



Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection

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ABSTRACT

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.

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1. 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 hypopharyngeal 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.

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2. Material and methods

2.1. 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.

2.2. 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).

2.3. 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.

2.4. Survival experiment

After strapping, the bees were fed once with either 0 μ l, 5 μ l, 10 μ l, 20 μ l, 30 μ l 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.

2.5. 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 2.5×10^5 or more (some bees had a spore count as high as 2.5×10^6 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).

3. Results

3.1. 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.

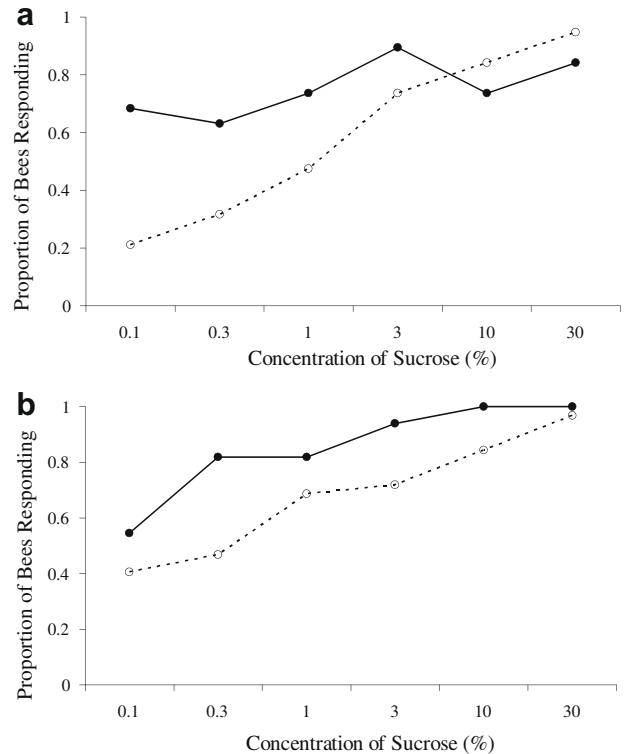


Fig. 1. Responsiveness of infected (●) and control (○) bees to sucrose solution of different concentrations in: (a) colony 1 (228 antennal probes from 19 control and 19 infected bees) and (b) colony 2 (390 antennal probes from 32 control and 33 infected bees). Proportion of responses is overall higher in colony 2 in comparison to colony 1 but the responsiveness of infected bees is higher than control bees within each colony.

3.2. 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.

3.3. 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

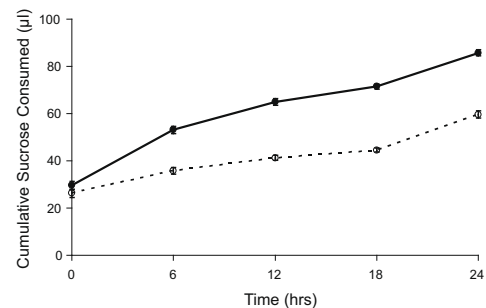


Fig. 2. Cumulative consumption of 30% sucrose solution by infected (●) and control (○) bees until satiation, measured every 6 h for 24 h. Data represent mean values for infected ($N = 57$) and control ($N = 44$) bees with standard error bars.

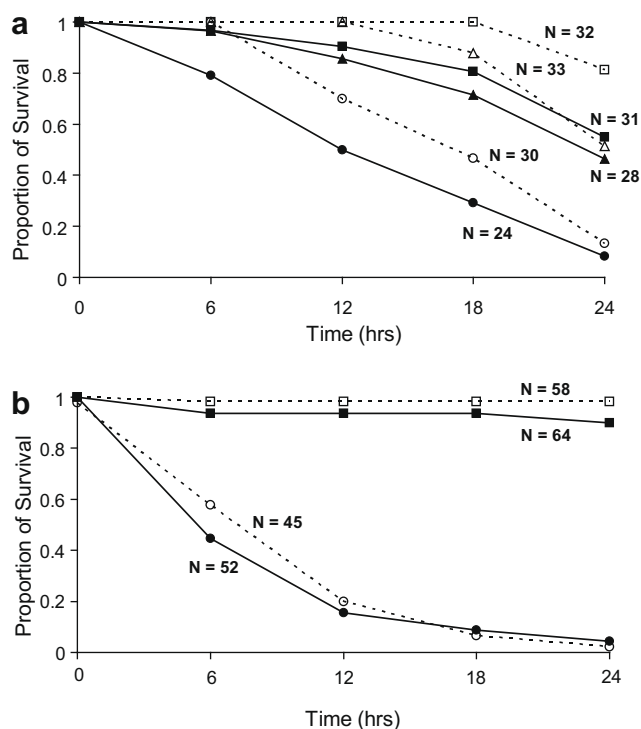


Fig. 3. Survival of infected (filled shapes) and control (empty shapes) bees fed with: (a) 5 µl (circles), 20 µl (triangles), and 30 µl (squares) and (b) 0 µl (circles) and *ad libitum* (squares), amounts of 30% sucrose solution. The number of bees tested to construct each survival curve is given against each line, the 10 µl amount is not shown for clarity but was included in the analysis.

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*.

4. 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 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 (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 hypopharyngeal gland (Hassanein, 1953; Wang and Moeller, 1971). If *Nosema* infected bees are indeed hungrier, the riskier foraging observed for such bees (Woyciechowski and Kozłowski, 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.

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