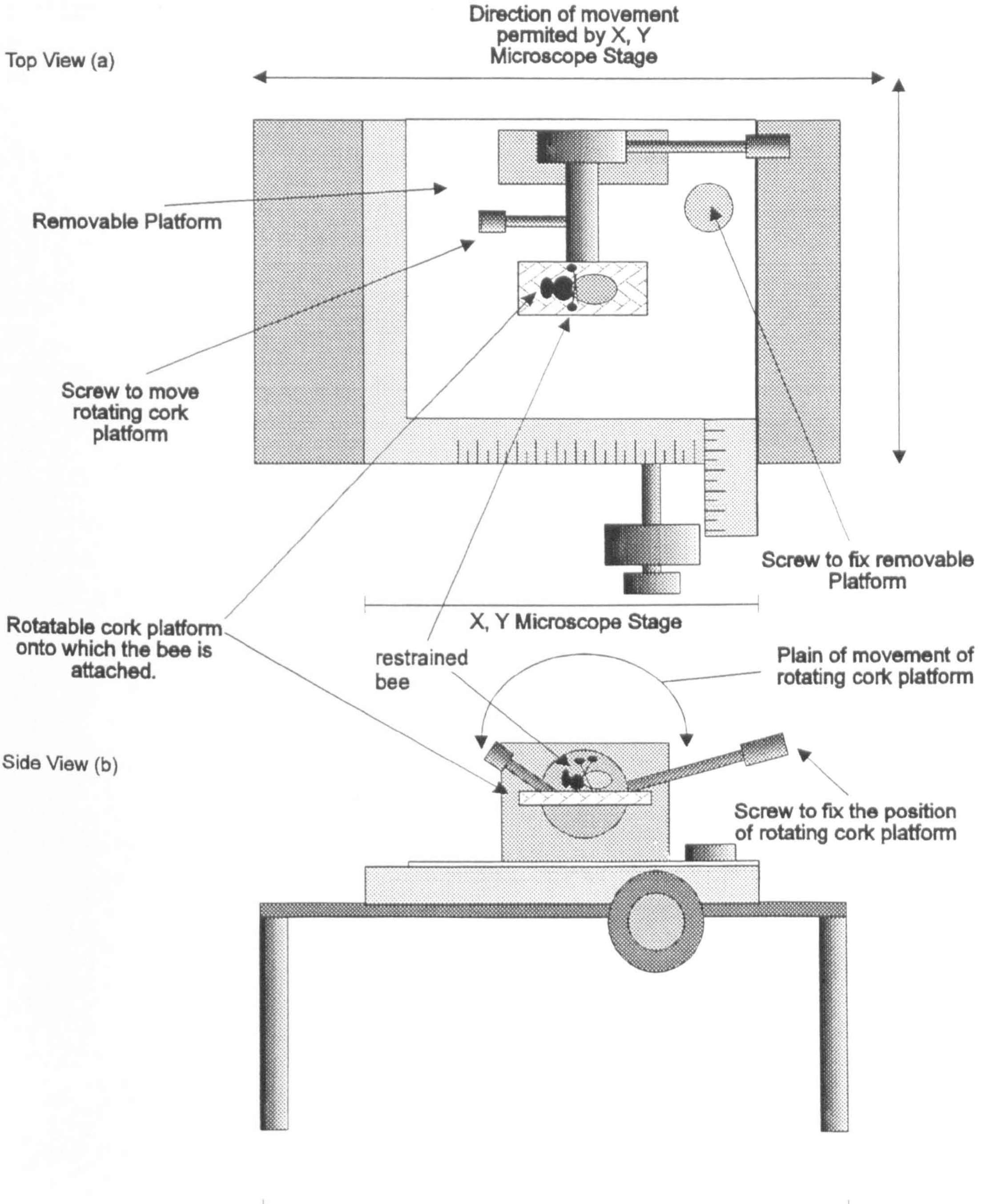
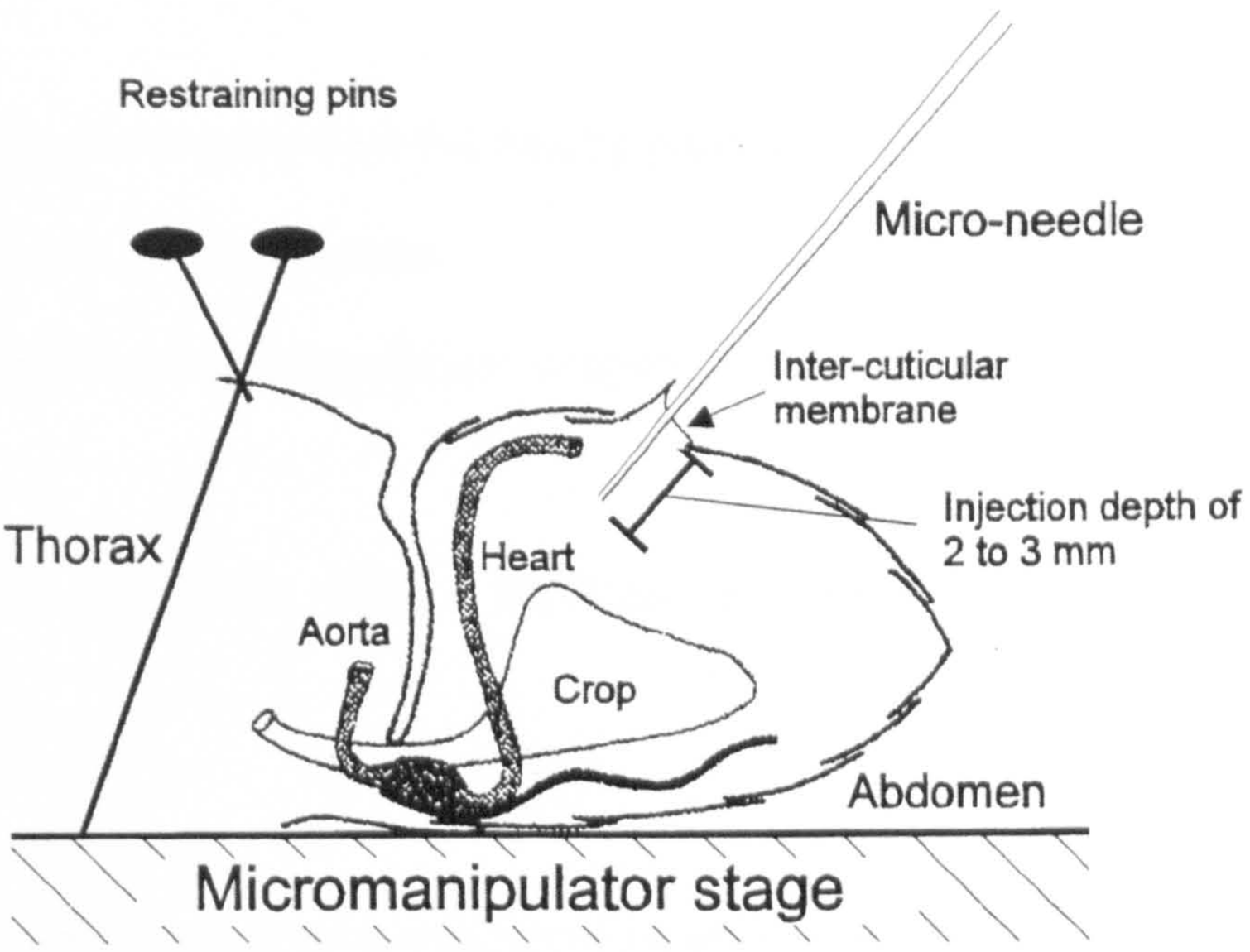


Figure 3.4.3.

B.terrestris were injected dorsally through the intersegmental membrane of the third and fourth segment of the abdomen. The micro-needle was inserted to a depth of 2 to 3 mm using the micro-manipulator, and the isotope administered using a Hamilton syringe and cheney adaptor, to which the micro-needle was attached.



3.5.0 Method for extracting Plasma from bumblebees

To analyze the isotopic composition of bumblebee plasma, haemolymph had to be extracted from the bee without significantly altering the ratio of light and heavy O and H isotopes, through processes such as fractionation (Haggarty, McGaw & Franklin, 1986; Schoeller *et al.*, 1986). This could be achieved in two ways.

1. Plasma could be removed from the bee by puncturing the cuticle and drawing haemolymph into a micro-pipette.
2. Plasma could be extracted by liquid nitrogen vacuum distillation (LNVD).

3.5.1. Removal of plasma from a bumblebee's haemoceol using micro-pipettes

Although haemolymph removal using micro-pipettes was possible, extraction of plasma volumes > 2µl was difficult (*pers. obs.*). This was due to blocking of the pipette by organic matter within the bee's haemocoel. As 2µl of plasma was not sufficient for analysis of the isotopic abundance of the bee's body water pool (Section 3.3.0), it was necessary to develop a new extraction technique.

3.5.2. Removal of plasma from a bumblebee's haemoceol by Liquid Nitrogen Vacuum Distillation (LNVD)

Extracting the entire haemolymph of the bee by liquid nitrogen vacuum distillation (LNVD) proved to be the most reliable way of extracting sufficiently large quantities of body water for subsequent analysis. This meant, however, that the bee had to be killed before extraction could be undertaken. As a result, it was only possible to use the LNVD technique for obtaining haemolymph when the bee was not required for further experimentation *e.g.* for isotopic analysis of final samples[1].

Bees were killed by dissection through the petiole, and the thorax flame sealed into a soda glass test tube. Flame sealing of the thorax prevented any loss or addition of water to the thoracic haemolymph, thus preventing significant contamination and fractionation of the thoracic body water. To prevent dilution of the body water from nectar within the crop, the abdomen was not used for further isotopic analysis. It was, however, possible that some nectar will have remained within the gut of the thorax, resulting in the dilution of the thoracic

[1] For a description of initial and final isotopic analysis see Section 3.2.1 & 3.2.4.

body water of the bee. However, any resultant errors in DLW estimates are likely to have been minimal, this was due to two factors:

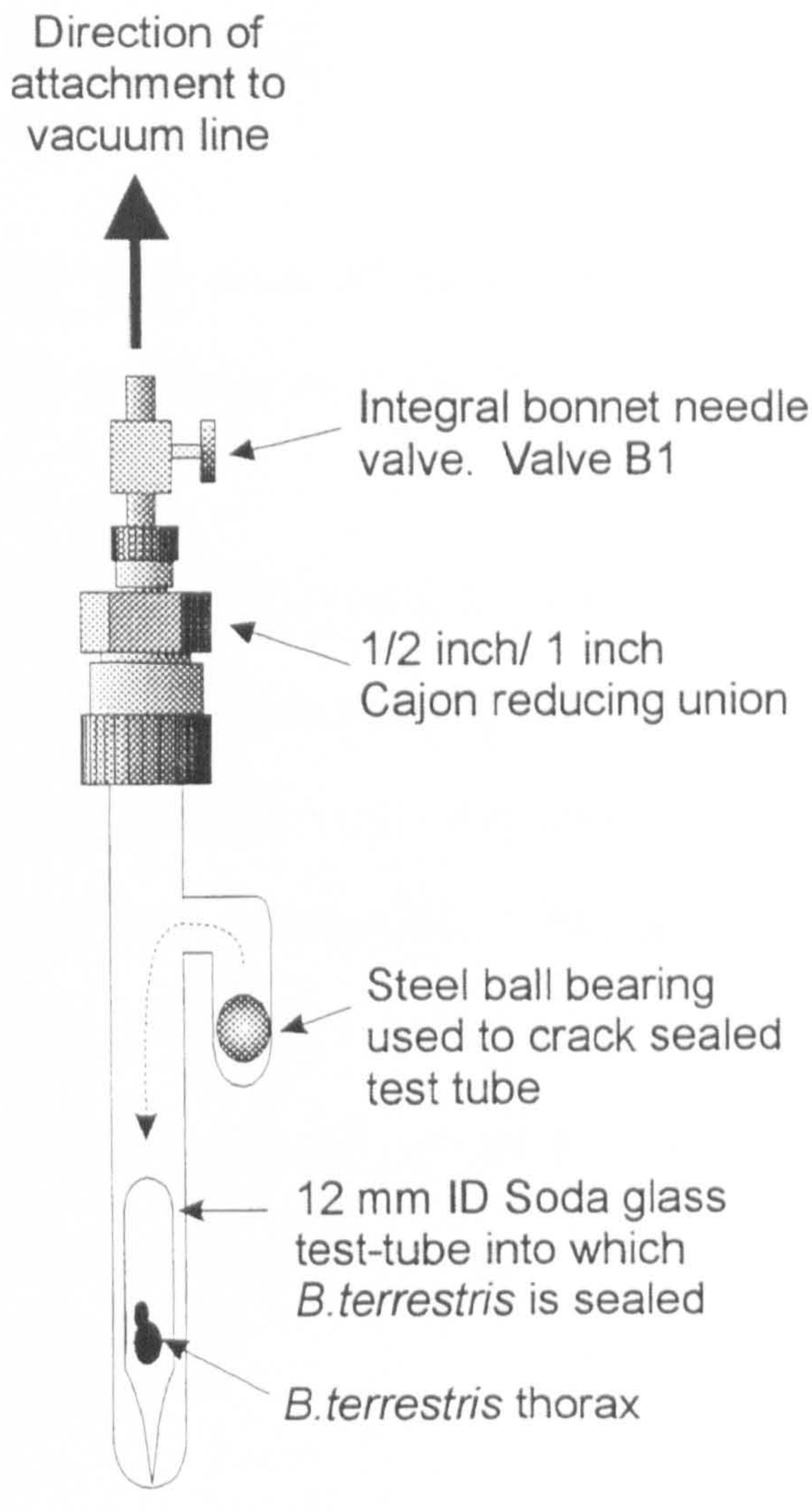
1. The volume of the thoracic gut is relatively small, accounting for circa 3±1%(n = 10) of the total volume of a *B.terrestris* thorax (*pers. obs*). Thus, any dilution of the thoracic body water pool from nectar within the gut will have been minimal.

2. Even if the thoracic body water pool is diluted by nectar within the gut, significant errors in DLW estimates of energy expenditure will only be created if the ratios of the light and heavy O and D isotopes are altered. It was, therefore, unlikely that the dilution of the bee's body water pool by relatively small volumes of nectar will have resulted in significant errors final DLW estimates of energy expenditure.

Once the thorax had been flame sealed into a test tube, it was placed in a specially designed "test tube breaker" with a steel ball bearing (Figure 3.5.1), which was placed on arm 2 of the UHV manifold via a Cajon union. A 6mm ID borosilicate tube was attached to one of the ports adjacent to the breaker, and any empty ports on arm 1 and 2 were sealed with glass stoppers (Figure 3.5.2.). Valves V1 to V5 and B1 were opened, and the UHV manifold, breaker and borosilicate tube evacuated to a pressure of ≤E-5 Mb.

Figure 3.5.1.

Breaker used to crack soda glass test tube into which *B.terrestris* was sealed, thus allowing liquid nitrogen distillation of plasma. To break the soda glass test tube, the breaker was first evacuated to a pressure of E-5 mb, valve B1 was then closed. Once sealed a magnet was used to drop the steel ball bearing onto the test tube, thus breaking the glass.



Once evacuated, valves V1 and V2 were closed and the test tube containing the thorax of the bee broken with the ball bearing. This was achieved by raising and dropping the ball bearing onto the test tube using a magnet.

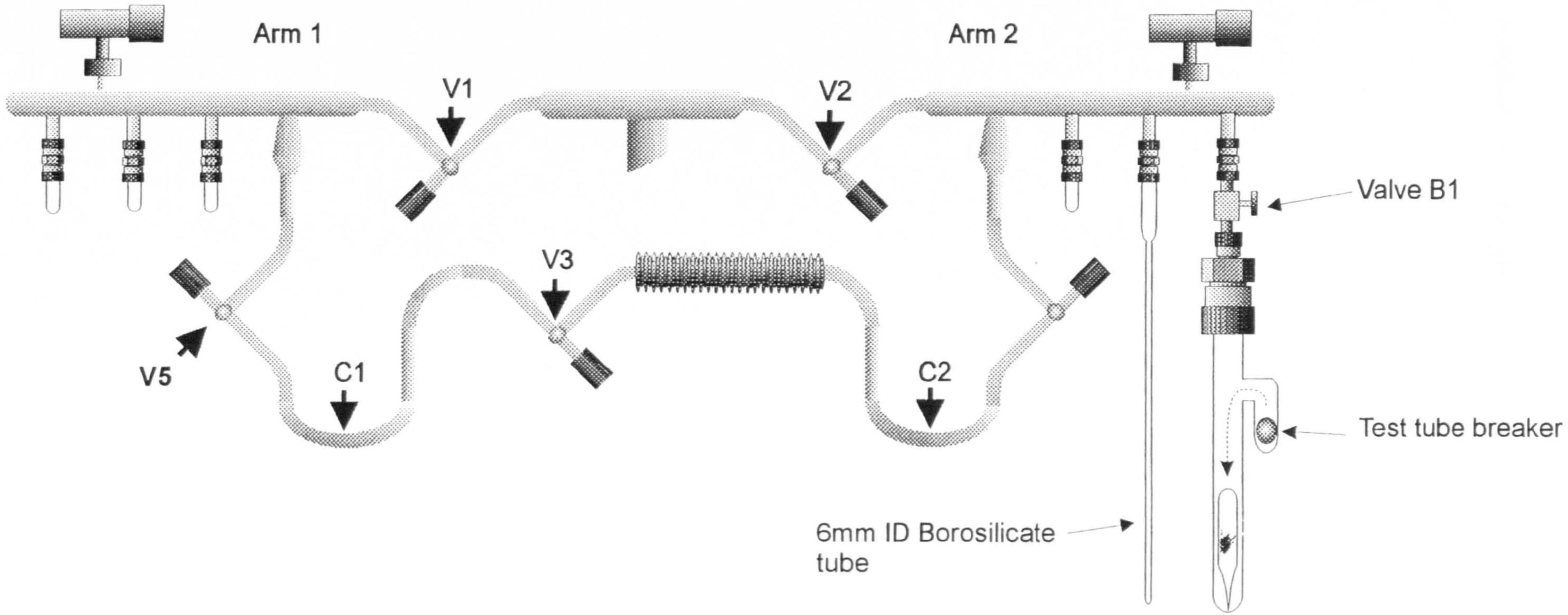
The breaking of the test tube exposed the thorax to the low pressures within the manifold, which facilitated the evaporation of the body water from the thorax. This was collected in the liquid nitrogen cold traps C1 and C2, thus condensing and freezing the evaporated plasma.

Non-condensable gases were removed by opening valve V1 until the pressure within the UHV manifold had returned to E-5 mb.

Following recovery of the vacuum, valve B1 was closed to prevent the bee's exoskeleton from re-absorbing / adsorbing any body water. Valve V1 was also closed and the dewers of liquid nitrogen removed from cold traps C1 and C2, thus allowing any frozen body water to melt. This was then LNV distilled into the bottom of the 6mm ID borosilicate tube (Figure 3.5.2.) and valve V2 opened to remove any remaining non-condensable gases. Finally, the borosilicate tube containing the sample was flame sealed under liquid nitrogen using a Microfine © hand held gas / oxygen torch.

The borosilicate tube was allowed to cool, broken and the water sample drawn into 5 µl Vitrex © micro-pipettes. Each pipette was flame sealed and stored for

Figure 3.5.2
Schematic diagram of UHV manifold during LNVD



subsequent preparation of oxygen and hydrogen gases for analysis (Section 3.2.1 & 3.2.4).

3.6.0 Validation of the Liquid Nitrogen Vacuum Distillation method of plasma withdrawal form bumblebees

Introduction

During LNVD extraction of haemolymph, ^{1}H, ^{16}O and ^{17}O isotopes evaporate from the thorax at a higher rate than the less volatile D and ^{18}O isotopes *i.e.* kinetic fractionation (Haggarty, 1991). At 37°C the rate of kinetic fractionation between ^{18}O and D isotopes with equilibrium water ($^{1}H_2{}^{18}O$) is 0.99 and 0.941 respectively (Dansgaard, 1964, Schoeller *et al.*, 1986).

Due to this fractionation, the ratio of ^{1}H / D and ^{16}O / ^{17}O / ^{18}O / ^{19}O isotopes in a bee's thoracic body water will change with time during LNVD extraction. If the isotopic composition of the extracted plasma is significantly different from the composition of the thoracic plasma prior to extraction, errors will result in final DLW estimates of rCO_2. This form of fractionation is only likely to result in significant changes in the isotopic abundance of extracted plasma if the plasma content of the thorax is not fully removed. Due to kinetic fractionation during extraction, any plasma remaining within the thorax would have an increased ratio of heavy to light hydrogen and oxygen isotopes, whilst the extracted plasma would have a reduced ratio of heavy to light isotopes. It was important,

therefore, to remove as much of the thoracic body water as possible, to minimize the effects of such fractionation[1] .

To determine whether the LNVD process resulted in significant kinetic fractionation of the plasma sample, the isotopic composition of haemolymph extracted by LNVD was compared to haemolymph samples taken using micro-pipettes (Weathers & Nagy, 1980; Cooper, 1983; Williams & Nagy, 1984; Masman & Klaussen, 1987; Speakman & Racey, 1988b; Brown, Perez-Mellado, Diego-Rasilla, Garcia, Naranjo & Speakman, 1992).

Plasma extracted by micro-pipettes should undergo minimal evaporation, and so kinetic fractionation of body water removed in this manner should be insignificant. If, therefore, the isotopic abundance of the plasma extracted using the two techniques was significantly different, this would indicate that significant kinetic fractionation was occurring during LNVD extraction.

3.6.1. Materials and methods

Twenty female *B. terrestris* were injected with 1 µl of 20% atom excess $D_2{}^{18}O$ (Section 3.4.4), and placed in a dark 20 x 20 cm wooden box for 15 minutes to allow equilibration with the body water (Section 3.7.0.). Full equilibration of the

[1] If the total body water content of the thorax could be extracted there would be no appreciable fractionation.

$D_2{}^{18}O$ was essential in order to prevent isotopic fractionation of the bee's plasma by equilibrium fractionation[2] (Haggarty *et al.*, 1988). Following equilibration, the bee was killed by dissection through the petiole.

3.6.1a. Pipette extraction of bumblebee plasma

5µl Vitrex © micro pipettes were used to pierce the inter-segmental membrane of the 4th and 5th segment of the bee's abdomen, and two 1µl samples of abdominal body water were drawn into the pipette by capillary action. The pipettes were then removed and flame sealed.

The body water from the thorax was LNVD extracted and sealed into micro-pipettes (Section 3.5.2.). The ^{18}O and D abundance of both micro-pipette and LNVD extracted samples were analyzed as described in Section 3.2.1 & 3.2.4

3.6.2. Results

Although ^{18}O abundances varied from 2237 ppm to 3635 ppm, and D abundances from 1053.28 ppm to 2271 ppm, there was little intra-bee variation in the isotopic composition of plasma extracted using the two methods. As

[2] Equilibrium fractionation occurs when poorly mixed water is evaporated. As the isotopic composition of the water is not homogenous, isotopes will evaporate dependent on their position relative to the evaporative surface rather than their atomic mass (Haggarty *et al.*, 1988).

Table 3.6.1 The effect of fractionation on the isotopic abundance of *B.terrestris* plasma extracted by LNVD. (All data is based on pairs of replicates).

	Isotopic composition of pipette extracted plasma	Isotopic composition of LNVD extracted plasma	
Bee ID	^{18}O ppm	^{18}O ppm	% difference in isotopic abundance of LNVD and pipette extracted plasma (Arsin transformed)
1	3489	3494	0.2
2	2237	2242	0.2
3	3629	3635	0.2
4	2791	2784	-0.2
5	2964	2974	0.4
6	2466	2469	0.1
7	2834	2831	-0.1
8	3255	3250	-0.2
9	3542	3549	0.2
10	2897	2906	0.3
		mean % difference	0.1%
		sd	0.2
	D ppm	D ppm	
11	2248	2271	1.0
12	1053	1067	1.3
13	1145	1132	-1.2
14	1263	1276	1.0
15	1127	1119	-0.8
16	1278	1288	0.8
17	1330	1324	-0.4
18	1642	1664	1.3
19	1476	1453	-1.6
20	1083	1095	1.01
		mean % difference	0.3%
		sd	1.1

shown in Table 3.6.1, the mean error between the two methodologies was only 0.1±0.2% for the ^{18}O analysis and 0.3±1.1% for the D analysis (paired *t*-test for ^{18}O and D respectively; $t_9 = -1.69$, ns & $t_9 = -0.88$, ns).

3.6.3. Discussion

Both methods of extraction provided close agreement, with variations between the two techniques falling within 1 Standard deviation of the analytical error[3]. It was, therefore, likely that the majority of the variation between the isotopic abundance of plasma extracted using the two techniques was due to analytical errors rather than to kinetic fractionation. Given the close agreement of the two methods and the advantages of LNVD extraction, discussed previously, the latter was used routinely in subsequent experiments.

[3] The analytical errors (observed in Section 3.3.2) equaled +0.05%±0.4 for the ^{18}O isotopic analysis, and +0.22±1.01 for the D isotopic analysis.

3.7.0 Equilibration of injected $D_2{}^{18}O$ isotope with the body water pool of bumblebees

Introduction

Failure to allow sufficient time for isotope equilibration following administration will create errors in DLW estimates of energy expenditure (Nagy 1980). The errors result largely from an unbalanced loss of isotopes from the bee's body. For example, if injected isotope equilibrated only with the abdominal water of the bee, the DLW measurements will only reflect the isotopic turnover of the abdomen and not the thorax. There is, moreover, no *ad hoc* method of predicting equilibration time as this varies widely between organisms (Table 3.7.1). Equilibration times can range from as little as 45 minutes (Utter & LeFebvre, 1973) to 24 hours (Gales, 1989), and there are no obvious physiological traits which determine body water / isotope mixing rates.

Most equilibration times have in any case been determined by the "plateau enrichment" method (Gales, 1989), which is inappropriate for small organisms such as bumblebees (Section 3.0.4)

Table 3.7.1. The time taken for $D_2{}^{18}O$ isotope to equilibrate with an animal's body water.

Species	Equilibration time (hours)	Source
Mammals		
Ruminants	7	Fancey *et al.*, 1986, Nagy, 1993
Humans	4	Goran & Poehlman, 1994
Bats (*Pipistrellus pipistrellus* & *Plecotus auritus*)	1.5	Speakman & Racey, 1988a
White Mice (MF1)	1.5	Speakman, Racey & Burnett, 1991
Marsupials		
Petauroides volan	4	Nagy, Foley, Kaplan, Meridith & Minagawa , 1990
Reptiles		
Podarcis lilfordi	3.5	Brown *et al.*, 1992
Aves		
Penguins *(Eudyptula minor)*	24	Gales, 1989
Eurasian Kestrels *(Falco tinnunculus)*	3	Masman & Klassen, 1987
Starlings *(Sturnus vulgaris)*	1	Ricklefs & Williams, 1984
Phainopepla nitens	1	Weathers & Nagy, 1980
Purple Martins *(Progne subis)*	0.75	Utter & LeFebvre, 1973
Arthropods		
Locust *(Locusta migratoria migratoriodes)*	8	Buscarlet *et al.*, 1978
Scorpions *(Hadrus arizonensis)*	4 to 6	King & Hadley, 1979
Tenebrionid Beetle (*Cryptoglossa verracosa*)	3	Cooper, 1983
Tenebrionid Beetle (*Eleodes armata*)	3	Cooper, 1983

Instead bumblebee equilibration time was estimated assuming bees had two water pools, the head / thorax and abdomen. As isotope was injected only into the abdomen (Section 3.4.4.), equilibration was assumed to be complete when the plasma removed from any point within the haemocoel had the same isotopic composition. For practical purposes, therefore, bees were injected with $D_2{}^{18}O$ and equilibration determined by comparing the isotopic composition of the abdomen (the injection site) with the head / thorax, at various time intervals after injection. When the isotopic abundance of these two pools were not significantly different, equilibration was taken to have occurred.

3.7.1. Materials and Methods

Forty five *B.terrestris* were removed from their colony and placed in a 20 x 20 cm wooden box for 30 minutes. During this period the bees were starved, thus ensuring their crops were empty prior to injection (Wolf *pers. com.*). This was important, as if nectar remained within the crop following dissection through the petiole, it would dilute the isotopes in the extracted plasma.

After 30 minutes, the bees were removed from the box and injected with 1µl of $D_2{}^{18}O$ (Section 3.4.4.). The injected bees were then placed in individual 20 x 20 cm wooden boxes, and the $D_2{}^{18}O$ allowed to mix with the body water for 2, 5, 10, 15, 30, 45 or 60 minutes respectively. Following mixing, the bee was removed from the box, killed and flame sealed into soda glass test tubes.

The body water was then extracted separately from both the thorax and abdomen (Section 3.5.2.) and the isotopic content analysed in duplicate (Section 3.2.1 & 3.2.4.).

3.7.2. Results

The difference in isotopic composition of the thoracic and abdominal body water varied with mixing time. The largest variation occurred at 2 and 5 minutes, with consistently higher isotopic enrichment in the abdomen than the thorax (Table 3.7.2 & 3.7.3). At these times, the mean % difference in isotopic contents, was 61±10.7% (n = 7) for the ^{18}O isotope and 46.1±13.4% (n = 6) for the D isotope. These differences were significant for both ^{18}O (paired *t*-test; $t_6 = 3.82$, $p = 0.009$) and D isotopes ($t_5 = 10.657$, $p<0.001$).

Variation in isotopic concentrations of the thoracic and abdominal body water were far lower for mixing times of 10, 15, 45 and 60 minutes. The mean difference between water pools was only -0.30±2.01% (n = 16) for the ^{18}O isotope and 0.7±1.21% for the D isotope, the differences being non-significant for both ^{18}O ($t_{15} = -0.54$, ns) and D ($t_{14} = 1.80$, ns) isotopes.

3.7.3. Discussion

Due to the significant differences observed in the thoracic and abdominal isotopic abundances, it was clear that equilibration of injected $D_2{}^{18}O$ (with the

Table 3.7.2. Equilibration time of injected ^{18}O with *B.terrestris* body water.

Isotopic equilibration time (minutes)	abdominal ^{18}O isotopic enrichment (ppm)	thoracic ^{18}O isotopic enrichment (ppm)	% difference between mean abdominal and thoracic ^{18}O isotopic enrichment	Paired *t*-test
2	3164	875	72.3	$^{\clubsuit}t_2 = 8.08$, p = 0.01
2	2517	1035	58.9	
2	2973	903	69.6	
5	9697	2843	70.7	$^{\clubsuit}t_3 = 2.68$, p = 0.04
5	5595	3204	42.7	
5	3164	1209	61.8	
5	3306	1532	53.7	
10	4418	4427	-0.2	$t_2 = 0.75$, ns
10	5226	5140	1.6	
10	3657	3664	-0.2	
15	5694	5726	-0.6	$t_3 = 0.40$, ns
15	2973	2986	-0.4	
15	3750	3750	0.0	
15	4068	3982	2.1	
30	3513	3503	0.3	$t_2 = -1.13$, ns
30	5241	5281	-0.8	
30	4575	4907	-7.2	
45	4608	4629	-0.4	$t_2 = -0.53$, ns
45	3459	3461	-0.1	
45	3657	3648	0.2	
60	4622	4583	0.8	$t_2 = 1.02$, ns
60	4357	4347	0.2	
60	3286	3294	-0.2	

ns = non significant.

$^{\clubsuit}$ = Denotes a significant difference in ^{18}O isotopic enrichment between thoracic and abdominal body water.

Table 3.7.3. Equilibration time of injected D with *B.terrestris* body water.

Isotopic equilibration time (minutes)	abdominal D isotopic enrichment (ppm)	thoracic D isotopic enrichment (ppm)	% difference between mean abdominal and thoracic D isotopic enrichment	Paired *t-test*
2	1894	875	53.8	*t_2 = 5.60, p = 0.03
2	1466	655	55.3	
2	2030	1497	26.3	
5	1536	574.	62.6	*t_2 = 9.24, p = 0.01
5	2481	1524	38.6	
5	1684	1005	40.3	
10	1329	1348	-1.4	t_2 = -1.44, ns
10	2505	2552	-1.9	
10	1793	1790	0.2	
15	2624	2661	-1.4	t_2 = -3.23, ns
15	2308	2330	-1.0	
15	1213	1225	-0.9	
30	2306	2342	-1.6	t_2 = -1.14, ns
30	1081	1077	0.3	
30	1660	1668	-0.5	
45	1484	1470	0.9	t_2 = -0.12, ns
45	2594	2549	1.7	
45	2321	2393	-3.1	
60	1730	1757	-1.5	t_2 = -1.86, ns
60	1115	1114	0.0	
60	1840	1858	-0.9	

ns = Non-significant.
* = Significant difference in D isotopic enrichment between thoracic and abdominal body water.

bumblebees body water pool) had not occurred between 2 and 5 minutes following injection. After 10 minutes, the variation in $D_2{}^{18}O$ isotopic composition of the two water pools was not significantly different; thus it was assumed that the injected $D_2{}^{18}O$ had equilibrated with the bees body water.

A 10 minute equilibration period was not dissimilar to that observed in previous honeybee studies. Crailsheim (1985) observed that ^{14}C markers, when administered to honeybees, equilibrated with the body water pool within 5 minutes. Crailsheim concluded that fast equilibration rates were driven by the high pulsation rates of the heart, a dorsal vessel which pumps haemolymph from the abdomen to the head (Snodgrass, 1956). As Heinrich (1980) recorded heart rates of 200 - 450 beats / minute in honeybees. and 350 - 600 beats / minute in bumblebees (Heinrich 1976b), the fast isotope equilibration times in bees were not surprising.

The equilibration times observed in bumblebees were, however. considerably shorter than has been observed in other arthropod studies. whose equilibration times ranged from three to eight hours (Table 3.7.1.). However, it should be noted that in previous arthropod studies, the method used to determine the time of isotope equilibration was not described in subsequent publications. If assumptions of equilibration times were predicted from previous vertebrate studies, it is possible that the true equilibration times of the arthropods may have been considerably shorter than reported.

3.8.0 Measurement of initial $D_2{}^{18}O$ isotopic composition of a bumblebee's body water pool

Introduction

To enable DLW estimates of energy expenditure, the rate of ^{18}O and D loss from an organism (isotopic turnover rate) must be determined (Section 3.0.2.). Turnover rates are normally measured by isotopic analysis of an organism's plasma at two points in time, often referred to as "initial" and "final" samples (Tatner & Bryant, 1989). Although LNVD extraction permitted sufficient volumes of plasma to be obtained for final sample analysis, the destructive nature of this technique prevented its use in extracting body water for isotopic analysis of the initial sample. Also, as discussed previously (Section 3.0.4 & 3.3.0), it was not possible to extract sufficient plasma to determine initial sample isotopic abundance, owing to the relatively small body water pool of the bees. It was, therefore, necessary to explore alternative methods for determining the isotopic abundance of the bees initial body water pool.

Three possible techniques were considered:

1. The single sample method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

The single sample method predicts the initial isotopic abundance of an animal's body water pool from the predicted dilution space of an organism (Nagy, 1983; Webster & Weathers, 1989; Tiebout & Nagy, 1991). Providing the volume of the body water pool and quantity and abundance of administered isotope are known, the initial isotopic abundance of an animal can be calculated. Although the quantity and abundance of administered isotope may be determined gravimetrically, the volume of an animal's body water pool cannot directly be measured. As a result, the volume of an animal's body water pool is predicted from the dilution space observed in other individuals of the same species. The dilution space is estimated by administering the animals with known volumes and abundance of ^{18}O isotopes. Following equilibration, a plasma sample is extracted and the ^{18}O content of the body water pool determined. The dilution of the administered ^{18}O isotope can then be used to calculate the volume of the animal's body water pool (Nagy, 1983). Although these organisms cannot be used in subsequent DLW experiments, least squares regression between the initial isotopic abundance and body mass can be used to predict initial isotopic composition in other organisms of the same species and of a similar body mass (Nagy, 1983; Webster & Weathers, 1989; Tiebout & Nagy, 1991).

As bumblebees maintain a constant body water volume and mass (Bertsch, 1984), they would appear an ideal species for the single sample method. However, although this technique has been validated in small birds (Webster & Weathers, 1989) it was far from clear whether this methodology would be applicable to bumblebees. As a result it was first necessary to validate the single sample procedure prior to applying it to bees.

2. The "urine" method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

When administered $D_2{}^{18}O$ has equilibrated with an organism's body water pool, plasma removed from any part of the animal should have an equal abundance of $D_2{}^{18}O$ isotopes. As a result, the isotopic composition of excreted urine should not significantly differ from that of an animal's body water pool from which the urine was excreted. As a result, the $D_2{}^{18}O$ isotopic content of excreted urine has been used to measure initial isotopic abundances in humans (Schoeller *et al.*, 1986; Goran, Poehlman & Danforth, 1994) and ruminants (Fancey *et al.*, 1986). As bumblebees can produce large volumes of urine (up to a third of their body water content per urination (*pers. obs*), it was theoretically possible to obtain sufficient urine to determine the initial isotopic abundance of the bee's body water pool. Furthermore, bees have been reported to urinate as a defensive measure (Winston 1991), thus making it possible to control the time of urination simply by agitating the bees (*pers. obs.*).

As the effect of excretion on the isotopic composition of a bee's urine was unknown, the use of urine to measure the $D_2{}^{18}O$ abundance of a bee's body water pool had to be validated. This required a comparison to be made between the isotopic composition of the urine, and the body water of the bee from which the urine was excreted. If the $D_2{}^{18}O$ abundances did not significantly differ, this would indicate that the isotopic abundance of urine could used to estimate the initial isotopic abundance of a bee's body water pool.

3. Prediction of initial D isotopic composition from initial ^{18}O abundance of a bee's body water pool

Although it was not possible to remove sufficient plasma from bumblebees for both initial ^{18}O and D isotopic analysis of initial isotopic abundance (Section 3.3.0), it was possible to remove sufficient plasma for small sample analysis of the ^{18}O isotope alone. A dilution series of $D_2{}^{18}O$ could then be used to predict the initial D plasma composition from the observed initial ^{18}O abundances. It was, however, unclear how accurate predictions of D abundance would be. To determine the accuracy of this technique it was, therefore, necessary to compare the abundances predicted by an ^{18}O dilution series with D abundances determined by isotopic analysis of a bees body water pool.

Although all three methods described above could potentially enable predictions of a bumblebee's initial body water isotopic abundances, the accuracy and viability of all procedures were unknown. As a result, it was necessary to

validate all three methodologies and compare their relative precision and viability in predicting initial $D_2{}^{18}O$ abundances.

3.8.1. Materials and methods

1. The single sample method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

45 female *B.terrestris* were removed from a Biobest © artificial hive and starved until their crops were empty (Section 3.4.4). The bees were then weighed using a 5 point Sartorius © top pan balance and injected with 1 µl of $D_2{}^{18}O$ (Section 3.4.4.). The injected bees were placed in a 10 x 10 cm wooden box for 15 minutes. Following equilibration, the bees were killed and flame sealed into a soda glass test tubes. The bees thoracic plasma was then LNVD extracted and the $D_2{}^{18}O$ isotopic content analyzed (Section 3.2.1 & 3.2.4). The relationship between initial $D_2{}^{18}O$ content and body mass of the bees were determined by least squared regression analysis.

2. The "urine" method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

20 female *B.terrestris* were removed from a Biobest © hive and starved until their crops were empty. The bees were then injected with 1 µl of $D_2{}^{18}O$ (Section 3.4.4.) and individually placed into soda glass test tubes. Following equilibration (Section 3.7.0), the bees were agitated by gently flicking the sides of the tubes. The bees were agitated in order to stimulate them to urinate as a defensive measure (Winston, 1991).

Following urination, the bees were removed from the test tube and killed. The excreted urine was drawn into two Vitrex © micro-pipettes by capillary action, and the pipettes flame sealed. The dissected abdomen and thorax were flame sealed into individual soda glass test tubes. The thoracic body water of the bees were then extracted, and the ^{18}O abundance analyzed for bees 1 to 10, whilst the D abundance was analyzed for bees 11 to 20 (Section 3.2.1 & 3.2.4). The $D_2{}^{18}O$ isotopic abundance of the excreted urine was analyzed in the same manner as the extracted thoracic body water.

3. Prediction of initial D isotopic composition from initial ^{18}O abundance of a bee's body water pool

To predict the initial D abundance of the bumblebees body water pools from the observed ^{18}O abundance, a series of ten $D_2{}^{18}O$ enriched standards were produced. The standards comprised of 1µl $D_2{}^{18}O$ diluted with 80, 100, 120, 140, 160, 180, 200, 220, 240 and 260 µl of distilled water respectively. Four 5µl samples were taken from each standard and flame sealed into four Vitrex © micro-pipettes. The $D_2{}^{18}O$ content of the 10 standards were then analyzed (Section 3.2.1 & 3.2.4), and the least squares regression between the observed ^{18}O and D content analyzed. The regression relationship between the ^{18}O and D content of the standards was then used to predict the initial D content of *B.terrestris* from the observed initial ^{18}O content.

To validate this procedure, the observed initial D isotopic abundances of 20 *B.terrestris* were compared to the D abundances predicted from observed initial ^{18}O isotopic content.

20 female *B.terrestris* were removed from a Biobest © hive and starved until their crops were empty (Section 3.4.4.). The bees were then injected with 1µl of $D_2{}^{18}O$ (Section 3.4.4.) and placed in a 10 x 10 cm wooden box during equilibration (Section 3.7.0.). A 1µl plasma sample was then extracted from the bee using a micro-needle with a 4 nm beveled point (Section 3.5.1.). The micro-needle was used to puncture the inter-segmental membrane between the third and fourth

segment of the bees abdomen. A 1µl plasma sample was then drawn into the needle by capillary action and the needle flame sealed. The bee from which the plasma sample had been taken was then killed and flame sealed into a soda glass test tubes. The thoracic body water was LNVD extracted, following which the D isotopic abundance was analyzed (Section 3.2.4). The ^{18}O isotopic content of the micro-needle plasma sample was analyzed as described in Section 3.2.1.

The ^{18}O abundance of the needle extracted plasma was then used to predict initial D abundance of the bees plasma. The accuracy of the prediction was determined by comparing the D content of the thoracic water with that predicted from the ^{18}O abundance.

3.8.2. Results

1. The single sample method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

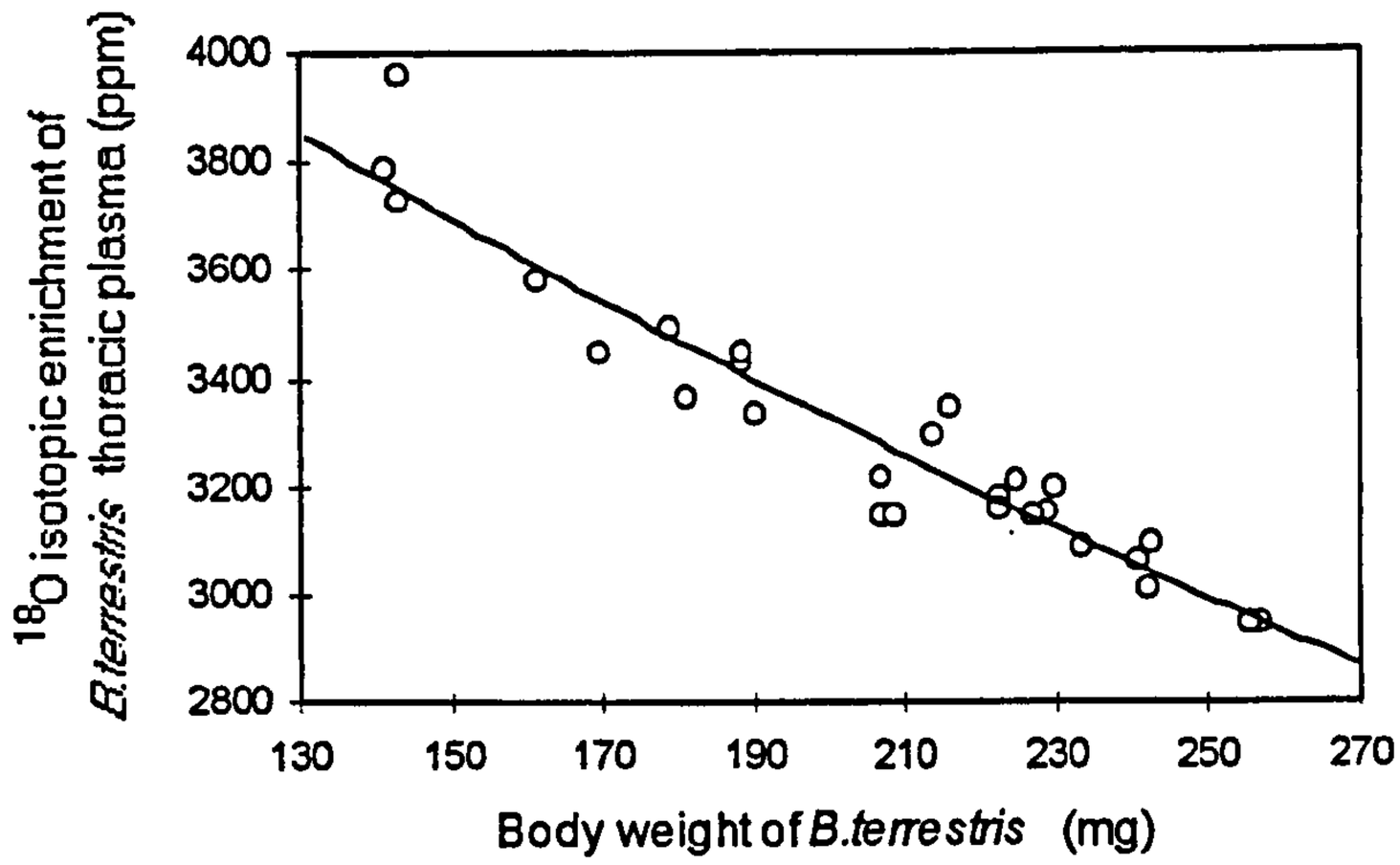
There was a good relationship between the body mass of *B.terrestris* and observed initial body water pool isotopic abundances of the bees (Figure 3.8.1). The relationship between starved body mass and initial ^{18}O abundance is described by equation 1, whilst the relationship between starved body mass and

Figure 3.8.1 Prediction of initial $D_2{}^{18}O$ abundance in the body water pool of *B.terrestris*, following injection and equilibration of 1µl of 20% atom excess $D_2{}^{18}O$.

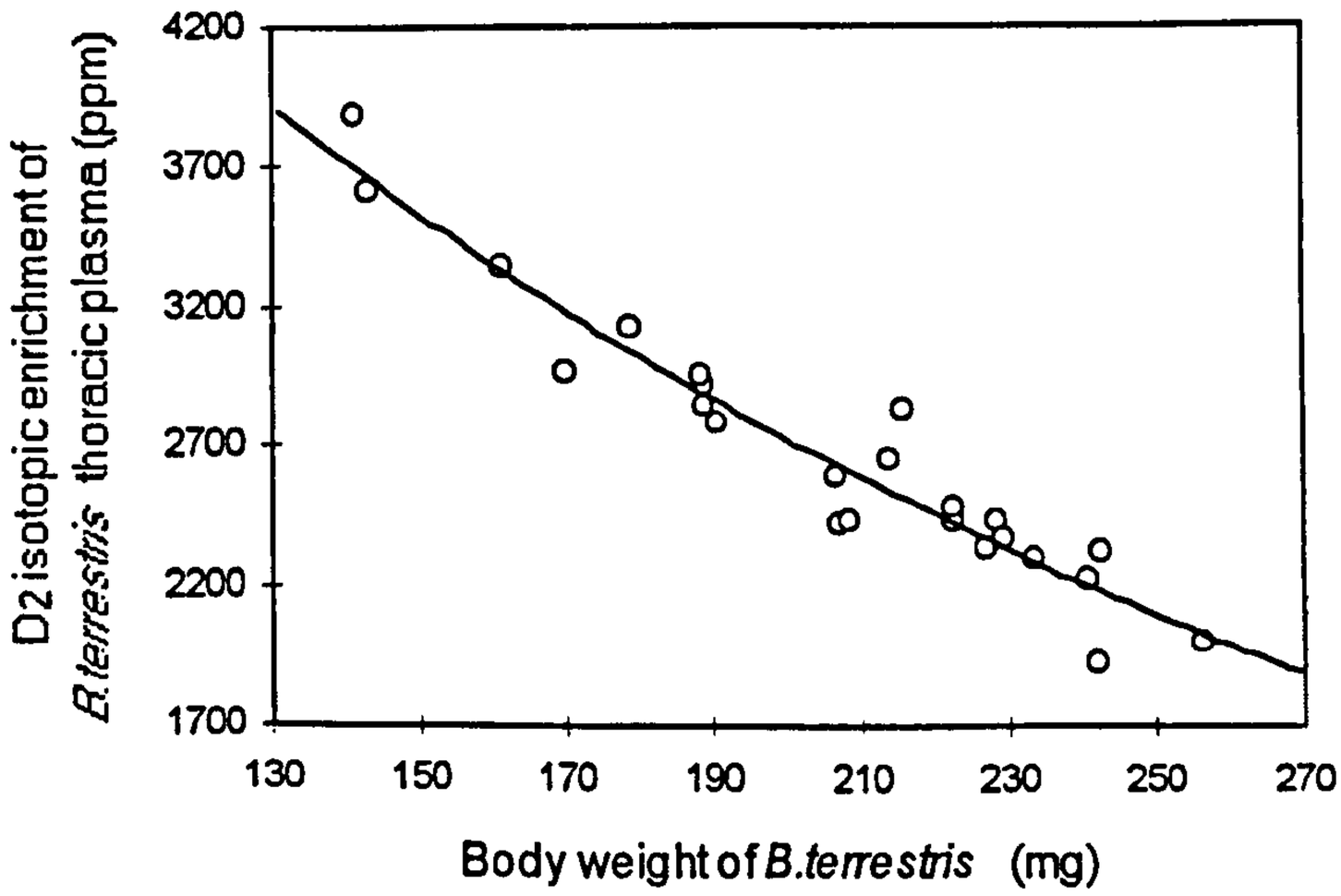
a) Prediction of initial ^{18}O istopic abundance of *B.terrestris* body water from starved body mass by least squared regression.

b) Prediction of initial D isotopic abundance of *B.terrestris* body water from starved body mass by least squared regression.

a)



b)



initial D abundance is described by equation 2:

$$Y = 507E^{-0.002x} (r^2 = 0.92) \quad (1)$$

where Y = initial ^{18}O isotopic abundance and x = starved body mass.

$$Y = 7710.9E^{-0.005x} (r^2 = 0.91) \quad (2)$$

where Y = initial D abundance.

The regression between body weight and initial isotopic composition of the bees body water pool was highly significant for both the ^{18}O ($F_{1,26} = 109.23$, $p < 0.001$) and D isotopes ($F_{1,23} = 231.22$, $p < 0.001$). It was, therefore, possible to use equations 1 & 2 to predict the initial $D_2{}^{18}O$ abundance of the body water pools of the bees in the current study (Table 3.8.1). When comparing predicted body water isotopic abundances with abundances observed by isotopic analysis, there was a mean error -0.64±2.1% (n = 27) for the ^{18}O isotope and -0.12±3.7% (n = 24) for the D Isotope (Table 3.8.1.). The maximum observed error was 4.7% for the ^{18}O isotope and 8.9% for the D isotope.

No significant differences were found between observed and predicted initial ^{18}O (*paired t-test*; $t_{26} = -1.33$, ns) and D isotopic abundances ($t_{23} = 0.02$, ns) of the bees body water pools.

Table 3.8.1 A comparison between the observed $D_2{}^{18}O$ isotopic abundances of a bumblebee's body water pool when injected with 1µl of 20% atom excess $D_2{}^{18}O$, and the abundances predicted from the body masses of the bees.

Bee ID	observed initial ^{18}O isotopic abundance of the bees body water pools (ppm)	predicted ^{18}O isotopic abundance of the bees body water pools (ppm)	% error between observed and predicted ^{18}O abundance	observed initial D isotopic abundance of the bees body water pool (ppm)	predicted D isotopic abundance of the bees body water pool (ppm)	% error between observed and predicted D abundance
1	3009	3079	-2.3	456.3	496.8	-8.9
2	sl	sl	sl	599.6	606.4	-1.1
3	2944	3003	-2.0	468.4	471.9	-0.7
4	3060	3085	-0.8	501.6	498.8	0.6
5	3155	3154	0.1	535.0	521.3	2.6
6	3959	3774	4.7	sl	sl	sl
7	3143	3164	-0.6	518.98	524.5	-1.1
8	3092	3077	0.5	518.30	496.1	4.3
9	3145	3288	-4.6	532.56	565.9	-6.2
10	3212	3177	1.1	sl	sl	sl
11	3431	3412	0.6	610.1	606.3	0.6
12	3090	3127	-1.2	513.9	512.6	0.3
13	3147	3277	-4.1	534.3	561.8	-5.2
14	3448	3551	-3.0	617.5	652.4	-5.6
15	3177	3190	-0.4	535.4	533.1	0.4
16	3158	3190	-1.0	541.4	533.1	1.5
17	3199	3149	1.6	525.1	519.8	1.0
18	2949	3008	-2.0	sl	sl	sl
19	3492	3483	0.3	642.6	629.8	2.0
20	3292	3243	1.5	569.1	550.6	3.2
21	3347	3230	3.5	596.3	546.5	8.4
22	3364	3467	-3.1	sl	sl	sl
23	3219	3289	-2.2	559.4	565.6	-1.1
24	3584	3620	-1.0	677.7	675.2	0.4
25	3451	3414	1.1	615.3	606.9	1.4
26	3330	3398	-2.1	589.7	601.8	-2.1
27	3786	3793	-0.2	760.2	732.9	3.6
28	3727	3774	-1.3	719.3	726.4	-1.0

sl = a plasma sample which was lost due to analytical error.

2. The "urine" method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

During the experiment, it was not possible to accurately control the time at which bees excreted. For this reason, urination occurred over a range of times following injection, from 8.3 to 25.9 minutes. This created an error in bee 8, which urinated before the injected isotope had equilibrated with the bee's body water pool. This resulted in the excreted urine not reflecting the true isotopic abundance of the body water pool of the bee, with a 47% difference in ^{18}O abundance of thoracic water and urine. Bee 8 was, therefore, not used in subsequent analysis.

Excreted urine had a similar $D_2{}^{18}O$ isotopic abundance to the thoracic body water of the bee from which it was excreted (Table 3.8.2). The mean difference between the isotopic composition of the urine and thoracic plasma was -0.44±1.4% (n = 9) for the ^{18}O isotope and 0.19±1.4% (n = 10) for the D isotope. The maximum error which occurred, when using urine to estimate initial isotopic abundances of *B.terrestris* body water, was -4.0% for the ^{18}O isotope and -1.87% for the D isotope.

There was a highly significant relationship between the ^{18}O isotopic abundance of the urine and the isotopic abundance of the bees thoracic water ($r_9 = 0.991$, $p < 0.001$), and this was also true for the D isotope ($r_{10} = 0.990$, $p < 0.001$).

Table 3.8.2. Variation in $D_2{}^{18}O$ isotopic abundances between *B.terrestris* urine and extracted thoracic body water.

Bee ID	Time of urination following injection of *B.terrestris* with $D_2{}^{18}O$ (min)	^{18}O Isotopic abundance of excreted urine (ppm)	^{18}O isotopic abundance of thoracic body water at the time of urination (ppm)	sd	% difference between isotopic abundance of urine and thoracic body water
1	18.2	2749	2747		-0.04
2	16.5	3064	3059		-0.16
3	23.3	3190	3181		-0.27
4	15.4	2837	2850		0.43
5	16.4	3235	3243		0.24
6	14.2	2930	2817		-3.98
7	16.4	2543	2542		-0.05
8*	8.3	2144	4090		47.57
9	15.3	3292	3280		-0.37
10	14.7	3410	3419		0.26

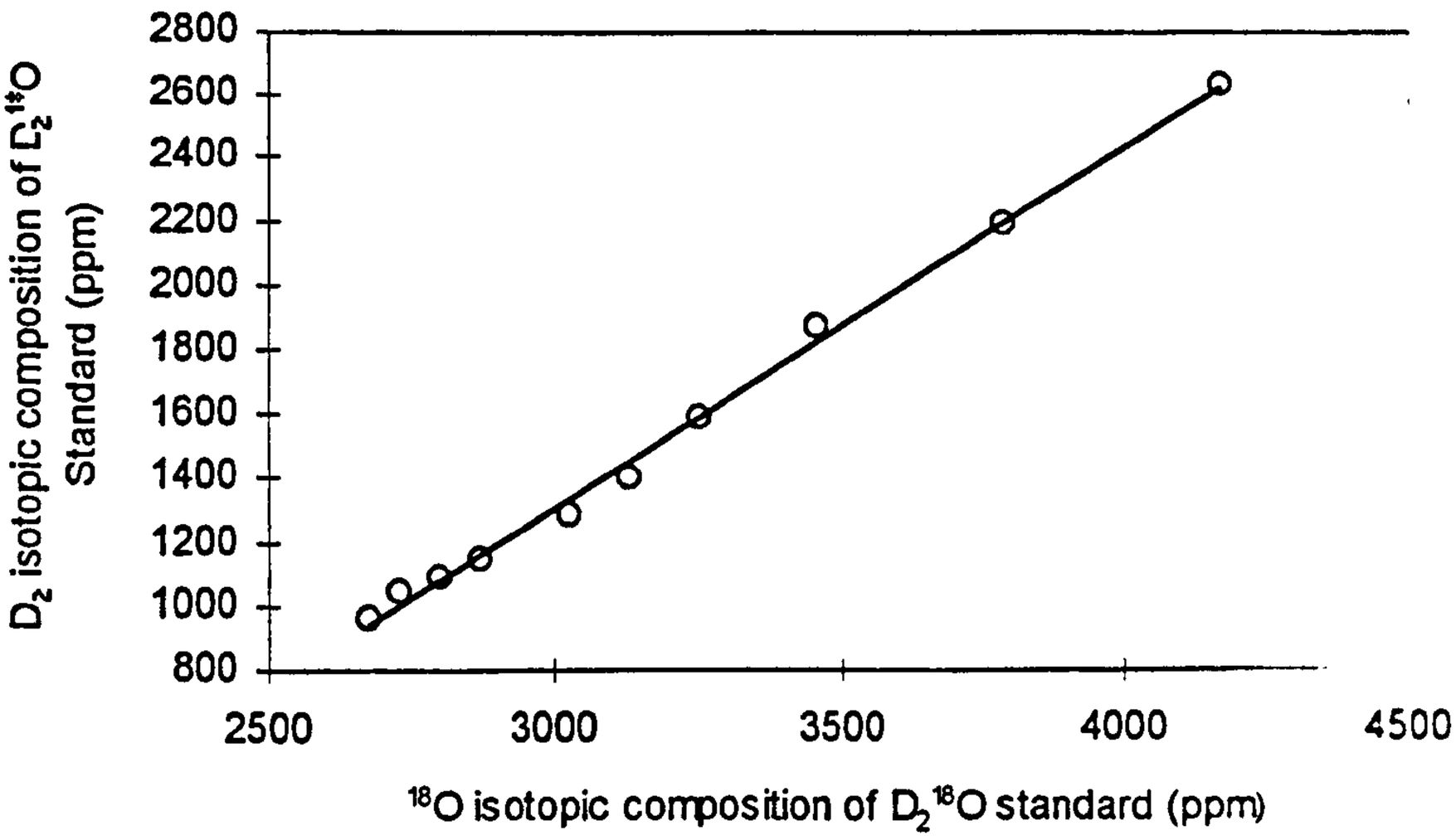
Bee ID	Time of urination following injection of *B.terrestris* with $D_2{}^{18}O$ (min)	D Isotopic abundance of urine (ppm)	D isotopic abundance of thoracic body water at the time of urination (ppm)	% difference between isotopic abundance of urine and thoracic body water
11	13.8	532	537	0.89
12	13.0	510	519	1.87
13	17.7	582	573	-1.67
14	13.7	553	548	-0.89
15	25.9	629	637	1.23
16	18.3	475	467	-1.81
17	21.0	557	563	1.00
18	11.6	521	527	1.10
19	10.8	540	547	1.33
20	14.9	650	643	-1.17

* Result rejected due to insufficient time between injection of isotope and urination for equilibration to occur.

3. Prediction of initial D isotopic composition from initial ^{18}O abundance of a bee's body water pool

There was a highly significant regression between ^{18}O and D isotopic abundances of the diluted standards ($f_{1,9} = 2384.46$, $p < 0.001$) (Figure 3.8.2).

Figure 3.8.2 The relationship between isotopic abundances of the ^{18}O and D isotopes in a range of 20 % atom excess $D_2{}^{18}O$ dilutions with distilled water.



The regression equation, derived from Figure 3.8.2, was then used to predict initial D abundances of *B.terrestris* body water, from the observed initial ^{18}O

composition of the bees plasma (Table 3.8.3) The equation used was:

$$Y = 1.13x - 2074.7 \ (r^2 = 0.997) \qquad (1)$$

where Y = predicted D initial isotopic abundance and x = the observed ^{18}O initial abundance of the bees body water pool.

The predicted and observed initial D abundances of *B.terrestris* were similar, with a mean error of only -0.14±0.6% (n = 10) and a maximum error of -1.12% (Table 3.8.3). There was a highly significant correlation between observed and predicted initial D abundances of the bees body water pools ($r_s = 0.999$, $p < 0.001$).

Table 3.8.3. The accuracy of predicting initial D isotopic abundance of bumblebees body water pool from the observed initial ^{18}O isotopic abundance.

Bee ID	Initial observed ^{18}O isotopic abundance of *B.terrestris* body water pool (ppm).	Initial observed D abundance of *B.terrestris* thoracic body water pool (ppm)	Initial D abundance predicted from observed initial ^{18}O abundance (ppm)	% error between observed and predicted initial D isotopic abundance
1	3091	1410	1418	0.53
2	3295	1660	1649	-0.68
3	2600	857	864	0.82
4	3490	1875	1869	-0.33
5	2805	1102	1095	-0.67
6	2315	540	542	0.41
7	2767	1064	1052	-1.12
8	2611	871	875	0.44
9	3505	1897	1886	-0.56
10	3482	1864	1860	-0.19

3.8.3. Discussion

1. The single sample method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

Regression analysis of the relationship between body mass and initial isotopic abundances of the bumblebees body water pool indicated a significant relationship between the two variables. Combined with the low mean errors between predicted and observed initial $D_2{}^{18}O$ abundances, it would appear reasonable to assume that initial isotopic abundance of a bees body water pool can be predicted from the bees body mass alone. This, however, does not take

into consideration the range of observed error around the mean. Although the maximum observed error in predictions of initial ^{18}O abundance (4.7%) remained within the 95% confidence limits of the data set, such an error would result in a 55.4% over estimate in rCO_2 for a typical bee[1]. Similarly, the maximum observed error in initial D abundances (8.9%) would result in under-estimates in rCO_2 of up to 54.9%. It was, therefore, clear that only small errors in estimates of initial isotopic abundances of the bees body water pool could lead to gross errors in final rCO_2 estimates of the bees energy expenditure. As the single sample method takes no empirical measurements of the isotopic abundance of the bees body water pool, it is particularly prone to errors which other techniques do not experience; these include:

1. **Fractionation of the $D_2{}^{18}O$ isotopes in the needle prior to injection.** This will result in altering the regression relationship between the body mass and initial isotopic abundances of the bees body water pool; leading to under-estimates in initial isotopic abundances.
2. **Injecting inaccurate volumes of $D_2{}^{18}O$ into the bee.** Injecting inaccurate volumes of isotope will again make regression analysis predictions of initial isotopic abundance unreliable. Due to the small volumes of isotope involved in the current study, it is highly likely that some errors in injection will have occurred.

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[1] The effect of errors in initial estimates of isotope abundance were calculated on an imaginary bee whose body mass, initial isotopic abundance and turnover rates equaled those predicted as mean values for *B.terrestris*. The initial ^{18}O abundance was predicted as 3802.572 ppm and the D abundance 2239.546, with a 50% ^{18}O turnover rate and 30% D turnover rate.

As no empirical measure of the bees body water isotopic abundances were taken, it was not possible to determine whether inaccurate volumes of isotopes were administered during injection, or if any fractionation of the injectate had occurred. As a result, it would not be clear in a field situation which results were subject to significant errors and which were reliable, thus making the single sample technique far from ideal.

2. The "urine" method of determining initial $D_2{}^{18}O$ isotopic abundance of a bee's body water pool

The $D_2{}^{18}O$ abundances of the bees urine were not significantly different to the abundance of the body water of the bee from which it was excreted. It was, therefore, possible to measure the initial isotopic abundance of *B.terrestris* from excreted urine. However, despite being able to stimulate the bees to urinate, the time of excretion was impossible precisely to control. This lead to bees urinating before the injected isotopes had fully equilibrated with the body water pool of the bee, thus producing large errors in the measurement of initial isotopic abundances. Also, to stimulate excretion, it was necessary to agitate the bee producing a defensive response. Subsequent experiments, involving bees foraging to artificial flowers (Section 5.0.0), showed that once a defensive response had been induced in a foraging bee, generally the bee would abandon the patch and forage at an alternative site or return to the hive (*pers. obs.*). As it was essential that bees continued to forage in a stable manner following extraction of an initial sample of body water, the urine technique was not appropriate for the current study. However, the urine technique may be of use in other studies where stable foraging behaviour is not a pre-requisite.

3. Prediction of initial D isotopic composition from initial ^{18}O abundance of a bee's body water pool

Prediction of initial D abundance from observed initial ^{18}O content resulted in the lowest mean and maximum errors in estimates of initial $D_2{}^{18}O$ plasma abundances of the three techniques.

Although the maximum observed error in D predictions (-1.12%) were larger than the error observed purely due to isotope analytical error (+0.22% (Section 3.3.2)), the mean observed error in predicting D abundances (-0.14%) was lower than that observed through analytical error alone. It is, therefore, possible that predicting D abundances from observed ^{18}O abundances, results in more accurate estimates of the isotopic content of a bee's body water pool than would direct isotopic analysis of the bee's plasma.

However, this technique still has one major draw-back, it requires the inter-segmental membrane of the bee to be punctured and plasma removed. Although the size of the needle wound was minimal (≈4-6 nm), and the volume of plasma extracted was small (≤1μl), the effect on the bees behaviour and physiology was far from clear. It was, therefore, important that careful controls were used in any subsequent experiments, in order to observe any deleterious effects this technique may have had on the bees.

3.8.4. Conclusion

Although all three methods can be used to predict initial $D_2{}^{18}O$ abundances of a bumblebee's body water pool, prediction of initial D abundance from observed initial ^{18}O abundances was the method subject to the least number of errors and resulted in the highest degree of precision. As a result, this technique was used in all subsequent DLW validation and field experiments.

Chapter 4

Validation of the bumblebee doubly labelled water protocol

4.0.0 Open circuit respirometry validation of the DLW protocol for bumblebees

Introduction

DLW measurement of bumblebees energy expenditure required several alterations to the protocol usually applied to large animals, these included:

1. Analysis of ^{18}O and D abundances of small plasma volumes (Section 3.3.0).
2. Injection of µl quantities of $D_2{}^{18}O$, without affecting the bees physiology or behaviour (Section 3.4.4).
3. Removal of µl quantities of plasma for analysis of initial ^{18}O abundances (Section 3.5.1).
4. Prediction of initial D plasma abundance from initial ^{18}O abundances (Section 3.8.1).
5. Removal of sufficient plasma for final analysis of $D_2{}^{18}O$ abundances (Section 3.5.2).

Although these modifications individually resulted in insignificant errors in estimates of initial and final isotopic abundances, the compound effect of any inaccuracy was unknown. Also, due to the small body size, high metabolic rate, and rapid water turn-over in bumblebees (Bertsch, 1984), the extent to

which bees violated the 6 basic assumptions of the DLW technique (Section 3.0.3) was also unknown. It was, therefore, essential that the developed protocol be validated.

This was achieved by comparing DLW estimates of *B.terrestris* CO_2 production with simultaneous open circuit respirometry measurements.

4.1.0. Materials and methods

4.1.1. Open circuit respirometry measurement of *B.terrestris* CO_2 production

An open flow respirometry system was simultaneously used with the DLW technique to measure *B.terrestris* rCO_2 (Figure 4.1.1). The experiment was carried out at Cambridge University insect flight group. The system collected expired CO_2 from the bee in an air tight perspex flight chamber (Figure 4.1.2), containing a rotating wire arm, onto which the bees were attached. The tethered bees could then fly along the path of rotation of the wire arm within the flight chamber. Tethered flying bees were used in preference to free walking or resting individuals, in order to increase the metabolic and water turn-over rates of the bees. This was necessary for two reasons:

1. To decrease the time required for the bees to turn-over C. 50% of the administered ^{18}O isotopes.

Figure 4.1.1.
Open circuit respirmometer system, enabling in-direct calormetric measurment of CO_2 production in *B.terrestris*.

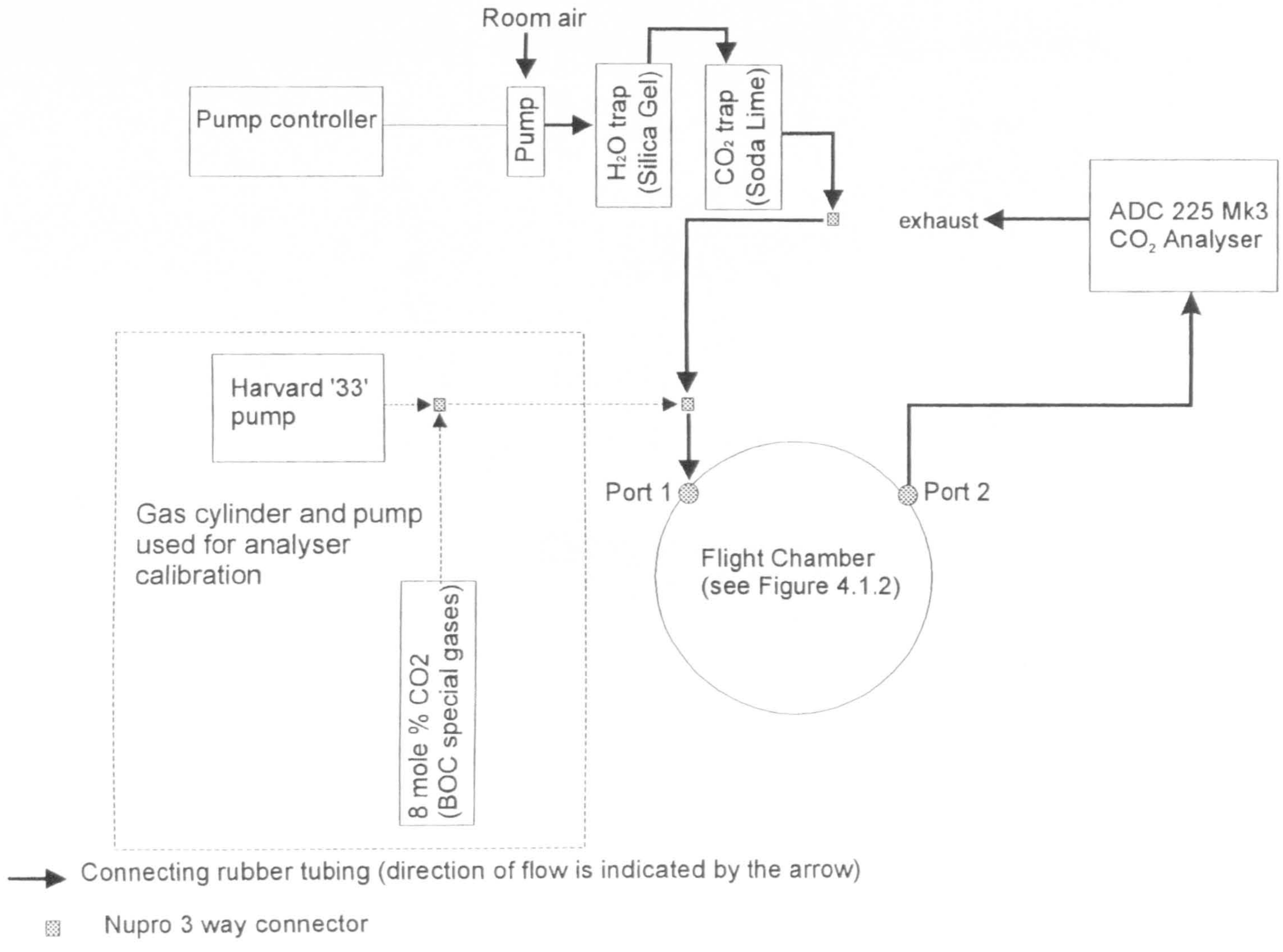
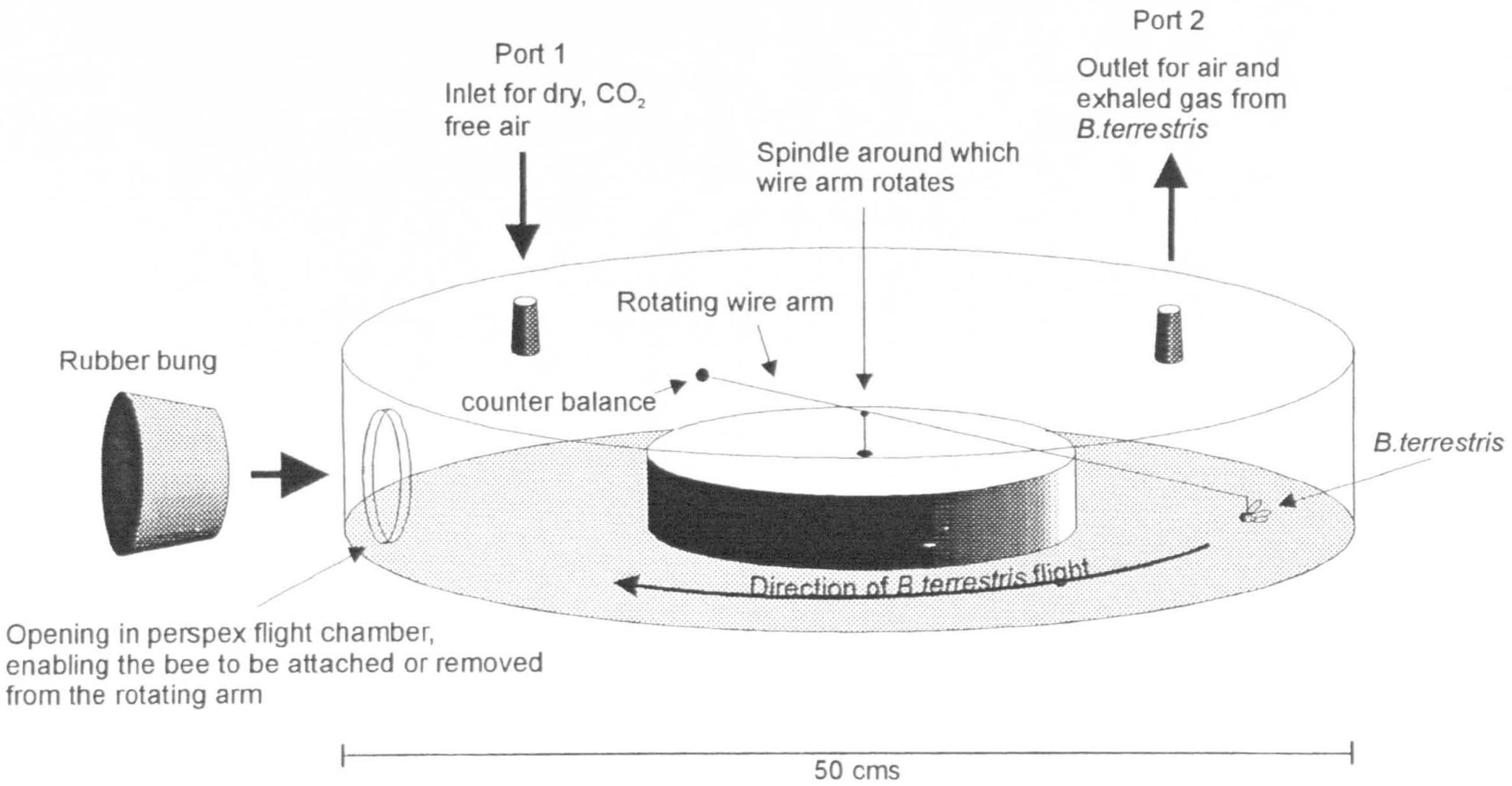


Figure 4.1.2
2.5 L volume perspex flight chamber, enabling collection of exhaled CO_2 from *B.terrestris* during tethered flight.



2. To ensure that the metabolic and water turn-over rates of the bees during the validation were as close as possible to those which would be experienced in a field situation.

CO_2 free, dry air[1] was pumped through the flight chamber at a rate of 1*L* minute^{-1}, maintaining a steady air flow across the bee. Air pumped into the flight chamber escaped through port 2 at low resistance (Figure 4.1.2), thus limiting any difference between the internal air pressure of the flight chamber and ambient air pressure. Samples of the "exhaust" gas from port 2 were passed through an infra-red CO_2 gas analyzer (ADC 225 Mk3)[2]. CO_2 levels were monitored on a chart recorder and integrated graphically to obtain total CO_2 production. Temperature and pressure were recorded at the beginning and end of the experiment, allowing all results to be adjusted for Standard Temperature and Pressure[3] (STP).

The CO_2 analyzer was calibrated prior to placing the bee in the flight mill, and again when the bee was removed at the end of the experiment. To calibrate the analyzer, 50 ml of 8±0.08 molar % CO_2 (BOC special gases) were injected into the flight chamber, at a flow rate of 4 ml min^{-1}, by a motorized Harvard '33' pump.

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[i] The room air was first passed through a silica gel trap to remove atmospheric water and a soda lime trap to remove CO_2.
[2] The CO_2 analyzer was not affected by water vapour (ADC 225 users handbook), thus, it was not necessary to dry air leaving the flight mill.
[3] Standard pressure was taken as 760 mm Hg and temperature as 273°K.

4.1.2. DLW estimates of *B.terrestris* CO_2 production

Female *B.terrestris* were captured when leaving the artificial hive, thus ensuring the bees were foragers and capable of flying. The captured bees were weighed and restrained between nylon mesh and the plunger of a syringe and injected with 1µl of $D_2{}^{18}O$ (Section 3.4.4)[4]. During equilibration, a queen marking tag was glued to the upper surface of the bee's thorax, and a small pin glued to the tag. When equilibration was complete, initial $D_2{}^{18}O$ plasma abundance was determined by the removal of a 1µl plasma sample (plasma **a**) with a micro-needle (Section 3.5.1). The sample was flame sealed into the micro-needle and refrigerated at $\approx 5^{o}C$ for later analysis.

The needle wound was sealed with melted dental wax and the bee fed *ad libitum* 66 % w/w sugar water. The bee was then attached to the rotating arm of the flight mill (Figure 4.1.2) by the pin glued to the bee's thorax The flight mill was sealed and simultaneous DLW and indirect calorimetry measurements of CO_2 production were taken. Bees were initially stimulated to fly by gently moving the rotating arm of the flight chamber with a magnet. As the metabolic rates of the flying tethered bees were lower than would have been expected during free flight, it was not possible to predict ^{18}O turnover rates from flight time. Bees were, therefore, encouraged to fly for 4 to 7 hours.

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[4]Bees were restrained rather than anaesthetized, thus preventing any deleterious physiological effects that rapid cooling may cause (the method previously used to anaesthetize the bee (Section 3.4.2)).

During the flight experiment, the flight chamber was opened approximately every 2 hours, and the bee removed and fed *ad libitum* 66% w/w sugar water. During feeding, it was not possible to take direct calorimetry measurements of the bees CO_2 production. Energy expenditure over this time period was graphically interpolated from CO_2 production of the bee prior to removal and following replacement into the flight chamber. Exogenouse air entering the flight chamber during feeding was pumped out of the system after 18 seconds.

Although this procedure will have created errors in CO_2 measurement, such estimates were only necessary for less than 2% of the total experimental time, and overall were unlikely to have significantly affected respirometry measurements of CO_2 production.

When the bee could no longer be stimulated to fly, she was removed from the flight chamber and killed. The thorax and abdomen were weighed and flame sealed into separate soda glass test tubes for subsequent plasma extraction and analysis.

4.1.3. Isotopic analysis of *B.terrestris* body water

Initial isotopic abundances of body water were determined by ^{18}O small sample analysis of plasma a (Section 3.2.1). Initial D abundances were predicted from the initial ^{18}O isotopic composition (Section 3.8.1). Final $D_2{}^{18}O$ concentrations were measured from the isotopic content of the bees thoracic water. The thorax water was LNVD extracted (Section 3.3.2) and the $D_2{}^{18}O$ content analyzed as described in Section 3.2.1 & 3.2.4. The background isotopic composition of the bees were estimated from the $D_2{}^{18}O$ content of three unlabelled female *B.terrestris* from the same hive. Body water content was predicted from the starved mass of the bee, using equation (1)

$$Y = 0.755x - 7.1;\ r^2 = 0.99;\ n = 40 \qquad (1)$$

where Y = body water content (mg) and x = starved body weight (mg).

4.1.4. DLW calculation of energy expenditure

DLW estimates of energy expenditure of *B.terrestris* were calculated using the Lusk (1928) equation (2):

$$EE = 22.4 \cdot rCO_2 \cdot (3.815 + 1.232RQ) \cdot 4.184 \quad (2)$$

where EE = energy expended (kJ h^{-1}), RQ = respiratory quotient[5] and rCO_2 = CO_2 production (mol CO_2 h^{-1}). rCO_2 was calculated using the Lifson and McClintock (1966) equation (3):

$$rCO_2 = \frac{N}{2.08} \cdot \left(K_O - K_D\right) - 0.015K_D \cdot N \quad (3)$$

where N = body water content of the bee (mol), K_O = the apparent fractional turnover rate of ^{18}O content of the bee and K_D = the apparent fractional turnover rate of the D content of the bee. K_O was calculated using equation (4):

$$K_O = \frac{\ln\left(I_O - B_O\right) - \ln\left(F_O - B_O\right)}{t} \quad (4)$$

Where I_O = initial ^{18}O isotopic abundance of *B.terrestris* body water (ppm), F_O = final ^{18}O isotopic abundance of *B.terrestris* body water (ppm), B_O =

[5] The RQ for *B.terrestris* was taken as 1 (Rothe & Nachtigall, 1989).

background ^{18}O isotopic abundance of *B.terrestris* body water (ppm), ln = natural log, and t = time between initial and final samples (hr).

K_D was calculated using equation (5):

$$K_D = \frac{\ln(I_H - B_H) - \ln(F_H - B_H)}{t} \quad (5)$$

where I_H = initial D isotopic abundance of body water (ppm), F_H = final D isotopic abundance of body water (ppm), B_H = background D isotopic abundance of body water (ppm), and ln = natural log.

4.2.0. Results

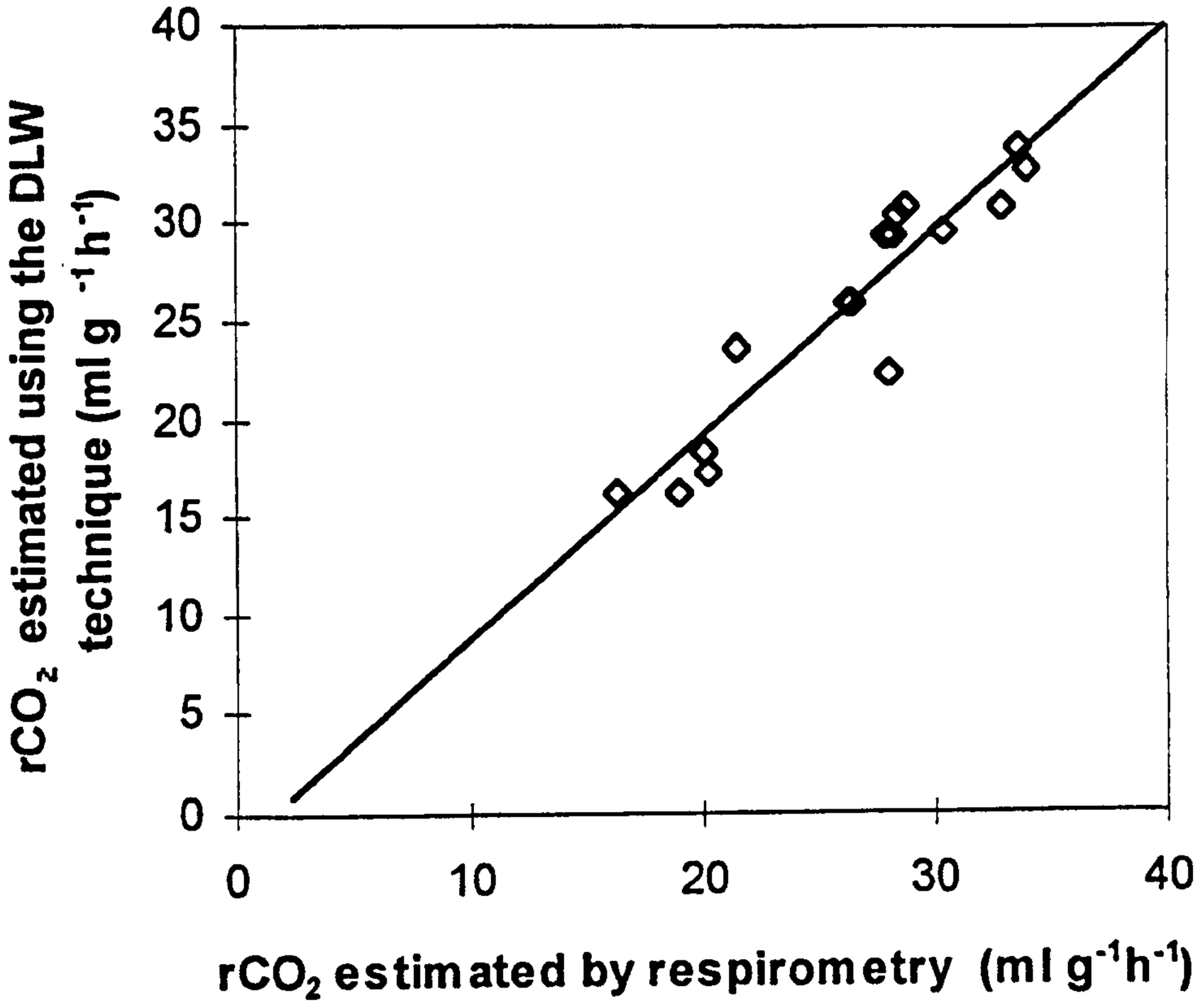
It was possible to encourage bees to fly within the flight mill for an average of 6.3±1.0 h, ranging from 4.5 h to 7.5 h (Table 4.2.1). This flight effort resulted in a mean ^{18}O turnover rate of 53.5±11.97%, ranging from 33.2% to 74.6% (Table 4.2.1). The relationship between flight effort (time spent flying in the flight mill) and ^{18}O isotope turnover was, as expected, highly significant (r_{14} = 0.80, $p<0.001$). Similarly, a significant correlation was found between flight effort and D isotope turnover rates (r_{14} = 0.70, $p<0.05$).

There was little difference in the DLW and calorimetry estimates of *B.terrestris* CO_2 production (Figure 4.2.1 & Table 4.2.1). The DLW estimates

Table 4.2.1. Measurements of energy expenditure of *B.terestris* in tethered flight.

Bee ID	Body mass of bee (mg)	Turnover time (h)	DLW measurement of VCO_2 (ml gh^{-1})	Calorimetry measurement of VCO_2 (ml gh^{-1})	Percentage difference	Percentage D turnover	Percentage $^{18}0$ turnover
1	248.4	5.3	33.6	33.9	-1.0	21.8	48.5
2	213.6	4.5	28.1	22.5	25.1	13.6	35.9
3	247.9	4.8	16.3	16.3	0	23.0	36.3
4	250.0	5.5	28.3	29.5	-4.2	19.3	44.1
5	228.4	7.2	26.3	26.1	1.1	25.5	52.4
6	252.9	7.0	28.3	30.5	-6.9	35.4	59.6
7	223.9	6.5	27.8	29.4	-7.2	30.6	54.4
8	190.5	6.7	19.0	16.3	16.3	32.7	50.6
9	270.1	7.5	21.4	23.7	-9.9	62.1	74.6
10	201.0	7.4	33.9	32.7	3.4	32.4	62.6
11	274.3	6.6	30.3	29.6	2.3	43.0	64.6
12	294.3	6.5	32.9	30.9	6.5	45.3	67.2
13	281.8	7.4	28.7	30.8	-6.9	41.9	64.9
14	282.5	5.4	26.3	26.1	0.6	34.6	53.5
15	228.6	5.2	20.0	18.4	8.6	14.6	33.2
16	292.0	7.5	20.3	17.3	16.9	33.5	53.6

Figure 4.2.1. The relationship between estimates of CO_2 production as measured using the DLW technique and open circuit respirometry. Best fit was calculated by reduced major axis regression and can be described as y = 3.12 + 0.90x (r^2 = 0.88, n = 16).



had a mean arithmetic difference of +2.8±9.9% from direct calorimetric measurements (Table 4.2.1). The largest underestimate was -9.9% and the highest overestimate +25.1%. However, 10 of the 16 bees had errors which fell within the range of -7% to +7%. In six cases the DLW technique underestimated of rCO_2, whilst there was no difference in estimates for one bee, and energy expenditure was overestimated for a further nine bees. The source of variation between DLW and direct calorimetry measurements of VCO_2 in different bees was not clear, with no significant correlations being observed between the variation and body mass of the bee (r_{14} = -0.29, ns), turnover time (r_{14} = -0.29, ns), turnover rate of the D isotope (r_{14} = -0.42, ns), or turnover rate of the ^{18}O isotope (r_{14} = -0.46, ns).

DLW estimates of CO_2 production were not significantly different from direct calorimetry measurements when comparing the results from all bees (Paired *t*-test; t_{15} = 0.84, ns).

4.3.0. Discussion

The degree of error observed between the two techniques compared favorably with similar validation studies on vertebrates (Table 4.3.1). The observed mean error of +2.8% was slightly higher than the mean error of -2.1% observed in 8 validation studies of 12 species of birds and reptiles, but was lower than the +3.1% error reported in 26 studies of 9 species of mammals (as described in Speakman & Racey, 1988_a). More importantly, the

Table 4.3.1. The accuracey of previous DLW measurments of rCO_2 production compared to direct respirometry measurments.

Species	n	Mean % error in DLW estimates	Range	Source
Mammals				
Man (adults) (*Homo sapien*)	5	+5.9	-6.5 to +14.1	Schoeller & Web, 1984
Man (infants)	4	+8.0	-5.3 to +14.5	Roberts, Coward, Norhia, Schingenseipen & Lucas, 1986.
Rat (*Rattus rattus*)	8	+1.8	-2.2 to +10.1	McClintock & Lifson, 1958
White mice	7	-4.0	-12.0 to +8.0	McClintock & Lifson, 1957
Pocket Gopher (*Thomomys bottae*)	6	+3.7	-8.7 to +14.5	Gettinger, 1983
Ground Squirrel (*Ammospermophilus*)	7	+0.8	-12.4 to +17.2	Karasov, 1981
Chipmunk (*Tamias*)	2	+4.5	+1.0 to +8.0	Little & Lifson, 1975
Perognathus	4	+8.5	-9.2 to +5.3	Mullen, 1970
Bats				
Pipistrelle (*Pipistrellus auritus*) (n = 7) & Long eared bats (*Plecotus auritus*) (n = 2))	9	+9.5	-14.3 to 28.6	Speakman & Racey, 1988[a]
Caribou (*Rangifer t. granti*)	3	-1.4	-4.8 to +5.8	Fancey *et al.*, 1986
Reindeer (*Rangifer tarandus*)	1	+1.1	-	
Sheep (*Ovis* spp.)	4	-14.6	-	Midwood, Haggarty, McGaw, Mollison, Milne & Duncan, 1994
Birds				
Budgerigar	9	-0.04	-5.2 to +6.2	Buttemer *et al.*, 1986
Gambels quail (*Callipepla*)	6	-6.0	-23.2 to +15.5	Goldstein & Nagy, 1985
Pigeon (*Columba*)	10	+4.0	-2.2 to +48.5	Lefebvre, 1964
Sparrow	13	-3.5	-17.0 to +4.2	
Four species *				
House martin (*Delichon urbica*)	4	+3.4	-5.1 to +14.0	Hails & Bryant, 1979
Sand Martin (*Riparia riparia*)	2	+4.4	+2.4 to +6.4	Westerterp & Bryant, 1984
Reptiles				
Lizard (*Sceloporous sp.*)	4	+3.2	-5.7 to +18.6	Congdon, King & Nagy, 1978.
Tortoise(Gopherus)	-	+2.2	-	Nagy, 1980

Table 4.3.1 continued

Arthropods				
Scorpion	5	+36.5	+11.1 to +71.7	King & Hadley, 1979
(*Hadrus arizonensis*)				
Locust (*Locusta migratoria*)	9	-4.1	-60.3 to +31.9	Buscarlet *et al.*, 1978
Beetles (*Eleodes sp.*)	4	+14.5	+12.7 to +74.2	Cooper, 1983
(*Cryptoglossa sp.*)	1	+25.9	-	
Bumble bee (*Bombus terrestris*)	16	+2.3	-9.9 to + 25.1	present study

♣ Song sparrow (*Melospiza melodia*), white throated sparrow (*Zonotrichia albicolis*), house sparrow (*Passer domesticus*) and savannah sparrow (*Passerculus sandwichensis*).

error was 12.3% lower than the mean error observed in 3 species of arthropods (Buscarlet *et al.*, 1978; King & Hadley, 1979; Cooper, 1983). The range of error was also far lower for *B.terrestris* than any previous arthropods studies (Table 4.3.1).

The turnover rates of $D_2{}^{18}O$ within *B.terrestris* were relatively high (Table 4.2.1), although this did not seem significantly to affect the accuracy of the DLW estimates of the bees rCO_2. A comparison with studies of vertebrate species with similarly high metabolisms shows not only comparable precision (Table 4.3.1), but also a striking similarity between the regression equations of the different species (Table 4.3.2).

Table 4.3.2. Least squares regression equations comparing DLW results (y) with respirometric or gravimetric estimates of energy expenditure (x). n = sample size, r^2 = regression.

Species	Equation	r^2	n	Source
Bats (2 species)	y = 3.45 + 0.87x	0.90	9	Speakman & Racey (1988a)
Hummingbirds	y = 5.67 + 0.81x	0.83	6	Tiebout & Nagy (1991)
bumblebees	y = 3.12 + 0.90x	0.88	16	Current Study

Overall, there have been 6 previous studies which have reported lower deviations between DLW and calorimetry estimates of rCO_2 than those observed in the current study (McClintock & Lifson, 1958; Nagy, 1980; Karasov, 1981; Buttemer, Hayworth, Weathers & Nagy, 1986; Fancey *et al.*, 1986; Masman & Klaassen, 1987). Despite this, the high level of accuracy in DLW predictions of calorimeter measurements of *B.terrestris* rCO_2 were unexpected, given the poor results from previous attempts to apply the DLW technique to invertebrates (Buscarlet *et al.*, 1978; King & Hadley, 1979; Cooper, 1983). The high level of accuracy in the present study is most likely to be due to two factors:

1. **An improved protocol for isotopic analysis of plasma.**

Due to an improved design of the vacuum manifold preparation line (Section 3.2.0), plasma samples were prepared and analyzed at pressures two orders of magnitude lower than had previously been attempted (Section 3.2.0). This greatly reduced sample contamination, halving the analytical error observed in previous inter-laboratory reports (Section 3.3.0). Although analytical errors are not the only limiting factor in the DLW technique, potentially they can result in massive errors in final rCO_2 estimates. Nagy (1980) demonstrated that errors in rCO_2 estimates were particularly sensitive to analytical errors in I_o and F_o, with the sensitivity to analytical errors increasing as water turnover decreases. Nagy showed that a 1% error in I_o and F_o, combined with a 50% water turnover rate,

would result in a 10% error in rCO_2 estimates, were as, if water turnover only equalled 10%, a 1% analytical error in I_o and F_o would result in a 70% error in final rCO_2 estimates.

As the mean ^{18}O turnover rate of the bumblebees, in the current study, equalled 53.5±12.0% (n = 16), according to Nagy's calculations a 1% analytical error in ^{18}O isotopic abundance of I_o and F_o would result in just under 10% errors in rCO_2 estimates. As Speakman *et al.* (1990) reported errors of -1.4±0.4% in inter-laboratory analysis of ^{18}O abundance of enriched water standards, the precision of this analysis would again have resulted in a mean error of over 10% in the rCO_2 estimates in the current study. However the observed error of analytical technique described in Section 3.3.2, was only 0.05±0.4%.

The effect of analytical error on rCO_2 estimates is well illustrated by bee 6. Bee 6 had an I_o of 3802.6 ppm and an F_o of 3212.1 ppm. If, therefore, the analytical errors experienced by I_o and F_o equaled -1.4% (as reported by Speakman *et al.* (1990)), the subsequent error in rCO_2 estimates would equal 3.2%. However, if I_o and F_o analytical error is reduced to 0.05% (as observed in Section 3.3.2.) the subsequent error in rCO_2 estimates is more than halved to 1.5%. As the current experiment used the same analytical procedure as was used in Section 3.3.2, it is reasonable to assume that the analytical error which occurred during I_o and F_o analysis did not significantly differ from 0.05%. As a result, it is likely that analytical error in the current study is lower than has been experienced in previous DLW

studies. It is, therefore, likely that the high degree of precision in the current study is partly explained by improved isotope analytical procedure.

2. The physiology and behaviour of bumblebees comply well to the six basic assumptions of the DLW method (Section 3.0.3):

Assumption 1 & 2) *Rates of flux of CO_2 production, water loss and body water pool are constant.*

Although the metabolic rates of the tethered bees were lower than would be expected during free flight (Cooper 1993), their rate of CO_2 production remained relatively constant throughout the validation experiment. Thus, any errors in DLW estimates of rCO_2, due to fluxes in CO_2 production, will have been minimal.

The flux rates in water loss and the body water pool of the bees could not be measured during the current experiment, so errors in rCO_2 estimates from this source could not be determined. However, it has previously been reported that male *B.lucorum* and *B.terrestris* maintain a constant water balance for periods of up to 24 hours (Bertsch 1984). Nicolson & Louw (1982) also found tha,t due to metabolic water production, foraging carpenter bees (*Xylocopa capitata*) exhibited no signs of water stress, even in hot desiccating environments. Nicolson & Louw also observed a highly significant relationship between O_2 consumption and evaporative water loss ($r = 0.73$,

with $p<0.001$). If a similar relationship also exists in bumblebees, the constant CO_2 flux rates observed in the current experiment will also indicate a relatively constant O_2 consumption rate and, therefore, a constant water flux. It is, thus, unlikely that the evaporative water loss or body water pool of the bees fluctuated significantly during the course of the validation experiment.

Assumption 3) *^{18}O and D are not incorporated into constituents other than CO_2 and H_2O*

One of the possible sources of error in the DLW protocol occurs when ^{18}O and D isotopes label compounds other than CO_2 and Water (Speakman & Racey, 1988_b). It is likely that this will occur most prevalently during anabolic metabolism, with isotopes being incorporated into fats and proteins as they are synthesized within an animal (Nagy, 1980). Although there is no direct evidence of non-aqueous assimilation of isotopes (Speakman *pers com.*), previouse studies have shown consistent overestimates of body water pool size when predicted from isotope dilution space (Schoeller & Webb. 1984). This would tend to indicate that isotopes are being bound in none aqueous substances, thus increasing the apparent dilution of administered isotopes. However, unlike other validated species, bees greatly reduce anabolic metabolism when they reach the adult instar (Winston, 1991). The rate of tissue synthesis in adult bees is reflected by the cessation of protein consumption following the larval instars, with adult bees consuming a pure carbohydrate diet (Rothe & Nachtigall, 1989). As no protein is available for

tissue synthesis, it is likely that any anabolic metabolism in an adult bee will be minimal, as will the incorporation of ^{18}O and D isotopes into non-aqueous compounds. This reduced assimilation of ^{18}O and D labels during protein metabolism will thus reduce overestimates of the isotope dilution space (Nagy, 1980) and isotopic turnover rate of the bees (Speakman & Racey, 1988_b).

As bumblebees are one of the few DLW validated species to have an RQ of 1, it is likely that the absence of significant anabolic metabolism is one of the reasons for the close correlation between DLW and open respirometry estimates of rCO_2.

Assumption 4) *^{18}O and D isotopes are lost from the organism only as CO_2 or H_2O*

Loss of ^{18}O and D isotopes via routes other then CO_2 and H_2O can occur without resulting in significant errors in rCO_2 estimates, providing both isotopes are lost in the same ratios (Tatner & Bryant, 1989). Concern has, however, been raised over the loss of ^{18}O isotopes during the synthesis of urea (Nagy, 1980, Speakman & Racey, 1988_a). It has been suggested that ^{18}O isotopes become irreversibly incorporated into urea during the ornithine - arginine synthesis cycle (McGilvery, 1970, Speakman & Racey, 1988_a). As a result, the apparent fractional turnover rate of the ^{18}O isotope will increase

relative to D, thus altering the ratio between the two isotopes and creating errors in rCO_2 estimates.

The effect of ^{18}O assimilation during urea synthesis in bumblebees is unknown. However, although small amounts of urea are synthesized in insects, the majority of nitrogenous waste is excreted in the form of uric acid (Chapman, 1985). Also, adult working caste bees have a protein free diet (Rothe & Nachitigall, 1989) and metabolize only small quantities of protein (Snodgrass 1956). The amount of nitrogenous waste a bee has to void will, therefore, be minimal. As urea synthesis is directly linked to the quantity of nitrogenous waste excreted, the rate of urea synthesis in bees is also likely to have been negligible. Given that urea synthesis in bumblebees is minimal, the quantities of ^{18}O assimilated into urea during the ornithine - arginine cycle will have been insignificant, and unlikely to have resulted in significant errors in DLW estimates of rCO_2.

Assumption 5) *H_2O and CO_2 exiting the body are in isotopic equilibrium with the organisms body water*

As CO_2 and H_2O exit an organism, there is a possibility that isotopic kinetic and / or equilibrium fractionation will alter the fractional turn-over rates of the two isotopes (Haggarty *et al.*, 1988). If fractionation does occur in the exiting CO_2 or H_2O, it may potentially result in significant errors in rCO_2 estimates of an organism (Nagy, 1980; Fancey *et al.*, 1986; Speakman & Racey, 1988_b).

There are two possible routes of $D_2{}^{18}O$ loss from a bee which may result in some form of kinetic or equilibrium fractionation:

i) Fractionation during Evaporative Water Loss (EWL).

Although EWL is one of the largest sources of H_2O loss from flying bees (Nicolson & Louw, 1982), it is generally considered that similar forms of water loss in vertebrates result in negligible levels of body water fractionation (Haggarty *et al.*, 1988). This is due to the H_2O lost from the animal not being evaporated directly from the animal's body water pool. Instead, body water must first pass from the body water pool to the outer surface of the skin. Once the water has past through the animal's epidermis, the water will begin to evaporate and undergo fractionation. However, as the fractionated water is normally unable to re-enter the animal's body (Haggarty *et al.*, 1988), it will have no effect on the isotopic composition of the body water pool. This assumption, however, may not hold true in bumblebees which can re-absorb small quantities of water through their cuticle and tracheal system by passive diffusion (Chapman, 1985). It was, however, not possible to determine whether bees did re-absorb fractionated water into the body water pool during the current experiment. As a result, the effect re-absorption of fractionated body water had on DLW estimates of the bees rCO_2 was unknown. However, as a close relationship was observed between respirometry and DLW estimates of energy expenditure, any errors resulting from isotope re-absorption will have been insignificant.

ii) Fractionation of the bees body water pool during urination

As has been discussed in assumption 4, it is possible that the synthesis of urea can lead to the fractionation of the ^{18}O isotope in the bees body water. However, due to low levels of urea synthesis, it is unlikely that this would result in significant errors in DLW estimates in the rCO_2 of bumblebees. Evidence for the low levels of fractionation in a bee's body water can also be found in Section 3.8.2, where comparisons between the isotopic abundance of the body water and voided urine of ten bees found no significant difference.

Assumption 6) *No labelled or unlabelled CO_2 enters the animal*

CO_2 is lost from the bee by diffusion from the body into the tracheae (Chapman, 1985). However, as the diffusion is passive, exogenous CO_2 may also diffuse in the opposite direction through the tracheae into the bee, resulting in underestimates of rCO_2. Absorption of environmental CO_2 into the bee was, however, unlikely to have resulted in significant errors during the current experiment. This was due to the air in the flight chamber being "scrubbed" with soda lime in order to remove any exogenous CO_2. Also, to prevent the build of expired CO_2 within the flight chamber, fresh CO_2-free air was continually pumped through the system. As the air within the flight chamber was completely replaced every 18 seconds, CO_2 levels did not exceed 200 ppm. As a result, it is highly unlikely that significant errors in

DLW estimates of rCO_2 will have resulted due to the absorption of exogenous CO_2.

Looking at the six assumptions of the DLW method, it is clear that *B.terrestris* are particularly well suited to this technique, with a constant water balance, low anabolic metabolism, low nitrogenous excretion, and an RQ of 1. The DLW protocol developed for *B.terrestris* resulted, therefore, in accurate rCO_2 measurements.

Chapter 5

Doubly labelled water measurement of energy expenditure in free ranging bumblebees: Do bumblebees maximize net energy efficiency when foraging for nectar?

5.0.0 Measurement of energy expenditure in free ranging *B.terrestris*: Do bumblebees maximize Net Energy Efficiency whilst foraging for nectar?

Introduction

Initial experiments indicated that current models of NEE resulted in poor predictions of *B.terrestris* behaviour whilst foraging for nectar (Section 2.3.1). The reason for the variation between the observed behaviour of the bees, and SH model predictions, could stem from two possible sources:

1) Bumblebees do not maximize NEE whilst foraging for nectar, or
2) Current model estimates of energy costs and gains are inaccurate

Before it was possible to determine whether *B.terrestris* maximize NEE or another currency, it was necessary to ensure that the inability of the SH model to predict foraging behaviour was not due to poor estimates of energy costs and gains incorporated within the model.

5.0.1. Accuracy of estimated energy gains by a foraging bee

As bumblebees can be trained to forage at artificial flower patches (Section 2.1.1), it was possible to use artificial corollas, attached to nectar pumps in

order to regulate nectar flow within the patch. This in turn permitted the quantity and sugar concentration of each nectar parcel collected by a forager to be accurately controlled and recorded. As a result, the energy gains of a bee foraging at the artificial flower patch could be simply and accurately determined from the equation 1:

$$G = nCw \qquad (1)$$

Where n = the number of flowers visited, C = weight specific energetic value of nectar (16.7 J mg^{-1}) and w = the mass of nectar collected on each flower visit (mg).

5.0.2. Accuracy in estimates of energy expenditure of a bee whilst foraging

Although energy gains can be determined by empirical measurement of the quantity and sugar concentration of nectar collected, it has previously not been possible to measure the energy costs of foraging bees directly. Instead, energy expenditure has been estimated by Time / Activity / Laboratory (TAL) predictions (Section 2.0.2) (Schmid-Hempel *et al.*, 1985; Seeley, 1986; Wolf & Schmid-Hempel, 1990), the accuracy of which are questionable (Section 2.3.3). It was, therefore, possible that errors in TAL estimates of energy costs were the source of the difference between the observed foraging behaviour of the bees and the behaviour predicted by the

SH model (Section 2.3.3). As a result, it was necessary to use the DLW technique to obtain direct empirical measures of the energy costs of foraging *B.terrestris*, thus removing any errors inherent in TAL estimates. Once reliable measurements of a foragers costs and gains were obtained, comparisons between observed and SH model predicted foraging behaviour could be used to determine whether *B.terrestris* maximize NEE.

5.1.0. Materials and methods

At the beginning of May 1994, one artificial colony of *B.terrestris* (Biobest Ltd, Kent) was placed at the Wandlebury nature reserve, situated 3 miles south of Cambridge city centre. Initially, the colony was provided with *ad libitum* nectar and pollen, and left undisturbed for seven days, allowing the colony to settle following re-location of the hive. To encourage workers to commence foraging, no further nectar or pollen was provided after the initial seven day period. The colony was then left undisturbed for a further seven days before foraging experiments commenced, allowing workers time to orientate to the local surroundings.

5.1.1. Training bumblebees to forage to the artificial flower patch

The DLW technique not only required the injection of foragers with heavy water isotopes, but also required the removal of haemolymph. As this was an invasive technique, it was necessary to determine whether stress or

physiological damage incurred during this procedure had a significant effect on the foragers subsequent behaviour. The nectar foraging behaviour of the bees was, therefore, recorded prior to the DLW treatment, permitting later comparisons to be made with behaviour following the DLW injection and plasma extraction procedure.

Training commenced at approximately 10 am each morning and would typically take 30 minutes to perform. Initially, foraging workers were trained to two artificial flowers (Section 2.1.0), situated 2m apart from one-another, and 20m from the hive. The flowers were used to simulate a non-depleting flower patch (Section 2.1.0, Figure 2.1.2). The volume and sucrose concentration of nectar collected on each flower visit was varied between bees; from 50% to 70% w/w sugar content, delivered in quantities varying from 1.2 μl to 2.5 μl. The variation in quantity and sugar content of the nectar permitted the effect of nectar load and gains on the bees behaviour to be observed.

Once trained to the flowers, the foraging behaviour of the bees was recorded using an Apple Macintosh ™ notepad and data logger program (written in Microsoft Basic ©). The foraging variables recorded were; 1) the number of flower visits per foraging cycle, 2) time spent in hive (s), 3) time taken to fly to the artificial flower patch (s), 4) time taken to collect each parcel of nectar (handling time) (s), 5) the time taken to fly between flowers (inter-flower time) (s), and 6) time taken to fly back to the hive (s).

The foraging behaviour of the bees during 10 foraging cycles was recorded, with only the final four cycles being used for subsequent analysis. The first six cycles were permitted to provide the bees with sufficient time to learn how to handle the nectar within the corollas of the artificial flowers (Schmid-Hempel *et al.*, 1985; Wolf & Schmid-Hempel, 1990). At the end of each foraging cycle, ambient temperature and humidity were recorded using an RS temperature and humidity gauge.

5.1.2. DLW analysis of foraging costs

On the eleventh foraging cycle, bees were captured prior to imbibing nectar, thus minimizing the risk of damage to the crop during isotope injection. The bees were then weighed, injected with 1µl of 20% atom excess $D_2{}^{18}O$ (Section 3.4.4), and released. Once released, the bees were permitted to continue foraging for a further fifteen minutes, allowing the injected isotope to equilibrate with the bees body water pool (Section 3.7.0). The foragers were then recaptured, restrained, and a 1µl haemolymph sample extracted through the inter-cuticular membrane of the abdomen (Section 3.5.1). The bees were then released and permitted to forage to the artificial flowers, during which time their foraging behaviour was recorded. The bees were allowed to forage from the artificial flowers for a period of 4 hours, after which time they were recaptured, weighed, and dissected through the petiole. The thoraces of the

bees were immediately flame sealed into soda glass test tubes for subsequent isotopic analysis.

Background $D_2{}^{18}O$ isotopic abundances of the bees were estimated from the mean abundance observed following gIRMS analysis of haemolymph LNVD extracted from 2 non-injected bees, collected from the same colony (Section 3.2.1 & 3.2.4).

Initial ^{18}O abundance was measured by gIRMS analysis of the initial 1µl haemolymph samples, initial D abundance was predicted from the initial ^{18}O abundance (Section 3.8.1), whilst final $D_2{}^{18}O$ abundance was determined by gIRMS analysis of LNVD extracted thoracic plasma (Section 3.2.1 & 3.2.4). The results of the isotopic analysis were then used to calculate the rCO_2 of all bees, using the Lifson and McClintock (1966) equation (Equation 3, Section 4.1.4). To allow comparison with SH model predictions, the DLW rCO_2 measurements were converted to mean J of energy expended per foraging cycle per bee (C_{DLW}).

5.1.3. Use of the SH model to compare observed and predicted foraging behaviour

Observed foraging behaviour of labelled bees was compared with predictions from the SH model of the same bees when maximizing NEE. Model

predictions were derived by combining observed foraging times[1] with TAL estimated costs (C_1), enabling the model to predict the optimum number of flowers a bee should visit, per foraging cycle, when maximizing NEE (Section 2.1.4). The observed number of flowers visits were then compared with the number predicted from the model.

5.1.4. Comparing DLW costs with the TAL costs predicted by the SH model, when predicted number of flower visits was altered to equal observed number of flower visits

As observed and SH model predictions of the number of flower visits were not always the same, direct comparisons between C_{DLW} and SH model TAL estimates were not possible. It was, therefore, necessary to derive TAL estimates for energy expenditure when the numbers of flower visits predicted by the SH model were altered to equal the observed numbers of flower visits. This was achieved using the SH model by incrementing the value of n from 1 to the observed number of flower visits. As the SH model then predicted the same number of flower visits as was observed in the field, the new TAL predicted costs (C_2) could then be used for direct comparison with C_{DLW}. These calculations were performed separately for each individual bee using the optimum foraging simulation program (Appendix 1).

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[1] Observed foraging times included; handling times, inter-flower times, hive times and flight times to the patch and from the hive. The values used in the calculations can found in a database included in the optimal foraging simulation program (Appendix 1).

5.1.5. Use of the SH model to determine whether the observed foraging behaviour of the bees conformed to bees maximizing Net Energy Efficiency

Although comparisons between C_2 and C_{DLW} enabled inaccuracies in TAL estimates to be quantified, such analysis did not determine whether the foraging bees were maximizing NEE. It was, therefore, necessary to determine what the foraging costs of each individual bee would have to be in order for the observed behaviour of the foragers to conform to maximization of NEE (C_3), *i.e.* the value of C_3 which satisfied equation 1:

$$\text{Maximum NEE} = \frac{(G - C_3)}{C_3} \qquad (1)$$

where G = mean observed gains per foraging cycle.

C_3 could then be compared to C_{DLW} and, assuming the bees were maximizing NEE, observed and predicted costs should not significantly differ from one another.

C_3 was calculated using the optimal foraging simulation program (Appendix 1), by incrementing the total costs per foraging cycle of the bee from zero to the value necessary for the SH model to predict the same number of flower

visits as was observed in the field[2]. These calculations were carried out separately for each individual bee.

5.2.0. Results

The effect on the bees foraging behaviour of being injected and having a haemolymph sample removed appeared to have been minimal. Following injection, the individual bees altered the mean number of flowers visited by only -0.6±5.7 (n = 6), with a range of -1.5 to +14 flowers (Table 5.2.1). Although the mean change in number of flower visits following injection was low, the range in number of flower visits and standard deviation were high. This was largely due to bee 5, which more than halved the number of flowers visited per foraging cycle following injection (from 26.8±1.9 (n = 4) to 12.8±1.9(n = 4) (Table 5.2.1)). When bee 5 was removed from the calculations, the mean change in number of flower visits decreased to -0.3±1.3 (n = 5), with a range of -1.5 to +1.8. The variation in number of flower visits prior to and following injection was non-significant in all bees except bee 5 (Table 5.2.2). Bee 5 also significantly altered handling and inter-flower times following injection (Table 5.2.2). As the DLW procedure significantly affected the foraging behaviour of the bee, bee 5 was not used in any subsequent analysis.

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[2] C_3 should not be confused with C_2, where the predicted number of flower visits was incremented until it equalled the observed number of flower visits, unlike C_3, where predicted costs were incremented until predicted number of flower visits equaled the observed number of flower visits.

Table 5.2.1. Observed foraging behaviour of *B.terrestris* when foraging to artificial flower's yielding varying volumes of nectar with varying sucrose concentrations.

				Mean number of flower's visited per foraging cycle ± sd (n = 4)		Mean handling times per foraging cycle ± sd (n = 4) (Sec.)		Mean inter-flower times per foraging cycle ± sd (n = 3) (Sec.)	
BeeID	Body weight of bee (mg)	Volume of nectar collected (µl)	% sucrose concentration of nectar collected (w/w)	Before injection	After injection	Before injection	After injection	Before injection	After injection
1	143	1.2	64	13.5 ±1.3	15.3 ± 1.7	7.2 ± 0.4	7.1 ± 0.4	2.5 ± 1.0	2.7 ± 1.7
2	168	1.2	64	13.8 ±1.3	12.3 ± 2.0	8.2 ± 0.6	9.6 ± 1.6	3.1 ± 0.9	1.8 ± 0.5
3	137	1.25	50	29 ±2.2	28 ± 2.5	3.4 ± 0.2	3.8 ± 0.2	2.5 ± 0.2	3.5 ± 0.6
5	186	2.5	50	26.8 ±1.9	12.8 ± 1.0	5.0 ± 0.3	6.8 ± 0.5	3.1 ± 0.1	3.9 ± 3.2
6	160	1.25	70	19.8 ±1.7	18.8 ± 1.7	11.1 ± 0.9	12.3 ± 2.4	5.3 ± 1.0	4.3 ± 0.4
7	105	2.5	50	11.5 ±2.1	11 ± 1.4	11.3 ± 1.0	15.9 ± 2.0	6.6 ± 0.9	5.4 ± 0.5

Table 5.2.2. Analysis of changes in foraging behaviour of *B.terrestris* prior to and following injection of 1µl of 20% atom excess $D_2{}^{18}O$ (df = 6).

Bee ID	Analysis of the difference in number of flower visits.		Analysis of the difference in handling time.		Analysis of the difference in inter-flower time.	
	Paired t-test		Paired t-test		Paired t-test	
	t-stat	P	t-stat	P	t-stat	P
1	-1.63	ns	0.42	ns	-0.21	ns
2	0.75	ns	-0.76	ns	1.17	ns
3	0.61	ns	-1.55	ns	-1.11	ns
5	13.34	p <0.001 **	-5.07	p<0.003 *	-3.95	p<0.008 *
6	0.83	ns	-1.37	ns	0.96	ns
7	0.34	ns	-4.08	p<0.007*	1.57	ns

* = Significant change in foraging behaviour following injection of 1µl of 20% atom excess $D_2{}^{18}O$.
** = Highly significant change in foraging behaviour following injection of 1µl of 20% atom excess $D_2{}^{18}O$.

When considering the foraging behaviour of all remaining bees, no significant difference was observed in mean number of flower visits prior to and following injection (*paired t*-test; $t_{19} = 0.70$, ns). Injection also had a minimal effect on the time taken by the foragers to collect nectar, with a mean change in handling times following injection of +1.5±1.2 seconds (n = 5). Only bees 5 and 7 significantly altered handling times following injection (Table 5.2.2). When observing the variation in handling times for all bees, the change in time taken to collect nectar following injection, was not significant ($t_{19} = -1.93$, ns). Inter-flower times were also unaffected following injection, with mean times changing by only -0.5±1.0 seconds. No bees, other than bee 5, were observed to significantly alter inter-flower times following injection ($t_{19} = 0.99$, ns) (Table 5.2.2).

Ambient temperature recorded during the experiment ranged from 24 to 41 °C, with a mean of 36±8 °C, whilst humidity ranged from 31 to 43% with a mean of 34±4%. Variations in ambient temperature did not, however, appear alter the foraging behaviour of the bees. This was illustrated by bee 2, which was subject to the greatest temperature and humidity range of any forager (temperature ranged from 24 to 40°C and humidity from 36 to 39%). Over 6 foraging cycles, when mean temperature equalled 26±1°C and humidity 39±0.2%, bee 2 visited a mean of 12.6±1.2 flowers per cycle, with a mean handling time of 7.3±0.8 seconds and inter-flower time of 2.6±0.6 seconds. When temperature increased to a mean of 38±2 °C and humidity decreased to 36±0.4%, mean number of flower visits (over 6 cycles) did not significantly

vary from the number observed at the lower temperature and higher humidity (*Paired t-test*: t_5 = <0.001, ns), this was also true for handling (t_5 = -0.54, ns) and inter-flower times (t_5 = 1.46, ns). The low standard deviation in number of flower visits, handling and inter-flower times of all bees (Table 5.2.1), indicated little intra-bee variation in foraging behaviour over the period of the experiment (approximately 5 hours in duration), irrespective of temperature and humidity. This would tend to suggest that foraging behaviour of the bees was not significantly affected by ambient weather conditions in the current study.

As in the previous field experiment (Section 2.0), there was no correlation between number of flower visits and nectar sucrose concentration (r_3 = - 0.07, ns). However, unlike the previous experiment, no correlation was observed between body mass of the bee and number of flower visits (r_3 = 0.34, ns).

When foraging at the artificial flower patch, all bees were observed to visit significantly more flowers than predicted by the SH models. The mean underestimate of the SH model was 10.9±6.9 (n = 20) flower visits, with a range of -2.5 to -11.3 flowers.

The SH model predictions of energy expenditure per foraging cycle (C_1) were consistently lower than C_{DLW} in all bees except bee 2, although the difference between the two estimates was non-significant for all bees (Table 5.2.3). The source of the variation between C_1 and C_{DLW} was, however, partially due to

Table 5.2.3. Variation between observed DLW costs of foraging, and costs predicted by the SH model (df = 1).

Bee ID	Variation between DLW measurements of costs per foraging cycle, and SH model predictions of costs per foraging cycle, assuming *B.terrestris* were to maximize NEE.		Variation between DLW measurements of costs per foraging cycle and SH model predictions of costs, when SH model predictions of number of flower visits per foraging cycle was altered to equal observed number of flower visits.		Variation between DLW measurements of costs per foraging cycle and the costs required for the SH model to predict the observed number of flower visits per foraging cycle, assuming *B.terrestris* were to maximize NEE (J).	
	G Stat		Stat		Stat	
	G	P	G	P	G	P
1	0.30	ns	0.04	ns	4.94	p<0.05 *
2	0.01	ns	0.05	ns	0.26	ns
3	0.81	ns	7.67	p<0.01 *	89.61	p<0.001 **
6	0.07	ns	3.37	ns	31.15	p<0.001 **
7	0.42	ns	6.71	p<0.01 *	68.79	p<0.001 **

* = Significant difference between observed DLW energy expenditure and predicted energy expenditure.
** = Highly significant difference between observed DLW energy expenditure and predicted energy expenditure.

the differences between predicted and observed number of flower visits (Table 5.2.4). This was because DLW estimates of energy expenditure reflected the costs of visiting the observed number of flower visits per foraging cycle, whilst C_1 reflected the TAL estimated costs of foraging to the SH model predictions of optimum number of flower visits. As there was a significant difference between the observed and predicted number of flower visits (Table 5.2.4.), it was not possible to compare directly C_1 and C_{DLW}. As a result, the accuracy of TAL estimates was determined by comparing C_{DLW} with C_2 (Table 5.2.5.). Although the differences between C_2 and C_{DLW} were greater than those observed between C_{DLW} and C_1 ($\bar{X}$ +5.9±5.2 J, ranging from +0.5 to +11.8), significant differences between C_2 and C_{DLW} estimates were only observed in bees 3 and 7 (Table 5.2.3.). However, when comparing the variation in all bees, a significant difference between C_2 and C_{DLW} was observed ($G_5 = 17.84$, $p<0.01$). The cause of the variation between these measurements was unclear, as there was no correlation between differences in estimates of costs and body mass ($r_3 = 0.45$, ns) or number of flower visits ($r_3 = 0.59$, ns).

To determine whether the bees were maximizing NEE, C_{DLW} was compared with C_3 (Table 5.2.5). C_3 estimates of costs were consistently higher than DLW estimates for all bees, with a mean over-estimate of +36.6±66.6 J, ranging from +1.2 J to +80.7 J. Except for bee 2, C_3 overestimates were significant for all bees (Table 5.2.3.), with the combined differences between C_{DLW} and C_3 for all bees also being highly significant ($G_5 = 194.75$, $p<0.001$).

Table 5.2.4. Variation between the observed number of flower visits per foraging cycle, and the number of flower visits predicted by the SH model if *B.terrestris* were to maximise NEE.

Bee ID	Mean observed number of flower visits per foraging cycle (mean of 8 foraging cycles*) (±sd)	Number of flower visits per foraging cycle predicted by the SH model if *B.terrestris* were to maximize NEE	Analysis of the difference between observed and predicted number of flower visits.
			Paired t-test (df = 14)
			t-stat P
1	14.4±1.7	8	10.70 p<0.001 **
2	13.3±1.8	11	3.47 p<0.004 *
3	28.5±2.2	7	27.59 p<0.001 **
5	19.9±7.5	4	6.01 p<0.001 **
6	19.2±1.7	8	19.06 p<0.001 **
7	11.3±1.7	3	13.98 p<0.001 **

* = Significant difference between observed and predicted number of flower visits.
** = Highly significant difference between observed and predicted number of flower visits.
* The number of observed flower visits was calculated using the final 4 cycles prior to injection and the final 4 four cycles following injection.

Table 5.2.5. Estimates of Costs and Gains of *B.terrestris* during one foraging cycle.

Bee ID	DLW measurements of total energy expenditure ($J\ gm^{-1}\ h^{-1}$)	DLW measurements of energy expenditure during one foraging trip (J), (Costs).	SH model predictions of energy expenditure during one foraging trip when maximizing NEE (J), (Costs). (C_1)	SH model predictions of energy expenditure if predicted number of flower visits per foraging cycle was altered to equal observed number of flower visits (J) (C_2)	Energy expenditure required for the SH model to predict the observed number of flower visits per foraging cycle if the bees were to maximize NEE (J). (C_3)	Energy value of nectar collected on each foraging trip (J), (gains).
1	408	3.2	1.9	3.7	11.4	192.4
2	198	2.3	2.6	2.8	3.6	153.9
3	380	3.9	1.8	15.7	83.6	292.3
6	222	3.7	3.1	10.5	36.7	277.6
7	256	2.8	1.5	12.5	63.5	229.6

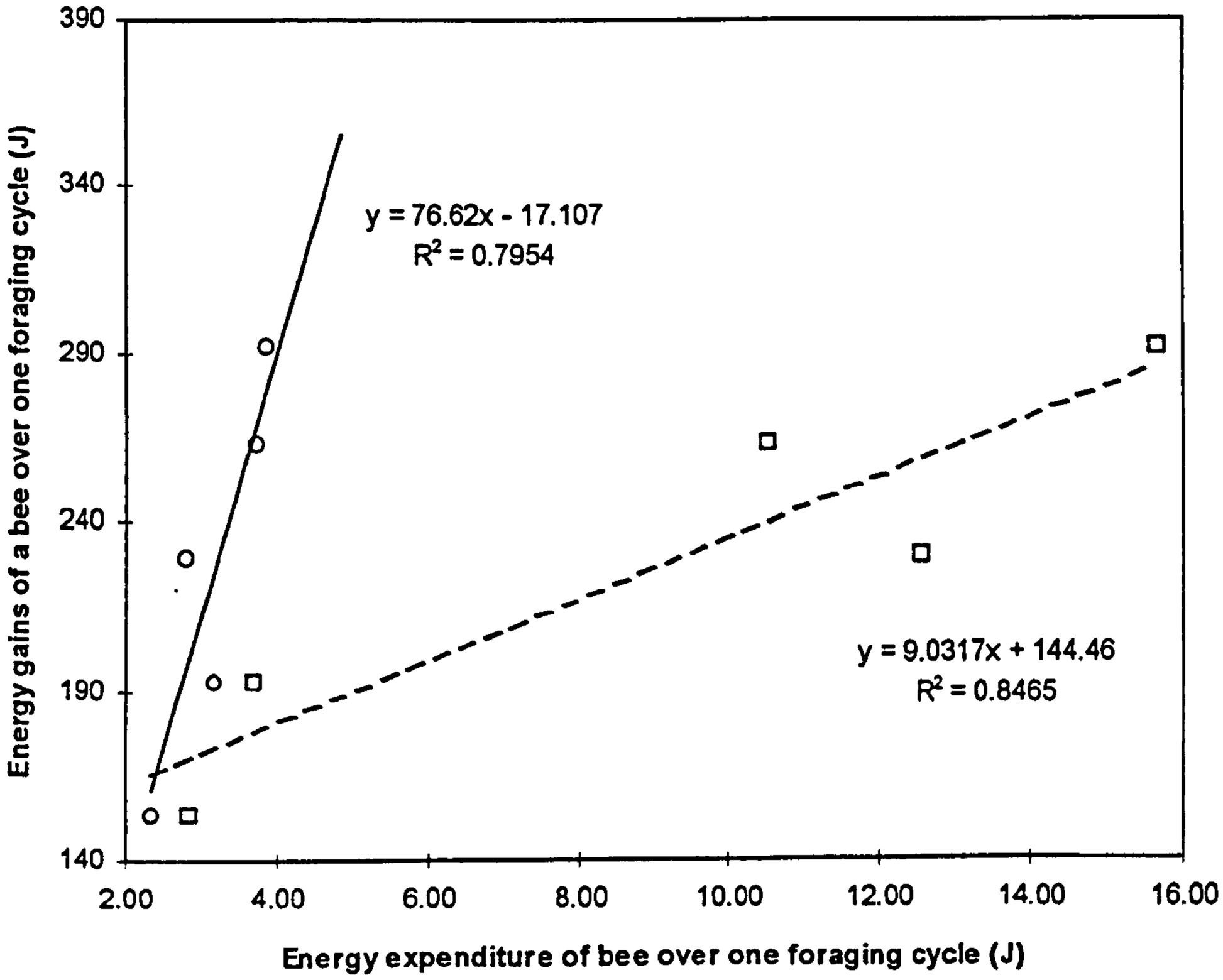
Mass of the bees can be observed in Table 5.2.1, page 202.

Although there was a significant difference between C_3 and C_{DLW}, significant relationships were observed between the costs and gains of the foraging cycle. In particular, a significant correlation was observed between C_{DLW} and observed mean gains per foraging cycle ($r_3 = 0.89$, $p<0.05$) (Figure 5.2.1.). A significant correlation was also observed between C_2 and the observed gains ($r_3 = 0.89$, $p<0.02$) (Figure 5.2.1), although no significant correlations were observed between C_1 and observed gains ($r_3 = 0.49$, ns) and C_3 and observed gains ($r_3 = 0.82$, ns). It should, however, be noted that a significant correlation was observed between C_{DLW} and number of flower visits ($r_{s\ 5} = 0.988$, $p <0.05$). As gains are linked to number of flower visits it was possible that any observed correlation between foraging costs and gains was simply due to the relationship between costs and number of flower visits, rather than an optimal foraging strategy by the bee.

5.3.0. Discussion

"Observation is likely to alter the properties and behaviour of the subject under study" (Capra, 1985). This statement is particularly true in the current experiment, where bees were not only passively observed whilst foraging, but also captured, restrained, injected with heavy water, and plasma samples removed from their haemocoel. However, even following this intrusive procedure, only bee 5 was observed to significantly alter her foraging behaviour during DLW measurements. It was, therefore, clear that DLW

Figure 5.2.1. Relationship between energy costs and gains of foraging bees over one foraging cycle.



□ = Predicted costs *vs* Observed Gains.
○ = Observed Costs *vs* Observed Gains.
-------- Linear regression, x = predicted costs, y = observed gains.
———— Linear regression, x = observed costs, y = observed gains.

estimates of energy expenditure can be obtained without significantly altering the behaviour of most *B.terrestris*. It should, however, be noted that future attempts to use the DLW protocol described in this study should also use careful controls in order to monitor any behavioural effects the procedure may have on the subjects.

As observed in the previous field experiment (Section 2.0.0), the SH model proved a poor predictor of *B.terrestris* foraging behaviour, with model estimates of the number of flower visits again being significantly lower than field observations. It was possible that this discrepancy was due to errors in the TAL estimates of energy expenditure used in the model, resulting in model estimates not reflecting the costs incurred by the bee in the field. This hypothesis was supported when comparing (C_2) and C_{DLW} measurements of foraging costs. Overall, a significant difference between the two estimates of energy expenditure was found, with C_2 estimates being consistently higher than C_{DLW} measurements. Assuming the C_{DLW} values are reliable, the difference between the two measures of energy expenditure indicate significant errors in TAL estimates of foraging costs. As SH model predictions of optimal number of flower visits depend on accurate estimates of foraging costs, the observed errors in TAL estimates will also have resulted in errors in model predictions of flower visiting behaviour. It was, therefore, possible that errors in cost estimates by the model were the source of the difference between observed and predicted foraging behaviour. As a result, TAL foraging costs were removed from the SH model, and replaced by

C_3. If the foraging bees were maximizing NEE, therefore, C_3 costs should not be significantly different from the observed C_{DLW} (assuming the C_{DLW} results were accurate). However, large differences between the two measures of energy expenditure were again found, with C_3 being significantly greater than C_{DLW} measurements in all but one bee. As a result, four possible conclusions may be drawn:

1. DLW field estimates of energy expenditure were inaccurate.

2. The design of the field experiment caused the bees to forage in a sub optimal manner.

3. C_3 overestimates of energy expenditure were due to foraging costs which could not be quantified by the DLW technique, *i.e.* non-fuel costs.

4. *B.terrestris* do not maximize NEE whilst foraging for nectar.

5.3.1.

1) Were DLW field estimates of energy expenditure reliable?

Although C_{DLW} estimates proved accurate during laboratory validations (Section 4.2.0), it would be incorrect to assume that the precision of field and laboratory DLW estimates were equal. In particular, it is important to consider what effect different environmental and behavioural conditions had

on the accuracy of DLW measurements when using tethered bees in the laboratory or free flying foragers in the field.

5.3.2. What effect did environmental conditions have on the accuracy of DLW measurements of metabolic rates?

During the validation experiment, bees were placed in a 2.5L volume flight chamber, through which H_2O and CO_2 free air was passed. As a result, the bees absorbed minimal quantities of exogenous H_2O and CO_2 during the validation experiment. As addition of exogenous H_2O and CO_2 to the bees body water pool increases the apparent turnover rates of the D and ^{18}O isotopes, potentially, high levels of ambient CO_2 (≥3.4%), combined with low humidity (3.8 mg H_2O/L air), could result in the apparent ^{18}O turnover rates increasing relative to D, resulting in large errors in DLW estimates of rCO_2 (errors of up to +81%) (Nagy, 1980; Tatner & Bryant, 1989) Due to the use of H_2O and CO_2 free air during validation, errors in rCO_2 estimates due to low humidity and high carbon dioxide levels will have been minimal Similarly, it is unlikely that H_2O and CO_2 levels encountered in the field will have approached the levels required to produce significant errors in rCO_2. This, however, may not have been the case during the time the bees were in the hive, where CO_2 levels may have been as high as 4.3% (Seeley, 1974). Despite high CO_2 levels, the humidity within the hive was also likely to have been high due to water evaporating from collected nectar in the honey pots. Nest ventilation by worker castes will have reduced the humidity, although it

is unlikely to have reached the levels described by Nagy (1980) (Wolf *pers. com.*). The combined effect of high carbon dioxide and humidity on the apparent isotope turnover rates within the bees body water pool was unclear, depending largely on the rates at which CO_2 and H_2O were absorbed by the bee. However, as CO_2 concentrations were likely to have been higher in the bees haemolymph than in the hives atmosphere (Chapman, 1985), the CO_2 gradient from the bee to hive will have limited the amount of carbon dioxide which the bees will have absorbed. The effect of CO_2 absorption will also have been limited by the length of time the bee spent within the hive, accounting for only ≈20% of the total foraging time of all bees (*pers. obs.*). Although the high humidity in the hive may have increased the apparent isotope turnover rates, it will not have altered the ratio between the ^{18}O and D isotopes, having increased the fractional turnover rates of ^{18}O and D isotopes equally. As a result, the effect of high humidity on the precision of DLW measurements will have been minimal.

Although caution must be taken when extrapolating the DLW precision from the validation experiment to the current field study, there is little evidence that differences in environmental conditions or behavioural traits of the bees significantly altered the accuracy of DLW rCO_2 measurements in the field experiments. It was, therefore, unlikely that errors in DLW measurements of energy expenditure can explain the inability of the SH model to predict the behaviour of *B.terrestris*.

5.3.3

2) Was the foraging behaviour of the bees affected by the experimental design of the artificial flower patch?

The experimental design resulted in three behavioural traits, which were counterintuitive to the behaviour that might by expected of a bumblebee foraging in a natural flower patch, These were:

i) Bees foraged from only one flower type (the artificial flowers).

ii) The bees were trained to revisit continually the same flowers during one foraging cycle.

iii) The nectar volume and sugar content encountered by the bees, on each flower visit, remained constant with time and number of flower visits (non-depleting).

i) Was the bees natural foraging behaviour affected by restricting the bees to forage from one floral type?

Although it may appear reasonable to assume that bees would visit many floral types in one foraging cycle, such behaviour is rarely observed[3] in the field (Heinrich, 1979a). It is well documented that bumblebees display flower

[3] Bumblebees have been observed to visit numerous flower types, although this is generally restricted to naive foragers or as a result of competition (Heinrich, 1979a).

consistency when foraging, normally visiting only one or two floral types, even when more rewarding flowers are available (Darwin, 1859; Heinrich, 1979a; Woodward & Laverty, 1992; Dukas & Real, 1993a; Laverty, 1993). The explanation for this behaviour appears to be linked to the bees poor ability to learn how to collect nectar from different floral types. As different flowers require different handling skills, the foragers limited memory restricts the number of floral types the bee is capable of learning how to forage from (Laverty, 1980; Dukas & Real, 1993b). It has also been shown that the handling skills learned from one flower type can also interfere with the skills learned from another flower type (Darwin's interference hypothesis) (Darwin, 1859; Woodward & Laverty, 1992).

As a flower constant forager will visit only a small number of floral types, the quantity and sugar content of the nectar encountered on each flower visit will be more predictable than when visiting multiple flower types. The handling costs incurred by the forager when visiting a small number of flower types will also be more constant. Thus, a forager is more likely to be able to "predict" foraging costs and gains when foraging in a flower constant manner. The ability of the forager to "predict" costs and gains is essential if the bee is to follow any energy-based rule of optimal foraging. It was, therefore, highly unlikely that the use of one floral type, in the current experiment, will have significantly affected the foraging behaviour of the bees.

ii) Did training bees to visit the same flowers repeatedly during one foraging cycle effect the bees natural foraging behaviour ?

Bees have been observed to revisit regularly rewarding flowers. Corbet, *et al.*, (1984) observed a modal revisit time for *Bombus* and *Apis* of only 3.25 minutes. Similar observations of bumblebees revisiting flowers in field situations have also been reported by Heinrich, 1979a; Dukas & Real, 1993a and Dreisig, 1995. It is, therefore, unlikely that training bees to revisit flowers will have significantly affected their foraging behaviour.

iii) Did providing bees with a non-depleting flower patch result in the bees altering their natural foraging behaviour ?

A non-depleting flower patch can be simply defined as: *a flower patch which will provide a constant supply of nectar, the sugar concentration and volume of which will not decrease over time or number of flower visits.* Although a bee will not encounter a natural flower patch which provides a truly *ad libitum* supply of nectar, it is possible that a worker may forage from flower patches which would, from the bees perspective, appear to conform to the above description. Such a situation would occur in a patch containing flowers of the same species, where the total nectar content exceeded the maximum quantity of nectar which could be collected by a forager over one cycle. As the flowers in the patch are of the same species, it is possible that nectar volume and sugar concentration would be similar on every flower visit. This would

mean that the forager would not encounter a decrease in nectar quality with each subsequent flower visit, providing the bee did not revisit flowers which it, or other bees had recently emptied. As this can be avoided by the foragers scent marking flowers containing no nectar (Cameron, 1981; Corbet *et al.,* 1984; Bertsch, 1990), foraging bees would not encounter a decrease in gains, or increase in costs, with number of flower visits or time. It should, however, be noted that the volume and sugar concentration of nectar in natural flowers does vary with time of day and ambient temperature (Willmer, 1988), as does the foraging behaviour of nectar feeding insects (Willmer, 1983). Under the current experimental methodology, no variation in nectar sucrose volume or concentration occurred with changes in temperature, humidity or time of day. This may explain the apparent insensitivity of the bees foraging behaviour to changes in ambient weather conditions. This, however, does not detract from the fact that bees may regularly forage at flower patches which would, from the bees perspective, appear none depleting. It would, therefore, appear unlikely that a bees behaviour would adversely be affected by the similar conditions encountered in the current experiment.

It is, thus, unlikely that there were any significant flaws in the current experimental design which could explain the inability of the SH model to predict the observed behaviour of the bees.

5.3.4.

3) Can the variation between C_{DLW} and C_3 be explained by the omission of non-fuel costs from the SH model of Net Energy Efficiency?

When comparing C_3 with DLW measurements of energy expenditure, C_3 consistently overestimated C_{DLW} costs. As there are costs involved in foraging which do not directly involve energy expenditure (non-fuel costs) (Seeley, 1986), these costs may affect the foraging decisions of the bee, but will not be measurable by the DLW technique. It is, therefore, possible that the bees in the current experiment were maximizing NEE whilst foraging, but non-fuel costs resulted in C_3 significantly overestimating C_{DLW}.

As discussed in Section 2.3.3, there are two main sources of non-fuel costs; i) depreciation costs of decreased life span due to foraging effort, and i) the costs of predator avoidance.

5.3.5. Do bumblebees include the non-fuel costs of decreased life span due to foraging effort, when determining Net Energy Efficiency

It has been shown that as the foraging effort of a bee increases, so the life expectancy of the bee decreases (Neukirch, 1982; Schmid-Hempel & Wolf, 1988; Cartar, 1992). Beauchamp (1992) produced a model which indicated that longevity of worker caste honeybees directly affected the fitness of the bees colony. Beauchamp's model indicated that colonies whose workers

maximized their life span, had larger worker populations at the end of a foraging season than colonies whose workers maximized their work loads (thus decreasing their life span). A large worker population in honeybees is a distinct advantage, as it permits more efficient temperature regulation of the hive during the winter (Seeley, 1985), and also permits foraging to commence immediately when weather conditions improve in the spring (Beauchamp, 1992). As a result, it is reasonable to assume that the non-fuel costs, to a foraging honeybee, resulting from decreased life span are high.

Although it may be true that Beauchamp's (1992) assertion that maximizing worker longevity increases colony fitness in honeybees, the benefits of this strategy are less obvious for bumblebees. This is largely due to the differences in over-wintering strategies between the two species Unlike honeybees, bumblebee colonies survive for only one year, with all workers and drones dying at the end of every foraging season and only fertilized queens surviving the winter through hibernation (Heinrich, 1979a) As a result, a large colony worker population at the end of a foraging season would be of no obvious benefit to the fitness of a bumblebee colony. Instead, the fitness of the colony is based on two other factors; i) the number of fertilized queens surviving the winter and establishing new colonies in the spring, and ii) the number of drones which successfully mate with queens from other colonies. It is, therefore, logical to assume that a colony wishing to maximize fitness would attempt to maximize the number of reproductives reared. As the amount of nectar and pollen required to raise reproductive

queen larvae is over four times that required to raise a worker larva[4] (Allen, Cameron, McGinley & Heinrich, 1978), the number of foragers in the colony must also increase in order to supply the reproductive brood with sufficient pollen and nectar for growth. Unlike honeybees, however, it would be most beneficial for bumblebees colonies to maximize worker numbers immediately prior to and during reproductive rearing, with numbers of workers decreasing as the colony nears the end of its cycle. A pattern similar to this was found by Allen *et al.* (1978), when studying a colony of *B.vosnesenskii.* The number of worker castes was found to peak at the same time as numbers of reproductive larvae. Also, the number of workers decreased as reproductive larvae hatched, and subordinates queens were then observed to assist in collecting nectar, although no subordinates were observed tending the brood. This kind of activity would tend to suggest that colony population size of workers is important, but at an earlier stage in the life cycle of the colony than required in honeybees.

As colony population size may be an important selective pressure, a high non-fuel cost may be incurred by foragers due to decreased longevity, resulting in limiting maximum colony worker numbers. However, foragers must expend energy in order to collect nectar and pollen to rear reproductive brood. The foragers must, therefore, optimize their behaviour to collect the maximum quantity of nectar and pollen whilst minimizing the costs of

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[4] Allen *et al.* (1978) reported that to raise a queen larvae 430 mg pollen and 200 mg of sugar were required, where-as only 100 mg of pollen and 43 mg of nectar were required to rear a worker.

decreased life span. Cartar (1992) suggested that this would result in bumblebees adopting "a currency that maximizes the ratio of net benefits to costs", *i.e.* NEE. Cartar's assertion may be correct, but as none of the current NEE models include the non-fuel costs of decreased life span, it is not possible to test this hypothesis on field observations of foraging behaviour.

5.3.6. Do bumblebees include the non-fuel costs of predator avoidance when maximizing Net Energy Efficiency

Predator[5] avoidance is often cited as a major consideration in the foraging behaviour of organisms (Abrams, 1994). As discussed previously (Section 2.3.3.), this may also be true of bumblebees. However, it is far from clear what effect such sensitivity would have on the foraging behaviour of the bees in the current study. As the greatest risk of predation occurs when a forager leaves the hive, it would be reasonable to assume that a predator sensitive bee would maximize time spent in the hive whilst minimizing foraging time (Alford, 1975). The bee, however, must also balance the gains of decreased predator risk with the costs of decreased nectar and pollen inputs to the hive. For such a strategy to be effective a bee should optimize time spent, i) in the hive, and ii) foraging, in order to acquire the maximum net gains over the forager's lifetime. Such a strategy would necessitate that the forager had some method of estimating the risk of predation, allowing the bee to alter hive and foraging times appropriately. It is, however, unlikely that a bumblebee is

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[5] The term predation, when used in the current context, also includes parasitization.

capable of acquiring sufficient information about predation risk in order to make any avoidance behaviour efficient (Alford, 1975). As a result, it may be more costly, in terms of nectar input to the hive, for a bee to decrease foraging effort than would be gained from a decreased risk of predation. There has also, to the best of my knowledge, been no published accounts of bumblebees altering their foraging behaviour as a result of predation risk. Anti-predatory strategies may also be counter productive, as is demonstrated by the reluctance of temperate honeybee and bumblebees to swarm and attack a predator en mass (Winston, 1991). This is because after a colony has swarmed, the hive is always abandoned (Seeley, 1985). For honeybees this will result in all collected nectar and pollen being lost, requiring new stores to be gathered prior to over-wintering. If the colony swarms close to the onset of winter, it is unlikely that sufficient stores can be collected prior to the end of the foraging season, resulting in the death of the colony (Winston, 1991). Tropical bees, however, do not have the seasonal constraints imposed on temperate bees, greatly decreasing the probability of the colony dying following a swarm (Seeley, Seeley & Akratanakul, 1982). As a result, many tropical species of honeybee are significantly more aggressive than temperate bees, displaying a high propensity to swarming (Seeley, 1985). Unlike honeybees, a bumblebee colony is not capable of re-establishing a colony following swarming, thus a colony which has swarmed will always die (Heinrich, 1979a). If swarming occurs towards the end of a season it is possible that sufficient reproductives will have been produced to ensure that

the genes of a colony will be carried onto the next generation. However, if reproductives have not been produced the colony, and its lineage, will die.

It is, therefore, clear that predatory avoidance strategies may be so costly to a colony that they are rarely employed. However, more work is required in this area, and it is feasible that some non-fuel costs were incurred by foragers in the current study. It would be possible to include an arbitrary value of none fuel costs into the SH model, as was carried out by Seeley (1986), to increase the NEE predicted costs of the foragers to equal DLW measurements. However, as this figure would be arbitrary and difficult to empirically test, any correlation between NEE model predictions and observed behaviour of the bees would be highly dubious (as discussed by Pierce & Ollason, 1987).

However, as honeybees are also subject to the same non-fuel costs as bumblebees (Beachamp, 1992) any discrepancies between observed and predicted foraging behaviour, resulting from non-fuel costs in bumblebees, should also be observed in honeybees. This, however, is generally not the case, with Schmid-Hempel *et al.* (1985) and Wolf & Schmid-Hempel (1990) finding no significant difference between observed and predicted foraging behaviour of honeybees (using models which did not include non-fuel costs). It would, therefore, appear that there is little evidence to support the theory that non-fuel costs account for the difference between DLW and predicted estimates of energy expenditure.

5.3.7.

4) Do *B.terrestris* maximize Net Energy Efficiency when foraging for nectar

The significant difference between DLW and predicted estimates of foraging costs, combined with the lack of any obvious explanation for the variation, strongly indicates that *B.terrestris* do not maximize NEE when foraging for nectar. This was unexpected, due to reports of honeybees conforming well to NEE models (Schmid-Hempel *et al.*, 1985, Wolf & Schmid-Hempel, 1990), and similarity in physiology and behaviour between honey and bumblebees.

There is, however, a number of important differences between honey and bumblebees, particularly the honeybees ability to communicate the location, quantity and energy content of nectar within a flower patch to one another (Frisch, 1967; Seeley, 1985, Gould & Gould, 1988, Winston, 1991). As a honeybee forager can obtain this information prior to leaving the hive, the forager will have some knowledge about the costs and gains it is likely to incur during a foraging cycle. This information could thus be used by the bee to help maximize its efficiency when in the patch.

Unlike honeybees, bumblebees are unable to communicate patch information to one another. Individual foragers have instead to rely on past flower visiting experience and possibly pheromone marking to determine the energetic costs and gains they may experience when foraging in a patch (Section 1.0.1).

However, it is clear that bumblebees have a limited ability to memorize previous flower rewards and foraging costs (Heinrich, 1976a; Laverty, 1980; Woodward & Laverty, 1992, Greggers & Menzel, 1993; Dukas & Real, 1993b). Also, the sucrose content and volume of nectar within natural flowers is highly variable, even within plants of the same species (Heinrich, 1979a; Pleasants & Zimmerman, 1979; Taubert & Barnes, 1979; Willmer, 1983; Zimmerman, 1981, 1983; Willmer, 1986; Real & Rathicke, 1988; Willmer, 1988; Creswell, 1990, Waser & Mitchel, 1990; Dukas & Real, 1993a). As a result, a foraging bumblebee will derive only a limited amount of information about the energetic costs and gains of foraging at any given patch from previous experience alone. Even if flowers within a patch have been scent marked, this will only indicate whether a flower is potentially rewarding or unrewarding, again providing no detailed information on the costs and gains of foraging at that flower. Other visual or chemical cues, used by bumblebees to estimate the prospective rewards which may be gained from foraging at a flower (Section 1.0.1), provide no information about the costs a forager will incur during nectar collection. It is, therefore, clear that bumblebees have only a limited perception of the costs and gains they will encounter when gathering nectar in a natural flower patch. This would make it unlikely that bumblebees had sufficient information to determine when, or if, they had maximized NEE. Although this situation can, to a certain extent, be ameliorated by flower consistent foraging, the lack of any knowledge of total available nectar in a patch would still limit the information required by bumblebee foragers required to maximize NEE. It is, therefore,

difficult to understand how an optimal foraging currency, dependent on accurate knowledge of foraging costs and gains, could have evolved when bumblebees are not able to obtain the information necessary to maximize NEE. Instead, it would appear more likely that natural selection will have acted on simple foraging variables which bumblebees were capable of quantifying.

5.3.8. If *B.terrestris* do not maximize Net Energy Efficiency, what currency, if any, do they use to optimize foraging behaviour ?

Although it would appear that bumblebees do not maximize NEE, a significant correlation was observed between the body mass of the forager and the total volume of nectar collected per foraging cycle ($r_{35} = 0.47$, $p<0.005$)[6] (Figure 5.3.1). The relationship between body mass and volume of nectar collected is described in equation 1:

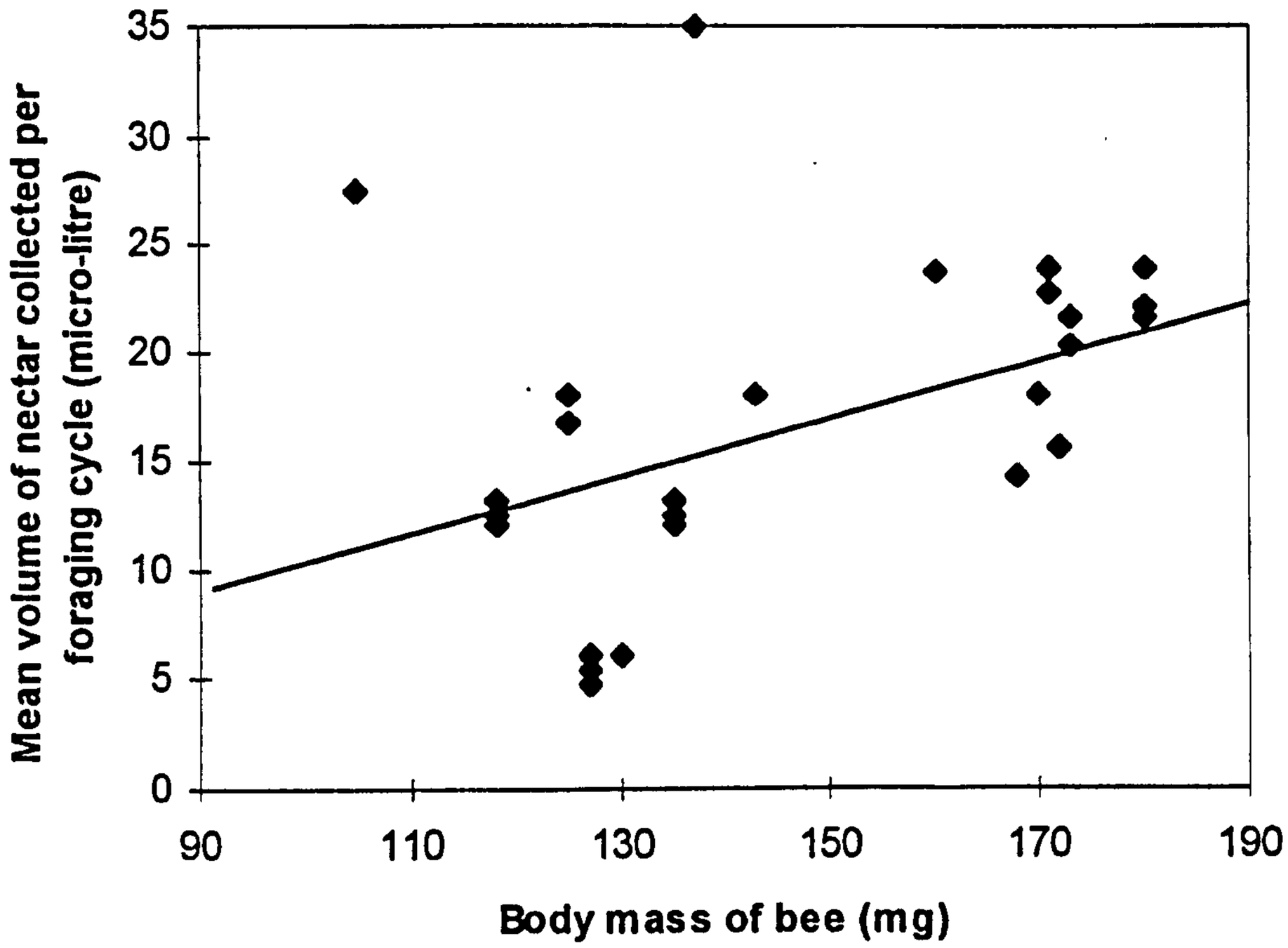
$$Y = 0.13x - 3.2 \ (r^2 = 0.23) \qquad (1)$$

A similar observation has also been reported by Shelly, Buchmann, Villalobos & O'Rouke, (1991) in the desert dwelling bumblebees *B. pennsylvanicus sonorus*, where a significant correlation between body mass and volume of nectar loads was observed (Spearman rank, $P<0.05$). It is, therefore, possible that such correlations indicate that bumblebees follow a

[6] The correlation analysis was carried out on the combined results obtained from the current experiment and the foraging experiment described in Section 2.0.0.

Figure 5.3.1.

The relationship the body mass of *B.terrestris* and the volume of nectar collected on each foraging cycle



simple volume threshold rule, collecting a given volume of nectar relative to their body mass, and abandoning the flower patch once this volume is reached. As a result, the bees do not have to make a cognitive decision as to when the patch should be abandoned, but can simply collect nectar until the nectar volume threshold has been reached, or no nectar remains within the patch. Such a strategy negates the need for the bees to obtain accurate information on the potential costs and gains of a given foraging strategy, an action which is likely to be impossible to *B.terrestris.*

Although a volume threshold theory may appear plausible, two major factors have yet to be determined, i) why the bees choose to abandon the patch at a given threshold volume, and ii) what is the evolutionary advantage to *B.terrestris* of following this foraging rule. Both of these questions are beyond the scope of this study. However, there is one current theory which may account for the observed behaviour.

5.3.9. Do *B.terrestris* limit the volume of nectar they collect in order to maintain a constant water balance?

When foraging, bees not only have to consider energetic costs and gains, but also have to take into account other physiological constraints, such as water balance (Bertsch, 1984; Willmer, 1986; Willmer, 1988). To date, only Bertsch (1984) has attempted to directly measure the water turn-over rate of flying bumblebees. Bertsch found that metabolism of sugar during flight

resulted in the production of large amounts of metabolic water (0.6 mg of water per mg of unloaded bee per 24 hr period) in male *B.lucorum*. Excluding water derived from collected nectar (0.6 mg of water per mg of 50% w/w sugar water), Bertsch estimated that the high production of metabolic water resulted in foraging effort being curtailed due to excess water production, rather than desiccation. It was calculated that in order to maintain a constant water balance, a forager would have to void its total body water content with every eight hours of flight. Observations by Bertsch, however, indicate that a foraging bee is only capable of expelling a maximum of 80% of its body water (through urination and evaporative water loss) over this period. This would, therefore, result in an overall increase in body water content of 20%, flooding the haemolymph and adversely affecting the foragers physiology. Thus, to maintain a constant water balance, a forager would have to limit flight effort in order to reduce the production of metabolic water. It is, therefore, possible that a volume threshold rule may be an attempt by a forager to limit work load, and hence metabolic water production, in order to maintain a constant water balance.

However, Bertschs observations have yet to be repeated, and other work appears to contradict many of Bertschs findings. In particular, Willmer (1986) observed the occurrence of water stress in the xerophilic bee *Chalicodoma sicula* whilst foraging for nectar. It was observed that the osmotic concentration of the bees haemolymph was greatly elevated when the forager arrived at a flower, suggesting excessive water loss by the bee whilst flying to

the patch. The osmotic concentration of haemolymph subsequently returned to normal after nectar within the flower had been imbibed. This would appear to suggest that water within the nectar was passing across the crop membrane, and entering the haemocoel, thus re-hydrating the forager (Willmer, 1986). Willmer also observed that *C. sicula* preferred to forage at flowers with more dilute nectar situated in areas of higher humidity. This would tend to suggest that far from producing excessive metabolic water, the foragers were under water stress, and selectively foraged to patches which minimized EWL and maximized water intake. It is, therefore, possible that water stress may act as a selective pressure in the co-evolution of flowers and specialist foragers (Willmer, 1988). This was demonstrated by Willmer (1988) when observing the foraging behaviour of two bees, *Xylocopa sulcatipes and X. pubescens*, at *Calotropis procera* (asclepiad). It was noted that *C. procera* varied nectar production throughout the day, producing more dilute nectar during periods when *X. pubescens* was thermally incapable of foraging. The smaller *X. sulcatipes*, however, was able to forage during this period, thus benefiting from the increased water content of the nectar. As increasing the water content of nectar is likely to be costly to *C. procera*, it would appear that this strategy is adaptive, and may stem from co-evolution with *X.sulcatipes* (Willmer, 1988). Conversely, the larger *X. pubescens* displayed profligate urination and was observed tongue lashing, a behaviour normally performed in order to evaporate excess water from imbibed nectar (Bertsch, 1984; Willmer, 1986). Such behaviour would tend to indicate that

the large, none specialized *X. pubescens* was suffering from excess water production (Willmer, 1988), as was found in male *B. lucorum* by Bertsch (1984). It would, therefore, appear likely that relatively large bees, foraging to none specialist flowers, are more likely to suffer from excess water production. Whether the foragers in the current study suffered from excessive water production is far from clear, as *B. terrestris* are relatively small in size compared to *Xylocopa* spp, but are not specialist foragers. It should, however, be noted that profligate urination was observed, normally immediately after imbibing nectar at the artificial flowers, as was tongue lashing. As similar behaviour observed in *X. pubescens*, would tend to suggest excessive water production, the same may also be true of the *B.terrestris* during the current study. As a result, the limited volume intake of nectar may be a strategy to reduce flight work load, thus decreasing metabolic water production, and to decrease the volume of water imbibed with the nectar. However, the evidence for this assumption is largely anecdotal and requires further investigation.

Conclusion.

DLW measurements of energy expenditure in foraging bees show that the costs required for current models of NEE to predict observed foraging behaviour are significantly higher than those observed. As there is no obvious explanation for the variation between observed and predicted costs, it is highly likely that *B.terrestris* does not maximize NEE when foraging for nectar. Instead, a significant correlation between mass of the bee and volume of total nectar load per foraging cycle was observed. This may indicate that *B.terrestris* follow a simple volume threshold rule when foraging, abandoning a flower patch once a threshold volume of nectar has been collected. However, the rules which determine the threshold volume at which the forager abandons the patch, and the evolutionary advantage of following this rule are unclear.

Future Work

Although the current study has developed and validated a technique for measuring energy expenditure in free ranging *B.terrestris*, energy costs do not appear to be the main consideration of foraging bees. As observations were made of *B.terrestris* foraging at artificial flower patchs, containing a relatively high sucrose content nectar, and situated close to the hive, it would be useful to repeat the experiment under more realistic conditions. This may include increasing hive-patch distance and decreasing nectar sucrose

concentrations. Such experiments would help clarify if *B.terrestris* are universally insensitive to foraging cost or whether insensitive is displayed only when costs are low and gains are high (as in the current study).

It would also be interesting to observe the affect on foraging behaviour of varying nectar rewards of a forager within one cycle *e.g.* changing nectar sucrose concentrations and volumes on subsequent flower visits.

Of particular importance is the need for further validation of the DLW protocol to enable its use in measuring the water turnover rate (water intake, metabolic water production and EWL) of a foraging bee. As has previously been discussed, water balance may be a major factor in the observed foraging behaviour of *B.terrestris* and many other species of bee It would, therefore, be of benefit to directly measure the water balance of a foraging bee, and use manipulative experiments to determine what effect water deficit / excess had on the foraging behaviour of *B.terrestris.*

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