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Changes in apolipophorin-III are related to the activation of phenoloxidase in the haemolymph of *Locusta migratoria* in response to injection of immunogens

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Abstract

In *Locusta migratoria,* activation of phenoloxidase in the haemolymph in response to injection of laminarin is age-dependent: being absent in fifth instar nymphs and newly emerged adults, and only becoming evident four days after the final moult. This pattern of change in phenoloxidase activation correlates with the pattern of change in the concentration of apolipophorin-III (apoLp-III) in the haemolymph. Injection of a conspecific adipokinetic hormone (*Lom*-AKH-I) has no effect on the phenoloxidase response in nymphs or newly emerged adults but, in adults older than four days, co-injection of the hormone with laminarin prolongs the activation of phenoloxidase in the haemolymph: a similar enhancement of the response to laminarin is observed in locusts that have been starved for 48 h but not injected with AKH-I. During most of the fifth stadium, injection of laminarin results in a decrease in the level of prophenoloxidase in the haemolymph; an effect that is not observed in adults of any age. Marked changes in the concentration of apoLp-III, and the formation of LDLp in the haemolymph, are observed after injection of laminarin (or LPS) and these are remarkably similar, at least qualitatively, to those that occur after injection of AKH-I. The involvement of apoLp-III in the activation of locust prophenoloxidase in response to immunogens is discussed.

Keywords: Apolipophorin-III, apoLp-III, development, fifth instar nymphs, laminarin, LDLp, LPS *Locusta*, *Lom*-AKH-I, nodules, phenoloxidase, prophenoloxidase, starvation

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1. Introduction

Insects lack specific immunoglobulins, instead relying upon binding proteins present in the plasma or haemocytes to recognise non-self material (Ratcliffe *et. al*., 1985). This recognition leads to activation of the immune system, involving cellular events such as phagocytosis and nodulation, and humoral events such as activation of a prophenoloxidase cascade in the haemolymph that produces effector molecules such as cytotoxic quinones (Ashida & Brey, 1998). Hormones such as corticosteroids regulate certain aspects of the mammalian immune response, and there is evidence that ecdysteroids, opiate peptides and biogenic amines exert effects on insect immune responses (Gillespie *et al*., 1997). All of this suggests that there may be considerable interaction between the insect endocrine and immune systems.

The lipophorins that carry lipids in the haemolymph are components of the clot that forms in response to wounding, and they also inhibit haemocyte adhesion in *Periplaneta* (Coodin and Caveney, 1992) and *Galleria* (Mandato *et al*., 1996). A further component of the lipid transport system, the exchangeable apolipophorin III (apoLp-III) has immune-stimulating ability in *Galleria* (Weisner *et al*., 1997; Halwani *et al*., 1999; Dettloff *et al*., 2001a). Two bacterial lipopolysaccharide (LPS) binding proteins were isolated from *Galleria* haemolymph, one of which may correspond to apoLp-III, based on its molecular weight (Dunphy & Halwani, 1997). The lipid transport system in locusts involves exchangeable apolipophorins, which associate with the lipophorins (see Goldsworthy, 1983; Wheeler & Goldsworthy, 1983ab; Goldsworthy *et al*., 1985) under endocrine control from adipokinetic hormones

(AKHs) released from the corpora cardiaca (see Goldsworthy *et al.,* 1972a). AKH causes mobilisation of lipid from the fat body by stimulating the conversion of triacylglcerols to diacylglycerols. Thus, if components of lipid metabolism are intimately involved in certain immune reactions, it seems likely that an investigation of the interactions between those endocrine mechanisms controlling lipid metabolism and the immune system could be appropriate.

Goldsworthy *et al*. (2002) demonstrated in the African migratory locust, *Locusta migratoria,* that co-injection of AKH with laminarin, (mainly a β,1-3 glucan similar to that found in fungal cell walls) or preparations of LPS from a number of gram-negative bacteria prolongs the activation of prophenoloxidase in the haemolymph *in vivo* in a dose-dependent manner. The effectiveness of AKH in bringing about a lipid mobilisation response is age-dependent in *Locusta,* with markedly poorer lipid mobilisation in response to AKH in fifth instars and newly emerged adults (Mwangi and Goldsworthy, 1977a). This may be due in part to the lower concentration of apoLp-III present in the haemolymph of fifth instar nymphs and young adults (Mwangi & Goldsworthy, 1977b; De Winther *et al*., 1996). The aim of this study was therefore to investigate if there are age-related changes in the activation of the prophenoloxidase cascade brought about by injection of immunogens, and to test whether these correlate with changes in the lipophorins and apolipophorins in the haemolymph.

2. Materials and Methods

2.1 Insects

A laboratory colony of *Locusta migratoria migratorioides* (R. & F.) was reared under crowded conditions at 30°C in a LD 12:12h photocycle, and fed daily with fresh grass and wheat seedlings supplemented with bran. Under these conditions the fifth stadium lasts 8 days. Male and female insects were separated after adult emergence. Male and female fifth instars, and male adults were used in this study. For starvation experiments, locusts were deprived of food for 48h prior to experiments and given access to water *ad libitum*.

2.2 Chemicals

Laminarin was purchased from Sigma Chemical Co. and 5mg/ml stock solutions were dissolved in insect saline (7.5g NaCl, 0.375g KCl /L). Lipopolysaccharide from *Pseudomonas aeroginosa* (phenolic extraction) was purchased from Sigma Chemical Co. (catalogue no. L-9143) and dissolved in insect saline at a concentration of 4 mg/ml. *Lom*-AKH-I was purchased from Novabiochem and stock solutions were made up in 80% methanol at a concentration of 20 pmol/ul. This stock solution gave a single peak on a reversed phase C_{18} HPLC column with the expected retention time of *Lom*-AKH-I, and was quantified by measuring the tryptophan fluorescence in an LS50B Fluorimeter (Ex 280 nm, Em 348 nm), and calibrating against a standard solution of tryptophan. Dopamine was purchased from Sigma Chemical Co. and was dissolved in 10mM phosphate buffer, pH 5.9 (3mg/ml) just before use.

2.3 Injections and samples of haemolymph

Injections of test materials were performed using plastic pipette tips with a stainless steel needle held in the bore by friction. Using these, 10µl volumes of laminarin or LPS, with or without AKH-I, were taken up accurately and injected into the haemocoel by inserting the needle between two abdominal terga and expelling the sample using an automatic pipettor. The abdomen was palpated gently after injection to mix the contents of the haemocoel. Samples of haemolymph were taken from fifth instar and adult locusts without cooling or anaesthesia, from a small puncture in the arthrodial membrane at the base of a hind leg. A calibrated capillary tube was used to take up 5µl of haemolymph immediately prior to injection, and either 90 min or 3 h after injection.

2.4 Measurement of phenoloxidase and prophenoloxidase activity

Phenoloxidase activity was measured by blowing 5µl of fresh haemolymph immediately into 95µl of 10mM sodium phosphate buffer, pH 5.9. After centrifugation (10,000 x g, at 4°C for 5 min), 40µl of this haemolymph/buffer supernatant were pipetted into a well of a microtitre plate. Phenoloxidase activity was assessed by determining the initial linear increase in absorbance at 492 nm over 40 min after addition of 160 µl of dopamine (3 mg/ml sodium phosphate buffer). Absorbances were read in a Labsystems Multiskan Bichromatic plate reader. Goldsworthy *et al*. (2002) demonstrated that the protein levels in the buffer/haemolymph supernatant remained unchanged after injection of laminarin whether or not locusts were co-injected with AKH-I, and so enzyme activity is expressed here in absorbance units (au) at 492 nm per minute per microlitre of

haemolymph. Prophenoloxidase activity was determined after activation *in vitro* by treatment with methanol: 40µl of the haemolymph/buffer supernatant was mixed with 40µl of absolute methanol and 10µl of the resulting solution was mixed with 190µl of dopamine in a microtitre plate well and enzyme activity recorded as before.

2.5 Isolation of apolipophorin-III (apoLp-III)

ApoLp-III was purified using a combination of the methods of Van der Horst *et al.* (1991) and Weisner *et al.* (1997). Haemolymph was taken from virgin female locusts > 15 days old and was immediately pooled in liquid nitrogen to prevent coagulation and melanisation. The frozen pellets of haemolymph were then transferred to microcentrifuge tubes and heated immediately at 96 °C for 20 min, after which centrifugation at 10,000 x g for 5 min yielded a clear yellow supernatant containing predominantly apoLp-III. The pellet was agitated and centrifuged a second and third time to maximise the yield of apoLp-III. The pooled supernatant was applied to a PD-10 (Pharmacia Biotech) desalting column, and the proteins eluted according to the manufacturer's instructions in 3.5 ml of deionised water and then centrifuged under vacuum to dryness overnight. The dried material was dissolved in 500µl of 20mM ammonium acetate and applied to a DEAE Sepharose column, equilibrated in 20mM ammonium acetate (pH 6.5), and eluted with 120mM ammonium acetate (pH 6.5) at a flow rate of 1 ml/min. Fractions containing apoLp-III were identified by native PAGE (see below), pooled and lyophilised.

2.6 Electrophoresis of haemolymph proteins

Vertical polyacrylamide gel electrophoresis (PAGE) was carried out on a LKB Midget Electrophoresis unit, according to the manufacturer's instructions. Native

PAGE was performed on 5% total solids content (%T), and 2% ratio of cross-linker to acrylamide monomer (%C) acrylamide gels, with a 3% T, 2% C stacking gel, using a continuous Tris-Glycine buffer system, pH 9.5, 500 V, 15mA at 10°C (Wheeler & Goldsworthy, 1983a). Protein bands were stained with Coomassie Blue R-250.

2.7 Isolation of high density lipophorin (HDLp), low density lipophorin (LDLp) and apoLp-III by selective precipitation

High and low density lipophorins and ApoLp-III in the haemolymph were selectively precipitated using the method of Goldsworthy et al. (1985). A 5µl sample of fresh haemolymph was blown into 200µl of heparin solution (0.375% heparin sodium salt in 25mM CaCl2, 3.8mM NaCl). After mixing, and centrifugation (13,000 x g, for 4 min) the pellet formed contained HDLp. The decanted supernatant was transferred to a separate tube and 100µl of 5% EDTA were added and mixed. After centrifugation (13,000 x g, for 4 min) of the EDTA/supernatant mixture, the pellet obtained contained LDLp. The decanted supernatant was then mixed with 600µl of acetone to precipitate the apoLp-III. After centrifugation (13,000 x g, for 4 min) the pellet was washed to remove EDTA and acetone (which interfere with the protein determination) before re-centrifugation. The final protein pellet was solubilised in either electrophoresis buffer or 0.5M NaOH.

2.8 Measurement of lipid concentration in HDLp and LDLp pellets

Total lipid (measured as vanillin-positive material) was measured as described previously (Goldsworthy et al., 1972b). Centrifuge pellets (from 5µl of haemolymph) were solubilised in 1 ml of concentrated sulphuric acid and transferred to glass tubes, in which the solution was heated for 10 min at 100°C. After cooling to room

temperature, 200µl of this solution were added to a fresh tube and 1 ml of vanillin reagent (1.98g vanillin, 668ml concentrated orthophosphoric acid, 332 ml water /L) was added, mixed immediately and left for 15 min. Lipid concentration was determined by reading the absorbance of each sample at 540 nm in a Labsystems Multiskan Bichromatic plate reader against a standard solution of cholesterol.

2.9 Measurement of protein concentration

The protein concentration in the acetone-precipitated pellet (apoLp-III) was measured using a modification of the method described by Schacterle and Pollack (1973). Precipitated protein was dissolved in 200µl of 0.5M NaOH, and 200µl of copper reagent (10% $NaHCO_3$, 0.1% $(CHOH.COOK)_2.½H_2O$), 0.05% $CuSO_4$) were added, mixed, and left for 10 min. A volume of 900µl of Folin-Ciocalteu phenol reagent (1M diluted x 9 with distilled water) was added and the resulting mixture heated for 5 min at 55°C. After cooling, sample colour development was determined at 620 nm, using a Labsystems Multiskan Bichromatic plate reader. ApoLp-III, purified as described above using DEAE Sepharose, was used to construct a calibration curve.

2.10 Statistical analysis

Data are expressed as means ± S.E. Pearson's sample correlation coefficients were calculated to examine the relationships throughout the fifth and adult stadia between: concentration of apoLp-III in the haemolymph; lipid mobilisation in response to injection of AKH-I; and the increase in phenoloxidase in the haemolymph in response to injection of laminarin with AKH-I. Data for LDLp and apoLp-III were analysed using paired t-tests or one-way ANOVA as appropriate. Nodule data were subjected to $\sqrt{p}$- transformation prior to analyses using one-way ANOVA. The level of significance was taken as $P \leq 0.05$ and all tests were undertaken using Minitab.

3. Results

3.1 Developmental changes in activation of phenoloxidase in the haemolymph in response to injection of laminarin

Fifth instar nymphs and adult male locusts were injected with 20μg of laminarin or with 20μg of laminarin plus 20 pmol of AKH-I. Figure 1 shows that injection of laminarin alone into adult locusts > 5 days after emergence resulted in activation of haemolymph prophenoloxidase, which remained elevated 3 h after injection. Co-injection of 20 pmol AKH-I resulted in the maintenance of even higher levels of phenoloxidase activity 3 h after injection. This response was not present in fifth instar nymphs and newly emerged adults, nor was there any phenoloxidase activation in such locusts earlier than 3 h after injection (data not shown). A response was present on day 4 of adult life and remained up to day 45 (Fig. 1). Figure 2 illustrates how the pattern of appearance of the phenoloxidase response to laminarin and AKH, correlates significantly ($r = 0.653$, $P = 0.016$) with the lipid mobilisation response to AKH in *Locusta* shown by Mwangi & Goldsworthy (1977a).

3.2 Changes in the concentration of apoLp-III in the haemolymph during development

The concentration of ApoLp-III in the haemolymph was measured throughout the fifth and adult stadia using heparin-EDTA-acetone precipitation. Native PAGE of the proteins in the pellet obtained from this precipitation showed the presence of two bands of apoLp-III, identical to those purified from haemolymph as described previously (data not shown). There was a low concentration (*c*. 2.5mg/ml) of these proteins present in the haemolymph at the beginning of the fifth stadium, but the concentration rose slowly during the course of the stadium (Fig. 2). The concentration

remained relatively low after the moult, but then increased steadily to reach a maximum of *c*. 25mg/ml at day 14, which was maintained for some days but declined slowly as the locusts aged further (Fig. 2). This pattern of change of the concentration of apoLp-III in the haemolymph is correlated with the lipid mobilisation response to AKH ($r = 0.804$, $P < 0.001$), and with the activation of prophenoloxidase in the haemolymph in response to injection of laminarin and AKH-I ($r = 0.643$, $P = 0.007$)

3.3 *Changes in prophenoloxidase in the haemolymph during development and in response to injection of laminarin with and without AKH-I*

Prophenoloxidase activity in the haemolymph was measured, after activation with methanol *in vitro*, alongside that of phenoloxidase prior to, and 3 h after injection. The levels of prophenoloxidase were generally an order of magnitude higher than those of phenoloxidase. There was no significant change in the resting level of prophenoloxidase in the haemolymph throughout the adult stadium, nor did injection of laminarin with or without AKH-I significantly change its activity. Resting levels of prophenoloxidase showed some variability throughout the fifth stadium, with no consistent trend (Fig. 3). Injection of laminarin (with or without AKH-I) in the first two days of the fifth stadium had little effect on prophenoloxidase activity but from day 3 onwards, a decrease in prophenoloxidase was measured after laminarin injection regardless of whether AKH-I was co-injected or not, and by day 6 onwards very little activity remained in the haemolymph after injection with laminarin.

3.4 The effect of starvation on the phenoloxidase activity in the haemolymph in response to injection of laminarin

Figure 4 shows that in adult locusts, starvation for 48h resulted in significantly greater increases in the phenoloxidase activity in the haemolymph ($P<0.001$, one-way ANOVA) in response to injection of laminarin, compared with the response to

injection of laminarin into fed animals. Phenoloxidase activation in starved adults in response to laminarin was of the same magnitude as that measured in response to injection of laminarin and AKH-I in fed adults (Fig. 4). Starvation did not significantly affect the phenoloxidase response to co-injection of laminarin and AKH-I in adults. Starvation had no significant effect on phenoloxidase activation in fifth instar nymphs in response to laminarin with or without co-injection of AKH- I (Fig. 4).

3.5 Electrophoresis of whole haemolymph before and after injection of laminarin or LPS

The purified apoLp-III used as a standard in these experiments comprised two protein bands, and this was consistent with the apoLp-III present in the haemolymph (see also Wheeler & Goldsworthy, 1983a). Native PAGE of whole haemolymph from adult males indicated a decrease in apoLp-III levels after injection with AKH-I as expected but, surprisingly, also after injection with laminarin or LPS (Fig. 5). Increased staining in a slowly-moving band that hardly entered the running gel was also observed after injection of AKH-I, laminarin or LPS. These gels were not stained for lipid, but the changes in this material are consistent with that of LDLp formation (see Wheeler & Goldsworthy, 1983ab). No such changes were observed after injection of saline.

3.6 Direct measurement of the changes in concentration of apoLp-III

The amounts of HDLp, LDLp and apoLp-III in haemolymph from locusts were determined by selective precipitation immediately prior to injection and 90 min after injection of 10μl saline (control), 20 pmol of AKH-I, 100 μg of laminarin or 100μg of

LPS. No changes in apoLp-III occurred after injection of saline. Injection of AKH-I caused a dramatic decrease in the level of apoLp-III in the haemolymph from 14μg/μl to 2.5μg/μl in 18 day-old male locusts (Fig. 6). Adult locusts injected with laminarin or LPS also showed significant decreases ($P = 0.001$, $P < 0.001$ respectively, paired t-tests) in the haemolymph concentration of apoLp-III, but these were less than those seen in those injected with AKH-I. Qualitatively similar changes (but smaller than those observed in adults) in the concentration of apoLp-III were seen in fifth instars in response to injection of AKH-I ($P < 0.001$, paired t-test) and laminarin ($P = 0.001$, paired t-test), but there was no significant change in response to injection of LPS (Fig. 6).

3.7 Changes in the lipid content of lipophorins in the haemolymph after injection of test materials

Precipitation with heparin of lipophorins in whole haemolymph from resting mature male locusts yielded a pellet representing the haemolymph HDLp fraction, and the decanted supernatant produced a second pellet representing the LDLp fraction when EDTA was added. The lipid content in both pellets was measured before and after injection of test materials. Figure 7 shows that in adults, saline injection produced no significant changes in the lipid content of either pellet, whereas injection of AKH-I resulted in a characteristic decrease ($P<0.05$, paired t-test) in the lipid associated with HDLp and a dramatic increase in the lipid associated with LDLp ($P<0.001$, paired t-test). Injections of laminarin ($P<0.001$, paired t-test) or LPS ($P=0.005$, paired t-test) resulted in similar, but less dramatic, changes in the levels of lipid associated with LDLp, but there were no significant changes (paired t-test) in the lipid content of HDLp. Qualitatively similar changes in LDLp were measured in fifth instars in

response to injections of AKH-I (P<0.001, paired t-test), laminarin (P<0.001, paired t-test) or LPS (P=0.031, paired t-test), but the amount of lipid associated with LDLp was always substantially lower than that in adults. The concentration of lipid associated with HDLp in fifth instars decreased significantly (paired t-tests) in response to injection of saline (P<0.001), AKH-I (P<0.001), laminarin (P=0.006), or LPS (P=0.013).

4. Discussion

In adult locusts, AKH-I prolongs the phenoloxidase response *in vivo* to injected laminarin, while injections of LPS on their own fail to activate prophenoloxidase activity unless combined with AKH-I (Goldsworthy *et al.*, 2002). The present study shows that the effects of injected laminarin are enhanced in locusts starved for 48h; starvation can thus substitute at least partly for the effects of injected AKH. Starvation for that period doubles the concentration of lipid in the haemolymph of adults but, although diglycerides are the major constituents of this elevated lipid, there is no evidence that this is as a result of increased titres of AKH in *Locusta* (Cheeseman *et al.*, 1976) or in *Schistocerca* (Candy, 2002).

The pattern of change of the phenoloxidase response to laminarin injection in the haemolymph of adult locusts correlates moderately well with that for lipid mobilisation in response to AKH shown by Mwangi and Goldsworthy (1977a), and with that for changes in the concentration of apoLp-III in the haemolymph. As shown in this study, the levels of apoLp-III in the haemolymph are greater in adults (12 -25 mg/ml in adults over 5 days old) than in nymphs and newly emerged adults (4-7mg/ml). Such changes in the concentration of the apolipophorin (see also De Winther *et al.*, 1996) may, in part, explain the age-related changes in both lipid mobilisation (Mwangi and Goldsworthy, 1977a) and may relate to the phenoloxidase responses to injection of laminarin seen here. Although the strongest correlation exists between the pattern of change in the concentration of apoLp-III and that of lipid mobilisation in response to AKH-I, it is significant that all three parameters increase 2-3 days after adult emergence.

There is no effect of co-injection of AKH-I on the phenoloxidase response to laminarin in fifth instar nymphs or young adults, in the way that Goldsworthy *et al*., (2002) have shown in mature adults and which is also a feature of this study. Although a detailed developmental study during the fifth stadium and onwards comparable with that described here has not been undertaken with LPS, results with that immunogen are broadly similar to those with laminarin seen here, except that injection of LPS never activates prophenoloxidase at any age. Thus, co-injection of AKH with LPS brings about a full phenoloxidase response in mature adults, but not in fifth instars or newly emerged adults (Goldsworthy *et al*., 2003b). The data presented here suggest that the lipid mobilisation that occurs due to injection of AKH-I, or as a result of starvation, may influence the activation of phenoloxidase.

What could be the relationship between levels of haemolymph lipid, apoLp-III and the phenoloxidase response? ApoLp-III is the name given to proteins that are major components of haemolymph protein in many insects. Although named C_L-proteins at the time, two haemolymph proteins were discovered by Mwangi and Goldsworthy (1977b) to play an important role in lipid transport in locusts: changes in their titre in the haemolymph of nymphs and adults were correlated with variations in the responsiveness of the lipid mobilising system to adipokinetic hormones (Mwangi and Goldsworthy, 1977a), and they were shown to be exchangeable apolipophorins (Wheeler and Goldsworthy, 1983ab; Goldsworthy *et al*., 1985). Similar exchangeable apolipophorins are present in a number of Lepidoptera, in which they have been studied extensively (see Narayanaswami and Ryan, 2000).

Molecules of apoLp-III comprise five elongated amphipathic helices connected by short loops in a cylindrical arrangement, with hydrophobic residues pointing inward, and the hydrophilic residues directed outward (see Kanost *et al.,* 1995; Narayanaswami and Ryan, 2000). In the lipid-free state, they behave as globular proteins with a loosely folded structure. However, when apoLp-III is lipid-associated, a major conformational change occurs; unfolding takes place around a putative hinge region (Niere *et al.,* 2001) to expose a large surface area of hydrophobic residues that can interact with lipids. ApoLp-III is thought to increase the capacity of lipophorin to incorporate diacylglycerol by stabilizing the lipid-water interface (Kawooga *et al.,* 1986). Increased lipid transport in the haemolymph during locust flight is triggered by the release of adipokinetic hormones that activate fat body triacylglycerol lipase, converting triacylglycerol to mono- and then diacylglycerol (see Goldsworthy, 1983).

Another function for apoLp-III in insect immunity, in addition to its role in lipid metabolism, has been postulated. Injected lipid-associated apoLp-III stimulates anti-microbial activity in the haemolymph of the wax moth *Galleria mellonella* (Wiesner *et al*., 1997; Dettloff *et al*., 2001a). The fact that only the lipid-associated form of the protein exerts this effect has led to the hypothesis that disturbances of the insect's homeostasis, evidenced by rearrangement of haemolymph lipophorins, could be recognised by a particular population of haemocytes, which would then be stimulated to activate the synthesis of anti-microbial peptides by the fat body (Dettloff *et al*., 2001a). This hypothesis is not entirely consistent with the observation that injection of AKH alone (even with the rearrangement of haemolymph lipophorins and resultant lipid association of apoLp-III) does not increase phenoloxidase activity (Goldsworthy *et al*., 2002).

Crude plasma from fifth instar locust nymphs precipitates two proteins when incubated with laminarin *in vitro* (Duvic & Brehélin, 1998). On the basis of their molecular weights, these proteins could correspond with locust lipophorins. However, no changes in the concentration of haemolymph total protein are observed after injections of laminarin or LPS in adults (Goldsworthy *et al*., 2002), or in nymphs (unpublished observations), which would be the case if lipophorins were precipitating. Furthermore, electrophoresis of haemolymph proteins from adults shows conversion of HDLp to LDLp after injection of laminarin or LPS, rather than any decrease in total lipophorins. The high concentration of two larval-specific storage proteins in haemolymph from nymphs (Ancsin & Wyatt, 1996), makes it difficult to identify the lipophorin bands by PAGE, but the small decreases in apoLp-III after injection of laminarin or LPS can be seen in the electrophoretograms (unpublished observations). Further, the quantitative analysis showis that the laminarin-induced changes in lipophorins and proteins in nymphs are qualitatively similar to those in adults. Furthermore, the changes in the lipid content of LDLp in response to laminarin or LPS injection parallel those observed after injection of AKH-I in both fifth instars and adult locusts. The changes in the lipid content of HDLp in both adults and fifth instars are inconsistent, but it should be noted that they result from two dynamic processes: conversion of HDLp to LDLp and the lipid loading initially of the HDLp and then of LDLp

It is tempting to suggest that these changes in lipophorins are indicative of the release of endogenous AKH-I as part of the locust immune response. However, such a hypothesis is not consistent with the fact that injection of LPS does not activate

prophenoloxidase in the haemolymph of adult locusts unless AKH-I is co-injected (Goldsworthy *et al*., 2002). Endogenous release of AKH-I also seems unlikely as no change in AKH-I titre has been detected in the haemolymph after injection of LPS, using either an enzyme-linked immunosorbant assay (G.J. Goldsworthy and M.E. Lightfoot, unpublished observations) or a radioimmunoassay (D.J. Candy and G.J. Goldsworthy, unpublished observations). The lipid-loading of haemolymph lipophorins in response to injection of laminarin or of LPS into locusts is consistent with the findings of Dettloff *et al*. (2001b) who demonstrated not only the formation of LDLp in *Galleria* larvae in response to injection of heat-killed bacteria, but also LDLp-induced superoxide radical production in isolated haemocytes. Significantly, apoLp-III-deficient HDLp did not enhance this superoxide radical formation (Dettloff *et al.*, 2001b). The precise mechanism for these changes remains to be elucidated.

Lipophorin has been implicated in reducing the toxicity of LPS in both mammals (Feingold *et al*., 1995, Kato *et al*., 1994b) and invertebrates (Kato *et al*., 1994a; Kitchens *et al*., 1999). Injection of LPS does not result in the activation of phenoloxidase in the haemolymph of resting locusts (Goldsworthy *et al*., 2002), when HDLp is the predominant lipophorin, perhaps because the circulating HDLp can accommodate/detoxify LPS thereby preventing prophenoloxidase activation. When LDLp becomes the predominant lipophorin under the influence of injected AKH-I this may no longer happen, possibly by the association of apoLp-III with LDLp preventing LPS from accessing the lipid interior of LDLp. ApoLp-III may also provide protection against LPS toxicity in *Galleria*. Two LPS-binding proteins have been identified in *Galleria* by Dunphy & Halwani (1997), one of which, according to its molecular weight, may correspond to apoLp-III. In *Galleria* larvae, this protein

removed the inhibition of prophenoloxidase activation by LPS. If apoLp-III functions in a similar way in *Locusta*, it is difficult to explain the absence of a phenoloxidase response to LPS injection alone (Goldsworthy *et al*., 2002), especially because the results presented here demonstrate clearly that lipid-loading of lipophorin in the haemolymph occurs in a manner similar to that after injection of laminarin (which does activate prophenoloxidase), with associated decreases in the concentration of free apoLp-III.

The conventional responses shown by insects to immune challenge comprise changes in cell activity: initially, phagocytosis, nodule formation, and activation of the prophenoloxidase cascade; and the eventual synthesis of antimicrobial compounds in the fat body. From this study it is clear that there are other, earlier, metabolic responses in the fat body that cause lipid-loading and rearrangement of the lipophorins in the haemolymph. This conversion of HDLp to LDLp, and the decrease in the concentration of free apoLp-III are the same changes that occur after injection of AKH-I or during starvation. Intriguingly, these latter experimental procedures, while not activating the immune system on their own, enhance the responses to immunogens: presumably by augmenting some of the naturally-occurring metabolic changes that are part of the immune response. The mechanisms by which the formation of LDLp or lipid-associated apoLp-III is stimulated by immunogens, or by which these molecules interact with the immune system remain to be investigated.

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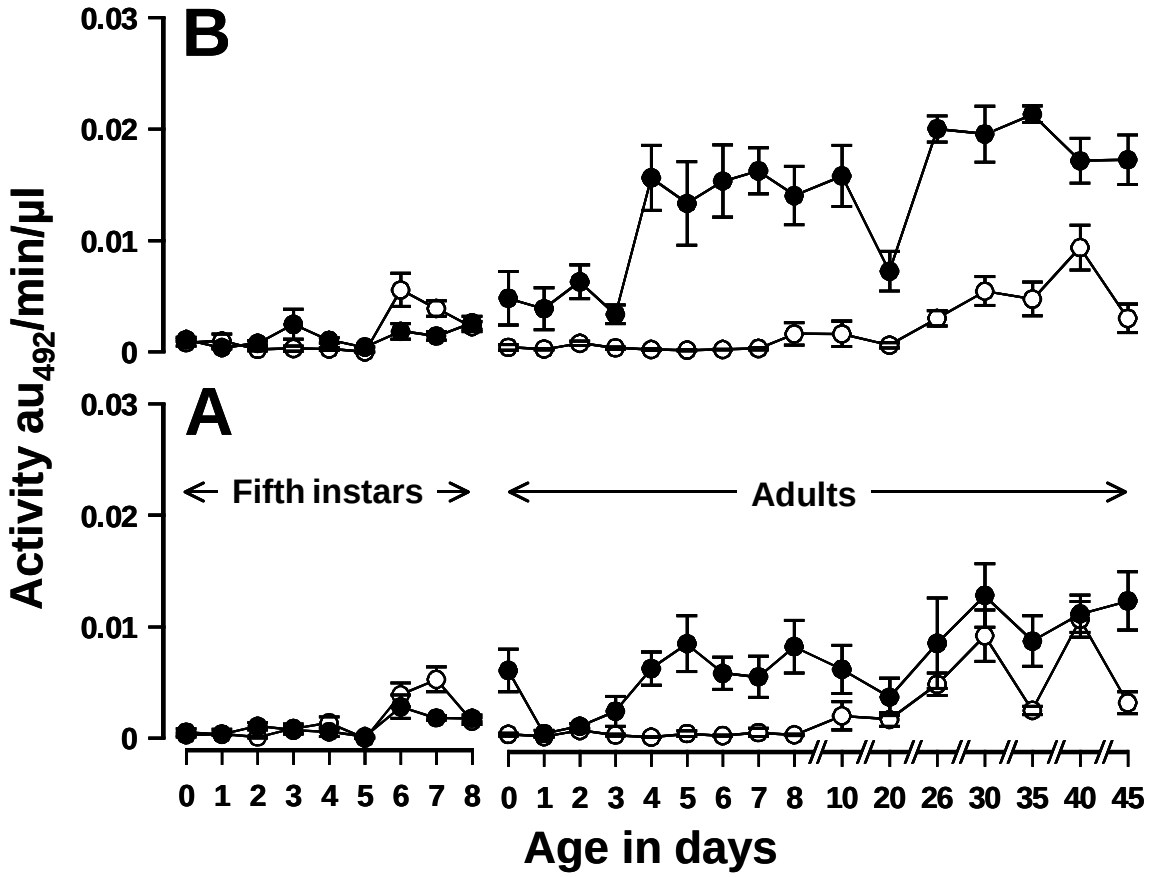

Figure 1 - Phenoloxidase activity in the haemolymph of *Locusta* adults and fifth instar nymphs before (open symbols) and 3 h after injection (solid symbols) of laminarin (A) and laminarin and AKH-I (B). Data points and vertical lines represent means ± S.E. (*n* = 10).

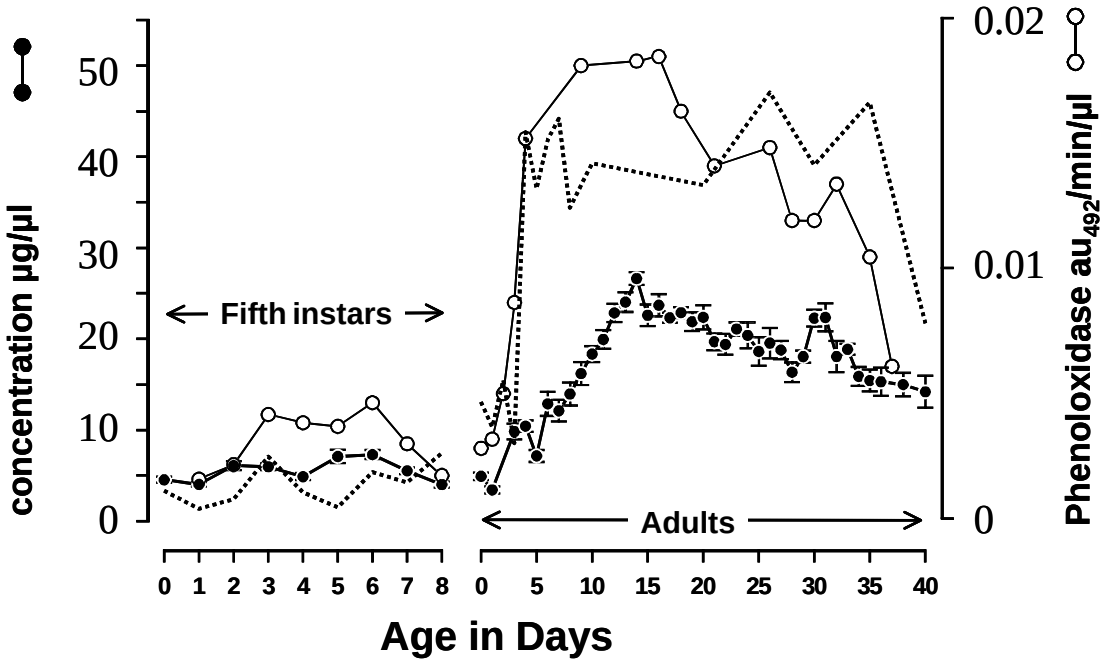

Figure 2 – Comparison of the changes in the concentration of apoLp-III in the haemolymph throughout development from the fifth stadium to day 45 of adult life with the lipid mobilisation response to AKH (open symbols, data redrawn from Mwangi and Goldsworthy, 1977a), and the changes in phenoloxidase activity in the haemolymph (between 0 and 3h) after co-injection with laminarin and AKH (broken line, data redrawn from Fig. 1). Solid symbols and vertical lines represent the means ± S.E. (*n*=10) for apoLp-III concentration in the haemolymph.

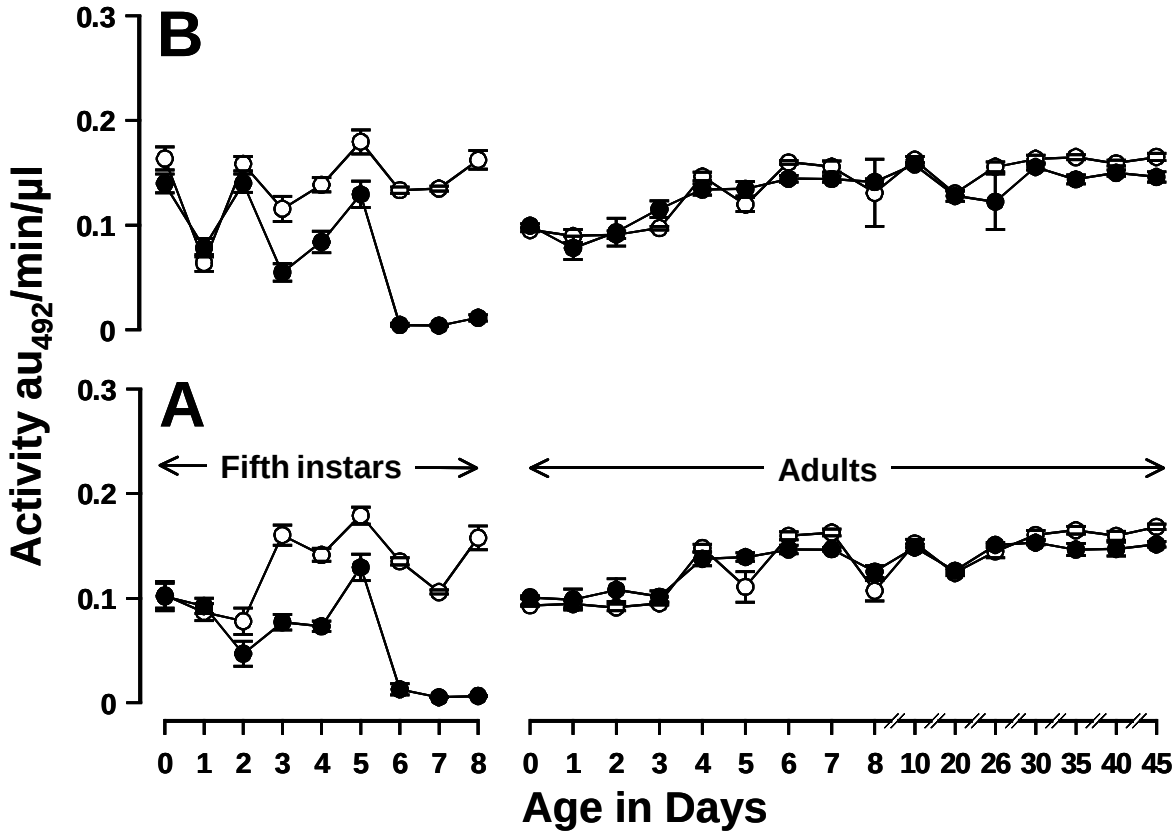

Figure 3 - Prophenoloxidase activity in the haemolymph of *Locusta* adults and fifth instar nymphs before (open symbols) and 3 h after injection (solid symbols) of laminarin (A), and laminarin and AKH-I (B). Data points and vertical lines represent means ± S.E. (*n* = 10)

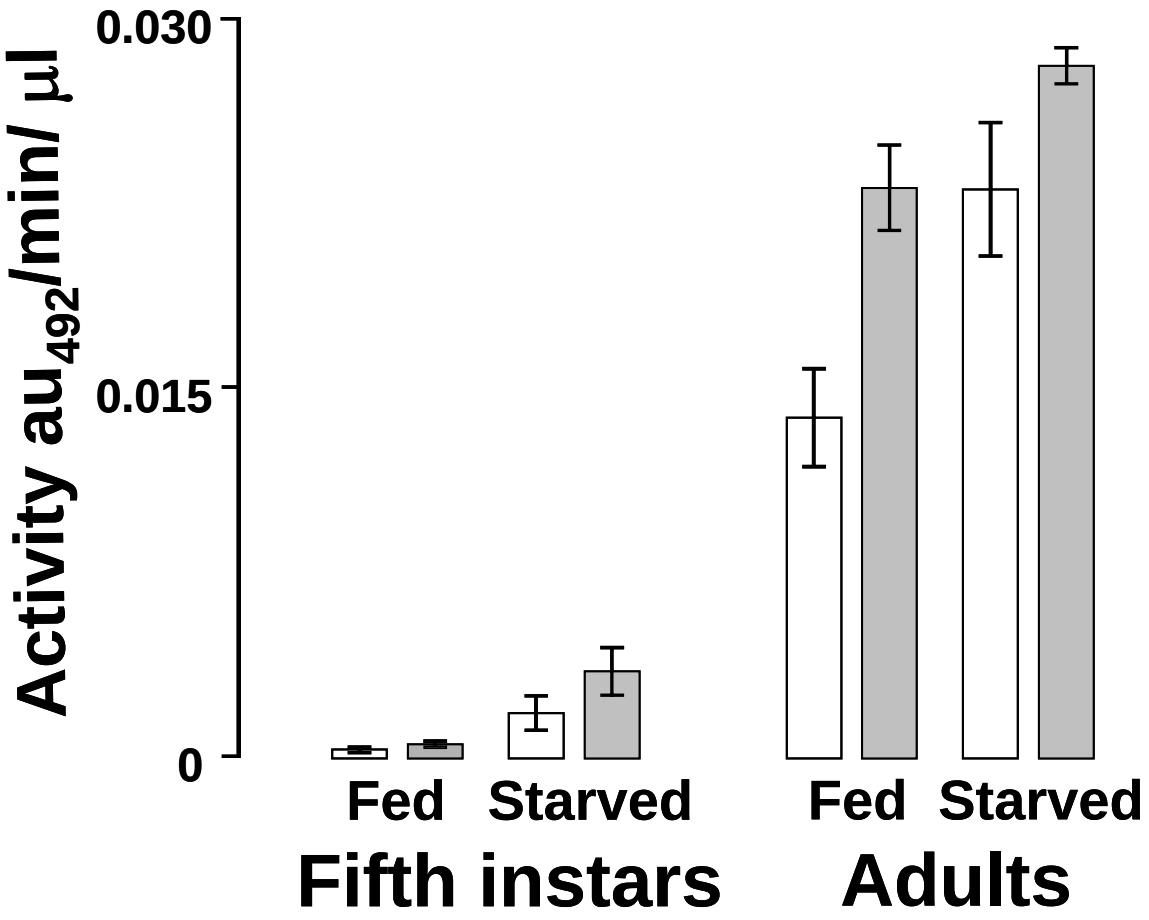

Figure 4 – The effect of starvation for 48h on the activation of phenoloxidase in the haemolymph in response to injection of laminarin (open bars), and laminarin and AKH-I (shaded bars) in fifth instar nymphs and adult locusts (age 15 - 18 days). Data from fifth instars and adults were analysed using a one-way ANOVA. The phenoloxidase response to laminarin in fed locusts is significantly different from all other adult data shown. There is no significant activation of phenoloxidase in the haemolymph of fed or starved fifth instars in response to injection of laminarin or of laminarin and AKH-I. Bars and vertical lines represent the means ± S.E. (*n*=10).

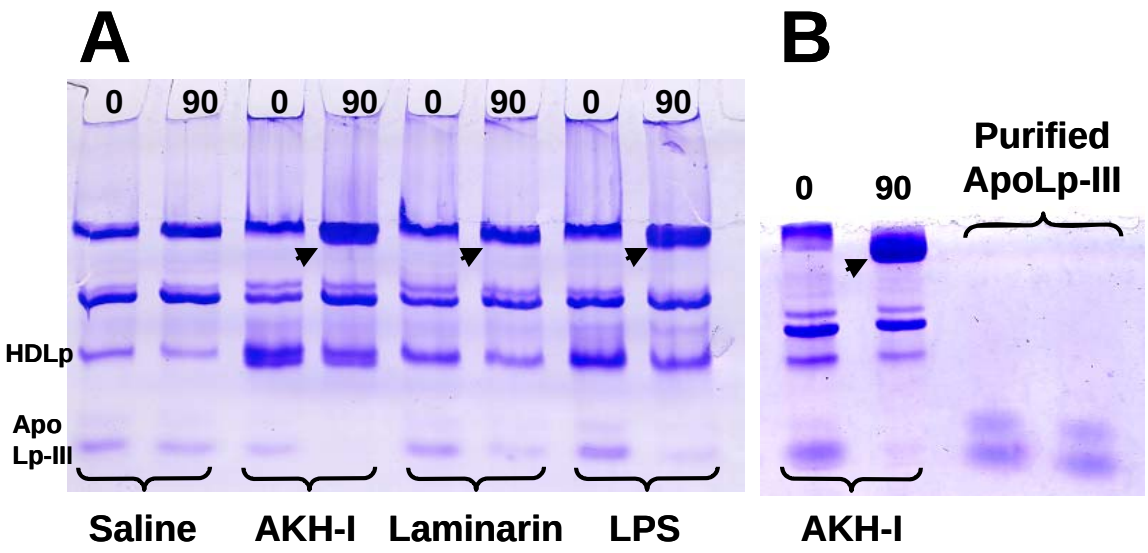

Figure 5 – Gel A - native PAGE of whole haemolymph from adult locusts injected with 10µl saline, 20 pmol AKH-I, 50µg laminarin or 40µg LPS Samples of haemolymph were taken immediately before and 90 min after injection. Gel B – native PAGE of whole haemolymph from adult locusts immediately before and 90 min after injection with 20 pmol AKH-I (first two lanes) and purified apoLp-III isolated from haemolymph (second two lanes). Arrowheads indicate bands of LDLp.

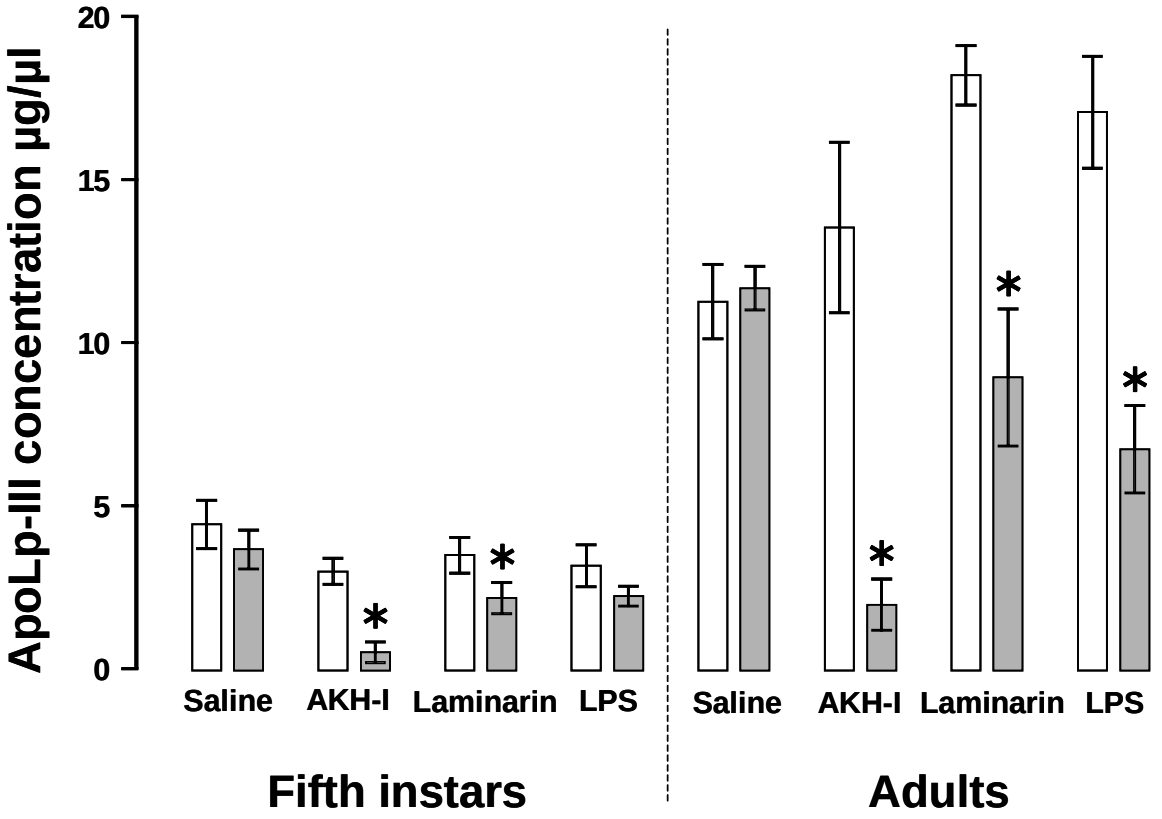

Figure 6 – Changes in the concentration of apoLp-III in the haemolymph before (open bars) and 90 min after (shaded bars) injection of test materials into fifth instar (age 5 days) and adult male locusts (age 18 days). Bars and vertical lines represent means ± S.E. (*n*=10). Asterisks denote statistically significant differences (using paired t-tests) between 0 and 90 min values (see text for *P* values)

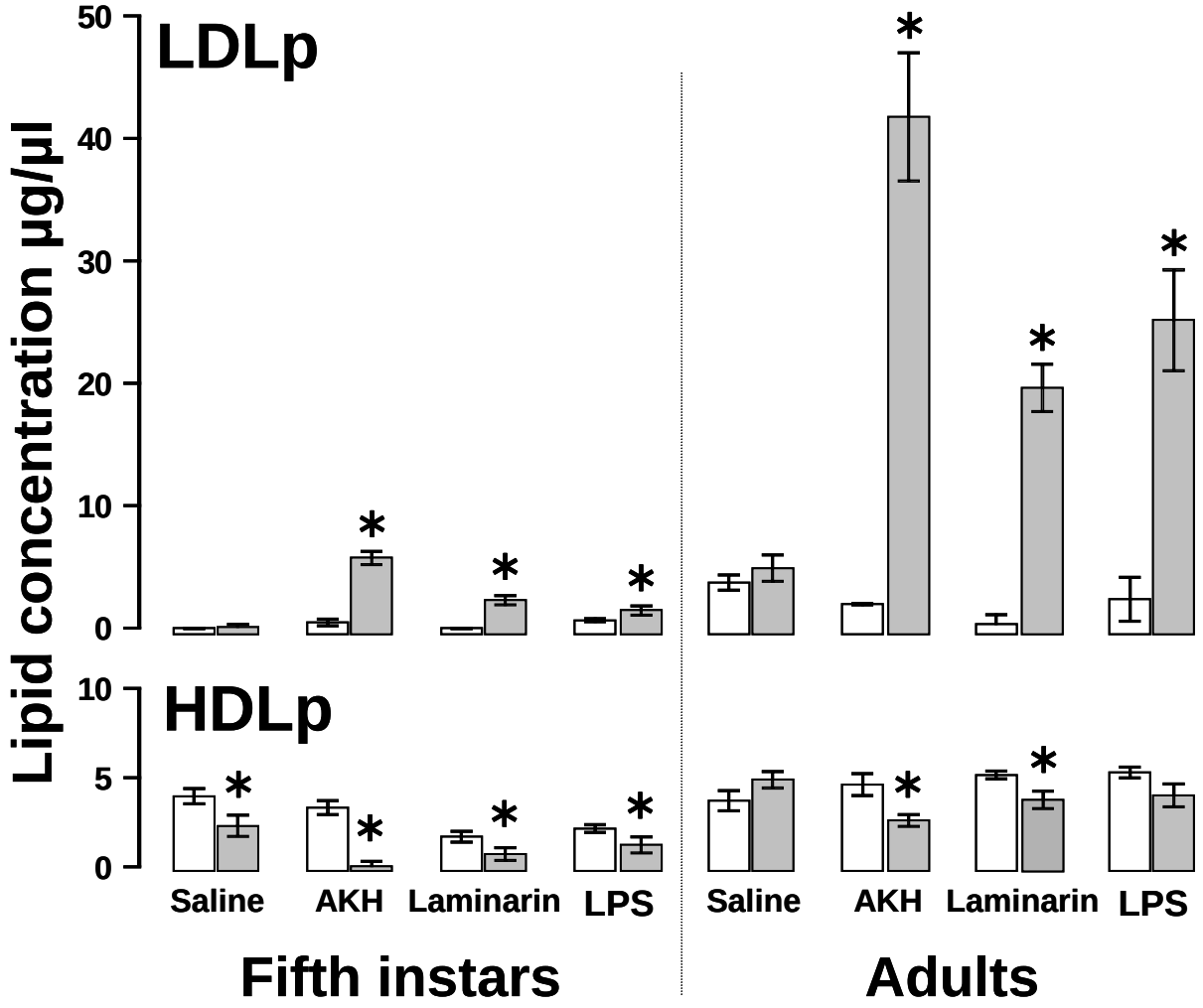

Figure 7 – Changes in the amount of lipid associated with HDLp (heparin precipitated material) and LDLp (EDTA precipitated material) in the haemolymph before (open bars) and 90 min after (solid bars) injection of test materials into fifth instars (age 5 days) and adult male locusts (age 18 days). Bars and vertical lines represent means ± S.E. (n=10). Asterisks denote statistically significant differences (using paired t-tests) between 0 and 90 min values (see text for *P* values).

Legends to figures

Figure 1 - Phenoloxidase activity in the haemolymph of *Locusta* adults and fifth instar nymphs before (open symbols) and 3 h after injection (solid symbols) of laminarin (A) and laminarin and AKH-I (B). Data points and vertical lines represent means ± S.E. (*n* = 10).

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