The Journal of Experimental Biology 211, 531-538
Published by The Company of Biologists 2008
doi:10.1242/jeb.013136

Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets

S. A. Adamo[1,*], J. L. Roberts[1], R. H. Easy[2] and N. W. Ross[2]

[1]Department of Psychology, Dalhousie University, Halifax, NS B3H 4J1, Canada and [2]Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, NS, Canada

*Author for correspondence (e-mail: sadamo@dal.ca)

Accepted 6 November 2007

SUMMARY

Intense physical activity results in transient immunosuppression in a wide range of animals. We tested the hypothesis that competition between immune function and lipid transport for the protein apolipophorin III (apoLpIII) can cause transient immunosuppression in crickets. Both flying, an energetically demanding behavior, and an immune challenge reduced the amount of monomeric (free) apoLpIII in the hemolymph of crickets. Because both immune function and flying depleted free apoLpIII, these two phenomena could be in competition for this protein. We showed that immune function was sensitive to the amount of free apoLpIII in the hemolymph. Reducing the amount of free apoLpIII in the hemolymph using adipokinetic hormone produced immunosuppression. Increasing apoLpIII levels after flight by pre-loading animals with trehalose reduced immunosuppression. Increasing post-flight apoLpIII levels by injecting purified apoLpIII also reduced flight-induced immunosuppression. These results show that competition between lipid transport and immune function for the same protein can produce transient immunosuppression after flight-or-fight behavior. Intertwined physiological systems can produce unexpected trade-offs.

Key words: Orthoptera, lipophorin, flight, *Gryllus texensis*, trade offs, disease resistance, *Serratia marcescens*.

INTRODUCTION

Intense physical activity results in transient immunosuppression in humans (Gleeson et al., 2004), other vertebrates (Ewenson et al., 2003; Thomas et al., 2005) and insects (Adamo and Parsons, 2006). This window of vulnerability appears maladaptive, suggesting that it is caused by a physiological constraint. For example, crickets (*Gryllus texensis*) are more likely to die from an infected wound after flight-or-fight behaviors (Adamo and Parsons, 2006). During an intense fight, crickets become less able to defend themselves against bacteria, even though intense fighting increases their chance of being exposed to bacteria through a wound. We hypothesize that this transient decline in disease resistance in crickets is caused, at least in part, by physiological interactions between the immune system and lipid transport. These two systems are intertwined in both vertebrates (Berbée et al., 2005; Wendel et al., 2007) and insects (Weers and Ryan, 2006).

In insects, two proteins, apolipophorin I and apolipophorin II combine to form high-density lipophorin (HDLp). HDLp ferries a variety of lipophilic compounds through the blood (hemolymph) (for a review, see Weers and Ryan, 2006). A third protein, apolipophorin III (apoLpIII), exists as a monomer at rest, but during energy-demanding behaviors, undergoes a conformational change and combines with HDLp to form low density lipophorin (LDLp) (Weers and Ryan, 2006). LDLp can carry the large amount of lipid (diacylglycerol) (Weers and Ryan, 2006), liberated from the fat body, needed to fuel flight in long-winged gryllid crickets (Zera et al., 1999).

However, lipid-free or monomeric apoLpIII also has immunological functions. It is thought to act as a pattern recognition molecule (Weers and Ryan, 2006). Apolipophorin III, like lipophorins in mammals (Wendel et al., 2007), can bind and detoxify lipopolysaccharides (LPS) (Dunphy and Halwani, 1997). It also binds to lipoteichoic acid and bacterial surfaces (Halwani et al., 2000), as well as to β-1,3-glucans and fungal conidia (Whitten et al., 2004). Once bound to pathogens or their components, apoLpIII is thought to undergo a conformational change that activates an immune response against the pathogen (Leon et al., 2006; Weers and Ryan, 2006). It then promotes cellular immune reactions such as phagocytosis (Wiesner et al., 1997) and an increase in antibacterial activity in the hemolymph (Wiesner et al., 1997; Dettloff et al., 2001a). Thus, apoLpIII appears to act as a circulating detector for bacteria (Kim et al., 2004).

ApoLpIII is required only intermittently for lipid transport and pathogen defense. Therefore, most of the time, these functions are not in conflict. However, because both lipid and LPS bind to the same position on apoLpIII (Leon et al., 2006), we hypothesize that apoLpIII cannot carry out both of its functions simultaneously. Once co-opted into lipid transport, it may no longer be available for immune surveillance. Reduced immune surveillance could explain the appearance of the transient period of immunosuppression that occurs immediately after flight-or-fight behavior (Adamo and Parsons, 2006).

To test this hypothesis, we first determined whether apoLpIII is depleted by both flying and an immune challenge in the cricket *G. texensis*, demonstrating the potential for conflict between lipid transport and immune function. We then tested whether disease resistance is related to the level of free apoLpIII in the hemolymph by reducing apoLpIII concentration using adipokinetic hormone

(AKH). AKH mobilizes lipid in the cricket *Acheta domesticus* (Woodring et al., 2002) and induces the formation of LDLp (Strobel et al., 1990). Finally we injected apoLpIII and tested whether we could prevent flight-induced immunosuppression by increasing apoLpIII levels.

MATERIALS AND METHODS

Animals

Crickets [long-winged *Gryllus texensis* (Cade and Daniel, 2000)] were originally collected near Austin, Texas and have been maintained as a laboratory colony for many generations. Crickets were reared at 28±2°C on a 12 h:12 h L:D cycle. Experiments were run at approximately the same time each day to avoid any circadian rhythm effects in hemolymph lipid levels (Das et al., 1993). Pellets of dry cat food and water were provided *ad libitum*. No cricket was used in more than one experiment. Tests were performed approx. 2 weeks (±3 days) after the molt to adulthood. At this age crickets are sexually mature and within their lifespan in the field (Murray and Cade, 1995). It is also well before the immune system begins to decline due to senescence (Adamo et al., 2001).

Hemolymph was removed by puncturing the pronotal membrane with a 10 μl Hamilton syringe needle and collecting 2 μl of hemolymph. Injections were also given through the pronotal membrane unless hemolymph was to be subsequently withdrawn. In that case, injections were given into the abdomen between the third and fourth caudal tergite.

Crickets used in experiments were isolated into individual opaque containers (10 cm in diameter) 1 day prior to use with food and water provided *ad libitum*. Unless otherwise indicated, groups were matched for sex.

All studies were approved by the Animal Care Committee of Dalhousie University and are in accordance with Canadian Council on Animal Care. All chemicals were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted.

Flying crickets

To test the effect of flight on immune function, crickets were tethered to a wooden applicator stick using low temperature wax and placed in a gentle air stream. Crickets were allowed to fly for 5 min. Control crickets were unhandled. Therefore, flying crickets experienced both a handling stress and a flight stress. Earlier work (Adamo and Parsons, 2006) demonstrated that physical activity, as opposed to handling stress, is required to produce immunosuppression in *G. texensis*. Crickets that did not fly during the trial were excluded from the study. In locusts, 5 min of flight activates the mobilization of energy reserves (Auerswald and Gäde, 2006), including the release of lipid 1 h after flight. Auerswald and Gäde (Auerswald and Gäde, 2006) hypothesize that after 5 min of flight, animals are hormonally committed to release lipid.

Measurement of total lipid content of the hemolymph

The effect of flight on total hemolymph lipid concentration was measured using the sulfuric-vanillin method (Barnes and Blackstock, 1973). 2 μl of hemolymph was added to 500 μl of 95% sulfuric acid heated to 95°C. Samples were heated for 10 min and cooled to room temperature on ice. 200 μl was then added to 1 ml vanillin agent (1.98 g vanillin, 668 ml orthophosphoric acid, 332 ml water). After 15 min, spectrophotometric readings were taken at 540 nm. Samples were run as duplicates. Diacylglycerol was used as the lipid standard. Standards were run with each trial.

Identification of free ApoLpIII using native PAGE and SDS-PAGE gels

ApoLpIII was identified using the methods of Smith et al. (Smith et al., 1994). ApoLpIII has little sequence homology among different orthopteran species (Strobel et al., 1990), and therefore sequence homology cannot be used to positively identify the band. Identification of apoLpIII was based on molecular mass and by the dramatic decline in the density of the band in response to adipokinetic hormone (Smith et al., 1994). Hemolymph from 2 crickets (4 μl total) was pooled in 20 μl of loading buffer with cricket anti-coagulant (loading buffer: 0.5 mol l^{-1} Tris–HCl pH 6.8, 10% SDS (w/v), 25% glycerol, 0.2% Bromophenol Blue; cricket anti-coagulant: phenothiocarbamide and protease inhibitor cocktail (a few crystals enough to form a supersaturated solution), 10 mmol l^{-1} EDTA, 0.15 mol l^{-1} NaCl, 10 mmol l^{-1} glutathione) kept on ice. The hemolymph–loading buffer mixture was spun at 2500 ***g*** for 10 min at 4°C. The supernatant was added to a 7% native-PAGE gel with 1 mmol l^{-1} EDTA. After running the sample on the native-PAGE gel, columns were cut and placed horizontally on top of a 12% SDS-PAGE gel. Gels were stained with silver stain (Swain and Ross, 1995). Molecular mass markers (Bio-Rad, Hercules, CA, USA) were run with the hemolymph samples.

To determine the effects of different treatments on the relative amount of free apoLpIII in the hemolymph, the darkness and size of the apoLpIII band was calculated using NIH Image software (ImageJ 1.38x). The darkness of this band was compared to the average darkness of two prominent unidentified bands (see Fig. 1, bands 1 and 2; ~65 kDa and 75 kDa) that did not differ in intensity depending on treatment (i.e. flying, immune challenge, AKH injection or pre-loading with trehalose; Kruskal–Wallis=2.6, P=0.45). The ratio of the darkness of the apoLpIII band was divided by the average darkness of bands 1 and 2 (see Fig. 1) in each gel and this ratio (band darkness ratio) was used to compare the relative amount of apoLpIII across samples. The amount of free apoLpIII in the hemolymph was quantified by comparing the darkness of bands of dilutions of known amounts of purified apoLpIII (see below) with the apoLpIII bands in the hemolymph. ApoLpIII standards were run on the same gel as the hemolymph samples.

Assessment of disease resistance

We estimated disease resistance by measuring the ability of crickets to withstand a challenge from the bacterium *Serratia marcescens*. Host resistance tests are the only way to determine whether a change in immune function has produced a change in disease resistance (Adamo, 2004a). Although individual immune assays can provide complimentary information, such assays are difficult to interpret without first establishing whether there is a biologically significant change in immune function (i.e. disease resistance). *S. marcescens* is a Gram-negative bacterium found world-wide in both soil and water and has been recovered from the bodies of crickets in the field (Steinhaus, 1959). The bacterium is not lethal unless it enters the hemocoel (Steinhaus, 1959). We gave an LD_{50} dose of *S. marcescens* by injecting crickets with approx. 1×10^4 cells per 2 μl in culture medium. Initially we determined bacterial cell number using a Petroff-Hauser cytometer and phase-contrast microscopy. Later bacterial cell number was estimated spectrophotometrically. We obtained bacteria from Carolina Biological Supply Co. (Burlington, North Carolina, USA). Injected crickets were housed individually in opaque containers (diameter 10 cm) with food and water *ad libitum*. We recorded mortality daily for the next 4 days after the bacterial injection, because mortality

due to *S. marcescens* occurs within 4 days of injection (Adamo et al., 2001).

Effect of flight and immune challenge on total lipid content and free ApoLpIII concentration of the hemolymph

To assess the effect of flight on total lipid concentration in the hemolymph, we tethered crickets and allowed them to fly for 5 min. Control crickets remained unhandled during this period. 2 μl of hemolymph was taken from control and flying crickets 15, 60, 90 or 120 min after the 5 min flight. Times were based on those of Woodring et al. (Woodring et al., 2002). Blood was taken only once from each cricket.

To determine the effect of immune challenge on hemolymph lipid concentrations, 2 μl of hemolymph was removed from crickets 90 min after injection of either heat-killed *S. marcescens* (1×10^5 cells) or sterile nutrient broth. Heat killed *S. marcescens* showed no growth when placed on agar plates. Injecting heat-killed *S. marcescens* induces an immune response in *G. texensis* (Adamo, 2004b). The time point of 90 min was chosen based on preliminary results and values in the literature (Mullen et al., 2004).

To determine the effects of flight on free apoLpIII levels, crickets were tethered and flown for 5 min. Crickets were then returned to their individual containers and 2 μl of hemolymph was collected from flown and control crickets 1 h later. The timing was based on the results of the effect of flight on lipid hemolymph levels (Fig. 2). The hemolymph was then run on a modified two-dimensional gel (native and SDS-PAGE) as described above, to determine the relative amount of free apoLpIII compared to controls.

To determine the effect of an immune challenge on the relative concentration of free apoLpIII, crickets were injected as described above. After the injection, hemolymph was removed 90 min later and the relative amount of free apoLpIII was assessed as described above.

Effect of adipokinetic hormone on total lipid, free ApoLpIII concentration and on resistance to *S. marcescens*

To test the effect of adipokinetic hormone (AKH; from *Gryllus bimaculatus*; Bachem, Bubendorf, Switzerland) on the lipid concentration in the hemolymph of *G. texensis*, we injected 20 pmol into crickets and measured the lipid content in 2 μl of hemolymph using the method described above. Control crickets were injected with 2 μl of the vehicle (80% methanol). Hemolymph was collected 90 min after injection. Using the procedures described above, hemolymph was tested for total lipid content and free apoLpIII levels.

To test the effect of AKH on disease resistance, crickets were given an injection of 20 pmol AKH followed by an LD_{50} dose of *S. marcescens*. The AKH solution was passed through a sterile 0.2 μm filter prior to injection. Hamilton syringes were cleaned with disinfectant prior to use.

Effect of pre-loading with trehalose on total lipid, free ApoLpIII concentration and on resistance to *S. marcescens*

Trehalose inhibits the release of lipid from the fat body in locusts (Thompson, 2003). Therefore, we preloaded crickets with trehalose prior to flight to reduce the release of lipid. To test whether trehalose can reduce the increase in lipid and the decrease in apoLpIIII that occurs after flight, crickets were randomly assigned to one of three groups. The first group was injected with 5 μl of trehalose (0.5 g ml^{-1} insect Ringer) just prior to being tethered and flown for 5 min. Insect Ringer was composed of 121 mmol l^{-1} sodium chloride, 4.1 mmol l^{-1} CaCl, 1.37 mmol l^{-1} dibasic potassium phosphate, 198 μmol l^{-1} monobasic potassium phosphate, and 38.6 mmol l^{-1} Tris–HCl, adjusted to pH 7.4. The second group were injected with 5 μl insect Ringer prior to flight. The third group, unhandled controls, were neither injected nor flown. Crickets had 2 μl of hemolymph removed 60 min (for total lipid measurement) or 90 min (for apoLpIII measurement) after they stopped flying. Total lipid and free apoLpIII concentration were assessed as described above. Prior to injection, the solution to be injected was passed through a sterile 0.2 μm filter. Hamilton syringes were cleaned with disinfectant prior to use.

To determine whether trehalose could prevent the decline in disease resistance in flying crickets, crickets were randomly assigned to four groups. The first two groups were injected with either trehalose or Ringer prior to flight, as described above. The second two groups were also injected with either trehalose or Ringer, but these two groups of crickets were not flown. After the 5 min flight, crickets were injected with an LD_{50} dose of *S. marcescens*. Crickets that were not flown were also injected with bacteria.

The effect of pre-loading with ApoLpIII on flight-induced immunosuppression

To determine whether injecting apoLpIII could reverse flight-induced immunosuppression, apoLpIII was isolated from cricket hemolymph using a procedure modified from published methods (Mullen and Goldsworthy, 2003; Halwani and Dunphy, 1999). Hemolymph was collected by making a shallow incision in the pronotal membrane and removing the hemolymph that welled up from the wound (approx. 10 μl). Hemolymph was immediately placed in an ice-cold microcentrifuge tube containing cricket anti-coagulant (0.15 mol l^{-1} NaCl, 10 mmol l^{-1} EDTA, 10 mmol l^{-1} glutathione, and a few crystals of phenylthiocarbamide and protease inhibitor cocktail). The anti-coagulant:hemolymph ratio was approx. 5:1). The hemolymph mixture was heated at 96°C for 5 min and then spun at 10 000 ***g*** for 5 min at 4°C. The supernatant was removed and stored at –20°C. The pellet was washed once and the wash was also collected. Pellets, wash and supernatant were shipped to Guild Biosciences (Charleston, South Carolina, USA) for apoLpIII purification. Briefly, heat treated supernatant was buffer exchanged by gel filtration using a Zeba 10 ml column equilibrated with 160 mmol l^{-1} ammonium acetate pH 6.5. The material was then partially purified by passing it through an Econo-Pac Q ion exchange column equilibrated with 160 mmol l^{-1} ammonium acetate (pH 6.5) using a Waters HPLC system with the separation monitored by absorbance at 280 nm. The flow through was collected and its purity was assessed by SDS-PAGE with silver staining. The flow-through material was concentrated using an Ultra 4 centrifugal concentrator then diluted with 0.4% w/v CHAPS containing 50 mmol l^{-1} Hepes pH 6.5. The material was then fractionated by size exclusion chromatography using a Zorbax GF-250 (4.6×250 mm, 4 μm) column equilibrated with 0.4% w/v CHAPS with 50 mmol l^{-1} Hepes pH 6.5 on a Waters HPLC system. Fractions were assessed by SDS-PAGE with silver staining (Swain and Ross, 1995). Protein concentration was determined using the Bradford assay with bovine albumin as the standard (Bradford, 1976).

Crickets were randomly assigned into four groups. The first group was injected with 15 μl apoLpIII (5 μg apoLpIII μl^{-1} insect Ringer). We used this concentration because it was above the amount required by Dettloff et al. (Dettloff et al., 2001a) to increase immune function. Control injected crickets (group 2) were injected

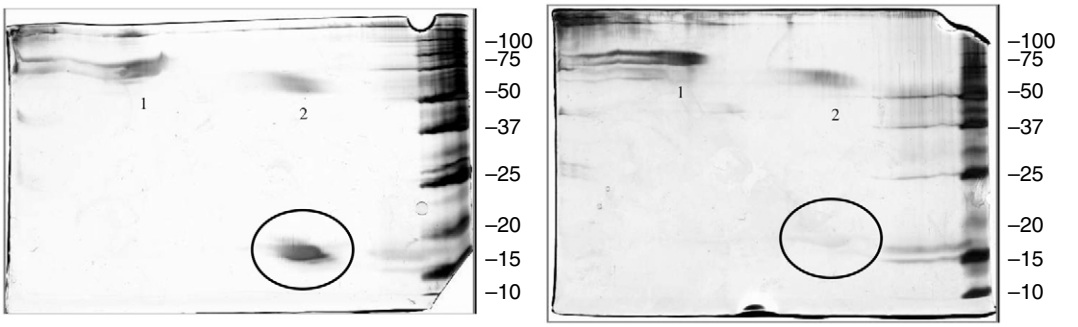

Fig. 1. SDS-PAGE gel (see Materials and methods) of cricket hemolymph from control and flying crickets. Molecular mass markers (kDa) are shown on the right. The circles denote the location of apoLpIII. The numbers 1 and 2 on each gel denote the bands that were used to determine the relative changes in the density of the apoLpIII band.

with 5 μl insect Ringer. Group 3 was injected with 2 μl of heat-killed *S. marcescens* prior to flight. The concentration injected is sufficient to induce an immune reaction in *G. texensis* (Adamo, 2004b). Crickets were then flown for 5 min. Unhandled controls (group 4) were neither injected nor flown. After the 5 min flight, crickets in all groups were injected with an LD50 dose of *S. marcescens*. Control crickets were injected at the same time, even though they were not flown.

Measurement of hemolymph volume

Hemolymph volume was measured using a modified version of the procedure of Ehler et al. (Ehler et al., 1986). Radioactive [^{14}C]inulin (Perkin Elmer, Waltham, MA, USA) was diluted in insect Ringer resulting in 56 000 d.p.m. per 5 μl. Crickets were given a 5 μl injection into the abdomen using a 10 μl Hamilton syringe. Injections were given between the third and fourth last abdominal tergites, 2 mm above the abdominal spiracles. 1 h after the injection (Ehler et al., 1986), 2 μl of hemolymph was collected through the pronotal membrane using a 10 μl Hamilton syringe. The hemolymph was added directly to 5 ml of scintillation fluid and gently vortexed for 5 s. Radioactivity was measured using a Winspectral 1414 scintillation counter (Perkin Elmer). Values were compared to both internal and external calibration curves. To measure the effect of 5 min of flight on hemolymph volume, crickets were injected with inulin, and after a 5 min rest period were flown for 5 min. Hemolymph was collected 50 min after the end of the flight. Control crickets remained in their containers for the entire 60 min period after their inulin injection. All crickets were weighed 2 h prior to the inulin injection. Crickets were matched by weight and assigned to either the flying or control group.

Statistics

Data were analyzed using Prism4 (Graphpad Software Inc.) software. When multiple comparisons were performed on the same data set, the alpha criterion was adjusted accordingly (Sokal and Rohlf, 1981). Non-parametric analyses were carried out according to Meddis (Meddis, 1984). Unless noted otherwise, values in the text are means ± standard deviation (s.d.).

RESULTS

On SDS-polyacrylamide gels ApoLpIII was visible as a dark band of approximately 17 kDa (Fig. 1), similar to that found for the crickets *Acheta domesticus* (Smith et al., 1994) and *Gryllus bimaculatus* (Tanaka et al., 1999). The apoLpIII concentration in the hemolymph of *Gryllus texensis* was 1.8±0.6 mg ml^{-1} (*N*=8).

Flight increased the amount of total lipid in cricket hemolymph 60 min later (ANOVA, $F_{(4,54)}$=4.0, *P*=0.007, Bonferroni *post-hoc* test, *P*<0.05; Fig. 2). Injection of heat-killed *S. marcescens* also resulted in an increase in hemolymph lipid concentration (7.8±2.4 mg ml^{-1} hemolymph, *N*=13) relative to that in nutrient broth-injected animals (6.7±2.7 mg ml^{-1}, *N*=14), 90 min after injection ($t_{(25)}$=2.2, *P*=0.03). Both flight (Mann–Whitney, *U*=16.0, *P*=0.001, *N*=12/group, 46% decrease from controls) and an immune challenge (Mann–Whitney, *U*=21.5, *P*=0.03, *N*=10/group, 63% decrease from controls) resulted in a decline in the amount of free apoLpIII in the hemolymph compared to controls. The increase in lipid during an immune challenge is less than that observed during flight ($t_{(22)}$=2.6, *P*=0.02). However, the reduction in free apoLpIII levels is greater during an immune challenge than it is during flight (Mann–Whitney, *U*=32.5, *P*=0.075).

Injecting AKH into *G. texensis* induced an increase in hemolymph lipid concentration (Fig. 3A, $t_{(7)}$=2.8, *P*=0.03) and a decrease in apoLpIII concentration relative to controls (Fig. 3B; Mann–Whitney, *U*<0.0001, *P*=0.03, *N*=4). AKH also produced a decline in resistance to the bacterium *Serratia marcescens* relative to vehicle-injected controls (Fig. 3C; Fisher's exact test, *P*=0.04).

Trehalose suppressed the lipid increase usually observed after flight (Fig. 4A; ANOVA, $F_{(2,33)}$=4.6, *P*=0.02, *post-hoc* Bonferroni test, *P*<0.05). Pre-loading flying crickets with trehalose also reduced the decline in free apoLpIII after flight [Fig. 4B; non-parametric test for trends (Meddis, 1984), *L*=108, *P*=0.001]. Crickets preloaded with trehalose survived a post-flight challenge with *S. marcescens* better than those preloaded with the vehicle [i.e. insect Ringer; Fig. 4C; test for trends (Meddis, 1984) *Z*=2.53, *P*<0.01]. Trehalose injections alone did not increase survival of a bacterial challenge in crickets that were not flown (Fig. 4C),

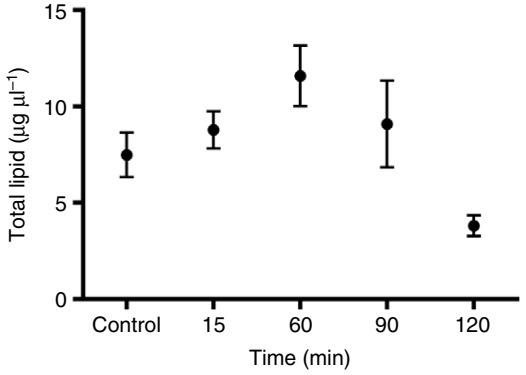

Fig. 2. Total lipid in the hemolymph increases 60 min after a 5 min flight. Values are means ± s.e.m. Control (not flown) *N*=13; 15 min, *N*=11; 60 min, *N*=11; 90 min, *N*=10; 120 min, *N*=10.

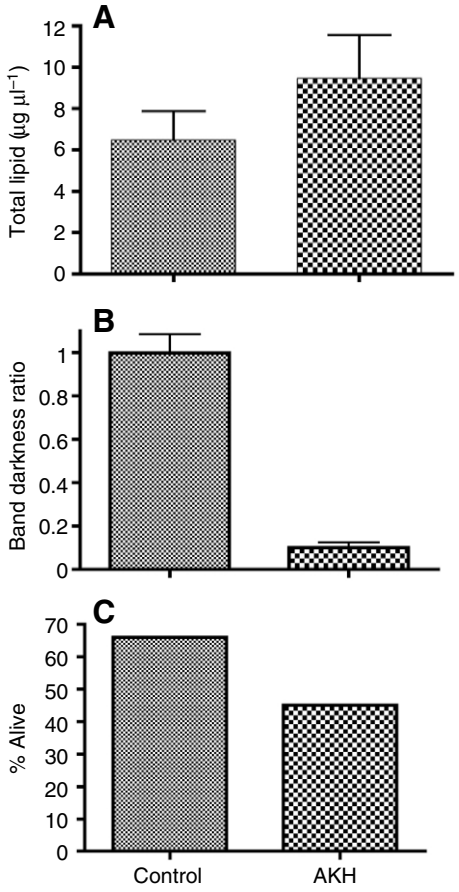

Fig. 3. The effect of adipokinetic hormone (AKH) on: (A) total lipid in hemolymph, control *N*=4, AKH *N*=5; (B) relative amount of apoLpIII, measured as the band darkness ratio (see Materials and methods); control *N*=4, AKH *N*=4; (C) resistance to infection with *S. marcescens*, control *N*=58, AKH *N*=58. Bars in A and B are means ± 95% CI. The differences between AKH and vehicle-injected controls are statistically significant in A, B and C (see text).

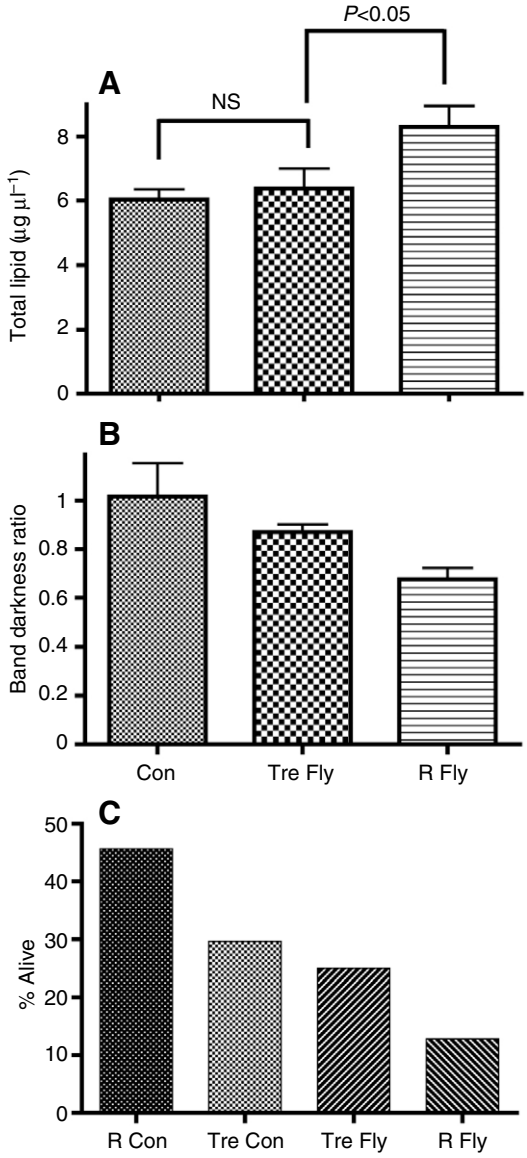

Fig. 4. The effect of preloading flying crickets with trehalose on: (A) total lipid in hemolymph of flying cricket preloaded with trehalose (Tre Fly) and of flying cricket preloaded with insect Ringer (R Fly) *N*=12, control (Con) *N*=10; (B) relative amount of free apoLpIII, measured as the band darkness ratio (see Materials and methods) *N*=4/group; (C) resistance to infection with *S. marcescens*, R Con *N*=35, Tre Con *N*=37, Tre Fly *N*=39, R Fly, *N*=39. Bars are means with 95% CI in A and B. Con, non-flying control; R Con, non-flying control preloaded with insect Ringer; Tre Con, non-flying cricket injected with trehalose. NS, not significantly different. In B and C there is a significant decreasing trend across groups (see text).

demonstrating that trehalose itself does not enhance resistance to *S. marcescens*.

Preloading crickets with apoLpIII prior to flight reduced immunosuppression (Fig. 5). An immune challenge did not enhance survival (Fig. 5).

Flying for 5 min had no significant effect on cricket hemolymph volume ($t_{(15)}$=0.342, P=0.74, 95% CI of the difference –16 to 23 μl g^{-1} wet mass). Flying crickets (N=8) had an average hemolymph volume of 162±12.8 μl g^{-1} wet mass whereas control (unflown) crickets (N=9) had an average hemolymph volume of 156±16.5 μl g^{-1} wet mass. This corroborates an earlier study on locusts that also found no effect of flight on blood volume (Beenakkers, 1973). Therefore, changes in lipid and free apoLpIII concentration observed after flight were not due to changes in blood volume.

DISCUSSION

The amount of free apoLpIII in the hemolymph declined after either flying or an immune challenge, demonstrating that there is the potential for conflict between these two functions. After flying, resistance to bacterial infection declined (Fig. 4C). When free apoLpIII levels in the hemolymph were lowered using the hormone AKH, resistance to bacterial infection also declined (Fig. 3C). Blocking the reduction in apoLpIII hemolymph concentration after flight by pre-loading crickets with trehalose (Fig. 4C), or by injecting crickets with additional apoLpIII (Fig. 5), prevented immunosuppression. These results support the hypothesis that shifting the function of apoLpIII from immune surveillance to lipid transport results in temporary immunosuppression.

After delivering lipid, LDLp is thought to release apoLpIII back into the hemolymph (Weers and Ryan, 2006) suggesting that lipid transport need not have a large impact on apoLpIII concentrations. Nevertheless, others have also found that AKH and/or an immune challenge result in a measurable decline in free apoLpIII

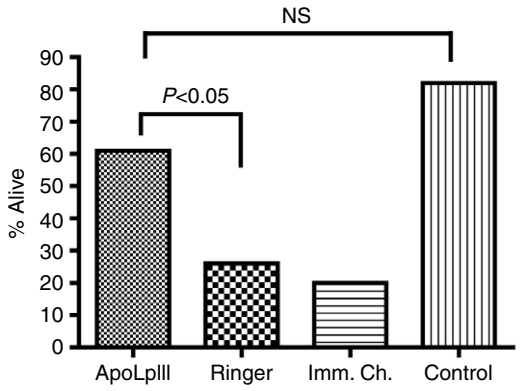

Fig. 5. Effect of preloading apoLpIII on flight-induced immunosuppression. ApoLpIII, crickets pre-loaded with apoLpIII (N=23); Ringer, crickets preloaded with insect Ringer (N=23); Imm. Ch., crickets injected with an immune challenge of heat killed *S. marcescens* (N=19); Control, crickets given the LD_{50} challenge, but not flown or injected (N=23). Mortality was significantly less in the apoLpIII group compared with Ringer-injected crickets (G=5.8, P<0.05) and was not significantly different from controls (G=2.45, P>0.1). n.s., not significantly different.

concentration (Mullen and Goldsworthy, 2003; Mullen et al., 2004). Up to 16 molecules of apoLpIII can associate with one particle of HDLp to form LDLp (Weers and Ryan, 2006). Therefore, even a small increase in LDLp could reduce apoLpIII concentrations.

The increase in disease resistance that we observed after an injection of apoLpIII supports earlier work that showed that injected apoLpIII increases antibacterial activity in the hemolymph of other insects (Lepidoptera) (e.g. Wiesner et al., 1997; Halwani and Dunphy, 1999; Kim et al., 2004). Wiesner et al. (Wiesner et al., 1997) found that the greater the amount of apoLpIII injected, the greater the increase in antibacterial activity in the hemolymph. This result supports our hypothesis that the amount of apoLpIII in the hemolymph determines, in part, the ability of the immune system to respond to bacterial challenges. Our apoLpIII results were not due to non-specific immune activation. Activating the immune system by injecting heat-killed *S. marcescens* (Adamo, 2004b) did not have a protective effect (Fig. 5). We do not know the fate of apoLpIII after we injected it. It is possible that it formed LDLp, and it was the LDLp that was responsible for the immune-enhancing effect (Dettloff et al., 2001a; Dettloff et al., 2001b). However, Halwani and Dunphy (Halwani and Dunphy, 1999) argue that apoLpIII remains in its native state when injected. Moreover, injections of LDLp do not enhance antibacterial activity in the hemolymph (Dettloff et al., 2001b), suggesting that it would not compensate for the loss of bacterial resistance induced by flight. Furthermore, flight results in the generation of LDLp (Chapman, 1998), and, therefore, if LDLp levels were critical for bacterial resistance, flight would not be expected to be immunosuppressive.

Paradoxically, AKH injections result in enhanced phenoloxidase (PO) activity in response to an immune challenge in locusts (Goldsworthy et al., 2003), despite the fact that it also reduces apoLpIII levels (Mullen and Goldsworthy, 2003) and decreases disease resistance (Goldsworthy et al., 2005). These results suggest that the immune-enhancing effects of AKH are smaller in magnitude than the immune-suppressing effects of AKH-induced apoLpIII reduction. We speculate that the function of the enhancing effects of AKH on phenoloxidase activity may be to help maintain adequate immune function despite the reduction in apoLpIII levels.

Trehalose not only inhibits the release of lipid, it also delays the release of AKH (Thompson, 2003). Therefore, it is possible that the effect of preloading flying crickets with trehalose was caused by reducing the effects of AKH on immunity and not by suppressing the decline in free apoLpIII. For example, AKH can reduce protein synthesis (Kodrík and Goldsworthy, 1995) and this may have immunosuppressive effects. However, if AKH were directly responsible for flight-induced immunosuppression, then injections of apoLpIII should not have reversed the effect of flight.

If our hypothesis is correct, an immune challenge occurring prior to flight should reduce an orthopteran's flying ability because of a reduction in lipid transport capacity. This would not be an energetic constraint *per se*. The animal may have substantial fat stores, but still run into an 'energy' shortage because of an inability to mobilize those stores due to a lack of apoLpIII. As predicted, immune challenged locusts show a decrease in flight ability (i.e. reduced flight time) that can be reversed by injecting trehalose (Seyoum et al., 2002). Moreover, locusts infected with fungus appear to have a decreased ability to raise their lipid levels in response to AKH (given as an extract of the corpora cardiaca) than do controls (Seyoum et al., 2002). This result is consistent with the hypothesis that as apoLpIII binds with fungal compounds, less is available to form LDLp. Therefore, less lipid would be able to enter the hemolymph during flight or in response to AKH. Other explanations are possible however, including a manipulative effect of the fungus (Seyoum et al., 2002).

ApoLpIII is only one of many pattern recognition molecules that exist in insects (Kanost et al., 2004). Why the decline in this particular molecule leads to such a strong deficit in disease resistance is unclear. Part of this confusion is due to our lack of understanding of the precise role apoLpIII plays in immune function. In fact, the roles of apoLpIII, LDLp and HDLp in immune function are still being assessed in insects (Zakarian et al., 2002; Mullen and Goldsworthy, 2003; Whitten et al., 2004; Park et al., 2005; Leon et al., 2006; Ma et al., 2006; Rahman et al., 2006). It is generally agreed that the immune system is activated when apoLpIII changes in configuration (e.g. Leon et al., 2006). However, it remains unclear how the immune system differentiates between the conformational changes that occur when apoLpIII is bound to lipid as part of LDLp and when apoLpIII is bound to LPS (Leon et al., 2006). It seems unlikely that the immune system is activated every time lipid is mobilized. Immune activation entails serious costs, including immunopathology (i.e. self-destruction) if inappropriately deployed (Sadd and Siva-Jothy, 2006). Leon et al. (Leon et al., 2006) speculate that there may be different protein conformations depending on what the protein is bound to. This issue deserves serious attention from insect immunologists.

Part of the difficulty in determining the immune functions of apoLpIII, HDLp and LDLp, is that they are probably to some extent species specific (Pratt and Weers, 2004). Their roles may vary depending on whether an insect relies on lipid, carbohydrates or other compounds as its major source of energy to fuel flight-or-fight behavior. For example, whether flight-or-fight behavior is immunosuppressive is likely to vary across, or even within, species. For example, AKH does not induce the formation of LDLp in solitary locusts because solitary locusts contain very low amounts of triacylglycerides in their fat body (Chino, 1997). In this case we would predict that injections of AKH will not be immunosuppressive in these animals. Other insects may have different points of conflict between lipid metabolism and immunity.

For example, in some insects, lipophorin transports carotenoids and hydrocarbons as well as lipid (Arrese et al., 2001). Using the same molecule for both transport and immune function is likely to produce physiological constraints, but the exact nature of these constraints may vary across species.

Similarly, which behaviors will produce a decline in apoLpIII and disease resistance is likely to depend on the species. In crickets, we predict that any behavior that results in an increase in circulating octopamine and AKH (and hence lipid) will produce a decline in apoLpIII and disease resistance. In crickets, fighting produces an increase in neurohormonal octopamine, but brief escape runs do not (Adamo et al., 1995). The fact that fighting reduces disease resistance (Adamo and Parsons, 2006), but brief escape runs do not (S.A.A., unpublished observations), supports our hypothesis. Furthermore, the band darkness ratio did not differ between unhandled controls during the trehalose trial and the vehicle-injected controls in the AKH trial ($t_{(5)}$=0.66, P=0.47). These results suggest that injection stress does not reduce apoLpIII concentrations. In crickets, only prolonged, physically intense behaviors are likely to induce a reduction in apoLpIII and disease resistance.

Immune challenge has been shown to induce a lipid increase and LDLp formation in other insects (e.g. locusts), although how the additional lipid is released is still unknown (Mullen et al., 2004). The increase in lipid may be needed to fuel the immune response (Dettloff et al., 2001b). In insects there is evidence that immune activation is costly (Siva-Jothy et al., 2005). However, in crickets, the increase in hemolymph lipid that occurs during an immune challenge appears insufficient to fully explain the decline in free apoLpIII levels. For example, the increase in hemolymph lipid is higher during flight than during an immune challenge, but the decrease in free apoLpIII is larger during an immune challenge. We suspect that the pronounced decline in apoLpIII levels during an immune challenge was produced not only by increased LDLp formation, but also by the binding of apoLpIII to bacterial components (Pratt and Weers, 2004). This issue requires further study.

These results demonstrate how different physiological systems can 'borrow' molecules from each other to serve intermittent needs. This perspective could help explain phenomena in a wide range of animals. For example, in vertebrates, lipoproteins [e.g. high density lipoprotein (HDL)] are important for both lipid (cholesterol) transport and for sequestering bacterial lipopolysaccharides (LPS) (Berbée et al., 2005; Wendel et al., 2007). During a bacterial challenge, the lipoprotein composition of HDL changes and reverse cholesterol transport declines (Wendel et al., 2007). At present, it is not known why the composition of HDL changes during a bacterial challenge. If the principle in this study applies to this question, then the changes in HDL lipoprotein composition may occur because they help shift the function of HDL from transporting cholesterol to participating in an immune reaction. The inability of HDL to simultaneously transport cholesterol and bind to LPS may explain, in part, why bacterial infection can accelerate atherosclerosis.

These results also demonstrate how multifunctional molecules can produce unsuspected trade offs. For example, the competition between lipid transport and immune surveillance for lipoproteins will result in trade offs between flight-and-fight behaviors and disease resistance in many insects. Long distance flight, male–male competition, and courtship (e.g. singing in crickets) may all exact a cost in terms of lowered disease resistance even in animals with substantial energy reserves.

LIST OF ABBREVIATIONS

AKH	adipokinetic hormone
apoLpIII	apolipophorin III
HDL	high density lipoprotein
HDLp	high density lipophorin
HPLC	high performance liquid chromatography
LDLp	low density lipophorin
LPS	lipopolysaccharide
PO	phenoloxidase

We thank J. Gallant for assistance in running the inulin samples and G. DiNovo at Guild Biosciences for the purification of the apoLpIII. We also thank K. Munro and N. Parsons for technical assistance. This research was supported in part by a grant from NSERC (Natural Sciences and Engineering Research Council of Canada) to S.A.A.

REFERENCES

Adamo, S. A. (2004a). How should behavioural ecologists interpret measurments of immunity? *Anim. Behav.* **68**, 1443-1449.
Adamo, S. A. (2004b). Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *J. Insect Physiol.* **50**, 209-216.
Adamo, S. A. and Parsons, N. M. (2006). The emergency life-history stage and immunity in the cricket, *Gryllus texensis*. *Anim. Behav.* **72**, 235-244.
Adamo, S. A., Linn, C. and Hoy, R. R. (1995). The role of neurohormonal octopamine during 'fight and flight' behavior in the field cricket *Gryllus bimaculatus*. *J. Exp. Biol.* **198**, 1691-1700.
Adamo, S. A., Jensen, M. and Younger, M. (2001). Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. *Anim. Behav.* **62**, 417-425.
Arrese, E. L., Canavoso, L. E., Jouni, Z. E., Pennington, J. E., Tsuchida, K. and Wells, M. A. (2001). Lipid storage and mobilization in insects: current status and future directions. *Insect Biochem. Mol. Biol.* **31**, 7-17.
Auerswald, L. and Gäde, G. (2006). The control of TAG lipase in the fat body of the migratory locust, *Locusta migratoria*. *Insect Biochem. Mol. Biol.* **36**, 759-768.
Barnes, H. and Blackstock, J. (1973). Estimation of lipids in marine animals and tissues: detailed investigation of the sulphophosophovanillin method for 'total' lipids. *J. Exp. Mar. Biol. Ecol.* **12**, 103-118.
Beenakkers, A. M. T. (1973). Influence of lfight on lipid metabolism in *Locusta migratoria*. *Insect Biochem.* **3**, 303-308.
Berbée, J. F. P., Havekes, L. M. and Rensen, P. C. N. (2005). Apolipoproteins modulate the inflammatory response to lipopolysaccharide. *J. Endotoxin Res.* **11**, 97-103.
Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of proteins using the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
Cade, W. H. and Daniel, O. (2000). *Gryllus texensis* n. sp.: a widely studied field cricket (Orthoptera: Gryllidae) from the Southern United States. *Trans. Am. Entomol. Soc. (Philadelphia)* **126**, 117-123.
Chapman, R. F. (1998). *The Insects: Structure and Function*. Cambridge: Cambridge University Press.
Chino, H. (1997). Physiological significance of lipid transport by lipophorin for long-distance flight in insects. *Comp. Biochem. Physiol.* **117B**, 455-461.
Das, S., Meier, O. W. and Woodring, J. (1993). Diel rhythms in adipokinetic hormone, fat body response, and haemolymph lipid and sugar levels in the house cricket. *Physiol. Entomol.* **18**, 233-239.
Dettloff, M., Kaiser, B. and Wiesner, A. (2001a). Localization of injected apolipophorin III in vivo-new insights into the immune activation process directed by this protein. *J. Insect Physiol.* **47**, 789-797.
Dettloff, M., Wittwer, D., Weise, C. and Wiesner, A. (2001b). Lipophorin of lower density is formed during immune responses in the lepidopteran insect *Galleria mellonella*. *Cell Tissue Res.* **306**, 449-458.
Dunphy, G. B. and Halwani, A. E. (1997). Haemolymph proteins of larvae of *Galleria mellonella* detoxify endotoxins of the insect pathogenic bacteria *Xenorhabdus nematophilus* (Enterobacteriaceae). *J. Insect Physiol.* **43**, 1023-1029.
Ehler, W. J., Espig, W. and Hoffmann, K. H. (1986). Blood volume of female adult crickets, *Gryllus bimaculatus* (Orthoptera) – methods and influence of temperature and reproductive activity. *Comp. Biochem. Physiol.* **83A**, 731-734.
Ewenson, E., Zann, R. and Flannery, G. (2003). PHA immune response assay in captive zebra finches is modulated by activity prior to testing. *Anim. Behav.* **66**, 797-800.
Gleeson, M., Nieman, D. C. and Pedersen, B. K. (2004). Exercise, nutrition and immune function. *J. Sports Sci.* **22**, 115-125.
Goldsworthy, G., Mullen, L., Opoku-ware, K. and Chandrakant, S. (2003). Interactions between the endocrine system and immune system in locusts. *Physiol. Entomol.* **28**, 54-61.
Goldsworthy, G. J., Opoku-ware, K. and Mullen, L. M. (2005). Adipokinetic hormone and the immune responses of locusts to infection. *Ann. N. Y. Acad. Sci.* **1040**, 106-113.
Halwani, A. E. and Dunphy, G. B. (1999). Apoplipophorin III in *Galleria mellonella* potentiates hemolymph lytic activity. *Dev. Comp. Immunol.* **23**, 563-570.
Halwani, A. E., Niven, D. F. and Dunphy, G. B. (2000). Apolipophorin III and the interactions of lipoteichoic acids with the immediate immune responses of *Galleria mellonella*. *J. Invertebr. Pathol.* **76**, 233-241.
Kanost, M. R., Jiang, H. and Yu, X. Q. (2004). Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol. Rev.* **198**, 97-105.

Kim, H. J., Je, H. J., Park, S. Y., Lee, I. H., Jin, B. R., Yun, H. K., Yun, C. Y., Han, Y. S., Kang, Y. J. and Seo, S. J. (2004). Immune activation of apolipophorin III and its distribution in hemocyte from *Hyphantria cunea*. *Insect Biochem. Mol. Biol.* **34**, 1011-1023.

Kodrík, D. and Goldsworthy, G. (1995). Inhibition of RNA-synthesis by adipokinetic hormones and brain factor(s) in adult fat-body of *Locusta migratoria*. *J. Insect Physiol.* **41**, 127-133.

Leon, L. J., Idangodage, H., Wan, C. L. and Weers, P. M. M. (2006). Apolipophorin III: Lipopolysaccharide binding requires helix bundle opening. *Biochem. Biophys. Res. Commun.* **348**, 1328-1333.

Ma, G., Hay, D., Li, D., Asgari, S. and Schmidt, O. (2006). Recognition and inactivation of LPS by lipophorin particles. *Dev. Comp. Immunol.* **30**, 619-626.

Meddis, R. (1984). *Statistics Using Ranks: A Unified Approach*. New York: Blackwell.

Mullen, L. and Goldsworthy, G. (2003). Changes in lipophorins are related to the activation of phenoloxidase in the haemolymph of *Locusta migratoria* in response to injection of immunogens. *Insect Biochem. Mol. Biol.* **33**, 661-670.

Mullen, L. M., Lightfoot, M. E. and Goldsworthy, G. J. (2004). Induced hyperlipaemia and immune challenge in locusts. *J. Insect Physiol.* **50**, 409-417.

Murray, A. M. and Cade, W. H. (1995). Differences in age structure among field cricket populations (Orthoptera: Gryllidae): possible influence of a sex-biased parasitoid. *Can. J. Zool.* **73**, 1207-1213.

Park, S. Y., Kim, C. H., Jeong, W. H., Lee, J. H., Seo, S. J., Han, Y. S. and Lee, I. H. (2005). Effects of two hemolymph proteins on humoral defense reactions in the wax moth, *Galleria mellonella*. *Dev. Comp. Immunol.* **29**, 43-51.

Pratt, C. C. and Weers, P. M. M. (2004). Lipopolysaccharide binding of an exchangeable apolipoprotein, apolipophorin III, from *Galeria mellonella*. *Biol. Chem.* **385**, 1113-1119.

Rahman, M. M., Ma, G., Roberts, H. L. S. and Schmidt, O. (2006). Cell-free immune reactions in insects. *J. Insect Physiol.* **52**, 754-762.

Sadd, B. M. and Siva-Jothy, M. T. (2006). Self-harm caused by an insect's innate immunity. *Proc. R. Soc. Lond. B Biol. Sci.* **273**, 2571-2574.

Seyoum, E., Bateman, R. P. and Charnley, A. K. (2002). The effect of *Metarhizium anisopliae* var acridum on haemolymph energy reserves and flight capacity in the desert locust, *Schistocerca gregaria*. *J. Appl. Entomol.* **126**, 119-124.

Siva-Jothy, M. T., Moret, Y. and Rolff, J. (2005). Insect immunity: an evolutionary ecology perspective. *Adv. Insect Physiol.* **32**, 1-48.

Smith, A. F., Owen, L. M., Strobel, L. M., Chen, H. D. R., Kanost, M. R., Hanneman, E. and Wells, M. A. (1994). Exchangeable apolipoproteins of insects share a common structural motif. *J. Lipid Res.* **35**, 1976-1984.

Sokal, R. R. and Rohlf, F. J. (1981). *Biometry*. New York: W. H. Freeman.

Steinhaus, E. A. (1959). *Serratia marcescens* (Bizio) as an insect pathogen. *Hilgardia* **28**, 351-380.

Strobel, L. M., Kanost, M. R., Ziegler, R. and Wells, M. A. (1990). Adipokinetic hormone causes formation of a low density lipophorin in the house cricket *Acheta domesticus*. *Insect Biochem.* **20**, 859-863.

Swain, M. and Ross, N. W. (1995). A silver stain protocol for protein yielding high resolution and transparent background in dodecyl sulfate-polyacrylamide gels. *Electrophoresis* **16**, 948-951.

Tanaka, S., Tanaka, K., Yasuhara, Y., Nakahara, Y. and Katagiri, C. (1999). Flight activity, flight fuels and lipophorins in a cricket, *Gryllus bimaculatus*. *Entomol. Sci.* **2**, 457-465.

Thomas, L. A., David, A. P. B. and Woods, J. A. (2005). Moderate exercise protects mice from death due to influenza virus. *Brain Behav. Immun.* **19**, 377-380.

Thompson, S. N. (2003). Trehalose – the insect 'blood' sugar. *Adv. Insect Physiol.* **31**, 205-285.

Weers, P. M. M. and Ryan, R. O. (2006). Apolipophorin III: role model apolipoprotein. *Insect Biochem. Mol. Biol.* **36**, 231-240.

Wendel, M., Paul, R. and Heller, A. R. (2007). Lipoproteins in inflammation and sepsis. II. Clinical aspects. *Intensive Care Med.* **33**, 25-35.

Whitten, M. M. A., Tew, I. F., Lee, B. L. and Ratcliffe, N. A. (2004). A novel role for an insect apolipoprotein (Apolipophorin III) in β-1,3-glucan pattern recognition and cellular encapsulation reactions. *J. Immunol.* **172**, 2177-2185.

Wiesner, A., Losen, S., Kopácek, P., Weise, C. and Götz, P. (1997). Isolated apolipophorin III from *Galleria mellonella* stimulates the immune reactions of this insect. *J. Insect Physiol.* **43**, 383-391.

Woodring, J., Lorenz, M. W. and Hoffmann, K. H. (2002). Sensitivity of larval and adult crickets (*Gryllus bimaculatus*) to adipokinetic hormone. *Comp. Biochem. Physiol.* **133A**, 637-644.

Zakarian, R. J., Dunphy, G. B., Albert, P. J. and Rau, M. E. (2002). Apolipophorin III affects the activity of the haemocytes of *Galleria mellonella* larvae. *J. Insect Physiol.* **48**, 715-723.

Zera, A. J., Sall, J. and Otto, K. (1999). Biochemical aspects of flight and flightlessness in *Gryllus*: flight fuels, enzyme activities and electrophoretic profiles of flight muscles from flight capable and flightless morphs. *J. Insect Physiol.* **45**, 275-285.