Animal Behaviour 84 (2012) 539–545

Contents lists available at SciVerse ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Potential evidence of parasite avoidance in an avian malarial vector

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ARTICLE INFO

Article history:
Received 1 November 2011
Initial acceptance 24 January 2012
Final acceptance 24 May 2012
Available online 4 July 2012
MS. number: 11-00885R

Keywords:
avian malaria
Culex pipiens
dual-choice olfactometer
great tit
host choice
mosquito
Parus major
vector-borne disease

Epidemiological studies of malaria or other vector-transmitted diseases often consider vectors as passive actors in the complex life cycle of the parasites, assuming that vector populations are homogeneous and vertebrate hosts are equally susceptible to being infected during their lifetime. However, some studies based on both human and rodent malaria systems found that mosquito vectors preferentially selected infected vertebrate hosts. This subject has been scarcely investigated in avian malaria models and even less in wild animals using natural host–parasite associations. We investigated whether the malaria infection status of wild great tits, *Parus major*, played a role in host selection by the mosquito vector *Culex pipiens*. Pairs of infected and uninfected birds were tested in a dual-choice olfactometer to assess their attractiveness to the mosquitoes. *Plasmodium*-infected birds attracted significantly fewer mosquitoes than the uninfected ones, which suggest that avian malaria parasites alter hosts' odours involved in vector orientation. Reaction time of the mosquitoes, that is, the time taken to select a host, and activation of mosquitoes, defined as the proportion of individuals flying towards one of the hosts, were not affected by the bird's infection status. The importance of these behavioural responses for the vector is discussed in light of recent advances in related or similar model systems.

Parasites are ubiquitous organisms and by definition impose fitness costs on their hosts. As a consequence, hosts have evolved a range of antiparasite defences. Together with the immune system (Wakelin 1996), behavioural responses are the most efficient defences that hosts have evolved to fend off the detrimental effects of parasites. Detecting and avoiding parasites is the first line of defence that has been proven efficient at curbing parasite spread. For example, birds have been found to avoid nest sites infected by fleas (Christe et al. 1994) and herbivores such as antelope (Ezenwa 2004) or kangaroos (Garnick et al. 2010) are known to select grass patches for grazing so as to minimize the risk of being infected by gastrointestinal parasites associated with faecal contamination. Hygienic behaviour (Arathi et al. 2006) and the incorporation of nest material that contains substances with antiparasite properties are other behavioural adaptations that animals have evolved to help prevent disease (Petit et al. 2002; Christe et al. 2003; Hart 2005; Castella et al. 2008). Behavioural defences against parasitism have the advantage of providing fast responses to environmental modifications. For example, Hawaiian birds have modified their behaviour in response to selection pressure imposed by the introduction of mosquito vectors and malaria parasites on the islands (van Riper III et al. 1986). It was shown that in a relatively short period of time some bird species had modified their sleeping postures to avoid being bitten by mosquitoes and had changed their daily movements between foraging and sleeping areas in order to minimize their temporal contact with malarial vectors (van Riper III et al. 1986).

Malaria parasites, sensu Valkiūnas (2005), belong to the genus *Plasmodium* (Apicomplexa: Haemosporidae) and represent a highly diversified, monophyletic group of blood protozoan parasites that can be divided into two well-supported clades, one containing parasites of mammals and the other parasites of reptiles and birds (Bensch et al. 2004; Martinsen et al. 2008; Witsenburg et al. 2012). These parasites exploit a large spectrum of host species that have a broad range of ecological niches, resulting in a world-wide distribution (Garnham 1966). Besides the requirement of a suitable vertebrate host species to multiply asexually, the *Plasmodium* parasite's life cycle involves haematophagous dipteran insects for both parasite transmission and sexual reproduction (Garnham 1966). Mammal-specific *Plasmodium* parasites are well known and have evolved to specialize within *Anopheles* mosquitoes (Garnham 1966; Martinsen et al. 2008). On the other hand, identification of vectors and their role in the epidemiology of avian malaria in the wild is poorly known. Some mosquito species are competent vectors

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0003-3472/$38.00
http://dx.doi.org/10.1016/j.anbehav.2012.06.004

in laboratory conditions (Huff 1965) but their vectorial capacity (i.e. the efficiency with which the vector transmits the parasite in natural conditions) has not been demonstrated or is based on a PCR detection of the parasite in the mosquito's body (Massey et al. 2007; Ishtiaq et al. 2008; Ejiri et al. 2009; Glaizot et al. 2012). However, only the presence of the infective stage of the parasite (sporozoites in the salivary glands) clearly demonstrates the competence and vectorial capacity of a given species (LaPointe et al. 2012).

Epidemiological studies or models of malarial disease first considered vectors as passive parasite transmitters (MacDonald 1957; Garrett-Jones 1964), with each host having the same probability of being infected (but see Kingsolver 1987; Kelly & Thompson 2000; Smith et al. 2007). However, several studies have challenged this assumption and have shown that hosts are not equally attractive to vectors (Kelly 2001). First, vectors may preferentially select to feed on a given species (Lord & Day 2000; Williams et al. 2003; Lefèvre et al. 2009; Simpson et al. 2009), depending on the diversity and the relative abundance of available host species (Lyimo & Ferguson 2009). Second, individuals of the same host species may represent different levels of attractiveness to the vectors. For instance, humans vary in their intrinsic attractiveness to mosquitoes (Knols et al. 1995; Mukabana et al. 2002; Smallegange et al. 2011) with pregnant women (Lindsay et al. 2000) or beer consumers (Lefèvre et al. 2010) being more attractive than other individuals. By increasing the attractiveness of an intermediate vertebrate host the parasite can improve its transmission to the vector. This can be regarded as a manifestation of the extended phenotype of the parasite (Dawkins 1982) and has been applied to parasites with trophic transmission (Moore 2002; Cezilly et al. 2010) as well as vector-borne diseases (Hurd 2003; Nacher 2005; Adedolapo & Olajumoke 2008). In this way, parasites may gain in terms of reproductive success by manipulating host attractiveness to the vectors. Even though *Plasmodium* parasites have an interest in keeping their vector alive until transmission to their vertebrate host has occurred, they may not accomplish their cycle without any collateral damage to the vector (Beier 1998; Ferguson & Read 2002; Hurd 2003; Lefèvre & Thomas 2008). Vectors may thus have evolved the ability to discriminate between infected and parasitized hosts to avoid being parasitized (Freier & Friedman 1976; Tomás et al. 2008; Martinez-de la Puente et al. 2009). Nonrandom host-feeding behaviour by the vector may therefore be at the heart of a conflict of interest between *Plasmodium* parasites and their vectors and may strongly affect the transmission dynamic of the disease (Dye & Hasibeder 1986; Kingsolver 1987; Dye 1992; Smith et al. 2007) as well as vector fitness (Lyimo & Ferguson 2009).

This study focused on a temperate avian malaria system involving great tit, *Parus major*, hosts naturally infected with malarial parasites, *Plasmodium* spp. (Richner et al. 1995; Oppliger et al. 1996, 1997; Christe et al. 2012) vectored by the ornithophilic mosquito *Culex pipiens* (Glaizot et al. 2012). The objective was to determine whether *Plasmodium* infection of great tits affects their attractiveness to wild *C. pipiens*. Host-seeking vectors were given the opportunity to orient themselves towards their preferred host in a dual-choice olfactometer baited with malaria-infected and uninfected birds. Attractiveness of the bird hosts and activation and reaction time of the mosquitoes were measured.

METHODS

General Procedure and Study Sites

The study took place in western Switzerland. Thirteen wild great tits were mist-netted in two forests situated 30 km apart (Dorigny: 46°31′N, 6°34′E; altitude: 400 m; La Praz: 46°66′N, 6°43′E; altitude: 871 m). Each bird was individually marked with a metallic ring, weighed to the nearest 0.1 g, sexed, aged (1 year old or older than 1 year based on plumage criteria) and blood sampled for later assessment of their malaria infection status (see below). Birds were then transferred to a mosquito-free animal room (20 °C, 50% relative humidity and a 16:8 h light:dark cycle) on the campus of the University of Lausanne in Dorigny, individually housed in aviaries (1×1 m and 2 m high) and provided with ad libitum access to water, mealworms and commercial seed for insectivorous passerines (Peddy seeds, Rolli-pet Tiernahrung GmbH, Hargelsberg, Germany). At the end of the experiment, individuals were blood sampled a second time to reassess their infection status.

Culex pipiens mosquitoes were collected as egg rafts in rainfall-collecting containers baited with live yeast and installed within the great tit population site at Dorigny. Containers were checked for egg rafts one to three times per week. Freshly laid eggs were transferred to the laboratory (25 °C, 70% relative humidity and a 14:10 h light:dark cycle) and allowed to hatch in plastic trays filled with 1.5 litres of spring water. Larvae were fed ad libitum with commercial fish flakes until pupation; pupae were then isolated in screened cages ($30 \times 30 \times 30$ cm) for adult emergence. Since *C. pipiens* are unable to mate in confined spaces (Vinogradova 2000), adult males and females, which had emerged within the same 72 h period, were transferred to bigger screened cages (30×30 cm and 90 cm high) to allow for vertical nuptial flights. A sex ratio of seven males to five females was maintained for 12–16 days (mean $\pm$ SD $= 15 \pm 1.3$) for mating and fed with a fresh 10% glucose solution renewed every 3 days to prevent fungi formation.

Ethical Note

Birds used in these experiments were mist-netted between 25 June and 12 July 2010 after the breeding season. They were carefully transferred from the field to the laboratory in muslin bird bags and travel time did not exceed 45 min. Individuals were kept in captivity for 8–28 days (average 16.4 days), including a period of 5–9 days after the last experiment, to ensure that birds were in good health before being released at their capture site.

Blood samples of 20 µl were collected into lithium-heparinized microvettes (Microvettes CB 300, Sarstedt, Germany) by puncturing the brachial vein with a sterile needle (Neolus 100, Terumo Europe, Heverlee, Belgium).

Bird captures and ringing were performed under licence number F044-0799 of the Swiss Federal Office for the Environment. Experiments with wild great tits were approved by the Veterinary service of the Canton de Vaud, licence number 1730.1.

Molecular Identification of Plasmodium *spp. from Birds*

Genomic DNA was extracted from the blood samples using a Qiagen BioSprint 96 workstation following the manufacturer's protocol (Qiagen, Hilden, Germany). Samples were screened for *Plasmodium* spp. infection using the nested PCR method refined by Waldenström et al. (2004) from the original protocol made by Bensch et al. (2000). Birds were deemed as malaria infected when a final PCR product of about 525 bp including primers was obtained. Stage of infection (chronic or acute) was not determined with this nonquantitative method. Positive controls were included in all PCR assays. Negative controls (purified PCR-grade water) were included every three to four samples. Reactions were run on a Biometra thermocycler (Biometra, Göttingen, Germany).

Olfactometer Set-up

Mosquito experiments took place in an olfactometer, consisting of a Y-shaped wind tunnel, as recommended by Besansky et al. (2004) and built with some modifications from the designs of Geier et al.