Mullen, Lisa M., Lightfoot, Mary E. and Goldsworthy, Graham J. (2004) Induced hyperlipaemia and immune challenge in locusts. *Journal of Insect Physiology* **50** (5) 409-417

Induced hyperlipaemia and immune challenge in locusts

Lisa M. Mullen, Mary E. Lightfoot, Graham J. Goldsworthy*

Department of Biology, School of Biological and Chemical Sciences, Birkbeck College, Malet Street, London WC1E 7HX UK

* Corresponding author: Professor G.J. Goldsworthy
Department of Biology, School of Biological and Chemical Sciences
Birkbeck College, Malet Street, London WC1E 7HX UK
Tel: 0207 631 6352
Fax: 0207 631 6246
Email: g.goldsworthy@bbk.ac.uk

Abstract

Injections of immunogens, such as β-1,3-glucan or lipopolysaccharide (LPS), bring about a marked hyperlipaemia with associated changes in lipophorins and apolipophorin-III in the haemolymph of *Locusta migratoria*. These changes are similar to those observed after injection of adipokinetic hormone (AKH). The possibility that endogenous AKH is released as part of the response to these immunogens is investigated using passive immunisation against AKH-I, and measurement of AKH-I titre in the haemolymph after injection of immunogens. The data presented show that, despite the similarity of the changes brought about by the presence of immunogens in the haemolymph to those brought about by AKH, there is no release of endogenous AKH after injection of laminarin or LPS. A direct effect of the immunogens on release of neutral lipids by the fat body cannot be demonstrated *in vitro*, and the mechanism by which hyperlipaemia is induced during immune challenge remains uncertain.

Key words: AKH, *Locusta*, immunogens, LPS, laminarin, ELISA, phenoloxidase, LDLp, apoLp-III, fat body, passive immunisation, β-1,3-glucan.

Abbreviations:
ELISA- enzyme-linked immunosorbent assay
LPS – lipopolysaccharide
AKH – adipokinetic hormone
ApoLp-III – apolipophorin-III

Introduction

The physiological effects of the neuropeptide adipokinetic hormones (AKHs), have been well characterised in the migratory locust, *Locusta migratoria*. The most well-known effects are lipid mobilisation and activation of glycogen phosphorylase in the fat body to provide energy for migratory flight (for review see Goldsworthy, 1990), although other actions include inhibition of the synthesis of protein, fatty acid and mRNA (Lee and Goldsworthy, 1995; Kodrik and Goldsworthy, 1994). Recently, however, it has been shown that in locusts injection of AKH can also influence immune responses (Goldsworthy *et al.*, 2002a; Goldsworthy *et al.*, 2003a; Goldsworthy *et al.*, 2003b; Mullen and Goldsworthy, 2003). The orthopteran prophenoloxidase system is apparently located within haemocytes (Brehelin *et al.*, 1989) and is activated by a serine protease cascade (Ashida, 1990) in response to recognition of non-self material within the haemocoel, and produces effector molecules such as cytotoxic quinones (for review see Ashida and Brey, 1998). Activation of prophenoloxidase in response to injection of immunogens such as bacterial lipopolysaccharide (LPS) or β- 1,3 glucan (laminarin) is influenced by co-injection of AKH-I: prolonging the activation of prophenoloxidase in the haemolymph in response to laminarin and enabling an activation of prophenoloxidase in response to LPS (Goldsworthy *et al.*, 2002a). Another aspect of the immune response, nodule formation, is also enhanced by co-injection of AKH-I with an immunogen such as LPS (Goldsworthy *et al.*, 2003a).

Intriguingly, the immunogen-induced changes in lipophorins and the exchangeable apoprotein, apolipophorin-III (apoLp-III) in the haemolymph of locusts shown previously (Mullen and Goldsworthy, 2003) are qualitatively identical to those brought about by AKH, including the mobilisation of diacylglycerol from the fat body, the appearance of low-density lipophorin (LDLp), and the virtual disappearance of free apoLp-III from the haemolymph. This provides strong circumstantial evidence to suggest that AKH could be released as part of the locust response to injected immunogens such as LPS or laminarin. The aim of this study is to investigate this possibility by measuring the AKH-I titre in the haemolymph after injection of immunogens, and to determine the effect of blocking the action of AKH-I by passive immunisation (injection of antibodies raised against AKH-I) on the responses to injection of laminarin or LPS.

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 12:12 h photocycle, and fed daily with fresh grass and wheat seedlings supplemented with bran. Male locusts between fifteen and twenty five days after the final moult were used in all experiments.

2.2 Materials

Laminarin was purchased from Sigma Chemical Co. and 5 mg mL^{-1} stock solutions were dissolved in insect saline (7.5 g NaCl, 0.375 g KCl L^{-1}). 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^{-1}. *Lom*-AKH-I was purchased from Novabiochem and stock solutions were made up in 80% methanol at a concentration of 20 pmol uL^{-1}, and 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 10 mM phosphate buffer, pH 5.9 (3 mg mL^{-1}) just before use. Rabbit polyclonal antibodies against Tyr^{1}-*Lom*-AKH-I were a generous gift from Dr. D. Candy (University of Birmingham). Immunoglobulin was prepared from this antiserum by selective ammonium sulphate precipitation (Harlow and Lane, 1988). The volume this immunoglobulin solution was adjusted to half that of the original serum (i.e. double the concentration). Initially, this IgG solution was diluted 10-fold, and 10 μL were injected into each locust that, assuming a total haemolymph volume of *c*. 250 μL (Jutsum and Goldsworthy, 1974) gave a final dilution of 1:500 in the animal. Subsequently, 10 μL of the neat IgG solution were injected into each locust, to give a final dilution of 1:25 in the animal. A sample of IgG purified from control serum taken from a rabbit that had not been immunised against AKH-I was prepared in a similar manner.

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, 10 μL volumes of test materials 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 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 a known volume of haemolymph immediately prior to injection, and 90 min after injection.

2.4 Measurement of phenoloxidase activity

Phenoloxidase activity was measured by blowing 5 μL of fresh haemolymph immediately into 95 μL of 10 mM sodium phosphate buffer, pH 5.9. After centrifugation (10,000 x g, at 4° C for 5 min), 40 μL 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^{-1} sodium phosphate buffer). Absorbances were read in a Labsystems Multiskan Bichromatic plate reader. Enzyme activity is expressed here in absorbance units (au) at 492 nm per minute per microlitre of haemolymph.

2.5 Extraction of AKH from the haemolymph

Fifty microlitres of haemolymph were collected from individual locusts without cooling or anaesthesia and blown immediately into 150 μL of chloroform / methanol (1:2). Chloroform (50 μL) and water (40 μL) were then added, and after mixing, the samples were centrifuged at 3000 g for 5 min at 4° C. The upper layer contained the AKH (Candy, 2002) and this layer, together with washings were dried *in vacuo* and taken up in 100 μL phosphate buffered saline (PBS – 8 g NaCl, 0.2 g KCl, 1.78 g $Na_2HPO_4.2H_2O$, 0.24 g KH_2PO_4 L^{-1}, pH 7.4) for quantitative measurement in the ELISA (see below) without further purification.

2.6 Measurement of AKH-I titre in the haemolymph by enzyme-linked immunosorbent assay (ELISA)

A competitive ELISA similar in design to that described previously (Goldsworthy *et al.*, 2002b), for the AKH from *Pyrrhocoris apterus*, was used. Briefly, a biotinylated probe was prepared from cys[1]*Lom*-AKH-I using Biotin Long Arm Maleimide (BLAM, Vector Laboratories, Peterborough, UK). Microtitre plates were pre-coated overnight at 4° C with a 1:14,000 dilution AKH-I IgG preparation, washed and blocked with non-fat dried milk powder solution. Test samples were added to specific wells, followed by 100 fmol of the biotinylated probe. Competition for the binding sites on the IgG bound to the plates was for 1h at 37°C with shaking. The wells were then washed and 100 μL of streptavidin conjugated with horseradish peroxidase solution (Vector Laboratories, diluted 1:500 in PBS/Tween), added to each well. After further incubation and washing, Ortho-phenylenediamine reagent (50 mg containing 2.4 μL of 30% hydrogen peroxide per 100 mL) was added to each well, incubated for 40 min, and then 50 μL of 0.5 M H_2SO_4 were added to each well and the absorbance values determined in a microtitre plate reader at 492 nm. One row of each plate contained a dilution series of *Lom*-AKH-I for the construction of a competition curve. This included one well with no material competing with the probe, to determine the maximum absorbance (MAX), and one well with an excess of 20 pmol of *Lom*-AKH-I to determine the non-specific absorbance (NSA). The percentage competition for each sample was calculated as: 100-100*(absorbance of the sample-NSA)/(MAX-NSA). Samples were run at least in duplicate on each plate.

2.7 Passive immunisation experiments

Locusts were injected with 10 μL of the IgG solution and, after 10 min, 2 pmol of AKH-I, and either 40 μg of laminarin or 50 μg of LPS were injected. Samples of haemolymph were collected after 90 min and the total lipid content, the lipid associated with LDLp, the concentration of free apoLp-III, and the phenoloxidase activity determined.

2.8 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 a method described previously (Goldsworthy *et al.*, 1985; Mullen and Goldsworthy, 2003). Briefly, a 5 μL sample of fresh haemolymph was blown into 200 μL of heparin solution (0.375 % heparin sodium salt in 25 mM $CaCl_2$, 3.8 mM NaCl) to precipitate the HDLp, followed by precipitation of LDLp in 5% EDTA from the supernatant. Finally, acetone was added to precipitate the apoLp-III.

2.9 Measurement of lipid concentration in HDLp and LDLp pellets

Total lipid (measured as vanillin-positive material) was measured as described previously (Mullen and Goldsworthy, 2003). Centrifuge pellets of HDLp and LDLp (from 5 μL of haemolymph) were solubilised in 1 mL of concentrated H_2SO_4. A 200 μL aliquot of this solution was heated and mixed with 1 mL of vanillin reagent. 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 diolein (Sigma Chemical Co.).

2.10 Measurement of protein concentration

The protein concentration in the acetone-precipitated pellet (apoLp-III) was measured as described previously (Mullen and Goldsworthy, 2003) using a modification of an earlier method (Schacterle and Pollack, 1973). Briefly, precipitated protein was dissolved in 200 μL of 0.5 M NaOH, and 200 μL of copper reagent (10% $NaHCO_3$, 0.1% $(CHOH.COOK)_2.½H_2O$), 0.05% $CuSO_4$) were added before the addition of Folin-Ciocalteu phenol reagent. Sample colour development was determined at 620 nm, using a Labsystems Multiskan Bichromatic plate reader. ApoLp-III, purified from locust haemolymph using DEAE Sepharose as described previously (Mullen and Goldsworthy, 2003) was used to construct a calibration curve.

2.11 Isolation and incubation of fat body in vitro

Pieces of fat body were dissected, washed, weighed and incubated for 1 h at 30° C in 100 μL of a 1:1 mixture of Schneider's and Minimum Essential Medium Eagle, pH 7.2 with 20 mM HEPES and 1% BSA, adjusted to 450 mOsm with sucrose (Lee and Goldsworthy, 1995). The medium was then removed and replaced with 100 μL of a 1:1 mixture of haemolymph proteins and the medium described above. Haemolymph proteins were prepared by collecting whole fresh haemolymph in 50% saturated $NH_4(SO_4)_2$, eluting from a PD10 (Pharmacia Biotech) desalting column in the Schneiders/MEM medium described above, and concentration to 50% of the original volume of haemolymph using an Amicon Ultra-15 filter unit purchased from Millipore. The pieces of fat body were incubated for 2 h at 30°C, the incubation medium removed, and a further 100 μL of the haemolymph protein/medium mixture added to each piece of fat body containing 20 pmol AKH-I, and either 40 μg of LPS or 50 μg of laminarin and incubated again for 2 h at 30° C.

2.12 Measurement of diacylglycerol

A 50 μL sample of the incubation medium from the final incubation was collected into 150 μL of chloroform / methanol (2:1), and 50 μL of chloroform and 40 μL of water were added. Two layers were formed after centrifuging for 5 min at 3000 g. A measured sample (equivalent to 10 μL of haemolymph / medium mixture) of the lower chloroform layer from the above extraction was loaded onto a TLC plate (silica gel 60 on glass, Merck KgaA, Darmstadt, Germany) and fractionated with toluene/ethyl acetate (4:1 v/v). Lipid fractions were detected with iodine vapour and marked, the iodine was allowed to sublime overnight, and the silica gel in the areas corresponding to 1,2-diacylglycerol, 1,3- diacylglycerol and triglyceride was transferred to glass tubes. Concentrated H_2SO_4 (200 μL) was added, the tubes were heated at 100° C for 10 min, and then 1 mL of phosphovanillin reagent was added to each. After 15 min the absorbance was read at 540 nm. The amount of acylglycerol was calculated by comparison of the absorbance with that produced by diolein (Sigma Chemical Co.).

2.13 Statistical analysis

Data are expressed as means ± S.E. Statistical analyses were undertaken using paired t-tests or one-way ANOVAs as appropriate using Minitab. The level for significance was taken as $P \leq 0.05$.

3. Results

3.1 Measurement of AKH-I titre in the haemolymph of poisoned locusts, flying locusts and locusts injected with LPS

The titre of AKH-I in the haemolymph was measured using a competitive ELISA. To ensure that this assay could detect AKH-I in the haemolymph of individual locusts, initial experiments were undertaken in which AKH-I titres were measured in locusts that had been poisoned by the injection of 100 μL of 50 μmol KCl. This resulted in a high titre of AKH-I in the haemolymph of 12.7 ± 3.3 (mean ± SE, n = 12) pmol mL^{-1}, compared with 1.7 ± 0.4 pmol mL^{-1} (mean ± SE, n = 12) detected after injection of an identical volume of saline. In the haemolymph of flying locusts, the amount of circulating AKH-I measured in the haemolymph increased steadily during flight, reaching 7.58 pmol mL^{-1} after 45 min (Table 1). However, in locusts injected with 100 μg of LPS there was no increase in AKH-I titre up to 90 min after injection. Injection of 50 μg of laminarin was also ineffective in increasing the titre of AKH-I in the haemolymph (data not shown).

3.2 The effect of passive immunisation against AKH-I on the changes in lipophorins and apolipophorin-III in the haemolymph of locusts injected with immunogens

Because of the possibility that the ELISA was not sensitive enough to detect very small changes in the titre of AKH-I in the haemolymph, passive immunisation against AKH-I was employed to render even small amounts of the hormone physiologically inactive by binding to the injected IgG. Figure 1 illustrates the effect on the total lipid concentration in the haemolymph of injecting IgG prepared against AKH-I five min prior to injection of 2 pmol of AKH-I. The increase in lipid concentration in the haemolymph observed after injection of AKH-I was completely blocked by prior injection of 10 μl of IgG: the control IgG was ineffective in blocking the effect of the hormone, as was a 20-fold diluted preparation of the IgG (Fig. 1). Having established that at high concentration the IgG could block the lipid-mobilising effect of AKH-I *in vivo*, the effect of the antibody on the lipid loading of LDLp in response to AKH, laminarin and LPS was investigated. Figure 2 (panel A) shows the dramatic increase in the lipid associated with LDLp after injection of AKH or immunogens. However, prior injection of the IgG blocked the lipid loading of LDLp in response to injection of AKH-I, but not in response to injection of laminarin or LPS (Fig. 2, panel B). The lipid-loading of LDLp also involves the association of apoLp-III, and the decrease in the concentration of free apoLp-III was measured after injection of AKH-I, laminarin and LPS (Fig. 3, panel A). IgG blocked the decrease in free apoLp-III in the haemolymph of locusts injected with AKH-I (Fig. 3, panel B), but not in the haemolymph of locusts injected with laminarin or with LPS.

3.3 The effect of passive immunisation against AKH-I on the activation of prophenoloxidase in the haemolymph of locusts injected with laminarin or LPS

Locusts injected with 50 μg of laminarin or 40 μg of LPS showed an increase in the phenoloxidase activity in the haemolymph (Fig. 4, panel A). This activation of prophenoloxidase in response to injection of these immunogens was not prevented by prior injection of 10 μL of the IgG raised against AKH-I (Fig. 4, panel B). Neither injection of AKH alone nor injection of AKH prior to injection of IgG activated prophenoloxidase (Fig.4, A,B).

3.4 The effect of AKH-I, laminarin or LPS on the release of lipid from locust fat body in vitro

The possibility that immunogens could act directly on the fat body to cause lipid mobilisation was tested by incubating pieces of locust fat body in a medium containing haemolymph proteins. Incubation with 20 pmol of AKH-I contained in this medium resulted in a significant increase in the concentration of 1,2 diacylglycerol in the incubation medium compared with pieces of fat body taken from the same locusts but incubated in medium alone (Fig 5). There was no significant release of either 1,3 diacylglycerol or of triacylglycerol in response to AKH-I. Incubation of fat body in the presence or absence (control) of laminarin or LPS did not elicit a significant release of any of the neutral lipid classes measured (see Fig. 5).

4. Discussion

The ELISA used here is the first assay in which it is possible to measure AKH titre in the haemolymph of individual locusts. Although overall the present ELISA is less sensitive than the radioimmunoassay described recently (Candy, 2002) it has considerable advantage in not requiring extensive clean-up of the haemolymph. Using this ELISA it is possible to detect increased levels of AKH-I after KCl poisoning or during flight. However, no increase in AKH-I titre was detected in the haemolymph after injection of LPS. This provides strong evidence that in *Locusta*, the immunogen-induced lipid mobilisation and associated changes in the lipophorins in the haemolymph are not mediated by the release of AKH-I. This conclusion is supported by the passive immunisation studies indicating that the changes in lipophorin and apoLp-III in the haemolymph brought about by injection of immunogens are not dependent on the action of AKH-I.

There are three AKHs in *Locusta*, designated AKH-I, II and III, with overlapping functions but varying potencies (Goldsworthy *et al.*, 1997). Co-injection of *Lom*-AKH-II with laminarin also results in a dose-dependent prolonging of phenoloxidase activity in the haemolymph, but this hormone is less potent in this effect than AKH-I (Goldsworthy *et al.*, 2002a). Passive immunisation experiments have been undertaken using IgG prepared against *Lom*-AKH-II (data not shown), and give similar results to those shown here for AKH-I. The possibility remains, of course, that the release of AKH-III is responsible for the changes in lipid metabolism induced by the immunogens, but this is considered very unlikely: the three AKHs in *Locusta* are thought to be released together (Harthoorn *et al.*, 1999; Flanigan and Gade, 1999), and AKH-III is present in the corpora cardiaca in the least quantities of the three neurohormones (Oudejans *et al.*, 1991; Oudejans *et al.*, 1993).

It is interesting to note that the particular preparation of LPS used here had unusual biological activity, in that it was able to activate prophenoloxidase in the haemolymph when injected alone: other commercial preparations of LPS from a variety of bacteria, including *Pseudomonas*, were reported to have no effect on the phenoloxidase activity in the haemolymph unless co-injected with AKH (Goldsworthy *et al.*, 2002a). A fresh sample of *Pseudomonas* LPS was purchased and tested for it's ability to activate prophenoloxidase when injected alone, and was completely ineffective in doing so (data not shown), which is consistent with the results presented earlier (Goldsworthy *et al.*, 2002a). Therefore, the physiological effects of commercial preparations of LPS should be interpreted with some caution, as in our experience, there are differences in physiological effects and in potency between preparations and between batches.

So what is the relationship between lipid status and activation of prophenoloxidase in the haemolymph in response to injection of immunogens? The lipid mobilisation that occurs in response to injection of AKH, during starvation, or after an immune challenge by laminarin or LPS involves the conversion of triacylglycerol to 1,2 diacylglycerol in the fat body, and its subsequent release into the haemolymph loaded onto particles of HDLp to form LDLp. The formation of LDLp also involves an association with apoLp-III. These rearrangements of the lipophorins and apoLp-III in the haemolymph are common to all three conditions: the presence of AKH in the haemolymph (Goldsworthy *et al.*, 1985), starvation (Cheeseman and Goldsworthy, 1979), and presence of immunogens (Mullen and Goldsworthy, 2003). Therefore, the effects of starvation or of co-injection of AKH with immunogens on phenoloxidase activity in the haemolymph are most likely explained by their effects on lipid mobilisation.

So, if AKH is not released as part of the immune response, how then do the immunogens bring about lipid mobilisation? The formation of low-density lipophorin (LDLp) and the decrease in apoLp-III in the haemolymph of locusts injected with laminarin or LPS indicates that immunogen-induced hyperlipaemia is a highly specific effect on the fat body, rather than one of indiscriminate damage of fat body cells by the immunogens (Mullen and Goldsworthy, 2003). However, the data presented here show that, at least *in vitro*, laminarin or LPS do not have a direct lipid-mobilising effect on the fat body in the way that AKH does; nor do the immunogens cause disruption of fat body cells *in vitro*, which would have resulted in an increase in the triacylglycerol content of the incubation medium. It is perhaps unsurprising that fat body cells do not have receptors for these immunogens. It is most likely that recognition of microbial compounds occurs in the haemolymph via pattern recognition proteins. The presence of such a protein has been described in *Manduca sexta* for β-1,3-glucan (Ma and Kanost, 2000) and for LPS in the larvae of *Galleria mellonella* (Dunphy and Halwani, 1997) and *Periplaneta americana* (Jomori *et al.*, 1990; Kawasaki *et al.*, 1993). The medium used for the fat body incubation experiments described here contained haemolymph proteins and lipophorins and it is assumed that any pattern recognition proteins in locust haemolymph would have been retained. However, it should be noted that the preparation of the haemolymph proteins meant that the haemocytes would not remain intact. The activation of the prophenoloxidase cascade is thought to occur via binding of immunogens to pattern recognition proteins and subsequent interaction with the haemocytes, which then induces activation of the prophenoloxidase cascade (Gillespie *et al.*, 1997; Soderhall and Cerenius, 1998). Thus, the absence of viable haemocytes in the incubation medium may explain why laminarin or LPS failed to bring about lipid mobilisation from fat body cells *in vitro*.

The formation of LDLp in the haemolymph of an insect in response to immune challenge was first demonstrated in larvae of the greater wax moth, *Galleria mellonella* (Dettloff *et al.*, 2001b). No experiments involving co- injection of AKH with microbes or immunogens were undertaken in *Galleria* larvae, most likely because the effect of AKH in lepidopteran larvae is to mobilise carbohydrate rather than lipid (Ziegler *et al.*, 1990). Therefore the appearance of LDLp in response to microbial challenge is unlikely to be mediated in *Galleria* by the release of endogenous AKH, and this is consistent with the findings in the locust as shown in this study.

Intriguingly, in *Galleria*, there is also an increase in anti-bacterial activity in the haemolymph in response to injection of apoLp-III, but only in the lipid-associated

form (Wiesner *et al.*, 1997; Dettloff *et al.*, 1991a). ApoLp-III is thought to be involved in early encapsulation responses in this insect (Whitten *et al.*, 2004), and has been shown to affect the adhesion of *Galleria* haemocytes to bacteria (Zakarian *et al.*, 2002). This protein may itself function as a LPS-binding protein (Dunphy and Halwani, 1997). It has been suggested that changes in the insects' metabolism and the appearance of LDLp (and disappearance of free apoLp-III) in the haemolymph acts as a stress signal, thereby activating immune function. However, this is obviously an over-simplification as in locusts, neither AKH nor starvation alone induce activation of prophenoloxidase: these physiological conditions affect prophenoloxidase activation only in the presence of an immunogen. It has been demonstrated that LDLp causes radical formation in isolated haemocytes from *Galleria* (Dettfoff *et al.*, 2001b), but this activation does not happen when haemocytes are incubated with HDLp. This suggests that *Galleria* haemocytes have receptors that recognise LDLp, but the involvement of the haemocytes in analogous interactions in *Locusta* has yet to be elucidated.

The precise relationship between lipid status and activation of prophenoloxidase is unclear. The activation of prophenoloxidase in response to injection of laminarin is observed within 5 min of injection (Mullen and Goldsworthy, 2003) and this would appear to indicate that prophenoloxidase activation occurs prior to lipid mobilisation. If this hypothesis is correct, could the presence of messenger molecules released via this pathway from the haemocytes, a component of the prophenoloxidase-activating pathway, or phenoloxidase itself in the haemolymph cause lipid mobilisation? It does seem likely that an intimate, if not causal, relationship between prophenoloxidase activation and lipid mobilisation in response to injection of immunogens exists, given that fifth instar nymphs have only a very weak lipid mobilisation response to injection of AKH (Mwangi and Goldsworthy, 1977) and are also seemingly unable to activate prophenoloxidase in the haemolymph in response to injection of laminarin (Mullen and Goldsworthy, 2003) or LPS (Goldsworthy *et al.*, 2003a).

Activation of the prophenoloxidase cascade in locusts presumably involves activation of the haemocytes, which could then release other signalling molecules such as cytokines or effector molecules such as reactive oxygen species. There is evidence for the presence of cytokines in insects, most notably plasmatocyte-spreading peptide and growth-blocking peptide in Lepidoptera (Strand *et al.*, 2000; Wang *et al.*, 1999). If cytokines are indeed released from the haemocytes in response to immunogens in locusts, these could be the effector molecules that bring about the hyperlipaemia observed after immune challenge. This possibility is under investigation.

Acknowledgements

This work was supported in part by the award of a Leverhulme Trust Research Fellowship to GJG, and a Jubber Studentship from the University of London to LM.

References

Ashida, M., 1990. The Prophenoloxidase Cascade in Insect Immunity. Research in Immunology 141, 908-910.

Ashida, M.B., Brey, P.T. 1998. Recent advances in research on the insect prophenoloxidase cascade. In Molecular Mechanisms of Immune Responses in Insects (ed P.T.H. Brey, D.), pp. 135-172. Chapman & Hall, London.

Brehelin, M., Drif, L., Baud, L.,Boemare, N., 1989. Insect Hemolymph - Cooperation between Humoral and Cellular Factors in *Locusta-Migratoria*. Insect Biochemistry 19, 301-307.

Candy, D.J., 2002. Adipokinetic hormones concentrations in the haemolymph of *Schistocerca gregaria*, measured by radioimmunoassay. Insect Biochemistry and Molecular Biology 32, 1361-1367.

Cheeseman, P. Goldsworthy, G.J., 1979. The release of adipokinetic hormone during flight and starvation in *Locusta.* General and Comparative Endocrinology 37, 35-43.

Dettloff, M., Kaiser, B.,Wiesner, A., 2001a. Localization of injected apolipophorin III *in vivo* - new insights into the immune activation process directed by this protein. Journal of Insect Physiology 14195 Berlin, Germany Free Univ Berlin, Inst Biol Zool, D-14195 Berlin, Germany 47, 789-797.

Dettloff, M., Wittwer, D., Weise, C.,Wiesner, A., 2001b. Lipophorin of lower density is formed during immune responses in the lepidopteran insect *Galleria mellonella*. Cell and Tissue Research 306, 449-458.

Dunphy, G. Halwani, A., 1997. Haemolymph proteins of larvae of *Galleria mellonella* detoxify endotoxins of the insect pathogenic bacteria *Xenorhabdus nematophilus* (Enterobacteriaceae). Journal of Insect Physiology 43, 1023-1029.

Flanigan, J.E. Gade, G., 1999. On the release of the three locust (*Locusta migratoria*) adipokinetic hormones: Effect of crustacean cardioactive peptide and inhibition by sugars. Zeitschrift Fur Naturforschung C-a Journal of Biosciences 54, 110-118.

Gillespie, J.P., Kanost, M.R.,Trenczek, T., 1997. Biological mediators of insect immunity. Annual Review of Entomology 42, 611-643.

Goldsworthy, G.J. 1990. Hormonal control of flight metabolism in locusts. In Biology of Grasshoppers (ed A. Chapman R.F. & Joern), pp. 205-225. John Wiley & Sons, New York.

Goldsworthy, G., Chandrakant, S., Opoku-Ware, K., 2003a. Adipokinetic hormone enhances nodule formation and phenoloxidase activation in adult locusts injected with bacterial lipopolysaccharide. Journal of Insect Physiology 49, 795-803.

Goldsworthy, G., Mullen, L., Opoku-Ware, K., Chandrakant, S., 2003b. Interactions between the endocrine and immune systems in locusts. Physiological Entomology 28. 54-61.

Goldsworthy, G., Opoku-Ware, K., Mullen, L., 2002a. Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. Journal of Insect Physiology 48, 601-608.

Goldsworthy, G.J., Kodrik, D., Comley, R., Lightfoot, M., 2002b. A quantitative study of adipokinetic hormone of the firebug, *Pyrrhocoris apterus*. Journal of Insect Physiology 48, 1103-1109.

Goldsworthy, G.J., Lee, M.J., Luswata, R., Drake, A.F.,Hyde, D., 1997. Structures, assays and receptors for locust adipokinetic hormones. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 117, 483-496.

Goldsworthy, G.J., Miles, C.M.,Wheeler, C.H., 1985. Lipoprotein transformations during adipokinetic hormone action. Physiological Entomology 10, 151-164.
Harlow, E. Lane, D. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, New York. p298-299.

Harthoorn, L.F., Diederen, J.H.B., Oudejans, R.,Van der Horst, D.J., 1999. Differential location of peptide hormones in the secretory pathway of insect adipokinetic cells. Cell and Tissue Research 298, 361-369.

Jomori, T., Kubo, T.,Natori, S., 1990. Purification and characterization of lipopolysaccharide-binding protein from hemolymph of American cockroach *Periplaneta americana*. European Journal of Biochemistry 190, 201-206.

Jutsum, A. R., Goldsworthy, G.J., 1974. Some effects of mermithid infection on metabolic reserves and flight in *Locusta*. International Journal of Parasitology **4**: 625-630.

Kawasaki, K., Kubo, T.,Natori, S., 1993. A Novel Role of *Periplaneta* Lectin as an Opsonin to Recognize 2-Keto-3-Deoxy Octonate Residues of Bacterial Lipopolysaccharides. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 106, 675-680.

Kodrik, D., Goldsworthy, G.J., 1994. Adipokinetic Hormones Inhibit RNA-Synthesis in the Fat-Body of Adult *Locusta-Migratoria*. Insect Neurochemistry and Neurophysiology 1993, 173-176.

Lee, M.J., Goldsworthy, G.J., 1995. Acetate Uptake Test - the Basis of a Rapid Method for Determining Potencies of Adipokinetic Peptides for Structure- Activity Studies. Journal of Insect Physiology 41, 163-170.

Ma, C.C., Kanost, M.R., 2000. A beta 1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. Journal of Biological Chemistry 275, 7505-7514.

Mullen, L., 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 Biochemistry and Molecular Biology 33, 661-670.

Mwangi, R.W., Goldsworthy, G.J., 1977. Age-related changes in the response to adipokinetic hormone in *Locusta migratoria*. Physiological Entomology 2, 37-42.

Oudejans, R., Kooiman, F.P., Heerma, W., Versluis, C., Slotboom, A.J.,Beenakkers, A.M.T., 1991. Isolation and Structure Elucidation of a Novel Adipokinetic Hormone (*Lom*-AKH-III) from the Glandular Lobes of the Corpus Cardiacum of the Migratory Locust, *Locusta-Migratoria*. European Journal of Biochemistry 195, 351-359.

Oudejans, R., Mes, T.H.M., Kooiman, F.P.,Vanderhorst, D.J., 1993. Adipokinetic Peptide-Hormone Content and Biosynthesis During Locust Development. Peptides 14, 877-881.

Schacterle, G.R., Pollack, R.L., 1973. A simplified method for the quantitative assay of small amounts of protein in biological materials. Analytical Biochemistry 51, 654-655.

Soderhall, K., Cerenius, L., 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Current Opinion in Immunology 10, 23-28.

Strand, M.R., Hayakawa, Y.,Clark, K.D., 2000. Plasmatocyte spreading peptide (PSP1) and growth blocking peptide (GBP) are multifunctional homologs. Journal of Insect Physiology 46, 817-824.

Wang, Y., Jiang, H.B.,Kanost, M.R., 1999. Biological activity of *Manduca sexta* paralytic and plasmatocyte spreading peptide and primary structure of its hemolymph precursor. Insect Biochemistry and Molecular Biology 29, 1075-1086.

Whitten, M.M.A., Tew, I.F., Lee, B.L., Ratcliffe, N.A., 2004. A Novel Role for an Insect Apolipoprotein (Apolipophorin III) in 1,3-Glucan Pattern Recognition and Cellular Encapsulation Reactions. The Journal of Immunology, 172: 2177-2185.

Wiesner, A., Losen, S., Kopacek, P., Weise, C.,Gotz, P., 1997. Isolated apolipophorin III from *Galleria mellonella* stimulates the immune reactions of this insect. Journal of Insect Physiology 43, 383-391.

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

Ziegler, R., Eckart, K., Law, J.H., 1990. Adipokinetic Hormone Controls Lipid Metabolism in Adults and Carbohydrate Metabolism in Larvae of *Manduca Sexta*. Peptides 11, 1037-1040

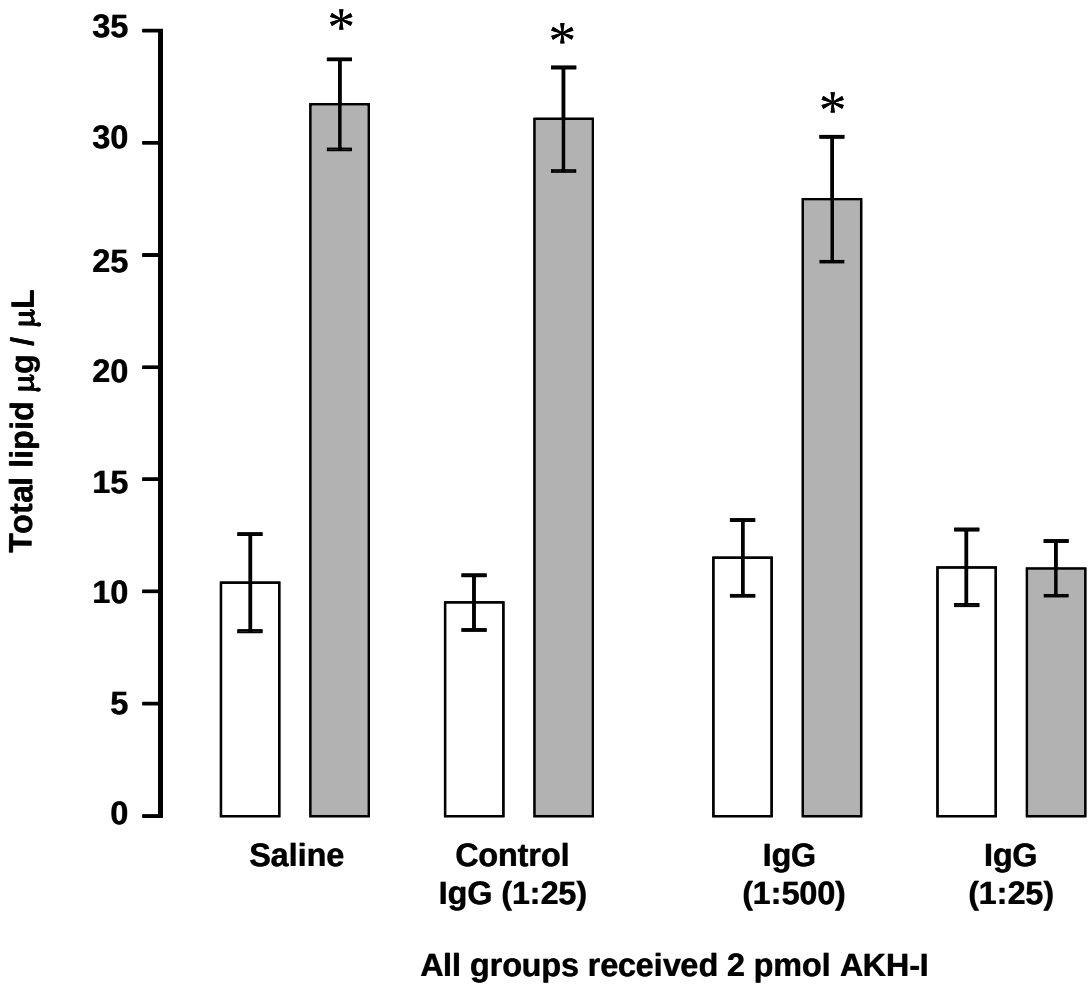

Figure 1 – The concentration of total lipid in the haemolymph of locusts injected with 2 pmol of AKH-I 5 min after injection of saline, control IgG at a 1:25 dilution in the haemolymph, or IgG raised against AKH-I at 1:500 and 1:25 dilutions in the haemolymph. Open bars represent concentrations of lipid in the haemolymph prior to injection and shaded bars represent the concentrations 90 min after injection. 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 (* $P < 0.001$).

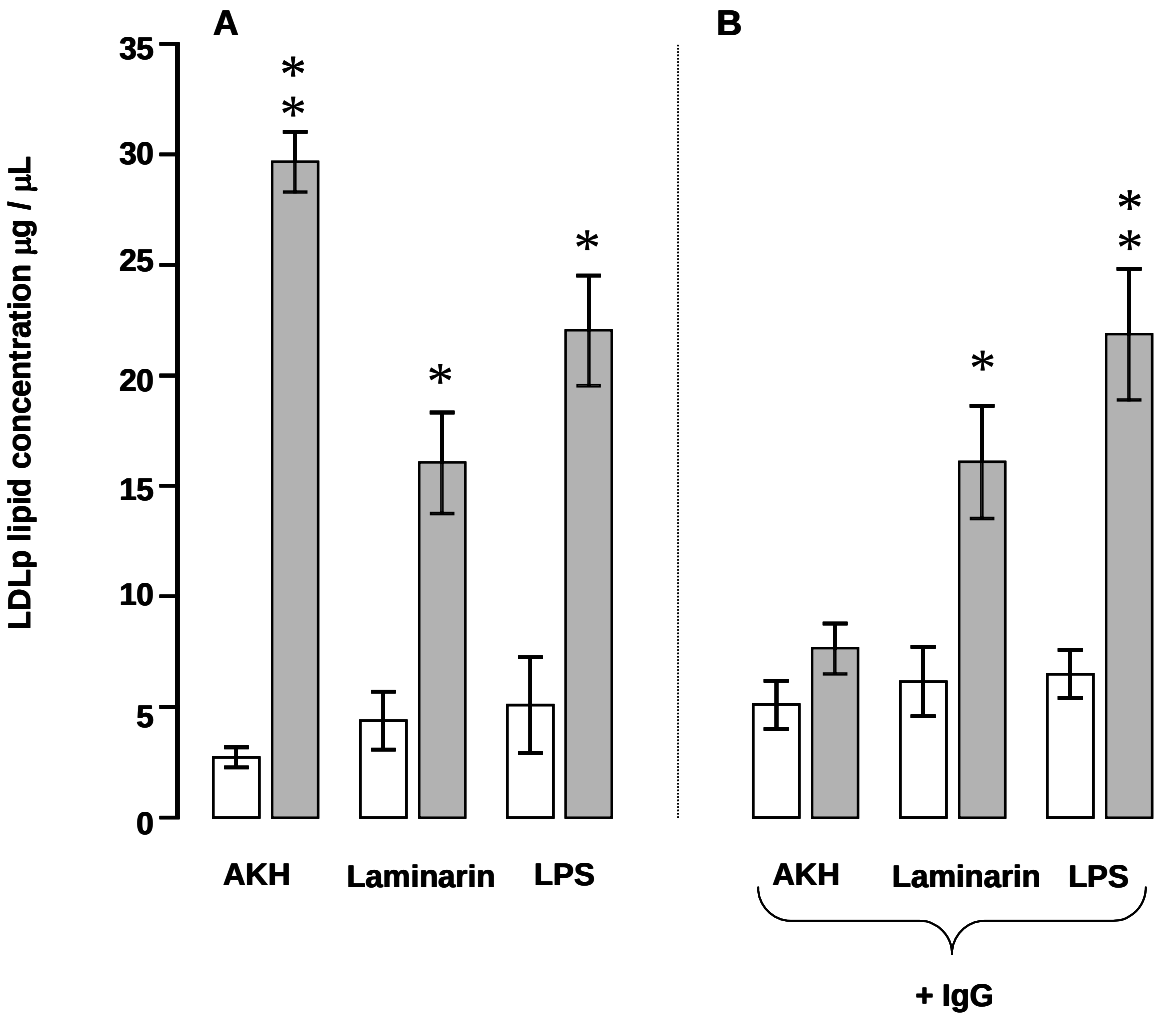

Figure 2 – Panel A: concentration of lipid associated with low density lipophorin prior to injection of 2 pmol of AKH, 50µg of laminarin or 40 µg of LPS (open bars) and 90 min after injection (shaded bars). Panel B: effect of prior injection of IgG raised against AKH-I on the concentration of lipid associated with low density lipophorin before injection of 2 pmol of AKH, 50 µg of laminarin or 40 µg of LPS (open bars) and 90 min after injection (shaded bars). 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 (* $P < 0.01$, ** $P < 0.001$).

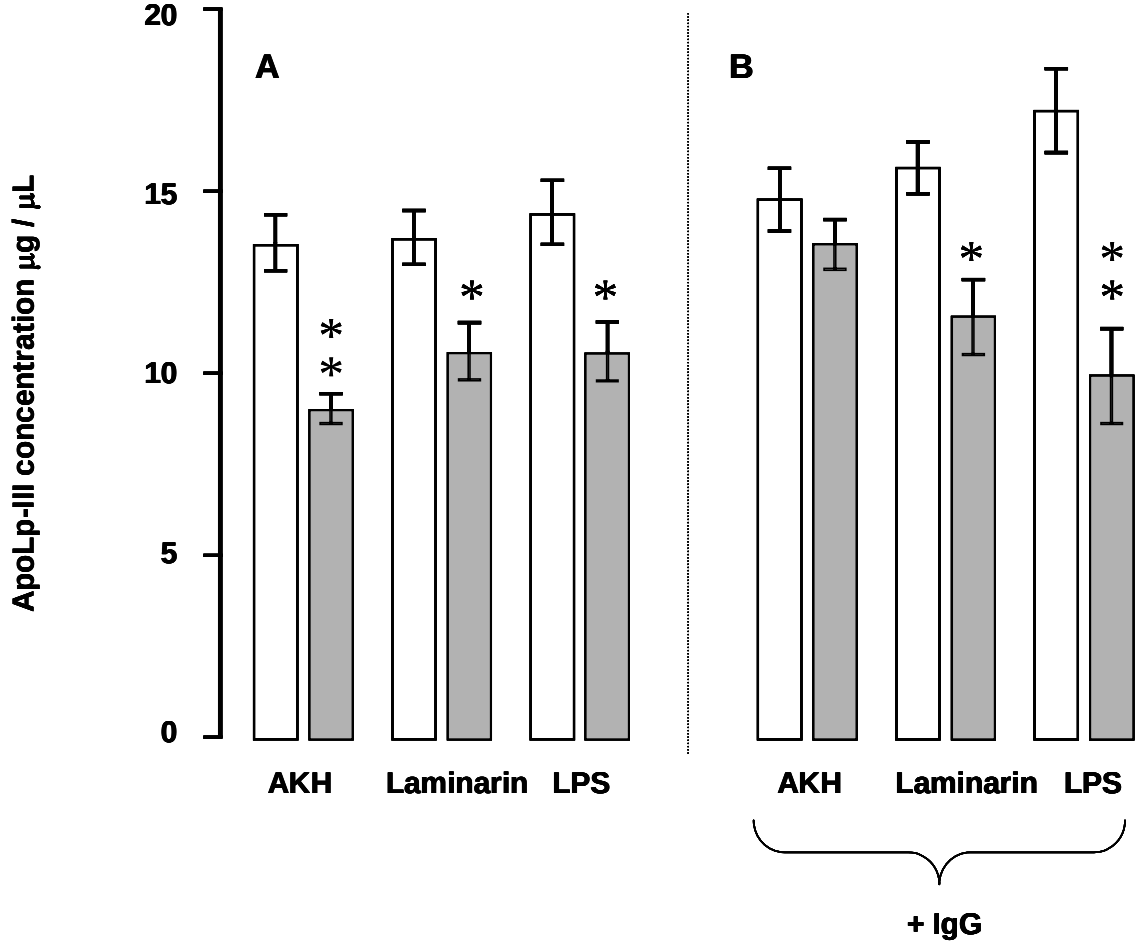

Figure 3 – Panel A: concentration of free apolipophorin-III before injection of 2 pmol of AKH, 50 µg of laminarin or 40 µg of LPS (open bars) and 90 min after injection (shaded bars). Panel B: effect of prior injection of IgG raised against AKH-I on the concentration of free apolipophorin-III before injection of 2 pmol of AKH, 50 µg of laminarin or 40 µg of LPS (open bars) and 90 min after injection (shaded bars). 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 (* $P < 0.01$, ** $P < 0.001$).

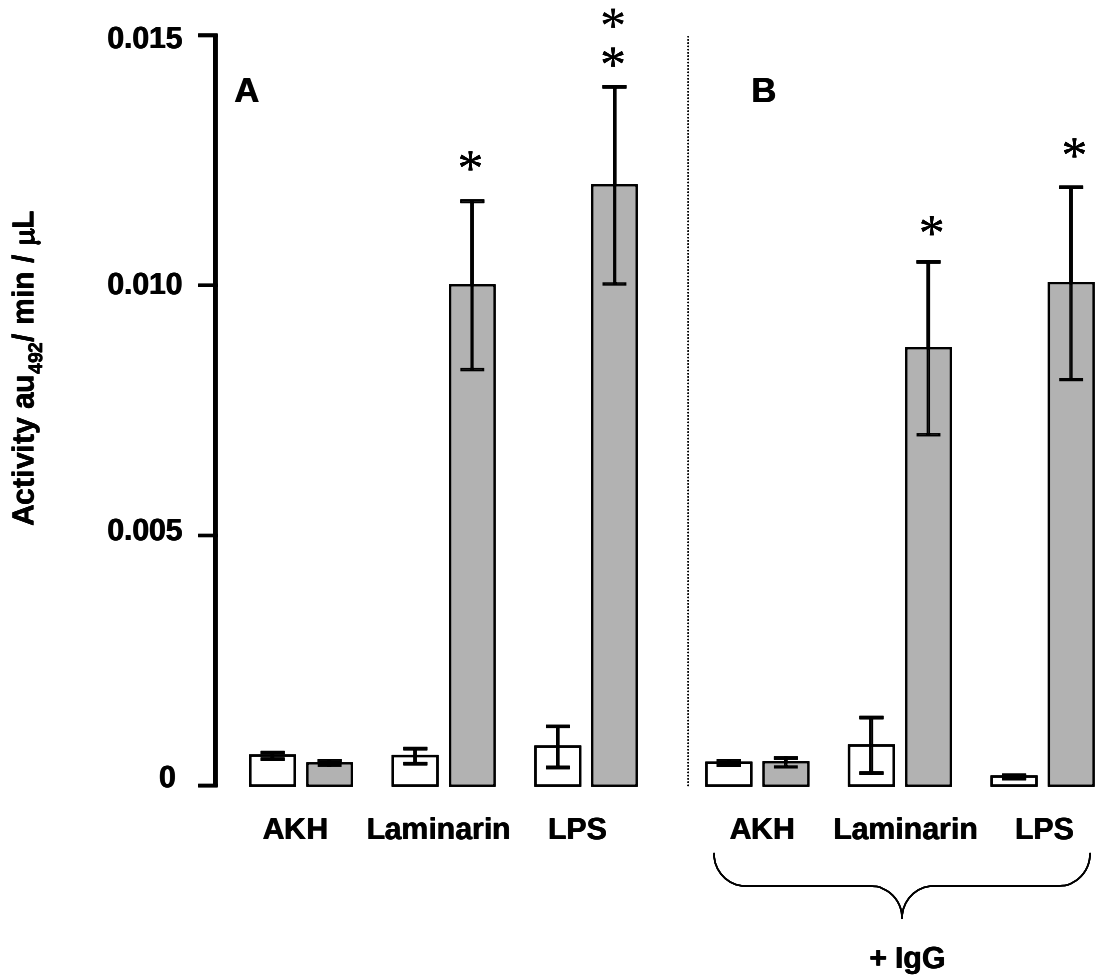

Figure 4 – Panel A: phenoloxidase activity in the haemolymph before injection of 2 pmol of AKH, 50 µg of laminarin or 40 µg of LPS (open bars) and 90 min after injection (shaded bars). Panel B: effect of prior injection of IgG raised against AKH-I on the phenoloxidase activity before injection of 2 pmol of AKH, 50 µg of laminarin or 40 µg of LPS (open bars) and 90 min after injection (shaded bars). 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 (* $P < 0.01$, ** $P < 0.001$).

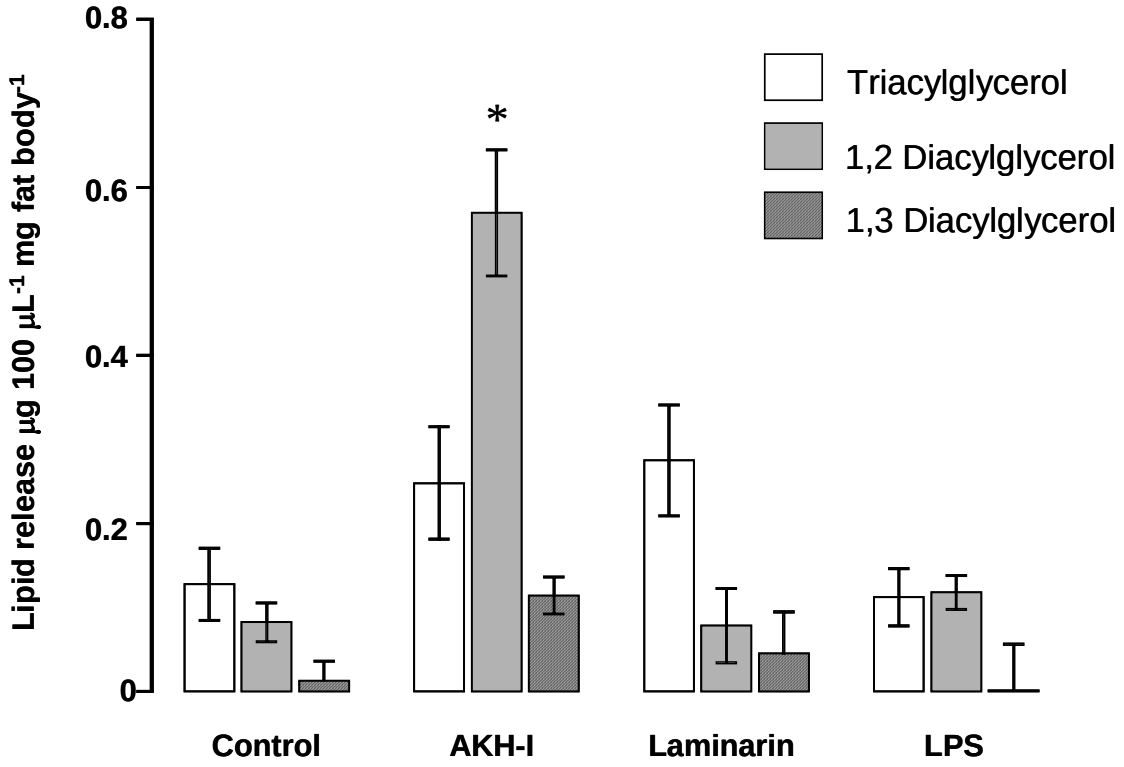

Figure 5 – The release of neutral lipids from fat body *in vitro* during a 2 h incubation, in response to 20 pmol of AKH, 50 µg of laminarin or 40 µg of LPS contained in 100 µL of incubation medium. Bars and vertical lines represent means ± S.E. (n = 10). Asterisk denotes statistically significant difference (using one-way ANOVA) between control and experimental group ($P < 0.001$).

Treatment Time after treatment (min)	**Saline-injection** **Mean AKH-I titre pmol mL^{-1} ± SE**	***n***	**LPS injected** **Mean AKH-I titre pmol mL^{-1} ± SE**	***n***	**Flown** **Mean AKH-I titre pmol mL^{-1} ± SE**	***n***
0	1.2 ± 0.2	15	1.2 ± 0.2	20	1.6 ± 0.3	4
5	-		-		1.7 ± 0.4	4
15	1.3 ± 0.3	15	1.3 ± 0.3	15	2.8 ± 0.7	4
30	1.1 ± 0.1	20	1.2 ± 0.4	15	3.4 ± 0.6	4
45	1.4 ± 0.3	5	-		7.6 ± 2.1	4
60	1.3 ± 0.4	10	1.2 ± 0.2	5	-	
90	1.2 ± 0.3	15	1.1 ± 0.1	15	-	

Table 1. The titres of AKH-I measured by competitive ELISA in the haemolymph of locusts injected with saline, or 100 μg of LPS, or flown for various periods. Each mean is derived from a different group of locusts.