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THE ROLE OF BAT FLIES (NYCTERIBIIDAE) IN THE ECOLOGY AND EVOLUTION OF THE BLOOD PARASITE POLYCHROMOPHILUS (APICOMPLEXA: HAEMOSPORIDA)

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pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Grégoire Millet

voor Opa en Oma,
die me hebben moeten missen

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RÉSUMÉ

La coévolution est l'évolution réciproque de deux organismes en interaction. Au sein d'une association hôte-parasite, ce sont ces interactions qui modèlent l'histoire de vie du parasite. Les parasites hétéroxènes requièrent deux hôtes pour compléter leur cycle de vie. Un fameux exemple de parasite hétéroxène est celui des parasites responsables de la malaria qui requièrent un vecteur diptère et un hôte vertébré. L'écologie évolutive des parasites responsables de la malaria est de ce fait déterminée par la biologie de l'hôte ainsi que celle du vecteur et peut être vue comme le produit de l'évolution d'une interaction triple. *Polychromophilus* spp. (Apicomplexa: Haemosporidae) sont des parasites responsables de la malaria qui utilisent de manière spécifique les chauves-souris comme hôte vertébré, et sont transmis par un ectoparasite, la nyctéribie (Diptera: Nycteribiidae). Cette thèse décrit l'écologie et l'évolution de *Polychromophilus* spp. et comment leur coévolution avec les nyctéribies a modelé ces processus.

Pour trouver l'origine évolutive de *Polychromophilus*, j'ai construit une analyse phylogénétique de l'Ordre des Haemosporidae, basée sur trois gènes. *Polychromophilus* est placé près de la base du clade des *Plasmodium* de sauropsidés. Cela suggère que *Polychromophilus* représente un second et indépendant évènement d'invasion des mammifères par un parasite Haemosporidae. Le changement de vecteur pour les Nycteribiidae a dû se produire après la spécialisation du parasite pour les chauves-souris.

La dispersion d'un parasite hétéroxène est supposée dépendre uniquement de son hôte le plus mobile. En utilisant des analyses microsatellites, je démontre que le vecteur nyctéribie a un niveau de dispersion à travers l'Europe supérieur à l'hôte chauve-souris. Cependant, après comparaison des distances génétiques par paire de l'ADN mitochondrial, ni le vecteur ni l'hôte ne corrèlent significativement avec le parasite. De ce fait, la structure de population d'un parasite transmis par un vecteur ne reflète pas simplement la structure de population de son hôte le plus mobile. La

distribution d'haplotypes du parasite suggère plutôt des effets fondateurs du parasite, un haut renouvellement des parasites ou une hétérogénéité du taux de dispersion des hôtes.

Les comportements et dynamiques du parasite, du vecteur et de l'hôte au sein d'une seule population sont déterminés par diverses interactions entre les trois acteurs. Des expériences de choix d'hôte et de survie montrent que les nyctéribies augmentent leur survie en se nourrissant préférentiellement sur des chauves-souris dont la parasitémie est plus faible. Néanmoins, la distribution naturelle des vecteurs au sein des hôtes est indépendante de la parasitémie de ces derniers. Bien que l'infection par *Polychromophilus* est liée à de plus faibles conditions corporelles chez les chauves–souris adultes, les effets pathologiques de l'infection restent méconnus, peut-être parce que les plus lourdes infections se retrouvent chez les jeunes.

Cette thèse démontre comment les Nycteribiidae influencent l'écologie évolutive de *Polychromophilus*. Cependant, la complexité des niveaux d'interaction, non seulement entre le parasite et ses deux hôtes, mais aussi entre les chauves-souris et leurs ectoparasites rendent difficile les prédictions sur l'épidémiologie de ce parasite.

SUMMARY

Coevolution is the reciprocal evolution of two interacting organisms. In a parasite-host association it is these interactions that shape the life history of the parasite. Heteroxenous parasites require two hosts to complete their life cycle. Malaria parasites are well known heteroxenous parasites which require a dipteran vector and vertebrate host to complete their life cycle. The evolutionary ecology of a malaria parasite is therefore determined by both host and vector biology and can be seen as the evolutionary product of this three-way interaction. *Polychromophilus* spp. (Apicomplexa: Haemosporida) are malaria parasites that have specialized on bats as hosts and are transmitted by an ectoparasite, the bat fly (Diptera: Nycteribiidae). This thesis describes the ecology and evolution of *Polychromophilus* spp. and how their coevolution with bat flies has shaped these processes.

To find the evolutionary origin of Polychromophilus, I construct a phylogenetic analysis of the order of Haemosporida, based on three genes. *Polychromophilus* is placed near the base of the sauropsid *Plasmodium* clade. This suggests that *Polychromophilus* represents a second, independent, invasion of mammals by a haemosporidian parasite. The vector switch to Nycteribiidae must have come after the parasite's move into bats.

The dispersal of a heteroxenous parasite is predicted to only depend on its most mobile host. Using microsatellite analyses I demonstrate that the bat fly vector has higher levels of dispersal through Europe than the bat host. However, when comparing mtDNA pairwise genetic distances, neither vector nor host correlate significantly with the malaria parasite. The population structure of a vector-transmitted parasite therefore does not simply reflect that of its most mobile host. The parasite haplotype distribution rather suggests parasite founder effects, high turnover of parasites or heterogeneity in host dispersal rates.

The behaviours and dynamics of parasite, vector and host within a single population are determined by many interactions among the three actors. A host-choice and survival experiment

reveals that bat flies increase their survival by preferentially feeding on bats with the lowest parasitemia. Yet the natural distribution of bat flies among hosts is independent of the host's parasitemia. Though *Polychromophilus* infection is linked to a lower body condition in adult bats, pathological effects of infection remain unclear, possibly since the heaviest infections occur in the young.

This thesis demonstrates how the Nycteribiidae influence the evolutionary ecology of *Polychromophilus*. However, the many higher-level interactions, not only between the parasite and its two hosts, but also between the bat host and its ectoparasitic vector make predicting its epidemiology still a challenge.

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Chapter 1

Introduction and background

COEVOLUTION

Evolution is the change in the inherited characteristics of populations over successive generations (Ridley 2004). A large part of these changes – though how large remains a matter of debate (Nei 2005) – can be attributed to nothing more than the random fluctuation of neutral genetic variation (i.e. drift). However, many characteristics are a response to external pressures. In this age where global warming is a hot topic, it is easy to think that these pressures consist purely of abiotic environmental conditions, e.g. temperature, rainfall, soil type. But the arguably larger part of an organism's environment consists of other organisms. These biotic external pressures cause evolutionary change in a focal species yet the pressures themselves may evolve as well in response to the focal's evolution.

Whenever two (or more) parties exert a selective pressure on each other, and thereby affect each other's evolution, coevolution is at play (Janzen 1980). It is important to note that not all types of evolutionary change triggered by other organisms automatically concern coevolution. Critical for coevolution is that both parties respond to each other's changes. Mimicry, for example, occurs when one species mimics the visual cues of another, often an unpalatable species. Any change in the appearance of the unpalatable species will induce evolutionary change in the mimicking species, yet the opposite is not true; the phenotype of the mimicking species will exert little evolutionary pressure on the original species (Schaefer and Ruxton 2009). These two species are therefore not coevolving. By contrast, the relationship between a species and its herbivore is of a coevolutionary nature; a change in phenotype of the plant induces adaptations in the herbivore, which in turn drives again the evolution of even further adaptations in the plant (Ehrlich and Raven 1964). One of the more spectacular examples of coevolution which does involve mimicry is the structures resembling *Heliconius* butterfly eggs grown by *Passiflora* spp. on their stems and leaves. *Passiflora* vines have chemical defences that deter most insects, but the *Heliconius* butterflies are one the few insects that have developed resistance against these metabolites. In response several *Passiflora* have, independently from each other, evolved structures resembling butterfly eggs. The butterflies, attempting to keep competition and cannibalism to a minimum for their caterpillars, refrain from laying their own eggs on these plants (Figure 1.1; Williams and Gilbert 1981).

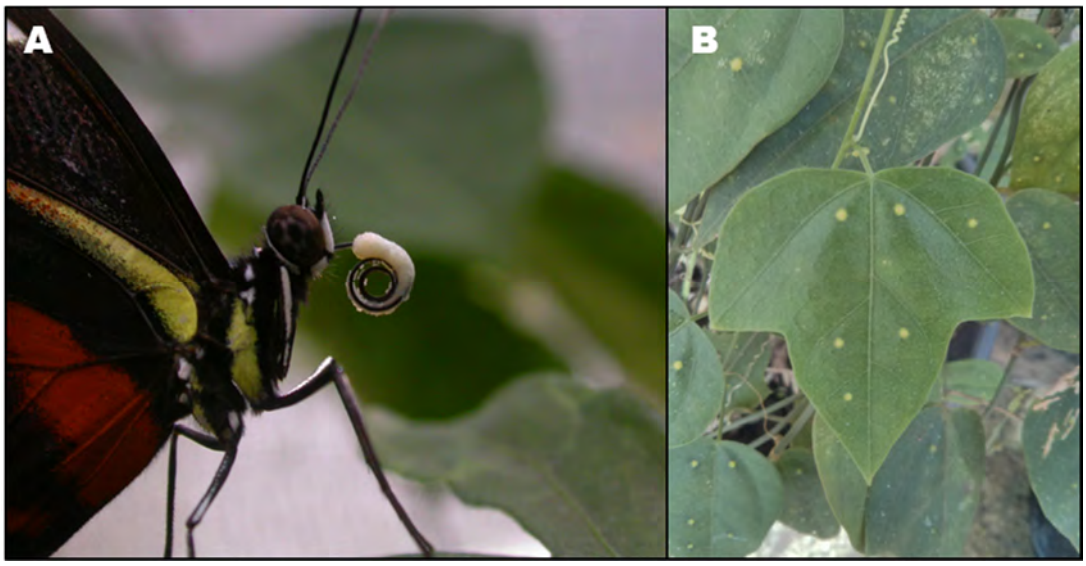

Figure 1.1 An example of coevolution: a herbivore, the *Heliconius* butterfly (A) can deal with the *Passiflora* metabolites, but is deterred from egg laying by the plant's egg-like structures (B).

Such arms races, where each party is trying to gain an edge on its counterpart, are a result of antagonistic coevolution. Predation, herbivory, competition and parasitism are all ecological processes that have the potential to induce antagonistic coevolution. The contrary, cooperative symbioses, may similarly cause coevolution. In these cases each partner develops ever more fine-tuned tools in response to the others needs and products, the result of which can be seen in certain pollination, nutrient exchange or defence symbioses. Despite the appeal of coevolution as a force, it is worth noting not all ecological relationships in which the parties have correlated characters, be they positive or negative, imply coevolution (Janzen 1980). Many other processes can result in correlated phenotypes, such as the non-reciprocal evolutionary adaptations of a species to another, or adaptations of both species to shared abiotic factors (Nuismer et al. 2010).

When many species interact, the kind of interaction between any two species may depend on other species in the community. For 'direct' coevolution to occur, the interaction and its outcome of any two populations are required to be genetically independent of other populations in the community. Because these types of isolated interactions are thought to be rare, most coevolution is instead considered 'diffuse', preventing any direct correlation of traits among species (Iwao and Rausher 1997).

Coevolution of parasites and host

Of all ecological processes, parasitism provides arguably the best conditions for coevolution. By definition, a parasite reduces the fitness of its host, and exerts therefore selective pressure on the host. In return, the host represents the parasite's source of energy as well as its hostile environment to which the parasite needs to adapt. Selection pressures thus go both ways in a parasite-host association, the main requirement for coevolution. Though parasites can be generalists, and hosts can be harbouring multiple parasites simultaneously, the possibilities to observe direct coevolutionary interactions – as opposed to diffuse coevolution – between specialist parasites and hosts are relatively abundant.

Parasites have evolved multiple times independently from non-parasitic ancestors and are therefore ubiquitous throughout the tree of life (Poulin 2007). Conservative estimates suggest that ~30% of current eukaryote species are parasitic at some stage in life (de Meeus and Renaud 2002). Moreover, though most bacterium species are considered non-pathogenic, when introduced into the wrong environment many will take advantage of the situation and reduce the host's fitness (Berg et al. 2005). Especially when viruses are taken into account, the number of opportunities to study parasite-host coevolution can be considered exorbitant.

The first study to mention coevolution was on herbivory (Ehrlich and Raven 1964) yet most early experimental studies on coevolution have focused on parasite-host interaction. Since parasites tend to be small and have short generation times they are ideal to keep in large numbers for many generations in the laboratory (Brockhurst and Koskella 2013). When studying parasite-host coevolution of larger, more slowly reproducing organisms, research necessarily turns more observational in nature. By using tools to either 'read' evolutionary history of both agents, or looking for correlated traits in natural experiments, one can identify causes and effects of coevolution.

Cophylogenies provide a tool to observe historical cospeciation events, indicated by identical branch splits in the host and parasite phylogenies. Moreover, cophylogenies also identify where coevolution broke down and hosts lost their parasites or parasites moved to a new host. Initial studies merely described the observed pattern of cospeciation (e.g.Paterson and Poulin 1999), but later studies also identified possible causes of cospeciation like habitat choice or migration (e.g. Bruyndonckx et al. 2009a, Jenkins et al. 2011).

Some species of parasites fail to speciate with each of their hosts and instead employ a generalist's strategy, infecting multiple host species at the same time and place. Interestingly, differentiation might actually still be occurring at the local scale. *Ixodes uriae* hard ticks feeding on oceanic birds demonstrated clear host-race formation, even though the different bird species shared the several oceanic islands, hundreds of miles apart (McCoy *et al.* 2005). On the other side, local differentiation does not necessarily imply local adaptation. The *Heliginosomoides* helminth parasites of *Apodemus* field mice showed clear patterns of differentiation through Europe. Yet this was not an adaptation to the local host species, but merely an effect of drift and isolation between the parasite populations (Nieberding et al. 2008).

The ecological effects of parasitism can be seen in the population dynamics of hosts. In one of the more spectacular examples, Hudson et al. (Hudson et al. 1998) interrupted the regular grouse population cycles by treating them against a prevalent nematode. By removing the nematode, host fecundity was no longer inhibited and a population crash was prevented. The population dynamics of a parasite are rather expressed in the epidemiological terms of prevalence (ratio of infected hosts), abundance (mean number of parasites on any host) and intensity (mean number of parasites on infected hosts; Rozsa et al. 2000). Parasite prevalence and abundance may fluctuate over the season (e.g. Locklin and Vodopich 2010), surviving the winter in low population numbers and showing a peak during the warm season, often timed with host peak reproduction when female hosts are immunocompromised and naïve newborns are available (e.g. Christe et al. 2000, Van Kuren et al. 2013).

Besides speciation, population differentiation and population dynamics, many behavioural traits of both parasite and host have been the result of their shared coevolution. As with important epidemiological parameters, such as virulence and resistance, these traits can be considered the 'combined phenotype' of parasite and host (after the 'extended phenotype'). However, as with all traits, the environment codetermines the phenotype and therefore all the before mentioned processes can only be fully understood once we know the host genotype x parasite genotype x environment three-way interaction (Lambrechts et al. 2006).

Using a second host

A heteroxenous parasite, or a parasite with a complex life cycle, is a parasite that needs a minimum of two hosts to complete its life cycle. Even though not all definitions require the second host to be of a different species (compare Clayton and Moore 1997, Poulin 2007), I could not find a single example where a parasite requires invasion of two individuals of the same species for it to reach maturity. The definitive host is defined as the host where sexual reproduction takes place (if any), whereas other hosts that it may parasitize before are intermediate hosts (Clayton and Moore 1997). Note that any organism that simply transmits the parasite, without incurring any costs from that parasite, is not considered a host but a carrier.

More often than not, the different hosts will not only be of different species, but even from different phyla. Large differences in host physiology and body plan demand often radical changes in the parasite. Pleiotropic effects should constrain the potential adaptions that the parasite can develop for each of its hosts (Ebenman 1992). Moreover, requiring a second host introduces risks associated with switching hosts. However, multi-host life cycles have evolved numerous times and should therefore have some fitness benefits. Parasite transmission, dispersal, reproduction and growth may all increase when the parasite adds a host to its life cycle, at least when transmission occurs trophically (Choisy et al. 2003, Parker et al. 2003). For other modes of transmission, for example transmission by a vector, the effects have not been studied and may or may not have similar benefits (Choisy et al. 2003).

Vectors are agents that transmit parasites from one host to another. The vectors themselves can be mere carriers of the parasites, or true hosts of the parasite (Clayton and Moore 1997). The parasite-vector-host system has two unique characteristics which distinguish it from other forms of heteroxenous parasitism. First of all the vector-transmitted parasites are obligate and permanent: they need both hosts and have no free-living stages, neither mobile nor immobile (e.g. eggs, cestodes). The second particularity is that the vectors themselves are parasitic, depending on resources of the host for at least part of their life cycle.

Speciation, population differentiation and population dynamics as well as many life history and behavioural traits of parasite, vector and host are determined by all three actors in concert. Though these processes have been studied in vector-transmitted systems, the vectors themselves are

often ignored (e.g. Fallon et al. 2003), or assumed to be simple transmission vessels. The current surge in vector research has taught us that vectors are not uniformly distributed agents (McCoy et al. 2005) moving in a Brownian fashion (Lalubin et al. 2012) unaffected by the parasite they carry (Waite et al. 2012). When we consider a vector-transmitted parasite, it is the four-way host x vector x parasite x environment interaction that is needed to fully understand the crucial role vectors play in the parasite's coevolutionary process.

The goal of the current thesis is to show how the vector influences these coevolutionary processes of the parasite, and how these processes may influence the vector. For this, we will focus on the malaria parasite *Polychromophilus* spp., their host the bats (Chiroptera) and their vectors the bat flies (Nycteribiidae).

THE STUDY SYSTEM

Malaria parasites

Malaria still wreaks havoc in great parts of the tropical world. Failure of its eradication, aside socio-economic reasons, is partly because the causative agent *Plasmodium* spp. is a very elusive pathogen; it has many dynamic ways to evade the host immune system (e.g. Ndungu et al. 2005, Jemmely et al. 2010). *Plasmodium* spp. are so effective that they managed to invade all of the terrestrial vertebrate groups, the only genus of the order Haemosporida to have done so (Garnham 1966).

The Haemosporida (Apicomplexa: Coccidea: Coccidia) consist of ten genera distributed over four families (Valkiũnas 2005). All are obligate, permanent heteroxenous parasites, requiring one stage in a dipteran insect, and one in a terrestrial vertebrate. The most infamous member is *Plasmodium falciparum*. It causes a severe form of malaria in humans, with short intense fever cycles and possible encephalitis. Like all *Plasmodium* spp. (with few exceptions) it is transmitted by Culicidae (mosquitoes). The genus *Plasmodium* is the only member of the Plasmodiidae family and can be subdivided into 11-14 subgenera, each of which is specialised on a specific class or order of vertebrates (Garnham 1966, Valkiũnas 2005).

Three other genera of Haemosporida are known to infect mammals, all member of the Haemoproteidae family. *Hepatocystis* is the best studied group, with over 25 known species. Their

main hosts seem to be arboreal mammals and hippopotamus (Garnham 1966), which may be partly an effect of the arboreal life style of their vectors, the Culicoides (biting midges). The other two genera are *Polychromophilus* and *Nycteria*. Both these genera have only been found in bats (Garnham 1966, 1973b). This makes bats unique among mammals having malaria parasites from four mammal-infecting haemosporidian genera (Garnham 1973b). The vector of *Nycteria* has not been identified, but *Polychromophilus* spp. are known to be transmitted by Nycteribiidae (bat flies; Corradetti 1936, Mer and Goldblum 1947).

Most members of the Haemoproteidae, together with the Leucocytozoidae and Garnidae, infect sauropsids, either birds or lizards (Valkiũnas 2005), with some exotic species infecting snakes and even marine turtles (Degiusti et al. 1973). For many species, the vectors are unknown and often extrapolated from the few species within a genus from which the vector is known. The Simulidae (black flies), Culicoides (biting midges), Hippoboscidae (louse flies) and Tabanidae (horse flies, for the marine turtle) have all been identified as vectors for some haemosporidian species.

Malaria is a disease accompanied by distinct signs and symptoms. Technically, only the five *Plasmodium* parasites causing these symptoms in humans are true 'malaria parasites'. However, many have taken to calling all members of the genus *Plasmodium* malaria. Others have argued that, because *Plasmodium* is paraphyletic, all members of the order Haemosporida should be known as malaria parasites (Perez-Tris et al. 2005), which is how I will use the term in this thesis.

Polychromophilus

Despite a worldwide distribution (Garnham 1973b), *Polychromophilus* is a little known genus of the order Haemosporida. *Polychromophilus* spp. are unique within the Haemosporida since their dipteran hosts are bat flies (Nycteribiidae), and secondarily because their vertebrate hosts are limited to insectivorous bats (Garnham 1966, 1973b).

When Dionisi (1899) was confronted with blood parasites with many pigmented black grains in their gametocytes (Figure 1.2), he immediately realized he was dealing with a new haemosporidian genus and named it *Polychromophilus*. Looking into different host bat species, he isolated and identified 2 different species of *Polychromophilus*: *P. melanipherus* (Dionisi, 1899) from the bent-winged bat (*Miniopterus schreibersii*) and *P. murinus* (Dionisi, 1899) from the parti-coloured bat (*Vespertilio*

murinus). Since then, only three more species have been described (Garnham et al. 1971, Landau et al. 1980b). This low species number is in stark contrast with their hosts the bats (20% of all extant mammal species) and bat-flies (Dick and Patterson 2007) which both are characterized by extreme species-richness. However, both sampling effort and species delineation issues may be responsible for the currently known low *Polychromophilus* diversity (Garnham 1973b). As this genus is increasingly studied, it is likely that there will be an increase in the number of recognized species in the future (Schaer et al. 2013).

The life cycle of *Polychromophilus* is similar to that of other haemosporidians, with the sexual phase in the dipteran host and asexual replication in the vertebrate body. Sexual gametocytes circulating in the bat's blood (Figure 1.2) are taken up by a bat fly during a blood meal. In the fly's gut, the male and female gametocytes ripen and release gametes. After fertilization, the zygote, the only diploid stage in a malaria life cycle, develops into an ookinete which subsequently penetrates the gut wall to develop into an oocyst (Figure 1.3; Garnham 1966 and references therein). Within the oocyst multiple sporozoites develop. After oocyst rupture the sporozoites migrate throughout the fly's body but end up in the salivary glands where they remain dormant until migration into the vertebrate host (Gardner and Molyneux 1988a).

The sporozoites are injected into the bat skin by the bat fly during a blood meal, but it is not known which cell type in their new host they invade first. However, exoerythrocytic schizogony (asexual multiplication in non-blood cells) has been observed in the macrophages of bone marrow, lung, kidney, spleen and liver (Landau et al. 1977). After an unknown amount of asexual cycles in these tissues, some schizonts invade red blood cells and form sexual gametocytes ready to invade the bat fly's gut (Garnham 1966 and references therein). As most Haemosporida spp., *Polychromophilus* has no cycles of asexual multiplication in the blood, a trait typical only of the *Plasmodium* spp. (together with the very elusive Garnidae spp.; Valkiunas 2005).

Like all Apicomplexa, *Polychromophilus* spp. have an apicoplast. This organelle is involved in the metabolism of fatty acids and therefore critical for the penetration of the host cell membrane. The plastid is thought to have been of green-algal origin (Lau et al. 2009) and - importantly for this study - carries its own genome. Though most of its genes have migrated to the nucleus, the ~1% of genes left in the plastid provide a strong phylogenetic signal (Gardner et al. 2002).

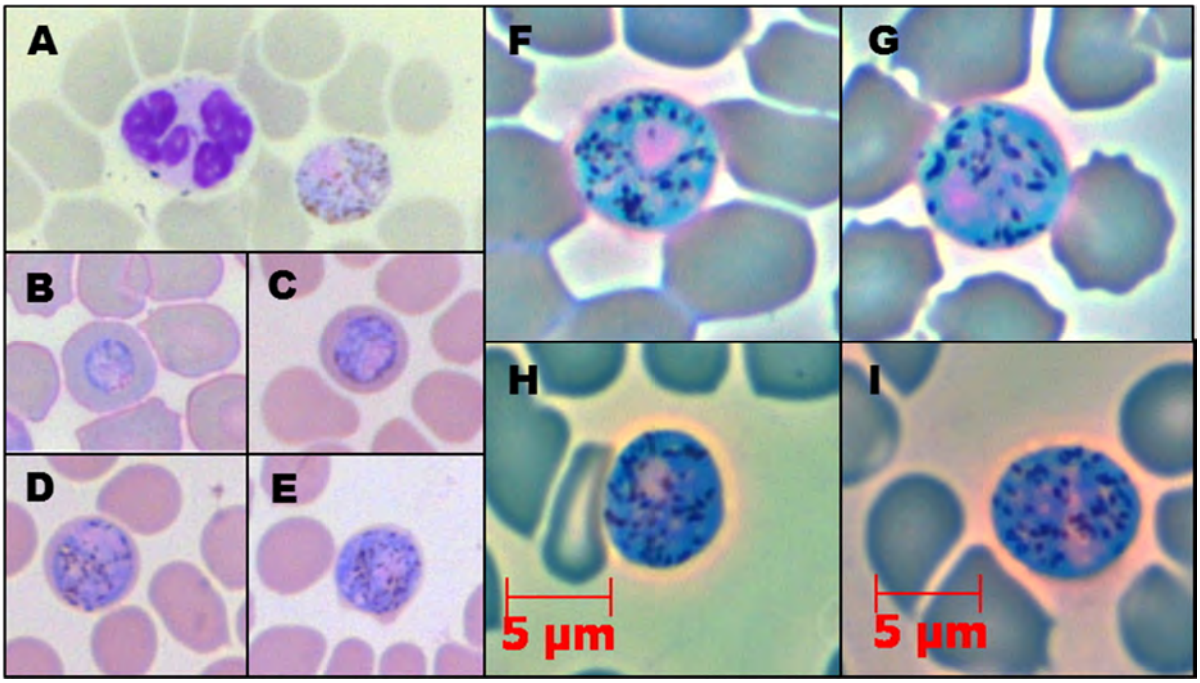

Figure 1.2 Different developmental stages of *Polychromophilus* gametocytes. (A) The pale, male, microgametocyte with leucocyte; (B-E) Development of gametocyte in erythrocyte; (F,H) Female, macrogametocyte, with dense nucleus; (G,I) Male, microgametocyte with diffuse nucleus. (A-G) *P. murinus*; (H,I) *P. melanipherus*. Thin blood films, giemsa staining, (A-E) 630x magnification (F-I) 1000x magnification with phase-contrast filter.

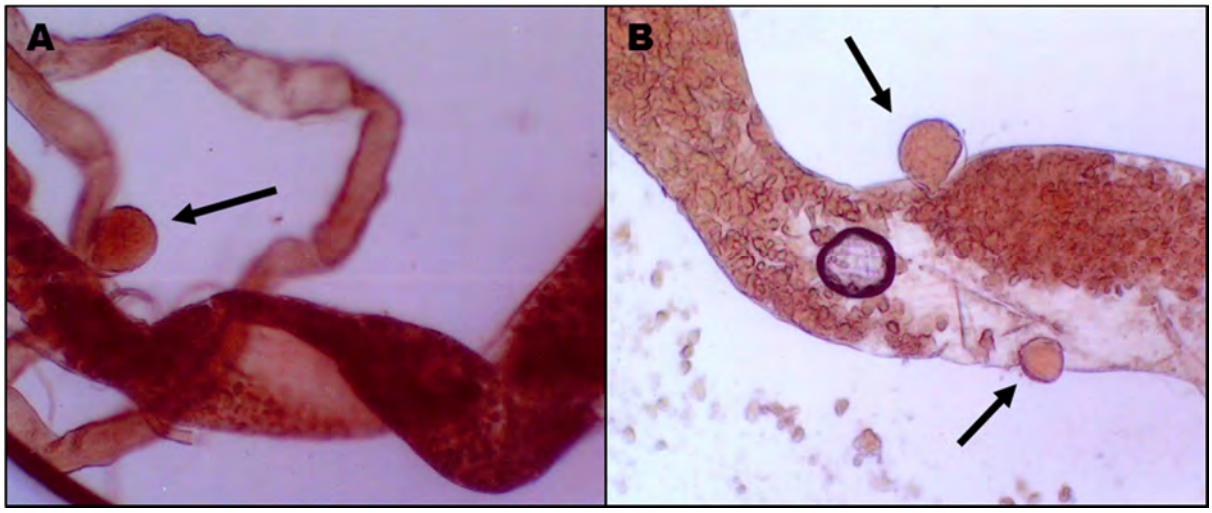

Figure 1.3 Oocysts of *P. murinus* on the gut wall of *Nycteribia kolenatii*. Oocysts are indicated by arrows. The filaments in the background of (A) are the Malpighian tubes. Fresh material, 100x magnification.

Nycteribiid bat flies

The Nycteribiidae (Diptera: Hippoboscoidea) are one of two bat fly families, the other being the Streblidae. Both families consist of obligate haematophagous ectoparasites that exclusively associate with bats. But whereas the Streblidae are mainly found in the New World, the Nycteribiidae are typically only Old World species (Dick and Patterson 2006). The Nycteribiidae show some extreme morphological adaptations to their parasitic life style. All nycteribiids are wingless and have a dorso-ventral flattened thorax with upward-protruding spider-like legs that end in hooking claws which allow swift movement through the fur (see Figure 1.4). On the front of the thorax and the base of the abdomen, they have developed comb-like structures, the cnetidia, which allow a firm grip in the fur, much like fleas (Theodor 1957). The relatively small head, which in many species lacks eyes, is folded backwards onto the thorax. Both males and females are haematophagous and unlike most other haematophagous Diptera, they do not engorge themselves. Instead, they take multiple blood meals per day, from once every hour up to every 8 minutes (Marshall 1970, Overal 1980, Fritz 1983).

Bat flies spend their whole life after emergence on hosts, except when females are ready to deposit larvae (Theodor 1967). Nycteribiidae are viviparous; the females nurture a single larva at a time in their abdomen through an intrauterine milk gland. When the larva has moulded twice, the female fly leaves the host temporarily to deposit the larva on the roost wall where it immediately pupates (Theodor 1957). Depending on the species of bat fly and the presence of hosts in the roost, the time until emergence can be between 22 (personal observation) and 451 days (Reckardt and Kerth 2006).

Three species of bat flies are important for the studies presented here: *Nycteribia kolenatii*, *N. schmidlii* and *Penicilidia conspicua*. The former species, *N. kolenatii* (Theodor & Moscana, 1954) is a small (2 mm) bat fly which mainly parasitizes the Daubenton's bat (*Myotis daubentonii*), but can be found on other members of *Myotis* (Müller and Ohlendorf 1984). It is expected to co-occur together with its host throughout its range, though it is curiously absent from Latvian populations of *My. daubentonii* (Jaunbauere et al. 2008). Its temperate zone habitat means that it spends the winter months on hibernating hosts, but keeps blood feeding during that period (Gardner and Molyneux 1988a). Both oocysts and sporozoites of *P. murinus* have been found in tissue of *N. kolenatii*, but how the infection affects the vector is unknown (Gardner and Molyneux 1988a).

Figure 1.4 The Nycteribiidae bat flies. (A) Unidentified *Nycteribia* sp. Both *N. kolenatii* and *N. schmidlii* look very similarly; (B) *P. conspicua*; (C) Two gravid female *N. schmidlii* in different stages of development (D) Eyeless head of *N. kolenatii*; (E) The head of *P. conspicua* with bristles and a single lens.

Neither *N. schmidlii* (Schiner, 1853) nor *P. conspicua* (Speiser, 1901) have been confirmed as vectors of *Polychromophilus* spp., though other members of their respective genera have been (Garnham 1973b). Both species' main host is the bent-winged bat (*Miniopterus schreibersii*). *Nycteribia schmidlii*, which resembles *N. kolenatii* in appearance, is very host specific and is hardly found on other bat species (Theodor 1957). *Penicilidia conspicua,* by contrast, is a much larger and robuster bat fly species (4mm) and will occasionally reside on other cave-roosting bat species, in particular *Myotis myotis* (Theodor 1957, Lanza 1999).

Chiroptera

Besides being the only flying mammal and living way too long for their size, bats have also been considered an immunological oddity (Wang et al. 2011). Indeed, recent comparative genomics of two bat species confirmed the absence of several genes normally involved in mammalian innate immunity

(Zhang et al. 2013). This finding could explain the apparent lack of pathology caused by many viruses that are harboured by bats as well as the overrepresentation of bats as sources of zoonotic diseases (Luis et al. 2013).

Bats are the known reservoir of many diseases and the suspected reservoir of many more (for an overview: Calisher et al. 2006). Studies on bat pathogens are driven by concerns for human and livestock health and consequently focus on potential emerging diseases. Yet often bats are in these cases merely the reservoir, biasing the spectrum of true bat pathogens. With the exception of the recent attention for White Nose Syndrome (Frick et al. 2010), studies of bat specialist pathogens are rare, yet these could provide valuable insights into true bat epidemiology.

Even though the Daubenton's bat (*Myotis daubentonii*: Kuhl, 1817; Vespertilionidae) is not the type host of *P. murinus,* several studies indicate it is an important, if not the primary host in the temperate regions of Europe (Gardner et al. 1987, Megali et al. 2011). They are small insectivorous bats (~7 g) that hunt over water and roost in tree holes. Their main habitat requirements therefore consist of trees and water and *My. daubentonii* is consequently found throughout Europe, as well as large parts of temperate Asia (Dietz et al. 2009). Nursery colonies of 20-50 females are formed in early summer. Males form their own groups, often in less productive habitats (Senior et al. 2005). Females give birth to one pup which after 4 weeks can hunt on its own. Hibernation sites can be enormous aggregations of several thousand individuals (Dietz et al. 2009). The rabies-causing European bat lyssavirus 2 originates from *My. daubentonii* (Amengual et al. 1997) and this bat species has also been identified as its reservoir in Switzerland (Megali et al. 2010).

The larger (~14 g) Schreiber's bent-winged bat (*Miniopterus schreibersii schreibersii*: Kuhl, 1817; Miniopteridae) is a Mediterranean insectivorous species. It is part of a species complex whose range extends from Western France to Australia and South Africa (Appleton et al. 2004). *Min. s. schreibersii* (from now on referred to as simply *Min. schreibersii*) roosts in karst caves which limits its distribution in Europe from the Iberian peninsula to coastal Anatolia, including the entire Balkan region as northerly as Slovakia. They are highly gregarious throughout the year, congregating in large numbers (100-1000 individuals) often close to or mixing with other cave-roosting species (Dietz et al. 2009). Apart from the hibernating period, *Min. schreibersii* perform extensive regional migrations, moving to caves with optimal thermal conditions for their reproductive cycle (Rodrigues and Palmeirim 2008).

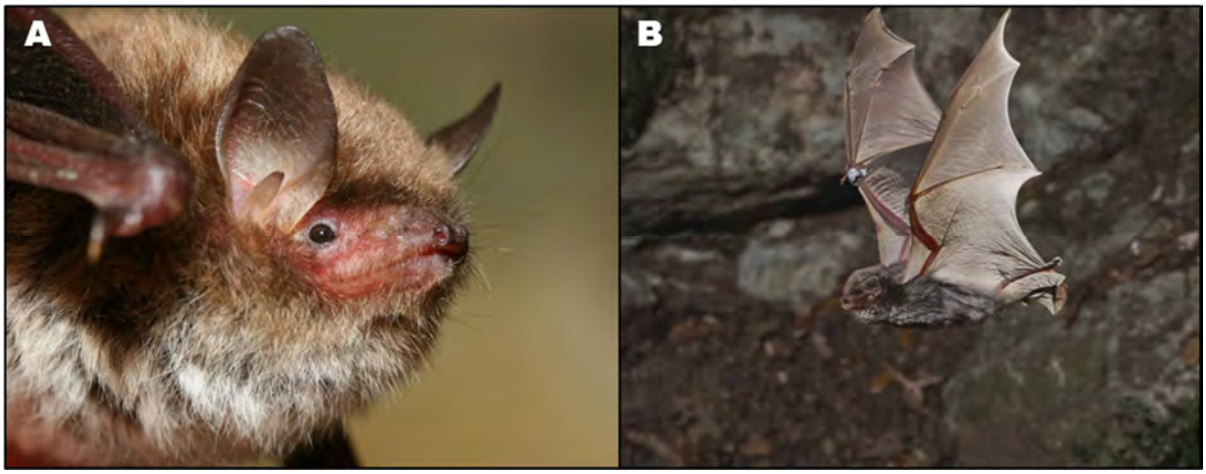

Figure 1.5 The two vertebrate hosts. (A) The Daubenton's bat *Myotis daubentonii*; (B) The bent-winged bat *Miniopterus schreibersii*.

Thesis goals and outlines

Apart from a few prevalence studies, very little of the ecology and evolution of *Polychromophilus* spp. is known. The goal of my thesis is to describe some of these processes for *Polychromophilus*: its speciation process, its pattern of population differentiation and its population dynamics i.e. epidemiology. As previously mentioned, these coevolutionary processes are dependent on all three actors of a parasite-vector-host system. In this thesis, I will in particular reflect on the role of the bat fly vector, how it has influenced the ecology and evolution of *Polychromophilus* and vice versa.

Chapter 2 will focus on the evolution of *Polychromophilus* as a genus. Which haemosporidian lineage is its closest relative? Where do *Polychromophilus*' origins lie? And what process led to the rise of this genus: was it the move to a new host species or to a new type of vector? Furthermore, by using *Polychromophilus* samples of both *P. melanipherus* and *P. murinus*, I can describe the level of differentiation between species of the genus.

In chapter 3 we focus on the parasite's spatial genetics and incorporate those of the vector and host. Specifically, I will use *Polychromophilus melanipherus*. Its host specificity allows accurate comparisons with its host *Min. schreibersii* as well as the host-specific *N. schmidlii*. Therefore, I will look into the process of population differentiation of *P. melanipherus* and, by comparative genetics, try to answer the question whether it is rather the vector or host that determines the parasite's distribution across Europe.

For my fourth chapter we change gears and species and focus on the ecology of a local population of *Polychromophilus*. Because *P. melanipherus* infects only the locally rare *Min. schreibersii*, I will instead focus for this study on *P. murinus* and its dynamics within its locally abundant host *My. daubentonii*. I will try to quantify some basic epidemiological traits such as parasite prevalence and abundance and their fluctuations throughout the season. Moreover, I will look at the susceptibility of different classes of hosts and provide a framework of how the infection is maintained in the host population.

The previous chapter does not consider the role of the vector in the epidemiology of *P. murinus*. Instead, the entire chapter 5 will focus on the behaviour of *Nycteribia kolenatii*. If an infection with *P. murinus* is harmful to its vector *N. kolenatii*, one would expect some adaptive behavioural responses of the vector to the threat of infection. In this chapter, I will identify the feeding preferences of the vector, determine if they are adaptive for its survival and compare these results with the natural feeding behaviours observed in the local population of *My. daubentonii*.

The final research chapter proposes and tests a method to facilitate the discovery of new species of haemosporidians and other blood parasites. Following the theme of this thesis, the method revolves around the use and identification of vectors and other ectoparasites.

In my final chapter I will synthesize the results of the previous chapters and formulate some overall conclusions on the evolutionary ecology of *Polychromophilus* spp. and its interactions with its bat fly vectors and chiropteran hosts. I will moreover provide some thoughts on themes concerning vectored parasites and *Polychromophilus*, but which do not fit in any of the previous chapters. These remarks, moreover, suggest exciting new directions in this field for future research.

Chapter 2

The evolutionary host switches of *Polychromophilus*: a multi-gene phylogeny of the bat malaria genus suggests a second invasion of mammals by a haemosporidian parasite

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Keywords: *Polychromophilus***,** malaria, Haemosporida, phylogenetic analysis, Chiroptera, *Plasmodium*, host switch, outgroup selection

ABSTRACT

The majority of Haemosporida species infect birds or reptiles, but many important genera, including *Plasmodium*, infect mammals. Dipteran vectors shared by avian, reptilian and mammalian Haemosporida, suggest multiple invasions of Mammalia during haemosporidian evolution; yet, phylogenetic analyses have detected only a single invasion event. Until now, several important mammal-infecting genera have been absent in these analyses. This study focuses on the evolutionary origin of *Polychromophilus,* a unique malaria genus that only infects bats (Microchiroptera) and is transmitted by bat flies (Nycteribiidae).

Two species of *Polychromophilus* were obtained from wild bats caught in Switzerland. These were molecularly characterised using four genes (*asl*, *clpc*, *coI*, *cytb*) from the three different genomes (nucleus, apicoplast, mitochondrion). These data were then combined with data of 60 taxa of Haemosporida available in GenBank. Bayesian inference, maximum likelihood and a range of rooting methods was used to test specific hypotheses concerning the phylogenetic relationships between *Polychromophilus* and the other haemosporidian genera.

The *Polychromophilus melanipherus* and *Polychromophilus murinus* samples show genetically distinct patterns and group according to species. The Bayesian tree topology suggests that the monophyletic clade of *Polychromophilus* falls within the avian/saurian clade of *Plasmodium* and directed hypothesis testing confirms the *Plasmodium* origin.

Polychromophilus' ancestor was most likely a bird- or reptile-infecting *Plasmodium* before it switched to bats. The invasion of mammals as hosts has, therefore, not been a unique event in the evolutionary history of Haemosporida, despite the suspected costs of adapting to a new host. This was, moreover, accompanied by a switch in dipteran host.

INTRODUCTION

Five genera belonging to the order of Haemosporida (Apicomplexa) are known to infect mammals: *Plasmodium, Hepatocystis, Polychromophilus*, *Nycteria* and *Rayella* (Garnham 1966, Dasgupta 1967). The dipteran vectors of the first three haemosporidian genera are represented by Culicidae (*Anopheles* spp.), Ceratopogonidae and Nycteribiidae respectively, while the vectors of *Nycteria* and *Rayella* are unknown (Garnham 1966, Dasgupta 1967). Culicidae and Ceratopogonidae also act as vectors of the avian and saurian Haemosporida (Garnham 1966, Valkiũnas 2005). These shared vectors suggest that haemosporidian parasites might have invaded mammals multiple times during their evolution. On the other hand, the switch to mammals is thought to have been an evolutionary demanding process for the parasite (Outlaw and Ricklefs 2010) and therefore a rare event (Yotoko and Elisei 2006).

Molecular phylogenetic studies to date have been able to detect only a single host switching event to mammals: mammalian *Plasmodium* and *Hepatocystis,* the main mammal-infecting genera, had a common origin and formed a monophyletic sister clade to sauropsid *Plasmodium* (Perkins and Schall 2002, Martinsen et al. 2008). However, these phylogenetic studies suffer from incomplete taxon sampling with most investigations including, besides the genera *Plasmodium* and *Hepatocystis*, only the avian *Haemoproteus* and *Leucocytozoon.* Consequently, with no knowledge of the evolutionary origin of the other mammalian haemosporidian groups (i.e. *Rayella*, *Nycteria, Polychromophilus*), a second move into mammals cannot be excluded.

One possible approach for resolving this standing question is to select a haemosporidian genus that could potentially have switched to mammal hosts independently of mammalian *Plasmodium*/*Hepatocystis*. A good candidate genus for this is *Polychromophilus* as it is well described, with the majority of its life cycle well documented, including its vector stage. Moreover, it infects mammals but is not transmitted by Culicidae like *Plasmodium*, nor Ceratopogonidae like *Hepatocystis*, but by Nycteribiidae (Diptera: Hippoboscoidea). Furthermore, *Polychromophilus'* vertebrate host species range is restricted to the insectivorous bats (Microchiroptera). A phylogenetic analysis of *Polychromophilus* can therefore elucidate whether it arose through an independent switch to mammal hosts (Carreno et al. 1997).

Only five species of *Polychromophilus* are known to exist. While they can be distinguished by their slight differences in ultrastructure, they are mainly classified based on host-type (Garnham 1973b, Landau et al. 1980b). Landau *et al.* (1980b) proposed dividing the genus into two subgenera based on their gametocyte morphology: 1) the subgenus *Polychromophilus,* with *P. (P.) melanipherus* as the type species, which has gametocytes similar to the type 'malariae'; 2) the subgenus *Bioccala* with type species *Polychromophilus (B.) murinus* whose gametocytes resemble the benign tertian parasites of birds and reptiles (Figure 1.2) (Landau et al. 1980b). Later, it was even suggested that the subgenus *Bioccala* be raised to genus level (Landau et al. 1984); however, this was not reflected in the literature (Gardner and Molyneux 1988a). Moreover, the morphological distinctions between the species have been described as 'slight' (Garnham 1973b) and how well they reflect the genetics of the genus has not been studied.

The Nycteribiidae vectors are also known as nycteribids or bat flies. These haematophagous flies are completely adapted to a parasitic lifestyle in the fur of bats in that they have lost their wings, have no or reduced eyes and possess hooking claws which allow them swift movements through the fur (Theodor 1967, Dick and Patterson 2006). Coradetti (1936) was the first to detect sporozoites in their salivary glands and later studies confirmed his finding (Mer and Goldblum 1947, Gardner and Molyneux 1988a).

When an evolutionary conservation of hosts is assumed, *Polychromophilus*' unique host-vector combination of Mammalia and Nycteribiidae gives rise to two hypotheses on its phylogenetic relationships: 1) it is monophyletic with the mammalian *Plasmodium/Hepatocystis* clade with which it shares the vertebrate host type, or 2) it shares its most recent common ancestor with the subgenus *Haemoproteus* (*Haemoproteus*), which has a similar vector. The genus *Haemoproteus* contains two avian subgenera which have different vectors. *H. (Parahaemoproteus)* spp. use biting midges as vectors, and *H. (Haemoproteus)* spp. are transmitted by Hippoboscidae, whose closest relatives are the bat flies (Petersen et al. 2007). A phylogeny based on ultrastructure and life-history traits grouped *Polychromophilus* together with both subgenera of *Haemoproteus* (Carreno et al. 1997). However, two recent molecular phylogenetic studies based on part of the cytochrome b sequence both suggest, despite their different topologies, a close relationship between *Polychromophilus* and sauropsid

Plasmodium (Megali et al. 2011, Outlaw and Ricklefs 2011). This fact provides a third hypothesis: 3) *Polychromophilus* is monophyletic with sauropsid *Plasmodium* (Figure 2.1).

The aim of this study was to test these three hypotheses against each other. Though previous studies on the phylogenetic relationships of *Polychromophilus* have been done, all used only a single gene. Different genes in a single organism can show different evolutionary patterns and it is therefore recommended to use multiple genes for accurate relationship estimation (Cummings and Meyer 2005). The four genes from three different genomes sequenced for this study represent two species of *Polychromophilus* (i.e. the two type species of the two proposed subgenera). These sequences were subsequently combined with an existing dataset of 60 species of Haemosporida to clarify the phylogenetic relationships and gain insight into the evolutionary host switches of *Polychromophilus*.

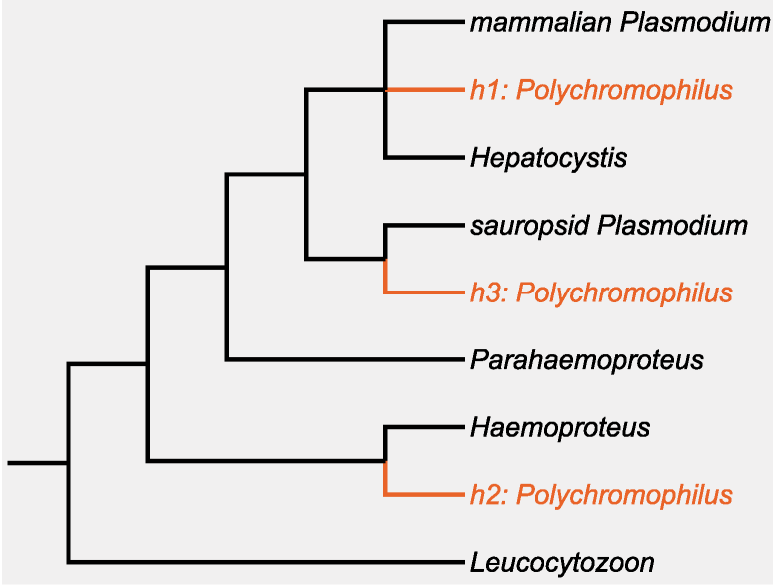


Figure 2.1 The hypothetical phylogeny of the genus *Polychromophilus* and the other Haemosporida. The hypothetical branches are marked in orange and based either on the conservation of the vertebrate host (hypothesis 1), the conservation of the dipteran vector (hypothesis 2), or based on previous molecular studies of the *cytb* gene (hypothesis 3).

METHODS

Sample collection and preparation

Four *Miniopterus schreibersii* (Schreibers' bent-winged bat) were caught using mist nets in the entrance of an abandoned mine in western Switzerland under authorization #2203 issued by the Veterinarian Service of canton Vaud, Switzerland. Blood was obtained by puncturing the uropatagial vein with a 0.5 mm gauge needle (Neolus). The blood beads that consequently formed on the uropatagium (between 10 and 30 µl total) were taken up in a microvette with EDTA (Sarstedt) and stored at 4ºC until further analysis. Haemostatic cotton was applied on the punctured vein until the bleeding had stopped before releasing any bats.

One drop of blood was applied to a glass microscope slide for later visual identification of the parasite species. After smearing the blood, the slide was dried and immediately submerged in 100% methanol for fixation. Finally 5% Giemsa-staining was applied for one hour to stain the cells. DNA was extracted from whole blood using the DNeasy Blood and Tissue kit (Qiagen). Megali *et al.* (2011) provided extracted DNA samples from blood of *Myotis daubentoni* (Daubenton's bat) which contained *P. murinus* infections. These infections were previously shown to be characterised by different cytochrome *b* haplotypes (Megali et al. 2011).

Molecular analysis

For the phylogenetic reconstruction, four genes were selected from the three cellular genomes: two mitochondrial DNA sequences, cytochrome *b (cytb,* 607 bp) and cytochrome oxidase subunit I (*coI,* 768 bp); one DNA sequence from the apicoplast, caseinolytic protease C (*clpc,* 502 bp); and one nuclear DNA sequence, adenylosuccinate lyase (*asl,* 186 bp).

All primer pairs used for the polymerase chain reactions were taken from Martinsen *et al.* (2008) with the exception of *coI nested Po*, which was designed during this study (see Supp. Table S2.1 for primer sequences). All reactions started with an initial denaturation phase at 94ºC for four minutes and one minute for the first and nested PCRs, respectively. The reactions ended with an annealing phase at 72ºC for seven minutes. All cycles started for 30 s at 94ºC, but the other cycle conditions and the number of cycles differed depending on the primer pair used (Supp. Table S2.1).

The 25 µl reaction volume contained 3 µl of extraction product, 0.25 U Taq polymerase, 0.3 mM of both primers, 0.25 mM dNTP's, 1x Qiagen PCR buffer and a total of 2 mM MgCl (except for the reactions with the *coI* primers, which had a total of 3 mM MgCl). The nested PCR reaction volume was similar except for the extraction product, which was replaced with 1 µl of product of the first PCR. For the *asl* amplification the first PCR product was purified, which resulted in a better performance of the nested reaction.

All successfully amplified samples were purified according to the manufacturer's protocol using the Wizard PCR Clean-Up system (Promega) or the Minelute PCR Purification kit (Qiagen) in the case of *asl*. DNA concentrations were estimated by visualisation on a 1.5% agarose gel with a 100 bp reference ladder (Roche). For the sequencing reactions ~20 ng of purified PCR product, 2 µl Big Dye Terminator v3.1 and 1 µl of 10 mM primer were mixed to a 10 µl volume. Sequence analysis was performed on an ABI Prism 3100 genetic analyser (Applied Biosystems). Sequence chromatographs were checked for ambiguities with Chromas Lite v2.01 (Technelysium).

Phylogenetic reconstruction

The obtained sequence data were combined with the same gene sequences of 60 other haemosporidian species obtained from GenBank (Supp. Table S2.2). These 60 species represent the major clades of the Haemosporida, i.e. *Leucocytozoon, Haemoproteus* (*Haemoproteus*), *Haemoproteus* (*Parahaemoproteus*), *Hepatocystis* and *Plasmodium* (including mammalian, avian and saurian). Sequences were aligned with ClustalW as implemented in MEGA version 5 (Tamura et al. 2011). The single-gene alignments were concatenated using FASconCAT (Kück and Meusemann 2010).

All phylogenetic reconstructions were done using both Maximum Likelihood (ML) analysis and Bayesian inference (BI). For ML analysis, the PhyML software (Guindon and Gascuel 2003) was used for the single-gene alignments. Since PhyML does not allow for partitioning of the data RAxML (Stamatakis 2006) was used for the concatenated alignment. Models of nucleotide substitution were GTR + Γ + I for *cytb, co1* and *clpc* and GTR + Γ for *asl,* as determined by MrAIC (Nylander 2004). For each analysis, the transition rates of the GTR model, the shape of the Γ-distribution and the proportion of invariable sites were estimated by the program. Both the RAxML and PhyML analyses were assessed by performing 1,000 bootstrap replicates.

For the Bayesian analysis the same models of character evolution as described for the ML analyses were implemented with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). In the concatenated analysis the data was again partitioned by gene, where each partition had its corresponding model and independent parameter estimations. The MCMC algorithm was done with four chains and was run for 20,000,000 generations, sampling every 1,000 generations. Two independent runs were performed to assess convergence to the correct posterior distribution. All parameters were checked for convergence using Tracer v1.5 and the first 10% of samples of each run was discarded as burn-in. All computations were performed on the Vital-IT cluster of the Swiss Institute of Bioinformatics.

Rooting the tree

Which outgroup to use has been a matter of debate lately. Perkins and Schall (2002) identified *Leucocytozoon* as the most primitive clade of the order, using *Theileria* as an outgroup in their analysis of *cytb* sequences. But a recent study by Outlaw and Ricklefs (2011) demonstrated that, when using a relaxed molecular clock, *Leucocytozoon* becomes the most derived group, effectively turning the tree inside-out. The authors argue that most ancient divergence should be between the mammal-infecting *Plasmodium* and *Hepatocystis* on the one side, and avian/saurian *Plasmodium*, both subgenera of *Haemoproteus* and *Leucocytozoon* on the other.

For the phylogenetic tree reconstructions, the *Leucocytozoon* spp. were initially selected as the outgroup, but these results were tested for their robustness by redoing the analyses using different rooting methods: 1) forcing the mammalian *Plasmodium/Hepatocystis* clade as outgroup instead of *Leucocytozoon*; 2) adding amino acid sequences of the more distantly related *Babesia* spp. as the outgroup (Supp. Table S2.2) and repeating the ML analyses; 3) using the molecular clock methods similar to Outlaw and Ricklefs (2011) but with varying priors: a Yule or birth-death tree prior, a strict, a log-normal relaxed or an exponential relaxed clock with a GTR + Γ + I substitution model, 20 million generations sampling every 2,000 generations and two independent MCMC runs using BEAST (Drummond et al. 2002, Drummond et al. 2006, Drummond and Rambaut 2007).

Table 2.1 The Kishino-Hasegawa topological test results.

	Single gene tree	**Concatenated 4 genes tree**	
	lnL	***lnL***	p_{KH}
asl	-3561.406	-3642.361	< 0.001*
clpc	-7718.852	-7728.157	0.2696
coI	-10062.16	-10074.33	0.2336
cytb	-6856.617	-6859.401	0.4323

For each gene the likelihood of the phylogeny of that gene was compared to the phylogenetic reconstruction based on all four genes. The log-likelihood values and p-values are shown per gene alignment. Only the *asl* alignment gives a significantly worse likelihood value for the tree based on the combined data, which indicates conflicting topologies.

Topological tests

The obtained Bayesian majority rule consensus tree was compared with each of the four Bayesian single-gene majority rule consensus trees to rule out any conflict in topology. The Kishino-Hasegawa tests (Kishino and Hasegawa 1989) were performed in Treefinder (Jobb 2008). The tests proved non-significant for all genes but *asl* (Table 2.1). This gene was therefore removed from the concatenated alignment and a new phylogenetic reconstruction was performed on the remaining genes only.

For each of the three hypotheses on the *Polychromophilus* origin a corresponding topology was constructed. This was done by restricting the placing of *Polychromophilus* during tree reconstruction in RAxML, forcing it either with the mammal-infecting *Plasmodium/Hepatocystis* clade (hypothesis 1), the *Haemoproteus (Haemoproteus)* clade (hypothesis 2) or with the sauropsid *Plasmodium* clade (hypothesis 3). These restricted topologies were then tested together with the topology produced by the maximum likelihood analysis using a Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) as implemented in PAML 4 (Yang 2007).

RESULTS & DISCUSSION

The stained slides showed erythrocytes infected with slightly oval-shaped gametocytes (Figure 1.2H). The granular appearance and pinkish staining at the nucleus fit the description of *Polychromophilus melanipherus* as given by Garnham (1966). The morphology of the observed gametocytes could therefore be linked to the molecular sequences obtained from the infections (for haplotype names and GenBank accession numbers, see Supp. Table S2.3).

None of the topologies obtained by independent analyses of the separate genes conflicted with the topology resulting from the concatenated alignment (Kishino-Hasegawa tests: *cytb*: Δlnl= 2.8, p_{KH}=0.432 , *coI*: Δlnl= 12.1, p_{KH}= 0.234, *clpc*: Δlnl= 9.3, p_{KH}= 0.270), except for *asl* (Δlnl= 81.0, p_{KH}<0.001). Despite this strong rejection, both the ML and BI trees of *asl* had only few supported nodes and only closely related pairs were recovered (data not shown). A possible cause of the incongruence detected could be positive selection events in the evolution of the *asl* nuclear sequence (Christin et al. 2012). However, analyses performed with Codeml (Yang 2007) did not show signs of positive selection on the nuclear gene (data not shown).

Although the reasons for this DNA region to be rejected by the topology tests are unclear, the length of the *asl* gene fragment sequenced in this study is very small (186 bp). This could suggest that random errors are responsible for creating the incongruences observed with this gene. Adding other, and especially longer, nuclear genes would certainly bring more information to test if the evolutionary relationships estimated from the different genomes are congruent or if specific gene trees best represent the evolution of each DNA regions. Different cellular genomes often have different evolutionary histories; even within a single genome not all genes show the same phylogenetic relationships (Cummings and Meyer 2005).

Figure 2.2 presents the reconstructed phylogenetic trees using the combined data of *cytb*, *coI* and *clpc* by ML and BI. The analyses produce no conflict on any of the major nodes. All major genera and subgenera are recovered and represented in the phylogenetic tree by separate monophyletic clades, with the exception of the sauropsid *Plasmodium* clade, which contains *Polychromophilus* within it.

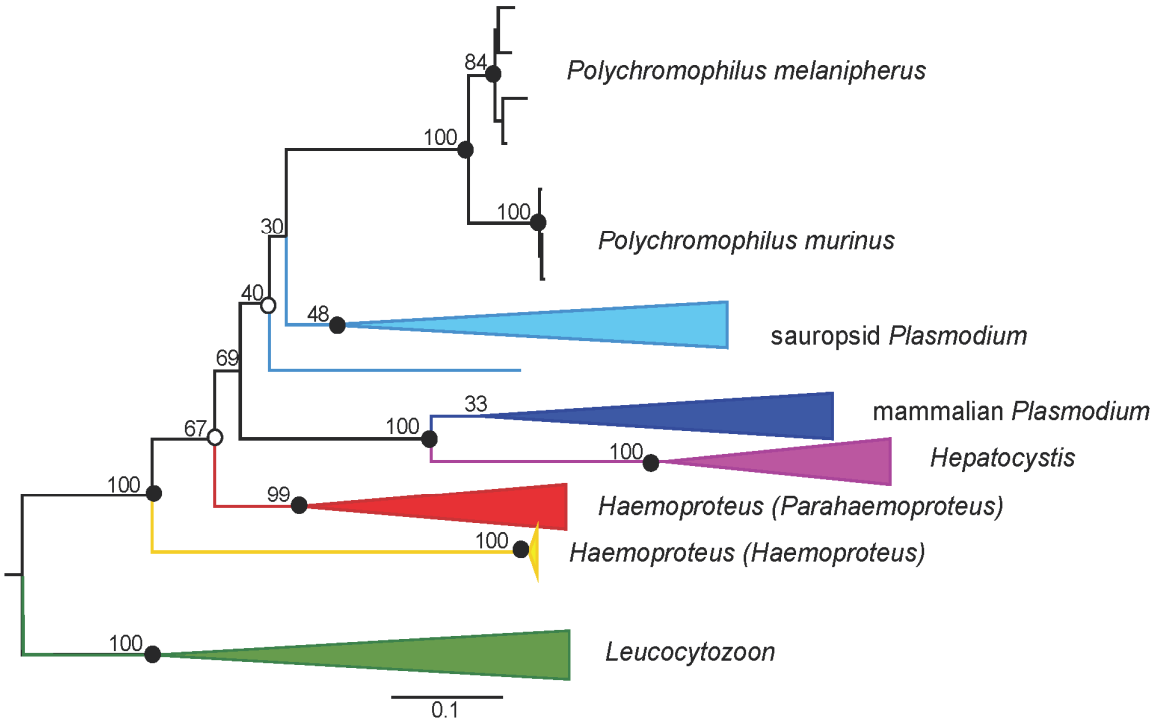


Figure 2.2 *Polychromophilus* shares its most recent common ancestor with avian and reptilian *Plasmodium*. Shown is the 50% majority-rule consensus tree from the Bayesian inference analysis. The phylogenetic reconstruction using maximum likelihood produced a similar tree. For clarity all clades except the *Polychromophilus* are collapsed and replaced by coloured triangles. Each colour represents a different haemosporidian group. The dots indicate Bayesian node support. Closed dots indicate a posterior probability ≥ 0.95, open dots a posterior probability ≥ 0.90. Node values indicate bootstrap values. Branch lengths represent the number of substitutions. The single blue branch belongs to a *Plasmodium* sp. infecting the skink *Egernia stokesii*.

Diversity of Polychromophilus species

Polychromophilus forms its own clearly defined clade in both the BI and ML reconstructions. Within this clade, the two species of *Polychromophilus* form well supported separate sister clades (Figure 2.2). The distinction between *P. melanipherus* and *P. murinus* has often been made based on host species, since *P. melanipherus* was by definition confined to *Miniopterus schreibersii* as hosts. This distinction, however, has been qualified as 'arbitrary' and 'unsatisfactory' (Garnham 1966). This study demonstrates for the first time that there is a clear genetic distinction between the two *Polychromophilus* species, confirming their taxonomic status of different species from a molecular point of view.

However, to determine whether this level of genetic divergence between *P. murinus* and *P. melanipherus* merits their placement in different subgenera (Landau et al. 1980b) or even different genera(Landau et al. 1984), other species of the genus should be added (e.g. *P. deanei* (Garnham et al. 1971) and *P. adami* (Landau et al. 1980b)). Without these supplementary species, the overall observed genetic diversity within the genus *Polychromophilus* is low; it is clearly less than that of the genera *Plasmodium* and *Haemoproteus* or even less than the diversity found in subgenera like *P. (Vinckeia)* and *H. (Parahaemoproteus)*. No critical level of genetic diversity exists as a precondition for the elevation of a subgenus, but the low diversity found within *Polychromophilus* does suggest that confirming *P. (Bioccala)* as a separate genus would cause a taxonomic asymmetry within the Haemosporida.

Two more haemosporidian genera infecting bats have been described: *Dionisia* (Landau et al. 1980a) and *Biguetiella* (Landau et al. 1984). Both contain only a single species and are described as 'little different' from *Polychromophilus (Polychromophilus)* spp. (Landau et al. 1980a) and as 'a vicariant form of' *Polychromophilus (Bioccala)* spp. (Landau et al. 1984), respectively. Whether their similarities to *Polychromophilus* spp. are because of convergence or shared ancestry can only be tested by combining the morphological data with molecular methods (Perkins et al. 2011). A big obstacle in studying these unfamiliar species however is the lack of observations. No other records of *Biguetiella* or *Dionisia* exist. Single descriptions of new parasite species found in a limited number of hosts are a problem encountered more often by parasitologists and can severely hamper classification (Perkins et al. 2011).

Table 2.2 The Shimodaira-Hasegawa topological test results comparing the three hypothetical topologies.

Tree	*lnL*	p_{SH}
Best tree (hypothesis 3)	-25126.753	-
Hypothesis 1	-25130.147	0.578
Hypothesis 2	-25154.740	0.023*

The best tree was the tree provided by the maximum likelihood analysis (Figure 2.2) and concurred with hypothesis 3. The log-likelihoods of the other two trees, based on hypothesis 1 and 2 (Figure 2.1), are compared with the best tree. The hypothesis 2 tree, which has *Polychromophilus* grouped with *Haemoproteus*, has a significantly worse fit and can be rejected.

Polychromophilus' placement in the phylogeny of Haemosporida

The bootstrap value (69/100) suggests that the *Polychromophilus* clade is restricted to the *Plasmodium* branch of the haemosporidian tree. Even though this node also appears in the Bayesian majority rule consensus tree, the support for it is actually very weak (posterior probability = 0.73). However, the alternative hypothesis 2, that *Polychromophilus* shares its most recent common ancestor with the subgenus *Haemoproteus (Haemoproteus)*, is clearly rejected (Shimodaira-Hasegawa test; Table 2.2).

It is less clear where within the *Plasmodium* clade *Polychromophilus* belongs. Neither phylogenetic method indicates that *Polychromophilus* originated from mammalian *Plasmodium/Hepatocystis* and both instead produced topologies suggesting a sauropsid origin (Figure 2.2). However, the actual support for the node separating the mammalian clade from sauropsid *Plasmodium/Polychromophilus* clade is low. The BI supports the monophyly of sauropsid *Plasmodium* and *Polychromophilus* (hypothesis 3) with a posterior probability of 0.92, but the ML support of that same critical node is absent (bootstrap value of 40/100). The topological test comparing the different phylogenetic scenarios did not provide more support for either hypothesis 1 or 3 (Table 2.2).

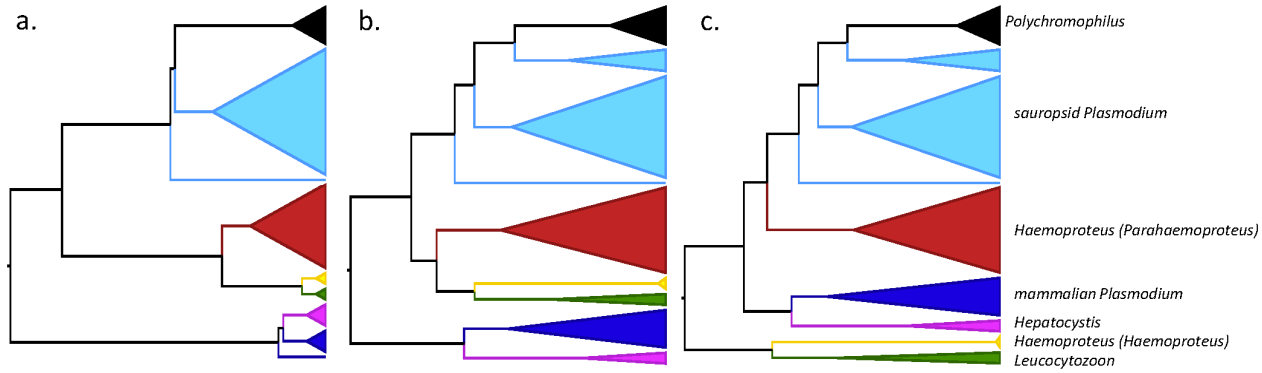


Figure 2.3 Changing topologies acquired by different methods of phylogenetic reconstruction. Irrespective of the root, *Polychromophilus* remains nested within the sauropsid *Plasmodium* clade. (a) The original best tree from maximum likelihood reconstruction, but now rooted with the mammalian *Plasmodium*/*Hepatocystis*, as suggested by Outlaw and Ricklefs (2011). Topologies b. and c. are acquired using a relaxed molecular clock with no predefined root. All nodes have clade credibilities > 0.5 (b) Topology acquired with the birth-death tree prior and an exponential relaxed clock. (c) Topology acquired with the Yule tree prior and a log-normal relaxed clock. The different haemosporidian clades are represented by the coloured triangles. The clade height represents the number of containing taxa.

Most of the alternative rooting methods favour hypothesis 3. Indeed, rooting the tree with the mammalian *Plasmodium* clade instead of *Leucocytozoon*, as suggested by Outlaw and Ricklefs (2011), validates the conclusion of a sauropsid origin of *Polychromophilus* in both BI and ML (Figure 2.3).

The choice of the prior distributions guiding either the distribution of mutation rates across the tree (log-normal *vs* exponential) or the divergence times (Yule *vs* birth-death) does not change the conclusion. All the molecular clock analyses place *Polychromophilus* within the sauropsid *Plasmodium* clade, with clade credibilities between 0.87 and 1. However the root itself does change depending on the prior set. The Yule and log-normal prior lead to the placement of the *Leucocytozoon* as the outgroup, whereas the mammalian *Plasmodium*/*Hepatocystis* clade is placed as the outgroup with the birth-death tree and relaxed clock exponential (Figure 2.3).

The ML analysis using *Babesia* as an outgroup produces a topology with very little support. All major nodes have bootstrap values of < 50/100, so no outgroup can be identified, nor can *Polychromophilus* be placed within the tree with any confidence (Supp. Figure S2.1). The genetic divergence between *Babesia* (Piroplasmida) and the Haemosporida is very high, which results in a very long branch leading to the *Babesia* lineages. This changes the rooting procedure to a problem of 'long-branch attraction' with all corresponding biases (Sanderson and Shaffer 2002), and these analyses should therefore be approached with caution (Outlaw and Ricklefs 2011).

None of the used phylogenetic methods reject our third hypothesis, stating that *Polychromophilus* is monophyletic with the sauropsid *Plasmodium* clade. ML and the topological test could not discriminate between hypothesis 2 and 3, but BI and molecular clock rooting methods gave more support for the latter hypothesis. These analyses are far from conclusive, but do suggest that *Polychromophilus* did not evolve from a mammal-infecting ancestor, but has instead invaded the mammalian class of hosts independently.

Our results show that the three DNA regions used in the combined matrix do not provide sufficient phylogenetic information to unambiguously place the *Polychromophilus* lineage. We are combining regions from different genomes, and this could introduce sufficient conflict to reduce the confidence in the reconstructed trees, even if the topology tests did not identified major incongruence. The way forward to clearly place the *Polychromophilus* lineage within the large *Plasmodium* clade is to sequence longer stretches of DNA regions, in particular from the nuclear genome, and to use gene tree approaches to identify the best evolutionary relationships at the species level (Ane et al. 2007, Heled and Drummond 2010).

Previous findings

The close relation between *Polychromophilus* and avian Haemosporida has been suggested before. Carreno *et al.* (1997) produced a phylogeny based on life-history and ultra-structure characters and concluded that *Polychromophilus* is most closely related to *Haemoproteus,* a hypothesis rejected by the current study. Megali *et al.* (2011) used a 705 bp *cytb* fragment and concluded that *Polychromophilus* shared its closest common ancestry with avian *Plasmodium*. However, the base of their tree was not well resolved. The authors themselves therefore recommended the use of multiple genes.

Duval *et al.* (2007) discussed bat Haemosporida but never identified the species. However, their molecular analyses, again using only *cytb*, grouped their samples clearly with sauropsid *Plasmodium*, leading to a similar conclusion as our current study. In the paper they cautiously did not name their collected species. However, the corresponding sequences that are available in GenBank have been identified as '*Hepatocystis* sp.'. Based on the work of Megali *et al.* (2011) it is very likely that part of those sequences are actually *Polychromophilus* species. Misidentification is a big obstacle in apicomplexan research as a whole (Morrison 2009) and haemosporidian research in particular (Valkiũnas et al. 2008). Therefore, caution is required when naming species for GenBank.

Switch of host, switch of vector

Parasitizing a new, mammalian host likely necessitated many adaptive changes, given their characteristic, non-nucleated red blood cells. The *cytb* DNA region sequenced here showed long branches of non-synonymous substitutions separating the avian from the mammal clade (Outlaw and Ricklefs 2010). Many lineages have become extinct over time during the evolution towards the mammalian and avian *Plasmodium* lineages (Ricklefs and Outlaw 2010). Nevertheless *Polychromophilus*' origin suggests that the switch to mammalian hosts happened at least twice during Haemosporida evolution. *Rayella* is thought to have originated from *Hepatocystis* (Mattingly 1983) and has been classified as such (Garnham 1966), but *Nycteria*'s origins are more elusive; whether it is a case of yet another independent host switch, or an ancient mammalian *Plasmodium* lineage that has survived the pruning on that branch, remains to be investigated.

Haematophagy has appeared multiple times in the evolution of the Diptera. It evolved once at the origin of the superfamily Hippoboscoidea and is shared by all its members (Petersen et al. 2007). Consequently, many Hippoboscoidea spp. are implicated in the transmission of diseases, most notably sleeping sickness (Glossinidae) and malaria (Hippoboscidae and Nycteribiidae). The relatively high relatedness of the latter two families (Petersen et al. 2007) is not reflected by their haemosporidian parasites. This study convincingly rejected the hypothesis that hippoboscid-transmitted *H. (Haemoproteus)* shares its most recent common ancestor with the nycteribid-transmitted *Polychromophilus*. A cospeciation event of these Haemosporida with their dipteran hosts can therefore clearly be excluded.

Instead, *Polychromophilus'* ancestor must have been vectored by of a member of the Culicidae, as are all modern *Plasmodium* species. Culicidae are one of the oldest members of the Diptera, an order with a higher radiation of species than all terrestrial vertebrates put together (Wiegmann et al. 2011). The phylogenetic distance between Culicidae and Nycteribiidae is one of the largest within the order (Wiegmann et al. 2011), yet the adaptations required for this new vector were seemingly acquired in parallel to those required for the new mammalian host.

Because the Nycteribiidae are completely specialised to bats, the first appearance of *Polychromophilus* in bats must have been mediated by either mosquitoes or via the hippoboscid flies. Many Culicidae spp. feed on both mammals and birds readily, and within the Hippoboscidae, the host switch from mammals to birds has happened several times (Petersen et al. 2007). Therefore, both could have been responsible for the first transmission. However, once *Polychromophilus*' ancestor was introduced in bats, adapting to the nycteribid vectors likely had large fitness advantages. Specifically, the haematophagous lifestyle of both males and females combined with their high prevalence on bats (Dick and Patterson 2006), and ease of moving between bat-hosts (unpublished observations), make the Nycteribiidae an ideal vector for the protozoan parasite. However, this same switch to Nycteribiidae also limited the potential range of *Polychromophilus* vertebrate hosts to the Chiroptera.

Conclusions

The phylogenetic reconstruction of three genes of *Polychromophilus* spp. demonstrates that the *P. melanipherus* and *P. murinus* are clearly two genetically distinct species. Only the addition of the other *Polychromophilus* spp. can validate the current division of *Polychromophilus* in separate subgenera. *Polychromophilus* is clearly not related to *Haemoproteus (Haemoproteus).* Instead Bayesian inference and molecular clock outgroup free phylogenetic reconstructions suggest that the *Polychromophilus* most likely had a bird- or reptile-infecting *Plasmodium* ancestor. The switch to mammalian hosts would therefore not have occurred once, but at least twice in the haemosporidian evolutionary past. This event was accompanied by the adaptation to a new, phylogenetically distant dipteran vector.

ACKNOWLEDGEMENTS

We would like to give a big thank you to Anne-Lyse Ducrest and Céline Simon for advice on and assistance with the molecular work. We would also like to give our gratitude to Emily Clark and two anonymous referees for helpful comments improving this manuscript. We thank the Swiss Institute of Bioinformatics for their support and the availability of the Vital-IT facilities. This research was supported by grant 31003A_120479 from the Swiss National Science Foundation.

SUPPLEMENTARY MATERIAL

Supplementary Table S2.1 Name, sequence and PCR conditions of the primers used.

Name		Primer sequence	annealing	extension	cycles
asl/outer	fw	GSKAARTTTAATGGKGCTGTWGG	47ºC, 30 s	72ºC, 50 s	35
	rv	GGATTAAYTTTATGAGGCATTG			
asl/nested	fw	GCTGATMAAAATRTTGATTGG	50ºC, 30 s	72ºC, 30 s	38
	rv	GAGGCATTGTACTACTWCC			
clpc/outer	fw	AAACTGAATTAGCAAAAATATTA	50ºC, 30 s	72ºC, 50 s	38
	rv	CGWGCWCCATATAAAGGAT			
clpc/nested	fw	GATTTGATATGAGTGAATATATGG	48ºC, 30 s	72ºC, 30 s	40
	rv	CCATATAAAGGATTATAWG			
coI/outer	fw	CTATTTATGGTTTTCATTTTTATTTGGTA	57ºC, 30 s	72ºC, 50 s	35
	rv	AGGAATACGTCTAGGCATTACATTAAATCC			
coI/nested Po	fw	AGCAATATCAATAGCTGCATTACCT	62ºC, 30 s	72ºC, 50 s	38
	rv	GATTTTCTTCAATATAATGCCTGGA			
cytb/outer	fw	TAATGCCTAGACGTATTCCTGATTATCCAG	55ºC, 30 s	72ºC, 50 s	35
	rv	TGTTTGCTTGGGAGCTGTAATCATAATGTG			
cytb/nested	fw	TCAACAATGACTTTATTTGG	55ºC, 30 s	72ºC, 50 s	40
	rv	TGCTGTATCATACCCTAAAG			

All denaturation and final extension periods are the same for all primer-pairs.

Supplementariy Table S2.2 Species name, host and accession numbers of sequences retrieved from GenBank for the phylogenetic reconstructions. This table contains additional host information and the GenBank accession numbers of all genes used for the phylogenetic analyses. Not all gene sequences are available for all species, missing sequences are denoted by '-'.

Species	Host species	*cytb*	*coI*	*clpc*	*asl*
Leucocytozoon					
Leucocytozoon sp.	*Buteo jamaicensis*	EU254518	EU254563	EU254609	EU254663
Leucocytozoon sp.	*Accipiter brevipes*	EU254519	EU254564	EU254610	EU254664
Leucocytozoon sp.	*Buteo lineatus*	EU254520	EU254565	EU254611	EU254665
***Plasmodium* (mammalian)**					
P. falciparum	*Homo sapiens*	AY2829721	M76611	X87631	AF033037
P. vivax	*Homo sapiens*	AY598137	AY598123	AF348344	AF262051
P. knowlesi	*Old World Monkeys*	EU880465	AY598141	AF348341	AF262052
P. yoelii	*Thamnomys rutilans*	EU254521	EU254566	EU254612	EU254666
P. berghei	*Grammomys surdaster*	EF011166	EF011199	AF348337	EU254670
P. vinckei	*G. surdaster*	EU254522	EU254567	EU254613	EU254667
P. atheruri	*Atherurus africanus*	EU254524	EU254568	EU254615	EU254669
P. chabaudi	*T. rutilans*	EF011167	EF011200	EU254614	EU254668
Hepatocystis					
Hepatocystis sp.	*Cynopterus brachyoti*	EU254526	EU254569	EU254616	EU254671
Hepatocystis sp.	*Nanonycteris veldkampii*	EU254527	EU254570	EU254617	EU254672
Hepatocystis sp.	*Nanonycteris veldkampii*	EU254528	EU254571	EU254618	EU254673
***Plasmodium* (avain and saurian)**					
P. mexicanum	*Sceloporus occidentalis*	AY099060	EU254572	EU254619	EU254674
P. floridense	*Anolis oculatus*	EU254530	EU254573	EU254620	EU254675
P. azurophilum R	*A. oculatus*	EU254532	EU254575	EU254622	EU254677
P. azurophilum W	*A. oculatus*	EU254533	EU254576	EU254623	EU254678
Plasmodium sp.	*Ameiva ameiva*	EU254537	EU254580	-	EU254684
P. giganteum	*Agama agama*	EU254534	EU254577	EU254624	EU254679
Plasmodium sp.	*Acridotheres tristis*	EU254542	EU254585	EU254636	EU254693
P. gallinaceum	*Gallus gallus*	NC_008288	EU254578	EU254625	EU254680
P. relictum	*Emberiza hortulana*	EF011193	EF011226	EU254627	EU254682
P. relictum	*Corvus corone*	DQ451404	EU254593	EU254645	EU254701
Plasmodium sp.	*Emberiza hortulana*	EF011194	EF011227	EU254628	EU254683
Plasmodium sp.	*Spizella passerina*	EF011176	EF011209	EU254632	EU254688
P. relictum	*Sialia mexicana*	EU254538	EU254581	EU254633	EU254689
P. relictum	*Zenaida macroura*	EU254536	EU254579	EU254626	EU254681
Plasmodium sp.	*Luscinia svecica*	EU254540	EU254583	EU254634	EU254691
Plasmodium sp.	*Larosterna inca*	EU254547	EU254590	EU254641	EU254698

continued on next page

Supp. Table S2.2, continued from previous page

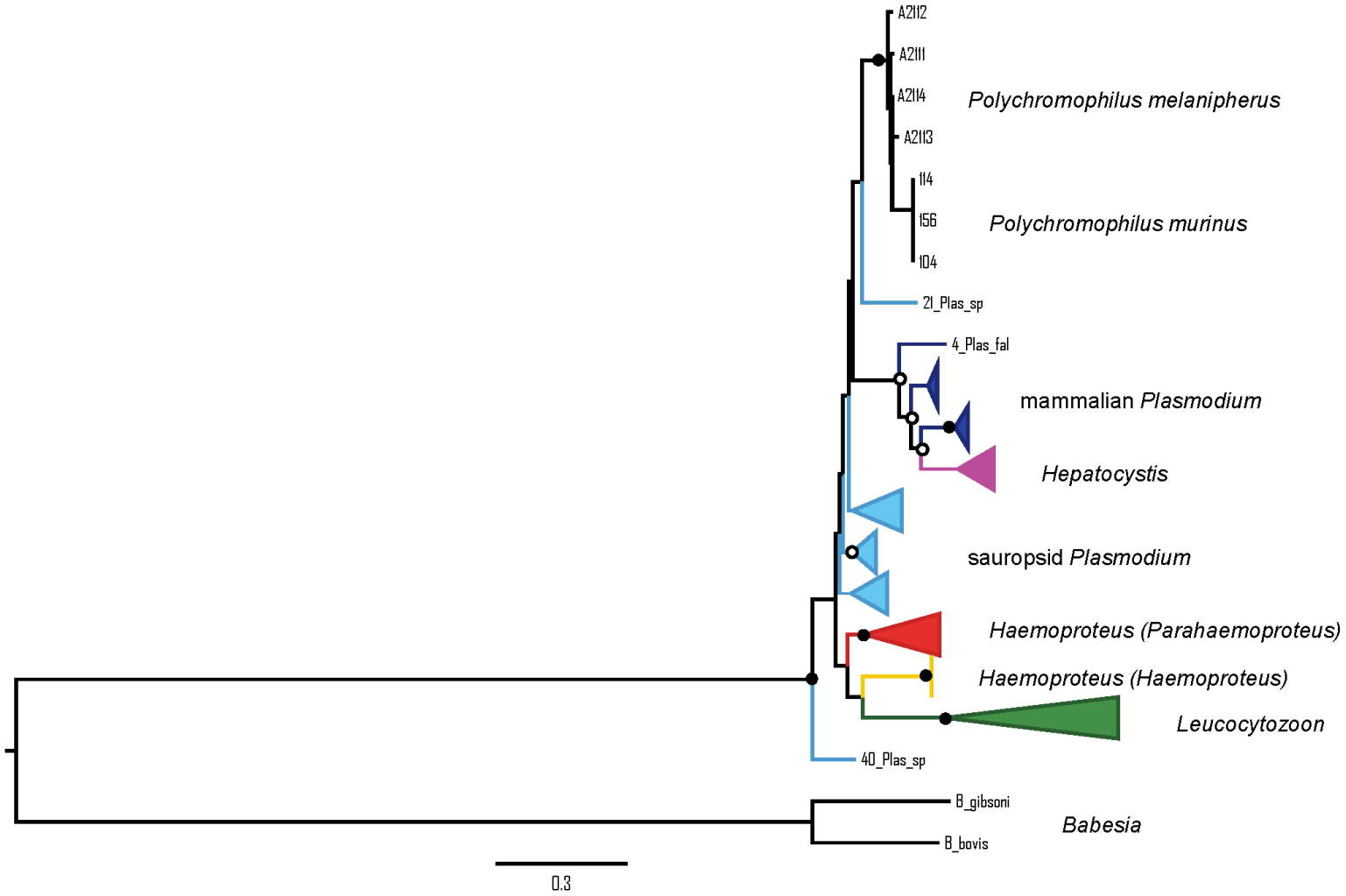
Plasmodium sp.	*Melospiza melodia*	EF011168	EF011201	EU254629	EU254685
Plasmodium sp.	*Aegolius acadicus*	EU254543	EU254586	EU254637	EU254694
Plasmodium sp.	*Accipiter striatus*	EU254539	EU254582	-	EU254690
Plasmodium sp.	*Ixobrychus minutus*	EU254541	EU254584	EU254635	EU254692
Plasmodium sp.	*Agelaius phoeniceus*	EF011171	EF011204	EU254630	EU254686
Plasmodium sp.	*Seiurus aurocapilla*	EF011173	EF011206	EU254631	EU254687
Plasmodium sp.	*Hylocichla mustelina*	EU254544	EU254587	EU254638	EU254695
Plasmodium sp.	*Turdus migratorius*	EU254545	EU254588	EU254639	EU254696
Plasmodium sp.	*Anthus trivialis*	EU254546	EU254589	EU254640	EU254697
Plasmodium sp.	*Egernia stokesii*	EU254531	EU254574	EU254621	EU254676
Haemoproteus (Parahaemoproteus)					
H. syrnii	*Strix selupto*	DQ451424	EU254591	EU254643	EU254700
H. turtur	*Streptopelia senegalensis*	DQ451425	EU254592	EU254644	-
H. picae	*Picoides pubescens*	EU254552	EU254597	EU254650	EU254706
Haemoproteus sp.	*Bonasa umbellus*	EU254555	EU254600	EU254654	EU254709
Haemoproteus sp.	*Mergus merganser*	EU254560	EU254606	EU254660	-
Haemoproteus sp.	*Bucephala clangula*	EU254561	EU254607	EU254661	-
H. magnus	*Fringilla coelebs*	DQ451426	EU254594	EU254647	EU254703
H. fringillae	*Zonotrichia albicollis*	EU254558	EU254604	EU254658	EU254711
H. belopolskyi	*Sylvia curruca*	DQ451408	EU254603	EU254657	EU254710
Haemoproteus sp.	*Vireo olivaceus*	EU254551	EU254596	EU254649	EU254705
H. coatneyi	*Dendroica coronata*	EU254550	EU254595	EU254648	EU254704
Haemoproteus sp.	*Dendroica caerulescens*	EU254562	EU254608	EU254662	-
H. passeris	*Passer moabiticus*	EU254554	EU254599	EU254653	EU254708
H. sanguinis	*Pycnonotus xanthopygos*	DQ451410	EU254598	EU254651	-
Haemoproteus sp.	*Chamaea fasciata*	EU254557	EU254602	EU254656	-
Haemoproteus sp.	*Dumetella carolinensis*	EU254559	EU254605	EU254659	-
Haemoproteus sp.	*Falco sparverius*	EU254556	EU254601	EU254655	-
Haemoproteus (Haemoproteus)					
H. columbae	*Columba livia*	EU254548	FJ168562	EU254642	EU254699
H. columbae	*Columba livia*	EU254549	-	EU254646	EU254702
H. columbae	*Columba livia*	EU254553	-	EU254652	EU254707
***Babesia* (Piroplasmida)**					
B. bovis	-	GQ214235	AB499088	NC011395	-
B. gibsoni	-	AB215096	AB499087	-	-

Supplementary Table S2.3 The haplotypes and corresponding accession numbers for GenBank per sequenced sample per gene**.**

	asl		***clpc***		***coI***		***cytb***	
ind.	**ht.**	**acc. nb.**	**ht.**	**acc. nb.**	**ht.**	**acc. nb.**	**ht.**	**acc. nb.**
104	Pmu1	JN990725	Pmu1	JN990723	Pmu1	JN990718	Pmu1	JN990712
114	Pmu1	..	Pmu2	JN990724	-	-	Pmu1	..
156	Pmu1	..	Pmu1	..	Pmu2	JN990719	Pmu2	JN990713
A2111	-		Pme3	JN990720	Pme3	JN990714	Pme3	JN990708
A2112	Pme2	JN990726	Pme4	JN990721	Pme4	JN990715	Pme4	JN990709
A2113	-	-	Pme5	JN990722	Pme5	JN990716	Pme5	JN990710
A2114	-	-	-	-	Pme6	JN990717	Pme6	JN990711

Samples 104, 114 and 156 are *Polychromophilus murinus*, sampled from *Myotis daubentoni* and shared some haplotypes. The samples A2111-A2114 are *Polychromophilus melanipherus* from *Miniopterus schreibersii* and never shared haplotypes. For each unique haplotype, the GenBank accession number is mentioned only once in the table. '..': accession number already mentioned. '-': sequencing was unsuccessful.

Supplementary Figure S2.1 A topology rooted with *Babesia* provides little information. The amino acid alignment provides too little contrast to construct a tree with high support as most nodes are unsupported. A very long branch separates the *Babesia* species from all Haemosporida. Shown is the best tree of a ML analysis using a JTT + Γ + I substitution model and bootstrapping a 1000 times. Closed dots: bootstrap value > 90; Open dots: bootstrap values > 50.



Chapter 3

How malaria gets around:

the genetic structure of a parasite, vector and host compared

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keywords: Haemosporida, Nycteribiidae, coevolution, codifferentiation, population genetics, epidemiology

ABSTRACT

Parasite population structure is often thought to be largely shaped by that of its host. In the case of a parasite with a complex life cycle, two host species, each with their own patterns of demography and migration, spread the parasite. However, the population structure of the parasite is predicted to resemble only that of the most vagile host species. In this study we tested this prediction in the context of a vector-transmitted parasite. We sampled the haemosporidian parasite *Polychromophilus melanipherus* across its European range, together with its bat fly vector *Nycteribia schmidlii* and its host, the bent-winged bat *Miniopterus schreibersii*. Based on microsatellite analyses, the wingless vector, and not the bat host, was identified as the least structured population and should therefore be considered the most vagile host. Genetic distance matrices were compared for all three species based on a mitochondrial DNA fragment. Both host and vector populations followed an isolation-by-distance pattern across the Mediterranean, but not the parasite. Mantel tests found no correlation between the parasite and either the host nor the vector. We found therefore no support for our hypothesis. Moreover, the parasite population did not seem to be an intermediate form of either host or vector. Instead, we hypothesize that the discrepancies might be caused by within-host-species dispersal heterogeneity linked to the parasite, as well as parasite founder effects or high parasite turn-over within the hosts' populations.

INTRODUCTION

Parasites are more and more being used as genetic tags of their hosts, which can give additional demographic information when a host population shows low genetic variability (Bruyndonckx et al. 2010). Moreover, parasites can reveal non-reproductive contact between different host populations or even different host species (Bruyndonckx et al. 2009a). Naturally, not all parasite species are as suitable as others. Certain mating systems, life-history traits or demographic characteristics can make them less convenient or positively misleading proxies for a host's genealogy (Nieberding and Olivieri 2007).

Parasites with complex life cycles are parasites which need a minimum of two hosts to complete their life cycle. Such heteroxenous parasites are potentially unsuitable tags since any genetic signal might come from either of the two host species. However, how the population structure of heteroxenous parasites is shaped by both hosts remains unclear. Is the pattern solely dependent on 'the most motile host' (e.g. Louhi *et al.* 2010)? Alternatively it is the definitive host, where sexual reproduction takes place, who determines the parasite's phylogeography. Or parasite dispersal might be mainly determined by the more numerous host, or, the host with the shortest life cycle.

Jarne and Théron (2001) proposed that, a priori, the population structure of a heteroxenous parasite should closely mimic that of its least structured host. The reasoning being that for its overall gene-flow, the parasite is dependent on the host with highest dispersal. Any potential signal of the more strongly isolated species would be erased by a more motile host with more frequent dispersal events. A second factor influencing parasite dispersal is its free-living stage. Though spores and eggs have the potential to be distributed over a large range, their effect on the parasites gene-flow has been studied only minimally (Jarne and Theron 2001).

Many socially and economically important diseases, like malignant malaria, chagas and lyme disease, are caused by vector-transmitted parasites. These can be considered a special class of heteroxenous parasites, for several reasons. First of all, the two host species required for completion of their life cycle are from much diverged taxa, different phyla in general. Moreover, these parasites are obligate, permanent parasites; they cannot live outside the host and have no free-living stages. As a consequence, for the parasite to complete its life-cycle, multiple encounters between the host and

vector species are needed. A last peculiarity of many vectored parasites is that one of the two hosts, 'the vector', is itself dependent on the other host for survival and can be considered a parasite itself.

Despite the peculiarities of the system, surprisingly few studies have looked into how vector and host populations interact in shaping the genetic structure of the parasite. Levin and Parker (2013) found a complete absence of differentiation in an island population of the haemosporidian parasite *Haemoproteus iwo.* The hippoboscid vector *Olfersia spinifera* had much lower population differentiation than the avian host *Fregata minor,* which might explain the lack of differentiation in *H. iwo*. However, the authors could not compare patterns of gene-flow due to the complete invariability of the parasite cyt*b* marker used. Gomez-Diaz *et al.* (2011) detected large genetic variability in populations of *Borrelia garinii* across an entire oceanic region but found it to be unstructured despite the presence of host-races within the tick vector across this region (McCoy *et al.* 2005). In both studies, the parasite was a generalist, and both vectors, the tick and hippoboscid fly, may feed on several species of bird. Dispersal patterns of parasite and hosts are therefore not expected to compare one-to-one; dispersal opportunities provided by unsampled host species could cause unexplainable discrepancies in the patterns of gene-flow.

The goal of the current study is to test the hypothesis that heteroxenous parasite dispersal patterns mimic those of its most vagile host (Jarne and Theron 2001) by comparing and quantifying the patterns of gene-flow of a specialist parasite, vector and host. To test this, we use a haemosporidian parasite, *Polychromophilus melanipherus* (Apicomplexa: Haemosporida), a malaria-like blood parasite of bats. In its European range, it mainly parasitizes *Miniopterus schreibersii* (Chiroptera: Miniopteridae), but is known to infect other members of the Miniopteridae in Africa (Garnham 1966, Lanza 1999, Duval et al. 2012). *Nycteribia schmidlii* (Diptera: Nycteribiidae) is a small wingless bat fly that is a host-specific ectoparasite of *M. schreibersii* (Theodor 1957), though can occasionally be found on other bat species sharing the same cave (Lanza 1999). Although it has never been formally demonstrated, it is the suspected vector of *P. melanipherus*, as it is the most common bat fly of *M. schreibersii* (Estrada-Peña and Serra-Cobo 1991), occurring throughout its European range (Theodor 1967) and is closely related to *N. kolenatii*, the confirmed vector of *P. murinus* (Gardner et al. 1987).

Using microsatellites, we first tested which is the more vagile species, the bat *M. schreibersii* or the bat fly *N. schmidlii*. Next, using mitochondrial markers, we tested the hypothesis whether the

dispersal patterns of *P. melanipherus* mimic more those of its vector or its host. Lastly, any absence of correlation between the parasite and host populations might be caused by the host population not behaving homogenously. Bats infected with *P. melanipherus* might show different dispersal patterns than the non-infected part of the population. We looked for any such patterns by separating the *M. schreibersii* based on their infection with *P. melanipherus*.

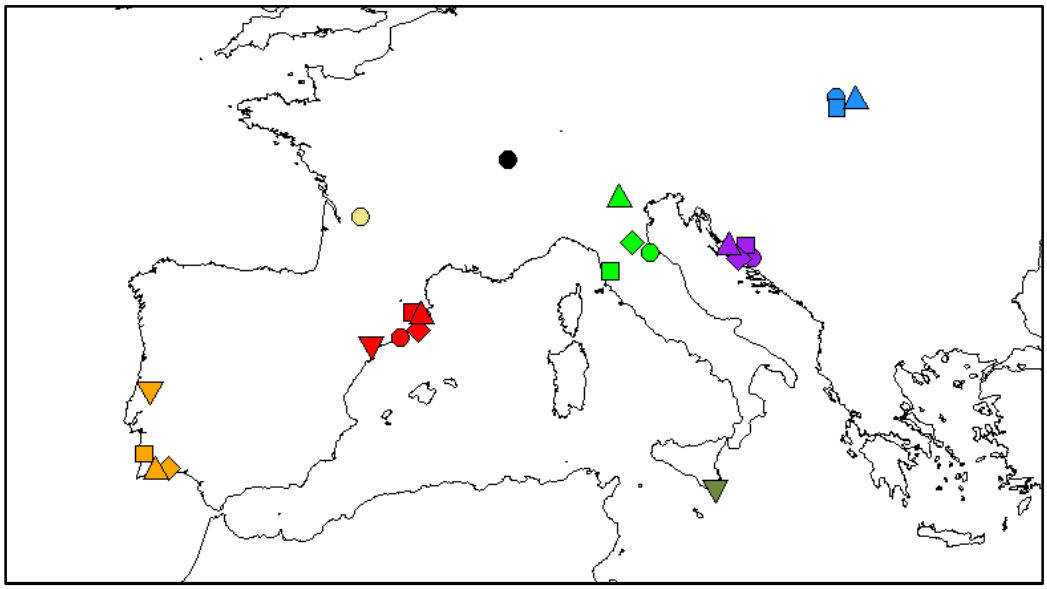

Figure 3.1 Map of Southern Europe with sampling sites. Symbols according to Table 3.1.

METHODS

Sampling

Sampling was done in 2011 and 2012, from May to September on 23 different sites across Southern and Central Europe (Table 3.1, Figure 3.1). *Miniopterus schreibersii* were captured at their roosts either upon emergence using mist nets and harp traps at dusk, or by entering the roosts at day time and collecting them while they were resting. Bat flies were looked for by blowing through the fur of the bat, collected using soft forceps and immediately stored on 96% ethanol.

A wing biopsy was taken using a 1 mm gauge punch (Stiefel) and stored on 96% ethanol. Approximately 15 µL of blood was taken by puncturing the uropatagial vein with a 0.5 mm gauge needle (Neolus, Terumo). Blood beads forming on the patagium were captured by pipetting or using a

heparinized glass microcapillary tubes (Marcel Blanc & Cie) and ejected on white blotting paper (3MM, Whatman) which was left to dry and later stored in separate envelopes. Wounds were treated with haemostatic cotton until bleeding had stopped after which bats were released at the site of capture. All captures were done in accordance with local regulations.

Table 3.1 Overview of sampling effort.

Name	Region	N	N inf.	N nyct.
Cercal	Portugal	15	12 (11)	6
Preguiça	Portugal	11	11 (11)	8
Soïdos	Portugal	11	10 (10)	5
Nabão	Portugal	15	15 (11)	22
Collserola	Spain	15	14 (11)	14
Savassona	Spain	15	11 (8)	9
Montnegre	Spain	14	13 (10)	21
Les Gavarres	Spain	14	14 (10)	7
Llaberia	Spain	15	15 (12)	24
St-Médard	France	15	7 (7)	3
Baulmes	Switzerland	16	9 (8)	0
San Marino	Italy	15	12 (12)	29
Monte Catini Val di Cecina	Italy	15	6 (4)	43
Riolo Terme	Italy	15	9 (8)	17
Velo Veronese	Italy	3	1 (1)	9
Marzamemi	Sicily	16	11 (11)	0
Rumin	Croatia	9	8 (5)	2
Kijevo	Croatia	15	13 (11)	5
Drnis	Croatia	15	12 (11)	9
Karin Gornji	Croatia	6	6 (6)	2
Nandraž Bradlo	Slovakia	15	4 (4)	16
Chvalovská jaskyňa	Slovakia	15	10 (9)	17
Drienovecká jaskyňa	Slovakia	15	8 (8)	10

N: number of *M. schreibersii* sampled, N inf.: number of bats infected with *P. melanipherus* (number of single clone infections), N nyct.: number of *N. schmidlii* bat flies collected.

DNA extraction and amplification

DNA was extracted from the blood-soaked blotting paper using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's tissue protocol with the following exceptions. A fragment of paper of ~10mm in diameter was cut in smaller pieces using sterile scissors, added to a 2 mL tube (Eppendorf) containing 360 µL of ATL buffer and incubated at 90°C for 15 min. After cooling down, 40 µL of Proteinase K was added to each sample and left for overnight digestion at 56°C. At the final

step 55 µL elution buffer was added to the filter and samples were incubated at 37°C for 15 min. before centrifuging. This step was repeated once to maximize recuperation. For a subset of individuals, extra DNA was extracted from the wing biopsy following the same tissue protocol with the addition of an initial rinsing phase where ethanol was removed from the tissue by soaking the biopsy in 1 mL of pure MilliQ water (Millipore).

For all collected bat flies, species was determined following the key by Theodor (1967). All specimens identified as *Nycteribia schmidlii* were rinsed of their ethanol by soaking in in 1mL of pure MilliQ water (Millipore) at room temperature for 2-3 hrs. Because females most often carry a developing larva, their abdomens were removed to prevent paternal contamination. Afterwards individual flies were triturated using sterile pestles. After an overnight digestion the standard Biosprint 96 tissue protocol (Qiagen) was followed.

Marker amplification

To determine the genetic structure of *M. schreibersii* in Europe, all individuals were genotyped at 14 polymorphic microsatellite loci (Supp. Table S3.1; Miller-Butterworth et al. 2002, Wood et al. 2011). Primers, PCR thermal profiles and reagent concentrations can be found in Supplementary Tables S3.1-S3.3. PCR products were controlled on an agarose gel 2% under UV light. Four post-PCR multiplexes were realized after different tests by adjusting the ratio of each reaction in a final volume of 3 µl (Supp. Table S3.1). PCR products were typed in an ABI Prism 3100 sequencer (Applied Biosystems).

For *N. schmidlii* 10 new microsatellite markers were developed commercially (Ecogenics, Zurich-Schlieren, Switzerland). Size selected fragments from genomic DNA were enriched for microsatellite content by using magnetic streptavidin beads and biotin-labeled CT and GT repeat oligonucleotides. The microsatellite-enriched library was analysed on a Roche 454 platform using the GS FLX titanium reagents. Reads were selected for tetra- or trinucleotide repeats of at least 6 units long or a dinucleotide repeat of at least 10 units long. Of these reads 1'173 were suitable for primer design, which resulted in primers for 10 new markers (Supp. Table S3.4).

The 10 new microsatellite markers for *N. schmidlii* were combined in 2 multiplexes, each reaction combining 5 loci each. See Supplementary Tables S3.2, S3.3 for the thermal profiles and

reagent concentrations respectively. Post-PCR reaction products were diluted 6x of which 3 µL was finally genotyped on an ABI prism 3100 sequencer.

The detection of *P. melanipherus* infection in the vertebrate host's blood was done by amplifying a 705 bp cytochrome *b* fragment (cyt*b*) of the parasite following a nested PCR protocol. Primers, reagents and PCR temperature profile were taken from Megali et al. (2011; Supp. Tables S3.2, S3.3). Bands ran on a 1% agarose gel and stained with ethidium bromide and were visualized under UV light. Each sample was tested in duplicate. Samples that gave ambiguous results were retested. Positive samples were either purified with the Wizard PCR clean-up system (Promega) and sequenced locally on a ABI Prism 3100 genetic analyzer (Applied Biosystems), or sent to a commercial agent for sequencing (Microsynth, Switzerland).

To compare the molecular variability of the parasite with that of the vector and host, also a mitochondrial gene fragment of vector and host was amplified. For *N. schmidlii,* 408 bp. of the 16S ribosomal subunit (16S) was amplified using the primers fw: 5'-CGC CTG TTT AAC AAA AAC AT-3' and rv: 5'-TGA ACT CAG ATC ATG TAA GAA A-3' (Petersen *et al.* 2007). For *M. schreibersii* 310 bp of the mitochondrial control region (CR, often referred to as 'd-loop') was amplified using the forward primer 5'-CAT CTG GTT CTT ACT TCA GG- 3' (Fumagalli *et al.* 1996) and a newly designed reverse primer 5'-GTG CAC AGT CGT AAT CTC-3'. Conditions and reagents for both amplifications can be found in Supp. Tables S3.2, S3.3. Purification and sequencing using the forward primers was performed by a commercial agent (Microsynth, Switzerland).

Microsatellite analyses

Microsatellite allele sizes were scored and sized by hand with the aid of the software GeneMapper (Applied Biosystems). Micro-Checker v2.2.3 (Van Oosterhout *et al.* 2004) was used to investigate large allelic drop out, stuttering and null alleles. The software FSTAT v2.9.3.2 (Goudet 2001) was used to test for linkage disequilibrium between loci. Deviation from Hardy-Weinberg equilibrium across all markers and populations was tested for with the X^2-test from the package hierfstat v0.04-10 (Goudet 2005) in R v3.0.1(R Core Team 2012). With the same package the observed heterozygosity, within population and overall gene diversity, allelic richness and within population heterozygote deficiency F_{IS} were calculated for each species. For both the bats and the bat flies, a hierarchical

population structure was assumed where sampling sites were clustered in regions (Table 3.1). To partition the components of genetic variation among regions and sites, a hierarchical analysis of variance (ANOVA) was performed using the function varcomp.glob of the hierfstat R-package. Significance of the variance components was tested using the likelihood ratio G-statistic permutation tests from the same package.

A pattern of isolation-by-distance between sampling sites was examined by looking at the relation between the pairwise F_{ST}'s, x / (1-x) transformed, and the natural logarithm of the overland geographical distances (Rousset 1997). The correlations between the genetic and geographical distance matrices were tested using a mantel test (1000 permutations) using the mantel function from the R-package ade4 v1.5-2 (Dray and Dufour 2007).

For both the bats and bat flies a principle component analysis (PCA) was performed on the population allele frequencies, weighted by population size to prevent small populations from having disproportional effects. The eigenvectors were then calculated using the function dudi.pca from the ade4 package. Significance of the inertia of the first two components was calculated by permuting the population assignment of each individual and recalculating the inertia using a custom script in R.

The most likely number of clusters in each species' European population was estimated within the baysian framework of Structure v2.3.4 (Pritchard et al. 2000). A model with admixture and correlated alleles (Falush et al. 2003) was used to assign individuals to any of the predestined number of clusters, ranging from 1 to 8, based on the number of regions sampled. The use of sampling site as a prior is recommended when the genetic signal is weak, but it also puts constrains on the analysis (Hubisz *et al.* 2009). Because the signal in our few loci was suspected to be low, we included site as a prior, but repeated the analysis without as to assess its impact on the result. Each simulation had a burn-in of 50'000 generations and data collection for 100'000 generations and 10 replicates were performed for each parameter setting. The most likely number of clusters was determined by looking at the raw loglikelihood, as well as ΔK, the ratio of the absolute rate of change of the loglikelihood and its standard deviation (Evanno et al. 2005), as implemented in Structure Harvester (Earl and Vonholdt 2012).

Mitochondrial sequence analysis

The chromatographs of all mtDNA sequences were manually checked, edited and aligned with the software Mega 5.03 (Tamura *et al.* 2011). Identification and characterization of haplotypes and the sequence diversity of the sampling sites were done with the aid of the pegas v0.4-5 package in R (Paradis 2010). A minimum-spanning network was created of the haplotypes for each of the three species in TCS v1.21 (Clement et al. 2000). Neutral evolution of each fragment was assessed by calculating Tajima's D and tested for significant deviation from zero using the function tajima.test from pegas, customized to take into account insertion-deletions (indels).

As with the microsatellite analyses, the components of genetic variation were partitioned hierarchically, though not only based on the local haplotype frequencies, but also on their molecular distances, in an analysis of molecular variance (AMOVA; Excoffier et al. 1992). To calculate the molecular distance between sequences two different models of sequence evolution were used: 1) the Kimura 2 parameter model of nucleotide evolution, ignoring indels (Kimura 1980) or 2) the absolute number pairwise differences, where the presence of different sized indels were considered a single difference, irrespective of their size difference in base pairs. Besides the AMOVA, a standard ANOVA (disregarding molecular distances among haplotypes) was performed allowing the identification of the effects of the evolutionary model, and tested using the AMOVA randomisation test from the ade4 package.

Arlequin v3.1 (Excoffier et al. 2005) was used to produce pairwise Φ_{ST} distance matrices for each species. These population distance matrices were based on both the frequency sequence data of the mitochondrial fragments and were used as measures of genetic distance between each sampling site for each species. These were used to test for isolation-by-distance between sites after transformation as described for the microsatellites. Moreover, the bat and bat fly matrices were compared with their microsatellite counterpart using the mantel test (1000 permutations) from the ade4 package. Lastly, the Φ_{ST} distance matrix of *P. melanipherus* was compared with both nuclear and mitochondrial distance matrices of both *M. schreibersii* and *N. schmidlii*. To test for different dispersal patterns based on infection, the mantel test was repeated for *M. schreibersii*, including only individuals of the host population infected with *P. melanipherus*.

To test for potential resistance among bat genotypes, we looked for association between infection status and the haplotype of the bat host. All sampling sites were pooled and only haplotype frequencies ≥ 5 were used to count observed haplotype frequencies of infected individuals. The expected frequencies were based on both haplotype frequencies and infection rates of the local sites. Using these, a standard goodness-of-fit X^2-test was performed.

In search of a genetic signature of the parasite in the European bat population, we split the *M. schreibersii* in infected and uninfected individuals and recalculated for both subpopulations the basic population genetic statistics (observed heterozygosity H_O, gene diversity H_S, allelic richness k_a, nucleotidic diversity π, haplotypic richness k_h). Significance of any difference was assessed by permutation of infection status and recalculating these statistics 1000 times. F_{ST}'s were calculated for both infected and uninfected bats. To arrive at similar sample sizes in each subset, only sites with infection rates between 0.3 - 0.7 were selected. The difference in the obtained F_{ST}'s was tested for significance by a 1000 permutations of infection status within sampling site and recalculating the difference in F_{ST} for the two subsets.

RESULTS

Miniopterus schreibersii and N. schmidlii: comparison of microsatellites

Three microsatellite loci of *M. schreibersii*, MM01, MM29 and MM34, had only a single allele and were removed from any further analyses, leaving the total number of markers at 11. No signs of stuttering, allelic drop out or null alleles were found in the bat microsatellite dataset and no loci showed signs of linkage. Eight population-locus combinations were not in Hardy-Weinberg equilibrium after Bonferroni correction. However, these were scattered randomly across the total 253 comparisons and were therefore left in the data set.

In the *N. schmidlii* data all markers were polymorphic. However, two loci, Ns134 and Ns719, produced null alleles consistently across populations. These loci were removed from any further analyses. No marker pair showed linkage and all markers were in Hardy-Weinberg equilibrium for each population.

Table 3.2 Hierarchical analysis of the variance components of genetic diversity of *M. schreibersii* and *N. schmidlii*.

		F-value	F'	CI	p
M. schreibersii	F_{ST}	0.0629	0.1391	0.0482 - 0.0798	0.001
	F_{SC}	0.0203	0.0449	0.0111 - 0.0311	0.001
	F_{CT}	0.0434	0.0986	0.0332 - 0.0534	0.001
N. schmidlii	F_{ST}	0.0053	0.0240	0.0012 - 0.0108	0.014
	F_{SC}	-0.0010	-0.0046	-0.0040 - 0.0015	0.497
	F_{CT}	0.0063	0.0284	0.0019 - 0.0137	0.002

N.B. CI: 95% confidence interval; p: p-value based on 1000 permutations

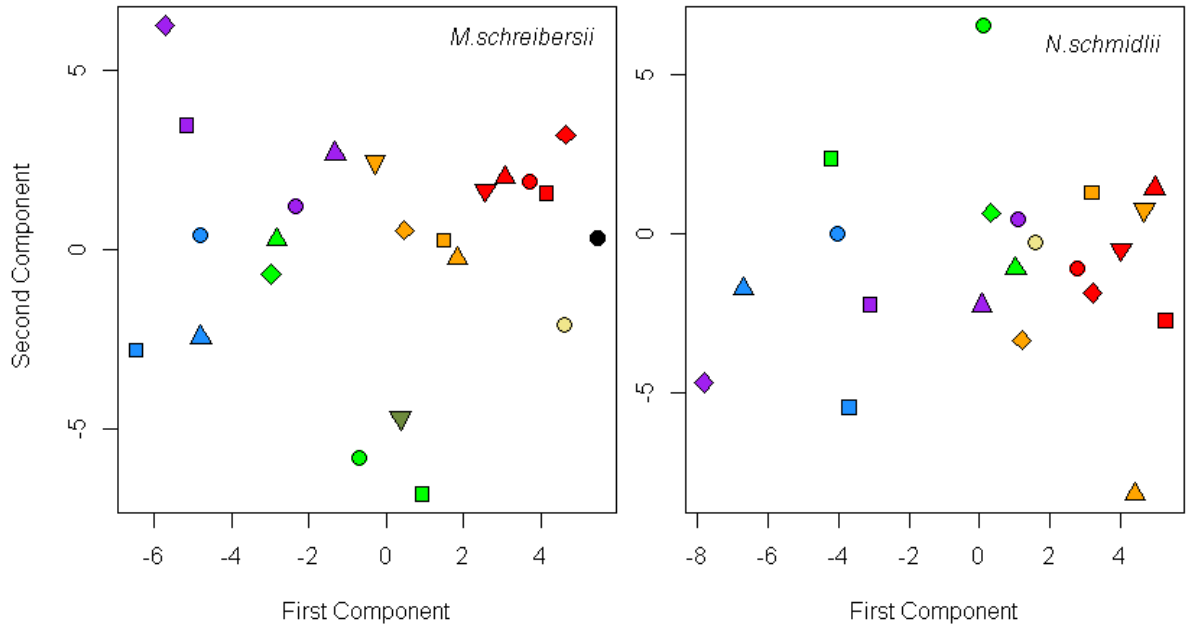

Figure 3.2 Ordinal plot of the principle component analyses of the microsatellite allele frequencies. Colouring according to sampling region (see Table 3.1). Left *M. schreibersii*, right *N. schmidlii*. PC1 was significant for both species, PC2 only for *M. schreibersii*.

The bat fly markers tended to be more diverse than those of the bats. Both the absolute number of alleles and gene diversities (H_S, H_T) were higher in the flies (Supp. Table S3.5). Bat flies showed slightly higher values of F_{IS}, both across markers and populations (Supp. Table S3.5, S3.6). At higher

hierarchical levels, F_{CT} and F_{ST} were 8-10 times higher in bats than bat flies (Table 3.2). Corrected for the lower gene diversity the bats still show a 6-fold higher level of isolation (Table 3.2: F'-values). Within region, the differentiation between sampling sites was only significantly different from zero in the bats, not in the bat flies. In both species, the structuring followed an isolation-by-distance pattern (mantel test; *M. schreibersii*: r=0.396, p=0.001; *N. schmidlii*: r= 0.275, p=0.002; Supp. Figure S3.2A-B) but had twice the explanatory power in the bat (r^2=0.157) than in the bat fly (r^2=0.076). In contrast, the population isolation patterns of each species did not correlate with each other (mantel test; *M. schreibersii* vs. *N. schmidlii*: r=0.020, p=0.434; Supp. Figure S3.2C).

For both *M. schreibersii* and *N. schmidlii*, the first principle component (PC1) explained a significant amount of the allelic variation observed in the European populations (permutation tests: *M. schreibersii*: inertia=0.148; p=0.001; *N. schmidlii*: inertia= 0.114; p= 0.006). In both species, this axis demonstrated an east-west pattern, except Portugal taking up a remarkably central position (Figure 3.2). Inertia of PC2 was significant for *M. schreibersii*, but not *N. schmidlii* (permutation tests: *M. schreibersii*: intertia = 0.105, p=0.001; *N. schmidlii*: inertia = 0.081; p=0.884). PC2 separates the more Western and Southern Italian sites (together with the Sicilian site), from the other *M. schreibersii* samples (Figure 3.2). Overall, the *M. schreibersii* samples were more clumped by region whereas the *N. schmidlii* sites were more mixed among regions.

Following the method of Evanno (2005), K=2 was indicated as the most likely number of clusters in the European population of *M. schreibersii* (ΔK_2 = 37.06, mean lnL= -8216.7, Figure 3.3A). When sampling site was left out as a prior, K=2 remained the most likely solution. According to the structure plot, the Spanish, French and Swiss sites formed one cluster and all sites more east a second. The Portuguese samples were identified as an admixture of the two clusters. Because Structure will only detect the highest hierarchical level in case of hierarchical clustering (Evanno et al. 2005), we created subsets of the data according to the sites' cluster assignment scores from the previous analysis. Subsets were made either 'strict', including only those sites clearly belonging to the cluster (assignment score > 0.66), or 'lenient' (assignment score > 0.33). The latter resulted in datasets including the 'hybrid' sites. Reanalysing these subsets (using the same parameter settings as before) resulted in a total number of subclusters ranging from k=5-9, depending on subset used (Figure 3.3B-C).

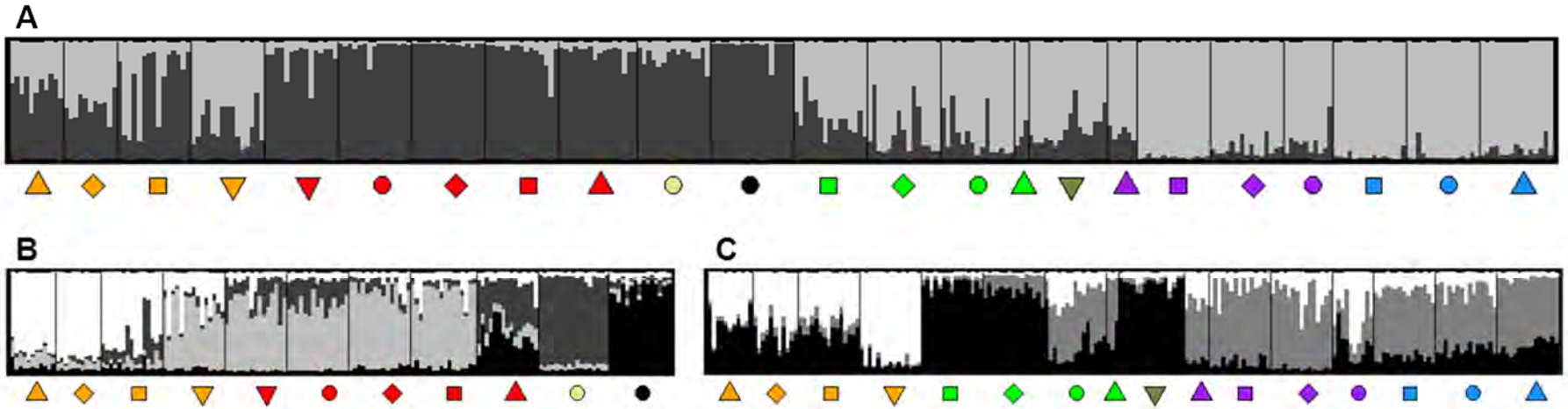

Figure 3.3 Structure assignment plots for *Miniopterius schreibersii* based on 11 microsatellites, including sampling site as a prior. (A) All sites; (B) the Western 'lenient' subset; (C) the Eastern 'lenient' subset, including Portugal. For symbol legend, see Table 3.1.

Table 3.3. Analysis of molecular variance of the mtDNA fragments, for three mutation models. Significant values are in bold.

		Equidistant				**Pairwise differences**			**Kimura 2p**		
		F-value	**F'**	**%**	**p-value**	**Φ-value**	**%**	**p-value**	**Φ-value**	**%**	**p-value**
M. schreibersii	F_{ST}	**0.1914**	**1**	80.86	0.0001	**0.3245**	67.55	0.0001	**0.3231**	67.69	0.0001
	F_{SC}	**0.0961**	**0.5067**	8.6	0.0001	**0.1089**	8.26	0.0001	**0.1084**	8.23	0.0001
	F_{CT}	**0.1054**	**1**	10.54	0.0001	**0.2419**	24.19	0.0001	**0.2407**	24.07	0.0001
N. schmidlii	F_{ST}	**0.0885**	**0.3318**	91.15	0.0001	**0.4068**	59.32	0.0001	**0.502**	49.8	0.0001
	F_{SC}	-0.0007	-0.0028	-0.07	0.6679	0.0265	1.61	0.2178	0.0338	1.74	0.2283
	F_{CT}	**0.0891**	**0.3336**	8.91	0.0007	**0.3906**	39.06	0.0002	**0.4846**	48.46	0.0001
P. melanipherus	F_{ST}	**0.163**	**0.4948**	83.7	0.0001	**0.1592**	84.08	0.0001	**0.1592**	84.08	0.0002
	F_{SC}	**0.0531**	**0.1456**	4.69	0.0475	0.0527	4.68	0.0755	0.0526	4.67	0.0752
	F_{CT}	**0.1161**	**0.4087**	11.61	0.0001	**0.1125**	11.25	0.0019	**0.1125**	11.25	0.0027

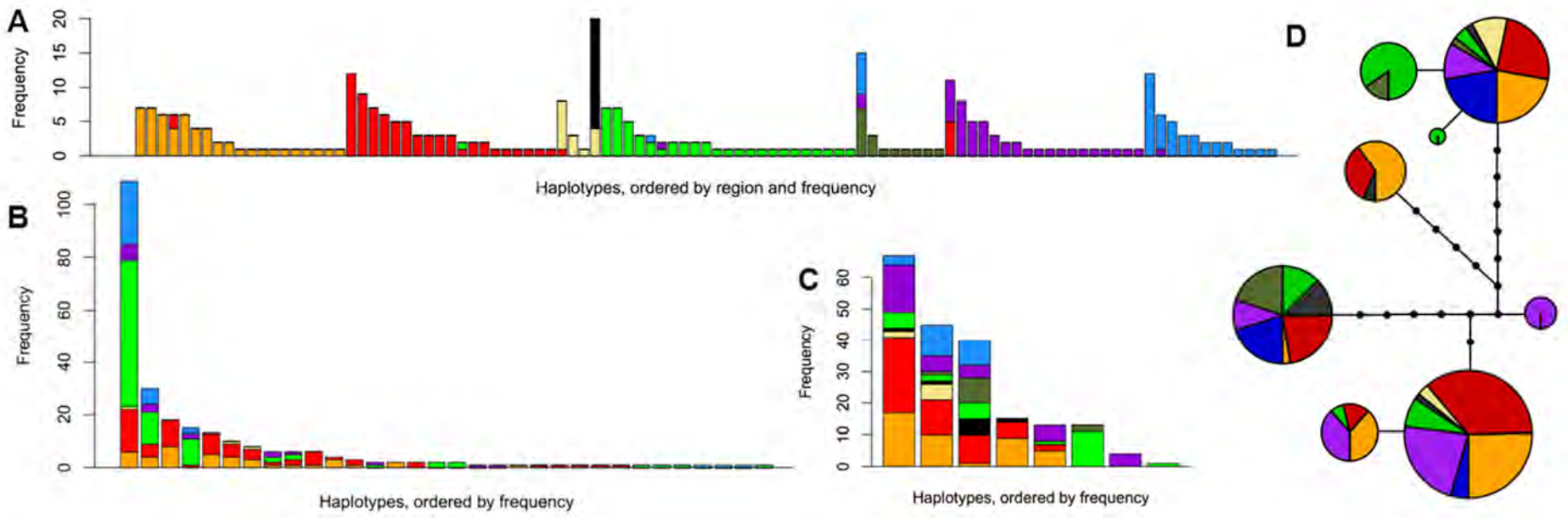

Figure 3.4 Frequency of each mitochondrial haplotype and distribution among the different regions sampled. Colour-coding according to region (Table 3.1). (A) 103 haplotypes of *M. schreibersii*; (B) 32 haplotypes of *N. schmidlii*; (C) 8 haplotypes of *P. melanipherus*. (D) A minimum spanning network of the 8 cyt*b* haplotypes of *P. melanipherus* in Europe. Haplotypes are represented by pie charts, the colour represent the regions where this haplotype was found and the size of the pie chart is relative to the total frequency of the haplotype. Each line represents a single nucleotide mutation, black dots represent unobserved hypothetical haplotypes.

In contrast, the most likely number of clusters for the *N. schmidlii* data was K=1, which was independent of use of the site-prior. Simulations with a single cluster produced the highest absolute log-probability (lnL = -9068.26), which decreased monotonically with increasing number of suggested clusters.

Host, vector, parasite: mtDNA sequence description

Of the 310 bats examined, 231 proved PCR-positive for *Polychromophilus melanipherus*, resulting in a cross-European infection rate of 74.5% (Table 3.1, Supp. Figure S3.1). After sequencing, 32 of the 231 infections (14%) had multiple ambiguous base calls, indicating these hosts were infected with multiple strains of *P. melanipherus* at the time of sampling. Because haplotypes could not be called unambiguously in these cases, the multiple infections were disregarded in the consecutive analyses.

Sequence analysis of the *P. melanipherus* cyt*b* fragment revealed 8 haplotypes in the European population, based on 22 segregating sites. The 310 bp CR fragment of *M. schreibersii* had 51 segregating sites and 103 haplotypes. The 455 bp 16S fragment of *N. schmidlii* had 32 haplotypes, based on 15 segregating sites plus 3 indels: a single adenine indel, an adenine-thymine indel and an indel consisting of 0-5 repeated thymines. Independent reamplification and resequencing confirmed these indels. For both *M. schreibersii* (51 out of 103) and *N. schmidlii* (15 out of 32) approximately half of the haplotypes were singletons, found in only 1 individual. All unique haploytpe sequences of *P. melanipherus* (acc.nb. KJ131270 – KJ131277), *M. schreibersii* (acc.nb. KJ131278 – KJ131380) and *N. schmidlii* (acc.nb. KJ131381 – KJ131412) were deposited at the publicly accessible online database GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Both haplotypic diversity and nucleotidic diversity were of the same order of magnitude for each species (Supp. Table S3.7). Despite having the shortest fragment, *M. schreibersii* had haplotypic diversity close to unity, whereas the vector and parasite had very similar, slightly lower values. The reverse trend had been observed in the microsatellites, where heterozygosity levels were higher in the vector than in the host. The vector *N. schmidlii*'s gene fragment had the lowest nucleotide diversity of the three species. However, this is partly an artefact caused by the indels in the sequence, which are ignored when calculating nucleotide diversity.

The minimum spanning network of *P. melanipherus* consisted of the eight haplotypes loosely connected by branches varying in length from 1-12 mutational steps with no alternative paths between haplotypes (Figure 3.4D). The most numerous haplotypes were also common across all regions, whereas a few rare haplotypes were specific to certain locations. Both the bat and bat fly minimum spanning networks formed large strongly interconnected nets. Most haplotypes were separated by only 1-3 mutational steps, and many alternative paths existed between haplotypes. Because of the many haplotypes and many connections, no clear minimum spanning network figures could be produced, however Figure 3.4A-C show the frequency and distribution of haplotypes across the different regions. None of the bats' CR haplotypes were shared among all sites; most of them were unique to a specific region. In contrast, the bat flies' 16S fragment had one extremely prevalent haplotype, followed by a few considerably less prevalent haplotypes, but all except the rarest were shared among regions (Figure 3.4B).

Selection might in part be responsible for the marked differences in minimum spanning networks. Indeed, the mtDNA fragment of *M. schreibersii* and *N. schmidlii* showed no signs of selection (*M. schreibersii*: D = -1.115, p= 0.265; *N. schmidlii*: D= -0.370, p=0.712). In contrast, the cyt*b* fragment of *P. melanipherus* had a high segregating-sites-to-haplotypes ratio (D=2.178, p=0.029), a sign of balancing selection or population contraction.

When not correcting for mutations, the host *M. schreibersii* showed the highest levels of population differentiation (F'-values, Table 3.3: 'Equidistant'), compared to vector and parasite. When mutations are considered, the contrast between the species' population differentiations becomes less pronounced (Table 3.3: 'Pairwise differences', 'Kimura 2p'). The vector *N. schmidlii* had the lowest (and non-significant) within-region differentiation (Φ_{SC}), but slightly higher levels of isolation among sites compared to parasite and host (Φ_{ST}, Φ_{CT}). The host *M. schreibersii* has the highest, significant, levels of within-region isolation (Φ_{SC}), whereas the parasite *P. melanipherus* is overall the least differentiated among sites. The two modes of nucleotide evolution differed very little in their effect on the Φ-statistics of parasite and host (Table 3.3). Notable exception was seen with the vector, where a clear difference is shown between the Kimura model (ignoring indels) and pairwise differences (including indels in the distance matrix).

Based on Tajima's D, Figure 3.4 and the AMOVA results, pairwise number of differences was used as the mutation model for the calculation of the pairwise Φ_{ST} matrices, except for *P. melanipherus* for which no mutation model was assumed. The patterns of population differentiation based on the mtDNA fragment agreed with the patterns based on microsatellites in both carrier species (mantel test, microsatellite F_{ST} – mtDNA Φ_{ST}; *M. schreibersii*: r=0.333, p=0.004; *N. schmidlii*: r=0.294, p=0.003). As with the microsatellites, the mitochondrial fragments of both the bat (mantel test; r=0.182, p=0.005) and the bat fly (mantel test; r=0.475, p=0.001) demonstrated an isolation-by-distance pattern (Supp. Figure S3.3A-B). The malaria parasite, however, showed no such geographical pattern (mantel test; r=0.072, p=0.191; Supp. Figure S3.3C).

Table 3.4 Mantel test results correlating the *P. melanipherus* pairwise genetic distances with those of *M. schreibersii* and *N. schmidlii*.

Dataset	**r**	**p-value**[1]
M. schreibersii		
microsatellites	0.0427	0.364
mtDNA	-0.0604	0.600
only infected bats		
microsatellites	0.0959	0.215
mtDNA	0.0462	0.431
N. schmidlii		
microsatellites	-0.0950	0.665
mtDNA	0.0740	0.182

[1]based on 1000 permutations

Comparisons between parasite and vector or host genetics

The parasite's genetic distance matrix did not correlate with either that of the host or the vector; not when the distances were based on microsatellites, nor on mtDNA (Table 3.4, Supp. Figure S3.4, Supp. Tables S3.8-S3.10). This was also true when only actual 'hosts' were used, that is, when only the bat host genotypes were included in the distance table that were actually infected with the parasite (Table 3.4). In contrast to their microsatellites, the host and vector mtDNA distance matrices did correlate significantly with each other (mantel test; r=0.529, p=0.001, r^2=0.280), though this correlation disappeared when corrected for geographic distance (mantel test; r=0.077, p=0.164).

No association was found between *M. schreibersii* haplotypes and infection with *P. melanipherus* (X^2-test: X^2=6.213, df=20, p=0.999). Moreover, no difference was observed in any of the population genetic statistics, when comparing the infected with uninfected population of bats (permutation test: ΔH_O=0.00897, p=0.663; ΔH_S=0.00398, p=0.648; Δk=0.349, p=0.126; $\Delta\pi$= 0.0013, p=0.199; Δk_h=5.96, p=0.122). Similarly, no difference was found in the level of population differentiation between infected and uninfected *M. schreibersii* (infected: N=61 F_{ST}=0.066286, F'_{ST}= 0.14374; uninfected : N=49, F_{ST}=0.065268, F'_{ST}=0.14246; permutation test: p=0.968). In contrast, on the mtDNA the infected bat population showed higher levels of isolation compared to the uninfected part population (infected: N=61, Φ_{ST} = 0.496; uninfected: N=49, Φ_{ST} = 0.193; permutation test: p=0.001).

DISCUSSION

Parasite gene flow is dependent on both vector and host dispersal behaviour. In this study, we determined the population genetic structure of the haemosporidian parasite *P. melanipherus* and tested the hypothesis that its structure mimics that of its most vagile host species. We demonstrated that it is the wingless vector *N. schmidlii* that is the most vagile and not the vertebrate host *M. schreibersii*. However, the pattern of genetic differentiation of *P. melanipherus* resembled neither that of the vector nor that of the host.

Which is the most vagile species?

When comparing the microsatellite data of the host *M. schreibersii* and the vector *N. schmidlii*, a clear pattern emerges. Though the F_{ST} was significant in both species, the detected levels of isolation were 10 times higher in the bat than in the bat fly, whose level of subdivision was close to zero. Even after correcting for the slightly higher levels of gene diversity in the bat fly, differentiation in bats remained higher. The clustering analysis and PCA confirm this pattern of regional clustering in the bat hosts, whereas the bat flies rather acted as a single European population. Lastly, both species showed a pattern of isolation-by-distance but this relation was much stronger in the bat. All this points to the

bat flies having stronger genetic exchange among populations and therefore being the more vagile species in Europe.

However, the lower level of differentiation observed between bat fly sites might also be due to a possibly larger population size, reducing drift. Indeed most *M. schreibersii* would be carrying multiple *N. schmidlii* in summer making the bat fly population size correspondingly larger compared to the bat. Yet when hosts were sampled in spring (Croatia) and autumn (Switzerland) lower numbers of bat flies were found, averaging less than one fly per host (Table 3.1). Though bat fly sampling was not exhaustive in this study, and geography might be a confounding factor, seasonal bottlenecks have been observed in *N. schmidlii* (Estrada-Peña and Serra-Cobo 1991) as well as other temperate *Nycteribia* species (Chapter 5). These severe bottlenecks should decrease the effective population size N_e and thereby increase relative levels of differentiation between bat fly populations. The shorter life cycle of temperate bat fly species (estimated at two generations per year; Reckardt and Kerth 2006) should also increase relative population differentiation compared to the more slowly reproducing bat host. Despite these two possible factors, *N. schmidlii* had a lower fixation values, strongly suggesting more migration between vector populations compared to the host.

The mitochondrial DNA confirms that within region the bat fly demes are well connected; much better than the bats at the same scale. But at a larger scale, when the role of migration is expected to diminish and that of mutation to increase, it is the mtDNA of bats that show lower levels of differentiation. Indeed, by comparing the effect of mutation model on the AMOVA results we observe that for the bat mtDNA fragment, mutation plays a much larger role than migration, whereas for the bat flies migration is more important.

Despite their extensive seasonal migrations between roosts, actual dispersal events of marked *M. schreibersii* to a different population have very rarely been observed (Rodrigues et al. 2010). Both males and females are philopatric, in this case meaning faithful to their natal group, rather than any geographic location, though females will always return to the same maternity roost (Rodrigues and Palmeirim 2008, Rodrigues et al. 2010). Despite the philopatry of both sexes, the Φ_{ST} of the maternally inherited CR fragment was much higher than the expected fourfold increase compared to the nuclear data. This implies that females disperse less than males (Ramos Pereira et al. 2009), which would contradict the behavioural studies. However, though males do not disperse to other

groups, the seasonally migrating *M. schreibersii* do meet other local populations and mating at these meetings have been observed (Rodrigues et al. 2010), allowing for the transfer of nuclear, but not mitochondrial, genes to the other population. Moreover, since the mitochondrial control region is the fastest evolving sequence of the mammalian genome (Fumagalli et al. 1996), our used mutation models might not have been able to correctly capture all of the observed variation, artificially inflating the fixation indices.

The exchange of bat flies appears much stronger than that of the bats' own genes. Though peak reproduction of bat flies is synchronized to that of the bat, *N. schmidlii* can reproduce throughout the season (Lourenço and Palmeirim 2008a). Moreover, hibernacula, shared among multiple subpopulations of *M. schreibersii* (Rodrigues and Palmeirim 2008), could facilitate bat fly dispersal to other groups. But contact of bat subpopulations needs not be direct for *N. schmidlii* dispersal to occur. Roosting in the same cave at different time points would already allow for the exchange of bat flies, which leave their pupae to develop on the cave walls.

The *N. schmidlii* microsatellites and mitochondrial sequences agree that within region all sites are strongly connected, with regular exchanges. But the between regions mitochondrial levels of fixation are 80 times the nuclear values. Again, our mutation model might not have effectively captured the mutations processes going on at the ribosomal RNA fragment. Yet, this large discrepancy also suggests a strongly reduced N_e, caused by processes such as skewed sex ratios in the bat flies or strong female fly philopatry. Skewed sex ratios in favour of males are known to occur in bat flies (Dick and Patterson 2008), but in this study, the opposite was the case (177 females vs. 124 males, X^2=9.33, *df*=1, *p*=0.002). Female bat flies have to leave their host frequently to deposit a pupa on the roost wall (Marshall 1970). This risky behaviour could lower the survival rate of females but should also increase their exchange rate between bat hosts, in case they cannot find their original host back. Sex differences in survival have not been observed in bat flies (Chapter 5, Marshall 1970). However, the performed experiments concerned short term off-host survival, yet these flies can live up to six months at least (Ryberg 1947 cited in Marshall 1970); sex differences in long-term survival might therefore very well be possible, and has been observed with regard to haemosporidian infections in hippoboscid flies (Waite et al. 2012). Females might also be less likely to survive the winter season, causing bottlenecks in the mitochondrial genome. Bat fly female philopatry could also

have caused the high level of differentiation of the mitochondrial gene, but to our knowledge, no study has looked into bat fly dispersal behaviour.

Population structure of Polychromophilus melanipherus

The cyt*b* fragment of *P. melanipherus* was the only fragment that showed signs of past selection. It was also the only protein-coding gene in this study which might explain the relatively few haplotypes separated by relatively long branches. The balancing selection indicates negative frequency-dependent selection, suggesting a coevolutionary alternation of defences between the parasite and either the vector or host. However, how the mitochondrial cytochrome *b*, involved in the trans-membrane transport of electrons and ultimately in ATP production, could be a target for host immune responses is not clear.

The four major haplotypes were shared among the different regions, with the other four being more geographically restricted. The bat fly also had its more frequent haplotypes shared among regions yet still had an isolation-by-distance pattern. The malaria parasite showed no such geographical pattern. Despite a relative low expected N_e (infection rates < 1, few multi-clone infections, high parasite inbreeding), and therefore high expected levels of differentiation among the parasite populations, the relative contribution of Φ_{CT} to the parasites' structuring was low compared with those of vector and host. The local region therefore seems to matter less to the parasite populations.

If differentiation exists among the parasite populations, but geography does not matter, the parasite should be efficiently distributed across Europe. Differentiation among sites might not arise depending on distance but on random effects such as drift and founder effects. Some avian haemosporidian parasites have only little 'turn over' in a host once they have colonized it (van Rooyen et al. 2013) which would make populations sensitive to historical contingency. On the other hand, seasonal bottlenecks in the parasite population can cause high turn-over of haplotypes and results in differentiated populations as well (Bruyndonckx et al. 2009b). Separating these two scenarios would require temporal sampling; a challenge considering the host is a regularly-migrating cave dweller.

Does the parasite mimic the vector's dispersal patterns?

As stated by Wright (1951) F_{ST} is inversely related to the estimated number of migrants *Nm.* With its lower F_{ST}, *N. schmidlii* provides a higher number of migrants between demes compared to *M. schreibersii*. Even if a majority of these migrants was male, as suggested by the mtDNA, both male and female Nycteribiidae take blood meals and can therefore transmit *P. melanipherus*. The bat fly *N. schmidlii* should therefore be the main agent transporting the parasite between demes, but only if infection rates among bats and bat flies are equal. Although we did not measure the infection status of *N. schmidlii* here, the infection rate of its sister species *N. kolenatii* with the *P. murinus* parasite was 17%, four times lower than of its host *M. daubentonii*. If similar rates were to apply to this system, the effective number of parasite dispersal events caused by vector or host might approach each other. However, the parasite's population structure neither resembled that of the bat fly vector, nor that of the bat host. And since the dispersal patterns of host and vector were correlated at the mitochondrial level, it is unlikely that the parasite's pattern would lie somewhere 'in the middle' of its two hosts. As discussed above, it rather has a structure of its own.

A possible cause for the lack of correlation between the population structures of the parasite and its host and vector might be a second vector. *Penicilidia conspicua* is a large bat fly species that can also be found on *M. schreibersii* (Theodor 1967). However, we found this species at lower intensities or not at all and it was therefore considered a less likely candidate-vector. If *P. conspicua* would also transmit *P. melanipherus*, we would expect an overall increase in gene flow for the parasite relative to the vector, which might be the case in our study.

Dispersal rate heterogeneity within species could have caused the discrepancy between host and parasite gene-flow. *Nycteribia kolenatii* carrying *P. murinus* have lower survival than their uninfected counterparts (Chapter 5), which should reduce dispersal rate of infected flies and with it the parasite dispersal. No clear effects of a *Polychromophilus* infection on its bat host have been found as of yet (Chapter 4), but a change in migration behaviour is not unlikely. No specific haplotype could be associated with the infection. However, we did find that the mitochondrial differentiation among the infected share of all *M. schreibersii* subpopulations was higher than among the uninfected share. This pattern could arise if outcrossed bats, with a mother from a different site, are less prone to

become infected owing to their increased heterozygosity (Hamilton et al. 1990). This, however, would not explain the overall discrepancy between parasite and host.

We cannot say how much the discrepancy between hosts and parasite is caused by the specific gene histories. As seen with *N. schmidlii*, the message from different types of markers can be markedly different. A comparison between parasite and hosts with microsatellites should give a higher resolution picture of gene flow patterns, but despite multiple efforts, we did not manage to develop microsatellite markers for *P. melanipherus*. Only once have microsatellites been used to compare dispersal patterns of a parasite with its two hosts (Prugnolle et al. 2005). Yet, as in our study, the authors found no correlation between their parasite (the trematode *Schistosoma mansoni*) and its little dispersing intermediate mollusc host *Biomphalaria glabrata*, nor with its definite host the rat *Rattus rattus*. Though undeniably dispersed by the rat, no apparent correlation existed between parasite and host gene flow. Only the genetic distance between the trematodes' infrapopulations showed a positive relationship with the shared allelic distance of each rat. Instead of a shared dispersal patterns, this might rather suggest a gene-by-gene model of coevolution between parasite and host (Prugnolle *et al.* 2005).

In conclusion

The parasite's genetic structure was clearly not a mere copy of that of one of its hosts, nor an intermediate of the two. We thus found no support for the hypothesis that a parasite's population structure resembles that of its most vagile host species. Our study system, with a specialized parasite and easily traceable vector would have been the ideal candidate to find such a pattern. We hypothesize that the discrepancies might be caused by within-host-species dispersal heterogeneity linked to the parasite, as well as parasite founder effects or high parasite turn-over within the hosts' populations. The genetics of *N. schmidlii* indicated that ample non-reproductive contact exists between European *M. schreibersii* subpopulations, which demonstrates the utility of using (ecto)parasites as an ecological 'tag' for host behaviour. In contrast, even though *P. melanipherus* is unquestionably 'tagged' onto both of its hosts, its complex message is much harder to read than any single-host parasite system. A future disentanglement of these effects should, however, prove to be some of the more valuable insights into epidemiology of any vector-transmitted disease.

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SUPPLEMENTARY MATERIAL

Supplementary Table S3.1 *M. schreibersii* microsatellites markers used in this study

marker name	T_a	multiplex	ratio	source
Mschreib2	58	1	1	Miller-Butterworth et al. 2002
Mschreib3	58	1	2	"
Mschreib4	55	2	1	"
Mschreib5	55	2	2	"
MM01	58	3	3	Wood et al. 2011
MM02	58	3	1	"
MM06	58	3	1	"
MM17	58	3	5	"
MM22	58	3	5	"
MM28	58	4	3	"
MM29	58	4	1	"
MM30	58	4	5	"
MM31	58	4	5	"
MM34	58	4	1	"

T_a: annealing temperature in °C; *multiplex*: all markers with the same number were analysed in the same multiplex; *ratio*: the relative concentrations of post-PCR product mixed together for genotyping.

Supplementary Table S3.2 Thermal profiles used for the amplification reactions in this study.

	microsatellites			mtDNA sequences		
	***M. schreibersii*, primers from[1] (multiplex 1/2)**	***M. schreibersii*, primers from[2]**	***N. schmidlii* (multiplex 1/2)**	***P. melanipherus* (parent/nested reaction)**	***M. schreibersii***	***N. schmidlii***
Initial denaturation	95°C, 2 min	94°C, 5 min	95°C, 15 min	94°C, 5 min	95°C, 5 min	94°C, 5 min
nb cycles	38x	35x	30x/38x	25x/35x	35x	39x
denaturation	95°C, 50 sec	94°C, 30 sec	94°C, 30 sec	94°C, 30 sec	94°C, 30 sec	94°C, 30 sec
annealing	58/55°C, 50 sec	58°C, 30 sec	57°C, 90 sec	55°C, 30 sec	50°C, 30 sec	50°C, 30 sec
elongation	72°C, 1 min	72°C, 40 sec	72°C, 1 min	72°C, 45 sec	72°C, 45 sec	72°C, 30 sec
final elongation	72°, 10 min	72°C, 10 min	60°C, 30 min	72°C, 10 min	72°C, 7 min	72°C, 10 min

[1] Miller-Butterworth, Jacobs & Harley 2002; [2] Wood, Weyeneth & Appleton 2011. N.B. Any values separated by '/' apply to the specific catergories separated by '/' as indicated in the description of the column.

Supplementary Table S3.3 Reagents and their respective concentrations used for the amplification reactions in this study.

	microsatellites			mtDNA sequences		
	***M. schreibersii*, primers from[1]**	***M. schreibersii*, primers from[2]**	***N. schmidlii*, (multiplex 1/2)**	***P. melanipherus* (parent/nested)**	***M. schreibersii***	***N. schmidlii***
PCR buffer	1x (Qiagen)	1x (Promega)	0.6x (Qiagen Multiplex PCR kit)	1x (Qiagen)	1x (Qiagen)	1x (Qiagen)
Taq polymerase	0.5 U (Qiagen)	0.5 U (Promega)	-	0.25 U (Qiagen)	0.75 U (Qiagen)	0.25 U (Qiagen)
$MgCl_2$ (extra)	0.25 mM	1 mM	-	0.5 mM	1 mM	0.5 mM
Primer (each)	0.1 µM	0.35 µM	see Supp.Table S3.4	0.3 µM	0.2 µM	0.3 µM
dNTPs	0.2 mM	0.2 mM	-	0.25 mM	0.5 mM	0.25mM
Solution Q (Qiagen)	-	-	-	-	1x	-
DNA template	2 µL	2 µL	3 µL	3 µL / 1 µL	2 µL	3 µL
final volume	10 µL	10 µL	8 µL	25 µL	25 µL	25 µL

Supplementary Table S3.4 Newly developed microsatellite markers for *N. schmidlii*.

marker	primer sequence 5‘- 3‘	repeat	size (bp)	multiplex	conc. (µM)	GenBank accession nb
Ns143	fw: AGC ACT ACA ACC GCA ATG TC rv: CTA GAG ATA CCC GCC GTC AG	(TCA)*9	81 – 117	1	0.125	KJ189112
Ns319	fw: CGT TGA CAG GAC TTT CGG C rv: TAT CGA ACC TCA GCA ACA GC	(TGT)*7	74 – 111	1	0.125	KJ189114
Ns358	fw: TGT CCT CGA GTC TCA TTG CC rv: GGC CCT CAG TGA ATT GGA TG	(ACAT)*8	128 – 161	1	0.25	KJ189117
Ns637	fw: CAT CAC CGT GCT TAG ATG AGG rv: CTG ATT CCA TTC GGC AAT AAA CG	(GT)*13	128 – 141	1	0.25	KJ189108
Ns840	fw: AGT TTG AAT CCG AAC ACC GC rv: TGT TGA CTT CGT TGT AGC CG	(CAA)*13	91 – 120	1	0.5	KJ189116
Ns134	fw: TGC ATT GAA ATC GAG CTG TG rv: TTA CCC GCC TTG CAT GTT TG	(GTT)*9	200 – 236	2	0.25	KJ189110
Ns301	fw: CGA TGC GGT ATC ATC GAA GC rv: TTT GTT GAG ACA ATC AGC CG	(ACA)*11	111 – 156	2	0.25	KJ189113
Ns354	fw: AAC AAT TGC TTT AGC GCC AC rv: GCT GTT GGC TGG AAA TTC GG	(CATC)*8	69 – 151	2	0.125	KJ189111
Ns719	fw: TGC CAT CAT ACT ACC GGC rv: TAT TCG TTG AAT GCC ACC GC	(TCAG)*10	80 – 129	2	0.313	KJ189115
Ns943	fw: GCT GCA AAT GGT TCT AGG AAA TG rv: GAC CGG ACA GTA CAT GCC TC	(GT)*14	96 – 147	2	0.625	KJ189109

multiplex: all markers with the same number were analysed in the same multiplex. *conc.*: the concentration of the primer in the multiplex PCR.

Supplementary Table S3.5 Molecular diversity indices based on microsatellite data for *M. schreibersii* and *N. schmidlii*. Total number of alleles, observed heterozygosities, within population gene diversity, overall gene diversity, observed F_{IS} per locus.

	nb.al.	**H_O**	**H_S**	**H_T**	**F_{IS}**
M. schreibersii					
Mschreib2	6	0.345	0.335	0.36	-0.028
Mschreib3	12	0.79	0.785	0.809	-0.006
Mschreib4	12	0.8	0.776	0.805	-0.031
Mschreib5	19	0.75	0.791	0.837	0.051
MM02	4	0.441	0.478	0.484	0.076
MM06	5	0.221	0.237	0.243	0.07
MM17	4	0.576	0.596	0.644	0.032
MM22	13	0.692	0.718	0.775	0.036
MM28	4	0.573	0.595	0.643	0.037
MM30	12	0.725	0.724	0.764	-0.001
MM31	3	0.029	0.029	0.029	-0.004
overall	**8.55**	**0.5402**	**0.5512**	**0.5811**	**0.02**
MM01	1	-	-	-	-
MM29	1	-	-	-	-
MM34	1	-	-	-	-
N. schmidlii					
Ns143	13	0.811	0.815	0.819	0.005
Ns301	35	0.878	0.944	0.943	0.069
Ns319	12	0.719	0.732	0.744	0.017
Ns354	17	0.847	0.866	0.876	0.022
Ns358	14	0.66	0.72	0.728	0.084
Ns637	7	0.452	0.486	0.495	0.069
Ns840	11	0.592	0.796	0.787	0.257
Ns943	20	0.911	0.868	0.866	-0.049
overall	**16.13**	**0.7337**	**0.7783**	**0.7822**	**0.0573**
Ns134*	12	0.302	0.742	0.774	0.592
Ns719*	15	0.403	0.862	0.857	0.532

*: Showed signs of null alleles and were removed from all analyses.

Supplementary Table S3.6 Basic population genetic statistics of *M. schreibersii* and *N. schmidlii*, averaged over 11, 8 microsatellites respectively.

	M. schreibersii				***N. schmidlii***			
pop	**k**	**H_O**	**H_S**	**F_{IS}**	**k**	**H_O**	**H_S**	**F_{IS}**
	4.271	0.558	0.588	0.051	16.125	0.771	0.794	0.019
	3.667	0.595	0.567	-0.069	16.125	0.812	0.786	-0.027
	4.022	0.702	0.59	-0.178	16.125	0.775	0.825	0.062
	3.602	0.467	0.487	0.039	7.634	0.777	0.812	0.041
	3.803	0.539	0.54	-0.007	6.868	0.73	0.78	0.068
	3.65	0.503	0.551	0.085	16.125	0.741	0.824	0.1
	3.853	0.55	0.535	-0.015	7.218	0.755	0.795	0.057
	3.957	0.541	0.563	0.089	16.125	0.786	0.801	0.007
	4.132	0.527	0.571	0.094	7.212	0.784	0.8	0.02
	3.91	0.493	0.493	-0.002	16.125	0.75	0.833	0.106
	3.905	0.585	0.542	-0.076	-	-	-	-
	4.088	0.455	0.511	0.084	7.278	0.739	0.781	0.066
	3.876	0.462	0.53	0.122	7.098	0.729	0.758	0.041
	4.071	0.582	0.599	0.025	7.008	0.711	0.755	0.06
	8.545	0.621	0.636	0.015	16.125	0.681	0.776	0.107
	3.616	0.485	0.514	0.07	-	-	-	-
	6.636	0.545	0.576	0.023	16.125	0.625	0.812	0.167
	4.34	0.552	0.58	0.048	16.125	0.7	0.722	0.015
	4.556	0.533	0.557	0.05	16.125	0.72	0.763	0.045
	8.545	0.503	0.518	0.011	16.125	0.625	0.656	0
	3.97	0.515	0.552	0.047	7.125	0.724	0.746	0.011
	4.102	0.594	0.555	-0.065	7.217	0.697	0.757	0.089
	4.034	0.518	0.539	0.025	8.75	0.774	0.792	0.014

pop: populations indicated by their symbol (Table 3.1); *k*: allelic richness; H_O : observed heterozygosity; H_S: expected heterozygosity; F_{IS} : heterozygote deficiency

Supplementary Table S3.7 Gene diversity measures of the parasite *P. melanipherus*, the host *M. schreibersii* and vector *N. schmidlii*, based on their mtDNA fragments.

	P. melanipherus				***M. schreibersii***				***N. schmidlii***			
pop	***N***	***n***	$\hat{h}$	π	***N***	***n***	$\hat{h}$	π	***N***	***n***	$\hat{h}$	π
All	198	8	0.782	0.010	312	103	0.982	0.016	253	32	0.787	0.005
	10	3	0.689	0.006	15	6	0.8	0.01	6	4	0.8	0.005
	11	4	0.673	0.009	11	9	0.964	0.017	8	6	0.929	0.006
	10	3	0.644	0.008	11	6	0.836	0.008	5	5	1	0.004
	11	4	0.782	0.011	15	3	0.648	0.007	21	11	0.919	0.006
	11	3	0.691	0.009	15	9	0.924	0.012	13	8	0.897	0.005
	8	4	0.75	0.009	15	10	0.914	0.011	9	6	0.833	0.007
	10	4	0.644	0.008	15	11	0.952	0.012	21	12	0.91	0.006
	10	5	0.867	0.01	15	10	0.924	0.012	5	3	0.8	0.007
	12	3	0.682	0.009	15	7	0.876	0.012	19	10	0.912	0.005
	7	2	0.476	0.007	15	4	0.714	0.015	3	3	1	0.008
	8	4	0.643	0.009	16	1	0	0	-	-	-	-
	12	4	0.636	0.009	14	12	0.978	0.015	29	8	0.611	0.001
	4	3	0.833	0.011	15	7	0.857	0.017	42	5	0.577	0
	8	4	0.786	0.011	15	5	0.705	0.01	14	5	0.593	0
	1	1	0	0	3	2	0.667	0.015	6	4	0.8	0.001
	11	3	0.473	0.007	16	8	0.8	0.013	-	-	-	-
	5	3	0.7	0.009	10	3	0.711	0.007	2	2	1	0.002
	11	4	0.6	0.004	15	11	0.952	0.015	5	5	1	0.001
	11	5	0.855	0.008	15	9	0.933	0.009	9	6	0.833	0.002
	6	2	0.333	0.000	6	5	0.933	0.017	1	1	0	0
	4	2	0.5	0.008	15	8	0.886	0.011	12	4	0.682	0
	9	3	0.556	0.008	15	8	0.838	0.01	17	4	0.419	0.001
	8	3	0.75	0.01	15	8	0.867	0.007	6	2	0.333	0

pop: populations indicated by their symbol (Table 3.1); *N*: sample size; *n*: number of haplotypes; $\hat{h}$: haplotype diversity; π: nucleotidic diversity.

Supplementary Table S3.8 Pairwise genetic distances between sampling sites of *M. schreibersii*. Upper triangle: pairwise F_{ST}'s based on microsatellites. Lower triangle: pairwise Φ_{ST}'s based on the mitochondrial CR fragment.

	-	0.004	-0.014	0.073	0.004	0.018	0.027	0.035	0.007	0.041	0.038	0.046	0.055	0.050	-0.006	0.029	0.064	0.046	0.060	0.027	0.046	0.061	0.051
	0.151	-	0.010	0.031	0.012	0.029	0.041	0.032	0.038	0.087	0.057	0.077	0.041	0.068	0.019	0.059	0.035	0.033	0.053	0.037	0.052	0.036	0.048
	0.069	0.193	-	0.063	-0.002	0.009	0.012	0.028	0.013	0.034	0.015	0.048	0.047	0.063	-0.012	0.046	0.044	0.040	0.041	0.025	0.049	0.058	0.049
	0.343	0.325	0.386	-	0.063	0.075	0.070	0.096	0.091	0.137	0.091	0.121	0.082	0.145	0.111	0.130	0.041	0.065	0.081	0.064	0.094	0.068	0.111
	0.266	0.289	0.288	0.392	-	-0.012	0.000	-0.002	-0.006	0.046	0.033	0.070	0.060	0.084	-0.001	0.088	0.047	0.054	0.055	0.050	0.071	0.063	0.056
	0.217	0.204	0.226	0.379	0.077	-	0.003	0.000	-0.006	0.026	0.020	0.073	0.082	0.107	0.027	0.083	0.038	0.068	0.063	0.068	0.088	0.088	0.075
	0.187	0.169	0.207	0.382	0.091	0.011	-	0.026	0.011	0.074	0.018	0.105	0.092	0.138	0.072	0.099	0.063	0.084	0.061	0.078	0.113	0.110	0.110
	0.152	0.149	0.167	0.369	0.165	0.020	-0.005	-	0.004	0.059	0.040	0.076	0.096	0.096	0.038	0.095	0.057	0.065	0.087	0.075	0.090	0.083	0.062
	0.154	0.148	0.189	0.356	0.131	0.012	-0.036	-0.040	-	0.045	0.039	0.047	0.074	0.071	-0.011	0.064	0.045	0.053	0.042	0.061	0.063	0.080	0.054
	0.318	0.287	0.333	0.426	0.324	0.231	0.280	0.239	0.206	-	0.040	0.052	0.122	0.096	0.045	0.063	0.106	0.102	0.116	0.057	0.091	0.107	0.103
	0.818	0.669	0.749	0.840	0.711	0.625	0.660	0.692	0.666	0.588	-	0.080	0.093	0.125	0.082	0.076	0.073	0.090	0.093	0.068	0.115	0.107	0.103
	0.323	0.229	0.302	0.427	0.324	0.219	0.260	0.277	0.260	0.302	0.504	-	0.081	0.004	0.011	0.032	0.083	0.054	0.093	0.040	0.034	0.054	0.042
	0.405	0.294	0.365	0.510	0.351	0.194	0.276	0.310	0.285	0.327	0.534	0.099	-	0.097	0.007	0.089	0.048	0.034	0.054	0.039	0.051	0.037	0.052
	0.313	0.226	0.308	0.459	0.317	0.153	0.207	0.214	0.205	0.302	0.547	0.222	0.151	-	-0.005	0.047	0.106	0.065	0.112	0.053	0.011	0.041	0.022
	0.404	0.199	0.336	0.516	0.361	0.247	0.308	0.336	0.303	0.343	0.895	0.086	0.146	0.104	-	0.037	0.023	-0.022	0.002	-0.016	-0.029	-0.006	-0.022
	0.349	0.306	0.323	0.498	0.380	0.242	0.296	0.315	0.307	0.340	0.548	0.202	0.195	0.209	0.268	-	0.103	0.050	0.094	0.038	0.054	0.066	0.076
	0.342	0.190	0.283	0.496	0.278	0.124	0.179	0.185	0.202	0.295	0.654	0.165	0.150	0.150	0.211	0.133	-	0.006	0.017	0.042	0.062	0.039	0.055
	0.295	0.201	0.263	0.433	0.267	0.120	0.167	0.182	0.186	0.292	0.492	0.162	0.136	0.158	0.217	0.129	-0.083	-	0.007	0.008	0.022	0.001	0.033
	0.389	0.275	0.367	0.529	0.363	0.153	0.221	0.242	0.232	0.335	0.589	0.202	0.146	0.182	0.348	0.122	0.030	0.005	-	0.041	0.055	0.052	0.069
	0.373	0.253	0.341	0.530	0.392	0.205	0.236	0.189	0.226	0.338	0.769	0.271	0.310	0.240	0.447	0.236	0.110	0.085	0.100	-	0.022	0.009	0.054
	0.467	0.312	0.444	0.569	0.464	0.285	0.342	0.352	0.346	0.391	0.622	0.161	0.218	0.219	0.307	0.113	0.149	0.128	0.117	0.276	-	-0.005	-0.005
	0.434	0.262	0.416	0.536	0.440	0.259	0.313	0.327	0.319	0.365	0.581	0.173	0.177	0.195	0.280	0.143	0.108	0.083	0.071	0.236	0.003	-	0.004
	0.563	0.384	0.522	0.644	0.536	0.372	0.421	0.437	0.426	0.447	0.672	0.228	0.307	0.301	0.435	0.170	0.264	0.224	0.224	0.387	0.020	0.133	-

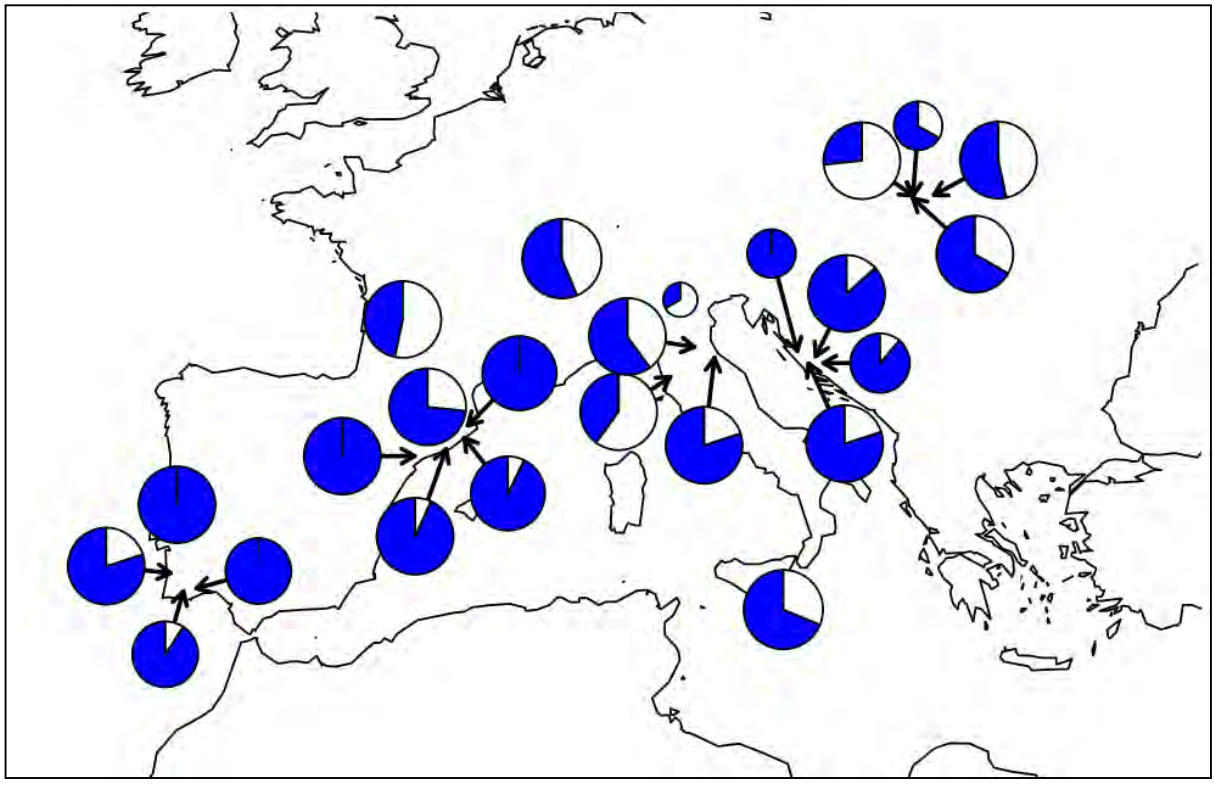
Supplementary Table S3.9 Pairwise genetic distances between sampling sites of *N. schmidlii*. Upper triangle: pairwise F_{ST}'s based on microsatellites. Lower triangle: pairwise Φ_{ST}'s based on the mitochondrial 16S rDNA fragment.

	-	0.027	0.028	0.018	0.018	0.001	0.024	-0.002	0.019	0.032	0.028	0.031	0.022	0.008	0.037	0.041	0.030	-0.005	0.004	0.026	0.013
	-0.098	-	0.013	0.009	-0.005	0.000	0.002	-0.007	0.012	0.005	0.004	0.004	0.001	-0.003	0.005	0.026	0.011	-0.031	0.015	0.015	0.001
	-0.159	-0.045	-	-0.011	0.000	-0.005	-0.017	-0.030	-0.008	-0.037	0.005	-0.006	0.003	0.002	0.006	0.049	0.006	-0.019	0.013	0.002	0.000
	-0.009	-0.061	0.033	-	0.006	-0.003	0.003	-0.007	-0.005	-0.026	0.014	0.008	0.006	0.006	0.040	0.044	0.020	-0.012	0.019	0.022	0.011
	-0.075	-0.079	-0.030	-0.024	-	0.000	-0.003	-0.014	0.008	-0.010	0.006	0.001	0.000	0.007	0.030	0.030	0.021	-0.023	0.013	0.016	0.008
	0.249	0.078	0.270	0.077	0.156	-	-0.005	-0.012	0.011	0.003	-0.001	0.000	0.005	-0.001	0.049	0.034	0.008	-0.012	0.002	0.016	-0.002
	-0.066	-0.058	-0.040	0.011	-0.024	0.155	-	-0.018	0.000	-0.023	0.001	-0.005	-0.004	0.000	0.032	0.029	0.005	-0.029	0.007	0.007	0.000
	0.162	0.009	0.169	0.032	0.094	-0.061	0.073	-	0.000	-0.013	0.003	-0.012	-0.002	0.005	0.024	0.019	0.002	-0.055	0.005	0.007	-0.002
	-0.096	-0.161	-0.082	-0.117	-0.108	0.007	-0.097	-0.083	-	-0.023	0.028	0.028	0.010	0.023	0.057	0.078	0.047	-0.005	0.042	0.033	0.033
	-0.140	-0.237	-0.113	-0.095	-0.158	0.057	-0.140	-0.047	-0.250	-	-0.006	-0.014	-0.010	-0.012	-0.017	0.005	-0.005	-0.069	0.001	-0.012	0.015
	0.777	0.626	0.759	0.522	0.610	0.277	0.575	0.328	0.602	0.713	-	-0.003	-0.001	0.001	0.037	0.016	0.003	-0.019	-0.002	-0.003	-0.009
	0.712	0.500	0.685	0.419	0.509	0.193	0.475	0.222	0.488	0.622	-0.020	-	-0.007	0.002	0.034	0.010	0.002	-0.030	0.006	0.000	0.001
	0.723	0.555	0.706	0.465	0.555	0.223	0.517	0.263	0.531	0.647	-0.003	-0.022	-	-0.006	0.030	0.013	0.005	-0.036	0.004	-0.003	-0.001
	0.582	0.342	0.565	0.308	0.405	0.040	0.374	0.072	0.305	0.434	-0.018	-0.047	-0.059	-	-0.016	0.013	0.014	-0.046	-0.010	-0.007	-0.006
	0.425	0.034	0.391	0.077	0.224	-0.435	0.158	-0.500	-0.133	-0.091	-0.762	-0.795	-0.821	-0.900	-	0.073	0.049	0.021	0.020	0.021	0.008
	0.549	0.303	0.540	0.265	0.379	0.018	0.375	0.066	0.256	0.377	0.108	0.043	0.061	-0.071	-0.600	-	0.003	-0.031	0.000	-0.001	0.032
	0.635	0.418	0.611	0.368	0.455	0.133	0.416	0.142	0.388	0.516	-0.013	-0.032	-0.031	-0.085	-1.000	0.051	-	-0.026	-0.007	0.001	-0.016
	0.400	0.101	0.413	0.104	0.232	-0.230	0.212	-0.198	0.015	0.118	0.203	0.176	0.088	-0.210	-1.000	-0.157	-0.026	-	-0.016	-0.030	-0.020
	0.756	0.551	0.729	0.443	0.536	0.221	0.499	0.250	0.543	0.680	0.003	0.015	0.007	0.013	-0.900	0.131	-0.004	0.241	-	-0.003	-0.014
	0.700	0.479	0.678	0.391	0.490	0.138	0.465	0.193	0.460	0.600	-0.009	0.005	0.007	-0.055	-0.636	-0.027	0.003	0.026	0.016	-	-0.003
	0.705	0.447	0.667	0.391	0.477	0.166	0.434	0.176	0.444	0.593	-0.031	-0.082	-0.053	-0.029	-1.000	0.149	-0.080	0.347	0.004	0.059	-

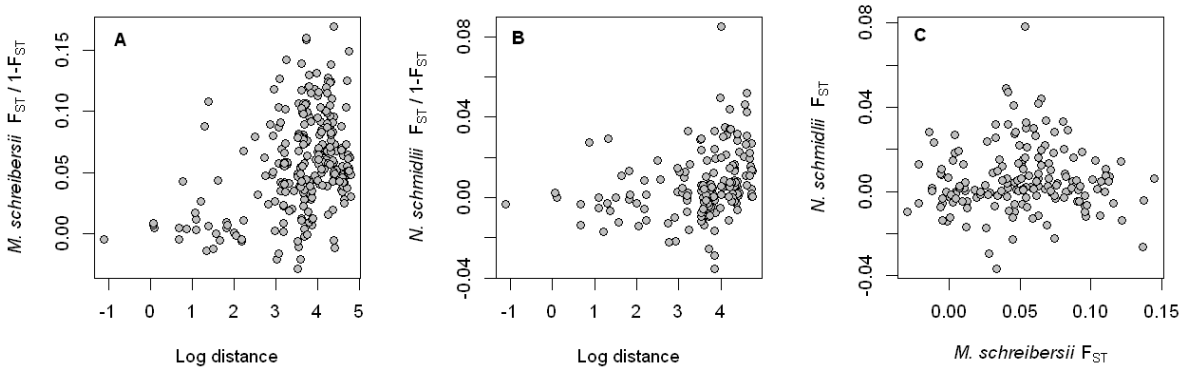
Supplementary Table S3.10 Pairwise genetic distances between sampling sites of *P. melanipherus*. Values represent pairwise Φ_{ST}'s based on the mitochondrial cyt*b* fragment.

	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.341	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.333	0.319	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.285	0.273	0.264	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.336	0.323	0.315	0.269	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.332	0.318	0.310	0.264	0.314	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.356	0.341	0.333	0.285	0.336	0.332	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.306	0.292	0.282	0.233	0.287	0.282	0.306	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.244	0.232	0.222	0.176	0.229	0.223	0.244	0.189	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.430	0.412	0.406	0.352	0.405	0.402	0.430	0.381	0.310	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.356	0.341	0.333	0.282	0.336	0.331	0.356	0.304	0.240	0.437	-	-	-	-	-	-	-	-	-	-	-	-
	0.286	0.269	0.257	0.199	0.264	0.257	0.286	0.216	0.146	0.381	0.282	-	-	-	-	-	-	-	-	-	-	-
	0.289	0.275	0.265	0.216	0.271	0.265	0.289	0.232	0.172	0.363	0.286	0.195	-	-	-	-	-	-	-	-	-	-
	0.360	0.346	0.339	0.292	0.341	0.337	0.360	0.312	0.253	0.431	0.361	0.294	0.296	-	-	-	-	-	-	-	-	-
	0.444	0.427	0.422	0.373	0.420	0.418	0.444	0.401	0.335	0.526	0.451	0.405	0.385	0.443	-	-	-	-	-	-	-	-
	0.484	0.463	0.459	0.402	0.454	0.453	0.484	0.438	0.360	0.591	0.496	0.458	0.419	0.481	0.581	-	-	-	-	-	-	-
	0.378	0.364	0.357	0.309	0.358	0.355	0.378	0.331	0.269	0.453	0.380	0.317	0.315	0.381	0.464	0.506	-	-	-	-	-	-
	0.248	0.236	0.227	0.182	0.233	0.227	0.248	0.194	0.139	0.313	0.244	0.154	0.178	0.257	0.336	0.361	0.273	-	-	-	-	-
	0.333	0.317	0.307	0.251	0.311	0.306	0.333	0.272	0.203	0.425	0.333	0.239	0.252	0.339	0.443	0.497	0.361	0.209	-	-	-	-
	0.399	0.383	0.376	0.326	0.376	0.373	0.399	0.350	0.285	0.481	0.402	0.341	0.333	0.401	0.489	0.539	0.421	0.288	0.386	-	-	-
	0.406	0.387	0.378	0.318	0.379	0.375	0.406	0.346	0.270	0.515	0.411	0.333	0.325	0.409	0.519	0.599	0.433	0.274	0.391	0.464	-	-
	0.306	0.292	0.282	0.233	0.287	0.282	0.306	0.250	0.189	0.381	0.304	0.216	0.232	0.312	0.401	0.438	0.331	0.194	0.272	0.350	0.346	-

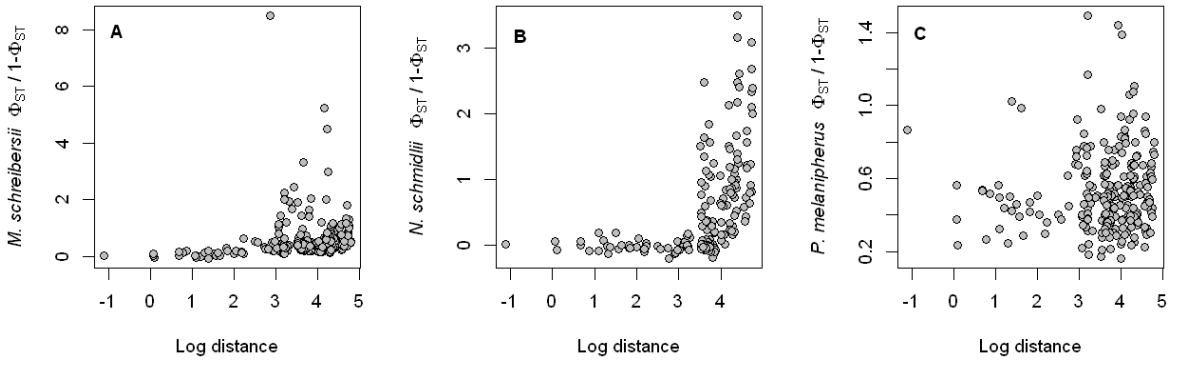
Supplementary Figure S3.1 Infection rates of *M. schreibersii* with the haemosporidian parasite *P. melanipherus* plotted per sampling site. White: uninfected, blue: infected. Size of the pie-chart is relative to sample size. N.B. A fourth site is shown in Slovakia. Though bats were captured at this site, they were merely visiting the location and originated from several other roosts. For the sake of infection rates, the site is included here, but excluded from all genetic analyses.



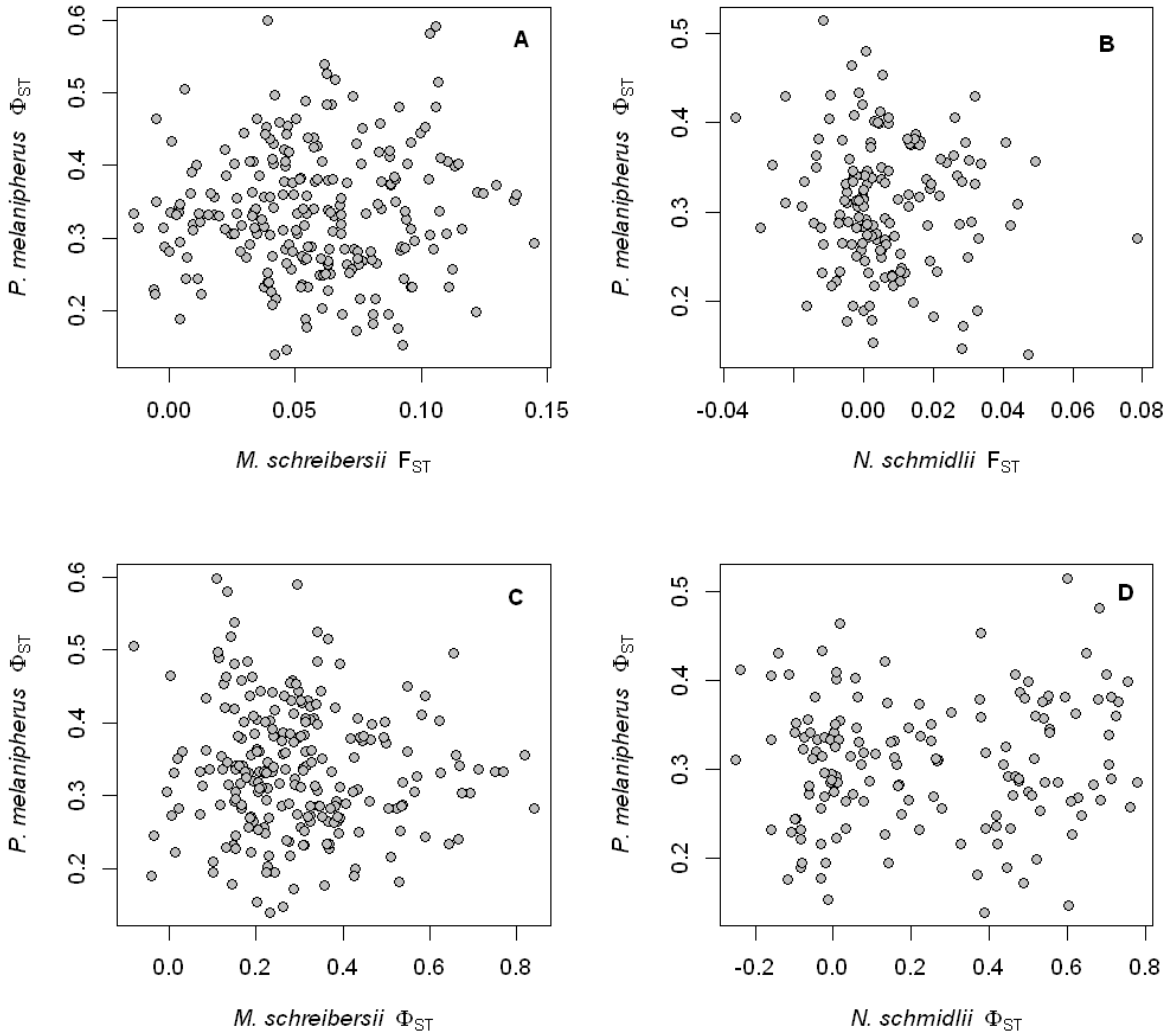
Supplementary Figure S3.2 Isolation-by-distance patterns for *M. schreibersii* (A) and *N. schmidlii* (B) based on microsatellite data. (C) No correlation between the vector and host population differentiation patterns.



Supplementary Figure S3.3 Isolation-by-distance patterns for *M. schreibersii* (A), *N. schmidlii* (B) and *P. melanipherus* (C) based on mitochondrial sequence data.



Supplementary Figure S3.4 Microsatellite (F_{ST}, top row A, B) and mitochondrial sequence (Φ_{ST}, bottom row C, D) based pairwise population genetic distances for *M. schreibersii* (left column A, C) and *N. schmidlii* (right column B, D) compared with the pairwise population genetic distances for *P. melanipherus*. Population differentiation patterns did not correlate between either host species and the malaria parasite. See Supp. Tables S3.8, S3.9, S3.10 for the raw pairwise distance tables.



Chapter 4

The epidemiology of *Polychromophilus murinus*

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Keywords: primary infection, ecology, Apicomplexa, Haemosporida, vector transmission, Nycteribiidae, population dynamics

ABSTRACT

An increasing number of diseases are classed as zoonoses, diseases that can be transmitted from animals to humans. Bats are implicated disproportionally as the source of these zoonoses and have therefore been well studied by wildlife epidemiologists searching pathogens with a high risk of spill-over to humans. However, this may cause a bias in our general knowledge of bat epidemiology since bat-specifc pathogens, with low risk to the human population, are rarely described. This study describes the seasonal dynamics of the epidemiological traits of *Polychromophilus murinus*, a malaria-like blood parasite, specialised on temperate-zone bats.

For three years we followed a population of *Myotis daubentonii* in Western Switzerland and screened them for the prevalence and parasitemia of *P. murinus*. In order to identify more susceptible classes of hosts, we measured, sexed and aged all individuals.

Juvenile bats demonstrated much higher parasitemia than any other age class sampled, suggesting that the first exposure to the parasite is very early in life when infection is most intense. Moreover, in subadults there was a clear negative correlation between body condition and intensity of the infection, which was inversed in adults, albeit not very strong. Neither body temperature, nor haematocrit, two proxies used for pathology, could be linked to the intensity of infection.

If both weaker condition and younger age are associated with higher infection intensity, then the highest selection pressure exerted by *P. murinus* should be at the juvenile stage. The yearly peak in both parasites and newly emerged vectors should facilitate the long-term maintenance of infection in the host population.

INTRODUCTION

Bats are the reservoir host of several severe human and livestock diseases (e.g. Ebola, rabies, Nipah viruses) and are suspected to harbour many more malignant agents (e.g. Middle Eastern Respiratory Syndrome (Ithete et al. 2013, Memish et al. 2013)). Parasites of bats –in particular their virosphere- have therefore received a lot of attention from epidemiologists (Calisher et al. 2006). Most studies so far have focused on bat parasites that cause zoonoses, *i.e.,* parasites of wildlife that are capable of cross-infecting humans or other, economically valuable, animal species. The notable characteristics of bat immunology observed in these studies (e.g. the frequent absence of virus-induced pathology (Baer and Bales 1967)) might therefore be a consequence of the research bias towards pathogens which require a reservoir.

Non-viroid parasites on the other hand, have received less attention. For instance, bats harbour both zoonotic and bat specific bacteria (Muhldorfer 2013) and they are also important hosts of protozoan parasites. Two recent studies suggest that bats might be the original mammalian host for both trypanosomes and malarial parasites (Hamilton et al. 2012, Schaer et al. 2013). Interest in these pathogens has been increasing, most studies focusing on the phylogenetic relationship between species (Duval et al. 2007, Cavazzana et al. 2010), prevalence (Raharimanga et al. 2003, Concannon et al. 2005, Maia da Silva et al. 2009, Megali et al. 2011, Drexler et al. 2012) or transmission (Gardner and Molyneux 1988b, Anez et al. 2009, Billeter et al. 2012). The natural epidemiology of these pathogens, their abundances, dynamics and ecology, however, has not been the focus of any study.

The present study describes the epidemiology of a bat-specific parasite and its dynamics in a local population of hosts. The protozoan *Polychromophilus murinus* (Dionisii, 1899) is a haemosporidian parasite (Apicomplexa: Haemosporida) closely related to the malaria causing *Plasmodium* spp. (Garnham 1966, Chapter 2). *Polychromophilus* spp. only infect bats, unlike *Plasmodium* spp, which have a host range encompassing at least 3 classes of vertebrates (Garnham 1966). *P. murinus* can be found in multiple bat species of the Palearctic (Garnham 1973b) and is well established in Western Switzerland where its main host is the Daubenton's bat *Myotis daubentonii* (Megali et al. 2011).

Myotis daubentonii (Chiroptera: Vespertilionidae) is a small brown-greyish bat that hunts insects over still water surfaces. During the summer season, males and females often live segregated, roosting in tree holes where the females form nursery colonies of 20-50 individuals. Mid-June, a single pup is born which after three weeks, when still being weaned, will start flying (Dietz et al. 2009).

Most life-cycle stages of *P. murinus* in the host have been described, as well as in the vector *Nycteribia kolenatii*, a bat fly of the family Nycteribiidae (Gardner et al. 1987). The distribution of *P. murinus* has been documented in a very few studies but since it has been observed in Italy, Switzerland and Great Britain (Corradetti 1936, Gardner et al. 1987, Megali et al. 2011) it is likely that *P. murinus* has established itself in populations of *M. daubentonii* across Europe. In contrast, we have little idea about the seasonal dynamics in prevalence as well as intensity of infection. The aim of the current study was therefore to determine these epidemiological parameters in a local population of *M. daubentonii.* Secondly, by observing the susceptibility of each host age and sex class, the most likely source of the epidemic can identified, which provides clues how the infection is maintained in the population.

By definition, a parasite should have a negative effect on the host's fitness. The direct effects of a malaria infection can be very severe (Garnham 1966, Valkiũnas 2005), but may depend heavily on the haemosporidian parasite and host species (Palinauskas et al. 2008). The only attempt at studying the physiological effects of *P. murinus* on bats failed when the artificially inoculated heterospecific host appeared unsusceptible to infection (Gardner and Molyneux 1988a). (The attempt to infect a human with *P. murinus* by injecting infected bat blood failed as well; though the test subject did develop a fever, this was probably caused by other pathogens introduced by the injection (Garnham 1966).) Our aim here is not to perform a clinical experiment, but to explore possible effects of *P. murinus* infection using two physiological characteristics that can be used as proxies for pathology: haematocrit for anaemia and body temperature for fever.

METHODS

Sample collection

Myotis daubentonii were captured during the seasons of 2010, 2011 and 2012 on the University of Lausanne campus, near Lake Geneva in Switzerland, using a harp trap positioned over the Sorge stream at dusk. While highly pregnant and lactating females were immediately released upon capture, all other bats were used for sampling. Each bat was ringed to prevent resampling. The age of the bat, either 'adult' or 'subadult' was determined by the presence of a dark spot on the lower lip, which fades after 1-2 years. Each bat's forearm length (to the nearest 0.1 mm) and weight (to the nearest 0.1 gram) were measured. We used the residuals from an OLS regression of body mass on forearm length as a measure of body condition (Schulte-Hostedde et al. 2005). In 2012, the haematocrit value (see below) and body temperature were also measured. Captured bats were immediately removed from the trap and body temperature was measured by inserting a lubricated probe into the rectum (RET-3 animal rectal probe and BAT-12 microprobe thermometer, Physitemp, Clifton, USA).

Blood was obtained by puncturing the uropatagial vein with a 0.5 mm gauge needle (Neolus). Between 5 and 30 µL of blood were collected using either microvettes with EDTA (Sarstedt; seasons 2010/2011) or heparinized glass microcapillary tubes (Marcel Blanc & Cie; season 2012). Samples were stored at -20°C until molecular analysis. Haematocrit was measured by centrifuging the microcapillary tubes containing fresh blood for 7 minutes at 12,800 rpm. The haematocrit value was calculated by dividing the length of the red blood cell column by the total length (measured to the nearest 0.1 mm).

After blood sampling, haemostatic cotton was applied to the punctured vein until bleeding ceased. All bats were captured under the licenses #1317 and #1656, authorized by the Cantonal Veterinarian Service of Vaud, Switzerland.

Blood parasite analyses

From each blood sample, one drop of fresh blood was applied to a microscope object glass to make a thin smear. Slides were subsequently dried and immediately submerged in 100% methanol for fixation. Finally, a 5% Giemsa-stain was applied for one hour to stain the cells. The abundance of

Polychromophilus murinus parasites, from now on referred to as *parasitemia*, was estimated by scoring the number of *P. murinus* gametocytes observed in each smear at 600x magnification for 15 minutes (see Appendix A for validation of this method).

DNA was extracted using the Blood and Tissue spin column kit (Qiagen, CA), following the manufacturer's tissue protocol, with an overnight digestion and eluted in 2 * 50 µL. To control for contamination, a negative control was always included during the extraction process. The infection status of the bat host was determined by detecting the presence of a 705 bp fragment of the mitochondrial cytochrome *b* gene (cyt*b*) following a nested PCR protocol. Primers, reagent concentrations and thermal profiles can be found in Megali et al. (2011). Amplified fragments ran on a 1.5% agarose gel and were visualised under UV light. For each 8 samples tested, one negative control was included in the amplification protocol. Each sample was tested in two independent tests. Samples with ambiguous results were tested again in duplicate. Samples remaining ambiguous were discarded from the data set.

Statistical analyses

Factors influencing *P. murinus* prevalence, as assessed by the nested PCR protocol, were identified by logistic regression. The original model included the variables *sex, age, body length* and *condition*, as well as *year* and *date*, the latter variable expressed as standardized days since April 1st. Interactions were included based on their biological relevance: *year x date*, *date x condition*, *sex x condition* and *age x condition*. Terms were removed by backward selection, based on AIC and non-significant residual deviances until a minimal adequate model was found.

Since the parasitemia data was zero-inflated and showed signs of overdispersion, a zero-inflated (i.e. 'mixed') negative binomial approach (ZINB) was implemented using the *pscl* package for R (Zeileis et al. 2008). To identify factors that influence *P. murinus* parasitemia in wild *M. daubentonii* hosts, this model considers the overly abundant zeros to come from two different processes, i.e. 'false zeros', caused either by poor observations or individuals that did not encounter the parasite, and 'true zeros', resulting from the covariates being unfavorable for the parasite (Zuur et al. 2009). For both the false-zero portion of the model, which attempts to discriminate between the two types of zeros, and the count portion, the same host-related covariates were used as for the previously described logistic

regression of infection status. However, the variable *date* was also included squared after graphical inspection of the data. A backward selection procedure was adopted wherein a term was dropped from either the false-zero or count portion of the model until no further decrease in AICc was observed. Each progressive model was tested for a significant change in log likelihood compared to its predecessor (Zuur et al. 2009).

Only in September and October 2012 juveniles were caught. These are fledged young-of-the-year, which can be recognized by the incomplete ossification of their finger joints. Because of their age, it is not recommended to capture them earlier in the season and they were therefore not included in the previously described analyses. A comparison between juveniles and the other two age classes was therefore done separately. An F-value was obtained by performing an ANOVA on parasitemia by age class, with *date* as a covariate. This F-value was compared to a null distribution obtained by randomizing *age* for 999 times.

The effects of parasitemia on bat hematocrit values and body temperatures were tested by linear regression. To linearize the relationship, *parasitemia* was $\log(x + 0.5)$ transformed. As female mammals can have lower hematocrit levels (Nemeth et al. 2010) and higher body temperatures (Cryan and Wolf 2003), sex was included as a cofactor in each analysis.

All statistical analyses were done in R version 3.0.1 (R Core Team 2012).

RESULTS

Of the 212 *M. daubentonii* tested, 157 (74.1%) were positive for the *P. murinus* cyt*b* fragment. Of the 186 individuals of which also the blood smear had been searched for parasites, 58 (31.1%) were positive. 80 individuals were negative by microscopic analysis but positive by PCR and 48 were negative according to both methods.

For 193 bats all required data for the logistic regression were available. Infection rates of *M. daubentonii* with *P. murinus* increased over the season, but only in 2010 a period of peak infection appeared, around July/August (Figure 4.1A) the other years showing either an unsteady increase (2011) or a flat trend (2012). The final model of *P. murinus* prevalence contained both *date*, *year* and its

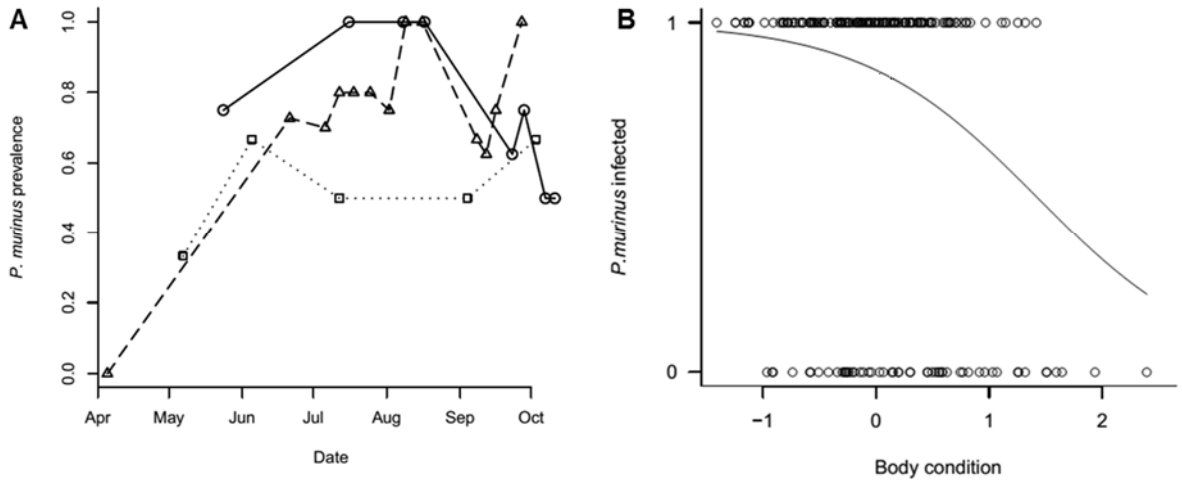

Figure 4.1 Results from the logistic regression. (A) Prevalence of *P. murinus* in the local population of *M. daubentonii* through the season, separated by year. Circles and continuous line: 2010; triangles and dashed line: 2011; squares and dotted line: 2012. (B) The possibility of infection with P. murinus reduces with increased condition of the bat.

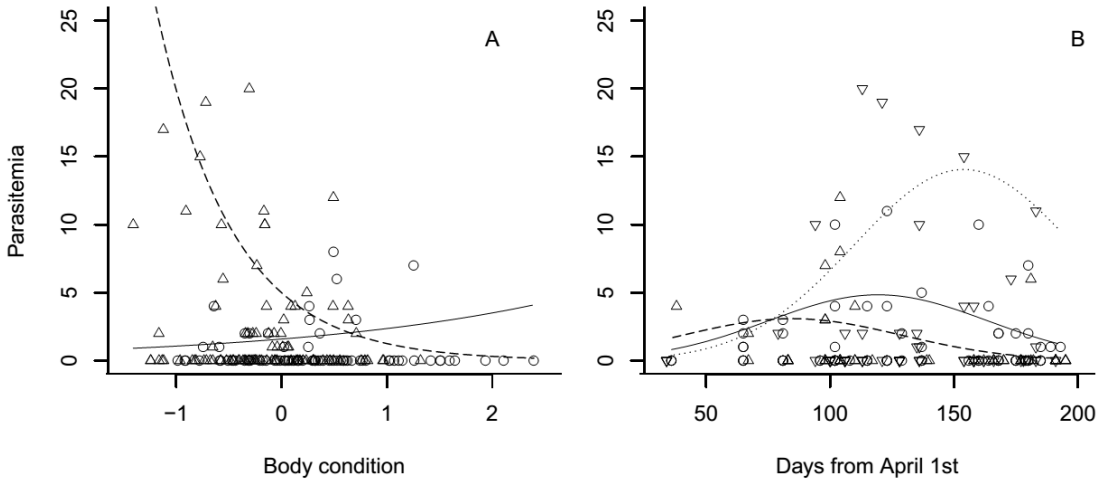

Figure 4.2 Predictors of the intensity of infection as approximated by parasitemia. A) The relation between body condition and parasitemia differs between age class. Circles and continuous trend line: adults; triangles and dashed trend line: subadults. B) Parasitemia changes through the season and interacts with body condition. Continuous trend line and circles: bats with mean body condition; dashed trend line and up-facing triangles: bats in high body condition (>mean + 0.5 s.d.); dotted trend lines and down-facing triangles: bats in low body condition (<mean - 0.5 s.d.).

Table 4.1 Estimates of the parameters and their significance for the logistic regression of prevalence.

Parameter	Estimate	Standard error	X^2	*p*
year (2011)	-0.7831	0.5449	-	-
year (2012)	-1.9964	0.6497	-	-
date	-0.2469	0.4173	-	-
sex (male)	-1.0610	0.4712	5.3190	0.02109
condition	-1.2975	0.3159	20.0147	$7.685*10^{-06}$
length	-0.3262	0.1833	3.3199	0.06845
date x year (2011)	1.0454	0.5060	6.3632	0.04152
date x year (2012)	0.1760	0.5404	”	”

interaction (Table 4.1). All other variables were retained as well, but not their interactions or *age* (Supp. Table S4.1). The goodness-of-fit test was not significant (Hosmer-Lemeshow, X^2= 8.558, p= 0.38), but visually many variables showed no pattern at all. Many intercorrelations existed between the predictors, which can make GLM solutions very sensitive to small variations in predictors (Quinn and Keough 2002). To assess the robustness of our solution, we randomly split the data in two and three subsets, each subset containing respectively 50% or 33% of all observations, and used these to retest our model. Upon retesting, many variables disappeared from the model. Only date appeared as a reliable predictor and, with the exception of one model, body condition as well (Figure 4.1B; Supp. Table S4.3).

Parasitemia data were collected from a total of 186 *M. daubentonii* for the analysis of blood parasite abundances. The minimal adequate ZINB model retained multiple terms in the count model, but none in the false-zero portion (Table 4.2, Supp. Table S4.2), though the difference between the last two models (with or without *age* in the false-zero part of the model) was only marginal (ΔAICc = 0.58; Supp. Table S4.2). The count portion of the ZINB model retained several variables. *Condition* and *age* had a significant interaction. In adults, parasitemia increased slightly with increasing body condition, whereas in subadults a strong negative relationship existed between *parasitemia* and *body condition* (Table 4.2, Figure 4.2A). In general, individuals in higher body condition had lower parasitemia, and the interaction with *date* indicated that peak parasitemia was reached sooner in bats in high body condition (Figure 4.2B). Though ranges overlapped, juveniles had higher median and

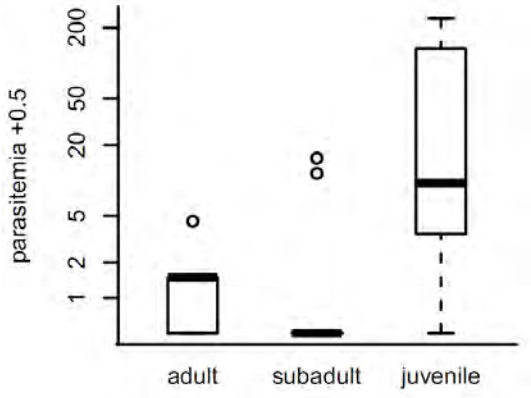

Figure 4.3 The abundance of *P. murinus* gametocytes in the blood (parasitemia) of *M. daubentonii* caught in September and October 2012. Young of the year (juveniles) have significantly higher parasitemia than older age classes. The y-axis, the number of blood parasites observed, is on a log scale. For visualisation purposes, 0.5 is added to parasitemia.

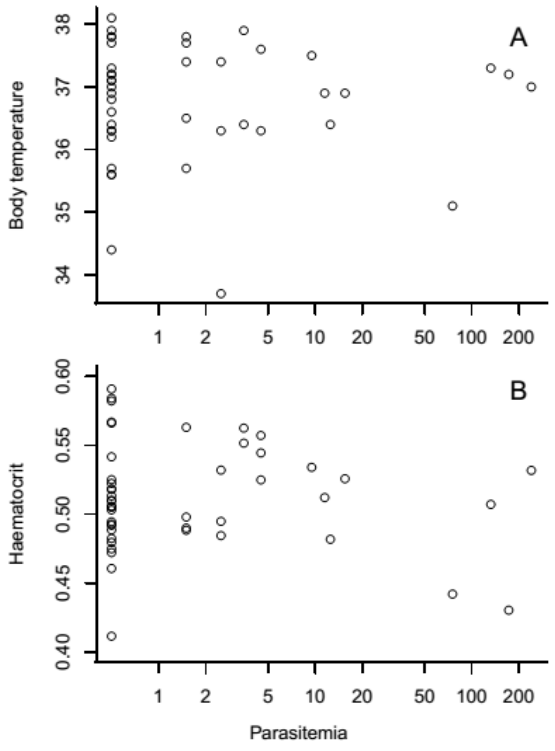

Figure 4.4 The infection of *P. murinus* had no clear physiological effect on the bats. (A) Body temperature in degrees Celsius; (B) Haematocrit, calculated as red blood cell volume fraction. The intensity of infection on the x-axis, expressed as parasitemia +0.5, is on a log scale.

Table 4.2 Estimates of the parasitemia statistical model parameters and their significance for the model.

Parameter	Estimate	Standard error	X^2	*p*
Count model μ				
date^2	-0.4717	0.1782	5.6189	0.01777
date	0.1894	0.1894	-	-
condition	0.3960	0.5253	-	-
age (subadult)	1.1437	0.3811	-	-
date x condition	-1.1296	0.3677	9.772	0.001772
age x condition	-1.7781	0.6792	6.3133	0.01198
False zero model π				
none	-	-	-	-

maximum parasitemia by one and two orders of magnitude respectively (Figure 4.3, randomized F-test: n=23, randomizations=999, p=0.014).

Parasitemia of *P. murinus* had no effect on the body temperature of the bats when corrected for sex (multiple linear regression: $F_{2,39}$=0.057; p= 0.943; Figure 4.4A). Hematocrit value did not appear to be influenced by the abundance of gametocytes either (multiple linear regression: $F_{2,44}$=0.078; p= 0.463; Figure 4.4B).

DISCUSSION

Polychromophilus murinus reached its highest abundances in juvenile *M. daubentonii*, which have to carry the heaviest burden of infection of the population. The zero-inflated model showed that across the whole season the level of infection was again much higher in subadults than adults. This suggests that with age, bats are more able to cope with infection.

The bat's body condition was linked to the chance of being infected and it correlated with the progression of infection as well as the maximum intensity of the infection. Notably, the effect of host body condition on the parasite intensity depended on the age of the host, for which several non-mutually exclusive processes might be responsible. Subadults with larger fat storages could be better equipped to mount a costly immune response (Lochmiller and Deerenberg 2000), though no direct

relation was found in the Brazilian free-tailed bat *Tadarida brasiliensis* (Allen et al. 2009). In *M. daubentonii* body condition increases with maturation (Encarnacao et al. 2006). The strong decline of parasitemia with condition seen in subadults might therefore actually represent maturing individuals learning to cope with infection.

However, in adults, the relationship between condition and parasitemia is slightly positive. Heavier adults might be trading off mass against immunity, though it is unclear why this would only effect adults (Lochmiller and Deerenberg 2000). Like other bat ectoparasites (Christe et al. 2003), the vector *N. kolenatii* is attracted to hosts in higher body condition (Chapter 5); these bats might therefore be more often exposed to new infections, which causes a slight increase in parasitemia. It might also be a sampling artefact: perhaps for bats with similar levels of infection, only those in good condition can tolerate it enough to go foraging at night when we caught them. Lastly, the positive correlation might be caused by pregnant females, which are relatively heavy for their size and also immunosuppressed (Christe et al. 2000). Though highly gravid females were never sampled, females in earlier stages of gestation are more difficult to recognize and might therefore be present in the data set.

It is worth noting however, that in our statistical analyses we have considered a bat's body condition as one of the causes predicting the likelihood and intensity of infection. Yet, in contrast to date or age, the reverse is just as likely. Mounting an immune response requires energy which should reduce fat reserves (Lochmiller and Deerenberg 2000). Loss of body mass might therefore very well be a symptom of infection with *P. murinus*. Only experimental infections under controlled conditions could resolve this question of cause and effect.

Apart from possible weight loss, we found no other signs of *P. murinus* pathology. *M. daubentonii* showed no signs of fever; whether fever is applied by heterotherms is still a debated issue (Canale and Henry 2011). The lack of anaemia on the other hand might be because of the biology of *P. murinus*. Unlike *Plasmodium* spp., *Polychromophilus* spp. have no asexual multiplication in the blood. The number of erythrocytes destroyed during an infection should therefore be much lower compared to other malaria species.

Over the three years we found an average infection rate of 74.1% which is the same rate as Megali et al. (2011) found in the same population in 2009 using the same screening method. Prevalence was not stable throughout the season, but this pattern seemed different each year suggesting either random emergent fluctuations prevalence or the influence of (unmeasured) climatic variables.

Prevalence was much higher based on PCR than based on the microscopy results. It is well established that nested PCR is more sensitive than pure visual control, though the two methods can approach each other in efficacy (Valkiunas et al. 2008). The proportion of 'false zeros' determined by the ZINB model did not correspond to the number of bats tested negative by PCR. When all PCR-negative individuals were removed from the parasitemia analysis, zero-inflation was still an issue. The false zero's were therefore not only caused by hosts that had not encountered the parasite, but also 'bad observations' i.e. false zero's. In our case, the ZINB found no factors influencing the appearance of false zeros, indicating that this rate of zero detection occurred rather constant across categories of hosts, without biases. In contrast with this the actual probability of being infected, as demonstrated by the logistic regression, mainly depends on time of season and the condition of the host.

To conclude, previous work has shown that the primary exposure to a haemosporidian parasite causes much higher parasitemia in hosts than any further encounters (Garnham 1966). Our study, demonstrating much higher parasite abundances in juveniles than other age classes, supports this observation. Bat ectoparasites synchronise their reproduction with their hosts (Lourenço and Palmeirim 2008a). After female bats have given birth, the ectoparasites massively move onto the pups (Christe et al. 2000). Blood parasites will thus be introduced to the neonates at a very early stage. The primary exposure to the haemosporidian parasite causes extreme high levels of parasitemia, which in turn increases the probability of establishing an infection in the abundant newly emerged bat flies. As bat flies are long lived and can overwinter (Gardner and Molyneux 1988a), this in itself might suffice to maintain the *P. murinus* infection.

Moreover, host condition was strongly correlated to the strength of infection. If both weaker condition and younger age are associated with a more intense infection, then the highest selection pressure exerted by *P. murinus* should be on the juvenile *M. daubentonii*. Weak young are pruned from

the population and any surviving bats should have developed strategies tolerating further infections of *P. murinus*. This could explain both the absence of any pathological symptoms in the adults and the ability of the parasite to remain present in the adult's blood stream throughout the year.

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SUPPLEMENTARY MATERIAL

Supplementary Table S4.1 Backward selection of the logistic regression of prevalence. The most unsignificant terms were dropped until minimum AIC had been reached.

Dropped variable	**AIC**	**df**	**Residual deviance**	X^2	***p***
None (initial model)	214.71	179	186.71	-	-
age x condition	212.71	180	186.71	$4.96*10^{-06}$	0.9982
sex x age	210.77	181	186.77	0.064221	0.7999
sex x condition	209.15	182	187.15	0.38227	0.5364
age	208.10	183	188.10	0.94743	0.3304
date x condition	207.68	184	189.68	1.5781	0.209

Supplementary Table S4.2. Backward selection of the parasitemia statistical model. Most unsignificant terms were dropped from the count- or false-zero model (respectively μ and π) until minimum AIC had been reached.

Dropped variable	**AICc**	**df**	**Loglikelihood**	X^2	***p***
None (Initial model)	537.03	29	-234.89	-	-
date x condition from π	534.30	28	-233.84	0.1065	0.7442
length from π	531.57	27	-233.88	0.0621	0.8032
length from μ	528.91	26	-233.92	0.0983	0.7539
$date^2$ *x year* from μ	525.51	24	-234.93	2.0146	0.3652
date from π	523.02	23	-235.02	0.1717	0.6786
sex x condition from π	521.15	22	-235.39	0.7532	0.3855
sex from π	519.86	21	-236.04	1.2984	0.2545
sex x condition from μ	519.68	20	-237.23	2.3795	0.1229
sex from μ	518.07	19	-237.69	0.9173	0.3382
year from μ	514.29	17	-238.28	1.1749	0.5557
year x date from π	511.39	15	-239.25	1.9413	0.3788
$date^2$ from π	509.52	14	-239.50	0.5067	0.4766
age x condition from π	509.01	13	-240.42	1.8424	0.1747
condition from π	506.81	12	-240.48	0.1124	0.7374
age from π	506.23	11	-241.34	1.7196	0.1897

Supplementary Table S4.3 Stability of the backwards selection procedure, by using subsets of the prevalence data set. Variables retained in the model after backward selection are indicated by 'P' or 'N' depending on whether the estimate was positive or negative respectively. '-' indicates the absence of this variable in the considered model. Subsets with lowest AIC are highlighted.

Subset	AIC	Year	Day	Year:day	Sex	Age	Condition	Length	Sex:age	Sex:condition	Condition:age	Condition:day
All	207.7	N	N	P	N	-	N	N	-	-	-	-
1st ½	114.9	-	P	-	-	-	N	-	-	-	-	-
2nd ½	94.16	N	N	P	-	-	N	-	-	-	-	N
1st ⅓	64.56	-	P	-	-	-	N	-	-	-	-	N
2nd ⅓	76.14	N	N	P	-	P	-	-	-	-	-	-
3rd ⅓	68.85	N	N	P	-	-	N	-	-	-	-	-

Chapter 5

Signs of a vector's adaptive choice: on the evasion of more infectious hosts and parasite-induced mortality

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Keywords: host choice, biting rates, vector survival, feeding preference, host attractiveness, host fidelity, Plasmodium, haematophagy, parasite manipulation, parasite transmission

ABSTRACT

Vector-borne diseases represent complex host-parasite associations with interactions among all three actors, i.e. the parasite, the vector and the host. Whether vectors are able to detect parasitized hosts and avoid them or are manipulated by and attracted to parasitized hosts remains a debated issue.

Laboratory and field experiments have demonstrated in many cases that malaria vectors do not feed randomly, but show important preferences either for infected or non-infected hosts. The preference or absence of preference may depend on the costs imposed by the parasites on both their vertebrate and dipteran hosts.

We used the natural associations between a malaria-like parasite *Polychromophilus murinus* (Apicomplexa: Haemosporida), the bat fly *Nycteribia kolenatii* (Diptera: Nycteribiidae) and a vertebrate host the Daubenton's bat *Myotis daubentonii* (Chiroptera: Vespertilionidae) to experimentally test host choice preference, frequencies of host switching and survival of vectors. Moreover we used bat fly abundance data from the field as an approximation of their actual feeding pattern and compared these with the experimentally preferences of the vectors.

Bat flies preferred hosts with the fewest infectious stages of the parasite. Behavioural observations revealed a relative high rate of host switches. In line with the hypothesis of costs imposed by parasites on their vectors, bat flies carrying parasites had higher mortality. However, in wild populations, bat flies were found feeding more on larger bats in good body condition, irrespective of host infection level.

The decreased survival of infected bat flies suggests that the preference for less infected hosts is an adaptive trait, but it is the complex ecological processes that ultimately determine the vector's biting rate. It is these processes rather than preferences per se, which need to be identified for successful epidemiological modelling.

INTRODUCTION

The success of a vector transmitted disease is, by its nature, not only defined by the ecology and behaviour of the host, but also by those of the vector. For malaria research this realization has meant that currently an unprecedented amount of research is focused on mosquitoes and their role in malaria epidemiology (e.g. Kelly 2001, Lyimo and Ferguson 2009, Farajollahi et al. 2011, Glaizot et al. 2012). One key aspect of the vector's biology is its blood-feeding behaviour. Anthropophilic mosquitoes do not feed indiscriminately but show a preference for some blood donors over others, within a single host species (Knols et al. 1995). Of particular interest for epidemiology is the feeding preference of the mosquito vector based on the host's level of infection. Such a preference is predicted to severely alter the dynamics and equilibrium level of infection of a given epidemiological system (Kingsolver 1987). This prediction, however, only holds if such a preference would actually result in differential biting rates of the more attractive hosts under natural conditions (Kingsolver 1987). The relation between the vector's preference and its natural feeding rate has, to our knowledge, never been tested.

Mosquito vectors are able to discriminate between hosts on the basis of the host infection status. Their feeding preference -or the 'host attractiveness'- has been experimentally tested for numerous host species. In humans (Lacroix *et al.* 2005), rodents (Day et al. 1983, Ferguson and Read 2004), as well as in birds (Freier and Friedman 1976, Lalubin et al. 2012, Cornet et al. 2013) these choice experiments demonstrated either attraction to or avoidance of malaria-infected hosts, or no effect of the parasite. These contradictory results could be due to the choice of methodology (e.g. olfactory cues versus restricted body contact), the study system (e.g. natural versus unnatural host species (Tripet 2009)), or generally to the geographic mosaic of coevolution that may lead to local adaptation. None of these studies, however, linked the found preference to the actual feeding rates of these vectors under natural conditions.

Measuring natural feeding rates of vectors on wild animals is methodologically challenging and we know of no study which has done this directly. Instead, approximations have been used such as relative local vector abundances. Tomás et al. (2008) demonstrated that biting midges (Ceratopogonidae), the natural vectors of avian *Haemoproteus* haemosporidians, were more abundant

in nests with malaria-treated blue tit females. Notably, the study could not demonstrate that the increased biting midge numbers were a direct consequence of the vector's preference, since the authors could not control for differential survival rates of the midges in the differently treated nests. As a result, a link between host preference and actual feeding patterns could not be established.

The aim of the present study was first to test under laboratory conditions the preference of the vector *Nycteribia kolenatii* for the host *Myotis daubentonii* based on the host's infection level with the haemosporidian parasite *Polychromophilus murinus.* A feeding preference for infected hosts might originate from many different processes, e.g. a host's lower anti-parasite behaviour or a parasite-based manipulation to increase its transmission success (Cezilly et al. 2010, Cator et al. 2012). Alternatively, a feeding preference for uninfected host would be a strong indication that the parasite has a detrimental effect on the bat fly's fitness. To investigate this point, an experiment was performed to test the effects of *P. murinus* infection on the survival of bat flies. We furthermore tested the host fidelity of the bat fly by quantifying their host switching behaviour. Finally, results of host choice preference obtained in laboratory conditions were compared to the natural feeding rate of the vector. We used the relative abundances of *N. kolenatii* in a wild population of *M. daubentonii* as the approximation of the bat flies feeding rate. Since bat flies rarely venture off-host and take very frequent blood meals, their presence on a certain host should directly translate to their relative feeding rate on that host.

METHODS

Studied species

Bat flies (Diptera: Nycteribiidae) are blood-sucking wingless ectoparasites which specialize on living in the fur of bats (Dick and Patterson 2006). Unlike most haematophagous Diptera, they do not engorge themselves, instead taking several blood meals per day, from once every hour up to every eight minutes (Marshall 1970, Overal 1980, Fritz 1983). Like all members of the Hippoboscoidea superfamily, they are viviparous (Petersen *et al.* 2007), i.e. all larval instar levels occur within the female abdomen. Their whole adult life is spent on-host, only female bat flies will temporarily leave

their hosts to deposit a single pupa on the bat roost wall, the only developmental stage of a bat fly spent off host (Marshall 1970). After emergence bat flies use carbon dioxide, as well as body heat and odour to locate their bat hosts (Lourenço and Palmeirim 2008b). If the same cues are used to discriminate between hosts is unknown.

Nycteribia kolenatii (Theodor & Moscona, 1954) is a relatively small bat fly with a length of 2-2.5 mm (Theodor 1967). It mainly parasitizes the Daubenton's bat *Myotis daubentonii* (Kuhl, 1817; Chiroptera: Vespertilionidae), a common Palearctic bat species which habitat spreads from Western Europe up to Japan. *N. kolenatii*, has been identified as a vector (Gardner et al. 1987) of *Polychromophilus (Bioccala) murinus* (Dionisi, 1899).

Polychromophilus spp. (Apicomplexa: Haemosporida) are a genus of malaria-like protozoan parasites that, though taxonomically classified under Haemoprotidae (Garnham 1966), are phylogenetically nested within the *Plasmodium* clade (Chapter 2). The dipteran part of the life cycle is similar to *Plasmodium* spp., with an oocyst developing on the gut wall (Mer and Goldblum 1947, Gardner and Molyneux 1988a). The vertebrate stage differs, however, in that there is no erythrocytic merogony, and only the gametocytes (the form infectious to the dipteran vector) develop in the blood (Garnham 1966). *Polychromophilus* spp. infect insectivorous bats globally (Garnham 1973a) and can reach high infection rates locally. In Western Switzerland 51% of the *M. daubentonii* population was found to be infected with *P. murinus* (Megali et al. 2011). Despite its large presence, little is known about the pathogenicity and virulence of the parasite for both vector and host (Corradetti 1936, Gardner et al. 1987).

Sample collection

Myotis daubentonii were captured during the seasons of 2010 and 2011 on the University of Lausanne campus, using a harp trap positioned over the Sorge stream at dusk. While gravid and lactating females were immediately released upon capture, all other bats were used for sampling and ringed to prevent resampling. Each bat's forearm length (to the nearest 0.1 mm) and mass (to the nearest 0.1 g) were measured. As a measure of body condition we used the OLS residuals from a regression of body mass on forearm length (Schulte-Hostedde et al. 2005). *Spinturnix andegavinus* wing mites were counted by inspecting the wing membranes and the uropatagium. Finally all bat flies were collected

from the fur, using soft forceps and by gently blowing carbon dioxide through the fur away from the bat's head. Bat fly species were identified by FW following Theodor (1967) and Aellen (1955).

Blood was obtained by puncturing the uropatagial vein with a 0.5 mm gauge needle (Terumo, Leuven, Belgium). Between 5 and 30 µL of blood were collected using microvettes with EDTA (Sarstedt, Nümbrecht, Germany). From each blood sample, one drop of fresh blood was applied to a glass microscope slide to make smears. Slides were subsequently dried and immediately submerged in 100% methanol for fixation. Finally, a 5% Giemsa-stain was applied for one hour to stain the cells. The *P. murinus* parasites were identified by FW following Garnham (1966) and their abundances, from now on referred to as 'parasitemia', were estimated by scoring the number of gametocytes observed in each smear at 600x magnification for 15 minutes (Appendix A).

After blood sampling, haemostatic cotton was applied to the punctured vein until bleeding ceased. If the bats were needed for the experiment described below, they were hand-fed with mealworms and released into an exterior aviary (1.8 m x 1.15 m x 2.45 m) equipped with roosting sites. Mealworms and water were provided ad libitum. Bats that were not needed for further experiments were released at the capture site on the same night. All bats were captured under the licenses #1317 and #1656, authorized by the Cantonal Veterinarian Service of Vaud, Switzerland. The collected bat flies were put in separate 2 mL screw cap tubes punctured with air holes and equipped with moist cotton. To prolong their survival, the flies were kept at 4°C for the survival experiment or until usage in the host choice assessment.

Host-choice assessment

The host preference of the bat fly *N. kolenatii* was assessed as follows. Two bats, which previously had been freed of their bat flies and with known parasitemia, were placed in a bat cage (50x40x30 cm) with a small slit as a roost site and with mealworms and water provided ad libitum. Pairs were created based on their dissimilar parasitemia and matched by sex and age as much as possible. At 09:00, two male and two female bat flies, which were all individually marked with UV-fluorescent dye (Aquacolor UV-Dayglo, Kryolan, Germany), were placed on each bat. These flies originated from other bats caught the same night. Every two hours (except at 19:00), both bats were scanned with a UV light to examine the presence and location of the marked bat flies. At 21:00, when the bats

would become active, the final position of each fly was noted and the bats released. In total, 35 of these experiments were performed, each with different bat flies and bats. Experimentation was performed under the license #2322, authorized by the Cantonal Veterinarian Service of Vaud, Switzerland.

To test the hypothesis that the bat flies discriminate between bats based on the hosts' parasitemia we performed a multiple logistic regression of proportions based on the number of bat flies on each host. The difference in parasitemia between the pair of bats was used as the predictor of this distribution of bat flies. Notably, this distribution can be influenced by other factors including differences in body size (as approximated by forearm length) and body condition (approximated by the residuals of the length-weight OLS regression), as well as the difference in the number of bat flies originally collected from the hosts. These terms were, therefore, included as co-predictors in the statistical model. For each term, a regression coefficient β is estimated which quantifies the change in probability to be on the partner bat for a given change in the predictor variable. Non-significant terms were dropped if the resulting reduced model did not significantly decrease the log-likelihood (χ^2 test) until the minimal Akaike's Information Criterion (AIC) had been reached.

Next, we tested if the bat fly preference based on host parasitemia, as identified in the choice experiment, codetermined the natural distribution of bat flies among bats in the wild. A generalized linear model was applied where the bat fly abundance (i.e. the total number of bat flies found on each bat) was modelled following a negative binomial distribution, and log-linked to the systematic component with host parasitemia as the predictor. Several other host-related factors that were expected to play a role in the bat fly distribution were included as predictors, including sex, age, number of *Spinturnix andegavinus* mites (a potentially competing ectoparasite), body condition, forearm length, year, day, and the interactions year*day, age*condition and sex*condition. A graphical inspection of 'day' (expressed as number of days since the first of April) suggested a possible quadratic relationship with the number of bat flies, and therefore a quadratic term was included as well. Non- significant terms were dropped from the initial model and compared with previous models using likelihood-ratio goodness-of-fit tests until no more significant reduction in AIC could be reached.

Bat fly survival

In June 2010 and 2012, all flies were removed from ten wild-caught *M. daubentonii*, and their survival off-host was monitored. To prolong their survival and thus gain resolution, the flies were kept at 4°C (Gardner and Molyneux 1988a). Each day at noon, the bat flies were moved to ambient temperature and examined for signs of life (e.g. movement of legs, pumping abdomen). Motionless bat flies were coaxed from inactivity by flicking their tubes. If this still elicited no response, the fly was gently prodded with a blunt needle. Unresponsive flies were deemed dead and either stored in 70% ethanol (2010) or frozen at -80°C (2012) until further analysis.

Like all Hippoboscoidea, and unlike mosquitoes, Nycteribiidae have their salivary glands in the abdomen, alongside the for- and midgut (Gardner and Molyneux 1988a). Attempts at isolating the salivary glands proved unsuccessful. Instead, *P. murinus* infection was detected by DNA extraction and amplification. First, flies stored in alcohol were soaked in Millipore water for 2 hours. All flies were then triturated with sterile pestles. For the DNA extraction and purification, the Biosprint 96 tissue protocol (Qiagen, Hilden, Germany) was followed, with an overnight digestion. Eleven blank samples were included during the extraction process to check for possible contamination. The detection of *P. murinus* infection was done by amplifying a 705 bp cytochrome b (cyt*b*) fragment of the parasite following a nested PCR protocol. Primers, reagents and PCR temperature profile can be found in Megali et al. (2011). Bands, ran on a 1% agarose gel and stained with ethidium bromide, were visualized under UV light. Each sample was tested in duplicate. Samples that gave ambiguous results were retested. Any sample that remained unclear was removed from further analysis.

To confirm that the *P. murinus*-positive bat flies were actively infected (i.e. with oocysts or sporozoites), and exclude the possibility that we were only detecting an infected blood meal in the gut, each sample was tested for presence of *M. daubentonii* mtDNA. A 195 bp fragment of the cyt*b* gene was amplified using a nested PCR protocol (see Supplementary Methods). The inner primer pair was designed to specifically amplify *M. daubentonii* DNA and no dipteran nor human material. Again, each sample was tested in duplicate and any samples with conflicting results were reanalysed or excluded from the study.

To test for statistical difference in survival between infected and uninfected bat flies, a Cox proportional hazards regression model (CoxPHM) was applied to the survival data using the survival package for R (Therneau 2013). Instead of directly using the survival data, the CoxPHM uses the underlying hazard function. Comparing this function between groups allows the calculation of the hazard ratio ($hr_{A/B}$), or the relative risk of group A compared to that of group B (Cox 1972). To deal with ties, Efron's approximation was used. We added year of the experiment (2010 or 2012) and the sex of the bat flies as covariates to the model, as well as the interaction between sex and infection status of the bat flies. All statistical analyses were done in R version 2.15.0 (R Core Team 2012).

RESULTS

Host choice assessment

Over the 12 hour period that bats roosted together, a median of three bat fly exchanges (*range* = 0 - 7) was observed, out of a potential 40 observable parasite exchanges per pair. The most common number of exchanges was two (Figure 5.1A). Of the 280 bat flies, 177 were never observed switching hosts (63.2%), the vast majority of those who did switched once (Figure 5.1B), and the average consecutive time spent on a specific host was 7.2 hours.

A total of 35 host choice experiments were performed with a total of 198 bat flies. The final model of the logistic regression only retained difference in parasitemia (N= 35, X^2=7.704, df=1, p=0.006) as a significant predictor of the distribution of flies between the two hosts. Bat flies tended to move to the host with the lowest parasitemia ($\beta_{parasitemia} \pm s.e.$ = -0.086 ± 0.034; Figure 5.2A). The original bat fly abundance of each host, the forearm length and body condition (Figure 5.2B) had no significant effect, and had therefore been removed from the model (Supp. Table S5.1). Similar results were obtained when we repeated the analysis with only those flies that had been observed to move at least once ($N_{exp.}$ = 35, n_{flies} = 88, parasitemia: X^2=4.555, df=1, p=0.033).

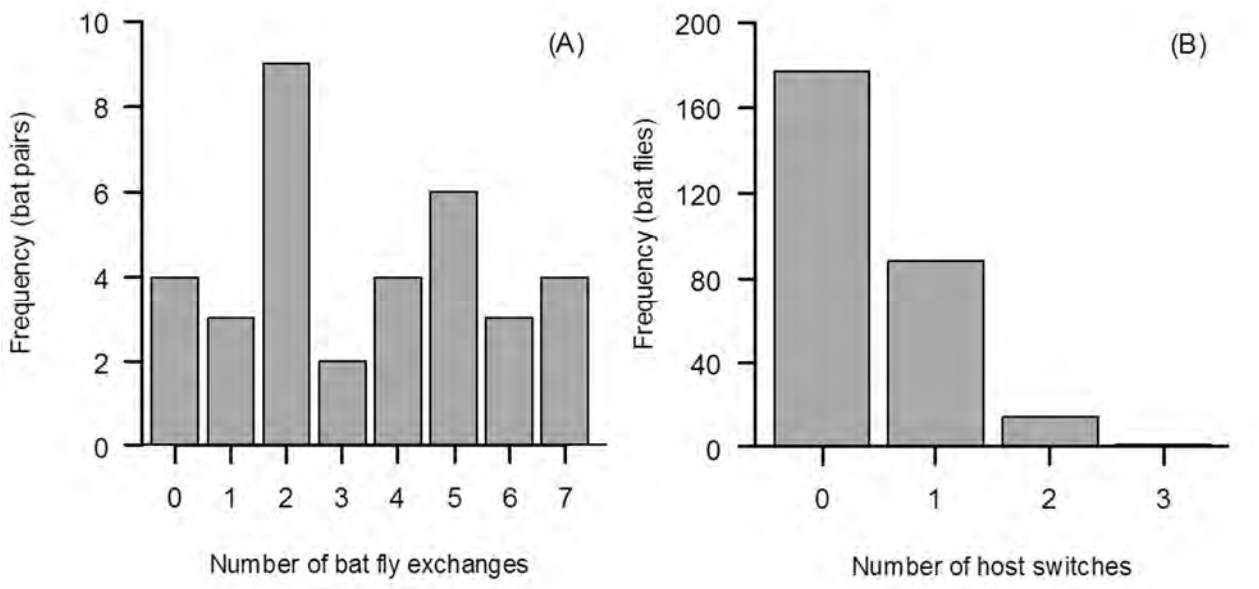

Figure 5.1 Bat fly host switching behavior. (*a*) From the host's perspective: The number of bat fly exchanges that occurred between the paired bats over the course of the 12-hour experiment. (*b*) From the bat fly's perspective: The number of times a bat fly was observed to change hosts over the course of the 12 hours experiment. A single individual was observed changing hosts three times.

Natural bat fly abundances

All 1,116 bat flies collected from the 163 captured *M. daubentonii* were identified as *N. kolenatii*. Bat fly numbers per host ranged from 0 – 26 flies with a median abundance of 6 flies (*mode*=2, *mean* ± *s.d.* = 6.84 ± 5.36). The bat fly abundance data of the wild host population was described best by a model containing four terms (Table 5.1, Supp. Figure. S5.1). Both body condition (Figure 5.2D) and forearm length (Supp. Figure 5.1B) of bats were positively correlated to bat fly abundance (Table 5.1). The number of bat flies a bat carried also increased over the season, with a peak mid-August (Supp. Figure S5.1A, Table 5.1). In 28% of bats gametocytes of *P. murinus* were detected, yet these parasitemia had no significant effect on the number of bat flies the host carried (X^2=1.752, *df*=1, *p*=0.186; Figure. 5.2C).

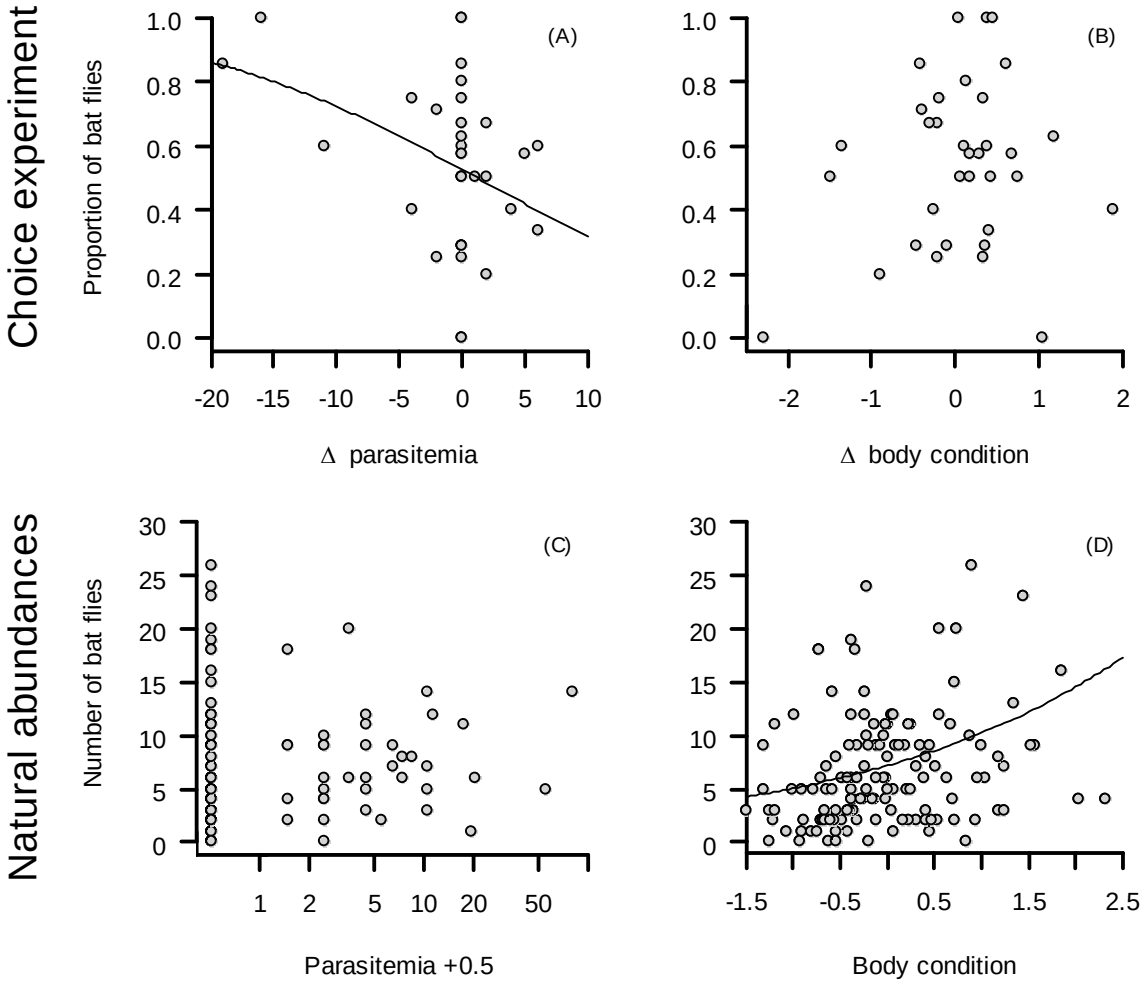

Figure 5.2 The contrasting patterns of bat fly distributions in the host choice experiment (top row) and bat fly abundance data from wild caught daubenton's bats (bottom row). Trend lines were created using averages for the other model parameters and only drawn when the relationship was significant. Note that the x-axes in the top row represent the difference (Δ) in value of that parameter for the paired bats, whereas the x-axes of the bottom row represent the absolute values of single individuals.

Table 5.1 Estimates and standard errors of each parameter regression coefficient for the minimal adequate model.

Parameter	**Estimate**	**Standard error**	X^2	***d.f.***	***p***
Day^2	$-7.450*10^{-5}$	$3.274*10^{-5}$	6.152	1	0.013
Day	$1.995*10^{-2}$	$0.851*10^{-2}$	6.520	1	0.010
Condition	0.351	0.091	14.475	1	0.0001
Length	0.157	0.059	7.508	1	0.006

Bat fly survival

A total of 177 *N. kolenatii* bat flies were monitored in the survival experiment (2010: n=115; 2012: n=62). In 13 individuals, the presence of malaria or host blood could not be unambiguously determined, and they were consequently excluded from any further analysis. The large majority (92.7%) of bat flies tested positive for *M. daubentonii* mtDNA, including all the 27 flies that were positive for *P. murinus* (16.4%). We decided to only analyse the individuals positive for *M. daubentonii* mtDNA to avoid any confounding effects. Nevertheless, repeating the analysis with all samples produced qualitatively the same results (data not shown). The sex of the bat flies had no effect on their survival (X^2=0.0737, df=1, p=0.786), nor its interaction with the infection status of the bat flies (X^2=1.769, df=1, p=0.184). Bat flies carrying *P. murinus* survived significantly less time, having an increased risk of dying ($hr_{present/absent}$ = 1.74, X^2=7.084, df=1, p=0.008; Figure 5.3). The year of the experiment also had a clear effect on the survival of the bat flies, with bat flies in 2012 surviving longer and enduring a lower relative hazard ($hr_{2012/2010}$ = 0.64, X^2=5.689, df=1, p=0.017; Figure 5.3).

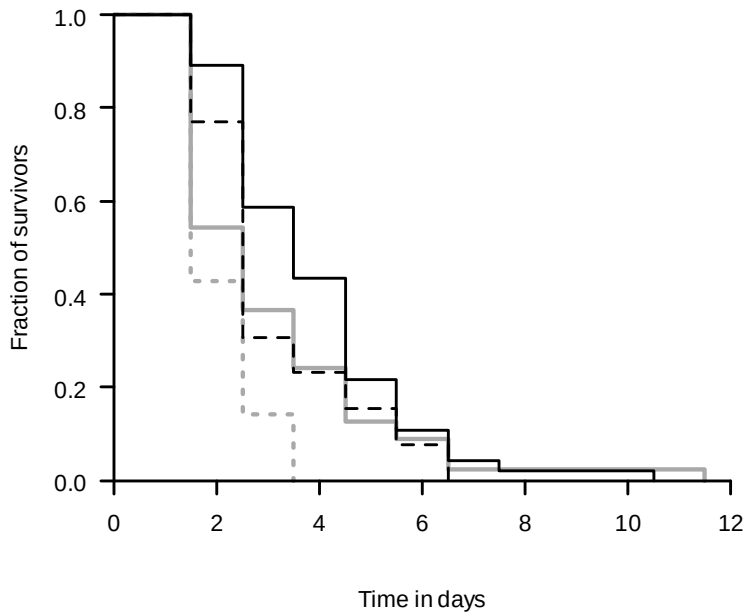

Figure 5.3 Survival of bat flies in relation to the presence of the parasite *Polychromophilus murinus* and the year of the experiment. Grey lines correspond to 2010, while black lines show data from 2012. Continuous lines indicate the absence of *P. murinus*, while dashed lines indicate the presence of *P. murinus*.

DISCUSSION

In this study, we tested if the dipteran vector of the malaria-like parasite *Polychromophilus murinus* showed a feeding preference for hosts depending on their infection status. The experimental results demonstrated that the bat fly *N. kolenatii* has a clear preference for hosts that carry the least infective stages of the haemosporidian parasite, *P. murinus*, in a laboratory setting. Moreover, we showed that batflies infected with *P. murinus* have a decreased survival. However, we also demonstrate that this apparent feeding preference does not explain the vector's actual feeding pattern in the wild. Specifically, parasitemia, the only effect with an influence on the preference of *N. kolenatii*, had no predictive power over the natural feeding pattern of bat flies. Conversely, the two terms that could explain variation in the natural distribution (host size and body condition) did not influence the vector in the host-choice experiment.

Choice experiment

Though not all bat flies were observed to change hosts, those that did move chose more often to leave the more infectious host in favour of the host with lower parasitemia. To our knowledge, this is the first study to demonstrate a preference of any member of the Hippoboscoidea based on the host's parasite status. Members of this superfamily are implicated in the transmission of a variety of endoparasites (e.g. *Trypanosoma* spp., *Haemoproteus* spp., *Bartonella* spp. Baker 1967), including the Glossinidae-transmitted human sleeping sickness. An experiment performed on *Trypanosoma congolense* infected cattle could not demonstrate a clear preference of the vector *Glossina pallidipes* (Baylis and Nambiro 1993). Our finding that these bat flies do adjust their host choice according to the presence of (potentially dangerous) blood parasites warrants more in depth studies in other members of the Hippoboscoidea.

The experimental setup could not prevent the bats from grooming themselves. Grooming could cause the observed bat fly distribution pattern if the healthier bats would somehow be less effective groomers. Although grooming has been hypothesized to be the main source of mortality for bat flies (Marshall 1970), its effectiveness has been questioned; Ter Hofstede and Fenton (2005) found no relation between ectoparasite load and the amount of grooming performed by the bat host. Moreover,

bats often seem oblivious to the presence of bat flies, showing no response to their biting (Dick and Patterson 2006) and several studies observed little to no response of bat flies to their hosts' grooming (Overal 1980, Fritz 1983).

Bat fly survival

Independent of the mechanisms used by bat flies to select the least-infected hosts, the survival experiment demonstrated that evading *P. murinus* infections would be an adaptive behaviour. Though oocysts have been found in the local population of *N. kolenatii* (see Figure 1.2), we were unable to demonstrate unambiguously any infection in the bat flies used for the survival experiment. Nevertheless, we found a negative effect of the presence of *P. murinus* on the survival of bat flies, regardless of whether the parasites resided in the blood meal in the gut or were present in the form of oocysts or sporozoites. This reduction in survival could, therefore, be either a direct effect of the parasite on the fly's physiology, or a more indirect effect, wherein the blood meal quality is lower in the presence of malaria.

The cost of infection to the dipteran vector has been a matter of on-going debate (Ferguson and Read 2002). While the survival cost could be mitigated by a higher fecundity, the cost of infection could also act on the fecundity directly (Hurd et al. 1995). Female bat flies have, due to their viviparous life history, many more resources allocated to reproduction as compared to males. Consequently, an infection is expected to act differently on the two sexes. Though reproduction was not measured, the present study found no differences between the sexes in survival, suggesting that *P. murinus* directly acts on survival. In contrast, the viviparous hippoboscid fly *Pseudolynchia canariensis* shows reduced survival in females when exposed to a haemosporidian parasite, but not in males (Waite *et al.* 2012).

The detection of *P. murinus* in the bat fly vector was low. Even if all the *P. murinus* PCR-positive bat flies were actually infected, the infection rate would only be 16%. In contrast, we observed blood stages of the parasite in 28% of the hosts, and a previous study, based on PCR amplification of a cyt*b* fragment of *P. murinus*, found an infection rate of 75% at the same site (Megali et al. 2011). The lower infection rate of the vectors might be caused by an effective immune response of the bat fly, enabling them to quickly clear the body of the haemosporidian parasite. However, this

study suggests a different mechanism, as revealed by the choice experiment. It is the adaptive feeding behaviour of the bat fly, avoiding highly infectious hosts, that minimizes the intake of blood parasites. When an infection reduces the fitness of the vector, as demonstrated in this study, such avoidance behaviour would be the outcome expected of a coevolutionary arms race between a parasite and any of its hosts.

Host switching behaviour

Like most bats, *M. daubentonii* are highly social animals and will often huddle together when roosting. Based on the extremely close body contact of the bats, and the bat fly's capacity for rapid movements when agitated (F. Witsenburg, unpublished observation), the expectation was to see *N. kolenatii* move between the two hosts in the experiment continuously over the day. If flies moved continuously between hosts without distinction, the current location of any bat fly would have provided us with very little information on its main source of blood meals. Instead 63% of bat flies were never observed to leave the bat host on which they were released, and therefore fed on that host exclusively.

Though observations often readily describe the ease with which bat flies move between hosts (Marshall 1970, Fritz 1983, Dick and Patterson 2006), only one other study tried to quantify these host switches in a bat fly, and found that on average 52% of bat flies changed hosts after 24 hours (Overal 1980). From an epidemiological perspective, this rate of vector exchange is extremely high, and any blood parasite transmitted by *N. kolenatii* should easily spread through the population.

Notably, the percentage of bat flies never leaving a particular host is probably a slight overestimate, since any bat flies that temporarily moved to the other host, but then returned before the subsequent observation, would not have been considered to have switched. Increasing the number observations, however, would have meant disturbing the bats more often. Since Nycteribiidae are more prone to leave hosts which are stressed (Marshall 1971), more disturbances could also have artificially inflated the observed number of host switches. The current level of disturbance, handling and marking of bat flies may have already caused changes in their behaviour, which we cannot fully take into account.

The natural distribution of bat flies

The temperate zone bat fly populations show a peak in their population size in summer, which is normally synchronized to the birth of the young bats (Lourenço and Palmeirim 2008a). The bat flies, together with other ectoparasites, then migrate en masse to the new born (Christe et al. 2000, Lourenço and Palmeirim 2008a). Whether this is also true for *N. kolenatii* and its host *M. daubentonii* is not known, but this study found the highest bat fly densities on adults and subadults in August, well after the birth of the new young in June. However, since this was not the primary aim of this study, sampling did not occur regularly throughout the season. June samplings were indeed avoided so as not to disturb the bats during this period.

Even after correcting for seasonal effects, parasitemia of the host had no effect on the *N. kolenatii* abundance on wild bats. Instead only host body size and body condition were found to influence natural bat fly load. The positive relation between bat fly numbers and body condition has been shown in a previous study and has been hypothesized to result from a preference of the bat flies (Reckardt and Kerth 2009). Choice experiments with Spinturnicidae wing mites and their bat hosts demonstrated a clear preference for well-fed individuals (Christe *et al.* 2003), whereas experiments with ornitophilic hippoboscid flies showed a preference for bird hosts in an average condition (Bize *et al.* 2008). In contrast, the choice experiment in the present study demonstrated no preference based on host condition.

The observed correlation between the body condition and bat fly abundance in the wild population might instead be explained by other factors than bat fly feeding preferences, e.g. an increased survival due to the higher nutritional value of well-fed hosts, a grooming-feeding trade-off for the bat or social and/or spatial isolation of weaker individuals of the bat population. In general, many processes apart from preference determine an ectoparasite's - or any vector's - distribution.

This study has demonstrated that the vector distribution cannot be predicted by the feeding preference of the vector. If preference cannot be translated into a relative increase of feeding on certain hosts, other methods will be required to estimate this important variable of epidemiological modelling (Kingsolver 1987). The increasing resolution of genetic typing already allows us to recognize not only host species, but known individual host blood donors from a vector's gut content (Cornet *et al.* 2013). The reducing costs of whole genome sequencing will soon make it feasible to

read most of a host's heritable characteristics from the vectors blood meal. Yet other phenotypic traits such as the host's age and body condition would require different 'low-tech' methods, allowing the observation of natural biting behaviour without disturbance of vector and host. The *Polychromophilus* model system conveniently allowed for these observations since the vector is an ectoparasite. Such observations are valuable when many of the world epidemics are still vector transmitted.

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SUPPLEMENTARY MATERIAL

Supplementary Methods:

Amplification of a 196 bp cyt*b* fragment of Myotis daubentonii

A new nested PCR protocol was designed to amplify a fragment of the *Myotis daubentonii* cyt*b* gene from the *Nycteribia kolenatii* material, similar to the protocol used for detection of the *Polychromophilus murinus* cyt*b* fragment.

All primers, save one, were designed with the aid of Primer3 (Rozen and Skaletsky 1998), and specificity was tested using Primer-BLAST (NCBI). Primers for the first reaction were fw-AATGACATGAAAAATCACCGTTGT (taken from Smith and Patton 1993) and rv-TTWTCAACRGAAAAGCCGCCTCA, which yielded a 500 bp long fragment. The nested primer pair consisted of fw-AACATTCGAAAATCCCACCC and rv-GGTGACTGAGTTAAAGGCTG, and produced a final fragment of 195 bp. The final product was designed to specifically amplify only the cyt*b* fragment of *M. daubentonii*, and not similar regions in Diptera or humans (the main potential sources of contamination).

Reactions were performed in a 25 µL volume containing: 1x PCR buffer (Qiagen, Hilden, Germany), 2.5 mM total $MgCl_2$, 0.25 mM of all dNTPs, 0.5 µM of each primer, 0.25 units Taq polymerase (Qiagen, Hilden, Germany). The first reaction contained 3 µL of *N. kolenatii* extraction as DNA template, and the nested reaction received 1 µL of PCR product from the first reaction as a template. The temperature profile for the first reaction started with 4 min at 94°C initial denaturation, followed by 25 cycles of: 45 s at 94°C, 30 s at 53°C, 45 s at 72°C, and a final elongation step of 10 min at 72°C. The nested PCR reaction started with 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, 45 s at 72°C, and ended with a final elongation step of 10 min at 72°C.

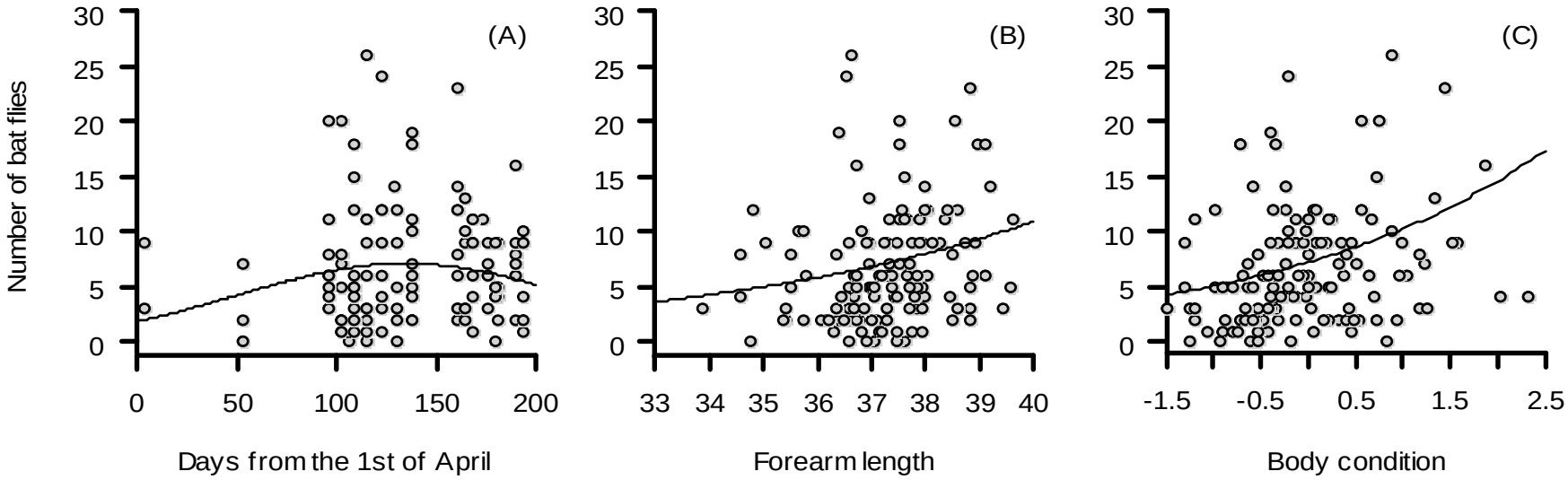
All reactions were performed in duplicate, with a single positive control consisting of the extraction product of *M. daubentonii* blood. Negative controls were also included, one for each 7 reactions. These negative controls included the extraction product of human saliva and legs from *N. kolenatii*.

Supplementary Table S5.1 Host choice experiment model selection. The log-likelihood ratio test (LRT) X^2-value and corresponding *p*-value always refer to the comparison with the smaller nested model below it. Note that when only flies that were observed to switch were included in the analysis, the model including the (non-significant) difference in condition has the lowest AIC value, yet its removal does not increase the residual deviance significantly.

Model with all flies	***AIC***	***deviance***	***LRT***	***p***
Δ length, Δ condition, Δ nycteribids, Δ parasitemia	188.40	45.423	1.0994	0.294
Δ condition, Δ nycteribids, Δ parasitemia	117.50	16.522	1.5814	0.209
Δ condition, Δ parasitemia	117.08	48.103	0.7497	0.387
Δ parasitemia	115.83	48.853		
Model with flies that moved				
Δ length, Δ condition, Δ nycteribids, Δ parasitemia	79.559	42.220	0.4484	0.503
Δ condition, Δ nycteribids, Δ parasitemia	78.007	42.668	0.3974	0.528
Δ condition, Δ parasitemia	76.404	43.066	2.5447	0.111
Δ parasitemia	76.949	45.610		

Supplementary Table S5.2. Model selection of *N. kolenatii* infestation. The most non-significant term is highlighted in bold. Interaction terms: YxD = year * day^2; SxC = sex * condition; AxC = age * condition. The log-likelihood ratio test (LRT) *X*-value and corresponding *p*-value always refer to the comparison the smaller nested model below it.

Model	AIC	Deviance	LRT	p
Maximal model: *Year, Day^2, Day, Condition, Length, Age, Sex,* ***Spinturnix,*** *Parasitemia, YxD^2, AxC, SxC*	773.76	145.83	0.0370	0.847
Year, Day^2, Day, Condition, Length, Age, Sex, Parasitemia, ***YxD^2****, AxC, SxC*	771.80	145.87	0.0810	0.776
Year*, Day^2, Day, Condition, Length, Age, Sex, Parasitemia, AxC, SxC*	769.88	145.95	0.0000	0.997
Day^2, Day, Condition, Length, Age, Sex, Parasitemia,, AxC, ***SxC***	767.88	146.00	0.2729	0.601
Day^2, Day, Condition, Length, Age, ***Sex,*** *Parasitemia, AxC*	766.15	146.28	0.0195	0.889
Day^2, Day, Condition, Length, Age, Parasitemia, ***AxC***	764.17	146.02	2.0237	0.155
Day^2, Day, Condition, Length, ***Age,*** *Parasitemia*	764.19	148.01	0.0348	0.852
Day^2, Day, Condition, Length, ***Parasitemia***	762.21	146.08	1.7515	0.186
Minimal adequate model: *Day^2, Day, Condition, Length*	761.96	147.82		



Supplementary Figure S5.1 The number of bat flies per bat at the time of capture, in relation to (A) date, (B) the size of the bat, as approximated by forearm length and (C) the body condition of the bat. The trend line in each graph was created using averages of the other parameter values.

Chapter 6

The utility of ectoparasites for blood parasite discovery and vector identification

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ABSTRACT

Bats are a known source of zoonotic outbreaks. To get a true grip on the diversity of parasites that bats harbour, sampling effort should be increased. However, blood sampling is stressful for the animals and often requires specific authorizations. Here we propose a less-invasive method of blood parasite screening by collection the haematophagous ectoparasites of bats and amplifying parasite DNA from their guts. An additional advantage of this method is that it immediately allows the identification of most likely vectors, a life stage of parasites often ignored.

We validate our method using *Nycteribia kolenatii*, a bat fly ectoparasite which vectors the haemosporidian parasite *Polychromophilus murinus*. 177 bat flies, collected from hosts of which the infection status was known. The flies are kept off-host until death after which they were stored in ethanol. All flies are tested for the presence of the blood parasite by nested PCR. In 17% of the flies we detect the parasite. This rate was higher in flies that died the first day and dropped to zero after 5 days off-host digestion.

We then apply the method to a sample of tropical bat flies collected in Ghana. The flies were collected from *Hipposideros cf. ruber, H. abae* and *Rousettus aegyptiacus* and the bat fly species were identified as *Penicillidia allisoni, Dipseliopoda biannulate* and *Eucampsipoda africana.* Isolation of the mid-guts provided a single individual with oocyst-like structures. However, we failed to amplify any haemosporidian genetic material. Our collected bat flies, and the hosts they had fed on, were therefore not infected with any malaria parasites. We discuss the vectorial capabilities of the found bat flies and possible pitfalls of our proposed method of parasite screening.

INTRODUCTION

Bats host a diverse range of ectoparasites: fleas, mites, ticks and bat flies (Theodor 1957). What all these ectoparasite have in common is that they are haematophagous. The potential for vector-transmitted pathogens is therefore high in the order Chiroptera. Indeed bats have a reputation as disease reservoirs, being confirmed overrepresented when considering sources of zoonotic viruses (Luis et al. 2013). Interestingly, the variety and often high prevalence of potential vectors is mostly ignored as a possible factor explaining the high pathogen incidence (e.g. Calisher et al. 2006, Muhldorfer 2013), though its role in Ebola transmission has been suggested (Dick and Patterson 2006).

Bats are the hosts to a large array of blood parasites (e.g. *Bartonella*, piroplasms, trypanosomes and microfillaria). In particular, bats are host to a unique collection of Haemosporida (Apicomplexa). Besides *Plasmodium* spp. and the rarer *Hepatocystis* spp., which infect several orders of Mammalia, bats host at least two unique genera which are not found outside the Chiropteran taxa (Garnham 1966), *Nycteria* and *Polychromophilus*, plus 2 other genera known from a single record (Landau et al. 1980a) and Biguetellia (Landau *et al.* 1984)). Up until now, *Nycteria* contains 7 described species (Rosin et al. 1978, Landau et al. 1984), *Polychromophilus* 5 species (Garnham 1966, Garnham et al. 1971, Landau et al. 1980b). These low numbers may rather be a reflection of sampling effort, than of true low diversity.

With the rise of molecular techniques, the description of parasite species has been partially replaced by the description of molecular variation. The mitochondrial cytochrome *b* gene (cyt*b*) is the fragment most often used in haemosporidian research when searching for natural haplotype diversity (e.g. Bensch et al. 2009, Thurber et al. 2013, van Rooyen et al. 2013). It has had some recent successes in finding new taxa of chiropteran haemosporidians (Duval et al. 2007, Schaer et al. 2013). To get a grip on the true extend of bat malaria diversity, though, sampling should be intensified.

Blood sampling is considered a traumatic experience for bats (Wimsatt et al. 2005). Moreover, for the field naturalist taking blood requires the acquirement of specific licences and authorizations, which only a small minority possesses. If sampling should be drastically increased, a simpler, faster and less invasive method of blood sampling would be desirable. Here, we use a method requiring no

blood sampling, in order to find new species of chiropteran Haemosporida. Instead of taking blood samples ourselves, we exploit the haematophagous life style of the bats' ectoparasites instead.

For a majority of malaria parasites, only the vertebrate host is known (Garnham 1966, Valkiūnas 2005). Modern blood parasite discovery relies heavily on taking vertebrate blood samples and amplifying parasite genes. This technique is very successful in finding new host-parasite associations, as exemplified by the expansion of the avian malaria MalAvi database (Bensch et al. 2009). However, for the majority of these parasites the vector remains to be a big unknown and little effort is put into the discovery of new vectors (but see: Kim and Tsuda 2012). A method of collecting and analysing ectoparasites therefore has the additional advantage of immediately identifying potential vectors of any newly discovered blood parasites.

This study will first validate the method by quantifying the detection probability of the haemosporidian parasite *Polychromophilus murinus* (Apicomplexa: Haemosporida) in its vector, the ectoparasitc bat fly *Nycteribia kolenatii* (Diptera: Nycteribiidae). Bat flies spent their entire adult life on their host and feed multiple times a day (Marshall 1970, 1971) making them very suitable for our goals. The main host of both endo- and ectoparasite is *Myotis daubentonii* (Chiroptera: Vespertilionidae), an insectivorous bat of the Palearctic.

All three actors have been found in Western Switzerland near the University campus of Lausanne (Megali et al. 2011) and which made it an ideal system for the current study. We applied the methods tested with our local parasite population on bat fly samples from Ghana, Western Africa, to discover new blood parasite diversity.

METHODS

Method validation

In 2010 and again in 2012, we captured a total of 22 *Myotis daubentonii* and removed all bat flies (identified as *Nycteribia kolenatii*) from their fur. The bat flies were immediately put in separate tubes, provided with moist cotton to prevent dehydration and put at 4°C. Because these flies initially took part in different experiment, the flies were only put in ethanol 70% once they had died, which ranged

from 2-11 days. All bats were blood sampled and had their blood tested for haemosporidian parasites by nested PCR as described in the methods section of Chapter 3.

For the DNA extraction, bat flies were soaked in Milliq water (Millipore) for 2 hours before being triturated using sterile pestles. For the DNA extraction and purification, the Biosprint 96 tissue protocol (Qiagen, Hilden, Germany) was followed, with an overnight digestion.

The presence of *P. murinus* in *N. kolenatii* was tested by amplifying a 705 bp cyt*b* fragment of the parasite following a nested PCR protocol. Primers, reagents and PCR temperature profile were taken from Megali et al. (2011). Per 7 samples tested, a negative control consisting of pure water was included, and two blood extractions of known positive bats (*Myotis daubentonii*) were included as positive controls. All amplified products ran on a 1% agarose gel. Bands were stained with ethidium bromide and visualized under UV light. Each sample was tested in duplicate for the malaria parasite and samples that gave ambiguous results were retested once.To check if the bat flies contained host blood and to track the degeneration of host DNA in the bat fly's gut, we amplified a 195 bp cyt*b* fragment of *M. daubentonii*. The nested-PCR protocol is described in the methods section of Chapter 5.

Samples from Ghana

From 3 locations in Ghana (Bouyem, Forikrom and Kwamang) bats were captured and pruned for bat flies. Collected bat flies were immediately stored on ethanol 70% and, once in the laboratory, at 4°C until dissection. Bat fly species were determined following the key by Theodor (1967) and its later published addendum (Theodor 1968). Each fly was observed under a magnification varying between 20x and 100x. Of each morphological characteristic, photos were taken with a mounted camera. For each identified species, one male and female individual were kept as reference samples and were therefore not dissected and further analysed.

The larger bat flies were dissected in an attempt to find oocysts, the main growth form in the vector of Haemosporida members. Before dissection, each fly was soaked in pure water for ~1 hour to rinse the alcohol. Dissection was performed in a drop of PBS buffer (pH 7.4) on a microscope object glass at 16x magnification. With sterile flamed tweezers and scalpel a small incision was made laterally on the abdomen. Next the abdomen was ripped open across this line, often lying bare the

whole gut which normally is positioned on the dorsal side of the abdomen. The whole gut was then transferred to a new drop of PBS and observed under a microscope at 400x magnification. The gut lining was searched for the presence of oocysts as described by Garnham (1971) and Adam et al. (1973). Moreover, presence of a blood meal in the gut was noted down. Next the whole abdomen (small bat flies) or its content (large bat flies) were transferred to a 1.5mL eppendorf tube and stored at -20°C until further processing.

Bat flies which had been dissected, were not triturated with pestle. As before, DNA was extracted and purified using the same protocol, except elution was in only 100mL buffer. To test if extraction had worked, a 408 bp fragment of the dipteran 16s ribosomal subunit (16s) was amplified for each sample (Petersen et al. 2007) using the same protocol as in Chapter 3. Next, the presence of haemosporidian parasites was tested using the same PCR-protocol as described above.

RESULTS

Method validation

From the 22 *M. daubentonii* we collected 177 *N. kolenatii*. In 155 bat flies (87.5%), host blood could be detected and this probability of detection did not decrease over the period of the experiment (Figure 6.1). The presence of *P. murinus* was detected in 30 individuals, making the overall detection rate 16.9%. In the bat host, *P. murinus* prevalence was 77.8%. Of the flies collected from infected hosts, 22 (15.5%) had detectable levels of *P. murinus*. However, the probability to detect the haemosporidian parasite was higher the first 5 days, after which detection dropped to zero (Figure 6.1).

The bat flies from Ghana

A total of 30 bats were caught: 23 *Hipposideros cf. ruber* (Noack's roundleaf bat), 1 *H. abae* (Aba roundleaf bat) and 6 *Rousettus aegyptiacus* (the Egyptian fruit bat), all from the Yinpterochiroptera suborder. From these hosts, 47 bat flies were collected of three different species. All 30 flies from the *Hipposideros* spp. were identified as *Penicillidia allisoni* (Theodor, 1968), a large, very setose insect (5mm), recognizable by its single lens, closed haltere groove, no abdominal ctenidium in both sexes and the presence of ~13 notopleural setae on the thorax (figure 6.2A). From *R. aegyptiacus* 13 bat flies

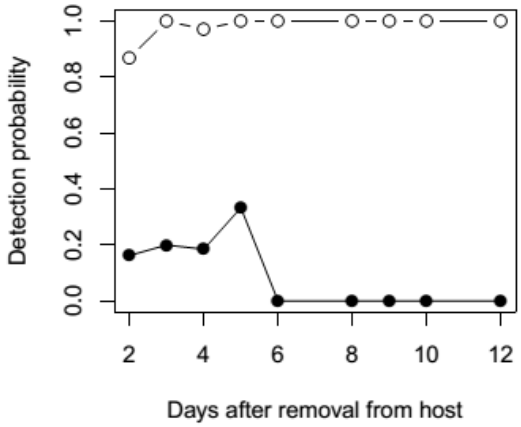

Figure 6.1 The probability to detect host or parasite material in the vector. Whereas host material stays detectable 12 days after the last blood meal, parasites detection drops to zero after day 5. Open circles: Probability to detect host DNA from *N. kolenatii*; Filled circles: Probability to detect haemosporidian DNA.

were identified as *Dipseliopoda biannulate* (Oldroyd, 1953), a small slender species (3.5 mm) with two eye lenses in a pigmented area, cylindrical tibia marked with 2 rings, 1 notopleural seta, 2 processes with clubbed spines on the male anal segment and a few long setae on the female abdomen dorsally (Figure 6.2B-C). Lastly, we identified 4 *Eucampsipoda africana* (Theodor, 1955), superficially similar in appearance as *D. biannulate* but with only a single eliptical lens (Figure 6.2D), a clearly opened oblique suture, only 2-4 long setae on the female dorsum of the abdomen placed posteriorly and the male parameres with hairs and claspers with pegs.

Because of time constraints, only for the large *P. allisoni* and 2 *D. biannulate* were the guts isolated. Of the 28 individuals dissected, 21 still had host blood in their guts. A single *P. allesoni* showed structures possibly representing oocysts (Figure 6.3), however, no haemosporidian DNA was detected in this individual. Overall none of the 40 bat flies tested for the haemosporidian cyt*b* fragment by PCR proved positive, but 35 did amplify their own 16s fragment (Table 6.1).

Table 6.1 Dissection and PCR amplification results for all bat flies.

	N	***host***	***oocysts***	***blood***	***malaria***
P. allisoni	30	*Hipposideros* spp.	1/26	21/26	0/24
D. biannulate	13	*R. aegyptiacus*	0/2	0/2	0/10
E. Africana	4	*R. aegyptiacus*	-	-	0/1

N: total number of bat flies collected. *host*: host species. *oocysts*: were oocyst-like structures observed? / total number of bat fly guts observed. *blood*: was host blood observed in gut? / total number of bat fly guts observed. *malaria*: number of bat flies positive for haemosporidian DNA / number of bat flies positive for bat fly DNA. N.B. Most *D. biannulate* and *E. Africana* were not dissected.

DISCUSSION

Though previous studies have used vector gut content to determine the blood donor species (e.g. Hellgren et al. 2008), this study was the first attempt to discover through host blood in the ectoparasites new haemosporidian parasites. Using a known vector, feeding from an infected host population, we managed to detect the parasite in 17% of the bat flies. After 5 days we could no longer detect the parasite. Because we had no problem detecting the host DNA until the end of the experiment, we believe that digestion of the parasite DNA was not an issue here. Rather than a reduction in detection rate, we rather see the effect of differential survival chances of those flies with and without the parasite (see Chapter 3 for a full discussion on vector survival).

One shortcoming of our experiment was that the flies were not kept at ambient temperatures, but significantly lower. Though bat flies in temperate zones more than likely will encounter such temperatures, the large majority of bat and their ectoparasites reside in the warmer (sub)tropics. Higher temperatures will most likely accelerate the process of DNA degradation. However, in our experiment we were hampered by the setup which was designed for another experiment. If the *N. kolenatii* had been stored on ethanol immediately after host removal, our detection rate would most likely have been much higher. The 17% detection rate should therefore be considered a conservative estimate.

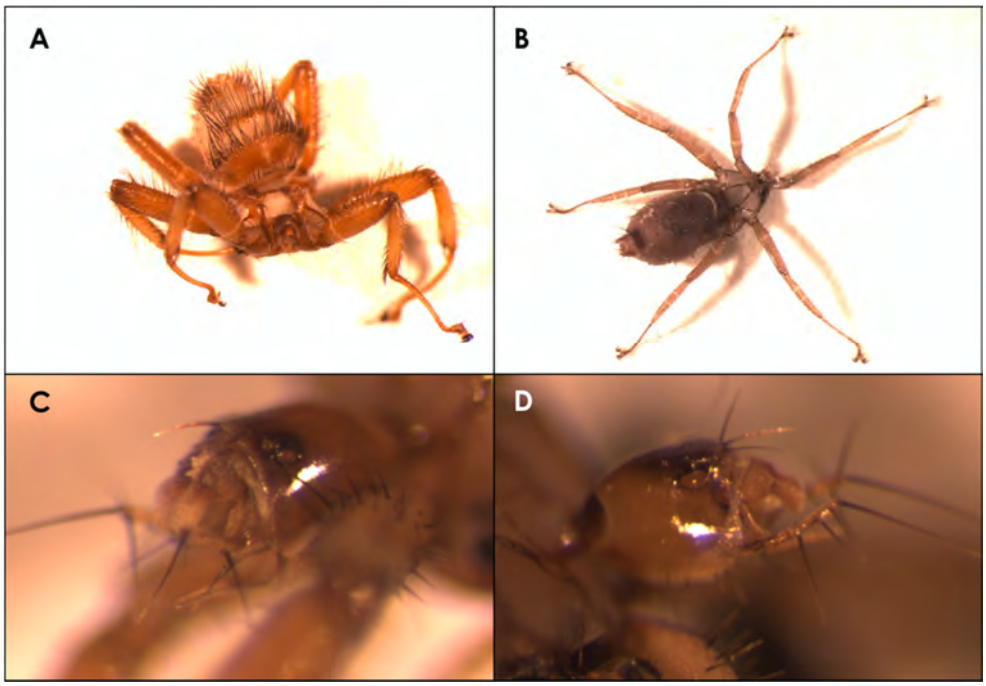

Figure 6.2 Bat flies from Ghana. (A) *Penicillidia allisoni* is a very robust built bat fly species; (B) *Dipseliopoda biannulate* is smaller and more slender. *Eucampsipoda africana* looks, superficially, very similar; (C) Detail of the head with two lenses on each eye patch of *D. biannulate*; (D) Head with single elliptical lens of *E. africana*.

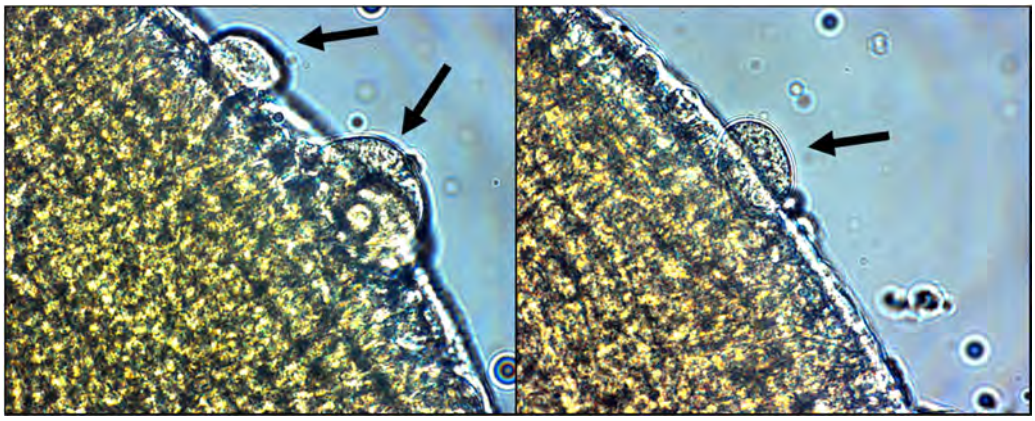

Figure 6.3 Oocyst-like structures observed during this study on the gut wall of a single individual. Immature oocysts do not yet have the characteristic 'pear shape' (compare with Figure 1.2). Failure to amplify malaria DNA indicates that these structures were no haemosporidian parasite. Suspected oocysts indicated by an arrow. Lower left of each image is the gut wall.

The field samples consisted of bat flies that had been immediately been put on alcohol. Still, only one sign of a haemosporidian parasite was found. The oocysts-like structures observed under the microscope must have been something else however, as the affected bat fly proved negative for malaria DNA. If it was a parasite, it must have been non-haemosporidian in nature, since the PLAS-primers are known to amplify all four major bat-malaria genera (Duval et al. (2007) amplified all four genera, though these were only correctly identified in Schaer et al. (2013)). All other bat flies similarly did not amplify the haemosporidian gene fragment. None of the collected bat flies were therefore carrying, nor vectoring any Haemosporida species.

The large majority of dissected bat flies still contained large quantities of blood in their digestive tracts. Indeed, most bat flies will be engorged when directly collected from the host (personal observation). It is consequently reasonable to assume that a majority of the unchecked bat flies will also have contained host blood. Moreover, based on the method validation and as discussed above, DNA degeneration was not an issue. Therefore, not only the bat flies, but also the bats on which these bat flies had fed were not infected with haemosporidian parasites, at least not with any blood stages. That notwithstanding, to conclude that none of our tested species of Vertebrata and Diptera is not a host to Haemosporida would be premature, seen the modest sample sizes.

Hipposideros spp. host a wide array of haemosporidian parasites; species of all six malaria genera have been observed in this bat genus (for an overview: Landau et al. 2012). However, *H. cf. ruber* itself has never been observed harbouring malaria parasites (Duval et al. 2012, Schaer et al. 2013). Despite several attempts, few malaria parasites have been found in *Rousettus* spp. Only twice have blood parasites been found and both times it turned out to be a new species of *Plasmodium: P. rousetti* and *P. voltaicum* (Garnham 1966). Only one recent study has looked at haemosporidian prevalence in *R. aegyptiacus* which, as in this study, found all individuals free of malaria (Schaer et al. 2013).

Penicilidia spp. have been implicated in the transmission of *Polychromophilus* spp.; sporozoites have been found on at least two occasions (Mer and Goldblum 1947, Adam and Landau 1973). Bat flies in general have long been suspected to vector *Nycteria* spp. (Garnham 1966), which, considering *Nycteria*'s ancient origin similar to *Polychromophilus* (Schaer et al. 2013), is most reasonable. We found no proof of any transmission by the bat flies, which makes sense if the hosts are also malaria free.

However, only *E. africana* has *R. aegyptiacus* as its primary host; *D. biannulate* is a general ectoparasite (Theodor 1967) and *P. allisoni* has only been described on other *Hipposideros* spp. (Theodor 1968). More generalist ectoparasites might transmit haemosporidians for other species of hosts.

Contemporary screening studies, looking for new species, hardly ever include vectors, which results in a growing collection of parasites with unknown transmission modes and stages. We argue that focus should move from the host, to both host and vector. By collecting and analysing the haematophagous arthropods from and around the host, both host and vector are analysed, which reduces both effort for the researcher and stress for the vertebrate host. One can increase sampling success by targeting more susceptible hosts (e.g. juveniles; Chapter 3) and sampling in periods of high parasite abundances (e.g. during the nursing season). When blood parasites are detected in the collected sample, specific hosts can be targeted for the acquirement of blood and tissue stages.

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Chapter 7

Synthesis and Discussion

Synthesis

The aim of this thesis was to look at the evolution and ecology of *Polychromphilus* spp. and identify the role of the bat fly vector in its natural history. The previous chapters have discussed the phylogenetics, population genetics and epidemiology of the parasite. In addition I have investigated whether including the vector's biology in the analysis increases our understanding of the parasite's evolutionary ecology.

In terms of phylogenetics, *Polychromophilus* spp. seems to be somewhere between the sauropsid and mammalian branches of haemosporidian parasites. The phylogenetic support favours a sauropsid origin but the evidence is not conclusive (Chapter 2). A more recent study included samples of the other elusive chiropteran haematozoon: *Nycteria* spp. (Schaer et al. 2013). Their analysis puts *Polychromophilus* at the root of the mammalian branch of Haemosporida, albeit again with low support. However, the second taxon to branch off the mammalian group is the other bat-specific genus *Nycteria*. This leads to the exciting hypothesis that *Polychromophilus* might not have made an independent switch to mammals, but that instead bats were the original hosts of all mammalian haemosporidian parasites (Schaer et al. 2013). A primeval vector with a broad host-range could have facilitated the radiation of that original parasite to the other orders of mammals, unlike the bat-specific Nycteribiidae. In fact, the switch to mosquitoes might hold the key to successful radiation of both the mammalian and sauropsid *Plasmodium* species (Martinsen et al. 2008).

On a more recent time scale, we find that the distribution of *P. melanipherus* genotypes cannot simply be predicted by the host or vector dispersal patterns (Chapter 3). However, knowledge of *N. schmidlii*'s population structure is essential to understand why the malaria's genotypes are spread equally across the European range. Without *N. schmidlii*, the ecological contact between *M. schreibersii* populations would have been invisible and therefore the parasite's apparent broad distribution pattern unintelligible.

Within a population of *M. daubentonii*, the *P. murinus* infections are most intense in the young (Chapter 4). This is a common physiological response of hosts to their primary infection (Garnham 1966) and the role for *N. kolenatii* in this process is probably minor. That said, bat flies do 'en masse' migrate to new born young (Christe et al. 2000), increasing the probability of infection for those young, and perhaps even the intensity, by the repeated release of sporozoïtes in the blood stream.

The second question of my thesis concerned how *Polychromophilus* spp. affect their Nycteribiidae vectors. *Nycteribia kolenatii* experience increased mortality when confronted with the parasite *P. murinus*. Moreover, they showed a behavioural preference for hosts with the lowest number of parasites in their blood, which in the light of their reduced survival could be considered adaptive (Chapter 5). However, even though we find theses links, we should be cautious with this conclusion. Instead of direct causation, common underlying factors also may cause these patterns observed in vector behaviour.

No effects were found of *P. melanipherus* on the population structure of *N. schmidlii.* However, we could not separate the infected from uninfected vectors in our experiment and look for genetic differences in the two groups (Chapter 3). If survival is affected by the infection, as it is in *N. kolenatii*, it is possible that the presence of the parasite increases isolation between bat fly populations by making it less likely for bat flies to be exchanged over longer distances. Only experimental manipulations, on a massive scale, could prove such a relation between parasite presence and vector gene flow.

On coevolution

The general theme of my thesis has been the coevolution between parasite, vector and host. The previous chapters revealed some striking links between *Polychromophilus* and its vector's behaviour (Chapter 5), but as already said we did not demonstrate *causation*. Moreover, for it to be coevolution, we also need to demonstrate that the parasite responses to the change in vector behaviour, e.g. by changing the attractiveness of the infected hosts (Lacroix et al. 2005).

We also observed a striking absence of correlation, when comparing the population genetics of species (Chapter 3). Thompson (2005) argued that due to gene flow and drift, correlations between parasites and hosts might not always be perfect or even be absent in certain sites, causing a 'geographic mosaic of coevolution'. The fact that the population structure of *P. melanipherus* does not correlate with that of *N. schmidlii* and *M. schreibersii* does not necessarily mean coevolution is not at play. The problem is that the null hypothesis of no coevolution predicts the same observations and therefore no sensible analysis can discriminate between these two scenarios. Only the identification of

genes under selection, as well as experimental manipulation could truly determine if coevolution is at play (Nuismer et al. 2010).

On Polychromophilus biology

This thesis used *Polychromophilus* as a model sytem, a little known species. But what new facets of *Polychromophilus* natural history has this thesis discovered? *P. melanipherus* is a cosmopolitan parasite and well established Europe-wide. Not a single *M. schreibersii* population we sampled in Chapter 3 was parasite-free. Whether *P. murinus* is also so widely distributed with its host *M. daubentonii* we do not know. Previous studies have found it in other species than *M. daubentonii* though (Lanza 1999, Megali et al. 2011), and during my thesis I once encountered *P. murinus* in *M. schreibersii* blood. However, the low frequencies of these cases suggest these are rather dead-end infections caused by spill over effects. How strongly *P. murinus* depends on specific bat fly species for transmission we do not know. One opportunity to test this can be found in Latvia; curiously, *M. daubentonii* there do not seem to be carrying *N. kolenatii* bat flies (Jaunbauere et al. 2008).

Surprisingly, for a malaria parasite of bats, the life cycle of *Polychromophilus murinus* has already been surprisingly well documented, even the vector stages (Garnham 1966, Gardner and Molyneux 1988a). But even though the physiology is well known, we know little of the effects of infection, especially the consequences of infection for the bat. The intensity of *P. murinus* infection is linked to a lower body mass of the host (Chapter 4), but only an experimental infection would allow the disentanglement of cause and effect. A next step would be to find out what such physiological effects have on the evolutionary ecology of *M. daubentonii* i.e. the fitness consequences of infection in the wild. As we have seen with *N. kolenatii*, the natural processes are not easily predicted based on laboratory findings (Chapter 5).

On haplotypes, lineages and morphospecies

This thesis treated two species of Polychromophilus: *P. murinus* and *P. melanipherus*. Both species have been genetically characterized at the cytochrome *b* gene. Within the 700 bp of this fragment, *P. murinus* had 7 different haplotypes in Western Switzerland (Megali et al. 2011), *P. melanipherus* 8 different haplotypes across Southern Europe (Chapter 3). For comparison, within a single European

region normally 5 haplotypes were present (in Spain, Portugal and Croatia). The richness of haplotypes seems therefore similar. However, the genetic distances between haplotypes were small among the seven Swiss *P. murinus* haplotypes, with 1 - 5 nucleotide differences. The Swiss *P. melanipherus*, by contrast, had 7 - 13 nucleotide differences between their 4 haplotypes (Figure 3.4). Cytochrome *b* is of course a protein coding gene and this could therefore mean that different selection pressures act on the different species of *Polychromophilus*. Alternatively it could be that it is the biology of the hosts and vectors that are responsible for the different haplotype distributions.

In avian malaria research, all cyt*b* haplotypes are considered distinct non-recombining genetic 'lineages', since they often have non-overlapping host distributions (Bensch et al. 2000) and show linkage with nuclear haplotypes (Bensch et al. 2004). In our case, the nuclear gene sequenced for *P. murinus* and *P. melanipherus* does not provide any conclusive evidence for or against recombination with the cyt*b* fragment (Supp. Table S2.2). Furthermore, both the *P. melanipherus* and *P. murinus* haplotypes are shared among their respective host species, with no geographical delineations (Chapter 3, Megali *et al.* 2011). Despite the relatively long genetic distances among the *P. melanipherus* haplotypes, the most likely scenario at this time is that these haplotypes are still recombining and should not be considered distinct lineages.

Methodological considerations

The rise of molecular techniques in parasitology also inadvertently meant a decline of light microscopy (Perkins et al. 2011), not only to describe new parasite species or lineages (but see: Palinauskas et al. 2007), but also to determine parasite prevalence and abundance. Though PCR and qPCR undeniably are much more efficient at analysing of hundreds of blood samples at a time, some pitfalls are associated with the use of molecular techniques; primers may be unsuitable for the parasite present (Perkins et al. 2011) or only dead-end stages, injected by the vector, are detected (Valkiunas et al. 2009), leading to the false conclusions that the host is uninfected or infected respectively. Moreover, the Haemosporida are characterized by a variable number of cyt*b* copies (Vaidya et al. 1989), hampering any interspecies comparison of qPCR results. However, the main advantage of light microscopy over both these methods is that is requires no initial setup and is applicable to any exotic parasite species under study.

Vector identification is still a problem in malaria research leading to unfounded generalizations of parasite-vector affiliations (Santiago-Alarcon et al. 2012). Though PCR screening is a good start for identifying potential candidates, it can never be the final proof of vectoring capabilities, especially when the candidate vector is a haematophagous ectoparasite of the host (e.g. Billeter et al. 2012). Instead, parasite gene amplification should be accompanied by dissection of the arthropod vector (Chapter 6) and tested for any traces of host blood (Chapter 5). In this thesis I made the assumption that *N. schmidlii* is the vector for *P. melanipherus* in Europe (Chapter 3). This is a major weakness of the study. Dissected *N. schmidlii* and *P. conspicua* have neither revealed any oocysts of the malaria parasite and isolation of the salivary glands, with potential sporozoites, has proven unsuccessful yet. Even screening the bat flies by PCR has, up to now, not provided unambiguous evidence of a *P. melanipherus* infection in those bat fly species. These screenings should therefore continue in the future, preferably on live samples.

Polychromophilus spp. as a study system

Why would one use *Polychromophilus* spp. as a study system? Unlike many exotic parasites, *Polychromophilus* can be found in on all six continents (Garnham 1973b) and should therefore easily be available to many researchers across the globe. Moreover, a single species, *P. melanipherus*, has a range that covers several climatic zones, from the tropical (Duval et al. 2012) to temperate (Chapter 3). Their main hosts, *M. schreibersii* and *M. daubentonii* are cosmopolitan species as well (Dietz et al. 2009). These distributions allow for studies of parasite colonization and local adaptation across an extremely large geographical range. Bats are not only the second largest order of mammals, they also have several peculiarities that set them apart from other mammals such as their flight-induced high metabolism, their aggregation in extremely high numbers and their relatively old maximum age. Studying different bat-specialised pathogens allows us to identify the effect that each of these factors may have on parasite-host coevolution.

Although working with *Polychromophilus* spp. allows the study of many interesting questions, there are some drawbacks to working with this system. First of all, though not all species are threatened, all bats in Switzerland are protected (Red lists of Switzerland 1994), which implies several restrictions when working with them. Secondly, *M. daubentonii* is a reservoir of European bat

lyssavirus (Amengual et al. 1997, Megali et al. 2010). Researchers should therefore take the necessary precautions when working with bats. Once roosts or flight routes have been identified, capturing bats is fairly straightforward. By contrast, bats do not respond well to long confinement; long captivity can result in a significant rise of leucocytes in *M. daubentonii* blood (personal observation). Moreover, bats in captivity should be hand fed, especially the first few days, since they will not recognize the provided food source.

Future directions

In each chapter of this thesis as well during the discussion above I have made recommendations for future research. For example in Chapter 2, the main direction would be to find the original vector of mammalian malaria. In Chapter 3 I point out the need to develop microsatellite markers for *P. melanipherus*. In Chapter 4 I suggest quantifying the fitness effects of *P. murinus* for *M. daubentonii.* Chapter 5 left us with the question of the low prevalence of *P. murinus* in *N. kolenatii*. In Chapter 6 the next step would be to use this method to uncover the true *Polychromophilus* species diversity (see also Chapter 2). In this last section of my thesis I will not discuss these further. Instead, I would here like to suggest a new avenue that could be explored.

During the screening of blood smears for this thesis, I have observed the presence of *Trypanosoma dionisii* in the Greater Mouse-eared bat *Myotis myotis*. *Trypanosoma* spp. are unicellular vector-transmitted blood parasites from the phylum Euglenozoa. As for the Haemosporida, their first mammalian hosts are suspected to be bats (Hamilton et al. 2012). And as with most bat-Haemosporida research, most of it focusses on discovering new species and their distribution (e.g. Maia da Silva et al. 2009, Marcili et al. 2009). Studies focussing on the evolutionary ecology and epidemiology are again lacking. Studying a second vector-transmitted protozoan in the same species (e.g. *M. schreibersii*; a PCR trial found 3 out of 8 bats positive for *Trypanosoma* sp.) allows comparative studies, identifying factors that influence the epidemiology and distribution of such parasites.

Known vectors of *Trypanosoma* spp. are the *Cimex* bugs, Triatominae assassin bugs and *Glossina* tsetse flies. Whether the closely related Nycteribiidae bat flies might play an additional part in the transmission of *Trypanosoma* spp. is not known. In fact, the potential for pathogen transmission is rather high for the Nycteribiidae. They might play a role in the transmission of Bartonella species

(Billeter et al. 2012) or even Ebola and Marburgvirus (Monath 1999). Finding proof for such transmission pathways does not only help understand the evolution of these pathogens, but also helps predict and curb the spread of such diseases.

Broadening our views to trypanosomes could be the start of cataloguing other protozoan parasites of bats. Are bats overrepresented as a host for these protozoans? And is there a link between the presence of protozoans and the load and diversity of ectoparasites that bats harbour? In the end, and as always, the question boils down to one which has been asked before (Wang et al. 2011, Luis et al. 2013), and, based on the unique biology of these mammals, will be asked many times more: "Are bats special?"

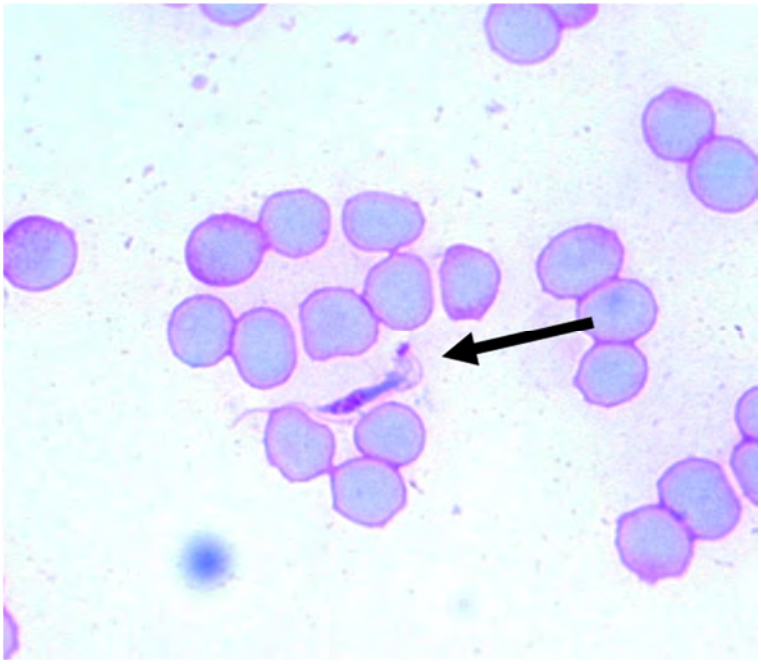

Figure 7.1 *Trypanosoma dionisii*, as seen in a thin blood smear of *Myotis myotis*, offers opportunities to perform comparative studies on bat blood parasites. Parasite indicated by the arrow. Thin blood smear, giemsa stained, 1000x magnification.

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All images were reproduced with permission, for which I would like to express my gratitude. Figures not mentioned below have been produced by myself.

Figure 1.1A-B	Bas Garcia	
Figure 1.4A	Gilles San Martin	http://www.flickr.com/photos/sanmartin/
Figure 1.4B	Adrià López-Baucells	
Figure 1.5A	Manuel Ruedi	
Figure 1.5B	Adrià López-Baucells	

APPENDIX A

Measuring Parasitemia

Several methods exist to measure and express blood parasite load by microscopy. Generally, the most accurate method is to observe a fixed number of red blood cells (RBC) and score the number infected. With high infection loads this number of observed RBC can be pretty low (2000; Godfrey et al. 1987). However, *Polychromophilus murinus* does not multiply in the blood and only the sexual gametocytes will invade erythrocytes. Infection loads are therefore generally low which would require counting a high number of RBC (~10'000). Since this can consume a lot of time, we wanted to compare this method with two other commonly used methods in their accuracy and speed.

Blood smears from twelve *Myotis daubentonii* were selected which, based on an initial scan of the smears, showed a range of infection intensities. We quantified parasite load by three measures of parasite density, based on number of RBC observed, number of microscope field observed and the number of minutes spent observing (referred to as 'per cell', 'per field' and 'per time' respectively). For the first two methods, 50 photos were taken of the blood smear at 630x enlargement. Photos were taken at regular intervals, in ten columns spread regularly across the smear, with 5 photos per column, resulting in a photographic cross section of the entire smear. Slightly enlarged RBC, like those infected with *Leucocytozoon* or *Polychromophilus*, tend to end up on the edges of a smear (Godfrey et al. 1987). To get an unbiased estimate of parasite density, it is therefore vital to observe all regions of the smear.

The resulting 50 photos were the 50 fields; the total number of *P. murinus* infected RBC observed on these photos was used for the 'per field' measurement. Next, all RBC on these photos were counted using the python script Plasmodium Autocount and corrected manually using the stand alone software Cell Counting Aid (Ma et al. 2010). The cell count correction was done per photo analysed, alternatively picking photos taken at the beginning and the end of the smear, until the threshold 10'000 RBC had been reached. The total number of infected RBC encountered on these analysed photos was taken as the 'per cell' measure. Depending on the thickness of the smear, the number of photos thus analysed per smear ranged between 25-45.

The last measure 'per time' was taken by scanning a blood smear at 630x for 15 minutes. The smear was traversed several times during the observation period, moving in a criss-cross pattern. Each *P. murinus* parasite encountered during this period was scored and the total number of parasites thus counted consisted of the measure 'per time'.

Counting 10'000 RBC took ~3 hours per smear, including taking the photographs. Observing the 50 fields -and photographing them- took approximately 1 hour per smear. Taking the photographs was not necessary for this method and could therefore be dropped, reducing the time per smear to ~30 min. The time-method was slightly quicker still requiring less than 20 min per smear.

The 'per time' and 'per field' method detected more infected RBC than the 'per cell' method, resulting in a wider range of parasite load values (Figure A.1). Since a few zero-counts were included, the data was $\log(x+0.5)$ transformed before Pearson's correlation test was performed. Both the 'per time'($r= 0.9417$, $df=9$, $p<0.0001$; Figure A.2A) and 'per field' ($r=0.9217$, $df=9$, $p<0.0001$, Figure A.2B) measurement correlated significantly with the 'per cell' count, but the explained variance was slightly higher for the 'per time' ($r^2= 0.8867$) than the 'per field' ($r^2= 0.8495$) method.

Since the 'per time' method was slightly more accurate and faster, we chose this method for measuring parasitemia in our study.

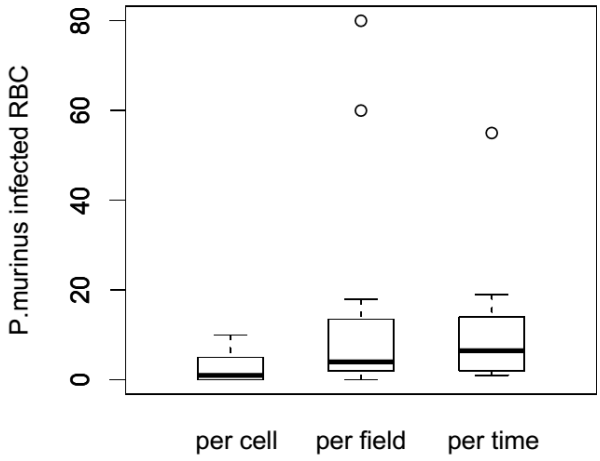

Figure A.1 The number of *P. murinus*-infected red blood cells encountered using the three different methods.

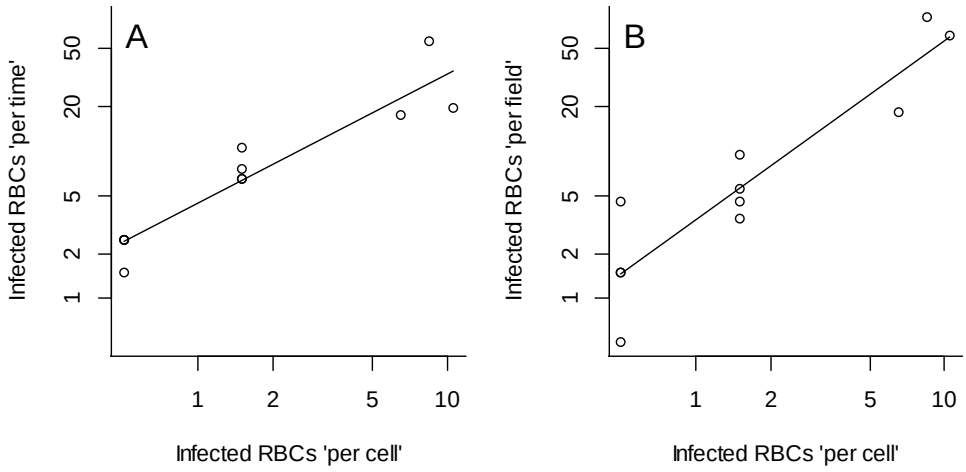

Figure A.2 Correlations between measures of blood parasite density. Both axes are on log-scale.

Sources:

Godfrey, R. D., A. M. Fedynich, and D. B. Pence. 1987. Quantification of Hematozoa in blood smears. Journal of Wildlife Diseases **23**:558-565.

Ma, C., P. Harrison, L. N. Wang, and R. L. Coppel. 2010. Automated estimation of parasitaemia of Plasmodium yoelii-infected mice by digital image analysis of Giemsa-stained thin blood smears. Malaria Journal **9**.