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Infection with *Haemoproteus iwa* affects vector movement in a hippoboscid fly—frigatebird system

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Abstract

Haemosporidian parasites, which require both a vertebrate and invertebrate host, are most commonly studied in the life stages occurring in the vertebrate. However, aspects of the vector's behaviour and biology can have profound effects on parasite dynamics. We explored the effects of a haemosporidian parasite, *Haemoproteus iwa*, on a hippoboscid fly vector, *Olfersia spinifera*. *Olfersia spinifera* is an obligate ectoparasite of the great frigatebird, *Fregata minor*, living among bird feathers for all of its adult life. This study examined the movements of *O. spinifera* between great frigatebird hosts. Movement, or host switching, was inferred by identifying host (frigatebird) microsatellite genotypes from fly bloodmeals that did not match the host from which the fly was collected. Such host switches were analysed using a logistic regression model, and the best-fit model included the *H. iwa* infection status of the fly and the bird host sex. Uninfected flies were more likely to have a bird genotype in their bloodmeal that was different from their current host's genotype (i.e. to have switched hosts) than infected flies. Flies collected from female birds were more likely to have switched hosts than those collected on males. Reduced movement of infected flies suggests that there may be a cost of parasitism for the fly. The effect of host sex is probably driven by differences in the sex ratio of bird hosts available to moving flies.

Keywords: frigatebird, *Haemoproteus*, Hippoboscidae, host switch

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Introduction

Arthropod-vectored diseases can profoundly affect human and wildlife populations. Historically, we have attempted to manage these diseases by focusing our control efforts on the vector, or alternatively, attempting to enhance host resistance (Elliot *et al.* 2003). These approaches inevitably have evolutionary consequences for vectors and hosts, and there is growing interest in understanding evolutionary forces and responses in these systems (e.g. Cohuet *et al.* 2009). In many cases, the invertebrate vector is a far more elusive target of study than the vertebrate host, and laboratory experiments in model systems are often only remotely similar to natural host–parasite or host–pathogen interactions (Tripet *et al.* 2008). This presents challenges to studying these interactions in their ecological and evolutionary contexts. Here we present a study of natural populations of a vertebrate host, the great frigatebird (*Fregata minor*), an invertebrate vector and obligate ectoparasite, the hippoboscid fly *Olfersia spinifera*, and the haemosporidian parasite, *Haemoproteus iwa*, that uses both the fly and the bird to complete its life cycle. One of the features that make this system tractable is the close association between arthropod vector and vertebrate host; *O. spinifera* have fully functional wings but live exclusively among bird feathers for all life stages except the late-instar larval and pupal stages. Therefore, our ability to understand the movement of flies between bird hosts and the subsequent transmission of the haemosporidian parasite is more straightforward than in free-living vector systems (e.g. *Plasmodium*—mosquito—vertebrate host).

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Haemoproteus iwa is a protozoan parasite that is found infecting frigatebirds throughout their tropical distribution (Levin *et al.* 2011). Based on *H. iwa* DNA amplification from *O. spinifera* thorax tissue (site of sporogony, the last developmental stage of the parasite in the invertebrate), we have strong evidence supporting *O. spinifera* as the vector (Levin *et al.* 2011). The fitness consequences of an *H. iwa* infection for frigatebirds are not well understood apart from evidence of immune or stress response as indicated in blood cell differentials (Padilla *et al.* 2006) and correlative evidence showing an association between infection with *H. iwa*, elevated testosterone and a poorer quality sexual ornament (gular pouch colour) important for mate attraction (Madsen *et al.* 2007). The impact of *H. iwa* on the hippoboscid fly vector is even less well understood. It is not surprising that we lack information about the impacts on the vector; after nearly a century of study, the impacts of *Plasmodium* spp. parasites (causal agent of malaria in humans) on their mosquito vectors are unresolved (Ferguson & Read 2002). It has generally been predicted that, along with the potential for higher virulence than in non-vector-borne parasites, vector-borne parasites should be less virulent to the arthropod hosts than to the vertebrate hosts (Ewald 1994). However, there is an alternative prediction stating that selection may act to increase the virulence of the parasite in the vector if that virulence translates to a higher chance of successful infection of another vertebrate host (Elliot *et al.* 2003). Identifying the effects of these parasites on their arthropod hosts is pivotal in advancing understanding of the biology of malaria and for disentangling population-level processes occurring between parasites, vertebrate hosts and arthropod vectors.

The best-studied haemosporidian parasite–vector system is *Plasmodium* spp. parasites and *Anopheles* spp. mosquitoes that cause millions of humans to become sick with malaria each year. There are several mechanisms by which *Plasmodium* parasites can damage the mosquito vector. First, passage of parasites through insect gut epithelia causes physical damage that can increase susceptibility to bacterial infection (Hurd & Carter 2004). In addition, there is evidence of physiological disruption in levels of mosquito digestive enzymes (Jahan *et al.* 1999) and resource depletion in the form of lower concentrations of amino acids (Beier 1998) and higher glucose usage (Hurd *et al.* 1995). Finally, there is evidence that mounting an immune response is costly to the mosquito (Tripet *et al.* 2008) and that some behavioural changes induced by infection, namely increased feeding and probing time, can result in increased risk of detection and consequently death of infected vectors (Ferguson & Read 2002).

This study examines the movements of *O. spinifera* between great frigatebird hosts. Movement, or host switching, is inferred by comparing genotypes at microsatellite loci in vector bloodmeals to those from the host (frigatebird) on which the flies were found. Using the most variable microsatellite markers, we are able to identify host genotypes in bloodmeals that do not match the host from which the fly was collected. These mismatched host and vector bloodmeal genotypes are then analysed in a predictive model incorporating host biological and spatial information and host and vector infection status. We predicted that: (i) if there is an impact of the parasite on the vector, we would expect infected flies to move less, assuming movement is energetically costly to the vector; and (ii) host switching by flies would be more likely in areas of high host density.

Materials and methods

Field sampling

Great frigatebirds (*F. minor*) were opportunistically sampled from five breeding colonies on different islands in the Galapagos archipelago, Ecuador (Darwin, Española, Genovesa, North Seymour, Wolf) in June and/or July of 2007, 2008 and 2010. Breeding adults were captured by hand at or near the nest. A blood sample was collected from the brachial vein and stored at ambient temperature in lysis buffer until DNA extraction. Hippoboscid flies (*O. spinifera*) were collected directly from the birds while sampling and stored in 95% ethanol at ambient temperature in the field and later at −20 °C in the laboratory until DNA extraction. Flies were not affected by pyrethrin-based flea and tick powder (I. Levin, personal observation), and therefore, we were unable to quantify fly infestation rates. A bird's sex was determined based on obvious sexually dimorphic plumage characteristics. Spatial data were collected for each breeding bird sampled using an Opti-Logic 400LH (Tullahoma, TN) laser rangefinder. These spatial measurements included the following: distance from the focal nest to the nearest nest, number of nests within 10 m and the number of neighbouring nests in 10 m that were occupied by conspecifics. Bird–fly pairs were excluded from the study if we lacked spatial information on the host (spatial information was collected only for breeding birds). Because sampled bird hosts were breeding individuals, approximately half were of each sex.

Frigatebird DNA extraction and microsatellite amplification

DNA extraction, PCR techniques used to amplify *H. iwa* parasite DNA and sequencing follow Levin *et al.* (2011). Eight microsatellite markers (Fmin1, Fmin2, Fmin4, Fmin6, Fmin8, Fmin10, Fmin11, Fmin18) described in

Dearborn *et al.* (2008) and used in Levin & Parker (2012a) were used to characterize host genotype. Protocols for microsatellite amplification and fragment analysis follow Levin & Parker (2012a). All individual genotypes were manually scored, 10% of the total samples were repeated across all loci, and approximately one-third of all homozygotes were rerun to ensure that we were not incorrectly assigning genotypes due to allelic dropout.

Fly DNA extraction and microsatellite amplification

In the laboratory, thoraxes of hippoboscid flies were separated from heads and abdomens. A Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, Germantown, MD, USA) was used to extract the DNA from each fly thorax. The standard protocol was followed, but DNA was eluted in half as much buffer due to assumed low concentrations of any parasite or host DNA. Protocols for PCR amplification and sequencing were as described in Levin *et al.* (2011). Fly thoraxes were tested for the presence of *H. iwa*. To ensure that the parasite amplification targeted DNA from sporozoites and not from undigested parasite-infected blood cells that might have persisted in the vector midgut as remnants of a bloodmeal, thoraxes of all flies were tested for bird mitochondrial *cytb* gene with primers and protocols used in Ngo & Kramer (2003). We interpreted the PCR-positive flies as carrying infective sporozoites only when they did not also test positive for bird DNA in the thorax extracts. DNA extractions were also performed on abdomens for bloodmeal analysis following the standard protocol recommended for the Qiagen DNEasy kit referenced above. Four of the frigatebird microsatellite markers described above (Fmin2, Fmin6, Fmin10, Fmin18) were run on either fly thorax (in cases where a fly was not infected with *H. iwa* and bird DNA was detected) or abdomen extracts (in cases where bird DNA was not detected in the thorax) using the same protocols described above. Thoraxes, rather than abdomens, were used in the specified cases due to financial constraints; we already had thorax DNA extractions from a related study. To confirm that we did not get conflicting results from the two different tissues, we extracted DNA from fly abdomens in 10 individuals that had host DNA in the thorax extraction and ran both tissue extractions in the analysis. The four microsatellite primer sets used were the most polymorphic in the bird host and therefore most informative for determining whether the bloodmeal in the fly matched the genotype of the host from which the fly was collected (Fmin6: 9 alleles, Fmin18: 8 alleles, Fmin10: 11 alleles, Fmin2: 17 alleles; see data accessibility accession for allele frequencies per locus). A subset of the flies (n = 10) whose genotype matched their host's genotype were analysed at six or all eight microsatellites to confirm that using the four most polymorphic markers was sufficient for identifying mismatched genotypes. Fly bloodmeal genotypes were scored without knowledge of the bird host genotype, and the data were coded as mismatch if at least one locus had different alleles in bloodmeal vs. host. If three or more alleles were found at any locus or loci (as was the case for some flies that had evidence of recently biting more than one host), we coded a mismatch (a recent host switch), even if there was a match for the host genotype among the 2+ bird genotypes in the fly. For all fly–bird genotype matches, the probability of misassignment (in this case, moving from one bird to another with an identical genotype) was calculated following Jeffreys *et al.* (1992). The average probability, standard deviation and range were calculated for genotype matches identified using four loci (n = 12) and greater than six loci (n = 10).

Logistic regression analysis

Logistic regressions were run using the package *glmulti* (Calcagno & de Mazancourt 2010) implemented in R v.2.14 (http://www.R-project.org). An exhaustive search was run on the seven parameters we postulated that could affect movement of vectors between individual hosts: island, infection status of the vector, infection status of the bird host, bird host sex, distance to the nearest nest, the number of nests within 10 m and the proportion of nests within 10 m that were conspecific. An additional model was tested using the parameters listed above and the interaction between bird host sex and fly infection status. We used the Akaike information criterion (AICc) (Akaike 1974) for model selection and Wald's tests to evaluate the significance of the parameters in the best model. To assess the goodness-of-fit of the best model, we ran a modified Hosmer–Lemeshow test in R using the package LDdiag (http://cran.r-project.org/src/contrib/Archive/LDdiag/).

Results

Of the 59 bird host–fly vector pairs analysed, 28 of the host birds were female and 31 were male. Samples per island ranged from two host–vector pairs from the island of Wolf to 21 pairs from North Seymour. Twenty-four of the 59 flies (41%) were infected with *H. iwa*, while prevalence in the frigatebird hosts was 33/59 (56%). In accordance with a larger study of *H. iwa* prevalence in great frigatebirds that reported 41% prevalence in adult females and 55% prevalence in adult males (Levin & Parker 2012b), male frigatebirds in our sample were more frequently parasitized by

Table 1 Best-fit logistic regression model as determined by the program *glmulti* run in R v. 2.14. Parameters used in the model search: Island, infection status of the vector, infection status of the bird host, bird host sex, distance to the nearest nest, the number of nests within and 10 m, and the proportion of nests within 10 m and that were occupied by conspecific individuals

	Outcome: fly (*Olfersia spinifera*) bloodmeal matches/mismatches bird host (*Fregata minor*) genotype				
Predictor	β (coefficients)	SE β	Wald's z-value	d.f.	*P*
Fly infection status	1.9919	0.6872	2.899	56	0.00375
Host sex	−2.2068	0.7275	−3.033	56	0.00242
Null model	0.7625	0.6246	1.221	58	0.22219

H. iwa (21/31) than females (12/28). Thirteen of the 24 infected flies were on infected males, while only two infected flies were on infected females. Thirty-seven of the 59 fly vectors (63%) had bird microsatellite genotypes that did not match the host they were collected from. Complete agreement was found between genotypes amplified from flies that had both abdomen and thorax tissue tested. More than one host genotype was unambiguously identified by three or more alleles at one or more loci in six of the 59 flies. The probability of misassignment calculated for fly bloodmeal–host genotype matches was low (For matches based on four loci: mean = 6.8×10^{-6}, standard deviation = 1.3×10^{-5}, range = 1.8×10^{-7} to 4.7×10^{-5}; for matches based on >6 loci: mean = 1.3×10^{-8}, standard deviation = 1.5×10^{-8}, range = 8.7×10^{-11} to 3.3×10^{-8}).

The best logistic regression model explaining host switching (determined by AICc values and residual deviances) included the infection status of the fly and the bird host sex (Table 1). Uninfected flies are more likely to have a bird genotype in their bloodmeal that was different from their current host's genotype (i.e. to have switched hosts) than infected flies (Fig. 1). Flies collected from female birds were more likely to have switched hosts than those collected on males (Fig. 2). A modified Hosmer–Lemershow test showed no evidence for a lack of fit with this model ($P = 0.57$). A Wald's chi-square test indicated that the z-scores for both fly infection status and host sex coefficients were significant (Table 1) and that this logistic regression model including both fly infection status and bird host sex demonstrated a better fit to the data based on significant improvement over the null (intercept-only) model. The model search that also included the interaction between bird host sex and fly infection status produced the same best model as before, including only bird host sex and fly infection status. The best model did not include any of the measured spatial parameters.

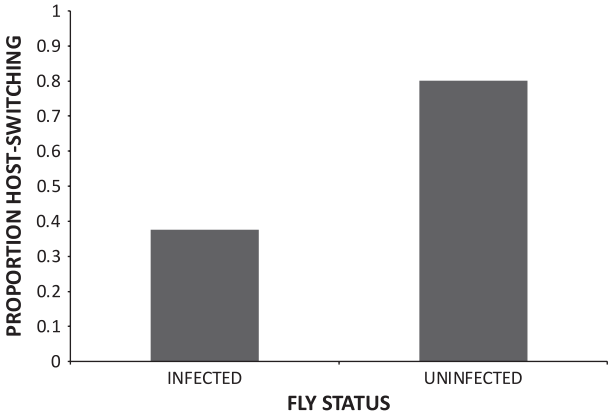

Fig. 1 Proportion of host switching *Olfersia spinifera* hippoboscid flies based on fly *Haemoproteus iwa* infection status. Host switching was determined by comparing frigatebird (host) microsatellite genotypes in the fly's bloodmeal to the genotype of the frigatebird from which the fly was collected.

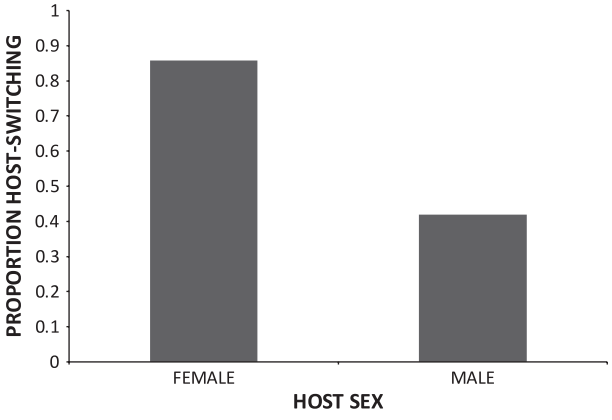

Fig. 2 Proportion of host switching *Olfersia spinferia* hippoboscid flies based on frigatebird host sex. Host switching was determined by comparing frigatebird (host) microsatellite genotypes in the fly's bloodmeal to the genotype of the frigatebird from which the fly was collected.

Discussion

The proportion of flies that had recently switched hosts was relatively high (37/59 or 62.7%). Previously, our only method of detecting potential host switches was the occurrence of an infected fly on an uninfected bird (13/105 cases), which we acknowledged as an estimate

of the lower bound of fly movement (Levin & Parker 2012b). This approach using polymorphic, bird-specific, genetic markers is far more precise and provides more information about the recent movement of this vector. There were at least six cases of multiple host genotypes detected in flies, and in one case, we were able to identify at least three bird genotypes in one fly. If fly movements between hosts are this frequent, it begs the question: why are some birds not infected with *H. iwa*? We argue that this could be a function of reduced movement by infected flies. However, one must keep in mind that the disparity between vector and host infection rate could be due to unidentified infections. The thorax is the most useful tissue for parasite detection in the vector because one can rule out digesting bloodmeals by the lack of bird DNA in the tissue extraction. Because salivary glands likely extend into the abdomen, we could potentially miss infections. Additionally, some flies in the population could be very young, and there may not have been sufficient time for the parasite to reach the sporozoite phase in the fly. Finally, very infected flies could be dying and therefore are not present in our sample.

Although the sample size is small, our results reveal a striking pattern in recent vector movement: infected flies were significantly more likely to have bloodmeals that matched the genotype of their current host than uninfected flies. Uninfected flies were more likely to have recently been on another bird host, indicating that they are more mobile. This suggests that there may be a cost of parasitism for the fly. From the parasite's perspective, an infected vector that is less likely to move may be problematic; however, we do document cases of recent movement of infected flies in nine of 24 cases. It is possible that the benefits to the parasite from the processes that result in reduced vector movement (e.g. replication of the parasite in vector tissue causing tissue damage and resource depletion) outweigh the cost of reduced connectivity between bird host individuals. In other words, selection may be acting to increase the virulence of the parasite in the vector if that virulence translates to a higher chance of successful infection of another vertebrate host. This contradicts the usual prediction of selective advantage in vectors less affected by infection (Cohuet *et al.* 2009), although whether these predictions of lower virulence to vectors have any empirical basis has been questioned (Elliot *et al.* 2003).

Recent work in a captive rock pigeon (*Columba livia*), hippoboscid fly (*Pseudolynchia canariensis*) and *H. columbae* system has demonstrated a fitness cost of *Haemoproteus* infection for female flies (Waite *et al.* 2012). Female *P. canariensis* that fed on *H. columbae*-infected pigeons had lower survivorship and produced fewer offspring than females that fed on uninfected hosts (Waite *et al.* 2012). Similar studies of *Anopheles* mosquitoes focus on the effects of *Plasmodium* on fecundity and survival, as both, especially survival, are expected to have large impacts on *Plasmodium* transmission. Because mosquitoes are free-living vectors, it is hard to compare the effects of parasitism on mosquito vector movement to that of our obligate ectoparasite. It has been established that *Plasmodium*-infected mosquitoes have a higher biting rate, presumably due to the high number of parasites in the vector that disturb the efficacy of blood feeding (Rossignol *et al.* 1984; Anderson *et al.* 1999). Infected mosquitoes were found to have less of a particular platelet inhibitor than uninfected mosquitoes, causing them to spend more time feeding (Simonetti 1996). If similar mechanisms are at work in our *Haemoproteus*–fly–bird system, we might predict that an infected fly could be reluctant to leave a host if it must feed at a higher rate. There is little information on the feeding rate of Hippoboscid flies, other than in *Crataerina pallida*, the obligate parasite of common swifts (*Apus apus*) that feed once every 5 days (Walker & Rotherham 2011). However, a feeding experiment in pigeons, *P. canariensis*, and *H. columbae* demonstrated that infection of *P. canariensis* with the parasite did not affect bloodmeal size (Waite *et al.* 2012). Female *P. canariensis* ingested larger bloodmeals than males and survived longer off the host (Waite *et al.* 2012).

Alternatively, the reduction in movement of infected flies could be mediated by the infection status of the host. Infected flies are most often found on infected birds (Levin & Parker 2012b), and *H. iwa* could also affect the health of the bird host. If infected birds preen less, this could make the host susceptible—and even attractive—to ectoparasites that can otherwise be removed via preening behaviour. There is some support for this hypothesis; Yorinks & Atkinson (2000) found that captive juvenile apapane (*Himatione sanguinea*) experimentally infected with *Plasmodium relictum* spent less time preening. We have little evidence for the effects of *H. iwa* on frigatebird hosts other than elevated heterophil-to-lymphocyte concentration ratios in infected great frigatebirds, indicating physiological stress and/or active response to infection (Padilla *et al.* 2006).

Another alternative, or associated, interpretation of reduced movement in *Haemoproteus*-infected flies is that it is a result of manipulation of the vector by the parasite. Host manipulation by parasites is well documented, and the altered host behaviour typically facilitates transmission of the parasite (Poulin 2010). As mentioned above, increased biting rate is a common form of manipulation that has been documented in several different species of infected haematophagous vectors (reviewed in Lefèvre & Thomas 2008). Activity

levels have also been shown to increase in infected vectors (e.g. intrathoracic Dengue 2 virus infected *Aedes aegypti* mosquitoes, Lima-Camara *et al.* 2011; *Ixodes persulcatus* infected with tick-borne encephalitis, Alekseev 1996). Reductions in locomotion, such as we document, have only rarely been shown, and potential explanations are not well explored (*I. persulcatus* infected with *Borrelia burgdorferi*, Alekseev 1996; *Trypanosoma rangeli*-infected *Rhodnius prolixus*, D'Alessandro & Mandel 1969). The difficulty is separating whether this apparent manipulation is adaptive or whether it is simply a by-product of pathology (Poulin 2010). The reduction in movement in *H. iwa*-infected flies observed here could facilitate transmission of the blood parasite if, by slowing down the fly, the parasite can pass through the vertebrate life stages and reinoculate the fly with more parasites for further transmission. This novel explanation has not been posited before; the only adaptive explanation of reduced movement in infected vectors involves facilitation of transmission through ingestion by an alternative host (Edman & Scott 1987; Alekseev 1996).

The other clear pattern we observed is the effect of bird host sex on the probability that the fly bloodmeal genotype matches that of its bird host. This could be driven by differences in the sex of bird hosts available to moving flies. During the breeding season (the time of sampling), female frigatebirds bear proportionally more of the reproductive effort as measured by time spent incubating the egg (Dearborn *et al.* 2001). Great frigatebirds on Tern Island in Hawaii spent, on average, 10 more of 57 days incubating the egg than males, and there is strong evidence that, when not incubating, the other member of the pair is not present in the colony (Dearborn *et al.* 2001). This translates to breeding females spending approximately 18% more time in the colony than breeding males and therefore being the more likely recipients of flies moving between individuals.

Using host-specific microsatellite markers on vector bloodmeals has proved to be a novel way to analyse recent vector movement. At this time, we cannot separate whether the reduction in movement by *Haemoproteus*-infected flies is a consequence of the pathology of infection or whether this is a case of parasitic manipulation of the vector, and this remains a challenge for future studies. This approach provides a wealth of information in our system where the vector is a host-specific, obligate parasite. Furthermore, it highlights *Haemoproteus* parasites and their hippoboscid fly and bird hosts as an ideal system to study host–parasite interactions, particularly for investigating the impacts of the haemosporidian parasite on the vector. Decades of laboratory research on mosquito–*Plasmodium* model systems have emphasized how specifically and intimately mosquito and parasite traits co-evolve and how context-dependent the outcomes can be (Tripet *et al.* 2008). Together, these highlight the need to work with these parasite–vector–host systems in natural settings.

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I.I.L. and P.G.P. designed the research, and I.I.L. collected the samples and performed the molecular analyses. I.I.L. analysed the data with input from P.G.P. I.I.L. wrote the paper with substantial input from P.G.P.

Data accessibility

Logistic regression data set for fly (*Olfersia spinifera*) bloodmeals and host (*Fregata minor*) genotype comparison, R code, great frigatebird (*F. minor*) allele frequencies used to calculate probability of misassignment, calculations of probability of misassignment: doi:10.5061/dryad.1d64r.