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Do malaria parasites follow the algebra of sex ratio theory?

Jos. J. Schall

Department of Biology, University of Vermont, Burlington, VT 05405, USA

The ratio of male to female gametocytes seen in infections of *Plasmodium* and related haemosporidian parasites varies substantially, both within and among parasite species. Sex ratio theory, a mainstay of evolutionary biology, accounts for this variation. The theory provides an algebraic solution for the optimal sex ratio that will maximize parasite fitness. A crucial term in this solution is the probability of selfing by clone-mates within the vector (based on the clone number and their relative abundance). Definitive tests of the theory have proven elusive because of technical challenges in measuring clonal diversity within infections. Newly developed molecular methods now provide opportunities to test the theory with an exquisite precision.

In evolutionary biology, the issue is always variation

In 1880, the physician C.L.A. Laveran looked through his microscope at the blood of a patient suffering from malaria and discovered something wonderful. Laveran observed exflagellation, the extrusion of flagellated gametes by a *Plasmodium* parasite's male cells, or microgametocytes [1]. This initiates the sexual cycle that is universal in malaria parasites (*Plasmodium*, *Parahaemoproteus* and *Haemoproteus*) and other Haemsporidia [2]. Malaria parasites replicate asexually as haploid cells within the vertebrate host, eventually producing the gametocytes in blood cells that develop into gametes within the blood-feeding insect vector. Male gametocytes yield two to eight gametes (their fecundity is the number of gametes produced), and female gametocytes develop into a single female gamete [3–5]. Intuition suggests the optimal gametocyte sex ratio for maximal transmission success would be just enough males to mate with all the females; each male gamete would be matched with a female gamete and no gametes would be wasted. This optimal ratio would depend on male fecundity. Although female-biased ratios are common for human malaria infections [6,7], there is also substantial variation among parasite species, among infections for any species and even over the course of individual infections [5]. For example, human *Plasmodium* infections vary considerably in sex ratio [6–9], and the malaria parasites of birds [10,11] and lizards [12–14] typically produce only slightly female-biased or equal sex proportions. Some species of lizard malaria parasites in the genus *Haemoproteus* produce bizarrely male-biased ratios of 15:1 [15]. For *Plasmodium* parasites of human, rodent, bird and lizard hosts, female

bias might be seen early in the infection but fades as more males appear over time [5,16–19]. Surely, partitioning reproductive effort to males and females is central to any species' life history and must be shaped by natural selection toward some optimal solution. What, then, accounts for this variation in gametocyte sex ratios? Sex ratio theory, a profoundly productive and successful effort in evolutionary biology, provides answers. The theory is elegant, but technical challenges have long hindered verification of quantitative predictions. Resolution of the issue will tell us whether a protist parasite can follow the elegant algebraic rules of evolutionary theory by evolving either local adaptations or phenotypic plasticity in a crucial life-history trait.

Sex ratio theory and *Plasmodium* gametocytes

Sex ratio theory originated with Darwin and Düsing [20], who noted that when one gender is less common in a population, this gender will have, on average, more off-spring per individual and, thus, higher fitness. Frequency-dependent selection will result in a 1:1 equilibrium sex ratio because the *per capita* contribution to the next generation will be equal for males and females. Thus, a population-level phenomenon (sex ratio) is driven by selection acting on individuals. This 1:1 equilibrium is often called a 'Fisherian' sex ratio (after R.A. Fisher, who restated the Darwin and Düsing argument) [21]. The novelty of this theory is that it focuses on three generations, with fitness of the mother based on the number of grand-offspring she enjoys. Nearly a century after Darwin, W.D. Hamilton recognized the theory applies only to large, randomly mating and genetically diverse populations, but for species distributed in patches in which brothers mate only with sisters, a mother should indeed produce just enough sons to mate with all daughters and, thus, there would be no wasted male gametes [22]. If additional females add their offspring to the patch, each mother should produce a greater proportion of male offspring to compete with the sons of other females for mating success. As more females enter the patch, the sex ratio will shift toward the Fisherian equal proportions.

The evolutionary theory of sex ratios is now mature and has been tested exhaustively. Entering 'sex ratio theory' into a search engine will yield thousands of papers for a lifetime of reading pleasure (for a succinct review, see Ref. [23]). Oddly, sex ratio theory was ignored by malaria researchers for a century (Düsing published in 1884, just before the *Plasmodium* life cycle became known). Perusal of two dozen major reviews on malaria parasites

Corresponding author: Schall, J.J. (jschall@zoo.uvm.edu).

Box 1. Sex ratio theory allows testing

For malaria parasites (*Plasmodium* and related haemosporidians), mating takes place only within blood-feeding vectors and only between male and female gametes released by gametocytes that were within the vertebrate host at the time the blood meal was taken. Sex ratio theory developed for this kind of patchy world recognizes that local mate competition [22] between brothers is severe if a single genotype of parasite is present in the blood meal and is reduced when just enough males are present to mate with all the females. Thus, the optimal sex ratio ($r*$) depends on the fecundity (f) of the male gametocyte cell or the number of male gametes produced (female gametocytes produce only one gamete). However, if multiple clones of a parasite are present in the blood meal, males also compete with other parasite genotypes, and the optimal sex ratio depends on both fecundity and the probability of selfing (s).

Figure I solves the algebraic solution of Hamilton for fecundity of one, four and eight gametes produced by a male gametocyte. For example, if there are six equally abundant clones of parasite in the vertebrate host's blood, and fecundity is four or eight gametes, then the probability of selfing would be 1/6, or 17%. (A range of numbers of equally abundant clones and the expected selfing is shown on the x axis.) For this example, the optimal sex ratio for each genotype (and, thus, overall in the infection) is (1 - 0.17) ÷ 2, or 42% males. However, for very low selfing rates, the calculated optimal proportion of males could be zero (which is clearly not optimal!), so the minimum number of males expected must be calculated as 1 ÷ (1 + f). Note that if only a single male gamete is produced per gametocyte, an equal proportion of male and female gametes is expected, but for higher fecundities, female bias is expected when few clones are present in the vertebrate and selfing rates are low.

A recent study established infections of the rodent malaria parasite, *P. chabaudi*, in laboratory mice [41]. When six clones of the parasite were mixed in infections, the sex ratio observed was ~45% (as measured accurately with a new quantitative real-time PCR assay), close to the predicted value (open point in Figure I). When clones with estimated fecundity of eight male gametes were matched two-by-two and their relative proportions determined, the sex ratios observed again closely matched the predictions (closed points). *P. chabaudi* follows the algebra of sex ratio theory, facultatively altering its production of male and female gametocytes. This exquisite fit to theory begs the question: how does this protist parasite monitor its own fecundity, relative proportion of the genotypes in an infection and probability of selfing within the blood-feeding vector?
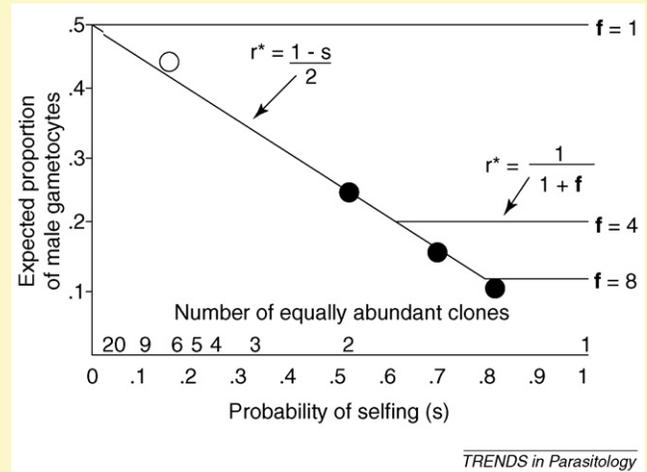

Figure I. Predicted gametocyte sex ratio based on probability of selfing and male fecundity. The figure is adapted from Refs [24,29].

published before 1990, including those devoted entirely to gametocytes, failed to find a single mention of sex ratios [13]. Even the initial publication to incorporate sex ratio theory into a study of *Plasmodium* in 1989 did not recognize the importance of the patchy mating within vectors and instead stressed a pure Fisherian perspective [13].

A.F. Read and colleagues [24] first recognized that malaria parasites reproduce in patches, as envisioned by Hamilton. If a vertebrate host carries only one genotype of parasite, then only clone-mates will cross during the brief sexual cycle in the vector. Thus, the optimal sex ratio for that clone will be female biased. If the infection houses a mixture of clones, Hamilton's simple algebraic treatment predicts the expected sex ratio based on the likely degree of selfing and fecundity of male gametocytes [24,25] (Box 1). One wrinkle in the theory for malaria parasites finds that infections producing very few gametocytes or transmitted by small vectors taking blood meals containing few gametocytes should produce more males (even for single-clone infections) because the mobile male gametes might have difficulty finding a female gamete within the few minutes open for mating [7,26,27]. Similarly, over time, the immune system would mount an attack that would reduce mobility of male gametes, reduce male fecundity and favor a shift toward males [5]. Note that this theory centers on the fitness of each genotype or clone and not the overall transmission success of all parasites within an infection.

The optimal gametocyte sex ratio could be reached in two nonexclusive ways [28,29]. First, selection could favor a locally adapted sex ratio based on the prevailing clonal diversity within infections and life-history traits (fecundity and density of gametocytes). Such a locally fixed sex ratio, however, seems a poor adaptive solution. Clonal diversity must have some variation among hosts and, thus, the parasites within many infections would produce an inappropriate gametocyte sex ratio. Ecological conditions change, and average clone number per infection might shift more rapidly than selection can respond. Most problematic, a geographic mosaic of local adaptation would be disrupted by gene flow [30]. Second, the parasite could evolve phenotypic plasticity and the ability to monitor its own male fecundity, density of gametocytes in the blood, intensity of likely gamete killing in the vector and likely degree of inbreeding. All of these factors certainly vary among infections and even within infections over time, so phenotypic plasticity would result in a very local (i.e. an individual host) adaptive sex ratio. Is it possible, though, for a protist living within its host's cells to gather the necessary information to reach the optimal sex ratio?

Testing the theory

Sex ratio theory is so compelling because the simple algebra both explains broad patterns and makes specific quantitative predictions (Box 1). For example, the near 1:1 gametocyte sex ratio typically seen for lizard malaria parasites can be explained if their male fecundity is low (compared to the *Plasmodium* parasites of humans that typically show female-biased sex ratios) and/or if clonal diversity is high within infections. In addition, variation in sex ratio among infections for any parasite species predicts specific male fecundity and inbreeding (Box 1). Creative attempts to the test the theory have included comparisons

among taxa with different life cycles [31], cross-population comparisons [32,33,27], comparisons among individual infections [12,14,19] and experimental manipulations of infections [29,34,35]. Results, however, have been equivocal. Until recently, a confounding factor has been lack of direct measures of actual clonal diversity and likely selfing within infections, clearly a major shortcoming. Instead, various surrogates for clonal diversity have been proposed.

A few examples show how investigators have struggled to test the theory while blind to actual clonal diversity within infections. First, blood from naturally infected lizard hosts of *Plasmodium mexicanum* was used to initiate experimental infections using blood from one to several donors. Infections started from multiple donors were assumed to be more genetically complex, but no difference was seen for sex ratio of 'simple' versus 'complex' infections [29]. Second, mixed-clone infections were assumed to lead to competition among clones and, thus, higher parasitemia [36]; natural infections of two lizard malaria parasites did, indeed, produce more male gametocytes when parasitemia was greater [12,14].

Perhaps the most striking outcome emerged from a study of *Leucocytozoon* (a clade of haemosporidians infecting birds [2]) at 11 sites that varied in prevalence [32]. A formal model was developed to predict proportion of mixed-clone infections based on prevalence and assumed transmission intensity. The results for *Leucocytozoon* fit the quantitative expectations well. Some sample sizes were small (only two to seven infections per site), yet the sex ratios of those sites also fit the prediction. This would be expected if there is local adaptation, with little variation among infections in the selfing rate. However, there was a mix of morphologically identified species among sites and a probably cryptic mix of species at local sites [37]. The underlying assumption of higher prevalence predicting average clonal diversity can also be questioned because prevalence and clonal diversity within infections are not correlated among sites for either human [38] or lizard [39] *Plasmodium* parasites. A similar study examined another avian malaria parasite (*Parahaemoprotus* [2]) but found no relationship between prevalence and gametocyte sex ratio [33].

New molecular techniques now provide very precise determinations of gametocyte sex ratio (the equivalent of counting millions of cells) [40] and enable the number of coexisting clones [39] and even their relative proportions to be measured [41]. Unfortunately, accurately measuring male fecundity remains challenging, but estimates can be inferred from sex ratio of solitary infections (although this is a reversed use of the theory to estimate a parameter in the equations).

In a cunning new study (the best we have in the study of sex ratio theory and malaria parasites), six laboratory-derived clones of a rodent malaria parasite, *Plasmodium chabaudi*, were used to induce infections in laboratory mice that carried one, two or six parasite clones [41]. Relative proportions of the clones were measured. The results fit the quantitative predictions from theory almost perfectly (Box 1). Indeed, few studies in evolutionary biology produce such clean results. The *P. chabaudi* experiments indicate that this malaria para-

site has a plastic phenotype, shifting sex ratio to an infection optimum. The parasite apparently monitors number of clones within infections, their relative proportions and even the age of the infection – an astounding finding. All of this is even more notable because the experiments used an unnatural parasite–host association. *P. chabaudi* is a parasite of thicket rats (*Thamnomys rutilans*) from the Central African Republic and Congo [42]. The laboratory mouse and *Thamnomys* are placed into the same subfamily of rodents (murinae) but must present different environments for the parasite. The ecology and evolutionary history of parasite and host, thus, has been lost, but the parasite still finds the appropriate cues and follows the predicted rules.

Concluding remarks and future perspectives

Aficionados of sex ratio theory would rejoice to learn that a protist parasite behaves as demanded by evolutionary theory, especially if the results salve the biologists' physics envy by matching precise quantitative predictions. However, public health warriors, who confront the devastation of human malaria, might appreciate elegant evolutionary theory and novel experimental approaches but could well ask how this is relevant to their efforts. What could be more central to the biology of any organism than how it allocates effort to reproductive cells? The theory, some studies that are blind to the likely degree of gamete selfing and, now, an impressive experimental study using molecular techniques conclude that gametocyte sex ratios are entwined with the clonal diversity of the parasite within infections (an infection trait important for pathology, population genetics, drug development and transmission). If there is phenotypic plasticity for sex ratio, based on male fecundity and likely inbreeding, we face two vexing, but fascinating, questions. Is there a genetic basis for the variation in male fecundity? This seems to be the case for both human and rodent malaria parasites [41,43]. A life-history trait as important as male fecundity should be under potent selection, so why should there be polymorphism among genotypes? And what cues could be used by the parasite to determine the number of coexisting clones and their relative abundance? An obvious candidate would be the diversity of antibodies cycling in the infection, yet the expected sex ratio in the *P. chabaudi* was seen early in the infection, before likely production of an immune response [41]. Identifying the cues could indicate new drug targets; instead of killing the parasite, just deprive it of mates! Once again, malaria parasites surprise – more than a century after Laveran first witnessed the beginnings of *Plasmodium* sex.

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