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Effects of malaria infection on vitellogenesis in *Anopheles gambiae* during two gonotrophic cycles

A. M. Ahmed,[1] R. Maingon,[1] P. Romans[2] and H. Hurd[1]

[1]*Centre for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Keele, Staffordshire, ST5 5BG, UK,* [2]*Department of Zoology, University of Toronto, Toronto, Ontario, M5S 3G5, Canada*

Abstract

We report changes in the abundance of vitellogenin (Vg) mRNA, and concentration of haemolymph Vg and ovarian vitellin (Vn) in *Anopheles gambiae* following infection with *Plasmodium yoelii nigeriensis*. A parasite-induced reduction in Vg mRNA abundance was first detected 24 h after feeding on an infective blood meal, when ookinetes were invading the midgut. During a second gonotrophic cycle post-infection, developing oocysts reduced Vg mRNA abundance by up to 33% and the effect was detected from 2 h post blood meal. Concentrations of Vg were initially reduced by infection during the second cycle, as predicted from Vg mRNA measurements. However, after 24 h, excess Vg had accumulated in the haemolymph. This accumulation may be due to impaired uptake, since ovarian vitellin accumulation was significantly decreased by infection during both gonotrophic cycles.

Keywords: *Anopheles gambiae*, vitellogenin mRNA, fecundity reduction, vitellogenesis, *Plasmodium yoelii nigeriensis*.

Introduction

In common with many parasitic infections of invertebrates, malaria induces changes in vector behaviour and physiology. These affect the fitness of the mosquito and include a consistent reduction in reproductive success (Hurd *et al.*, 1995; Ahmed *et al.*, 1999). Host fecundity reduction may be an adaptive strategy on the part of the host (Forbes, 1993) or the parasite (Obrebski, 1975; Read, 1990) whereby metabolic resources are directed away from host egg production, thus decreasing the virulence caused by nutrient robbery and increasing the potential life span of the parasite/host (Hurd & Webb, 1997).

The rodent malaria, *Plasmodium yoelii nigeriensis*, causes a significant reduction in the fecundity of both *Anopheles stephensi* (Hogg & Hurd, 1995; Jahan & Hurd, 1998) and *An. gambiae*, the major vector of *falciparum* malaria in sub-Saharan Africa (Ahmed *et al.*, 1999). The latter exhibited a 41% reduction in the number of eggs produced in the gonotrophic cycle immediately following an infective blood meal. This effect has also been observed in *An. gambiae* which has been naturally infected with *P. falciparum* (Hogg & Hurd, 1997). It is not the result of depleted nutrient intake as, in our studies, mosquitoes undergoing infection-associated fecundity reduction imbibe the same size of blood meal, and digestion proceeds normally (Hogg & Hurd, 1995, 1997; Jahan *et al.*, 1999; Taylor & Hurd, 2001). In infected *An. stephensi*, significant ovarian follicle atresia occurs midway through the gonotrophic cycle (Carwardine & Hurd, 1997). This has been associated with the induction of apoptosis in both follicular epithelial cells and nurse cells (Hopwood *et al.*, 2001). As a result of follicular resorption, the vitellin (Vn) content in the ovaries is reduced and vitellogenin (Vg) accumulates in the haemolymph of this species (Jahan & Hurd, 1998). However, no previous investigation has examined whether the effects of malaria infection on mosquito vitellogenesis extend to the fat body. The objective of this study was to determine whether *P. y. nigeriensis* infection affected Vg mRNA abundance and other aspects of vitellogenesis in *An. gambiae*, as a prelude to discovery of parasite and host molecular mechanisms underlying fecundity reduction.

The synthesis of nutritive egg proteins takes place in the mosquito fat body, the exclusive site of Vg synthesis in the great majority of insects (Hagedorn & Judson, 1972; Valle, 1993). Vg genes have been cloned from *An. gambiae* (Romans *et al.*, 1998; P. Romans, unpublished data). Briefly, the Vg gene family in this species consists of three or four tandem genes, three in genomes lacking the *Ikirara*1 insertion in an inter-Vg gene region, four in genomes containing it, and, regardless of the presence or absence of the transposable element, one additional dispersed Vg

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Correspondence: Dr Hilary Hurd, Centre for Applied Entomology and Parasitology, School of Life Sciences, Huxley Building, Keele University, Keele, Staffordshire, ST5 5BG, UK. Tel.: +44 1782 583034, fax: +44 1782 563516 e-mail: h.hurd@keele.ac.uk

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gene. All Vg genes are located at the same cytogenetic location, 18A/B on chromosome 2R. Southern blot analysis of the gene family and nucleotide sequence of the tandem genes (GenBank accession no. AF281078) has indicated that Vg genes are virtually identical in their protein coding regions. In addition, the predicted protein products of *An. gambiae* Vg genes are highly identical to that of the abundantly expressed *Aedes aegypti* VgA1 gene (Romans *et al.*, 1995; GenBank accession no. L41842).

In this study we used a cloned fragment from the *An. gambiae* Vg1 gene to investigate changes in Vg mRNA abundance in response to a blood meal and the effect of *Plasmodium* infection on this expression through two gonotrophic cycles. We also used antibodies raised against Vn to quantify Vg in haemolymph and Vn accumulation in the ovaries. In addition, we compared the fertility of oocyst-infected and uninfected *An. gambiae* to demonstrate that the molecular and physiological effects we were observing actually resulted in a reduced fecundity. Thus, we have monitored several aspects of the process of vitellogenesis in *An. gambiae* during two phases of malaria infection – the initial establishment of infection and the early growth of oocysts on the mosquito midgut.

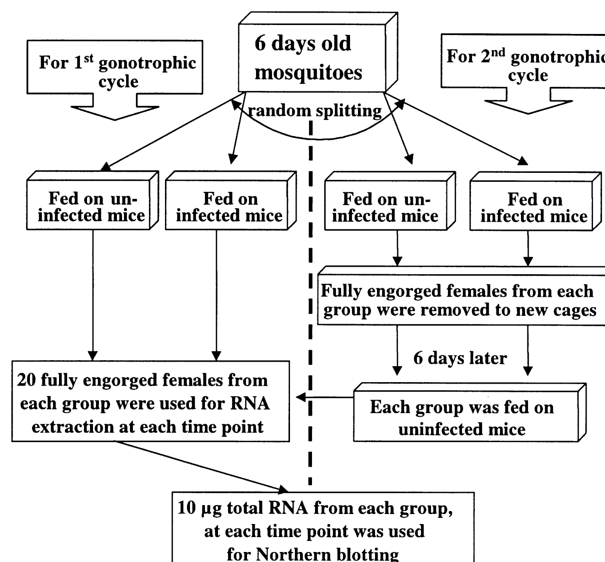

Figure 1. Experimental protocol for studying the effects of malaria infection on vitellogenesis in *An. gambiae*.

Results

The effect of Plasmodium *infection on Vg mRNA abundance*

Initially, we monitored the rise and fall of Vg mRNA abundance during the first gonotrophic cycle of uninfected *An. gambiae* by probing dot-blots with the cloned fragment of the Vg1 gene. Vg mRNA was initially detected at 1 h and increased in abundance to plateau between 28 h and 36 h (results not shown). However, although this profile is consistent, the timing of peak abundance varies slightly from one generation of mosquitoes to another (personal observations).

The experimental protocol we used to compare Vg mRNA abundance in uninfected and infected mosquitoes during two gonotrophic cycles post-infection is illustrated in Fig. 1. Prior to the first gonotrophic cycle, the mosquitoes were fed on either infected or uninfected mice whereas, prior to the second cycle, both infected and uninfected mosquitoes were fed on uninfected mice. In a first experiment, six time-points post blood meal (PBM) were selected for sampling whereas, in a repeat experiment, sampling was reduced to four time points. Figure 2 is an autoradiograph of a Northern blot showing changes in the temporal abundance of Vg and ribosomal protein S7 transcripts from uninfected and infected females during the second gonotrophic cycle. Ribosomal protein S7 mRNA (Salazar *et al.*, 1993) proved to be a reliable loading control, as had been found by Luckhart *et al.* (1998) in their studies of midgut gene expression, since approximately equal abundance was observed throughout the gonotrophic cycle.

During the first 24 h of the 1st gonotrophic cycle, no differences in Vg mRNA abundance were detected between control and infected mosquitoes. However, by 36 h PBM, there was an average 35.1% reduction in Vg mRNA abundance in infected mosquitoes. This difference was not statistically significant, possibly because of the large signal variance that was detected between different samples at this time (Fig. 3A). During the second gonotrophic cycle, after a non-infective blood meal, Vg mRNA abundance was significantly reduced in *Plasmodium*-infected females from 2 h through to 24 h PBM, the time at which levels peaked (Fig. 3B,C). The maximum reduction in Vg mRNA abundance was 32.75%, which occurred at 24 h. A second experiment, performed on a different generation of *An. gambiae* under similar infectivity conditions, confirmed these results (Fig. 3D,E). The maximum reduction recorded in this experiment was 26.9%. During the first cycle, Vg mRNA was again reduced midway through the first gonotrophic cycle ($P = 0.054$ at 24 h PBM).

Disease parameters relating to the mice used in these experiments, and mosquito size and infection data, are given in Tables 1 and 2, respectively. In all experiments, mouse weights, temperatures and blood PCV were within the narrow range known to cause no detectable difference to mosquito egg production because mosquito feeding behaviour and the protein content of the blood are not significantly affected at this stage of infection (Hogg & Hurd, 1995; Taylor & Hurd, 2001; Hurd, unpublished results). If the mosquitoes are allowed to feed to repletion, the size of

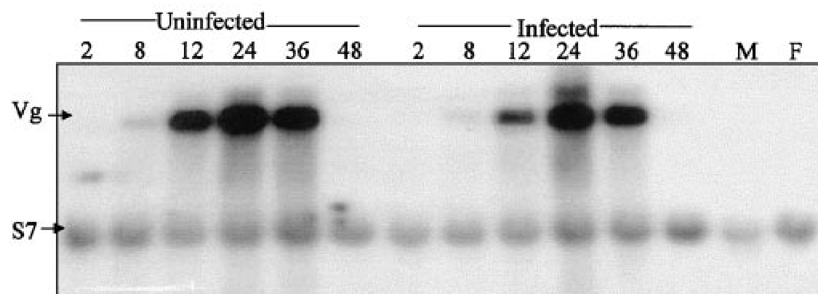

Figure 2. Autoradiograph of a Northern blot showing Vg mRNA levels in uninfected and infected female mosquitoes at various times post blood meal during the second gonotrophic cycle. The blot was hybridized with radiolabelled probes for Vg and ribosomal protein S7. Numbers indicate hours post blood meal. M: males, F: sugar-fed females.

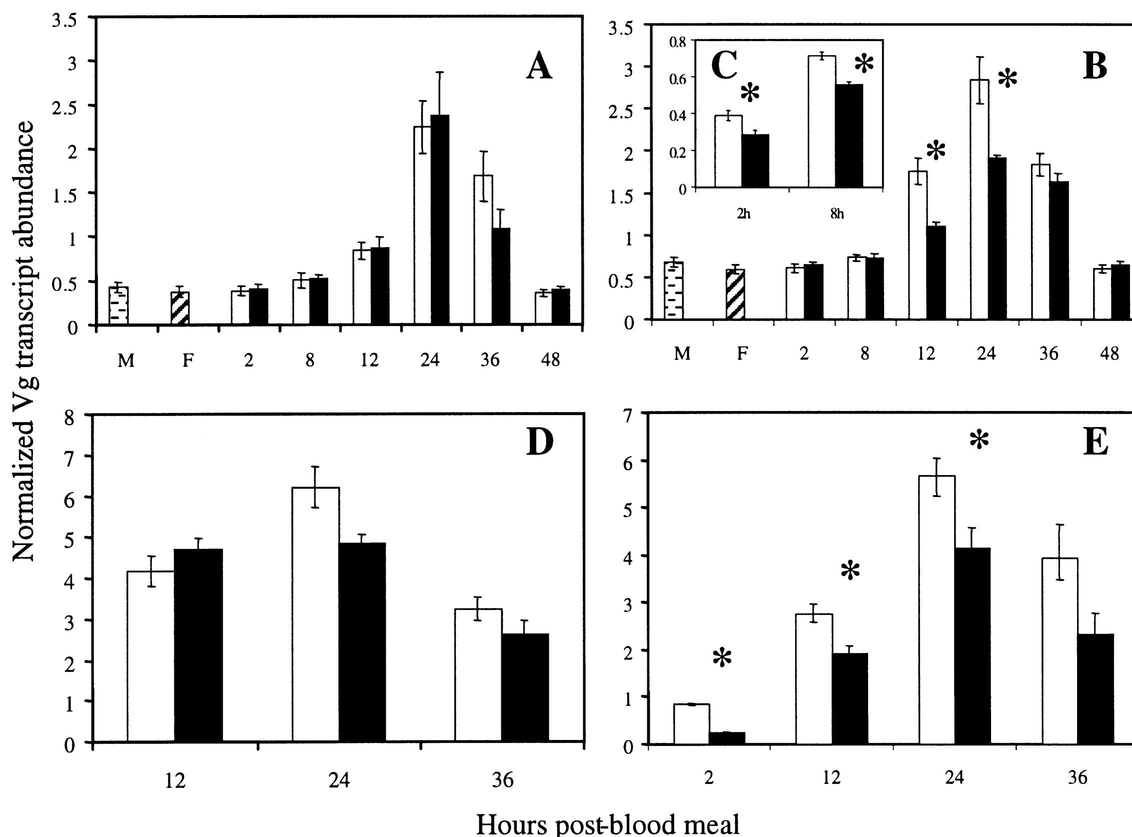

Figure 3. Vitellogenin mRNA abundance in uninfected (white bars) and *Plasmodium*-infected (black bars) *An. gambiae*, during the 1st and 2nd gonotrophic cycles (A and B). The experiment was repeated (D and E) for the 1st and 2nd gonotrophic cycles, respectively. Total RNA (10 μg) from whole male (M), sugarfed females (F) and blood-fed females (at different times post-blood meal) was assayed by quantification of signals on Northern blots after 2 h exposure to phosphor screens. The Northern blots used in (B) were also re-exposed to phosphor screens for 10 h in order to visualize the weak Vg mRNA signals at 2 h and 8 h (C). Vg transcript abundance was normalized against ribosomal protein S7 mRNA. Data from six treatment replicates (A, B, C and E) and 10 replicates (D) at each time point were analysed using two-way ANOVA. Error bars represent the mean ± SE. *Significant difference between group means (Tukey's test, $P < 0.05$).

the blood meal taken is related to their body size (Briegel, 1990), which can be assessed by measuring wing length. Mosquito size was used in this study as an indirect measurement of blood meal size, since the experimental protocol precluded the measurement of blood meals either by haemoglobinometry (Briegel *et al.*, 1979) or haematin excretion (Hurd *et al.*, 1995). Mosquito wing lengths did not differ significantly between treatment groups. Thus, the observed changes in Vg mRNA abundance should be due to *Plasmodium* infection rather than to intrinsic differences in mice, mosquitoes or blood meal size. Total parasitaemia in the mice ranged from 7.7 to 9.3% of erythrocytes infected. 90–95% of the mosquitoes became infected with oocysts.

The effect of Plasmodium *on Vg and Vn titres*

Haemolymph Vg concentration peaked earlier and was significantly reduced at 30 h following an initial infective blood meal compared with that of females fed on uninfected

Table 1. Physiological parameters of mice used to blood feed mosquitoes for the determination of Vg mRNA abundance during the 1st or 2nd gonotrophic cycle (see Fig. 1). In each case, two uninfected or infected mice (m1 and m2) were used to provide blood meals for the different groups of mosquitoes.

	For study of 1st gonotrophic cycle				For study of 2nd gonotrophic cycle*			
	Uninfected		Infected		1st BM Uninfected		1st BM Infected	
	m1	m2	m1	m2	m1	m2	m1	m2
W	43.0	41.5	44.6	44.2	41.5	40.0	42.0	43.0
PCV	42.0	40.0	43.0	42.0	42.0	43.0	43.0	44.0
T	37.0	36.5	37.3	37.0	36.7	37.2	37.0	37.5
Pa	–	–	8.2	9.3	–	–	7.7	8.2

W: body weight (g); PCV: blood packed cell volume (%); T: body temperature (°C); Pa: parasitaemia (% erythrocytes infected).
*Uninfected mice used for blood meals initiating the 2nd gonotrophic cycle had similar physiological parameters.

Table 2. Experimental parameters of mosquitoes used for determination of Vg mRNA abundance during the 1st or 2nd gonotrophic cycle.

	For first gonotrophic cycle			For second gonotrophic cycle		
	Uninfected	Infected	*P*	Uninfected	Infected	*P*
WL	2.9 ± 0.01	3.0 ± 0.01	0.20	3.0 ± 0.01	3.0 ± 0.01	0.50
Oo	0	89.0 ± 19.0	–	0	89.8 ± 21.2	–

WL: mean wing length (mm ± S.E). Oo: mean number of oocysts/midgut ± SE; *P*: probability derived from Student's *t*-test ($n = 20$).

mice. By 36 h PBM, Vg haemolymph titre was declining in both uninfected and infected females and the significant differences between the two groups had disappeared. Furthermore, infected mosquitoes had slightly, but non-significantly, more Vg remaining in the haemolymph at 48 h. By 36 h PBM, the ovarian Vn content of these infected females was significantly reduced relative to the uninfected ones (Fig. 4B).

In contrast, the presence of developing oocysts caused significant alterations in Vg concentrations throughout the second gonotrophic cycle. The early decreases in haemolymph Vg titre mirrored the reductions in Vg mRNA abundance. However, haemolymph Vg accumulated to significantly higher levels from 24 h to 36 h in infected mosquitoes (Fig. 4C). Furthermore, significantly less Vn accumulated in the ovaries of infected females relative to non-infected females at all time points during this second gonotrophic cycle (Fig. 4D).

Mouse disease parameters and data concerning mosquito size and infection were similar to those in Tables 2 and 3 and could not account for the differences in Vg and Vn that we observed.

Egg production

In common with a previous study of the effect of *P. y. nigeriensis* on the first gonotrophic cycle of *An. gambiae* (Ahmed *et al.*, 1999), the total number of eggs laid by parous females was significantly reduced in the presence of developing oocysts, as was the percentage of laid eggs that hatched to first instar larvae (Table 3). Thus egg viability, as well as fecundity, was significantly reduced when oocysts were present. All the infected females in this study contained more than fifty oocyst/midgut. The number of eggs matured by a mosquito is related to both the quantity and quality of the blood meal (Briegel & Rezzonico, 1985). It is also affected by the teneral reserves and size of the mosquito (Hurd *et al.*, 1995). In our experiments these parameters were standardized as far as possible. In particular, mosquito size, as this acts as an indirect indicator of blood meal size. Thus differences in the number of eggs produced could not be attributable to differences in the size of the control or infected mosquitoes, or to blood quality or the body temperature of the mice they fed on (data not shown, but similar to Table 1).

Discussion

Factors such as nutrient supply and mating status are known to modulate the production of eggs in many insects via alterations in the synthesis and/or uptake of yolk protein and resorption of developing follicles (Kubli, 1986; Bownes & Reid, 1990; Sondergaard *et al.*, 1995). Our results show that infection with the protozoan parasite *P. y. nigeriensis* also affects many aspects of the process of vitellogenesis in an important malarial vector, *An. gambiae*. Potential explanations for these changes include the possibility that *Plasmodium* infection may disrupt multiple stages of vitellogenesis in a malaria-stage dependent manner. Alternatively, the parasite may initiate an ovarian pathology that feeds back negatively on the fat body.

The impact of the parasite is not apparent immediately after the mosquito feeds on an infective blood meal because, initially, the process of vitellogenesis appears to proceed normally. This suggests that intrinsic differences in

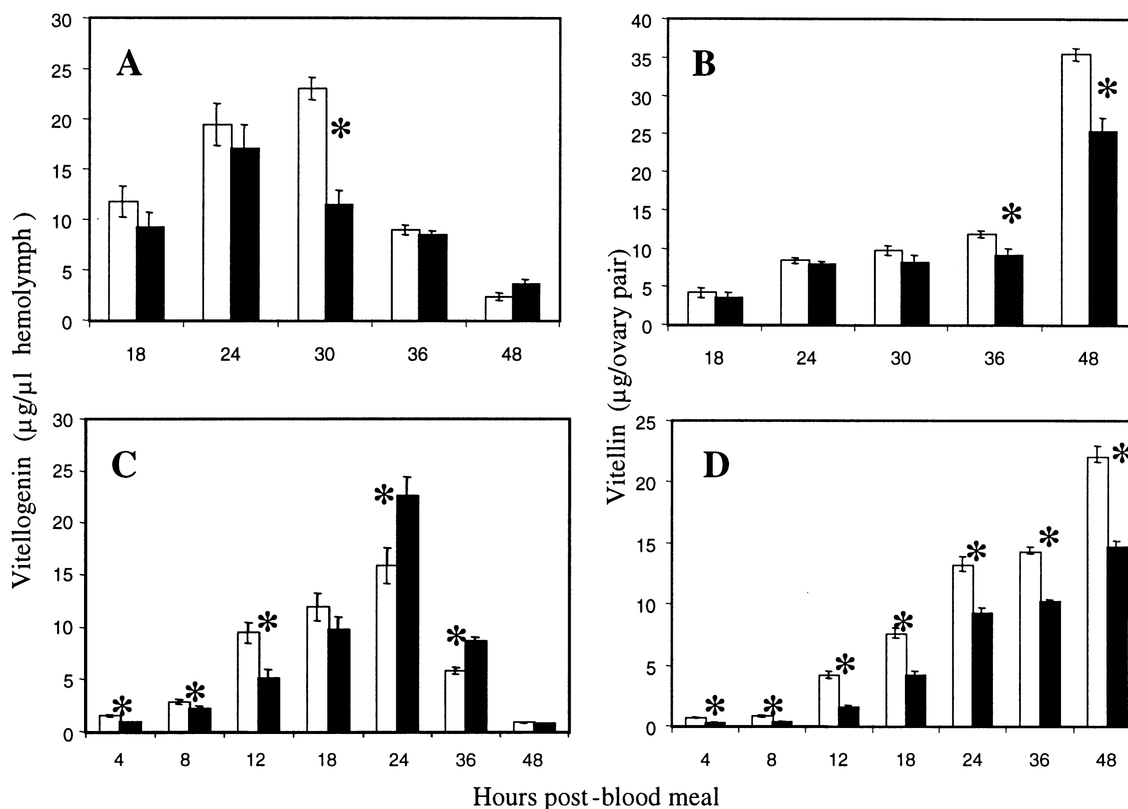

Figure 4. Effect of malaria infection on vitellogenesis in *An. gambiae.* Concentration of Vg and Vn in the haemolymph and ovaries of uninfected (white bars) and *Plasmodium*-infected (black bars) females, at various hours post blood meal, during first gonotrophic cycle (A and B) and 2nd gonotrophic cycle (C and D). Each bar represents the mean ± SE of 20 mosquitoes. *Significant differences between uninfected and infected groups ($P < 0.05$) as determined by Mann–Whitney *U*-test.

Table 3. Effect of malaria infection on the fecundity and fertility of *An. gambiae* during the 1st gonotrophic cycle (1st GC) (data from Ahmed *et al.*, 1999) and 2nd gonotrophic cycle (2nd GC).

	Wing length (mm, mean ± SE)		No. of eggs laid (mean ± SE)		% Egg Hatch (mean ± SE)	
	1st GC	2nd GC	1st GC	2nd GC	1st GC	2nd GC
U	3.02 ± 0.04	2.96 ± 0.02	89.7 ± 8.0	55.5 ± 3.8	78.4 ± 6.2	90.6 ± 1.8
I	3.00 ± 0.03	2.98 ± 0.01	37.0 ± 9.8	42.4 ± 4.6	46.3 ± 10.7	74.3 ± 6.6
P	0.96	0.47	0.005*	0.04*	0.02*	0.04*

U: uninfected; I: infected; *P*: probability derived from Student's *t*-test.
*Statistically significant difference ($n = 20$).

the plasma content of malaria-infected mouse blood, such as elevated cytokines or nitric oxide (Cao *et al.*, 1998; Fleck *et al.*, 1994) that could have an immediate effect on vitellogenesis, do not do so. Alternatively, if they do, the effects have a time lag. During these initial hours following an infective blood meal the parasites are not in contact with mosquito tissue as they are developing into the invasive, ookinete stage within the blood meal bolus (Killick-Kendrick, 1973). We observed that, under our experimental conditions, ookinete numbers in the midgut lumen of *An. gambiae* peaked at 20 h (data not shown). Ookinetes progressively invade the midgut from the time of first appearance in the blood bolus and transform into oocysts extracellularly between the basal labyrinth and the basal lamina at around 24 h PBM (Syafruddin *et al.*, 1991; Sinden, 1998). This invasion thus coincides with the time at which vitellogenesis is first affected. We propose that the invasion of midgut cells may initiate signals that directly or indirectly cause the changes we detect in the fat body and the ovary.

Ookinete invasion and transformation to oocysts has previously been shown to increase the abundance of several gene transcripts in the midgut and non-midgut carcass of *P. berghei* infected anopheline mosquitoes. Richman and co-workers observed a threefold induction of defensin and Gram-negative bacteria-binding genes in *An. gambiae* 24 h after feeding on infected blood (Richman *et al.*, 1997) and a strong induction of the *An. stephensi* nitric oxide synthase gene occurred in infected mosquitoes 1–3 days post blood meal (Luckhart *et al.*, 1998). The products of these genes are associated with the mosquito defence system.

Here we have shown, for the first time, that malaria infection can also cause a decrease in mosquito gene transcripts. We first detected a decrease in Vg mRNA steady state levels at 24 h PBM (Fig. 3A,D), when ookinetes were invading and transforming into oocysts. This difference was only significant at a *P*-value of 0.054, however. Due to the small sample size that we were able to analyse (initially six replicates and ten replicates in the repeat experiment), we believe that this decrease in Vg mRNA represents a biologically meaningful result and that, once the ookinetes begin to invade the midgut, the infection has a negative effect on vitellogenesis in the fat body.

Haemolymph Vg titres also remained unaffected until the parasite had made contact with the mosquito midgut, where after they were reduced significantly. This 50% drop in titre in infected mosquitoes was also noted in a pilot study (data not shown). It may be a result of the decrease in Vg mRNA abundance that is observed after 24 h, which would result in reduced Vg synthesis and secretion into the haemolymph of infected females.

Shortly after the decrease in haemolymph Vg, these initial stages of infection also resulted in a significant decrease in the Vn content of ovaries from infected mosquitoes. We have observed that this parasite has a similar effect on the ovarian Vn content of another malarial vector, *An. stephensi.* However, in the latter species, accumulation of Vn in the oocytes of normal females proceeded at a faster rate than in *An. gambiae* and the parasite-induced decrease in Vn content occurred earlier (Hogg *et al.*, 1997; Jahan & Hurd, 1998).

By 48 h PBM, ovarian Vn was reduced by 28.8% in *An. gambiae*. This is probably explained, at least in part, by the significant increase in the number of resorbing follicles detected in ovaries from *P. y. nigeriensis* infected *An. stephensi* (Carwardine & Hurd, 1997; Hopwood *et al.*, 2001) and *An. gambiae* (Tunnicliff & Hurd, personal observations).

Blood feeding on uninfected mice 6 days after the first blood meal initiated a second gonotrophic cycle. Mosquitoes, by now harbouring developing oocysts, contained significantly less Vg mRNA than uninfected females until after the peak in transcript levels in this cycle. As expected, titres of circulating Vg were also significantly reduced early in the 2nd gonotrophic cycle. However, this situation was later reversed and, by 24 h, Vg had accumulated to 29.9% above normal levels in infected mosquitoes. This accumulation might be explained by events occurring in the ovary, where significantly less ovarian protein is present at all stages observed (Fig. 4D). If the ovarian uptake of Vg is partially inhibited, it is likely to accumulate in the haemolymph. Thus a reduction in uptake of Vg could counterbalance a reduction in fat body synthesis and secretion into the haemolymph. Depending on the relative degree of inhibition in each tissue, this could result in a change in circulating Vg titre from a significant reduction to no difference and then, eventually, an increase in titre in infected mosquitoes. This we see in the results presented in Fig. 4C. There was also a trend towards this result during the first gonotrophic cycle, although the differences between infected and uninfected mosquitoes were not significant (Fig. 4A), probably because the parasite-induced effects were not initiated until midway through the gonotrophic cycle.

Findings from previous studies suggest two possible mechanisms underlying the changes observed in the ovary in this investigation. We have previously observed that the ovarian uptake of Vg in tapeworm-infected beetles occurs more slowly. This has been attributed to a significant decrease in the development of patency of the follicular epithelium cells that results from a parasite-induced competitive inhibition of juvenile hormone binding to these cells (Hurd & Arme, 1987; Webb & Hurd, 1995a,b; Webb & Hurd, 1997). We have some evidence that Vg uptake takes place more slowly in infected mosquitoes because development of the terminal follicles in malaria-infected *An. stephensi* is retarded compared to uninfected females (Carwardine & Hurd, 1997). In addition, we have observed that a proportion of follicles in infected mosquitoes do not complete their development but are resorbed during the first gonotrophic cycle of *An. stephensi* (Carwardine & Hurd, 1997; Hopwood *et al.*, 2001) and in the first and second gonotrophic cycle in *An. gambiae* (Tunnicliff & Hurd, unpublished observations). Whichever underlying mechanisms are responsible, it is likely that vitellogenin accumulates in the haemolymph because uptake by the ovaries is reduced.

The reduction in ovarian protein content that occurred during the first and second gonotrophic cycles clearly affected egg total production and fertility; therefore, malaria infection has considerably negative effects upon the reproductive fitness of its vector.

The mosquitoes in our study had high mean intensities of infection compared to mosquitoes in the field, which are usually infected with just one or two oocysts (Hogg & Hurd, 1997). However, we believe that the changes we observed in Vg transcript levels and Vg and Vn titres were at least qualitatively similar to what would be observed in wild-infected mosquitoes for two reasons. First, a previous study demonstrated that significant reduction in fecundity (17.5%) also occurs in field-caught *An. gambiae* infected with *P. falciparum* (Hogg & Hurd, 1997) Second, laboratory infected *An. stephensi* exhibiting a mean intensity of infection of four oocysts also laid significantly fewer eggs (18% fewer during the first gonotrophic cycle and 27% during the second cycle) than uninfected females (Hogg & Hurd, 1995). Fecundity reductions of the same order of magnitude are thus produced, despite large differences in parasite burden. In our beetle/tapeworm model some parasite density-dependent effects occurred but a critical threshold is reached at ten metacestodes, above which no further effect is caused (Webb & Hurd, 1996). It is possible that

this phenomenon also occurs in malaria/mosquito infections. Moreover, parasites may employ multiple strategies to affect fecundity reduction, only some elements of which may be density related.

Mosquito reproductive success has been linked to blood meal size (see Clements, 1992). However, we propose that the parasite-induced effects on vitellogenesis reported here are not due to nutrient deprivation. The blood meal haemoglobin imbibed by mosquitoes fed on gametocytaemic mice with parasitaemias and PCVs similar to those in this experiment is actually elevated in comparison to mosquitoes fed on control mice, and plasma protein levels are unchanged (Taylor & Hurd, 2001). Furthermore, protein digestion proceeds normally in infected mosquitoes (Jahan *et al.*, 1999). Once oocysts are present, it is conceivable that they could compete with the vector for trace-level nutrients. However, we think it is unlikely that our fully engorged infected mosquitoes lacked sufficient protein to mature a complete egg batch because Briegel (1990) calculated that only 19% of the blood meal protein is incorporated into the ovaries during normal vitellogenesis in *An. gambiae*.

The transcription of mosquito Vg genes is activated in response to a blood meal but the details of their activation have not been completely elucidated. It has been shown that 20-hydroxyecdysone (20-E) stimulates Vg synthesis in *An. stephensi* and *Ae. aegypti* (Redfern, 1982; Hagedorn, 1985). In *Ae. aegypti*, the action of 20-E, bound to the heterodimeric ecdysteroid receptor/ultraspiracle directly activates transcription of a Vg gene (Martin *et al.*, 2001). It is not known whether malaria impacts upon this control mechanism to cause a decrease in Vg mRNA abundance. Indeed, our current data do not allow us to determine whether gene transcription rates are down-regulated or mRNA turnover rate increased, or both. Future *in vitro* studies on the dynamics of fat body Vg synthesis may help to elucidate this, and also to determine whether the effect of the parasite is direct or indirect.

Vg gene promoters have been identified as potentially useful tools for regulating the expression of malaria toxin genes in transgenic mosquitoes (Gwadz, 1994). This general strategy has now been tested successfully using bacteria-infected transgenic *Ae. aegypti* expressing a defensin gene from the VgA1 promoter (Kokoza *et al.*, 2000). With the recent germ-line transformation of *An. stephensi* (Catteruccia *et al.,* 2000), it could now be tried in a vector of human malaria. Our work suggests that it would not be rendered less useful in newly infected mosquitoes if the toxin targeted stages prior to the very early oocyst. However, achieving sufficient expression of an inserted foreign gene following a subsequent blood meal might not be as efficient as in an uninfected female. Candidate genes for the production of incompetent vectors should be chosen with this in mind.

Ovarian uptake of Vg does not appear to control fat body synthesis (Van Handel & Lea, 1984; Clements, 1992; Bownes & Reid, 1990). Nor does the Vg titre in the haemolymph initiate or regulate yolk uptake (see the review by Sappington *et al.*, 1998). Thus, it is unlikely that a reduction in circulating Vg titres could be responsible for the decrease in Vn accumulation in ovaries of infected females. We postulate that we have observed a parasite-induced modulation of two separate events, namely; impairment of ovary function, partly or wholly as a result of follicle resorption, and a decrease in Vg mRNA levels that leads to a decrease in protein synthesis in the fat body. However, the reduction in the number of follicles and hence the amount of ecdysone secreting tissue may result in a decrease in circulating ecdysone during the latter part of the first gonotrophic cycle and throughout the second gonotrophic cycle post-infection. This could further impact Vg mRNA transcript levels.

Parasite-induced curtailment of reproductive success is common, particularly amongst invertebrate hosts that devote a large amount of their resources to reproduction. This study extends our earlier work on fecundity reduction in *P. y. nigeriensis* infected *An. stephensi* (Hogg & Hurd, 1995), *Onchocerca lienalis*-infected *Simulium ornatum* (Renshaw & Hurd, 1994) and *Hymenolepis diminuta*-infected *Tenebrio molitor* (Hurd, 1998). In each of these associations, Vg synthesis is reduced, ovarian uptake impaired and follicle resorption induced. This is the first investigation to show an effect of parasites on insect Vg mRNA abundance. This effect may also be observable in the other systems.

Fecundity reduction may be a pathological response by the mosquito to infection. An alternative hypothesis is that fecundity reduction is an adaptive strategy employed by the parasite to manipulate the host, such that more resources are available for the parasite/host complex over a longer period of time. The latter strategy is likely to enhance the probability of parasite transmission (Hurd, 2001).

Experimental procedures

Mosquito maintenance and Plasmodium *infections*

Hatched larvae of *An. gambiae* (*KIL*) were reared under standardized conditions to produce adults of uniform size (Ahmed *et al.*, 1999). Access to glucose solution was denied 12 h prior to blood feeding. *P. y. nigeriensis* Killick-Kendrick (N67), was maintained in CD male mice, as described (Ahmed *et al.*, 1999). Blood packed-cell volume (PCV) was determined immediately prior to mosquito feeding and parasitaemia was assessed in thin blood smears. Only infected mice containing exflagellating microgametocytes (seen in thick blood smears) were used in the study.

Experimental design

Discrete generations of mosquitoes were used to investigate the effect of infection on fecundity, Vg mRNA abundance, Vg

haemolymph and ovarian Vn titres. The effect of infection on fertility was investigated as previously described (Ahmed *et al.*, 1999), except that egg production and hatch rate were investigated following a second, uninfected, blood meal given 6 days after the first, infective, blood meal; thus developing oocysts were present from the beginning of the cycle. The eggs laid by individual infected or uninfected mosquitoes were counted. The larvae that hatched from each of these egg batches were counted and the percentage of eggs that hatched was calculated.

Figure 1 shows the protocol used to compare aspects of vitellogenesis. A group of twenty mosquitoes from each experiment was maintained for 8 days post-infection to determine the prevalence and intensity of infection and mosquito body size (wing length measurement as in Ahmed *et al.*, 1999).

Probes

Vg mRNA abundance was measured using a probe derived from the central 3.3 kb Sal 1 fragment common to all *An. gambiae* Vg genes cloned into pBluescript II KS– (Stratagene). It hybridizes to a 6.5 kb mRNA, present only in adult females after a blood meal. M13 forward (5′-GTT TTC CCA GTC ACG AC-3′) and reverse (5′-AGC GGA TAA CAA TTT CAC ACA GGA-3′) primers (Pharmacia Biotech) were used to amplify this Vg insert. S7 ribosomal forward (5′-GGC GAT CAT CAT CTA CGT GC-3′) and reverse (5′-GTA GCT GCT GCA AAC TTC GG-3′) primers (Salazar *et al.*, 1993) were used in RT-PCR with total RNA of *An. gambiae*, extracted from females 24 h PBM to amplify an S7 DNA fragment for labelling. Amplified fragments were randomly labelled with [α-^{32}P]-dCTP, ≈ 3000 Ci/mmol (ICN) using Ready-To-Go DNA Labelling Beads (-dCTP) (Amersham Pharmacia Biotech).

RNA quantification

Total RNA was extracted from groups of fifteen whole mosquitoes using an Rnaid RNA purification kit (BIO 101) following the manufacturer's instructions. Northern blots were prepared essentially as described (Sambrook *et al.*, 1989) after electrophoresis of 10 µg of total RNA per well (15 µg at 2 h PBM) on formaldehyde agarose gels. They were simultaneously probed with both Vg and S7 DNA probes. Hybridization was carried out overnight at 65 °C in 5 × SSPE containing 5 × Denhardt's solution and 0.5% (w/v) SDS. Blots were washed in SSPE (up to 0.1×) containing 0.1% SDS (w/v) at 65 °C and exposed to phosphor screens for 2 h (12–48 h PBM) or 10 h (2–8 h post second blood meal) at RT. The screens were scanned using a Molecular Dynamics PhosphorImager 445 SI Scanner, and the Vg mRNA signals in the resulting images quantified using ImageQuant software, following the procedures outlined in the instruction manual. The resulting signals were normalized to those of S7.

Measurement of Vg and Vn concentrations

To produce a standard curve for Vg and Vn quantification immunoassays, protein was extracted from 100 pairs of *An. gambiae* ovaries as previously described (Renshaw & Hurd, 1994) and the ovarian supernatant dialysed (pore size: 12–14 kDa) against 100 volumes of 20 mM Tris-HCl, pH 7.5 for 18 h at 4 °C. The concentration of soluble protein was measured using the Coomassie Blue binding assay (Bio-Rad), using bovine serum albumin as a standard. The dialysate was adjusted to 20 µg/µl protein and stored at –80 °C. A sample was subjected to SDS-PAGE analysis, which revealed two predominant subunits of 200 and 66 kDa, as described in *Ae. aegypti* (Raikhel & Bose, 1988; data not shown). Western blots revealed that a polyclonal antibody raised against the Vn of *An. stephensi* (Jahan & Hurd, 1998) recognizes *An. gambiae* Vn but not *Ae. aegypti* Vn (data not shown). Therefore this heterologous antibody could be used in *An. gambiae* Vg and Vn ELISAs.

Haemolymph was collected from chilled mosquitoes using a precalibrated capillary needle inserted into the thorax below the base of the wing (Renshaw & Hurd, 1994). Clear haemolymph (0.05 µl) was drawn up and immediately used to measure the Vg concentration. Vn was extracted from the ovaries of the same mosquitoes (Renshaw & Hurd, 1994). ELISAs, employing an antibody capture assay with indirect detection (Jahan & Hurd, 1998), were used to measure Vg from the haemolymph of individual mosquitoes and Vn from 5 µl aliquots of ovary supernatant. Protein concentrations were calculated by reference to a standard curve of Vn. Negative, antigen-free controls, were also tested in each assay.

Statistical analysis

Comparisons of mean wing lengths of experimental groups, mean egg production per mosquito and arcsine transformed percentages of eggs hatching were performed using Student's *t*-test. Initially, means of Vg mRNA abundance were normalized against S7 for each time point of each group during the 1st and 2nd gonotrophic cycles and analysed by two-way ANOVA. Tukey's test was used to compare differences between treatment groups at specific times. In addition, a two-way ANOVA was followed by unplanned paired comparisons between means using Holm's (1979) adjusted *P*-values as recommended by Wright (1992) for the analysis of data presented in Fig. 3D. When this experiment was repeated, the variances obtained in the second gonotrophic cycle were not homogenous. Therefore these data were compared using Student's *t*-test for individual pairs (Fig. 3E). Concentrations of Vg and Vn were compared using the Mann–Whitney *U*-test with the significance limit set at $P < 0.05$. All statistical analyses were performed using Minitab software (Minitab, State College, PA).

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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.

Bow
nes, M. and Reid, G. (1990) The role of the ovary and nutritional signals in the regulation of fat body yolk protein gene expression in *Drosophila melanogaster*. *J Insect Physiol* **36**: 471–479.

Briegel, H. (1990) Fecundity, metabolism and body size in *Anopheles* (Diptera: Culicidae), vectors of malaria. *J Med Entomol* **27**: 839–950.

Briegel, H., Lea, A.O. and Klowden, M.J. (1979) Haemoglobinometry as a method for measuring bloodmeal size of mosquitoes (Diptera: Culicidae). *J Med Entomol* **15**: 235–238.

Briegel, H. and Rezzonico, L. (1985) Concentration of host blood protein during feeding by anopheline mosquitoes (Diptera: Culicidae). *J Med Entomol* **22**: 612–618.

Cao, Y.-M., Tsuboi, T. and Torii, M. (1998) Nitric oxide inhibits the development of *Plasmodium yoelii* gametocytes into gametes. *Parasitol Internat* **42**: 157–166.

Carwardine, S.L. and Hurd, H. (1997) Effect of *Plasmodium yoelii nigeriensis* infection on *Anopheles stephensi* egg development and resorption. *Med Vet Entomol* **11**: 265–269.

Catteruccia, F., Nolan, T., Loukeris, T.G., Blass, C., Savakis, C., Kafatos, F.C. and Crisanti, A. (2000) Stable germline transformation of the malaria mosquito *Anopheles stephensi. Nature* **405**: 959–962.

Clements, A.N. (1992) Vitellogenesis. *The Biology of Mosquitoes*, Vol. 1, pp. 360–379. Chapman & Hall, London.

Fleck, S.L., Butcher, G.A. and Sinden, R.E. (1994) *Plasmodium berghei*: Serum-mediated inhibition of infectivity of infected mice to *Anopheles stephensi* mosquitoes. *Exp Parasitol* **78**: 20–27.

Forbes, M.R.L. (1993) Parasitism and host reproductive effort. *Oikos* **67**: 444–450.

Gwadz, R.W. (1994) Genetic approaches to malaria control: how long the road? *Am J Trop Med Hyg* **50** (Suppl. 6): 116–125.

Hagedorn, H.H. (1985) The role of ecdysteroids in reproduction. *Comprehensive Insect Physiology Biochemistry and Pharmacology* (Kerkut, G.A. and Glibert, L.I. eds), Vol. 8, pp. 205–262. Pergamon Press, Oxford.

Hagedorn, H.H. and Judson, C.L. (1972) Purification and site of synthesis of *Aedes aegypti* yolk protein. *Exp Zool* **182**: 367–377.

Hogg, J.C., Carwardine, S.L. and Hurd, H. (1997) The effect of *Plasmodium yoelii nigeriensis* infection on ovarian protein accumulation by *Anopheles stephensi*. *Parasitol Res* **83**: 374–379.

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.

Hogg, J.C. and Hurd, H. (1997) The effect of natural *Plasmodium falciparum* infection on the fecundity and mortality of *Anopheles gambiae s.l.* in north east Tanzania. *Parasitology* **114**: 325–331.

Holm, S. (1979) A simple sequentially rejective multiple test procedure. *Scan J Statist* **6**: 65–70.

Hopwood, J.A., Ahmed, A.M., Polwart, A., Williams, G.T. and Hurd, H. (2001) Malaria-induced apoptosis in mosquito ovaries: a mechanism to control egg development. *J Exp Biol*, in press.

Hurd, H. (1998) Parasite manipulation of insect reproduction: who benefits? *Parasitology* **116**: S13–S21.

Hurd, H. (2001) Host fecundity reduction: a strategy for damage limitation? *Trends Parasitol*, in press.

Hurd, H. and Arme, C. (1987) *Hymenolepis diminuta*: effect of infection upon the patency of the follicular epithelium in the intermediate host, *Tenebrio molitor*. *Invert Pathol* **49**: 227–234.

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

Hurd, H. and Webb, T.J. (1997) The role of endocrinologically active substances in mediating changes in insect hosts and insect vectors. *Parasites and Pathogens: Effects on Host Hormones and Behaviour* (Beckage, N.E., ed.), pp. 179–197. Chapman & Hall, New York.

Jahan, N., Docherty, P.T., Billingsley, P.F. and Hurd, H. (1999) Blood digestion in the mosquito *Anopheles stephensi*: the effect of *Plasmodium yoelii nigeriensis* on midgut enzyme activity. *Parasitology* **119**: 535–541.

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.

Killick-Kendrick, R. (1973) Parasitic protozoa of the blood of rodents, I: the life cycle and zoogeography of *Plasmodium berghei nigeriensis* sub sp. nov. *Ann Trop Med Parasitol* **67**: 261–277.

Kokoza, V., Ahmed, A., Cho, W.-L., Jasinskiene, N., James, A.A. and Raikhel, A. (2000) Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proc Nat Acad Sci USA* **97**: 9144–9149.

Kubli, E. (1986) The *Drosophila* sex peptide: A peptide phenomenon involved in reproduction. *Advances in Developmental Biochemistry* (Wasserman, P. ed.), Vol. 4, pp. 99–128. JAI Press, New York.

Luckhart, S., Vodovotz, Y., Cui, L. and Rosenberg, R. (1998) The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proc Natl Acad Sci USA* **95**: 5700–5705.

Maier, W.A., Becker-Feldman, H. and Seitz, H.M. (1987) Pathology in malaria infected mosquitoes. *Parasitol Today* **3**: 217–218.

Martin, D., Wang, S. and Raikhell, A.S. (2001) The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. *Mol Cell Endocrinol* **173**: 75–86.

Obrebski, S. (1975) Parasite reproductive strategy and the evolution of castration of hosts by parasites. *Science* **118**: 1314–1316.

Raikhel, A.S. and Bose, S.G. (1988) Properties of mosquito yolk protein: a study using monoclonal antibodies. *Insect Biochem* **18**: 565–575.

Read, A.F. (1990) Parasites and the evolution of host sexual behaviour. *Parasitism and Host Behaviour* (Barnard, C.J. and Behnke, J.M., eds), pp. 179–201. Taylor & Francis, London.

Redfern, C.P.F. (1982) 20-Hydroxyecdysone and ovarian development in *Anopheles stephensi*. *J Insect Physiol* **28**: 97–109.

Renshaw, M. and Hurd, H. (1994) The effects of *Onchocerca linealis* infection on vitellogenesis in British blackfly, *Simulium ornatum*. *Parasitology* **109**: 337–343.

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

Romans, P., Bhattacharyya, R.K. and Colavita, A. (1998) *Ikirara*, a novel transposon family from the malaria vector mosquito, *Anopheles gambiae*. *Insect Mol Biol* **7**: 1–10.

Romans, P., Tu, Z., Ke, Z. and Hagedorn, H.H. (1995) Analysis of a vitellogenin gene of the mosquito, *Aedes aegypti and* comparison to vitellogenins from other organisms. *Insect Biochem Molec Biol* **25**: 939–958.

Salazar, C.E., Mills-Hamm, D., Kumar, V. and Collins, F. (1993) Sequence of a cDNA from the mosquito *An. gambiae* encoding a homologue of human ribosomal protein S7. *Nucl Acids Res* **21**: 4147.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sappington, T.W., Hebling, P., Koller, C.N. and Raikhel, A. (1998) Activation *in vitro* of vitellogenin uptake by the oocytes of the mosquito, *Aedes aegypti. Physiol Entomol* **23**: 158–164.

Sinden, R.E. (1998) Gametocytes and sexual development. *Malaria: Parasite Biology, Pathogenesis, and Protection*. (Sherman, I.W., ed.), pp. 25–48. ASM Press, Washington DC.

Sondergaard, L., Mauchline, D., Engetoft, P., White, N., Wulff, P. and Bownes, M. (1995) Nutritional response in a *Drosophila* yolk protein gene promoter. *Mol Gen Genet* **28**: 25–32.

Syafruddin, R.A., Kamimura, K. and Kawatoma, F. (1991) Penetration of the mosquito midgut wall by the ookinetes of. *Plasmodium yoelii nigeriensis. Parasitol Res* **77**: 230–236.

Taylor, P. and Hurd, H. (2001) The influence of host haematocrit on the blood feeding success of *Anopheles stephensi*: implications for enhanced malaria transmission. *Parasitology* **122**: 491–496.

Valle, D. (1993) Vitellogenesis in insects and other groups. A review. *Mem Inst Oswaldo Cruz* **88**: 1–35.

Van Handel, E. and Lea, A.O. (1984) Vitellogenin synthesis in blood-fed *Ae. aegypti*, in the absence of the head, thorax and ovaries. *J Insect Physiol* **30**: 871–875.

Webb, T.J. and Hurd, H. (1995a) The use of a monoclonal antibody to detect parasite induced reduction in vitellin content in the ovaries of *Tenebrio molitor*. *J Insect Physiol* **41**: 745–751.

Webb, T.J. and Hurd, H. (1995b) *Hymenolepis diminuta*-induced fecundity reduction may be caused by changes in hormone binding to *Tenebrio molitor* ovaries. *Parasitology* **114**: 175–179.

Webb, T.J. and Hurd, H. (1996) *Hymenolepis diminuta*: metacestode-induced reduction in the synthesis of the yolk protein, vitellogenin, in the fat body of *Tenebrio molitor*. *Parasitology* **112**: 429–436.

Webb, T.J. and Hurd, H. (1997) The interplay between patency, microsomal Na^+/K^+ ATPase activity and juvenile hormone in *Tenebrio molitor* parasitized *Hymenolepis diminuta. J Insect Physiol* **43**: 337–343.

Wright, S.P. (1992) Adjusted *P*-values for simultaneous inference. *Biometrics* **48**: 1005–1013.