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Natal Philopatry in Song Sparrows (*Melospiza melodia*) Predicts Female Cellular Versus Humoral Immune Function, But Does Not Consistently Predict Parasitism

Heather L. MacGillivray
The University of Western Ontario

Supervisor
Dr. Elizabeth A. MacDougall-Shackleton
The University of Western Ontario

Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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NATAL PHILOPATRY IN SONG SPARROWS (*MELOSPIZA MELODIA*) PREDICTS FEMALE
CELLULAR VERSUS HUMORAL IMMUNE FUNCTION, BUT DOES NOT CONSISTENTLY
PREDICT PARASITISM

(Thesis format: Monograph)

by

Heather Louise MacGillivray

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biology

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Parasites vary geographically and dispersing host individuals may encounter different strains and wider varieties than their philopatric counterparts. Previously observed lower parasite counts in philopatric than dispersing birds have been suggested to be the result of local adaptation to parasites, but I suggest an alternative: these patterns may result from differing immune function. I used genetic assignment tests to infer natal philopatry of song sparrows (*Melospiza melodia*), and tested whether this predicted parasitism and innate and adaptive immune function. I found no relationship between parasitism and philopatry, in contrast to previous findings. In females, philopatry was associated with higher cellular innate immunity and lower humoral innate immunity. I also detected a negative relationship between measures of cellular adaptive immunity and humoral innate immunity. These results suggest a sex-specific, philopatry-mediated immune tradeoff that occurs along the cellular-humoral axis, rather than the innate-adaptive axis traditionally focused on in ecophysiological life history.

Keywords

Philopatry, dispersal, parasite, coevolution, haematozoa, immunocompetence, song sparrow, *Melospiza melodia*

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1 Introduction

1.1 Host-parasite interactions

A parasite is an organism that lives in or on a host for at least part of its life cycle, deriving nourishment and habitat from the host and causing the host harm (Anderson and May, 1978). Parasites are a diverse group taxonomically, encompassing ten phyla (Poulin and Morand, 2000), including taxa ranging from bacteriophages (Buckling and Rainey, 2002) to fungi (Ahmed et al., 1995) to invertebrate (Lively, 1999) and vertebrate animals (Bence et al., 2003). Indeed, parasitism appears to be the most common strategy for consumers on earth (Gómez and Nichols, 2013), with parasites comprising about 40% of described species (Dobson et al., 2008).

By definition, parasites divert resources from their host, often resulting in reduced fitness of the host individual in many different taxa. In some cases, parasitic infection reduces host sexual ornamentation (Hamilton and Zuk, 1982). For example, male three-spined sticklebacks (*Gasterosteus aculeatus*) experimentally infected with parasites showed reduced brightness and hue of red colouration, even after infections were cleared (Milinski and Bakker, 1990). Other harmful effects of parasitic infection include reduced reproductive success. For example, female blue tits (*Cyanistes caeruleus*) treated with an antimalarial drug had higher hatching success, fledging success and provisioning rates to their offspring relative to control females that were untreated and presumably parasitized (Knowles et al., 2010). Similarly, female red squirrels (*Tamiasciurus hudsonicus*) had heavier offspring when ectoparasites were removed before the breeding season, and their offspring were more likely to survive (Patterson et al., 2013). An observational study of great tits (*Parus major*) revealed that females infected with parasites of the genus *Trypanosoma* had lower hatching success and produced smaller offspring in worse body condition than did unparasitized females (Dufva, 1996a). In addition to their effects on ornamentation and reproductive success, parasites can also reduce host survivorship. For example, flies (*Drosophila putrida* and *D. neotestacea*) experimentally infected with a nematode parasite (*Howardula*

aoronymphium) showed greater mortality than uninfected counterparts (Jaenike et al., 1995). Female blue tits that were experimentally treated with an antimalarial drug to reduce *Haemoproteus* sp. infection were more likely than control females to be recaptured in subsequent years (Martínez-de la Puente et al., 2010). This finding suggests enhanced survivorship of treated females and thus a harmful effect of parasites on survival.

In addition to, and because of, parasite effects on the fitness of individual hosts, interactions with parasites also affect the evolution of host populations. For example, parasites have been hypothesized to promote the evolution of extravagant sexually selected traits in host species, because such traits may allow females to indirectly assess heritable disease resistance in potential mates (Hamilton and Zuk, 1982). In red grouse (*Lagopus lagopus scoticus*), the condition dependence of a secondary sexual characteristic was stronger at sites and years with higher prevalence of infections by nematodes (*Trichostrongylus tenuis*) (Vergara et al., 2012). Parasites may also promote the maintenance of population genetic diversity, if genetically diverse individuals are better able to avoid or control parasitic infection. For example, mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*) infected with haematozoan (blood-borne) parasites had lower neutral-locus (microsatellite) heterozygosity than did their uninfected counterparts (MacDougall-Shackleton et al., 2005). Similarly, juvenile harbour seals (*Phoca vitulina*) with lower microsatellite heterozygosity had higher lungworm burden (Rijks et al., 2008). In some cases, parasite-mediated promotion of genetic diversity can be ascribed to heterozygote advantage at specific coding loci, most notably at the major histocompatibility complex (MHC). For example, among mice (*Mus domesticus*) experimentally infected with multiple *Salmonella* and *Listeria* strains, MHC heterozygotes had higher survivorship than MHC homozygotes (Penn et al., 2002).

Because hosts represent an important component of their parasites' environment, hosts may impose selection pressures on parasites just as parasites do upon hosts. For example, the evolution of host resistance to highly virulent parasites is expected to place selection on parasites favouring the evolution of reduced virulence (Ebert, 1994).

Another way in which hosts may influence the evolutionary trajectories of their parasites is through co-speciation events (Sorenson et al., 2003). Host switching events can induce speciation in parasite lineages. For example, in the brood-parasitic African indigobird (*Vidua* spp.), host switching leads to significantly differentiated allele frequencies in populations with different hosts (Sorenson et al., 2003). This supports the hypothesis that parasites may speciate along with their host species. Thus, hosts and parasites can impose important and reciprocal selective pressures.

1.2 Coevolutionary arms races and local adaptation in host-parasite systems

The selective pressures that host and parasite populations impose upon one another can give rise to a coevolutionary arms race. This term refers to an escalation of reciprocal selection pressures in two phylogenetically unrelated populations with conflicting conditions for best fitness (Dawkins and Krebs, 1979; Jenkins et al., 2011). Coevolutionary arms races occur over a multiple-generation rather than an individual time scale. Moreover, because natural populations vary geographically, arms races between hosts and parasites play out on a spatial scale. Thus, host-parasite interactions have important consequences for local adaptation, in which a population has higher fitness at its native site than at any other site, and in which no introduced population has higher fitness than the native one (Oppliger et al., 1999; Savolainen et al., 2013).

1.2.1. Parasite local adaptation: Within the context of local adaptation, there are three possible outcomes of host-parasite interactions. First, parasites may become adapted to their local hosts, such that parasites are better able to infect or exploit sympatric host populations than allopatric host populations. In this case, sympatric hosts are those from the same area as the parasite, and allopatric hosts are from a different area. It is expected that parasite local adaptation should be common, because parasites typically have faster generation times and larger population sizes than their hosts. This may provide parasites with an evolutionary advantage over their hosts (Gandon and Michalakis, 2002). Indeed, a recent review of local adaptation in host-parasite systems suggests that parasite local adaptation to hosts is widespread (Greischar and Koskella,

2007). Examples of parasites having greater success on sympatric than allopatric hosts include a fungal parasite *Mycosphaerella graminicola* on local populations of its wheat host (*Triticum aestivum*, Ahmed et al., 1995); a trematode parasite (*Microphallus* sp.) on local populations of its freshwater snail host (*Potamopygrus antipodarum*, Lively, 1989); a hoverfly parasite (*Microdon mutabilis*) on local populations of its ant host (*Formica lemani*, Schönrogge et al., 2006); and a tick parasite (*Ixodes uriae*) on local populations of its seabird host (*Rissa tridactyla*, McCoy et al., 2002). In all cases, the parasite showed evidence of being particularly well adapted to local host populations, relative to more distant host populations, or relative to more distant parasites combined with the same host population, usually as measured by incidence or intensity of parasitic infection.

1.2.2. No local adaptation by either party: A second potential outcome of coevolutionary arms races between hosts and parasites is that neither party becomes locally adapted to the other. This can be inferred in systems in which a parasite performs equally well (same risk or intensity of infection) on sympatric and allopatric hosts. Such a pattern presumably reflects similar rates of local adaptation by each party, not necessarily a lack of coevolution. Examples of systems showing no local adaptation are taxonomically diverse, such as a bean (*Phaseolus coccineus*) and its fungal parasite (*Colletotrichum lindemuthianum*), in which infectivity and aggressivity of the fungus were the same for sympatric and allopatric plant-fungus combinations (Sicard et al., 2007). Additionally, a pea aphid (*Acyrtosiphon pisum*) was not parasitized more by its parasitoid wasp (*Aphidius ervi*) based on whether the wasp was sympatric or allopatric (Hufbauer and Via, 1999). A bumblebee (*Bombus terrestris*) suffered no significantly greater mortality or mass loss from its trypanosome parasite (*Crithidia bombi*) at a single-region scale (Imhoof and Schmid-Hempel, 1998). In vertebrates, great tit nestlings (*Parus major*) experienced no difference in the effects of sympatric versus allopatric flea parasites (*Ceratophyllus gallinae*) in terms of mortality and morphometric condition (Dufva, 1996b).

1.2.3. Host local adaptation: Finally, coevolutionary arms races between hosts and parasites may result in hosts becoming adapted to their local parasites. That is, hosts

may be better able to avoid or control infection, or both, by sympatric rather than allopatric parasite strains. Theoretical simulations (Gandon and Michalakis, 2002) and meta-analyses (Greischar and Koskella, 2007) suggest that host local adaptation is most likely to occur when the dispersal ability of hosts exceeds that of parasites. Examples of hosts showing local adaptation to their parasites include a herbaceous host plant (*Silene latifolia*) and its fungal pathogen (*Microbotryum violaceum*; Kaltz et al., 1999), a bacterial host (*Pseudomonas fluorescens*) and its bacteriophage (phage SBW25 ϕ 2; Buckling and Rainey, 2002), a snail (*Lymnaea truncatula*) and its liver flukes (*Fasciola hepatica*; Gasnier et al., 2000), and a Canarian lizard (*Gallotia galloti*) and its blood parasites (*Haemogregarina* spp.; Oppliger et al., 1999).

The studies reviewed above examined local adaptation in the context of host-parasite interactions by experimentally exposing hosts to either their local (sympatric) parasites or to parasites from another area (allopatric). In addition, several correlational studies of free-living birds and their parasites have found lower parasite loads in more philopatric individuals. This pattern is consistent with hosts becoming adapted to local parasite strains. Systems in which this pattern has been observed include mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*) and their haematozoan parasites (*Haemoproteus* spp.; MacDougall-Shackleton et al., 2002), song sparrows (*Melospiza melodia melodia*) and their haematozoan parasites (usually *Haemoproteus* spp.; Stewart and MacDougall-Shackleton, 2008), and barn swallows (*Hirundo rustica*) and haematophagous louse flies (*Ornithomya biloba*; Saino et al., 2014). Although such correlational findings are consistent with adaptation by hosts to their local parasites, there are alternative explanations. Individuals that move far from their natal origin to breed may simply differ from philopatric individuals in body condition, social stress, or investment in immune function that is independent of any prior adaptation, and that may each influence their susceptibility to parasitism in general, without indicating local adaptation to a specific parasite.

1.3 Natal dispersal

Natal dispersal occurs when individuals move away from their natal population to breed in a new population (Burgess et al., 2012; Saino et al., 2014). Natal dispersal influences population connectivity and population genetic variation (Burgess et al., 2012). Moreover, natal dispersal has costs and benefits likely to influence individual fitness. Benefits include avoiding competition, especially with related individuals. For example, increasing population density was associated with increased production of dispersing-type seeds by a daisy (*Heterosperma pinnatum*, Martorell and Martínez-López, 2014), and higher rates of dispersal in lizards (*Lacerta vivipara*, Cote and Clobert, 2010) and other animals (Travis et al., 1999). Dispersal may also allow individuals to escape harsh or stressful conditions: for example, daisies (*H. pinnatum*) subjected to water stress produced more dispersing-type seeds (Martorell and Martínez-López, 2014). Another benefit of natal dispersal involves avoiding inbreeding. For example, male Townsend voles (*Microtus townsendii*) whose mother and sisters remained on their natal range dispersed further than those whose female relatives were no longer in the area (Lambin, 1994). Similarly, juvenile meadow voles (*Microtus pennsylvanicus*) released into a plot with their siblings were more likely to disperse than were those released into a plot with non-siblings (Bollinger et al., 1993).

However, natal dispersal also incurs costs, including direct mortality. For example, snakes of several species are more likely to be killed in times of year corresponding to dispersal events (Bonnet et al., 1999). Moreover, dispersal may incur energetic costs that result in reduced body condition. Perhaps as a result, screech owls (*Otus asio* and *O. kennicottii*) disperse only if they are in relatively good condition (Belthoff and Dufty, 1998). Natal dispersal may also result in social stress upon arrival at the new population. For example, olive baboons (*Papio anubis*) herd females away from immigrant males and sometimes wound the immigrant males (MacCormick et al., 2012).

1.3.1. Fitness consequences of natal dispersal by hosts. The fitness consequences of natal dispersal by host individuals may depend in part on the outcome of coevolutionary

arms races between hosts and parasites. If parasites are locally adapted to their hosts (parasite local adaptation), then individual hosts that disperse away from their natal population may benefit by escaping their native parasites. The enemy release hypothesis (Keane and Crawley, 2002) posits that avoiding or escaping locally adapted natural enemies may explain the success of some invasive species. Freed from the burden of parasitism and the need to allocate energy to immune defence, invasive populations may outcompete their native counterparts, and be more successful in their introduced range than in their native range (Keane and Crawley, 2002). For example, experimentally treating an invasive shrub (*Clidemia hirta*) with pesticide and fungicide increased survival in its native range, but not in its introduced range, suggesting that parasites are a limiting factor only in the shrub's native range (DeWalt et al., 2004).

On the other hand, non-dispersing hosts may be adapted to local parasites (host local adaptation). Because parasite assemblages can vary geographically (Merino et al., 2008; Fallon et al., 2005), host individuals that breed far from their natal area may encounter a greater variety of specific unfamiliar parasites, parasites which may vary widely depending on the breeding location. Natal dispersal may thus increase the risk of parasitic infection. This pattern may arise because dispersing individuals are immunologically naïve and lack previous experience with parasites in their new location, or because individuals dispersing to a variety of unfamiliar breeding grounds are not genetically adapted to resist parasites in their new location, or both. Thus, in systems in which hosts are adapted to their local parasites, philopatric host individuals may be less likely to be parasitized.

1.4 Immunity

The harmful effects that parasites may exert on host survival, reproductive success, and secondary sexual trait expression (reviewed in section 1.1) make the ability to avoid, control or tolerate parasitic infection a critical aspect of host fitness. Adaptations for avoiding, controlling or tolerating parasitic infection include behavioural defences, such as avoiding contact with infected conspecifics (Kiesecker et al., 1999), as well as anatomical and physiological defences referred to as immunity.

1.4.1. The vertebrate immune system. Vertebrate animals have evolved a complex immune system, with many components that collectively are capable of dealing with a variety of challenges (Lee, 2006). The function of the immune system is to differentiate between self and non-self components, and to eliminate the latter (Demas and Nelson, 2012). The immune system comprises two main branches, termed innate and adaptive immunity. Each of these branches includes cellular and humoral components (Figure 1.1).

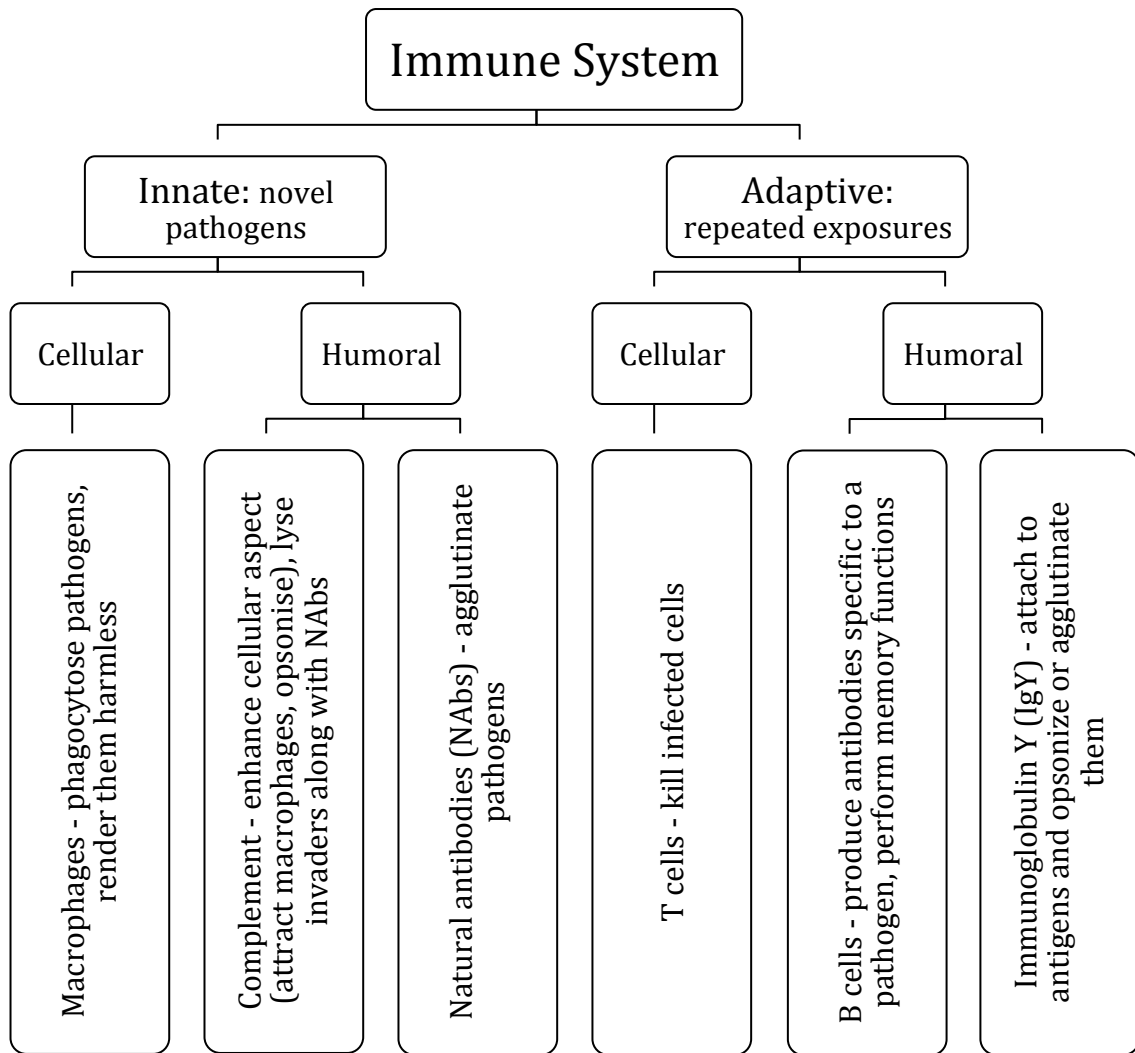


Figure 1.1. Components of the vertebrate immune system. Adapted from Demas and Nelson, 2012, with information from Matson et al., 2005; Schmid-Hempel, 2011.

Innate immunity. The innate branch of the vertebrate immune system is a fast-acting, non-specific system that constitutes the first line of defence against parasites and other pathogens. Innate immunity includes anatomical defences such as mucus, competitive exclusion of invaders by the body's normal flora, plus both cellular and humoral components (Demas and Nelson, 2012). Cellular components include phagocytic cells such as macrophages, which engulf and destroy pathogens; natural killer cells, which quickly induce apoptosis in infected cells; and proteins that act as inflammatory mediators. An important component of humoral innate immunity is the complement system, which is a collection of small circulating proteins that eliminate pathogens through the following means: opsonisation of infected cells or pathogens, lysis of pathogens using the membrane attack complex, promotion of inflammation, and activation of the adaptive immune system (Schmid-Hempel, 2011). An advantage of innate immunity system (relative to adaptive immunity) is its capacity for mounting a rapid, non-specific response. Innate defences can thus respond well to novel pathogens as well as to pathogens that have been encountered before. However, innate immunity has the disadvantage of being energetically expensive to maintain on a constant basis (Klasing, 2004; Matson et al., 2005).

Adaptive immunity. The adaptive branch of the vertebrate immune system is slow to develop, specific in its response, requires a first encounter with a pathogen, but can rapidly and efficiently deal with repeated encounters of a previously-encountered pathogen (Schmid-Hempel, 2011). Adaptive immunity includes both cellular and humoral components: the former includes lymphocytes, primarily T cells and B cells. T cells kill infected cells and pathogens, and direct the activity of other cells. B cells, while presenting a pathogen's antigens to other cells, also contribute to humoral adaptive immunity by producing antibodies, used to identify and emphasize pathogens and infected cells (Schmid-Hempel, 2011). B cells are also responsible for the long-term memory functions of the adaptive immune system, which confers long-term immunity to repeat encounters with familiar pathogens. B cells also produce cytokines, which function to recruit the innate immune system to the site of infection. Relative to innate immunity, adaptive immunity has the advantage of a specific memory that allows rapid responses to previously-encountered pathogens, which can greatly reduce the impact of

infection. However, adaptive immunity is costly to develop and is not immediately useful in defending against novel pathogens.

1.4.2. Importance of assessing immune allocation: When characterising local adaptation in the context of host-parasite systems, it is important to also evaluate host immune function. Variation among host individuals in their investment in immunity may influence the prevalence or severity of parasitic infection. Moreover, an individual's investment in immunity may also be influenced by its likelihood of encountering familiar or unfamiliar parasites, or both.

Some evidence indicates that the diversity of parasites encountered may influence optimal investment in immune function. Møller and Erritzøe (1998) compared the size of immune-related organs (spleen and bursa of Fabricius) in closely-related bird species that differed in migratory status. Migratory species, in which individuals presumably encounter a greater diversity of parasites, had larger immune-related organs than did non-migratory species, suggesting greater immune investment in species that encounter a greater diversity of parasites (Møller and Erritzøe, 1998). Although this study drew comparisons across species rather than among individuals within a species, and examined migratory status rather than natal dispersal, it raises the possibility that individual variation in natal dispersal tendency may influence optimal immune investment, which is the rationale for my thesis. Like birds that migrate seasonally, individuals that disperse from their natal area to breed in a new population presumably encounter a greater diversity of parasites, and are more likely to encounter novel parasites, than individuals that remain to breed close to where they were born.

Philopatry may be negatively associated with immune function, if individuals that disperse compensate for the risk of encountering unfamiliar parasites by increasing investment in immunity. Similarly, if natal dispersal increases the risk of parasitic infection, the subset of individuals that successfully disperse and survive to breed may be highly immunocompetent because less immunocompetent dispersers do not survive (Lucas et al., 1994). Conversely, philopatry may be positively associated with immunity if philopatry is condition-dependent. That is, if individuals in good condition are less likely to disperse, philopatric individuals may have higher immunocompetence and

thus enjoy lower parasite loads or infection rates or both, relative to dispersers. For example, American kestrels (*Falco sparverius*) that were more immunocompetent as nestlings were more likely to return to their natal site as breeding adults (Tella et al., 2000).

1.5 Study system

1.5.1. Song sparrows. Song sparrows (*Melospiza melodia*) are common throughout North America. Across this range, song sparrows show considerable variation in morphology (Arcese et al., 2002), song (Searcy et al., 2002), and genetics, with analysis of mtDNA indicating a dispersal distance of approximately 6 km (Zink and Dittmann, 1993). Like many birds in which song varies geographically, female song sparrows prefer locally common song types (Searcy et al., 2002). Male song sparrows that sang locally common songs and syllables were relatively similar genetically to other members of the population (Stewart and MacDougall-Shackleton, 2008). Locally typical singers also had reduced indicators of physiological stress, including lower parasite loads, compared to males that sang less typical songs and were presumed to have immigrated from elsewhere (Stewart and MacDougall-Shackleton, 2008). Stewart and MacDougall-Shackleton hypothesized that female preferences for local song may allow them to select mates that are better able to defend against the local parasite fauna.

1.5.2. Study population. I examined natal philopatry, parasite load, and immune function in a long-term study population of song sparrows (*M. melodia*, subspecies *melodia*) breeding at a field site near Newboro, Ontario, Canada. The study site is not isolated from other surrounding suitable breeding habitat, and genetic analyses indicate that individuals in this population vary substantially in natal philopatry (Sarquis-Adamson and MacDougall-Shackleton, unpublished; Stewart and MacDougall-Shackleton, 2008). High overwinter return rates of adults (45-50%; MacDougall-Shackleton et al., 2009) suggest very high breeding philopatry. About 10% of nestlings banded at the study site return to breed at the site the following year (Stewart and MacDougall-Shackleton, 2008), providing a minimum estimate of natal philopatry.

Previous studies of this population, as well as of other breeding populations of song sparrows within 50 km of the study site, have found a negative relationship between natal philopatry and haematozoan parasite load (Stewart and MacDougall-Shackleton, 2008; Sarquis-Adamson and MacDougall-Shackleton, unpublished). However, these studies did not investigate the degree to which an individual invests in immune defenses. If philopatric and non-philopatric individuals differ in their immune investment, this could bias estimates of local adaptation of song sparrows to their haematozoan parasites. Further evidence suggesting that birds in the study population are adapted to their local haematozoan parasites comes from a cross-infection experiment (Sarquis-Adamson and MacDougall-Shackleton, unpublished). Song sparrows captured from Newboro or from London, Ontario were exposed to *Plasmodium* parasites cultured either from Newboro or from London, and parasites had lower infection success on their sympatric than on allopatric hosts (Sarquis-Adamson and MacDougall-Shackleton, unpublished). Again, however, this study did not directly measure immune function.

1.5.3. Haematozoan parasites. Blood-borne parasites (haematozoa) have important negative effects on the fitness of their avian hosts, including reduced survival (Martínez-de la Puente et al., 2010) and reproductive success (Dufva, 1996a; Marzal et al., 2005; Knowles et al., 2010). Avian haematozoa are globally widespread and collectively infect approximately 70% of bird species (Dufva, 1996a; Isaksson et al., 2013). Four genera of haematozoa are particularly relevant to my study population, in terms of being abundant in the population (*Plasmodium*, *Haemoproteus*, *Leucocytozoon*) or having particularly detrimental effects on host fitness (*Trypanosoma*).

Plasmodium spp. (phylum Apicomplexa) are unicellular protozoa and intracellular parasites of red blood cells in the vertebrate host. The life cycle of *Plasmodium* is complex, involving alternating generations between invertebrate and vertebrate hosts (Valkiūnas, 2005; Ventim et al., 2012). Generally, a mosquito vector (usually *Culex* spp.) becomes infected by consuming a blood meal from an infected bird. The parasite leaves the mosquito's midgut and migrates, after reproducing, to the salivary gland, where it can infect the avian source of the mosquito's next blood meal. In the avian host,

infectious gametocytes mature in red blood cells, ready to be consumed by another mosquito vector. *Plasmodium* can cause fever and anemia, and in severe cases, swelling of the liver and spleen, as well as capillary blockage leading to anoxia and tissue death (Valkiūnas, 2005).

Haemoproteus spp. (phylum Apicomplexa) are also unicellular protozoa and intracellular parasites of the vertebrate host's red blood cells. The life cycle of *Haemoproteus* is similar to that of *Plasmodium*, but its vectors are the louse flies (Hippoboscidae), biting midges (Ceratopogonidae) and black flies (Simuliidae) (Fatima et al., 2014; Valkiūnas, 2005). *Haemoproteus* can cause fever, anemia, and swelling of the liver and spleen (Valkiūnas, 2005).

Leucocytozoon spp. (phylum Apicomplexa) are closely related to *Plasmodium* and *Haemoproteus*, and are also unicellular protozoa and intracellular parasites, capable of parasitizing the red and white blood cells of the vertebrate host (Valkiūnas, 2005). The life history of *Leucocytozoon* is similar to those of *Haemoproteus* and *Plasmodium*, and these parasites are vectored by black flies (Simuliidae; Jenkins and Owens, 2011; Valkiūnas, 2005). *Leucocytozoon* can cause fever, anemia, liver and spleen swelling, and respiratory disruption due to gametocytes in the alveoli (Valkiūnas, 2005). Together, these three genera comprise the most commonly observed haematozoan parasites in the study population.

Less commonly observed in my study population, but noteworthy for their intense effects on host fitness at least in other bird species (Dufva, 1996a) are parasites of the genus *Trypanosoma* (phylum Euglenozoa). Like the three genera reviewed above, *Trypanosoma* has a complex life cycle, involving alternating generations between vertebrate and invertebrate hosts. Although it is a unicellular protozoan, *Trypanosoma* differs from genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, in that *Trypanosoma* remains extracellular for its entire life cycle. *Trypanosoma* is transmitted by a variety of arthropods, including louse flies (Hippoboscidae), biting midges (Ceratopogonidae), black flies (Simuliidae), mosquitoes (Culicidae) and mites (Dermanyssidae; Valkiūnas, 2005; Valkiūnas et al., 2011). *Trypanosoma* can cause fever and swelling of lymphatic organs in its avian hosts (Valkiūnas, 2005).

1.6 Research objectives, hypothesis, and predictions

With rising average global temperatures, parasites are predicted to shift or expand their ranges, or both (Lindgren et al., 2000). As a consequence, the affected parasite populations are expected to encounter new populations and species of hosts. Climate change may also affect vector ranges and abundance (Russell, 1998), host density, timing of breeding and migration, and other factors affecting parasitism (Polley et al., 2010). In the current era of climate change, understanding parasite-host interactions is more important than ever.

In this thesis, first I will examine the relationship between parasite load and philopatry in free-living song sparrows. Based on previously observed patterns of lower parasite loads in more philopatric birds (Stewart and MacDougall-Shackleton, 2008; Sarquis-Adamson and MacDougall-Shackleton, unpublished), I hypothesize that song sparrows (the host in this system) are consistently locally adapted to their haematozoan parasites. If my hypothesis is correct, then I predict a lower parasite load in more philopatric birds this year.

Second, I will examine the relationship between inferred philopatry and several measures of innate and adaptive immunity. Natal philopatry should be associated with a lower diversity of parasites encountered and increased encounters with the same pathogen. Adaptive immunity provides efficient protection against repeated encounters with the same pathogens, so I hypothesize that natal philopatry will increase optimal investment in adaptive immunity and decrease optimal investment in innate immunity. If my hypothesis is correct, then I predict that relatively philopatric birds will invest more into adaptive than into innate immunity. Conversely, because innate immunity is an important first line of defence against unfamiliar pathogens, less philopatric birds should invest more heavily into innate than into adaptive immunity.

2 Methods

2.1 Study site and field methods

I examined philopatry, parasite load and immune function in a population of song sparrows (*Melospiza melodia melodia*) breeding near Newboro, Ontario, Canada (latitude 44.644, longitude -76.333), on land owned by the Queen's University Biological Station. The study site includes a mixture of fields, small ponds, wetlands and forest edge, making it appropriate breeding habitat for song sparrows. It is not physically isolated from other patches of suitable breeding habitat. Based on recapture of birds banded as nestlings in previous years, up to 10% of breeding adults each year are known to have originated from the study area (Stewart and MacDougall-Shackleton, 2008). These data provide a minimum estimate of 10% for natal philopatry.

Between 15 April and 14 May 2013, and assisted by other members of the research team, I captured a total of 56 adult song sparrows (N= 29 male, 27 female). This time period corresponded to the mate attraction, nest-building, laying and early incubation periods. All birds were captured between 06:30 and 10:30, using Potter traps baited with millet and checked at least once per hour.

I equipped each bird with a numbered Canadian Wildlife Services leg band, together with a unique combination of coloured plastic leg bands to permit field identification (Environment Canada banding and marking permit 10691B). I used dial calipers to measure the unflattened length of the right wing chord, and the length of the left tarsus, to the nearest 0.1 mm. I determined sex by the presence (male) or absence (female) of a cloacal protuberance, and measured mass to the nearest 0.1 g using a spring scale.

Together with another member of the research team, I also collected up to 200 μ L of blood from each bird's brachial vein. These samples were taken within 15 minutes of approaching the trap to reduce the bird's stress, and collected into heparinized capillary tubes, using aseptic technique (Millet et al., 2007) to avoid contamination from external pathogens and debris. This involved plucking a few feathers from the puncture site if necessary, wiping down the skin twice with 70% ethanol, and using the ethanol to

direct remaining feathers away from the area. The brachial vein was punctured approximately 10 s after applying ethanol, to permit the skin to dry. Blood was used for genetic, immune, and parasite analyses that are described below. After confirming that bleeding had stopped, I released birds at the site of capture.

2.2 Genetic analysis

2.2.1. Molecular Methods: Immediately after collecting blood from each bird, I blotted approximately 10-15 μ L of whole blood onto high wet-strength filter paper (Whatman, no. 1) and allowed it to air-dry for subsequent genetic (microsatellite) analysis. Once dried, blood blots were treated with a drop of 0.5 M Na-EDTA, pH 8.0 (Ambion, Cat. no. AM9260G) as a preservative and allowed to air-dry again. Blots were kept separated from one another to avoid cross-contamination.

I stored blood blots at room temperature for 18-22 weeks after collection before isolating DNA. I cut each blot into small (~ 1 mm²) sections using scissors sterilized in bleach solution, in preparation for extraction. I used an ammonium acetate-based protocol to extract DNA from blots. Briefly, this involved incubating blood-saturated filter paper in 5 mM proteinase K in cell lysis buffer for two hours at 55°C -65°C. I used ammonium acetate to precipitate proteins out of solution, centrifuged, and retained the supernatant that contained dissolved DNA. I precipitated DNA with cold isopropanol, centrifuged to generate a solid pellet of DNA at the bottom of a microcentrifuge tube, and discarded the supernatant. I then gently washed the DNA pellet with 70% ethanol, poured off the ethanol, allowed the tube to dry until any excess ethanol had evaporated, then suspended the DNA pellet in 50 μ L 1X TE (Tris buffer and EDTA).

I genotyped each bird at 13 microsatellite loci developed for use in song sparrows and other Emberizid species (Table 2.1). One primer from each pair was fluorescently labeled (Life Technologies) and for each individual I multiplexed up to 4 loci in a single PCR reaction. I conducted PCR in a final volume of 10 μ L, including 10 mM Tris-HCl, 0.2 mg/mL BSA, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.1 mM of each primer (except Sosp 14, for which I used 0.4 mM of each primer), 0.5 U *Taq* polymerase (Invitrogen, Cat. no.18038-042), and 1 μ L of genomic DNA. Thermal cycling

conditions included an initial step of 180 s at 94°C; followed by 28 cycles of 30 s at 94°C, 90 s at the annealing temperature, and 60s at 72°C; and a final step of 270 s at 72°C. Annealing temperatures were 57°C for Mme 1, Mme 12, Pdoμ 5, Zole CO2, Sosp 2, 3, 4, 9, 13, and 14; and 55°C for Sosp 1, 5, and 7.

PCR products were separated by capillary electrophoresis on an Applied Biosystems automated genotyper (ABI 3730) at the London Regional Genomics Centre (Robarts Research Institute, University of Western Ontario). I analysed fragment lengths with reference to the internal size standard LIZ using Genemapper 4.0 software to visualize and manually identify alleles. I tested for deviations from Hardy-Weinberg equilibrium, for evidence of null alleles, and for linkage disequilibrium using Genepop for the Web (<http://genepop.curtin.edu.au>; dememorization number=1000, number of iterations=1000) and found no evidence of any of these (all $P > 0.10$). A small number of values were missing for some loci for a few birds, but those individuals were still used for further analysis.

2.2.2. Inference of Natal Philopatry: For 47 individuals, I used three methods of estimating natal philopatry, described below. I was unable to obtain genetic data on the remaining 9 individuals, so they were only used to examine relationships between different immune measures.

a. Mean Genetic Similarity: For this approach, I used the GenALEX add-in to Microsoft Excel (Peakall and Smouse, 2006, 2012) to calculate pairwise coefficients of genetic similarity (i.e. “relatedness”; Queller and Goodnight, 1989) between each individual and all of the other 46 individuals in my sample. This metric ranges between -1 and 1, with -1 representing total genetic dissimilarity between two individuals and 1 representing two individuals that are genetically identical across all genotyped loci. For each individual, I then calculated the average across all of their pairwise relatedness coefficients as an estimate of their mean genetic similarity (hereafter, MGS) to all others in the sample. This approach does not require knowledge of allele frequencies in other surrounding populations, and assumes that higher values of MGS reflect more philopatric individuals (e.g. Stewart and MacDougall-Shackleton, 2008).

b. Genetic assignment testing in STRUCTURE: As a complementary approach, I used the software STRUCTURE 2.3.4 (Pritchard et al., 2000) to detect population genetic structuring and to determine the likelihood that a given individual originated from the site in which it was sampled (i.e. from my study site). In addition to microsatellite genotypes from my study subjects, for this analysis I also used genotypes from an additional 308 adult song sparrows captured between 2009-2011 at my study site and at 10 additional sites within 50 km of my study site (Sarquis-Adamson and MacDougall-Shackleton, unpublished). These additional 308 birds had already been genotyped at the same 13 loci that I used in this study. I used the admixture model in STRUCTURE, in which individuals can derive some proportion of their genome from ancestors in multiple populations, and did not use prior location information. Settings included a 'burnin' length of 100,000 iterations and a run length of 1,000,000 iterations, which I confirmed were sufficient for model convergence. To confirm consistency between runs, I performed five replicates for each value of K between K=1 and K=11. I determined the optimal value of K, reflecting the most likely number of distinct genetic clusters sampled, by calculating the posterior probability for each value of K using ln-likelihood scores (Pritchard et al., 2000). Provided that two or more genetic clusters are detected, STRUCTURE calculates for each individual the proportion of ancestry in each genetic cluster, which can be interpreted as a measure of philopatry, that is, the likelihood of having originated in the site in which it was sampled (Pritchard et al., 2000).

c. Genetic assignment testing in GeneClass2: As a third way of inferring philopatry, I entered genotypes of my study subjects, together with the additional 308 sampled at sites throughout eastern Ontario (see above; Sarquis-Adamson and MacDougall-Shackleton, unpublished) into the program GeneClass2 (Piry et al., 2004). I used the program's L_home option to detect first-generation migrants. L_home ranges from 0 to 1 and represents the probability of a specific genotype occurring in the population in which it was sampled, and does not require that all potential source populations have been sampled (Piry et al., 2004). I used the probability computation option to generate the probability that each of my study subjects originated from the site in which it was sampled, i.e. my study area. I used Rannala and Mountain's (1997) criterion for

computation and the resampling algorithm described by Paetkau et al. (2004), as recommended by Piry et al. (2004). I used 10,000 simulated individuals in the run.

2.3 Immune assays

2.3.1. Innate Immune System: Phagocytosis Assay: The phagocytosis assay (Millet et al., 2007) measures activity of the cellular component of the innate immune system, by testing the phagocytic activity of cells in whole blood when challenged with microbes. Following methods established for this species by Kubli and MacDougall-Shackleton (2014; adapted from Millet et al., 2007) I used 10 μ L of whole blood to perform the phagocytosis assay in the field within 45 minutes of blood collection. At the beginning of the field season (that is, up to five weeks in advance of use), I reconstituted fluorescently labeled BioParticles of heat-killed *Escherichia coli* (Invitrogen E-2864) to 20 mg/mL in phosphate buffered saline (PBS) and 2 mM sodium azide. I stored this suspension at 4°C until use.

In the field, I diluted blood into cell media solution (4 μ M L-glutamine, 100 U/mL penicillin-streptomycin, 0.05 mL/mL fetal bovine serum in CO₂-independent media) to a 1:20 ratio, then added 20 μ L of this blood-media dilution to each of four wells of a chamber slide (EMD Millipore, Cat. no. PEZGS0816). To ensure sterility, all field pipetting and incubation steps were conducted inside a Plexiglass dead-air box equipped with a HEPA filtration system. I added 75.75 μ L of *E. coli* BioParticles (diluted to achieve a target ratio of 100 particles per leukocyte; Kubli and MacDougall-Shackleton, 2014) to each well. I incubated slides at 41°C for 15 minutes, then ended phagocytosis by placing the slide on ice for five minutes. After 2 washes with cell media (4°C) to remove non-adherent cells, I added ice-cold methanol and placed the slide on ice to fix the cells and end phagocytic activity. To preserve the fluorescence of BioParticles, I minimized exposure to light throughout the field assay and stored slides in a light-proof case awaiting examination (approximately 9-15 weeks after assays were performed).

Under the 60x objective of a fluorescent microscope (Leica DMLB, excitation/absorption spectrum of 505/513 nm), I examined adherent cells (mainly

macrophages; Millet et al., 2007). I counted the number of macrophages that had engulfed at least one fluorescent particle, out of the first 100 macrophages I saw in each of the four wells, for a total of 400 cells per individual.

Hemolysis-Hemagglutination Assay: The hemolysis-hemagglutination assay of innate immunity (Matson et al., 2005) measures the efficacy of natural antibodies and the complement system. Natural antibodies (NAbs) are antibodies secreted by B-cells, and combat early-stage infections by recognizing, opsonizing, and enhancing antigen presentation of invaders, as well as by activating the complement enzyme cascade that ends in cell lysis (Mendes et al., 2006). The lysis component of the assay assesses this interaction. The agglutination component of the assay reflects the ability of NAbs to agglutinate foreign cells (Matson et al., 2005).

All blood that was not used whole (for blots for microsatellites, the phagocytosis assay, or for thin-film blood smears) was kept at ambient temperature for no more than 3 hours, then spun for 10 minutes at 13,000 rpm in a microcentrifuge. I extracted the plasma supernatant then stored this at -20°C for up to 3 months.

I used 50 µL of freshly-thawed plasma for the hemolysis-hemagglutination assay (adapted from Matson et al., 2005). Using chicken plasma (Sigma, P3266) alongside as a positive control, I serially diluted song sparrow plasma with PBS using one row of 12 wells per bird in a 96-well round-bottomed plate (Corning Costar #3798). Plasma concentrations ranged from 100% plasma (column 1) to 0.098% (column 11) as well as a negative control containing entirely PBS and no plasma (column 12), such that each subsequent dilution was made from equal parts of the previous dilution and PBS. I added 25 µL of a 1% solution of rabbit red blood cells (Cedarland Laboratories, 7206403) to each well, then incubated the plate at 37°C for 90 minutes. After incubation, I placed the plate on a 45° angle at room temperature for 20 minutes, then scanned it immediately using a top-lit flatbed scanner (EPSON Perfection 4990 Photo scanner) to observe agglutination. I laid the plate flat and incubated it for 70 minutes more at room temperature, then scanned it again as above to observe lysis. For both agglutination and lysis, I recorded scores as the last well (i.e. weakest dilution of plasma, ranging from 1-11) in which I observed the target phenomenon, such that

higher scores reflect greater agglutination or lytic activity. However, lysis did not occur in any of my samples even at the strongest concentrations of plasma. Thus, following Kubli and MacDougall-Shackleton (2014) I consider only agglutination scores in this study.

2.3.2. Adaptive Immune System: IgY Assay: IgY is the most highly concentrated serum immunoglobulin in the avian system, with functions including mediation of anaphylactic reactions, opsonization, and complement fixation. The IgY assay is informative regarding the state of the humoral, B-cell mediated component of adaptive immunity (Munhoz et al., 2014). I used 2 μ L of thawed plasma in the IgY assay (adapted from Bourgeon et al., 2006).

Determining Appropriate Concentration: To find the best plasma dilution to use for this assay in song sparrows, I first conducted a pilot study using excess plasma collected from some study subjects. I serially diluted plasma samples with dilution solution (a mixture of NaCO_3 and NaHCO_3 solutions at pH 9.6; see Appendix B) to achieve plasma:diluent ratios ranging from 1:500 through 1:64000. One row of pure dilution solution served as a negative control. This pilot test revealed that a plasma:diluent ratio of 1:4000 had the greatest sensitivity (defined as the greatest change in final result from one concentration to the next). Accordingly, I used this ratio for all IgY assays.

Performing the Assay: I conducted dilutions of 2 μ L of freshly-thawed plasma from each individual to achieve a 1:4000 ratio of plasma:diluent, then placed 100 μ L of diluted plasma solution into each well of a 96-well flat-bottomed plate (Corning Costar # 3370). I incubated plates at 37°C for 1 hour, followed by a 24-hour incubation at 4°C. I then washed the wells twice with 200 μ L PBS-Tween and added 100 μ L of 5% powdered skim milk solution, allowing the samples to incubate in the milk for 1 hour at 37°C. After washing twice again with PBS-Tween, I added 100 μ L of anti-IgY (Anti-chicken IgY peroxidase conjugate, Sigma Catalogue No. A9046) diluted 1:250 in PBS-Tween. I incubated the samples at 37°C for 2 hours, then washed again twice with PBS-Tween. I added a revealing solution of 31% hydrogen peroxide diluted in 1:1000 ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] and incubated for 1 hour at 37°C,

while the anti-IgY-conjugated peroxidase became coloured. After the incubation, I transferred the plate to a plate reader (BIORAD iMark™ Microplate Reader) and read it at 405 nm using the included software. This assay was conducted in duplicate for each individual, and I calculated the average of the two replicate wells.

2.4 Parasite load

Immediately after collecting a blood sample in the field, I placed a small drop of whole blood (~4 µL) onto a clean glass microscope slide and prepared a thin-film blood smear by gently pushing the blood across the slide using a second slide. I allowed smears to air-dry, then fixed them by immersing for 1 minute in 100% methanol, and air-dried again before storing smears at room temperature.

Following the field season, I treated smears using Harleco® Stain (Hemacolor Stain Set, Millipore, 65044/93) according to the manufacturer's protocol, in order to visualize blood cells. I examined the smears using light microscopy, under 1000x magnification using oil immersion, to assess haematozoan parasite load for each individual. I took digital photographs at regular intervals from the tail end of the smear until 10,000 erythrocytes per individual had been visualized. I then counted the number of haematozoan parasites observed on the photographs as a measure of infection severity (haematozoa per 10,000 erythrocytes). I noted the genera of haematozoa with reference to Valkiūnas (2005). I also categorized individuals as being infected (one or more haematozoa observed per 10,000 erythrocytes) versus uninfected (no haematozoa observed in a scan of 10,000 erythrocytes). Analyses of infection severity included only those individuals categorized as infected, that is, zero values were excluded.

2.5 Data analysis

2.5.1. Normality Tests, Transformations and Nuisance Variables: Prior to performing the linear regressions, I visually assessed Q-Q plots of all variables to test for normality. When variables were not normal, I transformed them to achieve normality. Specifically, I transformed mass to $\arcsin(\log_{10}\text{mass})$, phagocytosis score to

$\arcsin(\log_{100}\text{phagocytosis})$, HL-HA score to $\arcsin(\log_{10}\text{HLHA})$, IgY to $\log_{10}\text{IgY}$, and parasite load to $\arcsin(\log_{1000}\text{parasiteload})$.

I used linear regression, implemented in JMP 10.0.0 (SAS Institute, 2012) to identify any confounding relationships between the variables of interest for this study (inferred philopatry measures, parasite load and infection status, and immune measures) and potential 'nuisance' variables that I wanted to control for. Nuisance variables included date of capture, time of capture, and body condition (defined as the residual of mass on tarsus length, e.g. Whitfield et al., 1999). In cases where a nuisance variable was a significant predictor of a variable of interest, I calculated the residuals of this linear regression and used these to characterize the variable of interest corrected for the nuisance variable. Accordingly, I corrected phagocytosis scores for date of capture and corrected HL-HA for body condition. Two nuisance variables, date of capture and time of capture were significantly negatively correlated with one another (Pearson's $r_{1, 72} = -0.36$, $p = 0.002$), because sunrise occurred progressively earlier throughout the season and capture times were based upon the timing of sunrise. Consequently, phagocytosis scores were corrected only for date of capture, although these scores were also significantly predicted by time of capture.

A growing body of evidence (Buehler et al., 2008; Palacios et al., 2012; Kubli and MacDougall-Shackleton, 2014) suggests that relationships among different immune measurements are complex. I used Pearson's correlation to investigate potential relationships among phagocytosis scores, HL-HA scores, and IgY levels.

2.5.2. Regressions: I performed simple linear regressions in JMP 10.0.0 (SAS Institute, 2012), with inferred philopatry (estimated by either L_home or MGS) as the independent variable, and an immune variable (HL-HA, phagocytosis, IgY) or parasite load as the dependent variable. I performed a t-test, also in JMP 10, between inferred philopatry of birds with different infection statuses (infected versus uninfected). Sample sizes varied between tests as I was unable to complete all immune measures on all birds.

2.4.3. Sex and infection status differences: I used bifactorial ANOVAs to investigate effects of sex and infection status on variables of interest, in order to determine whether to pool data across sexes and across infection classes. Specifically, I ran a separate bifactorial ANOVA to examine the effects of sex, infection status and their interaction for each immune variable (phagocytosis, HL-HA, IgY), and measure of inferred philopatry (L_home, MGS). I used a univariate ANOVA to examine the effect of sex on parasite load, because by definition this variable could only be examined for the subset of infected birds.

2.5.4. False Discovery Rate Adjustments: To correct for an increased likelihood of type I error associated with performing multiple tests on the same data set, I calculated the Benjamini-Hochberg False Discovery Rate (FDR) adjustment as recommended by García (2004). Adjustment was done using the FDR P-Value add-in for JMP 10.0.0 (Source: support.sas.com). For each dependent variable (i.e. phagocytosis, HL-HA, IgY, and parasite load), I grouped the P-values for the regression using L_home and the regression using MGS together, and corrected the P-value accordingly (i.e. two tests of each hypothesis).

Table 2.1. Microsatellite loci used to assess natal philopatry in song sparrows, including repeat motifs, product size range, and observed heterozygosity for the 47 birds genotyped in my study.

Locus Name	Repeat Motif	Product Size Range (bp)	Observed Heterozygosity	Source
Mme 1	(TG) _n TC(TG) _n	130-172	0.850	Jeffery et al., 2001
Mme 12	(CCCACA) _n	180-250	0.907	Jeffery et al., 2001
Pdo μ 5	(CA) _n	221-269	0.975	Griffith et al., 1999
Sosp 1	(GGAT) _n GCAT (GGAT) _n	225-280	0.882	Sardell et al., 2010
Sosp 2	(CTGT) _n (GT) ₃	130-182	0.522	Sardell et al., 2010
Sosp 3	(CTGT) _n	178-226	0.894	L. Keller, Pers. comm. To E. A. M-S.
Sosp 4	(TGTC) _n	155-210	0.826	Sardell et al., 2010
Sosp 5	(GACA) _n GACT (GACA) _n	80-139	0.690	Sardell et al., 2010
Sosp 7	(GACA) _n	60-120	0.391	Sardell et al., 2010
Sosp 9	(GACA) _n	80-130	0.267	Sardell et al., 2010
Sosp 13	(GATA) _n	172-228	0.851	Sardell et al., 2010
Sosp 14	(CTAT) _n	223-299	0.891	Sardell et al., 2010
Zole CO2	ATCC _n	180-250	0.891	Poesel et al., 2009

3 Results

3.1 Inferring philopatry

Mean genetic similarity (MGS) ranged from -0.18 to 0.086 in the 47 birds I genotyped (average = -0.021, SE = 0.0081). L_Home ranged from 0.46 to 1 (average = 0.91, SE = 0.016). For assignment testing using STRUCTURE, in all five replicates the program consistently identified an optimal K of 1 (posterior probability = 1 in all cases). Thus, I could not obtain measures of inferred philopatry from STRUCTURE and subsequent analyses were done only on MGS and L_home.

Philopatry as inferred from MGS was significantly positively correlated with philopatry as inferred from L_home (Pearson's $r_{1,45}=0.61$, $P<0.0001$; Figure 3.1). There was no difference in inferred philopatry between sexes, either using L_home (t-test, $t_{43,6}=-0.34$, $P=0.73$) or MGS (t-test, $t_{41,4}=-0.20$, $P=0.84$).

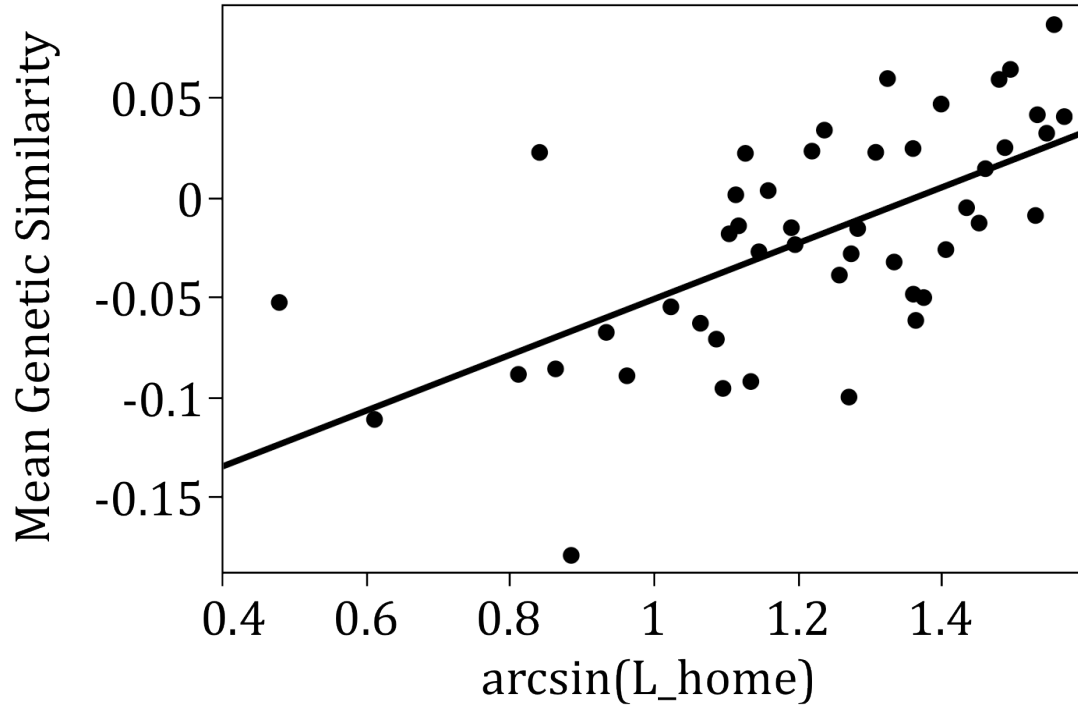


Figure 3.1. Relationship between two measures of inferred philopatry, L_{home} and mean genetic similarity, in 47 song sparrows. Inferred philopatry as estimated by L_{home} has been arcsin transformed. The solid line represents a linear regression and is plotted for visualization purposes only.

3.2 Parasite Load and Philopatry

Among song sparrows classified as infected ($n=25$ or 45%), haematozoan parasite load ranged from 1 to 522 parasites per 10,000 erythrocytes (average = 60.9, SE = 127.6). Males and females did not differ in parasite load (t-test, $t_{22.8}=0.86$, $P=0.93$) so I pooled data from both sexes.

Parasite load was not significantly predicted by L_{home} (GLM; $R^2=0.024$, $F_{1,21}=0.52$, FDR-corrected $P=0.48$, Fig 3.2a) or by MGS ($R^2=0.038$, $F_{1,21}=0.82$, FDR-corrected $P=0.48$, Figure 3.2b).

Inferred philopatry did not differ between infection statuses - that is, whether haematozoa were observed at all in a scan of 10,000 erythrocytes, either using L_{home} (t-test, $t_{39.1}=-0.43$, $P=0.67$, Figure 3.3a) or MGS to infer philopatry (t-test, $t_{37.7}=0.63$, $P=0.53$, Figure 3.3b).

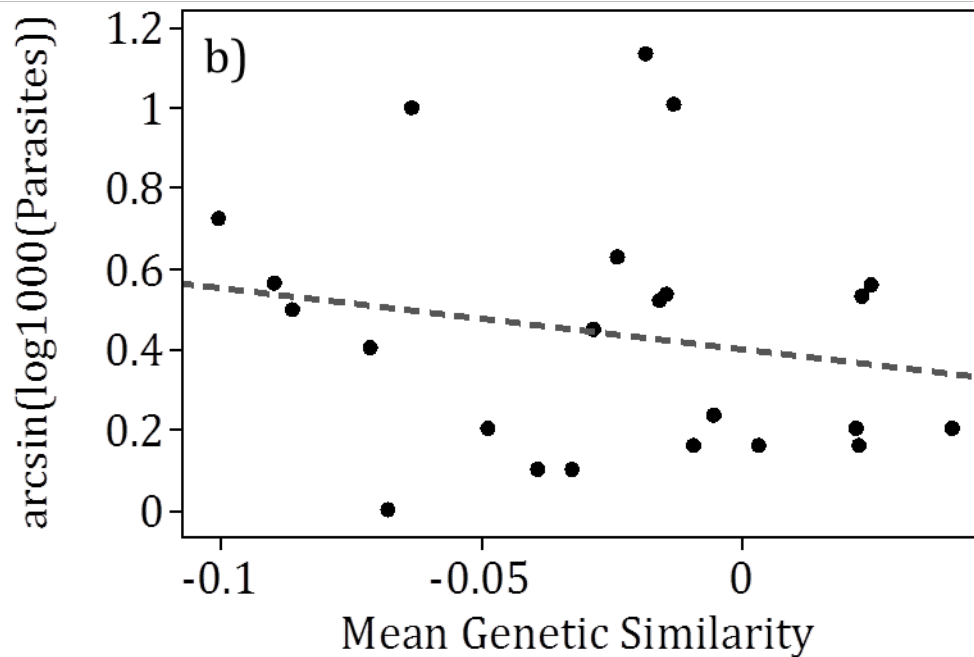
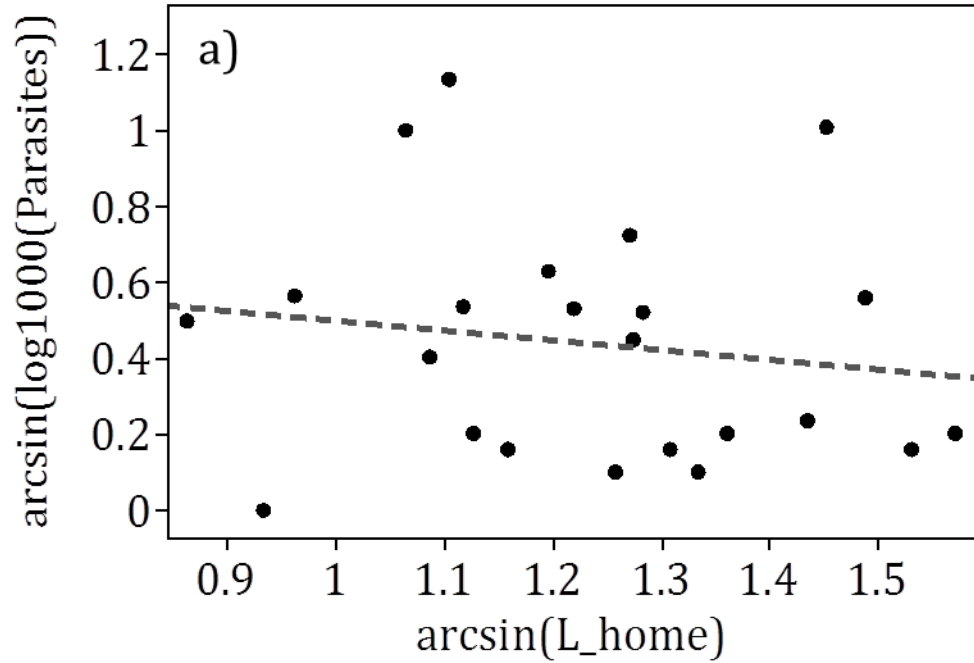


Figure 3.2. Parasite load per 10,000 erythrocytes ($\arcsin(\log_{1000})$ transformed) as a function of philopatry as inferred by a) L_{home} and b) mean genetic similarity in 31 song sparrows infected by haematozoa. The dashed lines are linear regressions for illustrative purposes only. Neither measure of philopatry significantly predicted parasite load.

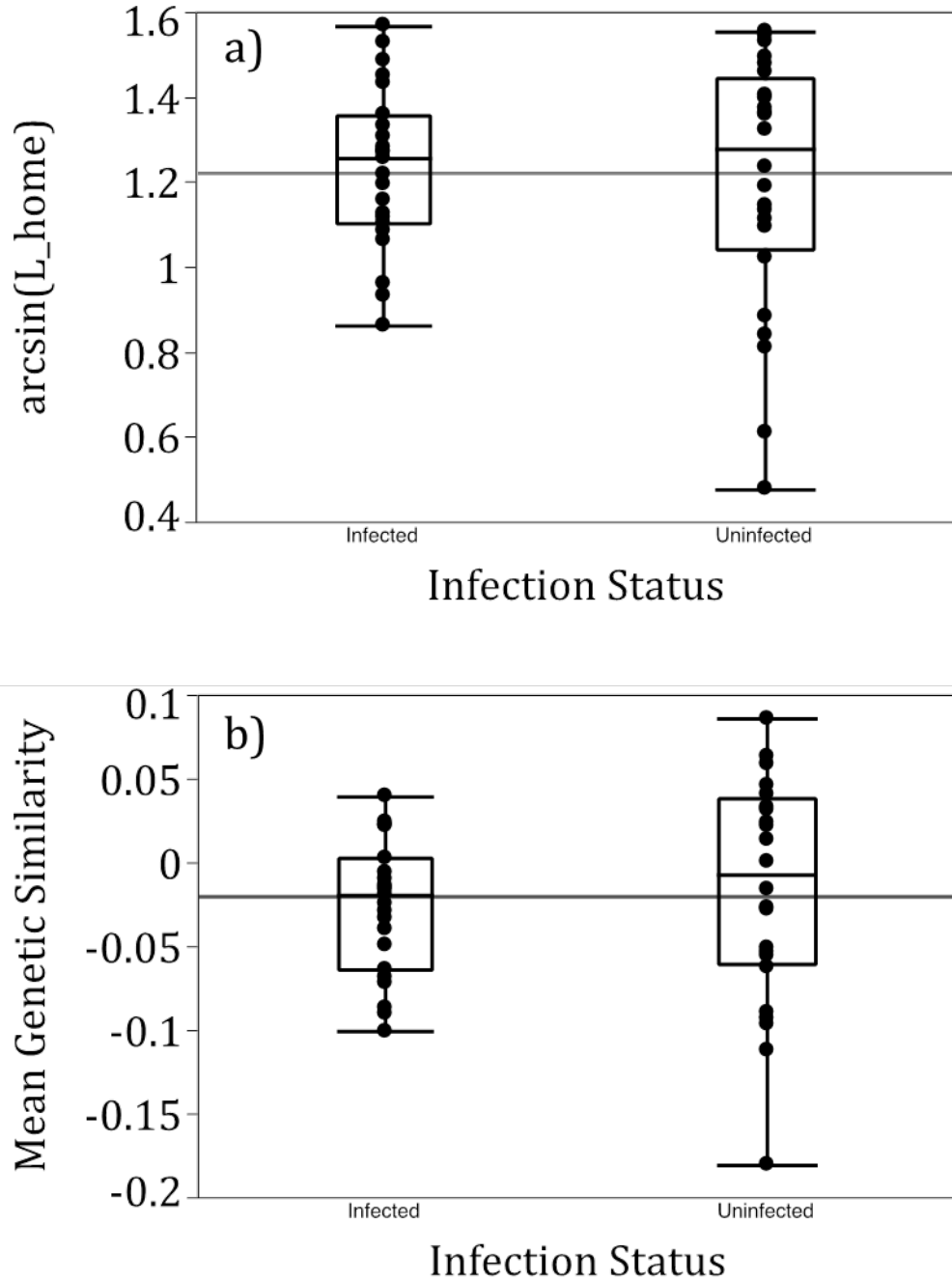


Figure 3.3. Comparison of inferred philopatry as measured by a) L_{home} and b) mean genetic similarity between 47 song sparrows infected and uninfected by haematozoa. Inferred philopatry does not differ significantly between infection statuses for either measure of philopatry. The horizontal gray line is the grand mean, and each boxplot contains the 90th percentile, 75th percentile, median, 25th percentile, and 10th percentile.

3.3 Immune measures and Philopatry

Phagocytosis scores did not differ between sexes or with infection status, nor was a significant interaction observed (sex: $F_{1,53}=2.46$, $P=0.12$; infection status: $F_{1,53}=0.14$, $P=0.70$; sex×infection status: $F_{1,53}=2.53$, $P=0.12$). Similarly, HL-HA (agglutination) score did not differ across sexes or with infection status, nor was a significant interaction observed (sex: $F_{1,51}=1.10$, $P=0.30$; infection status: $F_{1,51}=0.22$, $P=0.64$; sex×infection status: $F_{1,51}=2.59$, $P=0.11$). Likewise, IgY levels did not differ across sexes or with infection status, nor was a significant interaction observed (sex: $F_{1,53}=0.48$, $P=0.49$; infection status: $F_{1,53}=0.71$, $P=0.40$; sex×infection status: $F_{1,53}=0.34$, $P=0.56$). Thus, for each immune measure I pooled data across sexes and across infection statuses. However, in some cases I ran additional tests for each sex separately to investigate sex-specific patterns.

The two measures of innate immunity, phagocytosis and HL-HA score, are not significantly related to one another (Pearson's $r_{1,50}=-0.067$, $P=0.64$; Figure 3.4a). Phagocytosis was significantly negatively related to levels of IgY, a measure of adaptive immunity (Pearson's $r_{1,52}=-0.36$, $P=0.012$; Figure 3.4b). HL-HA score and IgY level were not significantly correlated with one another (Pearson's $r_{1,51}=0.025$, $P=0.86$; Figure 3.4c). Differences in degrees of freedom reflect differences in sample size, resulting from limited amounts of whole blood or plasma available from some individuals.

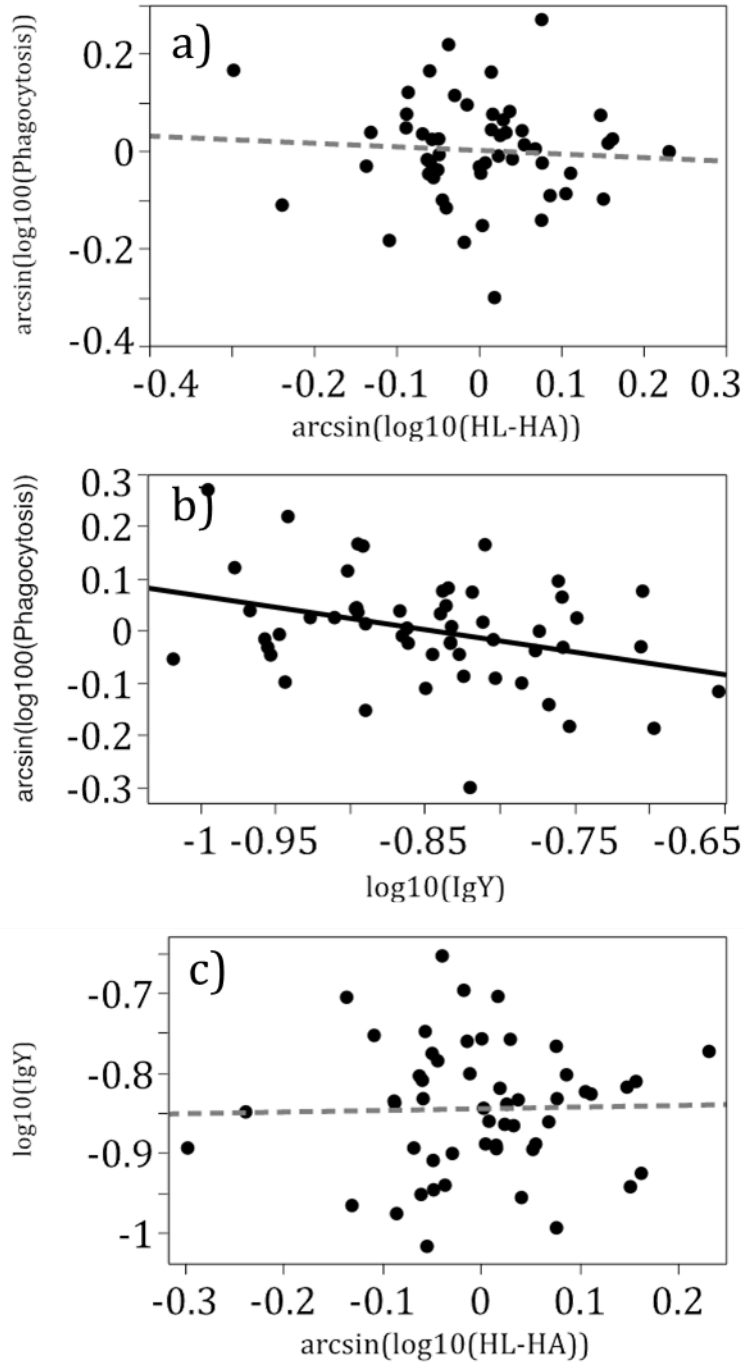


Figure 3.4. Correlation between a) hemolysis-hemagglutination (HL-HA) score, corrected for body condition, and phagocytosis score, corrected for date, in 52 song sparrows, b) immunoglobulin Y (IgY) levels and phagocytosis score, corrected for date, in 54 song sparrows, c) HL-HA score, corrected for body condition and immunoglobulin Y (IgY) levels in 53 song sparrows. Dashed lines represent non-significant linear regressions and the solid line represents a significant linear regression. All lines are for illustrative purposes.

Overall, inferred philopatry did not predict any of the three immune measures when data were pooled across sexes and across infection statuses. However, some sex-specific patterns (restricted to females) were observed.

When data for both sexes were combined, phagocytosis was not significantly predicted by L_{home} (GLM: $R^2=0.031$, $F_{1,45}=1.41$, FDR-corrected $P=0.48$, Fig 3.5a) or by MGS ($R^2=0.00075$, $F_{1,45}=0.033$, FDR-corrected $P=0.86$; Figure 3.5b).

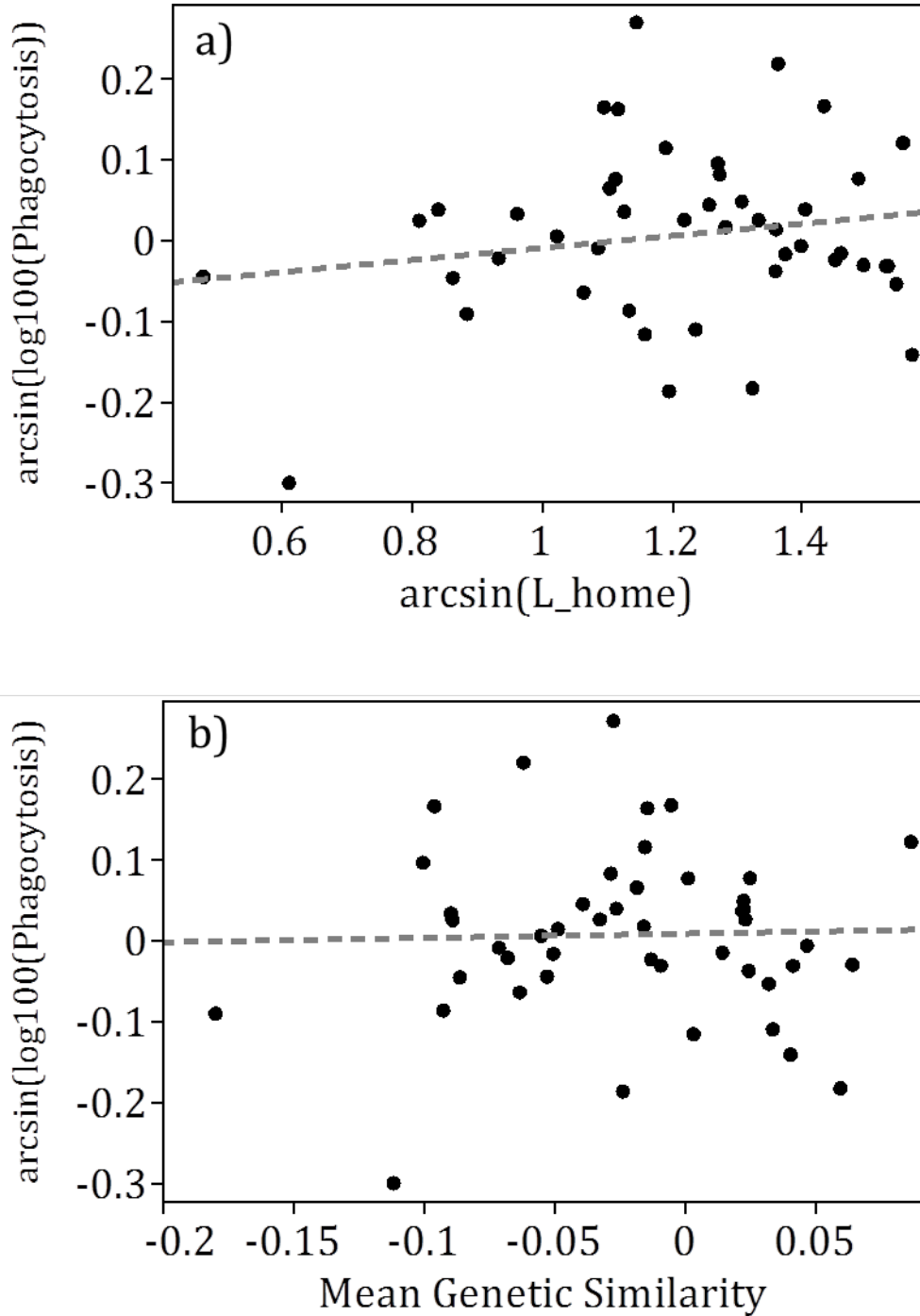


Figure 3.5. Phagocytosis score (corrected by date) as a function of inferred philopatry as estimated by a) L_{home} and b) mean genetic similarity in 47 song sparrows. Dotted lines are linear regressions and are for illustrative purposes. Phagocytosis score was not predicted by either measure of inferred philopatry.

In females alone, inferred philopatry as estimated by L_home predicted macrophage phagocytosis (GLM, $R^2=0.24$, $F_{1,22}=6.91$, FDR-corrected $P=0.031$; Figure 3.6) such that increasing L_home was associated with higher phagocytosis scores. However, this relationship did not occur in males ($R^2=0.0045$, $F_{1,20}=0.091$, FDR-corrected $P=0.77$). Inferred philopatry as estimated by MGS did not predict phagocytosis in either females ($R^2=0.058$, $F_{1,22}=1.35$, FDR-corrected $P=0.26$) or males ($R^2=0.075$, $F_{1,20}=1.61$, FDR-corrected $P=0.44$).

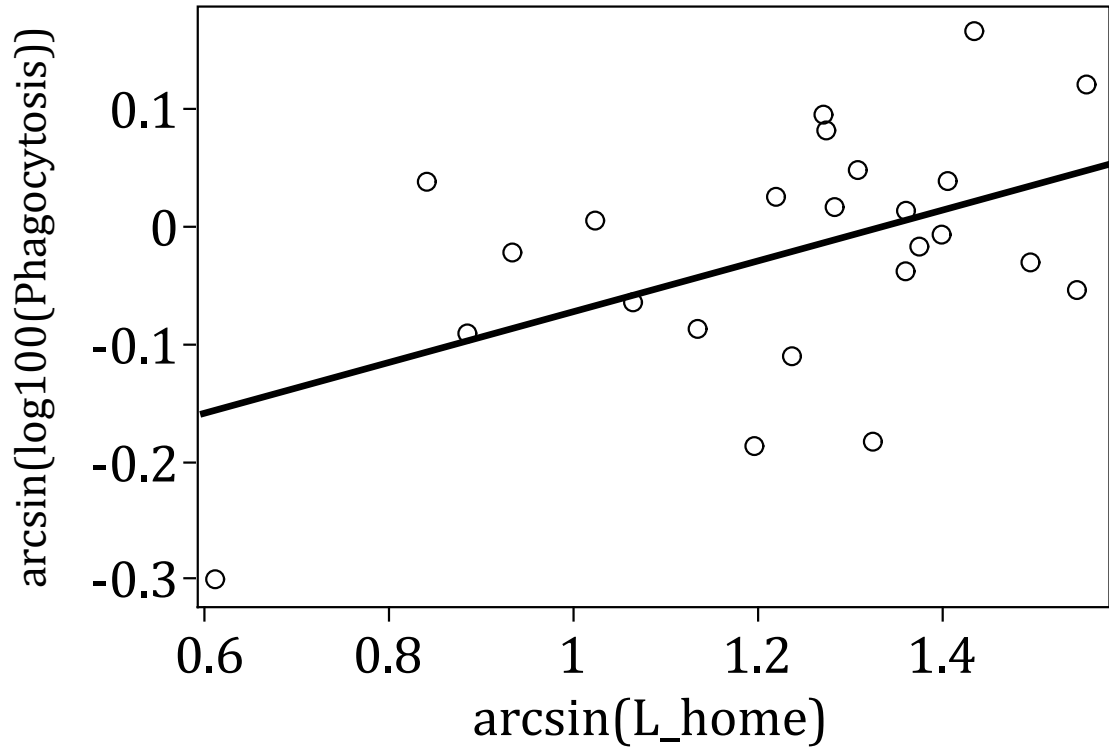


Figure 3.6. Phagocytosis (corrected by date) as a function of inferred philopatry as estimated by L_{home} in 22 female song sparrows. The solid line represents a linear regression and is for illustrative purposes. Inferred philopatry significantly predicts phagocytosis score in females.

When data for both sexes were combined, condition-corrected agglutination (HL-HA) score was not significantly predicted by L_home (GLM; $R^2=0.046$, $F_{1,43}=2.08$, FDR-corrected $P=0.16$, Figure 3.7a) or by MGS ($R^2=0.078$, $F_{1,43}=3.66$, FDR-corrected $P=0.13$, Figure 3.7b). Sex-specific analyses showed that for females, both measures of inferred philopatry were negatively associated with condition-corrected agglutination (L_home: $R^2=0.17$, $F_{1,21}=4.41$, FDR-corrected $P=0.048$, Figure 3.8a; MGS: $R^2=0.17$, $F_{1,21}=4.45$, FDR-corrected $P=0.0479$, Figure 3.8b). For males, neither L_home nor MGS significantly predicted condition-corrected agglutination (L_home: $R^2=0.037$, $F_{1,20}=0.77$, FDR-corrected $P=0.42$; MGS: $R^2=0.033$, $F_{1,20}=0.68$, FDR-corrected $P=0.42$).

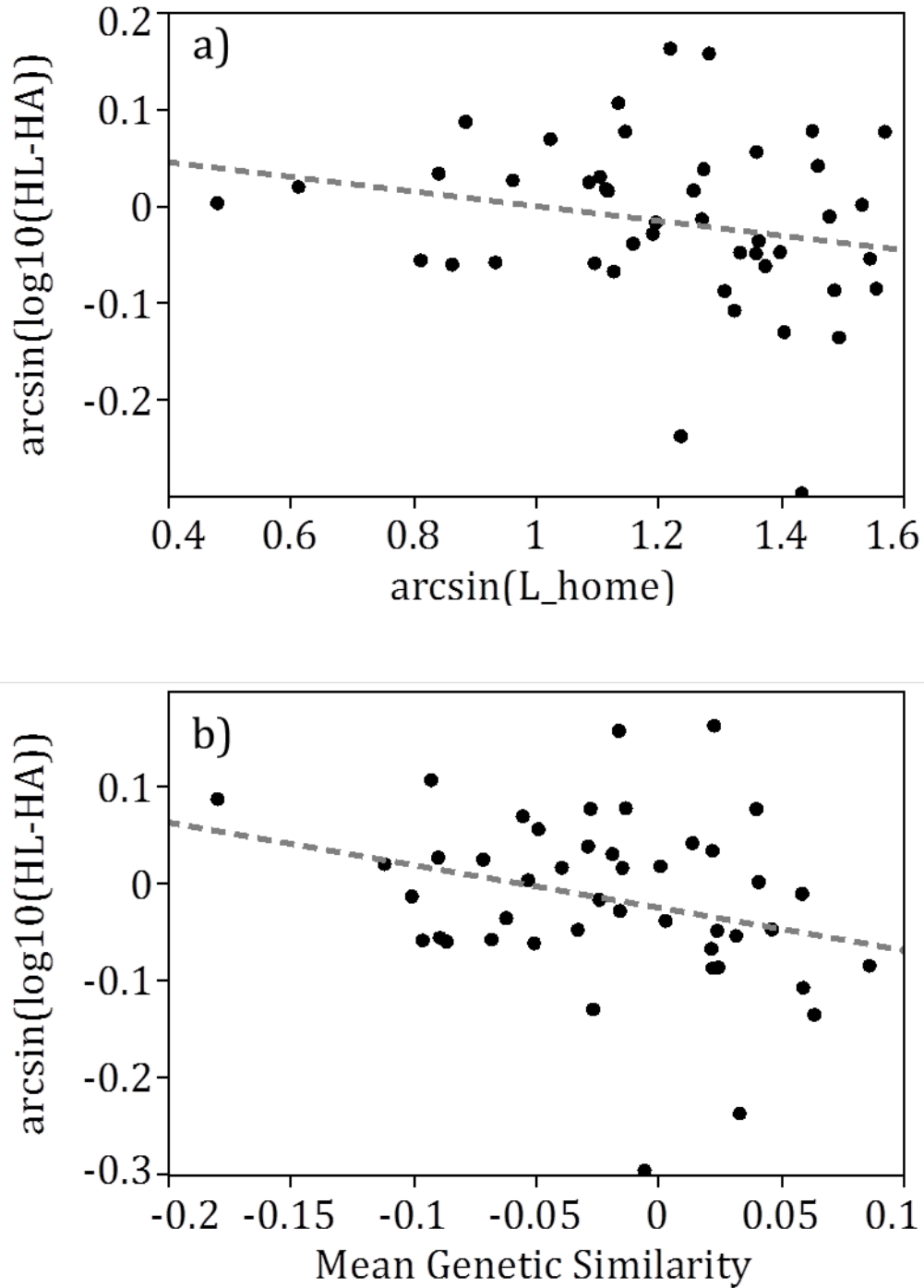


Figure 3.7 Agglutination score (HL-HA; corrected by body condition) as a function of inferred philopatry as estimated by a) L_{home} and b) mean genetic similarity in 47 song sparrows. Dashed lines are linear regressions and are for illustrative purposes. Agglutination score for both sexes was not predicted by either measure of inferred philopatry.

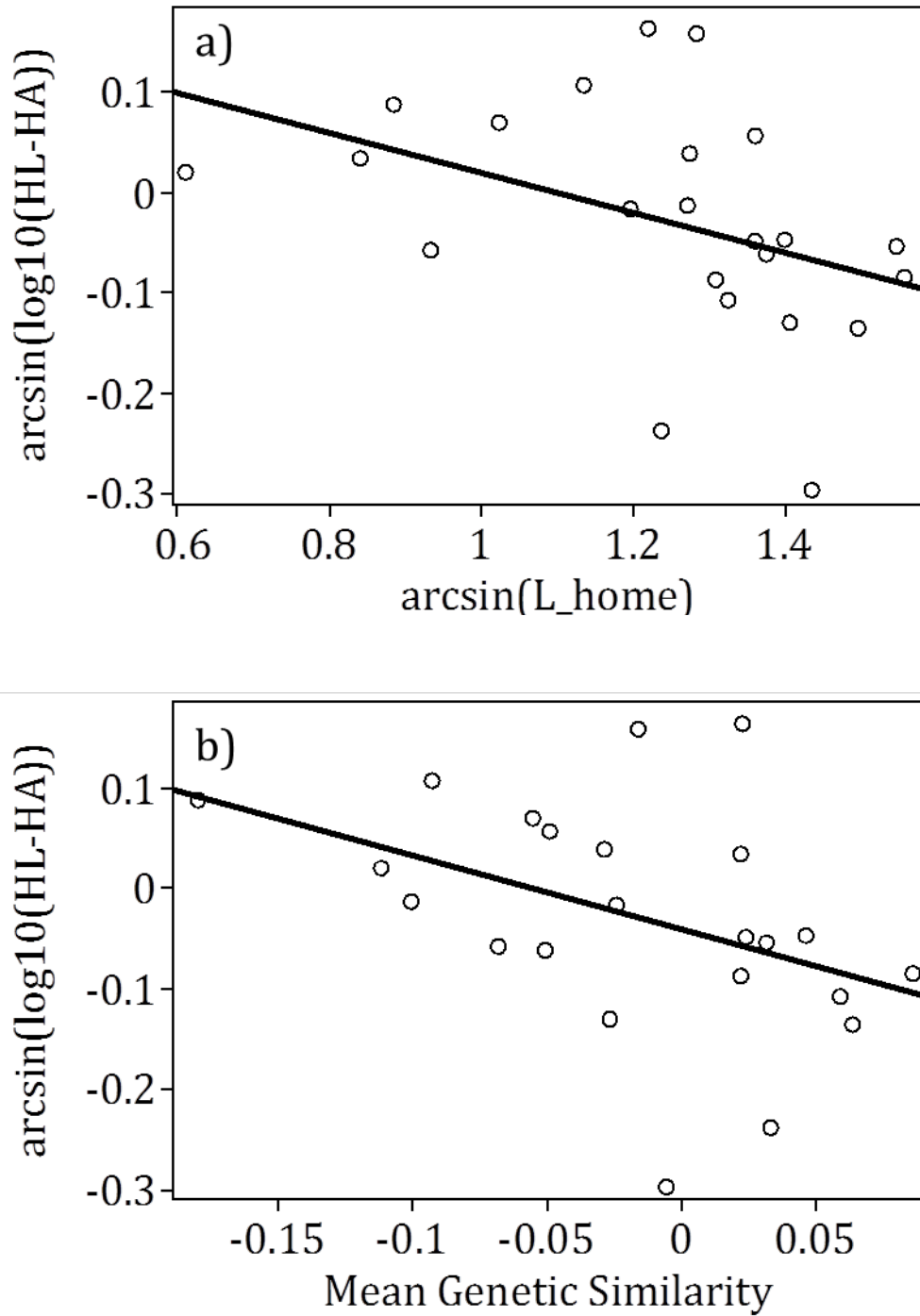


Figure 3.8. Agglutination score (HL-HA; corrected by body condition) as a function of inferred philopatry as estimated by a) L_{home} and b) mean genetic similarity in 23 female song sparrows. Solid lines are linear regressions and are for illustrative purposes. Agglutination score for females was significantly predicted by both measures of inferred philopatry.

Agglutination ability may vary with infection status (Matson et al., 2005), so as a complementary analysis I further subdivided birds according to whether or not they were infected with haematozoan parasites. Uninfected birds (both sexes combined) showed a trend (albeit not statistically significant after correcting for multiple tests) towards decreasing condition-corrected agglutination with increasing philopatry as inferred from L_home (GLM; $R^2=0.15$, $F_{1,22}=3.81$, FDR-corrected $P=0.064$, Figure 3.9a) and a significant relationship along the same lines as inferred from MGS ($R^2=0.22$, $F_{1,22}=6.38$, FDR-corrected $P=0.039$, Figure 3.9b). Among infected birds, however, condition-corrected plasma agglutination did not vary with either L_home ($R^2=0.000060$, $F_{1,19}=0.0011$, FDR-corrected $P=0.99$, Figure 3.10a) or MGS ($R^2=0.00000020$, $F_{1,19}=0.0000$, FDR-corrected $P=0.99$, Figure 3.10b).

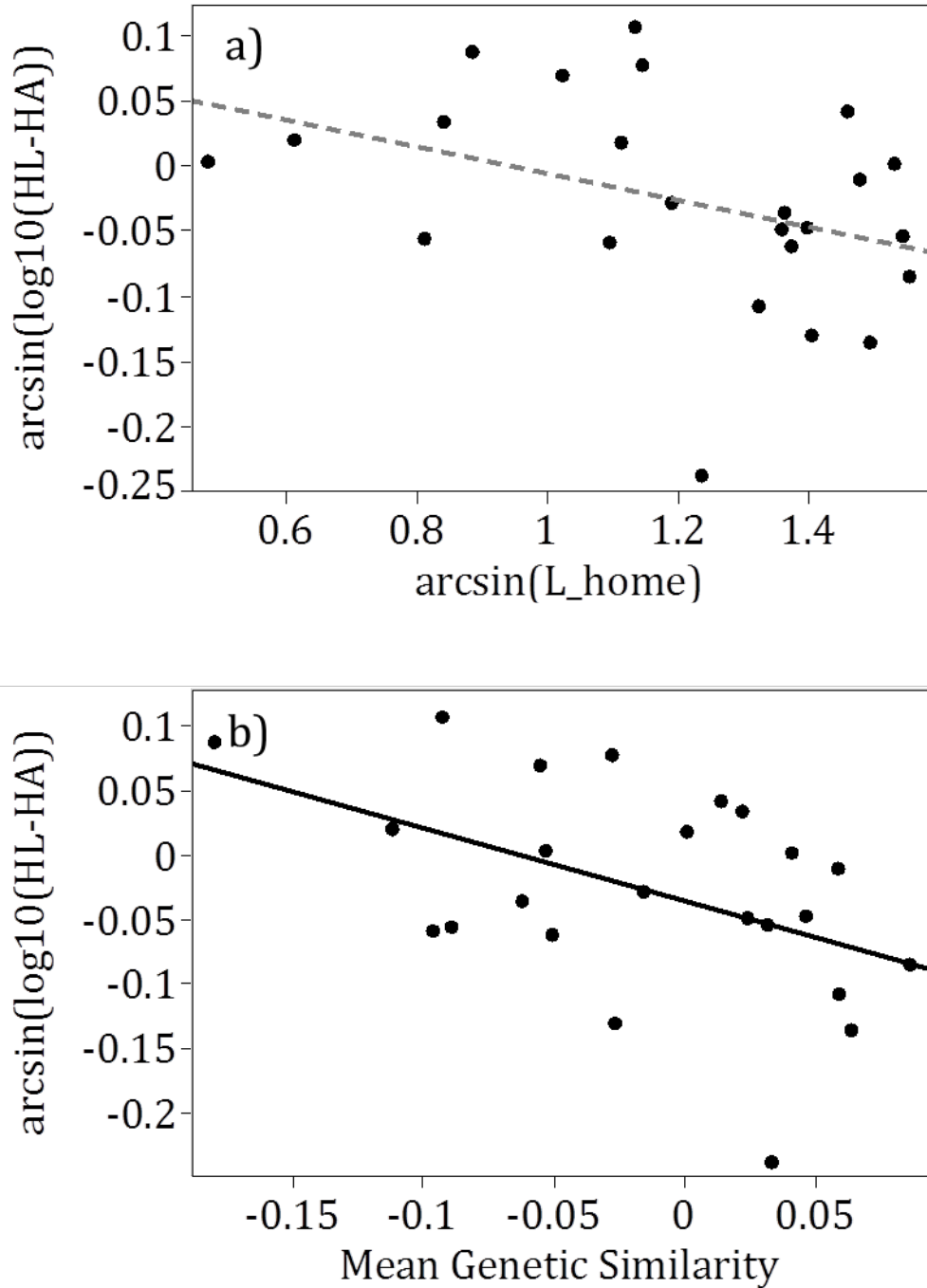


Figure 3.9. Agglutination score (HL-HA; corrected by body condition) as a function of inferred philopatry as estimated by a) L_home and b) mean genetic similarity in 24 uninfected song sparrows. The solid line is a significant linear regression, the dashed line an insignificant linear regression, and both are for illustrative purposes. Agglutination score for uninfected song sparrows was significantly predicted by mean genetic similarity but not L_home .

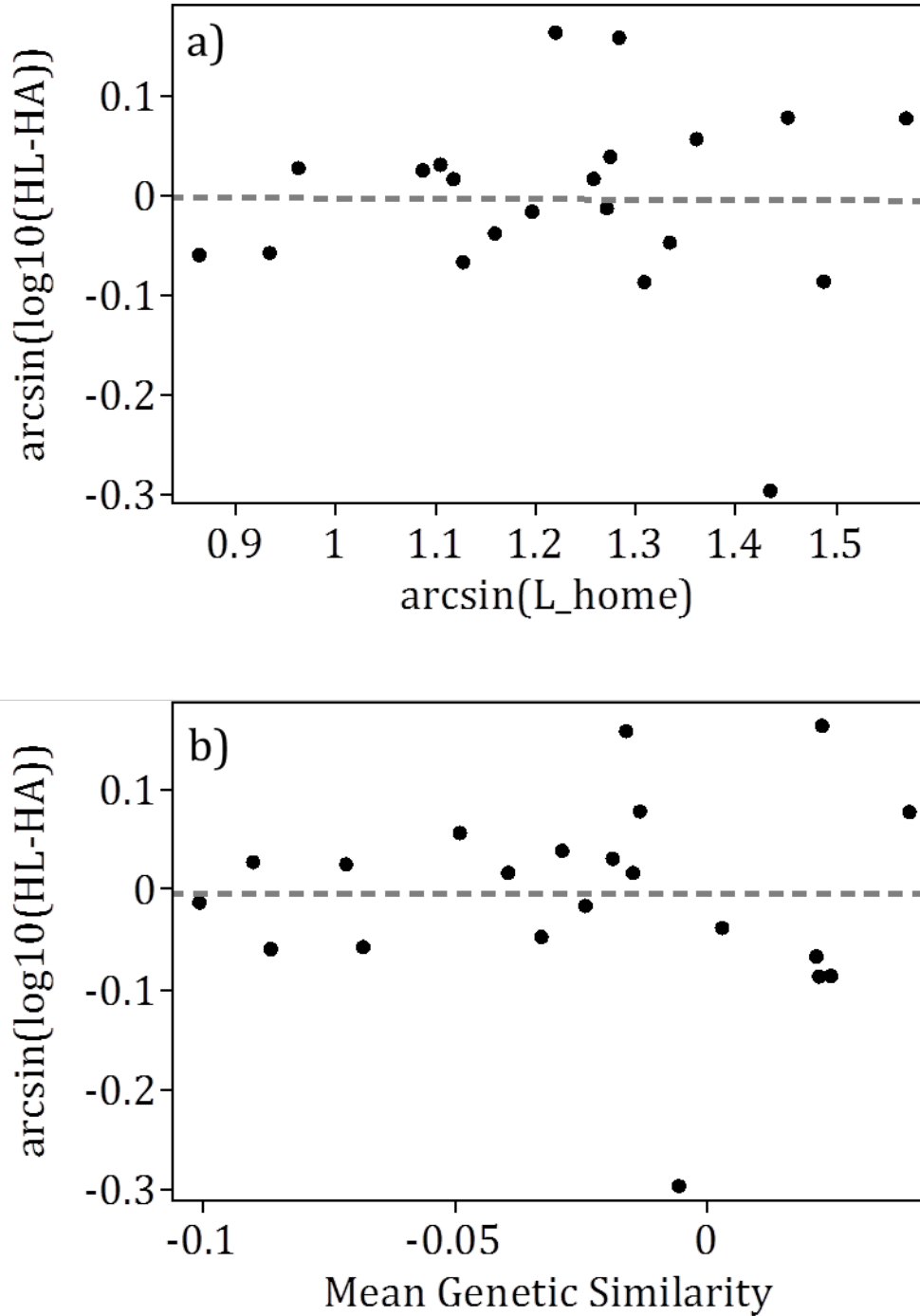


Figure 3.10. Agglutination score (HL-HA; corrected by body condition) as a function of inferred philopatry as estimated by a) L_{home} and b) mean genetic similarity in 21 infected song sparrows. The dashed lines are insignificant linear regressions and are for illustrative purposes. Agglutination score for infected song sparrows was not significantly predicted by either measure of inferred philopatry.

Plasma levels of IgY were not predicted by either L_home (GLM; $R^2=0.023$, $F_{1,44}=1.03$, FDR-corrected $P=0.63$, Figure 3.11a) or by MGS ($R^2=0.0025$, $F_{1,44}=0.11$, FDR-corrected $P=0.74$, Figure 3.11b).

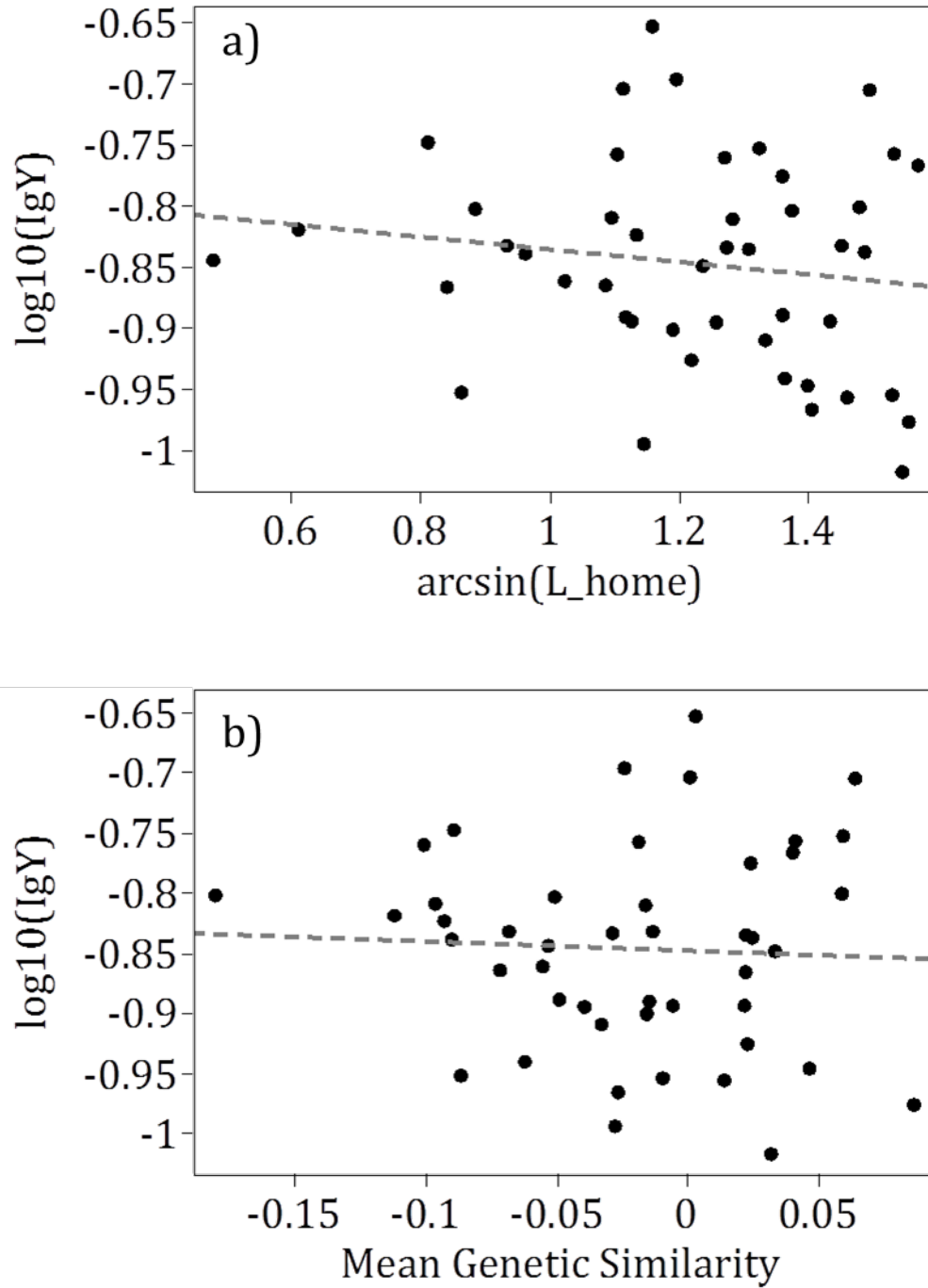


Figure 3.11. IgY levels as a function of inferred philopatry as estimated by a) L_{home} and b) mean genetic similarity in 46 song sparrows. The dashed lines are insignificant linear regressions and are for illustrative purposes. IgY levels were not significantly predicted by either measure of inferred philopatry.

4 Discussion

4.1 Summary of Findings

In exploring the relationships between natal philopatry, parasitism and immune function, I used different approaches to infer natal philopatry, and assessed multiple measures of immune function. My two informative measures of natal philopatry, mean genetic similarity (MGS) and L_{home} , were significantly positively correlated, suggesting that MGS may provide an acceptable substitute for genetic assignment tests such as L_{home} when allele frequencies from surrounding sites are not readily available. Moreover, whereas some studies (e.g. Råberg et al., 2003; Greenman et al., 2005) still assess only a single measure of immunity and from this draw general conclusions about “immunocompetence”, and thus implicitly assume that all measures of immunity are positively correlated, I found evidence for negative relationships between different measures of immunity. Phagocytosis (a measure of cell-mediated innate immunity) was negatively related to IgY levels (a measure of humoral adaptive immunity), and negatively related to HL-HA scores (humoral innate immunity), although this latter relationship was only a trend, and not significant. HL-HA scores and IgY levels were not significantly related to one another.

Contrary to previous findings in this population, natal philopatry was not associated with either reduced haematzoan parasite load or decreased likelihood of infection. In female song sparrows, natal philopatry was associated with greater phagocytosis but lower HL-HA. These patterns were sex-specific, as similar trends were not observed in males. In the subset of birds (both sexes) that were not infected with haematzoa, higher natal philopatry again tended to be associated with lower HL-HA, although not always significantly so. Contrary to my expectation that philopatry should be positively associated with investment in adaptive immunity, philopatry did not predict IgY. Thus, in contrast to my prediction that philopatry should affect the balance between adaptive and innate branches of immunity, I instead found an association between philopatry and allocation to humoral versus cell-mediated innate defences.

4.2 Inferring Philopatry

I attempted three different approaches to infer natal philopatry from microsatellite allele frequencies. Of these, only two proved to be informative, as the program STRUCTURE could not detect any population structure at the geographic scale examined in this study (sites within 50 km of my study population). STRUCTURE uses assumptions of Hardy-Weinberg equilibrium to determine whether a population consists of subpopulations, and if so how many, before assigning individuals to subpopulation (Pritchard et al., 2000). This approach can underestimate population genetic subdivision, so that subtle structuring may not be detected (Krutovsky et al., 2009). Moreover, STRUCTURE may fail to detect population subdivision when a large proportion of individuals are drawn from the same site (Kalinowski, 2011), as was the case in this study in which 191 of 364 birds genotyped were captured at the Bracken field site. Underestimation of population genetic subdivision is particularly likely when using the ecologically appropriate, but less powerful, admixture option (Pritchard et al., 2000) as I did in this study. Even in starfish (*Astroides calycularis*), an organism with limited dispersal capacity (approximately 1 km dispersal distance on average; Casado-Amezúa et al., 2012), STRUCTURE had difficulty assigning clusters when subpopulations were highly admixed (Casado-Amezúa et al., 2012). This method of inferring philopatry may be of limited use for highly mobile animals such as birds, particularly for species that, like song sparrows, occupy an ephemeral forest-edge habitat that may necessitate frequent dispersal.

The other two approaches for inferring natal philopatry that I used, L_home and mean genetic similarity (MGS), were significantly and positively correlated to one another. Estimating L_home makes use of more information than does MGS, because assignment testing requires sampling individuals and characterizing allele frequencies not just at the target site but also at other potential source locations (Piry et al., 2004). Thus, L_home should presumably provide a more accurate estimate of natal philopatry. However, an important advantage of the MGS approach is the ability to sample only at the target site. Confirming a significant positive relationship between these two measures suggests that genetic sampling at a single site and using MGS to infer

philopatry may be a reasonable substitute for larger, landscape-scale sampling efforts at least in this study system. However, it remains to be determined whether this relationship also applies to less mobile organisms or to those inhabiting more patchy environments.

4.3 Philopatry and Parasites

Inferred natal philopatry did not predict haematozoan parasite load or likelihood of infection, contrary to previous findings in this population (Stewart and MacDougall-Shackleton, 2008; Sarquis-Adamson and MacDougall-Shackleton, unpublished). This discrepancy between years at the same study site may reflect oscillations in the evolutionary arms race between hosts and parasites, and thus variation over time in the fitness consequences of natal philopatry versus dispersal. Simulations show that evolutionary arms races between hosts and parasites have different front-runners at different points in time, even when the general tendency is for local adaptation by either host or parasite (Gandon and Michalakis, 2002). Thus, comparing parasite loads of philopatric versus dispersing individuals at any single point in time may not reflect the trend over several years. In addition, unicellular parasites generally have short generation times, such that the relative abundance of different parasites may vary from year to year (e.g. Olson et al., 2004). Temporal variation in parasite abundance may influence the relationship between philopatry and susceptibility to parasites. Indeed, if dispersal were consistently associated with an increased risk or severity of parasitism, heritable variation in dispersal would not be maintained over evolutionary time.

The lack of relationship between inferred philopatry and parasitism is in contrast to findings from other systems as well. In mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*), males that sang nonlocal song types (and thus assumed to have immigrated from elsewhere) had greater haematozoan parasite loads than did males singing the local dialect and thus inferred to be of local origin (MacDougall-Shackleton et al., 2002). Ecological differences between song sparrows and the closely related white-crowned sparrows may help to explain differences between my findings and those of MacDougall-Shackleton et al. (2002). In white-crowned sparrows, natal

philopatry is greater for males than for females (Morton, 1997), whereas I found no sex differences in inferred natal philopatry in song sparrows. Moreover, mountain white-crowned sparrows breed in scattered meadows that are ecologically similar to islands (Morton, 1997), whereas song sparrows inhabit a relatively continuous and less fragmented landscape (Knapton and Krebs, 1974).

My findings also contrast with patterns seen in barn swallows (*Hirundo rustica*), in which natal dispersal was positively associated with louse fly load in females but negatively associated with chewing lice load in males (Saino et al., 2014). Again, the difference between my findings and those of Saino et al. (2014) may be explained by ecological differences: barn swallows are colonial breeders, which may enhance transmission of ectoparasites (Tella, 2002) and intensify selection pressure on hosts and/or increase the likelihood that philopatric individuals have had previous experience with the local parasite fauna. However, the different relationships between philopatry and parasitism in male versus female barn swallows (Saino et al., 2014) are somewhat similar to my findings of relationships between philopatry and immunity in females but not in males.

My findings are similar in outcome to those found in great tits (*Parus major*) and their flea parasites (*Ceratophyllus gallinae*; Dufva, 1996b), a system in which neither host nor parasite appears to show local adaptation to the other. Dufva (1996b) suggested that the lack of local adaptation may be explained by a relatively low virulence of the fleas on the hosts: this explanation may also extend to my study system, as the haematozoan parasites I studied often incur little harm to their avian hosts (Valkiūnas, 2005). However, it should be noted that the study by Dufva (1996b) used experimental infection and was thus different in methods and approach from my study. Still, low parasite virulence may be an important contributor to the outcome of host-parasite interactions in my study system.

Conclusively determining whether the haematozoan infections examined here were acquired on the wintering grounds, at stopover sites throughout migration, or on the breeding grounds, was beyond the scope of my project. However, in the case of birds showing acute infections (relatively high parasite load) it seems reasonable to assume

that these infections were acquired on the breeding grounds, because birds that are heavily infected with bloodborne parasites delay migration (van Gils et al., 2007). Similarly, based on the timeframe of infection (parasites reach peak load approximately 3 weeks after initial infection), it is reasonable to assume that individuals with high parasite loads have been infected recently (Valkiūnas, 2005). Conversely, simply classifying individuals according to infection status may not accurately reflect susceptibility to parasites on the breeding grounds, as haematozoan parasites can remain at low levels within an infected host (chronic infections) throughout the host's lifetime (Valkiūnas, 2005).

A limit of any observational study of parasitism, like this one, is the problem of unsampled individuals. That is, field-based studies of naturally occurring variation in philopatry and parasitism may draw from a non-representative subset of birds that either avoid or survive infection with parasites. It is possible that other individuals may have attempted to immigrate to the study site, but upon arrival, may have become infected and died, and were thus never sampled in my study. The ability to track survivorship and infection status of all individuals is one of the benefits of an experimental infectivity approach, which is the logical follow-up to my study. Experimentally exposing birds to their local parasites versus parasites from another area (e.g. Sarquis-Adamson and MacDougall-Shackleton, unpublished) should help to assess whether philopatric birds have an advantage in dealing with the local parasite fauna.

4.4 Philopatry and Immune Measures

4.4.1. Correlations between immune measures: I observed a significant negative relationship between phagocytosis (reflecting cell-mediated innate immunity) and levels of IgY (reflecting humoral adaptive immunity). I did not find a similarly negative association between phagocytosis and HL-HA scores (reflecting humoral innate immunity). The two measures of humoral immunity, HL-HA and IgY (representing innate and adaptive branches, respectively) were not significantly related to one another.

The negative relationship between phagocytosis (innate and cellular) and IgY (adaptive and humoral) might reflect trade-offs between the innate and adaptive branches of the immune system, and/or between cellular and humoral types of immunity. The lack of relationship between HL-HA (innate and humoral) and IgY, suggests trade-offs between cellular and humoral defences, rather than between innate and adaptive branches of immunity. Consistent with this idea, Kubli and MacDougall-Shackleton (2014) also found negative relationships between humoral and cellular components of innate immunity in my study population.

In birds, humoral defences require investment (i.e. antibody repertoire development) within the first few months of life, before the bursa of Fabricius regresses, at 10-12 weeks after hatch in some species (e.g. ring-necked pheasant *Phasianus colchicus*, Mercer-Oltjen and Woodard, 1987; Kubli and MacDougall-Shackleton, 2014). This implies that only individuals in relatively good condition as juveniles can invest heavily in humoral immunity. In other taxa, similarly, even short-term stressors during the juvenile stage can cause reduction in humoral immunity (as in Atlantic cod, *Gadus morhua*, Caipang et al., 2014). Once established, however, humoral immunity is relatively efficient to maintain later in life (Valtonen et al., 2010). By contrast, cellular defences have no specific early-life window for investment, and are thus available even to individuals that were in poor condition during early life (Lee, 2006; Buehler et al., 2008). However, cellular defences incur high energetic and inflammatory costs (Buehler et al., 2008). Previous work on this population of song sparrows found that males with complex song repertoires (a trait acquired during early life, and thus thought to reflect early-life condition) invested more in humoral and less in cellular aspects of innate immunity (Kubli and MacDougall-Shackleton, 2014), raising the possibility that the balance between cellular and humoral immunity is influenced by condition during early life.

4.4.2. Philopatry and Immunity: Among female song sparrows (but not males), philopatry was positively associated with phagocytosis, and negatively associated with HL-HA. Thus, females that were more philopatric invested more in cell-mediated innate defences and less in humoral innate defences. This pattern suggests that at least for

females, the optimal balance between humoral and cell-mediated innate defences may depend on philopatry, or vice versa. However, if humoral immunity reflects good condition during early life as suggested by Kubli and MacDougall-Shackleton (2014), this would suggest that among females, relatively philopatric individuals are those that were in poor condition during early life. If so, this would represent a rare exception to general patterns of condition-dependent dispersal. In most taxa that have been studied, juveniles in good condition tend to remain at their natal site, while those that disperse tend to be in relatively poor condition (Mestre and Bonte, 2012; but see Massot and Clobert, 1995 for an exception). Alternatively, the relationship between philopatry and investment in cellular versus humoral defences may be mediated by factors other than early-life condition. For example, humoral defences might be more effective than cellular defences against novel pathogens, which would account for enhanced investment in humoral defences by dispersing birds. The relative efficacy of cellular versus humoral defences against novel versus familiar parasites remains unexplored, but may help to explain the patterns in immune investment I observed.

Philopatry was associated with the balance between cellular innate and humoral innate immunity in female song sparrows but not in males. Several factors may contribute to this apparent sex-specificity. In many vertebrate species, males exhibit reduced immune function overall due to the immunosuppressant effects of testosterone, particularly during the breeding season (Zuk and McKean, 1996). Individual variation among males in testosterone levels may thus obscure any relationships between philopatry and immunity, although it should be noted that I found no sex differences in any measure of immunity and this undermines the above explanation. Females may also have more limited energy budgets than males during breeding (e.g. Biedenweg, 1983; Hinsley and Ferns, 1994), and this limitation may intensify any trade-offs between cellular and humoral immunity more in females than in males. Importantly, in birds of both sexes that were not harbouring haematozoan infections, greater philopatry was associated with lower HL-HA. Because levels of natural antibody and thus HL-HA scores can be influenced by immune challenges such as parasitic infections (Matson et al., 2005), the relationship between philopatry and immune allocation may be obscured in infected birds, but males were not more likely than females to be infected.

Contrary to my original prediction, philopatry did not predict IgY levels (a measure of adaptive immunity). However, Palacios and colleagues (2012) caution against using only one measure for any branch of the immune system, and particularly recommend at least two for the adaptive branch: one for T-cell responses (cellular) and one for B-cell responses (associated with humoral immunity, such as the IgY assay). IgY assays rely on a single snapshot of antibody levels, and thus may be affected by an individual's current infection status (Ziglari et al., 2009) or other factors. The assay does not directly assess the individual's ability to create antibodies in response to a pathogen threat, and this limitation reduces the ecological validity of this assay. An alternative assay of adaptive immunity, also conducted *in vitro*, is the lymphocyte proliferation assay, which measures lymphocyte division in response to T-cell stimulating phytohemagglutinin and B-cell stimulating lipopolysaccharide (Cunnick et al., 1994; Palacios et al., 2012). This alternative may give a more complete picture of the adaptive immune system and be less vulnerable to distortion by recent immune events like infection, yet is still performable using a single sample *in vitro*. However, I cannot dismiss the possibility that philopatry does not influence adaptive immune investment.

4.5 Conclusions

Overall, my findings suggest that previous observations of home-field advantage in song sparrows (i.e. lower parasite loads in philopatric than dispersing individuals; Stewart and MacDougall-Shackleton, 2008; Sarquis-Adamson and MacDougall-Shackleton, unpublished) are not stable over time. Parasite assemblages vary over time (Gandon and Michalakis, 2002; Olson et al., 2004), so it seems reasonable that whether philopatric or dispersing host individuals have an advantage in dealing with parasites might likewise vary from year to year. Temporal variation in selection pressures imposed by parasites may help to maintain additive genetic variation in dispersal tendency.

Previous observations of reduced parasite loads in philopatric song sparrows (Stewart and MacDougall-Shackleton, 2008; Sarquis-Adamson and MacDougall-Shackleton, unpublished) do not seem to be attributable to philopatric sparrows having generally

stronger immune function. Although female philopatry was positively related to a cell-mediated measure of innate immunity, it was negatively related to a humoral measure of innate immunity. Moreover, these patterns were restricted to females, and I found no relationship between philopatry and adaptive immune function in either sex. Given that during my field season (spring 2013) there was no relationship between philopatry and parasite load or likelihood of infection, previously observed patterns of home-field advantage may be ephemeral, reflecting temporal variation in host-parasite arms races and/or temporal variation in parasite assemblages. Variation over time in the selective environment may maintain variation in dispersal tendency in this population. My findings also suggest that philopatry may also influence optimal investment in innate immunity through increasing the balance of cellular relative to humoral components of innate immunity in females, but may have little influence on this balance in males, or on adaptive immune investment in either sex. Finally, my findings highlight the importance of measuring multiple aspects of immune function to better characterize immunocompetence as a whole.

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Appendices

Appendix A: Animal Use Protocol

AUP Number: 2008-054-05

AUP Title: Mating Signals, Gene Flow, and Disease Resistance in Songbirds

Yearly Renewal Date: 06/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2008-054-05 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS: Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D on behalf of the Animal Use Subcommittee

Appendix B: ELISA Protocol for IgY Assays

ELISA Protocol for IgY Assays

Supplies:

- Costar 96 Well Flat bottom plates (Corning Cat. No. 3370)
- Lids (come with plates)
- Parafilm
- Plastic loading trays for filling multi channel pipetters
- Multichannel pipettor (8 channels)
- Pipet man
- Sodium carbonate (Na_2CO_3 ; Caledon Cat. No. 5968-11-6)
- Sodium bicarbonate (NaHCO_3 ; Caledon Cat. No. 144-55-8)
- Citric acid, anhydride (Caledon Cat. No. 77-91-9)
- Sodium phosphate (Na_2HPO_4 ; Caledon Cat. No. 7558-79-4)
- Tween (Sigma Cat. No. P1379)
- Sodium chloride (NaCl)
- Potassium phosphate, monobasic (KH_2PO_4 ; Caeldon Cat. No. 7778-77-0)
- Hydrogen peroxide, 31% (H_2O_2 ; Sigma Cat. No. H1009)
- 2,2-azino-di-3-ethylbenzaloin sulfonic acid (ABTS; Sigma Cat. No. A1888)
- Anti-chicken IgY (whole molecule) peroxidise conjugate (Sigma Cat. No. A9046)
- Fat free (Skim) powdered milk (grocery store)
- 15 mL Falcon tubes (Science stores)

Solution Preparation

Before beginning your solution preparation, make sure that you have calibrated and zeroed the pH meter. Instructions on how to do this are in the manual for the pH meter.

A.) Dilution Solution

1. Make 0.25L of 0.1M Na_2CO_3 : Place 2.65g of Na_2CO_3 in a 250mL flask and fill to the 250mL mark with deionised water. Cap and mix.

2. Make 0.5 L of 0.1M NaHCO_3 : Place 4.2g of NaHCO_3 in a 500mL flask and fill to the 500mL mark with deionised water. Cap and mix.

3. Add the Na_2CO_3 solution slowly to the NaHCO_3 solution until a pH of 9.6 is reached.

4. *Store in +4°C fridge. Solution will last for months.*

B) PBS-Tween Solution

Suggested volume to make: 8L. You can adjust this accordingly for 4L, 2L or 1L (but you use a lot in this assay so 4L is probably enough unless you are running more than one field season's worth of samples).

1. Make 8L of PBS Solution. First make the following three solutions separately:

a.) Add 35.06g of NaCl to 4L of deionized water

b.) Add 9.59g K_2HPO_4 0.96L of deionized water

c.) Add 64.73g of Na_2HPO_4 to 3.04L of deionized water.

2. Mix all of the above solutions to get 8L of PBS. Then add 4mL of Tween to the

PBS. Use the pipetman to pipet up the Tween – it is thick and you will not be able to pipet it with a typical pipettor like a P1000 or P5000.

3. *Store in +4°C fridge. Solution will last for months.*

C) Milk Solution

1. For 1 assay (or plate): mix 0.75g of powdered milk in 15mL of PBS-Tween solution.

2. *Do not store, make as needed.*

D) Antibody Solution

1. For 1 assay (or plate): mix 44 μ L of chicken anti-body with 11mL of PBS-Tween. Fill 15 mL Falcon tubes with 11 mL of PBS-Tween, and add antibody to each tube. Date and label the tubes with solution name.

2. Store pre-prepared tubes in -80°C freezer. Unfreeze as needed.

E) Revealing Solution

1. Make 0.1M citric acid: 9.607g in 0.5L of deionized water.

2. Make 0.1 Na_2HPO_4 : 17.907g in 0.5L of deionized water

3. Carefully add 0.1M citric acid to 0.1M Na_2HPO_4 until you reach a pH of 5.

Note: 500 mL of citric acid is not enough to bring the pH down to 5. You have two options: make 1L of citric acid and add to 500 mL of Na_2HPO_4 or make 500 mL of each solution, but add 500 mL of citric acid to 250 mL of Na_2HPO_4 , depending on which you feel more comfortable with.

4. Add 0.05g of ABTS (2,2-azino-di-3-ethylbenzalonic sulfonic acid) to 100mL of the above solution.

5. Prepare frozen samples by measuring out 11mL of the mixture containing the ABTS in tubes labelled "REVEAL" and store in -20°C freezer. Unfreeze as needed.

6. For one assay (one plate): Unfreeze on sample of 11mL of the ABTS revealing solution and add 11 μ L of the hydrogen peroxide. Mix well (i.e. vortex 5-10 seconds) before use.

Preliminary Tests

Before you begin measure the immunoglobulin content of blood plasma, you must determine the appropriate dilution of plasma according to the species being studied. To do so, you must work with a range of plasma dilutions from 1/500 to 1/64000.

1. To test for the necessary dilution, begin by diluting 12 plasma samples at 1/500 and place 200 μ L of this diluted plasma in the first row (row A) of your Elisa plate (**always**

be careful not to touch the bottom of the plate with your fingers; avoid this by wearing gloves at all times when handling your plate).

2. Fill the rest of the rows (rows B through H) with 100 μ L of the dilution solution (free of any plasma samples).

3. Next, using the 8 tip multi-channel pipettor, take 100 μ L from row A and add it, while mixing in circular motions, to row B. Then take 100 μ L of row B and add it will mixing in circular motions to row C. Continue doing this, making sure that the diluted plasma is well mixed. Once you have added the 100 μ L to row H, remove 100 μ L and discard it. ***(Note – you do not need to change pipette tips during this procedure).***

Because we do not currently have a 12 tip multi-channel pipettor, you will have to do 8 wells across first, and then the remaining 4 second.

In order to determine which dilution was appropriate, follow the procedure outlined in the next section. You will then need to calculate the average quantity of immunoglobulin for the 12 samples for all of the dilutions and plot these data as a graph. The dilution you should use will be the one where the slope of this graph is steepest.

Assay Procedures

*This procedure is to be completed in 2 days. The first day will require about 2 hours while the second will require about 5-6 hours. If you do not want to be in the lab forever, I suggest getting your plates into the fridge by no later than 11am the first day. You will be able to test **47 samples per plate**. Do not touch the bottom of the plate with your bare fingers!! To avoid this, **wear gloves at every single step you are handling a plate.***

A) Dilute blood plasma (Day 1)

Before beginning the day's procedures – turn on your incubator and set it to 37°C.

1. Using a beaker, pour out that day's aliquot of dilution solution. Allow it to warm to room temperature (~30 minutes).
2. Dilute the plasma using the serial dilution data you have generated for your species, or use a dilution from the table below.

Table 4.1. Optimal plasma dilution for selected species for IgY assay

Species	Final dilution (done in 2 steps)	1st dilution step	2nd dilution step
Starling adult	1/8000	2µL plasma + 1000µL of dilution solution	62.5µL of 1 st dilution + 937.5µL of dilution solution
Starling chick	1/4000	2µL plasma + 1000µL of dilution solution	125µL of 1 st dilution + 875µL of dilution solution
Eider adult	1/32000	2µL plasma + 1000µL of dilution solution	15.65µL of 1 st dilution + 984.35µL of dilution solution
King penguin adult	1/32000	2µL plasma + 1000µL of dilution solution	15.65µL of 1 st dilution + 984.35µL of dilution solution
Zebra finch; Ring-billed gull chick	1/8000	2µL plasma + 1000µL of dilution solution	62.5µL of 1 st dilution + 937.5µL of dilution solution

3. If you have n samples to perform, label n tubes with one coloured pen. Fill n tubes with 1000µL of dilution solution.

4. Add 2 μ L of plasma to each tube, making sure to change the pipette tip for every sample.
5. Close all tubes. Be sure to record which plasma sample (band number, code number, or what have you) corresponds to which tube number (using 1, 2, 3 etc.).
6. Depending on which dilution you are using, label a second set of tubes with a different coloured pen. Fill this second set of tubes (using n tubes if you have n samples) with the required amount of dilution solution (e.g. for adult starlings, this would be 937.5 μ L). Ensure you know which colour pen is which dilution.
7. ***Vortex all samples of your first dilution!!*** If you forget, you will **have to start over.**
8. Add the required volume of the first dilution sample to the tubes you have prepared in step 4 (e.g. For starling adults, add 62.5 μ L of the first dilution to the tubes you prepared containing 937.5 μ L of dilution solution).

Keep step 4 dilutions as a precaution in case you lose or make mistakes with the step 8 dilutions. This way you can just remake a step 8 dilution using what is left from step 4.

B) Filling Plate with Diluted Samples (Day 1)

1. Vortex your diluted plasma samples for 5-10 seconds. **Make sure you are using the samples which have been diluted twice, from step 8 in the previous section.**
2. Place 100 μ L of the first diluted plasma sample in the first well of the plate, as well as in the second well.
3. Keep filling the wells in order with 100 μ L of diluted samples, making sure you have 2 wells for every sample. **Note: you should loading the samples in tube order (e.g. 1, 2, 3) so that you can trace your samples back to each bird.**
4. Once you have filled in your 47 samples, fill the last two wells of the plate with 100 μ L pure dilution solution (this will be your control).

5. Cover the plate with Parafilm (ensure that the parafilm stretches and covers each well) and place the lid on top of the parafilm. Place for 1 hour in the oven at 37°C.
6. After incubation, place the plate overnight in the 4°C fridge.

C) Adding Antibodies and Revealing Solution (Day 2)

1. In a beaker, pour out that day's aliquot of PBS-Tween and let it warm to room temperature (~ 30 minutes). Have a plastic loading tray ready (label it PBS-Tween) and pour into the tray once the solution has warmed to room temperature.
2. Before beginning any steps, label a plastic loading tray with Milk solution. Then proceed to make the milk solution (in a small beaker) and pour into the plastic loading tray. Cover with parafilm and leave on lab bench.
3. Turn on your incubator and set it for 37°C.
4. The next day (about 24 hours after leaving the plate in the fridge – just be consistent for all your samples) empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.**
5. Once the wells have been emptied, blot the plate upside down on paper towels 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***
6. Using the multi-channel pipettor, place 200µL of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip. This is your first rinse.
7. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to***

next step rapidly.

8. Using the multi-channel pipettor, place 200 μ L of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip. This is your second rinse.

9. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***

10. Add 100 μ L of the milk solution to each well using a multi-channel pipettor.

11. Cover the plate with parafilm and place the lid on top. Put the plate in the incubator for 1 hour at 37°C.

12. While the plate is in the oven, defrost your antibody solution. Label another plastic loading tray “antibody”.

13. After the incubation is over, take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***

14. Using the multi-channel pipettor, place 200 μ L of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip. This is your first rinse.

15. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any

remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***

16. Using the multi-channel pipettor, place 200 μ L of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip. This is your second rinse.

17. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***

18. Invert antibody solution gently to mix, then pour into plastic loading tray. Using the multi-channel pipettor, add 100 μ L of the antibody solution into each well. Be careful not to scratch the bottom of the plate with the pipet tips.

19. Cover with parafilm and place the lid on top. Put the plate in the incubator for 2 hours at 37°C.

20. While the plate is in the oven, prepare the revealing solution. Defrost the revealing solution on the bench. Label another plastic loading tray “Reveal”. Just before the plate is about to come out, add the 11 μ L of hydrogen peroxide and mix well before pouring into the loading tray.

21. After the incubation is over, take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***

22. Using the multi-channel pipettor, place 200 μ L of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must

make sure that you do not scratch the walls of the wells with the pipette tip. This is your first rinse.

23. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***

24. Using the multi-channel pipettor, place 200 μ L of PBS-Tween solution in each well – this rinses the plate so you do not need to be super careful with volumes but you must make sure that you do not scratch the walls of the wells with the pipette tip. This is your second rinse.

25. Take the plate back to the sink. Empty the wells by forcefully shaking the liquid out of the wells over the sink. **Do this with gloves on to prevent fingerprints on the underside of the plates.** Blot on a piece of paper towel 3 times to get rid of any remaining liquid. ***You do not want to leave the wells exposed to air, so proceed to next step rapidly.***

26. Using the multi-channel pipettor, add 100 μ L of the revealing solution in each well.

27. Cover with parafilm and place the lid on the plate. Put the plate in the incubator for 1 hour at 37°C.

28. While the plate is in the incubator, turn on the plate reader to let it warm up. Because the plate reader software is fickle, you may have to “re-install” the software and re-input the serial numbers.

29. Open up the plate reading program and select a file type at random (you are only reading absorbances so it really doesn't matter which one you select).

30. When the incubation time is up, remove plate from oven and place in plate reader. Read the plate using a wavelength of 405nm.

31. Save the outputs as a text file. Your IgY readings are the average absorbance of the two wells you loaded per sample.

Note: This protocol is set for IgY, but the procedures can really be used to measure anything. If you wish to measure IgG, for example, you would just need to order the anti-IgG chicken antibody from Sigma.

Curriculum Vitae

Name: Heather MacGillivray

Post-secondary Education and Degrees: Dalhousie University
Halifax, Nova Scotia, Canada
2005-2012 B.A.Hons

University of Western Ontario
London, ON, Canada
2012-2015 M.Sc. (in progress)

Honours and Awards: Dalhousie University Entrance Scholarship
2005-2006

Related Work Experience

Research Assistant
Dalhousie University
May 2011-July 2011

Research Assistant
Dalhousie University
May 2012-August 2012

Teaching Assistant
The University of Western Ontario
2012-2015

Publications:

Phillmore, L.S., MacGillivray, H.L., Wilson, K.R., and Martin, S. (2015). Effects of sex and seasonality on the song control system and FoxP2 protein expression in black-capped chickadees (*Poecile atricapillus*). *Dev. Neurobiol.* 75, 203–216.