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The early sporogonic cycle of *Plasmodium falciparum* in laboratory-infected *Anopheles gambiae*: an estimation of parasite efficacy

Louis Clément Gouagna[1,3], Bert Mulder[1,3], Elisabeth Noubissi[1,3], Timoléon Tchuinkam[1,3], Jan Peter Verhave[4] and Christian Boudin[1,2,5]

1 *Malaria Department, OCEAC, Yaoundé, Cameroun*
2 *LAF 302, Laboratoire Associé Francophone, Cameroun;*
3 *Department of Animal Biology and Physiology, University of Yaoundé I, Cameroun*
4 *Microbiology Department, University of Nijmegen, The Netherlands*
5 *ORSTOM, Institute Français de Recherche Scientifique pour le devélopment en coopération, Paris, France*

Summary This study investigated the successive losses in the parasite densities of *Plasmodium falciparum* stages during the early sporogony in laboratory-reared *Anopheles gambiae* infected by membrane feeding with blood from naturally infected gametocyte carriers (>50 gametocytes/mm³). The developmental stages of *P. falciparum* in the mosquito were studied from zygote to oocyst, by immunofluorescent method using monoclonal antibodies against the Pfs25 protein present on the surface of newly formed gametes. This method allows for assessment of the various sporogonic stages before, during and after passage of the midgut wall. Parasite densities were determined within the entire blood meal at 3 h (zygotes and macrogametes) and 24 h (ookinetes) post-infection. At 48 h after the mosquito blood meal, midguts were checked for the presence of early oocysts. For the mid-size oocysts count, classic microscopy examination was used at day 7 post-infection. The parasite efficacy was estimated by following successive losses in parasite densities between different early stages of the sporogonic cycle in *A. gambiae*. Thirty-seven experimental infections were realized with high gametocyte densities, ranging from 64 to 2392 gametocytes/mm³. All gametocyte carriers showed infection with round forms 100%; ookinetes were found in 91.9%. The prevalences of infections with oocysts were 48.6% at day 2 (young oocyst) and 37.8% at day 7 (mid-size oocyst). The mean densities per mosquito for each parasite stage were 12.6 round forms, 5.5 ookinetes, 1.8 young oocyst and 2 mid-size oocysts. Significant correlations were found between two consecutive parasite stages (round forms/ookinetes, ookinetes/young oocysts, young oocysts/mid-size oocysts) and between round forms and mid-size oocysts. The mean parasite density significantly decreased between round forms and ookinetes (yield Y1 = 41.6%) and between ookinetes and young oocysts (Y2 = 61.4%). By contrast, no significant decrease was observed between young oocysts and mid-size oocysts (Y3 = 91.2%). The overall yield of the early sporogonic cycle (from round form to oocyst at day 7) was equal to 25.7%, indicating that almost 3/4 of the total parasites were lost during the early step of the sporogonic cycle, from 3 h post-infection to day 7.

keywords immunofluorescence, Pfs25, sporogonic cycle, *Plasmodium falciparum*, *Anopheles gambiae*

correspondence Louis Clément Gouagna, Department of Malaria Research, OCEAC, PO Box 288 Yaounde, Cameroun

Introduction

The estimation of malarial infectivity to the mosquito vector of a population living in an endemic area is of epidemiological importance. Studies are usually carried out by direct feeding or membrane feeding of laboratory-reared mosquitoes with blood from gametocyte carriers. Mosquitoes are examined later, at day 7 post infection, for oocyst detection (Draper 1953; Muirhead-Thomson 1957; Collins *et al.* 1977; Boudin *et al.* 1993; Tchuinkam *et al.* 1993) or at day 12–14 for sporozoites (Collins *et al.*1977; Boudin *et al.* 1989).

However, numerous gametocyte carriers do not give positive infection in mosquitoes in spite of a suitable gametocyte density (Rutledge *et al.* 1969). The two preceding methods for measuring infectivity to mosquitoes do not take into account the biological mechanisms of parasite mortality which arise during the early steps of the sporogonic cycle.

The infectivity of malaria parasites to their vectors is generally determined by both the availability of the gametocytes to the vectors and their intrinsic capacity to infect. Ingested by the anopheline vector, male and female gametocytes of *P. falciparum* rupture from their erythrocyte envelope in the midgut of the mosquito vector. They activate and transform into gametes due to environmental changes (temperature, bicarbonate and mosquito factors) (Sinden & Smalley 1976; Nijhout & Carter 1978; Nijhout 1979). Exflagellation of the microgamete and subsequent fertilization of the macrogamete lead to a spherically shaped zygote. It protudes into a retort form which becomes an elongated and motile ookinete. During all these changes, parasites are exposed to blood factors (antibodies, white blood cells, complement and cytokines) ingested with the blood meal. These factors can inhibit or block fertilization or parasite development (Rener *et al.* 1980; De Naotunne *et al.* 1991). Subsequently, the ookinete has to cross epithelial cells of the midgut wall and sometimes the peritrophic membrane (according to the mosquito species) and forms a cyst under the basal membrane. During this step, one can expect a great loss of parasites trapped in midgut content or tissue (Sieber *et al.* 1991). Oocysts can eventually mature and burst, liberating sporozoites.

These morphological changes are accompanied by the disappearance and appearance of different surface antigens including Pfs25 (Vermeulen *et al.* 1985; Kumar & Carter 1985; Alano 1991). This newly formed protein expressed at 3 h postfeeding, persists on zygotes (3–5 h post-infection) and ookinetes (18–30 h) until young oocysts appear (36–48 h and even longer) (Lensen *et al.* 1992). This 25 kD protein can therefore be used as marker that permits detection of early stages of parasite development in the mosquito midgut by using a fluorescein isothiocyanate (FITC) labelled anti-25 kD monoclonal antibody.

Vaughan *et al.* (1992, 1994) presented the results of quantitative studies of sporogonic development of cultured *P. falciparum* in *A. gambiae*. These studies were realized by using simple light microscopic examination which has a low sensitivity. Another study using immunoflurescence for the detection of preoocyst stages of *P. falciparum* in anophelines was performed in Cameroon recently (Robert *et al.*1995) and shown to be more sensitive. We have used this sensitive fluorescent test to estimate the loss in successive life stages of *P. falciparum* in cultured *A. gambiae* infected by membrane feeding and to define the key steps in parasite development.

Materials and methods

Gametocyte carriers

Gametocyte carriers were selected among patients at the urban dispensary of Messa (Yaoundé) during consultations or among school children in a rural area near Yaoundé. Thick smears were stained for 20 min with 10% Giemsa and examined with a light microscope (100× oil immersion lens) for *P. falciparum* gametocytes. All gametocyte carriers with malaria species other than *P. falciparum* or with low gametocyte density (<50 gametocytes/mm3) were excluded.

Selected carriers or their parents were informed about the purpose of the study and were asked to cooperate. From those who consented, 2 ml of venous blood was collected into a heparinized vacutainer tube.

Experimental infections

A strain of *A. gambiae* sl., originating from Yaoundé (Tchuinkam *et al.* 1993), has been adapted to feeding on a Parafilm® membrane feeder (Ponnudurai *et al.* 1989) and was reared in the OCEAC insectary. Three-day-old mosquitoes were used for experimental infections. Fifty to 100 female mosquitoes were allowed to feed for 15 min on a membrane feeder with blood from a gametocyte carrier. Unfed mosquitoes were removed and others were kept in the insectary with daily access to a 10% saccharose solution.

Detection of sexual stages of *P. falciparum*

Detection of preoocyst stages.

Six mosquitoes per lot were dissected and their midguts examined for the presence of round forms (RF, which are zygotes indistinguishable from macrogametes) and ookinetes (OOK, including retort forms) at 3 and 24 h postfeeding, respectively. The pooled engorged midguts were delicately crushed by repeated pipetting in a vial containing 10 µl phosphate buffered saline (PBS, pH 7.2)/midgut. A quantity of 10 µl/midgut FITC-labelled anti-25 kD monoclonal antibody in 0.05% Evans blue was added to the solution which was homogenized by pipetting (Robert *et al.* 1995). The tube was incubated in the dark for 30 min at room temperature. The solution was washed with one ml of PBS (pH 7.2) and centrifuged at 5000 g for 2 min.

The pellet was resuspended in 10 µl/midgut PBS and mounted between a slide and a cover glass, with a layer of vaseline on the periphery of the cover glass in order to avoid desiccation. Preparations were examined for round forms and ookinetes with a Leitz microscope under epifluorescent light (at 60×, oil immersion lens).

Detection of young oocysts (OOC2)

Forty-eight hours after infection, 20 mosquitoes were dissected in a drop of distilled water and their midguts incubated at room temparature in FITC-labelled anti-25 kD monoclonal antibody in 0.05% Evans blue. After incubation, midguts were washed in PBS (pH 7.2), mounted individually and examined in the above mentioned conditions.

Detection of mid-size oocysts (OOC7)

At day 7 postfeeding, surviving mosquitoes (about 30) were dissected. Midguts were stained with 2% mercurochrome and the presence and the number of oocysts were detected by normal light microscopy (10×). As only gametocyte carriers with density >50 gametocytes/mm³ were included, we expected high densities of RF and OOK and a pool of only 6 mosquitoes per batch were dissected. By contrast, we expected low densities of OOC2 and OOC7 and therefore 20–30 mosquitoes were dissected individually at days 2 and 7, respectively.

Data analysis

For each infection mean parasite densities and prevalences of positive infections were recorded. Comparisons of averages were done with the Kruskall-Wallis' test (for more than 2 means) and Wilcoxon's test (for 2 means of paired data, *W*). An alpha risk of 5% was chosen. The correlations between two consecutive life stages were established using Spearman's test (non-parametric analysis) or, when the distribution of the data was normalized after log transformation, a linear regression test.

The loss of parasites between two consecutive stages 1 and 2 was estimated by the population mortality coefficient (K_i) on quantitative data (mean of parasites per mosquito). This estimate was compared with that obtained by the linear regression model when possible. The mean parasite densities per mosquito were expressed as logarithms. The difference between the means of two consecutive stages was computed for each experiment and the average and standard deviation of these differences were calculated. This parameter was an estimation of K_i (SD) between two consecutive stages (Vaughan *et al.* 1992). K_i was subdivided into 3 steps namely $k_1 = \Sigma[\log(\text{round forms}) - \log(\text{ookinetes})]/n$, $k_2 = \Sigma[\log(\text{ookinetes}) - \log(\text{young oocysts})]/n$, and $k_3 = \Sigma[\log(\text{young oocysts}) - \log(\text{mature oocysts})]/n$. Yield between two consecutive stages was calculated by inverse antilog of mean K_i [Y_i = 1/antilog (mean K_i)]. The sum of K_i (K_T) for each experiment was computed and the total parasite loss during the sporogonic cycle was estimated by [Y_T = 1/antilog (mean K_T)].

Results

In all, 37 experimental infections of mosquitoes were realized. The mean gametocyte density was 433.5 gametocytes per mm³ (range = 64–2392). At 3 and 24 h postfeeding, the observed prevalences of RF and OOK were 100% and 91.9%, respectively. Mean parasite densities varied from 12.6 RF per mosquito (range = 0.2–77.3) to 5.5 OOK per mosquito (range = 0–35.7). At days 2 and 7, the observed prevalences of OOC2 and OOC7 were 48.6% and 37.8%, respectively. Mean parasite densities varied from 1.8 OOC2 per mosquito (range = 0–13.4) to 2.0 OOC7 (range = 0–16.5) (Table 1). These low parasite densities did not lead to a particular mortality of mosquitoes during the period following the infection of *A. gambiae*.

Prevalences of RF and OOK were closely related ($c^2 = 3.1$, $P = >0.05$) while their mean densities per mosquito were significantly different ($W = 5.1$, $P < 10^{-4}$). There was a significant decrease between OOK and OOC2, as well for prevalences ($c^2 = 18.2$, $P < 10^{-4}$) as for mean densities ($W = 3.6$, $P < 10^{-3}$). By contrast, the differences in prevalences and mean densities of OOC2 and OOC7 were not significant ($c^2 = 0.89$ and $W = 0.51$).

Positive correlations were found between RF and OOK densities, OOK and OOC2, OOC2 and OOC7 (Figure 1a–c).

Table 1 Prevalences of infected mosquitoes, mean parasite densities per mosquito and interstage yields of the sporogonic development of *P. falciparum* in laboratory infected *A. gambiae*

	GAM	RF	Y_1	OOK	Y_2	OOC2	Y_3	OOC7	Y_T
Prevalence	—	100%		91.9%		48.6%		37.8%	
Average	433.5	12.6		5.5		1.8		2	
SD	456.1	16.2		7.7		3		4.2	
Yield			41.6% (20–85)		61.4% (22–169)		91.2% (54–154)		25.7% (7–83)

GAM, gametocyte; RF, round form; OOK, ookinete; OOC2, young oocyst (day 2); OOC7, mid-size oocyst (day 7). Y_1 = 1/antilog k_1 with $k_1 = \Sigma[\log(\text{RF}) - \log(\text{OOK})]/n$; Y_2 = 1/antilog k_2 with $k_2 = \Sigma[\log(\text{OOK}) - \log(\text{OOC2})]/n$; Y_3 = 1/antilog k_3 with $k_3 = \Sigma[\log(\text{OOC2}) - \log(\text{OOC7})]/n$; Y_T = 1/antilog k_T with $k_T = \Sigma(k_1 + k_2 + k_3)/n$, represents the total parasite loss from round form to oocyst day 7.

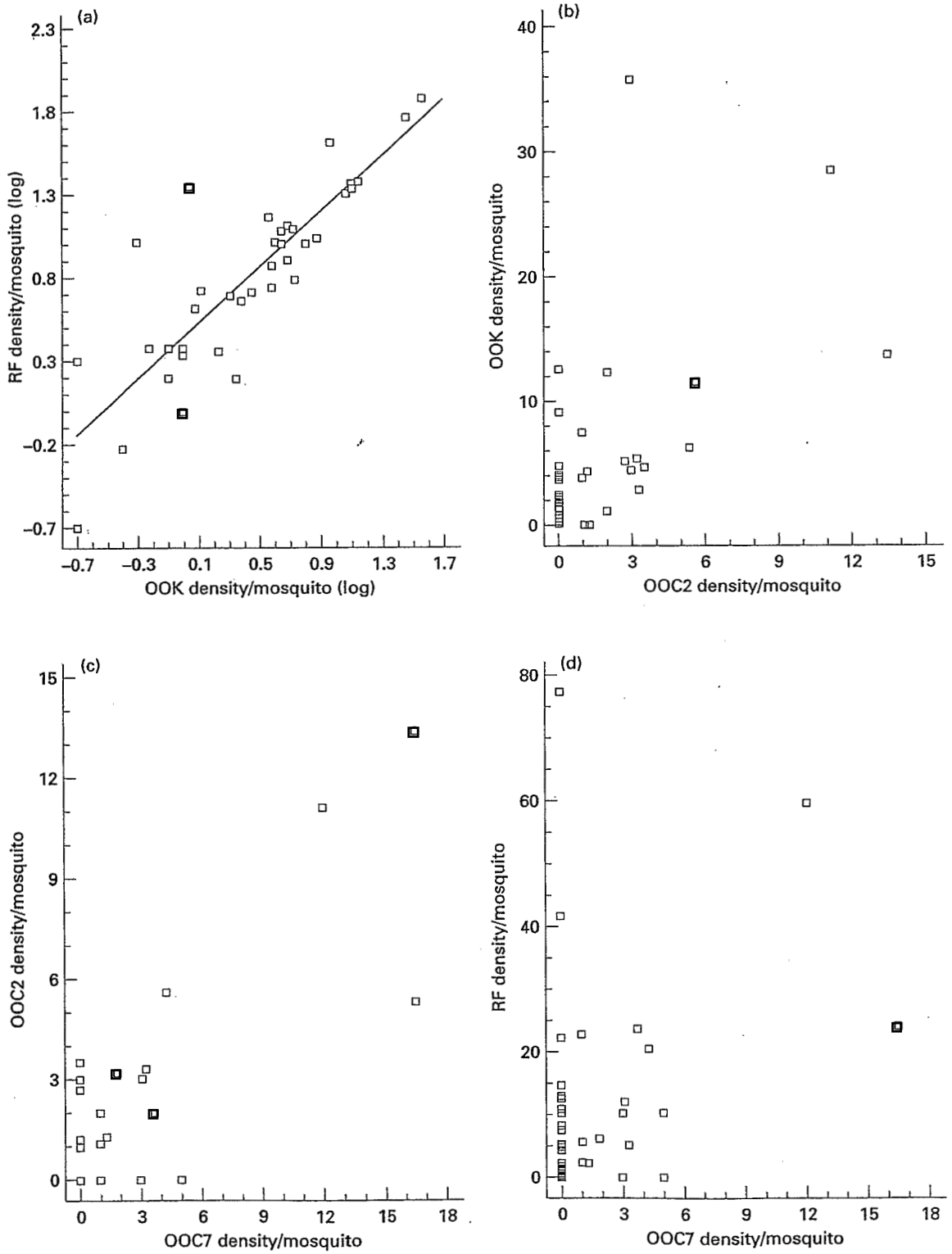

Figure 1 Relationship between densities of *P. falciparum* sporogonic stages in laboratory infected *A. gambiae*. a, Round form (RF) and ookinete (OOK); b, OOK and young oocyte (OOC2); c, OOC2 and midsize oocyte (OOC7); d, RF and OOC7.

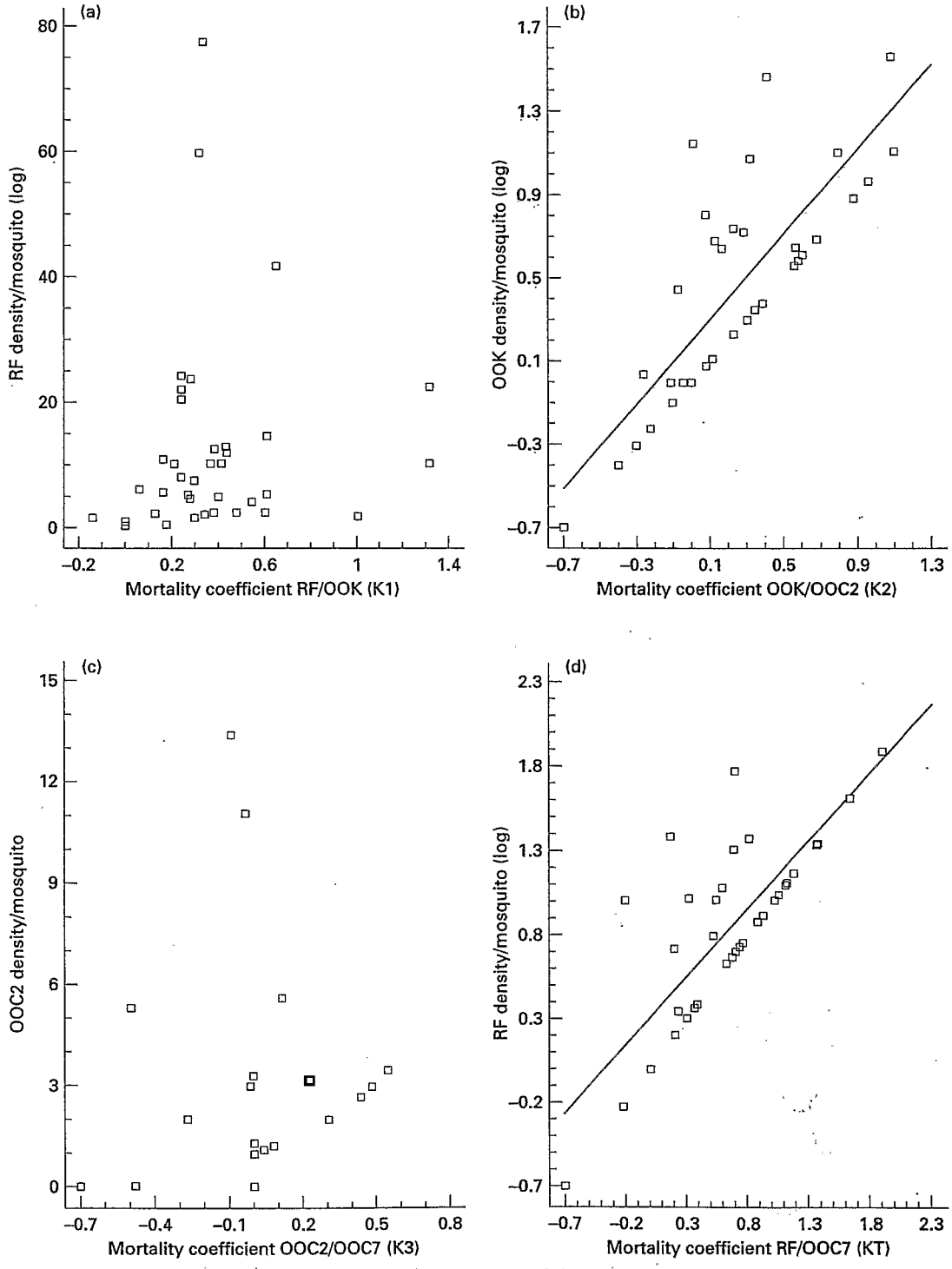

Figure 2 Relationships between densities of *P. falciparum* sporogonic stages and their mortality rates in laboratory-infected *A. gambiae*. a, round form (RF) density and mortality rate (k_1); b, ookinete (OOK) density and mortality rate (k_2); c, young oocyst (OOC2) density and mortality rate (k_3); d, RF density and total mortality rate ($K_T = k_1 + k_2 + k_3$); NS, non significant; $k_1 = \Sigma[\log (RF) - \log (OOK)]/n$; $k_2 = \Sigma[\log (OOK) - \log (OOC2)]/n$; $k_3 = \Sigma[\log (OOC2) - \log (OOC7)]/n$; $K_T = \Sigma(k_1 + k_2 + k_3)/n$, represents the total parasite loss from round form to oocyst day 7.

An overall significantly positive correlation was observed between RF and OOC7 densities (Spearman coef. = 0.37, $P < 0.05$, Figure 1d). Only RF and OOK densities were normally distributed after log transformation. A linear model ($y = 0.84\,x + 1.03$) was defined. It needed about 2 RF to obtain 1 OOK and the estimated yield was 53.4%.

Using mortality coefficients (K_i) we have been able to estimate a yield (Y_i) between two consecutive parasite stages. During the first transition (RF and OOK), a mean yield (Y_1) of 41.6% (confidence limits from 20% to 85%) was observed. This yield was closely related to the one estimated by a linear regression model (53.4%). During the second transition (OOK and OOC2), a mean yield (Y_2) of 61.4% (confidence limits from 22% to 169%) was observed. There was no substantial loss of parasites between OOC2 and OOC7 ($Y_3 = 91.2\%$; confidence limits from 54% to 154%). The overall parasite outcome from RF to OOC7 (Y_T) was about 25.7% (confidence limits from 7% to 83%). This result indicates that 7% to 83% of newly formed RF survive and develop in a mosquito until 7 days after the infective blood meal (Table 1).

A positive correlation was found between the OOK densities and the mortality coefficient K_2 (OOK to OOC2) and between the RF densities and the total mortality coefficient K_T (RF to OOC7). By contrast, there was no linear relationship either between the RF densities and the K_1 coefficient (RF to OOK) or between the OOC2 densitis and the K_3 coefficient (OOC2 to OOC7) (Figure 2).

Discussion

The different developmental stages of natural *P. falciparum* strains were successfully detected with the immunofluorescent assay. Three hours after an infective blood meal, spherically shaped cells (RF) of light green colour were observed in the midgut contents. They were either activated macrogametes or fertilized zygotes (Robert *et al.* 1995). Twenty-four hours post-infection, some RF plus retorts and elongated OOK were still detectable. At day 2 after infection, OOC2 were observed (Lensen *et al.*1992; Ponnudurai *et al.*1988). They were of different types ranging from elongated-shaped during the crossing of midgut wall, to perfectly round young oocysts encysted under the basal lamina. All these forms were characterized by a double membrane and pigment granules of distinct patterns in the lumen of the cyst (Shute & Maryon 1952).

A significant decrease of parasite densities was observed between RF and OOK. The parasite outcome calculated from the mortality coefficient ($Y_1 = 41.6\%$) did not seem different from the yield estimated by the mathematical model of linear regression (53.4%). This reduction was mainly of quantitative importance and had little effect on the prevalence of positive infections with RF and OOK. There was no correlation between RF density and the mortality coefficient K_1 (RF to OOK). Both high and low RF densities can develop into OOK. 15% to 80% of RF failed to develop into OOK, with an important heterogeneity among gametocyte carriers. The loss in transition from RF to OOK may be brought about by several biological mechanisms that are active in the mosquito blood meal, such as: antibodies against sexual stages (Rener *et al.* 1980; Carter *et al.* 1984; Vermeulen *et al.* 1985), complement (Grotendorst & Carter 1987), cytokines (Naotunne *et al.*1991), and phagocytosis (Sinden & Smalley 1976). These mechanisms are more or less important according to host immune state and might explain the extreme heterogeneity among gametocyte carriers (Mulder *et al.* 1994). The low digestion speed and the destructive action of digestive enzymes on immature stages of the malaria parasite might also explain part of the observed parasite loss (Gass 1977; Ponnudurai *et al.* 1988; Billingsley & Rudin 1992).

A decrease, concerning either mean densities or prevalences, was also observed between OOK and OOC2. The parasite outcome estimated by the mortality coefficient was $Y_2 = 61.4\%$. Interestingly, there is a strong positive linear relationship between OOK densities and the K_2 mortality coefficient (OOK to OOC2). The higher the OOK density, the higher is the mortality rate between OOK – OOC2. More than half of the OOK failed to develop into OOC2. Host immune factors probably lose their effect after the ookinete migration through the midgut wall. It is established that the penetration of the peritrophic matrix and the midgut wall by the ookinete constitutes a rate-limiting step which can lead to the ookinete deterioration (Feldmann *et al.* 1990; Billingsley & Rudin 1992; Medley *et al.* 1992; Shahabuddin *et al.* 1993). The intensity of inflammatory reactions in the midgut wall probably reduced the OOK/OOC2 density by blocking the crossing of numerous OOK through the midgut wall. This biological phenomenon might explain the positive correlation between OOK densities and the mortality coefficient K_2 (OOK to OOC2) might be explained this way. Suprisingly, the observed yield of 61.4% between OOK and OOC2 is not as low as expected. It even seems to be higher than that observed between RF and OOK ($Y_1 = 41.6\%$). Two factors might explain this observation: a good adaptation of the parasite to its vector and a relatively low density of parasites in mosquitoes, which does not lead to major inflammatory reaction in the midgut wall (Klein *et al.* 1986). There was no particular mosquito mortality in the course of the round form/ookinete development or of the ookinete penetration, at the beginning of the oocyst growth. This observation may be related to the low parasite density and may also be consistent with an excellent tolerance of *A. gambiae* to *P. falciparum* infections.

After day 2 post-infection, no substantial reduction in OOC2 densities or in prevalences was recorded (Y_3 equal to 91.2%). As the OOC2 densities were relatively low, *P. falciparum* has grown in optimal conditions. In fact no competition for available nutrients among parasites in the mosquito midgut was found (Rosemberg & Koontz 1984; Ponnudurai *et al.* 1989). Despite the possibility of underestimation due to sample preparation for microscopy, we noted fluorescent tracks which could be the traces of antigenic coat left by the ookinete while migrating toward the basal membrane. This observation indicates that certain parasites might shed their Pfs25 coat and can therefore not be detected shortly after the crossing of the stomach wall. Therefore, the mortality between OOC2 and OOC7 could have been overestimated and the natural outcome in this transition is probably more efficient, almost 100%. The discrepancy between the two methods of detection might explain the failure of linear correlation between these parasite life stages.

The overall parasite yield between RF stages and OOC7 was almost 25%, indicating that three-quarters of the RF were lost during the early step of the sporogonic cycle, from 3 h post-infection to day 7. Vaughan *et al.* (1992) observed a very low yield between macrogametocyte and OOC day 9–12 (0.03%). However, these results are not comparable to ours because they used a bioassay with cultured *P. falciparum* and a nonimmune pool of sera, high gametocyte densities, Giemsa-stained thick smears for macrogamete detection and a phase contrast examination for ookinete detection. These techniques are less sensitive than the immunofluorescent assay (Chege & Beier 1994), and high parasite densities in mosquitoes might decrease the parasite efficacy.

This is one of the first studies in which *A. gambiae* are experimentally infected with malaria parasite from naturally infected individuals. The immunodetection of malaria parasites in the mosquito midgut allows for the expansion of investigations in three ways: firstly, to follow in different population groups (from different malaria transmission areas) the mean parasite outcome in naturally infected vectors. Therefore it would be possible to detect subjects with inhibiting or blocking bloodfactors that can prevent the success of RF/OOK transition. Secondly, this method of detection is also suitable for comparing different vector competences in *P. falciparum* infections, where the most competent vector would have the highest yield during the OOK-OOC2 transition. Thirdly, the use of a specific fluorescent marker to gametocyte surface antigens can make it possible to quantify the amount of functional gametocytes ingested during the mosquito blood meal, with the objective to estimate the rate of gametocyte/round form transformation.

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