INTERACTIONS BETWEEN IMMUNITY AND REPRODUCTION IN THE CRICKET, *GRYLLUS TEXENSIS*

by

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for the degree of Doctor of Philosophy

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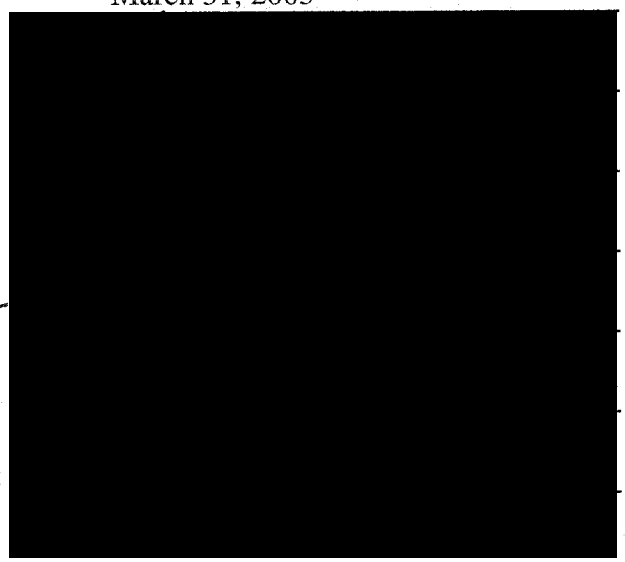

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To my beautiful Kathryn Grace

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ABSTRACT

Physiological trade-offs describe the negative relationship between two fitness traits of an individual, which occur based on competition between the two traits for energetic and other resources. A recent trend in the study of trade-offs involves the potential physiological trade-off between reproduction and immunity, which are both assumed to be energetically costly. This trade-off suggests that an increase in reproductive effort will result in a decrease in immune system activity, and conversely, an increase in immune system activity will result in a decrease in reproductive effort. While many studies of insect models suggest that both immunity and reproduction are costly, few studies directly investigate the relationship between the two. Evidence for this potential trade-off was examined here in the female cricket, *Gryllus texensis*. Four separate experiments were conducted. I predicted that: 1) infection of *G. texensis* with a live insect pathogen, *Serratia marcescens*, would lead to an adaptive increase in egg laying at the expense of immunity; 2) chronic immune system activation would lead to a decrease in the number and/or quality of eggs laid; 3) higher levels of reproductive activity would lead to decreases in immunocompetence, assayed by phenoloxidase (PO; an enzyme involved in insect immunity) activity of the hemolymph, and a functional test measuring survival following *S. marcescens* infection; and 4) higher levels of reproductive activity would lead to a reduction in somatic maintenance (including immunity) and result in reduced lifespan. I found no evidence to support these predictions, suggesting that no physiological trade-off exists between reproduction and immunity for this cricket. Additionally, I determined that PO provides a poor estimate for immunocompetence. While the lack of evidence for a trade-off could be explained in various ways, I suggest that under nutrient-rich conditions, reproduction may not be costly enough to reduce investment in somatic maintenance, including immunity. This possibility, along with the possible confound of PO activity measurements (a popular assay), forces a reexamination of previously published work within the field of insect ecological immunology.

LIST OF ABBREVIATIONS

LPS	lipopolysaccharide
m.a.d.	mean absolute deviation
PO	phenoloxidase
pPO	prophenoloxidase

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CHAPTER 1: GENERAL INTRODUCTION

Trade-offs, very generally, describe the negative association between two fitness traits, which include such elements as current reproductive output, somatic growth, lifespan, and offspring condition. The idea that reproduction is costly, and therefore leads to decreases in somatic function, has been central to the concept of trade-offs (Williams, 1966). Stearns (1992) summarizes the various types of trade-off studies that have been conducted, which include those between current reproduction and parental survival, current reproduction and future reproduction, current reproduction and parental growth, current reproduction and parental condition, and number and size of offspring, to name the most abundant types of studies.

This thesis examines a more recent trend in the study of trade-offs: the potential physiological trade-off between reproduction and immunity, both believed to be energetically expensive (Sheldon and Verhulst, 1996). The essence of this trade-off is that when an animal is sick, energy normally reserved for reproduction is diverted to the immune system. Alternatively, increased reproductive activity may drain resources normally reserved for immunity. Evidence documenting negative relationships between reproductive effort and immunity is abundant in birds (for review, see Gustafsson et al., 1994; Norris and Evans, 2000). For example, Nordling et al. (1998) found that experimental increases in brood size of the collared flycatcher led to a decreased antibody response to Newcastle disease virus.

Less is known about this trade-off in insects, although the literature on this subject is quickly growing (see below). In this thesis, I will search for evidence of this trade-off in the female cricket, *Gryllus texensis*. To provide the reader with an appropriate context for reading the dissertation, I will briefly review several relevant areas of literature: trade-offs generally, insect reproduction, insect immunity, trade-offs between reproduction and immunity, *G. texensis* as a model species, and finally, my experimental questions.

Trade-offs

The ecological concept of trade-offs is typically divided into two main categories: microevolutionary trade-offs and physiological trade-offs. While both imply a negative correlation between two fitness traits, the method of study and the conclusions reached can be different.

Microevolutionary trade-offs

Microevolutionary trade-offs describe the negative phenotypic correlations of fitness traits **within a population** (Stearns, 1992). For example, within a population, one might observe that some animals invest heavily in reproduction while others do not, but those animals investing heavily in reproduction have shorter lives. This describes a trade-off between reproductive effort and longevity.

Microevolutionary trade-offs by definition have a genetic basis, and because of this, it is possible to measure the relationship of two fitness traits following the creation of selected genetic lines. For example, Kraaijeveld and Godfray (1997) found that

Drosophila melanogaster selectively bred for increased resistance against the endoparasitoid, *Asobara tabida*, had reduced larval competitive ability relative to controls. Boots and Begon (1993) showed that the Indian meal moth, selected for increased resistance to granulosis virus, had a reduction in egg viability. These trade-offs might be the result of resource rededication between the fitness traits, or could imply negative pleiotropy, in which a single gene affects the expression of two phenotypes.

Physiological trade-offs

Physiological trade-offs occur based on competition between fitness traits for energetic, nutrient, or other resources **within an individual** (Stearns, 1992; Williams, 1966). These resources are required for activities such as maintenance metabolism (e.g. respiration and energy storage), foraging, growth, and reproduction, all of which are critical in determining an animal's fitness. The fundamental assumption within this area of research is that with limited resources, an increased demand by one fitness trait necessitates a decrease in the resources allocated to another trait. Physiological trade-offs are measured primarily through phenotypic correlations or by manipulation experiments. While phenotypic correlations allow for study on more natural populations, they may yield limited information, since many variables are uncontrolled and it is difficult to determine if the negative relationship is causal (Zera and Harshman, 2001). Therefore, manipulation experiments, both physiological and environmental, are superior. For example, a common experimental paradigm in birds is to manipulate reproductive effort by manipulating brood size by adding or removing eggs from the clutch. Other fitness traits are then measured correspondingly (e.g. Nordling et al., 1998).

My thesis is concerned with physiological trade-offs, and specifically with trade-offs between reproduction and immunity in crickets. Before discussing these trade-offs, I will first review some of the basic aspects of these physiological systems.

Insect Reproduction

The literature on insect reproduction is large and complex, and encompasses topics such as pair formation, sexual selection and courtship, mating, egg production, and their underlying physiological bases. For the purposes of this thesis, however, a brief and general overview will be restricted to egg production, mating, and oviposition, with an emphasis on cricket behaviour and physiology. The detail provided here should be sufficient to demonstrate how reproductive costs in the female can accumulate.

Egg production

Much of the information below on egg production has been derived from Davey (1985), Engelmann (1970) and Evans (1984). Unless otherwise specified, details are from these sources.

Within the abdomen of the female there are two ovaries, which are subdivided into ovarioles. Ovarioles, in turn, are subdivided into three regions: the germarium at its most anterior, the vitellarium, and the pedicel at the posterior end. The number of ovarioles present in the ovary can vary depending on the number of eggs a female of a species will lay. That is, a female laying many eggs will have more ovarioles than a

female laying relatively few. A female cricket, in a lifetime, may lay upwards of 1500 eggs (*G. texensis*, Chapter 2).

Insect ovarioles can be either panoistic, in which all oogonia differentiate into ooctyes (see below), or meroistic, in which trophocytes (nurse cells) are present in the germarium, and oogonia differentiate into both oocytes and nurse cells. Since orthopterans, including crickets, have panoistic ovarioles, the review below will describe oocyte development along a panoistic ovariole.

Development of an egg begins at the tip of an ovariole, the germarium, where an oogonium differentiates to form a primary oocyte, and mesodermal cells differentiate to form prefollicular tissue. The prefollicular cells surround the oocyte. The oocyte passes posteriorly down the length of the ovariole, and at the vitellarium, the prefollicular cells develop into the follicular epithelium. In this region, vitellogenin, synthesized in massive quantities from fat body (Kunkel and Nordin, 1985), is incorporated into the egg as yolk. Oocyte maturation is completed with the formation of the vitelline membrane and finally the chorion (Margaritis, 1985). The egg then sheds the follicular epithelium as it passes through the pedicel and into the lateral oviduct. In crickets, mature eggs are maintained in the lateral oviducts until oviposition is released by mating, and cannot be resorbed.

Mating

In crickets, the male attracts and courts a female through song, generated by rubbing its thickened forewings (tegmina) together (reviewed in Loher and Dambach, 1989). The decision to mate is made by the female, based on a number of assessments, such as male song quality (e.g. *Gryllus integer*, Hedrick, 1986), male age (*G. veletis* and

G. pennsylvanicus, Zuk, 1987), and mate location/predation risk (*G. integer*, Hedrick and Dill, 1993).

Once the female cricket has decided to mate, she mounts the male (most crickets maintain a female-above male mating posture), and the male passes a spermatophore to her. A spermatophore is a structure consisting of an ampulla (containing sperm and other mating compounds and accessory substances), an anchor plate by which the male attaches the spermatophore to the female, and a tube by which the sperm passes from the ampulla to the female. The ampulla remains outside the female, and the tube is guided into the spermathecal duct aperture. While the spermatophore remains attached, ampulla contents migrate into the female's reproductive tract. Sperm are stored and maintained by the female in the spermatheca. Mature eggs from the lateral oviducts are fertilized upon oviposition.

While the sperm provided by a single mating can be adequate to fertilize a lifetime of eggs (*A. domesticus*, Murtaugh and Denlinger, 1985), female insects, including *G. texensis* (Chapter 5), often mate repeatedly. Some crickets will mate as early as the day of eclosion (*G. integer*, Solymar and Cade, 1990; *Teleogryllus commodus*, Loher and Edson, 1973), and many times over the course of a day (*G. lineaticeps*, Wagner et al., 2001), likely to protect against unsuccessful matings (Sakaluk and Cade, 1980), replace deteriorating or diminishing numbers of sperm (Murtaugh and Denlinger, 1985), and/or increase genetic diversity of offspring (Fedorka and Mousseau, 2002).

Cricket oviposition

Oviposition itself is a complex behaviour, and has been examined in detail for *Teleogryllus commodus* (Sugawara and Loher, 1986). Briefly, the female prepares for egg deposition by searching the substrate for suitable moisture content. To oviposit, the female first raises her abdomen and angles her ovipositor down to make contact with the substrate surface. She then pushes her body backwards to push her ovipositor into the substrate. Penetration is aided through the coordinated movements of the ovipositor's four valvulae. When the ovipositor is fully buried in the substrate, the female slightly retracts her ovipositor, and at this time, a mature egg is expelled from the lateral oviduct through peristaltic muscular contractions into the genital chamber. At the same time, muscles deflect and retract the chamber. Then, there is a rest phase, at which time the egg is fertilized by a single spermatozoan that arrives via the spermathecal duct. The egg appears at the base of the valvulae, and the valvulae all move separately back and forth to carry the egg down their length. The female withdraws her ovipositor from the substrate, either partially or completely, and repeats the steps again. The process of substrate penetration, egg fertilization (rest phase) and egg deposition can take upwards of 30 seconds per egg, and a female can easily lay 100 eggs overnight (*G. texensis*, personal observations)

Evidence that reproduction is costly

The costs of reproduction can potentially be great from both an ecological and physiological standpoint. Perhaps the greatest ecological cost of mating and egg laying is the increased risk of predation (reviewed in Magnhagen, 1991). For example, song used

by male crickets, *Gryllodes supplicans*, to attract and court females may also attract predators, such as the gecko, that can then intercept approaching females (Sakaluk and Belwood, 1984). Another ecological cost is one of time. A female laying 100 eggs overnight, at 30 seconds per egg, would spend almost one hour laying eggs. More time might also be spent searching for the appropriate substrate. Time spent laying eggs is time that cannot be spent engaging in other activities, such as foraging.

More important within the context of this thesis are the physiological costs of reproduction, such as the commitment of resources and energy for eggs, and the risks associated with mating. The resources required to produce eggs are substantial. Eggs are relatively large (egg dry weight in *G. texensis* is 0.27 mg, Chapter 3) and quite complex, composed of yolk proteins predominantly, but also hormones, lipids, glycogen, vitamins, and other substances required to support the development of a new larval body (Kunkel and Nordin, 1985; Sander et al., 1985). While ultimately these resources are acquired through ingestion of food, their immediate source is internal. For example, massive quantities of vitellogenin are synthesized within the fat body, and proteins and lipids within the ovary (reviewed in Koeppe et al, 1985). The hundreds or thousands of eggs a female may produce in a lifetime (Hinton, 1981) can multiply this investment. This could lead to a costly drain of internal resources, especially if food, or some particular limiting nutrient, is scarce.

These resource costs are accompanied by energetic costs. Energy is required for increased foraging to supply the female with adequate materials to make the eggs. Energy is required for the metabolic machinery that processes the food, synthesizes vitellogenin and other products, and eventually creates the egg. Muscular energy is required to

transport the egg within the reproductive tract and along the ovipositor. These increased metabolic demands could lead to detrimental effects such as oxidative damage to DNA, proteins, and lipids, and to an accumulation of free radicals that may accelerate senescence and death (see Nilsson, 2002).

Copulation carries its own costs. Firstly, it could result in physical injury. While to my knowledge, this has not been documented in crickets, it is not inconceivable, and certainly has been noted in other species. For example, in the bean weevil (Crudgington and Siva-Jothy, 2000) and the dung fly (Blanckenhorn et al., 2002), internal damage from copulation can be seen in female genital tracts as sclerotized scars, and females mated multiply showed decreased lifespan as a result. The cause of death could possibly be a result of increased metabolic costs for wound repair, or perhaps increased risk of infection through the wound. Copulation, and internal fertilization generally, can also expose the female to parasites, pathogens, and spermatophore toxins. For example, toxins in the seminal fluid of *Drosophila melanogaster* can reduce female lifespan (Chapman et al., 1995).

These reproductive costs of can be translated into decreased lifespan. Arnqvist and Nilsson (2000) reviewed 122 experimental studies on insects in the literature, and found that for female insects without nuptial feeding (like *Gryllus texensis*), mating more frequently increases fitness (i.e. lifetime offspring production) but reduces lifespan, suggesting very strongly that reproduction carries costs. *Drosophila melanogaster* provides a particularly compelling example. Partridge et al. (1987) manipulated access to egg-laying substrate, and showed that mated female *D. melanogaster* lived longer when they laid fewer eggs, suggesting a cost to egg-laying. Lifespan was also significantly

reduced under continuous exposure to males compared with those given limited access, even though egg-laying rates were the same, suggesting a cost to mating per se. Virgin female fruit flies, *Ceratitis capitata*, have been shown to live significantly longer than non-virgins (Chapman et al., 1998).

Insect Immunity

Insects can protect themselves from microbial attack through behavioural, physical, and physiological mechanisms. One behavioural mechanism is behavioural fever. For example, the cricket, *Acheta domesticus*, showed a preference for increased temperature after inoculation with the intracellular parasite *Rickettsiella grylli* (Adamo, 1998). Physical protection is afforded by the sclerotized cuticle and peritrophic membrane, which protects the animal from microorganisms ingested with food (Dunn, 1990). Microorganisms that breach the insect hemocoel trigger the immune system. Unlike the vertebrate immune system, which has both acquired and innate components (Roitt et al., 1996), the invertebrate system relies on innate immunity alone. Innate immunity in the insect is well developed and sophisticated, and comprises both cellular and humoral components, which act in concert to protect the animal from pathogenic attack (for review see Gillespie et al., 1997; Lackie, 1988b).

Cellular immunity: hemocytes

Hemocytes (blood cells), by their morphological, ultrastructural, and behavioural characteristics, can be classified as prohemocytes (considered to be stem cells from which other hemocyte classes develop), granulocytes, plasmatocytes, spherulocytes, and

oenocytoids (reviewed in Lackie, 1988a; Lavine and Strand, 2002). They are responsible for three important immune functions in the insect: phagocytosis, nodulation, and encapsulation.

Phagocytosis describes the process of a single hemocyte, usually a plasmatocyte, engulfing entities recognized as non-self, including bacteria, yeast, and abiotic particles such as latex beads and lipopolysaccharides (LPS).

Nodulation is defined by the aggregation of many hemocytes surrounding greater densities of bacteria, yeast, abiotic materials, and protozoan parasites (e.g. *G. assimilis*, Miller et al., 1999; Gunnarsson and Lackie, 1985). Within minutes of pathogenic entry into the hemocoel, granulocytes begin to degranulate and entrap the particles of non-self, and plasmatocytes begin to aggregate around the perimeter. Melanization sometimes, but not always, occurs following nodulation.

Encapsulation is a hemocytic response to objects of non-self that are too big to be phagocytized or trapped within nodules, such as parasitoids or nematodes. Hemocytes aggregate in a multilayered fashion. The inner layer of the capsule is composed of necrotic and melanized hemocytes, probably granulocytes. Surrounding that are layers of flattened interdigitated cells, probably plasmatocytes. The outermost layer of the capsule comprises an extracellular material originally laid down as plaques. These capsules remain in the hemocoel until the animal dies (Gupta, 1991).

The cellular response may temporarily or permanently remove hemocytes from circulation (da Silva et al., 2000; Geng and Dunn, 1989). In exopterygotes, such as the cricket, hemocytes are continuously produced and differentiated in the hemopoietic tissue, from which they are released (Hoffmann et al., 1979). In the cricket, *Gryllus*

bimaculatus, this tissue is located in the abdomen on both sides of the dorsal vessel (Hoffmann et al., 1979).

Humoral immunity

Humoral immunity includes the rapid synthesis and release of antimicrobial proteins and peptides (Bulet et al., 1999; Kanost et al., 1990), mostly from fat body tissue, and lysozyme (e.g. *Gryllus bimaculatus*, Schneider, 1985) and cytotoxic compounds (e.g. hydrogen peroxide) from activated phagocytes (Nappi and Vass, 2001). These factors are broadly active against numerous pathogens, including Gram-positive bacteria, Gram-negative bacteria, fungi, and yeast. The number of inducible proteins and peptides that have been described are many, and are often species-specific. For this reason, generalizations about their activity cannot be made. For example, attacin, described in lepidopterans, increases the permeability of the outer membrane of Gram-negative bacteria (e.g. Carlsson et al., 1998), and cecropins, also described in lepidopterans, are active against Gram-positive and Gram-negative bacteria. Some inducible proteins, such as lectins, are capable of recognizing carbohydrates and may assist in hemocyte recruitment and ultimate destruction of the pathogen (Natori, 2001).

Humoral immunity also includes the enzyme phenoloxidase (PO), which is a copper-containing enzyme activated from its precursor prophenoloxidase (pPO) by a serine protease cascade following pathogenic insult (Ashida and Brey, 1997). PO activation is rapid (less than 1 hr, *Melanoplus sanguinipes*, Gillespie and Khachatourians, 1992), and is stimulated by factors such as β 1,3-glucans from fungal cell walls (Leonard et al., 1985), peptidoglycan and LPS.

PO is responsible for the oxidation of phenols to quinones, and ultimately to the production of melanin, which is deposited around nodules and encapsulated objects (Sugumaran, 2001), and therefore physically isolates pathogens from the host. Additionally, the biochemical pathway initiated by PO can lead to the production of toxic quinines and other compounds believed to be toxic to microorganisms (Nappi and Vass, 2001; Söderhäll et al., 1996).

Evidence that immunity is costly

The idea that immunity is costly has received increasing attention recently (Sheldon and Verhulst, 1996), and evidence of immune trade-offs in insects is accumulating. The cost of immunity can be expressed in several ways.

Firstly, an active immune system requires energy input. Siva-Jothy and Thompson (2002) found that short-term starvation in *Tenebrio molitor* resulted in a down-regulation of PO, which plays a role in humoral immunity (above). Feder et al. (1997) found in *Rhodnius prolixus* that larvae fed only on plasma, and not whole blood, had reduced measures of cecropin and lysozyme, reduced numbers of hemocytes, and decreased ability to combat infection. Finally, Anderson et al. (1973) found that hemocytes require an increase in glycogen consumption during active phagocytosis.

Activation of the immune system results in hemocyte damage, which in turn requires hemocyte replacement to maintain the pre-infection concentration. da Silva et al. (2000) found a decline in hemocyte counts in the hemolymph of *Acheta domesticus* following injection of bacteria or LPS, and four hours post-injection, the number of damaged hemocytes was measured at 58%.

Cytotoxic molecules that are byproducts of immune reactions (see above) are not only harmful to invading microorganisms, but also to the host cells. Therefore, initiating an immune response, while combating the invader, may possibly cause damage to the host that initiated it (reviewed in Nappi and Vass, 2001; Råberg et al., 1998).

Finally, the cost of immunity can be revealed through potential trade-offs with other components of fitness. For example, Doums and Schmid-Hempel (2000) showed that in the bumblebee, *Bombus terrestris*, increased foraging activity resulted in a decreased immune response to an experimental challenge with a nylon filament. Kraaijeveld and Godfray (1997) found that *Drosophila melanogaster* selectively bred for resistance against the endoparasitoid, *Asobara tabida*, had reduced larval competitive ability relative to controls. Moret and Schmid-Hempel (2000) found that starved bumblebees, *Bombus terrestris*, experimentally infected with non-pathogenic lipopolysaccharide or sterile micro-latex beads, had significantly reduced lifespans compared with those that were not infected, suggesting a cost to immune system activation.

General statement about trade-offs between immunity and reproduction

Despite the evidence that immunity and reproduction are costly in insects, few studies directly examine a trade-off between the two, although there is a widespread belief that one exists. While much of this belief stems from the elegance and simplicity of trade-off theory generally, it is also supported by the possibility that a common underlying biological mechanism links these two physiological systems. For example, eicosanoids (reviewed in Stanley, 2000) play a role in both stimulating egg-laying

behaviour in female crickets (Loher et al., 1981) and mediating nodulation reactions to bacterial infections (Miller et al., 1999). Furthermore, juvenile hormone levels, upregulated following mating, may mediate concurrent declines in PO activity in *Tenebrio molitor* (Rolff and Siva-Jothy, 2002). Exactly how these compounds might contribute to the expression of a trade-off, however, is not currently understood.

While it has been shown in many circumstances that infection can lead to a decrease in female fecundity in invertebrates (reviewed by Hurd, 2001), it is unclear whether this reduction is pathogen- or host-mediated. This thesis will examine the potential physiological trade-off between immunity and reproduction in *G. texensis*. Existing examples demonstrating trade-offs between reproduction and immunity in insects are listed in Table 1.

Table 1 Examples of studies explicitly investigating relationships between reproduction and immunity in insects.

Authors	Finding
Ahmed et al. (2002)	Infection with LPS in the mosquito, *Anopheles gambiae*, led to decreases in egg production and ovarian protein.
Boots and Begon (1993)	The Indian meal moth, *Plodia interpunctella*, selected for increased resistance to granulosis virus, had a reduction in egg viability.
Fellowes et al. (1999)	*Drosophila melanogaster* that survived parasitism by *Asobara tabida* had reduced egg production as adults.
Hosken (2001)	Selective breeding (through enforced monogamy or polyandry) in the dung fly, *Scathophaga stercoraria*, resulted in males and females with different sizes of reproductive organs. A negative correlation was found between the size of the reproductive organ and PO activity in the blood.
Rolff and Siva-Jothy (2002)	Adult mealworm beetles, *Tenebrio molitor*, had decreased PO levels after a single mating.
Ryder and Siva-Jothy (2000)	A positive relationship was found between energetically expensive calling song in male crickets and immunocompetence (i.e. disease resistance), measured by hemocyte load.
Siva-Jothy et al. (1998)	Mating decreased encapsulation of a nylon filament in damselflies.

Field cricket, *Gryllus texensis*, as a model

Several aspects of immunity and reproduction make an insect, and especially the cricket, an excellent model for the study of trade-offs.

The main advantage of using an insect model for the study of immunity is that its immune system is relatively simple, compared with that of a vertebrate, in that it lacks an acquired component (see Roitt et al, 1996). While the invertebrate immune system relies mostly on the activity of hemocytes, the hemopoietic organ and fat body, the vertebrate immune system recruits additional molecules (e.g. antibodies), cells (e.g. lymphocytes), and tissues (e.g. spleen, bone marrow, etc.) in its immune response. These additional immune mediators, while presumably adaptive, make it more complicated to clearly estimate immunocompetence, especially when immunocompetence is usually assessed through indirect measures (e.g. white blood cell counts, Ots et al., 2001). Because it cannot be assumed that all immune factors increase or decrease in a similar way to the same immune challenge, then the more "players" there are, the greater the number of measures that must be made in order to evaluate immunocompetence (Norris and Evans, 2000). Moreover, acquired immunity adds another layer of complexity to studies of immunity, because subsequent immune challenges with the same pathogen yield a more rapid and efficient immune response than the first (Roitt et al., 1996). Without rearing animals axenically, it is often impossible to determine whether the experimental animal has previously encountered the particular pathogen. Finally, *G. texensis* yields an advantage over other insect models in that its large size allows for fast and easy collection of hemolymph for physiological analysis.

There are many advantages to using the field cricket for reproductive studies. Because the female cricket can store enough sperm for a natural lifetime worth of eggs (Murtaugh and Denlinger, 1985), it allows the female to be held and examined in isolation for the duration of an experiment, instead of requiring constant pairing with a male. In addition to imparting an extra level of control, it also means that a decrease in egg output cannot be interpreted as a female's unwillingness to mate. Secondly, because *G. texensis* exhibits no parental care, increases in reproductive output translate directly into increased fitness, and decreases in reproductive output cannot be interpreted as an adaptive response based on an ill mother's inability to care for her future young. Moreover, animals with parental care might be less likely to make any trade-offs that reduce survival, because survival in these animals is crucial for reproductive success. Thirdly, because the natural lifespan of the female is only one breeding season, less than 30 days (Murray and Cade, 1995), her lifetime fitness can be more easily assessed than in other animals, such as birds. Finally, because the female has multiple offspring per reproductive bout (Loher and Dambach, 1989; personal observations) and produces eggs continuously (*A. domesticus*; Woodring et al., 1979), increases or decreases in reproductive output are easy to quantify.

Questions for the thesis

In this thesis, I will examine interactions between reproduction and immunity in the cricket, *Gryllus texensis*, in an effort to find evidence for a potential physiological trade-off.

In Chapter 2, I examine whether infection with the bacterium, *S. marcescens* (an insect pathogen), will increase oviposition in *G. texensis*. Fighting an infection might be expected to cause a decrease in egg laying. However, given that fitness is dependent on survival, oviposition in female crickets might be sensitive to factors that predict reduced life expectancy, such as immune system activity that accompanies infection with a potentially lethal pathogen. Therefore, a host-mediated increase in oviposition following infection could compensate for potential losses in future reproductive output. Adamo (1999) observed such an effect in the cricket, *Acheta domesticus*.

In Chapter 3, I examine whether chronic immune system activation, induced by regular injections of lipopolysaccharide into the hemocoel of the female, will lead to a reduction in the number or quality of eggs that a female produces. Ahmed et al. (2002) found that in the mosquito, *Anopheles gambiae*, there was a significant reduction in egg production and ovarian protein 24 hours following a challenge with LPS. Egg quality in my experiments will be measured by egg weight, protein content, fertilization success, and hatching success.

In Chapter 4, I investigate whether a decrease in reproductive activity will lead to increased immunocompetence (i.e. disease resistance) in female crickets. For example, Siva-Jothy et al. (1998) found that female damselflies are less immunocompetent following oviposition. In my experiments, levels of reproductive activity will be manipulated through mating (virgin vs. mated) and oviposition (substrate preferences) experiments. Immunocompetence will be assayed functionally by injection with *S. marcescens*, and through measurement of phenoloxidase in the hemolymph.

In Chapter 5, I will examine whether mating and oviposition decrease lifespan in female *G. texensis*. Lifespan is an indicator of overall body condition (including immunity). I predict that virgin females will live longer than mated females, because their reproductive costs over time have been minimized by not mating and by laying fewer eggs. For example, Chapman et al. (1998) found that virgin female fruit flies, *Ceratitis capitata*, live significantly longer than mated flies. Chapters 2, 3, and 4 will be discussed in the context of the results of Chapter 5.

Table 2 summarizes the manipulation experiments to be conducted.

Table 2 Summary of experiments in the dissertation

Manipulate	**Measure**	**Chapter**
Immune system by injecting bacteria or LPS	Egg output (number and/or quality)	2, 3
Reproductive activity • Virgin vs. mated • Mated laying few vs. mated laying many	Immunocompetence • Functional assay • Phenoloxidase activity	4
Reproductive activity	Lifespan	5

CHAPTER 2: REPRODUCTIVE DECISION-MAKING IN THE CRICKET *GRYLLUS TEXENSIS* (FORMERLY *GRYLLUS INTEGER*) FOLLOWING ACUTE INFECTION

Summary

To maximize fitness, the rate of offspring production should be sensitive to the animal's changing internal and external conditions. I predicted that acutely activating the immune system in the cricket (*Gryllus texensis*), signaling the possibility of decreased life expectancy, would lead to an immediate increase in reproductive effort. I found that lifetime reproductive output varies among individual crickets, and that female crickets were able to alter the number of eggs laid depending on substrate conditions (moist sand or cotton), suggesting that they have the capacity to modify oviposition rates. However, exposing female crickets to a potentially lethal pathogen, *Serratia marcescens*, did not increase or decrease egg laying. Therefore, activating the immune system with a sub-lethal dose does not affect egg laying.

Introduction

Because the timing, duration, and expression of reproductive behaviour play a critical role in determining an animal's reproductive success, it is reasonable to expect that the regulation of reproductive behaviours has been shaped through evolution in order to maximize lifetime reproductive output. For example, internal and external environmental factors can influence parent and offspring survival/success, which can consequently have an impact on fitness. Animals that are sensitive to conditions that predict offspring and/or parental survival, and can modify their reproductive behaviour accordingly, will have a selective advantage.

Cricket oviposition is no exception to such regulation. Although egg laying may appear to be a relatively stereotyped behaviour (described for *Teleogryllus commodus* by Sugawara and Loher, 1986), the underlying central control is complex and is regulated by a variety of neurohormones/neurotransmitters (Sefiani, 1987) and sensory input (Sugawara, 1993). This complexity allows for flexibility in egg-laying behaviour, which increases the cricket's ability to maximize lifetime reproductive success.

The cricket, *Gryllus texensis*, is an ideal animal in which to examine the regulation of reproductive effort. It has multiple offspring per reproductive bout (Loher and Dambach, 1989; personal observations), so that increases or decreases in reproductive output are easy to quantify. *G. texensis* exhibits no parental care (personal observations) so that reproductive success can be estimated solely by the number of eggs laid. It lives for only one breeding season: up to 22 (male) or 27 (female) days in the field (Murray and Cade, 1995). Adult females produce eggs continuously (*A. domesticus*;

Woodring et al., 1979) and carry mature eggs in the lateral oviducts. These eggs are fertilized during oviposition using stored sperm, and in *A. domesticus*, a related cricket, one mating can supply enough sperm for a lifetime of eggs (Murtaugh and Denlinger, 1985). Because of the existence of mature eggs and stored sperm within the female, an increase in the number of eggs laid can occur immediately upon receipt of relevant stimuli. In most other species an increase in offspring production first requires the synthesis of more eggs and the location of a mate, introducing additional energetic costs and a time delay between the stimulus and the response.

Given that fitness is dependent on survival, in female crickets oviposition should be sensitive to factors that predict reduced life expectancy. Carrying mature eggs may be beneficial if the cricket is able to lay them in the future, but if the chance of future reproduction declines, it would be to the female's advantage to increase current reproduction. One such factor that could predict decreased lifespan is the presence of a potentially lethal pathogen in an insect's hemocoel.

Although recently there has been a heightened interest in the trade-offs involving reproductive effort and immune defense (Norris and Evans, 2000; Sheldon and Verhulst, 1996), only a handful of studies have examined how animals increase reproductive effort following a potentially lethal infection. For example, Polak and Starmer (1998) found that male Drosophila, *D. nigrospiracula*, increased mean courtship activity when infected with the parasitic mite *Macrocheles subbadius*, a parasite shown to reduce longevity in these animals. Minchella and Loverde (1981) also found an increase in egg production in the snail when exposed to a particular parasite, regardless of whether the snail became infected.

Recent work has shown that female crickets (*A. domesticus*) injected with an LD_{50} dose of *S. marcescens* laid significantly more eggs the day following infection than those females injected with saline, suggesting that the crickets increase reproductive effort when the potential opportunity for future reproduction declines (Adamo, 1999). They also increased egg output following injection with *S. marcescens* lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria that elicits an immune response (e.g. Gunnarsson and Lackie, 1985) but is not itself intrinsically poisonous (Rietschel and Brade, 1992). Adamo (1999) suggested, therefore, that the egg-laying response was an adaptive host-mediated one based on the activity of the immune system, and further implied a functional immune/neural connection.

In this paper, I examine whether the presence of *S. marcescens* in the hemocoel of *G. texensis* affects oviposition. *S. marcescens* is a gram-negative bacterium found worldwide in water and soil, and is a potentially lethal pathogen in orthopterans in the field (Stevenson, 1959). Insects have external physical barriers to protect them from infection (Gillespie et al., 1997), but pathogens that overcome this barrier and enter the hemocoel activate a variety of immune responses, both cellular and humoral (for reviews see Gillespie et al., 1997; Lackie, 1988b; Sugumaran, 2001). Such immune activation may have been the cue that resulted in increased reproductive output in *A. domesticus*.

I predict that female *G. texensis* will also increase reproductive effort (i.e. number of eggs laid) following injection with *S. marcescens*. If immune activity alone is the cue for increased oviposition, then a sublethal dose may be sufficient to induce this effect. In the following experiments, a dose less than the LD_{50} is used. Additionally, as a first step to addressing the question, some preliminary descriptive data on egg laying will be

collected, including the modification of egg laying in response to different substrate conditions.

Materials and methods

Crickets

Crickets, *G. texensis*, were obtained from a colony maintained at Dalhousie University. Experiments were approved by the Animal Care Committee of Dalhousie University, and adhered to the guidelines of the Canadian Council on Animal Care. Crickets were reared on a 12:12 light:dark cycle at 28 ± 2 °C with food and water provided *ad libitum*. I removed newly emerged adults daily from nymph cages and gave them the designation "day 0" (d0). They remained in the colony until d7-d15, by which time most females should have mated (Solymar and Cade, 1990) and successfully filled their spermathecae (Chapter 4). I then isolated females into individual clear plastic containers (18 x 14.5 x 9 cm) with opaque lids. In all experiments described below, I provided females with food and water *ad libitum*, as well as a dome-shaped cover (an opaque cup from a paper egg-carton: diameter 4.5 cm). In some experiments, I weighed females before isolating them.

Oviposition over a female's lifespan

To determine patterns of egg laying over time and to assess an appropriate age group for further experiments, I isolated 15 females for a maximum of 57 days, and gave each female a large water bottle with loosely packed cotton for egg laying (see below). Each day, I replaced water bottles and counted both the eggs in the cotton and those found on the floor of the cage.

In another group of 23-29 day old adult females, hatching success of eggs laid in cotton in large water bottles was assessed. Eggs were collected daily for four days, and were incubated at room temperature in vermiculite. Females had access to males until they were 21-27 days of adult age.

Effect of substrate conditions on the number of eggs laid

To determine whether the daily number of eggs laid by female *G. texensis* could be modified by substrate composition, I gave females either cotton or sand in which to lay their eggs. I prepared moist sand by combining 12 mL of water with 37 mL of sand (sifted to a grain size smaller than egg size) in a small paper cup (50 mL volume). I counted eggs in sand by first allowing the sand to dry and then re-sifting it three times. Eggs were retained in the sieve (0.7 mm mesh size).

I prepared cotton substrate by packing cotton into either a small or large bottle full of water. Small water bottles were 6 mL in volume (4.4 cm long, 1.3 cm diameter), and a single large cotton ball produced a tightly packed, drier, cotton substrate. Large water bottles were 37 mL in volume (9.5 cm long, 2.2 cm diameter), and two large cotton balls produced a loosely packed, wetter, cotton substrate. I counted eggs in the cotton by unrolling each cotton ball into a thin layer where eggs were clearly visible.

I isolated females for four days into containers provided with either a small water bottle, large water bottle, or moist sand. In both cotton conditions, the water bottle provided both the egg-laying substrate and the water source for the cricket. In the sand condition, I provided females with a small water bottle as a water source.

I removed the egg-laying substrate (and the small water bottle in the sand condition) daily from each cage and replaced it with fresh substrate. I counted both the eggs in the substrate and the eggs found and removed daily from the cage floor. During counting, an effort was made to leave the females undisturbed. The mean number of eggs laid per day over four days was described as the baseline egg count.

Effect of infection on the number of eggs laid (Figure 1)

I gave females cotton in large water bottles as an egg-laying substrate for four days. At the end of day four, I made an estimate of the mean number of eggs laid per day for each female, based on a daily egg count over the first three days and an estimate of egg count on day four. I initially estimated the number of eggs on day four by a less accurate but rapid count (visual inspection without unrolling the cotton) so that I could perform injections immediately after I replaced the daily water bottle. Based on these estimated means, I matched females into three groups: saline-injected, bacteria-injected, and unhandled. Following injections (see below), an accurate count of the eggs laid on day four was completed.

I gave females in the saline-injected group a 5 μL dose of endotoxin-free saline using a 10 μL Hamilton syringe. All injections were given through the membrane beneath the pronotum. Saline was composed of 121 mM sodium chloride, 4.1 mM calcium chloride, 1.37 mM dibasic potassium phosphate, 198 μM monobasic potassium phosphate, and 38.6 mM Tris-hydrochloride (pH 7.4). Saline was made endotoxin-free by centrifugal filtration at a 5000 nominal molecular weight limit (Millipore Corporation,

Bedford MA). After the injection, I returned females to their individual cages for a fifth day.

I gave females in the bacteria-injected group a 5 μL dose of 8.75×10^3 cells of the bacterium *S. marcescens*. This dose is less than 1/10 the LD_{50} dose (Adamo et al., 2001). I used a different 10 μL Hamilton syringe than the one used for saline injections to avoid contaminating the saline syringe with bacteria. I obtained *S. marcescens* commercially from Carolina Biological Supply Company (Burlington, North Carolina), and calculated bacterial concentration using a Petroff Hausser counting chamber and phase-contrast microscopy. After injection, I returned females to their cages for a fifth day.

Unhandled females were left undisturbed for a fifth day.

At the end of the fifth day, I counted all eggs in the cages, and made comparisons between the baseline egg count (mean number of eggs laid per day for the first four days), the number of eggs laid the day before the injection (day 4), and the number of eggs laid the day after injection (day 5).

To determine whether substrate could affect the egg-laying response following infection, I repeated the above experiment using both moist sand and cotton in small water bottles as the egg-laying substrate. In the moist sand condition I did not match females to groups because sand needed several days to dry before eggs could be accurately counted. Thus, an estimate of baseline egg-laying rate could not be calculated. For the sand group only, I randomly distributed females to groups.

I conducted a second study to determine whether a larger dose, closer to the LD_{50}, would affect egg-laying. I repeated the experiment described above, except I infected females in the bacteria group with 10^5 cells/5 μL. For ease of counting, and the ability to

match females to groups, I used cotton in large water bottles only. Additionally, I counted eggs for three days following the injection, instead of one day, to determine whether there was a delayed response to bacterial injection.

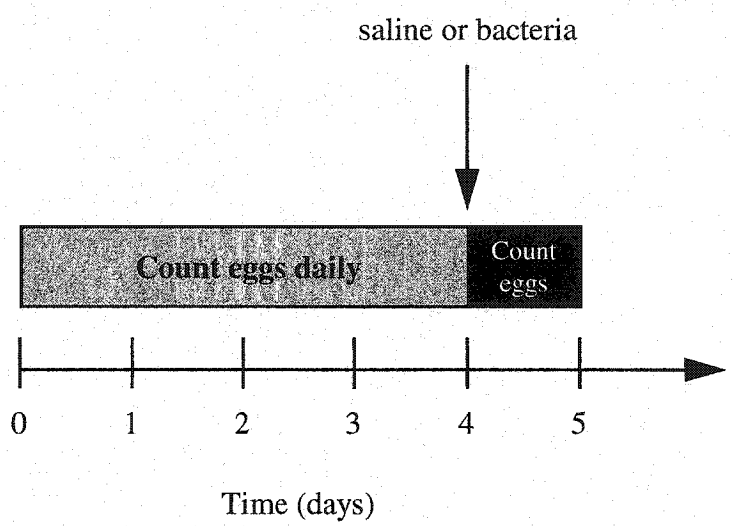


Figure 1 Diagram illustrating the method in Chapter 2. This procedure was repeated for females laying eggs in large water bottles, small water bottles, and moist sand.

Statistics

Because egg-laying data were not normally distributed, I used the median as the measure of central tendency, and the mean absolute deviation (m.a.d.) to describe dispersion about the median. I compared groups using nonparametric statistics as described in Meddis (1984), Sokal and Rohlf (1981) and Zar (1984). Eggs laid before and after infection were statistically compared using the nonparametric matched pairs

exact test (Meddis, 1984), which allowed me to directly examine responses of individuals within a group, without the influence of variability between individuals.

Results

Oviposition over a female's lifespan

All 15 animals lived for at least 18 days after isolation. Ten animals died between day 18 and day 57.

Median egg output of surviving females was high until approximately 31-36 days of adult age (day 22), which exceeds the approximate life expectancy of *G. integer* in the field (Murray and Cade, 1995). Egg output gradually fell over time after day 22 (Figure 2). Because of this trend, I continued to use only females between one and two weeks of adult age for all subsequent experiments. The median egg count per day per female until day 22 of the study was 9.5 ± 5.5 eggs. During the lifetime of the female, total egg output was variable between animals. The median total egg output over a female's lifetime was 541 ± 347.6 eggs, with the lowest total being 39 eggs (over 57 days) and the highest being 1530 eggs (over 54 days).

Daily egg output within animals was highly variable. For example, one female laid 60 eggs on one day, 206 the next, and 16 eggs the following day. The median change in egg output from one day to the next (during the period from day 1 to 15, excluding the one animal that laid no eggs most days) was 6 ± 7.0 eggs.

Eight of the 15 animals in this experiment had a median egg score of two or less, indicating that these animals laid very few eggs for most days of their lives. Nine animals laid no eggs for 4-20 days in a row.

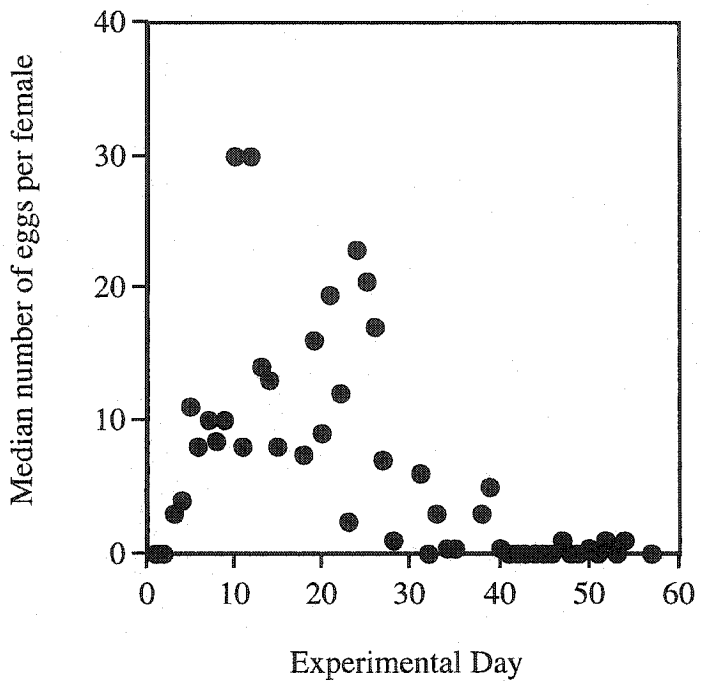


Figure 2 The median number of eggs laid per female per day over 57 days. Females (starting N = 15) were 9-14 days old on Experimental Day 1.

For the 10 animals that died before completion of the experiment, only two laid more eggs on the day before death or the second last day before death than they laid on average over their lifetime.

Hatching success, based on eggs laid by 23-29 day old females, was 52.6 ± 20.4% (n = 32). I found no evidence for a relationship between the number of eggs laid by a female, and the hatching success of those eggs (Pearson product moment correlation, r = -0.139, n = 28, P = 0.5).

Relationship between female weight and number of eggs laid

Heavier females (7-15 days of age) tended to lay more eggs (Spearman rank correlation, r=0.346, N=62, P<0.01; Figure 3).

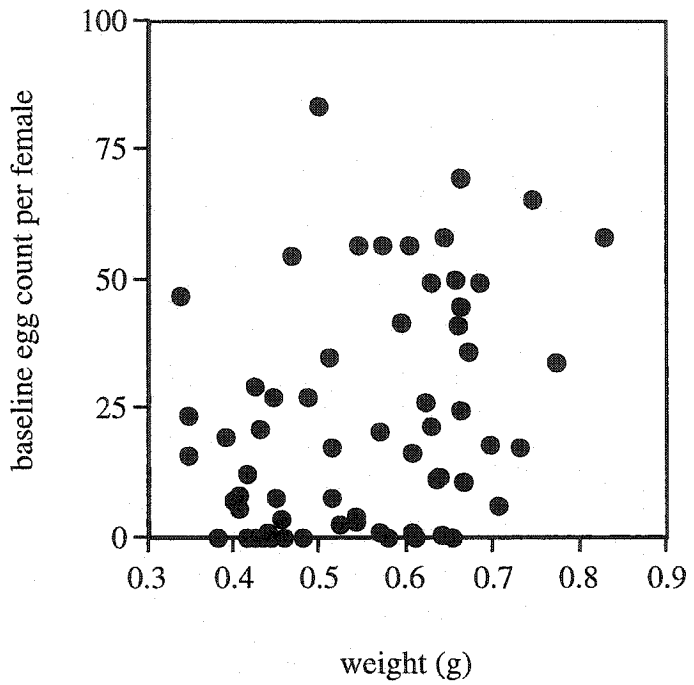


Figure 3 Correlation between female weight and baseline egg count in large water bottles (Spearman rank correlation r = 0.346, N = 62, P < 0.01). Each data point represents one female.

Effect of substrate conditions on number of eggs laid

There was a significant difference between baseline egg counts for sand (N=48), large water bottles (N=59) and small water bottles (N=56) (Kruskal-Wallis test, H_2=70.48, P<0.05). Nonparametric Tukey-type comparisons revealed that there were significant differences between all three groups (sand vs. small bottles: Q_3=8.29, P<0.001; sand vs. large bottles: Q_3=5.607, P<0.001; small vs. large bottles: Q_3=2.901, P<0.02) (Figure 4).

In the small bottle condition, 57% of the females had a baseline egg count of zero. In the large bottle condition, 36% of the females had a baseline egg count of zero. In the sand condition, only 8% of the females had a baseline egg count of zero, and no eggs were laid in the small bottle provided as a water source. The proportion of crickets laying no eggs was significantly different between the groups (G-test, G=29.74, P<0.001).

That females preferred sand to the other two substrates was additionally clear by the fact that they laid fewer eggs on the cage floor in the sand condition. Of the 44 animals in the sand condition that laid at least one egg, only one female ever laid eggs on the cage floor. In the large bottle condition, of the 38 females that laid at least one egg, 17 laid at least one egg on the floor. In the small bottle condition, of the 24 females that laid at least one egg, 23 laid at least one egg on the floor. These differences are significant (G-test, G=69.74, P<0.001).

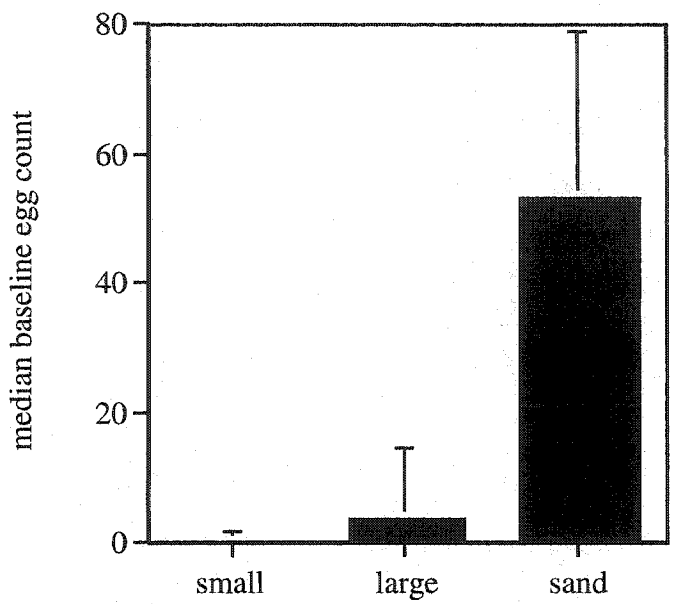


Figure 4 Median baseline egg count (± m.a.d.) for females laying in small bottles with cotton (n = 56), large bottles with cotton (n = 59), and sand (n = 48).

Effect of infection on the number of eggs laid

In the large water bottle group (Figure 5A), small water bottle group (Figure 5B) and moist sand group (Figure 5C), I found no significant increase in the number of eggs laid on the day after injection in the unhandled, saline, or bacteria (8.75×10^3 cells) groups (all comparisons, matched pairs exact test, NS). All females survived infection with 8.75×10^3 cells of *S. marcescens*.

To confirm that the low egg counts in the small bottle condition were not due to the females being virgins, I counted eggs for a sixth day following the experiment, and then gave all females a large water bottle to lay eggs in on day 7. Females laid significantly more eggs on d7 than they did on d6 (matched pairs exact test: unhandled, L=27, N=14, P<0.001; saline, L=26, N=13, P<0.001; bacteria, L=23, N=12, P<0.01).

Figure 5 Median egg counts (± m.a.d.) for the baseline before injection of saline/low dose bacteria (black bars), the day before injection of saline/low dose bacteria (white bars), and the day after injection of saline/low dose bacteria (hatched bars). A group of unhandled females served as a control. Females laid eggs in (A) large water bottles; unhandled N=19, saline N=20, bacteria N=20, (B) small water bottles; unhandled N=18, saline N=19, bacteria N=19, and (C) sand; unhandled N=16, saline N=16, bacteria N=16. Bacterial dose was 8.75×10^3 cells/5 µL.

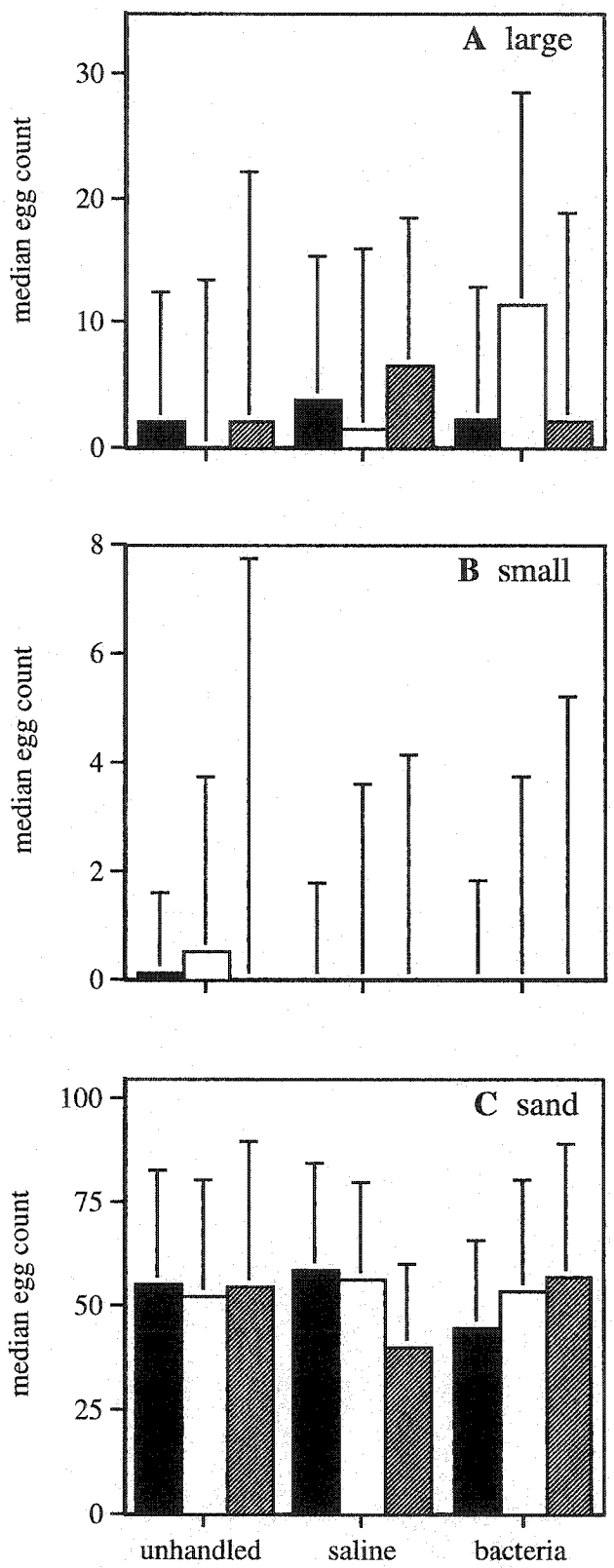

A large
median egg count
30
20
10
0
B small
median egg count
8
6
4
2
0
C sand
median egg count
100
75
50
25
0
unhandled
saline
bacteria

Figure 5

I repeated the experiment with a higher dose ($1x10^5$ cells/5μL), although still less than the LD_{50} (one bacteria-injected animal died out of 21). In no group did I find a significant increase in egg laying the day following injection (matched pairs exact test, NS; Figure 6). I continued to count eggs in this experiment for three days following injection, and observed no increases in egg laying on any of these days (matched pairs exact test, NS; data not shown).

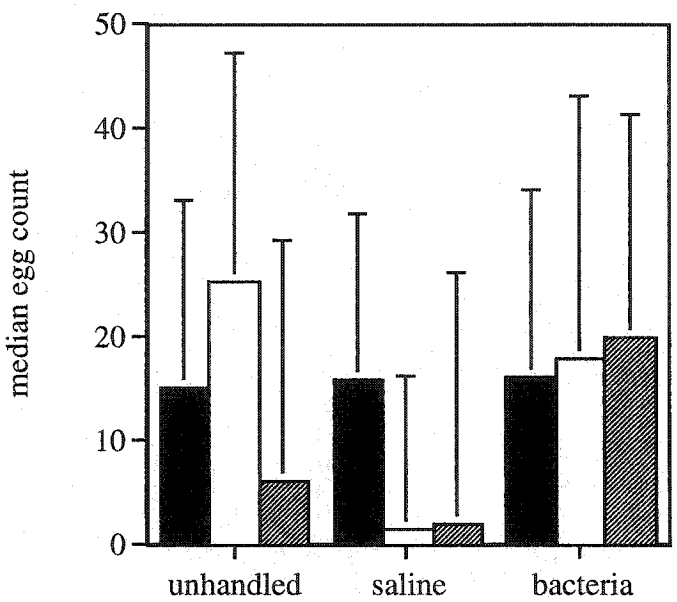


Figure 6 Median egg counts (± m.a.d.) for the baseline before injection of saline/high dose bacteria (black bars), the day before injection of saline/high dose bacteria (white bars), and the day after injection of saline/high dose bacteria (hatched bars). A group of unhandled females served as a control. Females laid eggs in large water bottles. Unhandled *N*=22, saline *N*=22, bacteria *N*=22. Bacterial dose was 1 x 10^5 cells/5 μL.

Discussion

Selection will favour animals that can alter the rate at which they produce progeny in response to the changing probability of maternal and/or offspring survival. I have found some evidence that crickets can do this, at least in some contexts. Crickets do not lay the same number of eggs daily. Instead, females retain mature eggs and lay variable numbers daily. Therefore, they have the potential to alter oviposition adaptively. I found that female *G. texensis* can vary the number of eggs laid depending on substrate composition. I did not, however, see any modification in the number of eggs laid in response to infection, a potentially life-shortening event.

The ability of *G. texensis* females to modify egg laying in response to substrate condition may indicate an adaptive preference. Substrate conditions can have an impact on female fitness because they are critical for hatchling success. For example, moisture is required for egg development (Masaki and Walker, 1987), yet excessive moisture reduces egg-hatching success (Gaylor and Frankie, 1979). Indeed, eggs left to hatch in large water bottles, which held "wet" cotton substrate, were often observed to rot (personal observations). Soil moisture also influences hatchling survival in the cricket *Allonemobius socius* (Bradford et al., 1993). Not surprisingly, oviposition substrate preferences have been documented in many insects (British grasshopper, Choudhuri, 1958; Japanese beetles, Allsopp et al., 1992), including crickets (Destephano et al., 1982; Howard and Harrison, 1984; Clifford and Woodring, 1990). Sensory receptors on female ovipositors (Sugawara and Loher, 1986) may mediate these preferences.

Although females show a capacity to modify egg output on a daily basis, I found no evidence suggesting that they do so in response to infection. Animals that cannot store mature eggs or embryos (e.g. most birds and mammals) prior to oviposition or parturition might not be expected to increase reproductive rate in response to an acute event such as infection. For example, Williams et al. (1999) found no increase in the clutch size of European Starlings after inducing an immune response, and several correlational reports in birds demonstrate a negative relationship between brood size and immune defense (reviewed in Norris and Evans, 2000; Sheldon and Verhulst, 1996). However, in animals such as the cricket, *G. texensis*, an increase in reproductive rate in response to infection would not be unexpected, since the female already holds a supply of mature eggs. In addition to the experiments conducted by Adamo (1999), Minchella and Loverde (1981) found that the snail *Biomphalaria glabrata* increased egg laying following exposure to the parasite *Schistosoma mansoni*, regardless of whether the snail became infected, and this was interpreted as the host's response to declining future reproduction. Cen-Aguilar et al. (1998) found that naturally infected *Boophilus microplus* ticks laid significantly more eggs on the first day of oviposition than uninfected ticks, although total lifetime egg production of infected females was not different.

In a closely related cricket species, *A. domesticus*, females laid more eggs following infection with *S. marcescens*, even when the infection was not lethal (Adamo, 1999). Adamo (1999) also observed this effect when *A. domesticus* was injected with only LPS derived from *S. marcescens*, suggesting that the response is host-mediated and likely induced by simple immune activation. Increased oviposition was not observed following

physical stress (tumbling or enforced exercise), short-term food deprivation, saline injections, or following infection by the parasite *Ormia ochracea*.

Unlike *A. domesticus* , *G. texensis* did not show an increase in egg laying following infection, regardless of substrate composition. Interestingly, I also did not see an increase following a large (100 μg) dose of LPS derived from *S. marcescens* (Chapter 3). It may be argued that since mortality rates were low the females may have been able to "assess" that death was not imminent, and therefore an increase in egg output was to no specific advantage. However, I saw no effect even at the high dose, which resulted in some (1/21) mortality under optimal lab conditions (i.e. ad lib food and water). Additionally, activating anti-microbial defenses with LPS was enough to trigger increased oviposition in *A. domesticus* (Adamo, 1999).

It is difficult to explain why *G. texensis* showed no modification in egg laying in response to infection while *A. domesticus* did (Adamo, 1999). Both *G. texensis* and *A. domesticus* occur in the Southern United States (Weissman et al., 1980), although *A. domesticus* most likely originated in Asia (Ghouri, 1961). However, they are found in different ecological niches, and this difference may have led to different reproductive strategies in response to infection. For example, *G. texensis* lives in lower density populations (Cade and Cade, 1992; Adamo, personal communication) in grassy fields and lawns (Cade and Otte, 2000), while *A. domesticus* lives in higher densities in more cosmopolitan areas, including garbage dumps (Bate, 1969). In addition to each species being exposed to a different subset of pathogens, which might lead to selection for different immune strategies, individuals living in high densities often have greater levels of pathogen resistance (e.g. Reeson et al., 1998), likely due to the increased probability of

horizontal transmission of disease between conspecifics (Freeland, 1983). This increased risk of disease may have put additional selection pressure on *A. domesticus*, and ultimately resulted in an adaptive egg-laying response to infection. Alternatively, it is possible that *G. texensis* only increases oviposition in response to massive infections (close to the LD_{50}). More work is needed to determine whether either possibility is true. If the latter explanation proves to be correct, it suggests that simple immune activation is not the trigger for increased oviposition.

It is also possible that the relationship between immunity and reproduction is extremely sensitive and specific, and as a result, not always easy to demonstrate. For example, Minchella and Loverde (1981) found that the snail *B. glabrata*, increased egg laying following exposure to infection with the trematode parasite, *S. mansoni*, but Crews and Yoshino (1989) did not find this effect. Cen-Aguilar (1998) found that naturally-infected *B. microplus* ticks laid significantly more eggs on the first day of oviposition, and found no difference in lifetime egg production, but Davey (1981) found that experimentally-infected ticks had significantly reduced egg output. These contradictory results, along with those presented in this chapter, emphasize the complexity of these physiological interactions.

Perhaps different females have different strategies in response to infection, and in taking an average of egg-laying output across a sample, evidence of these changes is lost. For example, some females might lay more eggs after infection while others lay fewer. Different strategies could result from a variety of factors that were not measured here, such as current nutritional status, the number of mature eggs present in the lateral oviducts, and the number of matings prior to isolation. However, correlational analyses

of egg-laying data show that the type of variation in egg-laying output on a day-to-day basis is similar to the type of variation seen before-and-after infection. Additionally, the variance in each sample is not significantly different before-and-after infection (Bartlett's test for homogeneity of variance). Although this does not negate the possibility that individual females have different strategies, it does suggest that any potential differences are subtle, or at least cannot be predicted by the recent egg-laying history of the female.

Finally, it is important to note that I did not see any evidence for an alternative reproductive strategy in response to illness: a decrease in egg-laying rate. Such a response could be the product of a physiological trade-off, in which an increased allocation of energy towards immune activation would lead to a decreased allocation of energy towards reproduction (Forbes, 1993; Sheldon and Verhulst, 1996). Correlational and experimental evidence of this negative trade-off is abundant in birds (e.g. Oppliger et al., 1997; Nordling et al., 1998). Possibly because crickets do not require an increased energy investment in egg production to lay stored eggs, and because they can store a large number of mature eggs in the lateral oviducts (approximately 120, see Chapter 4), mobilizing the immune system does not decrease the number of eggs laid. Instead, storing so many eggs would buffer the cricket against short-term effects of immune activation.

Cricket oviposition can be stimulated or inhibited in response to sensory cues, and yet it remains highly variable both between and within animals. This degree of variability is somewhat surprising given the strong selection pressures on this behaviour. More work remains to be done before we understand how these cues regulate oviposition.

CHAPTER 3: PHYSIOLOGICAL TRADE-OFFS AND CHRONIC INFECTION IN THE CRICKET, *GRYLLUS TEXENSIS*

Summary

In Chapter 2, I found no evidence for an adaptive increase in egg laying in the female cricket, *Gryllus texensis*, following simple activation of the immune system. However, at the end of Chapter 2, I noted that activation of the immune system might instead lead to a decrease in egg laying, because the costs of activating the immune system would rob energetic resources normally allocated for reproduction (physiological trade-off). Although I observed no evidence for this in Chapter 2, I suggested that the large numbers of eggs that can be stored in the lateral oviducts (approximately 120) could buffer females from the short-term effects of infection.

To accurately assess whether immune system activity makes a physiological trade-off with egg production, I activated the immune system in *G. texensis* females over a period of 12 days with regular injections of LPS derived from *S. marcescens*. By this time, the stored eggs should be depleted (*G. texensis* females can lay 600 in this length of time), and the effects of immune system activation on egg production should be obvious. I also assessed egg quality by measuring total protein of eggs laid, hatching and fertilization success, and the weight of individual eggs before and after chronic infection.

Although injections of LPS led to an immune response, I found no evidence that the immune response led to a decline in the number or quality of eggs produced.

Introduction

A physiological trade-off can be defined as the negative relationship between two physiological systems that are competing for the same limited energetic resources within an individual (Stearns, 1992). Two physiological systems that are likely expensive and therefore likely to make trade-offs with one another are the immune and reproductive systems (Sheldon and Verhulst, 1996). When an animal gets sick, the increased energetic needs of the immune system may drain energetic resources normally reserved for reproduction. Evidence documenting negative relationships between reproductive effort and immunity is abundant in birds (for review, see Gustafsson et al., 1994; Norris and Evans, 2000). For example, Nordling et al. (1998) found that experimental increases in brood size of the collared flycatcher led to a decreased antibody response to Newcastle disease virus.

Such a physiological trade-off may also be true in insects, since both reproduction and immunity are thought to be expensive (see General Introduction). Reproduction comprises many components, including finding a mate, copulation, egg production and oviposition. In the following investigation, I focus on egg production, which in insects, requires a large investment of resources. Eggs are relatively large and quite complex, composed of yolk proteins predominantly, but also hormones, lipid, glycogen, vitamins, and other substances required to support the development of a new larval body (Kunkel and Nordin, 1985; Sander et al., 1985). Additionally, proteins are secreted around the egg to form the chorion (Regier and Kafatos, 1985), which protects the embryo from desiccation. The hundreds or thousands of eggs a female may produce in a lifetime can multiply this investment. For example, in *Acheta domesticus*, adult female growth is

mostly ovarial, and between the ages of 10 and 30 days, the female invests 12.2 mg of dry weight into egg growth per day (Woodring et al., 1979). This is substantial considering the dry weight of the body without the ovaries is about 110 mg.

Immunity is also thought to be expensive in insects. The invertebrate immune system is simpler than that of the vertebrate, lacking acquired immunity and instead relying on innate immunity comprising both cellular and humoral components (for review see Gillespie et al., 1997; Lackie, 1988b). Cellular immunity depends on hemocytes, or blood cells, which can phagocytize small particles, form multicellular aggregates around large numbers of bacteria (nodulation), or encapsulate larger objects by forming multiple layers around them, and sometimes depositing melanin (for review see Gupta, 1991). Humoral immunity includes the synthesis of antimicrobial proteins and peptides (Bulet et al., 1999; Kanost et al., 1990), mostly from fat body tissue, and the activation of prophenoloxidase which is important in nodulation and melanization (Sugumaran, 2001). Potential energetic costs to resistance have been demonstrated by Doums and Schmid-Hempel (2000), who showed that in the bumblebee, *Bombus terrestris*, increased foraging activity resulted in a decreased immune response to an experimental challenge with a nylon filament. The potential fitness costs of immunity have been demonstrated through selective breeding experiments and through experimental immune challenge. For example, Kraaijeveld and Godfray (1997) found that *Drosophila melanogaster* selectively bred for resistance against the endoparasitoid, *Asobara tabida*, had reduced larval competitive ability relative to controls. Moret and Schmid-Hempel (2000) found that experimental infection had lifespan costs in *B. terrestris* when the insect was denied access to food.

Despite the fact that both immunity and egg production are believed to be energetically costly to the insect, few experiments have demonstrated that increased immune activity results in a host-mediated decline in reproductive output (but see Ahmed et al., 2002). While many studies show that infection with a live pathogen leads to decreases in reproductive output (see Hurd, 2001), it is difficult in these cases to distinguish whether the host or the pathogen triggered the decrease. For example, Davey (1981) found that experimental infection of *Boophilus microplus* ticks led to a significant decrease in egg output. While this decrease could have been the result of a host-mediated physiological trade-off, it may have also been a consequence of detrimental metabolic products of the parasite, or parasite-generated tissue damage.

In Chapter 2, I investigated whether acutely activating the immune system in the female cricket, *Gryllus texensis*, would lead to an adaptive increase in egg output. This prediction was based primarily on the findings of Adamo (1999), who found that injecting female crickets, *A. domesticus*, with either live bacteria, *Serratia marcescens*, or lipopolysaccharide (LPS) from *S. marcescens* cell walls, led to increases in oviposition the day after the injection. Chapter 2 documents that no such effect can be found in *G. texensis*. However, an alternative hypothesis presented at the end of Chapter 2 was that an increase in immune activity, as the result of an infection, might lead to a decrease in egg-laying, due to a physiological trade-off. While this result was not apparent in Chapter 2, it was possible that the large number of eggs a female can store in her lateral oviducts (approximately 120, personal observations) buffered her from the immediate effects of infection. In other words, decreases in egg production might not be obvious until the stored eggs are depleted.

To address this possibility, I injected female crickets, *G. texensis*, with LPS derived from *S. marcescens* chronically over a period of 2 weeks, to determine whether upregulation of immune system activity might make trade-offs against egg production. During this time, each female should be capable of laying 600 eggs, which will deplete internal stores. However, because mated females continuously produce and lay eggs, then over the course of 2 weeks, the lateral oviducts should be filled with eggs that were produced during the course of injections. The number of eggs laid immediately before and immediately after the chronic infection paradigm will be compared. Egg quality might also be affected by immune system activation. For example, in the damselfly, a positive correlation was found between larval size (based on head width), and the number of ectoparasites present on the mother (Rolff, 1999). Based on this observation, I also made measures of egg quality before and after the course of injections. I measured dry weight, hatching and fertilization success, and total protein content of eggs laid.

LPS is a glycolipid which makes up the outer surface of the outer membrane of a gram-negative bacterium, and it was chosen as the antigen for three reasons: 1) it has been shown to lead to an immune response in insects (e.g. *Zophobas atratus*, Bedick et al., 2000; *Schistocerca gregaria* and *Periplaneta americana*, Gunnarsson and Lackie, 1985); 2) *S. marcescens* is a gram-negative bacterium found world-wide in water and soil, and is a potentially lethal pathogen in orthopterans in the field (Stevenson, 1959); 3) LPS is not intrinsically poisonous (Rietschel and Brade, 1992) and, unlike a live pathogen, does not generate its own metabolic products. Therefore, any changes in egg production will be due to immune activation alone.

Based on its reproductive physiology, *G. texensis* makes an excellent model for addressing this type of question. Firstly, females can store enough sperm to fertilize stored eggs for at least two weeks (see results), and because of this, any decrease in egg output cannot be interpreted as a female's unwillingness to mate. Secondly, *G. texensis* exhibits no parental care, and therefore, any decreases in egg output cannot be interpreted as an adaptive response to the mother's inability to care for her future young. Finally, because the natural lifespan of the female is less than 30 days (Murray and Cade, 1995), her lifetime fitness can be more easily assessed than in other animals, such as birds.

Methods

Animals

Female crickets, *G. texensis*, were obtained from a colony maintained at Dalhousie University. Crickets were reared on a 12L:12D cycle at 28 ± 2 °C with food and water provided *ad libitum*. Newly emerged adults were removed from nymph cages daily. In all experiments described below, I provided females with food and water *ad libitum*

Positive control

Before beginning the chronic infection procedure, I established an appropriate dose for treatment. Two doses of LPS, a low dose (20 μg) and a high dose (100 μg), were selected for these experiments based on previously published physiological and behavioural findings. Gunnarsson and Lackie (1985) found that locusts, *Schistocerca gregaria*, and cockroaches, *Periplaneta americana*, had significantly increased nodule counts in the hemolymph in response to 25 μg *S. marcescens* LPS and 10 μg *E. coli* LPS, respectively. Larger doses have been shown to have behavioural responses. For example, Adamo (1999) found that injecting the cricket *A. domesticus* with 100 μg of LPS from *S. marcescens* resulted in a significant increase in eggs laid. McClain et al. (1988) found that 50-150 μg of LPS from *E. coli* resulted in expression of behavioural fever in the beetle, *Onymacris plana.*

To confirm that female crickets responded to 20 μg of LPS derived from *S. marcescens*, I coinjected females with an LD_{50} dose of live *S. marcescens* and 20 μg of LPS. If females launch a hemocytic immune response to LPS (as in Gunnarsson and

Lackie, 1985), then coinjection of LPS and live bacteria should result in increased mortality compared to females that receive live bacteria without LPS.

I chose females from the colony that were 16-23 days of adult age, and placed them at random into two groups. Each female in the first group received a 5 μL injection of 4.2×10^5 cells of *S. marcescens* (Carolina Biological). Bacterial concentration was calculated using a Petroff-Hausser counting chamber and phase contrast microscopy, and it was adjusted by mixing with insect saline (121 mM sodium chloride, 4.1 mM calcium chloride, 1.37 mM dibasic potassium phosphate, 198 μM monobasic potassium phosphate, and 38.6 mM Tris-hydrochloride; pH 7.4). Females in the second group also received a 5 μL injection of live *S. marcescens* (4.2×10^5 cells), but LPS derived from *S. marcescens* (Sigma), was also added to the bacterial mixture, resulting in a 20 μg dose of LPS per injection. Females were then isolated into small plastic containers with dry cat food, water, and moist sand. All injections were given through the membrane beneath the pronotum with a 10 μL Hamilton syringe. Mortality was monitored for four days.

Effects of chronic infection on egg laying (Figure 7)

Females remained in the colony until 9-15 days of adult age, by which time most of them should have mated (Solymar and Cade, 1990; Chapter 4). Females were then isolated into individual clear plastic containers (18 × 14.5 × 9 cm; day 0 in Fig. 1) with opaque lids, food and water *ad libitum*, an opaque cup from a paper egg-carton (diameter 4.5 cm) for cover, and moist sand for egg laying. Moist sand was prepared by combining 12 mL of water with 37 mL of sand (sifted to a grain size smaller than egg size) in a small plastic cup, and it was replaced every two days for the duration of the experiment.

Females were designated randomly to one of three groups: unhandled, saline-injected, and LPS-injected. On the fourth day of isolation (Figure 7), females were first weighed, and the first injection was given. Females were injected every third day for 12 days (5 injections) with 5 μL of either LPS solution or endotoxin-free saline (Figure 7; unhandled females were never injected). Injections were given through the membrane beneath the pronotum with a 10 μL Hamilton syringe, and syringes were dedicated to either saline or LPS injections to avoid potential cross-contamination. The LPS dose was further subdivided into a low dose (20 μg LPS / 5 μL) or a high dose (100 μg LPS / 5 μL). Females were weighed again prior to the final injection on day 16, and after the final injection, all females remained unhandled for the duration of their lives. Females remained isolated, and were given fresh food and water when required. Lifespan was monitored.

To determine the effect of chronic infection on the number of eggs laid, eggs were counted on both days 4 and 18, and since sand was collected every second day, the number of eggs counted reflected the number of eggs laid over two days (days 3 and 4, and days 17 and 18). Comparisons between "before" and "after" were made for the number of eggs laid per day, and for female body weight (days 4 and 16).

Effects of chronic infection on egg quality (Figure 7)

Egg quality was estimated by measuring hatching success, fertilization success, protein content of eggs laid, and dry weight of eggs.

Hatching success was measured after chronic infection (day 16 in Figure 7) by first allowing the eggs to develop within the sand for 11 days. At this time, the sand (still containing the eggs) was dried slowly at room temperature. Moisture content of the sand was carefully monitored to prevent desiccation of the eggs. While the sand was still slightly moist, it was passed through a sieve (0.7 mm mesh size). Eggs were retained in the sieve. All eggs were counted and assessed for presence of eyespots before being incubated in moist vermiculite. Hatchlings emerged approximately 3 days later, and they were counted over seven days. Hatching success was calculated by dividing number of hatchlings by the number of eggs incubated with eyespots. Fertilization success was calculated by dividing the number of eggs with eyespots by the total number of eggs counted.

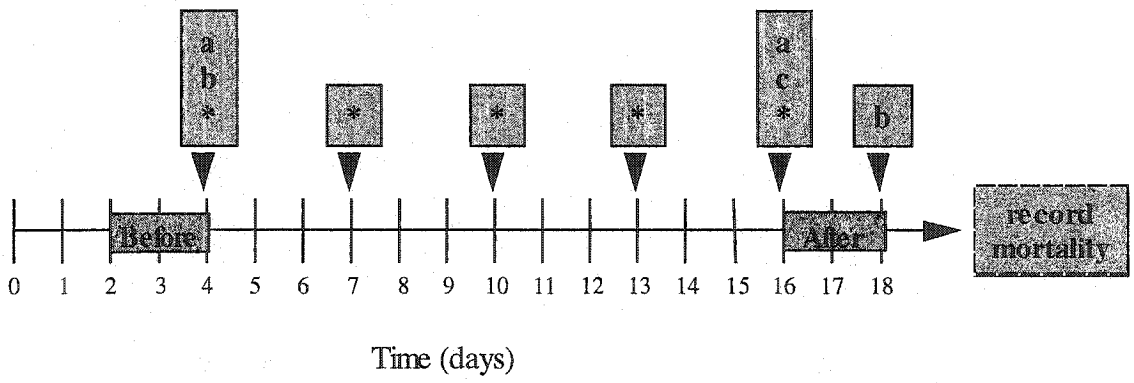
Protein content of eggs laid after chronic infection was measured by first collecting one to five eggs from the moist sand of each female on day 18 (Figure 7). Each egg was placed separately into a microfuge tube with 110 μL of phosphate-buffered saline (Sigma) at room temperature. The egg was crushed with the tip of a sonicator probe, and then the solution was sonicated for five seconds. 50 μL of the homogenate was added to a spectrophotometry cuvette with 950 μL of Bradford Reagent (Sigma; Bradford, 1976). After approximately 20 minutes of incubation at room temperature, absorbance of the sample was measured spectrophotometrically at 595 nm. Protein content was compared to a standard calibration curve calculated using bovine serum albumin (Sigma). For those

females from which more than one egg was collected, the median value for protein content was used.

Dry weight of eggs laid was measured by allowing eggs from day 18 to dry several days in the sand. Eggs were collected by sifting the sand (eggs were retained in the sieve, mesh size 0.7 mm). All eggs in the sand were counted and collected, and care was taken to ensure that all grains of sand were removed from the eggs. Eggs were weighed to the nearest 0.1 mg, and this weight was divided by the total number of eggs weighed to give the weight per egg.

Statistics

Because egg-laying data were not normally distributed, the median and mean absolute deviation (m.a.d.) about the median were used to describe numbers of eggs laid. All other values report the mean and standard deviation. I used parametric and nonparametric statistics when appropriate (Meddis, 1984; Zar, 1984). ANOVA and Kruskal-Wallis analyses were conducted using StatView 5.0.1. Mortality analysis was done using a Cox-Mantel test (Lee, 1980).



a = female weight
b = count eggs, measure total protein and dry weight of eggs
c = hatching success/fertilization success
* = injection of saline or LPS ("unhandled" females received no injection)

Figure 7 Diagram illustrating the chronic infection paradigm, and the times at which egg measurements were made. Females were 9-15 days old on day 0, and were placed at random into one of three "injection" groups: unhandled, saline, or LPS. Sand was replaced every 2 days, on "even" days. Comparisons for variables **a** and **b** were made "before" (day 4) and "after" (day 16 or 18) the chronic infection procedure. The five asterisks indicate the five injection times.

Results

Positive control

Coinjection of 20 μg of LPS and 4.2×10^5 cells of live *S. marcescens* led to significantly greater mortality than the injection of live bacteria alone, suggesting that LPS imposes an additional immune cost to the insect (Figure 8; Cox-Mantel test for survival distribution, $C = 1.91$, $P < 0.05$, $n = 16$/group).

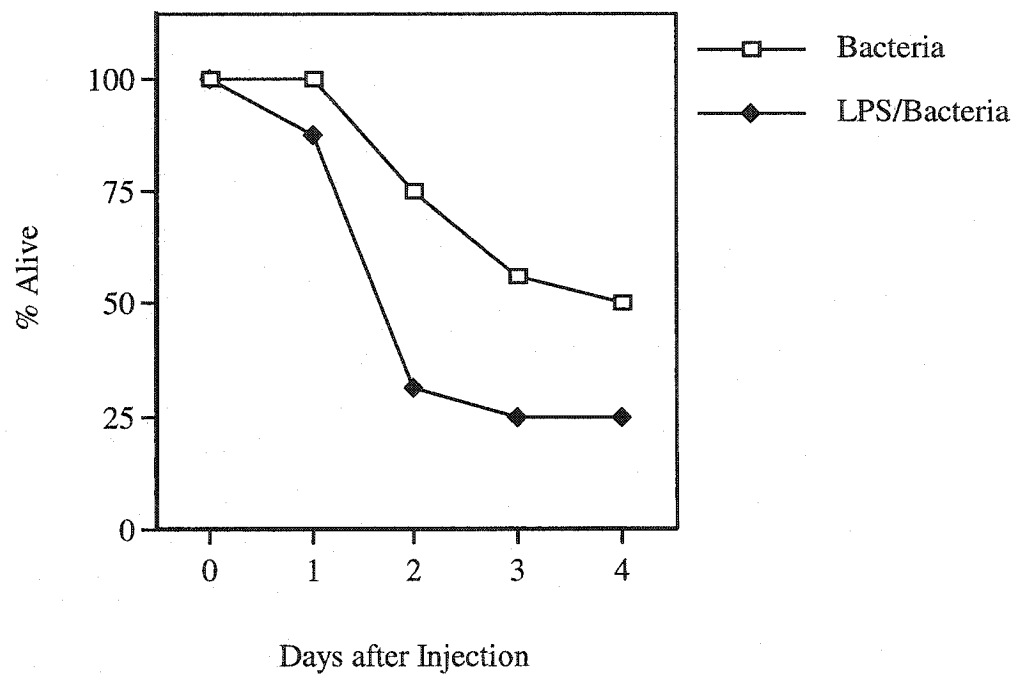


Figure 8 Survival distributions for females injected with bacteria, or coinjected with bacteria and LPS. n = 16 per group.

Effect of chronic infection on egg numbers, female weight, and lifespan

I found no evidence for a decrease in egg output following chronic infection with 20 μg doses (Figure 9A; G-test, $G = 2.58$, $P > 0.25$) or 100 μg doses (Figure 9; G-test, $G = 4.08$, $P > 0.1$) of LPS.

I found no evidence for a change in female weight following either low dose (Figure 10A; Repeated measures ANOVA, $F = 0.13$, $P = 0.88$) or high dose (Figure 10B; Repeated measures ANOVA, $F = 0.76$, $P = 0.48$) LPS injections.

I found no evidence for decreased lifespan following either chronic low dose or high dose LPS injections (Figure 11; Low dose, Kruskal-Wallis, $H = 0.086$, $P = 0.96$; High dose, Kruskal-Wallis, $H = 4.994$, $P = 0.08$). Females in the low LPS dose experiment lived 59.8 ± 17.8 days ($n = 47$), while females in the high LPS dose experiment lived 52.1 ± 16.3 days ($n = 22$).

In a comparison of mean eggs laid per day before the first injection and mean eggs laid on the two days after the first injection, I found no evidence for an increase in egg output for the low LPS group (G-test, $G = 2.09$, $P > 0.25$), or the high LPS group (G-test, $G = 1.79$, $P > 0.25$; data not shown), a possibility hypothesized in Chapter 2.

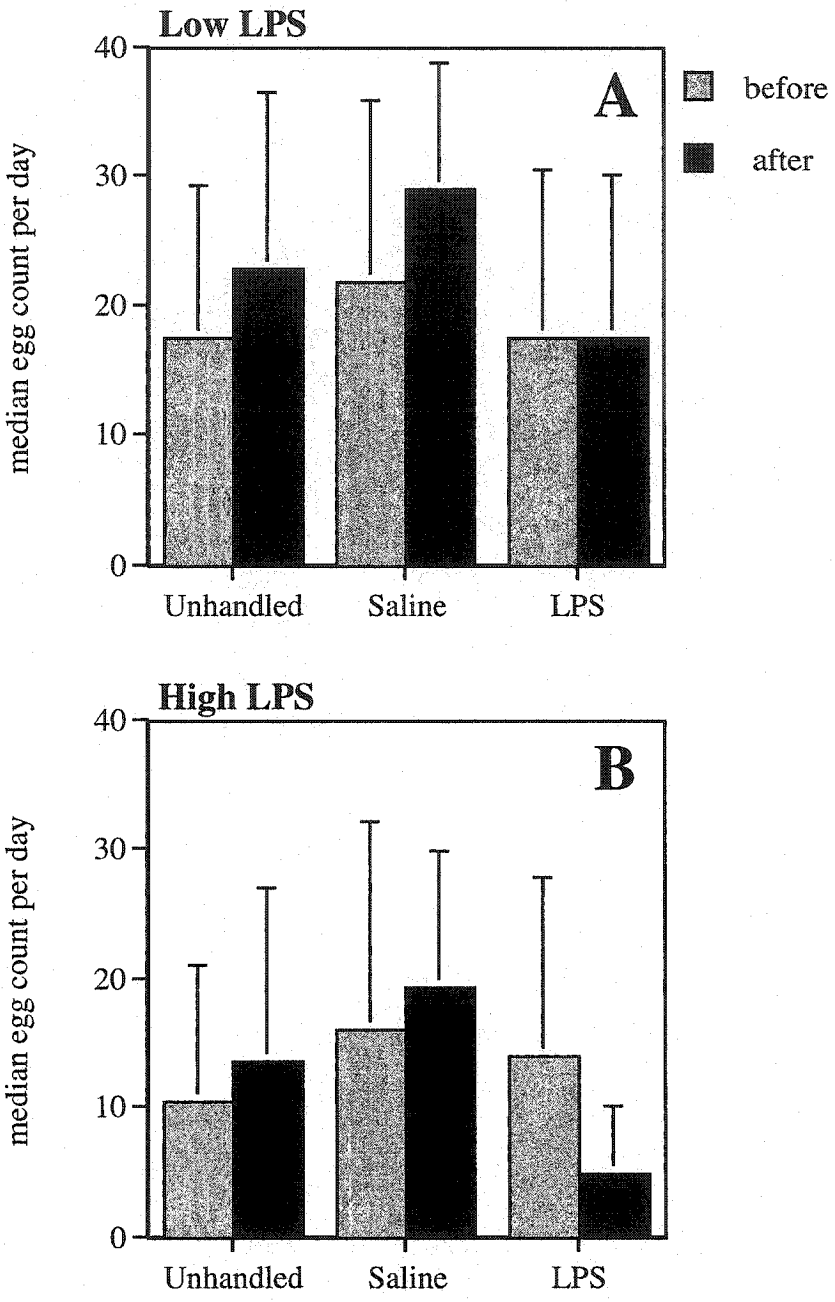


Figure 9 Median egg output per day over the 2 days immediately before beginning chronic infection, and the 2 days following the final injection. A) Egg output in unhandled (n = 20), saline-injected (n = 22) or LPS-injected (20 μg/injection; n = 24) crickets. B) Egg output in unhandled (n = 14), saline-injected (n = 14) or LPS-injected (100 μg/injection; n = 14) crickets.

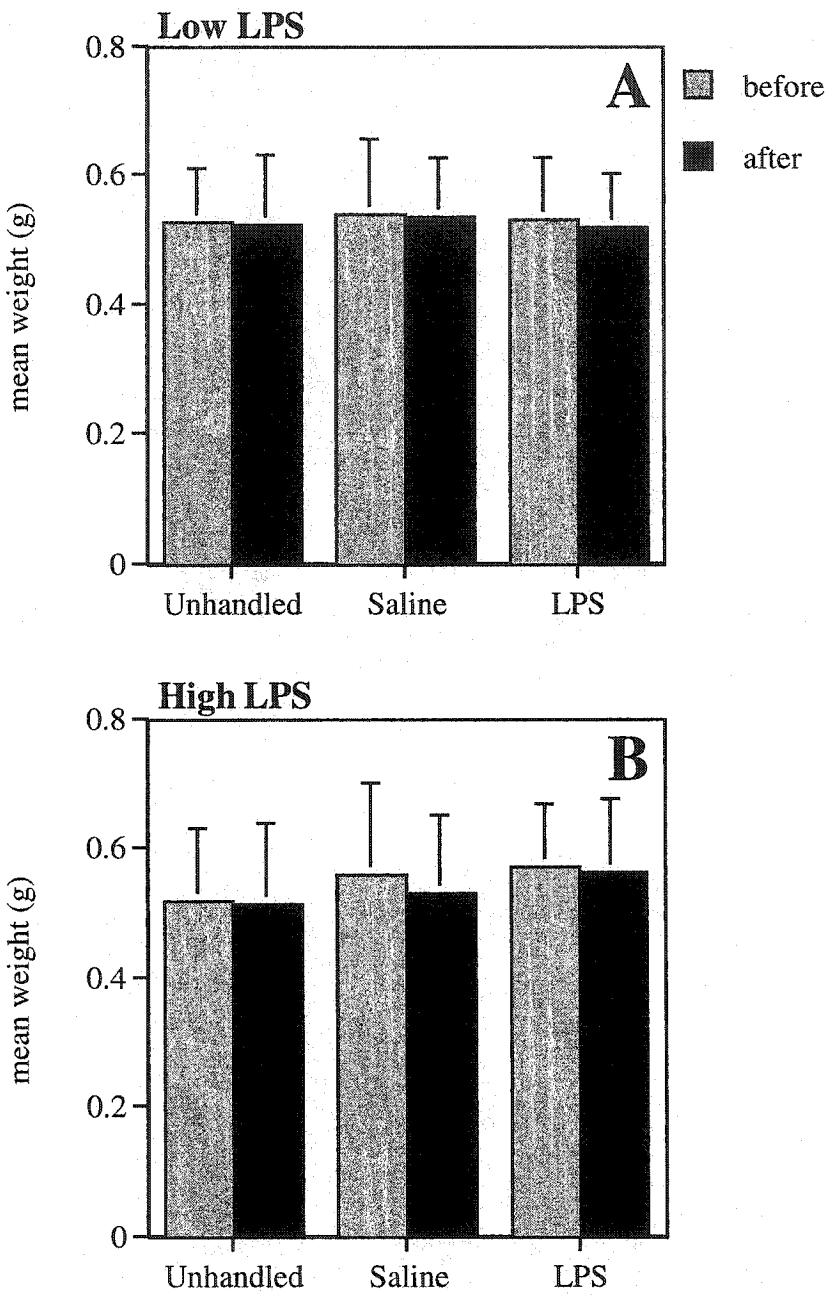


Figure 10 Mean weight of females immediately before beginning chronic infection (day 4; before), and immediately prior to the 5th injection (day 16; after). A) Low-dose (20 µg/injection) experiment. Unhandled n = 20, Saline n = 22, LPS n = 24. B) High-dose (100 µg/injection) experiment. All groups n = 14.

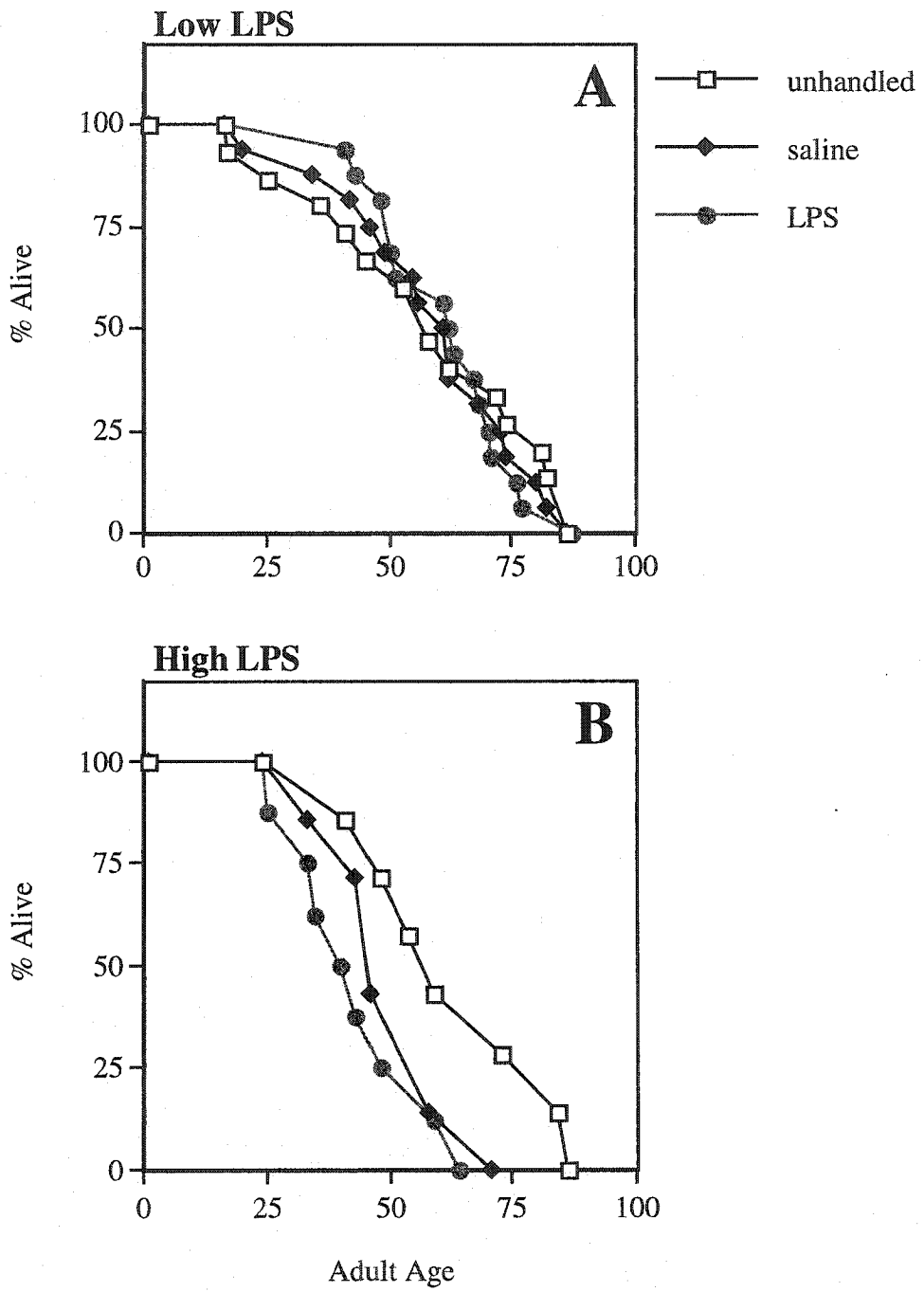


Figure 11 Lifespan distribution curves for females receiving chronic injections. A) females that have been injected with saline (n = 16), LPS (20 μg/injection; n = 16), or that have been left unhandled (n = 15) B) females that have been injected with saline (n = 7), LPS (100 μg/injection; n = 8), or that have been left unhandled (n = 7).

Effect of chronic infection on egg quality

Egg quality was assessed by total protein per egg, dry weight of eggs, fertilization success, and hatching success.

In an analysis of total protein per egg, I found no evidence for a difference between groups on day 18 (Figure 12; ANOVA, $F = 1.198$, $DF = 3$, $P = 0.34$). The average total protein per egg was 87.5 ± 7.6 μg per egg. For those females that yielded data from both before and after the experiment, I found no evidence for a change in total protein per egg over time (paired t-test, $DF = 14$, $t = 1.419$, $P = 0.18$).

In an analysis of dry egg weight before and after infection, I found no evidence for a change in egg weight in any of the groups (Figure 13; Repeated measures ANOVA, $F = 0.740$, $DF = 3$, $P = 0.5447$).

In an analysis of fertilization success, I found no evidence for a difference between groups on day 16 (Figure 14A; Kruskal-Wallis, $H = 4.79$, $P = 0.19$). Average fertilization success was 86.3 ± 10.5 %. I also found no evidence for a difference between hatching success, calculated from those eggs collected on day 16 (Figure 14B; Kruskal-Wallis, $H = 3.350$, $P = 0.34$). Average hatching success was 91.2 ± 7.7 %.

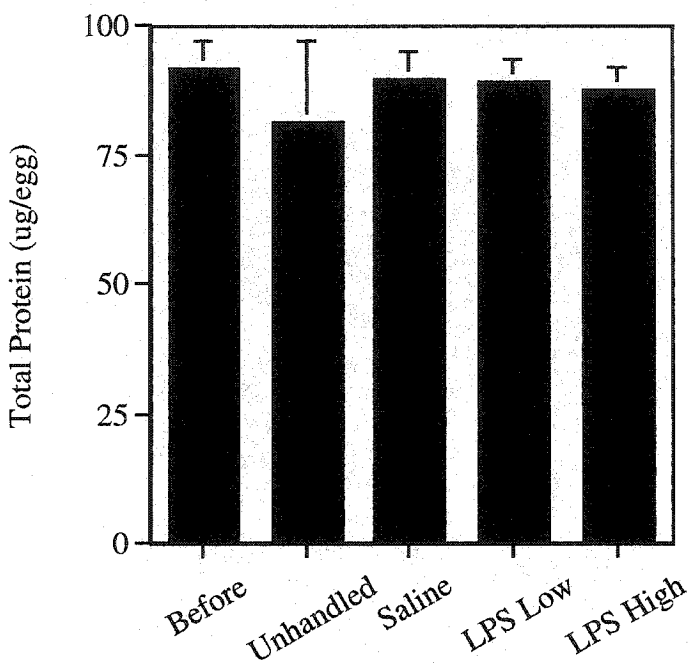


Figure 12 Total protein of individual eggs laid in sand. Values have been calculated based on standards made with bovine serum albumen. "Before" (n = 17) represents values from eggs laid on d4, prior to the first injection, while unhandled (n = 4), saline (n = 7), LPS Low (n = 6), and LPS High (n = 6) represent values from eggs laid on d18 following all 5 injections.

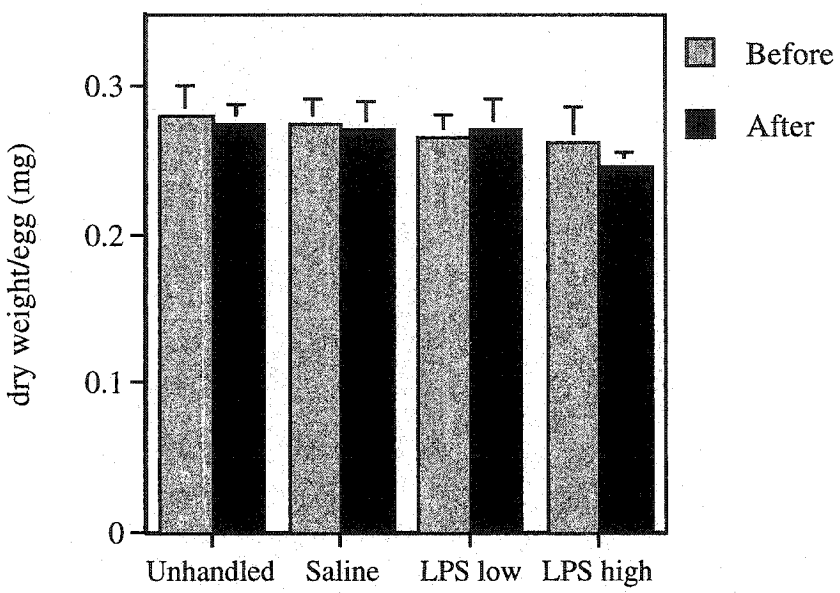


Figure 13 Dry weight of eggs laid in sand before and after the experimental protocol. Unhandled n = 5, Saline n = 6, LPS low n = 4, LPS high n = 4.

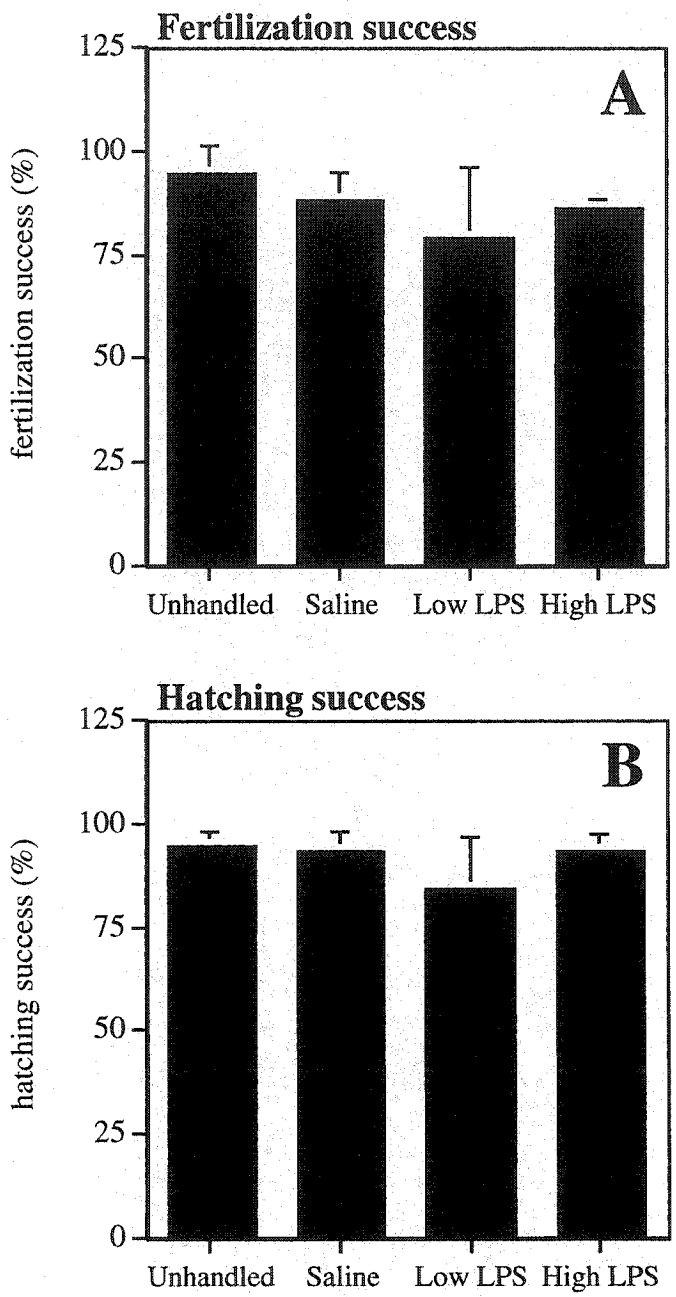


Figure 14 Fertilization success (A) and hatching success (B) for females laying eggs in sand. Unhandled n = 3, Saline n = 7, Low LPS n = 5, High LPS n = 4.

Discussion

Twenty micrograms of LPS probably led to an immune response, because coinjection of both LPS and live *S. marcescens* resulted in greater mortality in the female cricket compared to the injection of *S. marcescens* alone. The most likely explanation for this is that in the coinjected crickets, immune resources that may have been more suitably directed towards the bacteria were split between targeting both bacteria and LPS, leaving the female to inadequately cope with the live bacteria. Nevertheless, the immune response to LPS did not appear to make any obvious trade-offs with reproduction, as measured by egg number and quality. Further evidence for the absence of energetic trade-offs is that immune activation had no effect on female body weight or lifespan, even at the high dose (100 μg). This last result also supports the conclusion that neither dose of LPS was toxic.

It is not surprising that 20 μg of LPS induced an immune response. Evidence in the literature suggests that very small quantities of LPS in insects can elicit a response. For example, da Silva et al. (2000) found that in fifth instar male and female *A. domesticus*, a related cricket, injection of 1 μg/nymph of *Xenorhabdus nematophilus* LPS caused a dramatic drop in hemocyte count at 60 minutes postinjection, and initiated an increase in lysozyme activity lasting at least 60 minutes. Moret and Schmid-Hempel (2000) found that in the bumblebee, *Bombus terrestris* L., LPS from *E. coli* induced antibacterial activity in the hemolymph at levels as low as 2.5 μg/insect. Gunnarsson and Lackie (1985) found that locusts, *Schistocerca gregaria*, showed significant increases in nodule counts in the hemolymph in response to 25 μg injections of LPS from *S.*

marcescens, and the cockroach, *Periplaneta americana*, showed increases in nodule counts in response to LPS of *E. coli* at just 10 μg per insect.

Despite the potential costs of activating an immune response (e.g. Moret and Schmid-Hempel, 2000), there appeared to be no obvious trade-offs with (or between) egg number or quality in *G. texensis*. Below are possible explanations, not mutually exclusive, for the results presented here:

1. Systems that are tightly and directly associated with fitness, such as egg production, are protected from any energetic trade-offs with immunity in this animal. Instead, immunity may make trade-offs with other somatic systems within the individual, such as fat body storage or tissue maintenance. However, if somatic systems were not adequately maintained, then I would expect reduced longevity in challenged females relative to controls, and this is not the case.

Immunity could also make a trade-off with the activity level of the cricket (e.g. foraging). Hart (1988) argues that during times of illness, decreased levels of activity in behaviours such as foraging and grooming save energy for fueling expensive immunological responses. Although his review focuses on fever in mammals, there is no reason that the same relationship would not be present in other animals, including insects. Further experiments would be required to determine whether the animals in my experiments are less physically active following an injection of LPS.

2. With food and water *ad libitum*, females can adequately fuel both reproduction and immunity, and therefore, any potential physiological trade-offs would not occur because

there is no competition for resources. Support for this possibility comes from work by Moret and Schmid-Hempel (2000), who found that in the bumblebee, *B. terrestris*, immune activation will lead to decreases in lifespan when food is denied, but nonstarved animals show no decrease in lifespan in response to immune activation. These results suggest that insects can compensate for immune costs by increased food intake. Also, Burpee and Sakaluk (1993) found that crickets, *G. sigillatus*, can live 12 days with no food, while continuing to lay some eggs, suggesting that females have substantial reserves within the body. Woodring et al. (1979) in a study of food utilization in the cricket, *A. domesticus*, concluded that ovarial growth was powered by absorbed food, while energetic reserves of the fat body might be used for somatic growth, and they proposed that fat body reserves (up to 28 mg dry weight) may be reserved for times of starvation stress (or in this case, possibly immune activity). Together, these results suggest that crickets fed *ad libitum* have enough resources to fully fuel immunity and reproduction. Because crickets also have substantial fat body in the field (S.A. Adamo, personal communication), it is also reasonable to expect that crickets in a natural setting do not make trade-offs between egg production and immunity.

3. Mounting an inducible immune response is not energetically costly to the cricket. It is important here to make a clear distinction between inducible immunity and resistance. Resistance is the ability of an animal to combat a pathogen (that is, its state of "readiness", regardless of whether it ever becomes infected). Inducible immunity is the response that is "turned on" in an animal when it is forced to combat a pathogen. It may be appropriate here to draw an analogy between the immune system and an army:

resistance is analogous to maintaining an army, while inducible immunity is analogous to actually going to war.

Because gram-negative bacteria, and therefore LPS, are common pathogens world-wide, this cricket may have evolved a high level of resistance to them (genetically driven). High levels of resistance could also be caused by rearing insects in densities greater than those expected in nature (Reeson et al., 1998). This improved resistance could be mediated, for example, by increased numbers of hemocytes or inducible proenzymes in the hemolymph (Fellowes and Godfray, 2000; Reeson et al., 1998). While maintaining increased resistance may be energetically costly, inducing an immune response to a particular challenge may not be (Råberg et al., 2002; Rigby et al., 2002). Perhaps there is a negative relationship between costs of resistance and costs of inducible immunity.

Animals selected for high levels of resistance may suffer fitness costs. For example, in artificial selection experiments in *Drosophila*, Fellowes et al. (1998) found that breeding flies for increased resistance to a particular endoparasitoid led to decreased competitive ability of fly larvae, which ultimately meant increased mortality rates in fly larvae with greater resistance (interestingly, Fellowes et al. (1998) found no differences in fecundity or egg viability between adults from the same lines). Therefore, in my crickets, high levels of resistance to gram-negative bacteria may have led to some fitness costs (including egg production) over evolutionary time, yet these costs would be the same for all the animals in my experiments. Further challenges to the immune system may not result in further fitness costs because compared to the energy required to maintain the immune system, the energy required for an immediate response is minimal.

Support for this argument comes from Tiën et al. (2001), who examined larval competitive ability in two species of *Drosophila*, one of which was unable to mount an immune response to parasitoids. Following parasitization, there was a small reduction in competitive ability of larvae that were able to mount an immune response, suggesting that it was associated with some cost, possibly energetic. However, the reduction in competitive ability was much lower than that described in *Drosophila* lines selected for increased resistance (Kraaijeveld and Godfray, 1997). Taken together, these two studies suggest that the costs of maintaining an efficient immune system is substantially more than the costs of actually using it (Tiën et al., 2001). If this were true in my own experiments, then I might find evidence for trade-offs between inducible immunity and reproduction in a different cricket species that had less evolutionary pressure for high levels of resistance.

Studies investigating the direct relationship between immune system activation and reproductive output in insects are scarce, and their results are not easily compared with those presented here. For example, Siva-Jothy et al. (1998) found that in the damselfly, there was a decrease in immune system function (assessed by encapsulation of a nylon filament implanted into the hemocoel) following reproductive activity (copulation or oviposition). However, this same trend was not observed following energy-demanding fighting in males, suggesting that the trade-offs observed between immunity and reproduction were not energetic in nature. More importantly here, however, is that Siva-Jothy et al. (1998) did not investigate the converse hypothesis: that decreases in reproductive activity are a result of increased immune system function. Whether or not

an energetic trade-off is observed in one direction (energy from immunity to reproduction), it does not automatically follow that a trade-off will be observed in the other direction (energy from reproduction to immunity). For example, it may take a substantial investment of resources to make small increases in reproductive output, but it may take only small amounts of energy to have a large impact on immunity (i.e. upregulating immunity is not costly, see argument 3.).

Another recent paper by Ahmed et al. (2002) showed that in the mosquito, *Anopheles gambiae*, there was a significant reduction in ovarian protein 24 hours after a single challenge with LPS, along with a significant decrease in egg production. LPS did not increase immediate mortality in the mosquito, indicating that the dose of LPS was not exceedingly toxic (although this may be argued, see General Discussion). However, sham injections also induced declines in ovarian protein that were not reported to be statistically different from LPS-induced declines, suggesting that the injections themselves may cause internal damage. Additionally, like Siva-Jothy et al. (1998), Ahmed et al. (2002) suggest that the nature of the trade-off may not be energetic, because *A. gambiae* probably consumes more food than is necessary to sustain egg production and immune defense.

It is even difficult to make comparisons within insect subfamilies. Adamo (1999) found that injecting female crickets, *A. domesticus*, with 100 μg of LPS resulted in increases in the number of eggs laid during the day following injection, another result that was not observed in my experiments (I looked over 2 days following injection).

Taken together, it is clear that there is still no convincing evidence for a predictable energetic impact of immune activity on reproduction, or consequently fitness, in the insect.

CHAPTER 4: PHENOLOXIDASE, REPRODUCTION, AND IMMUNITY IN THE CRICKET, *GRYLLUS TEXENSIS*

Summary

Since reproduction and immunity are both costly functions, there may be selection pressures for animals to trade one against the other to maximize reproductive success. I tested whether female crickets, *Gryllus texensis*, when manipulated to invest more energy in reproduction, had decreased immunocompetence by 1): analysing mortality following injections of *Serratia marcescens*, and 2): measuring phenoloxidase (PO) activity of the hemolymph. Although mated females had greater reproductive costs relative to virgins, I found no evidence that they had decreased immunocompetence as measured by LD_{50} or PO activity of the hemolymph. In fact, based on LD_{50} measures, virgins may be less immunocompetent than mated females. Since I found no evidence for decreased immunocompetence with increased egg output in mated females only, then differences in immunocompetence between virgins and mated females may be due only to the fact that virgins are not mated, and not due to egg-laying costs. These results are inconsistent with predictions in the literature suggesting that virgin females should have extra resources to invest in immunity, but are consistent with the possibility that mated cricket females receive fitness benefits from male ejaculates.

Introduction

Animals cannot invest their resources maximally into all physiological functions. Instead, they must allocate their resources prudently between many activities to optimize fitness. Generally, these activities can be divided into two broad categories: reproductive activity and somatic growth and maintenance (Williams, 1966). For the most part, studies on the trade-offs between these activities have investigated the relationships between current reproduction and survival, or current reproduction and future reproduction (Stearns, 1992).

More recently, there has been a growing interest in the physiological trade-offs between reproduction and immunocompetence (Norris and Evans, 2000; Owens and Wilson, 1999; Sheldon and Verhulst, 1996). Because both processes are assumed to be costly, animals must "choose" to sacrifice reproductive investment for improved immune readiness, or sacrifice immune readiness for increased reproductive effort. Many of the studies investigating these relationships have been in birds (see Norris and Evans, 2000), sometimes with conflicting results. For example, Nordling et al. (1998) found that in the collared flycatcher, increased reproductive effort, accomplished by manipulating brood size, decreased the ability of females to mount an antibody response against Newcastle disease virus vaccine, and also increased the intensity of *Haemoproteus* infections. However, Williams et al. (1999) found that in female European starlings, there was no change in size of a second clutch following injection with a non-pathogenic antigen. Part of the difficulty in finding evidence for trade-offs between immunocompetence and reproduction may stem from the complexities of these two physiological systems in vertebrates. The vertebrate immune system is very complex, comprising both acquired

and innate immunity (Roitt et al., 1996). Likewise, reproductive effort of vertebrates is complex, often involving multiple breeding seasons, parental care, and long lifespan. For these reasons, studying this relationship in insects can prove to be a useful model because of the relative simplicity of their reproductive and immune systems. Insects do not often exhibit parental care, and often have a single breeding season and lay multiple eggs per reproductive bout (Evans, 1984), allowing for fast and easy quantification of lifetime reproductive output. Finally, the invertebrate immune system is simpler than the vertebrate immune system, lacking acquired immunity and instead relying on innate immunity composed of both cellular and humoral components (for review see Gillespie et al., 1997; Lackie, 1988a,b). Cellular immunity depends on hemocytes, or blood cells, which can phagocytize small particles, form multicellular aggregates around large numbers of bacteria (nodulation), or encapsulate larger objects by forming multiple layers around them, and sometimes depositing melanin (for review see Gupta, 1991). Humoral immunity includes the synthesis of antimicrobial proteins and peptides (Bulet et al., 1999; Kanost et al., 1990), mostly from fat body tissue. Humoral immunity also includes the enzyme phenoloxidase (PO), which is activated from its precursor prophenoloxidase (pPO) by a serine protease cascade. PO is responsible for the oxidation of phenols to quinones, and ultimately to the production of melanin, which can be deposited around nodules and encapsulated objects (Sugumaran, 2001). Additionally, components of the pPO cascade (e.g. quinones) are believed to be toxic to microorganisms (Söderhäll et al., 1996). PO activity is capable of adaptive plasticity in some insects (e.g. Barnes and Siva-Jothy, 2000; Reeson et al., 1998).

Work in the past has suggested that female reproduction is costly because of the costs associated with the mating itself, and the costs of egg production and laying. For example, Partridge et al. (1987) showed that mated female *Drosophila melanogaster* had longer lifespans when egg-laying rate was experimentally reduced by removing appropriate egg-laying substrate, suggesting a cost to egg-laying. Lifespan was also significantly reduced under continuous exposure to males compared with those given limited access, even though egg-laying rates were the same, suggesting a cost to mating per se. Virgin female fruit flies have been shown to live significantly longer than non-virgins (*Ceratitis capitata*, Chapman et al., 1998). With regards to immunity, Moret and Schmid-Hempel (2000) found that starved bumblebees, *Bombus terrestris*, experimentally infected with non-pathogenic lipopolysaccharide or sterile micro-latex beads, had significantly reduced lifespans compared with those that were not infected, suggesting a cost to immune system activation.

Despite the fact that insects could prove to be a useful model for examining the relationship between immunocompetence and reproductive activities, only a handful of studies examine them. For example, Adamo et al. (2001) showed that immunocompetence in male crickets (*Gryllus texensis*), measured separately by PO activity and bacterial infection (*Serratia marcescens*), decreased significantly at the onset of sexual behaviour. However, the immunocompetence hypothesis in females, proposing that females made a trade-off, was not supported in this study. Siva-Jothy et al. (1998) showed, using an encapsulation assay, that females are less immunocompetent following 28 minutes of oviposition, and males are less immunocompetent following a day of mating.

In this chapter, I investigate the relationship between immunocompetence and reproduction in cricket females that are 1): virgins, 2): mated and laying eggs in a preferred substrate, and 3): mated and laying eggs in a non-preferred substrate. In crickets, there are several physiological differences between virgin and mated females (for review see Loher and Zaretsky, 1989; Strambi et al., 1997). Following the moult to adult, both virgin and mated females begin the process of egg development, and mature eggs are stored in the lateral oviducts. Following mating, titres of juvenile hormone (JH: an important insect hormone associated with ovarian development) and egg production increase, and mated females release, fertilize, and lay many eggs. These eggs can translate into a substantial material cost for the female, as the dry weight of an individual egg is approximately 0.27 mg (Chapter 3), and the female can lay 1500 eggs in a lifetime (Chapter 2). Enforced virginity leads, among other things, to a decrease in JH titre (*Teleogryllus commodus*, Loher et al., 1983), an increase in JH esterase activity (*Acheta domesticus*, Woodring and Sparks, 1987), a decrease in egg production, an increase in the number of stored eggs in the lateral oviducts, and a low egg-laying rate. Because the reproductive costs are lower for virgins than for mated females, I predict that virgins will be more immunocompetent than mated females. I also predict that mated females laying many eggs in a preferred substrate will have decreased immunocompetence compared with mated females laying few eggs in a non-preferred substrate. These predictions are based on the hypothesis of physiological trade-offs: a decreased energy investment in reproductive activity will liberate energetic resources for other metabolic functions, including immunity.

In this study, immunocompetence will be measured in two ways. PO activity in the hemolymph will be measured because it plays an important role in arthropod immunity (see above) and because decreased PO activity is thought to reflect decreased immunocompetence (Dunphy, 1991; Reeson et al., 1998). I will also monitor mortality of virgin and mated females following challenge with a bacterial pathogen, *Serratia marcescens*, which has been shown to infect orthopterans in the field (Stevenson, 1959).

Methods

Animals

Crickets, *Gryllus texensis*, were obtained from a colony maintained at Dalhousie University. They were reared on a 12L:12D cycle, at 28 ± 2 °C. Dry cat food and water were provided *ad libitum*.

Immunocompetence of virgin and mated females (Figure 15)

In experiments comparing virgin and mated females, newly moulted adults were removed from nymph cages daily, and were given the age designation "day 0" (d0). Females and males were housed separately. On d5-12, females were placed, at random, into one of two large clear plastic containers for 5-7 days. One container housed 5 adult males (at least 7 days old) while the other housed 5 male nymphs. By the end of the time females were housed with males, they should have mated (Solymar and Cade, 1990), and successfully so (see below). Then, on d10-18, females were isolated into individual clear plastic containers (18 x 14.5 x 9 cm) with opaque lids for 5 days. During these 5 days, eggs were counted for most females (on days 2, 4, and 5). Females were given moist sand for egg-laying (12 mL of water with 37 mL of sand, sifted to a grain size smaller than egg size, in a small paper cup), a small water bottle (6 mL volume, 4.4 cm long, 1.3 cm diameter) plugged with cotton for drinking, an egg cup for cover placed over the oviposition site, and cat food *ad libitum*. I counted eggs in sand by first allowing the sand to dry and then re-sifting it three times. Eggs were retained in the sieve (0.7 mm mesh size). Then, on d15-23, females were weighed, blood was sampled for PO activity and

total protein concentration, and they were injected with *S. marcescens*. A preliminary unpublished experiment that I conducted found no difference in survival distribution of virgin and mated females when given an almost 100% lethal injection of *S. marcescens*. Therefore, for this experiment, I injected females with an LD_{50} dose (approximately 3 x 10^5 cells/5 μL) of *S. marcescens*.

Examination of stored eggs and flight muscle status of virgin and mated females

In another group of virgin and mated females (16-20 days of adult age), handled the same way as already described but given large water bottles for egg-laying, mature eggs in the lateral oviducts were counted, and colour of the thoracic dorso-ventral flight musculature was noted as either pink or white. Flight muscle colour is strongly associated with its degeneration/histolysis in many insects, including the cricket, *Gryllus firmus*, with pink muscle weighing more, having more and larger muscle fibres, and having higher respiration rates than white muscle (Stirling et al., 2001; Zera et al., 1997). It has been suggested that there are physiological trade-offs between muscle maintenance and reproductive investment (e.g. Zera et al., 1998).

Immunocompetence and oviposition manipulation (Figure 15)

Newly moulted adults were removed from nymph cages daily, and were given the age designation "day 0". Females and males were housed together. On d8-18, females were isolated into individual clear plastic containers (18 x 14.5 x 9 cm) with opaque lids for 5 days, and were randomly assigned to one of three groups. One group ("large") was given a large water bottle for egg laying and drinking (37 mL volume, 9.5 cm long, 2.2

cm diameter, plugged with cotton). The second group ("small") was given a small water bottle for egg laying and drinking (6 mL volume, 4.4 cm long, 1.3 cm diameter, plugged with cotton. Eggs in the first two groups were counted by unrolling the cotton where the eggs were clearly visible. The third group ("sand") was given moist sand for egg-laying (see above for details of sand procedure). All females were given an egg cup for cover, and cat food *ad libitum*, and eggs were counted for 5 days, on days 2, 4, and 5. After these 5 days, on d13-23, PO activity as well as total protein concentration of the hemolymph were measured. Three to five hours after beginning these measurements, females were injected with approximately 2.5 x 10^5 cells/5µL of *S. marcescens*.

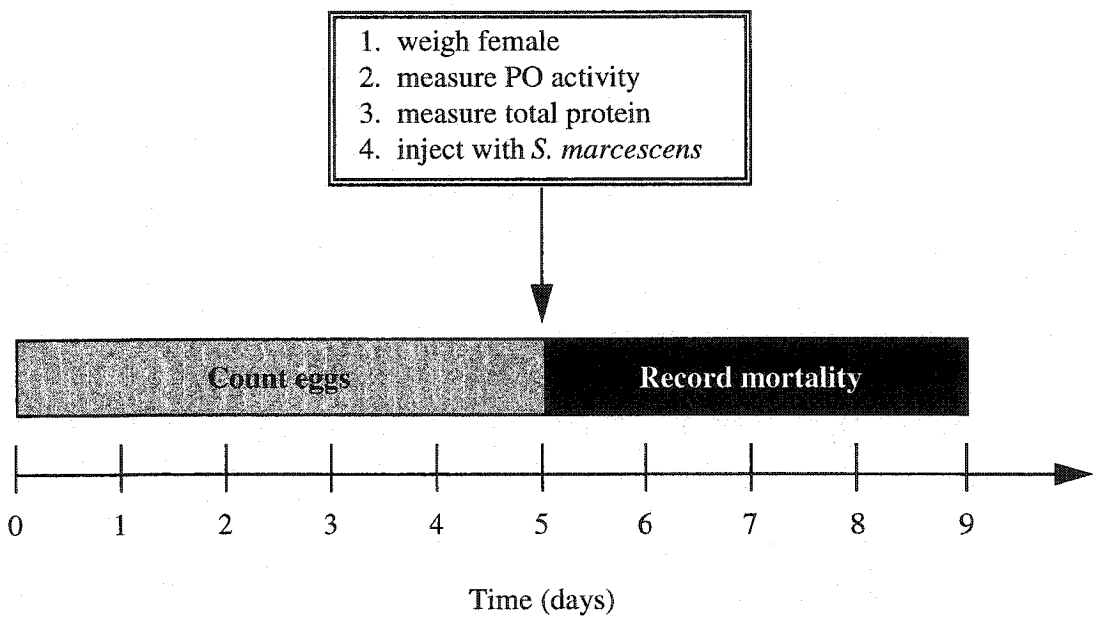


Figure 15 Diagram illustrating the method in Chapter 4. This procedure was used to compare virgin and mated females laying eggs in moist sand, and to compare mated females laying eggs in either moist sand, large water bottles, or small water bottles.

Phenoloxidase measurements

Hemolymph was sampled from beneath the pronotum using a 10 μL Hamilton syringe. 3 μL of whole hemolymph was vortexed with 100 μL of phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4: Sigma). Samples were split into 50 μL aliquots to measure both total protein (see below) and PO activity. To measure PO activity, one 50 μL aliquot was incubated with 70 μL of bovine pancreas α-chymotrypsin (1.3 mg/mL solution, Sigma) for 20 minutes at room temperature. Then, 900 μL of 10mM L-dihydroxyphenylalanine (L-DOPA; Sigma) was added to the PBS-hemolymph solution, and the solution was vortexed again. Through a series of biochemical steps, PO converts L-DOPA to dopachrome (Marmaras et al., 1996), and thus PO levels in the hemolymph can be monitored indirectly by the formation of dopachrome over time. The solution was incubated in darkness at room temperature, and absorption measurements were made spectrophotometrically (Novaspec II, Biochrom Ltd.) at 1, 10, 20, and 30 minutes at a wavelength of 475 nm. The reaction is linear over the observed time period (personal observations), and PO is expressed in units, where one unit is defined as the amount of enzyme required to increase absorbance by 0.001 per minute.

Total protein concentration

Total protein concentration of the hemolymph was assayed based on the procedure described by Bradford (1976). The 50 μL aliquot of the hemolymph/PBS mixture (see above) was incubated with 900 μL Bradford Reagent (Sigma) in disposable cuvettes for

30 ± 10 minutes. The protein-dye complex is stable for up to 60 minutes (Bradford, 1976). Absorbance of the complex was measured spectrophotometrically at 595 nm, and values were compared to a standard calibration curve made from a bovine albumin (Sigma).

Bacterial injections

Bacteria, *S. marcescens*, were obtained commercially from Carolina Biological Supply Company (Burlington, North Carolina), and bacterial concentration was determined using a Petroff Hausser counting chamber and phase-contrast microscopy. The bacterial solution was diluted to the appropriate concentration with insect saline (121 mM sodium chloride, 4.1 mM calcium chloride, 1.37 mM dibasic potassium phosphate, 198 μM monobasic potassium phosphate, and 38.6 mM Tris-hydrochloride, pH 7.4). Females were given a single 5 μL bacterial dose through the membrane beneath the pronotum with a 10 μL Hamilton syringe. After injection, females were returned to their cages for at least 3 days, where they were inspected each day for survival. Most crickets that die from an *S. marcescens* injection do so within 3 days (Adamo et al., 2001).

Statistics

Simple statistical measures and tests were conducted using SYSTAT for the Macintosh, Version 5.2, and SuperANOVA 1.11 (Abacus Concepts, Inc.). Parametric and non-parametric analyses were also conducted according to Zar (1984) and Lee (1980).

Results

Bacterial infections and LD_{50} (virgin vs. mated)

To determine whether virgins were more immunocompetent than mated females, I infected both groups with an LD_{50} dose of *S. marcescens*. The total starting sample size was 43 mated females and 44 virgins, representing the cumulative numbers from four replicates. The age of virgin and mated crickets within replicates was not different. Over the 4 day observation period, there was a significant difference in the survival distributions of virgin and mated females, with significantly more virgins succumbing to the infection (Figure 16; Cox-Mantel test, $C = 3.215$, $P < 0.05$).

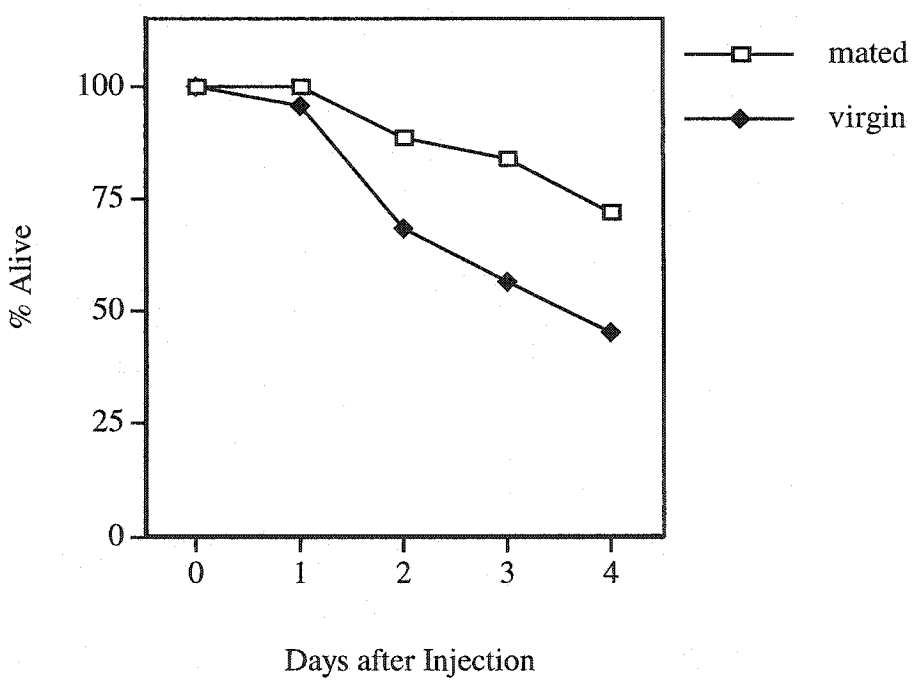


Figure 16 Survival distribution for mated (n = 43) and virgin (n = 44) females following an LD_{50} dose on day 0 with *S. marcescens*.

There were no significant differences in body weight between virgin (n = 43) and mated (n = 43) females (t-test, t = 0.768, df = 84, P = 0.45). Females weighed 0.526 ± 0.081 g.

Mated (n = 34) females laid significantly more eggs than virgin (n = 34) females (Figure 17; Mann-Whitney U = 1081.5, df = 1, P < 0.001). I also found that virgins (n = 55) had significantly more eggs stored in the lateral oviducts than mated (n = 46) females (Table 1: t-test, t = -3.56, df = 99, P = 0.0002). Individual fresh eggs weighed approximately 0.77 mg. Additionally, virgin females (n = 51) were more likely to have pink dorso-ventral flight muscles than mated females (n = 43). 62.7% of virgins had pink muscles, while only 16.6% of mated females had pink muscles. This difference is significant (G-test, G = 19.55, P < 0.001). During these dissections, I confirmed that 93% of females in the mated group (n = 43) had clearly filled spermathecae, indicating that by 11-14 days of adult age, these females had successfully mated (Loher and Dambach, 1989).

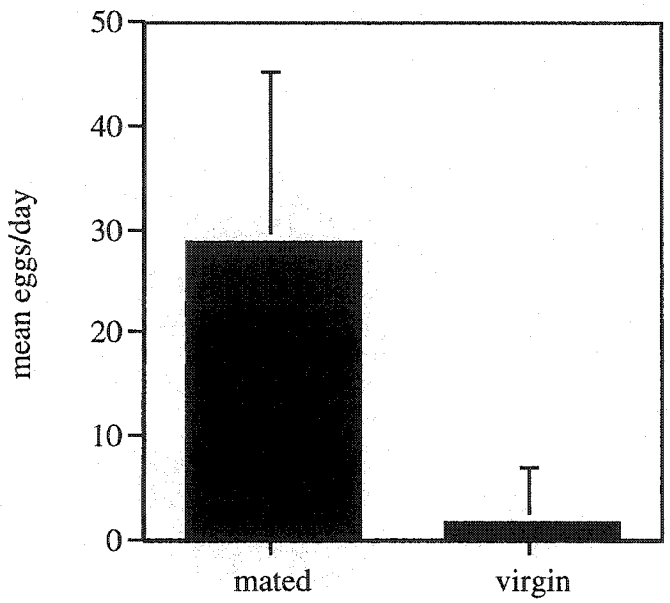


Figure 17 The number of eggs laid in sand per day (over 5 days) for both mated (n = 34) and virgin (n = 34) females.

Total protein concentration and phenoloxidase activity in the hemolymph (virgin vs. mated)

I found that virgin females had significantly greater total protein concentration in the hemolymph than mated females (Table 3: virgin n = 22; mated n = 23; t-test, $P < 0.001$), but I found no significant difference in PO activity between virgin and mated females (Table 3: virgin n = 28; mated n = 29; t-test, $P = 0.13$). Calculating the ratio of PO units per milligram protein did not yield a different result.

I also found no correlation in mated females between the number of eggs laid over 5 days and PO activity ($r = 0.117$, $P > 0.5$). For mated females that survived or succumbed to infection, there was no difference between the number of eggs laid per day (Mann-Whitney, $U = 122$, $P = 0.28$) or weight (t-test, $t = -0.932$, $P = 0.36$).

Table 3 Results of t-tests comparing total protein of hemolymph, PO activity of hemolymph, and eggs stored in the lateral oviducts of virgin and mated females

	Virgin	Mated	t	df	P
Total Protein (mg/mL)	20.57 ± 2.51 n = 22	16.81 ± 3.45 n = 23	-4.167	43	0.001
PO units	8.29 ± 3.95 n = 28	6.77 ± 3.56 n = 29	1.525	55	0.13
Eggs stored in Lateral Oviducts	202.2 ± 111.8 n = 55	119.5 ± 120.6 n = 46	-3.56	99	0.002

Bacterial infections and LD_{50} (substrate manipulation for mated females)

I found no difference in survival distributions for mated females laying eggs in moist sand, large water bottles, or small water bottles following infection with *S. marcescens* (Figure 18; K-sample test (Lee, 1980), $X^2 = 0.672$, n.s.), even though these substrates resulted in differences in the number of eggs laid per day (Figure 19; Kruskal-Wallis, H=32.630, df = 2, $P < 0.001$; non-parametric multiple comparisons: sand vs. small, $Q = 5.53$, $P < 0.001$; large vs. small, $Q = 4.14$, $P < 0.001$; sand vs. large, $Q = 1.45$, $P > 0.05$).

I found no significant difference in weight between females laying eggs in moist sand, large water bottles, or small water bottles (ANOVA, $F = 0.171$, df = 2, $P = 0.84$). Females weighed 0.526 ± 0.090 g.

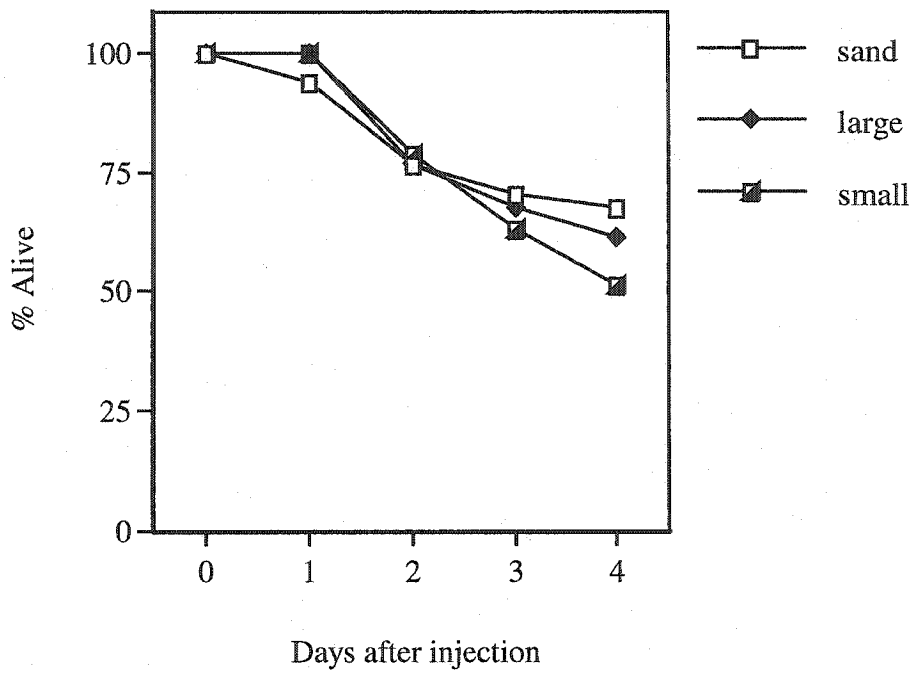


Figure 18 Survival distribution following an LD_{50} dose of *S. marcescens* for mated females laying eggs in sand (n = 34), large water bottles (n = 34) or small water bottles (n = 33)

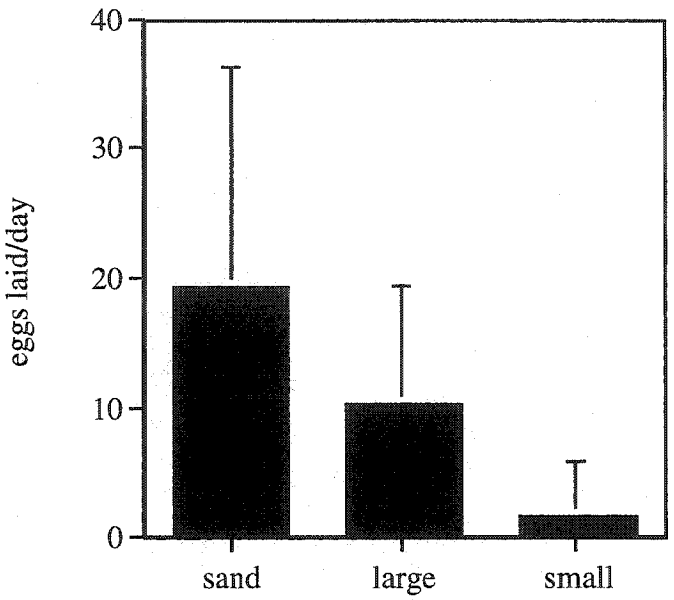


Figure 19 The number of eggs laid per day (over 5 days) for mated females laying in moist sand (n = 34), large water bottles (n = 35), or small water bottles (n = 31).

Total protein concentration and phenoloxidase activity in the hemolymph (substrate manipulation for mated females)

For females induced to lay more or fewer eggs, I found no difference in total protein concentration in the hemolymph (Table 4; P = 0.71). I also found no difference between groups for PO activity (Table 4; P = 0.60).

Table 4 Results of ANOVA comparing total protein and PO activity of hemolymph between mated females laying eggs in either moist sand, large water bottles, or small water bottles

	Moist Sand	Large Bottles	Small Bottles	F	P
Total Protein (mg/mL)	17.05 ± 5.29 n = 10	18.76 ± 6.96 n = 10	17.08 ± 1.74 n = 9	0.343	0.71
PO units	7.45 ± 3.36 n = 20	8.49 ± 3.30 n = 25	7.83 ± 3.77 n = 19	0.519	0.6

Discussion

Immunocompetence was measured by two standard assays: a functional assay using live bacteria, and a PO activity assay of the hemolymph. In my experiments, I found no evidence to support the hypothesis that virgin females are more immunocompetent than mated females, or that mated females manipulated to lay few eggs are more immunocompetent than mated females manipulated to lay many eggs.

That PO activity did not correlate with *S. marcescens* susceptibility is not completely surprising. Other studies have shown that PO correlates imperfectly with other measures of immunocompetence. For example, Adamo et al. (2001) found that two-week old female *G. texensis* had greater PO levels than 1-3 day old females, but did not show greater resistance to *S. marcescens*. Barnes and Siva-Jothy (2000) found that mealworm beetles, *Tenebrio molitor*, raised in high larval densities had increased resistance to the fungus *Metarhizum anisopliae*, but there was no correlation with PO activity. Wilson et al. (2002) found that locusts, *Schistocerca gregaria*, reared under crowded conditions were more resistant to *M. anisopliae* than those reared under solitary conditions, but there was still no correlation with PO activity. Drawing a correlation between PO activity and immunocompetence may be complicated, especially in adult female insects, because PO may play a role in egg production. McFarlane (1960) found that eggs of *Acheta domesticus* likely contain tyrosinase (PO) in the inner endochorion, the serosal cuticle, and probably in the egg yolk. Li and Christensen (1993) found evidence for a role of PO in the tanning of mosquito, *Aedes aegypti*, eggs. Additional indirect evidence suggests that PO may be involved in reproductive activity/egg production. For example, Adamo et al. (2001) showed in a time series experiment with

G. texensis, that PO activity in the hemolymph increased significantly at two weeks of adult age, when females are reproductively active, and actively laying eggs (pers. obs.), suggesting that PO could be important in egg production, at least in some capacity. Siva-Jothy et al. (2001) also found that female damselflies, early in the reproductive season, had significantly greater PO activity than males, which indirectly suggests that PO could be involved in egg production. Finally, Ahmed et al. (1999) found in the mosquito, *Anopheles gambiae*, that there was no up-regulation of mRNA for the prophenoloxidase (PPO) gene, AgPPO1, following a bacterial challenge, yet the *A. gambiae* PPO-producing cell line showed increased AgPPO1 transcription following treatment with physiological concentrations of 20-hydroxyecdysone, an important steroid hormone that functions in insect reproduction. Together, these data suggest that PO, while not unimportant in insect immunity, may also play a role in other insect systems, and as such, may provide a poor estimate of immunocompetence, at least in the female insect.

Interestingly, my results suggest that virgins may be more susceptible to *S. marcescens* infection than mated females. This difference is probably not simply due to the low egg output of virgins, since mated females manipulated to retain eggs do not show a similar susceptibility to infection, although this comparison was not directly made here. Current theory would suggest that since immunity and reproduction are both expensive activities, then with a finite energy budget, animals should trade one against the other (e.g. see Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). Some evidence in the literature suggests that invertebrates will make such trade-offs between immunocompetence and other components of fitness. For example, Moret and Schmid-Hempel (2000) studied trade-offs between survival and immunocompetence in workers of

the bumblebee *Bombus terrestris* by measuring lifespan following immune challenge. They found that, in starved workers, lifespan was significantly reduced when bees were challenged with non-pathogenic materials like lipopolysaccharide and micro-latex beads, suggesting that there is a cost to immune function. Kraaijeveld and Godfray (1997) found that in *D. melanogaster*, flies bred for improved resistance to *Asobara tabida* showed reduced larval competitive ability. Moreover, mating is thought to entail its own costs (Chapman et al., 1998), and in *Drosophila*, for example, virgins outlive mated females. Both these lines of research should predict that virgins will be less susceptible to disease.

Three possibilities could explain my observation that virgin females are more susceptible to infection than mated females. Enforced virginity may not be a natural state for *G. texensis* at two to three weeks of adult age, and therefore, the difference I observed could be a pathological one. I do not believe this explanation can entirely account for their lack of relative disease resistance, because if virgins are in a pathological state, they should have shorter lifespans than mated females. I have found that virgins do not have a reduced lifespan compared to mated females (Chapter 5). Moreover, Burpee and Sakaluk (1993) found that virgin female crickets (*Gryllodes sigillatus* and *Gryllus veletis*) given food *ad libitum* lived as long as females given unlimited access to males.

A second possibility is that virgins are unable to take in as much energy (food) as mated females. Although I provided my crickets with food *ad libitum*, Clifford and Woodring (1986) found that food consumption in virgin *A. domesticus* decreases to very low levels because the lateral oviducts, swollen with eggs, compress the gut. Such decreased food intake is possible in my animals, given that virgin and mated females have the same body weight, yet virgins store an additional 100 eggs in the lateral oviducts, at

an approximate weight of 0.77 mg each. Therefore, while mated females may be compensating for the costs of immune challenge by eating more, virgins may be unable to substantially increase their food intake. However, I do not believe this explanation can entirely account for my results, again because virgin females live as long as mated females, which would be unlikely if somatic maintenance could not be maintained in virgins. Additionally, despite food intake, virgin females retain greater reserves of fat body than mated females (based on data from *A. domesticus*, Clifford and Woodring, 1986), which might give them an immune advantage, since fat body is the tissue primarily responsible for the synthesis of antimicrobial proteins and peptides (Gillespie et al., 1997).

I favour the possibility that mated females are receiving fitness benefits from male ejaculates. Wagner et al. (2001) found that female crickets (*G. lineaticeps*) given continuous access to males lived significantly longer than females given limited access, suggesting that there are benefits from mating. Burpee and Sakaluk (1993) also investigated this relationship, and found that females given continuous access to males live as long as virgin females (*G. sigillatus* and *G. veletis*), suggesting that the costs of reproduction can be offset by mating. Substances in the seminal fluid of males transferred during mating may be responsible for this observation (Wagner et al., 2001), and based on the present experiments, these substances may act by giving the female an immune boost, or alternatively, a vaccination/immunization. Whatever the mechanism, mating in crickets appears to provide the female with a fitness advantage. Therefore, it is possible that in my experiments, in which females had ready access to food, mating provides females with benefits that allow them to maintain a robust immune response and

a large reproductive output. Mated females may also have an additional energetic savings relative to virgins because their flight muscle has already been histolyzed, and is therefore less expensive to maintain.

These results suggest that, under optimal lab conditions, trade-offs between reproduction and immunity may be difficult to uncover, especially when both systems, although simpler in invertebrates, are still very complex. Because both are critical for protecting fitness of the animal, energetic resources may not be diverted from either when food is abundant.

CHAPTER 5: GENERAL DISCUSSION — THE COST OF REPRODUCTION IN *GRYLLUS TEXENSIS*

Summary

Ecological immunology proposes that reproductive and immune investments will trade-off against one another, based on the assumption that both of these activities are metabolically demanding. However, experiments described in this thesis reveal no evidence that this is the case, suggesting that the underlying assumption is invalid. To test whether reproduction carries costs in terms of reduced somatic maintenance, I conducted a lifespan study in virgin and mated female crickets. Since virgins invest less in reproduction than mated females, they should have increased energy for somatic function and thus have an increased lifespan compared with mated females. While virgin females laid significantly fewer eggs than mated females, I found no evidence that they live significantly longer, suggesting little cost to reproduction. This result, along with those from previous chapters, casts doubt on some of the assumptions within the field of insect ecological immunology, and in turn, raises doubts concerning the conclusions of previously published data.

Introduction

Reproduction requires energy. In mammals, for example, energy and other resources are required for activities such as mating, nursing offspring, and parental care. With a limited energy budget, however, increased energetic investment in reproduction necessarily results in energy being withheld or withdrawn from other physiological processes, such as somatic growth and maintenance. Therefore, while current reproduction positively impacts on an animal's fitness, it is also associated with a fitness loss, or "cost", mediated by a decreased investment in other fitness components. The idea of reproductive costs, or trade-offs, was proposed by Williams (1966), and has since been the subject of many reviews (e.g. Bell and Koufopanou, 1986; Reznick, 1985, 1992; Stearns, 1992; General Introduction). Specifically, increases in reproductive investment will lead to decreases in somatic investment, which may leave an animal in poorer condition for reproduction in the future. Conversely, decreases in reproductive investment will allow for increases in somatic investment, resulting in superior physical condition and longer lifespan, increasing potential for reproduction in the future. For example, in female *Drosophila melanogaster* that were equally exposed to males, those that laid fewer eggs because they were denied a suitable egg-laying site lived longer, suggesting a potential physiological trade-off between reproduction and somatic maintenance (Partridge et al., 1987).

Immunity also requires energy, for such processes as activating immune cells, and synthesis of antimicrobial proteins and peptides. Based on its energetic requirements, increased immune system activity, like increased reproduction, may also be costly, and result in trade-offs with other fitness components within an individual (Sheldon and

Verhulst, 1996; General Introduction). For example, up-regulation of immune system activity could carry costs that are measured as decreased lifespan (Moret and Schmid-Hempel, 2000), body condition (Fellowes et al., 1999), or reproductive output (Ilmonen et al., 2000).

If both reproduction and immunity are costly and, as such, make trade-offs with other components of fitness, then experimental manipulation of one should have a measurable impact on the other. This technique for assessing physiological trade-offs is widely applied in the literature (Zera and Harshman, 2001), and has been described throughout this thesis.

In Chapter 2, I investigated whether experimental infection of the female cricket, *Gryllus texensis*, with the live bacterium *Serratia marcescens*, would lead to an adaptive increase in reproductive output. Since *S. marcescens* is potentially lethal, the theoretical basis for this proposal was that the cricket would increase current reproductive output to compensate for possible reproductive losses in the future. Implicit in this argument is an underlying trade-off, in which a female up-regulates current reproduction at the expense of somatic maintenance (i.e. immunity) and consequently future reproduction. Although an adaptive increase in egg-laying was found in the female cricket, *Acheta domesticus* (Adamo, 1999), I found no evidence for this response in female *G. texensis*.

In Chapter 3, I investigated whether injection of LPS from the cell wall of *S. marcescens* into female *G. texensis* would lead to a decrease in the number or quality of eggs laid, based on a more typical physiological trade-off, in which the energetic expense of immune system activation would rob resources normally allotted for reproduction. I injected crickets chronically over a period of two weeks with LPS, to determine whether

energy spent on immunity would have a negative effect on the number or quality of eggs produced and laid. I found no evidence for such a trade-off.

In Chapter 4, I investigated whether manipulating the reproductive output of females would lead to a change in immunocompetence. I monitored mortality between virgin and mated *G. texensis* females following an infection with *S. marcescens*, and proposed that virgins would outlive mated females because their reproductive costs are lower. I found no evidence for this (in fact, mated females survived longer!). Additionally, survival did not correlate with PO activity, a widely used assay for immunocompetence (e.g. Rolff and Siva-Jothy, 2002). I also manipulated egg output of mated females by providing them with different egg-laying substrates, and found that the LD_{50} did not differ between females laying many eggs and females laying few eggs. Therefore, I found no evidence for a trade-off.

The lack of evidence for physiological trade-offs in my own experiments leads me to investigate the soundness of the underlying assumptions. To be sure, cricket reproduction is associated with energetic expense. For example, egg production in insects requires a large investment of resources, such as yolk proteins, hormones, lipid, glycogen, vitamins, and other substances required to support the development of a new larval body (Kunkel and Nordin, 1985; Sander et al., 1985). Additionally, proteins are secreted around the egg to form the chorion (Regier and Kafatos, 1985), which protects the embryo from desiccation. The hundreds or thousands of eggs a female may produce in a lifetime can multiply this investment. In the cricket, *A. domesticus*, adult female growth is mostly ovarial, and between the ages of 10 and 30 days, the female invests 12.2

mg of dry weight into egg growth per day (Woodring et al., 1979). This is substantial considering the dry weight of the body without the ovaries is about 110 mg.

That immunity requires energy is not disputed either. The immune response of insects is complex, and involves the degranulation (and subsequent replacement of) hemocytes (Lavine and Strand, 2002), the up-regulation of antimicrobial protein production (Bulet et al., 1999; Kanost et al., 1990), and the activation of prophenoloxidase (Söderhäll and Cerenius, 1998).

Therefore, while it is clear that reproduction and immunity use energy, it is not clear that increased investment in one leads to decreased investment in the other, based on observations from my own data. How could this be possible? One explanation is that for a trade-off to occur, it would be necessary for both reproduction and immunity to be overwhelmingly expensive, so that the female, through increased food consumption or release of stores from fat body, could not energetically afford to up-regulate one without down-regulating the other. Alternatively, for a trade-off to occur, reproduction and immunity would have to be "low priorities" for the insect, so that it would be willing to sacrifice investment in one to fuel the other. A greater understanding of energy flow/utilization within an animal would be required to determine if either explanation is likely.

In the following simple experiment, I will assess the "costliness" of reproduction in the cricket, *G. texensis*, by conducting a lifespan study of virgin and mated females. Lifespan is an indicator of overall body condition, of which immunity is a part. If reproduction is generally costly, enough to reduce somatic maintenance, then virgin females should outlive mated females.

Methods

Animals

Crickets, *Gryllus texensis*, were selected from a colony maintained at Dalhousie University. They were reared on a 12L:12D cycle, at 28 ± 2 °C, with dry cat food and water *ad libitum*. For the experiments described below, newly moulted adults were removed from nymph cages daily. Females were housed separately from males until they were 6-9 days of adult age.

Lifespan of virgin and mated females: cotton substrate

When females were 6-9 days old, they were randomly assigned to one of two groups. The "mated" group was housed in a large, clear plastic container (23 × 36 × 15 cm) with five sexually mature males for five days. The "virgin" group was housed in a similar container with five large male nymphs for five days. Then, females were isolated into individual clear plastic containers (18 × 14.5 × 9 cm) with opaque lids. Each female was provided with food *ad libitum* and an opaque egg cup for cover. A water bottle (37 mL volume, 9.5 cm long, 2.2 cm diameter) plugged at the end with cotton provided the female with both a water source and an oviposition site. The water bottle was replaced every 5-10 days, and on those days, the eggs laid in the cotton were counted, up to a maximum count of 100 eggs. Females laying more than 100 eggs in that time were recorded as laying 100+ eggs. Females were weighed occasionally throughout the course of the experiment, and were checked daily for mortality.

Lifespan of virgin, singly mated and multiply mated females: sand substrate

When females were 6-9 days old, they were matched to one of three groups based on weight: 1) virgin, 2) singly mated, and 3) multiply mated. Singly mated females were allowed to mate once, while multiply mated females were allowed to mate three times, each time with a different male (see below). Females were housed individually in small clear plastic containers (18 × 14.5 × 9 cm) with opaque lids. Each female was provided with food *ad libitum* and an egg cup for cover. They were provided with a small water bottle (6 mL volume, 4.4 cm long, 1.3 cm diameter) plugged with cotton as a water source, and moist sand for oviposition. Moist sand was prepared by combining 12 mL of water with 37 mL of sand, sifted to a grain size smaller than egg size (0.7 mm mesh size), in a small plastic cup.

Virgin females were paired with large male nymphs for at least 1.5 hours. Females in the singly and multiply mated groups were paired with sexually mature males for at least 1.5 hours daily, or until a successful mating occurred. At that time, I left the spermatophore attached for 30 minutes, and then I removed the entire spermatophore carefully with forceps and discarded it, and the male was removed from the cage. Removing the spermatophore allowed me to control for potential benefits that the females might receive from male sperm or seminal fluid (Wagner et al., 2001), as well as potential benefits gained from eating it (Burpee and Sakaluk, 1993). I allowed at least 24 hours to elapse between matings. Half of the multiply mated females completed three matings over four days, and all but one female mated three times during the course of a week. All females mated at least once by the time they were two weeks old.

Once the required number of matings was achieved, the female remained in isolation for the duration of her life. Sand was replaced and the eggs were counted every second day. To count the eggs, the sand was first dried, and then I re-sifted it three times. Eggs were retained in the sieve (0.7 mm mesh size). Females were weighed every 7 days, and were checked daily for mortality.

Statistics

The average number of eggs laid per day was calculated by dividing the total number of eggs laid by a female in her lifetime by the number of days in her adult life. Parametric and nonparametric statistics were conducted as appropriate using StatView 5.0.1 for PowerPC (SAS Institute Inc.) and Zar (1984).

Results

Lifespan

I found no significant differences in lifespan between virgin and mated females laying eggs in moist cotton (Table 5), or between virgin, singly mated, or multiply mated females laying eggs in moist sand (Table 5).

Table 5 Lifespan data for virgin and mated females laying eggs in either wet cotton or moist sand.

Oviposition Substrate	**Group**	**n**	**Lifespan (mean ± SD)**	**P value**
Cotton Substrate	Virgin	13	73.8 ± 14.5	Mann-Whitney
	Mated	13	61.7 ± 23.4	U = 114
				P = 0.129
Moist Sand	Virgin	15	56.9 ± 14.7	Kruskal-Wallis
	Singly Mated	15	49.7 ± 11.0	statistic = 2.656
	Multiply Mated	14	53.9 ± 9.3	P = 0.26

Eggs laid per day

In the large water bottles, mated females laid significantly more eggs than virgin females (Table 6), and multiply mated females laid significantly more eggs in moist sand than either virgins or singly mated females (Table 6).

There was no relationship between the number of eggs laid per day and lifespan in multiply mated females (Spearman Rank Correlation, r = -0.169, n = 14, P = 0.54). Additionally, there is some suggestion that there is a limiting factor to oviposition, as 80% of multiply mated females laid essentially no eggs in the last 2-4 weeks of life.

Table 6 Average number of eggs laid per day by virgin and mated females in either wet cotton or moist sand.

Oviposition Substrate	**Group**	**n**	**Eggs laid per day* (median ± m.a.d.)**	**P value**
Cotton Substrate	Virgin	13	1.25	Mann-Whitney
	Mated	12	>6.96	U = 130
				P = 0.005
Moist Sand	Virgin	15	2.29 ± 3.10	Kruskal-Wallis
	Singly Mated	15	2.53 ± 3.54	H = 13.2
	Multiply Mated	14	13.79 ± 3.25	P = 0.0013[1]

* Calculated by dividing the total number of eggs laid in a lifetime by the female's lifespan

[1] Nonparametric multiple comparisons: virgin vs. singly mated P > 0.05; singly vs. multiply mated P < 0.02; virgin vs. multiply mated P < 0.005.

Discussion

In this cricket, I found no evidence to support the idea that virgins live significantly longer than mated females, and this suggests little cost to reproduction. Although it is true that the difference in lifespan between virgin and mated females laying eggs in cotton is bordering on statistical significance, the variability of data about the mean is high. Assuming no change in the mean or variability of the data, it would require approximately 55 animals per group to reach a significance level of $P = 0.05$ with high power (0.9). Additionally, one female within the mated group displayed unusual behaviour, laying no eggs in her 20-day adult life. Without this female, the difference between the groups is decreased, and it would then require a sample size of more than 90 per group to achieve statistical significance. Moreover, a recent review describing lifespan consequences of mating reveals that out of 41 studies of insects without nuptial feeding, 75% use fewer than 30 animals per group (average sample size approximately 24; Arnqvist and Nilsson, 2000). In two cricket studies investigating lifespan consequences of mating, sample sizes of 15 (Burpee and Sakaluk, 1993) and 19 (Wagner et al., 2001) were required to achieve statistical significance. Therefore, in my own experiments, the difference in lifespan between virgin and mated females in the cotton experiment, if real, is small. While this difference is less dramatic in the sand experiment, it is probably not appropriate to compare the results of the cotton and sand experiments because the effect on lifespan between these two substrates was not explicitly tested, egg numbers were only approximated for the cotton experiment, and the data were collected from two different times of year (cotton, March – May 2001; sand, Oct 2001 – Jan 2002).

The results presented here show similarities to trends that have been previously published in the literature. Burpee and Sakaluk (1993) found in *Gryllus veletis* and *Gryllodes sigillatus*, that females given continuous access to males over their lifetime lived as long as virgin females, and lived significantly longer than females given only limited access to males (first 15 days of adulthood). Wagner et al. (2001) found that female crickets that mate repeatedly have significantly longer lifespans than those that mate only once. As a consequence of their increased lifespan, they also show increased lifetime fecundity (*Gryllus lineaticeps*, Wagner et al., 2001). As discussed by Burpee and Sakaluk (1993) and Wagner et al. (2001), these results suggest that while mating (and subsequent reproductive activities) may be costly, the costs can be offset by mating more.

The mechanism underlying the apparent compensation of mating costs has been discussed by both Burpee and Sakaluk (1993) and Wagner et al. (2001). Certainly, consumption of the spermatophore could influence female fitness (Burpee and Sakaluk, 1993; Simmons, 1988) if the spermatophore contained important nutrients, and there may also be benefits derived from the male ejaculate. While the nature of the benefit is unclear, Wagner et al. (2001) suggest that there could be substances transferred in male seminal fluid, that either directly or through digestion, impart to the female nutrients that are scarce in her normal diet. They also suggest that ejaculates could give the female an energetic advantage, but do not favour this explanation because females in their experiments were fed *ad libitum*, and therefore, singly mated females had access to as much energy as they required. Alternatively, the act of mating per se may cause a physiological change in the female that results in a lifespan benefit. For example, mated

G. texensis females fed *ad libitum* are more resistant to *S. marcescens* than virgins (Chapter 4), suggesting that mating might improve immunity.

It is important to note that the potential mechanism underlying the lifespan benefit gained by multiply mated females is not the focus here. Instead, the essence of this chapter is that mating and egg production do not induce an obvious decline in somatic maintenance that results in decreased lifespan in *G. texensis*. This has important implications for the preceding three chapters, since one of the two critical underlying assumptions for all three is that reproduction is costly, and therefore, reduces energy for somatic maintenance, including immunity.

A re-examination of the literature reveals little convincing evidence supporting a physiological trade-off between reproduction and immunity in insects, despite the common assumption that one exists. Table 7 lists some of the studies that attempt to identify this trade-off, and for each I give a reason why the conclusions should be accepted cautiously as evidence. This list is not meant to be exhaustive, but instead provide the reader with a taste for the complexity within the field of ecological immunology.

Table 7 Examples of studies explicitly investigating relationships between reproduction and immunity in insects: a critical view

Authors	Finding	Criticism
Ahmed et al. (2002)	Infection with LPS in the mosquito, *Anopheles gambiae*, led to decreases in egg production and ovarian protein.	It is unclear whether the dose of LPS was toxic to the mosquito. They claimed that LPS was not toxic because those females injected with LPS did not suffer from increased mortality, but mortality was only monitored for two days. Without this knowledge, it cannot be determined whether their results are not simply the result of pathology. Additionally, saline (sham) injections also induced large declines in ovarian protein and increases in lysozyme concentration in the hemolymph, which were not reported to be statistically different than the changes induced by LPS. Injections performed on such small animals may lead to extensive internal damage, including damage to reproductive organs.

Authors	Finding	Criticism
Boots and Begon (1993)	The Indian meal moth, *Plodia interpunctella*, selected for increased resistance to granulosis virus, had a reduction in egg viability.	Physiological trade-offs were not examined here. Instead, this experiment tested genetically-based microevolutionary trade-offs, in which selection for an increase in one fitness trait (e.g. resistance) leads to a decrease in another fitness trait (e.g. egg viability). Microevolutionary trade-offs do not necessarily involve resource reallocation, but instead could be explained by negative pleiotropy.
Fellowes et al. (1999)	*Drosophila melanogaster* that survived parasitism by *Asobara tabida* had reduced egg production as adults.	Alternative explanations are possible. For example, attack by adult parasitoids per se or action of immature parasitoids may damage the host.
Hosken (2001)	Selective breeding (through enforced monogamy or polyandry) in the dung fly, *Scathophaga stercoraria*, resulted in males and females with different sizes of reproductive organs. A negative correlation was found between the size of the reproductive organ and PO activity in the blood.	The trade-off described here is microevolutionary, not physiological. Additionally, PO may be a poor measure of immunocompetence (Chapter 4), and no correlation was found between size of reproductive organ and hemolymph antibacterial properties.

Authors	Finding	Criticism
Rolff and Siva-Jothy (2002)	Adult mealworm beetles, *Tenebrio molitor*, had decreased PO levels after a single mating.	One of the assumptions of their study is that mating is costly, which may not be true in *T. molitor* (Worden and Parker 2001). Additionally, immune function was only assessed by PO activity, which may be a poor measure of immunocompetence (Chapter 4). They found no difference in hemocyte load following mating, and did not examine other components of immunity. Most importantly, a functional assay, in which an animal responds to a live pathogen as a measure of immunocompetence, was not conducted.
Ryder and Siva-Jothy (2000)	A positive relationship was found between energetically expensive calling song in male crickets and immunocompetence, measured by hemocyte load.	Trade-offs usually imply a negative relationship. Additionally, hemocyte numbers in the hemolymph may be a poor measure of immunocompetence, since in some orthopterans (e.g. crickets), circulating hemocytes may play a minor role in engulfing bacteria compared to the reticular cells of the hemopoeitic organ, where hemocytes are produced (Hoffmann et al., 1979). A functional assay would be a superior measure of immunocompetence.
Siva-Jothy et al. (1998)	Mating decreased encapsulation of a nylon filament in damselflies.	No difference was found in encapsulation following fighting in males, suggesting that the decrease found following mating was not the result of an energetic trade-off. Additionally, no functional assay was conducted.

Two important points from Table 7 should be addressed here. The first is that the observation of a microevolutionary trade-off does not automatically imply a physiological trade-off (Stearns, 1992; Zera and Harshman, 2001). Unlike physiological trade-offs, microevolutionary trade-offs are the genetically-based result of selection pressure. Over evolutionary time, animals may be selected for higher levels of resistance or reproduction, and other fitness traits may show a correlated decrease, which may or may not be the result of resource reallocation, and which may or may not imply a functional relationship (e.g. genetic linkage). The fact that these negative associations can evolve within a species does not mean that the individuals within a population can, or will, reallocate internal resources in response to an immediate change in reproductive status or severity of infection.

The second point is that an accurate assessment of immunocompetence (i.e. "strength" of the immune system) cannot be made through measures of single immune elicitors. While PO, hemocyte load, or antibacterial protein levels are important measures, these factors may not always correlate with level of resistance. For example, in Chapter 4 I found no difference between PO activity levels in the hemolymph of virgin and mated females, yet virgins are significantly more susceptible to succumbing to an *S. marcescens* infection. This is likely because PO is important for various other functions in the insect body, including cuticle formation and egg production (see Chapter 4), and as such, gives a poor estimate of immunocompetence. Levels of circulating hemocytes may also be a poor measure of immunocompetence, especially in orthopterans, since the hemopoeitic organ, where hemocytes are produced, may have a more prominent role in engulfing invading bacteria than the hemocytes themselves (Hoffmann et al., 1979).

Additionally, the immunoredistribution hypothesis (Braude et al., 1999) suggests that immune cells could be temporally, spatially and reversibly redistributed throughout the body in response to fluctuating hormone or activity levels. Therefore, hemocyte levels may not accurately reflect immunocompetence, depending on where and when they are measured. For example, Siva-Jothy et al. (1998) found that damselflies had a decreased encapsulation response following mating, but not following fighting, both suspected to be energetically demanding activities. Perhaps neither activity leads to a decline in hemocyte number, but simply results in immunoredistribution, where hemocytes during mating are redistributed to reproductive organs, while hemocytes during fighting remain in general circulation. Since encapsulation was always monitored in the same abdominal segment, this possibility could be overlooked, and a false conclusion might be reached regarding the immune status of the individual. The only clear way to determine an animal's level of immunocompetence is through a functional assay, in which the animal is forced to respond to an ecologically relevant and potentially lethal pathogen.

If there is little convincing evidence for a physiological trade-off between immunity and reproduction, why then does the theory persist? In the words of Bell and Koufopanou (1986), discussing the costs of reproduction:

> "The elegance of the theory ... has tended to distract attention from the empirical question of whether or not the premise on which it is founded is generally true".

Indeed, the elegance of the theory has even distracted attention from the empirical data that suggest it isn't true, and has instead led to an abundance of explanations for the

existence of trade-offs despite the lack of evidence. Several of these have been explained in Stearns (1992). For example, age of the animal could play a role in determining how it adaptively responds to both reproductive and immune challenges. A young, reproductively naïve animal with high future reproductive potential would probably have more to gain from investing in physiological traits that extend lifespan (such as immunity) than would an older animal that has already had considerable reproductive success. Instead, the older animal might devote its remaining resources to reproduction, perhaps at the expense of immunity. While this logic is reasonable in a longer-lived animal, it becomes less important for insects like *G. texensis* that live less than one month in the field. Moreover, because egg laying only begins at approximately seven days of adult age regardless of the age at first mating (*G. texensis*, personal observations; *A. domesticus*, Clifford and Woodring, 1986), her reproductive lifespan is shorter yet. The animals considered in my experiments began at 7-12 days of adult age, and were therefore only beginning to reliably lay eggs. They all had considerable reproductive potential of 1000 eggs or more (Chapter 2). As my experiments extended up to 18 days (the entire natural reproductive lifespan of the female), there would have been a question of ecological validity had the animals been any older at the start. Furthermore, if this logic (described above) were true in my female crickets, then I would expect virgin females to invest more heavily in immunity than mated females, since their reproductive success is critically dependent on surviving infection and extending lifespan. This result was not found (Chapter 4). Therefore, while age and residual reproductive value may play a role in the life-history strategies of other animals, it is likely not a factor is the experiments described within this thesis.

It is also possible that potential physiological trade-offs will only be observed under conditions of food stress (Zera and Harshman, 2001). Logically speaking, if an animal can acquire sufficient resources from food to fuel all processes within the body, and compensate for increases in metabolism due to infection or reproduction by eating more or drawing from storage, then physiological trade-offs would not occur. An experimental study on the bumblebee, *Bombus terrestris*, provides an example of an immune cost that is not evident until resources are scarce. In this insect, immune activity, initiated by injection of LPS or Sephadex beads into the hemolymph, makes trade-offs with lifespan, but only when the animal is deprived of food (Moret and Schmid-Hempel, 2000). Similar injections in bumblebees fed *ad libitum* had no impact on lifespan. Moreover, it is possible that crickets do not deplete internal resources for the production of eggs. Clifford and Woodring (1986), in an analysis of food consumption and fat body mass in the cricket, *A. domesticus*, found that the mass of eggs ovulated was approximately five times the mass of fat body lost, and concluded that most of the resources used for egg production came from food consumed. If this is true in *G. texensis*, then only in situations of food stress, when the animal relies more on internal stores for reproduction, might a trade-off between reproduction and immunity occur.

While it is true that nutrient deprivation might reveal negative correlations where none existed previously, it is important to recognize that short-term nutrient deprivation could lead to a variety of undesirable physiological and metabolic responses (e.g. production of ketone bodies, Downer, 1985), which could in turn alter both reproduction and immunity independently, and uncouple them from the mechanism that linked them in a trade-off in the first place (Zera and Harshman, 2001). While the phenotypic correlation

might be negative, it would be premature to conclude that the correlation implies a trade-off, since a trade-off specifically refers to a functional reallocation of internal resources from one fitness function to another (Zera and Harshman, 2001). Moreover, if trade-offs only exist during times of food stress, then this life-history strategy is not as general as supposed, but can instead be considered as a subset of mechanisms that animals have evolved to deal with stressful situations.

In their natural life history, it appears that crickets are not normally faced with conditions of food deprivation. Crickets in the field, including those with ormiine parasitoids, have ample fat body reserves (Adamo, Murray, and Cade, unpublished data), and Burpee and Sakaluk (1993) found that female *Gryllus veletis* can survive 20 days with no food, suggesting that they allocate resources aggressively to storage when they can. For many species in which food supply is normally adequate, other forces may preclude the evolution of trade-offs. In crickets, the greatest selection pressure is likely predation (e.g. Sakaluk and Belwood, 1984), not starvation. If female *G. texensis* have sufficient fat reserves such that trade-offs between reproduction and immunity have no selective value, and if their life history has been greatly shaped by the life-shortening forces of predation, then it becomes interesting to speculate as to why these females are not investing ***more*** into current reproduction. The theory of trade-offs assumes that animals are selected to maximize reproduction. Any resources not invested in increasing reproduction directly should be invested indirectly (e.g. increasing lifespan). Taken together, these arguments suggest the possibility that there are physical or physiological constraints, independent of energy constraints, which limit investment in reproduction. For example, egg production may be limited by a particular nutrient that is scarce in a

normal diet. Alternatively, accumulation of detrimental metabolic products, which would exact some fitness cost, may be generated by bouts of high egg production. Regardless of the details, if reproduction is capped to a maximal limit by other constraints, then trade-offs between reproduction and immunity may be rare in animals not suffering from food shortage.

There also exists the possibility that reproduction and immunity do not always require excessively large energetic investments, which would make energetic trade-offs less necessary. In addition to the example in this chapter that reproduction may not be costly in crickets (see above), Worden and Parker (2001) found that in *Tenebrio molitor*, mating with five mates leads to a significant increase in egg production, but there was no difference in lifespan. This suggests that there is no ultimate cost, enough to reduce somatic maintenance, in this species. A recent review suggests that increased immunity may not be costly either. Rigby et al. (2002) discuss various mechanisms by which immunity may not be costly. For example, a host response to infection could be to decrease, as opposed to increase, the expression of a factor that is required for success of the pathogen. Alternatively, the host could make trade-offs within its own immune system in response to infection. For example, the animal might increase hemocyte production while decreasing prophenoloxidase production. This would enable the animal to optimize its own immune system for immediate use, with minimal expense to other fitness components. Finally, the largest immune expense may be due to anti-pathogen defenses that are maintained constantly such as hemocyte number or antimicrobial production in cuticle. Inducible immunity, such as that studied in this thesis, may be very inexpensive by comparison (e.g. Råberg et al., 2002). This is supported by data from

Chapter 3, in which chronic injections of LPS led to an immune response, but had no measurable effects on egg number, quality, or female lifespan.

Since the energetic expense of reproduction and immunity is unknown, it has been suggested that measures of metabolic rate, such as oxygen consumption measured by flow-through respirometry, be taken during these activities. While this has been done in some experimental situations, results are difficult to interpret. For example, Clifford and Woodring (1986) showed that virgin female *A. domesticus* have lower metabolic rates than mated females, and that oxygen consumption is directly and negatively proportional to the number of eggs stored in the oviducts. While it is simple to conclude from this that reproduction requires energy investment, it is difficult to determine where the metabolic activity is greatest. For example, increased metabolism could be a result of egg-laying behaviour, egg production, or even digestive activity, as eggs no longer compress the gut. Moreover, it provides no insight into the origin of the metabolic substrate.

While no experiments have investigated the energy required to launch an immune response in insects, some have been conducted in vertebrates, again with results that are difficult to interpret. For example, Martin II et al. (2003) found that injection of house sparrows, *Passer domesticus*, with the nonpathogenic compound phytohaemagglutinin, resulted in a cell-mediated immune response that raised the basal metabolic rate by 29%. In contrast, Svensson et al. (1998) showed that in blue tits, *Parus caeruleus*, there was no significant increase in basal metabolic rate in response to injections of killed diptheria-tetanus virus.

While information regarding basal and active metabolic rates for *G. texensis* would be useful generally, it would be very difficult to interpret within the context of this

thesis because oxygen consumption is such a crude measure of the numerous metabolic processes that are occurring within the body. For example, an increase in basal metabolic rate after infection with LPS would suggest a cost to inducible immunity, but would not reveal the proportion of the daily energy budget that is invested in immune maintenance, or resistance. Levels of standing resistance may play an important role in the cost of inducible immunity (see Chapter 4 discussion). Furthermore, it would fail to reveal: 1) the potential costs involved in recovery from the immune response, including hemocyte replacement; 2) whether all or just some of the components of the immune system were activated; and 3) the potential physiological trade-offs that are non-energetic in nature. To complicate matters, the reproductive state of the female could influence the substrate for metabolic energy (i.e. proportion of carbohydrate, lipid, and protein), which would in turn influence the measures of oxygen consumption. Careful control would be required to gauge the potential effects of activity levels, age, body size, nutrient intake, and egg production history.

Most importantly, energetic requirements for physiological functions like reproduction or immunity do not automatically translate into increased fitness costs.

A superior approach would be to determine how energy flows within the animal (see Rivera and Casas, 1999). How is food is consumed by the animal? How is it assimilated in the body? How much food is required to support basic body maintenance? How much is required to support maximal reproduction? What proportion of egg production do internal stores support? Do "sick" insects eat more? How and when is fat body used? Which aspects of metabolism take "priority" (see below)? Since the theory of energetic trade-offs relies on the idea of energy budgets of individuals, then a summary

of how an individual of any species actually allocates its energy would prove valuable. For example, *Asobara tabida* females use fat reserves to build eggs and sustain locomotory activity (Ellers and van Alphen, 1997; Ellers et al., 1998), while in the *Heliconius* butterfly, the use of incoming reserves were preferred to use of stored reserves (Boggs, 1997). These types of differences in energy utilization, which are poorly understood at present, could determine whether different components of fitness will trade-off against one another.

Zera and Harshman (2001) review the idea of "priority rules", suggesting that functions, such as reproduction, growth, basic metabolism, and metabolism of physical activity are physiologically "ranked", and this ranking controls how energy is distributed within the animal. For example, Zera et al. (1998) found that in the cricket, *G. assimilis*, energy allocation to maintenance or storage took precedence over allocation to reproduction in nutrient-poor conditions. The life histories of different animals, including typical lifespan, type of reproduction (continuous vs. discrete), number of breeding seasons, population density during development, and individual genetic differences, will shape these priority rules, and determine whether immunity is traded off for reproduction. For example, in *G. texensis*, reproduction and immunity may both be high priority functions, and additional energy requirements to fuel them may be drawn from elsewhere. Alternatively, additional energy may be evenly drawn from all fitness functions, and therefore changes measured in only one or two of these might be small and difficult to measure.

Together, these difficulties underscore the problem of definitively testing whether or not a trade-off is occurring. The theory, while elegant and simple, is horrendously

difficult to test in practice. For example, Zhao and Zera (2001) conducted radiotracer studies in the wing-polymorphic cricket, *Gryllus firmus*, and showed that the flight-capable genotype synthesized a greater amount of lipid and triglyceride which is important for somatic tissue maintenance (including flight musculature), while the flightless genotype synthesized a greater amount of phospholipid for ovary development. While this result is suggestive of an energetic trade-off mediated through lipid biosynthesis, it could also be the result of antagonistic control, whereby accumulation of one biosynthetic product downregulates synthesis of the other (Zera and Harshman, 2001). Therefore, even in studies that find effects consistent with trade-off theory, plausible alternatives remain.

From data that are inconsistent with theory, my thesis illuminates some of the largest methodological and theoretical problems within the field of insect ecological immunology, which may have an enormous impact on the interpretation of results. The largest methodological problem is the consistent use of PO and hemocyte levels as measures of immunocompetence. PO plays a multifunctional role in the insect (e.g. wound repair and cuticle formation) and circulating hemocytes may not accurately reflect the number available to respond to a pathogen. Additionally, the immune system is complex and dynamic, involving the coordinated response of all its components. Measuring a single factor cannot provide a complete picture of immune system activity. Instead, conducting a functional assay with a live pathogen is the only clear way of assessing it.

Along with the methodological problems, the largest theoretical problems within the field of insect ecological immunology are the virtually untestable underlying assumptions: that immunity and reproduction are costly. These assumptions are based on yet another assumption: a limited energy budget. Therefore, only in cases of food deprivation might immunity and reproduction trade-off against one another, and even then, the ecological and functional validity of the results are compromised. Insects are highly evolved creatures, and have most likely adapted to handle the costs of both reproduction and immunity. With an adequate food supply, it is only reasonable to expect that the animal will have sufficient resources to fuel all its reproductive and somatic processes. More work on the energetic requirements of immunity and reproduction, and their source of fuels, would be required to determine whether this is true.

The information within this thesis makes two important points about insect ecological immunology: 1) assays of PO activity for immunocompetence should be abandoned in favour of a functional test, and 2) physiological studies of energy budgets, including reproductive costs, must be conducted for individual species before conclusions about trade-offs can be made. While these two issues will hopefully guide future work, they also suggest that some of the published data in this field need reinterpretation, and question the accepted dogma in this field as a whole. Until these issues are addressed and resolved by the scientific community, the significance of previous work will be in limbo.

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