OIKOS 97: 371–377, 2002

The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*

A. M. Ahmed, S. L. Baggott, R. Maingon and H. Hurd

Ahmed, A. M., Baggott, S. L., Maingon, R. and Hurd, H. 2002. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. – Oikos 97: 371–377.

We have used *Anopheles gambiae*, a major vector of malaria in Africa, to test the hypothesis that the operation of a surveillance or immune system against microorganisms and parasites can be costly to the reproductive success of the host. Blood-fed mosquitoes were challenged with an immune elicitor, lipopolysaccharide (LPS), and their resultant antimicrobial activity, accumulation of yolk protein in the ovary and egg production was monitored. Humoral activity against the Gram-positive bacterium *Micrococcus luteus* was induced by LPS injection in a dose-responsive manner. LPS treatment also caused a concomitant significant reduction in the accumulation of protein in ovaries 24 h after injection and in the production of eggs during the same gonotrophic cycle. Unlike immune stimulation, reduction in reproductive fitness was not dose responsive. Oral administration of LPS also significantly reduced ovarian protein content although we could not detect the presence of anti-*M. luteus* activity in the gut tissue by using an inhibition zone assay. These findings indicate that immune stimulation imposed reproductive fitness costs on mosquitoes.

A. M. Ahmed, S. L. Baggott, R. Maingon and H. Hurd, Center for Applied Entomology and Parasitology, School of Life Sciences, Keele Univ., Staffordshire, ST5 5BG, UK (h.hurd@keele.ac.uk).

Advancement in the area of ecological immunology has been accompanied by the realization that the evolution and deployment of defence mechanisms might impose some costs on other fitness components of the organism. It is argued that this is because the operation of any surveillance or immune function requires resources (Gustafsson et al. 1994, Sheldon and Verhulst 1996, Kraaijeveld and Godfray 1997, Moret and Schmid-Hempel 2000). Although few investigations have assessed the energetic costs of defence they have been demonstrated in antigen-stimulated mice and Blue Tits (Demas et al. 1997, Svenson et al. 1998). Finite resources will result in trade-offs between development and deployment of defence systems and other life-history traits such as longevity and reproduction. Here we focus on the potential costs to reproductive fitness of deployment of immune defence.

Many species of insects act as competent vectors of parasites of medical and veterinary importance despite the array of defence mechanisms that they possess (Lowenberger 1996). In these cases, insect immune systems may keep parasite infections within bounds but do not eliminate them. This may be because they will tolerate infections if the costs involved in eliminating them outweigh the benefits. Furthermore, the response made to pathogens may be linked to phenotypically plastic traits (Barnes and Siva-Jothy 2000) and resource allocation to defence may depend upon the degree of parasite virulence that occurs in a particular strain of host, the particular environmental conditions and the fitness costs to the host associated with changes in various life-history traits (Kraaijeveld et al. 1998, Brown et al. 2000, Moret and Schmid-Hempel 2000).

Accepted 3 December 2001

ISSN 0030-1299

Energetic trade-offs have been reported to be resolved in favour of reproduction in several cases (reviewed by Sheldon and Verhulst 1996). Studies of lactating bighorn ewes (Festa-Bianchet 1989) and breeding great tits (Norris et al. 1994, Richner et al. 1995) demonstrated that investment in reproductive effort led to increased parasite burden as less resources were available for defence. This is also seen in insects, where a forced increase in parental effort correspondingly decreased immune response (Sheldon and Verhulst 1996, Kraaijeveld and Godfray 1997).

In these latter studies, a putative relationship between reproduction and immune function is presumed such that reproduction takes precedence over defence. However, many studies of parasitized insects demonstrate the opposite, namely that infection is associated with a reduction in reproductive effort (Hurd 1990, 2001). In addition, the fixed costs of selection for resistance to infection have been associated with a loss of reproductive effort in a snail-schistosome host-parasite system (Webster and Woolhouse 1999) and a mosquito/filarial nematode association (Ferdig et al. 1993). Although many studies describe parasite-induced fecundity reduction and parasites clearly stimulate a defence response there are few studies that demonstrate a clear physiological link between infection, immune costs and reduced reproduction (but see Ferdig et al. 1993).

Both immune and reproductive systems are controlled by a multitude of signal molecules. It is thus difficult to demonstrate a causal relationship between such complex systems. We have, therefore, designed experiments that simplify the putative infectious agent/immune system/reproduction pathway by removing one aspect, the infectious agent. Thus the hypothesis that direct stimulation of the immune system results in costs to reproductive success can be tested. We have chosen an insect model, the mosquito *Anopheles gambiae*, in which malaria infection causes fecundity reduction (Ahmed et al. 1999) to perform this test.

Both bacterial and malaria infection causes activation of the mosquito humoral defence system. mRNA of the *A. gambiae* homologue of Gram-negative bacteria binding protein from *Bombyx mori* is upregulated after infection with bacteria (Dimopoulos et al. 1997, 2001) and malaria (Richman et al. 1997). Richman and co-workers (1997) showed that levels of mRNA of the antimicrobial peptide defensin were also upregulated following malaria infection although Lowenberger et al. (1999), using RT-PCR, were unable to detect a significant increase in defensin transcript following infection with the same species of rodent malaria. Defensins are active against Gram-positive bacteria and form voltage-dependent channels in the cytoplasm membrane of *Micrococcus luteus* (Cociancich et al. 1993). Their activity can be detected with the anti-*M. luteus* activity assay used in this study.

We have used the non-pathogenic microbial immune elicitor, lipopolysaccharide (LPS), from Gram-negative bacteria to stimulate the humoral immune response of blood-fed *A. gambiae* females and monitored the upregulation of antimicrobial peptide production in parallel with reproductive fitness. LPS induces several pathways of the insect immune system and has been shown strongly to upregulate the promoter of an *A. gambiae* defensin gene in cultured cells, possibly *via* the NF-κB/Rel pathway (Eggleston et al. 2000). LPS is innocuous to most cells and tissues (Beutler 2000) and it is thus unlikely that it is toxic to cells of the ovarian follicular epithelium.

Our previous experience of monitoring fitness of anopheline mosquitoes (Hurd et al. 1995, Jahan and Hurd 1998, Ahmed et al. 1999) has led us to select two fitness parameters to measure. These were the accumulation of yolk protein (vitellin) in the ovaries and the production of mature eggs during the gonotrophic cycle following blood feeding and immune stimulation. Here we describe the effect of artificial immune stimulation upon the reproductive success of *A. gambiae* during one gonotrophic cycle.

Materials and methods

A stock colony of *A. gambiae* (KIL strain) was maintained in standardised conditions designed to produce adults of similar size (Ahmed et al. 1999). Mosquito size affects egg production (Hurd et al. 1995) and each treatment group was therefore checked to verify that they were of similar size range by measuring wing lengths (Briegel 1990).

Blood feeding and immune stimulation

Nulliparous (not having blood fed or produced an egg batch), 6 day-old mosquitoes, were starved for 12 h before blood feeding on an anaesthetised CD strain mouse and fully engorged females were randomly assigned to one of three groups. Mosquitoes were either injected with filter sterilized *Aedes* physiological saline (APS) (Ahmed et al. 2001), to act as a trauma control, or lipopolysaccharide (LPS) (0.08 mg/ml APS) or left as uninjected controls. Intrathoracic injections were performed using a sterile, finely drawn glass capillary needle, pre-calibrated to inject 0.25 μl/mosquito. Mosquitoes were maintained in cages ($6 \times 6 \times 6$ cm) in standard insectary conditions. No difference in the mortality of LPS or sham-injected mosquitoes was observed.

To investigate the effect of different doses of LPS, fully engorged mosquitoes were divided randomly into

5 groups immediately after blood feeding (30 mosquitoes each). Three groups were injected with LPS (10, 20 or 40 ng), one group was injected with APS alone (sham injected) and one group was not injected (control). Groups were kept separately in small cages prior to haemolymph extraction at 24 h post-feeding/injection.

To administer LPS orally, two groups of approximately 100 mosquitoes were randomly selected and starved as above. Each group was fed on freshly collected mouse blood via a membrane feeder (Hemotek Membrane Feeding System). The blood in one feeder was supplemented with 0.5 mg LPS/ml. Fully engorged mosquitoes from each group were maintained ($6 \times 6 \times 6$ cm cages) for 24 h.

Antibacterial activity assay

Micrococcus luteus (NCTC 2665) (Sigma, UK) was incubated in nutrient broth (13 g/l) at 37°C for 48 h (Nimmo et al. 1997). After incubation, 1 ml of *M. luteus* culture was mixed with 7 ml nutrient agar and then poured into a sterile Petri dish (∅ 9 cm; Sterilin). On setting, 1 mm wells were punched in the agar at positions equidistant from the edge and any remaining liquid was removed. The dishes were used for inhibition zone assays.

Individual samples of haemolymph from experimental mosquitoes ($n = 5$ per group) were subjected to an inhibition zone assay, to measure humoral activity against Gram-positive bacteria (*M. luteus*). Haemolymph was collected from chilled mosquitoes 24 h post-blood feeding/injection using a pre-calibrated capillary needle inserted into the thorax below the wing. Clear haemolymph (0.5 µl) was drawn up and immediately added to 500 µl sterile anticoagulant II solution (Mead et al. 1986). The haemolymph dilution factor was determined as optimal during a pilot study (details not shown).

Lysozyme was used as a standard for activity against *M. luteus* at doubling dilutions ranging from 1000 ng/µl down to 16.6 ng/µl. One µl of the diluted haemolymph was added to each well on the bacterial seeded agar dishes and incubated for 16 h at 37°C. The diameters of optical clearing zones were measured. Test haemolymph and standards were assayed in each Petri dish ($n = 5$ dishes). Antibacterial peptide activity was calculated as lysozyme equivalents using a standard curve produced from measurements of the inhibition zone diameters produced around the wells.

Midguts from the oral administration experiment were dissected and washed in APS. Fifty midguts from control blood-fed or from LPS blood-fed mosquitoes were pooled in 300 µl of 200 mM sodium acetate, homogenised and then further disrupted by ultrasound sonication for 15 sec on ice. The homogenates were boiled for 5 min, cooled and centrifuged for 10 min at 1300 g. Supernatants (µl) were assayed for antimicrobial activity and activity compared with lysozyme as described above.

Measurements of ovarian protein

A crude preparation of *A. gambiae* vitellin, to be used as a standard, was prepared from gravid females 48 h post-blood meal and purified as outlined in Ahmed et al. (2001). The concentration of soluble proteins in the standard was measured using a Coomassie Blue binding assay (BioRad), with bovine serum albumin as standard.

The total protein content of ovaries from the same mosquitoes as used for inhibition zone assay (24 h post-feeding/injection) was measured as above. The sample concentration was adjusted to 100 ng/µl, and then stored at −80°C. Ovary pairs from 2 mosquitoes were pooled and ten replicates from LPS-injected, sham-injected or control blood fed uninjected mosquitoes were analysed.

Ovaries from 50 orally stimulated or control mosquitoes were pooled into groups of 5 pairs and the protein content of 10 groups of ovaries, from treated or control mosquitoes, was also determined.

The effect of LPS on egg production

Groups of 20 fully engorged females were either injected with LPS (20 ng), sham injected or uninjected and the maintained individually in small cages ($6 \times 6 \times 6$ cm). Plastic dishes containing water were provided for oviposition from day 2 onwards. The number of eggs laid per female was counted and ovaries were dissected to count the retained eggs that had fully developed. Total egg production was calculated by summing the laid and retained eggs.

Statistical analysis

All statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA). Data were tested for normality and variance homogeneity prior to further analysis. To determine overall effects of LPS on antimicrobial activity and fecundity comparisons between test and control groups were made using one-way ANOVA and the Kruskal-Wallis test. The Mann-Whitney *U* test (for individual comparison) was used for analysing the non-parametric data resulting from the measurement of the ovarian protein content and wing lengths.

Results

The effect of LPS on mosquito antimicrobial activity

Humoral activity against *M. luteus* was detected in haemolymph from blood-fed uninjected females 24 h post-feeding that was equivalent to the activity of 12.61 ± 2.96 ng lysozyme (Fig. 1A). Injection of blood fed mosquitoes caused an overall significant effect (one-way ANOVA: $F_{2,12} = 11.84$, $p < 0.001$, $n = 5$ per treatment). A single dose of 20 ng LPS, injected immediately after a blood meal, caused a mean increase of 160% in anti-*M. luteus* activity (Tukey's pairwise comparison; $p < 0.05$). Sham injection of saline resulted in a slight, but non-significant increase in anti-*M. luteus* activity compared with constitutive activity in blood-fed control mosquitoes (Tukey's pairwise comparison, $p > 0.05$).

No antimicrobial activity could be detected in the aliquots of midgut extract that were tested after oral administration of LPS.

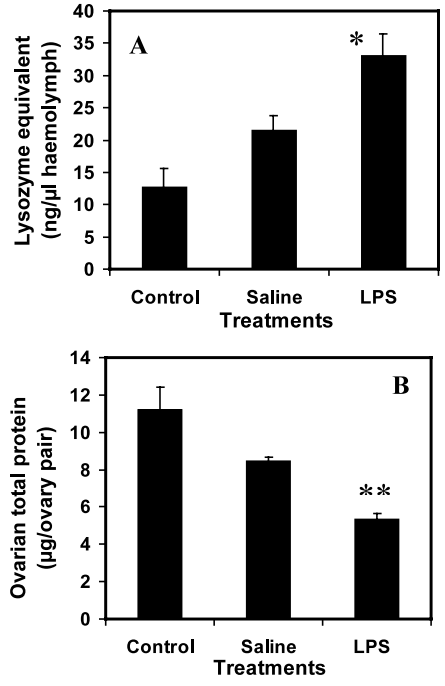

Fig. 1. Anti-*M. luteus* humoral activity in *A. gambiae* haemolymph and ovarian protein content as a result of injecting 20 ng/LPS mosquito. A: Values for lysozyme equivalent antibacterial activity were obtained using an inhibition zone assay against *M. luteus*. Error bars = SEM, in each case; $n = 5$ samples, each pooled from 5 mosquitoes; * = significantly different from the control (ANOVA followed by Tukey's pairwise comparison $p < 0.05$). B: Ovarian protein content was measured using a Coomassie Blue BioRad assay 24 h post-feeding/injection. ** = significantly different from control (Student's *t* test, $p < 0.0001$, $n = 10$ for each treatment).

The effect of LPS on mosquito ovarian protein

Ovaries from control *A. gambiae* 24 h post bloodmeal contained a mean concentration of 11.2 ± 1.2 µg protein/ovary pair (Fig. 1B). Injection caused an overall significant change in protein content (one-way ANOVA: $F_{2,12} = 16.0$, $p > 0.05$, $n = 10$ for each treatment). Ovarian protein content decreased significantly by 52.2% when 20 ng LPS was injected (Tukey's pairwise comparison, $p < 0.05$) whereas injection of saline into the haemolymph caused a non-significant, decrease in ovarian protein (Tukey's pairwise comparison, $p > 0.05$). The reduction in ovarian protein content could not be attributed to body size differences between the control and injected mosquito groups since the mean wing length of groups did not differ significantly (control, 2.77 ± 0.02 mm; sham injected, 2.78 ± 0.02 mm; LPS injected, 2.73 ± 0.02 mm, Mann-Whitney *U* test, $p > 0.05$, $n = 20$ in each case)

The mean total protein content of ovaries from mosquitoes that had been fed on blood containing LPS was significantly reduced by 29.45% compared with the group that had been fed blood without LPS (Student's *t* test: $p < 0.0001$; $n = 10$).

The dose effect of LPS

LPS was shown to affect the humoral defence system in a dose-dependent manner (one-way ANOVA: $F_{4,20} = 16.91$, $p < 0.05$) (Fig. 2A). Mean activity against *M. luteus* was again significantly increased by injection of 10 ng LPS compared to sham injected or control mosquitoes (Tukey's pairwise comparison, $p < 0.05$). Increasing the LPS dose to 40 ng significantly increased the immune response compared with 10 ng LPS (Tukey's pairwise comparison, $p < 0.05$). In contrast, no dose-dependent effect of LPS was seen in the ovarian total protein content; all doses of LPS produced a similar, significant, decrease in ovarian protein compared to non-injected or saline injected controls (Mann-Whitney *U* test, $p < 0.05$, $n = 10$) (Fig. 2B).

The effect of immune stimulation on fecundity

Total egg production was reduced by 18.5% in the group of mosquitoes that were immune stimulated with LPS injection (one-way ANOVA: $F_{2,55} = 5.16$, $p < 0.05$). Sham injection had no significant effect upon egg production (Tukey's pairwise comparison, $p > 0.05$) (Table 1). The reduction in production could not be attributed to body size differences between the control and injected mosquito groups since there was no significant difference in wing lengths between groups (data not shown but similar to previous measurements). Throughout the study only three mosquitoes retained a few eggs whilst ovipositing the majority of the egg

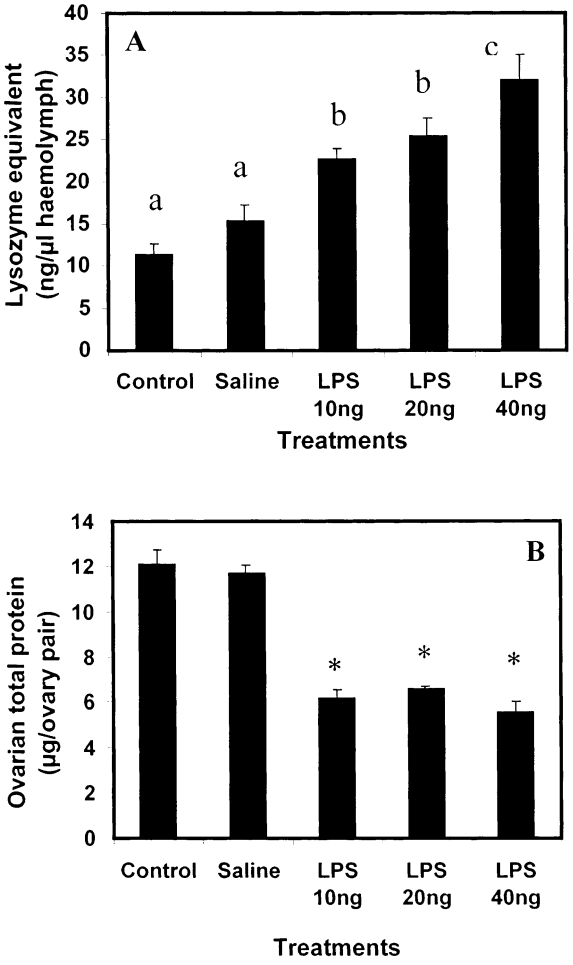

Fig. 2. Dose-dependent anti-*M. luteus* humoral activity and ovarian protein content in *A. gambiae* as a result of injecting three different concentrations of lipopolysaccharide A: Values for lysozyme equivalent antibacterial activity were obtained using an inhibition zone assay. Bars with different letters are significantly different from each other (Tukey's pairwise comparison, $p < 0.05$). Error bars = SEM, in each case; $n = 5$ samples, each pooled from 5 mosquitoes. B: Ovarian protein content was obtained using a Coomassie Blue BioRad assay 24 h post-infection/injection. Error bars = SEM; * = significantly different from the control (Mann-Whitney *U* test, $p < 0.05$, in each case $n = 10$ samples pooled from 5 mosquitoes).

batch. However, mosquitoes maintained individually are reluctant to oviposit, even when suitable sites are provided (personal observation) and approximately 40% of the females in each groups contained a complete, fully mature batch of eggs. Treatment had no significant effect on oviposition (Kruskal-Wallis test; $p = 5.00$).

Discussion

Our data demonstrate that immune stimulation results in the allocation of fewer resources to reproduction in *A. gambiae*. This conclusion is based on the assumption that LPS, in the doses administered here, is not toxic to the mosquito because injection did not increase mortality relative to sham-injected mosquitoes. It would thus appear that, in this mosquito, operation of a defence response is costly and that a life-history strategy exists whereby maximum fitness is achieved by balancing competing needs, such that mortality is not affected (Hurd 2001).

In this study we have assessed the variable costs associated with a general stimulation of the humoral system and the resultant production of antimicrobial peptides. Reduced fecundity was also demonstrated to be a variable cost when a cellular response, resulting in parasitoid encapsulation, was demonstrated in *Drosophila melanogaster* (Fellows et al. 1999). Both artificial stimulation of the cellular defence system and LPS injection have also been shown to incur costs, in terms of survival, in bumblebees under starvation conditions (Moret and Schmid-Hempel 2000) and a trade off between immune activity and longevity appears to operate in these insects (Doums and Schmid-Hempel 2000).

Our findings offer a different perspective to other studies, where resources appear to be diverted from defence towards reproduction when reproductive effort was forced to be larger (see review by Sheldon and Verhulst 1996 and Deerenberg et al. 1997). However, whichever way resource allocation operates, physiological links between immune stimulation, infection and fecundity reduction clearly exist, although they remain undefined. In our model, there could be direct competition between the defence and reproductive systems for nutrients required for the synthesis of molecules involved in the immune cascade, as proposed by Ferdig et al. (1993) for a mosquito/filarial nematode system. However, unless specific limiting factors are involved, this would seem unlikely as only 19% of nutrients obtained from a blood meal are utilized for egg production in *A. gambiae* (Briegel 1990) and the excess is likely to be greater than that required to synthesise antimicrobial molecules. Svensson and co-workers concluded that energy or nutrient limitation may not be the basis for a trade-off between energetically costly activities and immunocompetence in immunized Blue Tits in their study (Svenson et al. 1998).

An alternatively explanation is that induction of an immune response via one or more "danger signals" (Bendelac and Fearon 2000) may also induce, directly or indirectly, effects that are unrelated to the defence system but result in reduced egg production. For example, by inducing follicle cell apoptosis as seen in malaria-infected *A. stephensi* (Hopwood et al. 2001) or the synthesis of antigonadotrophin molecules such as the trematode-induced release of schistosomin seen in a snail, infected with *Trichobilharzia ocellata* (De Jong Brink et al. 1997).

Table 1. Effect of LPS injection (20 ng/mosquito) on *A. gambiae* fecundity.

	Control	Sham injected	LPS injected
Total no. of eggs laid per female	87.00 ± 3.68	84.67 ± 4.89	70.90 ± 3.45*
Wing length (mm)	2.78 ± 0.021	2.80 ± 0.025	2.73 ± 0.026
No. of mosquitoes	20	18	20

* = significantly less than control mosquitoes (ANOVA: $F_{2,55} = 5.16$, $p < 0.05$) followed by Tukey's pairwise comparison ($p < 0.05$).

The observation that LPS stimulated the immune system in a dose-responsive manner, whereas fecundity reduction was not related to degree of defence response, also suggests that direct competition for resources is not involved. This latter observation is in keeping with the findings of Moret and Schmid-Hempel (2000) who reported that LPS induced the production of antibacterial molecules in a dose-related manner in worker bumble bees, but no dose response was observed in the LPS-induced reduction in the survival of food-deprived workers.

This lack of a relationship between LPS concentration or parasite biomass and virulence suggests that these factors are decoupled, as is also seen in trypanosome-infected bumblebees (Brown et al. 2000) and parallels observations of several parasite-infected insects that have been studied. Malaria-infected mosquitoes and blackflies infected with a filarial nematode both exhibit fecundity reduction that is not correlated with parasite density (e.g. Renshaw and Hurd 1994, Hogg and Hurd 1995). A possible explanation for the fitness cost that we have demonstrated to be associated with malaria infection in anopheline mosquitoes (Ahmed et al. 1999) is that infection initiates a defence response that is, in itself, costly to the mosquito. This defence response then contributes in part or entirely to a reduction in egg production even though the link is indirect and not related to nutrient competition.

Mosquitoes are known to mount a defence response to malaria parasites and antimicrobial molecules have been shown to be toxic to the parasite stages present in mosquitoes (e.g. Shahabuddin et al. 1998). These observations have led to the proposal that mosquitoes could be genetically modified to be incompetent vectors by enhancing some element of the defence system such as defensin production (e.g. Kohoza et al. 2000). If this strategy is adopted, the modified mosquitoes might pay a price for enhanced immune competence in terms of reduced reproductive fitness.

In addition to variable costs caused by immune stimulation, such as those described here and elsewhere (e.g. Doums and Schmid-Hempel 2000), fixed costs are also involved in developing and maintaining a defence system (Kraaijeveld and Godfray 1997). The manner of the resolution of these short and long term trade-off positions associated with defence costs will be a result of the selection pressures imposed by parasites and pathogens on their hosts. A better understanding of the costs and benefits of mounting a defence response will help us to understand the co-evolution of parasites and their hosts and vectors.

Acknowledgements – We are indebted to the Egyptian Government for funding for A. M. A. and to Mrs. F. H. Zolkipli and Mr. N. Bowers for technical assistance.

References

Ahmed, A. M., Taylor, P., Maingon, R. and Hurd, H. 1999. The effect of *Plasmodium yoelii nigeriensis* on the reproductive fitness of *Anopheles gambiae*. – Invertbr. Reprod. Dev. 36: 217–222.

Ahmed, A. M., Maingon, R., Romans, P. and Hurd, H. 2001. Effect of infection on vitellogenesis in *Anopheles gambiae* during two gonotrophic cycles. – Insect Molec. Biol. 10: 347–356.

Barnes, A. I. and Siva-Jothy, M. T. 2000. Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. – Proc. R. Soc. Lond. B Biol. Sci 267: 177–182.

Bendelac, A. and Fearon, D. T. 2000. Innate immunity receptors and efforts of innate immunity. – Curr. Opin. Immunol. 12: 11–12.

Beutler, B. 2000. Tlr4: central component of the sole mammalian LPS sensor. – Curr. Opin. Immunol. 12: 20–26.

Briegel, H. 1990. Fecundity, metabolism and body size in *Anopheles stephensi* (Diptera: Culicidae) vector of malaria. – J. Med. Entomol. 27: 839–856.

Brown, M. J. F., Loosli, R. and Schmid-Hempel, P. 2000. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. – Oikos 91: 421–427.

Cociancich, S., Ghazi, A., Hetru, C. et al. 1993. Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus*. – J. Biol. Chem. 268: 19239–19245.

Deerenberg, C., Apanius, V., Daan, S. and Bos, N. 1997. Reproductive effort decreases antibody responsiveness. – Proc. R. Soc. Lond. B Biol. Sci. 264: 1021–1029.

Demas, G. E., Chefer, V., Talan, M. C. and Nelson, R. J. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. – Am. J. Physiol. 273: 1631–1637.

Dimopoulos, G., Richman, A. M., Muller, H. - M. and Kafatos, F. C. 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. – Proc. Natl. Acad. Sci. USA 94: 11508–11513.

Dimopoulos, G., Muller, H.-M., Lavashina, E. A. and Kafatos, F. C. 2001. Innate immune defence against malaria infection in the mosquito. – Curr. Opin. Immunol. 13: 79–88.

Doums, C. and Schmid-Hempel, P. 2000. Immunocompetence in workers of a social insect, *Bombus terrestris* L., in relation to foraging activity and parasitic infection. – Can. J. Zool. 78: 1060–1066.

Eggleston, P., Lu, W. and Zhao, Y. 2000. Genomic organization and immune regulation of the defensin gene from the mosquito, *Anopheles gambiae*. – Insect Mol. Biol. 9: 481–490.

Fellows, M. D. E., Kraaijeveld, A. R. and Godfrey, H. C. J. 1999. The relative fitness of *Drosophila melanogaster* (Diptera, Drosophilidae) that have successfully defended themselves against the parasitoid *Asobara tabida* (Hymenoptera, Braconidae). – J. Evol. Biol. 12: 123–126.

Ferdig, M. T., Beerntsen, B. T., Spray, F. J. et al. 1993. Reproductive costs associated with resistance in a mosquito-filarial worm system. – Am. J. Trop. Med. Hyg. 49: 756–762.

Festa-Bianchet, M. 1989. Individual differences, parasites and the costs of reproduction for bighorn ewes (*Ovis candensis*). – J. Anim. Ecol. 58: 785–795.

Gustafsson, L., Nordling, D., Andersson, M. S. et al. 1994. Infectious-diseases, reproductive effort and the cost of reproduction in birds. – Philos. Trans. R. Soc. Lond. B Biol. Soc. 346: 323–331.

Hogg, J. C. and Hurd, H. 1995. *Plasmodium yoelii nigeriensis*: the effect of high and low intensity of infection upon the egg production and blood meal size of *Anopheles stephensi* during three gonotrophic cycles. – Parasitology 111: 555–562.

Hopwood, J. A., Ahmed, A. M., Polwart, A. et al. 2001. Malaria-induced apoptosis in mosquito ovaries: a mechanism to control vector egg production. – J. Exp. Biol. 204: 2773–2780.

Hurd, H. 1990. Physiological and behavioural interactions between parasites and invertebrate hosts. – Adv. Parasitol. 29: 271–317.

Hurd, H. 2001. Host fecundity reduction: a strategy for damage limitation? – Trends Parasitol. 17: 363–368.

Hurd, H., Hogg, J. C. and Renshaw, M. 1995. Interaction between blood-feeding, fecundity and infection in mosquitoes. – Parasitol. Today 11: 411–416.

Jahan, N. and Hurd, H. 1998. Effect of *Plasmodium yoelii nigeriensis* (Haemosporidia: Plasmodiidae) on *Anopheles stephensi* (Diptera: Culicidae) vitellogenesis. – J. Med. Entomol. 35: 956–961.

De Jong Brink, M., Hoek, R. M., Lageweg, W. and Smit, A. B. 1997. Schistosome parasites induce changes in their snail host by interfering with two regulatory systems, the internal defence system and the neuroendocrine system. – In: Beckage, N. E. (ed.), Parasites and pathogens effects on host hormones and behaviour. Chapman and Hall, pp. 57–75.

Kohoza, V., Ahmed, A., Cho, W.-L. et al. 2000. Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. – Proc. Nat. Acad. Sci. USA 97: 9144–9149.

Kraaijeveld, A. R. and Godfray, H. J. C. 1997. Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. – Nature 389: 278–280.

Kraaijeveld, A. R., Van Alphen, J. J. M. and Godfrey, H. C. J. 1998. The coevolution of host resistance and parasitoid virulence. – Parasitology 116: S29–S45.

Lowenberger, C. A. 1996. Insect immunology: mosquito immune responses. – Lab. Animal 25: 30–33.

Lowenberger, C. A., Smartt, C. T., Bulet, P. et al. 1999. Insect immunity: molecular cloning, expression, and characterization of cDNAs and genomic DNA encoding three isoforms of insect defensin in *Aedes aegypti*. – Insect Mol. Biol. 8: 107–118.

Mead, G. P., Ratcliffe, N. A. and Renwrantz, L. R. 1986. The separation of insect haemocyte types on percoll gradients: methodology and problems. – J. Insect Physiol. 32: 167–177.

Moret, Y. and Schmid-Hempel, P. 2000. Survival for immunity: the price of immune system activation for bumblebee workers. – Science 290: 1166–1168.

Nimmo, D. D., Ham, P. J., Ward, R. D. and Maingon, R. 1997. The sand fly *Lutzomyia longipalpis* shows specific humoral responses to bacterial challenge. – Med. Veter. Entomol. 11: 324–328.

Norris, K., Anwar, M. and Read, A. F. 1994. Reproductive effort influences the prevalence of haematozoan parasites in great tits. – J. Anim. Ecol. 63: 601–610.

Renshaw, M. and Hurd, H. 1994. The effect of *Onchocerca* infection on the reproductive physiology of the British blackfly, *Simulium ornatum*. – Parasitology 109: 337–345.

Richman, A. M., Dimopoulos, G., Seeley, D. and Kafatos, F. C. 1997. *Plasmodium* activates the innate immune response of *Anopheles gambiae* mosquitoes. – EMBO 16: 6114–6119.

Richner, H., Christie, P. and Oppliger, A. 1995. Parental investment affects prevalence of malaria. – Proc. Natl. Acad. Sci. USA 92: 1192–1194.

Shahabuddin, M., Fields, I., Bulet, P. et al. 1998. *Plasmodium gallinaceum* differential killing of some mosquito stages of the parasite by insect defensin. – Exp. Parasitol. 89: 103–112.

Sheldon, B. S. and Verhulst, S. 1996. Ecological immunology: costly parasite defenses and trade-offs in evolutionary ecology. – Trends Ecol. Evol. 11: 317–321.

Svenson, E., Rabert, L., Koch, C. and Hasselquist, D. 1998. Energetic stress, immunosupression and the cost of an antibofy response. – Funct. Ecol. 12: 912–919.

Webster, J. P. and Woolhouse, M. E. J. 1999. Cost of resistance: relationship between reduced fertility and increased resistance in a snail-schistosome host-parasite system. – Proc. R. Soc. Lond. B 266: 391–396.