Commitment to sexual differentiation in the human malaria parasite, *Plasmodium falciparum*

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SUMMARY

The differentiation of the two sexes in the gametocytogenesis of *Plasmodium falciparum* was investigated using a plaque assay and antibodies specific for various stages and sexes of gametocytes. Immunofluorescence assays on plaques of cultured parasites grown in monolayers of erythrocytes revealed that the merozoites released from a single sexually-committed schizont became either all male or all female gametocytes. Thus, the commitment of this species to differentiate into one sex or the other is likely to occur prior to the nuclear division of the sexually-committed schizont. The characteristic female-biased gametocyte sex ratio observed for many *Plasmodium* species is manifested in *P. falciparum* by a greater percentage of schizonts that produce female gametocytes (67–71 %) than those that yield males. From the plaque assay, it was determined that the number of gametocytes produced per sexually-committed schizont was similar for both sexes, indicating that allocation of parasite resources was equal for each sex of gametocyte. The timing of sexual differentiation and features of the gametocyte sex ratio is discussed in relation to previous observations on *P. falciparum* and related malaria parasites.

Key words: gametocytogenesis, malaria, plaque assay, *Plasmodium falciparum*, sex differentiation, sex ratio.

INTRODUCTION

Plasmodium species undergo an indeterminate number of cycles of asexual intraerythrocytic schizogony in their vertebrate hosts. After each cycle, a proportion of merozoites differentiates into gametocytes, a process that involves the formation of mature microgametocytes (males) and macrogametocytes (females). Gametogenesis occurs in the gut of a suitable mosquito after it takes a bloodmeal from a vertebrate containing gametocytes. Each male gametocyte undergoes exflagellation to produce 8 microgametes, whereas each female gametocyte differentiates into a macrogamete that may be fertilized by one microgamete (Garnham, 1988).

Unlike many other Apicomplexa, which undergo an exact number of asexual cycles before entering the sexual phase, the switch of *Plasmodium* species from asexual to sexual development is not genetically predetermined (Barnwell & Galinski, 1998). The timing, induction and mechanism of control of this switch, as well as that of the differentiation of the two sexes, are poorly understood (Lobo & Kumar, 1998; Sinden, 1998). Sexual differentiation is not the result of the segregation of specific sex chromosomes, since cloned lines of *P. falciparum* originating from a single haploid parasite produce both male and female gametocytes (Trager *et al*. 1981).

Many *Plasmodium* species have female-biased gametocyte sex ratios in natural infections of the vertebrate host (Schall, 1989; Read *et al* . 1992) and in culture (Ranford-Cartwright *et al*. 1993). Female-biased sex ratios are predicted by evolutionary theory when matings take place between one or a small number of clones (Read *et al*. 1992). However, these models of optimal gametocyte sex ratios in malaria parasites and other apicomplexans are based on the assumption that allocation of resources is equal for both sexes (Read *et al*. 1992, 1995; Pickering *et al*. 2000; West, Smith & Read, 2000).

Work by Bruce *et al*. (1990*a*) revealed that all of the merozoites produced by a single schizont of *P. falciparum* were destined to produce further asexual stages or to develop into gametocytes. However, it is not known at which stage in the life-cycle *Plasmodium* species differentiate into males or females and whether the progeny of one sexually-committed schizont produce gametocytes of 1 sex or a mixture of 2 sexes. In this report, the differentiation of the sexes of *P. falciparum* was investigated using a modified plaque assay and an IFA with antibodies specific for different sexes of gametocytes. The aims of this study are to determine when during the life-cycle sexually-committed parasites switch to de-

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veloping into either male or female gametocytes, to ascertain the relationship between this time of commitment and the gametocyte sex ratio, and to investigate the relationship between the gametocyte sex ratio and the allocation of parasite resources in the production of the different sexes.

MATERIALS AND METHODS

Cultures of Plasmodium falciparum

Plasmodium falciparum clones 3D7A (Walliker *et al*. 1987) and HB3B-B2 (Bhasin & Trager, 1984; Walliker *et al*. 1987) were cultured *in vitro* using standard procedures (Trager & Jensen, 1976) with the following modifications. Cultures were grown at a 5% haematocrit in 50 ml tissue culture flasks at 37 °C in complete medium that consisted of RPMI 1640 supplemented with 5·94 g/l HEPES, 50 mg/l hypoxanthine, 50 mg/l gentamycin sulphate, 42 ml/l 5·0% (w/v) $NaHCO_3$ and 10% pooled heat-inactivated human serum. Group O, RhesusD-positive erythrocytes were obtained as whole blood from the Edinburgh and Southeast Scotland Blood Transfusion Service. Cultures were gassed with a mixture of 3% CO_2, 1% O_2 and 96% N_2, and maintained in a shaking incubator at 60 r.p.m. to minimize the number of erythrocytes infected with more than a single parasite.

Preparation of erythrocyte monolayers

Falcon tissue culture dishes (35 mm) were coated for 30 min at 37 °C with 0·5 ml of 60 mg/l of a recombinant protein, Protein L (Actigen Limited, Cambridge) in 1 × phosphate-buffered saline (PBS). After washing 3 times with PBS, dishes were incubated for 30 min at 37 °C with 0·5 ml of an anti-RhesusD IgM antibody solution (Edinburgh and Southeast Scotland Blood Transfusion Service) that had been dialysed in PBS for 12 h to remove sodium azide and EDTA. Dishes were washed 3 times with PBS and blocked for 30 min at 37 °C with 0·5 ml of 3% bovine serum albumin (BSA) in PBS. Following 3 washes with PBS, dishes were incubated for 1 h with 1·5 ml of a 1% haematocrit solution of washed group O, RhesusD-positive blood cells in incomplete medium (medium without serum). Unbound erythrocytes were removed by directly drawing off the medium and adding 1·5 ml of fresh incomplete medium. This procedure was repeated 10 times until the only erythrocytes that remained were those attached to the dish as a monolayer. The structure of this novel erythrocyte monolayer is illustrated in Fig. 1.

Infection of erythrocyte monolayers with parasite cultures

Dishes containing monolayers were overlaid with 1·5 ml of parasite culture at 10–15% parasitaemia with a final haematocrit of 0·01% (3 μl of a 5% haematocrit culture). The dishes were incubated at 37 °C for 24 h in a modular incubator gassed with the previously described gas mixture. During this period, schizonts in the overlay burst and resulting merozoites invaded erythrocytes in the adjacent monolayer, forming a cluster of parasites called a plaque. Overlays were then gently resuspended and drawn off, and the monolayers were washed twice with complete medium to remove all unbound erythrocytes and parasites. A total of 1·5 ml of complete medium was then added to the monolayers, which were allowed to incubate at 37 °C for an additional 72 h with daily changes of medium and gassing as before. To verify that parasites were growing, medium was drawn off and monolayers on a proportion of the dishes were fixed in ice-cold methanol for 1 min prior to staining with Giemsa. The remaining monolayers were fixed in the same manner for IFA.

Antibodies

Two antibodies were used in the assay; the first a monoclonal antibody specific to an antigen expressed in gametocytes, the second a polyclonal antiserum (raised in rabbit) specific to male gametocytes. The monoclonal antibody 93A3 (Bruce *et al*. 1990*b*), raised in mice (isotype IgG1) and provided as ascitic fluid, targets Pfs16, a sexual stage-specific parasitophorous vacuole membrane protein that can be detected in gametocytes of *P. falciparum* 35 h after merozoites invade erythrocytes (Baker *et al*. 1994; Bruce *et al*. 1994). The rabbit polyclonal antiserum targets a COOH-terminal synthetic peptide sequence (NH2-CEGEDEGYEY-COOH) of α-tubulin II (Guinet *et al*. 1996), expressed only in male gametocytes and gametes of *P. falciparum* (Rawlings *et al*. 1992). Rawlings *et al*. (1992) reported detection of α-tubulin II as early as stage III gametocytes (5 days after merozoites invade erythrocytes). We were able to detect this protein as early as stage II (72 h after merozoite invasion). Antibodies 93A3 and anti-α-tubulin II were used at concentrations of 1:20 and 1:1000 in PBS, respectively.

Immunofluorescence assay

A 1·5 cm × 1·5 cm square was drawn with nail polish on each fixed monolayer to form a chamber. The portion of the monolayer within the square was then incubated for 5 min at room temperature with block buffer containing 1% BSA and 0·7% Triton X-100 in PBS. The detergent was required for the specificity of the anti-α-tubulin II antibody as reported by Rawlings *et al*. (1992). After washing with PBS, a total of 50 μl of a 1:1 mixture of the two antibodies, diluted as described above, was applied for 20 min at room temperature. After washing with PBS, the

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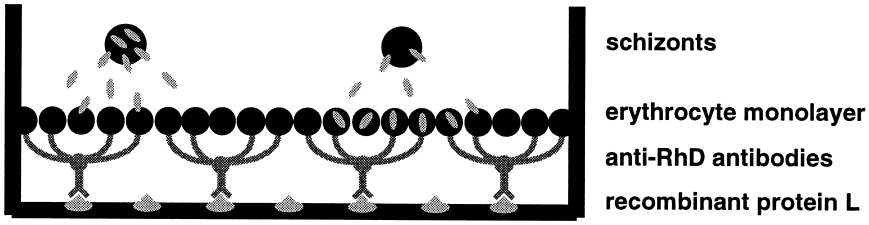


Fig. 1. Diagram of the plaque assay used in this study. Tissue culture dishes, shown in cross-section, were coated with the recombinant protein (Protein L), followed by an incubation with an anti-Rhesus D IgM monoclonal antibody. The light chains of the Fab sites of the IgM antibody bind to the Ig-binding domains of Protein L. Rhesus D-positive human erythrocytes then bind to the Fab sites of anti-RhesusD antibodies to form a monolayer that adheres to the bottom of the dish. Schizonts infecting red blood cells in the overlay release merozoites (grey ovals) that infect adjacent cells in the monolayer, forming a cluster of parasites that appears as a plaque.

dishes were incubated for 20 min at room temperature with 70 μl of a mixture of 1:25 dilutions of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Scottish Antibody Production Unit) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates Inc.), each containing 100 ng/ml DAPI (4, 6-diamidino-2-phenylindole). A drop of glycerol containing 2·5 mg/ml of the anti-fade agent 1, 4-diazabicyclo (2, 2, 2) octane (DABCO) was added to the monolayer within the square after washing with PBS. A 22 cm × 22 cm cover-slip was applied over the square and sealed at the edges with nail polish.

Fluorescence microscopy and photography were carried out on a Leitz Dialux 20 microscope with ultraviolet illumination, fitted with an Olympus PM-30 automatic photomicrographic system. The nuclei of all parasites appeared blue using a Leitz filter block A due to DAPI staining. All gametocytes appeared red using a Leitz filter block N2 as a result of the binding of the TRITC-conjugated secondary antibody with 93A3, whereas male gametocytes additionally appeared bright green using a Leitz filter block I2 due to a binding of the FITC-conjugated secondary antibody with the anti-α-tubulin II antibodies. Hence, female gametocytes were determined indirectly as reacting positively with TRITC and negatively with FITC. The number of plaques containing only male or only female gametocytes, as well as those containing a mixture of sexes, were counted. The number of gametocytes within each plaque was also scored.

RESULTS

Fluorescence microscopy of the monolayers revealed 3 types of gametocyte plaques. One type contained gametocytes that fluoresced brilliant green with the FITC-conjugated secondary antibody (Fig. 2A, top) and fluoresced red with the TRITC-conjugated secondary antibody (Fig. 2A, bottom). Gametocytes in these plaques were considered to be males. The second type of plaque consisted of gametocytes that fluoresced dull green due to the lack of α-tubulin II and the presence of background fluorescence (Fig. 2B, top) but fluoresced red as a result of the presence of Pfs16 (Fig. 2B, bottom). These gametocytes were identified as females. A third type of plaque contained some parasites that stained bright green and others that stained only faintly (Fig. 2C, top). The positive reaction of these with 93A3 (Fig. 2C, bottom) indicated that all such parasites were gametocytes. These plaques were considered as mixed males and females.

Without previous knowledge as to the mode of sexual differentiation in this malaria species, it can be reasoned that mixed plaques may result from (1) one schizont producing a mixture of male and female progeny, or (2) two schizonts within a single erythrocyte in the overlay, one of which produces only male gametocytes and the other only female gametocytes. To test these hypotheses, the expected number of mixed plaques from an overlaid culture was determined and compared to the observed number of mixed plaques on each monolayer (see Table 1).

In most cases, all gametocytes in a plaque were at the same stage in development. As such, only those plaques that contained stage II or later gametocytes that could react with anti-α-tubulin II were counted. In addition, only those gametocytes that reacted with 93A3 were recorded in counts of males and females; the remainder were considered dead. Data for the number of gametocyte plaques on each dish that contained only males, only females and a combination of the two are given in Table 1. If each mixed plaque is considered to arise from an erythrocyte infected with both a male-producing schizont and a female-producing schizont, then redistribution of the data gives 28·9% of gametocyte-producing schizonts of 3D7A (and 33·2% of those of HB3B-B2) as giving rise to males, with the remainder yielding females.

Data for the number of gametocytes in all plaques on each dish were also compiled (Table 1). Using clone 3D7A as an example, there was an average of 3·68 male gametocytes per plaque derived from a male-producing schizont and 3·80 female gametocytes per plaque arising from the rupture of female-

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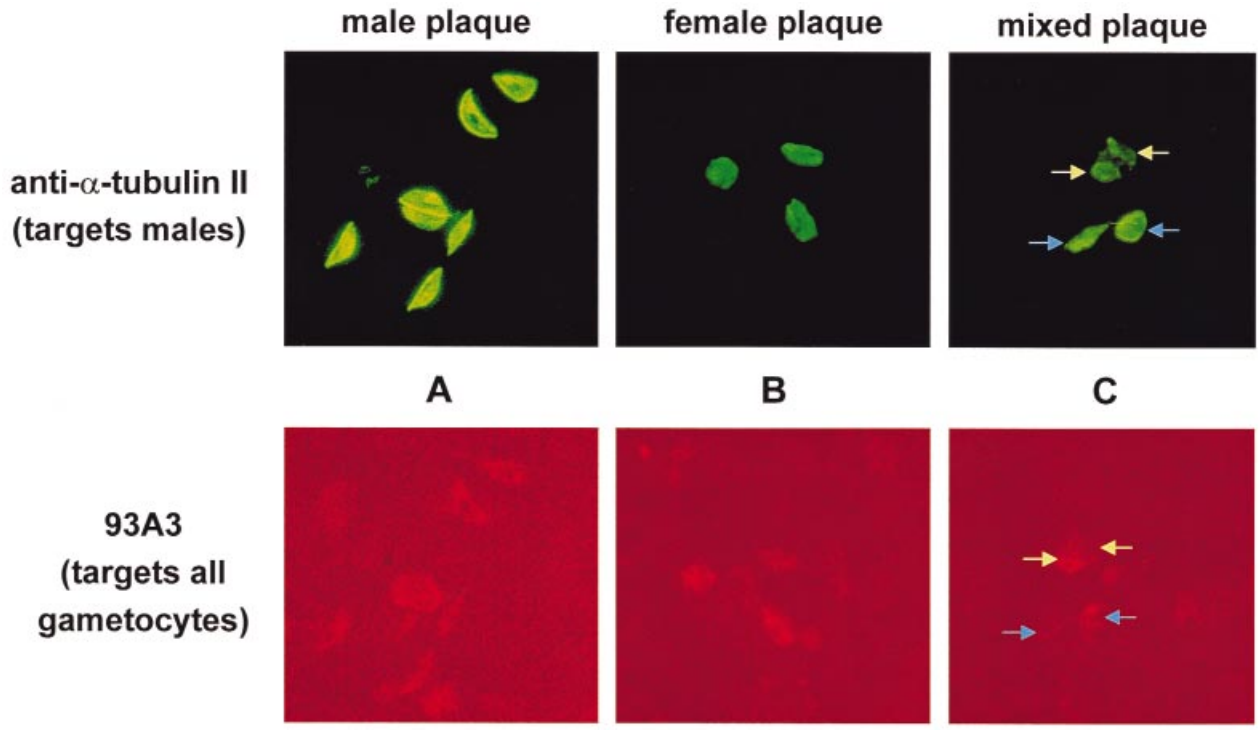


Fig. 2. Fluorescence micrographs of gametocyte plaques of *Plasmodium falciparum* clone 3D7A. (A) A plaque containing 7 male gametocytes, displaying positive fluorescent reaction with an antibody specific for α-tubulin II (top) and with the monoclonal antibody 93A3 specific for Pfs16 (bottom). (B) A plaque containing 3 female gametocytes, showing only background fluorescence with FITC (top) but reacting positively with TRITC (bottom). (C) A mixed plaque revealing 2 male gametocytes (blue arrows) that react positively and 2 female gametocytes (yellow arrows) that display only background fluorescence with FITC (top). All gametocytes react positively with TRITC.

Table 1. Composition of gametocyte plaques of *Plasmodium falciparum* clones

(A total of 7 dishes were scored for each clone (% Mix O – mixed plaques observed, % Mix E – mixed plaques expected from a range of the percentage of multiply-infected erythrocytes using method by Bruce *et al.* (1990*a*), M/P – males per plaque; F/P – females per plaque).)

Plaques Clone	Total	Male	Female	Mixed	% Male	% Mix O	% Mix E
3D7A	430	97	306	27	28·9	6·3	2·1–4·8
HB3B-B2	339	98	219	22	33·2	6·5	3·4–5·5
Gametocytes Clone	**Total**	**Male**	**Female**	**% Male**	**M/P**	**F/P**	
3D7A	1720	456	1264	26·5	3·68	3·80	
HB3B-B2	1334	485	849	36·4	4·04	3·52	

producing schizont. As a result of these data and the percentage of male and female plaques, the gametocyte sex ratio (conventionally given as the percentage of males) was 26·5% for 3D7A and 36·4% for HB3B-B2.

DISCUSSION

One aim of this study was to determine the point in the life-cycle of *P. falciparum* at which sexually-committed parasites commit to male or female gametocytogenesis. The high percentage (93%) of plaques of a single sex indicates that all of the merozoites emerging from a single sexually-committed schizont of *P. falciparum* are destined to form only male gametocytes or only female gametocytes. The number of mixed plaques observed was similar to that expected using the method of Bruce *et al.* (1990*a*), indicating that mixed plaques most likely arise from parasites emerging from erythrocytes infected with at least 1 schizont of each sex and not from 1 schizont producing gametocytes of both

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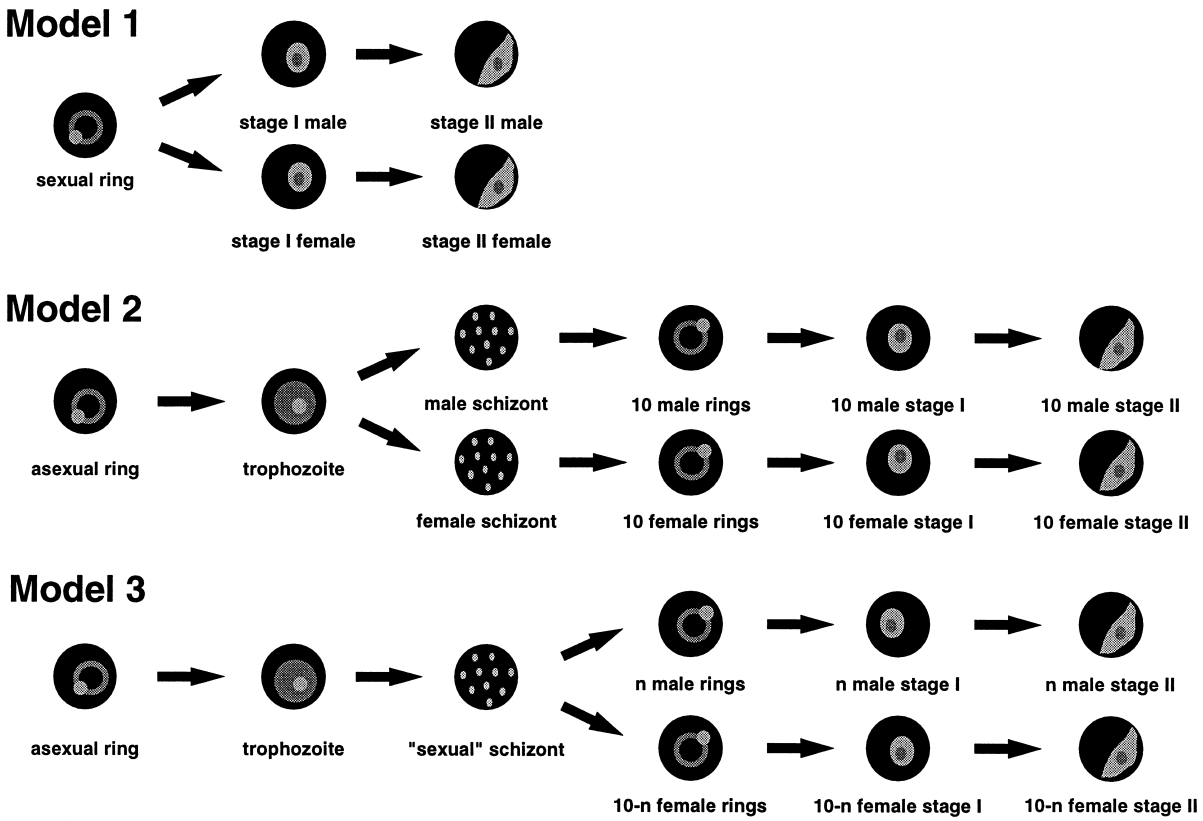


Fig. 3. Three models of sexual differentiation of *Plasmodium falciparum* (modified from Carter & Miller, 1979). Assume that the decision for an asexually reproducing parasite to enter the sexual cycle occurs in either the ring or trophozoite stage prior to the formation of the schizont that produces gametocytes (Carter & Miller, 1979; Bruce *et al*. 1990*a*), and that there are 10 merozoites produced by each schizont. In model 1, merozoites emerging from sexually-committed schizonts do not differentiate into either sex until the ring, trophozoite or early gametocyte stage. In Model 2, commitment to one sex or the other occurs in the ring or trophozoite stage that gives rise to the sexual schizont and thus all merozoites emerging from the schizont will develop into one sex or the other. In Model 3, commitment to one sex or the other occurs in the schizont stage, such that 'n' merozoites are male, whereas the remainder ($10-n$) are female. This work has revealed that Model 2 most probably applies for *P. falciparum*.

sexes. These observations suggest that the second model in Fig. 3, whereby all of the merozoites in each schizont are committed to one sex or the other, is applicable for *P. falciparum*. Thus, the timing of the commitment to sexual differentiation is likely to occur in the ring stage or trophozoite that develops into the gametocyte-producing schizont. It is also possible that a diffusible signal to switch to one sex or the other occurs during schizont formation or after merozoites in the schizont have already formed.

The gametocyte sex ratio observed in the study varied from 26·5% for clone 3D7A to 36·4% for clone HB3B-B2. Similar gametocyte sex ratios of 27·4% and 32·8% were obtained on blood films of cultured 3D7A by Baker *et al*. (1995) using an mRNA probe that detected transcripts of the female gametocyte-specific protein Pf77, and by Severini *et al*. (1999) (see also Alano *et al*. 1995) using rat antisera to the female gametocyte-specific protein Pfg377, respectively. However, these ratios are significantly higher than those of 8·3% (3D7A) and 17·9% (HB3B-B2) obtained on Giemsa-stained blood films of cultured *P. falciparum* (Ranford-Cartwright *et al*. 1993). One explanation for these disparate values is that mature male gametocytes may have been incorrectly identified as immature stage III or IV gametocytes on Giemsa-stained blood films instead of being recorded as mature males, resulting in a more female-biased sex ratio than that recorded with methods that utilize antibodies and IFA on plaques or slides containing earlier stages of gametocytes. Potential problems with estimating the maturity of gametocytes of *Plasmodium* species are outlined by Schall (1989).

The number of gametocytes in plaques containing only males or only females was approximately equal, indicating that both male-producing and female-producing schizonts contain similar numbers of merozoites. These data, coupled with the assumption that sexual differentiation of *P. falciparum* follows the second model, in Fig. 3, indicate that the female-biased sex ratio observed in this study is manifested by a greater number of schizonts producing female gametocytes. Such female-biased sex ratios are predicted by evolutionary theory when fertilization events occur between 1 or a small number of clones

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(Read *et al*. 1992). Specifically, the optimal proportion of male gametocytes would be 0·11 (1 male to 8 females) for complete selfing, which would provide just enough male gametocytes to fertilize all of the available females if each male produced 8 microgametes. This gametocyte sex ratio would increase to 0·5 in a population with frequent outcrossing, since genotypes with sex ratios producing more males would be favoured by selection and would thus be disproportionately represented in more zygotes (Read & Day, 1992).

The observation that male-producing and female-producing schizonts contain similar numbers of merozoites suggests an equal investment of parasite resources in the production of each sex of gametocyte. This study represents the first test of the assumption made in models predicting the optimal sex ratio of apicomplexan parasites that allocation of resources is equal for both sexes (Read *et al*. 1992, 1995; Pickering *et al*. 2000; West *et al*. 2000). This observation for *P. falciparum* differs from the one made by Schall (1989) that female gametocytes of 3 *Plasmodium* species of lizards have a significantly larger 2-dimensional area than do male gametocytes and thus are possibly more costly to produce.

Little is known about the molecular basis of the switch of *Plasmodium* species from asexual to sexual development or the differentiation of the two sexes during gametocytogenesis. In terms of sexual differentiation, it is known that haploid clones of *P. falciparum* produce male and female gametocytes (Trager *et al*. 1981), and thus sexual differentiation must result from differentially-induced gene expression that occurs at some point before parasites express sexual traits (Ranford-Cartwright & Carter, 2000). It can be surmised from this study that induction could occur as early as the ring stage prior to the formation of the gametocyte-producing schizont, whereas detectable expression of the sexual trait occurs early in gametocyte formation.

The plaque assay used in this study was developed to enable gametocytes of *P. falciparum* to grow for at least 4 days in erythrocytes on a stable monolayer. The use of the anti-Rhesus D IgM monoclonal antibody to provide an attachment between Rhesus D antigens on erythrocytes and a protein that adheres to the culture dish allowed the structural integrity of each erythrocyte to be maintained. This was thought to be important for the elongation of gametocytes and the expression of structural proteins. Monolayers in previous plaque assays have been attached directly using lectins such as Concanavalin A (Inselburg, 1983; Bruce *et al*. 1990*a*) or a polyphenolic protein tissue adhesive, Cell-Tak, derived from marine mussels (Williams, 1999). Concanavalin A, initially used in this study, was found to distort red blood cells and gametocytes and to prevent the expression of α-tubulin II. In addition, it was not possible to grow viable gametocytes beyond 70 h. Although gametocytes grown on monolayers attached using Cell-Tak appeared to grow normally (Williams, 1999), it is unknown whether the expression of α-tubulin II or other structural proteins is affected by this adhesive.

Subsequent to obtaining the results in this study, rat antisera to the sexual stage-specific protein Pfg377 has been reported to differentiate female gametocytes as early as stage III (Severini *et al*. 1999). This reagent provides a second sex-specific antibody that should make it possible in the future to verify our results by defining females with a direct positive reaction instead of with the negative reaction used in the present study (Alano, Silvestrini & Williams, personal communication).

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