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Adipokinetic hormone enhances nodule formation and phenoloxidase activation in adult locusts injected with bacterial lipopolysaccharide.

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Abstract

Interactions between the locust endocrine and immune systems have been studied *in vivo* in relation to nodule formation and activation of the prophenoloxidase cascade in the haemolymph. Injection of bacterial lipopolysaccharide (LPS) extracted from *Escherichia coli* induces nodule formation in larval and adult locusts but does not increase phenoloxidase activity in the haemolymph. Nodule formation starts rapidly after injection of LPS and is virtually complete within 8h, nodules occurring mainly associated with the dorsal diaphragm on either side of the heart, but sometimes with smaller numbers associated with the ventral diaphragm on either side of the nerve cord. Co-injection of adipokinetic hormone-I (*Lom*-AKH-I) with LPS stimulates greater numbers of nodules to be formed in larval and adult locusts, and activates phenoloxidase in the haemolymph of mature adults but not of nymphs. The effect of co-injection of *Lom*-AKH-I with LPS on nodule formation is seen at low doses of hormone; only 0.4 pmol of *Lom*-AKH-I per adult locust is needed to produce a 50% increase in the number of nodules formed. When different components of LPS from the *E. coli* Rd mutant are tested, the mono- and the diphosphoryl Lipid A components have similar effects to the intact LPS. Remarkably, detoxified LPS activates phenoloxidase in the absence of *Lom*-AKH-I, although co-injection with hormone does enhance this response. Both diphosphoryl Lipid A and detoxified LPS induce a level of nodule formation that is enhanced by co-injection of *Lom*-AKH-I, but monophosphoryl Lipid A does not initiate nodule formation even when injected with hormone. Co-injection of a water-soluble inhibitor of eicosanoid synthesis, diclofenac (2-[(2, 6-dichlorophenyl)amino] benzeneacetic acid), reduces nodule formation in response to injections of LPS (both in the absence and presence of hormone) in a dose-dependent manner, but does not prevent activation of phenoloxidase in adult locusts. It is shown that nodule formation and activation of the prophenoloxidase in locust haemolymph can both be enhanced by *Lom*-AKH-I, but it is argued that these processes involve distinct mechanisms in which eicosanoid synthesis is important for nodule formation, but not for the increased phenoloxidase activity.

Keywords: adipokinetic hormone; *Lom*-AKH-I; diclofenac; eicosanoids synthesis; immune response; Lipid A; nodule; phenoloxidase; lipopolysaccharide; *Locusta migratoria*; *Schistocerca gregaria*

1. Introduction

The insects' immune system against potential pathogens involves both cellular and humoral responses (see Lavine and Strand, 2002). The cellular mechanisms involve phagocytosis and encapsulation, where haemocytes engulf and/or entrap foreign bodies or invading microbes, and aggregate to form nodules (see Ratcliffe *et al*., 1985). The humoral responses include activation of prophenoloxidase, and the eventual synthesis by the fat body of antimicrobial peptides that are released into the haemolymph to fight infection (see Gillespie *et al*., 1997).

Phenoloxidase catalyses the oxygenation of monophenols to diphenols and quinones, which are toxic to microbes. The enzyme is present in the haemolymph as an inactive zymogen, prophenoloxidase, which is activated by a Ca^{2+}-dependent serine protease cascade in response to microbial invasion (Ashida and Brey, 1998). It has been shown

recently that not only can this cascade be activated in the locust *in vivo* by injection of immunogens such as laminarin (mainly β-1,3 glucan), but also that adipokinetic hormone (*Lom*-AKH-I) prolongs this response to laminarin. Intriguingly, in the case of injections of bacterial lipopolysaccharide (LPS), co-injection of *Lom*-AKH-I brings about an activation of prophenoloxidase activity that is not seen in the absence of the hormone (Goldsworthy *et al*., 2002).

The lack of prophenoloxidase activation when locusts are injected with LPS in the absence of *Lom*-AKH-I does not necessarily mean that the immune system of the locust does not respond at all to such a challenge. Indeed, it is known that locusts form nodules in response to injections of bacterial products or LPS (Hoffmann *et al*., 1974, Gunnarsson and Lackie, 1985; Brookman *et al*., 1989ab; Ratcliffe *et al*., 1991). The present study was undertaken to determine whether nodule formation in locusts in response to injections of LPS is influenced by signalling molecules like *Lom*-AKH-I and eicosanoids; the latter are known to mediate the cellular response to bacterial infections in a number of insects (Stanley-Samuelson *et al*., 1991; Miller *et al*.1996, 1999; Bedick *et al*., 2000; Miller and Stanley, 2001).

2. Materials and methods

2.1. Insects

Locusta migratoria migratorioides (R. & F.) and *Schistocerca gregaria* (Forskal) were taken from laboratory colonies 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. Except where indicated otherwise, all experiments were conducted on adult males between 12 and 25 days after adult emergence.

2.2. Injection of materials into the haemolymph of locusts

Injections of test materials were made using specially adapted plastic pipette tips within the bore of which a short length of stainless steel needle was held by friction. Using these, small volumes (usually 20 µl) could be taken up accurately and injected into the haemocoel by inserting the needle between two abdominal terga and expelling the sample using an automatic pippettor.

Stock solutions of *Lom*-AKH-1 were made up in 80% methanol (usually *c*. 20 pmol/µl) and quantified by measuring the tryptophan fluorescence in an LS50B Fluorimeter (Excitation wavelength, 280 nm; Emission wavelength, 348 nm) and calibrating against a standard solution of tryptophan. Unless otherwise stated, a standard dose of 20 pmol of *Lom*-AKH-I was injected. All chemicals were purchased from Sigma Chemical Co., except for diclofenac (Calbiochem) and *Lom*-AKH-I (Novabiochem). The commercial preparations of LPS used were prepared by phenolic extraction from *E. coli*, serotype 0111, B4 (L 2630, Sigma). For some experiments, preparations of LPS components from the *E. coli* Rd mutant were purchased from Sigma and tested for their ability to stimulate nodule formation and/or activate the phenoloxidase cascade. These were, LPS from *E. coli* F583 Rd Mutant (L 6893); monophosphoryl lipid A (L 6638) and diphosphoryl lipid A (L 5399) from *E. coli* F583 Rd Mutant; and LPS from *E. coli* serotype 0111, B4 (L 3023) detoxified by alkaline hydrolysis. All stock solutions of LPS were made in insect saline (7.5 g NaCl and 0.375g KCl/litre) at 8 mg/ml and, unless otherwise stated, 100 µg were injected into each locust. A stock solution of diclofenac (2-[(2, 6-dichlorophenyl)amino] benzeneacetic acid**,** sodium salt) was

prepared in saline at 16 mg/ml. Stock solutions of *Lom*-AKH-I, LPS and diclofenac were stored at –15 ºC until needed.

2.3 Samples of haemolymph
Samples of haemolymph were taken from locusts without cooling or anaesthesia, by making a small puncture in the arthrodial membrane at the base of a hind leg. A calibrated capillary tube was used to take up 5 µl of haemolymph. The haemolymph was taken 3 h after administration of the test material and blown immediately into plastic 1.5 ml centrifuge tubes containing 95 µl of phosphate buffer (10 mM, pH 5.9).

2.4. Measurement of phenoloxidase activity
Phenoloxidase activity was measured by a slight modification of the procedure described by Goldsworthy *et al*. (2002). Briefly, 5 µl of fresh whole haemolymph was blown into 95 µl of phosphate buffer. After centrifugation (10000 x g, at 4°C for 5 min), 40 µl of the supernatant was pipetted into a well of a microtitre plate and 160 µl of dopamine (3mg/ml phosphate buffer) added as substrate (instead of the *L*-dopa used previously) because of its high solubility in aqueous media. Phenoloxidase activity was assessed by determining the initial linear increase in absorbance at 492 nm over 30 min using a Labsystems Multiskan Bichromatic platereader. Enzyme activity is expressed in absorbance units (au) at 492 nm per minute per microlitre of haemolymph (see Goldsworthy *et al*., 2002).

2.5 Assessment of nodule formation
Insects were killed by decapitation, and a mid-ventral longitudinal incision was made so that the abdomen could be pinned out on a cork board for examination under a binocular microscope. The number of nodules in the first abdominal segment was counted. Except where stated, nodules were examined 24 h after injection of LPS. No account was taken of any variations in the size of individual nodules.

2.6 Statistical analysis
Data are expressed as means ± S.E. Nodule count data were subjected to √p-transformation prior to statistical analysis. ANOVA (two-way) was used to analyse the relationship between the numbers of nodules formed, the amount of LPS injected, and the effect of co-injection with *Lom*-AKH-I. A one-way ANOVA with Tukey's pairwise comparison, was employed with all other data. The level of significance was taken as $P \leq 0.05$ and all tests were undertaken using Minitab.

3. Results

3.1 Nodule formation in response to injection of LPS into adult locusts.
Adult locusts were injected with 20 µl of saline containing 100 µg of LPS or 100 µg of LPS and 20 pmol of *Lom*-AKH-I. A control group was injected only with 20 µl of saline. After 24 h all insects were killed and examined for nodules. A small number of nodules were found in saline-injected animals (Fig. 1A and B), whereas nodules were usually absent in non-injected locusts (not shown). When nodules were observed in LPS-injected locusts, they were not randomly distributed within the haemocoel or tissues, but were exclusively in the anterior portion of the abdomen, associated with the dorsal diaphragm, and concentrated on either side of the heart, although sometimes smaller numbers of nodules were seen associated with the ventral diaphragm and along

either side of the nerve cord. After injection of LPS, the total number of nodules present in any locust was too large to determine routinely. Correspondingly, in all experiments only the number of nodules in the first abdominal segment was counted for the data presented here. For both *Locusta* and *Schistocerca*, the numbers of nodules formed after injection of LPS increased dramatically when *Lom*-AKH-I was co-injected with the LPS. Injections of *Lom*-AKH-I alone did not induce nodule formation (data not shown). Overall, the numbers of nodules formed in *Locusta* or *Schistocerca* after these treatments were broadly comparable (Fig. 1B).

The relationship between time after injection of LPS and the numbers of nodules formed was investigated in a group of adult male *Schistocerca* (Fig. 1A). The numbers of nodules formed were determined at 0, 1, 2, 4, 8, 18 and 24 h after injection of LPS and *Lom*-AKH-I. It can be seen in Figure 1 that nodule formation began very soon after injection, and was more or less complete after 8 h. However, for convenience, nodule formation was assessed in subsequent experiments 24 h after injection.

3.2 The dose-response relationships for LPS and Lom-AKH-I and their effects on nodule formation

The effect of varying the dose of LPS injected on nodule formation was determined in adult locusts (Fig. 2). One group of locusts received only LPS in increasing amounts, whereas a second group were injected with these different amounts of LPS and a constant dose of 20 pmol of *Lom*-AKH-I. Figure 2 shows that increasing the dose of LPS caused proportionally more nodules to be formed both in the presence and absence of *Lom*-AKH-I. A two-way ANOVA showed that the effect of increasing doses of LPS on nodule formation was highly statistically significant ($P<0.001$ both with and without AKH), and that the effect of co-injection of the AKH was also statistically significant ($P=0.05$). In these experiments the greatest dose of LPS used was 120 µg, and even at this level there was no suggestion that nodule formation had reached a plateau: greater amounts of LPS were not tolerated by the locusts, which died within 24 h of injection. When the effect of varying the amount of hormone co-injected with the LPS was examined, a classical sigmoidal dose-response relationship was established, with a calculated ED_{50} of 0.4 pmol of *Lom*-AKH-I (Fig. 3).

3.3 Responses to injection of LPS into fifth instar nymphs or adults

The numbers of nodules formed in response to injections of LPS into fifth instar nymphs or young adults were similar to those formed in mature adults, and co-injection of *Lom*-AKH-I with LPS caused significantly ($P<0.001$) more nodules to be formed in all three age-groups (Fig. 4B). Whether or not hormone was co-injected, there was no activation of prophenoloxidase in the haemolymph of nymphs or newly emerged adults equivalent to that seen in mature adults in response to co-injection of LPS and AKH (Fig. 4A).

3.4 Responses to injection of LPS into starved adult male locusts

As reported previously (Goldsworthy *et al*., 2002), LPS did not bring about a significant activation of prophenoloxidase in fed *Locusta migratoria* unless *Lom*-AKH-I was co-injected (see also Fig. 4B). Surprisingly, in locusts starved for 24 h, phenoloxidase activity was high 3 h after injection of LPS even without injection of hormone (Fig. 5A). However, starvation had no effect on nodule formation in response to injections of LPS or on its enhancement by co-injection of *Lom*-AKH-I (Fig. 5B).

3.5 Effect of diclofenac on nodule formation and activation of prophenoloxidase by LPS

Figure 6 shows the effect of diclofenac on nodule formation by LPS in adult male *Locusta migratoria*. In locusts injected with LPS, diclofenac reduced nodule formation to a level similar to that seen in saline-injected locusts (Fig. 6B). However, when *Lom*-AKH-I was co-injected with the LPS, the degree of inhibition of nodule formation by diclofenac was dependent on the dose of diclofenac injected and, even at the highest dose tested, the number of nodules formed was similar to that when LPS was injected on its own (Fig. 6A,B). Phenoloxidase activity in the haemolymph was measured in the same animals at 3 h after injection. Whether or not hormone was co-injected with the LPS, diclofenac had no inhibitory effect on the activation of prophenoloxidase (Fig. 7). Furthermore, when data from experimental and control locusts were pooled, there did not appear to be any clear relationship between the levels of phenoloxidase measured in the haemolymph, and the numbers of nodules present (Fig. 7B).

3.6 Responses to different components of LPS

Nodule formation and activation of prophenoloxidase in response to injections of various components of LPS from the *E. coli* Rd mutant were studied in adult locusts (Fig. 8). In activating phenoloxidase in the haemolymph only when *Lom*-AKH-I was co-injected, the mono- and the diphosphoryl Lipid A components behaved in a similar way to the intact LPS from the Rd mutant. Remarkably, however, detoxified LPS activated phenoloxidase in the absence of *Lom*-AKH-I, although co-injection of the hormone produced an even greater response. Both diphosphoryl Lipid A and detoxified LPS induced a level of nodule formation that was enhanced by co-injection of hormone, but monophosphoryl Lipid A did not cause nodule formation even when injected with *Lom*-AKH-I (Fig. 8).

4. Discussion

In a previous study (Goldsworthy *et al.*, 2002) it was shown that co-injection of adipokinetic hormone (*Lom*-AKH-I) with laminarin or bacterial lipopolysaccharide (LPS) prolongs or facilitates respectively the activation of prophenoloxidase in the haemolymph of adult locusts. The present study shows that *Lom*-AKH-I can affect another aspect of innate immunity, by increasing the formation of nodules in response to injections of LPS. The hormone also increases the numbers of nodules formed in response to injections of laminarin (unpublished observations). It is somewhat surprising, but well documented, that nodules are formed in response to injections of solutions of LPS or laminarin (Gunnarsson and Lackie, 1985). When nodules are formed in locusts in response to injection of laminarin (unpublished observations) or LPS (this study), their distribution is very striking. Nodules form in a pattern that appears to reflect the distribution of haemopoietic tissue associated with the dorsal diaphragm on either side of the dorsal blood vessel as described by Ogel (1959) and Hoffmann (1970); a slight difference being the formation of a smaller number of nodules aligned on either side of the ventral nerve cord. Whether there are small numbers of previously unrecognised reticular cells in these latter sites remains to be determined. In *Locusta migratoria*, freely circulating haemocytes may not play a major

part in the phagocytosis of foreign materials; injected particles of Indian ink or bacteria, for example, are phagocytosed by cells of the haemopoietic tissue (Ogel, 1959; Hoffmann, 1973; Hoffmann *et al*., 1974). It seems that as a result of massive uptake by the reticular cells of foreign material, such as the LPS in this study, nodules form in a very defined pattern reflecting the distribution of the reticular cells.

The effects of different doses of LPS and of co-injected *Lom*-AKH-I in relation to activation of the prophenoloxidase cascade have already been described (Goldsworthy *et al*., 2002), but in the present study these were also studied for their effects on nodule formation. Increasing doses of LPS caused progressively greater numbers of nodules to be formed, but it was not possible to test the effects of doses of LPS in excess of 150 µg per locust on nodule formation because of the associated high mortality. Nevertheless, within the range of doses of LPS tested, co-injection of hormone always enhanced the level of nodule formation, and the ED_{50} for this effect of the hormone was 0.4 pmol of *Lom*-AKH-I. Nodule formation in response to injection of LPS is therefore at least as sensitive to the hormone as the lipid mobilisation response seen in adult locusts, which has an ED_{50} of 0.8 pmol of *Lom*-AKH-I (see Goldsworthy *et al*., 1997). It is intriguing that, even at doses in excess of 100 µg of LPS, nodule formation shows no evidence of becoming maximal. Injecting doses of LPS (from *Serratia marcescens*) up to 200 µg into the beetle *Zophobas atratus,* Bedick *et al*. (2000) showed that formation of cellular microaggregates in the haemolymph (an early stage of nodule formation) was still increasing at the highest dose. These observations suggest that recruitment of haemocytes from the haemopoietic tissue occurs in response to injections of LPS (see discussion by Lackie, 1988), otherwise the number of haemocytes in the haemolymph would drop dramatically, which they do not do according to Hoffmann *et al*. (1974), and severely limit further nodule formation.

Injection of inhibitors of eicosanoid synthesis reduces nodule formation in a number of insects, and this has led to the suggestion that products of cyclo- and lipoxygenases may regulate some aspects of the insect immune response (Miller *et al*., 1994; 1996; 1999; Bedick *et al*., 2000; Miller and Stanley, 2001; Dean *et al*., 2002). In the present study, diclofenac was chosen as an inhibitor of the arachidonic acid cascade in preference to dexamethasone, which has been used extensively in other laboratories. In terms of nodule formation in the locust, dexamethasone produces similar results to diclofenac (G.J. Goldsworthy and S. Chandrakant, unpublished observations), but it is not water-soluble, and is usually dissolved in 95% ethanol before injection. To measure prophenoloxidase activation and nodule formation in the same animals, alcoholic solvents were avoided because of their possible direct effects on prophenoloxidase activation. For this reason the water-soluble diclofenac was preferred, which in mammals is a potent inhibitor of cyclooxygenase at micromolar concentrations, but at higher concentrations also inhibits lipoxygenase pathways (Ku *et al*., 1985). Diclofenac is extremely effective in inhibiting nodule formation in the locust, with an ID_{50} of 1 nmol (*c*. 4 µM in the haemolymph) but it has no inhibitory effect on phenoloxidase activity; strong evidence that eicosanoids are important for nodule formation in the locust, but not for activation of the prophenoloxidase cascade. Further, as suggested by Brookman *et al*. (1989ab), and exemplified by the data provided here (see Fig. 4B), there is not a direct relationship between activation of the prophenoloxidase cascade and nodule formation. Thus, fifth instar nymphs and adults respond to injection of LPS by forming nodules, whereas activation of prophenoloxidase in the haemolymph of

adult locusts (when *Lom*-AKH-I is co-injected) is observed only in mature adults, not in newly emerged adults or fifth instar nymphs.

During adult maturation, changes in responsiveness to injections of laminarin occur that are similar to those observed with injections of LPS: nodules are formed in response to laminarin at all ages of final stage nymphs and adults, whereas only mature adults show activation of prophenoloxidase (Mullen and Goldsworthy, in preparation). This pattern of changes in prophenoloxidase sensitivity to immune stimulators correlates with the pattern of change in the lipid mobilisation response to AKH described by Mwangi and Goldsworthy (1977ab). Starvation causes lipid mobilisation in locusts (Jutsum *et al*., 1975), so the effect described here of starvation facilitating the phenoloxidase response after injection of LPS in the absence of AKH, is consistent with a link between lipid mobilisation and activation of the prophenoloxidase cascade. However, starvation has no effect on nodule formation in locusts, so whatever the involvement of lipid mobilisation in activating prophenoloxidase, it does not appear to be important in nodule formation or its stimulation by *Lom*-AKH-I.

The present study is the first to indicate a possible interaction between a neuropeptide hormone and nodule formation in insects. However, the precise mechanisms of action of AKH in enhancing nodule formation (or phenoloxidase) in locusts remain to be determined. It could be that the interaction between AKH and the immune systems studied here are entirely indirect effects of alterations in the locust's metabolism. Messenger molecules other than AKHs are claimed to affect energy metabolism in insects, and Baines *et al*. (1992) showed that injections of millimolar concentrations of octopamine or 5-hydroxytryptamine stimulate nodule formation in *Periplaneta americana*. Subsequently, Dunphy and Downer (1994) suggested that octopamine is released into the haemolymph during bacterial infection in *Galleria mellonella* and accelerates the removal of injected bacteria or fungal conidia by a combination of two actions: direct action on the haemocytes to enhance nodule formation; and an indirect increase in entrapment by binding to the surface of bacteria to facilitate removal from the haemolymph. Interestingly, these authors found also that the effects of octopamine on nodule formation occurred without activation of prophenoloxidase in the haemolymph. In locusts, however, co-injections of octopamine or 5-hydroxytryptamine with LPS or laminarin have no effect on nodule formation or prophenoloxidase activity (G.J. Goldsworthy, L. Mullen, and S. Chandrakant, unpublished observations). The increased sensitivity of the locust prophenoloxidase activating mechanism after 24 h starvation was initially surprising. In *Rhodnius prolixus*, a change of diet from human blood to plasma reduces nodule formation in response to an immune challenge, but appears to have no effect on the activation of prophenoloxidase (Feder *et al*., 1997). However, in *Lacanobia oleracea* starvation for 48 h causes a doubling of the numbers of circulating haemocytes (Richards and Edwards, 1999). It seems likely, therefore, that starvation affects the immune system in different ways in different species of insect, and this is an area in which more work is needed. In the locust, starvation mimics partly the effect of injecting *Lom*-AKH-I alongside immunogens because of the associated hyperlipaemia: nodule formation is unaffected, but prophenoloxidase activation is enhanced.

Different batches of commercial LPS from the same microbial source can vary considerably in their potency to cause either nodule formation or prophenoloxidase activation (unpublished observations). Furthermore, the commercial preparations of

LPS used here were not subjected to any further purification. Thus the studies undertaken to test different components of LPS extracted from the Rd mutant of *E. coli* should be interpreted with caution. Nevertheless, it seems that while the intact LPS from the Rd mutant activates the prophenoloxidase cascade and initiates nodule production, it is slightly less potent in these respects than the 'standard' preparation of LPS (from *E. coli* serotype 0111, B4) used in other experiments described here. It is intriguing that while the toxic (naturally-occurring form) diphosphoryl Lipid A and the detoxified LPS induce nodule formation, and this is enhanced by co-injection of *Lom*-AKH-I, the non-toxic monophosphoryl (modified) Lipid A does not induce nodule formation, even when injected alongside the hormone. The mono- and the diphosphoryl Lipid A components behave as the intact LPS from the *E. coli* Rd mutant in activating phenoloxidase in the haemolymph only when hormone is co-injected but, remarkably, detoxified LPS activates phenoloxidase in its absence, and co-injection with *Lom*-AKH-I produces a greater response. In insects, as in vertebrates (see Holst *et al*., 1996), there is evidence that it is the Lipid A portion of LPS that is recognised by LPS-binding proteins (Koizumi *et al*., 1997, 1999), although one such insect protein, hemolin (Daffre and Faye, 1997), also has binding sites for the immunogenic core carbohydrate of LPS (Yu *et al*., 2002). The present data are in general support of these views, and it is intriguing that the detoxified LPS (in the absence of *Lom*-AKH-I) activates the prophenoloxidase cascade in a manner reminiscent of the action of the carbohydrate laminarin when it is injected, and the kinetics of its action are currently being investigated.

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Figure 1A. The time course for the formation of nodules after injection of adult male *Schistocerca gregaria* with 20 µl of saline containing 100µg of LPS and 20 pmol of ***of*** *Lom*-AKH-I . The control locusts received only 20 µl of saline. Each point and vertical line represents the mean ± SE for 5 observations. Figure 1B shows a comparison of the number of nodules formed in *Schistocerca* and *Locusta* injected with 100 µg of LPS, with and without *Lom*-AKH-I (20 pmol). The bars and vertical lines represent the means ± SE for 10 observations.

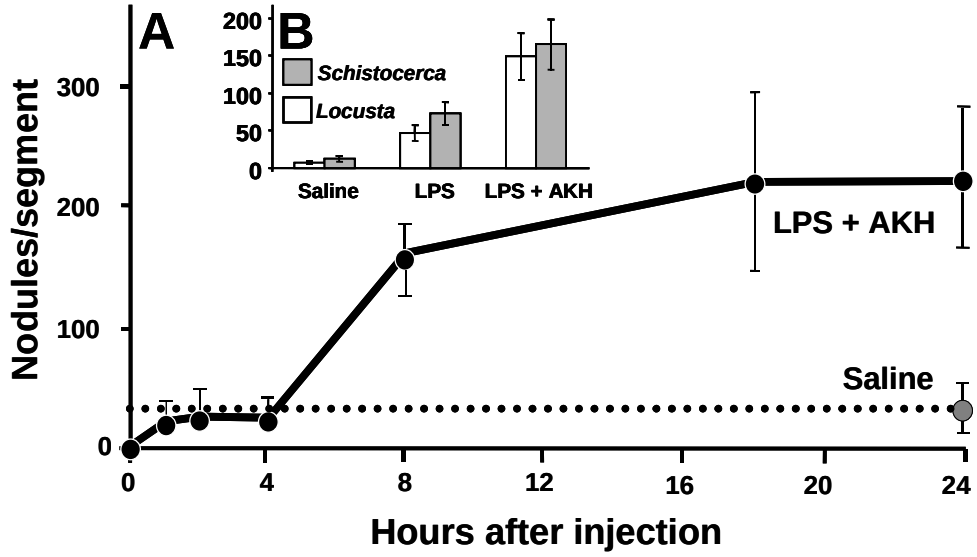

Figure 2. The effect of increasing doses of LPS on nodule formation in adult male *Locusta migratoria*. Two groups of locusts were studied. In both, the concentration of LPS was increased progressively from 0 – 150 µg, and in one group (solid circles) the concentration of *Lom*-AKH-I co-injected was kept constant at 20 pmol. The second group (open circles) did not receive *Lom*-AKH-I. Each data point represents the nodules counted (Mean ± SE) 24 h after injection for 5 locusts at each concentration of LPS.

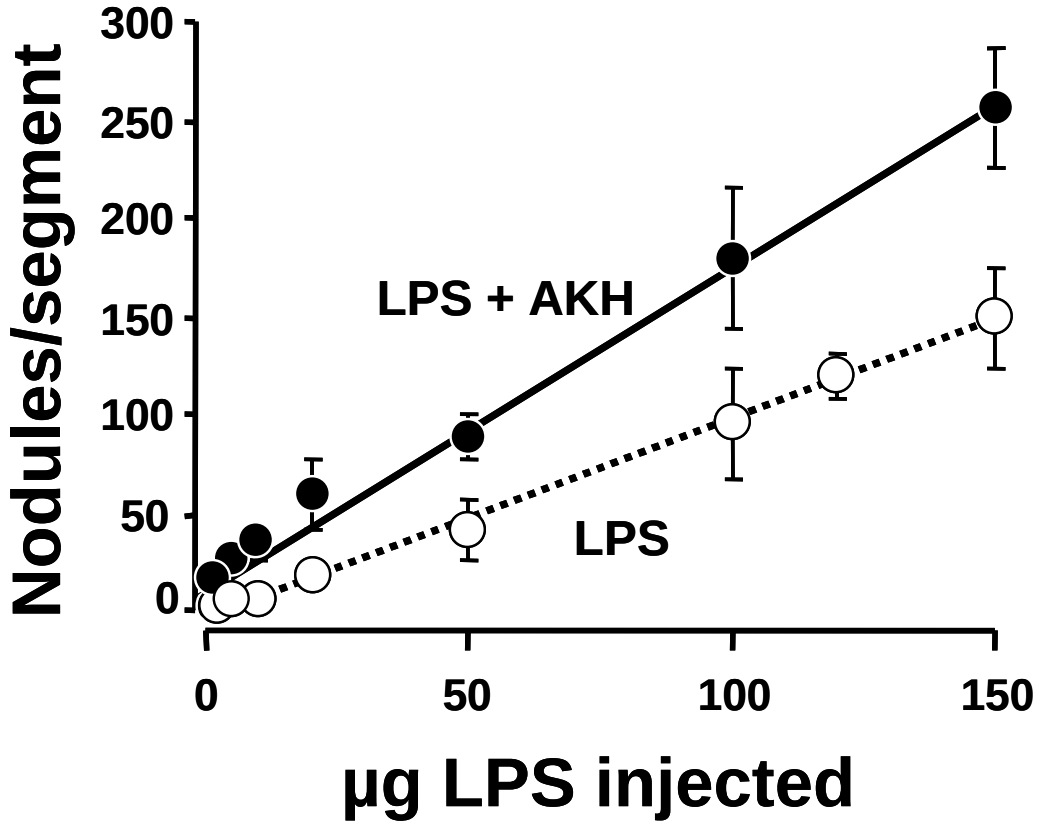

Figure 3. The effect of increasing doses of *Lom*-AKH-I on the formation of nodules in adult male *Locusta migratoria* in response to injection of 100 µg of LPS. The points represent the nodules counted (Mean ± SE) 24 h after injection; 10 observations at each dose of hormone. The curve was fitted as a Hill Plot in FigP (Biosoft), which was also used to estimate the ED_{50} value.

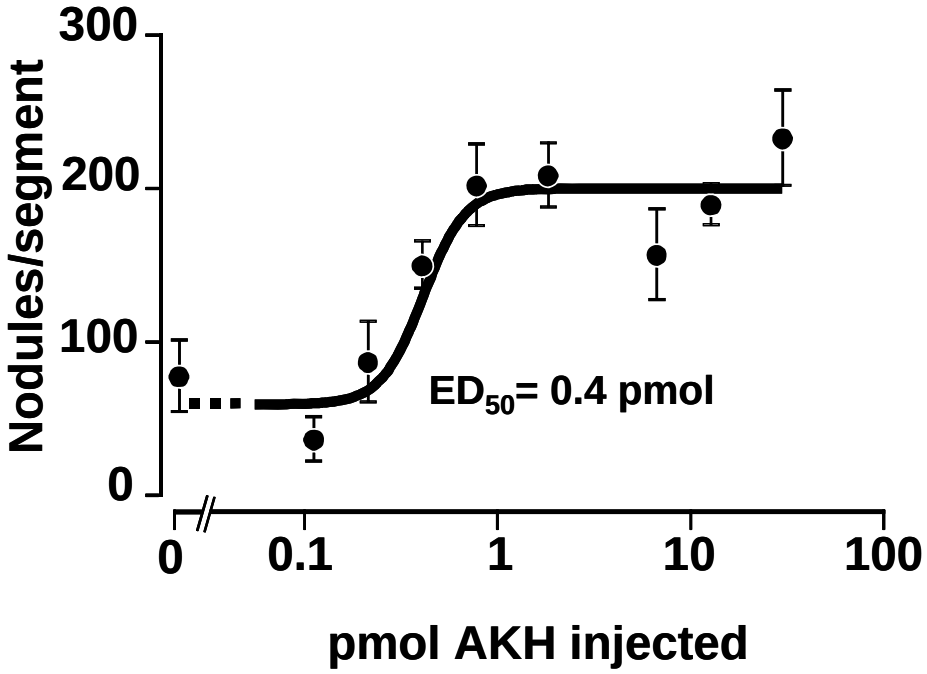

Fig 4
Phenoloxidase activity and nodule formation in fifth instar nymphs (3-6 days old), newly emerged adults (3-6 days old), and mature adult male *Locusta migratoria* (12-16 days old) after injection of LPS (100 µg) with and without *Lom*-AKH-I (20 pmol). Samples of haemolymph were taken 3 h after injection for measurement of phenoloxidase activity in **A**, and nodules were counted 24 h after injection (in the same animals) in **B**. Bars and vertical lines represent the mean ± SE for the number of observations indicated above each bar. Bars with different letters are significantly different (P<0.001 for both nodule formation and phenoloxidase activity).

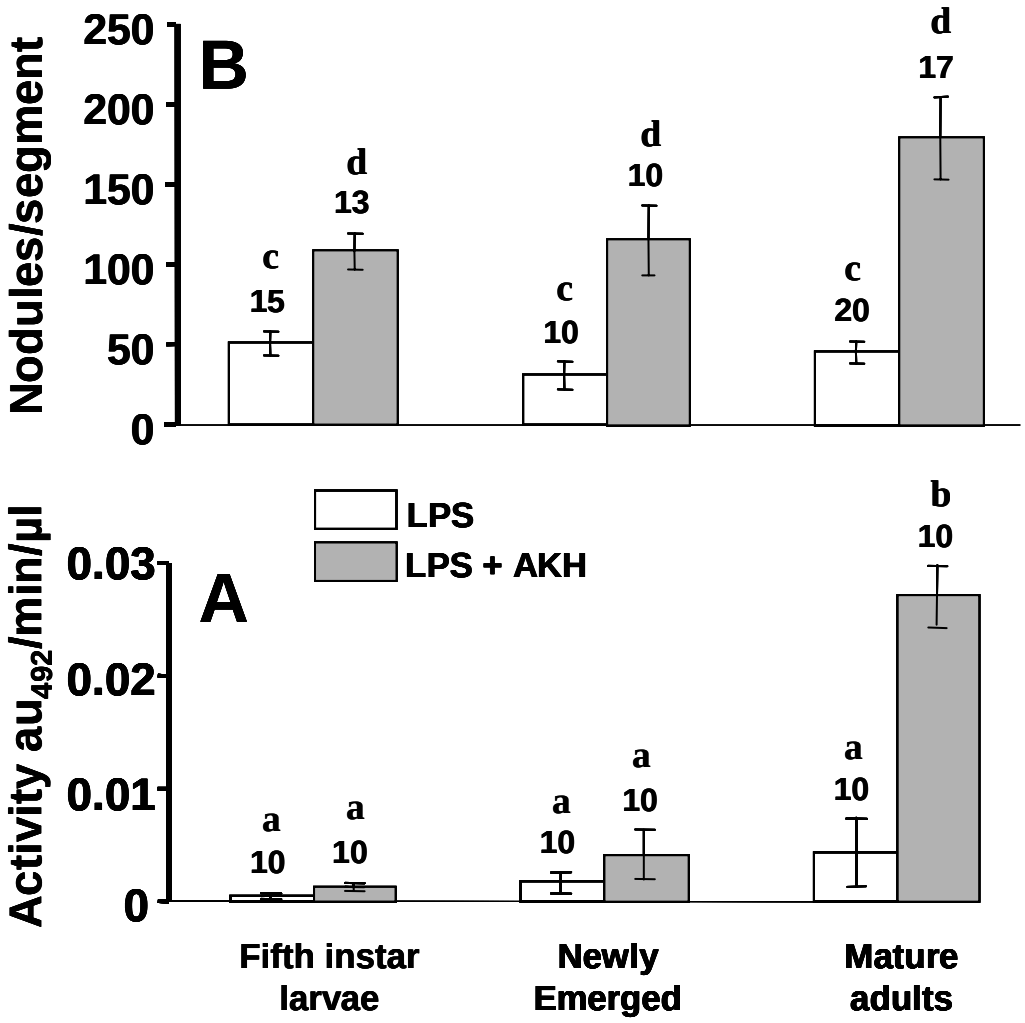

Figure 5. The effects of starvation on phenoloxidase activity and nodule formation in adult male *Locusta migratoria* after injection of LPS with and without *Lom*-AKH-I. Starved locusts were deprived of food for 24 h and given access to water. Groups of these starved locusts, and fed controls of the same age, were injected, and samples of haemolymph taken 3 h later for measurement of phenoloxidase activity in **A**. In **B**,

nodules were counted 24 h after injection in the same animals as in A. Bars and vertical lines represent the mean ± SE for each of 10 observations. Sal=saline; 100 µg of LPS were injected; and AKH= *Lom*-AKH-I (20 pmol injected). Bars with different letters are significantly different from each other (P<0.001).

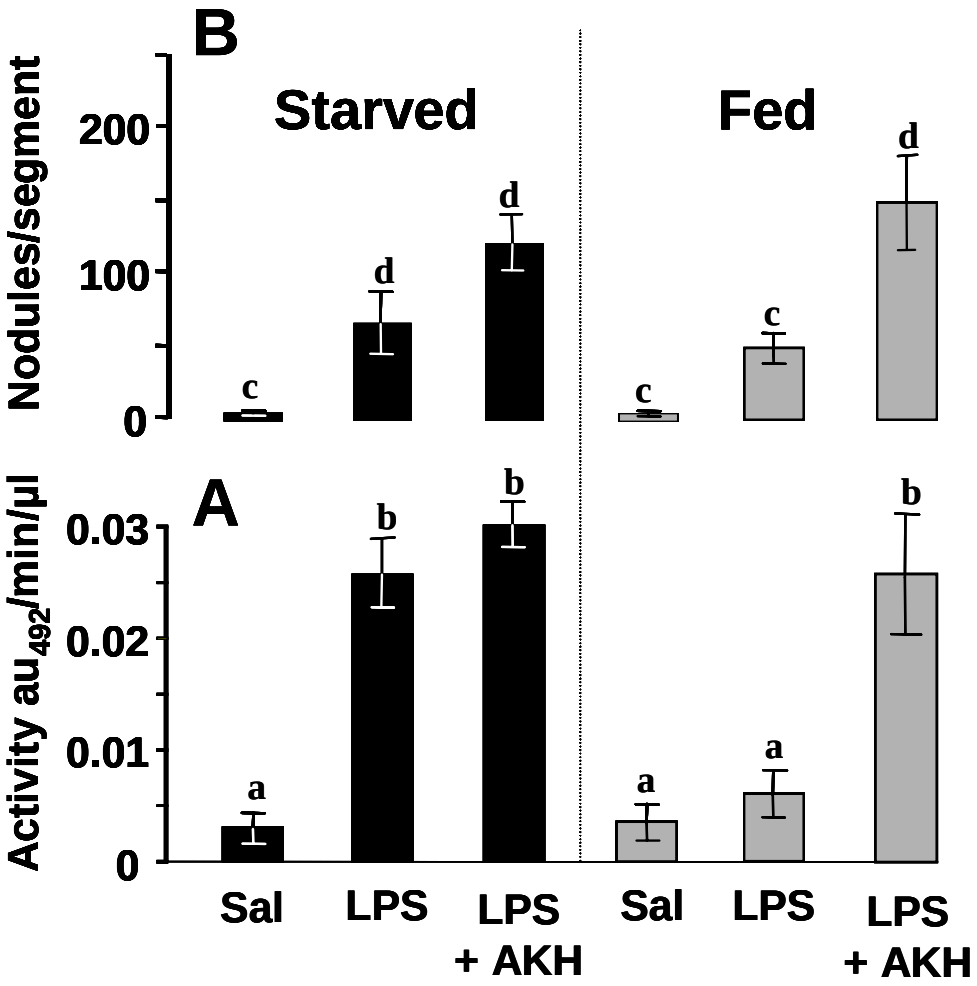

Figure 6. The effect of diclofenac on LPS-induced nodule formation in adult male *Locusta migratoria*. In **A**, increasing doses of diclofenac (D) progressively inhibited nodule formation in response to the injection of LPS and *Lom*-AKH-I. The curve was fitted as a Hill plot in FigP (Biosoft) and used to calculate an ID_{50} of 1 nmol. In **B**, the effect of diclofenac (D, 10 nmol) on LPS-induced nodule formation in the presence and absence of *Lom*-AKH-I is shown, S=saline. The bars and vertical lines represent the means ± SE for 10 observations. Bars with different letters are significantly different from each other (P<0.001). In both A and B the doses of LPS (L) and *Lom*-AKH-I (AKH) were 100 µg and 20 pmol respectively.

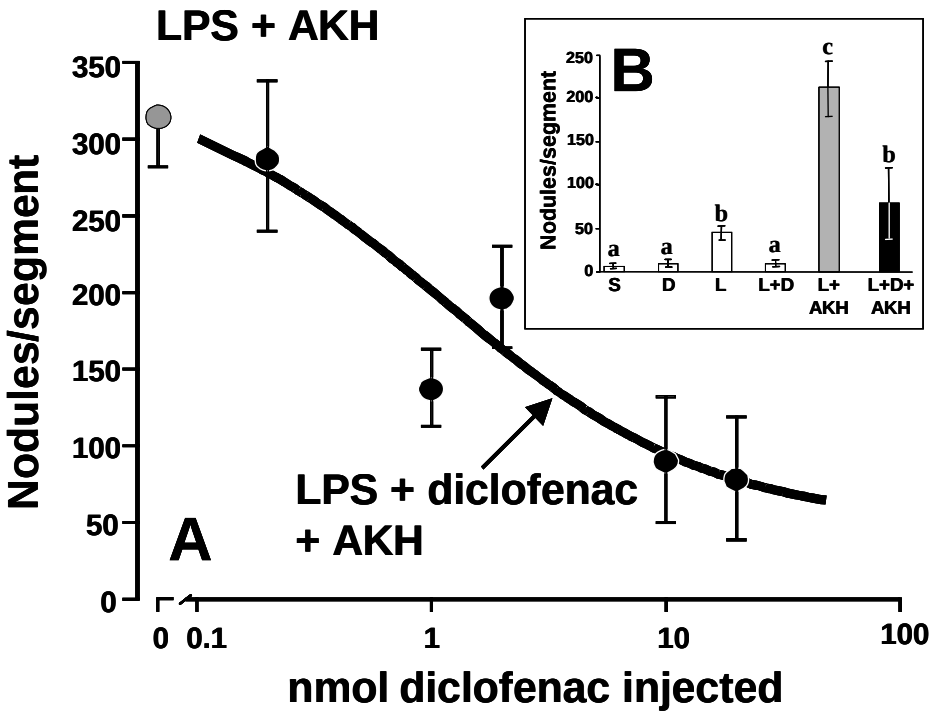
LPS + AKH
Nodules/segment
350
300
250
200
150
100
50
0
A
LPS + diclofenac
+ AKH
0
0.1
1
10
100
nmol diclofenac injected
B
Nodules/segment
250
200
150
100
50
0
a
a
b
a
c
b
S
D
L
L+D
L+
AKH
L+D+
AKH

Figure 7. The effect of diclofenac (D) on phenoloxidase activity in adult male *Locusta migratoria* injected with LPS (L, 100 µg) with and without *Lom*-AKH-I (K, 20 pmol). In **A**, the injection of 10 nmol of diclofenac failed to affect the LPS-induced changes in the activity of phenoloxidase in the presence and absence of hormone: Sal = Saline; Dfc = diclofenac. The bars and vertical lines represent means ± SE for 10 observations. Bars with different letters are significantly different from each other (P<0.001). In **B**, mean values (for at least 5 locusts at each point) for phenoloxidase activity from a range of experiments are plotted against numbers of nodules counted 24 h later.

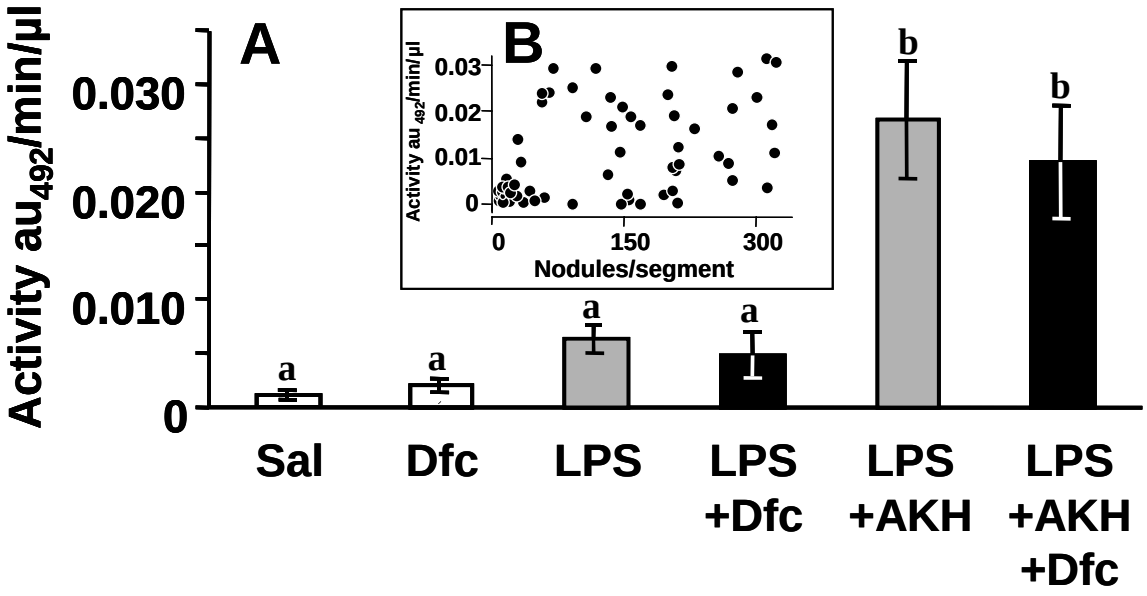

Figure 8. The effect of injecting different components of LPS (100 µg) with (grey-shaded bars) and without (open bars) *Lom*-AKH-I (20 pmol) on phenoloxidase activity (3 h after injection) and nodule formation (24 h after injection) in adult male *Locusta migratoria*. The bars and horizontal lines represent means ± SE for at least 10 observations. Bars with different letters are significantly different from each other (P<0.001 for both nodule formation and phenoloxidase activity).