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# The effects of infection with *Plasmodium yoelii nigeriensis* on the reproductive fitness of the mosquito *Anopheles gambiae*

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**Summary**

Malaria parasites are known to affect the fecundity of several species of mosquitoes but the effect of the parasite on the next generation of the vector had not been investigated. Here we report the results of a laboratory study designed to determine the effects of a rodent malaria parasite, *Plasmodium yoelii nigeriensis*, on the reproductive fitness of females feeding on an infected blood meal. Female *Anopheles gambiae* from the same generation and of the same age and size were fed on a gametocytaemic or non-infected mouse, and the reproductive fitness of individual females monitored. Fecundity (total number of eggs produced) and fertility (number of larvae hatched) were significantly reduced by 41.2% and 61.8%, respectively. In the resulting offspring, there was no significant difference in the survivorship of the larvae. The sex ratio, wing length and symmetry, and the blood meal size of the $F_1$ adults were also unaffected, suggesting that parasite-induced reduction in fitness is not carried over to the succeeding generation.

***Key words***: *Anopheles gambiae, Plasmodium yoelii nigeriensis* reproductive fitness, survivorship, malaria

## Introduction

Anopheline mosquitoes are vectors of several important diseases including malaria, and as such, their control remains a major health problem in tropical Africa (Coluzzi, 1992). New methods of mosquito control are being sought and these will rely upon a better understanding of the interactions between the parasite and the vector. During a period of maturation and replication in the mosquito gut, and sporozoite invasion of the salivary glands, *Plasmodium* alters longevity, blood feeding behaviour, flight activity, haemolymph metabolites and the reproduction of mosquitoes (Kittayapong et al., 1992; Mack et al., 1979; Maier, 1987; Hurd, 1993).

Mosquito reproductive fitness is reduced by several parasites including the nematodes *Dirofilaria repens* and *Brugia pahangi* (Javadian and MacDonald, 1974), and *Aedes aegypti* infected with *P. gallinacium* (Hacker, 1971; Freier and Friedman, 1976; Rossignol

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et al., 1986) produced fewer eggs. Fecundity reduction was associated with the ingestion of a smaller blood meal of lower haematocrit due to parasite-induced anaemia (Hogg and Hurd, 1995a). However, *P.y. nigeriensis* also caused significant reduction in egg batch size of *A. stephensi* when haematocrit was high and there was no difference in the blood meal size between infected and non-infected females (Hogg and Hurd, 1995b). Infected females produced between 18–26% fewer eggs compared to non-infected ones (Hogg and Hurd, 1995a), a similar reduction occurred at both high and low oocyst burdens (Hogg and Hurd, 1995b). Using the same model, Jahan and Hurd (1997) reported a significant reduction in reproductive fitness which was higher in larger mosquitoes than in smaller ones. Field studies showed that the presence of *P. falciparum* oocysts in *A. gambiae* resulted in 17% fewer eggs in infected individuals (Hogg and Hurd, 1997).

This report is an extension of previous studies to determine whether *P.y. nigeriensis* has an adverse effect on the fecundity and fertility of *A. gambiae*, and to determine whether the first generation ($F_1$) of infected females were also affected. Reproductive fitness in this study was assessed by counting the number of eggs produced per individual egg batch, which is a measure of fecundity (Clements, 1992), and by assessing egg hatching rate and larvae production as a measure of overall fertility during one gonotrophic cycle.

## Material and Methods

### *Mosquito maintenance*

*A. gambiae* s.l. were reared at 24°C, the optimum temperature for *P.y. nigeriensis* development (Killick-Kendrick, 1973), and 80% RH in a 12:12 light/dark photoperiod. Stock larvae were reared according to Jahan and Hurd (1997) in trays at a density of 300 larvae/800 ml de-ionized water. Two drops of Liquifry® No. 1 (Interpet, Dorking UK), was added to each tray for the first 3–4 days; then TetraMin® flake fish food (Tetra Werke, Melle, Germany), was supplied until pupation (9–11 days post hatching). Male and female adults emerging 24–36 h after pupation were kept together in cages (30 $cm^3$) for mating and were provided with 10% glucose solution containing 2.8 ml per liter of a stock solution of antibiotics (10,000 units penicillin and 10 mg streptomycin/ml 0.9% sodium chloride) per liter and water *ad lib.*

### *Parasite maintenance*

*P.y. nigeriensis* Killick-Kendrick (N 67) was maintained in CD male mice in controlled conditions at 18±2°C and a 14:10 photoperiod. To infect animals, cryopreserved mouse blood, containing predominately ring stages and young trophozoites, was thawed rapidly to 37°C, suspended in citrate saline (1.5% tri-sodium citrate in 0.85% sodium chloride), and injected interperitoneally at 0.1 ml/mouse.

To promote the malaria infection, mice were supplied with water containing 0.05% para-amino-benzoic acid (PABA) *ad lib.* (Peters and Ramkaran, 1980). When parasitaemia reached about 10%, as assessed by Giemsa-stained thin blood smears, a blood passage was performed to infect experimental mice which produced gametocytes in about 36–48 h. This was confirmed by observing exflagellation in thick blood smears (×1000). Blood packed cell volume (PCV) for infected and non-infected mice was determined immediately prior to mosquito feeding.

### *Experimental protocol*

*Parental generation*

Mosquitoes from the same generation were randomly divided into two groups on day 6 post-emergence and starved for 12 h before blood feeding. The control group was allowed to feed on a non-infected mouse, and the other on an infected, gametocytaemic mouse for 30 min, the mice being litter-mates of similar weight and PCV. After feeding, 20 fully engorged females from each group were transferred individually to small cages (6 $cm^3$) and provided with 10% glucose solution in 0.05% PABA water, to enhance infection. After two days a Petri dish (4.5cm in diameter furnished with moist cotton wool and covered with filter paper) was placed in each cage for egg laying. The Petri dish was kept moist until egg laying was completed. Two females from each group died within two days of blood feeding. These were replaced with other fully engorged females from the same blood-feeds that had been kept in identical conditions. The number of eggs per female were counted and immediately transferred for hatching in small trays 12 $cm^2$ (20 eggs/tray), containing 55 ml distilled water. The percentage of eggs hatched was calculated. Two days after completing egg laying (7 days post-feeding), female size was assessed by measuring wing length, and the ovaries dissected to count the retained eggs [i.e., Christopher's stage V; (Clements and Boocock, 1984)]. Total egg production was calculated by summing the laid and retained eggs.

The midgut of infected female mosquitoes was dissected to check for the presence of oocysts. Non-infected females were removed from the study.

*Offspring ($F_1$)*

The fitness of the $F_1$ generation from both the infected and non-infected groups was assessed. For each female the daily survival of larvae was monitored, and the percentage of larvae reaching pupation was recorded. Water volume in larval trays was topped up each day; equal amounts of food were given to each group (as before). Pupae were transferred to small pots containing distilled water in small cages (6 $cm^3$) for adult emergence, and the percentage of the resulting males and females was recorded. The adult mosquitoes were provided with 10% glucose plus antibiotics as before, until blood feeding.

*Blood meal size*

Six days post-emergence the adult progeny from each infected female were collected together and placed in a single cage (30 $cm^3$). The same treatment was applied to the non-infected progeny, and all mosquitoes were starved for 12 h prior to blood feeding. Both groups were allowed to feed on non-infected mice of approximately the same weight and PCV (38 g and 45% and 40 g and 45% for the progeny of non-infected and infected groups, respectively) as described above. Two hours post-feeding, the blood-fed females were frozen and stored at −20 °C till use. Blood meal size was measured according to Briegel et al. (1979) using haemoglobinometry. The whole abdomen of each blood-fed mosquito was placed in 500 ml of Drabkin's reagent (1.0 g $NaHCO_3$, 0.1 g $K_2CO_3$, 0.05 g KCN and 0.2 g $K_3Fe(CN)_6$/litre). After sonication for 30 s to homogenize and elute the haemoglobin, the samples were incubated at room temperature for 20 min. Five hundred ml of chloroform was added to each sample, which were then centrifuged at 2900 g for 5 min. The optical density of 200 ml of the supernatant from each sample was read on a microplate reader (Labsystems Mulliskan Multisoft) at 540 nm. The abdomens of nine unfed females were treated as above, and their mean value subtracted from each sample, as the components of the abdomen contribute slightly to the readings.

To calculate total haemoglobin, a standard curve was produced using a concentration (50 mg/ml) of mouse haemoglobin (Sigma-Aldrich, Dorset UK), in the range of 0.05–1.5 mg, and optical densities of the samples compared with standards.

*Body size*

The body size for each female mosquito was calculated using their wing length by measuring both wings from the distal end of the allula to the tip of the wing, excluding the fringe (Briegel, 1990; Jahan and Hurd, 1997), using a calibrated dissecting microscope. Wing lengths were compared to assess symmetry.

*Statistical analysis*

All statistical analysis was carried out using the MINITAB® computer program. Comparison of means for significance was analyzed by a Student's *t-test*. The Mann-Whitney *U* test was used for non-matched paired analysis. A two-tailed *P* value <0.05 was considered significant.

## Results

Difference in egg production could not be attributed to size differences between the control and infected parental groups (control group n=20, wing length 3.02 ± 0.04 mm; infected, n=20, wing length 3.004 ± 0.03 mm; $p = 0.96$). The infected group was fed on a gametocytaemic mouse, PCV 50%, parasitaemia 12% and weight 36 g. All infected females contained more than 50 oocysts /midgut. The control group was fed on a normal mouse with PCV 48% and weight 40 g.

Total egg production was significantly reduced by infection (Table 1). Egg retention was rarely observed in non-infected females (0.07% of eggs); however, 38.6% of the eggs produced by infected females were not laid within the period of 7 days post blood feeding. This *Plasmodium*-induced reduction in total egg production and oviposition, coupled with a significant decrease in egg hatch, led to a significant decrease in fertility or number of live larvae produced (Table 1).

Although the percentage of larvae from infected group that survived to pupate was less than that of the control one, no statistically significant difference was found and survival rate was more variable amongst offspring from infected females (Fig. 1). The infection status of the parent mosquitoes had no effects upon the success of pupation or adult emergence (Table 2).

The first generation of adults from experimental insects did not vary in mean wing size (Table 3), and wing length was found to be symmetrical in offspring from each group. The ratio of males to females was approximately 50% in both groups (Table 3). Similarly, the infection status of the parent did not affect the ability of the resulting adult to obtain a blood meal (Table 3).

Table 1. Effect of *P.y. nigeriensis* on the reproductive fitness of *A. gambiae*

| Variables | Uninfected | Infected | *P* value |
|---|---|---|---|
| No. of females examined | 20 | 20 | |
| Mean wing length (mm) ± SE | 3.02 ± 0.04 | 3.00 ± 0.03 | 0.96 |
| Mean no. of eggs produced/female±SE | 89.82 ± 8.04 | 52.80 ± 8.10 | 0.001 |
| Mean no. of eggs laid/female ±SE | 89.76 ± 8.03 | 37.00 ± 9.84 | 0.0005 |
| Mean no. of larvae/female ±SE | 77.29 ± 8.43 | 29.47 ± 8.5 | 0.001 |
| Mean % of egg hatching/female ±SE | 78.42 ± 6.25 | 46.30 ± 10.7 | 0.025 |

Table 2. Effect of *P.y. nigeriensis* infection on the viability of immature stages of *A. gambiae*

| Variables | Uninfected | Infected | *P* value |
|---|---|---|---|
| Mean % of larvae surviving to last larval instar ± SE | 82.95 : 3.06 | 72.47 ± 6.88 | 0.39 |
| Mean % of last larval instar pupating ± SE | 67.55 : 9.12 | 73.70 ± 13.30 | 0.25 |
| Mean % of adult emergence ± SE | 83.65 : 1.45 | 90.85 ± 4.48 | 0.20 |

Table 3. Effect of *P.y. nigeriensis* on the first progeny of *A. gambiae*

| Variables | Uninfected | Infected | *P* value |
|---|---|---|---|
| Mean wing length (mm) ± SE | 3.30 ± 0.01 | 3.34 ± 0.02 | 0.16 |
| Mean of haemoglobin (mg) ± SE | 0.51 ± 0.015 | 0.53 ± 0.02 | 0.16 |
| Mean proportion of males (%) ± SE | 49.31 ± 3.14 | 49.84 ± 4.19 | 0.95 |

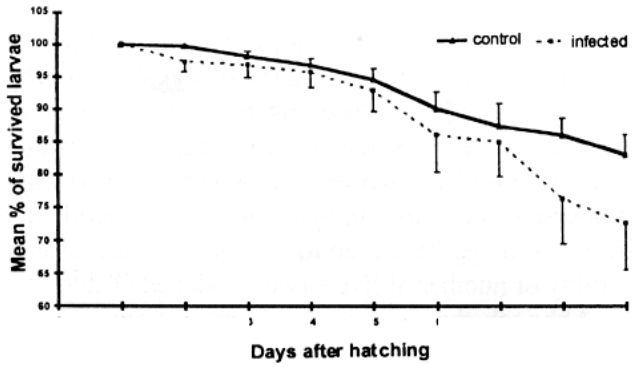


Fig. 1. Survivorship curve of the larvae of infected and control females of *A. gambiae* infected with *P.y. nigeriensis* during the larval period (no significant difference).

## Discussion

*Plasmodium* has a highly significant effect on reproductive fitness of anopheline mosquitoes under ideal laboratory conditions where continuous food supply is given. When Hogg and Hurd (1995b) infected *A. stephensi* with *P.y. nigeriensis*, the infection results in a fecundity reduction of 28% with a high oocyst number (>75/midgut) and of 20% with a lower oocyst number (4.4/midgut) over three gonotrophic cycles. No relationship between parasite density and fecundity could be demonstrated. Hacker and Kilama (1974) also found that the mean number of eggs produced by each *Plasmodium*-infected female mosquito was reduced. Here also, increased parasite densities in the mosquito, or in the host, did not lead to further reduction in mosquito fecundity. Jahan and Hurd (1997) found that the fertility of *A. stephensi* was reduced by 38.3% in smaller females with relatively larger parasite burdens and by 48.81% in the larger females.

In the field, Hogg and Hurd (1997) found that *P. falciparum* significantly decreased the fecundity of wild caught *A. gambiae*; the infection results in 17% reduction in egg production. In the present study, our results demonstrate that *P.y. nigeriensis* also showed a great impact on the reproductive fitness of *A. gambiae*. Fecundity and fertility are significantly reduced (by 41.2% and 61.8%, respectively).

Freier and Friedman (1976) attributed the reduction in egg production to the female *Ae. aegypti* taking smaller blood meals from *Plasmodium*-infected

chickens than from a non-infected chicken. However, work in the laboratory has not been able to demonstrate a significant reduction in blood meal size associated with infection (Hogg and Hurd, 1995b).

After digestion of the blood meal, protein is broken down into its constituent amino acids which are then absorbed into the haemolymph of the mosquito, increasing the total amino acid concentration. Protein digestion has been showed to proceed normally in *P.y. nigeriensis*-infected *A. stephensi* and enzyme activity is not affected (Jahan and Hurd, personal observation). However, specific amino acids such as L-isoleucine could be limiting to oogenesis [reviewed by Clements (1992)] and may be affected by malaria infection in the mosquito. Significant changes in the amino acid concentration of haemolymph in infected mosquitoes have been recorded (Gad et al., 1979). Follicular development in the ovaries of infected and non-infected mosquitoes is initially similar, but by 24h post-infection, large numbers of follicles are undergoing resorption and retardation in the development of the remaining follicles occurs (Clements and Boocock, 1984; Carwardine and Hurd, 1997). The latter may affect the viability of eggs which are produced and the former explains the reduction in fecundity. Accumulation of the yolk protein in the ovaries of infected females proceeds normally for the initial part of the gonotrophic cycle, but by 24h post-feeding, vitellin content was significantly reduced and a concomitant increase in haemolymph vitellogenin occurred (Jahan and Hurd, 1998). Yolk protein supply is thus not limiting in the later stages of the gonotrophic cycle. We have yet to identify the cause of egg resorption in infected females.

Evidence from other parasitized insects suggested that fecundity reduction is a result of changes in vitellogenin synthesis by the fat body and uptake by the ovary (Renshaw and Hurd, 1994; Webb and Hurd, 1995). Production of vitellogenin is controlled by a complex hormonal system (Dhadialla and Raikhel, 1994) and investigation of a cestode-coleopteran relationship indicate that the parasite is capable of interacting with the vitellogenic endocrine system (Hurd, 1998). We are seeking a full understanding of the mechanisms underlying the *Plasmodium*-induced reduction in fecundity and have now begun investigations of the relationship between *Plasmodium* infection and the transcription of the vitellogenin gene in the fat body of *A. gambiae*.

Reduction in fertility may be attributed to fewer eggs being fertilized or from failure of the embryos to develop. Factors that might reduce egg hatching are unknown. Mating occurred before infection and thus could not be affected, although fertilization of individual eggs may be reduced. Briegel (1990) found that the energy content of eggs of four species of mosquitoes was constant despite the changes in the blood meal size. If the vitellin content of eggs from infected females were reduced, this could affect the embryo development. We had considered that eggs from infected females that do hatch may still produce offspring with reduced fitness if the nutritional content of these eggs were inferior. However our data demonstrate this is not so.

Malaria parasites have a significant effect on mosquito reproductive fitness in the laboratory and the field. Although this effect does not carry over to the next generation, it is likely to exhibit an evolutionary pressure in areas of high parasite prevalence. Any pressures have not, however, led to the selection of refractory strains of *A. gambiae* in the wild. We suggest that there may be costs associated with refractoriness that would reduce the fitness of refractory mosquitoes and act against their selection in the field. We were unable to demonstrate any significant effect of infection on the survival of larvae, pupation success, $F_1$ adult size, sex ratios or blood feeding. However, larval survival and pupation from individual females were highly variable.

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