A Comparative Approach to Studying Sickness Mediated Behavioural Changes

by

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I dedicate my work to my grandfather.

Thank you for everything.

You are remembered.

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Abstract

Immune activation alters behaviour in animals across phyla (i.e. sickness behaviour) and is thought to aid recovery from infection. Hypotheses regarding the adaptive function of different sickness behaviours (e.g. decreased movement and appetite) include the energy conservation and predator avoidance hypotheses. These hypotheses were originally developed for mammals (Hart, 1988), however similar sickness behaviours are also observed in insects (e.g., crickets). Based on these hypotheses, we predicted that immune-challenged crickets (*Gyrllus texensis*) would reduce general activity and increase shelter use. We found evidence of illness induced anorexia in adult and nymph insects, consistent with previous research (Adamo, et al., 2010) and increased grooming (contrary to expectations), but no evidence that crickets decreased general activities (e.g., locomotion or exploration) or increased shelter use in response to immune challenge. We should expand upon Hart's hypotheses for a more complete understanding of the adaptive nature of sickness behaviour.

List of Abbreviations and Symbols Used

AKH	Adipokinetic Hormone
CI	Confidence Interval
IL-1	Interleukin-1
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
LPS	Lipopolysaccharide
TNF	Tumour Necrosis Factor

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Chapter 1 - Introduction

Animals change their behaviour during illness. A sick animal typically runs a fever and becomes anti-social, lethargic and disinterested in food (Dantzer & Kelley, 2007). While this is generally considered common knowledge, it is only since the 1980's that we have begun to understand the significance of these behavioural changes and the role they play in aiding recovery. Hart (1988) was among the first to suggest that the behaviour of sick animals, termed "sickness behaviours" (Dantzer & Kelley, 1989), could be adaptive. Hart (1988) suggested that this common behavioural shift was a host-mediated response to enhance disease resistance and not an unfortunate side effect of the immune system or a direct effect of the triggering pathogen. But to understand the behavioural changes associated with sickness behaviour we must first understand the physiological mechanisms that facilitate recovery from infection.

1.1 Fever

Fever was once thought to be a direct effect of bacteria (Beeson, 1947), as injection of bacteria in the periphery would cause body temperature to rise. The mechanism was thought to be through secretion of a pyrogenic factor from bacteria (pyrogens) due to the almost universal accompaniment of fever with bacteria and infection (Beeson, 1947; Beeson, 1948). However, injection of sterile fluids was also reported to induce fever and inflammation, albeit shorter in duration than the fever and inflammation caused by injection of bacteria (Bennett, 1948b; Bennett & Beeson, 1950). Thus while bacteria may induce fever, some factors secreted by the host in response to foreign substances could also elicit the febrile response. Later these factors would be

isolated (Dinarello, Goldin & Wolff, 1974; Murphy, Chesney & Wood, 1974) and known as cytokines.

Cytokines are immunomodulatory proteins that regulate immune function (Kelley, et. al., 2003) (see figure 1.1). Proinflammatory cytokines (e.g., interleukin 6 (IL-6), IL-1 and tumour necrosis factor (TNF)) are typically released by immune cells (e.g., macrophages) in response to injury or invading pathogens and facilitate inflammation and fever during illness (Dinarello, 2000). A series of experiments by King and Wood (1958a; 1958b; 1958c) suggested that proinflammatory cytokines are necessary to convey information about peripheral infection to the brain. The hypothalamus, which is the thermoregulatory center of the brain (Morrison, & Nakamura, 2011), is a key cytokine target (Dantzer, 2001). Cytokines appear to stimulate afferent nerves to convey information about infection in the periphery to the brain (Konsman, Kelley & Dantzer, 1999; Layé, et. al., 1994; Maier & Watkins, 1999; Watkins, Maier & Goehler, 1995). Thus, fever is the result of the hypothalamus increasing the body's thermal homeostatic set point, in response to information sent to the brain via proinflammatory cytokines.

Endothermic animals (e.g. mammals) can increase heat production via shifts in their metabolism (metabolic fever) and maintain it through upregulation of metabolism or conservation of heat. Ectotherms (e.g. reptiles and invertebrates) experience fever as well, but because they are dependent on external sources for heat an increase in body temperature requires a shift in thermoregulatory behaviour (Deen & Hutchison, 2001) (e.g., such as seeking out warmer places), in what is known as behavioural fever (Kluger, 1978). Behavioural fever was first discovered by Vaughn, Bernheim and Kluger (1974) in the desert iguana (*Dipsosaurus dorsalis*). In their experiment, immune-challenged

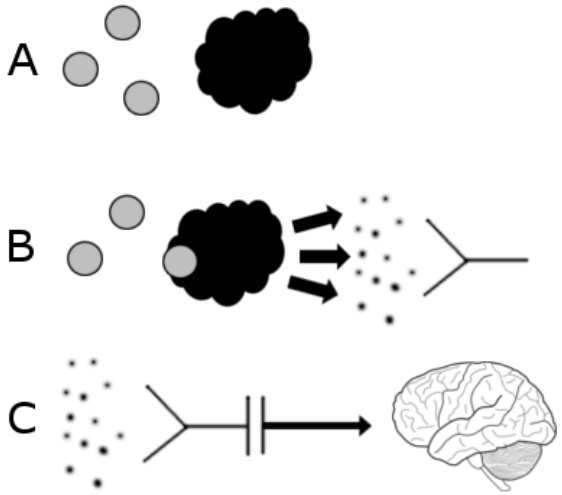

Figure 1.1. (A) Bacteria is detected by macrophages. (B) Macrophages produce cytokines when they have consumed bacteria. (C) Cytokines signal infection in the periphery to the brain. Adapted from Kelley et al. (2003).

desert iguanas were introduced to a long arena with a temperature gradient that ranged from room temperature up to 50°C and were allowed to move freely while their temperature was recorded. Compared to controls, immune challenged desert iguana reached body temperatures comparable to fever in mammals (Vaughn, Bernheim & Kluger, 1974) which was found to decrease mortality in response to live infection (Bernheim & Kluger, 1976).

Sickness behaviour and fever are closely linked as they are both induced by an immune response (Hart, 1988) and research on fever and immune mechanisms has demonstrated that robust immune/neural connections exist in animals (Khardori, Adamski & Khardori, 2007; Ottiviani & Franceschi, 1998). Hart (1988) noted that the febrile response was one of the most energetically expensive responses mammals made during infection. Metabolic fever is indeed a costly endeavour and it has been reported that increasing body temperature by 1 °C requires a 13% increase in metabolism (Baracos, Whitmore, & Gale, 1987; Kluger, 1991). For animals in the wild with limited

resources, Hart (1988) reasoned that fever coupled with the decreased desire to seek out resources could lead to a deathly fuel drain. Concerns over the costs of fever drove many of Hart's hypotheses about the adaptive nature of sickness behaviour.

1.2 Hart's hypotheses on the adaptive nature of sickness behaviour

Cytokines are able to alter behaviour because neurons have receptors for them (Dantzer & Kelley, 2007). Cytokine-mediated behavioural changes include lethargy (decreased general activity and sleepiness), anorexia (decreased food intake), adipsia (decreased water intake), depression or anhedonia (decreased interest in pleasurable or social activities), decreased grooming (often negatively affecting physical appearance as a result) and decreased libido or fecundity (decrease in sexual activity or decreased number or health of total offspring). These behavioural changes are often also accompanied by fever (as previously discussed) and a number of other physiological changes such as hyperalgesia (increased sensitivity to pain) and a reduction in blood micronutrients (such as iron or zinc).

Hart (1988) proposed two theories of how sickness behaviour could be adaptive. The first hypothesis, the energy conservation hypothesis, states that sickness behaviours cause the animal to conserve energy. Heat conservation is important to reduce the energetic expense involved with fever (Hart, 1988; Kluger, 1991). Therefore, decreasing the desire for resources such as food, water or mates, should encourage animals to remain sheltered and curled up, preventing heat loss and diverting available energy into the immune response. Illness-induced anorexia is suggested to decrease the exposure to contaminated food to and conserve energy that would have been expended searching for food (Hart, 1988), but it may play some other role in survival as well. Murray and

Murray (1979) found that mice that were force fed during an immune challenge had a significantly higher mortality than immune challenged mice who were allowed to feed freely. Hart (1988) suggests that illness induced anorexia may also be adaptive because it restricts the supply of micronutrients, such as iron, that may aid in the proliferation of pathogens. During inflammation, iron is stored in reserve causing a notable decrease in blood iron content (Hershko, Cook & Finch, 1974), suggesting that avoiding even trace amounts of micronutrients may have an effect on recovery. The second hypothesis proposed by Hart, the predator avoidance hypothesis, states that sickness behaviours are adaptive because they decrease the risk of predation. Sickness is a period of vulnerability and certainly by decreasing the motivation to seek out resources there is a reduced risk of predation while the animal is lethargic and perhaps not able to respond to threats as quickly. Aubert (1999) suggests that if sickness behaviour is a motivational shift, then it follows that animals should be able to adapt their motivations to the situation and even anticipate future needs, even going so far as to supress sickness behaviours if necessary. While sickness behaviour could be suppressed in order to flee from a predator, it is still in the best interest of a sick animal to avoid predators entirely (Aubert, 1999; Hart, 1988).

Aubert et al. (1997) subjected rat mothers rearing pups to warm and cold temperatures while facing an immune-challenge and observed the effect on nest building behaviour and retrieval of pups. Immune-challenged mothers reduced nest building while in warm temperatures but increased nest building in colder temperatures. Regardless of temperature though, immune-challenged mothers consistently retrieved their pups when they were moved from her. The pups are dependent on the mother for body temperature thus regardless of the temperature or condition of the mother, so she must protect them

(Aubert et al., 1997). Likewise, there is more pressure on the mother to have a warmer nest in a colder environment. The energy investment involved in nest building during colder temperatures would enhance energy conservation in the long run as it would prevent the loss of body heat, allowing her and her pups to survive. Thus, Aubert et al. (1997) suggest this change in behaviour is an adaptive motivational shift and ultimately does conform to Hart's hypotheses.

Durazzo, Proud and Demas (2008) found that Siberian dwarf hamsters (*Phodopus sungorus*) decrease feeding when faced with an immune challenge, which is consistent with the energy conservation hypothesis, except that they continue to hoard food. Durazzo et al. (2008) suggest that different physiological processes may regulate food intake and food hording, which could be adaptive as this would allow the animal to have a readily available source of food for when they overcome the illness. However, even if fighting off illness drains the hamster of energy to the point that later foraging becomes risky, the act of foraging while sick could just as easily expose the animal to predators at a time of weakness and expend energy that could have been used to fight the illness. Alternatively, if the animal does not perceive any potential risk of predation perhaps it is more likely to take a risk to forage for food while sick. Furthermore, if food is scarce in their natural environment, it may not be feasible for them to give up time foraging, even when sick. Regardless, while this behaviour may be adaptive and shows a motivational shift based on the anticipation of future needs (Aubert, 1999), it is questionable whether it is in line with either of Hart's hypotheses.

Stress or otherwise physically demanding situations are also known to suppress immune function (Råberg, et. al., 1998). Owen-Ashley, Hasselquist and Wingfield (2004)

found that in response to an immune challenge, song sparrows (*Melospiza melodia*) decreased territorial behaviour in the winter and supressed sickness behaviour during the spring, which is the mating season for this species. Owen-Ashley et al. (2004) suggest this seasonal suppression of sickness behaviour must be an adaptive response to illness; if the animal suspends territorial behaviour and misses out on mating, it has to wait until next year for the chance to mate again. In this way the risks involved with supressing immune function must be outweighed by the potential gain in fitness during the mating season. Administration of testosterone has also been found to supress sickness behaviour in white crowned sparrow (*Zonotrichia leucophrys gambelii*) (Owen-Ashley, et al., 2009). Similarly, Boonstra, McColl and Karels (2001) found that during mating season, male arctic ground squirrels (*Spermophiluts parryii plesiuts*) show extreme stress-induced immunosuppression and often do not survive past this time. However, Boonstra et al. (2001) suggest that this behaviour may be adaptive. This is a strategy called terminal reproductive investment, in which the animal emphasizes reproductive fitness over survival. In this case, trading off immune function, capitalizing on limited reproductive opportunities, but potentially reducing its lifespan. Suppression of sickness behaviour must be adaptive in circumstances where it enhances reproductive output (regardless of the costs) and is further example of how sickness behaviour is a motivational shift.

While fever is one of the most ubiquitous responses of the innate immune system to illness, Owen-Ashley and Wingfield (2007) note that some species instead employ hypothermia in response to an immune challenge. While some birds do exhibit fever in response to illness (Bonneaud, et al., 2003; Johnson, et al., 1993), passerine birds often

maintain a higher body temperature (41°C) than mammals. Thus Owen-Ashley and Wingfield (2007) speculate that for some birds, raising body temperature through a febrile response may not be feasible. Passerines experience a high rate of heat loss because of their small surface to body ratio (Owen-Ashley & Wingfield, 2007), something that is also experienced by newborn chickens and rats due to their inability to thermoregulate (Aubert, et al., 1997). Bats also do not appear to exhibit fever when infected, but instead seem to experience febrile core body temperature when in flight (O'Shea, et. al., 2014). Bats also experience regular torpor, which is an energy conservation mechanism that decreases metabolism to as low as 1% of baseline and body temperature to subfreezing (Bouma, Carey & Kroese, 2010). While it is unclear if this has an effect on pathogen tolerance or resistance, the lack of fever and the expenditure of energy involved in stimulating febrile temperatures violates Hart's energy conservation hypothesis. Furthermore, behavioural fever (in the case of ectotherms) violates both of Hart's hypotheses. Seeking out warm environments expends energy that could be diverted towards the immune response and the act of increasing body temperature requires the animal to expose itself to open, heated areas, therefore exposing it to predators as well. These exceptions to Hart's hypotheses that suggest there may be gaps in our understanding of sickness behaviour.

Sickness behaviour is well researched and documented in mammals and while there is certainly evidence to support Hart's hypotheses (Aubert, 1999; Dantzer & Kelley, 2007; Johnson, 2002) sickness behaviour is observed in animals across phyla (Adamo, 2006; Llewellyn, et. al., 2005; Maier & Watkins, 1999; Ottiviani & Franceschi, 1998). There is also evidence that cytokine-like molecules influence behavioural and neural

function in invertebrates, similar to vertebrates (e.g., Adamo, 2008a) but while these cytokine-like molecules have a similar function to cytokines, structurally they are unrelated (Beschin et al., 2001). The existence of sickness behaviour in animals across phyla (See table 1.1) suggests that connections between immune systems and nervous systems are either very ancient, or have evolved independently in several phyla. Regardless, it appears that these connections are critical for animals. Below I review the similarities and differences in sickness behaviour in animals across phyla in search for potentially conserved adaptive functions.

1.3 Sickness Behaviour across Phyla

1.3.1 Lethargy

Lethargy is easily observed and is characterised by reduced locomotion or general activity, sometimes through inducing or increasing sleep. Injection of heat-killed bacteria (Toth & Krueger, 1988; 1989) and TNF (Kapas & Krueger, 1992) have been found to alter sleep patterns in rabbits, increasing non-rapid eye movement sleep. Inhibition of TNF suppresses the changes in sleep pattern (Takahashi, Kapas & Krueger, 1996; Takahashi & Krueger, 1997) suggesting that illness-induced lethargy is driven by proinflammatory cytokines. Lethargy is found in humans (Vollmer-Conna et al., 2004), rodents (Hopwood, Maswanganyi, & Harden, 2009; Hübschle, et al., 2006) and among nearly every mammal investigated (Hart, 1988) and is almost as ubiquitous a response to illness across phyla as fever.

Chickens (*Gallus gallus domesticus*) (Johnson et al., 1993) and house sparrows (*Passer domesticus*) (Bonneaud et al., 2003) run a fever and become lethargic in response to LPS injection. Immune challenged zebra finches (*Taeniopygia guttata*) become

Table 1.1: *Comparison of sickness behaviour across multiple animal species. Citations selected as notable experiments or reviews. Notable exceptions or contradictions are in included in brackets. All selected research included an inert pathogen to show a behavioural change.*

Vertebrates	**Lethargy**	**Anorexia**	**Decreased Grooming**	**Decreased Fecundity**	**Shelter Use**
Mammalia	1	2	3	4, 5 [6]	6
Aves	7, [8, 9]	10, 11	9	11, [10]	12
Reptilia	[13]	14	-	15	-
Amphibia	16	16	-	-	-
Teleostei	17	18	-	-	-
Selected Insect Orders					
Diptera	-	19	20	19	-
Lepidoptera	21	21, 22	-	-	-
Hymenoptera	[23, 24]	[23]	[24*]	-	[25]
Orthoptera	-	26	-	27 [28, 29]	[30]

1. Hopwood et al. (2009)
2. Aubert et al. (1995)
3. Tikhonova et al. (2011)
4. Yirmiya et al. (1995)
5. Yirmiya (1996)
6. Aubert et al. (1997)
7. Burness et al. (2010)
8. Lee et al. (2005)
9. Marais et al (2013)
10. Owen-Ashley et al. (2006)
11. Bonneaud et al. (2003)
12: Nord et al (2014)
13. Deen & Hutchison (2001)
14. Schumacher (1997)
15. Meylan et al. (2013)
16. Llewellyn et al. (2011)
17: Bjørge et al (2011)
18. Volkoff and Peter (2004)
19. Bashir-Tanoli & Tinsley (2014)
20. Yanagawa, et al. (2014)
21. Dunn et al. (1994)
22. Adamo et al. (2007)
23. Tyler et al. (2006)
24. Aubert & Richard (2008)
25: Alaux et al (2012)
26. Adamo et al. (2010)
27. Stahlschmidt, et al., (2013)
28. Adamo (1999)
29. Adamo, et al. (2015)
30: Otti et al (2012)

** No change in self grooming behaviour and increase in grooming behaviour of nestmates*

lethargic but also increase sleep when subjected to colder environments compared to those who were immune challenged and subjected to warmer temperatures (Burness, et al., 2010). Adelman, et al (2010a) studied two populations of song sparrows that lived in California (warmer native climate) and Washington (cooler native climate) and brought them into a laboratory environment. In response to the same LPS injection, song sparrows from a warmer climate presented with greater fever and a prolonged period of lethargy (over 24H) than song sparrows from the cooler climate, which had a shorter period of lethargy (less than 24H) and presented with less fever. Adelman, et al (2010a) suggested that this may be for territorial purposes and that there may be stronger

territorial pressure for the Washington population than the California population (Adelman, et al., 2010b). At first this may appear to be a case of stress-induced immunosuppression, but corticosterone levels were elevated in both populations of immune challenged animals and not significantly different between the populations (Adelman, et al., 2010a). Adelman et al (2010a) note that in response to immune challenge, the California population had a higher level of circulating IL-6 compared to the Washington population. Thus they concluded that selective regional pressures may have altered immune function of the two populations which in turn resulted in variation in behavioural patterns as well.

As previously discussed, ectotherms, such as reptiles, experience behavioural fever and must seek out warmer areas in order to raise their body temperature. If doing so would expose them to predators, it stands to reason that lethargy would be a maladaptive behavioural change. But lethargy is reported as a common response to many infections in reptiles (Schumacher, 1997). Despite this, there has been limited research on sickness behaviour in reptiles outside of behavioural fever. However, similar to birds, reptiles sometimes respond differently to an immune challenge depending on the temperature they have been acclimatised to while in their youth. Deen and Hutchison (2001) acclimatized juvenile green iguanas (*Iguana iguana*) to warm and cold environments before administering an immune challenge. Animals in the warm environment exhibited behavioural fever, as would be expected from previous research. But animals in the cold environment lowered their body temperature, exhibiting behavioural hypothermia. While the energy cost of fever is less in ectotherms than that in endotherms there is still a cost (Seebacher & Franklin, 2005). Deen and Hutchison (2001) note the greater the mean

mass and size of the juvenile iguana, the more likely it was to employ fever over hypothermia, regardless of its previously acclimated environment. Thus larger iguanas may be better suited to handle the metabolic cost involved in fever and are large enough to maintain their body heat, while less suited iguanas employ hypothermia. If this were true, then it is likely that healthy adult iguanas would employ fever over hypothermia regardless of their previously acclimated environment. It is also unclear how behavioural hypothermia would affect mortality in a live challenge in iguanas, but this strategy is employed by other reptiles in response to infection. Dunlap and Church (1996) found that western fence lizards (*Sceloporus occidentalis*) injected with IL-1β, a proinflammatory cytokine released during the innate immune response, spent more time burrowed in sand than controls. Though they did not measure body temperature in this study, Dunlap and Church (1996) note based on previous research, that burrowed fence lizards do experience cooler body temperatures than those that are exposed (Sinervo & Dunlap, 1995). Shelter and cover (i.e., burrowing) come with cooler temperatures than direct sun, decreasing the risk of exposure to predators and conserving energy that can be diverted to the immune response by decreased overall activity. In a way, behavioural hypothermia is more in line with Hart's hypotheses than behavioural fever, as it facilitates reduced activity in a way that would reduce predation risks. However it is not a universal strategy and seems to be contingent on the size of the animal.

Behavioural fever is also observed in amphibians (Bicego et al., 2010) and in fish (Cabanac & Laberge, 1998). Research on sickness behaviour is limited in these animals, but exposure to live bacteria is reported to induce lethargy in fish (Harikrishnan et al., 2010) and in African clawed frogs (*Xenopus laevis*) (Fremont-Rahl, et al., 2011;

Hubbard, 1981). Interestingly, Llewellyn et al. (2011) found that while the cane toad (*Bufo marinus*) becomes lethargic in response to an immune challenge, the animal would not move from its starting position and thus would become lethargic instead of demonstrating behavioural fever. Llewellyn et al. (2011) speculated that the cane toad may favour energy conservation over inducing febrile temperatures. While illness induces behavioural fever and lethargy, they may sometimes be mutually exclusive; reduced activity level is not conducive to seeking out warmer environments, which again is not conducive to avoiding predators.

Research on invertebrate sickness behaviour is even more limited. Immune challenge is reported to induce lethargy in the common octopus (*Octopus vulgaris*) (Locatello, et al., 2013) and live pathogen exposure is reported to induce lethargy in a variety of crustaceans (Crockford, 2001; Klaphake, 2009). Contrary to this behaviour, Arthurs and Thomas (2001) found that exposure to a fungal pathogen initially increased locomotion in desert locusts (*Schistocerca gregaria*) but subsequently decreased their ability to escape a simulated predator attack. Arthurs and Thomas (2001) indicate that their experimental design did not accommodate for behavioural fever (i.e., temperature remained constant) and thus the initial increase in locomotion in the desert locusts could have been an attempt to find an appropriate place to increase their body temperature. But even if the desert locusts found an appropriate place to raise their body temperature, doing so would come at the increased risk of predators, which Arthurs and Thomas (2001) indicate would be a legitimate concern.

1.3.2 Anorexia and Adipsia

Reduction in appetite is a predictable response following infection and is well studied in mammals (Exton, 1997; Khardori, et al., 2007; Langhans, 1996; Vollmer-Conna, et al., 2004). Adipsia, a reduction in water intake, is also a common behavioural change in mammals in response to illness (Cross-Mellor, et al., 2000; Hübschle, et al., 2006; Vollmer-Conna, et al., 2004). Aubert et al. (1995) found that immune challenged rats reduced their overall dietary intake relative to controls. But when given access to protein-, fat- and carbohydrate-based diets, immune challenged rats ate more carbohydrates and less protein (Aubert, et al., 1995). Rats and mice injected with the proinflammatory cytokine IL-1 also reduce their appetite in a similar fashion to immune-challenged animals (Aubert, et al., 1995; Kent, et al., 1996) suggesting that loss of appetite is a host response. As previously discussed, illness induced anorexia also appears to be a motivational shift, as it inhibits appetite but not hoarding behaviour in Siberian hamsters (Durazzo, et al., 2008). In rats, the anorexia observed in response to illness is still present even when fever is supressed with medication (McCarthy, Kluger & Vander, 1984).

Illness induced anorexia and adipsia are found in birds as well (Webel, Johnson & Baker, 1998). Burness et al. (2010) studied the effects of immune challenge on zebra finches in two temperature conditions, neutral and cold, hypothesizing that animals in the cold environment would have a reduced immune response as a trade-off for staying warm. While they found that animals in both environmental conditions had reduced body mass, immune-challenged animals in both temperature groups showed reduced appetite. Owen-Ashley et al. (2006) found that white crowned sparrows show reduced appetite and water intake in response to an immune challenge, however, males also show seasonal

variations in their immune response. Greater weight loss was observed in males that experienced longer days than males that experienced shorter days. Adelman et al. (2010a; 2010b) found regional variations in lethargy and fever between two populations of song sparrows, but both populations had the same pattern of reduced appetite. Testosterone has been found to supress sickness behaviour in white crowned sparrow, returning appetite and water intake to those of control animals (Ashley, et al., 2009). Thus, the regional and seasonal variations in immune response in birds must be related to the selective pressures put on the animals by a short mating season, which they respond to by supressing immune function (Adelman, et al., 2010b; Owen-Ashley, et al., 2006).

Reduced appetite is reported as a common result of infection in reptiles (Schumacher, 1997), frogs (Fremont-Rahl, et al., 2011; Hubbard, 1981) and fish (Harikrishnan, et al., 2010). Llewellyn et al. (2011) found that an immune-challenge reduced appetite in cane toads, but drinking could not be measured directly in cane toads, as these animals take in water through the skin. Instead, level of hydration was measured and was found to remain unchanged across conditions. Volkoff and Peter (2004) found that there was a dose dependent effect of immune-challenge on appetite in goldfish (*Carassius auratus*), with higher doses leading to greater decrease of appetite. However, instead of adipsia, Best, Eddy and Codd (2003) observed increased drinking in response to an immune challenge in rainbow trout (*Oncorhynchus mykiss*). Drinking was assessed by measuring the water content of the gut, with immune challenged animals having higher gut water content than saline injected controls. They suggested that this may not actually be an adaptive behaviour, as increased drinking would increase potential

exposure to toxins, and potentially increase the rate of exposure of the pathogen to the rest of the population through increased water exhalation.

Illness induced anorexia is reported in crustaceans (Crockford, 2001; Lightner, & Redman, 1985). Locatello, et al. (2013) tested the effect of an immune challenge on behaviour in the common octopus. After receiving an immune challenge they did not exhibit illness induced anorexia and still accepted food readily after the experiment (Locatello, et al., 2013). Despite this, immune-challenged animals had reduced body mass compared to controls. Ayres & Schneider (2009) found that fruit flies (*Drosophila melanogaster*) became anorexic in response to a dose of live bacteria. However, while anorexia reduced mortality in response to a dose of *Salmonella typhimurium*, anorexia increased mortality in response to a dose of *Listeria monocytogenes*, suggesting a complicated effect of anorexia on different live pathogens (Ayres & Schneider, 2009).

There has been extensive work investigating illness induced anorexia in lepidopterans. Retnakaran, Lauzon and Fast (1983) found that live *Bacillus thuringiensis* induced anorexia in the spruce budworm (*Choristoneura fumiferana*). Dunn, Bohnert and Russell (1994) reported that in response to an immune challenge, the tobacco hornworm (*Manduca sexta*) consistently reduced feeding and developed slower, an effect they referred to as "malaise syndrome." Further investigation of *M. sexta* revealed a distinct pattern of reduced eating, weight loss in 6 hours, and decreased excretion of frass (feces) leading to softer or liquid frass that returned to normal after 48 hours. They suggested that this change in frass coupled with illness induced anorexia may be a method to empty the gut and therefore reduce continued exposure to ingested pathogens (Dunn, et al., 1994). However, Adamo et al. (2007) found that immune challenged *M. sexta* did not

actively avoid their current food source and were not more susceptible to infection when force-fed food containing live bacteria. Furthermore force-feeding did not affect the mortality of the immune challenged animals, thus providing evidence against the conclusions of Dunn et al. (1994). But Adamo et al. (2007) did find that force-feeding a high lipid diet decreased mortality in immune challenged animals, but not controls. Adamo et al. (2007) suggested there may be a conflict between immune function and the digestion of fats and therefore illness-induced anorexia helps to prevent the animal from further ingestion of fats.

In orthopterans, Adamo et al., (2010) found that the Texas field cricket (*Gryllus texensis*) shifted its dietary preference in response to immune challenge. Adamo et al. (2010) hypothesized that immune challenged crickets should reduce fat intake in their diet, due to the limited availability of the multifunctional protein apolipophorin III. Apolipophorin III functions as an immune surveillance molecule, recognizing and binding to bacteria in order to enhance immune function (Halwani, Niven & Dunphy, 2001). But apolipophorin III also functions as a lipid transporter during times of stress; stress hormones (e.g., Octopamine and Adipokinetic Hormone (AKH)) release lipid reserves to compensate for increased energy consumption (Weers, & Ryan, 2006). Therefore, while apolipophorin III is functioning as a lipid transporter, it does not serve its role in immune surveillance. Amidst a variety of food choices, Adamo et al. (2010) found that immune challenged crickets shifted their preference towards foods containing low fat. This behavioural shift, manifesting as a preference for low lipid food, would allow apolipophorin III to continue perform its role in immune function, thus enhancing

survival. This suggests that sickness mediated behavioural changes are an adaptive response to physiological limitations.

1.3.3 Depression, Anhedonia and Social Behaviour

Depression is a feeling of low mood and is a general term that covers a broad range of negative emotions and symptoms (American Psychiatric Association, 2013). But as it pertains to sickness behaviour, depressive behaviour is often defined as a decrease in the desire for social interaction along with anhedonia, which is a reduced desire for pleasurable stimuli (Willner, Muscat & Papp, 1992). Depression is reported in humans in response to the onset of chronic illness, such as cancer (Myers, 2008) and HIV (Brown, et al., 1992), though in such cases this may have less to do with the immune system and more to do with the negative mental impact this knowledge has on the patients. However, studies have found a link between proinflammatory cytokines and depression (Aubert, 1998; Schiepers, Wichers & Maes, 2005; Yirmiya, et al., 1999), reporting that people suffering from major depression have increased levels of proinflammatory cytokines (TNF-a & IL-6) compared to healthy controls (Dowlati, et al., 2010). In fact, suppression of proinflammatory cytokines in otherwise healthy patients has suggested to be an alternative treatment to depressive mood disorder (Miller, Maletic & Raison, 2009) and a great deal of research has been directed into investigating this method of treatment and the interaction between depression and the immune system (Dantzer, 2001; 2006; Dantzer & Kelley, 2007; Schiepers, et al., 2005).

Beyond humans, depression becomes harder to study. We cannot ask a rat or a finch if they are feeling depressed. However, we can observe whether they exhibit anhedonia or decrease their social activity in response to treatment and use these

observations as an approximation for the depressive behaviour seen in humans (Willner, Muscat & Papp, 1992; Yirmiya, 1996). In rats anhedonia is often measured by observing the animal's preference for water mixed with saccharine (a sweet substance that has no caloric value) over regular water; rats that are not experiencing depressive-like symptoms will show a preference for saccharine water, while rats experiencing depressive-like symptoms will show no preference (Willner, 1997). Yirmiya (1996) found that immune challenged rats still drank water but had no preference for saccharine water, in contrast to the controls which showed a preference for saccharine. Immune challenged animals also showed decreased sexual behaviour, and social exploration. However, administration of anti-depressant medication (imipramine) reversed the observed depressive behaviour in immune challenged animals, so that their level of preference for saccharine water, social exploration and sexual behaviour were no different from the control's. In contrast to the research in mammals, Lopes et al. (2012) found that the sickness behaviour of immune challenged zebra finches in a social environment was suppressed, while isolated immune challenged zebra finches expressed sickness behaviour normally.

While there have been no studies to assess depressive-like behavioural changes in reptiles, amphibians or fish, there have been a few studies on changes in invertebrate social behaviours. In a study by Locatello et al. (2013), common octopus were tested in chambers with a shelter and a window into another chamber containing a conspecific, which was another octopus not used in the experiment. Locatello et al. (2013) found that controls spent a portion of their time interacting with the conspecific through the window, while immune challenged subjects secluded themselves in shelters, significantly decreasing visual interaction with conspecifics. As previously discussed it is in the best

interest of a sick animal to find shelter away from potential predators at a time of weakness (sometimes even away from members of their own species), thus Locatello et al. (2013) suggest this is an adaptive response to illness.

In insects, Rueppell, Hayworth and Ross (2010) found that when honey bees (*Apis mellifera*) were exposed to lethal dose of a pathogen they abandoned their colony, resulting in what they referred to as altruistic suicide. Due to the altruistic nature of colony building hymenopteran insects, this is considered to be adaptive; one sick individual removing itself to avoid spreading infection is better for the reproductive fitness of the colony as a whole (Rueppell. et al., 2010). While Rueppell et al. (2010) did not include the use of bacterial pathogens, it seems logical to assume that a bacterially infected honeybee would engage in the same behaviour in order to avoid infecting its kin.

In contrast though, Traniello, Rosengaus and Savoie (2002) found that termite (*Zootermopsis angusticollis*) nymphs that were isolated following exposure to a fungal pathogen had higher mortality compared to exposed nymphs who were introduced back into a group. Traniello et al, (2002) suggest that this species may develop a shared immunity, wherein the more individuals that have been exposed and survive a particular pathogen, the better chance the colony has as a whole to survive. Traniello et al. (2002) note there are other benefits from social interaction, such as grooming and social food transfer that may impact immune function in an infected individual. It is unclear if group immunity would enhance immune function against other pathogens (e.g., bacteria).

1.3.4 Grooming

Decreased grooming is a common response to illness in mammals (Hollis, et al., 2006; Tikhonova, Kulikov and Kulikov, 2011). Hart (1988) suggests that decreased grooming may be an energy conservation tactic, as grooming is not essential for survival. Reducing grooming may also be a method for conserving water, which is expended during the grooming process and may be vital for sick animals to conserve (Hart, 1988). In birds, Burness et al. (2010) found that immune challenged zebra finches exposed to high temperatures reduced their grooming behaviour compared to the amount of grooming they performed at baseline, while zebra finches exposed to cooler temperatures did not change their grooming behaviour, though at baseline the finches exposed to cooler temperatures groomed less than finches exposed to warmer temperatures.

There has been a little research on immune activated changes in grooming behaviour with invertebrates. Lightner and Redman (1985) have observed reduced grooming in several species of shrimp in response to a viral infection, which is in line with the energy conservation hypothesis. In contrast, Hurst, Stevenson and Wright (2014) have reported that, in response to injection or ingestion of toxic substances, honey bees decrease their general activity, but increase the amount of time they spend grooming. While this is in contrast to what is seen in other animals, Hurst et al. (2014) indicate that grooming in insects is used to remove ectoparasites, thus it may still be in the best interest of the animal to continue grooming if they can survive their encounter with the pathogen.

1.3.5 Libido and Fecundity

Libido, the interest or participation in sexual behaviour, is often decreased in mammals in response to immune challenge. For instance, Yirmiya et al. (1995), found that injection of IL-1 decreased sexual activity in female rats, while males acted normally. They speculated that it would be adaptive for females to reduce sexual activities while sick, as the energy requirements and complications that could arise from pregnancy while ill were too great. Yirmiya (1996) found that an immune challenge was sufficient to decrease sexual activity in male rats. Yirmiya (1996) did not include female rats in this part of their study, but given the results of Yirmiya et al. (1995) it seems likely that females would also decrease their sexual activity, since IL-1 is released during the immune response. Together, these results suggest rats suppress their sexual behaviour in response to a perceived threat to health.

Outside of mammals, the focus is less on the impact of immune challenge on libido, but instead on fecundity: the reproductive rate or success of an animal. Bonneaud et al. (2003) found that immune challenged female house sparrows were more likely to abandon their offspring than controls, which they determined was dependent on number of offspring; the fewer offspring they had, the more likely immune challenged mothers were to abandon them. Bonneaud et al. (2003) also found that immune challenged mothers that remained performed less parental care (e.g., feeding offspring), though male mates would take over more parental care to compensate. In reptiles, Uller, Isaksson and Olsson (2006) observed the effect of immune challenge on pregnant female comb-basking lizards (*Ctenophorus fordi*) and found that while the number of offspring was not affected by immune challenge, the lizards produced smaller eggs and smaller hatched

offspring compared to controls. Meylan et al. (2013) observed the same outcome of immune challenge on pregnant female common lizards (*Zootoca vivipara*), producing smaller offspring but not fewer. Meylan et al. (2013) notes that after gestation begins, the number of offspring is unlikely to be affected. Since the eggs are already fertilized and maturing, the mother decreases reproductive effort by allocating less resources to the development of their offspring.

In contrast, Adamo (1999) found that female house crickets (*Acheta domesticus*) increased the number of eggs they laid in response to a dose of live or heat-killed bacterial, but not in response to parasitic infection. Adamo (1999) suggests that this is a host response, as it is triggered by heat-killed bacteria and indicates that the house cricket must be increasing the rate of matured eggs to offset potential mortality caused by infection. A study by Shoemaker and Adamo (2007) looked at several aspects of egg quality, such as protein content, fertilization and hatchling success in immune challenged female Texas field crickets and found no effect of immune challenge on egg quality compared to controls. Also, Copeland and Fedorka (2012) found that southern ground crickets (*Allonemobius socius*) did not decrease their singing, which they use to attract mates, in response to an immune challenge. Furthermore, when given the opportunity to mate, male crickets will supress their sickness behaviour in to compete with other males, but the absence of a potential mate, immune challenged crickets do not engage other males (Adamo, et al., 2015).

1.4 Overview of the Cricket

Crickets are a member of the orthopteran order of insects, well known for their chirping and singing (with some exceptions) (Huber, 1989). Their song is performed by males and is produced by rubbing the forewings together in a specific pattern, called stridulation (Bennet-Clark, 1989). The male cricket can perform short chirps or calling songs, which are used to attract mates. Not all crickets produce song, but songs are unique to each calling species (Bennet-Clark, 1989). Crickets hatch from eggs and as they grow they shed their exoskeleton (moulting). Each stage of development before the animal reaches sexual maturity is referred to as an instar and with each instar the appearance of the pre-adult (nymph) cricket becomes closer to their adult form. The number of instars a cricket goes through over its lifespan varies between species, but can also be altered within species by environmental factors such as diet and temperature (Walker & Masaki, 1989). A common developmental pattern in many cricket species is the appearance of the wing buds in the second to last instar and partial development of the fore and hindwings in the final instar (Walker & Masaki, 1989).

Crickets are considered omnivorous as they accept and thrive off of a variety of foods in the laboratory. Crickets usually have a herbivorous diet in the wild, as cricket species are rarely predatory, but crickets will engage in cannibalism if they meet a smaller, defenseless prey (e.g., another moulting cricket) (Walker & Masaki, 1989). Crickets are prolific breeders and have an r-selected population growth direction, selecting for maximum possible growth or quantity of offspring over quality, (i.e., crickets live fast and die young), in contrast to a K-selected population which selects for quality offspring over quantity, as is the case for many mammalian species (Pianka,

1970). It is necessary for crickets to maximize their population size as they are at the bottom of the food chain and have many natural predators (Walker & Masaki, 1989). Not surprisingly crickets have a very short life expectancy in the wild; in addition to predation, they are also susceptible to a variety of diseases and parasites (Walker & Masaki, 1989), with some species of parasitic wasps specializing in laying their larvae on crickets (Adamo, et al., 1995). It may be a wonder they can survive at all with all the odds stacked against them, but crickets do have a few natural defenses against predators. Commonly crickets seek shelter in order to hide from predators. Crickets sometimes utilize natural camouflage or engage in a periods of immobilization when disturbed; both strategies allow the cricket to avoid detection (Walker & Masaki, 1989). Very often though, crickets will flee and are well equipped to evade predators and laboratory students alike! As such, the cricket will often flee to shelter but may also become immobile or attempt to take advantage of camouflage.

1.4.1 Nervous and Endocrine System

The insect nervous system has a distributed organization. The central nervous system is comprised of the brain, the supraesophageal ganglion and a number (varying between species) of ventral ganglia distributed along the nerve cord. The insect brain is comprised of three segments: the protocerebrum, the deutocerebrum and tritocerebrum. The protocerebrum is the largest and most complex of the three brain segments and appears to have a multitude of functions. Notably, the protocerebrum is responsible for coordinating motor activity and for processing visual and olfactory sensory information (Cooter, 1975; Gillott, 2005). The protocerebrum also contains the mushroom bodies, which are important for insect learning and memory (Unoki, Matsumoto & Mizunami,

2005). The deutocerebrum is largely comprised of the antennal lobes which take in sensory information from the antennae and transmit it to the protocerebrum (Rospars, 1988). The tritocerebrum is the smallest of the three brain sections and integrates sensory input from the other parts of the brain (Ayali, 2004). It also connects to the frontal ganglion, which controls the foregut (Ayali, 2004). The supraesophageal ganglion contains nerves that control the mouth parts, neck and salivary glands, and is responsible for maintaining locomotion (Gillott, 2005).

Two important glands of the insect endocrine system are the corpora cardiaca and the corpora allata. The corpora allata secretes juvenile hormone that is critical for development (Gillott, 2005). The corpora cardiaca is responsible for the storage, production and release of a number of hormones, including AKH (Loher & Zaretsky, 1989). AKH is released during insect flight as part of the stress response, and its purpose is to mobilize lipid during a period of high energy consumption in the animal (Loher & Zaretsky, 1989). Octopamine, which is similar to noradrenaline (Roeder, 1999) functions as a neurotransmitter in insects (Evans & O'Shea, 1977). Octopamine is found in the dorsal unpaired median (DUM) cells of insects (Dymond & Evans, 1979), which are distributed across the insect nervous system (Stevenson & Spörhase-Eichmann, 1995) and are thought to have a neuromodulatory role (Pollack, Ritzmann, & Westin, 1988). Octopamine, like noradrenaline, is released in response to stressful stimuli (Davenport & Evans, 1984) and thus is considered an insect stress hormone (Roeder, 1999).

1.4.2 Immune System

The immune system of insects is very different from that of mammals. The most notable difference in immune function between insects and mammals is the insect's lack of an acquired immune system (Lavine & Strand, 2002). But speaking in broad terms there are some similarities between their innate immune system function. The primary (i.e. barrier) defenses in mammals are the epithelium of the skin and gut, and in insects they are the endoskeleton and gut epithelium (Dunn, 1986). Pathogens that get past the primary defenses encounter the secondary defenses, which are comprised of humoral and cellular defenses in insects (Gupta, 1986). The recognition of foreign particles through the secondary defenses informs the immune system that there is a potential threat, which in turn upregulates immune function (Schmidt, Theopold & Beckage, 2008).

Humoral defenses are primarily antimicrobial enzymes (e.g., lysozyme) that target non-eukaryotic pathogens, such as bacteria or fungus and are used to disrupt and destroy these invading microbes (Schmidt, et al., 2008). These enzymes are present in the hemolymph of insects without infection, but are substantially upregulated in response to invading pathogens (Dunn, 1986). For invertebrates, phenoloxidase is an important immune-enzyme and is necessary in order for the melanization cascade to occur, as disruption of phenoloxidase in freshwater crayfish (*Pacifastacus leniusculus*) led to reduced nodule formation (explained shortly) and increased mortality (Liu, et al., 2007).

Cellular defenses in insects primarily use hemocytes (phagocytes) that identify and consume foreign particles (phagocytosis) to destroy them and prevent further harm to the host (Lavine & Strand, 2002). Hemocytes are analogous to the macrophages found in mammals (Ottaviani & Franceschi, 1998). Hemocytes recognize the polysaccharide

molecules inherent to bacteria (e.g., Lipopolysaccharide or LPS) and other pathogens (Gillespie, Kanost & Trenczek, 1997). When a hemocyte consumes bacteria, after endocytosis the hemocyte goes through a process called nodulation, wherein the hemocyte becomes melanized and permanently adheres to tissue of the insect, restricting the movement and growth of the pathogen (Dunn, 1986). Encapsulation has an energy cost in the cricket, reportedly increasing their metabolism by 28% (Ardia, et al. 2012). Nodulation is thought to be mediated by eicosanoids (Miller, Nguyen & Stanley-Samuelson, 1994) which are lipid mediators with an immunomodulatory role (Rocha, Plumb & Coffman, 2003). Eicosanoids are also thought to induce behavioural fever in insects (Adamo, 2008b). Particles that are too large to be consumed by one hemocyte alone are sequestered by multiple hemocytes in a process called encapsulation (Strand, 2008). Hemocyte activation can initiate the innate immune response (Schmidt, et al., 2008) resulting in a number of proinflammatory mediated behavioural and physiological changes as previously discussed.

There is strong evidence for bidirectional communication between the immune system and the brain in mammals via proinflammatory cytokines, as previously discussed (Dantzer, 2004a; 2004b). Given the similar pattern of sickness behaviour and the physiological similarities in the immune system observed across phyla, bidirectional communication between the nervous system and immune system has been suggested to exist in insects, but this is still not quite fully understood (Adamo, 2008b). Adamo and Parsons (2006) applied several stressors to crickets (*G. texensis*) and found that stress affected the immune function of crickets enough to cause a decline in disease resistance (stress induced immunosupression). Specifically, exercise, restraint and agonistic

behaviour increased susceptibility to infection. Injection of octopamine, the insect stress hormone, also decreased disease resistance, suggesting that in crickets stress or strenuous activities are immunosuppressive, something which is also observed in mammals (Råberg, et. al., 1998).

Recent work suggests that stress induced immunosuppression is adaptive. Octopamine induces a release of lipid into the hemolymph (Orchard, Loughton, & Webb, 1981) as does the stress hormone AKH (Loher & Zaretsky, 1989). Apolipophorin III is limited and when it is transporting lipid, it is not enhancing immune function (Weers, & Ryan, 2006). Adamo et al. (2007) found that both flight and immune function depleted available reserves of apolipophorin III, suggesting a functional conflict for the protein. Injection of AKH following an immune challenge also supressed immune function (Cheeseman & Goldsworthy, 1979). This is presumably due to apolipophorin III being coerced into lipid transport over immune function, due to the abundance of AKH released lipid (Adamo, et al., 2007).

When given an opportunity to mate, immune-challenged male crickets fought and won as often as healthy crickets, supressing their immune function for the purpose of agonistic displays (Adamo et al., 2015). In the absence of potential mates though, crickets invested in their immune function normally and either lost fights or avoided fighting altogether. This would appear to be against the energy conservation hypothesis suggested by Hart (1988), but it is still likely an adaptive response. Due to the short life span of the cricket, mating opportunities are limited, thus it is logical that they would supress their immune function when given the chance to mate, as a missed mating opportunity could mean that the animal never has the chance to mate at all. This is not unlike the terminal

reproductive investment behaviour seen in mammals (e.g., arctic ground squirrels) (Boonstra et al., 2001), though crickets appear to show conditional suppression of immunity as is seen in birds (Owen-Ashley, et al., 2004).

Otti et al. (2011) found that immune challenged male crickets showed lower survival than controls in the presence of a predator. Immune challenged crickets spent more time out of their burrow and reacted slower to a predator attack and thus increased immune investment decreased predator avoidance and overall survival. They speculated that investment in immunity diverts energy and resources from optimal survival strategies and suggested the reason that the crickets spent more time out of their burrows when immune challenged, despite the risk of predation, was because they were exhibiting behavioural fever. However, previous experiments suggest that the pathogen they used, *Serratia marcescens*, does not elicit behaviour fever in crickets (Adamo, 1998). Regardless, they concluded that optimal immune defense may differ in relation to abundance and threat of predators.

1.5 Objectives

Sickness mediated behavioural changes are observed in animals across all phyla (See Table 1.1). The first aim of the research at present is to test the range of behaviours expressed by sick insects (i.e. crickets). Specifically, is there evidence to support the energy conservation and predator avoidance hypotheses in our model organism, the Texas field cricket, *G. texensis*?

Insect physiology differs from that of the mammals on which Hart based his hypotheses. For example, the energy conservation hypothesis hinges on conserving resources to enhance fever. Insects experience behavioural fever, but crickets do increase

metabolic energy consumption during illness (Ardia, et al. 2012). Previous research has also found crickets have slower reaction time and reduced survival in the presence of a predator (Otti, et al., 2012). Thus it is still in their best interest to conserve energy and avoid predators during illness, as Hart suggests. The suggested function of illness-induced anorexia is to reduce the motivation to seek resources (Hart, 1988) and it is reasonable to assume it would serve a similar purpose in crickets.

Therefore, we predict that crickets will decrease their grooming, exploration, foraging and locomotion, to conserve energy in order to compensate for the increased energy cost of immune function. We also predict that immune challenged crickets will increase their shelter use in order to enhance predator avoidance strategies during this period of vulnerability.

Chapter 2 - Experiments

2.1 Animals

Experiments used two populations of Texas field crickets (*G. texensis*). The first population was originally collected near Austin, Texas and was maintained in a laboratory colony for many generations. The second population was collected near San Antonio, Texas and has been maintained as a laboratory colony for less than five generations. Crickets were maintained at 25+/-2°C with 65% relative humidity on a 12:12 light:dark cycle and fed dried cat food pellets and water ad libitum. Crickets used in experiments were isolated into white opaque plastic tubs (10cm diameter x 10cm depth) at least 24h before treatment and stored in the colony room so as not to cut off olfactory or auditory communication with the rest of the colony. Isolated crickets received food and water ad libitum. All crickets used were 12-18 days into adulthood unless otherwise noted. No animals were used for more than one experiment. All studies were approved by the Animal Care Committee of Dalhousie University (I-11-025) and are in accordance with the Canadian Council of Animal Care.

2.2 Immune Challenge

To examine the effect of an immune response on different behaviours, crickets were given an injection of heat-killed bacteria (*S. marcecens*) suspended in nutrient broth. Heat-killed *S. marcescens* induces an immune response in *G. texensis* (Adamo, 2004; Adamo et. al., 2008a) but cannot synthesize compounds. Therefore, changes in cricket behaviour cannot be ascribed to bacterial secretions or active manipulation by the bacteria. Immune challenged crickets were injected with 2 µl of heat-killed *Serratia marcescens* (approximately 10% of the LD50 dose of live bacteria) suspended in nutrient

broth (Adamo et. al., 2001). Sham-injected crickets were given 2 µl of nutrient broth in order to control for the minor immune response elicited by the wound caused by the injection (Wigby, et. al., 2008). Control crickets received no injection or handling manipulations. All injections were delivered through the pronotal membrane using a 10 µl Hamilton syringe. Separate syringes were used for administering sham treatment and heat-killed bacteria treatments and were thoroughly rinsed with ethanol and water in between injections. All tests were performed 60-90 minutes post-injection unless otherwise indicated in the study. Physiological (Adamo, et al., 2008a) and behavioural changes (i.e. illness-induced anorexia, Adamo, et al., 2010) are known to occur at this time.

2.3 Effect on general activity level

2.3.1 Methods

Hart's energy conservation hypothesis suggests that animals should conserve energy by reducing general activities, such as locomotion, in response to an immune challenge. We expect that an immune challenge would lower general activity level in crickets. In this design, the arena (72 cm x 126 cm x 38 cm) was marked off in a 2 cm by 2 cm grid. A single shelter made from part of an egg carton (~5cm L x 5cm W x 4 cm H), 3 cat food pellets and 1 water vial were all placed against the length of one side of the arena. An electric thermometer was used to confirm there was no difference in temperature inside and outside of provided shelter. Crickets from population one (n = 64) were randomly assigned to one of the three treatment groups: control, sham-injected and immune challenged. Animals were placed in a cup and transferred to the centre of the arena. After 1 minute the cup was removed and total locomotion (time spent moving

outside of shelters) as well as number of lines crossed was assessed over a 30-minute trial. This experiment also assessed feeding time (time spent in contact with and eating the food provided), number of drinking bouts (contact with and drinking from water vial, for a maximum of 1 minute per bout), shelter use (time spent with at least four legs in the provided shelter) and number of grooming bouts (each time the animal used its mandibles to groom antennae or legs).

2.3.2 Results

There was uneven sampling of sex in this study (males = 78, females = 20). Females were not equally distributed among groups (control = 9, sham-injected = 4, immune challenged =7) and preliminary tests showed no significant sex differences for shelter ($F_{1,92} = 0.44$, $p = 0.51$) locomotion, ($F_{1,92} = 0.17$, $p = 0.69$) or grooming ($\chi^2 = 2.10$, $p = 0.15$), so the data was pooled and sex was not included in further analysis.

Preliminary analysis revealed that shelter ($F_{(2, 95)} = 4.44$, $p = .014$) and locomotion ($F_{(2, 95)} = 6.91$, $p = .002$) did not have equal variances, which could not be corrected with data transformation. However the data for each measure did not deviate from a normal distribution so the decision was made to use Brown-Forsythe F correction for analysis of these measures. Extreme outliers were identified by using the outlier labeling rule (Hoaglin & Iglewicz, 1987), which did not result in any values being removed.

There was a significant effect of treatment on time spent locomoting ($F_{(2, 75.99)} = 7.71$, $p < .001$) (see figure 2.1). Immune challenged animals increased locomotion compared to control ($p < .001$, 95% CI [84.50, 425.97]) and sham-injected animals ($p < .01$, 95% CI [56.69, 425.04]). Locomotion of sham-injected animals was not significantly

different from control animals (p = .98, 95% CI [-165.47, 196.21]). There was a significant effect of treatment on time spent in shelter ($F_{(2, 88.90)} = 6.63$, $p < .01$) (see figure 2.2). Immune challenged animals decreased their shelter use compared to control (p < .01, 95% CI [-914.14, -133.09]) and sham-injected animals (p < .01, 95% CI [-968.53, -121.04]). Shelter use of sham-injected animals was not significantly different from control animals (p = .99, 95% CI [-394.91, 437.24]). Feeding was normally distributed and met all assumptions. One-way ANOVA revealed no significant effect of treatment on feeding time ($F_{(2, 95)} = 0.81$, $p = .43$) (See figure 2.3). These results are contrary to previous studies (e.g., Adamo, et al., 2010) and may be because animals did not go through a period of food deprivation before the experiment.

Poisson regression was used to analyse the effect of treatment on grooming bouts, lines crossed and drinking bouts during the trial. There was a significant effect of treatment on grooming ($\chi^2_{(2, 2)} = 17.47$, p < .001) (see figure 2.4). Immune challenged animals groomed significantly more than controls (p < .001, 95% CI [0.49, 1.35]), while grooming behaviour of sham-injected animals was not significantly different from controls (p = .41, [-0.20, 0.48]). There was not a significant effect of immune challenge on drinking ($\chi^2_{(2, 2)} = 0.26$, p = .88), or lines crossed ($\chi^2 = 5.06$, p = .08) (see figures 2.5 and 2.6).

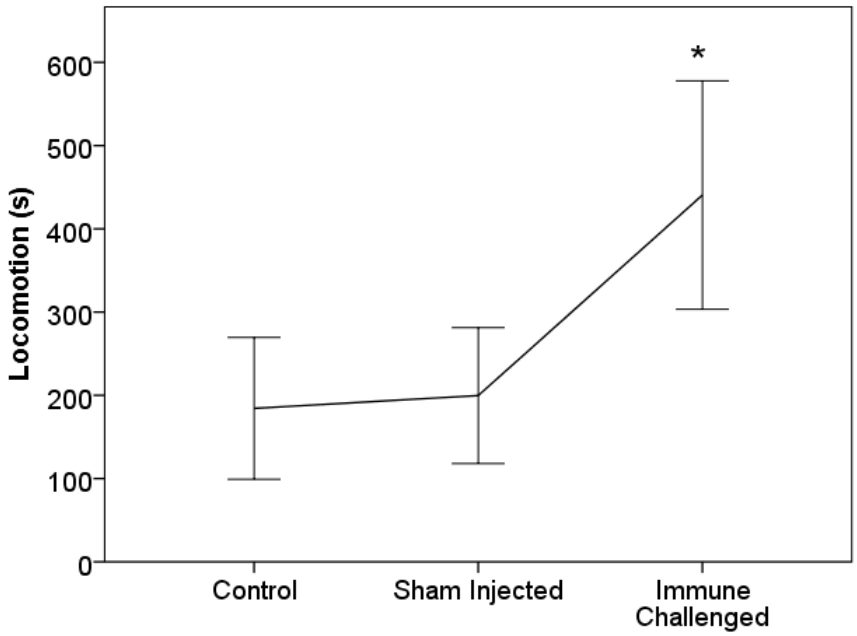

Figure 2.1. Mean locomotion (in seconds) for control (n = 37), sham-injected (n = 27) and immune challenged (n = 34) animals. Error bars represent 95% confidence interval. * represents significant result.

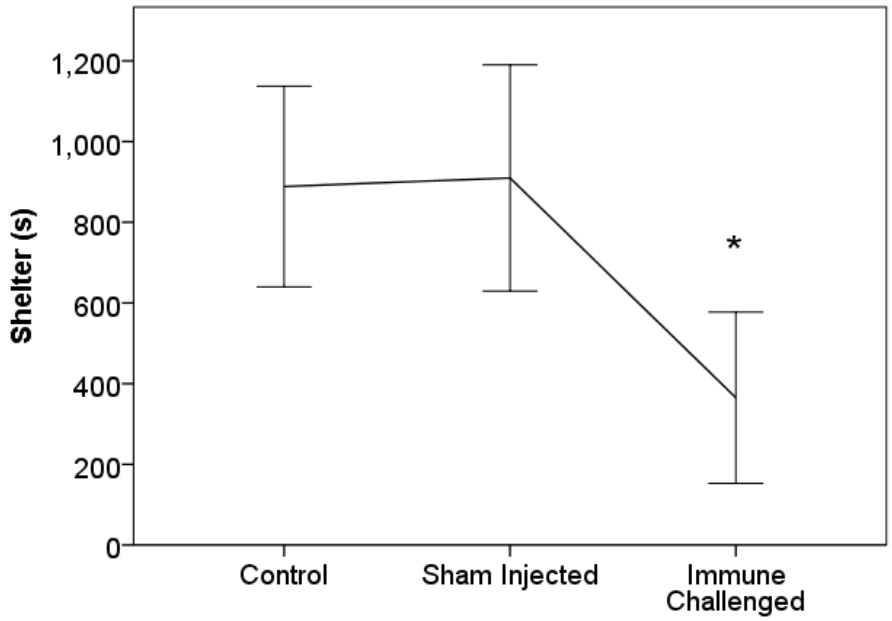

Figure 2.2. Mean time spent in shelter (in seconds) for control (n = 37), sham-injected (n = 27) and immune challenged (n = 34) animals. Error bars represent 95% confidence interval. * represents significant result.

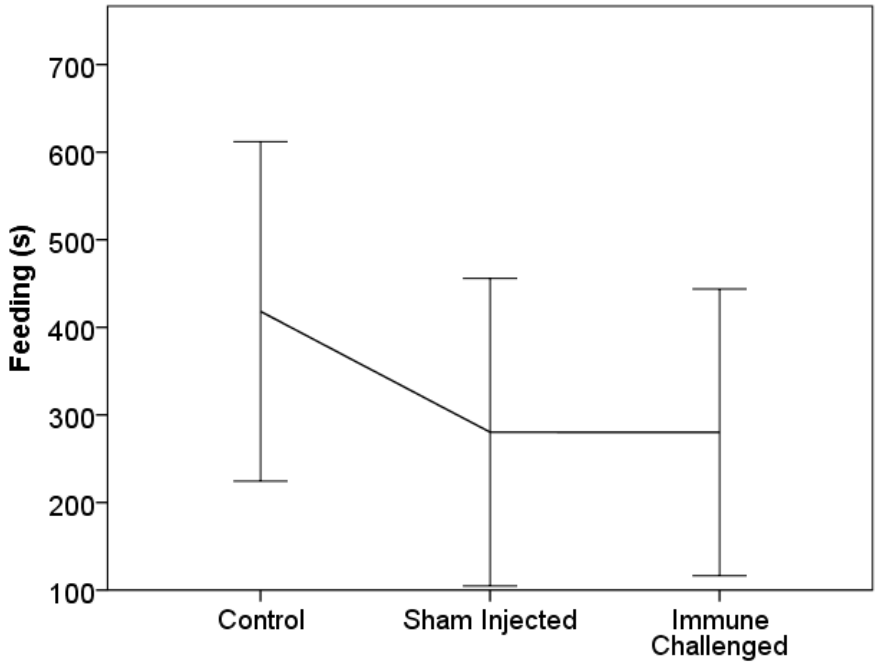

Figure 2.3. Mean time spent feeding (in seconds) for control (n = 37), sham-injected (n = 27) and immune challenged (n = 34) animals. Error bars represent 95% confidence interval.

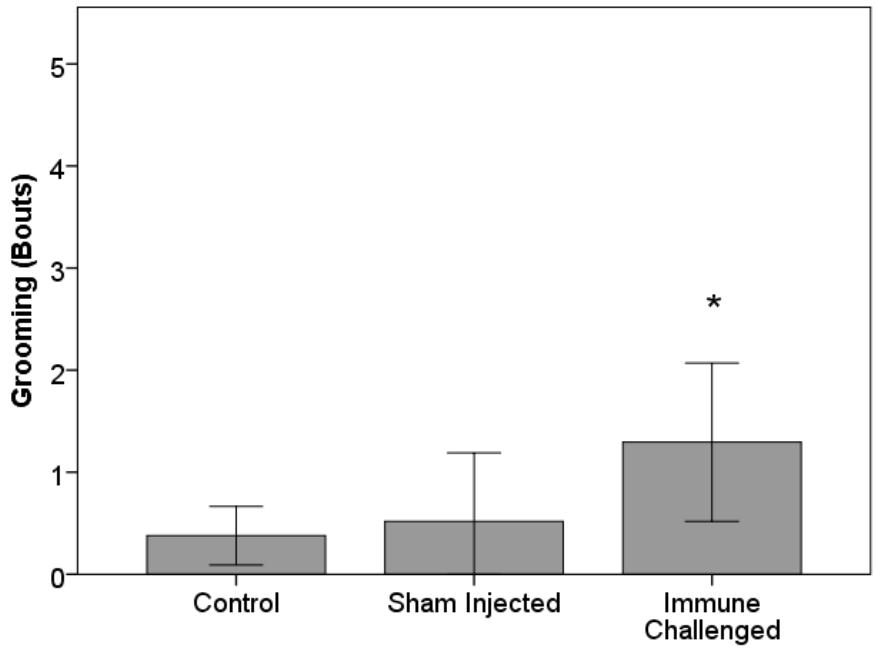

Figure 2.4. Mean number of grooming bouts for control (n = 37), sham-injected (n = 27) and immune challenged (n = 34) animals. Error bars represent 95% confidence interval. * represents significant result.

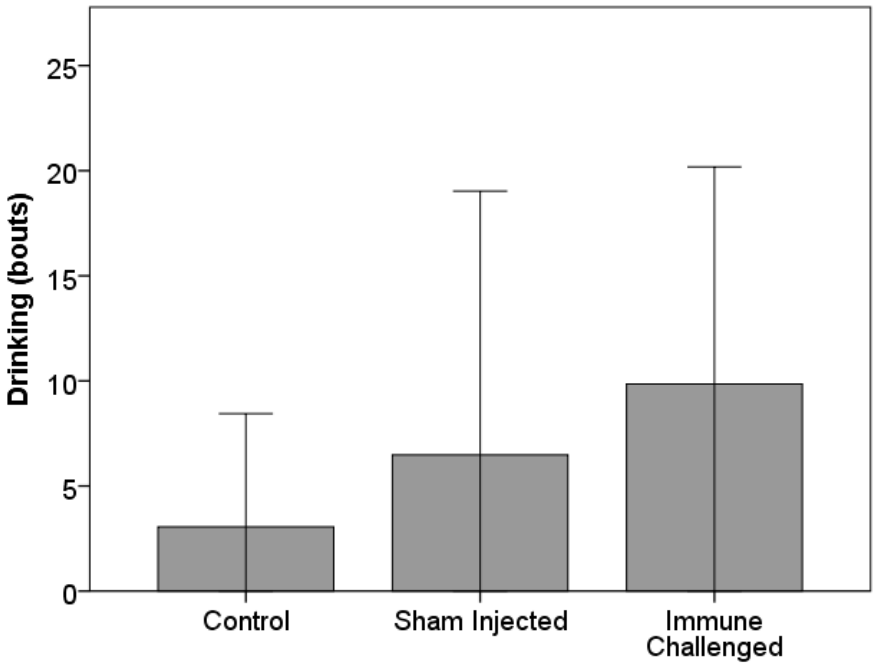

Figure 2.5. Mean number of drinking bouts for control (n = 37), sham-injected (n = 27) and immune challenged (n = 34) animals. Error bars represent 95% confidence interval.

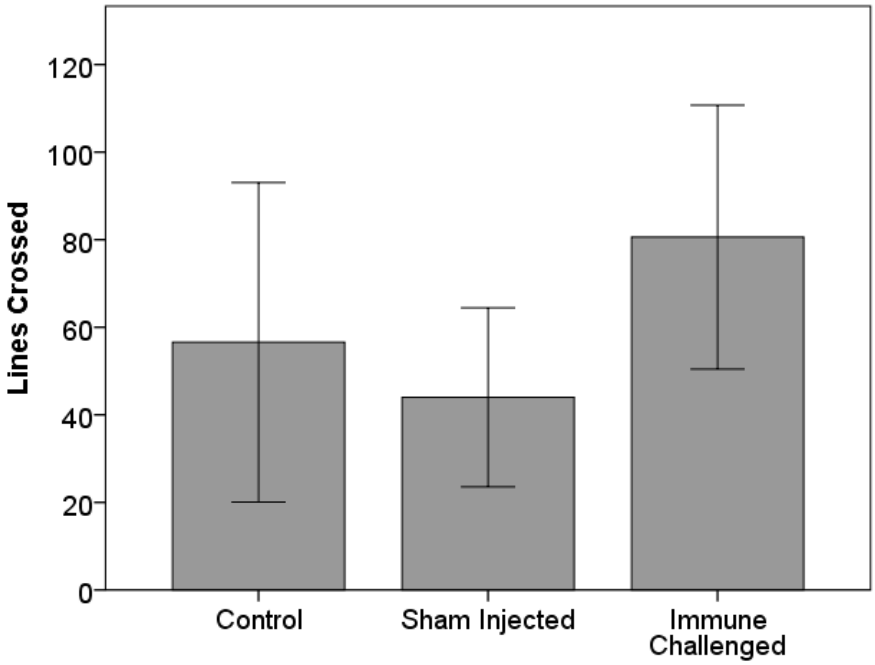

Figure 2.6. Mean number of lines crossed for control (n = 37), sham-injected (n = 27) and immune challenged (n = 34) animals. Error bars represent 95% confidence interval.

2.4 Effect on shelter use, feeding and drinking

2.4.1 Methods

Crickets from population one (n = 195) were randomly assigned to one of the three treatment groups. Food was removed 3 hours before the start of the trial. Animals were placed in a cup (6 cm in diameter) and transferred into a clear plastic arena (17cm L x 15cm W x 9.5cm H). This arena included a water vial, a shelter made from a large water vial (9.5 cm L x 2.5 cm diameter) wrapped in tape to make it opaque and one piece of cat chow. The cat chow was presented at the end of a needle and held in place with a piece of adhesive Velcro so the animal could not drag the food from the presentation area (see figure 2.7). After 1 minute the cup was removed and the animal was scored on feeding time (time spent in contact with and eating the food provided), drinking (contact with and drinking from water vial, for a maximum of 1 minute per bout) and shelter use (time spent with all six legs inside the shelter provided) by reviewing a recording of the 3 hour and 45 minute trial.

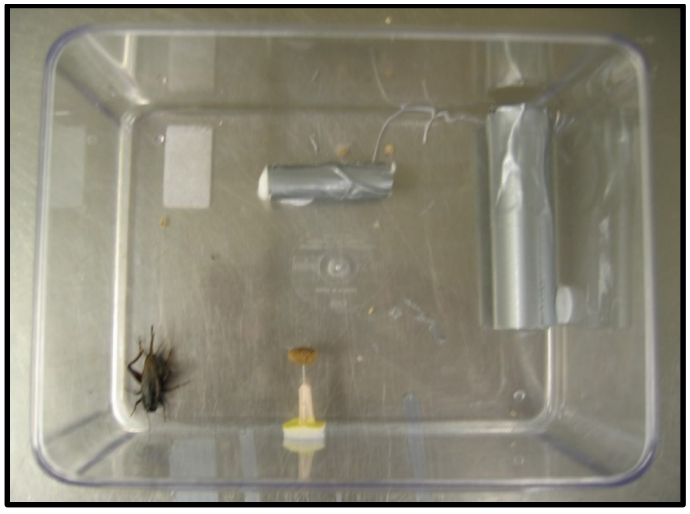

Figure 2.7. Picture of the arena used in the shelter use, drinking and feeding experiment.

2.4.2 Results

A two-way ANOVA revealed a significant effect of treatment on feeding ($F_{2, 189}$ = 7.39, $p < 0.001$) (See figure 2.8). A post hoc Tukey test revealed that immune challenged animals ate significantly less than control ($p = 0.009$, 95% CI [-24.96, -2.97]) and sham injected ($p = .001$, 95% CI [-27.60, -5.61]) animals. There was no apparent effect of sex on feeding ($F_{1, 189} = 0.46$, $p = .50$) and no interaction effect ($F_{2, 189} = 0.33$, $p = 0.72$). There was no effect of treatment ($F_{2, 189} = 1.99$, $p = 0.14$) or sex ($F_{1, 189)} = 0.50$, $p = 0.48$) on shelter use. But there was an interaction effect between sex and treatment ($F_{2, 189}$ = 3.05, $p = 0.05$). A post hoc Tukey test revealed that immune challenged males spent significantly less time in shelter than control males ($p < 0.05$, 95% CI [-79.72, -4.90]) or sham injected males ($p < 0.05$, 95% CI [-80.10, -5.28]).

Poisson regression was used to analyse the effect of treatment on drinking bouts during the trial. Males drank so infrequently that their variance was approximately 0, therefore only females were included in analysis. There was a significant effect of treatment on drinking ($\chi^2 = 17.47$, $p < .001$) (see figure 2.10). Sham-injected animals drank significantly less than controls ($p < .001$, 95% CI [0.05, .173]). Drinking behaviour of immune challenged animals was not significantly different compared to controls ($p > .05$, [0.29, 1.14]).

Some results of this experiment disagree with the results from the previous experiment. For one, crickets in the previous experiment did not exhibit illness-induced anorexia, but exhibited the behaviour change in this experiment. As previously described, food deprivation was not implemented in the previous experiment. The results of this experiment are in line with previous research showing that immune challenged crickets

exhibit illness induced anorexia (e.g., Adamo, et al., 2010). In this experiment we found that sham-injected crickets drank less than both controls and immune challenged crickets but in the previous experiment we found no difference across groups. The previous experiment was shorter in length had far fewer observed drinking bouts than this study. It is possible the effect is difficult to observe over a shorter time period. Regardless, immune challenged animals performed no differently from controls in both experiments. Finally, we found no difference in shelter use across groups in this experiment, while in the previous experiment we found that immune challenged animals used less shelter. Looking at the data, there does appear to be a trend in this study that the mean shelter use of immune challenged animals is lower than control or sham injected animals, but it is possible this effect is small enough that it is lost over a longer trial. Shelter use would have to be further investigated.

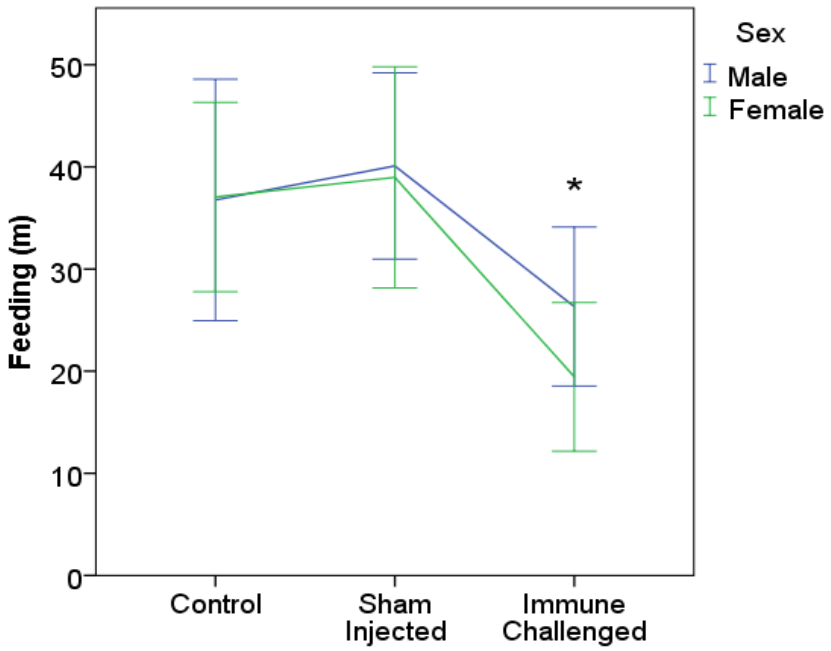

Figure 2.8. Mean time spent feeding (in minutes) for control (n = 65), sham injected (n = 65) and immune challenged groups (n = 65). Error bars represent 95% confidence interval. * represents significant result.

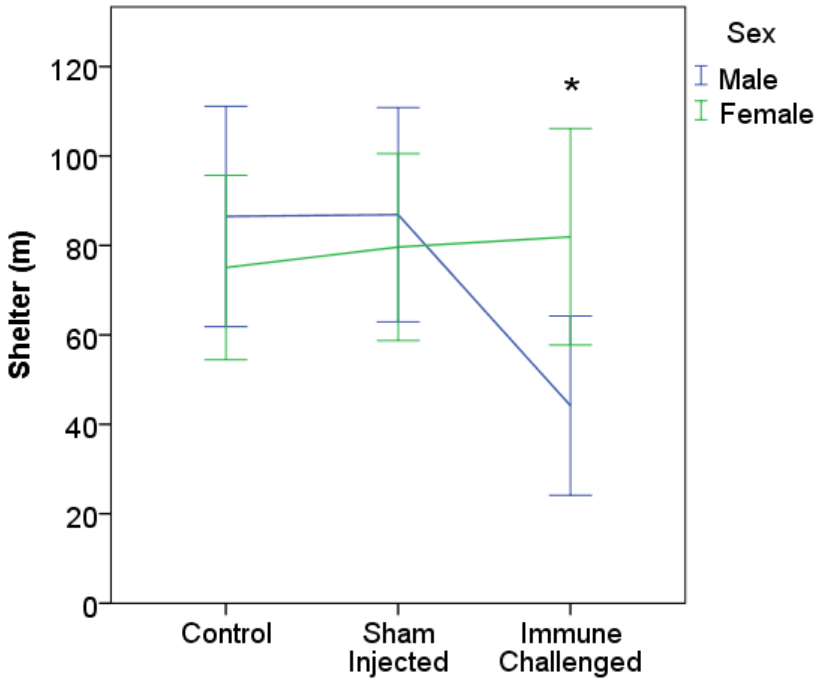

Figure 2.9. Mean time spent in shelter (in minutes) for control (n = 65), sham injected (n = 65) and immune challenged groups (n = 65). * represents significant result.

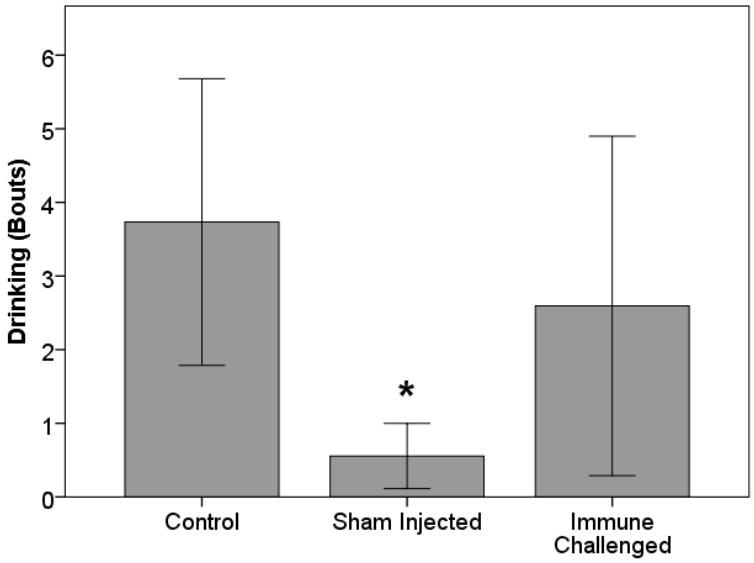

Figure 2.10. Mean drinking bouts for control (n = 65), sham injected (n = 65) and immune challenged groups (n = 65). Error bars represent 95% confidence interval. * represents significant result.

2.5 Effect on the latency to become active in a novel environment

2.5.1 Methods

Hart's predator avoidance hypothesis suggests that immune challenged animals should be motivated to decrease the amount of time they spend out in the open to reduce the risk of predation. Crickets from population one (n = 180) were isolated in clear plastic containers (17cm L x 15cm W x 9.5cm H) and randomly assigned to one of three treatment groups. Animals were gently placed into an opaque shelter (5 cm L x 3 cm diameter) plugged with cotton. This shelter was gently transferred into an empty clear plastic arena (17cm L x 15cm W x 9.5cm H) and secured with Velcro. After 5 minutes of habituation, the cotton plug was gently removed and animals were measured on their latency to exit the shelter over a 10 minute trial. This study was repeated (n = 180) except that at the start of the trial a 5 pound sand bag was dropped from a height of 58 cm to create vibrations and a loud disturbance. The crickets were then observed to determine their latency to exit the shelter.

2.5.2 Results

Preliminary analysis revealed that the data for this experiment were not normally distributed. Kruskal-Wallis tests were used to analyse the data by sex. There was no effect of treatment on the latency to exit shelter without a disturbance in males ($\chi^2 = 1.28$, $p = 0.53$) or females ($\chi^2 = 1.24$, $p = 0.54$) (see figure 2.11) or with a disturbance in males ($\chi^2 = 4.78$, $p = 0.09$) or females ($\chi^2 = 0.08$, $p = 0.96$) (see figure 2.12).

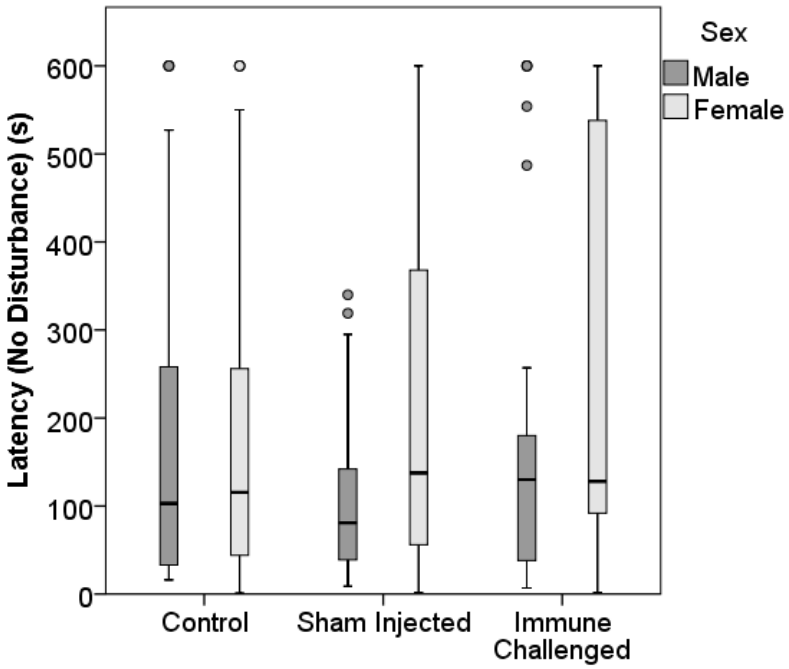

Figure 2.11. Mean latency to become active without a disturbance (in seconds) for control (n = 60), sham injected (n = 60) and immune challenged groups (n = 60). Error bars represent 95% confidence interval. Circles represent non-significant outliers.

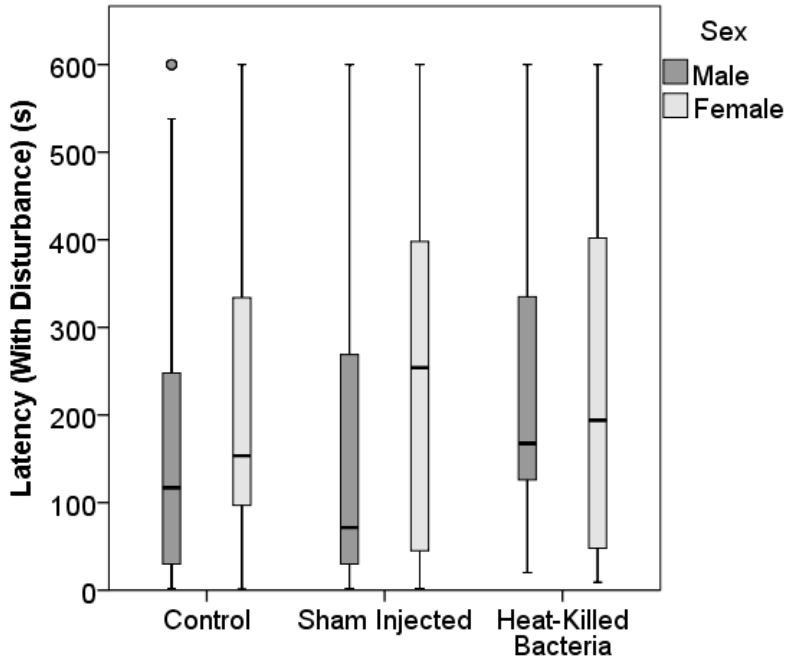

Figure 2.12. Mean latency to become active with a disturbance (in seconds) for control (n = 60), sham injected (n = 60) and immune challenged groups (n = 60). Error bars represent 95% confidence interval. Circles represent non-significant outliers.

2.6 Effect on predator avoidance latency

2.6.1 Methods

Crickets from population one (n = 297) were randomly assigned to one of three treatment groups. Animals were gently placed in a transparent container (4 cm L x 2.5 cm W by 3 cm H) and placed on the starting end of a runway (34 cm L x 11 cm W x 14 cm H) similar to Adamo and Baker (2011) (see figure 2.13). The far end of the runway was covered with a 10 cm length of card stock raised 2 cm from the surface to create a darkened space to provide cover. A robotic hamster (ZhuZhu Pets, Cepia, St. Louis, MO) was placed in a separate chamber (21.5 cm L x 16.5 cm W X 14 cm H) adjacent to the starting area of the runway. After 1 minute the robotic hamster was activated and it hit the wall behind the cricket, causing both noise and vibration. An infrared diode was placed 5 cm in front of the starting area and a second one was placed immediately in front of the covered area. These sensors were used to automatically record latency to begin escaping (time to cross the first sensor) and escape time (time to cross the second sensor) (Python Software 2.6.5, Python Software Foundation, Wolfeboro Fall, NH). Crickets were tested 5 minutes after treatment (n = 99), 90 min after treatment (n=124) or 24 hours after treatment (n = 74).

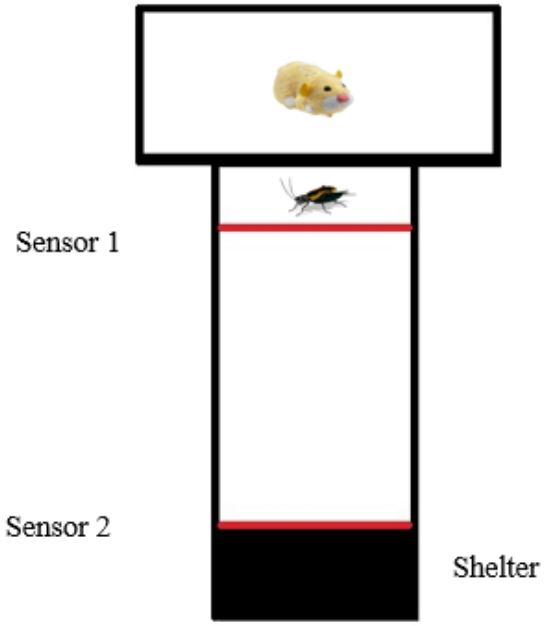

Figure 2.13. Picture of the runway design used in the predator avoidance latency study.

2.6.2 Results

Preliminary analysis revealed that the data were not normally distributed. Kruskal-Wallis tests were used for analysis and only females were used in this study. Animals tested 90 minutes post treatment (n = 124) showed a significant effect of treatment on escape time ($\chi_{(2)} = 6.94$, $p = 0.03$) but not on latency to escape ($\chi_{(2)} = 1.836$, $p = 0.34$) (See figure 2.14). A post hoc Dunn's test revealed that immune challenged ($Z = -2.27$, $p = 0.02$) and sham injected animals ($Z = -2.23$, $p = 0.03$) had significantly increased escape time compared to controls. However, immune challenged and sham injected animals were not significantly different ($Z = -0.04$, $p = 0.97$). There was not a significant effect of treatment on crickets tested 5 minutes post treatment (n = 99) on either latency to escape ($\chi_{(2)} = 0.51$, $p = 0.77$) or escape time ($\chi_{(2)} = 0.44$, $p = 0.80$) (See figure 2.15). There was also not a significant effect of treatment on animals tested 24 hours post treatment (n = 74) on latency to escape ($\chi_{(2)} = 2.17$, $p = 0.34$) or escape time ($\chi_{(2)} = 0.48$, $p = 0.79$) (See figure 2.16).

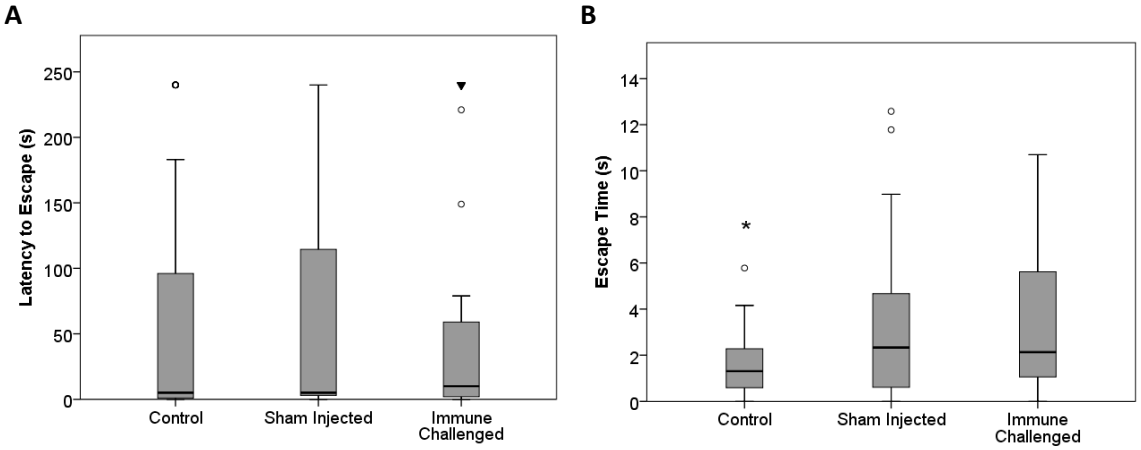

Figure 2.14. Means of ranked data for animals tested at 90 min for control (n = 46), sham injected (n = 39) and immune challenged (n = 39) in A) latency to escape and B) escape time in seconds. Error bars represent 95% confidence interval. * represents significant result. Circles represent non-significant outliers, while triangles represent significant outliers.

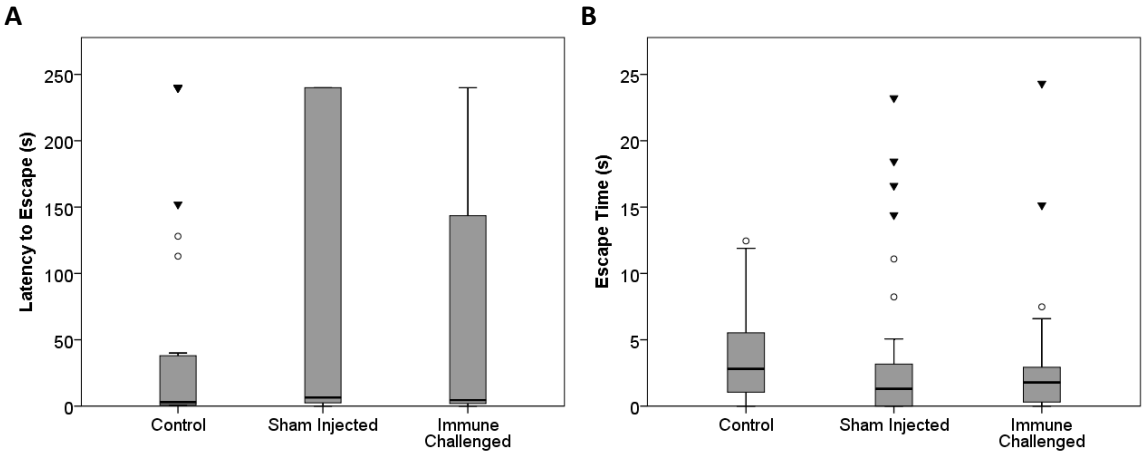

Figure 2.15. Means of ranked data for animals tested at 5 min for control (n = 35), sham injected (n = 32) and immune challenged (n = 32) in A) latency to escape and B) escape time in seconds. Error bars represent 95% confidence interval. Circles represent non-significant outliers, while triangles represent significant outliers.

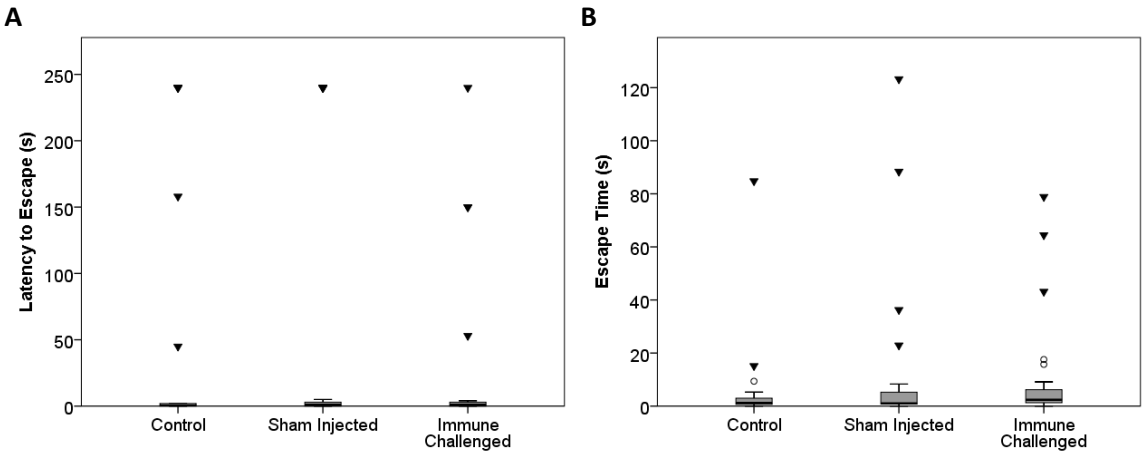

Figure 2.16. Means of ranked data for animals tested at 24 hours for control (n = 26), sham injected (n = 25) and immune challenged (n = 23) in A) latency to escape and B) escape time in seconds. Error bars represent 95% confidence interval. Circles represent non-significant outliers, while triangles represent significant outliers.

2.7 Effect on exploratory behaviour

2.7.1 Methods

Crickets from population two in their second to third lab generation (n = 68) were randomly assigned to one of three treatment groups. Animals were tested in a modified plus maze similar to Adamo et al. (2013), composed of opaque black acrylic and shaped like a plus sign (see figure 2.17) with four arms (14 cm L x 8 cm W x 6.5 cm H) and an open central area (8 cm L x 8 cm W x 6.5 cm H). Each arm was marked with a white line, 9.5 cm from the end. During the trial, two opposing arms were covered up to the white line with black card stock to create areas of cover. Crickets were tested in the plus maze to establish a behavioural baseline. Crickets received treatment 24 hours later and were retested 90 minutes post-treatment. Crickets were gently placed in a cup (6 cm diameter) and placed in the center of the plus maze. After 1 minute the cup was removed and the animal was assessed on initial immobilization (time spent initially frozen in place, ended by any motion whatsoever), locomotion (time spent moving during the trial), arms

entered (number of times all six legs crossed a white line entering an arm of the plus maze) and time under covered arms over a 10 minute trial. In between trials the plus-maze was rotated clockwise in order to control for group preferences for specific arms across all animals. The plus maze was also thoroughly cleaned with ethanol and allowed to completely dry between trials in order to remove any scents left by previous animals that could potentially affect behaviour.

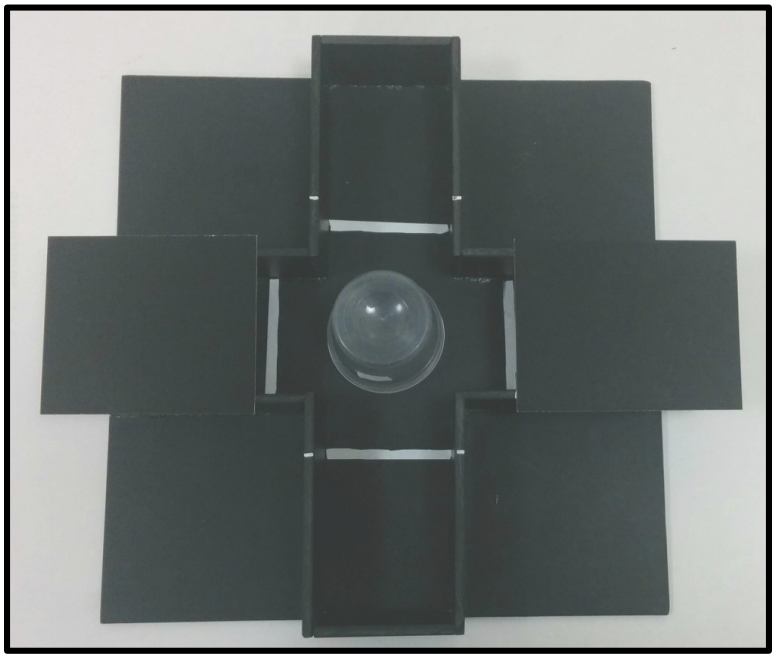

Figure 2.17. Picture of the arena used in the plus maze trials.

2.7.2 Results

Preliminary analysis revealed that the data for all four measures were not normally distributed. The data could not be normalized using normal data transformations. A delta transformation (b – a = Δ) was applied to each of the four measures, subtracting pre-treatment scores (a) from post-treatment scores (b) resulting in delta scores (Δ) representing change in behaviour as a result of treatment (positive scores indicating an increase from baseline, negative scores indicating a decrease from

baseline). Further analysis revealed that the delta transformation had normalized data for total locomotion and time under covered arms (Shapiro-Wilk $p > 0.001$), but not initial immobilization. Extreme outliers were identified by using the outlier labeling rule (Hoaglin & Iglewicz, 1987), resulting in 1 score removed from total locomotion. At this point the decision was made to exclude arms crossed from analysis; after removing outliers the resulting data had too little variability.

Two-way ANOVAs were performed. There was no effect of treatment ($F_{2, 61} = 0.92$, $p = 0.40$) or sex ($F_{1, 61} = 0.18$, $p = 0.68$) on Δ total locomotion, and no interaction effect ($F_{2, 61} = 0.09$, $p = 0.92$) (See figure 2.18). There was no effect of treatment ($F_{2, 62} = 0.80$, $p = 0.46$) or sex ($F_{1, 62} = 0.02$, $p = 0.89$) on Δ time spent under covered arms, and no interaction effect ($F_{2, 62} = 0.21$, $p = 0.81$) (See figure 2.19). Kruskal-Wallis was also performed, revealing no significant effect of treatment on Δ initial immobilization time in males ($\chi^2 = 5.54$, $p = 0.06$) or females ($\chi^2 = 2.88$, $p = 0.24$).

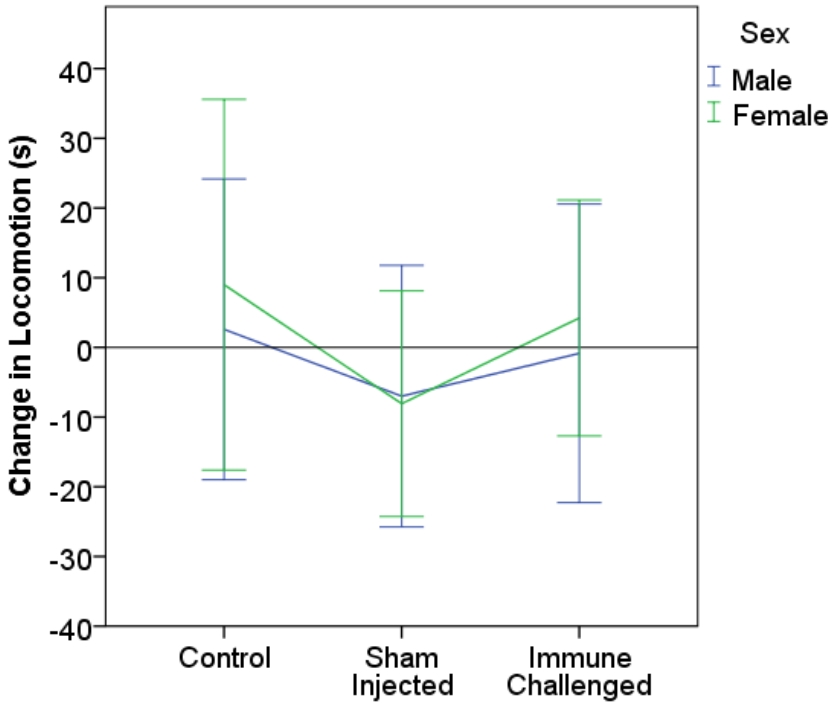

Figure 2.18. Mean change in total locomotion (in seconds) for control (n = 16), sham injected (n = 23) and immune challenged groups (n = 28). Error bars represent 95% confidence interval.

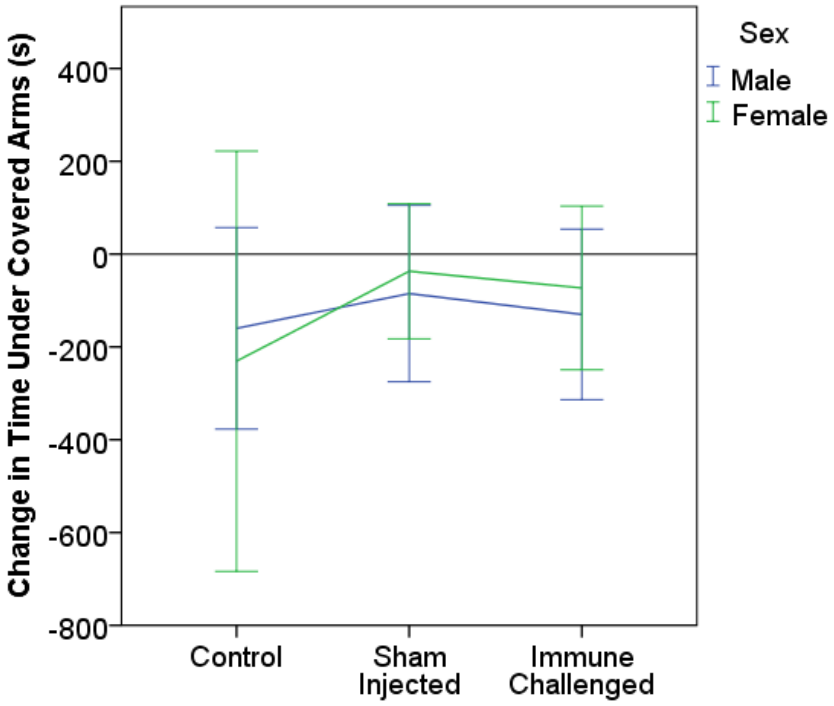

Figure 2.19. Mean change in time under covered arms (in seconds) for control (n = 16), sham injected (n = 24) and immune challenged groups (n = 28). Error bars represent 95% confidence interval.

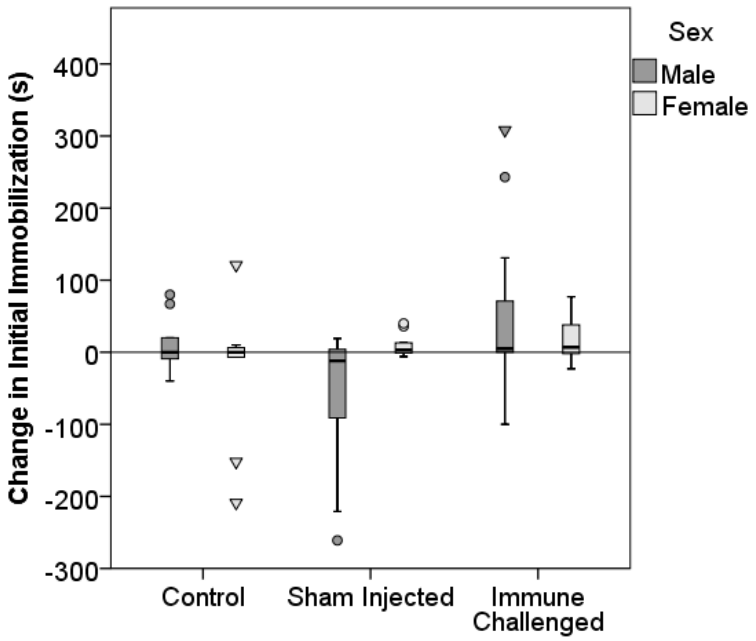

Figure 2.20. Means of ranked data of change in initial immobilization for control (n = 16), sham injected (n = 24) and immune challenged groups (n = 28). Error bars represent 95% confidence interval. Circles represent non-significant outliers, while triangles represent significant outliers.

2.8 Effect of immune challenge on feeding in nymphs

2.8.1 Methods

Growth is vital for the reproductive success of larval insects (Chown & Nicholls, 2004). We tested the effect of immune challenge on food consumption on nymphs one instar prior to adulthood. Crickets from population one (n = 60) were first tested 48 hours after molt and randomly assigned to one of the three treatment groups. Food was removed from isolation tubs 24 hours before the start of the trial. Animals were gently placed in a cup (6 cm diameter) and placed in the centre of the trial arena (17cm L x 15cm W x 9.5cm H). After 1 minute the cup was removed and the animal was assessed on time spent feeding over the 30 minute trial. The test chamber was cleaned thoroughly between trials and the food was replaced for each trial.

2.8.2 Results

A two-way ANOVA was performed, revealing a significant effect of treatment on time spent feeding ($F_{2, 54} = 9.14$, $p < 0.001$). A post hoc Tukey test revealed that immune challenged animals ate significantly less than control ($p < 0.001$, 95% CI [-9.70, -1.65]) and sham injected ($p < 0.001$, 95% CI [-10.54, -2.49]) animals (See figure 2.21). There was also a significant effect of sex on time spent feeding ($F_{2, 54} = 5.75$, $p = 0.02$), with females (mean = 16.40) spending more time eating overall than males (mean = 13.11). But there was no significant interaction effect of treatment and sex ($F_{2, 54} = 0.91$, $p = 0.41$).

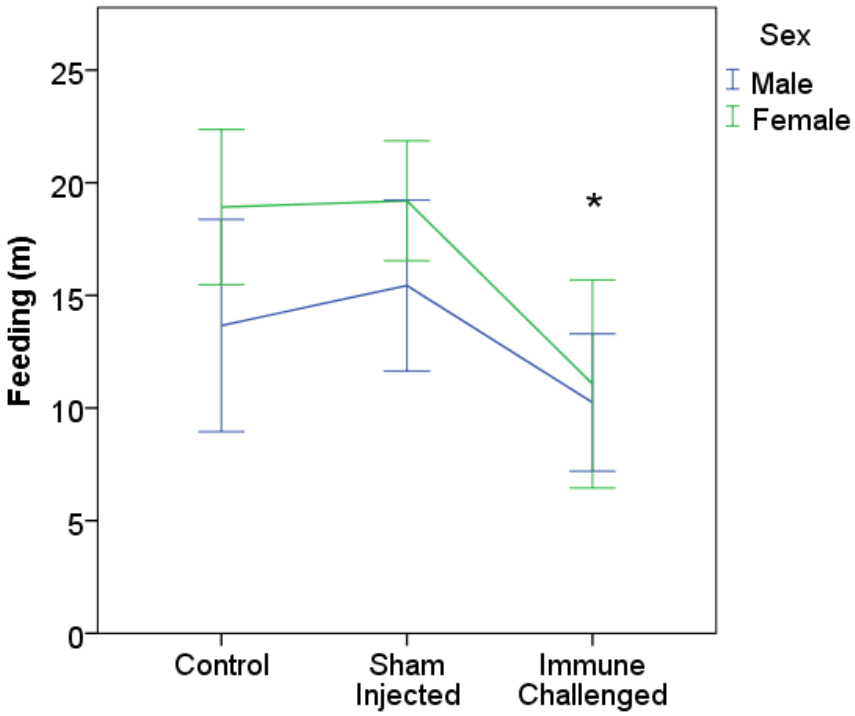

Figure 2.21. Mean time spent feeding (in seconds) for control (n = 20), sham injected (n = 20) and immune challenged groups (n = 20). Error bars represent 95% confidence interval. * represents significant result.

2.9 Effect of immune challenge on exploratory behaviour in nymphs

2.9.1 Methods

Adult and nymph crickets show decreased feeding in response to an immune challenge but adult crickets do not appear to exhibit other behavioural changes consistent with sickness behaviour. Adult crickets are motivated to find a mate and this motivation could alter the expression of sickness behaviour, as is seen in other invertebrate species (González-Tokman, et al., 2013). Nymphs however cannot mate until adulthood and should be motivated by survival rather than finding a mate, making them more likely to conserve energy and seek shelter while ill.

Nymphs from population two were isolated by the third or fourth instar (n = 76). Animals were randomly assigned to one of the three treatment groups two instars before their adult molt, when the wing buds and ovipositor (females only) are first apparent (see figure 2.22). Animals were first tested in the plus maze design (as previously described) 48 hours after molt to establish a behavioural baseline (see figure 2.23). Animals received treatment 24 hours later and retested 90 post-treatment. Juvenile animals received half the dosage (1 μl) of heat killed bacteria and nutrient broth described for adult treatment, as the weight of nymphs at this instar is about half that of adults. Crickets were gently placed in a cup (6 cm diameter) and were placed in the center of a plus maze (see previous plus maze description), with two open arms and two covered arms. After 1 minute the cup was removed and initial immobilization time, total locomotion, number of arms entered and total time spent under covered arms was measured over the 10 minute trial (as described in the previous plus maze study).

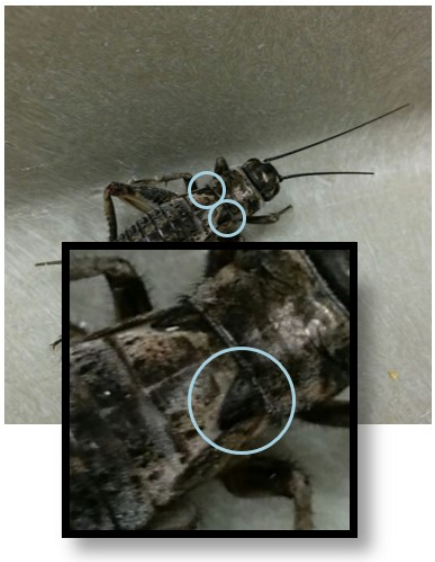

Figure 2.22. Wing buds appear 2 instar before final adult molt.

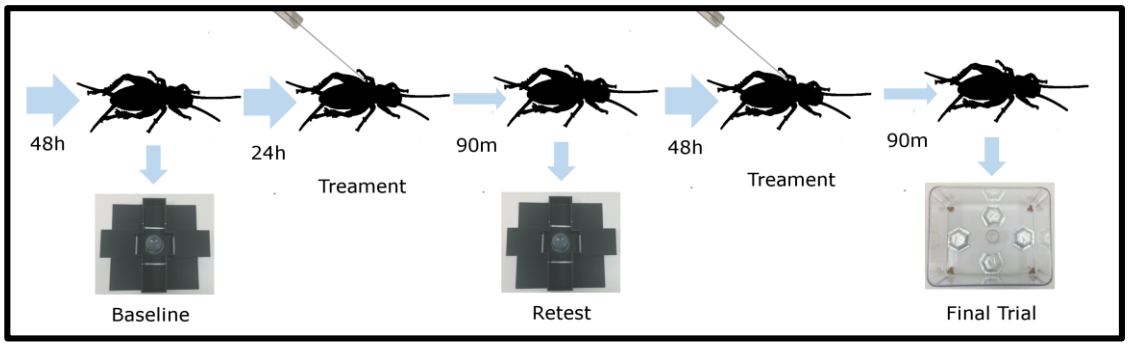

Figure 2.23. Time line of treatment and testing investigating exploratory behaviour in nymphs.

To further understand whether the effect of immune challenge on nymph anti-predator behaviour, animals were tested in a shelter use arena 48 hours after final plus maze trial. Treatment was administered to nymphs based on their previous treatment group and nymphs were tested 90 minutes post-treatment. Animals were gently placed in a small plastic vial (2 cm diameter) in the center of a clear plastic arena (17cm L x 15cm W x 9.5cm H). One piece of cat chow was placed in each corner and one shelter was placed against the center of each wall (see figure 2.24). The shelter consisted of an

inverted hexagonal weigh boat (3.8 cm long, 2.5 cm per side, 1 cm depth) with one side removed and covered in tin foil to be opaque. After 1 minute the cup was gently removed and the animal was assessed on shelter use (Animals that spent less than a minute in shelter were rated as low sheltering animals, while animals that spent more than a minute were rated as high sheltering animals) and feeding bouts (contact with mandibles and eating food, for a maximum of 1 minute per bout) over the 30 minute trial. The test chamber was cleaned thoroughly and the food was replaced between trials.

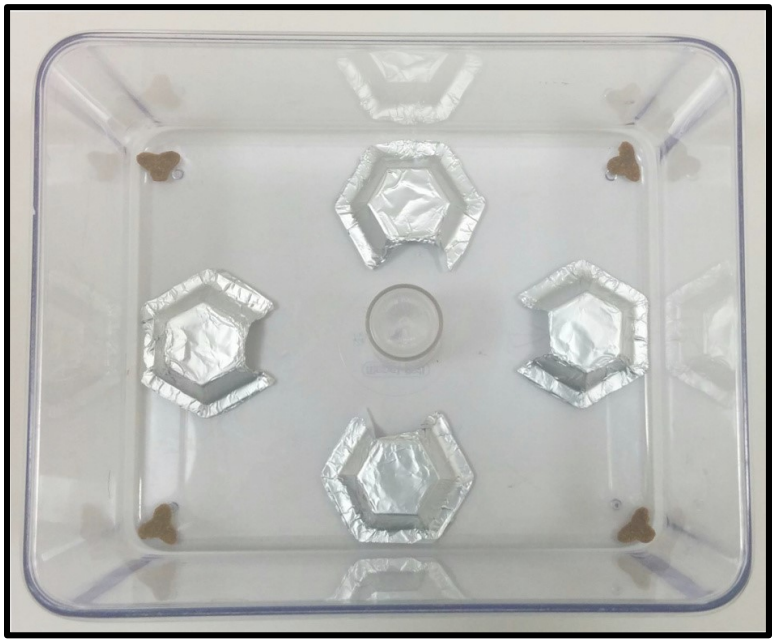

Figure 2.24. Picture of the shelter use arena used in the final trial.

2.9.2 Results

Preliminary analysis revealed that the data for the four plus maze measures were not normally distributed. Data were transformed using a delta transformation (as described in section 2.7.2). Further analysis revealed that the delta transformation had normalized data for all four measures. Extreme outliers were identified by using the outlier labeling rule (Hoaglin & Iglewicz, 1987), resulting in 1 score removed from

Δ total locomotion, 11 scores removed from Δ initial immobilization and 5 scores removed from Δ arms crossed.

A two-way ANOVA was performed for each measure. There was no effect of treatment ($F_{2, 66} = 0.31$, $p = 0.73$) or sex ($F_{1, 66} = 0.62$, $p = 0.43$) on Δ total locomotion, and no interaction effects detected ($F_{2, 66} = 1.76$, $p = 0.18$) (See figure 2.25). There was no effect of treatment ($F_{2, 70} = 0.81$, $p = 0.45$) or sex ($F_{1, 70} = 0.28$, $p = 0.60$) on Δ time spent under covered arms, and no interaction effects detected ($F_{2, 70} = 1.44$, $p = 0.24$) (See figure 2.26). There was no effect of treatment ($F_{2, 66} = 0.40$, $p = 0.68$) or sex ($F_{1, 66} = 2.86$, $p = 0.10$) on Δ initial immobilization, and no interaction effects detected ($F_{2, 66} = 0.74$, $p = 0.48$) (See figure 2.27). There was no effect of treatment ($F_{2, 65} = 0.19$, $p = 0.82$) or sex ($F_{1, 65} = 0.02$, $p = 0.88$) on Δ lines crossed, and no interaction effects detected ($F_{2, 65} = 0.59$, $p = 0.56$) (See figure 2.28).

Shelter use was analysed with the Kruskal-Wallistest. There was no effect of treatment on shelter use in males ($\chi^2 = 2.08$, $p = 0.35$)or females ($\chi^2 = 2.89$, $p = 0.24$) (See figure 2.29). Poisson regression was used to analyse the effect of treatment on feeding bouts during the final trial (See figure 2.30). Extreme outliers were identified by using the outlier labeling rule (Hoaglin & Iglewicz, 1987), resulting in 4 male scores (2 in sham-injected, 2 in immune challenged) and 0 female scores removed. Analysis revealed an effect of treatment on feeding bouts ($\chi^2 = 21.78$, $p < 0.001$). Immune challenged animals ($p = 0.004$, 95% CI [0.28, 0.78]) ate significantly less than controls. Sham injected animals were not significantly different from controls ($p = 0.35$, 95% CI [0.38, 1.41]). There was an effect of sex on feeding bouts ($\chi^2 = 6.39$, $p = 0.011$) with female having

2.32 feeding bouts for every 1 feeding bout of males, but there was no significant interaction between treatment and sex ($\chi^2 = 1.62$, $p = 0.44$).

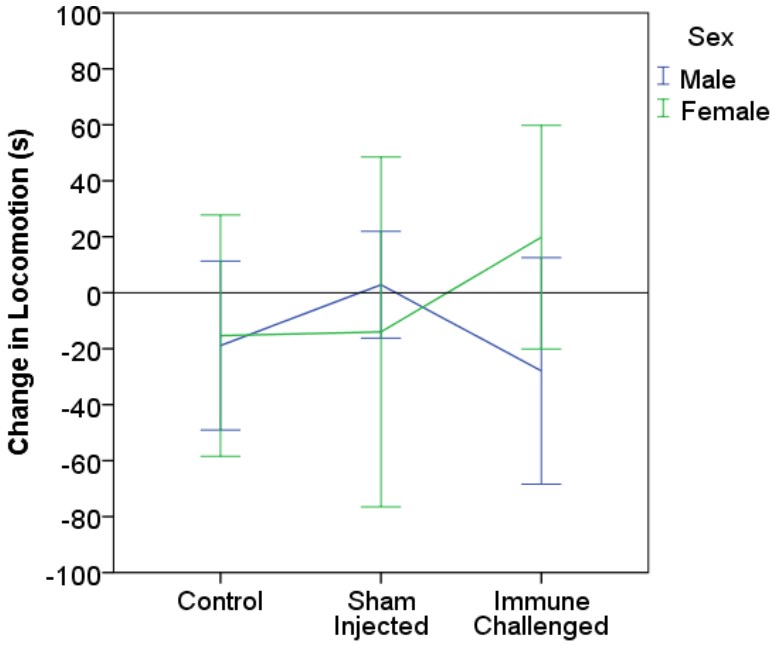

Figure 2.25. Mean change in locomotion (in seconds) for control (n = 24), sham-injected (n = 23) and immune challenged (n = 25) animals. Error bars represent 95% confidence interval.

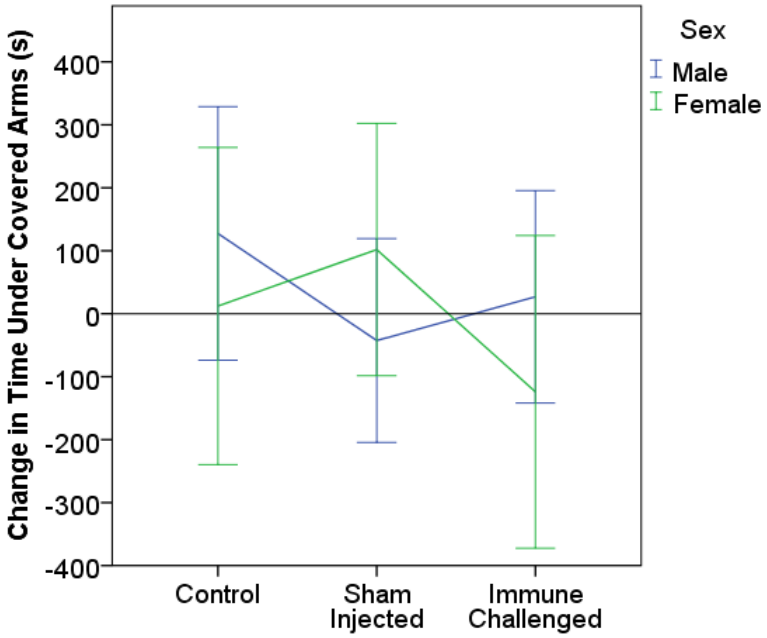

Figure 2.26. Mean change in time spent under covered arms (in seconds) for control (n = 24), sham-injected (n = 23) and immune challenged (n = 24) animals. Error bars represent 95% confidence interval.

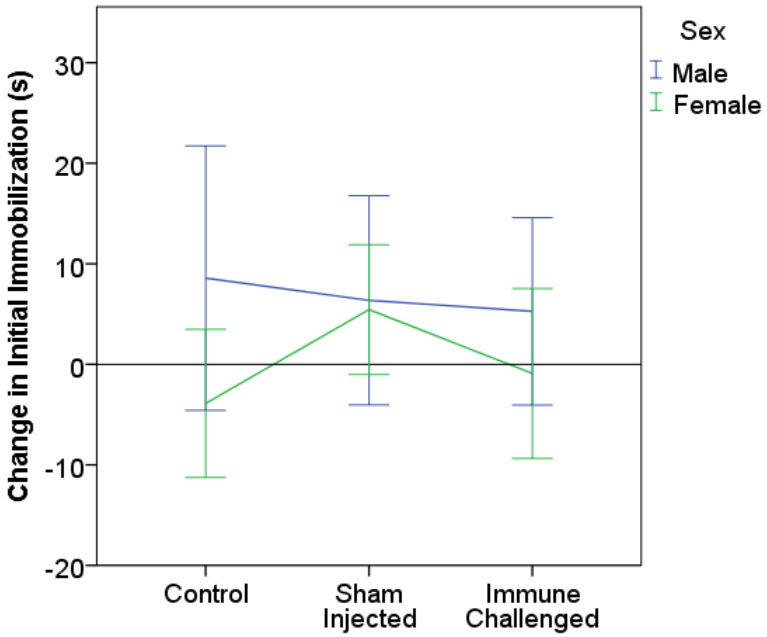

Figure 2.27. Mean change in initial immobilization time (in seconds) for control (n = 23), sham-injected (n = 20) and immune challenged (n = 22) animals. Error bars represent 95% confidence interval.

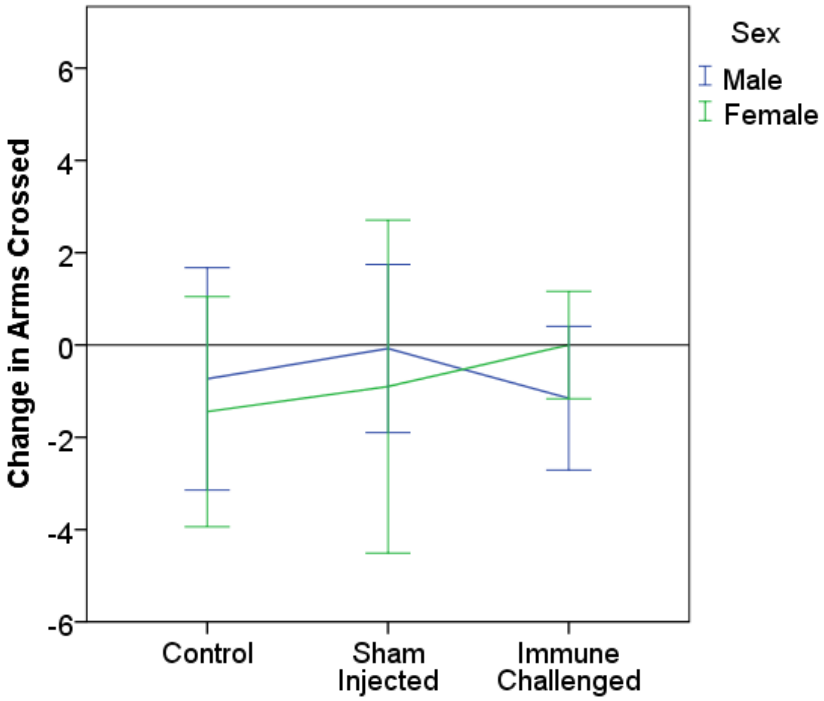

Figure 2.28. Mean change in total number of arms crossed, for control (n = 24), sham-injected (n = 23) and immune challenged (n = 24) animals. Error bars represent 95% confidence interval.

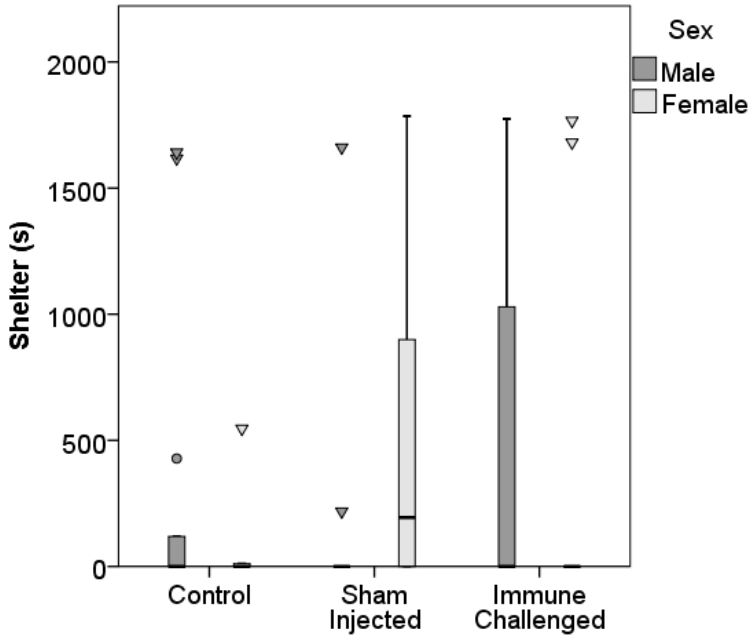

Figure 2.29. Comparison of animals counted using high or low shelter for control (n = 24), sham-injected (n = 24) and immune challenged (n = 24) animals. Circles represent non-significant outliers, while triangles represent significant outliers.

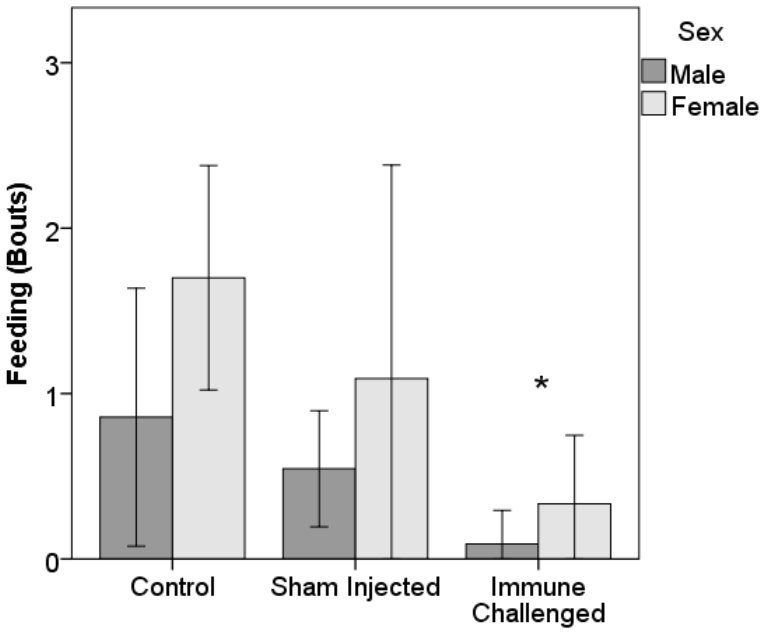

Figure 2.30. Mean feeding bouts for control (n = 25), sham-injected (n = 25) and immune challenged (n = 26) animals. Error bars represent 95% confidence interval. * represents significant result.

Chapter 3 - Discussion

3.1 Evidence for Energy Conservation Strategies

In response to an immune challenge, crickets increased grooming bouts, which is contrary to the behaviour observed in mammals and birds. Honeybees also increase grooming in response to injection or ingestion of lethal amounts of toxic substances (Hurst, et al., 2014), so this result is not without precedent. But the increase in grooming behaviour we detected was in response to a dose of non-lethal heat-killed bacteria. Grooming in insects is used to remove ectoparasites (Hurst, et al., 2014) to which sick insects may be more susceptible. Therefore increased grooming could be preventative, allowing them to not have to fight off multiple challenges at once. Regardless, this provides evidence that crickets increase grooming behaviour during immune activation.

We expected crickets would decrease their locomotion or general activity during an immune challenge, as reduced general activity is a hallmark of the lethargy seen in mammalian sickness behaviour and is a crucial part of the energy conservation hypothesis. However, across several studies, observing both adult and nymph crickets, we found no evidence that immune challenged crickets decreased their locomotion, reduced their exploration or reduced their general activities. In fact we found evidence to the contrary: immune challenged animals increased their locomotion compared to control and sham-injected animals (See section 2.3). This is similar to the results found by Arthurs and Thomas (2001), where immune challenged locusts initially increased their locomotion. This suggests that that crickets do not conserve energy by reducing their general activity.

We also did not find any evidence for adipsia in immune challenged crickets in either of the experiments this was tested in. We did find further evidence of illness induced anorexia in crickets, which has been established in previous research (Adamo, et al., 2010). We also observed illness induced anorexia in nymphs, which has not been previously demonstrated in the literature. Nutrition at the pre-adult stages is related to the number of molts and length of time it takes for the animal to reach adulthood (Merkel, 1977), so for nymphs to reduce their food intake at such a crucial stage underlines the importance of illness-induced anorexia. Our results show no evidence that Hart's energy conservation hypothesis is supported in crickets.

3.2 Evidence for Predator Avoidance Strategies

The most surprising result of this research is that crickets did not increase shelter use during an immune challenge. Crickets that experience high predation risk in the wild increase their shelter use compared to crickets from low predation environments (Hedrick & Kortet, 2006); therefore crickets can shelter use. In the nymph shelter-use trials the majority of animals were scored as low shelter use regardless of condition. It is possible that nymphs traded off shelter use for foraging behaviour. The longer crickets stay in their pre-adult stages of development (going through extra molts) the greater risk of predation (Merkel, 1977), and there is a relationship between adult size and reproductive success (Adamo, et al., 2015). So there is overall cost to fitness the longer they remain a nymph and thus a motivation to continue foraging even while sick.

Since nymphs show illness-induced anorexia, foraging seems like a counterproductive behaviour that only increases the risk of predation. But our research did not account for the potential scarcity of food in the wild. If food scarcity is an issue

for crickets, then it is advantageous to forage instead of staying put, similar to the Siberian dwarf hamster which continues to hoard food despite showing illness-induced anorexia (Durazzo, et al., 2008). In adults it also may not be adaptive to increase shelter use in response to illness. Due the short life span of crickets and the narrow window for mating opportunities in their life time, an increase in shelter use could mean missed mating opportunities for male crickets, which would markedly decrease their reproductive fitness. Immune challenged male crickets supress their sickness behaviour and fight just as hard as healthy males when presented with a mating opportunity, at the risk of their own immune function (Adamo, et al., 2015). Similarly, following an immune challenge, Nielsen and Holman (2012) found that male mealworm beetles (*Tenebrio molitor*) put greater investment into the pheromones they use to attract females implying an investment in fitness over survival. Alternatively, food scarcity may be a consideration for adults as well. The quality of eggs of chronically immune challenged female crickets is no different from controls when given free access to food and water (Shoemaker & Adamo, 2007) and chronic immune challenge reduces the fecundity of female crickets regardless of food availability (Stahlschmidt, et al., 2013). Food limited adult female crickets are more likely trade off shelter (anti-predator behaviour) for food, when choosing sites to lay their eggs (Stahlschmidt, O'Leary & Adamo, 2014). But in this way, they are also trading off fecundity (reproductive success) for food. Therefore, foraging with the risk of predation, even during immune challenge, must be a consideration for adult crickets as well.

A potential concern is that our study did not account for behavioural fever. While including a temperature gradient to account for temperature motivated locomotion could

give us more insight into sickness mediated behavioural shifts in crickets, we would actually expect to see a decrease in shelter use in crickets that experience behavioural fever, not an increase. Also, ss previously shown by Adamo (1998), even though crickets respond to a number of pathogens with behavioural fever, *S. marcescens* does not elicit behavioural fever, so it is unlikely that behavioural fever motivates behavioural shifts in the crickets in our study. Across several studies we could not find any evidence that crickets increased shelter use in response to an immune challenge, approaching the subject with several methods. The only evidence for a shift in shelter use behaviour we found was that immune challenged crickets decreased shelter use, which is consistent with the findings of Otti et al. (2012), but this result was not replicated in our further studies. Therefore, we did not find any evidence for increased shelter use as a predator avoidance strategy in immune challenged crickets.

In plus maze trials, immune challenged adult crickets were not significantly different than controls in their locomotion or initial immobilization. Immune challenged animals had significantly greater initial immobilization time compared to sham-injected animals, but were not significantly different from control animals. The stress of injection, without the immune challenge, may have altered the anti-predator behaviour of the adult sham-injected animals. But there was no significant difference in nymphs across all three groups. However, there is some evidence for a shift in other anti-predator behaviours in response to immune challenge. Both immune-challenged and sham-injected crickets had a longer total escape time than controls when subjected to a simulated predator. This is different from the results found by Otti et al. (2012), who found that the escape time of immune challenged European field crickets (*Gryllus campestris*) was not affected by

immune challenge. Otti et al. (2012) found that the reaction time of in response to a simulated predator was worse in immune challenged crickets. In our studies, immune challenged crickets did not differ from controls in initial immobilization (in adults or nymphs), or the latency to begin escaping in response to a simulated predator. Otti et al. (2012) found immune challenged animals were significantly more likely to remain outside of shelter, which is in line with the results of our first experiment, but was not replicated in our other experiments. Furthermore, Otti et al. (2012) found that immune challenged animals were more likely to succumb to predation when left in an environment with a live predator. Otti et al. (2012) suggests that their crickets were utilizing behavioural fever, but as previously discussed, *S. marcescens* is not known to elicit behavioural fever in crickets (Adamo, 1998), therefore it is unlikely this is a motivation for the shift in shelter use behaviour observed. Regardless, we did not find any evidence that crickets increase their shelter use in response to an immune challenge.

Use of shelter is an important predator avoidance strategy and since immune challenged crickets are more likely to succumb to predation (Otti, et al., 2012) enhancing predator avoidance strategies is crucial for survival. We have provided some evidence for how crickets change their anti-predatory behaviours in response to an immune challenge but we do not have evidence that these shifts in behaviour are adaptive. Therefore, we did not find any evidence that directly suggests Hart's predator avoidance hypothesis is supported in crickets, but there is some evidence for a shift in anti-predator behaviour.

3.3 Conclusions

Hart's hypotheses are well supported in mammals and have formed the foundation for our understanding of the adaptive nature of sickness behaviour (Dantzer & Kelley, 2007). Hart's hypotheses make sense in the context of mammals (Aubert, 1999; Johnson, 2002) but as previously discussed, even within mammals there are exceptions to the hypotheses. Furthermore, sickness mediated behavioural changes are seen in animals across all phyla, not just mammals. Hart's energy conservation hypothesis hinges on maintaining fever, but this is not applicable to ectotherms (e.g., insects) and behavioural fever is antithetical to shelter use, which is crucial to the predator avoidance hypothesis. However, the essence of Hart (1988), that animals change their behaviour during illness in order to enhance recovery, is supported in many animal species. Thus, we should expand upon Hart's hypotheses in order to accommodate for these exceptions.

Aubert (1999) has suggested that sickness behaviour is a shift in the motivational state of the animal, which is a better way to explain why animals suppress their sickness behaviour than Hart's hypotheses. Owen-Ashley and Wingfield (2007) have suggested two more hypotheses: the limited energy hypothesis (animals will modulate their immune function in response to the resources and seasonal limitations of their environment) and the life-history trade-off hypothesis (animals will trade-off immune function for other needs if it enhances their reproductive efforts). While their proposed hypotheses are based on their research on passerine species of birds, Owen-Ashley and Wingfield's hypotheses are a bit more accommodating to account for sickness behaviour across phyla; they account for sickness behaviour as a motivational shift and do not use fever as an explanation for behavioural shifts. For example, the life-history trade-off hypothesis

considers that animals will shift their behaviour to avoid predators, but may take risks if there is a chance it will increase their overall fitness. While Hart's hypotheses form the foundation of our understanding of the adaptive nature of sickness behaviour, they are not able to account for the exceptions even within mammals. Therefore, we should consider amending or appending to Hart's hypotheses for a more complete explanation of the adaptive nature of sickness behaviour across phyla.

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