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Changes in *Haemoproteus* sex ratios: fertility insurance or differential sex lifespan?

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There is little direct evidence of the fitness effects of changes in malaria gametocyte sex ratio. Gametocyte sex ratios in haemospororin parasites (phylum Apicomplexa) are usually female skewed. However, in some cases and especially in *Haemoproteus* parasites, less female-biased and even male-biased sex ratios are encountered. The 'fertility insurance hypothesis' tries to explain these biases as an evolutionary strategy to facilitate gamete encounter. Thus, the hypothesis predicts that, if there is a reduction in gametocyte density (intensity of infection) or other factors preventing gametes from meeting, a change to a higher proportion of male gametocytes may be favoured. By contrast, a change in sex ratio may be caused by other non-adaptive mechanisms, for example differential survival of the gametocytes of each sex. We study within-host changes in *Haemoproteus majoris* sex ratios following an experimental reduction in the density of the parasites in the blood in a breeding population of blue tits (*Parus caeruleus*). Medication with the antimalarial drug primaquine induced a significant reduction in *Haemoproteus* gametocyte infection intensity in two different breeding seasons and under two different doses of medication. Sex ratios became male skewed following the experimental treatment in agreement with the predictions of the 'fertility insurance' hypothesis. Also in support of the hypothesis, a significant change towards male-biased sex ratios emerged for non-medicated birds in one year, probably owing to the natural immune reduction of the density of the parasites in the blood. The alternative possibility that changes are caused by different lifespans of gametocytes is not supported by changes in sex ratios in control hosts, where new production and release of gametocytes occur.

Keywords: gametocyte sex ratios; intensity of infection; *Haemoproteus majoris*; haemospororin parasites; malaria parasites; *Parus caeruleus*

1. INTRODUCTION

The phylum Apicomplexa forms a large and cosmopolitan assemblage of protozoan parasites. Within this phylum are the haemospororins, or malaria parasites, which infect blood-feeding dipterans as vectors, and several classes of vertebrates. The life cycle of these parasites includes sexual and asexual stages. Within the vertebrate hosts, haploid infective stages (sporozoites) infect host tissues and form feeding stages (trophozoites). These undergo asexual proliferation to become multinucleated meronts (or schizonts), which rupture to produce merozoites; some of these transform into sexual stages called gametocytes. Once in the dipteran vector, a macrogametocyte produces a single female gamete and the microgametocytes each produce up to eight male gametes (Atkinson & Van Riper 1991; Desser & Bennett 1993). Mating usually occurs within a few minutes after the vector takes a blood meal containing the parasite gametocytes, and mated gametes tend to originate from a single blood meal and therefore from a single host. In the insect, the diploid zygote undergoes meiosis, restoring the haploid state. The resulting cell divides mitotically to yield the infective sporozoites, thus completing the complex life cycle.

For most species of the parasite, the sex ratio in the blood sexual stage is readily identifiable in blood smears, and a number of both theoretical and empirical studies have pursued the adaptive significance of sex ratio (defined as the proportion of male gametocytes) in malaria parasites (West *et al.* 2001; Read *et al.* 2002). By producing several gametes, one male gametocyte is able to fertilize several female gametes, and intuition suggests that a female skew in sex ratio would maximize successful transmission by optimizing zygote production (Ghiselin 1974; Read *et al.* 1992; Paul *et al.* 1999; West *et al.* 2001). In the extreme, if mating occurs between the gametes of a single clone, the unbeatable strategy is to produce just enough male gametes to fertilize the female gametes. By contrast, if one male gametocyte can produce multiple gametes, the sex ratio is expected to be female biased and limited only by the fecundity of the male gametocytes (Hamilton 1967; Charnov 1982).

Conflicting with this expectation, unbiased gametocyte sex ratios and even male-skewed sex ratios have been observed, especially for *Haemoproteus* species, which are common parasites of birds worldwide (Shutler *et al.* 1995; West *et al.* 2001). Less female-biased sex ratios are predicted under several circumstances. In cases in which outbreeding is high, such as when infections include several or many genetically distinct clones, those clones that produce more male gametocytes will obtain a larger number of mates and thus make a greater contribution to the next generation. In these cases an unbiased sex ratio is expected (Fisher 1930; Hamilton 1967; Read *et al.* 1992). An alternative 'fertility insurance hypothesis' is presented by West

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et al. (2002), who note that under low gametocyte densities male gametes would have difficulty meeting female gametes, and natural selection would favour a less female-biased sex ratio to ensure mating of female gametes. A reduced probability of a male gametocyte meeting a female gamete can result from a low number of gametocytes in the blood or reduced gamete mobility or viability owing to the host immune response. An extreme case would result in the average number of successful male gametes produced by a gametocyte falling to less than one, and thus selecting for a male-biased sex ratio (Gardner *et al.* 2003).

The fertility insurance hypothesis predicts that an infection should produce a greater proportion of male gametocytes when gametocyte density falls or if male gametes suffer increased mortality. Paul *et al.* (1999) found such a pattern in the bird malaria parasite *Plasmodium gallinaceum*. However, such changes in gametocyte sex ratio can be produced by differences in the survival of male and female gametocytes. In a laboratory study, Reece *et al.* (2003) have shown that for the rodent malaria parasite (*Plasmodium chabaudi*) female gametocytes have a shorter lifespan than males. Thus, in the absence of production of new gametocytes and their release to the blood, a male-biased sex ratio is produced, accompanied by a reduction in the density of gametocytes in the host blood (but see Smalley & Sinden 1977). We present the results of an experiment that sought to test the fertility insurance hypothesis and, for the first time to distinguish between differential mortality of gametocytes by sex and an adaptive shift in sex ratio. The experiment reduced the within-host density of the parasites and documented the shift to a male-biased sex ratio in *Haemoproteus majoris* in a wild population of breeding blue tit (*Parus caeruleus*) hosts.

2. MATERIAL AND METHODS

The study was carried out in a Pyrenean oak (*Quercus pyrenaica*) deciduous forest located in Valsain (Segovia, central Spain, 40°54′ N, 4°01′ W, 1200 m above sea-level). The high prevalence of infection by blood parasites in a population of blue tits (*P. caeruleus*) breeding in next-boxes has been under study to determine the impact of infection on host reproduction (Fargallo & Merino 1999; Merino *et al.* 2000).

In the 1999 breeding season, nests were randomly assigned to one of two treatment groups. Female blue tits were trapped at nest-boxes when their nestlings were 3 days old, and injected subcutaneously with either 0.01 mg of primaquine (Sigma, St Louis, MO, USA) diluted in 0.1 ml of saline solution (medicated hosts) or with the same volume of saline solution (control hosts). Primaquine is an antimalarial compound that has been employed successfully to reduce blood parasitization in the bird species under study (Merino *et al.* 2000). In addition, primaquine has been shown to have effects on gametocytes of several species of *Plasmodium*, including *P. falciparum*, where a reduction in gametocyte density is observed within 72 h (López-Antuñano 1999; WHO 2001). Apparently, primaquine acts by binding and modifying the parasite's DNA (López-Antuñano 1999), as well as by disrupting parasite mitochondrial membranes (Baird & Rieckmann 2003). All of the birds were treated during the same phase of their reproduction (young hatchlings in the nest), thus avoiding differences caused by variation in hormone levels and/or immune response of the birds in different breeding states.

Immediately after capture and before the primaquine injection, we obtained blood from the brachial vein of each bird with a capillary tube (initial sample). One drop of this blood was smeared on a slide for detection of blood parasites (see below). Ten days later, we recaptured female blue tits, and a second sample was obtained (final sample). This allowed us to determine the post-treatment density of parasites in the blood (parasitaemia) and the gametocyte sex ratio of the parasite. In the spring of 2001 the experiment was repeated, but medication was increased 10-fold (0.1 mg of primaquine diluted in 0.1 ml of saline solution) to try to attain a higher reduction in the density of the parasites in the blood. In both years, the dose of primaquine allowed some production and release of parasite blood stages during the interval between captures.

Blood smears were immediately air dried and later fixed with ethanol (96%) and stained with Giemsa (1/10 v/v) for 45 min. *Haemoproteus majoris* parasites were detected using a magnification of ×1000 (Merino & Potti 1995; Merino *et al.* 1997). The density of parasites was estimated as the number of infected cells per 2000 erythrocytes (Godfrey *et al.* 1987). Sex ratios (proportion of male gametocytes) were obtained by scoring 100 gametocytes (see Schall 1989 and Read *et al.* 1992). All sex ratio counts were carried out by the same person (C.F.), who was blind to the treatment group from which the samples came. Only smears for which we were able to count 100 gametocytes in both initial and final samples were included in the analysis. Gametocyte gender was easily determined based on differential morphological and staining characteristics (Peirce 1981).

(a) *Statistical analyses*

Our aim was to compare changes in parasite sex ratios within host (the change in sex ratio between initial vs. final sample for each individual host) between groups of hosts subjected to different treatments (hosts medicated vs. hosts control) as this allows us to control for both initial sex ratio in each individual host and for host environment. Thus, the best way to test for these changes is with a paired or repeated-measures (initial versus final samples of each individual) test. To our knowledge, a repeated-measures ANOVA is the best way to analyse the pairwise nature of our experiment, having the advantage of the robustness of within-individual paired comparisons. Sex ratios were arcsine square-root transformed and the density of the parasites was logarithmically transformed prior to using parametric tests.

Transformed values were normally distributed. A repeated-measures ANOVA was conducted with initial and final sex ratio and density of the parasites in the blood as dependent (repeated-measures) variables, and year and treatment as factors, to look for between-year and treatment differences and their interactions. The analysis identified differences in the initial and final sex ratios according to treatment. More importantly, it tested for an interaction between treatment and the timing of sex ratio samples. Non-significant interactions were backwards eliminated. Residuals of the model were tested for normality.

3. RESULTS

Haemoproteus majoris was the most common blood parasite infecting female blue tits in both years (76.9% in 1999 and 68.8% in 2001; Merino *et al.* 2000). Median *Haemoproteus* intensities of infection for initial and final samples

Table 1. Median density (number of parasites (infected cells) per 2000 erythrocytes) of the *Haemoproteus majoris* parasites in the blood for initial and final samples of different experimental groups in both years.
(Sample size (*n*) and range are also shown.)

	control			medicated		
year	*n*	median	range	*n*	median	range
1999						
initial	13	18	7–115	19	15	3–68
final	13	17	4–46	19	11	2–33
2001						
initial	15	10	2–88	17	11	1–62
final	15	12	1–49	17	10	2–26

Table 2. Median *Haemoproteus majoris* sex ratio for initial and final samples of different experimental groups in both years.
(Sample size (*n*) and range are also shown.)

	control			medicated		
year	*n*	median	range	*n*	median	range
1999						
initial	13	0.47	0.39–0.55	19	0.46	0.37–0.56
final	13	0.54	0.37–0.61	19	0.58	0.38–0.68
2001						
initial	15	0.52	0.38–0.67	17	0.48	0.42–0.59
final	15	0.49	0.39–0.71	17	0.58	0.36–0.76

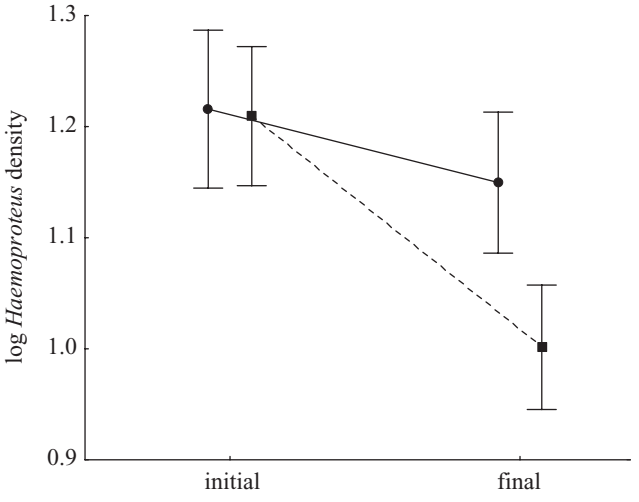

Figure 1. Change between initial and final *Haemoproteus majoris* densities according to treatment for both years as a result of repeated-measures ANOVA conducted with density of the parasites in the blood as the dependent variable and year and treatment as factors (control, filled circles; medicated, filled squares). Bars show s.e.

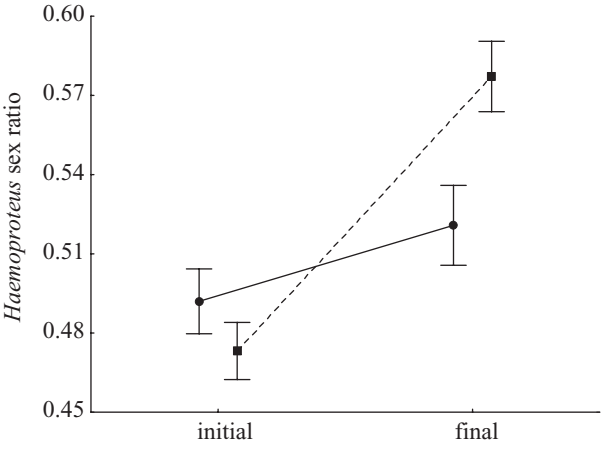

Figure 2. Change between initial and final *Haemoproteus majoris* sex ratios according to treatment for both years as a result of repeated-measures ANOVA conducted with initial and final sex ratios as the dependent variable and year and treatment as factors (control, filled circles; medicated, filled squares). Bars show s.e.

from hosts of both treatments in both years are shown in table 1. The repeated-measures analysis shows between-year differences, with intensities of infection being higher in 1999 than in 2001 ($F_{1,61} = 4.07$, $p = 0.048$; table 1). The same analyses also showed that final intensities of infection are significantly lower than initial ones ($F_{1,61} = 16.38$, $p = 0.0002$). In addition, a significant interaction between change in intensity and treatment exists, implying that the reduction in intensity from initial to final samples is more marked for medicated than for control hosts ($F_{1,61} = 4.37$, $p = 0.041$; figure 1).

The repeated-measures analysis for initial and final sex ratios showed that final sex ratios are higher than initial ones ($F_{1,61} = 23.32$, $p < 0.0001$; table 2). The effect of the year is not significant ($F_{1,61} = 3.21$, $p = 0.08$). There was also a significant interaction between change in sex ratio and treatment, implying that final sex ratios increase more in medicated hosts than in controls ($F_{1,61} = 7.36$, $p = 0.009$; figure 2). By introducing final and initial intensities of infection as covariates in the analyses, we find only a significant positive relationship between final intensity and initial sex ratio ($F_{1,59} = 4.17$, $p = 0.046$). Other relationships are not significant (data not shown).

4. DISCUSSION

Changes in gametocyte sex ratio toward a greater proportion of males can be an adaptive response of 'fertility insurance' (West *et al.* 2002) if gametocyte density declines or male gametocytes experience a reduction in their fecundity, or they can be a non-adaptive consequence of higher mortality of female gametocytes (Reece *et al.* 2003).

Our results show that the sex ratio of *H. majoris* increases (an increasing proportion of males) both in the untreated control infections and in the infections treated with primaquine. In both cases, the increase in the proportion of male gametocytes is associated with a reduction in gametocyte density. Reece *et al.* (2003) used a curative dose of chloroquine, a drug that kills all asexual and very young gametocytes, leaving an infection composed only of mature non-dividing gametocytes. These authors show that time of survival of female gametocytes is lower than that of males in *P. chabaudi* infections in laboratory mice, thus producing a male-skewed sex ratio. In our experiments, we treated infected birds with primaquine, which kills gametocytes (López-Antuñano 1999; WHO 2001). It is unlikely that the medication affects female gametocytes differentially because of the primaquine mode of action (López-Antuñano 1999; Baird & Rieckmann 2003). Thus, after a reduction in the density of gametocytes in the blood, new gametocytes are being produced, and changes in sex ratio can be caused only by differential production of male and female gametocytes. In the control infections, it is highly improbable that the immune system attacks female gametocytes more than males, although it is possible that a frequency-dependent removal of gametocytes by the immune system may reduce a higher proportion of the more common sex simply because they are more common (Paul *et al.* 1999).

If the adaptive explanation is correct (fertility insurance), the parasite should respond as soon as it receives the cue that conditions could deteriorate and the response may be independent of the severity of the shock. The time between capture and recapture of the birds in our experiment (10 days) was short, yet would allow production and release of new gametocytes. The period between initial infection and release of gametocytes into the blood for *Haemoproteus* is *ca*. 12–13 days (Fallis & Bennett 1961; and S. Merino, G. Tomás, J. Moreno, J. J. Sanz, E. Arriero and C. Folgueira, unpublished data), and this time should be considerably reduced when internal organs are already infected, as in our experiment. The observed results were similar for both years of the experiment despite the 10-fold difference in the dose of the medication, demonstrating that the shift in sex ratio is independent of the severity of the shock received by the infection.

Several previous studies demonstrate that malaria parasites shift their life histories in response to changing host conditions. *Plasmodium* parasites infecting rodents will speed up the production of gametocytes when shocked by several kinds of antimalarial drugs (Buckling *et al.* 1997). Thus, the parasites have the ability to detect a general threat to the infection and to alter the timing of reproductive events as predicted by life-history theory. Paul *et al.* (2000, 2002) found that the proportion of male gametocytes increases in infections of the bird malaria parasite *P. gallinaceum* when gametocyte density declines and survival and mobility of male gametes is reduced by the host immune attack. This host response is mediated by erythropoietin (Paul *et al.* 2000), a hormone controlling erythropoiesis, and this may be the proximate cue used by the parasite when the host immune system begins to eliminate infected cells. This observed response supports the predictions of the fertility insurance hypothesis.

Haemoproteus majoris infections normally relapse during host breeding, peaking during laying and decreasing throughout the nestling-rearing phase (Atkinson & Van Riper 1991). This reduction is attributable to an increase in immune function associated with a downregulation of hormone levels (Weatherhead & Bennett 1991). We also observed a reduction in the density of parasites in the control birds during both years of the study (figure 1), but this change was slight compared with that experienced by the medicated birds. A shift in sex ratio of the control birds was observed for only the first year of the study, and the shift was small. Shutler *et al.* (1995) reported a lack of variation over the course of the season for *Haemoproteus* gametocyte sex ratios in several species of passerines, and in only one population of infected pied flycatchers, *Ficedula hypoleuca*, was there a shift to a less female-biased sex ratio later in the season. Based on our results we suggest that a natural change in sex ratio later in the season may be observed depending on the magnitude of change in infection intensity throughout the breeding season. Only when hosts substantially reduce a high-intensity infection during the season may a change in parasite sex ratio be observed.

The fertility insurance hypothesis focuses on two events during infection that could place female gametes in danger of not being fertilized: a reduction in the viability of the male gametes or simply a low gametocyte density in the blood (Read *et al.* 1992; West *et al.* 2002; Gardner *et al.* 2003). Our results show that an experimentally induced precipitous decline in blood gametocytes results in a rapid shift towards a higher production of male gametocytes as predicted by the hypothesis. Studies of gamete production in the insect vectors for malaria parasites, the number of gametocytes per blood meal and the reproductive success of different gametocyte sex ratios are needed to reveal the adaptive importance of the observed shift in parasite sex allocation in both natural and experimentally altered infections. In addition, the study of factors inducing adaptive changes in malaria parasites will ultimately be of value in efforts to control human and animal disease.

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REFERENCES

Atkinson, C. T. & Van Riper III, C. 1991 Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. In *Bird–parasite interactions: ecology, evolution and behavior* (ed. J. E. Loye & M. Zuk), pp. 19–48. Oxford University Press.

Baird, J. K. & Rieckmann, K. H. 2003 Can primaquine therapy for vivax malaria be improved? *Trends Parasitol.* **19**, 115–120.

Buckling, A. G. J., Taylor, L. H., Carlton, J. M.-R. & Read, A. F. 1997 Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. *Proc. R. Soc. Lond.* B **264**, 553–559. (DOI 10.1098/rspb.1997.0079.)

Charnov, E. L. 1982 *The theory of sex allocation*. Princeton University Press.

Desser, S. S. & Bennett, G. F. 1993 The genera *Leucocytozoon*, *Haemoproteus*, and *Hepatocystis*. In *Parasitic protozoa*, vol. 4, 2nd edn (ed. J. Kreier), pp. 273–307. New York: Academic Press.

Fallis, A. M. & Bennett, G. F. 1961 Sporogony of *Leucocytozoon* and *Haemoproteus* in simuliids and ceratopogonids and a revised classification of the Haemosporidiida. *Can. J. Zool.* **39**, 215–228.

Fargallo, J. A. & Merino, S. 1999 Brood size manipulation modifies the intensity of infection by haematozoa in female blue tits *Parus caeruleus*. *Ardea* **87**, 261–268.

Fisher, R. A. 1930 *The genetical theory of selection*. Oxford University Press.

Gardner, A., Reece, S. E. & West, S. A. 2003 Even more extreme fertility insurance and the sex ratios of protozoan blood parasites. *J. Theor. Biol.* **223**, 515–521.

Ghiselin, M. T. 1974 *The economy of nature and the evolution of sex*. Berkeley, CA: University of California Press.

Godfrey Jr, R. D., Fedynich, A. M. & Pence, D. B. 1987 Quantification of hematozoa in blood smears. *J. Wildl. Dis.* **23**, 558–565.

Hamilton, W. 1967 Extraordinary sex ratios. *Science* **156**, 477–488.

López-Antuñano, F. J. 1999 Is primaquine useful and safe as a true exo-erythrocytic merontocidal, hypnozoitocidal and gametocidal antimalarial drug? *Salud Pública de México* **41**, 410–419.

Merino, S. & Potti, J. 1995 High prevalence of hematozoa in nestlings of a passerine species, the pied flycatcher (*Ficedula hypoleuca*). *Auk* **112**, 1041–1043.

Merino, S., Potti, J. & Fargallo, J. A. 1997 Blood parasites of some passerine birds from central Spain. *J. Wildl. Dis.* **33**, 638–641.

Merino, S., Moreno, J., Sanz, J. J. & Arriero, E. 2000 Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proc. R. Soc. Lond.* B **267**, 2507–2510. (DOI 10.098/rspb.2000.1312.)

Paul, R. E. L., Raibaud, A. & Brey, P. T. 1999 Sex ratio adjustment in *Plasmodium gallinaceum*. *Parasitologia* **41**, 153–158.

Paul, R. E. L., Coulson, T. N., Raibaud, A. & Brey, P. T. 2000 Sex determination in malaria parasites. *Science* **287**, 128–131.

Paul, R. E. L., Brey, P. T. & Robert, V. 2002 *Plasmodium* sex determination and transmission to mosquitoes. *Trends Parasitol.* **18**, 32–38.

Peirce, M. A. 1981 Haematozoa of British birds. V. Redescription of *Haemoproteus majoris* (Laveran) from the great tit, *Parus major*. *J. Nat. Hist.* **15**, 151–154.

Read, A. F., Narara, A., Nee, S., Keymer, A. E. & Day, K. P. 1992 Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* **104**, 387–395.

Read, A. F., Smith, T. G., Nee, S. & West, S. A. 2002 Sex ratios of malaria parasites and related protozoa. In *Sex ratios: concepts and research methods* (ed. I. C. W. Hardy), pp. 314–332. Cambridge University Press.

Reece, S. E., Duncan, A. B., West, S. A. & Read, A. F. 2003 Sex ratios in the rodent malaria parasite, *Plasmodium chabaudi*. *Parasitology* **127**, 419–425.

Schall, J. J. 1989 The sex ratio of *Plasmodium* gametocytes. *Parasitology* **98**, 343–350.

Shutler, D., Bennett, G. F. & Mullie, A. 1995 Sex proportions of *Haemoproteus* blood parasites and local mate competition. *Proc. Natl Acad. Sci. USA* **92**, 6748–6752.

Smalley, M. E. & Sinden, R. E. 1977 *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* **74**, 1–8.

Weatherhead, P. J. & Bennett, G. F. 1991 Ecology of red-winged blackbird parasitism by haematozoa. *Can. J. Zool.* **69**, 2352–2359.

West, S. A., Reece, S. E. & Read, A. F. 2001 Evolution of gametocyte sex ratios in malaria and related apicomplexan (protozoan) parasites. *Trends Parasitol.* **17**, 525–531.

West, S. A., Smith, T. G., Nee, S. & Read, A. F. 2002 Fertility insurance and the sex ratios of malaria and related hemospororin blood parasites. *J. Parasitol.* **88**, 258–263.

WHO 2001 *The use of antimalarial drugs: report of an informal consultation*. Geneva: World Health Organization.