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Original article

Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis

Ashraf M. Ahmed [a], Hilary Hurd [b,*]

[a] *Department of Zoology, Faculty of Sciences, University of El-Minia, El-Minia, Egypt*
[b] *Center for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Staffordshire CW3 9EY, UK*

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Abstract

The employment of defense mechanisms is recognized as a costly life-history trait. In the malaria vector *Anopheles gambiae*, reproductive costs have been associated with both humoral and cellular innate immune responses and also with malaria infection. The resorption of developing oocytes associated with malaria infection is preceded by the programmed cell death, or apoptosis, of follicular cells. Here we demonstrate that apoptosis in ovarian follicular epithelial cells also occurs when mosquitoes are subjected to artificial immune-elicitors that induce a melanization response or humoral antimicrobial activity. Caspases are key cysteine proteases involved in apoptosis. Caspase-like activity was detected in epithelial cells in approximately 4.0% of the developing ovarian follicles of untreated, blood-fed, mosquitoes. Lipopolysaccharide injection resulted in a significant increase in anti-*Micrococcus luteus* humoral activity and a significant increase of 257.7% of follicles exhibiting apoptosis compared to results after saline injections. Melanization also triggered follicular apoptosis, which increased by 106.25% or 134.37% in Sephadex C-25 or G-25 bead-inoculated mosquitoes, respectively, compared to that in sham-injected ones. Ovaries from *Plasmodium yoelii nigeriensis*-infected mosquitoes exhibited a significant increase in follicular apoptosis of 440.9% compared to non-infected ones. Thus, at the time point investigated, infection had a much greater effect than artificial immune-elicitors. Death of follicular epithelial cells has been shown to lead to follicle resorption and hence a decrease in egg production. We propose the trade-off between reproductive fitness and immune defense in *A. gambiae* operates via the induction of apoptosis in ovarian follicles and that different immune responses impose costs via the same pathway.

Keywords: Defense; Fitness costs; Apoptosis; Antimicrobial activity; Melanization; *Anopheles gambiae*; *Plasmodium yoelii nigeriensis*

1. Introduction

Despite the obvious benefits of eliminating infectious agents, the deployment of defense systems can be costly, and may result in a reduction in the organism's fitness [1]. Insects provide good examples of the principle that the fitness of an organism may be traded-off against immune defense. Although these costs have been recognized in many systems [2–6], with a few exceptions [7,8], we lack an understanding of the mechanism by which they are imposed.

The mosquito, *Anopheles gambiae*, has now provided the first evidence of how immune stimulation can cause a reduction in fecundity by inducing the resorption of ovarian follicles during oocyte development. *A. gambiae* is autogenous and thus requires the nutritious content of a blood meal to produce eggs. Following a blood meal, the follicular epithelial cells of the terminal follicles in the mosquito ovary become patent and yolk proteins can then be imported into the developing oocytes from their site of synthesis, the fat body; details are reviewed in [9]. Egg batch size is regulated by resorption of developing follicles if blood meals are inadequate [10]. If a follicle resorbs, its oocyte stops developing, its yolk content disperses and it undergoes atresia, thus no egg is produced. This resorption occurs as a result of apoptosis of patches of follicular epithelial cells [11,12]. Insufficient nutrient intake is one, but not the only, circumstance in which follicle resorption is initiated. We have previously shown that up to 35% of the follicles in anopheline mosquitoes that have taken adequate blood meals, but are infected with the malaria

* Corresponding author. Tel.: +44 1782 58 3034; fax: +44 1782 58 3516.
E-mail address: h.hurd@keele.ac.uk (H. Hurd).

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parasite, also undergo resorption during their development, resulting in a concomitant decrease in egg production [13–15]. This follicular atresia is initiated by the programmed cell death, or apoptosis, of cells in the epithelium. Apoptosis is first detected 16 h post-infection, and can be reversed by the injection of a caspase inhibitor [11]. Previously, we have detected apoptosis by identifying cells containing nuclei with condensed chromatin and fragmented DNA. Very early events indicative of apoptosis could be demonstrated by monitoring the activation of a family of cysteine proteases, the caspases, which are central to apoptotic pathways in the metazoan cell [16]. Activation of these enzymes is an early signature of apoptosis that can be detected by inhibitors that specifically bind to the active site of the molecule.

Two different innate immune responses, namely, antimicrobial peptide production and a melanization response, have also been associated with loss of reproductive fitness in mosquitoes [6,17]. The former can be stimulated by inoculation of the non-pathological microbial immune elicitor, lipopolysaccharide (LPS). LPS provokes a strong, dose responsive, immune response which is governed by a number of recognition molecules. It has been found to induce several pathways of the insect immune system, possibly by up-regulating the immune genes via the NF-κB/Rel pathway [18] and results in the production of antimicrobial peptides such as defensin in *A. gambiae*. LPS injection reduces the protein content of developing ovaries, and consequently the number of eggs that mature, in a manner that is not dose responsive [17]. The melanization response can be initiated by injection of a negatively charged, CM C-25, or neutral, CM G-25, Sephadex® bead [19,20]. Immune costs have been related to the type of antigen, negatively charged beads reducing fecundity in *Aedes aegypti* but not neutral beads [6].

Here we have investigated the hypothesis that the loss of reproductive fitness, associated with mosquito immune responses, operates in the same manner as that which occurs when mosquitoes have not fully fed, or are infected with malaria. We propose that both the antimicrobial response and melanization are traded-off against egg production via the induction of apoptosis in the ovarian follicular epithelial cells and hence resorption of developing follicles.

2. Materials and methods

2.1. Mosquitoes

The malaria vector, *A. gambiae* (KIL strain), was maintained in standardized conditions to produce adults of similar size, as outlined in Ahmed et al. [15]. Small mosquitoes have ovaries with fewer follicles and imbibe smaller blood meals; factors which subsequently affect vitellogenesis and fecundity [21,22]. Small blood meals have also been associated with the induction of apoptosis in mosquito ovaries [12]. Each treatment group was therefore checked to verify that they were of similar size range by measuring their wing lengths from the distal end of the allula to the tip, excluding the fringe.

Six-day-old mosquitoes were starved for 12 h before feeding on the blood of an anesthetized CD strain mouse. All mosquitoes within an experiment were fed on the same mouse to control for mouse effects, with the exception of the experiment comparing the effect of malaria infection. After feeding, fully engorged females were randomly selected and assigned to different groups for the experimental procedures. Immediately after treatments, mosquitoes were maintained in small cages in their rearing insectary and allowed access to 10% glucose solution.

2.2. Testing LPS toxicity

The toxicity of LPS used in this work was initially tested on a mosquito cell line derived from *A. gambiae* Suakoko strain (Sua 4.0) [23] which was kindly provided by Hans-Michael Müller (The European Molecular Biology Laboratory, Heidelberg, Germany). Cells were maintained at 27 °C in Schneider's insect medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich). Cells were counted before use and cultured at 1×10^5 cells/100 μl medium. LPS was added at 500 ng/100 μl to mimic the approximate concentration circulating in experimental mosquitoes (on the assumption that the injected dose will be diluted in the hemolymph to ~ 5 ng/μl). APS alone was added at 25 μl/100 μl medium as a control. Cells were kept for 24 h incubation at 27 °C and dead cells were then visualized by adding 100 μl of Trypan blue (0.4%, Sigma-Aldrich). Numbers of dead cells (stained blue) were immediately counted and % of death was calculated.

2.3. Antimicrobial peptide induction and ovarian apoptosis

Two groups of fully engorged mosquitoes (50 mosquitoes each) were injected as detailed in Ahmed et al. [15], with filter sterilized *Aedes* physiological saline (APS) to act as a trauma control, or with LPS (10 ng/0.25 μl APS). A final group was un-injected. Initial sample sizes were high as a precaution because several mosquitoes in each group did not survive the injection procedure and a variable number of mosquitoes were alive but unable to fly. It proved difficult to perform all the necessary assays within an acceptable time frame if the number of mosquitoes to be monitored exceeded 10. Hemolymph was collected from 10 fully active mosquitoes, collected at random, 18 h post-LPS injection and used immediately for an inhibition zone assay against *Micrococcus luteus* (NCTC 2665) (Sigma, UK), as outlined in Ahmed et al. [24], to determine activation of an antimicrobial response. Ovaries from the same mosquitoes were dissected and used for monitoring apoptotic follicles, as were ovaries from control groups.

2.4. Melanization response and ovarian apoptosis

Two groups of fully engorged mosquitoes (50 mosquitoes each) were each inoculated with negatively charged CM

C-25 or neutral CM G-25 Sephadex® beads (Sigma-Aldrich, UK) re-hydrated in APS. A bead (in 0.25 µl APS) was inoculated into the thoracic hemocoel (one bead/mosquito) using a calibrated, heat pulled, microcapillary needle. Only beads that were between 40 and 80 µm in size were used to reduce the injection wound as much as possible. A third group was injected with APS (0.25 µl per mosquito) as a trauma control and a fourth group was untreated. As before, large numbers of mosquitoes were injected because many did not survive the injection procedure, or were not able to fly 18 h later.

Ten fully active mosquitoes were randomly selected and dissected 18 h later. Beads were recovered and monitored for melanization. At this time post-inoculation, beads were melanized to differing degrees that were difficult to determine accurately. Bead melanization was therefore scored in three categories: no visible melanization (white bead), patchy (dotted or partly melanized bead) and complete melanization (dark black beads) [25]. Ovaries from the same mosquitoes were dissected and used for monitoring apoptotic follicles in parallel with control groups.

2.5. Malaria infection

Six-day-old mosquitoes were allocated randomly to two groups (≈ 100 mosquitoes each) and starved for 12 h prior to feeding on an anesthetized non-infected or malaria infected mouse (gametocytaemic and exflagellating) as detailed in Jahan and Hurd [14]. Mice were litter mates and the packed cell volumes of control and infected mice were measured prior to mosquito blood-feeding to match mice with similar hematocrits and remove the likelihood of blood meals differing in total protein content. Immediately after feeding, males and partially fed or unfed mosquitoes were removed and fully engorged females from the two groups were maintained in two separate cages and allowed access to 10% glucose solution. Eighteen hours later, ovaries from mosquitoes from each group were dissected for monitoring apoptotic follicles. A group of 20 infected mosquitoes was separated and retained for later checking for prevalence and intensity of malaria infection by counting oocysts developing on the midgut (at day 8 post-infected blood meal). Prevalence of infection exceeded 90%, and oocyst burden was over 50 per mosquito (data not shown).

2.6. Ovary dissection and caspase-like activity detection

The presence of activated caspase molecules in the cytoplasm of follicular epithelial cells is indicative of the irreversible initiation of the apoptotic pathway and hence the ultimate resorption of the follicle. The number of follicles per ovary that contained cells undergoing apoptosis can be counted by incubating ovaries with an artificial caspase substrate, tagged with fluorescein, that becomes bound to activated caspase-like molecules.

Ovaries were dissected in phosphate buffered saline (PBS), 18 h post-treatment and kept on ice. Immediately after dissection, ovaries were used for detecting caspase-like activity using CaspaTag™ Fluorescein Caspase (VAD) (Intergen Company) according to manufacturer's instructions. Briefly, FAM-Peptide-FMK was added directly to each ovary pair, mixed gently and incubated for 1 h at 37 °C under 5% CO_2 and protected from light. Ovaries were then washed, mounted in Vectashield® medium and immediately examined under a fluorescence microscope using a band pass filter (excitation 490 nm, emission 520 nm). It was neither possible to count the number of individual dying cells per follicle nor determine the degree of caspase-like activity per cell. Thus, whole follicles that contained cells displaying fluorescence, and therefore exhibiting caspase-like activity, were counted and the % per ovary pair was calculated.

2.7. Statistical analysis

All statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA). Data were tested for normality (Anderson-Darling Normality test) and variance homogeneity prior to any further analysis. Data pertaining to the effects of LPS on the mosquito cell line, antibacterial activity and follicular caspase-like activity were all normally distributed and had homogeneous variances and thus, comparisons between treatments were made using one-way analysis of variance (ANOVA) and differences between individual pairs of data were analyzed using the multiple comparisons Tukey test [26]. Non-parametric data from bead inoculations were arcsine transformed because they were %s and all were less than 30%, again tested for normality and variance homogeneity and, as they were then normally distributed, they were analyzed as above using ANOVA. Wing lengths were log transformed but data were still non-parametric and was thus analyzed by the stacked ANOVA method of Kruskal–Wallis to test for differences between treatment groups and multiple comparisons made using the Mann Whitney *U*-test. A two-sample *t*-test (for individual comparison) was used for comparing differences between malaria infected and uninfected mosquitoes. Repeated experiments were carried out using new generations of mosquitoes and different mice as blood sources.

3. Results

3.1. LPS-toxicity effect and antimicrobial activity assay

The toxicity of the LPS dose (10 ng per mosquito) used in this study was tested against the *A. gambiae* Sua 4 cell line. Less than 8% of cells were dead after 24 h in treated or un-treated cultures. One-way ANOVA showed a non-significant difference in the % of cell death between LPS- and APS-treated and un-treated cell cultures ($F_{2,12} = 0.83$, $P > 0.05$, $N = 5$ per treatment) (Fig. 1). This indicates that the concentration of LPS injected into mosquitoes (10 ng per mosquito) in this study is unlikely to have a marked toxic

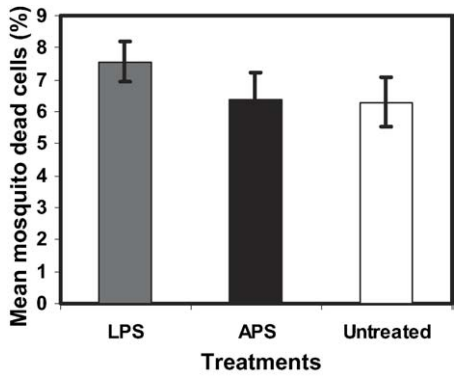

Fig. 1. Assessment of the toxicity of LPS to *A. gambiae* Sua 4 cells 18 h post-treatment. The percentage of cell death after addition of 5 ng LPS per µl cell medium was compared with APS-treated or un-treated cells. Error bars represent the standard error of means of five replicate experiments. No significant change in cell death was noted.

effect on mosquito. No significant difference in the mortality of LPS and sham-injected mosquitoes was seen (data not shown).

There was no significant difference in the wing size of mosquitoes in the three experimental groups (non-injected = 2.88 ± 0.02 mm; ASP injected = 2.82 ± 0.01 mm; LPS injected = 2.87 ± 0.01 mm; $N = 20$ and $P > 0.05$ in each case). Hemolymph from blood-fed non-injected mosquitoes showed humoral activity against *M. luteus* at 18 h post-feeding which was equivalent to the activity of approximately 210 ng of lysozyme (Fig. 2). Different treatments gave rise to significant differences in anti-*M. luteus* activity (one-way ANOVA: $F_{2,12} = 23.85$, $P < 0.001$, $N = 5$ per treatment). LPS injected immediately after a blood meal (10 ng per mosquito) caused a mean increase of 43.0% in anti-*M. luteus* activity (Tukey's pairwise comparison; $P < 0.05$; Fig. 2). Sham injection of APS resulted in a slight (16%), but non-significant, increase in anti-*M. luteus* activity compared with blood-fed non-injected mosquitoes (Tukey's pairwise comparison, $P > 0.05$; Fig. 2).

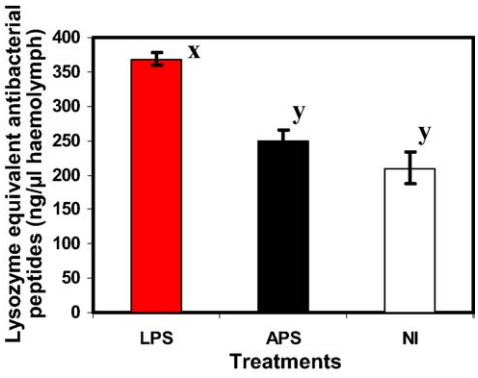

Fig. 2. Anti-*M. luteus* humoral activity in *A. gambiae* hemolymph 18 h post-LPS injection. Hemolymph from injected mosquitoes was subject to an inhibition zone assay using lysozyme as a standard. Blood-fed mosquitoes were injected with LPS, APS, or non-injected (NI). Error bars represent the standard error of means of five replicates of each treatment. Different letters above bars indicate significant differences.

3.2. The effect of LPS injection on follicular caspase activity

Each *A. gambiae* ovary consists of a variable number of ovarioles (approximately 90 per ovary) and is served by an extensive tracheal system. Following a blood meal, the terminal follicle in each ovariole begins to develop. The follicle consists of an oocyte and nurse cells surrounded by a single layer of epithelial cells (see Fig. 4). Caspase-like activity was detected within the follicular epithelial cells in a very small number of developing follicles (4.45 ± 0.5%) in most ovaries from un-treated mosquitoes (Figs. 3 and 4).

This activity was detected in patches of epithelial cells of the developing follicles but no activity was detected in the nurse cells (Fig. 4). No fluorescent activity was detected in negative control ovaries in which FAM-Peptide-FMK was omitted from the incubation medium (data not shown).

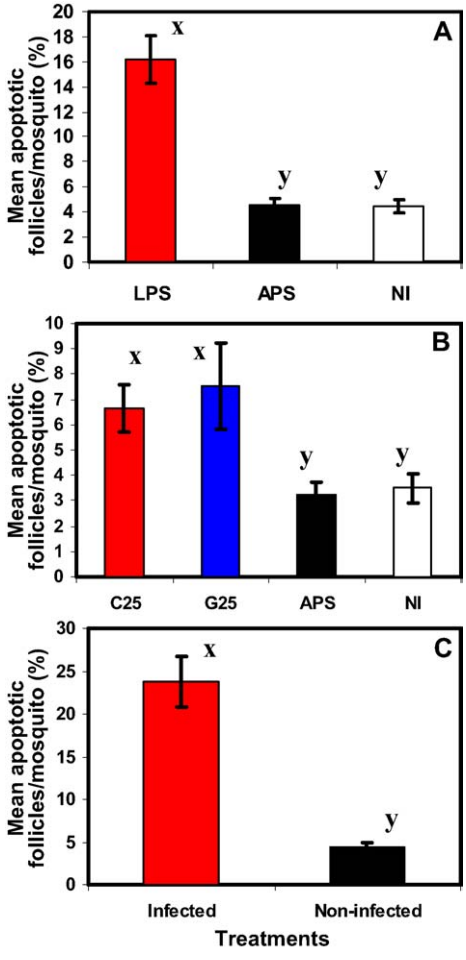

Fig. 3. Caspase-like activity in mosquito ovaries 18 h post-treatment. The percentage of apoptotic follicles was calculated following observation of fluorescent labeling with FAM-Peptide-FMK. A; ovaries from LPS injected, APS injected or non-injected (NI) mosquitoes. B; ovaries from mosquitoes inoculated with C-25 or G-25 Sephadex® beads, APS alone or non-injected (NI). C; ovaries from mosquitoes fed on *P. y. nigeriensis*-infected or uninfected mouse blood. Error bars represent the standard error of means of 10 mosquitoes in every case. Within each experiment different letters above bars represent significantly different percentages (A, B $P < 0.05$, C $P < 0.001$).

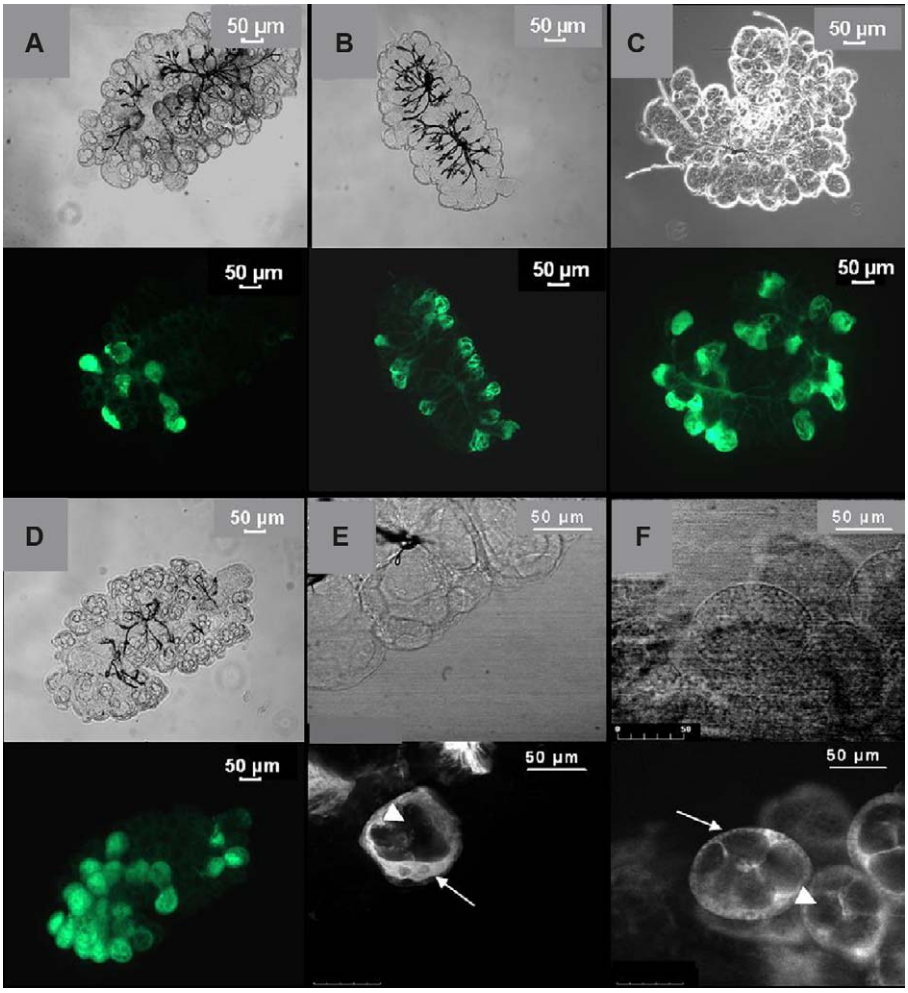

Fig. 4. Caspase-like activity detected in the ovarioles of *A. gambiae*. Upper panels were observed under phase contrast and lower panels show fluorescent labeling with FAM-Peptide-FMK. A, an ovary from an un-treated mosquito. B, an ovary from a C-25 Sephadex® bead-inoculated mosquito. C, an ovary from an LPS injected mosquito. D, an ovary from a *P. y. nigeriensis*-infected mosquito. E, F, follicles from an infected mosquito, long arrows indicate follicular epithelial cells surrounding the oocyte (fluorescing) and white arrow heads indicate nurse cells (not fluorescing) within the follicle.

LPS injection caused an overall significant change in the % of apoptotic follicles (one-way ANOVA: $F_{2,27} = 32.98$, $P < 0.05$, $N = 10$ for each treatment) (Fig. 3A). Follicular cell apoptosis in LPS injected mosquitoes was significantly increased by 265.9% compared to un-injected control and by 257.7% compared to APS sham-injected ones (Fig. 3) (Tukey's pairwise comparison, $P < 0.05$). This experiment was repeated, using a different mosquito generation and different mouse, and a significant increase of 249.1% of apoptotic follicles was detected in LPS injected mosquitoes compared to APS injected ones.

3.3. The effect of bead melanization on follicular caspase-like activity

Mosquitoes within each treatment group had similar wing sizes (non-injected = 2.88 ± 0.02 mm; APS injected = 2.82 ± 0.01 mm; G-25 bead injected = 2.86 ± 0.02 mm; C-25 bead injected = 2.85 ± 0.01 mm; $N = 20$ in each case and $P > 0.05$). Amongst mosquitoes inoculated with negatively charged C-25 beads, 70% partially melanized the bead (patchy) and 30% did not melanize them (white beads) (Fig. 5). However, 80% of the neutral G-25 beads were completely melanized (dark black) and 20% partially melanized (patchy) by mosquitoes (Fig. 5; $N = 10$). These results suggest that the melanization response against neutral G-25 beads was stronger than that against the negative C-25 ones at 18 h post-inoculation.

Ovaries from the same mosquitoes were used for detecting the effect of melanization on follicular cell apoptosis. Control mosquitoes (un-injected, blood-fed) showed a mean % of apoptotic follicles of 3.50 ± 0.56 per ovary pair at 18 h post-blood meal (Figs. 3B, and 4). Bead inoculation caused an overall increase in the % of follicular apoptosis (ANOVA: $F_{3,36}$; $P = 0.005$; Fig. 3B). Individual comparison using Tukey's test showed a significant increase of 100.0% in C-25-inoculated and 120.5% in G-25-inoculated mosquitoes compared to blood-fed un-injected (control) ones ($P < 0.05$, $N = 10$ in each sample; Fig. 3B). Moreover, this increase was 106.2% and 134.3%, respectively, compared to APS injected mosquitoes (sham-injected) ($P < 0.05$ $N = 10$) (Fig. 3B). Individual comparison between C-25- and G-25-inoculated mos-

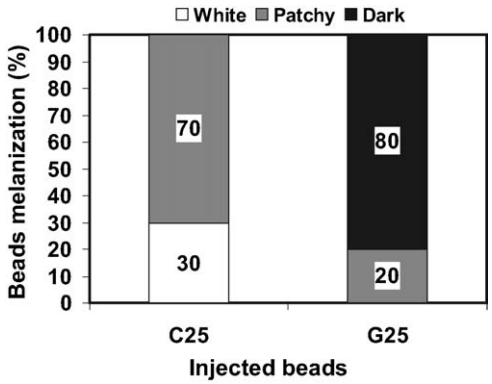

Fig. 5. Degrees of melanization of C-25 or G-25 Sephadex® beads 18 h post-inoculation. Mosquitoes were inoculated with one bead immediately after a blood meal and dissected 18 h later for scoring bead melanization. Bars represent the percentage of mosquitoes ($N = 10$) within each melanization category. Based on the intensity of melanin, melanization was categorized as; no melanization (white bar), patchy (dotted bar) and completely melanized (black bar).

quitoes showed no significant difference ($P > 0.05$ $N = 10$) in the % of follicular apoptosis, despite the differences in the degree of bead melanization. No significant difference was detected in the % of follicular apoptosis between sham-injected and control mosquitoes (Fig. 3B). In a repeat experiment, a significant increase of 254.05% and 218.91% of apoptotic follicles was detected in C-25 and G-25 inoculated mosquitoes, respectively, compared to APS injected ones (one-way ANOVA was used for analysis as data were normally distributed and variances were homogeneous). Again, no significant difference was detected between G-25- and C-25-inoculated mosquitoes.

3.4. Follicular caspase-like activity of malaria infection

There was no significant difference in the mean wing size of infected and uninfected mosquitoes (uninfected = 2.88 ± 0.015 mm; infected = 2.84 ± 0.013 mm; $N = 20$ and $P > 0.05$ in each case). Ovaries from malaria infected mosquitoes showed a significant increase in the % of apoptotic follicles of 440.9% (Student's *t*-test, $P < 0.05$, $N = 10$; Figs. 3C and 4). This was the highest % of follicles containing cells demonstrating caspase-like activity in this study.

4. Discussion

In this study we have obtained a better understanding of the mechanism(s) underlying the reproductive costs that result from immune stimulation in *A. gambiae*. Stimulation of both the cellular and the humoral arms of the mosquito defense system results in the induction of apoptotic pathways in ovarian follicular epithelial cells. By comparison with our work on the effect of malaria infection on mosquito ovaries [11] we think this is likely to be a major, and possibly the only, mechanism for reducing egg production in immune stimulated females. Previous work has shown that apoptosis of follicular epithelial cells results in follicle atresia and a consequent reduction in egg production [11,12]. The difference in the degree of melanization seen between beads with different charges did not produce a concomitant difference in the proportion of apoptotic follicles, indicating that this was not a dose-related response such that increased deposition of melanin did not increase the costs of this immune response. Similarly, we previously showed that fecundity reduction induced by LPS injection was not a dose-dependent effect [15].

Infection with the murine malaria *Plasmodium yoelii nigeriensis* likewise induces activation of a caspase-like response in ovarian follicles; a response which appears to be more pronounced than that induced by LPS or bead injection. Once malaria gametocytes have been imbibed with a blood meal they differentiate to gametes. Gamete fertilization rapidly ensues within the blood bolus in the midgut and a motile ookinetes is formed. These begin to transit through the midgut epithelial cells within 16 h, about the time that our previous time course study first detected apoptosis in the ovaries of infected females [11]. Both *P. berghei* and *P. falciparum* have been shown to cause severe pathology in invaded cells, resulting in their apoptosis and expulsion from the epithelium [27,28]. Midgut invasion is associated with a robust immune response initiated in the midgut cells and systemically [29–31], including transcriptional upregulation of mosquito immune genes such those coding as defensin and nitric oxide synthase e.g. [29,32]. It is thus conceivable that the detrimental effect that malaria infection has on egg production is mediated via the induction of an immune response and what we see are actually costs associated with mounting an immune response. Though, of course, this effect is indirect as the parasites do not invade or damage the ovaries. The apparent difference in the magnitude of response between parasite infection and inanimate immune-elicitors may be due to a timing effect. Response to LPS or beads may be rapid whereas it may not be initiated by the parasite until it begins to cross the midgut epithelium, 16 h later. It is thus possible that a short-lived response, such as caspase activation, may have been largely missed 18 h after stimulation. It is noteworthy that our previous work has shown that injection of LPS only results in an 18.5% reduction in total egg production [15], a figure that approximates the percentage of follicles found to contain apoptotic cells after LPS treatment in this study. It is thus likely that additional apoptosis-inducing stimuli are present in the malaria-infected mosquito [33].

A melanotic encapsulation occurs in response to infection in the genetically selected *A. gambiae* strain L3-5, in which parasites are melanized as they exit on the basal side of midgut epithelial cells. This strain is refractory to infection. The *A. gambiae* KIL strain, used in this study, is susceptible to *P. y. nigeriensis* infection and no melanization of invading parasites occurs. However, it is possible that infection upregulates proteolytic activation of prophenoloxidases in this mosquito strain and that this response also results in follicular cell apoptosis, as we see in our experimental stimulation of the melanotic encapsulation response. How the effect of

induction of a melanization response acts on the ovarian follicles is not known. Toxic compounds such as quinones are generated during the melanization response [34] but these have not been reported to induce apoptosis.

Although we have demonstrated that LPS injection, and the subsequent antimicrobial peptide synthesis, is associated with the induction of apoptosis, we are uncertain of the exact pathways involved. We are confident that LPS is unlikely to be toxic to mosquito ovarian cells as no toxicity was detected when our cell line was exposed to LPS [35,36]. Some perspective may be gained from a study of the *immune deficiency* (*imd*) gene in *Drosophila*, that controls antibacterial defense and encodes a protein with a death domain that plays a role in both NF-κB activation and apoptosis. Georgel et al. [37] showed that it functions upstream of the DmIKK signalosome and the caspase DREDD. Over-expression of *imd* in *Drosophila* led to transcription of *reaper* (*rpr*) and apoptosis of a large number of fat body cells, both responses being blocked by the coexpression of P35, an anti-apoptotic protein. Although *imd* mutants were more resistant to UV radiation-induced apoptosis evidence did not point to a role of apoptosis in *Drosophila* fat body response to bacterial challenge [37]. *Rpr*-induced apoptotic death of follicle cells leads to death of the follicles of *Drosophila* [38] but we have no information concerning the activators of apoptosis in mosquito follicles and *rpr* has not been identified in the *Anopheles* genome [29,39]. There is clearly much more to investigate before we elucidate the signaling pathways involved in this response to immune stimulation but it is particularly interesting that both arms of the immune response converge where costs are paid.

Zuk and Stoehr [40] identified two types of cost associated with immune responses i) costs related to the allocation of limited resources and ii) option costs that are not paid in the currency of resources but in the structural or functional components of an organism. The trade-off between immune response and fecundity is usually ascribed to the repartitioning of resources. For instance, the melanotic encapsulation of filarial worms by mosquitoes requires tyrosine, a melanin precursor, which is also needed for other biological events, such as formation of egg-chorion. Thus the fecundity reduction associated with this melanization response has been attributed to a competition for nutrients [41]. However, it is unlikely that competition for tyrosine could induce apoptosis in follicle cells as tyrosine is required later in the gonotrophic cycle, when the egg-chorion is laid down. Furthermore fully engorged mosquitoes obtain more dietary protein than is completely utilized for oogenesis and a substantial portion is excreted [42] thus nutrients may not be limiting. We consider it unlikely that the small amount of resources required to melanize a single bead of this size, or to synthesize antimicrobial peptides, would represent a significant drain on resources so soon after the supply provided by a blood meal. Thus we propose that mosquito immune responses may incur option costs via the indirect induction of pathways leading to apoptosis.

We have generated further evidence to support the view that infection and defense are costly and have begun to elucidate the mechanism by which these cost operate at the cellular level. However, as yet we have no understanding of the triggers that initiate apoptosis. Other immune molecules such as serine proteases, pattern recognition molecules and the hemocyte-specific complement-like protein TEP1 are known to be involved in the mosquito response to malaria [30,43,44] and upregulation of these may also incur fitness costs. In view of the interest in immune effectors as candidates for manipulation via mosquito transgenic technology, it is clearly imperative that we separate individual immune response and evaluate their respective costs in order to maximize the fitness of any genetically modified mosquitoes that are produced.

Acknowledgements

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