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Physiological trade-off between cellular immunity and flight capability in the wing-dimorphic sand cricket, *Gryllus firmus*

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ABSTRACT

The sand cricket, *Gryllus firmus*, is a wing-dimorphic species with long-wing (LW) and short wing (LW) morphs. The LW forms have very well developed wings and flight muscles and their SW counterparts have reduced wings and flight muscles, coupled with greater resource allocations to reproduction. This is the well-known oogenesis-flight syndrome, in which resources are differentially allocated to reproduction or flight. Insects have also evolved very sophisticated and robust immune systems, understood as a major adaptation that partly accounts for the long-term biological success of insects. Nodulation is the predominant cellular immune reaction to microbial infections, responsible for clearing most infecting microbes from hemolymph circulation in the first two hours after an infection. This is a biologically expensive process because in large infections millions of hemocytes are invested into melanotic nodules. These lost hemocytes are later replaced in hematopoietic organs. Honey bees and a few other insect species redirect resources from cellular immunity to another biologically expensive activity, foraging flights. These reallocations are conceived as physiological trade-offs, in which the costs of one biological function are traded off for another. Based on previous work, we posed the hypothesis that the sand cricket, *Gryllus firmus*, already known to reallocate resources in the oogenesis-flight syndrome, also reallocates resources from immune functions to flight. Here, we report that relative to long-wing morphs, the short-wing forms have higher immune capacity, registered in terms of nodulation reactions to the same challenge, higher hemocytic phospholipase A_2 activity (an enzyme involved in immune signaling) and relatively reduced age-related immunosenescence. We infer from these data that sand crickets, and likely many insect species, reallocate resources between flight, reproduction and immunity. Such trade-offs may support other functions, such as foraging and mate finding, as well.

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Introduction

Many insect species exhibit wing dimorphism, which often operates in a physiological trade-off between flight and reproduction. Migratory morphs generally have well developed wings and flight muscles, and non-migratory morphs have reduced wings and flight muscles, coupled with greater resource allocations to reproduction. This is generally known as the oogenesis-flight syndrome, although members of some wing dimorphic species somehow develop flight capacity and well-developed ovaries (Lorenz, 2007). The sand cricket, *Gryllus firmus*, occurs in two morphs, a long-winged (LW), flight-capable morph with fully developed wings and flight muscles and a short-winged (SW), flightless morph with enhanced reproductive potential (Roff and Fairbairn, 2007). This is taken as a physiological trade-off in which resources are differentially allocated between flight capability and fecundity (Zera et al., 2011). Development and maintenance of large flight muscles and storage of flight fuel are demanding in terms of energy (Zera and Denno, 1997), indicated by the increased metabolic rate of the LW morph (Crnokrak and Roff, 2002).

Aside from reproduction and flight, insects invest considerable resources into robust innate immune systems that provide protection from many foreign invaders such as microbes and parasitoids. Insect immunity is generally assorted into two classes, humoral and cellular immunity, although there is overlap between the two (Stanley and Kim, 2014). Humoral immunity involves induced biosynthesis of antimicrobial peptides (AMPs), such as attacin, cecropin, and lysozyme, which appear in hemolymph at 6–12 h post-infection (Kanost et al., 2004). Cellular immunity is characterized by direct interactions between circulating hemocytes and invaders in hemolymph; these interactions are initiated immediately after detection of infection or invasion. Cellular immune reactions include nodulation, phagocytosis, and encapsulation (Strand, 2008). Small invaders such as bacterial cells can be cleared from hemolymph circulation by phagocytosis and nodulation, which entrap bacteria in hemocyte aggregations (Stanley and Kim, 2014); larger invaders are cleared by encapsulating invaders in

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layers of hemocytes. Encapsulation and nodulation processes are completed by a melanization process, which produces reactive oxygen species that act in chemically killing invaders (Nappi and Christensen, 2005). Many insect immune reactions are mediated by eicosanoids, including prostaglandins (PGs) and various lipoxygenase products, summarized in Discussion (Stanley, 2000; Stanley and Kim, 2014). Research into ecological and evolutionary forces responsible for shaping invertebrate immune systems indicates immune effector mechanisms are not fixed features of insect biology (Rolff and Siva-Jothy, 2003). Although robust immune defense mechanisms are vital to insects, they are also costly physiological functions (Schmid-Hempel, 2003). Nodulation reactions lead to loss of thousands or even millions of hemocytes, which entails high replacement costs (Miller et al., 1994). In some insects, the biological resources invested in immunity are reallocated to several other physiological parameters, including reproduction and foraging. For specific examples, adult honeybees abandon biologically expensive cellular immunity when they reach the foraging stage of adulthood, while retaining their phenoloxidase-based immunity (Bedick et al., 2001; Schmid et al., 2008). Adult crickets, *Gryllus texensis*, reallocate resources between immunity and reproductive behavior (Adamo et al., 2001), and bumblebees, *Bombus terrestris*, redistribute energy between foraging and immunity (König and Schmid-Hempel, 1995). We infer from these studies that the various immune mechanisms are physiological functions subject to reallocations according to temporary optimizations that vary within a single lifespan.

The reallocation of resources in the oogenesis-flight syndrome and between foraging and immunity may give the impression that physiological trade-offs operate in a bi-modal pattern, directing resources one way or another. Here, we consider the idea that resources can be devoted to a broader range of physiological functions, differentially favoring flight, reproduction and immune functions. We tested the idea with the wing dimorphic sand cricket, *G. firmus*, already known to operate an oogenesis-flight syndrome. In this paper we report that SW adult male crickets have more robust immunity, compared to the LW form, from which we infer that flight capacity draws on resources invested into oogenesis and immunity.

Materials and methods

Organisms

Wing dimorphic sand crickets, *G. firmus*, were obtained from a laboratory colony maintained by Dr. Anthony Zera, University of Nebraska. The crickets were maintained on oatmeal and tap water at 25 ± 1 °C, 16L:8D photoperiod, and RH 60 ± 5% in the incubator. Long-winged (LW) and short-winged (SW) adult male crickets were used in this study were taken from an artificially selected population of *G. firmus* maintained in culture for at least 5 generations. The age of crickets was recorded starting on the day of adult eclosion. Only crickets of known age were used in this study. Each cricket was sampled only one time and treated crickets were maintained individually until sampled.

LPS dose–response curve: nodulation

The nodulation assay was done following general protocols developed by Miller and Stanley (1998). Crickets were anesthetized by chilling on ice and surface-sterilized with 95% ethanol. Lipopolysaccharide (LPS), prepared from the bacterium *Serratia marcescens,* was used to stimulate nodulation (Bedick et al., 2000). Briefly, lyophilized bacterial cells were suspended in 90% phenol with constant stirring at 50 °C. After 24 h, chloroform and petroleum ether were added. After 30 min the mixture was centrifuged at 5000 ×g and the combined supernatant layers were removed under vacuum. LPS aggregates were precipitated by adding deionized water to obtain 85% phenol, and then aggregates were pelleted by centrifugation. The final pellet was dissolved in phosphate buffered saline with sonication (PBS, pH 7.5) and injected into the hemocoels of crickets between second and third abdominal sclerites. All injections were done in 4 μl volumes with a Hamilton 701 microsyringe (Hamilton, Reno, NV, USA).

Both adult morphs (1 day after adult emergence) were injected with 0, 50, 100, or 200 μg LPS/cricket, $n = 5$ crickets/dose and kept individually. At 24 h post-injection (PI), the crickets were anesthetized by chilling on ice, and then the hemocoels were exposed. Melanized, dark nodules were counted under a stereomicroscope at 60 × (Olympus, Tokyo, Japan). After the initial count in abdomens and thoraces, the alimentary canal was removed and nodulation was assessed in the newly exposed areas. The internal tissues, including alimentary canal, Malpighian tubules and accessory glands were then checked for possible nodules. Nodules were distinct and direct counting reliably reflects the extent of nodulation reactions to challenges (Miller and Stanley, 1998).

LPS time course: nodulation

Individuals from both morphs (1 day after adult emergence) were injected with 100 μg of LPS, $n = 5$ crickets per time point. Nodulation was assessed at 0, 1, 6, 12, and 24 h PI. Controls were injected with 4 μl PBS.

Influence of wing-dimorphism and aging on nodulation

LW and SW adult males of ages 1, 7, 14, 21, 28 and 35 days were challenged with 100 μg LPS in 4 μl/cricket, $n = 5$ crickets per age. At 24 h PI, the crickets were anesthetized and numbers of nodules were determined as just described.

Hemocytic PLA_2 preparation and enzyme activity assay

Cricket hemolymph was collected by the method of Tunaz et al. (2003) and was centrifuged at 1300 ×g for 15 min. Pellet was rinsed three times with saline buffer (SB: 1.7 mM Pipes, 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2$, 3 mM $CaCl_2$, 243 mM sucrose, 15 mg/l polyvinylpyrrilidone, pH 7.5) and transferred to a fresh 1.5 ml tube. Hemocytes were homogenized by sonication with eight 0.5 second bursts at 60 W using a VibraCell sonicator (VibraCell, Danbury, CT). The tubes were centrifuged at 1300 ×g for 10 min at 4 °C. Final supernatants were used in PLA_2 activity assays. Protein concentrations were determined using the bicinchoninic acid reagent (Pierce, Rockford, IL) against bovine serum albumin as quantitative standard at 562 nm using a BioTek microtiter plate reader.

PLA_2 reactions were started by adding aliquots of hemocyte preparation (200 μg total proteins) to tubes containing 0.05 μCi of radiolabeled phosphatidylcholine vesicles as a PLA_2 substrate. After vortexing the tubes for 15 sec, the reaction mixtures were incubated at 28 °C in a shaking water bath for 30 min. The reactions were terminated by adding 0.6 ml of acidified extraction solvent (chloroform:methanol 2:1, *v*/*v*, amended with 50 μl of 2 N HCl). The tubes were vortexed for 15 sec and centrifuged at 735 ×g for 2 min. The lower organic fractions were transferred to fresh 1.5 ml tubes and dried under N_2 gas. The reaction products were separated on TLC plates (silica gel G, 20 × 20 cm, 0.25 mm thick, Sigma-Aldrich, St. Louis, MO) and developed in a mixture of petroleum ether: diethyl ether:glacial acetic acid (980:20:1, *v*/*v*/*v*). The amounts of radioactivity in each fraction were estimated by counting in EcoLite scintillation cocktail (ICN Biomedicals, CA) on a LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Pharmacia, Finland) at 96% counting efficiency for ^{14}C. PLA_2 activity was calculated from the scintillation counter data.

LPS dose–response curve: PLA_2 activity

The LPS doses (0, 50, 100, or 200 μg) were added to each reaction tube containing 250 μl of hemocyte PLA_2 preparation from LW or SW

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