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THE HUMORAL ANTI-BACTERIAL RESPONSE OF *ANOPHELES GAMBIAE* AND THE IMMUNITY-REPRODUCTION TRADE-OFF: BETWEEN THE HOPE AND LIMITATION OF THE MALARIA IMMUNO-CONTROL STRATEGY

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ABSTRACT

In this study, induction of the humoral anti-bacterial activity of the African human malaria vector, *Anopheles gambiae*, and the mechanisms of the concomitant fecundity reduction were investigated. The blood meal induced a humoral anti-*Micrococcus luteus* activity, which was detectable at 12h, peaked around 24h and become hardly detectable at 48h post-feeding. This humoral activity was also detected in sugar-fed mosquitoes at 18h post-intrathoracic injection with the immune elicitor, lipopolysaccharide (LPS, 10ng/mosquito). Moreover, the blood meal significantly enhanced the immune-stimulatory effect of LPS when injected into blood-fed mosquitoes, as a significant higher anti-*M. luteus* activity was detected compared to that of LPS-injected sugar-fed mosquitoes. These data suggest an immune enhancive effect of blood meal, which could be in the favour of malaria immuno-control strategy. On the other hand, induction of this immune activity did impose a reproductive cost, as LPS-injected mosquitoes showed a significant 28.8% fecundity reduction compared to those injected with *Aedes* physiological saline (APS). Follicular apoptosis and resorption were investigated in this study as two suggested mechanisms of this fecundity reduction. On one hand, follicular apoptosis was clearly detectable as early as 18h post-LPS injection, which seems to occur within epithelial cells not in nurse cells. On the other hand, follicular resorption was detected 18h post-LPS injection, which was 44.5% significantly higher than that in APS-injected mosquitoes. Based on these data, this study suggests that involving the humoral immune response in the battle against malaria would be costly in terms of fecundity reduction. Thus, as part of the scenario of immunity-reproduction

conflict, the discussion of these data concludes by suggesting that the impact of immune stimulation on the vector fecundity would participate as a limiting factor to the success of malaria immuno-control strategy.

Key words: *Anopheles gambiae*, Lipopolysaccharide, anti-bacterial activity, follicular resorption, follicular apoptosis, fecundity reduction.

INTRODUCTION

Insect immune peptides represent an important line of defence against a broad spectrum of bacterial and fungal pathogens (reviewed by Brey and Hultmark, 1998) as well as against malaria parasites (Somboon *et al*.; 1999 and Dimopoulos *et al*., 2001). Efforts are currently taking place to utilize the vector immune responses to block malaria development in its gut as an alternative to the recently ineffective chemical control (firstly suggested by Curtis, 1994). This strategy is being rapidly evolving as a result of the emergence of mosquito resistance to pesticide and the rapid spread of multiple anti-malarial drug-resistance of *Plasmodium*. Thus, immuno-control strategy takes place *via* three different parallel hypotheses; a) genetically selecting malaria refractory strains that melanize ookinetes in its gut (Somboon *et al*.; 1999), b) driving immune-genes from the refractory strain to the susceptible one (Kokoza *et al*. 2000) or c) transmission blocking strategy, which relies on a single-chain antibody fragment (scFv)* directed to Pbs21 (*Plasmodium bergheii* surface protein 21) on the surface of the ookinetes and eventually kills the parasite (Yoshida *et al*.; 2001). All these hypotheses are aiming at developing a mosquito that is incompetent of malaria transmission.

On the other hand however, innate refractoriness or mounting immune responses in malaria vector was proved to be costly in terms of most fitness

* It is a single-chain antibody produced by *E. coli* and possesses both binding specificity for *Plasmodium bergheii* ookinetes and inhibitory activity against oocysts development in the mosquito midgut. Thus, it works as targeting moiety.

parameters (Yan *et al.*, 1997, Moreira, *et al*., 2004 and Irvin, *et al*., 2004). Reproductive fitness, in particular, has been significantly reduced as a result of both melanizing parasites (Ferdig *et al*., 1993), and mounting humoral antibacterial activities (Ahmed *et al*., 2002). Hence, better understanding of the costs of mounting immune responses in general, could, in my opinion, help in developing a novel way of blocking malaria transmission.

It has been proved that the two immune responses that working effectively against *Plasmodium* are mainly the melanization response (in refractory mosquitoes), and the humoral anti-bacterial one (Gwadz, *et al*., 1989 and Shahabuddin *et al*. 1998). Thus, intensive studies on these two particular responses are urgently needed to better understand their costs and benefits upon the vector before thinking about involving them in the malaria immuno-control strategy. Therefore, in a previous study, I have shown the efficiency of melanization against the Sephadex beads model and the mechanism underlying the concomitant fecundity reduction in *An. gambiae* (Ahmed 2005). In the current study here, I used the same vector to investigate the second response, the humoral anti-bacterial activity, as a result of LPS injection and the enhancive role of blood meal on it. Moreover, the concomitant reproductive costs and its underlying mechanism were also investigated.

MATERIALS AND METHODS

Rearing experimental mosquitoes:

A stock colony of *A. gambiae* (KIL strain) was maintained in standardized conditions designed to produce adults of similar size as detailed in Ahmed *et al*., (1999) in the laboratory of Vector Biology in Biological Sciences Department at Keele University, UK). As mosquito size affects vitellogenesis (Hurd *et al*., 1995), each treatment group was therefore checked to make sure that their body sizes were similar based on wing length measurements (Briegel 1990).

Blood feeding, injections, haemolymph collection and inhibition zone assay:

Six days old mosquitoes were assigned to experimental groups, allowed to feed on either 10% glucose solution or mouse blood and intra-thoracic injected with either filter sterilized APS (to act as a trauma control) or LPS (10ng/mosquito) (Sigma, UK), or left as non-injected controls as detailed in Ahmed et al., (2002). Mosquitoes were then maintained in cages (6×6×6 cm) in standard insectary conditions. No difference in the mortality of LPS- or sham-injected mosquitoes was observed. Haemolymph was collected from the thoraces of experimental mosquitoes and was subjected to an inhibition zone assay, to measure the humoral activity against the Gram-positive bacteria, *Micrococcus luteus* (NCTC 2665, Sigma, UK), as detailed in Ahmed *et al*., (2002).

Experimental designs:

Six days old mosquitoes, were used to study the hypothesis that inducing the humoral antibacterial activity could be costly in terms of fecundity reduction (total number of eggs produced), and follicular apoptosis and resorption at 18h post-treatments *via* the following experimental designs:

I)- Time profile of the stimulatory effect of blood meal on antibacterial activity:

A group of 6 days old mosquitoes (100 mosquitoes) were starved for 12h prior to feeding on the blood of anesthetized CD mouse for 20 minutes. After feeding, 50 fully engorged females were randomly selected and moved into a small cage (6 x 6 x 6 cm). Haemolymph was collected individually from 5 mosquitoes (n = 5) at the time point chosen for this experiment (12, 24, 36 and 48h post-feeding) and subjected to an inhibition zone assay to measure the anti-*M. luteus* activity.

II)- Effect of blood meal on the LPS-induced humoral antibacterial activity:

As illustrated in Fig. (1), mosquitoes were randomly divided into 3 groups (100 mosquitoes each). Group A was allowed to access 10% glucose solution, and group B & C were allowed to feed on the blood of an anesthetized CD mouse for 20 minutes. Immediately after feeding, fully engorged females from each of the group A & B were randomly divided into two subgroups (30 mosquitoes each) and injected with either LPS (10ng in 0.25 µl APS/mosquito) or APS (0.25 µl/mosquito). Group C was kept non-injected (control). The idea of having a forth-negative control group (sugar-fed non-injected) was neglected in this experiment based on a pilot study that showed no humoral activity as the immune system is not triggered. Haemolymph was freshly collected from treated mosquitoes 18h post-treatments and was immediately subjected to an inhibition zone assay to measure the humoral anti- *M. luteus* activity.

III)- Effect of antibacterial activity on the development of ovarian follicles:

This experiment was designed to study the effect of this immune response on the follicular development in the ovaries of the same mosquitoes that used for carrying out inhibition zone assay. Thus, after collecting haemolymph, these vitellogenic mosquitoes were kept on ice until used for ovaries dissection half an hour later in order to investigate the follicular apoptosis and resorption as following:

a)- Follicular apoptosis:

A sample of 5 ovaries were dissected in Phosphate Buffered Saline (PBS) and immediately moved into clean sterilized eppendorf tubes (one ovary pair/100 µl PBS) and used for detecting the caspases activity in its developing follicles using CaspaTag^{TM} Fluorescein Caspase (VAD) Activity kit (Intergen Company, UK) according to its manual instructions and as detailed in Ahmed (2005).

b)- Follicular resorption:

Ten ovary pairs were dissected in APS and immediately dipped in Neutral Red [0.5% (w/v) solution in citrate-phosphate buffer (0.1 $moll^{-1}$ citric acid/0.1 $moll^{-1}$ sodium citrate) at pH 6 for 1 min to assist the visualization of resorped follicles (Clements and Boocock, 1984) as detailed in Ahmed (2005). Percentages of resorped follicles were then calculated (per ovary pair) under the microscope.

V)- Effect of the antibacterial activity on total egg production (fecundity):

Vitellogenesis competent mosquitoes (6 days old) were starved for 12h prior to feeding on the blood of CD muse for 20 minutes. Immediately after feeding, fully engorged females were randomly divided into 3 groups (30 mosquitoes each). The first or second group was injected with filter sterilized LPS (10ng/mosquito) or APS (0.25 µl/mosquito) respectively. The third group was left non-injected as control. Mosquitoes within each group were then kept individually in small cages (6 x 6 x 6 cm) (one mosquito/cage) and supplied with 10% glucose solution. On the third day, mosquitoes were allowed to lay eggs on filter paper flooding on the surface of distilled water. Laid eggs were counted on day 4 and 5, and ovaries of the same mosquitoes were dissected on day 6 for counting retained eggs. Total number of eggs produced was counted as the number of eggs laid + the number of eggs retained. At least, 20 alive and active mosquitoes within each treatment were successfully laid eggs and included in this experiment.

Statistical analysis:

All statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA, Version 13.1, 2002). Data from all experiments were first tested for normality using Anderson Darling test, and for variances homogeneity prior to any further statistical analysis. Data from the experiments of immune activity and follicular resorption were normally distributed, and variances were homogeneous. Thus, One-way ANOVA was used to determine overall

effects of treatments followed by individual comparison using Tukey's Pairwise comparison. Data from the experiment of egg laying and wing measurements were not normally distributed. Thus, Kruskal-Wallis was used to test the overall differences prior to individual comparisons within treatments using the non-parametric test, Man-Whitney *U*.

RESULTS

Time profile of the blood meal-stimulatory effect on antibacterial activity:

Humoral activity against *M. luteus* was detected in haemolymph from blood-fed mosquitoes at different time points post-blood feeding. This activity was equivalent to the activity of 140.9 ± 3.1, 235.6 ± 4.1, 156.2 ± 4.9 and 89.78 ± 1.4ng Lysozyme at 12, 24, 36 and 48h post-blood feeding respectively (Fig. 2) and also see Fig. (3). This indicates that blood meal has a stimulatory effect to this immune activity, which was detectable at 12h and peaked around 24h then declined again by 48h post-blood feeding. This activity was hardly detectable before 12h and after 48h post-feeding. Thus, based on these data, 18h post-treatments was the time chosen at which the hypotheses of this study were achieved.

Enhancive effect of blood meal to the LPS-induction of the antibacterial activity:

Injection of sugar-fed and blood-fed mosquitoes with LPS caused an overall significant effect on the anti-*M. luteus* activity at 18h post-injection (One-way ANOVA: $F_{4,20} = 76.86$, $P < 0.001$, n = 5 mosquitoes per treatment). In sugar fed mosquitoes, APS-injection induced a week anti-*M. luteus* activity of 83.3 ± 12.5ng Lysozyme equivalent (Fig. 3B & 4A). This indicates that stress (the damage caused by injection) could cause an immune response that has growth-inhibition effect of *M. luteus*. Injection of a single dose of 10ng LPS/mosquito induced a mean significant increase of 66.7% in anti-*M. luteus* activity compared

to APS-injection (250ng *v.* 83.3ng) (Tukey's Pairwise comparison; $P < 0.05$) (Fig. 3A & 4A).

In blood-fed non-injected (control) mosquitoes, the blood meal induced a significant 43% increase of anti-*M. luteus* activity compared to APS-injected sugar-fed ones (146ng *v.* 83.3ng) (Fig. 3B & 4A). On the other hand however, APS-injected blood-fed mosquitoes showed a significant 65.2% (239.36ng *v.* 83.3ng) or 39% (239.36ng *v.* 146ng) increase in anti-*M. luteus* activity compared to APS-injected sugar-fed or control mosquitoes respectively (Tukey's Pairwise comparison; $P < 0.05$) (Fig. 4A). Furthermore, the anti-*M. luteus* activity induced by LPS injection was significantly 25.8% higher in blood-fed mosquitoes compared to that in sugar-fed ones (336.6ng *v.* 250ng). These results indicate that blood meal not only induce humoral antibacterial activity but also enhance the effect of LPS on the same activity. Mosquitoes body sizes were assessed by measuring wing lengths (from the distal end of the allula to the tip of the wing, excluding the fringe) (Briegel, 1990). No significant difference was detected between wing lengths within treated groups (Man-Whitney *U*, $P > 0.05$, Fig. 4B).

Effect of the antibacterial activity on the development of ovarian follicles:

Humoral antibacterial activity was found to affect follicular development in terms of triggering follicular apoptosis and resorption as early as 18h post-immune induction (Fig. 5). Follicular apoptosis was detected as caspases activity in the developing ovarian follicles. This activity was visualized as fluorescing follicular epithelium, but nurse cells appeared normal (Fig 5 A, B, C & D). Moreover, induction of anti-*M. luteus* activity in blood fed mosquitoes by LPS injection caused an overall significant increase in follicular resorption (One-way ANOVA: $F_{2,12} = 13,6$, $P < 0.001$, n = 10 per treatment) (Fig 5E & F and 6A). Blood-fed non-injected (control) mosquitoes showed 7.4 ± 0.87% of follicles undergoing resorption per ovary pair, which was similar to that in APS-injected mosquitoes (Tukey's Pairwise comparison; $P > 0.05$) (Fig. 6A). However,

induction of anti-*M. luteus* activity by LPS injection showed a significant 48.6% (14.4% *v.* 7.4%) or 44.5% (14.4% *v.* 8%) increase in follicular resorption compared to control or APS-injected blood-fed mosquitoes respectively (Tukey's Pairwise comparison; $P < 0.05$) (Fig. 6A and 5E & F). These results indicate that induction of humoral antibacterial activity may triggers follicular apoptosis and resorption. No significant difference was detected between wing lengths within treated groups (Man-Whitney *U*, $P > 0.05$) (Fig. 6B).

Effect of the antibacterial activity on total egg production (fecundity):

Humoral antibacterial activity was found to reduce the total number of eggs produced (laid eggs + retained eggs). Kruskal-Wallis test has shown an overall significant effect of LPS-injection on the total number of eggs produced ($P < 0.05$). No significant difference was detected in fecundity between control and APS-injected mosquitoes (98.12 ± 3.00 and 92.67 ± 4.80 eggs/mosquito respectively (n = 20/ each treatment) (Man-Whitney *U*, ($P > 0.05$) (Fig. 7A). However, LPS-induced anti-*M. luteus* activity resulted in a significant 32.8% (65.9 *v.* 98) or 28.8% (65.9 *v.* 92.6) reduction in the total number of eggs produced compared to control or APS-injected ones respectively (Fig. 7A and 8A & C). No egg retention was detected in APS-injected mosquitoes (Fig 8B), however, 10% of LPS-injected mosquitoes were retaining some of their fully developed eggs in their ovaries (Fig. 8D) until dissected at 2 days after complete egg laying (6 days post-blood meal). These results may indicate that LPS-induced humoral antibacterial activity has significantly reduced mosquito fecundity. No significant difference was detected between wing lengths within treated groups (Man-Whitney *U*, $P > 0.05$, Fig. 7B).

DISCUSSION

This study shows the blood meal as an immune-inductive factor as the blood-fed *An. gambiae* showed a humoral anti-*M. luteus* activity, which peaked

around 24h post-blood meal. This, in fact, may explain the finding of Kelly and Edman (1996) who recorded significant lower malaria virulence in the gut of *Aedes aegypti* when allowed to access an additional blood meal shortly after a malaria infective one. Furthermore, the current study showed an anti-*M. luteus* activity in sugar-fed mosquitoes as a result of LPS-injection, which was significantly stronger in blood-fed ones. This is in consistence with Ahmed (2005) who also showed an enhancive role for the blood meal in melanizing Sephadex beads model by the same vector. Hence, this may indicate that blood meal acts as an enhancive factor for melanization and humoral anti-microbial responses, which are the two main immune responses working effectively against malaria parasite in the refractory mosquitoes in nature (Collins, *et al*., 1986 and Shahabuddin, *et al*., 1998). This, in theory, could be deleterious to malaria development in the vector midgut if it is infected. I would, therefore, suggest this to be in the favor of the malaria immuno-control strategy that aiming at blocking *Plasmodium* development in the midgut of its vector. (James, *et al*., 1999). Evidence for this is the 90% reduction in oocysts number when mosquito immune system is stimulated immediately after a malaria infective blood meal (Ahmed 2004). It is currently under investigation to know which type(s) of anti-bacterial peptides is expressed here in this study. It would also be interesting to know whither or not these immune molecules induced in the haemolymph by LPS-injection are capable of crossing the intracellular junctions of the mosquito gut to process anti-ookinetes/young oocysts response, like what is happening in case of refractory mosquitoes in nature (Collins, 1986).

The humoral anti-bacterial activity induced by LPS injection could be attributed to a fixed number of recognition molecules that are specifically targeting LPS molecule (reviewed by Hultmark, 1993). Moreover, LPS has been found to induce several pathways of the insect immune system by up-regulating the promoter immune genes *via* a particular pathway (Eggleston *et al.*, 2000) resulting in the production of antimicrobial peptide(s). Hence, the enhancive role

of blood meal could be occurring *via* strengthening, directly or indirectly, these recognition molecules and/or enhance the promoters of the related immune genes. This is possibly by enriching the fat body (the main site of immune molecules/genes) with the nutritional resources needed for the induction and manufacture of the immune genes and molecules respectively. This, in dead, is a kind of re-allocation of the nutritional resources that should otherwise be mainly allocated for maintenance and reproduction (Fig. 9, and also see Sheldon and Verhulst, 1996 for more details). Follicular apoptosis and resorption were also investigated as two possible mechanisms underlying fecundity reduction in this study.

Basically, apoptosis is a genetically regulated programmed cell death (PCD) as the cell will be able to self-destruct *via* activation of a cell suicide program (Kerr *et al*., 1972). Caspases, the biochemical core of the apoptosis mechanism (Alnemri *et al.*, 1996), act in a site-specific process as a cascade to bring about the PCD. Besides, they are playing an important role in the innate immune responses in vertebrates and insects (Slee, *et al*., 1999 and Kumar & Doumanis (2000). And hence, inducing the innate antibacterial immune response by LPS injection may be the trigger of caspases that caused follicles undergone apoptosis. Vega and Epel (2004) categorized the apoptotic process into three stages; a) early stage, which can be monitored by detecting increased permeability of cell membrane, b) mid-stage, which can be monitored by detecting caspases activity and c) late stage, which can be monitored by detecting DNA fragmentation. Based on this categorization, I have detected the caspases activity, the mid-stage in this study under fluorescence microscope (Fig. 5B & D). Studying this stage within follicular epithelial cells in more details using confocal microscope is currently under investigation (A. M. Ahmed and H. Hurd, unpublished data). Based on this finding, as well as on a previous one (Hopwood, *et al*., 2001, and Ahmed, 2005), I would conclude that apoptosis is occurring in the follicular epithelial cells (18h post-treatment), which is a process proceeding

follicular resorption. The proportion of apoptotic follicles is currently under investigation as it was noticeably higher in the ovaries of immunized mosquitoes comparing to that of controls (A. M. Ahmed and H. Hurd, unpublished data).

Follicular resorption is a well-known process as it regulates the mosquito fecundity. This process occurs at Christophers' stage III in very few numbers, (as seen in blood fed non-injected mosquitoes of this study, Fig. 5E & F and 6A) from 12-20h post-blood meal (Clements and Boocock, 1984). Blood meal quantity and quality are two main factors play an important role in inducing this process (Clements, 1992 and Carwardine & Hurd, 1997). Hence, to standardize blood quantity and quality, I used mosquitoes of similar body sizes (in terms of wing sizes) and a single CD mouse to feed all experimental mosquito groups at once. I would, therefore, suggest resorption-inducing, specific or non-specific signals, which to my knowledge, no body knows their nature, which may, in turn, affect vitellogenesis process *via* three routs. The first, is interrupting vitellogenesis in its sole site, the fat body, by somehow. The second, is re-directing most of the resources in the fad body to produce antibacterial peptides at the expense of resources available for vitellogenin production, and hence, not enough vitellogenin produced and reached the follicles for their development to proceed (see Fig. 9). And the third is decreasing the ability of the ovarian follicles to take up the vitellogenin from the haemolymph, and as a result, some undergoing resorption or do not fully develops and hence, retained in the ovary (Fig. 5E & F and 8D). I would, therefore, refer the 28.8% fecundity reduction (the prise paid by the vector as a result of mounting anti-*M. luteus* activity) to one or all of the above mentioned routs.

Data of this study come in consistence with those proved that operating the immune system gives rise to a decrease in the mosquito reproductive output (Ferdig, *et al*., 1993; Sheldon, and Verhulst, 1996, Yan, *et al*., 1997, Ahmed, *et al*., 2002 and Ahmed, 2005). Although melanization and the antibacterial activity

are the main responses working against malaria parasite, they, on the other hand, represented a strong immunity-reproduction trade-off conflict. As illustrated in figure (9), and in the evolutionary point of view, it seems that mosquito fecundity reduction is an adaptive strategy selected as a result of the trade-off that exists between reproduction and immunity. Diversion of resources away from reproduction and towards defense may serve to increase the life span of the vector as it produces more defensive molecules against deadly microorganisms (in this case, anti-bacterial peptides against injected LPS) or melanization against the non-biological agent, Sephadex beads (Ahmed, 2005).

I do believe that the concomitant reproductive stress detected in this study as a result of mounting anti-bacterial response is not in part as a result of a toxic effect of LPS. This is based on studies of Samakovlis, *et al*., (1992) and Wittwer, *et al*., (1997) who proved that LPS is a non-toxic immune stimulator to insect haemocytes *in vitro.* Furthermore, Beutler, (2000) proved a non-toxic effect of LPS on most mammalian cells and tissues. This is confirmed [on the dose administered as that of this study (10 ng/mosquito)] as LPS induced humoral anti-*M. luteus* activity and has no toxic effect on *An. gambiae* cell line *in vitro* where no significant difference was detected in cells mortality between APS- and LPS-treated cells (A. M. Ahmed and H. Hurd, un-published data). Besides, in this study, LPS injection did not increase mosquitoes mortality compared to sham-injection (personal observation and data not shown). Thus, it is very likely that follicular apoptosis and resorption, and hence fecundity reduction, could have happened solely as a result of the induced anti-*M. luteus* activity not as a result of LPS toxicity.

In conclusion, melanization and humoral antimicrobial activity are the two main responses targeted by the studies aiming to motivate the malaria immuno-control strategy (Collins *et al.;* 1986, Shahabuddin *et al*., 1998, James *et al*., 1999, and Kokoza *et al.*, 2000). However, they are costly in terms of reproductive

fitness, which in fact, limits the success of this strategy. Thus, a factor of a dual effect (enhances the immune system and reduces reproductive cost) remains to be looked for. Thus, a suggested immune-enhancive natural product, the black seed oil, has already tested effectively against malaria (Ahmed, 2004). Testing whether or not it has a concomitant reproductive costs, is currently under investigation.

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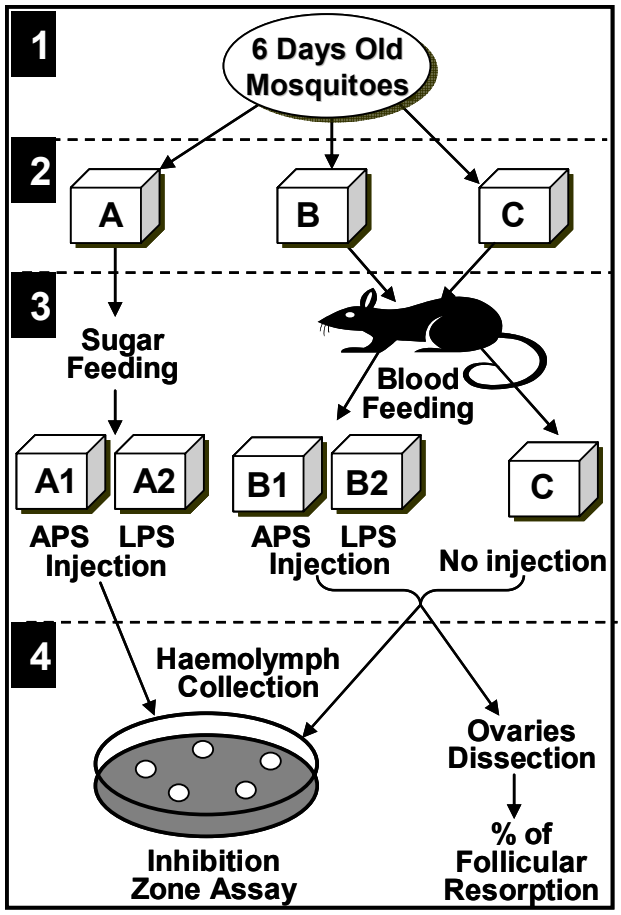

Fig. 1. An illustration showing, in steps, the experimental design of studying the effect of blood meal on the LPS-induced antibacterial activity in *An. gambiae*.

Step 1: Six-days old mosquitoes were used in this study.

Step 2: Mosquitoes were randomly divided into 3 groups, **A**, **B**, and **C**.

Step 3: After feeding of group **A** on 10% glucose, and group **B** on the blood of CD mouse, each was immediately subdivided into two groups (**A1** & **A2** and **B1** & **B2**) then injected with LPS or APS. Group **C** was kept untreated after blood feeding.

Step 4: After treatments, haemolymph was separately collected from thoraces at 18h post-feeding/injection and subjected to inhibition zone assays. Ovaries of mosquitoes from groups **B1**, **B2** and **C** were dissected and tested for follicular apoptosis and resorption.

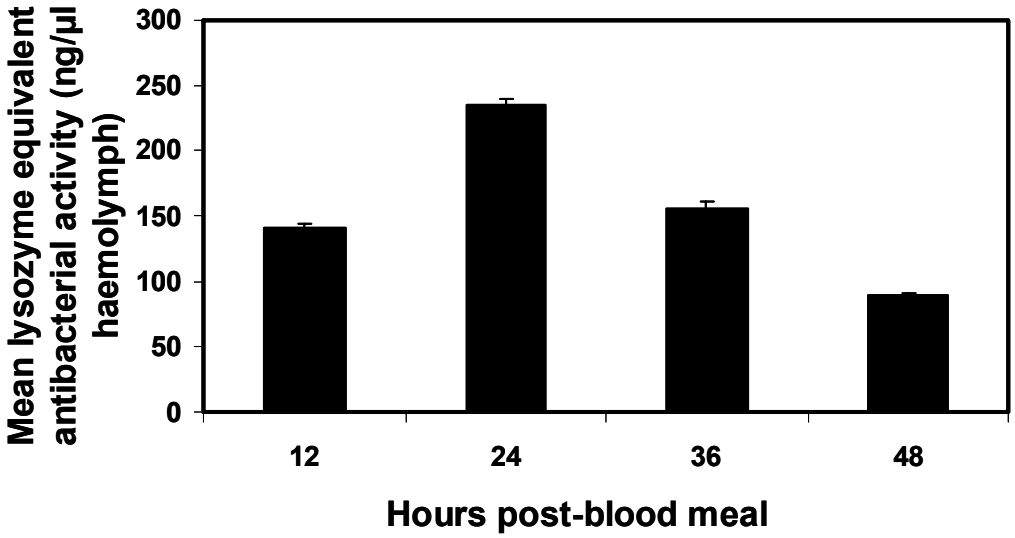

Fig. 2: Time profile for the stimulatory effect of the blood meal on the humoral antibacterial activity in *An. gambiae*. Haemolymph was collected from mosquitoes at different time points post-blood meal, and assigned to inhibition zone assay against *M. luteus* with reference to Lysozyme as a standard. Error bars represent means of five replicates (n = 5 mosquitoes) at each time point.

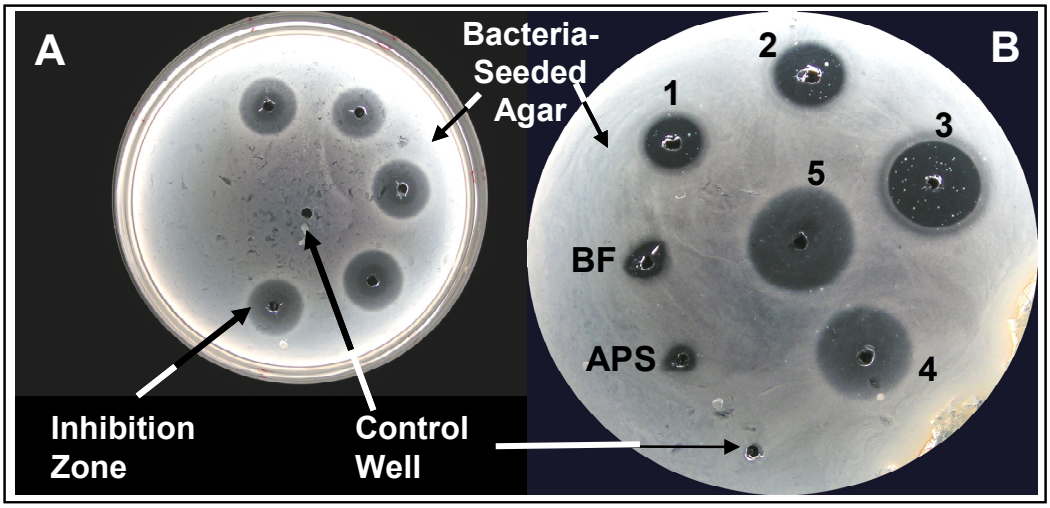

Fig. 3: An example of Petri dishes (9 cm; Sterilin) containing *M. luteus*-seeded agar representing inhibition zone assay, after incubation for overnight at 30ºC, showing the antibacterial activity of *An. gambiae*. **A**: represents 5 replicates of inhibition zones after loading fresh haemolymph [2 µl/well of diluted haemolymph in sterile anticoagulant II solution (Mead et al. 1986)] from LPS-injected sugar-fed mosquitoes 18 h post-injection. Inhibition of bacterial growth appears as a clear zone, which contains the antibacterial peptides. The zone diameter depends on the concentration of antibacterial peptides exist in the loaded haemolymph. Similar dish was loaded with haemolymph from APS-injected mosquitoes at the same time. **B**: represent a similar dish loaded, at the same time, with 5 ascending concentrations of Lysozyme (1:100 – 1:16, numbered from **1 - 5**) as a standard (Ahmed (2004), and an example of two wells loaded with haemolymph from blood-fed (**BF**) or APS-injected sugar-fed mosquitoes (**APS**). A negative control well (loaded with haemolymph from non-injected sugar-fed mosquitoes) was used in each dish. Diameters were measured directly by naked eyes using ruler and concentrations of peptides were calculated as Lysozyme equivalents.

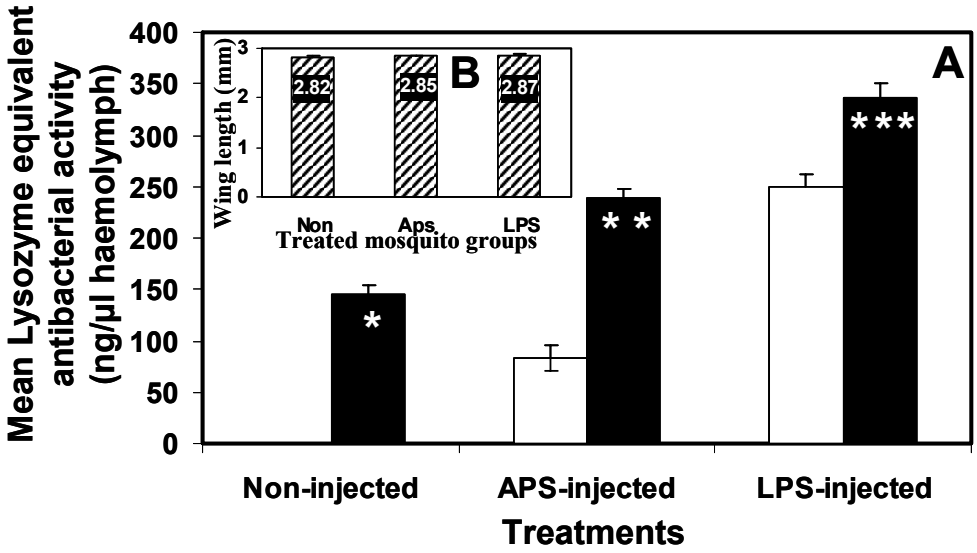

Fig. 4. A: Anti-*M. luteus* humoral activity in sugar-fed (**White bars)** and blood-fed **(black bars)** *An. gambiae* as a result of injecting 10ng of LPS/mosquito at 18h post-feeding/injection. Values of Lysozyme equivalent antibacterial activity were obtained using an inhibition zone assay against *M. luteus* (Fig. 3**B**). Data were first tested for normality using Anderson Darling test and for variances homogeneity prior to One-way ANOVA. Tukey's Pairwise comparison was used to compare between treatments. Error bars represent standard errors of five replicates in each case (n = 5 mosquitoes). White stars on bars represent significant higher activities in blood-fed mosquitoes compared to sugar-fed ones ($P < 0.05$). **B:** Mean wing length (mm) (shown in numbers on bars) of non-injected (Non), APS- and LPS-injected groups. No significant difference in wing sizes was detected (Man-Whitney U, $P > 0.05$, n = 20 mosquitoes).

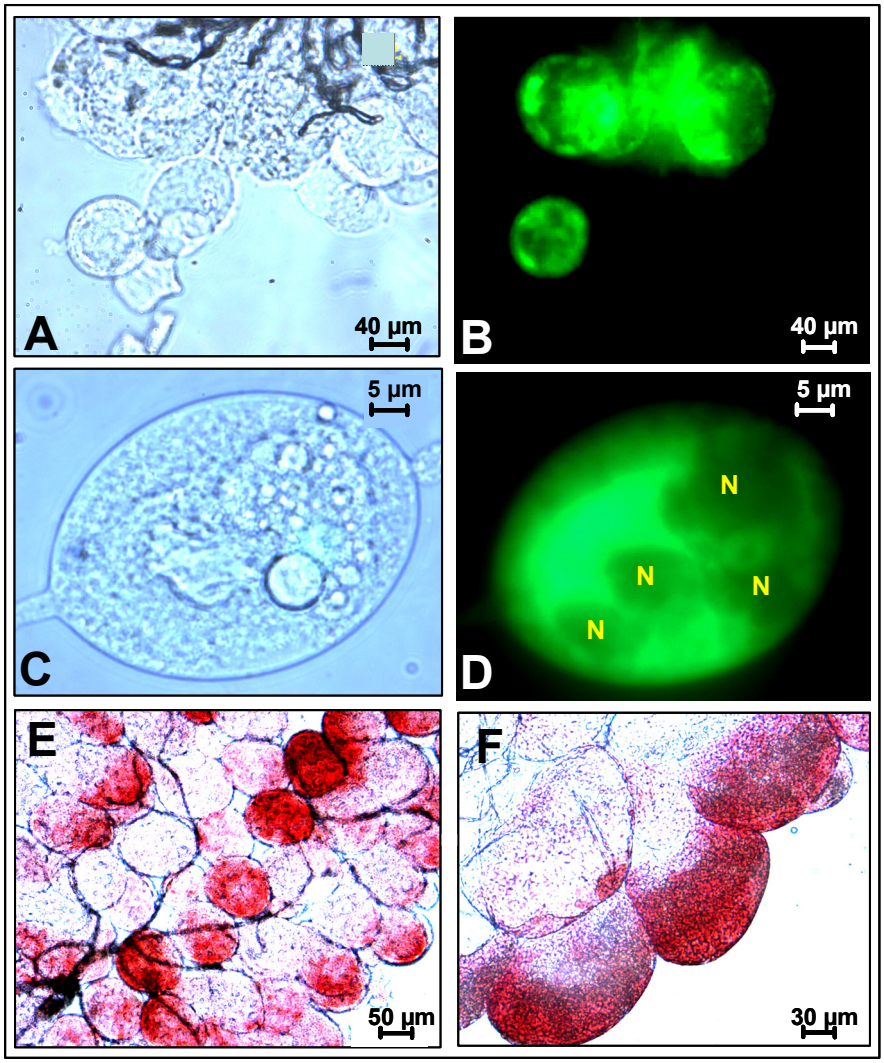

Fig 5. A colour plate showing apoptosis and resorption processes within developing follicles in the ovaries of vitellogenic *An. gambiae* at 18h post-blood meal/LPS-injection (10ng/mosquito). Freshly dissected ovaries from injected and control mosquitoes were used for monitoring follicular apoptosis or resorption. Apoptotic follicles (**green**) were examined under a fluorescence microscope to visualise green fluorescence. **A** and **B** represent an apoptotic part of an ovary from LPS-injected mosquitoes as seen under light and fluorescence microscopes respectively. **C** and **D** represent a higher magnification of an apoptotic follicle, under light and fluorescence microscope respectively, showing apoptotic (fluorescing) outer epithelium but normal nurse cells (**N**). **E** and **F** represent part of an ovary from LPS-injected mosquitoes showing two different magnifications of resorped follicles, which appear with inner contents stained in dark red as a result of up-taking Neutral Red.

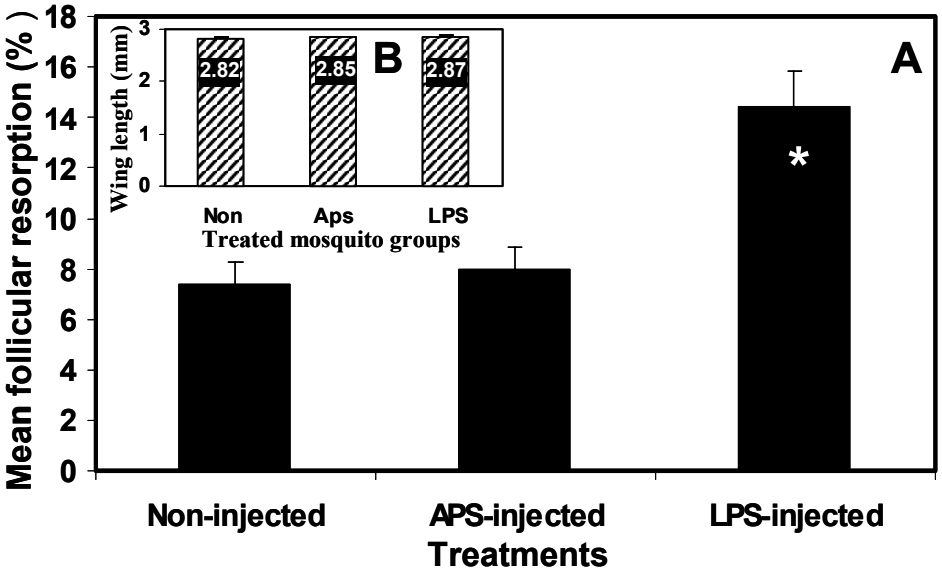

Fig 6. **A:** Follicular resorption in ovaries of blood-fed *An. gambiae* at 18h post-blood meal/injections. Percentages of resorping follicles were calculated from ovaries of LPS-, APS-injected and non-injected (control) mosquitoes. Data were first tested for normality using Anderson Darling test and for variances homogeneity prior to ANOVA. Tukey's Pairwise comparison was used for individual comparison within treatments. Error bars represent the standard error of means of 10 replicates (n = 10 ovary pairs) in each case. White star represent significant higher % of follicular resorption compared to controls ($P < 0.05$). **B:** Mean wing length (mm) (shown in numbers on bars) of non-injected (Non), APS- and LPS-injected groups. No significant difference in wing sizes was detected (Man-Whitney U, $P > 0.05$, n = 20 mosquitoes).

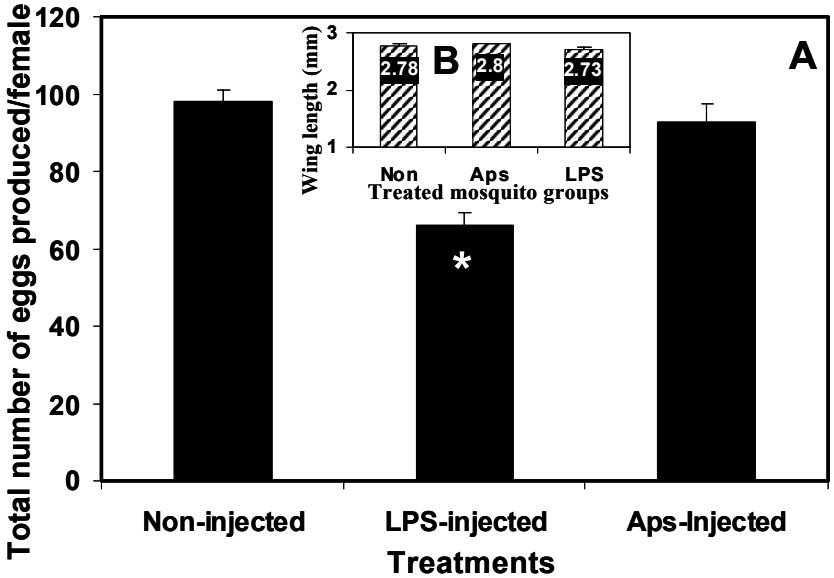

Fig. 7: A: Effect of LPS injection (10ng/mosquito) on *An. gambiae* fecundity. Total number of eggs (number of laid eggs + number of retained eggs) was counted 3-4 days post-blood meal/injection. Data were first tested for normality using Anderson Darling test prior to further statistical analysis. For this nonparametric data, Kruskal-Wallis test showed an overall significant difference between treatments ($P < 0.05$) and Man-Whitney U test was used for individual comparisons within treatments. Error bars represent standard errors of 20 means (n = 20 mosquitoes) for each treatment. White stars represent significant fewer eggs compared to controls ($P < 0.05$). **B:** Mean wing length (mm) (shown in numbers on bars) of non-injected (Non), APS- and LPS-injected groups. No significant difference in wing sizes was detected (Man-Whitney U, $P > 0.05$, n = 20 mosquitoes).

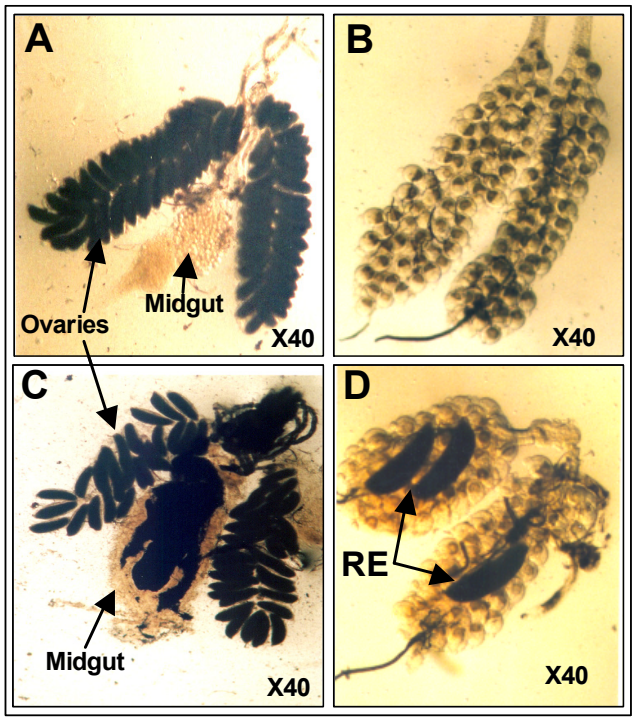

Fig. 8. Examples of freshly dissected ovaries from experimental mosquitoes before and after egg laying. Ovaries were dissected in APS few hours before or 2 days after egg laying. **A** and **B** represent ovaries from APS-injected mosquitoes before and after egg laying respectively showing no retained eggs in **B**. Figures **C** and **D** represent ovaries from LPS-injected mosquitoes before and after egg laying respectively showing some retained eggs (**RE)** in **D** at 2 days after egg laying completion. It appears clearly that ovaries from LPS-injected mosquitoes (**C**) contain fewer fully developed eggs compared to those in APS-injected ones (**A**).

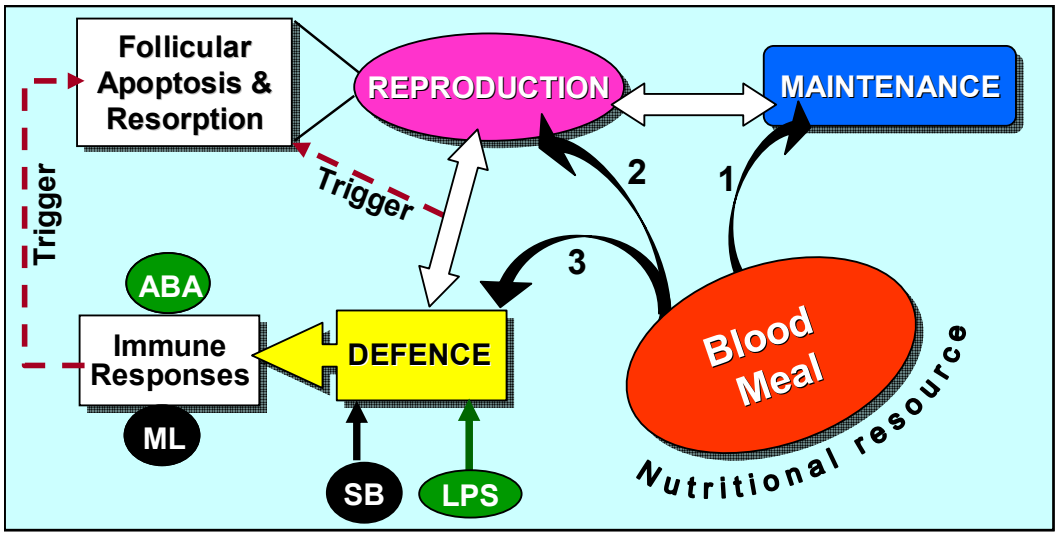

Fig 9. A speculative illustration model showing the trade-off positions (double headed white arrows) in the resource management in control and immunized female mosquito. The blood meal constitutes the main nutritional resource, which is mainly allocated for **MAINTENANCE** and **REPRODUCTION** in normal mosquito (black arrow 1 & 2). Triggering humoral antibacterial activity (**ABA**) or melanization (**ML**), by lipopolysaccharide (**LPS)** injection or Sephadex beads (**SB**) inoculation respectively, may cause re-allocation of some resources towards **DEFENCE** system on the cost of reproduction (black arrow 3), and hence, net resources for reproduction shrink. This enforced new trade-off position, and probably the induced immune responses themselves, may act as triggers of follicular apoptosis and resorption (dotted arrows), and consequently, reduction in fecundity.

الإستجابة المَصْلِية المُضادة للبكتريا فى أنُوفِيلِيس جامْبِيا واالمُفاضَلة بين المناعة والتكاثر: بين الأمل و الحَدِ من استراتيجية المُقاوَمَة-المناعية للملاريا

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فى هذه الدراسة، تم حَث النشاط المَصْلى المُضاد للبكتريا فى البعوضة الناقلة للملاريا، أنُوفِيلِيس جامبيا، ودراسة مكيكانيكية ضعف التناسل الناتج عنه. حثَّت وجبة الدم النشاط المَصْلى ضد بكتريا مَيْكْرُوكُوكَاس لُوتياس، والذى أمْكَنَ قياسه عند ١٢ ساعة، وبلغ ذروته عند ٢٤ ساعة، واصبح صَعْب القياس عند ٤٨ ساعة بعد التغذية. و تم أيضا قياس هذا النشاط فى البعوض المُغْتَذِى على مَحْلول السُّكر عند ١٨ ساعة بعد الحَقن بالمُحَفِز المناعى عَدِيد السْكر الدّهنى، LPS، بتركيز ١٠ نانوجرام/بعوضة. و علاوةً على ذلك، فقد وُجِد أن وَجْبة الدم تؤدى إلى تحفيز نوعى لتأثير LPS عند حَقْنِه فى البعوض المُغتذى على الدم، حيث أنه تم تسجيل نشاط مَصْلى أعلى نوعيا ضد بكتريا مَيْكْرُوكُوكَاس لُوتياس مقارنة بتلك فى المغتذى على السكر بعد حَقْنِه بـ LPS. هذه النتائج تَقْترح أن وَجْبة الدم لها تأثيرا مُحفِزا للمناعه، والذى قد يكون فى مَصْلَحة استراتيجية المقاومة المناعية للملاريا. من ناحية أخرى، فإن حَث هذا النشاط المناعى قد فَرَضَ تكلُفة تناسلية، حيث أن البعوض المحقون بـ LPS أظهر نقصا نوعيا فى الخُصوبة بمقدار ٢٨,٨% مقارنة بتلك فى البعوض المحقون بالمحلول الفيسيولوچى APS. ولقد تم دراسة عَمَلِيَّتَىْ اضْمِحْلال الحُوَيْصلات البَيْضية النامية و الموت المُبَرْمَج لخلاياها كَمِيكانِيكِيَّتَيْن مُقترَحَتَيْن للنقص فى الخُصوبة. فمِن ناحية، تم الكشف عن عملية الموت المُبَرمَج بخلايا الحويصلات البيضية النامية عند ١٨ ساعة بعد الحَقن بـ LPS، والتى تَبْدو وكأنها تَحْدُث فى خلايا الطبقة الطلائية لها. و من ناحية أخرى، تم الكشف عن عملية اضْمِحْلال الحويصلات البيضية النامية أيضا عند ١٨ ساعة بعد الحقن بـ LPS، والتى كان حُدوثها أكثر نوعيا بنسبة ٤٤,٥% من تلك فى البعوض المحقون بـ APS. بناءا على هذه النتائج، فإن هذه الدراسة تَقْترح أنه فى حالة استخدام الإستجابة المناعية المَصْلية فى المعركة ضد الملاريا فإنها قد تكون مُكَلِفة من حيث ضعف الخُصُوبة. ولهذا، وكجزء هام فى سيناريو تصادم المناعة بالتكاثر، فإن مناقشة هذه النتائج تنتهى باقتراح أن تأثير التحفيز المناعى على الخُصوبة قد يشترك كعامل مُحِد لنجاح استراتيجية المقاومة المناعية للملاريا.