DISSERTATION

BEHAVIORAL ALTERATION IN THE HONEYBEE DUE TO PARASITE-INDUCED

ENERGETIC STRESS

Submitted by

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ABSTRACT

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ENERGETIC STRESS

Parasites are dependent on their hosts for energy and honeybee foragers with their high metabolic demand due to flight are especially prone to an energetic stress when they are infected. The microsporidian gut parasite *Nosema ceranae* is relatively new to the honeybee, *Apis mellifera* and because it is less co-evolved with its new host the virulence from infection can be particularly high. Using a series of feeding and survival experiments, I found that bees infected with *N. ceranae* have a higher appetite and hunger level, and the survival of infected bees is compromised when they are fed with a limited amount of food. However, if fed *ad libitum* the survival of infected individuals is not different from that of uninfected bees, demonstrating that energetic stress is the primary cause of the shortened lifespan observed in infected bees. I then developed a high throughput colorimetric assay to analyze hemolymph sugar levels of individual bees to demonstrate that the parasite mediated energetic stress is expressed as lower trehalose levels in free-flying bees, which suggests that infected bees are not only likely to have a reduced flight capacity but they are also unable to compensate for their lower energetic state.

One of the ways in which the changing energetic state of an individual is predicted to impact its behavior is its sensitivity to risk although this has never been convincingly demonstrated. According to the energy budget rule of Risk Sensitivity

Theory, it is adaptive for an animal to be risk averse when it is on a positive energy budget and be risk prone when it is on a negative budget because the utility of a potential large reward is much higher in the latter case. By constructing an empirical utility curve and conducting choice tests using a Proboscis Extension Response assay in bees that have been variously manipulated with respect to their energy budgets, I comprehensively demonstrated that bees shift between risk averse to risk prone behavior in accordance with the energy budge rule. Even more importantly, I showed that this shift is contingent upon a change in the energy budget as bees maintained on constant high or low energy budgets were found to be risk indifferent. Given that *Nosema* infected bees have been seen to forage precociously and inclement weather, my results suggest that such risky foraging might be a consequence of the lower energetic state of infected foragers.

As these previous results suggest that parasitism, by lowering their energetic state could have a significant influence on how infected bees forage, I decided to test if the energetic state of an individual can regulate its foraging independent of the colony level regulation of foraging. I uncoupled the energetic state of the individual from that of the colony by feeding individual bees with the non-metabolizable sugar sorbose, thereby creating hungry bees in a satiated colony. I found that these energy depleted bees initially compensate for their lower energetic state by being less active within the colony and taking fewer foraging trips, but not by feeding more within the colony. However, with further depletion in their energetic state, these bees increase their foraging frequency showing that foraging is still partly regulated at the individual level even in a eusocial animal such as the honeybee.

My research therefore shows that the energetic stress from a parasite could be a general mechanism that leads to significant behavioral alterations in infected individuals. Since the energetic state of an animal is a fundamental driver of its behavior, such a mechanism underlying behavioral alterations could have a significant impact on the life history of the host and transmission dynamics of a disease. More specifically, these results also suggest that a parasitic infection leading to energy depleted bees going out to forage in a risky manner also provides a plausible mechanism that explains the recent observations of bees disappearing from their colonies.

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her zany personality and her ability to craft a scientific document with creativity which still conveys an important underlying scientific message. I would also like to thank David Stephens for sharing his theoretical insights and having a great sense of humor. I admire him for being so brilliant but yet down to earth at the same time. I appreciate Boris Kondratieff's friendliness which makes me comfortable enough to discuss important issues with him. I will always admire how he is perceived by the local community. I thank him for always having an eye for detail and facilitating my connections that have developed into a working relationship with the local beekeeping community. Last but not least, I would like to thank Arathi Seshadri for all of her support over the years. Even though she was technically not on my committee she played the role of a committee member. She served as a sounding board over the years, gave me useful second opinions throughout my time as a graduate student, and became a valuable part of my experience here at Colorado State University.

Graduate student co-workers played a vital role in mentally supporting me over the years. I would like to thank Craig Feigenbaum the other Masters student in the lab that joined when I first started. He was a great person to talk to about the hurdles of graduate school which were especially prominent when we were mentally fragile in the first few years joining the lab. He was always encouraging me to not get too caught up with work and helped me keep a balance between work and play, so that I did not initially get burnt out from overworking myself in the lab. Jacob Scholl was another Masters student in the lab during my tenure at Colorado State University. We became very good friends while traveling around and seeing much of the United States. We also spent many hours supporting each other during the hardships that come with graduate school. I

appreciate his work ethic that he brought to the lab, especially when helping me with my second chapter and I thank him for pushing me to stand up for myself to fight for what is right.

I feel that the undergraduates in any research lab when it comes to producing quality research are the man, behind the man, behind the man. What I mean by this saying is that the undergraduates are in the lab helping with the experiment day in and day out and without them quality research could not be produced, but at the same time they are twice removed when it comes time to present the research, so it is often that their contributions go unnoticed. I would therefore like to take the time to thank each undergrad that I have worked with while producing quality research that constitutes my dissertation.

I would like to thank Ann Gibbs for introducing me to the lab when I first arrived and showing me the ropes with her cheeriness and her upbeat personality. I appreciated the devotion Jeremy Gendleman exhibited by putting in the work needed to get the project done and without his help the completion of my second chapter of my dissertation would not be possible. He was a pleasure to work with and we became good friends outside of work. I thank Sarah Jaumann for her long term commitment to the lab and her willingness to come in at all hours of the night to assist in collecting bee data for the third chapter of my dissertation. In addition, I really enjoyed the company of Amanda Stammer and Robin Scudelari in the lab and during lab meetings. Amanda's insights on projects and intellectual contributions in lab meetings will be missed and I admire Robin's independence, as she was great at troubleshooting experiments and being resourceful all on her own. I thank Kira Terry because even though she realized research

DEDICATION

For my parents, Lynn Anne Mayack and David Thompson Mayack, who are parents that I am grateful for never pushing me into a certain career direction, but rather just supported my career decisions in whatever I had a passion for.

TABLE OF CONTENTS

CHAPTER 1:

ENERGETIC STRESS IN THE HONEYBEE *APIS MELLIFERA* FROM *NOSEMA*

CERENEA INFECTION

SUMMARY

Parasites are dependent on their hosts for energy to reproduce and can exert a significant nutritional stress on them. Energetic demand placed on the host is especially high in cases where the parasite-host complex is less co-evolved. The higher virulence of the newly discovered honeybee pathogen, *Nosema ceranae*, which causes a higher mortality in its new host *Apis mellifera*, might be based on a similar mechanism. Using Proboscis Extension Response and feeding experiments, we show that bees infected with *N. ceranae* have a higher hunger level that leads to a lower survival. Significantly, we also demonstrate that the survival of infected bees fed *ad libitum* is not different from that of uninfected bees. These results demonstrate that energetic stress is the probable cause of the shortened life span observed in infected bees. We argue that energetic stress can lead to the precocious and risky foraging observed in *Nosema* infected bees and discuss its relevance to colony collapse syndrome. The significance of energetic stress as a general mechanism by which infectious diseases influence host behavior and physiology is discussed.

INTRODUCTION

Parasites typically compete with their hosts for nutrition and exert an energetic stress on them. There are two different mechanisms by which the energetic stress is imposed, the parasite either directly draws energy from the host for its own metabolic needs or the host needs to expend energy for mounting an immunological response, which is known to be an energetically expensive process (Schmid-Hempel, 2005). The energetic stress placed on the host as a result of an infection can compromise the effectiveness of the immune response itself and allow other pathogens to invade the host, setting off a cascading effect. Such severe and continued stress might lead to complex changes in host feeding behavior as they seek to meet this nutritional shortfall (Thompson and Redak, 2008). Some pathogens such as microsporidians are particularly severe on their hosts in terms of exerting an energetic stress because they lack mitochondria and therefore have little metabolic ability themselves (Agnew and Koella, 1997). *Nosema* is a microsporidian pathogen that infects the honeybee gut and is known to cause a suite of metabolic changes in the host (Bailey, 1981). Infected bees are known to have lower levels of protein, resulting in a reduced hypopharengeal gland (Malone and Gatehouse, 1998; Wang and Moeller, 1970; Wang and Moeller, 1971), as well as altered fatty acid composition in the hemolymph (Roberts, 1968). It has been less commonly suggested that *Nosema* also uses carbohydrates from the epithelial cells of the honeybee gut lining (Higes et al., 2007; Liu, 1984). The demand placed on the host with respect to carbohydrate is especially interesting because it is the most fundamental source of energy and bees, due to their high metabolic rates that come with flight (Neukirch, 1982), have a high demand for it. It is also important to note in this context that the foragers, which are

likely to have the highest energetic demand, are also the ones with the highest *Nosema* load (El-Shemy and Pickard, 1989; Higes et al., 2008). The idea that *Nosema* places a substantial energetic demand on the host is supported by the observation that infected bees in cages consumed significantly more sugar–water although the lower oxygen consumption that accompanied it (Moffet and Lawson, 1975) suggests that infected bees are probably not able to utilize the extra carbohydrates. A newly reported *Nosema* species, *Nosema ceranae*, has recently jumped hosts to the European honeybee (Higes et al., 2006) and is currently replacing *Nosema apis* throughout the world (Klee et al., 2007). The observations that *N. ceranae* causes a higher mortality than N. apis in caged bees despite the same pathogen load (Paxton et al., 2007) and that colonies infected with *N. ceranae* die if left untreated (Higes et al., 2008) suggest that the new species possibly has a higher virulence. While this means that *N. ceranae* could cause a particularly severe metabolic stress in its new host, there is little information on its physiological and behavioral effects in infected bees. Therefore, the major motivation for this study was to investigate if *N. ceranae* imposes an energetic demand on its host, causing infected bees to display an increased hunger and a lower survival as a direct consequence of it. We focus our study on the foragers because they are likely to incur the highest energetic stress due to an infection for the reasons discussed above.

MATERIALS AND METHODS

Forager collection

We monitored the *N. ceranae* infection status of two full-sized honeybee colonies in the field by regularly sampling foragers for the microsporidian spores. We collected

returning foragers from these two colonies with a vacuum after placing a wire-mesh screen over the hive entrance and released them into a cage.

Proboscis extension response (PER) experiment

We placed each bee in a glass vial, chilled it on ice until the individual became immobile and strapped her within a 4.5 cm long plastic drinking straw with a small strip of tape on her thorax. Testing began 45 min after the last bee was strapped to allow the bees to get acclimated. The antennae of a strapped bee were touched with a droplet of sucrose and whether she responded by fully extending her proboscis – a Proboscis Extension Response (PER) – was recorded. Each bee was assayed with a concentration series of 0.1%, 0.3%, 1%, 3%, 10%, and 30% sucrose solution by weight and between every two successive concentrations, the antennae were touched with water to control for possible sensitization from repeated stimulation (Bitterman et al., 1983).

Hunger level experiment

Bees were strapped and fed 30% sucrose solution *ad libitum* every 6 h for 24 h and the amount consumed by each bee was recorded at each time point. The bees were kept in an incubator set at 25 °C and 70% RH during the entire period.

Survival experiment

After strapping, the bees were fed once with either 0 μl, 5 μl, 10 μl, 20 μl, 30 μl at the beginning of the experiment, or *ad libitum* and their survival was monitored every 6 h for 24 h. The bees were kept in an incubator similarly as in the previous experiment.

Infection status

After the conclusion of each experiment, the subjects were freeze-killed, their entire gut was removed and homogenized in water and the number of *Nosema* spores in each bee was quantified on a hemacytometer. Infected bees had a spore count of 2.5 x 10^5 or more (some bees had a spore count as high as 2.5 x 10^6 or more). The species of *Nosema* seen was confirmed using the multiplex PCR and electrophoresis method (Martín-Hernandez et al., 2007). Infected bees produced a DNA fragment length in the 218–219 bp range but no fragment lengths in the 312 bp range, indicating that *N. ceranae* was the only *Nosema* species present. None of the two fragment lengths were present in uninfected bees (negative controls).

RESULTS

Proboscis extension response (PER) experiment

Infected bees were significantly more responsive to sucrose than uninfected bees in each colony tested: colony 1 (G test of independence: G = 7.23, N = 228, P = 0.01, Fig. 1a) and colony 2 (G = 16.36, N = 390, P < 0.0001, Fig. 1b), especially at the lower concentrations, indicating that infection with *N. ceranae* increased their appetite. As the difference in response between control and infected bees were consistent between the two colonies, data from them were pooled in the next two experiments.

Hunger level experiment

Infected bees consumed a significantly higher amount of sucrose over the 24 h period tested (repeated measures ANOVA: $F_{1,99}$ = 27.44, P < 0.0001, Fig. 2). The amount

fed by the bees significantly decreased with time ($F_{1,99}$ = 108.80, P < 0.0001) but there was a significant interaction effect ($F_{1,99}$ = 5.96, P = 0.016) indicating that infection not only increases overall hunger but also the rate at which bees starve.

Survival experiment

Survival of bees significantly depended on the amount of food consumed (repeated measures ANOVA: $F_{4,5}$ = 13.25, P = 0.007, Fig. 3a), with almost no bees surviving for more than 24 h when fed with specific amounts of sucrose. Infected bees survived significantly less than uninfected bees (Wilcoxon Signed Rank test: Z = 3.52, N = 20, P < 0.0001) at all given amounts of food but their survival was not significantly different when either fed with nothing or fed until satiation (Wilcoxon Signed Rank test: Z = 1.96, N = 10, P = 0.05, Fig. 3b). Almost all bees survived after 24 h when fed *ad libitum*.

DISCUSSION

The results support our initial hypothesis that the microsporidian N. ceranae imposes an energetic stress on infected bees, revealed in their elevated appetite and hunger level. Our direct measure of hunger determined by the total sucrose consumed definitively shows that infected bees attempt to compensate for the imposed energetic stress by feeding more, which is correlated to their higher appetite as seen by their PER responses. Such pathogen imposed energetic stress might be a general effect of a number of infections since even Deformed Wing Virus was incidentally found to increase the PER response of infected bees (Iqbal and Mueller, 2007). A number of other studies of

6

parasitic associations involving insect hosts have demonstrated alterations in host nutrition (Thompson and Redak, 2008) and increased rates of feeding (Grimstad et al., 1980; Rahman, 1970). Such nutritional interactions between the parasite and the host have a significant effect on insect hosts where the parasite biomass often represents a significant proportion of the host–parasite complex. Parasites are known to influence host feeding by affecting the level of nutrients in the hemolymph (Cloutier, 1986; Cloutier and Mackauer, 1979). Appetite and hunger in hymenopterans is regulated by not only the carbohydrate level in the hemolymph but also by the mechanoreceptors that monitor the volume of the foregut (crop) and midgut (Stoffolano, 1995). Bees infected with *N. apis* have a reduced metabolic efficiency due to the degeneration of the ventricular epithelium and lower secretion of digestive enzymes (Liu, 1984; Malone and Gatehouse, 1998). We also noticed the crops and midguts of infected bees to be somewhat smaller in comparison to those of uninfected ones. This suggests that both the regulatory pathways could be involved in increasing the hunger level in infected bees. The lower survival of infected bees shows that *N. ceranae* has important fitness consequences on its host. From our observation that this decrease is apparent only when infected bees are fed with limited amounts of sucrose, we contend that the lower survival of bees infected with *N. ceranae* is mainly due to the energetic stress imposed upon them by the pathogen. It is remarkable that infected bees survived almost to the same extent as uninfected ones when they were fed with *ad libitum* sucrose. It seems therefore that the lower survival of *Nosema* infected bees observed in a number of other studies (Bailey, 1981; Hassanein, 1953; Higes et al., 2007) is largely due to the impairment of metabolic functions as the reduced longevity cannot be explained by any other pathogenic effects of this infection

7

(Liu, 1984; Muresan et al., 1975). This idea is also consistent with the observation that infected bees show no outward differences from uninfected bees (Bailey, 1981). The energetic stress induced by the newly reported *N. ceranae* is likely to be even higher because it is less co-evolved with the host. It is probably therefore less efficient in its physiological integration in the host–parasite complex (Thompson, 1990) and is required to draw more food from its host due to a lower conversion efficiency. This could explain the lower survival observed for bees infected with *N. ceranae* compared to those with *N. apis* (Paxton et al., 2007). The increased hunger of infected bees might be even larger in a natural setting than what was observed in our data because the bees in our experiment were kept harnessed at an ideal temperature. Active foragers are bound to have a much higher energetic demand given that flight is a metabolically expensive process and that honeybees are synchronous fliers who use only carbohydrates as fuel (Sacktor, 1970). Foragers are likely to burn sugar even faster on cold windy days when simultaneous energetic cost for thermoregulation and flight is the highest (Harrison et al., 2001; Woods et al., 2005). Increase in hunger could have a number of behavioral effects at both the individual and the colony level that have implications for the epidemiology of *Nosema* disease. It could lead to higher trophallactic rates within the colony, potentially increasing the transmission of the pathogen within the colony. An elevated hunger could also increase foraging rates, thus increasing the potential for horizontal transmission of the pathogen via flowers (Colla et al., 2006; Durrer and Schmid-Hempel, 1994). One could also speculate that the precocious foraging observed in *Nosema* infected bees is partly driven by hunger in addition to the physiological changes associated with the atrophy of the hypopharengeal gland (Hassanein, 1953; Wang and Moeller, 1971). If *Nosema*

infected bees are indeed hungrier, the riskier foraging observed for such bees (Woyciechowski and Kozlowski, 1998) could be an outcome of the energy budget rule of Risk Sensitivity Theory (Stephens and Krebs, 1986). It is important to note that in honeybees and other social insects, foraging is regulated not only by colony demand but also by the hunger level of the individuals (Howard and Tschinkel, 1980; Toth et al., 2005). Risk-prone foraging by bees that are already in a lower energetic state due to infection by *N. ceranae* could play a role in the recently observed disappearance of bees from hives because such bees would have a lower likelihood of making it back to the colony. *N. ceranae* has already been found to be a major contributor to the depopulation of colonies (Higes et al., 2007, 2008), the most typical symptom of colony collapse syndrome (Oldroyd, 2007). Nutritional stress imposed on a host by a pathogen, especially by those that are new and are less co-evolved with the host, could be a general mechanism that applies to a number of emerging infections. An understanding of pathophysiological mechanisms and their impact on host behavior can give us important insights into host–parasite interactions.

FIGURES
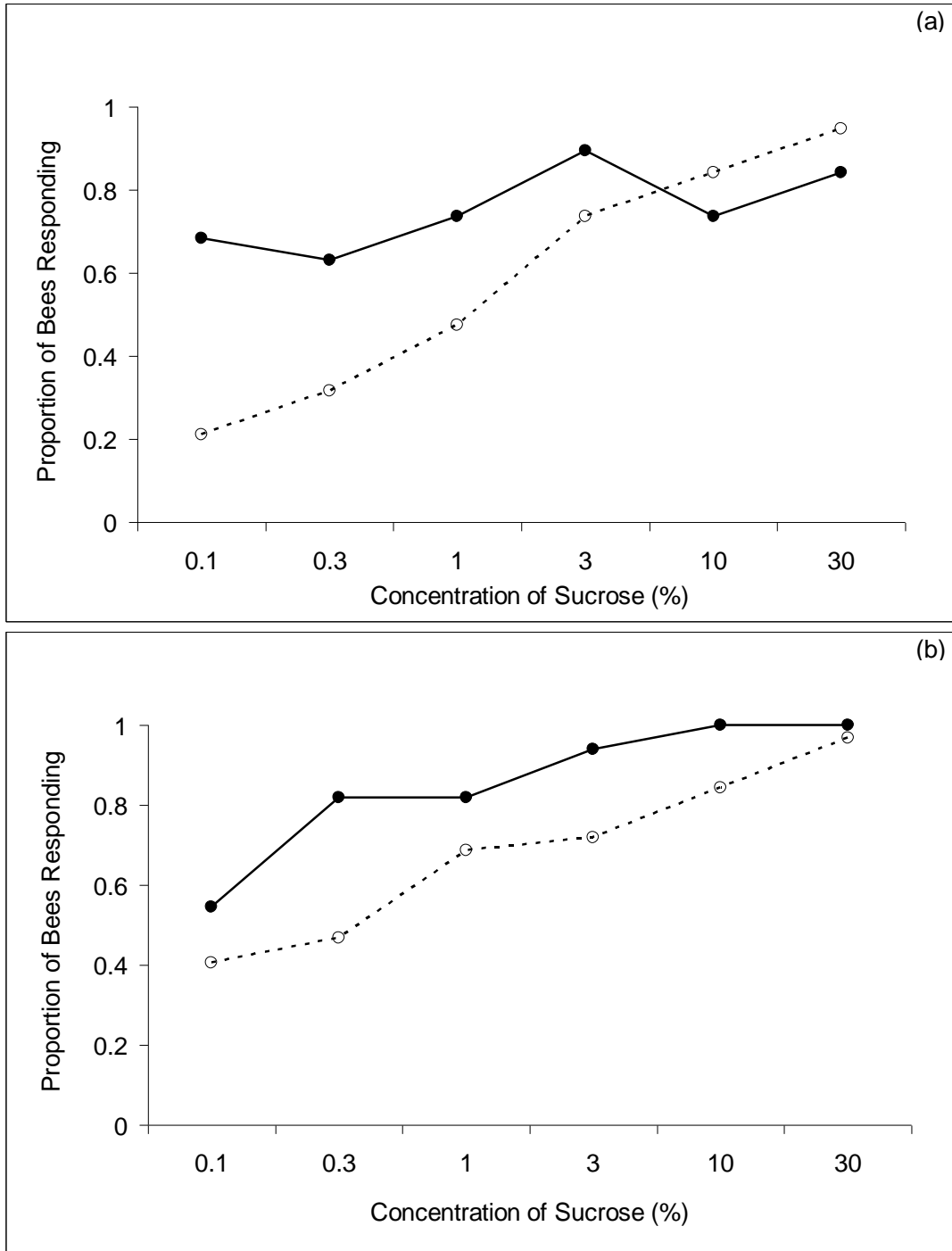
Fig. 1.1. Responsiveness of infected (●) and control (○) bees to sucrose solution of different concentrations in (a) colony 1 (228 antennal probes from 19 control and 19 infected bees) and (b) colony 2 (390 antennal probes from 32 control and 33 infected bees). Proportion of responses is overall higher in colony 2 in comparison to colony 1 but the responsiveness of infected bees is higher than control bees within each colony.
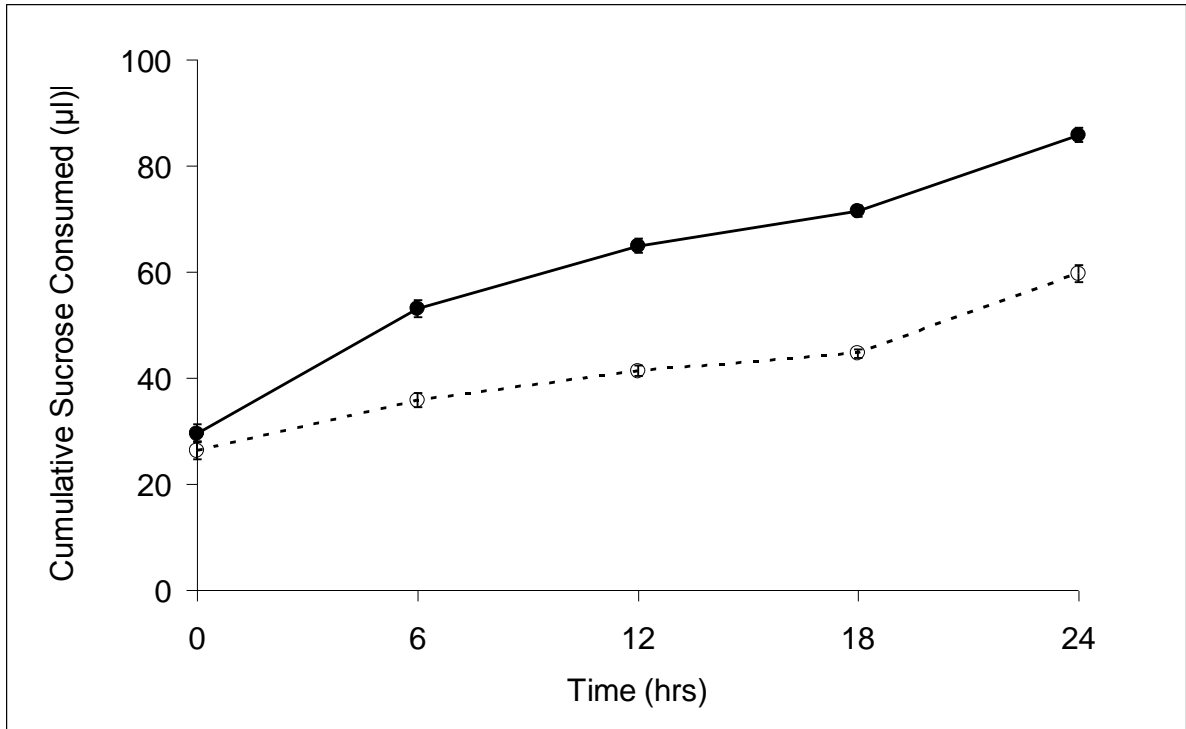
Fig. 1.2. Cumulative consumption of 30% sucrose solution by infected (●) and control (○) bees until satiation, measured every 6 hours for 24 hours. Data represent mean values for infected (N = 57) and control (N = 44) bees with standard error bars.
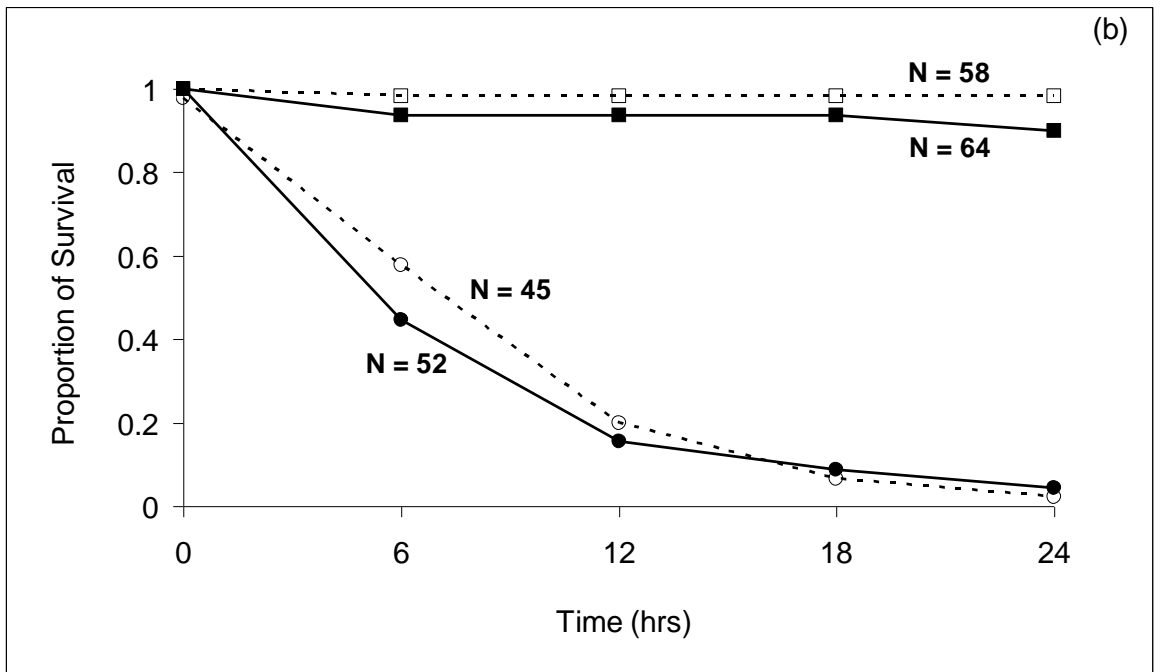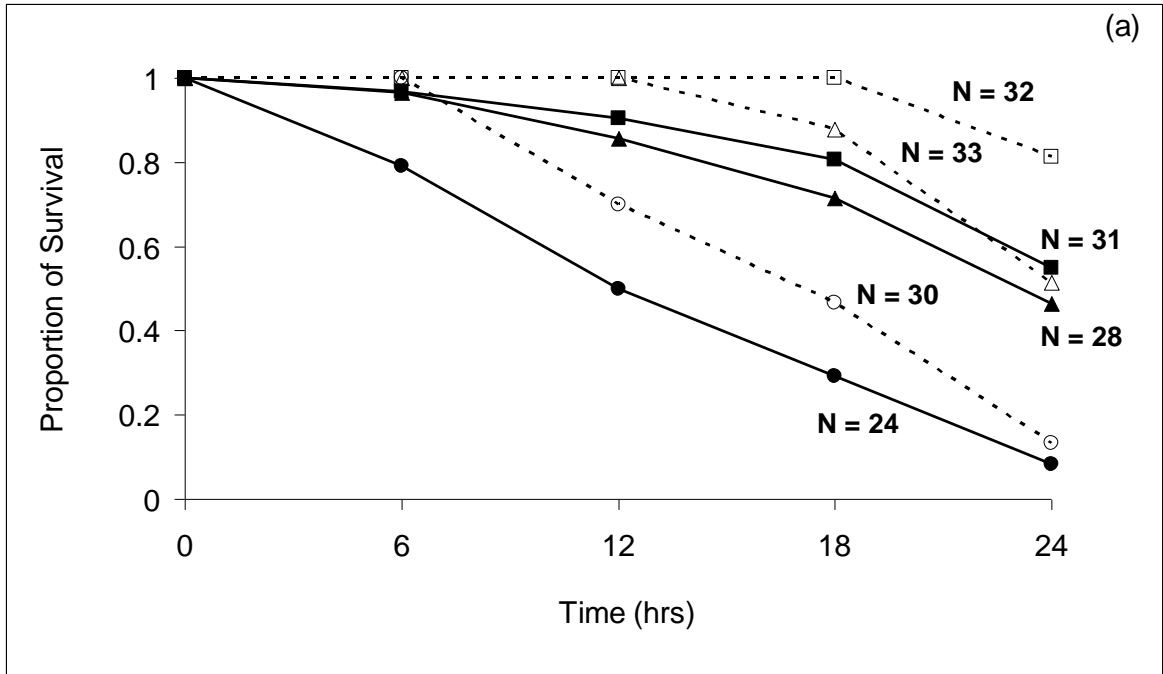
Fig. 1.3. Survival of infected (filled shapes) and control (empty shapes) bees fed with (a) 5 µl (circles), 20 µl (triangles), and 30 µl (squares), and (b) 0 µl (circles) and *ad libitum* (squares), amounts of 30% sucrose solution. The number of bees tested to construct each survival curve is given against each line, the 10 µl amount is not shown for clarity but was included in analysis.

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CHAPTER 2:

PARASITIC INFECTION LEADS TO DECLINE IN HEMOLYMPH SUGAR LEVELS

IN HONEYBEE FORAGERS

SUMMARY

Parasites by drawing nutrition from their hosts can exert an energetic stress on them. Honeybee foragers with their high metabolic demand due to flight are especially prone to such a stress when they are infected. We hypothesized that infection by the microsporidian gut parasite *Nosema ceranae* can lower the hemolymph sugar level of an individual forager and uncouple its energetic state from its normally tight correlation with the colony energetic state. We support our hypothesis by showing that free-flying foragers that are infected have lower trehalose levels than uninfected ones but the two do not differ in their trehalose levels when fed until satiation. The trehalose level of infected bees was also found to decline at a faster rate while their glucose level is maintained at a quantity comparable to uninfected bees. These results suggest that infected foragers have lower flying ability and the intriguing possibility that the carbohydrate levels of an individual bee can act as a modulator of its foraging behavior, independent of social cues such as colony demand for nectar. We discuss the importance of such pathophysiological changes on foraging behavior in the context of the recently observed colony collapses.

INTRODUCTION

Parasites typically draw nourishment from their hosts and can cause a nutritional stress in them, especially when the parasite biomass is significant with respect to that of the host (Holmes and Zohar, 1990). As resources are generally limited, this can lead to a significant effect on the behavior of the host as it attempts to meet this increased demand. Flying insects with their characteristically high energetic demands are more likely to display such behavioral changes due to parasitism. Honeybee foragers, which have some of the highest recorded sugar levels of any insect (Fell, 1990), show evidence of an energetic stress when they are parasitized by the microsporidian *Nosema ceranae* (Campbell et al., 2010; Mayack and Naug, 2009). Lysing the epithelial cells of the midgut (Higes et al., 2007; Liu, 1984), these microsporidians are in an ideal position to draw away the glucose and fructose that are produced from the breakdown of dietary sucrose and as a result reduce the synthesis of trehalose, the principal carbohydrate in insect hemolymph. Carbohydrates form the majority of the adult honeybee diet and power their flight (Candy et al., 1997; Sacktor, 1970). Nevertheless, foraging by individuals is traditionally considered to be a socially regulated behavior with colony demand playing a critical role in modulating it (Seeley, 1995). A few studies have however shown that colony energetic state, defined by the honey storage levels, has no effect on the nectar foraging rates of individual bees (Fewell and Winston, 1996). We suggest that such conflicting observations could result from the possibility that the energetic state of the colony and that of the individual play independent roles in the regulation of foraging activity. While the normally tight correlation between the two in most situations make such contrasts rare and difficult to understand, recent experimental

17

work has shown that the nutritional state of the individuals can be uncoupled from that of the colony and can act independently of social cues in causing bees to forage (Schulz et al., 1998; Toth et al., 2005; Toth and Robinson, 2005). This leads us to suggest that a parasitic infection can influence the foraging behavior of an infected honeybee by causing a reduction in its trehalose level. More fundamentally, energetically stressed infected bees thus provide an opportunity to investigate the possible role of the individual energetic state on the foraging behavior of honeybees by dissociating it from the colony energetic state. With the goals of measuring the energetic stress caused in an infected bee and evaluating the role of energetic stress in honeybee foraging, we compared the trehalose and glucose levels in the hemolymph of free-flying foragers that were uninfected with those infected with *N. ceranae*. In addition, by monitoring the sugar levels in these bees over a period of 24 h, we determined the rate at which *N. ceranae* draws energy from its honeybee host.

MATERIALS AND METHODS

We collected returning foragers from two colonies that had both uninfected bees and bees infected with *N. ceranae* by placing a wire screen to block the entrance of the hive. Hemolymph was extracted from some of these free-flying foragers right after their capture and the rest were strapped inside plastic straws. We fed the strapped bees with 30% sucrose solution until they stopped extending their proboscis to feed. These satiated bees were randomly assigned to one of five groups, 0, 6, 12, 18, or 24 h, based on the time at which hemolymph was going to be extracted from them. Any bee that died before its pre-determined extraction time was not used. The bees were kept in an incubator set at

25 °C and 70% RH for the entire duration of the experiment and up to 40 bees were tested at a time. At the end of the experiment, all bees were dissected and their infection status was determined by counting the number of spores in their guts. Infected foragers were found to contain 2.5×10^4 to 3.4×10^7 spores per bee. The species of *Nosema* was confirmed using the multiplex PCR and electrophoresis method (Martín-Hernández et al., 2007). In infected bees, DNA fragment lengths were only produced in the 218–219 bp range as opposed to the 312 bp range indicating that *N. ceranae* is the only *Nosema* species present, while neither of the two bands was evident in uninfected bees.

Hemolymph extraction

The bees were freeze killed and the guts were removed to assay their infection status. In addition, their mouth parts were glued shut to prevent any possible contamination of the hemolymph sample to be extracted. The distal ends of the antennae were then clipped with scissors and each bee was placed upside down in a centrifuge tube and spun at 16,000 RCF for 30 s. The hemolymph trickled out from the cut ends of the antennae and 2 µl of this hemolymph was diluted with 58 µl of distilled water and the samples were placed in a -20 °C freezer. The extraction process was carried out over ice to prevent any degradation of the sugars.

Glucose and trehalose quantification

The amount of glucose in 5 µl of each diluted sample was quantified using a Quantichrome Glucose Assay Kit (Bioassay Systems, Hayward, CA, USA). Each sample was placed in a well of a 96-well microplate and read by a microplate reader set at 630

nm wavelength for maximum absorbance. A glucose standard curve was constructed for each run and was used to quantify the amount of glucose present. Another 5 μl of the diluted sample was used to quantify trehalose which was broken down into glucose within a microplate well by adding 2.7 μl of trehalase (Sigma–Aldrich, St. Louis, MO, USA) in 9 μl of citrate buffer (pH 5.7). The microplate was placed in the microplate reader, shaken for 5 min and then incubated for 1 h at 37 °C. Trehalose standards were run in the same way in triplicate to make a standard curve. The amount of trehalose was quantified by subtracting the amount of glucose that was previously quantified in the same sample from the total glucose measured after trehalose breakdown.

Statistical analysis

One-way ANOVAs were used to compare the trehalose and glucose levels between infected and uninfected foragers. A two-way ANOVA was used to compare the decline in sugar levels over time in uninfected and infected foragers followed by a post hoc Tukey–Kramer multiple comparison test that compared the decline between different time points within each group. A regression analysis followed by a Tukey–Kramer comparison of slopes (Sokal and Rohlf, 1995) was used to compare the rates of decline of trehalose and glucose within each group.

RESULTS

There was a significantly lower amount of trehalose in the hemolymph of free-flying foragers infected with *N. ceranae* in comparison to uninfected foragers (one-way ANOVA: $F_{1,75} = 6.93$, $P = 0.01$), but the glucose levels in the two groups were similar

($F_{1,75} = 0.01$, P = 0.90, Fig. 1A). When fed to satiation, the infected foragers were not significantly different from uninfected foragers in terms of either their trehalose levels (one-way ANOVA: $F_{1,59} = 1.75$, P = 0.19) or their glucose levels ($F_{1,59} = 0.002$, P = 0.96, Fig. 1B). A two-way ANOVA with infection status and time from satiation as fixed factors showed that infected foragers had significantly lower trehalose levels than uninfected foragers over the entire 24 h period ($F_{1,300} = 20.70$, P < 0.0001). There was a significant interaction effect ($F_{4,300} = 4.40$, P = 0.002, Fig. 2A), indicating that the trehalose levels of infected bees declined at a faster rate in comparison to uninfected bees. However, the glucose levels of infected and uninfected bees were not significantly different over the same period ($F_{1,300} = 0.84$, P = 0.36) and there was no significant interaction with infection ($F_{4,300} = 0.67$, P = 0.61, Fig. 2B). A linear regression analysis followed by a comparison of regression coefficients showed that there was no significant difference between the rates at which trehalose and glucose declined over time within a group, in either uninfected (trehalose: y = -1.20x + 39.88, glucose: y = -1.14x + 32.05, MSD = 4.44, P > 0.05) or infected bees (trehalose: y = -0.72x + 24.91, glucose: y = -1.04x + 29.68, MSD = 2.92, P > 0.05). A multiple comparison across the different time points using the Tukey–Kramer method showed that in uninfected bees the levels of trehalose and glucose are not significantly different in the first 12 h while the amounts of both these sugars were found to start declining during the same period in infected bees.

DISCUSSION

The results of this study showing that infected honeybee foragers have lower trehalose levels lend support to our previous finding that foragers infected with *N.*

ceranae have a higher hunger level than uninfected foragers (Mayack and Naug, 2009). It also shows that infected foragers are not somehow able to compensate for this energetic stress and that a parasitic infection such as *Nosema* can dissociate the energetic state of the individual from that of the colony. It is also important to note that if fed until satiation, the trehalose levels of infected bees are similar to those of uninfected bees, supporting the idea that a critically important pathological effect of *N. ceranae* infection is the energetic stress imposed by the parasite. Our previous study shows that infected foragers survive just as well as uninfected foragers when fed *ad libitum*, indicating that energetic stress is the primary cause of lower survival in infected bees. The finding that both uninfected and infected bees have similar glucose levels despite having different trehalose levels is consistent with the earlier result of Blatt and Roces (2001), who found that glucose levels in the hemolymph are maintained at the expense of trehalose. Under increased metabolic demands, the rate of trehalose synthesis in the fat body cannot keep up with the rate at which it is broken down (Woodring et al., 1994). Unlike the infected bees, uninfected bees which presumably are under lower energetic demand were able to maintain their trehalose levels for the first 12 h after being satiated. This difference cannot be explained by a difference in the crop emptying rates between the two groups because the crop volume in satiated foragers is known to decline to about 7 ml in the very first hour (Roces and Blatt, 1999) and at this rate the crop would be completely empty well before 12 h. The increase in trehalose levels seen in infected foragers after 18 h, although a non-significant trend, can possibly be attributed to the mobilization of glycogen reserves due to the large decline in hemolymph sugar levels by this point. To the best of our knowledge, this is the first study to measure the sugar levels of honeybee

foragers at regular intervals for a 24 h period starting from when they are completely satiated. As the subjects were kept immobilized at a constant and ideal temperature of 25 °C in the laboratory, this is a close approximation of their basal metabolic rate. The noticeably large variability in sugar levels observed in our study therefore suggests that there are intrinsic differences among individuals in their basal metabolic rates. However, the difference in trehalose levels between infected and uninfected foragers may be even greater than what was observed in this study if one controls for variation in the age of the foragers in the sample. Infected bees more likely being older (Higes et al., 2008) would have higher sugar levels on account of their age (Harrison, 1986), thus skewing the infected average a bit to the higher side. The lower trehalose level in bees infected with *N. ceranae* is likely to lead to a lower flying ability. These energetically stressed infected foragers are also most likely to see the additive detrimental effects of increased energetic demand due to their poor thermoregulatory ability (Campbell et al., 2010), propensity to forage on cold windy days (Woyciechowski and Kozlowski, 1998), and heavier body weight (Vance et al., 2009) if they are also precocious (Wang and Moeller, 1970). In our study, the mean trehalose and glucose levels of infected and uninfected foragers were 8.5 mg/ml and 16.98 mg/ml respectively. Using these amounts, the fact that trehalose is made up of two glucose molecules, and the assumption that the level of fructose is similar to that of glucose (Blatt and Roces, 2001), one can approximate the total amount of sugar in the hemolymph. This gives 50.96 mg/ml of glucose an infected forager has, and 75.76 mg/ml of glucose an uninfected forager has, available for flight. Using a metabolic rate of about 700 mW/g at 20 °C or 450 mW/g at a more ideal environmental temperature of 35 °C (Woods et al., 2005), an infected forager can be estimated to have the ability to fly

about only two-thirds the distance compared to an uninfected forager on any given day. It would be interesting to test whether reduced trehalose level is also responsible for causing precocious foraging seen in infected bees, given the fact that lipid depletion has been shown to advance the age at onset of foraging (Toth et al., 2005; Toth and Robinson, 2005). The repercussions of this decreased flying ability are critical considering the rapid decline in area that is suitable as foraging habitat for the honeybees (Naug, 2009). Studies have shown that foragers infected with *N. ceranae* (Higes et al., 2008; Kralj and Fuchs, 2010) or tracheal mites (Harrison et al., 2001) have a lower ability to return to the colony, especially on cold days, and fatigue has been suspected as the cause for it. The results of this study support our earlier suggestion that pathogen imposed energetic stress and increasing difficulty in finding food could be a general mechanistic explanation for bees dying outside their colonies (Mayack and Naug, 2009; Naug, 2009), the typical characteristic of the recently observed colony collapse in honeybees. This study shows how the pathophysiological consequences of a disease can have far reaching implications on the behavior of an animal and how understanding such mechanisms can contribute to our knowledge about the epidemiology of a disease.

FIGURES
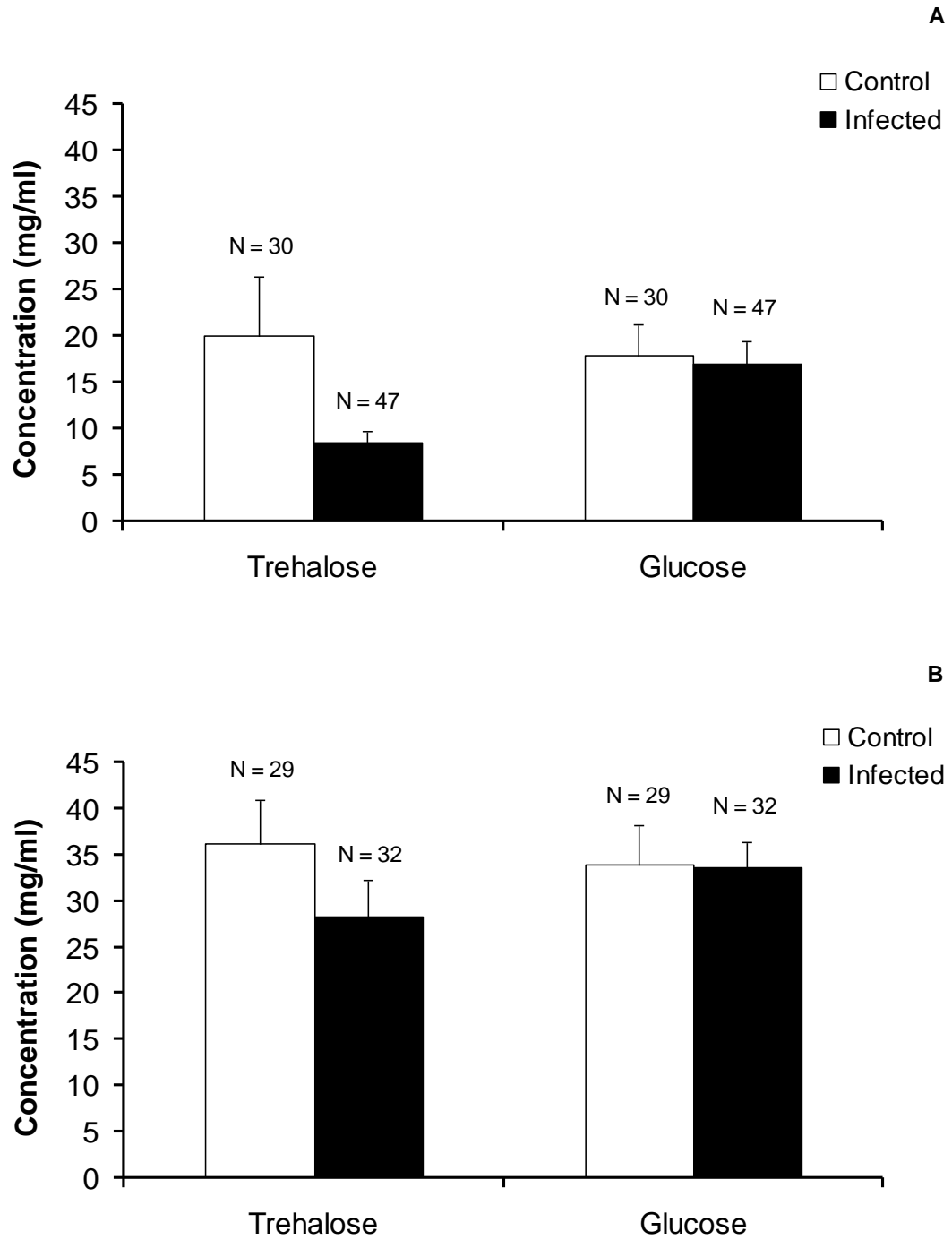
Fig. 2.1. Trehalose and glucose levels (mean ± s.e.m.) of (A) free-flying and (B) satiated honeybee foragers that are infected or uninfected with *Nosema ceranae*. The number above each bar indicates the sample size of the group.
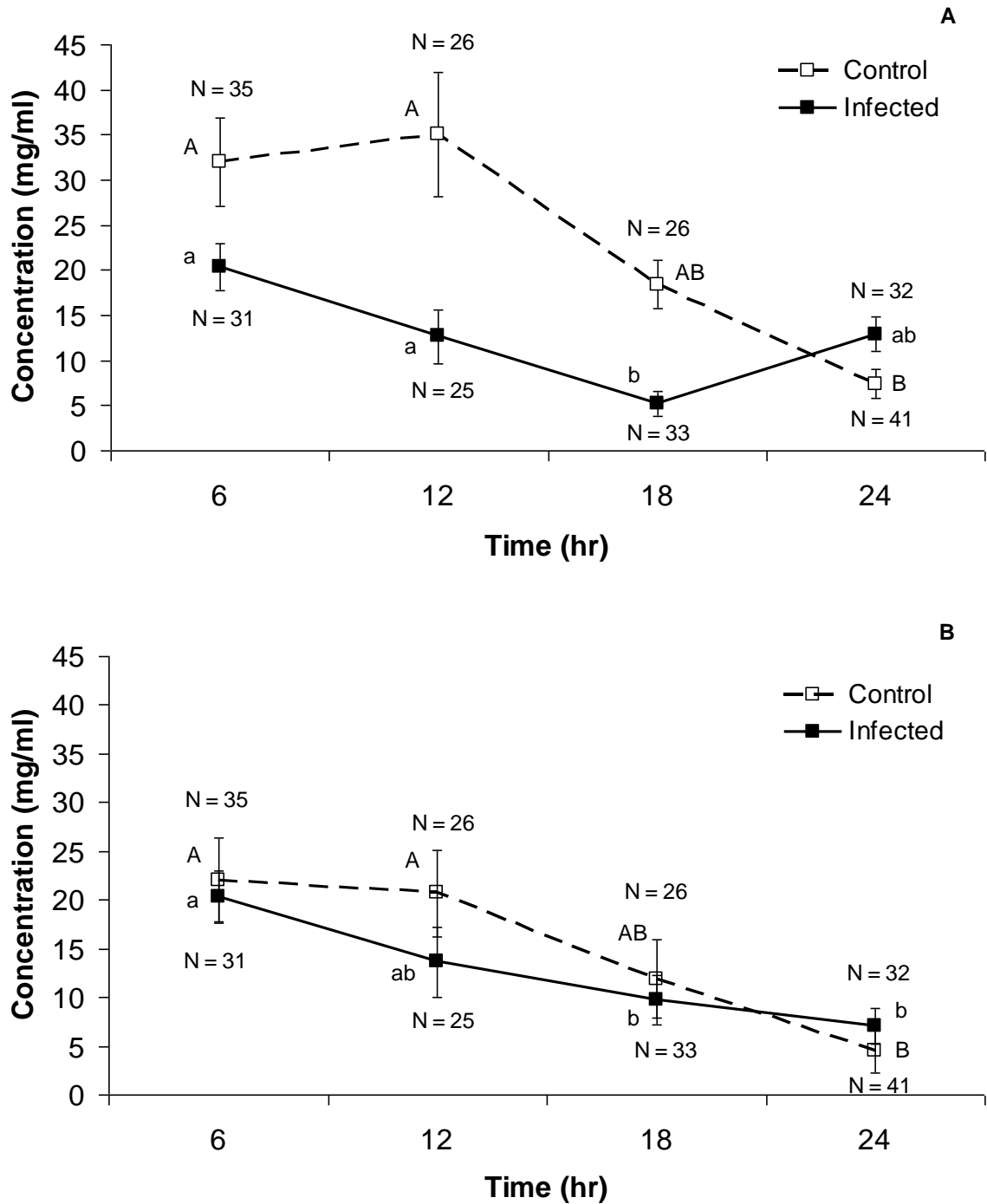
Fig. 2.2. Amounts (mean ± s.e.m.) of (A) trehalose and (B) glucose measured every 6 hours for uninfected and infected honeybee foragers fed until satiation at the start of the experiment and starved for 24 hours. Multiple comparisons within each group across different time points using a Tukey post-hoc test are presented with different letters (upper case for uninfected and lower case for infected bees) indicating a significant difference at P < 0.05 level. The number above and below each point indicates the sample size of the group.

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CHAPTER 3:

A CHANGING BUT NOT AN ABSOLUTE ENERGY BUDGET DICTATES RISK-

SENSITIVE BEHAVIOR IN THE HONEYBEE

SUMMARY

Animals are sensitive to risk or the variability of a reward distribution, and the energy budget rule of risk sensitivity theory predicts that it is adaptive for an animal to be risk averse when it is on a positive energy budget and to be risk prone when it is on a negative budget, because the utility of a potential large reward is much higher in the latter case. It has, however, been notoriously difficult to find conclusive empirical support for these predictions. We performed a comprehensive test of the energy budget rule in the honeybee, *Apis mellifera*, by constructing empirical utility functions and by testing the choice of bees for a constant or variable reward with an olfactory conditioning assay subsequent to manipulating their energy budgets. We demonstrate that a decline in energetic state leads to an increasing choice for a variable reward, while an increase in energetic state leads to an increased choice for a constant reward. We then show that subjects maintained on constant high or low energy budgets are risk indifferent, which suggests that an animal must perceive a change in its energetic state to be risk sensitive. We discuss the challenges of finding empirical evidence for the energy budget rule and the necessity of integrating physiological assays in these tests. Based on our previous results showing that parasitic infections cause an energetic stress in honeybees, we also

discuss the possibility of energetic shortfall being responsible for the observed display of risky behavior in infected bees.

INTRODUCTION

Foraging animals, faced with the formidable challenge of dealing with the intrinsic heterogeneity of the natural environment, must be sensitive not only to the average energy gain from a resource distribution but also to the variability associated with it. The energy budget rule of risk sensitivity theory (Caraco et al. 1980; Stephens 1981; Stephens & Krebs 1986), also referred to as variance sensitivity theory in the recent past (Ydenberg 2008), proposes that foragers on a negative energy budget should prefer higher variability (be risk prone) because under such a budget there is an accelerating fitness gain with each unit of energetic intake. In contrast, foragers on a positive energy budget gain a diminishing fitness return from each unit of energetic intake and are therefore predicted to prefer lower variability (be risk averse). In one of the earliest and most comprehensive experimental tests of the energy budget rule, dark-eyed juncos, *Junco hyemalis*, were shown to prefer a variable reward when their rate of energetic gain did not satisfy their energetic costs and to prefer a constant reward when they gained energy faster than what was required to meet their energetic costs (Caraco 1981). However, subsequent studies have provided a mixed variety of results, and there is a lack of robust support for the energy budget rule (reviewed in Kacelnik & Bateson 1996). This has led to a number of alternative hypotheses, largely based on cognitive mechanisms, to explain the observed sensitivity of animals to reward variability (Kacelnik & Bateson 1997). However, none of these alternative hypotheses can address

the unique predictions of the energy budget rule with regard to a change in risk sensitivity with a change in energetic state. One of the major shortcomings in most experimental tests of the energy budget rule is an insufficient understanding of the actual energetic state of the animal and how it relates to fitness, the utility function, even though Caraco et al. (1980) strongly pointed out that it is meaningless to test the energy budget rule without this knowledge. While this weakness is admittedly due to the challenges involved in precisely measuring the energetic state of the subjects, especially in vertebrate systems, it has resulted in studies using energy budget manipulations that are somewhat arbitrary. Studies using natural foraging are particularly prone to this problem as they cannot control the energetic state of the subjects as they forage or the reward distributions as they change across the course of the experiment (Hurly 2003; Bacon et al. 2010; Ratikainen et al. 2010), also making it difficult to show changes in risk sensitivity within the same individuals. A comprehensive test of the energy budget rule requires an integration of experimental methods in behavior and physiology and an animal model that allows such an integrative design. Honeybee foragers are ideal models for such an experiment because their high metabolic rate, powered primarily by carbohydrates (Sacktor 1970) and small fat stores, not only make them likely to be subject to strong selection for minimizing energetic shortfall but also allows one to accurately quantify their energetic states and construct empirical utility functions. Honeybees are also ideal subjects for precisely controlled decision-making studies in the laboratory, and Shafir et al. (1999), using a forced-choice proboscis extension response (PER) protocol, found that bees are risk averse in response to variability in reward amount when the reward distribution consists of both a zero reward and a high coefficient of variance (CV). This

makes the important point that CV rather than absolute variance is how animals probably perceive variability, and a precise control of such parameters is critical in any test of risk sensitivity (Shafir et al. 2005; Drezner-Levy & Shafir 2007). In this study, we first constructed empirical utility functions for bees at positive and negative energetic states by measuring their respective increment in survival as a function of each unit of energetic intake. We then tested the energy budget rule by examining whether there is a shift in the preference for variability within individual subjects as their energetic state is experimentally manipulated from positive to negative or vice versa. In the final set of experiments, keeping subjects under a constant high or low energy budget, we examined whether an animal's absolute energetic state or a change in its energetic state is responsible for driving risk-sensitive decisions.

MATERIALS AND METHODS

We collected returning honeybee, *Apis mellifera*, foragers by placing a wire screen to block the entrance of the hive, using four different colonies for the entire experiment to control for possible colony effects. The captured individuals were released in a cage and brought back to the laboratory. Each bee was then placed in a vial, chilled on ice to the point of immobilization, and strapped in a 4.5 cm plastic drinking straw with a small strip of tape around its thorax. All the subjects were kept in an incubator at 25 °C and 70% relative humidity at all times outside an experimental procedure.

Utility Function Experiment

Up to 40 bees were strapped at a time and randomly assigned to one of following feeding treatments: bees were fed with 0, 5, 10 or 20 μl of 30% sucrose solution and their survival was monitored every 6 h for 24 h.

Risk Sensitivity and Energy Budget Experiments

We performed four treatments: (1) decreasing energy budget, (2) increasing energy budget, (3) constant high-energy budget and (4) constant low-energy budget. For each treatment replicate, 14-16 bees were strapped at a time and divided into two groups. We assigned half of the bees to the energy budget experimental group, and extracted hemolymph samples from them to assay energy budgets. The other half formed the risk sensitivity experimental group, which we assayed for risk-sensitive behavior. Both the groups were maintained and fed exactly in the same schedule so that energetic state and risk sensitivity could be measured in parallel and data from the two groups could complement each other in each case (see Fig. 1 for details).

Risk Sensitivity Experiment

Conditioning trials for training bees to two reward distributions

A forced-choice proboscis extension response (PER) assay (Shafir et al. 1999) was used to train the bees in the risk sensitivity experimental group to associate two different odors, each a conditioned stimulus (CS), with two different distributions of a 30% sucrose reward, an unconditioned stimulus (US). This assay consists of presenting the subject simultaneously with two different CSs from two directions and forcing it to

34

choose between the two, a choice that is measured by the orientation of the subject's head towards one or the other stimulus. Hexanol and octanone were used as the two odors and were paired with either a constant or variable reward, interchanging the pairing as well as the direction of the two reward distributions between experimental replicates to account for odor and side biases. The odor delivery system consisted of an air pump connected to two odor cartridges (glass syringes containing a filter paper soaked in 5 μl of pure odor) through a set of computer controlled valves that presented each of the two odors twice in alternating 0.2 s pulses to the subject on either side of its head, 1 cm away at a 30° angle. Based on the orientation of its head at the end of the four odor pulses, a choice for one of the two odors was scored for the subject. The chosen odor was presented once again to the subject in a 2 s pulse along with the appropriate reward associated with it. If the subject chose the odor paired with the variable reward, it received either a high reward of 0.4 μl or a low reward of 0 μl in a predetermined pseudorandom sequence in which the probability of obtaining each was 0.5. If the subject chose the odor paired with the constant reward, it always received a 0.2 ml reward (there was no significant difference in the handling time of the three reward volumes; see Supplementary Material, Fig. S1). This resulted in two reward distributions, both giving an average gain of 0.2 ml but with a CV of 100 in the variable one. A subject underwent 20 such conditioning trials, with an intertrial interval (ITI) of 7-9min. The bees in the four treatments learned the two reward distributions at similar levels, as seen by their proboscis extension responses (see Supplementary Material, Fig. S2).

Retention tests to measure choice for one of the two rewards

The subjects were scored for their choice for one of the two odors corresponding to the two rewards during retention tests that involved presenting only the alternating series of 0.2 s pulses of the two odors (CS) and recording the final orientation of the head. For the decreasing and the increasing budget treatments, each subject was tested every 6 h for 24 h, while for the constant budget treatment, the subjects were tested once per hour for 6 h. All the retention tests were conducted in the blind with the observer having no knowledge of the odor-reward pairing.

Energy Budget Experiment

For the decreasing and increasing energy budget treatments, all bees were fed until satiation with 30% sucrose solution to equalize their energy budgets, and then starved for 24-30 h to increase motivation for the conditioning trials in the risk sensitivity group. Following these trials, in the decreasing energy budget treatment, the bees were starved for 24 h, whereas in the increasing energy budget treatment, each bee was fed 8 μl of 30% sucrose solution every 6 h for 24 h. In each treatment, two to three bees from the energy budget experimental group were freeze-killed at each 6 h mark, and their hemolymph was assayed for trehalose, the primary carbohydrate in insects, as an indicator of their energetic states. For the two constant energy budget treatments, all bees were starved for 3 h before conditioning trials began in the risk sensitivity group (there was no significant difference in the energetic states of bees from the four treatments at this initial time point; see Supplementary Material, Fig. S3). Upon the completion of the conditioning trials, bees in the low treatment were fed nothing, while those in the high

36

treatment were fed until satiation with 30% sucrose solution, after which bees in both groups were fed 1 μl of 30% sucrose solution every hour for 6 h. Two to three bees from the energy budget experimental group were extracted at each 1 h mark for hemolymph samples. Each 2 μl of hemolymph sample was diluted with 58 μl of distilled water and divided into two subsamples. Using an o-toluidine colorimetric glucose assay, one subsample was quantified for glucose without trehalase and the other one was quantified for glucose with trehalase, which breaks down trehalose into glucose. The amount of trehalose present in the sample was calculated by subtracting the amount of glucose in the first subsample from that in the second (Mayack & Naug 2010).

Statistical Analysis

Utility curves were constructed and analyzed using a nonlinear regression analysis with the proportion of survival calculated across all trials as the dependent variable and amount fed as the independent variable. Risk sensitivity of the bees was analyzed for each energy budget treatment separately using a repeated measures logistic regression, with the proportion of choices made for the constant reward during the retention tests as the dependant variable and time as the independent variable. The energy budgets of bees in different treatment groups were compared using ANOVAs, with hemolymph trehalose levels of bees as the dependent variable and time as the independent variable.

RESULTS

Utility Function Experiment

Survival as a function of amount fed showed a concave function for bees that were starved 6 h and were on a positive energy budget ($Y = -0.002X^2 + 0.074X + 0.487$, $R^2 = 0.63$, $F_{2,18} = 15.71$, $P < 0.0001$), but the same relationship produced a convex function for bees that were starved 24 h and were on a negative energy budget ($Y = 0.001X^2 + 0.006X + 0.033$, $R^2 = 0.51$, $F_{2,18} = 9.41$, $P = 0.002$; Fig. 2).

Risk Sensitivity and Energy Budget Experiments

The bees in the decreasing energy budget treatment progressively shifted from choosing the constant reward (being risk averse) to choosing the variable one (being risk prone) (repeated measures logistic regression: $\chi^2_4 = 19.07$, $P = 0.001$; Fig. 3a), corresponding with their declining energy budgets (trehalose levels) over time (one-way ANOVA: $F_{1,146} = 29.51$, $P < 0.0001$; Fig. 3b). Conversely, bees in the increasing energy budget treatment shifted from being risk prone to being risk averse (repeated measures logistic regression: $\chi^2_4 = 24.18$, $P < 0.0001$) as their energy budget became positive (one-way ANOVA: $F_{1,48} = 19.36$, $P < 0.0001$). Bees maintained at a constant high or low budget showed no preference for either reward, or were risk indifferent, and did not differ significantly from one another (repeated measures logistic regression: time*treatment interaction: $\chi^2_{11} = 4.32$, $P = 0.96$; treatment main effects: $\chi^2_1 = 0.04$, $P = 0.83$; Fig. 4a). The energy budget of bees in both these groups remained constant (two-way ANOVA: time*treatment interaction: $F_{5,119} = 1.03$, $P = 0.40$), and trehalose levels of bees on the

high budget were significantly higher than those on the low budget (treatment main effects: $F_{1,119} = 217.0$, $P < 0.0001$; Fig. 4b).
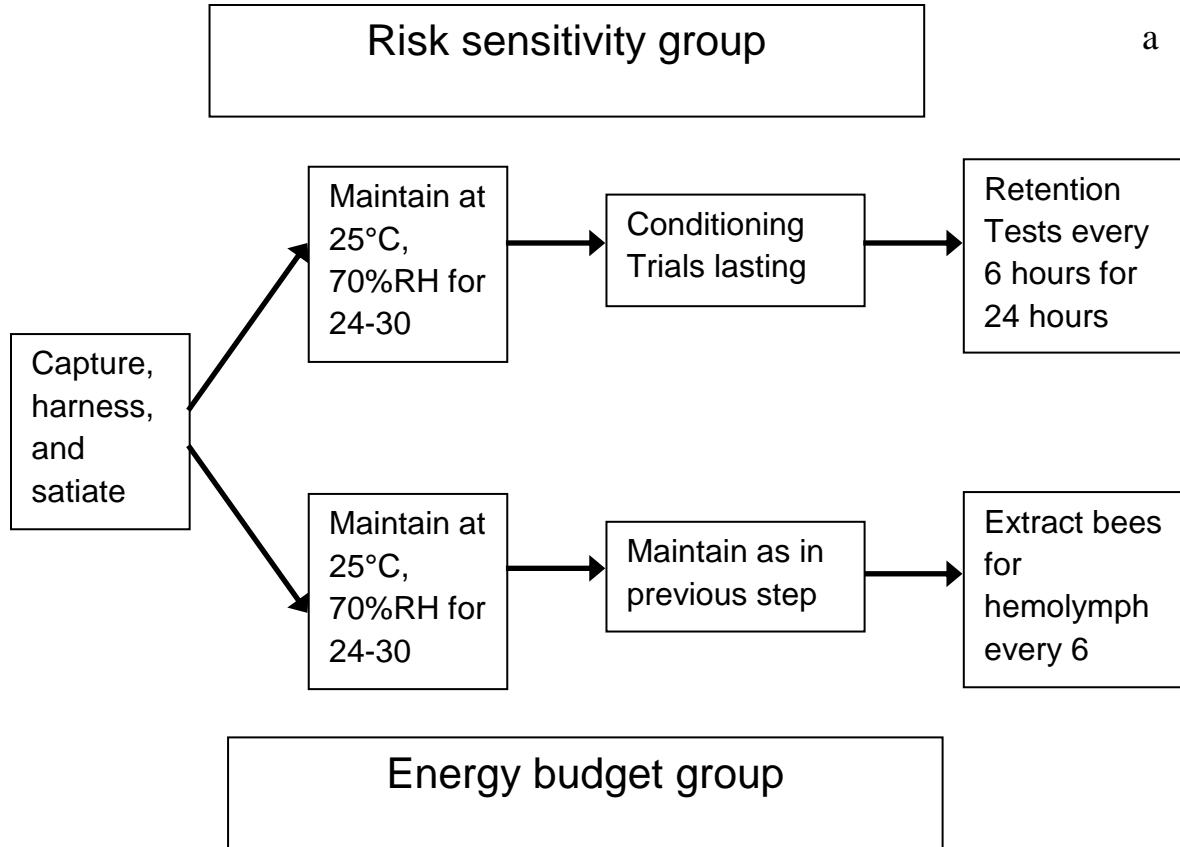
DISCUSSION

This study represents one of the most comprehensive tests of the energy budget rule and convincingly demonstrates that honeybees shift from risk-averse to risk-prone behavior with a decline in their energy budgets and vice versa. The observed changes in risk-sensitive behavior correspond to what is predicted by the observed utility functions for the two groups. An accelerating utility function for bees on a negative energy budget and a decelerating utility function for bees on a positive energy budget satisfy one of the necessary conditions of the energy budget rule, which otherwise has rarely been demonstrated. Unlike in many other experiments, a shift in risk sensitivity was observed within the same individuals rather than in different groups of subjects under different energy budgets. Forcing the subjects to first learn the two reward distributions and then measuring their choice between the two as their energetic state was manipulated constitutes an ideal approximation of the theoretical idea, something that is missing in most studies. The observed risk sensitivity cannot be explained by alternative mechanisms such as a difference in handling times for the different rewards, which were not significantly different from each other. There are only a handful of studies that have demonstrated a significant switch in risk sensitivity in terms of reward amount with a direct manipulation of the energy budget (Caraco et al. 1980, 1990; Caraco 1981; Croy & Hughes 1991). Most studies, in contrast, have found only weak or inconsistent support for the energy budget rule (Barkan 1990; Cartar & Dill 1990; Cartar 1991; Reboreda &

Kacelnik 1991; Banschbach & Waddington 1994; Abreu & Kacelnik 1999). While it is difficult to directly compare across studies because of the variety of procedures used to alter the energy budget and to measure choice, we offer a few suggestions that should be taken into consideration for testing the energy budget rule. The results of our constant budget experiments revealed that bees maintained at a constant high or low energy budget were equally risk indifferent, suggesting that an energy gain or loss rather than an absolute energetic threshold is what dictates their risk-sensitive decisions. Risk sensitivity has been commonly modeled using a dynamic state variable (Houston & McNamara 1985; McNamara & Houston 1992; Dall & Johnstone 2002), and our results provide empirical support for this approach. Our results also suggest that it may be of utmost importance to consider carefully how the energetic state of an animal is exactly affected by any budget manipulations, because both prior and current energetic conditions of the subject can influence its risk-sensitive decisions (Bacon et al. 2010). Using a change in energetic state rather than an absolute energetic threshold to dictate risk sensitivity makes adaptive sense because it provides a mechanism that allows the animal to adjust these decisions according to the time remaining to forage. Our finding that an individual at a constant low budget is risk indifferent may seem maladaptive at first, but it is equally important to note that the individual is not heading into an energetic shortfall that needs a risk-prone response. Similarly, an animal on a constant high budget can afford to be risk indifferent without paying a cost. The amount of interindividual variation in the energetic states and metabolic rates should also be an important consideration in studies of the energy budget rule because basal metabolic rates have been found to be important predictors of risk-sensitive behavior (Mathot et al. 2009). We have found substantial

intrinsic variation in the energetic states among bees even when they are maintained under constant conditions (Mayack & Naug 2010). However, our experimental protocol of using immobilized bees that were first fed until satiation allowed us to equalize their energy budgets and considerably reduce such intrinsic interindividual variability. Such variability in energetic states among individuals could explain why different individuals from the same group have been found to be either consistently risk averse or risk prone (Fülöp & Menzel 2000). The problem of interindividual variability in energetic states can get further compounded when only a few subjects are tested, which is common for studies conducted with vertebrates. Our results also indicate that animals must face severe energetic shortfall before displaying risk-prone behavior. Bees in our experiment showed risk-prone behavior only after their trehalose level dropped to about half of what it had been when they were satiated. This might explain why it has been generally easier to observe risk-averse rather than risk-prone behavior (Kacelnik & Bateson 1996). Based on our earlier results showing that the microsporidian *Nosema ceranae* can create an acute energetic stress in the honeybee that can be seen as a faster drop in trehalose levels in infected bees (Mayack & Naug 2009, 2010), parasitic infections could be one mechanism that can create a severe energetic shortfall in animals. While *Nosema* infected bees are known to display riskier foraging strategies, such as foraging earlier in life and foraging in inclement weather, whether it is a consequence of an altered life history strategy dictated by their shorter life span (Woyciechowski & Kozlowski 1998), or whether the energy budget rule has something to do with it is an intriguing question that remains to be tested. Some authors have argued that social animals such as honeybees are unlikely to show risk sensitivity because by foraging individually at many flowers and in

large numbers preclude them from being substantially affected by the variability in the resource distribution (Banschbach & Waddington 1994). However, several studies have pointed out that animals, being constrained by their cognitive capacity, frequently make decisions over small sample sizes (Real et al. 1990; Bateson & Kacelnik 1996; Stephens & Anderson 2001; Naug & Arathi 2007; Buchkremer & Reinhold 2010). In addition, recent data suggest that despite the regulatory mechanisms operating at the social level, foraging behavior in honeybees is still partly regulated by the energetic state of the individual (Toth & Robinson 2005; Mayack & Naug 2010). Our experimental protocol, which tested individual bees at different energetic states making a single choice at a time, allowed us to control for the social influences and show that individual bees make risk-sensitive decisions in accordance with the energy budget rule when they are trying to maximize their short-term energetic gain. The design of this study shows how it is critically important to integrate detailed physiological assays in any test of the energy budget rule, and serves as a reminder to behavioral ecologists not to ignore the proximate mechanisms that underlie a behavior.

	Risk sensitivity group		a

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                    ┌──────────────┐      ┌──────────────┐      ┌──────────────┐
                    │ Maintain at  │      │ Conditioning │      │ Retention    │
                 ┌─▶│ 25°C,        │─────▶│ Trials       │─────▶│ Tests every  │
                 │  │ 70%RH for    │      │ lasting      │      │ 6 hours for  │
                 │  │ 24-30        │      └──────────────┘      │ 24 hours     │
┌──────────────┐ │  └──────────────┘                           └──────────────┘
│ Capture,     │ │
│ harness,     │─┤
│ and          │ │
│ satiate      │ │  ┌──────────────┐      ┌──────────────┐      ┌──────────────┐
└──────────────┘ │  │ Maintain at  │      │ Maintain as  │      │ Extract bees │
                 └─▶│ 25°C,        │─────▶│ in           │─────▶│ for          │
                    │ 70%RH for    │      │ previous step│      │ hemolymph    │
                    │ 24-30        │      └──────────────┘      │ every 6      │
                    └──────────────┘                           └──────────────┘
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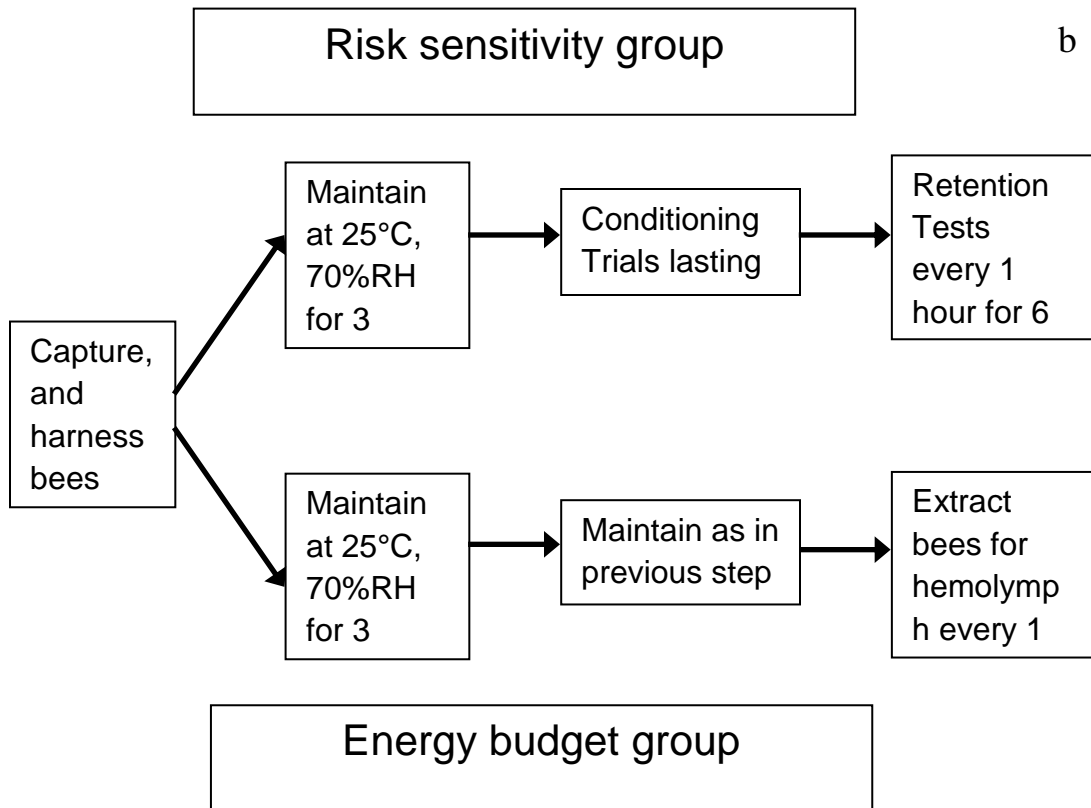
Energy budget group

Fig. 3.1. (a) In the decreasing energy budget treatment, both groups were satiated at the end of the conditioning trials and then starved for 24 hours while in the increasing energy budget treatment, both groups were fed nothing at the end of the conditioning trials but were fed 8 µl of 30% sucrose solution every 6 hours following each retention test and extraction procedure. (b) In the constant high energy budget treatment, both groups were satiated at the end of the conditioning trials and then fed 1 µl of 30% sucrose solution every 1 hour for 6 hours while in the constant low energy budget treatment, both groups were fed nothing at the end of the conditioning trials and then fed 1 µl of 30% sucrose solution every 1 hour for 6 hours.
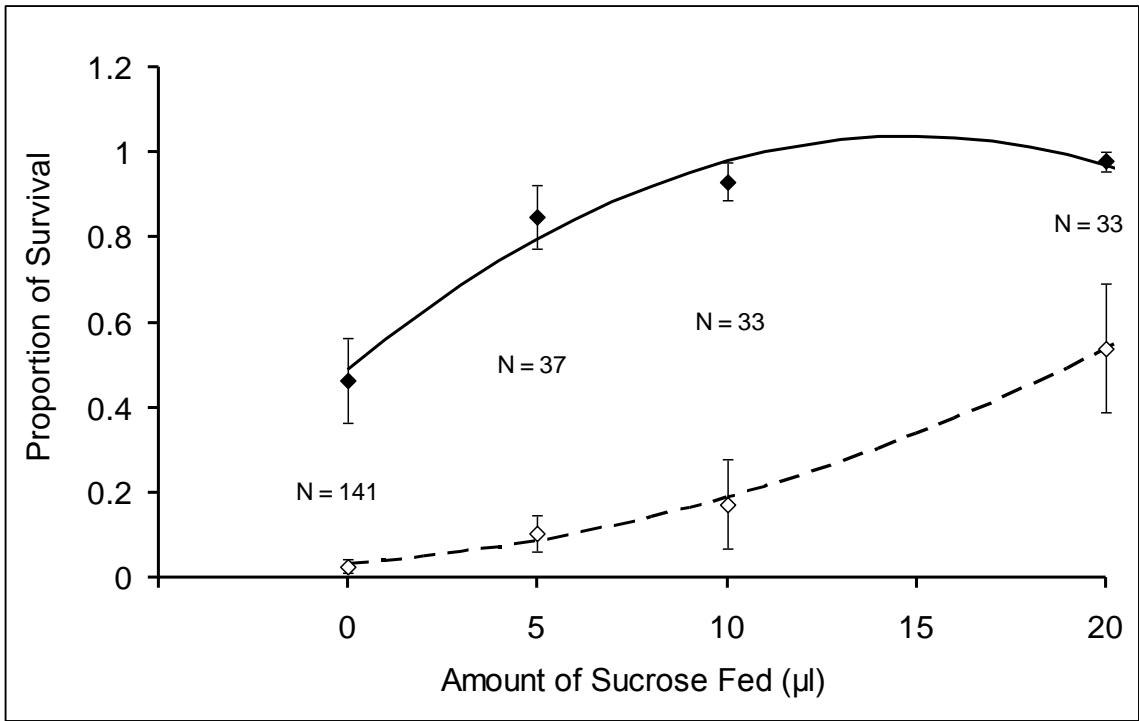
Fig. 3.2. Utility functions for honeybees on a positive and a negative energy budget, measured as proportion of bees surviving after 6 hours (—♦—) and 24 hours (- ◊ -) of starvation respectively, as a function of different amounts of food consumed, with corresponding best-fit lines.
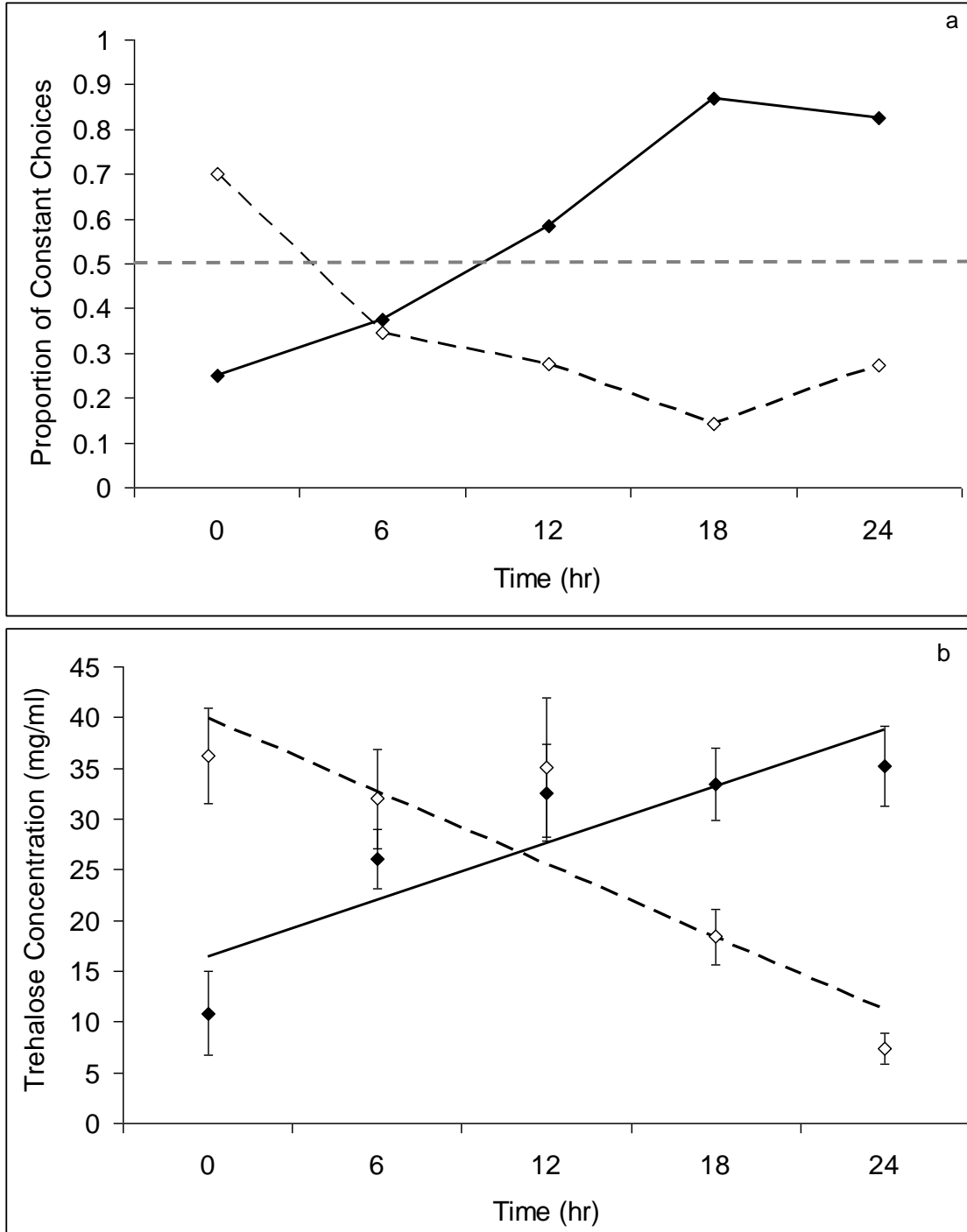
Fig. 3.3. (a) Risk-sensitivity of bees on a decreasing (- - -) and an increasing energy budget (———), given by the proportion of choices made for the constant reward. Data consist of 30 bees for the decreasing budget and 24 bees for the increasing budget. The dotted gray line represents the predicted probability of constant choice if choice is random or risk-indifferent. (b) Corresponding energetic states of bees on a decreasing (- ◊ -) and an increasing budget (—◆—), given by their hemolymph trehalose levels (mean ± S.E.) with respective best-fit lines given by y = -1.19x + 39.87, R^2 = 0.16, and y = 0.76x + 17.74, R^2 = 0.29.

Fig. 3.4. (a) Risk-sensitivity of bees on a constant low (- - -) and a constant high energy budget (———), given by the proportion of choices made for the constant reward. Data consist of 30 bees for each energy budget treatment. (b) Corresponding energetic states of bees on a constant low (- ◊ -) and a constant high energy budget (—♦—), given by their hemolymph trehalose levels (mean ± S.E.) with respective best-fit lines given by y = 0.48x + 12.48, $R^2 = 0.01$, and y = 1.02x + 39.84, $R^2 = 0.01$.
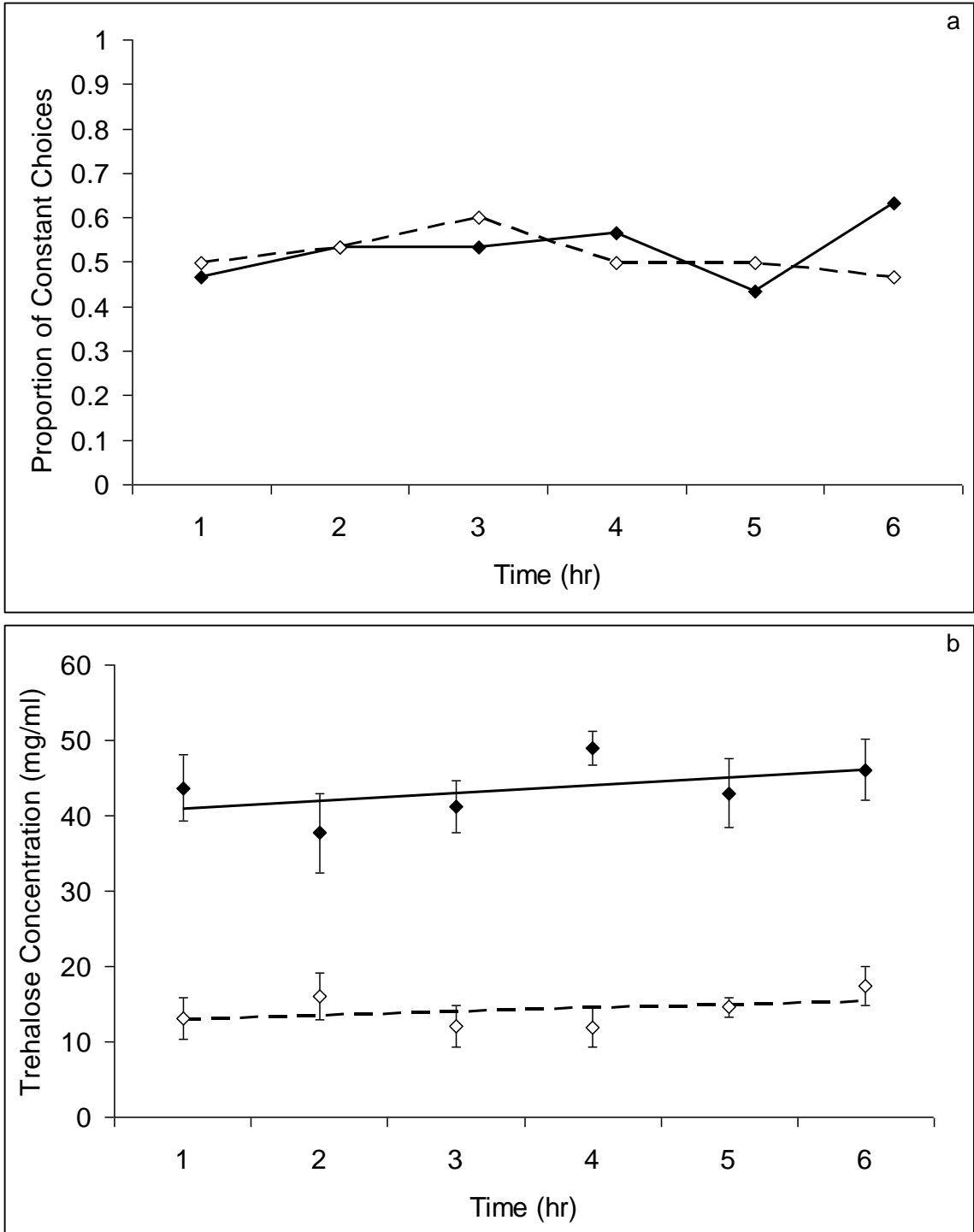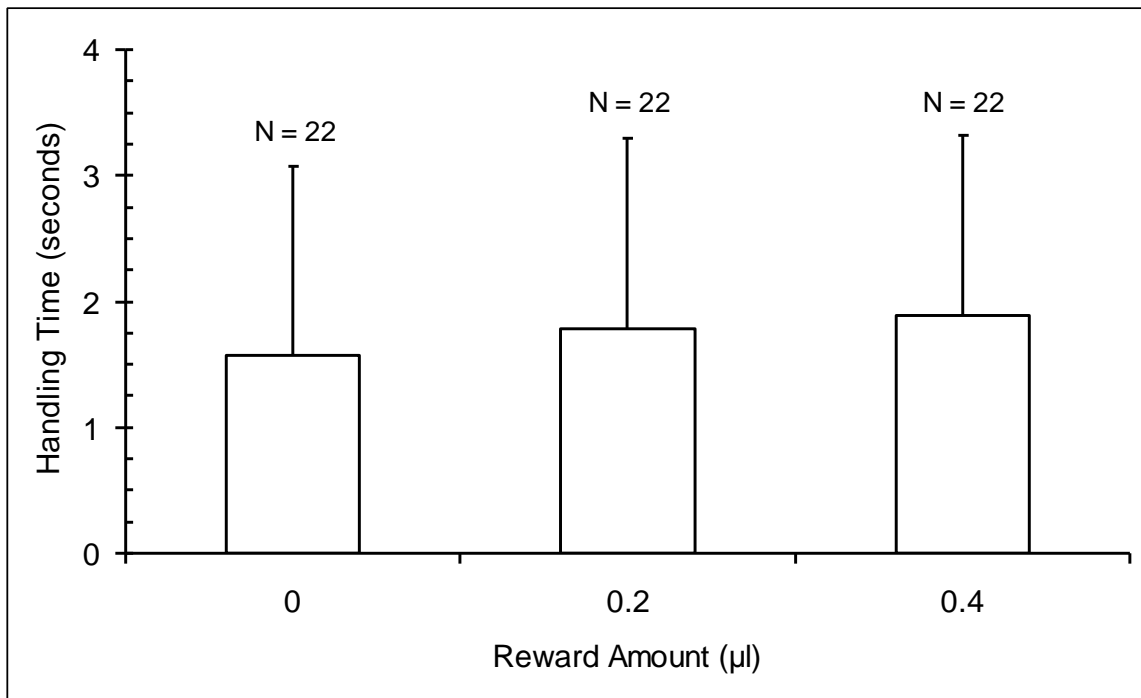
Fig. 3.S1. Handling times for the three reward amounts during the conditioning trials. Each bar represents a mean with standard deviation and there is no significant difference among the three handling times (repeated measures ANOVA: $F_{1,2} = 0.67$, $P = 0.51$). Handling time was calculated as the duration when the bee had the proboscis extended in response to the odor pulse.
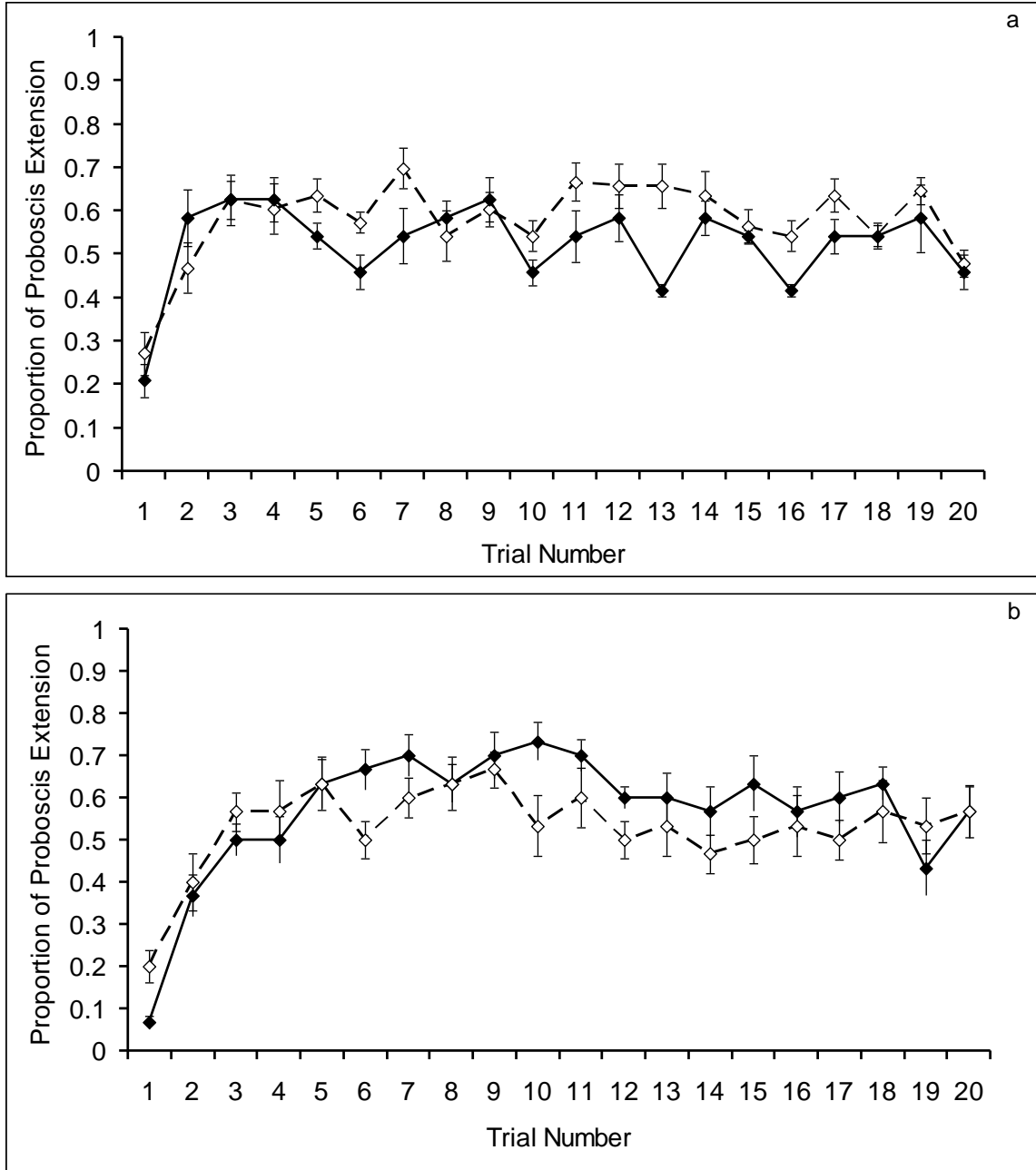
Fig. 3.S2. Proportion of bees that extended their proboscis during the conditioning trials in the (a) increasing (♦, N = 24) and decreasing (◊, N = 30) energy budget treatments, and (b) constant high (♦, N = 30) and constant low (◊, N = 30) energy budget treatments. Each data point represents the proportion averaged across all trials with corresponding standard error bars. There is no significant difference in learning among the four groups (repeated measures logistic regression: $\chi^2_3 = 0.40$, P = 0.94).

Fig. 3.S3. Hemolymph trehalose levels measured after 3 hours of starvation without being fed and after 24 hours of starvation after being fed until satiation with each bar indicating mean with standard deviation. There is no significant difference between the two groups (one-way ANOVA: $F_{1,55} = 0.73$, $P = 0.40$).

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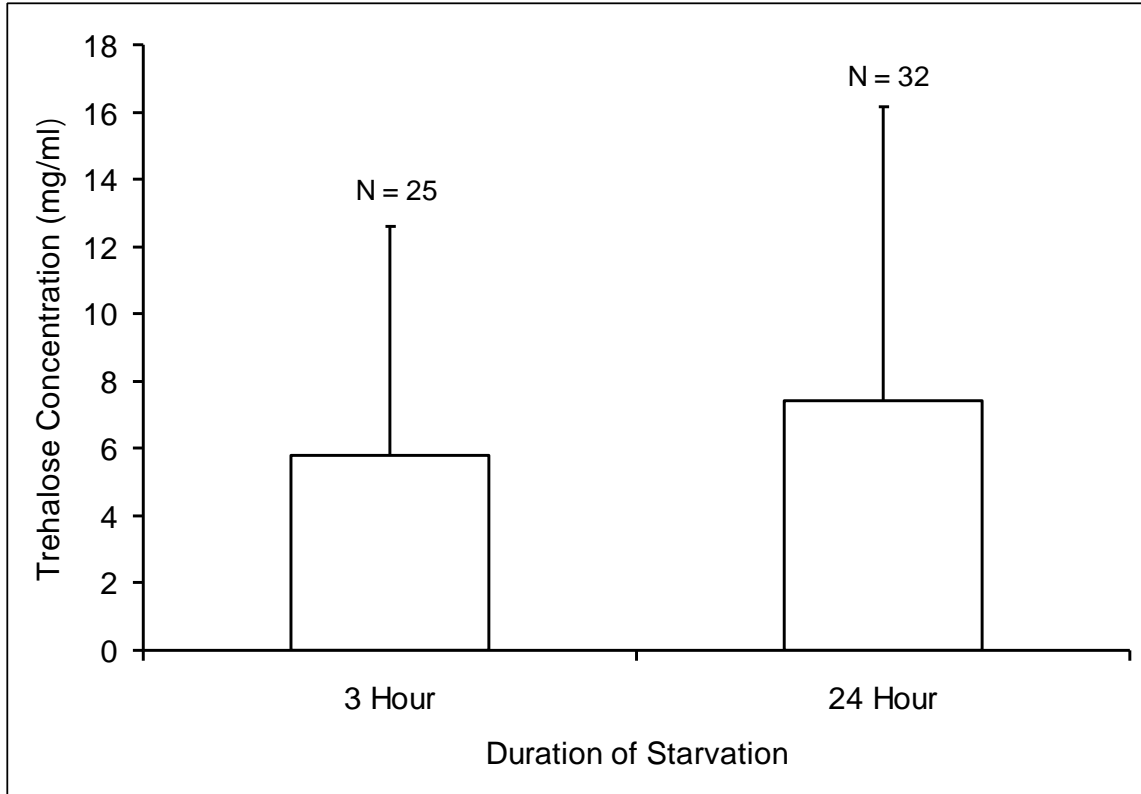Bacon, I. E., Hurly, T. A. & Healy, S. 2010 Both the past and the present affect risk-sensitive decisions of foraging rufous hummingbirds. *Behavioral Ecology* **21**, 626-632.

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CHAPTER 4:

HONYBEE WORKERS USE "WEAKLY SELFISH" STRATEGIES TO

COMPENSATE FOR ENERGETIC DEPLETION

SUMMARY

The energetic state of an individual is a fundamental driver of its behavior. However, the regulation of the energetic state of an individual in a eusocial group such as the honeybee consists of two distinct components, the individual level based on the amount of carbohydrates in the hemolymph and the colony energetic state given by the amount of nectar stores in the hive. The two are normally coupled and the predominant view is that food acquisition behavior in eusocial groups is socially regulated by the colony state. We uncouple the energetic state of an individual honeybee from its colony by feeding it with a non-metabolizable sugar and show that these energy depleted bees in a colony with full food stores use "weakly selfish" strategies to compensate for energetic shortfall. They are initially less active within the colony and take fewer foraging trips, but do not feed from colony food stores. Further energy depletion causes these bees to increase foraging, demonstrating that food acquisition is partly regulated at the level of the individual, even in eusocial groups. These bees also experience higher mortality during foraging and we discuss how energetic depletion can play a role in the recent observation of bees disappearing from their colonies.

54

INTRODUCTION

Energy is a fundamental requirement for maintenance and growth in all animals and is therefore a primary driver of their behavior. Energetic demands are highly dynamic and animals alter their behavior in numerous ways in order to meet this constantly changing demand. For example, in order to prevent an energetic shortfall, animals may increase their overall search activity to find food (Lee and Park 2004; Mailleux et al 2010a), incur a greater risk of predation to gain access to food patches (Abrahams and Dill 1989; Croy and Hughes 1991), or prefer food patches with a higher variance (Caraco 1981; Stephens 1981). The analogous situation in social animals is somewhat more complex because they not only forage to meet individual energetic demands but they also share food with other group members and hoard food in a communal storage as a resource for inclement times. Consequently, an individual in a eusocial group such as a honeybee colony is subject to two energetic states that can potentially dictate its food acquisition behavior, the individual state based on its own nourishment level and the colony state given by the amount of food stores in the hive. However, due to the expected intrinsic correlation between these two states, it is difficult to evaluate the role of these two possible kinds of regulatory control on the decision-making related to food acquisition in social animals (Ydenberg et al 1994).

In eusocial insects such as the honeybees, foraging is generally considered to be regulated at the social level because the colony is viewed as the unit of selection (Ydenberg and Schmid-Hempel 1994, Seeley 1985). While this might be true for a resource like pollen, which is collected primarily to feed the brood and is regulated by a feedback loop based on the amount of brood and stored pollen in the colony (Fewell and

55

Winston 1992; Camazine 1993; Sagili and Pankiw 2007), nectar foraging might be regulated differently since nectar supplies energy to the adult individuals in the colony, including the foragers themselves. This suggests that foraging behavior can be potentially dictated by either the individual or the colony energetic state acting independently or together in concert (Schmid-Hempel et al 1993; Fewell and Winston 1996). However, the discovery of various social level signals that regulate foraging (von Frisch 1967; Seeley 1986, 1989, 1992) and observations such as starvation at the colony level leads to increased foraging (Howard and Tschinkel 1980; Schulz et al 1998; Mailleux et al 2010b) have led to the general view that forager behavior is regulated at the colony level, even at the expense of an individual forager (Eckert et al 1994). This has resulted in a traditional and continuing focus on the role of social regulatory factors in most research related to foraging in social insects (Seeley 1995; Gordon et al. 2008; Jarau and Hrncir 2009).

However, it is important to note that the above studies cannot rule out whether there are regulatory factors operating at the individual level that also influence the food acquisition behavior of a social insect forager. In certain situations, such as when an individual is parasitized, its energetic state may become uncoupled from that of the colony (Mayack and Naug 2010), making it adaptive for the individual to alter its foraging behavior independent of the colony state in order to compensate for its own depleted energetic state. A few recent studies have provided some evidence regarding such a possibility. The lipid level of an individual honeybee has been shown to play an important regulatory role in dictating the ontogeny of when it begins foraging, acting independently of age, experience, and social cues (Toth and Robinson 2005; Toth et al 2005). Lipid levels have also been found to be the best predictor of which individuals

leave the nest to forage in ants (Blanchard et al 2000), a few lean foragers perform the majority of the foraging activity for the entire colony (Robinson et al 2009). However, these findings only address how long-term energetic depletion can alter the ontogeny of food acquisition behavior in social animals. On a shorter time scale, hemolymph trehalose titer was found to be correlated with activity in ants under a starvation treatment, suggesting that it may be an important behavioral modulator in an individual responding to energetic depletion (Schilman and Roces 2008). In insects, trehalose is known to serve as a constant monitor of the internal energetic state (Thompson 2003) and the amount of trehalose in the hemolymph is an important regulator of feeding behavior in solitary insects (Simpson and Raubenheimer 1993; Friedman et al 1991), suggesting that it could also play a critical role in regulating food acquisition behavior.

In social insects, attempts to understand the factors regulating foraging behavior have traditionally relied upon experimental designs consisting of treatments administered at the colony level. Such designs would however fail to uncouple the energetic state of the individual (trehalose levels) from that of the colony (amount of food stores), thereby masking the role of any regulatory mechanism operating at the individual level independent of the colony state. Some recent research has shown that trehalose levels can have a significant effect on the behavioral decisions of honeybee foragers that have been isolated from their social environment (Mayack and Naug 2011). Yet, it is not clear if a similar effect would be observed in the presence of a social context that includes competing colony level regulatory cues. The objective of this study is therefore to determine if a honeybee forager can alter its food acquisition behavior in response to energetic depletion at the individual level even when the colony as a whole is in a

positive energetic state. First, we experimentally uncoupled the energetic state of the individual from that of the colony by feeding experimental bees with Sorbose, a non-metabolizable sugar, to lower their trehalose levels. We then quantified the foraging and in-hive behaviors of these energy depleted bees to address the broader question of whether mechanisms related to nutritional physiology and foraging in solitary insects have been co-opted to regulate altruistic foraging in social insects (Toth et al. 2005).

MATERIALS AND METHODS

Observation Hive Set Up

We set up a three-frame observation hive consisting of two brood frames, a stored honey frame, a laying queen, and about 7,000 bees. The hive was located in a dark room with diffuse light, maintained at approximately 25° C, and was connected to the outside environment through a tube. Blocks were placed inside the hive such that bees could enter and exit the colony from only one side, the one facing the observer. The front glass pane of the observation hive was marked with 5 x 5 cm grid to assist behavioral and spatial sampling of the experimental bees. In order to ensure that the colony energetic state remained the same throughout the experiment, we replaced the honey frame with one from a source colony when needed. We performed four replicates of the experiment in four consecutive weeks to control for changing colony and outside environmental conditions.

Energy Depletion Treatment

We created an energetic depletion in individual bees by feeding them with 30% sucrose solution mixed with 2% sorbose, a non-metabolizable simple sugar that is passively absorbed through the midgut of the bee and reduces trehalose levels in the hemolymph (Blatt and Roces 2002). Using a series of experiments which involved feeding harnessed individual bees various dosages of sorbose followed by measuring their trehalose levels, it was determined beforehand that a 2% dosage administered daily for three days was sufficient to create and maintain a significant reduction in energetic state without causing large, immediate changes in survival.

On the morning of Day 1 of the experiment around 9:00 A.M., just when the bees are starting to forage, after temporarily blocking the entrance of the observation hive, we captured returning nectar foragers with empty pollen baskets individually, five at a time, and chilled them immediately on ice until immobility. Using tags of two different colors to divide the bees into two groups, we put a unique number tag on each bee and then fed her *ad libitum*, feeding the bees in the control group with 30% sucrose solution and feeding the ones in the treatment group with 30% sucrose solution mixed with 2% sorbose. We recorded the amount of food consumed by each bee and placed each group of bees in a separate flight cage. We repeated the entire procedure until there were 25 foragers for each group, and 30 minutes after the last bee was fed (to allow for crop emptying and reduce the chances of these bees engaging in trophallaxis with others), we released all the bees outside the entrance to the observation hive so that they could fly back into the colony.

59

On the mornings of day two and three of the experiment, before foraging started for the day, all tagged foragers found in the hive were individually re-captured, chilled, fed, and released in the same way as the first day. At the end of the third day, all the remaining tagged bees were captured, freeze killed, and their hemolymph was extracted and assayed for trehalose and glucose (for details see Mayack and Naug 2010).

Behavioral Observations

On each day of the experiment, starting 30 minutes after the two groups of foragers were released and allowed to go back into the colony, we conducted behavioral observations consisting of a 3-4 h session of focal animal sampling and an equally long session of focal behavior sampling, from about 12-7 pm, resulting in a total of 36 h of behavioral observations across all the replicates. The aim of focal animal sampling was to quantify the proportion of time spent by tagged bees in specific in-hive behaviors (Standing, Walking, Head inside nectar cell, Trophallaxis, and Dancing), while the focal behavior sampling was conducted on the hive entrance to quantify their foraging frequency. For focal animal observations, we selected a specific grid using a random number and if a single tagged bee was present within this square, for 10 mins we recorded her behavior with a scan every 15 s. If no bee or multiple bees were present in the selected square, another square was randomly chosen. In order to ensure equal representation of the two groups in the behavioral sample, bees from each group was chosen alternately in successive focal animal sessions. Observations were terminated for a bee before the 10 min period if she left for foraging or went to the other side of the observation hive. From this data, the spatial location of each bee was also classified in

terms of either being present close to the entrance (on the bottom frame) or being away from it at the interior of the hive (on the top two frames) and its speed of movement was calculated as the sum of the shortest distance between the squares it was located at during the entire duration of her focal sample divided by the total duration of her focal sample.

Focal behavior sampling consisted of observing the entrance tube of the observation hive and recording the time a tagged bee left or entered the hive. From this data, foraging frequency, and time spent outside and inside the hive was calculated. At the end of each day, we performed a census of the tagged bees present in the colony and from this data we calculated the number of foragers lost from each of the two groups.

Statistical Analysis

One-way ANOVAs were used to compare the control and energy depleted bees in terms of amount fed, trehalose levels, the time spent inside and outside the hive as well as the proportion of time spent in the five in-hive behaviors after arc sine transformation. The spatial data was analyzed using a G-test of independence. Due to the ordinal nature of the data, a Kruskal Wallis test with a Scheirer-Ray-Hare extension (Sokal and Rohlf 1995) was used to analyze the foraging frequencies of the two groups and test for an interaction effect. There were no significant differences across the three days and across the four replicates with regard to the proportion of in-hive behaviors, spatial locations, and the amount of food consumed, so data were pooled for analysis.

RESULTS

Energy Depletion Treatment

Bees fed with the sucrose solution mixed with 2% sorbose consumed significantly more food (one-way ANOVA: $F_{1,396} = 3.87$, P = 0.003; Fig. 1a) and their trehalose levels were significantly lower at the end of the experiment (one-way ANOVA: $F_{1,67} = 5.77$, P = 0.02; Fig. 1b) than control bees.

In-hive Behaviors

Energy depleted bees spent a significantly higher proportion of time standing (one-way ANOVA: $F_{1,119} = 9.28$, P = 0.003) and correspondingly a significantly lower proportion of time walking ($F_{1,119} = 4.43$, P = 0.04; Fig. 2a) compared to control bees. However, there was no significant difference between the two groups in terms of the proportion of time spent dancing ($F_{1,119} = 0.0007$, P = 0.98), engaging in trophallaxis ($F_{1,119} = 2.00$, P = 0.16) or with their head inside nectar cells ($F_{1,119} = 0.70$, P = 0.40). Energy depleted bees were also found disproportionately more frequently at the interior of the hive away from the entrance in comparison to the control bees (G test of independence: G = 27.06, N = 3450, P < 0.0001; Fig. 2b) even though there was no significant distance in the walking speed between the two groups ($F_{1,119} = 0.50$, P = 0.48).

Foraging Behavior

There was a significant change in the foraging frequency with time in the two groups (Kruskal Wallis test: $H_{2,138} = 11.31$, P = 0.004), with a significant interaction between time and treatment (Scheirer-Ray-Hare extension: $H_{2,138} = 21.69$, P < 0.0001,

Fig. 3a) with an increase in foraging by energy depleted bees relative to control bees. However, the times spent by the two groups inside ($F_{1,245} = 2.76$, $P = 0.10$) and outside the hive ($F_{1,256} = 0.08$, $P = 0.78$) were not significantly different. There was also a significantly higher proportion of cumulative forager loss in the energy depleted group (Wilcoxon Signed Rank test: $Z = 1.96$, $N = 12$, $P = 0.05$; Fig. 3b).

DISCUSSION

Our results confirm that the energetic state of an individual in a eusocial group can indeed be uncoupled from that of its colony and can dictate its behavior independently of the colony energetic state (Mayack and Naug 2010, 2011). This study, to the best of our knowledge, is the first one to successfully implement an energetic depletion at the level of the individual in a eusocial group without altering the colony energetic state, as indicated by the effectiveness of the sorbose treatment in significantly lowering trehalose levels and increasing the amount of sucrose solution consumed by the treatment group.

Surprisingly, energy depleted foragers did not compensate for their lower energetic state by changing their social behavior within the hive. Individuals in a social insect colony can potentially feed from the colony food storage or acquire food from nestmates *via* trophallaxis to meet an energetic shortfall and some previous studies have documented an increase in trophallaxis with starvation treatments imposed at the colony level (Howard and Tschinkel 1980, 1981). Instead, energy depleted foragers were seen to reduce their activity level within the hive, which included more standing and less walking, presumably to conserve energy. A similar reduction in activity levels was found

to be an effective strategy in conserving energy in ants, where individuals that did not move at all had enough energy to survive for an additional 22 h compared to others in the colony (Schilman and Roces 2008). Inactivity as a compensatory response to energy depletion is somewhat surprising as solitary insects have been found to induce hyperactivity in response to the same contingency, probably to increase the search area for food (Lee and Park 2004). However, such starvation-induced hyperactivity occurs in a fairly short burst, which also makes it difficult to demonstrate (Renault et al 2003). Although such observed inactivity might be counterproductive to colony ergonomics, it may be an effective short term strategy at the individual level. With their significantly higher metabolic rates than ants (Woods et al 2005; Schilman and Roces 2006), honeybees are likely to show such energy depletion induced inactivity sooner or with less severe starvation.

Energy depleted bees were also found to stay away from the nest entrance, which might allow them to avoid the typical bustle found at the entrance of the hive. While this might be a subtle strategy that further allows them to conserve energy, it would also make these bees have less access to social information related to food resources outside. In an experiment where ants were starved at the colony level, more individuals were found by the nest entrance, presumably to gain such social information (Mailleux et al 2011). While the two experiments are not strictly comparable, this could be a reason why energy depleted bees showed lower foraging at first, which then gradually increased over the course of the experiment, such a delayed response being typical for bees starved at the colony level (Schulz et al 2002). It seems the energy-depleted bees initially try to compensate for their reduced energetic state by reducing their activity level and only

64

resort to foraging when their energetic state falls further. On the other hand, control bees continuously decreased their foraging over the course of the experiment as their trehalose levels increased from being fed until satiation everyday with sucrose. A role of trehalose in driving individual foraging behavior is also indicated by a trend for a negative correlation that was found between the trehalose level of a forager and its foraging frequency measured on the last day of the experiment ($r = -0.37$, $N = 21$, $P = 0.10$). While one could hypothesize that energy depleted foragers may also take shorter trips to save energy (Schilman and Roces 2006), in our study the time spent by the two groups outside and inside the hive did not significantly differ. Foraging trip time however, is a function of both the distance a forager flies and the speed at which she flies, and we speculate that the negative influence of energy depletion on both these variables could result in a net lack of effect on trip time in comparison to control bees, but further investigations would be necessary to resolve these effects.

Using individual energetic state as a reference for the overall colony energetic state and making foraging decisions based on it has some advantages because the two are generally coupled in a normal colony and relying on social information for finding food is also costly, especially when food is spaced heterogeneously or ephemeral (Dechaume-Moncharmont et al 2005). The uncoupling of the individual and the colony energetic states can be brought about by a number of agents that cause energetic stress in individuals, such as poor nutrition (Abou-Seif et al 1993), parasites (Mayack and Naug 2009, 2010), or sublethal exposure to pesticides (Alaux et al 2010). Our results suggest that foragers with such prolonged exposure to energy depletion might leave the colony at an increased frequency, and given that such foragers are also likely to have poor

thermoregulatory ability (Campbell et al 2010), cognitive impairments (Mery and Kawecki 2005) and risk-prone behavior (Mayack and Naug 2011), all of these factors can synergistically act to increase their mortality rate outside the colony. Such mortality could be further compounded by the fact that recent reductions in suitable habitat might be forcing bees fly further distances from the hive to find forage (Naug 2009). Energetic stress can therefore be an underlying mechanism that explains the recently observed depopulation and weakening of honeybee colonies known as colony collapse. The importance of nutritional physiology in potentially causing such colony depopulation has recently been demonstrated (Dussutour and Simpson 2012).

However, one question that arises here is why would an energy depleted individual in a satiated colony go foraging when there are sufficient nectar reserves within the colony to meet its energetic demand? Previous research has found that bees have two different brain biogenic amine pathways that correspond with the two different ways of gaining satiation, one from individual feeding and the other from food sharing (Wada-Katsumata et al 2011). This suggests that there are two independent pathways at the neuronal level that regulate food acquisition behavior in social insects. Therefore, an energy depleted individual could be equally likely to choose either of these two behaviors and it would be interesting to ask what regulates the activation of these two pathways and the interaction between them.

In summary, in a social insect colony, the individual energetic state is typically co-opted with the colony state and work together in concert to maximize colony fitness. However, the uncoupling of these two states due to a range of factors can lead to behavioral alterations that in turn could lead to some complex and unexpected

consequences. The behavioral alterations observed due to energy depletion in this study could be considered as "lazy" and therefore selfish from the perspective of colony ergonomics. Energetic depletion could lead to selfish behavior in an even broader sense because being social is known to be energetically costly as it requires complex neural processing to override natural selfish behaviors (Gailliot and Baumeister 2007). Energy depleted humans have been shown to decrease their altruistic behaviors (DeWall et al 2008) and even exhibit a loss of "self-control", which leads to behaviors that are not beneficial for the "greater good" or society (DeWall et al 2011). It has been proposed that the amount of self-control negatively correlates with metabolic rate (Tobin & Logue, 1994) and the fact that bees seem to defy this idea and typically exhibit "self-control" (Cheng et al 2002), is probably the reason why energy depleted bees use "weakly selfish" strategies, such as reducing activity to conserve energy. This suggests that instead of eating communal food stores and damaging the "greater good", acting more selfish in more subtle ways may be a more effective and general strategy in eusocial animals. An explanation for such weak selfish behavior in eusocial insects probably lies in the fact that while natural selection acts more strongly at the colony level, it still acts at the level of the individual to increase its survival while at the same time preventing the loss of colony fitness from depleting communal food stores.
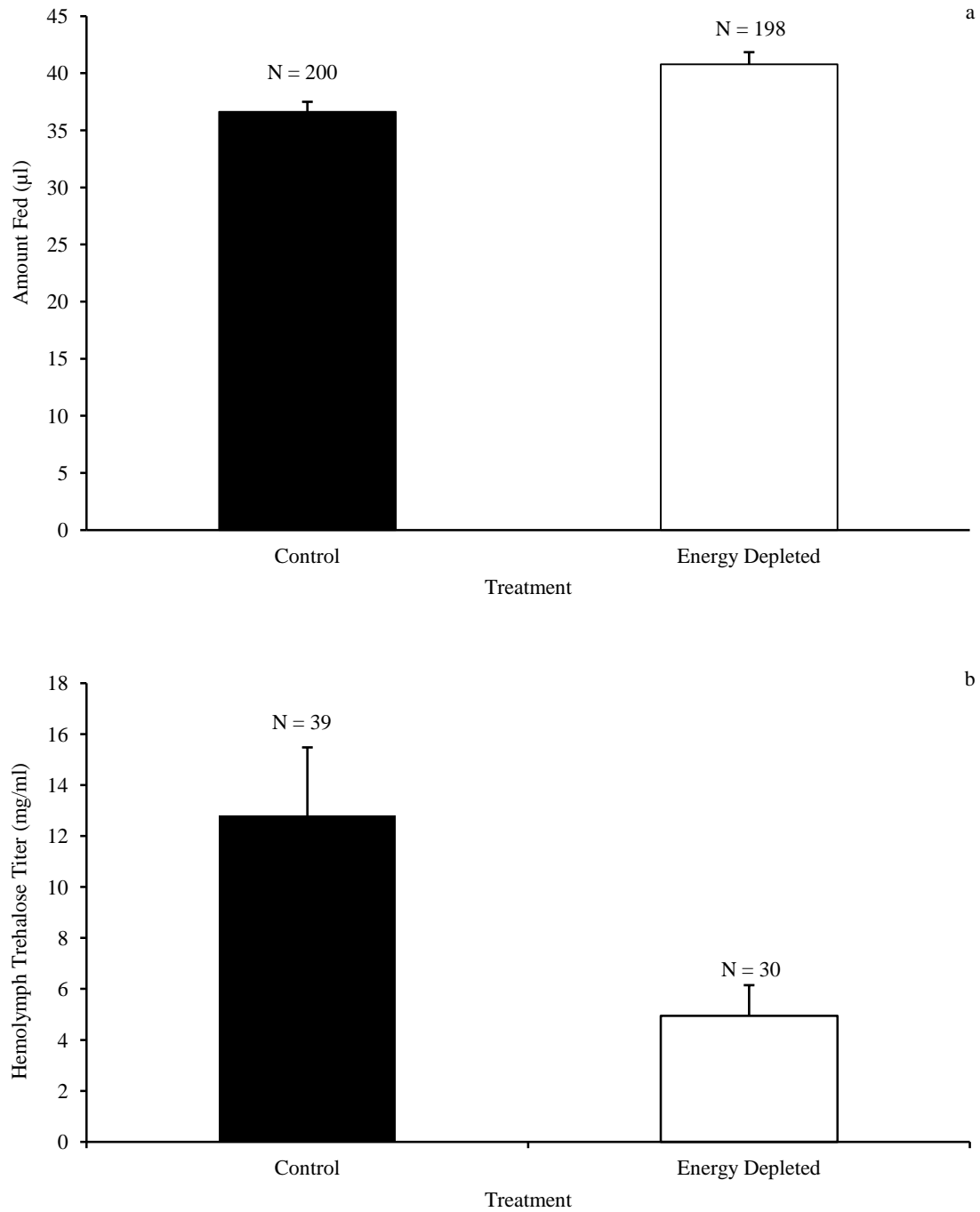
Fig. 4.1. Energetic states of control bees fed with 30% sucrose solution, and energy depleted bees with 30% sucrose solution mixed with 2% sorbose, in terms of (a) amount of sucrose solution consumed per day, and (b) hemolymph trehalose levels at the end of the experiment. In each case, data represent means with standard error bars with the respective sample sizes indicated above each bar.
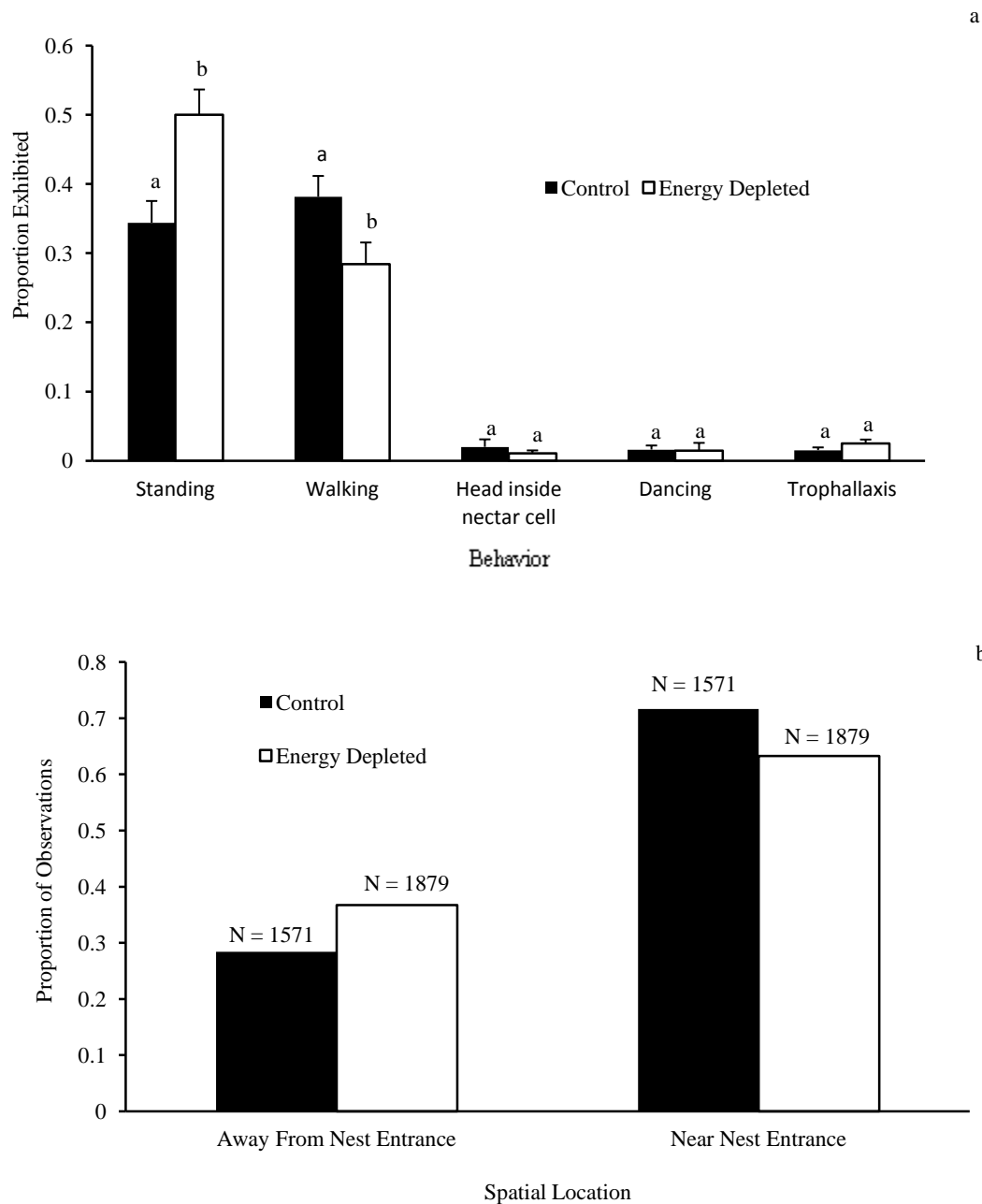
Fig. 4.2. Proportion of (a) various in-hive behaviors performed by bees in the control (N = 59) and energy depleted group (N = 62) with data representing means with standard errors and the letter above each bar representing significant differences between the two groups at α = 0.05, and (b) observations in which control and energy depleted bees were found near and away from the entrance of the hive with the number of observations given above each bar.
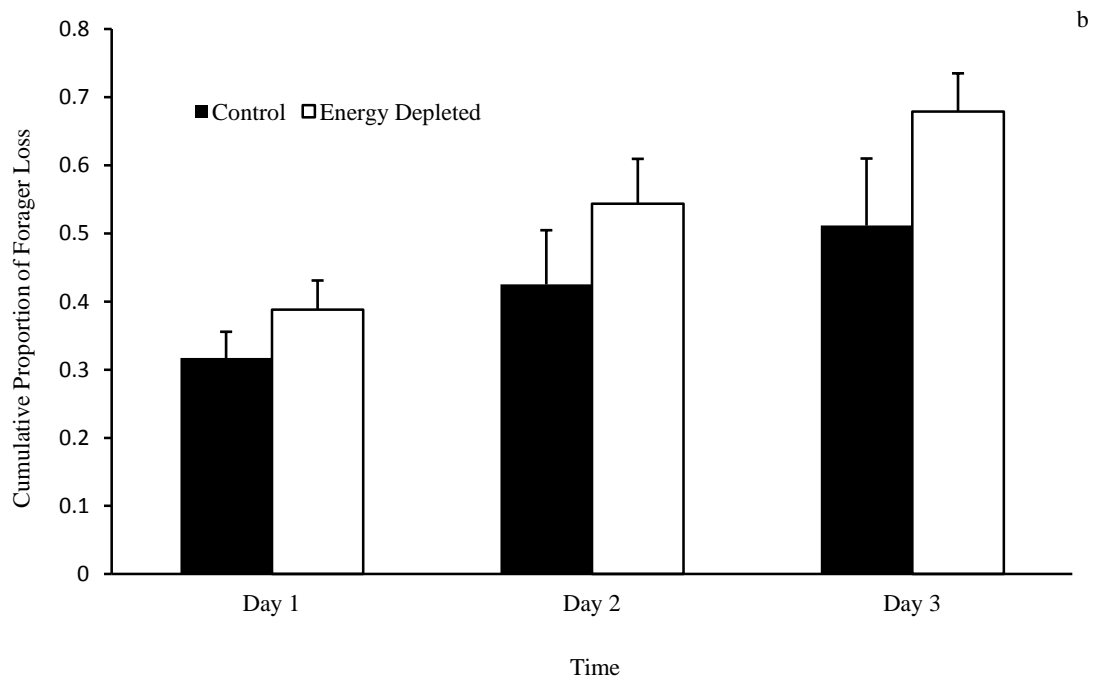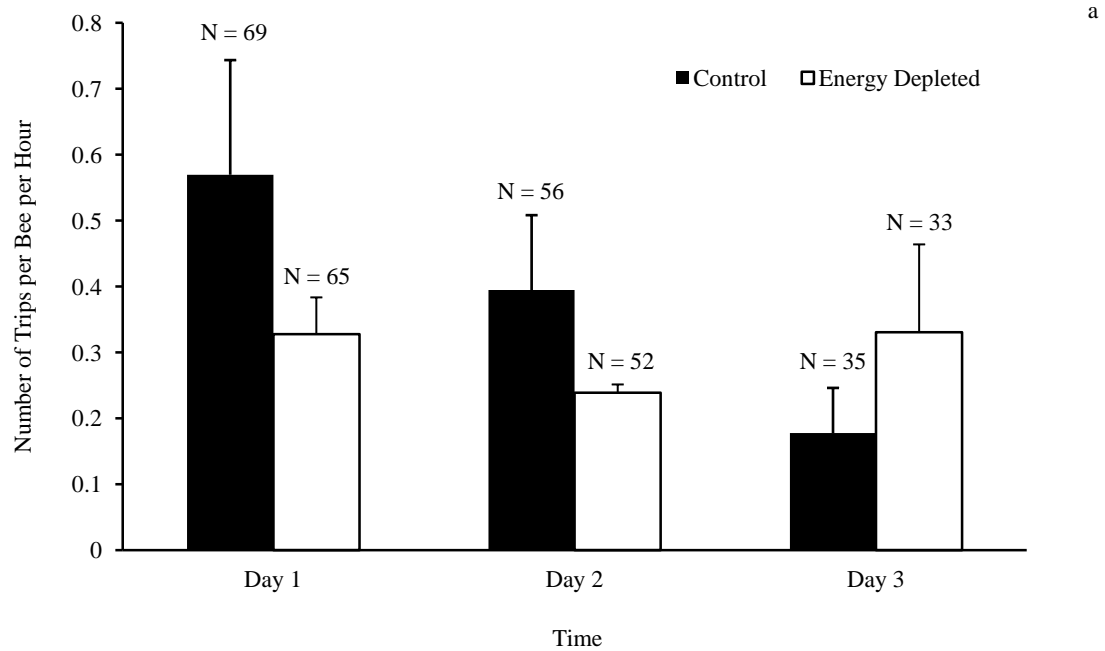
Fig. 4.3. Foraging behavior of control and energy depleted bees across the three days of the experiment in terms of (a) mean foraging frequency per bee per hour, and (b) proportion of forager loss. Data represent means across all the experimental replicates with corresponding standard error bars.

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