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ORIGINAL PAPER

Attraction between sexes: male–female gametocyte behaviour within a *Leucocytozoon toddi* (Haemosporida)

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Abstract Understanding the breeding systems of *Plasmodium,* and the closely related *Haemoproteus* and *Leucocytozoon* (Apicomplexa: Haemosporida), is fundamental to virulence and transmission research. We report an unusual binding behaviour between gametocytes of *Leucocytozoon toddi*. This aggregative behaviour was notably characterised by a disparity in the likelihood of clustering by female and male gametocytes. Thus, indicating a possible difference in the 'stickiness' of gametocytes per sex. Overall, 12% of gametocytes in this high-parasitaemia infection (0.269 gametocytes per 100 red blood cells (RBCs)) were incorporated into aggregations involving substantial contact. The gametocyte sexual combinations within aggregations varied significantly from expected according to the background 0.49 sex ratio within this sample, with female–female contacts occurring more and male–male contacts occurring less frequently than expected. A second *L. toddi* (identical for 709 bp of the cyt *b* mitochondrial gene) with lower parasitemia (0.035 gametocytes per 100 RBCs) showed no significant binding. Interestingly, the ratios of male gametocytes in both of these parasites were greater than expected under sex-ratio theory and similar to the 50% observed in species with syzygy breeding strategies. We discuss the ramifications of this observation in terms of sex-ratio theory and breeding strategies and provide speculative explanations for this unusual gametocyte behaviour.

Introduction

Haemosporidians (phylum Apicomplexa, order Haemosporida) are common protozoan parasites of vertebrates, some of which are responsible for widespread illness and death. The most widely known of this group are the human malaria parasites from the genus *Plasmodium* (within which genus all the true malaria parasites are placed). Parasites of

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the genera *Haemoproteus* and *Leucocytozoon* are closely related to *Plasmodium* (e.g. Perkins and Schall 2002) and these three genera fall within the same order or the same family, depending on the authors (Garnham 1966; Levine 1988; Valkiunas 2005).

Plasmodium, Haemoproteus and *Leucocytozoon* are each common in birds. These parasites are obligatory sexual organisms and require two hosts, a haematophagous dipteren vector and a vertebrate host. They must undergo a round of sexual reproduction in their vector in order to produce stages that can be transmitted to vertebrate hosts. The three genera have similar life cycles, with schizogony and gametocytogenesis within their vertebrate host leading to gametogenesis, zygote formation and sporogony within their insect vectors. Differences between their life cycles include the type of blood cells that are parasitised (erythrocytes for *Plasmodium* and *Haemoproteus* and leucocytes for avian *Leucocytozoon*) and the type of vector (mosquito for *Plasmodium*, midge and other biting Diptera for *Haemoproteus*, black fly for *Leucocytozoon*). However, the process of fertilisation is similar among these genera: After a period of asexual proliferation in a vertebrate host, transmission to the vector occurs via the uptake of dioecious haploid pre-sexual stages (the gametocytes) in a blood meal. Activation of the gametocytes inside the vector takes the form of exflagellation of male gametes, with several gametes arising from one male gametocyte, and the production of a single female gamete from each female gametocyte. Fertilisation produces a zygote in the lumen of the insect vector's midgut. This zygote undergoes multiple divisions (sporogeny) to form and release haploid sporozoites that migrate into the vector's salivary glands. Infection of a vertebrate host occurs when sporozoites are injected with saliva as the vector takes a blood meal (e.g. see Paul et al. 2003 for detailed ecology of *Plasmodium*).

Multiple clones are not needed for successful transmission and sexual reproduction. This is because a single haemosporidian parasite clone can produce self-compatible male and female gametes (e.g. West et al. 2000a), and parasite species can differ in the range of gametocyte sex ratios produced (Read et al. 1995).

Central topics in the study of apicomplexan breeding strategies include patterns of gametocyte distribution and production within the vertebrate host, gametocyte aggregative behaviours (e.g. syzygy) and applying sex-ratio theory to gametocyte sex ratios (e.g. Hamilton's 1967 theory of local mate competition). These are important themes because the number, distribution and sex ratios of gametocytes within the peripheral blood of the vertebrate affect the likelihood of successful transmission of the parasite to its vector and subsequent sexual reproduction.

Sex ratio is an excellent model trait for testing the validity of important components of 'Darwinian medicine' (applying evolutionary principals to etiological agents of disease; West et al. 2001) and may provide insights into clinical and epidemiological factors pertaining to human malaria (Read et al. 2002), for instance, by attempting to explain and predict gametocyte sex ratio in terms of what would be favoured by natural selection. The predictions for gametocyte sex ratios arising from the principles of local mate competition outlined in the review by West et al. (2001) include: (1) the sex ratio should be 0.5 or female biased and never male biased, (2) the extent of female bias observed in the sex ratio of a population of species should be related to the inbreeding rate, (3) across species and populations, the sex ratio should be negatively related to the inbreeding rate. Promising trends have emerged through this field of research. For instance, the average sex ratio in human malaria has been shown to correlate to levels of inbreeding (as reviewed by West et al. 2001). Furthermore, Read et al. (1995) found a correlation between gametocyte sex ratios and prevalence of *Leucocytozoon* spp. (incorporating a likely minimum of seven *Leucocytozoon* species), thereby supporting a theoretical relationship between these two life history characteristics.

Within the phylum Apicomplexa, syzygy has been known to occur within the adeleorins, gregarines and haemogregarines (subclass Gregarinasina) and piroplasms (suborder Piroplasmasina) but has not previously been known in the Haemosporida (Read et al. 2002). Syzygy occurs when a single male and a single female gamonte pair together physically, or in close proximity, prior to fertilization (West et al. 2000b). A gamonte is pre-gamete stage synonymous with a gametocyte and, therefore, the term syzygy is applied to haemosporidian parasites in this paper as the physical pairing of male and female gametocytes prior to fertilization. Interestingly, West et al. (2000b) also predicted that within species in which syzygy occurs, sex ratios would be unlikely to differ significantly from 0.5 and that, consequently, local mate competition theory would also not apply within these groups. By implication, if local mate competition can be successfully applied to haemosporidian parasites, this would preclude the likelihood of syzygy within this group.

Counts of *Plasmodium falciparum* gametocytes in the blood meals of anophelines have revealed a nonrandom distribution and the likely presence of gametocyte clusters within the vertebrate host's peripheral blood (Pichon et al. 2000). Subsequently, it has been hypothesised that not only may there be a clustering of gametocytes within the vertebrate host capillaries but that clustered gametocytes may also potentially be able to bind with one another, possibly even preferentially with those of the opposite sex (Gaillard et al. 2003; Pichon et al. 2000). However, these authors also recognised that no convincing microscopic evidence had yet been found for such behaviour.

We conducted thin blood smear surveys for avian *Plasmodium, Haemoproteus* and *Leucocytozoon* in Mada-

gascar. Here, we present a novel occurrence of gametocyte binding behaviour encountered in a single sample during this research. We contrast this gametocyte behaviour with that exhibited on smears from standardised comparison samples and discuss the results in the context of parasite breeding strategies for increasing chances of successful transmission and sexual reproduction.

Materials and methods

A total of 812 wild-caught birds were sampled during a succession of large-scale surveys for the avian haemosporidian parasites *Plasmodium, Haemoproteus* and *Leucocytozoon* in Madagascar, from 1994 to 2004. These surveys yielded an example of unusual gametocyte aggregative behaviour exhibited by a *Leucocytozoon toddi* within a female sparrowhawk (*Accipiter francesii*). We compared the gametocyte behaviour, sex ratio and parasitaemia on the two study slides taken from this sample with those from a second *A. francesii* collected during the same survey, also containing *L. toddi*. The bird hosting the study *L. toddi* was sampled once during this survey on January 7th 2004, whilst the bird hosting the comparison *L. toddi* was sampled twice, on December 21st 2003 and January 8th 2004.

The slides taken from the two comparison samples provided the closest possible replicate for the study slides in that they shared the same bird host species, parasite species, collection location, collection time-period and slide preparation process. Furthermore, these two bird hosts were also sampled in the exactly same manner by the same researcher: they were mist-netted, the brachial vein under the wing was lightly pierced and a small amount of blood was collected with untreated capillary tubes. Two thin blood smears were made from each sample then any remaining blood was stored in liquid nitrogen. Smears were air-dried then immediately fixed in absolute methanol. The time taken between piercing the brachial vein and making the thin blood smears is estimated to be less than 20 s in each instance. The only key known environmental difference between the study and comparison slides was the fact that the study slides were from a female *A. francesii,* whilst the comparison slides were from a male.

Slides were stained with May-Grünwald-Giemsa (RAL 555®; Bordeaux Technopolis, Martillac, France) or Gurr's Giemsa Improved R66 stain, examined for the presence of haematozoa at ×200 and ×1,000 with a compound light microscope, and photographs were taken. One study slide and one comparison slide are now in the collection of Protistology, Muséum National d'Histoire Naturelle, 61 rue Buffon, Paris, under the accession numbers 28ZS (study slide) and 29ZS (comparison slide).

The sex of each gametocyte was unambiguously determined by the pinkish colouration of the cytoplasm of males and blue of females. The total number of clusters and the sexual composition of each of these were recorded from the study and two comparison samples (two slides examined per sample) to determine if there was preferential or random binding between gametocytes.

Repeated nonoverlapping counts of approximately 1,000 individual gametocytes or alternatively complete slide surveys (when the total number of gametocytes per slide approximated or was less than 1,000) were conducted to determine the proportion of the available male and female gametocytes that were incorporated into clusters and to estimate the sex ratio of gametocytes per sample. Parasite gametocyte density per sample was calculated as the number of gametocytes detected per 100 red blood cells. In all cases, the results from each pair of slides were combined to yield a total per sample (one study and two comparison samples).

These *L. toddi* samples were collected from an isolated forest fragment system within the Malagasy highlands, incorporating the protected Réserve Spéciale d'Ambohitantely. The largest forest remnant within this fragmented system is approximately 1,250 ha (Langrand and Wilmé 1997) and the next significant native forest, Andranomay-Anjozorobe, is situated approximately 90 km to the east (Goodman and Raherilalao 2003). Therefore, the *A. francesii* hosts surveyed in this study comprise part of an isolated and likely small population of sparrowhawks.

To ascertain relatedness between the *L. toddi* parasites from the study and comparison slides, these parasites were sequenced to 709 bp mitochondrial cytochrome *b* (Accession numbers AY762076 (RB58) and AY762077 (RB7)) with the following primers designed by our research group, PLAS1 (5′-GAGAATTATGGAGTGGATGGTG-3′) and PLAS2 (5′-TGGTAATTGACATCCAATCC-3′).

We used Chi-square goodness-of-fit to test for differences in the sexual composition of aggregations within the study sample—firstly, for only paired clusters and, then, for all gametocyte aggregative contact surfaces. We also tested whether the expected proportion of male and female gametocytes were incorporated within aggregations according to overall sample gametocyte sex ratios. Finally, we used the Chi-square test for homogeneity to determine if there were significant differences in the proportion of gametocytes occurring in aggregations between the samples.

Results

Sequencing of these two *L. toddi* isolates revealed that the study and comparison parasites were identical for 709 bp

mitochondrial cytochrome *b*. Both of these sequences were clean, indicating that a single individual parasite appeared to occur within each bird.

Similar sex ratios were found within the study sample and two comparison samples (study=0.49; comparison=0.46 and 0.45, respectively). However, the study sample carried a remarkably greater level of parasitaemia than the two comparison samples (study=0.269, comparison=0.035 and 0.034 gametocytes per 100 red blood cells). The aggregation apparent in the study sample (Fig. 1), in which 12% of gametocytes were incorporated in aggregations (661/4,767), was not exhibited within the comparison samples, where 0.9% (16/1,736) of gametocytes in the comparison sample was incorporated within clusters on Dec 21st and 1% (10/889) on January 8th.

The study sample slides held 536 aggregations between pairs of gametocytes and a further 47 multiple aggregations (some of which were complex, i.e. when a single gametocyte could be pressed against a number of other gametocytes; Fig. 1j–l). Whereas, in contrast, a total of eight paired aggregations occurred in the comparison sample from December 21st (male (m) + female (f)=4; m + m=1; f + f=3) and five within the January 8th comparison sample (m + f=1; m + m=2; f + f=2). No multiple aggregations occurred within either of the two comparison samples. Most gametocytes observed within aggregations were not in simple contact with one another but in a bond that incorporated substantial contact, often involving the longest available gametocyte surface from end to end (Fig. 1). None of the gametocytes occurring in these aggregations showed signs of undergoing transformation into gametes.

When considering whether the sexual composition of this gametocyte binding on the study slides was random or preferential, we first addressed only paired clusters as they featured a single clear contact surface. A total of 536 paired clusters were counted, of which 52.7% were heterosexual (283), 16.9% were same-sex male (91) and 30.2% were same-sex female pairings (162). Goodness-of-fit testing indicated that the sexual composition of these paired clusters was not random (p=0.0004, Chi-square=15.55, degrees of freedom (*Df*)=2) and m + m pairings occurred

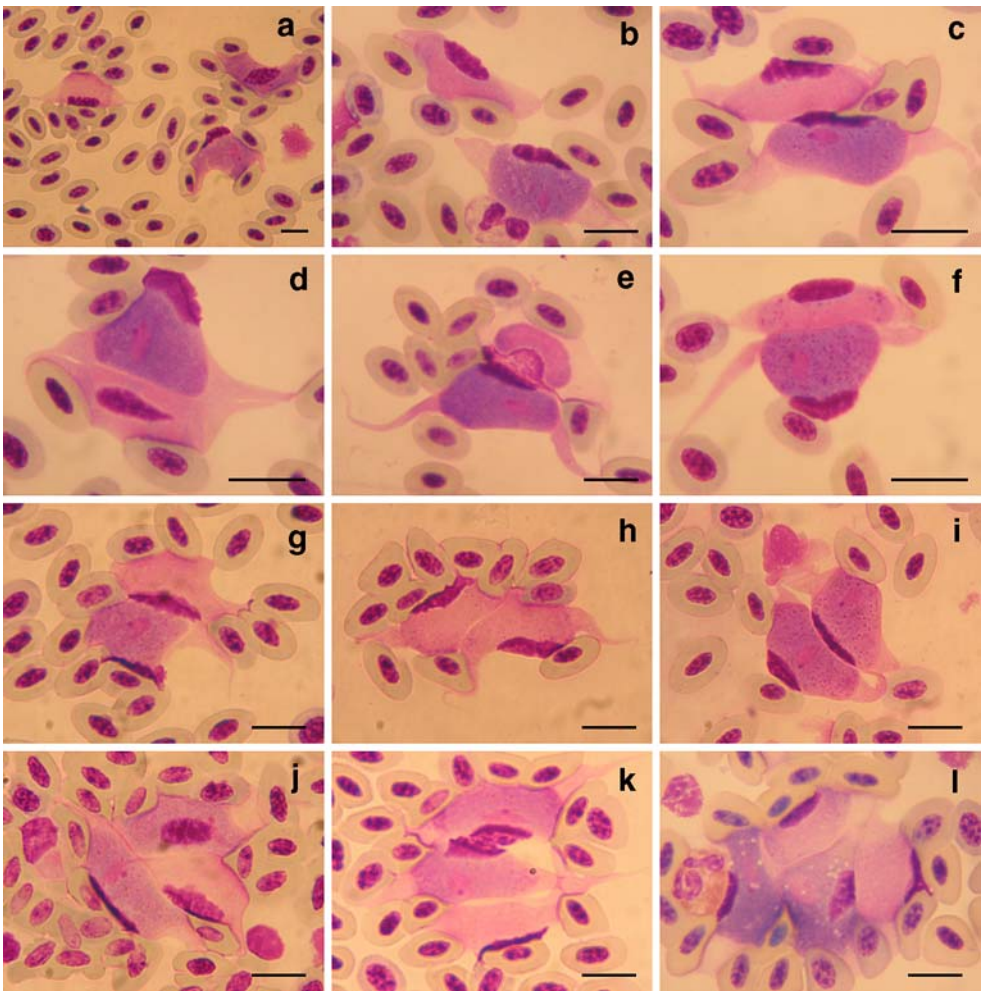

Fig. 1 Gametocytes of *Leucocytozoon toddi* in a thin blood smear from an *Accipiter francesii*. The male gametocyte has pinkish colouration of the cytoplasm and the female blue. **a**, **b** Gametocyte without contact, as usually observed in the blood of birds. **c**–**g** bound male and female gametocytes; **h** cluster of two males; **i** cluster of two females; **j**, **k** cluster of two females and one male; **l** cluster of two males and two females. *Scale bar* 10 mm

less frequently, while f + f pairings more frequently than expected. The number of heterosexual (m + f) contacts closely reflected the expected number of contacts given the background 0.49 sex ratio.

The results were very similar when multiple clusters (including complex aggregations) were incorporated into the analysis by including the sexual composition of every gametocyte–gametocyte contact surface. These totalled 638 contacts, of which m + f=54.23% (346); m + m= 16.45% (105); f + f=29.3% (187). This difference in sexual composition of contacts departed significantly from values expected with random aggregation (p=0.0001, Chi-square=20.13, Df=2).

The proportion of individual female and male gametocytes incorporated within aggregations (including complex clusters) also differed significantly from expected proportions according to the sex ratio of gametocytes within the blood stream (p=0.0004, Chi-square=12.48, Df=1), with more female gametocytes than expected (observed=678; expected=616.59) and fewer males (observed=531; expected=592.41) than expected within aggregations.

In conclusion, these results indicate that although gametocytes may randomly come into contact with each other, there is a difference in the probability of a male or female gametocyte remaining in contact (or adhering) to another gametocyte. Female gametocytes are more likely, and males less likely, to remain in contact with another gametocyte.

Discussion

Leucocytozoon toddi is considered the sole valid named species of leucocytozoids parasitising falconiform birds (Greiner and Kocan 1977). Sehgal et al. (2006) has recently revealed structure within this taxonomic species but confirmed that the parasite sequence reported in this paper is part of this widely distributed *L. toddi* species complex.

The parasites of the study and comparison slides were identical for 709 bp cyt. *b*. Because it has been established that mitochondrial DNA lineage diversity reflects intra- and inter-specific variation (Bensch et al. 2004), this result indicates that these two parasites were genetically very similar, if not identical. Yet, despite this genetic relationship and the standardisation of environmental and ecological factors relating to the origin and collection of these two parasites, the comparison slides did not exhibit the binding behaviour apparent in the study slides.

The main identifiable difference between these parasites was the level of parasitaemia, and it is not unreasonable to assume that the higher parasitaemia within the study sample afforded greater opportunity for gametocytes to come in contact with each other. However, this high parasitaemia alone is not adequate for explaining the aggregations evident in this slide as such gametocyte behaviour has not previously been reported in instances of high gametocytaemia.

The slide-making process may have increased the incidence of clusters on the study slides if cells that were already close together in the peripheral blood were pushed against each other. However, because binding was visible in all regions of the smears (both thin and thick) and included instances of complete contact between gametocytes (as per Fig. 1d), we argue that at least a proportion of clusters were already formed within the peripheral blood before sampling.

The aggregative behaviour on the study slide is unlikely to be an artefact of the blood-sampling process (i.e. the exposure of blood to the air), as it only took a matter of seconds to make the thin blood smears after venipuncture, and the slides dried immediately. Moreover, the natural development pathway of gametocytes would suggest that any gametocyte response to the sampling process would likely to have been ‘activation’ (i.e. commitment towards gamete formation) rather than a binding behaviour, and this was not seen within either the study or comparison slides.

The high proportion of heterosexual pairing observed in the study sample was reflective of the 50% chance of random heterosexual contact within a sex ratio that was approximating 50:50. However, the binding behaviour observed here has revealed a different likelihood of male and female gametocytes being incorporated into, or remaining within, a cluster. This indicates a possible difference in the ‘stickiness’ of gametocytes according to sex, making it more likely for a female gametocyte to adhere to another gametocyte. Thus, the aggregative behaviour observed in the study slide allowed us to see a process otherwise undetectable with low parasitaemia.

The binding behaviour observed here fulfils predictions by Gautret et al. (1996a, b), Gaillard et al. (2003) and Pichon et al. (2000) that aggregation of gametocytes is possible. However, this observation did not fulfil the speculative prediction of possible sexual preference in such clustering. Moreover, counter to predictions by Gaillard et al. (2003), in this instance, the aggregation of gametocytes occurred in conjunction with high gametocytaemia rather than as an adaptation to low gametocytaemia.

This observation of gametocyte binding or aggregation is, thus far, also a unique documentation within *Lecucocytozoon* and haemosporidians. The closest breeding strategy equivalent within Apicomplexa is syzygy; however, no such sygyzy-like process has been known, thus far, within the order Haemosporida. Moreover, West et al. (2000b) predicted that limited mate competition theory will not apply to the groups in which syzygy occurs and sighted data from four adeleorin species showing sex ratios not

significantly different from 0.5. Therefore, it is interesting to note that the unusual syzygy-like clustering occurring here took place when sex ratios were close to 0.5. The multiple gametocyte aggregations exhibited by *L. toddi* on the study slides in this study were also a behavioural characteristic previously observed in syzygy. For example, the species *Gregarina polymorpha* (Gregarinidae) have been observed in aggregations incorporating three and four partners (Devauchelle 1968; Nelson and Smith 1926).

It is important to note that the binding described by this study should not be confused with the simultaneous occurrence of male and female gametocytes inside a single host blood cell. Such double gametocyte infections have recently been documented in *Plasmodium, Haemoproteus, Leucocytozoon* and *Hepatozoon* with cases of male–female double gametocyte infection in *Haemoproteus* of birds and reptiles and in *Leucocytozoon* of birds (Jovani et al. 2004). In these instances, this close proximity of the gametocytes has also been mooted to be an aid to fertilisation within the arthropod vector, although experimental evidence is lacking (Martinez et al. 2006).

It is also interesting to note that the high gametocyte sex ratios exhibited within the study and comparison samples were not what were expected according to sex allocation theory. Previous work by Read et al. (1995) suggests that *Leucocytozoon* sex ratios appear to relate to prevalence and, thereby, the likelihood of outbreeding. The population of *Leucocytozoon toddi* within our study is located within an isolated and small population of sparrowhawks. This and the fact that the two sparrowhawks tested carried either identical or closely related clones support the assertion that these *L. toddi* are from a depauperate population with a strong probability of inbreeding. Generally, greater female biases should occur with greater probability of inbreeding (West et al. 2001), and the theoretical optimum dictates that when there is complete inbreeding, the minimum number of males required to fertilise the available female gametes should be produced (Gardner et al. 2003). Consequently, one would expect to find a strongly female-biased sex ratio within these two *L. toddi*. However, this is not the case. The study sample carried a ratio of male gametocytes of close to 50% (0.49; count of 5,428 gametocytes) and the comparison sample slides exhibited a ratio of approximately 45% (0.46, 0.45; count of 1,752 and 899 gametocytes, respectively). Such sex ratios approximating 50% should be theoretically favoured when there is no inbreeding (West et al. 2001).

However, there is great intricacy in haemosporidian breeding systems. Factors such as stress from host immunity, drug-pressure or competing parasites in the host appear to be a general enhancer of sexual conversion in the human *P. falciparum* (Talman et al. 2004). Also, as per Reece et al. (2005) and Gardner et al. (2003), parasites can respond when fertilisation success is compromised, including through a less female-biased sex ratio than predicted by their inbreeding rate alone, due to fertility insurance. The high parasitaemia and the aggregative behaviour described here would help to guarantee self-fertilisation in the event of gametocytes being taken in a blood meal, due to the extreme proximity of the male and female gametocytes. Therefore, in this instance, we suggest that one explanation for both the high gametocyte sex ratios and syzygy-like binding may be a hereto unseen, or novel, response to extreme fertility insurance, as a result of isolation and inbreeding.

In conclusion, because such clustering behaviour of gametocytes in the peripheral blood has not been reported by generations of microscopists working for more than a century on these parasites, it is hard to argue that this is a regularly occurring phenomenon within the Haemosporida. Rather, its rarity suggests that it is unlikely to be a long-term state. However, this observation does show that this important group of parasites is capable of adopting a 50% sex ratio and clustering breeding strategy ordinarily restricted to other orders within Apicomplexa. Furthermore, our observation of this unusual behaviour introduces the suggestion that male gametocytes are more likely to adhere to female than other male gametocytes, and female gametocytes tend to be more likely to adhere to another gametocyte, regardless of sex. These two factors influence transmission success and, therefore, will be of interest to those researching breeding strategies of this important group of organisms. What is certain is that these observations afford important new examples of gametocyte behavioural plasticity, while also highlighting the underlying complexity of Haemospridian breeding systems.

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