Host-parasite interactions: bird immune genes, blood parasites and climate change implications

Thesis submitted by

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To my parents:

Dora y Carlos

To my sister and brother:

Eva y Carlos

In memory of

Ross H Crozier†

Ethics statement

This research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004 and the Qld. Animal Care and Protection Act, 2001. The proposed research study received animal ethics approval from the JCU Animal Ethics Committee Approval Number A-1120.

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General Abstract

It is predicted that rising temperatures, and changes in other climatic variables as a consequence of global warming, will increase the global distribution of infectious diseases. In contrast, many potential host species will experience reductions in geographical range size and abundance and changes in their distribution to higher altitudes or latitudes as a result of climate change. The potential interactions between climate change and disease dynamics is of significant importance to both natural biodiversity and human health. There is a vital need to evaluate the potential effect of climate change on host-parasite interactions. Understanding how environmental factors, especially temperature, affect parasite distribution and how these two variables directly or indirectly affect host immunity in comparative studies is one approach to fill this gap in current knowledge. The aim of this thesis was to study a model system including temperature, vector-borne diseases (VBD) and host Major Histocompatibility Complex (MHC) genes along altitudinal gradients, and predict the effects of climate change on these host-parasite interactions. The avian community of the Australian Wet Tropics (AWT) was analyzed as a model case system, in which I investigated blood parasite pressure in relation to temperature and host MHC genes along altitudinal gradients. MHC genes were used because they show extreme polymorphism within populations and this gene diversity is thought to arise via interactions of host MHC gene products and parasites. I used PCR screening of cytochrome b to investigate the prevalence and lineage diversity of four of the main genera of blood parasites (*Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* (Haemosporida) and *Trypanosoma spp*. (Kinetoplastida)) in birds of the Australian Wet Tropics. I found that parasite prevalence and lineage richness were positively and strongly associated with temperature. The phylogenetic relationships among parasite lineages were analyzed to determine the host specificity of each parasite genus. I found that *Plasmodium*

spp. and *Trypanosoma spp.* displayed low specificity, whereas *Haemoproteus spp.* seemed to display specificity at host family level. I also amplified a 173 bp fragment of the second exon of the MHC class II β gene of fifteen species from two bird families (Acanthizidae and Meliphagidae) in order to analyze their allele diversity and reveal evidence for selection (average d_N/d_S ratio and number of positive selected sites; NCBS). MHC diversity and selection were positively correlated with prevalence of blood parasites. The results suggest that the stronger the parasite pressure the higher the MHC allele diversity average d_N/d_S ratio and NCBS. It appears that higher parasite prevalence imposed stronger selective pressure in the host immune system, therefore the higher MHC allele diversity and selection allowed them to tolerate higher parasite prevalence. These results suggest an interaction between temperature, parasite prevalence and lineage richness, and bird MHC diversity and selection on MHC genes. Higher temperatures in lowland areas promote the development of parasites. This strong parasite pressure on the host immune system promotes higher diversity and selection of MHC genes. As elevation increases both temperature and parasite prevalence decreases. The lower temperature of highland areas inhibits development of parasites, creating a low-parasite environment and hence lower MHC diversity and selection in birds. To understand the possible effects of climate change on these host-parasite interactions, I used the regression of overall parasite prevalence and temperature documented to estimate increases in prevalence of parasites with temperature rise. This relationship predicts an increase of about 10% in the prevalence of parasites, for each 1^oC increment in temperature. The shifts of host distribution along the elevation gradient that would be required to hold parasite prevalence to current values were determined using parasite prevalence data from this study. For each 1^oC increase in temperature, bird distributions would need to ascend 200 m in elevation. Given a 4^oC temperature increase, only birds that currently live at 400 m or below would be able to offset increases in parasite prevalence by shifting their distributions upwards; for birds currently living above 400 m, some increase in parasite prevalence would

be unavoidable. It was shown that upland birds have lower MHC diversity, and rapid adaptation of their immunity could be unlikely due to the long life cycles of birds. My results also predict that lineage richness will increase with temperature, and that *Plasmodium spp.* and *Trypanosoma spp.* may have greater opportunities for host-switching than other more host-specific genera like *Haemoproteus spp*. Increased parasite pressure are expected to have negative effects on the bird populations of the region, particularly those inhabiting the upland areas and populations unable to shift upwards. The predicted increase of parasite prevalence and lineage richness could interact with, and further exacerbate, the projected impacts of climate change on this bird community, leading to an increased risk of extinction for many bird species. In conclusion, temperature is one of the main variables driving patters of distribution of avian haematozoa in this avian community. *Plasmodium spp. and Trypanosoma spp.* showed low specificity and as such higher host-switching potential than *Haemoproteus spp.* Blood parasites are driving selection and diversity of bird MHC genes. Increasing parasite pressure was predicted with rising temperature as a consequence of climate change. Shifts upwards of bird distributions along the elevation gradient can help to reduce the impact of increment of parasite pressure in this community, but upland bird communities and populations unable to shift upwards will be susceptible to the consequences of increased parasite pressure.

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	Sample collection	IZV, SEW
	Data analyses	IZV
	Manuscript preparation	IZV, CNJ
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	Study design	IZV, CNJ, SEW, JAE, RES
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	Data analyses	IZV, CNJ, SEW, SFC
	Manuscript preparation	IZV, CNJ, SEW, JAE, RES

IZV = Itzel Zamora-Vilchis; CNJ = Chris N Johnson; SEW = Steve E Williams; DB = David Blair; JAE = John A Endler; RES = Rodrigo Esparza-Salas

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Conference Presentations

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CHAPTER **1**

General Introduction

Introduction

Parasites can be significant threats to humans and wildlife. They can have negative effects on host populations, reducing the growth and fitness of infected animals and causing higher mortality and/or lower reproductive rates (Donovan et al. 2008; Marzal et al. 2005; Norte et al. 2009; Van Riper et al. 1986). Climate change is predicted to increase the global distribution and prevalence of infectious diseases by providing more favorable conditions for the transmission and development of many pathogens (Benning et al. 2002; Kovats et al. 2001; Lindsay and Birley 1996; Patz and Reisen 2001). In contrast, many hosts are likely to experience reductions in geographical range size and abundances and shifts in distribution to higher altitudes or latitudes as a consequence of climate change (Foufopoulos et al. 2011; Gasner et al. 2010; Green and Pickering 2002; Hickling et al. 2006; Peterson et al. 2002; Thomas et al. 2004; Tryjanowski et al. 2005).

To understand the full implications of climate change for biodiversity, we need to evaluate the potential effects of climate change on host-parasite interactions. Understanding how environmental factors, especially temperature, affect parasite infectivity and distribution, and how these two variables directly or indirectly affect host immunity, is one possible approach to fill this gap in current knowledge. Immunity is one of the most important life history traits of organisms, but this trait has not been incorporated into models to predict the effects of climate change on host populations. The aim of this introduction is to propose one approach to study a model system including temperature, vector-borne diseases (VBD) and host Major Histocompatibility Complex (MHC) genes along altitudinal gradients to predict the

consequences of climate change for host-parasite interactions. The avian community of the Australian Wet Tropics (AWT) will be analyzed as a model case system, and their blood parasite pressure in relation to temperature and host MHC genes along altitudinal gradients will be investigated.

Here, I will first provide a general framework describing the relationships of temperature to VBD and give some examples of distribution of this group of parasites along altitudinal gradients. I will then discuss the way in which parasites mediate selection on MHC genes, consider the costs of the immune system and give some examples of behavioural strategies used by hosts to avoid parasites. I will further discuss some possible mechanisms of host response to increases in parasite pressure. Finally, I will discuss how temperature, VBD and host MHC genes can be integrated as a model system along altitudinal gradients to study how climate change will affect host-parasite interactions.

Epidemiology of vector-borne diseases

VBDs are pathogenic microorganisms transmitted from an infected individual to another by an intermediate agent - the vector - which is usually an arthropod. Haematozoans are a common group of VBDs that live, feed and reproduce in host blood. Their life cycle includes a vertebrate host for the asexual stages of the parasite and a vector where the sexual stages occur. Some of the common vectors of Haematozoa are Dipterians (Culicidae, Hippoboscidae, Cerotopogonidae) biting midges and ticks (Atkinson et al. 2008). Much of what we know about the life cycle of Haematozoa such as avian *Plasmodium spp.* is based on experiments by Cay Huff and co-workers on *Plasmodium gallinaceum* (Hemosporida) (Huff 1951; Huff and Coulston 1944). The general life cycle of *Plasmodium gallinaceum* begins with a susceptible avian host being inoculated with sporozoites by a vector. This is followed by multiple cycles of asexual reproduction within host erythrocytes and tissues (merogony) that lead to production of gametocytes within circulating red blood cells. These gametocytes can be

ingested by a vector where they will continue their development. Gametogenesis takes place in the vector when it ingests infected blood containing both micro and macrogametocytes, then exflagellation occurs and finally fertilization. After fertilization an ookynete stage is present, this stage penetrates the midgut wall of the vector where an oocyst is developed. Inside the oocyst many oocyst sporozoites are developed (sporogony). The duration of sporogony depends on temperature. At optimal temperatures sporozoites of *P. gallinaceum* can mature within seven days. Once mature, the oocyst ruptures and sporozoites migrate from the hemolymph to the salivary glands of the vector. Sporozoites will pass with the saliva into a new avian host to initiate infection (Valkiunas 2005) (Figure 1.1)

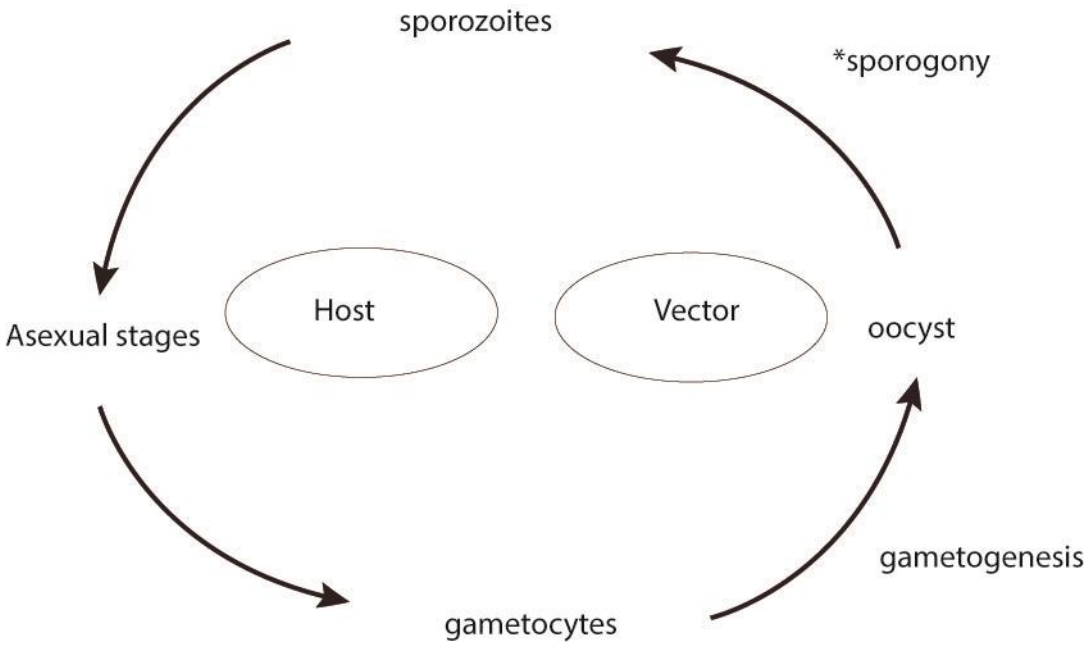

Figure 1.1. The general life cycle of *Plasmodium gallinaceum*
Life cycle of Haematozoa illustrated by the life cycle of avian *Plasmodium spp.* *Temperature dependent stage.

Another important genus of blood parasites is *Trypanosoma spp.* (Kinetoplastida). The life cycle of *Trypanosoma spp.* includes a vertebrate and invertebrate host. Here, I will describe the general life cycle of *Trypanosoma avium baker* within the mosquito *Aedes albopictus* and an avian host. The cycle begins with a Trypomastigote stage within the mosquito fore-gut after

it ingests blood from an avian host. An epimastigote stage is formed after asexual reproduction of Trypomastigote. Epimastigote will undergo binary fission to form a metacyclic form that will become a Trypomastigote in the hind gut of the mosquito. An avian host will be inoculated with Trypomastigotes that will migrate to its bone marrow. Trypomastigotes will divide to form an amastigote stage. After that, an epimastigote stage will be produced followed by the trypomastigote stage. A new vector will ingest blood from this avian host to initiate the cycle (Chatterjee 1983).

Influence of temperature on vector-borne diseases

VBDs are temperature dependent organisms, in other words their reproduction and development are directly affected by ambient temperature. For example, in avian *Plasmodium spp.,* sporogonic development was completed at constant laboratory and mean field temperature between 30 and 17^{o}C, but development, prevalence (proportion of infected hosts) and intensity (number of parasites found in the infected host) decrease significantly below 21^{o}C (LaPointe et al. 2010). Optimal temperatures for *Plasmodium spp.* development were around 28-30^{o}C, whereas temperatures lower than 16^{o}C greatly inhibited parasite development (LaPointe et al. 2005). Warmer temperatures also increase vector reproduction, development and frequency of blood feeding (Liang et al. 2002; Patz et al. 2000). Some arthropod vectors may undergo more generations per year at higher temperatures and shorter or milder winters will increase parasite survival (Sutherst 2001). In Hawaii, the abundance of mosquitoes is high at lower elevations, where temperatures are high, and declines with increasing elevation; mosquitoes are absent at high elevations (Van Riper et al. 1986). Some studies have demonstrated that both vectors and parasites are influenced by the daily temperature variation. It has been shown that temperature fluctuation can substantially alter the incubation period of malaria parasites within the mosquito and hence influence malaria transmission rates. This study found that temperature fluctuation around mean temperatures

of <21°C could speed parasite development, whereas fluctuation around means >21°C could slow development, compared with constant temperatures (Paaijmans et al. 2009). Other studies have showed that temperature fluctuations around low mean temperatures speed up parasite infection, whereas fluctuation around high mean temperatures acts to slow infection down (Paaijmans et al. 2010). It has been also demonstrated that a large diurnal temperature range (≈ 18°C daily swing) extended immature development time of dengue viruses in their vector *Aedes aegypty*, lowered larval survival and reduce adult female reproductive output (Carrington et al. 2013).

Other environmental variables like rainfall, seasonality and large-scale meteorological phenomena such as ENSO may also alter the quality and quantity of breeding sites for vectors. Higher precipitation can increase the number of breeding sites for vectors such as mosquitoes. Expansion of human malaria was linked to the high precipitation during the 1991-1992 El Niño Southern Oscillation in South America (Carter 2008). Transmission of many parasitic diseases is confined to the rainy season. Small changes in seasonality may be very important because transmission rates tend to increase exponentially rather than linearly through the season (Patz et al. 2000). Nevertheless, seasonality might be more important for both vector and parasite development in temperate regions, but not such limitation can be expected in more tropical areas, where climate is more or less stable throughout the year (MacDonald 2000) and vector development could takes place year round .

Influence of parasites on vectors

Parasites can directly affect not only host but also vectors (Valkiūnas and Iezhova 2004; Valkiunas et al. 2014). In a study of biting midges (*Culicoides impunctatus*), it was found that individuals non-infected with *Haemoproteus spp.* survived significantly more than infected individuals (Valkiūnas and Iezhova 2004). Other studies examined the effects of different species of *Haemoproteus spp.* on the survival of the Eurasian mosquito (*Ochleratatus*

cantans). They found higher survival rates in noninfected mosquitoes compared to mosquitoes with high parasitemia (Valkiunas et al. 2014). These studies suggest that increments of parasites can not only affect host survival but also can have negative effects on vectors.

Altitudinal gradients and vector-borne diseases

Altitudinal gradients are among the most powerful natural experiments for testing ecological and evolutionary responses of wildlife to environmental variables such as temperature (Michalet et al. 2014; Mori et al. 2013; Narins and Meenderink 2014; Wagner et al. 2013). Environmental conditions change along the gradient and organisms are commonly well adapted to the conditions of the portions of the gradient that they occupy. This offers ideal opportunities to explore evolutionary adaptation over short distances (Korner 2007). The study of parasite distributions in relation to climate gradients is important in helping us to understand how host species might be affected by alterations of parasite pressure under climate change. Altitudinal gradients provide an excellent framework for such research, because temperature is closely related to elevation and elevational differences can cause large changes in temperature over short geographic distances (Korner 2007). Research on bird Haematozoa distribution along altitudinal gradients in Dominican Republic and Madagascar showed that parasite prevalence tends to decrease with elevation, but these changes along the gradient were attributed to variation in composition of the avian community and temperature was not incorporated as a variable in their models (Latta and Ricklefs 2010; Savage et al. 2009).

MHC genes and causes of immune diversity

A parasite is an organism that exploits resources that are part of another organism, which serves as its host. The challenge from parasites can promote selection for changes in the host that reduce the impact of the parasite on host fitness. In turn, effective defense selects

for change in the parasite leading to an evolutionary arms race, or Red Queen effect (Van Valen 1973). These interactions can generate rapid evolutionary change and genetic polymorphism in both host and parasites, and have been identified as important factors generating diversity within and between species (Anderson and May 1982). One of the crucial life history traits affecting fitness of an organism is the immune system. Host-parasite coevolution is one of the main causes of immune diversity since this relationship promotes a molecular arms race between host immune genes and parasites (Renaud et al. 1996). Most research on genetic effects on vertebrate hosts of interaction with parasites has focused on genes of the Major Histocompatibility Complex (MHC). The MHC is a set of genes that bind peptides from pathogens and present these to T cells for initiation of the immune response. MHC genes show extreme polymorphism within populations (Klein et al. 1993). The diversity of these genes at the population level is thought to arise via interactions of host MHC gene products and parasites (Potts and Wakeland 1990) a phenomenon known as parasite-mediated selection (PMS). For PMS to occur on any trait, that trait must show a clear connection with the number or diversity of parasites, or with their effects (Goater 1997).

There are at least three theories proposing mechanisms by which PMS could maintain MHC diversity: 1) “heterozygote advantage”, 2) “rare allele advantage” or “frequency-dependent” selection, and 3) “fluctuating selection”. According to the heterozygote advantage theory, heterozygosity at MHC loci increases the range of parasites that can be recognized by the immune system, so that individuals with high heterozygosity have higher fitness (Doherty and Zinkernagel 1975). A classic example supporting this theory is that heterozygosity of a locus in humans confers protection from persistent hepatitis B virus infection (Thursz et al. 1997). In chickens, heterozygosity seems to confer resistance to Rous sarcoma virus (Senseney et al. 2000). In natural populations of yellow-necked mice (*Apodemus flavicollis*), populations with larger numbers of different MHC alleles had lower parasite pressure than populations with few different MHC alleles (Meyer-Lucht and Sommer 2009). Also, in sticklebacks

(*Gasterosteus aculeatus)* and salmon (*Salmo salar)*, high MHC allele diversity in individuals was associated with low parasite pressure (Dionne et al. 2007; Reusch et al. 2001; Wegner et al. 2003). However, in a comparative study of parasites and MHC diversity of Eurasian kestrels (*Falco tinnunculus* and *Falco naumanni*), lower pathogen pressure and less MHC diversity were found in island species than mainland species where both parasite pressure and MHC diversity is higher (Alcaide et al. 2010). These apparent contradictory results arise from the fact that in the kestrel's study different host species are compared rather than individuals within one host species as many studies do. Moreover, immune response is a costly life-history trait that needs to be in trade-off with other fitness characters like reproduction, growth and maintenance (Langand et al. 1998). There are energetic costs associated to evolve immune response. In theory, immune diversity needs to evolve proportionally to the parasite pressure that each host species had experience along its evolutionary history (Lochmiller and Deerenberg 2000). The high parasite pressure that mainland kestrel species has exposed along its evolutionary history have decisively contributed to its larger MHC diversity, whereas island species have cope with less parasite pressure (Alcaide et al. 2010).

The second parasite-mediated selection theory is "rare allele advantage" or "frequency-dependent selection". Given that there is strong selection on host immune systems to overcome the resistance of pathogens, this theory proposes that new rare alleles of hosts that confer resistance against parasites will offer a selective advantage and will be favored by selection. However, as these rare alleles increase in frequency, the parasite resistance will also increase and new rare alleles will need to be selected. The result is an arm race process in which pathogens and MHC alleles fluctuate in frequency (Clarke and Kirby 1966). In the following examples, it was demonstrated that one or more allele variants on host conferred resistance against parasites, as host individuals carrying these alleles tended to resist infection or have better survival rates than individuals without the variant. In house sparrows (*Passer domesticus*) one MHC I allele variant was associated with reduced infection by *Haemoproteus*

spp., but at the same time an increase in the risk of infection with *Plasmodium spp.* (Loiseau et al. 2008; Loiseau et al. 2011). Other studies in chickens have shown that individuals carrying the MHC haplotype B21 were resistant to an ectoparasite (Owen et al. 2008). In natural populations of vertebrates like Soay sheep, specific MHC alleles were associated with both juvenile survival and resistance to intestinal nematodes (Paterson et al. 1998). In a study of the MHC II of hairy-footed gerbil (*Gerbillurus paeba*) one specific allele was found to confer resistance against helminth parasites (Harf and Sommer 2005). These two theories - heterozygote and frequency-dependent advantage - are not mutually exclusive, as shown by a long-term study of reed warblers in which individuals carrying either large number of MHC alleles or a specific MHC allele were protected against malaria infection (Westerdahl et al. 2005).

The third theory, "fluctuating selection", proposes that pathogens can drive MHC diversity by fluctuation in the intensity of selection they exert. If the pathogen regime faced by an organism varies in space and time, the intensity of selection at MHC genes will also fluctuate. This means that different subsets of MHC alleles will be selected at different points in space and/or time, which can explain genetic diversity across subpopulations (Hill 1991). All these assumptions indicate that selection is directional instead of cyclical and pathogen fluctuations are determined by ecological variables. This theory has been studied mainly by theoretical models, because experimental or natural models require long term studies to encompass the fluctuations of parasite pressure in host populations. These models predict that MHC diversity could be maintained by fluctuating selection, even in the absence of heterozygote or rare allele advantage theories (Hedrick 2002; Hedrick et al. 1987).

It is not clear which of these three selection models is most important, or if all have a role at times. In fact, the three types of balancing selection may be overlapping and rare allele and heterozygote advantage could be taking place at a local level in small geographical distances and short terms and fluctuating selection could occurs at large geographical ranges

and long term. This can generate a mosaic of coevolution for different populations of the same species.

The three theories have been based on the study of MHC genes diversity at the allelic level. However, other PMS studies have also been based on the identification of selection of MHC genes. The d_N/d_S ratio is a widely used method to quantify selection pressure in populations. The measure is a rate between the synonymous sites, silent sites that are normally neutral and the non-synonymous, non-silent sites which possible experience selection. An excess of nonsynonymous relative to synonymous polymorphism is a clear signal of balancing selection whereas a lack of nonsynonymous relative to synonymous polymorphism is indicative of purifying selection (Kimura 1977; Yang and Bielawski 2000). Studies of the d_N/d_S ratios for the whole MHC sequence or codons putatively involved in peptide interactions with parasites are usually calculated and compared to other phylogenetically related species and/or different levels of parasite pressure. For example, Hawaiian honeycreeper populations have been strongly affected by the introduction of avian malaria (Van Riper et al. 1986). It has been shown that d_N/d_S ratios of MHC genes in honeycreepers are significantly higher than in Darwin´s finches species, their close relatives (Jarvi et al. 2004). In wild salmon, d_N/d_S ratios of MHC of the peptide binding region were positively related to bacterial diversity (Dionne et al. 2009). All these studies are correlative that means that other parasites different from the ones sampled could be causing identified differences in d_N/d_S ratios.

Trade-offs of immunity with other life history traits

The immune system is a costly life-history trait and as such it generates trade-offs with other fitness characters like reproduction, growth and maintenance (Langand et al. 1998). The energetic cost associated with the immune response are due to the metabolic requirements of immune cells and the indirect consequences of immune up-regulation (e.g. acute inflammatory response) (Lochmiller and Deerenberg 2000). This can be divided in two types of

cost: 1) the cost associated with evolving immunity, and 2) the physiological cost of maintaining and utilizing immune systems. Evidence for the cost associated with evolving immunity is provided by research on *Drosophila melanogaster*, in which selection for resistance against parasitoids produces the correlated response of reduced larval ability to acquire food (Kraaijeveld and Godfray 1997). Evidence of the physiological cost of maintaining and utilizing immune systems includes research on the house martin *Kelichon urbica,* where adults treated with primaquine showed reduction of the levels of infection with the blood parasite *Haemoproteus prognei.* The reduction of parasites with the treatment increased the clutch size in these birds (Marzal et al. 2005). Another similar study found that experimental reduction of the nematode parasite *Trichostrongylus tenuis* in red grouse (*Lagopus lagopus scotica*) increased the clutch size, hatching success and survival of grouse chicks (Hudson 1986).

Behavioural defense to avoid parasites

Because the immune system is a costly trait, other mechanisms such as behavioural avoidance and physical barriers that minimize the population of parasites are important to allow potential hosts to make more economic use of resources. Many potential hosts actively avoid exposure to parasites or vectors, altering their behaviour to reduce parasite risk. For example, preening or grooming with the bill in birds is thought to be effective in removing ectoparasites. Clayton and Cotgreave (1991) found that house sparrows that were missing part of their upper beak had higher loads of lice and mites than normal sparrows. Other examples include fly-repelling behaviours that are indirect means for avoiding parasites in birds. These activities take the form of bill snapping, foot stamping, head shaking, and wing flapping, which are very effective in reducing bites by mosquitoes (Clayton 1991; Clayton and Cotgreave 1991).

A second mechanism to avoid infectious diseases is the use of physical barriers that minimize the attacks by parasites or vectors. For example, by moving to higher altitudes during summer grazing, caribou are able to avoid the intense mosquito activity to which they are

exposed at lower altitudes (Downes et al. 1986; Mooring et al. 2007; Mooring et al. 2003). In Hawaii, two species of honeycreepers (*Himatione sanguinea* and *Vestiaria coccinea*) make daily evening movements from lowlands to uplands and it is believed that these movements allow them to escape from mosquitoes that can transmit malaria (Van Riper et al. 1982).

One of the most interesting examples of microhabitat selection in birds comes from work on the Hawaiian Islands that were home to more than 100 endemic species and subspecies of birds that are now either extinct or endangered due to the introduction of mosquitoes and non-native birds carrying malaria and avian pox. Native birds were very susceptible to these diseases. For those that did not become extinct, malaria became the limiting factor restricting their abundance and distribution. Introduced bird species took over the lowland areas, which had high numbers of mosquitoes. Some native bird species survived by re-locating out of the lowlands which had become a malaria zone, to higher elevations where malaria was absent. Other birds unable to shift died due to parasite infection. (Van Riper et al. 1986). However, more recent studies have probe that populations of one of those bird species (*Hemignatus virens*) have been recovering at low elevations (Woodworth et al. 2005). Further studies have found that lowland individuals of *Hemignatus virens* have acquired tolerance (withstanding the infection while paying a low fitness cost) rather than resistance (parasite clearing) (Atkinson et al. 2013).

Climate change and vector-borne diseases

The rising global temperature due to climate change is predicted to directly affect both vectors and parasites. There are two main reasons for this: abundance and activity of most species of vectors is directly affected by temperature (Lindsay and Birley 1996); and for most VBDs, rates of development, reproduction and transmission can be enhanced by higher ambient temperature to a certain higher limit (LaPointe et al. 2005). For many diseases, there are predictions of expanded distribution and increased prevalence under climate change. For

example, recent research on the relationship of malaria (*Plasmodium relictum*) and environmental factors in house sparrow (*Passer domesticus*) in France showed that prevalence of malaria was highly correlated to temperature. Loiseau et al. (2013) predicted that under climate change scenarios, *Plasmodium spp.* occurrence will spread to regions in northern France, and that prevalence levels are likely to increase in locations where transmission already occurs . In a meta-analysis of *Plasmodium spp.* of more than 3,000 bird species, it was found that the infection rate of malaria has increased in parallel with climate change, especially during the last 20 years (Garamszegi 2011). Other examples include the bird community of the Hawaiian Islands. Future projections of the distribution of malaria and its vectors have suggested that increases in temperature will allow the spread of vectors to even higher elevations, increasing the range of parasites and restricting even more the distribution of native species (Benning et al. 2002). Other studies have already found evidence of higher infection or wider distribution of *Plasmodium spp.* associated with increased temperature. A recent study showed that increased temperature in the past 30 years has already played an important factor in the exacerbation of VBDs. In a highland region of East Africa, records of malaria cases have increased since 1970, at the same time of an increase of 1^{o}C of temperature (Alonso et al. 2011). A recent study looked for evidence of a changing spatial distribution of human malaria with varying temperature for over a decade in highland regions of northwest Colombia and central Ethiopia has found that increases in temperature across years has extended the spatial distribution of malaria cases to higher elevations (Siraj et al. 2014). In other study, future projection under climate change scenarios predict an increase in the annual person-months at risk to contract human malaria (Caminade et al. 2014). Nevertheless, other studies have shown that the distribution of two of the main vectors for human malaria *Anopheles pseudopunctipennis* and *Anopheles albimanus* has reduced compared with past records of these mosquitoes and future projections under climate change scenarios predict that this tendency will be hold and there will be reductions in the distribution

of these vectors in Veracruz, Mexico (Beltrán-Aguilar 2011). However, this study only includes information of a few climatic stations in only one state of Mexico, so the model has low capacity of prediction.

Studies in other VBDs like African trypanosomiasis have found that a large shift of up to 60% in the geographical extent of the range for this parasite with increase temperature. The model also predicts that 46-77 million additional people may be at risk of exposure by 2090 (Moore et al. 2012). However, for most VBDs the knowledge of competent vectors is poor. It would be important to study the specific vectors for each VBDs in order to improve the future projections of geographic distribution of parasite associated to climate change.

The expected expansion of diseases will also provide favorable conditions for host-switching for species with low host specificity, increasing the parasite pressure on host communities even more. Studies of host-switching among fungal pathogens infecting plants indicate that geographical proximity and opportunities for cross-species transmission, rather than genetic changes in the parasites themselves, are primarily responsible for the origin of new host-parasite combinations (Antonovics et al. 2002).

Climate change and mechanisms of host-parasite interaction response

Even though it may be difficult to predict the consequences of redistribution of host and parasites associated with climate change, it is clear that these changes will affect the global host-parasite dynamics leading to a disruption of adaptive processes and if parasite pressure increase, it is likely that more energy will be allocated to host immune system affecting other life history traits. Some of the possible mechanisms of host response to increments of parasites could be as follows.

First, host immune systems could adapt to the higher parasite pressure. However, parasite adaptation is usually faster than host counter-adaptation due to the parasite`s shorter generation time, its usually larger population size and its often haploid genome (Hamilton et

al. 1990). As discussed earlier, this option is likely to have costs associated with the evolution or utilization immunity. Furthermore, other life history traits like reproduction and growth are very likely to be affected.

Second, there could be increased mortality rates and/or lower birth rates in host populations as a result of infection, reducing population density. Decreased reproductive success has been associated with high infection of *Haemoproteus spp.* and *Leucocytozoon spp.* in passerine birds (Marzal et al. 2005; Norte et al. 2009). *Haemoproteus spp.* can also cause severe disease and high mortality in avian hosts (Donovan et al. 2008).

Third, birds could shift their distributions to hold parasite prevalence and intensity constant. This option is likely to occur because as discussed early, immunity is a costly trait and mechanisms like behavioural avoidance that minimize the population of parasites allow more economic usage of resources.

One different scenario from the other three exposed above is that parasites can decrease as an effect of climate change. This can relax parasite pressure on host and could have negative effects on host immune genes because there will be no parasite pressure to keep genetic diversity on these genes.

Influence of climate change and host MHC genes-VBD interaction

It is clear that there is a direct influence of temperature on vectors and parasites, and in turn that parasites have a direct effect on the selection of host MHC genes. Nevertheless, little is known about the effects of climate change on this host-parasite interaction, especially in view of the projected increases of parasite pressure and distribution (Figure 2.2). Are host immune systems prepared to respond to higher parasite pressure? Addressing this gap will require interdisciplinary research in well documented ecosystems. Moreover, it will be necessary to find a suitable model with a vertebrate community that displays different parasite pressures as an effect of temperature. The Australian Wet Tropics region offer ideal

conditions. Temperature promotes the existence of different levels of infection along altitudinal gradients which can generate different parasite pressure on quite small spatial scales (Zamora-Vilchis et al. 2012). The Australian Wet Tropics bioregion (AWT) is located in far North Queensland between 19°30'S and 15°30'S. The region is dominated by tropical rainforest, which covers an area of 10,000 km^2 and is primarily distributed along the mountain ranges (MacDonald 2000). In this region, temperature is one of the most important variables driving trends of distribution of many species along elevational gradients of the mountain systems (Williams et al. 2010a). Mean annual rainfall in the region varies between 1500 mm and 3300 mm (Williams and Middleton 2008), with approximately 75-90% falling between November and April (MacDonald 2000). There are more than 240 vertebrates in this region, many of which are regionally endemic (Williams et al. 2010b). The bird community shows strong trends of assemblage structure along the elevational gradient with high levels of regional endemism in the uplands (Shoo et al. 2005b; Shoo et al. 2005c). Both species richness and bird abundance exhibit a humped-shaped pattern with elevation, with highest values found between 600 m and 800 m (Williams et al. 2010a).

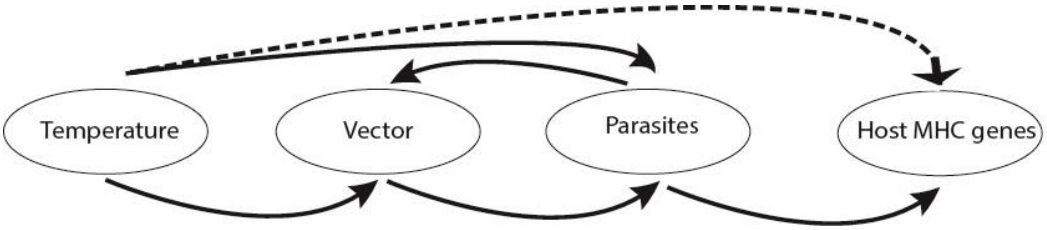

Figure 1.2. Relation of environmental and biological variables affecting host-parasite interaction
Describes how temperature directly influences the development of both vectors and parasites. Vectors have a direct relation with parasites and parasites have also a direct relation with vectors. In turn, parasites have a direct influence on host MHC genes. Host MHC genes are indirectly receiving the influence of temperature. Bold arrows are used for direct effect of variables whereas dotted arrows indicate an indirect effect.

Research aims

The overall goal of the current thesis is to study a model system including temperature, bird blood parasites and bird MHC genes along altitudinal gradients within the AWT to predict the consequences of climate change on host-parasite interactions. The thesis is organized as individual research papers (Chapters 2, 3 and 4) that are interconnected.

In Chapter 2, I used PCR screening of cytochrome b and 18S rRNA genes to investigate the prevalence of four genera of blood parasites (*Plasmodium spp.*, *Haemoproteus spp.*, *Leucocytozoon spp.* (Hemosporida) and *Trypanosoma spp.* (Kinetoplastida)) in 403 birds. I determine relations between prevalence of blood parasites, temperature, and seasonal rainfall in a bird community of the Australian Wet Tropics along an elevation gradient using simple regression models. I used the regression of overall parasite prevalence and temperature to predict the increase of parasite prevalence with temperature. The shifts of host distribution along the elevation gradient that would be required to hold parasite prevalence to current values were also determined.

In Chapter 3, I determine relationships of parasite lineage richness with environmental factors (temperature, elevation and rainfall) and host ecological and morphological traits (geographic range size, population density, population size, group size and body mass). I also described the phylogenetic relationships among the parasite lineages and examined the host specificity of each parasite genus. I discussed implications of climate change on these relationships.

In Chapter 4, I amplified a 173 bp fragment of the second exon of the MHC class II β gene of fifteen species from two bird families (Acanthizidae and Meliphagidae) in order to analyze their allele diversity and selection (average d_N/d_S ratio and Number of Codons under Balancing Selection; NCBS) in relation to parasite prevalence and other important variables like elevation, total population size and range size. I also contrasted the patterns of variation at MHC with those of neutral loci. For this, three intron sequences were amplified in all the

individuals of the fifteen bird species: α-Enolase (ENOL), Laminin (LAM) and Glyceraldehide-3-phosphate dehydrogenase (GAPDH). I discussed implications of climate change on the host-parasite interactions.

In Chapter 5, I summarized the results of chapters 2, 3 and 4 and discuss their implications on current and future conservation of the AWT.

CHAPTER **2**

Environmental Temperature Affects Prevalence of Blood Parasites of Birds on an Elevation Gradient: Implications for Disease in a Warming Climate

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Introduction

Many studies have described trends in the structure of assemblages along elevational gradients, and have found temperature to be one of the main variables controlling elevational distribution across a diverse taxonomic and ecological range of species (Meik and Lawing 2008; Oommen and Shanker 2005; Wilson et al. 2007). However, little is known about the distribution of pathogenic organisms on these gradients. Vector-borne diseases are widely distributed pathogens transmitted to hosts by arthropod vectors such as biting flies (Atkinson et al. 2008). The rising global temperature is predicted to expand the distribution of vector-borne diseases (Patz and Reisen 2001). There are two reasons for this: abundances of most vectors are positively related to temperature (Lindsay and Birley 1996); and for most vector-borne diseases, transmission may be enhanced by higher ambient temperature. The development of *Plasmodium spp.*, for example, can occur between 16-30^{o}C, with optimal temperatures around 28-30^{o}C, whereas temperatures lower than 16^{o}C greatly inhibit parasite development (LaPointe et al. 2005).

In contrast to predictions for vector borne parasites, many studies have reported reductions in geographical range size and abundance, and shifts to lower latitudes or high altitudes, in a wide range of organisms that are potential hosts for these parasites (Foufopoulos et al. 2011; Gasner et al. 2010; Hickling et al. 2006; Tryjanowski et al. 2005). Range expansions for human malaria, avian malaria and African trypanosomiasis are already taking place or are predicted to occur worldwide (Alonso et al. 2011; Benning et al. 2002; Caminade et al. 2014; Garamszegi 2011; Loiseau et al. 2013; Moore et al. 2012; Siraj et al. 2014). This extension in the spatial distribution of vector borne parasites may increase their prevalence in many host populations. Increased parasite pressure can have negative effects on host populations, reducing growth and causing higher mortality and/or lower birth rates (Donovan et al. 2008; Marzal et al. 2005; Norte et al. 2009; Van Riper et al. 1986). These effects could amplify the risk of extinction for many already threatened species. Other environmental variables like rainfall are also predicted to be affected in association with climate change (Stocker et al. 2013). Changes in rainfall may alter the quality and quantity of breeding sites for vectors. Higher precipitation can increase the number of breeding sites for vectors such as mosquitoes. Expansion of malaria was linked to the high precipitation during the 1991-1992 El Niño Southern Oscillation in South America (Carter 2008). Recent studies have found that daily temperature variation is an important variable affecting the incubation period of malaria parasites within the mosquito and hence influence malaria transmission rates (Paaijmans et al. 2009). In general, daily fluctuation around cooler temperatures acts to speed up rate processes relative to the mean, fluctuation around warmer temperatures acts to slow them down, and fluctuation around intermediate temperatures tends to have little net effect (Carrington et al. 2013; Paaijmans et al. 2010).

The study of parasite distributions in relation to climate gradients is important in helping us to understand how host species might be affected by changing parasite prevalence under climate change. Elevational gradients provide an excellent framework for such research,

because temperature is closely related to elevation and elevation differences can cause large changes in temperature over short geographic distances (Korner 2007).

The main aim of this study was to determine how temperature and rainfall influence prevalence of blood parasites in tropical birds. To do this I studied bird communities along elevation gradients in the Australian Wet Tropics. This bioregion is one of the best-studied tropical rainforests in the world. It consists of a strip of coastal plains and a series of adjacent mountain systems, with an altitude range from sea level to 1600 meters above sea level (MacDonald 2000). Species distribution models predict that under impending temperature rises many bird species in this region could experience significant range reductions, increased population fragmentation and declines in population size, and therefore heightened risk of extinction (Shoo et al. 2005a; Shoo et al. 2005c; Williams et al. 2003; Williams and Middleton 2008). However, there has been no study of elevational distribution of bird parasites and how climate change could affect their prevalence.

I present data on the prevalence and lineage diversity of four genera of blood parasites (*Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* (Haemosporida) and *Trypanosoma spp*. (Kinetoplastida)) in birds of the Australian Wet Tropics in relation to elevation. I test for relations between parasite prevalence, elevation, temperature, and seasonal rainfall. These studies are not only important to implement future models on how increase of temperature will affect parasite pressure but also how host communities could be affected by parasites.

Methods

Study area and bird community

The Australian Wet Tropics bioregion (AWT) is located in far North Queensland between 19°30'S and 15°30'S. The region is dominated by tropical rainforest, which covers an area of 10,000 km^2 and is primarily distributed along the mountain ranges (MacDonald 2000). In this region, temperature is one of the most important variables driving trends of distribution

of many species along elevation gradients of the mountain systems (Williams et al. 2010a). Mean annual rainfall in the region varies between 1500 mm and 3300 mm (Williams and Middleton 2008), with approximately 75-90% falling between November and April (MacDonald 2000). The bird community shows strong trends of assemblage structure along the elevational gradient with high levels of regional endemism in the uplands (Shoo et al. 2005b; Shoo et al. 2005c). Both species richness and bird abundance exhibit a humped-shaped pattern with elevation, with highest values found between 600 m and 800 m above sea level (Williams et al. 2010a).

Data collection

Data were collected during 2005 and 2006 from two localities of the region: the South Johnston/Atherton Tablelands area (17.62ºS; 145.72ºE) and the Carbine Range (Lat; Long 16.56 ºS; 145.28 ºE). These localities are around 125 km apart. Nevertheless, they are within the same bioregion and have similar vegetation structure and almost identical bird faunas (Tracey 1982; Williams et al. 2010a). There is a strong relationship with bird assemblages across elevation in the two localities, and that relationship is similar in both (Williams et al. 2010a). Bird blood samples were collected at different elevation sites (Table 2.1). Mean annual temperature for each elevational site located at every 200 m of elevation in each locality was measured using data loggers maintained by the Centre for Tropical Biodiversity and Climate Change at James Cook University. Each logger consists of five sensors, which measure air temperature, relative humidity, soil moisture, soil temperature, and condensation at 15 min intervals. Mean monthly rainfall for each elevational site at each locality was estimated using daily rainfall data extracted from the Australian Water Availability Project http://www.bom.gov.au/jsp/awap/. Temperature decreased at an approximate rate of 1^{o}C per 200 m altitude and there was approximately 1^{o}C difference between the two areas sampled at the same elevation (Figure 2.1A). The monthly average rainfall indicated that the dry season

began in May and was extended and acute until November or December when the rainy season began. The highest values of rainfall were between February and May (Figure 2.1B).

Table 2.1 Localities of sampling in the AWT

Elevation, mean annual temperature and number of individual birds sampled are indicated for each locality.

Localities	Elevation (m)	Subpopulation abbreviation	Mean annual temperature (°C)	Birds sampled
Carbine Range	100	100CR	21.8	14
Carbine Range	400	400CR	20.9	27
South Johnston	400	400SJ	20.5	102
South Johnston	800	800SJ	17.1	18
Carbine Range	1000	1000CR	17.3	190
Carbine Range	1200	1200CR	16.4	52
Total	-	-	-	403

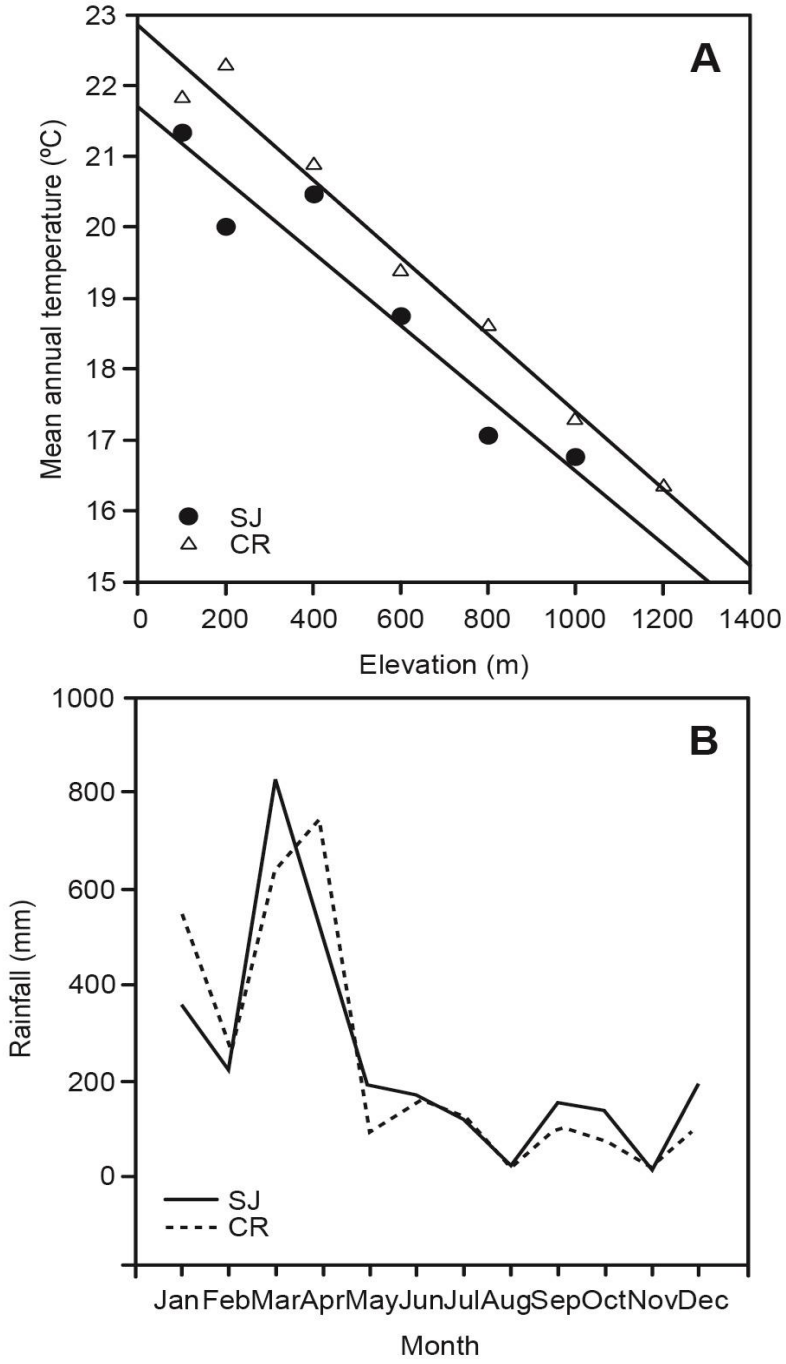

Figure 2.1 Variation of temperature and rainfall at the AWT.

A) Predicted variation of Mean annual temperature as a function of elevation. Temperature decreased at an approximately rate of 1ºC per 200 m altitude and there was approximately 1ºC difference between the two localities sampled at the same elevation and B) Monthly variation of rainfall at the two localities within the region indicated that the dry season began on May and was extended and acute through November or December when the rainy season began. The highest values of rainfall were between February and May. Localities: South Johnston (SJ) and Carbine Range (CR) (See also Table 2.1 for detailed information on localities).

Study species

I collected blood samples from 403 individual birds belonging to 40 species in sixteen different families: Acanthizidae, Alcedinidae, Climacteridae, Columbidae, Dicaeidae, Dicruridae, Estrildidae, Eupetidae, Meliphagidae, Muscicapidae, Nectariniidae, Pachycephalidae, Paradisaeidae, Petroicidae, Ptilonorhynchidae and Zosteropidae (all host species and details of frequency of detection are listed in Appendix I). None of the bird species used here migrates to different geographic regions except *Rhipidura rufifrons*. Some species have seasonal local migrations within the region (Higgins and Peter 2002; Higgins et al. 2006; Higgins et al. 2001). However, most of the bird species show specific trends of distribution along the elevation gradient (Williams et al. 2010a). Birds were caught in mist nets, and approximately 50 to 75 µl of blood was collected by puncture of the brachial vein. Blood samples were stored in Queens lysis buffer (Seutin et al. 1991) for subsequent analysis.

Molecular analyses

DNA was extracted from all samples using silica fines (Elphinstone et al. 2003). Two nested-PCR protocols were used to detect four genera of blood parasites: one nested PCR assay for *Plasmodium spp., Haemoproteus spp.* and *Leucocytozoon spp.* targeting a 478 bp section of the mitochondrial cytochrome b gene (Hellgren et al. 2004), and another assay for *Trypanosoma spp.* targeting a 326 bp section of 18S rRNA gene (18S) (Sehgal et al. 2001). These two nested-PCR protocols are highly repeatable (Hellgren et al. 2004; Sehgal et al. 2001). For *Plasmodium spp., Haemoproteus spp.* and *Leucocytozoon spp.* the first PCR step was carried out in a 10 µl reaction, using approximately 50 ng of DNA, 1x GoTaq Green Master Mix (Promega) and 0.5 µM of each primer (Table 2.2). Cycling conditions included an initial denaturation step at 94°C for 3 min, followed by 20 cycles of 30 s at 94°C, 30 s annealing at 50°C and 45 s extension at 72°C; and a final extension step of 10 min at 72°C. PCR products from the first reaction were used as a template for two other reactions: one that amplifies

specific cytochrome b sequences for the genera *Plasmodium spp.* and *Haemoproteus spp.*, and another for *Leucocytozoon spp.* Reactions were carried out in a 25 µl volume containing 1x GoTaq Green Master Mix, 0.6 µM of each of the respective primers (Table 2.2) and 2 µl of the PCR product from the initial reaction. Cycling conditions were identical to the first PCR but performed for 35 cycles instead of 20. The first reaction for *Trypanosoma spp.* was carried out in a 10 µl volume containing 1x GoTaq Green Master Mix, 0.5 µM of each primer (Table 2.2) and approximately 50 ng of template DNA. Cycling conditions included an initial denaturation at 95°C for 5 min followed by five cycles at 95°C for 1 min, 45°C for 30 s, 65°C for 1 min, and 35 cycles at 95°C for 1min, 50°C for 30 s, 72°C for 1 min; and a final extension at 65°C for 10 min. The second reaction included 1x GoTaq Green Mastermix, 0.6 µM of each primer (Table 2.2) and 1 µl of PCR product from the initial reaction. Cycling conditions included an initial denaturation at 96°C for 3 min, followed by 35 cycles at 96°C for 30 s, 63°C for 1 min, 72°C for 30 s and a final extension at 74°C for 7 min. To identify parasite lineages, all the positive products were bidirectionally sequenced. Sequences were edited and aligned using the program Sequencher 4.8. Sequences were deposited in both MalAvi database (Bensch et al. 2009) http://mbio-serv4.mbioekol.lu.se/avianmalaria and GenBank (Accession numbers JX021535-JX021582; see Supplement II for details).

Table 2.2. Primer sequences used for the two PCR step reactions to detect blood parasites

Primers used to amplify Cytochrome b (Cyt-b) in Plasmodium spp. (Pla), Haemoproteus spp. (Hae) and Leucocytozoon spp. (Leu), and 18S rRNA (18S) in Trypanosoma spp. (Try). Universal base inosine (I)

Gene	Step	Target genera	Primer name	Primer sequence	Reference
Cyt-b	1st	*Pla, Hae, Leu*	HaemNFI	5'-CATATATTAAGAGAAITATGGAG-3'	(Hellgren et al. 2004)
			HaemNR3	5'-ATAGAAAGATAAGAAATACCATTC-3'	
	2nd	*Pla, Hae*	HaemF	5'-ATGGTGCTTTCGATATATGCATG-3'	(Bensch et al. 2000)
			HaemR2	5'-GCATTATCTGGATGTGATAATGGT-3'	
	2nd	*Leu*	HaemFL	5'-ATGGTGTTTTAGATACTTACATT-3'	(Hellgren et al. 2004)
			HaemR2L	5'-CATTATCTGGATGAGATAATGGIGC-3'	
18S	1st	*Try*	S762	5'-GACTTTTGCTTCCTCTA(A/T)TG-3'	(Sehgal et al. 2001)
			S763	5'-CATATGCTTGTTTCAAGGAC-3'	
	2nd	*Try*	S755	5'-CTACGAACCCTTTAACAGCA-3'	(Sehgal et al. 2001)
			S823	5'-CGAA(T/C)AACTGC(C/T)CTATCAGC-3'	

Statistical analyses

I used the program Quantitative parasitology 3.0 (Rózsa et al. 2000) to calculate mean prevalence and 95% confidence intervals for each elevation site for the different parasite genera. I also performed a χ^2 and Fisher's exact test looking for differences between elevation sites.

Results

Prevalence of parasites

Of the 403 individual birds screened, 130 (32.3%) tested positive for one or more parasite genera. The predominant parasite was *Haemoproteus spp.* with 80 infected birds (19.9%). *Trypanosoma spp.* and *Leucocytozoon spp.* showed very similar prevalence with 28 (6.9%) and 25 (6.2%) infected birds respectively, whereas *Plasmodium spp.* was present in only

7 (1.7%) birds. An additional 15 (3.7%) individuals were infected with *Haemoproteus spp.* and/or *Plasmodium spp.* but the parasite could not be identified to genus due to low PCR amplification, poor-quality sequence or unresolved multiple infections. Among well-sampled host families (i.e. >15 individuals sampled per family, Table 2.3), prevalence of *Haemoproteus spp.* ranged from 2.1% (Estrildidae) to 60.3% (Petroicidae). The family with the highest prevalence of *Plasmodium spp.* and *Trypanosoma spp.* was Pachycephalidae with 3.1% and 15.6% respectively, whereas Dicruridae had the highest prevalence of *Leucocytozoon spp.* with 16.3%. Prevalence of the four genera of parasites was similar across different host families.

Table 2.3 Parasite prevalence of well represented families and other families

Percentage of total number of birds infected and number of birds infected by each parasite genus (%) (*Hae: Haemoproteus spp., Pla: Plasmodium spp.*, Unknown: either *Haemoproteus spp.* and/or *Plasmodium spp.*, *Leu: Leucocytozoon spp.* and *Try: Trypanosoma spp.*)

Host family	% of total infected birds	% Hae	% Pla	Unknown % Hae and/or Pla	% Leu	% Try	Sample sizes
1. Petroicidae	76.7	65.8	1.4	6.8	0	11	73
2. Pachycephalidae	43.8	31.3	3.1	3.1	0	15.6	32
3. Dicruridae	30.2	9.3	0	2.3	16.3	11.6	43
4. Meliphagidae	22.1	4.4	2.9	5.9	10.3	4.4	68
5. Acanthizidae	15.3	8.1	1.8	0.9	0.9	2.7	111
6. Estrildidae	8.3	2.1	0	4.2	4.2	0	48
7. Others	42.9	17.9	3.6	3.6	28.6	14.3	28
Total	32.3	19.9	1.7	3.7	6.2	6.9	403

Lineage diversity

A total of 48 unique lineages of parasites (including the four genera) was detected (See Supplement II for details). *Haemoprotueus* was the genus exhibiting the highest number of lineages (30). *Trypanosoma* and *Leucocytozoon* presented 7 and 6 lineages respectively. Finally, for *Plasmodium* only 5 unique lineages were detected. Analysis of parasite lineages along the elevation gradient showed that most of the lineages were present only in certain elevation sites. This was probably due to the observed high lineage diversity and the specific

trends of host distribution along the gradient. Only a few lineages of two well-represented families (Petroicidae and Pachycephalidae) were distributed along the entire gradient. Nevertheless, sample sizes of each of these well-distributed lineages are not large enough to determine significant trends of distribution in relation to elevation, temperature or rainfall.

Temperature and prevalence of bird blood parasites on an elevation gradient

The overall prevalence of infection (of all four parasite genera) was negatively related to elevation ($F_{1,4} = 52.45$, $P < 0.002$, $R^2 = 0.93$) and positively to mean annual temperature ($F_{1,4} = 438.98$, $P < 0.00003$, $R^2 = 0.99$; Figure 2.2A). A multiple regression model of parasite prevalence on both elevation and temperature was highly significant ($F_{2,3} = 164.63$, $P < 0.02$, $R^2 = 0.99$, Adjusted $R^2 = 0.98$) but only temperature contributed significantly to the model (temperature: Beta = 0.99 P = 0.02; elevation: Beta = 0.004 P > 0.98). I checked for relationships of parasite prevalence to host characteristics, including each species' geographic range size, body mass and body size, but found no significant relationships (Table 2.4; only species with more than 5 individuals were used in the analysis).

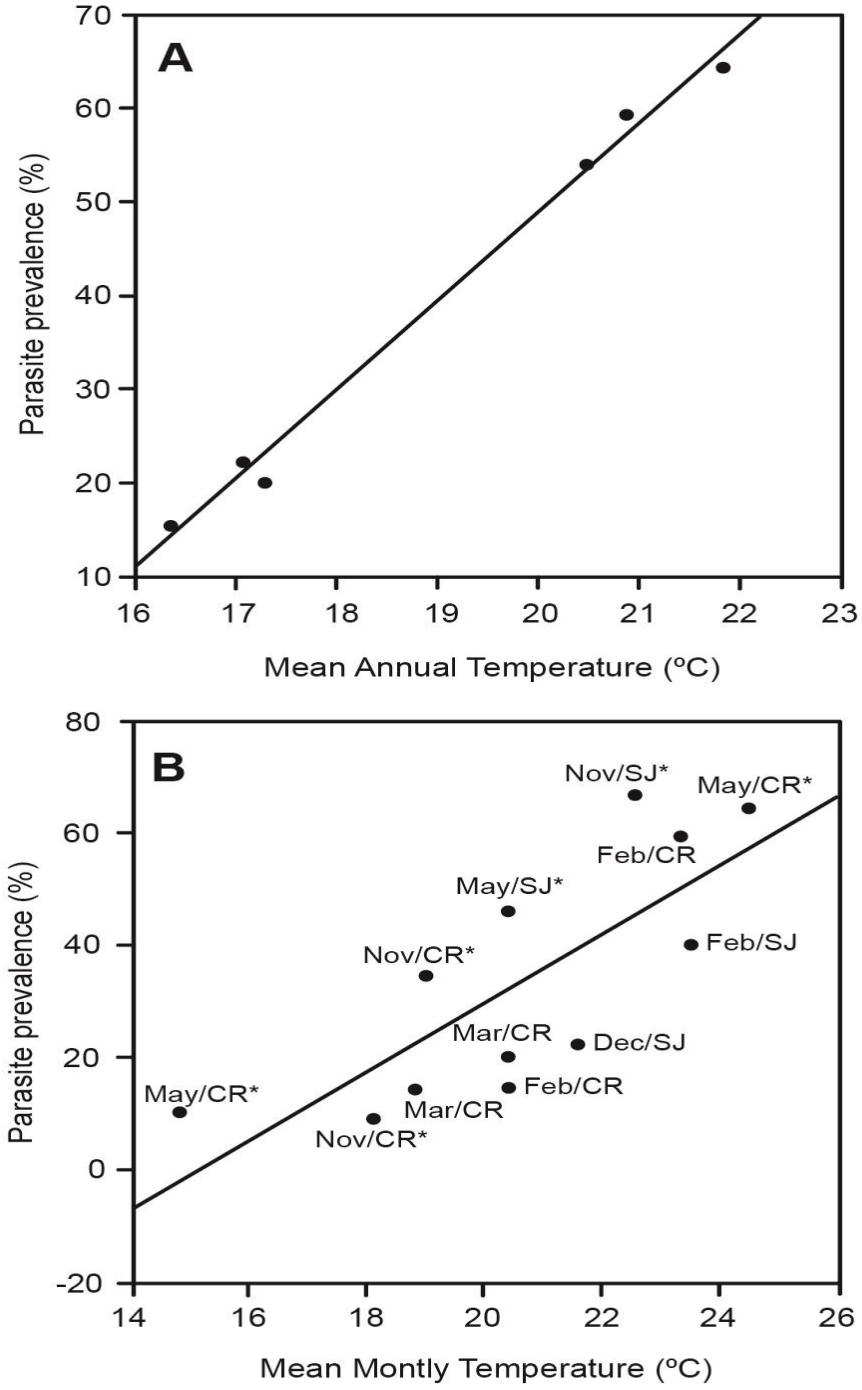

Figure 2.2 Relationship between overall parasite prevalence and temperature.

Predicted variation of overall parasite prevalence as a function of A) Mean annual temperature and B) Mean monthly temperature. Month, year and locality (SJ = South Johnston and CR = Carbine Range) are indicated for each point. Dry season months are marked with asterisks.

Table 2.4 Regressions between parasite prevalence and host ecological variables

Relationships between Parasite prevalence and host: a) Geographic range size, b) Body mass and c) Body size. All regressions are low and non-significant.

Variable		F	*P*	R^2
a)	Range size	(1,16)=1.59	0.23	0.09
b)	Body mass	(1,16)=4.02	0.06	0.20
c)	Body size	(1,16)=2.45	0.14	0.13

Relationships of overall parasite prevalence to temperature in well sampled families (represented by >15 individuals and sampled from at least 3 elevations) were positive in Acanthizidae ($F_{1,3} = 10.67$, $P < 0.05$, $R^2 = 0.78$) and Dicruridae ($F_{1,3} = 14.53$, $P < 0.05$, $R^2 = 0.83$), whereas Meliphagidae ($F_{1,2} = 2.19$, $P > 0.05$, $R^2 = 0.52$) and Pachycephalidae ($F_{1,1} = 1.36$, $P > 0.05$, $R^2 = 0.58$) displayed positive relationships that were not significant. Finally, Petroicidae was divided into the two species that make up this family and both showed a positive but statistically non-significant relationship of parasite prevalence to temperature: *Tregellasia capito* ($F_{1,1} = 33.22$, $P > 0.05$, $R^2 = 0.97$); and *Heteromyias albispecularis* ($F_{1,1} = 14.74$, $P > 0.05$, $R^2 = 0.88$).

Testing relationships of temperature to prevalence for each genus of parasite showed that prevalence of the predominant parasite *Haemoproteus spp.* was positively related to temperature ($F_{1,4} = 37.621$, $P < 0.003$, $R^2 = 0.90$). Relationships for *Leucocytozoon spp.* ($F_{1,4} = 4.90$, $P < 0.09$, $R^2 = 0.55$), *Trypanosoma spp.* ($F_{1,4} = 4.45$, $P < 0.1$, $R^2 = 0.53$) and *Plasmodium spp.* ($F_{1,4} = 0.54$, $P < 0.5$, $R^2 = 0.12$) were also positive but were not statistically significant.

I calculated the mean prevalence and 95% confidence intervals for each elevation site for the different parasite genera. I found significant differences between sites for the overall parasite prevalence and all the parasite genera except for *Plasmodium spp.* (Table 2.5). In general, lowland sites (100-400 m) presented higher parasite prevalence than upland sites (800-1200 m) (Figure 2.3).

Table 2.5 Differences between parasite prevalence along elevation gradient

Differences between mean parasite prevalence for each elevation site along elevation gradient for the overall parasite prevalence and four parasite genera using χ^2 test and Fisher's exact test.

	χ^2 test				Fisher's exact test
	χ^2	df	Min. expected count	*P*	*P*
Overall parasites	61.329	5	4.5	0	0
Haemoproteus spp.	61.220	5	2.8	0	0
Plasmodium spp.	29.605	5	0.2	0	0.004
Leucocytozoon spp.	8.494	5	0.9	0.131	0.144
Trypanosoma spp.	12.213	5	1.0	0.033	0.04

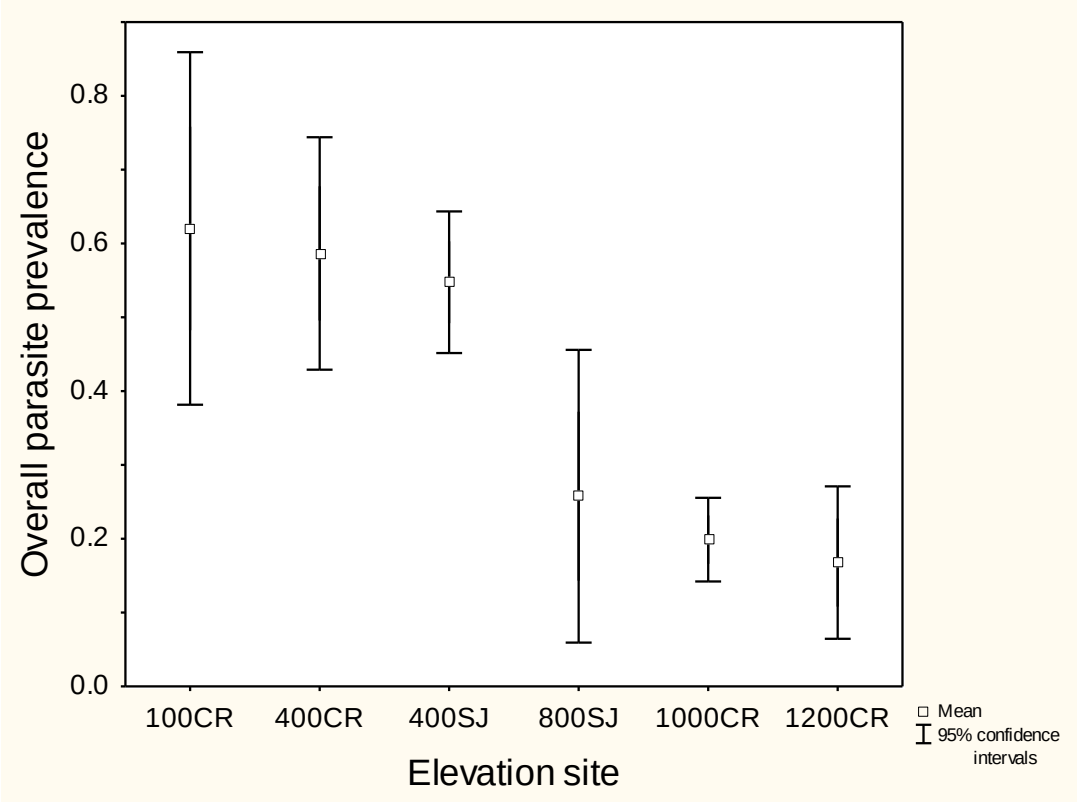

Figure 2.3 Mean parasite prevalence along elevation sites

Mean parasite prevalence for the overall parasite prevalence and 95% confidence intervals along elevation sites (See Table 2.1 for elevation sites abbreviations and details).

Seasonal changes of parasite prevalence

The positive relationship between parasite prevalence and temperature held even when the data were divided into monthly averages ($F_{1,10} = 14.44$, $P < 0.003$, $R^2 = 0.59$; Fig 2B), but the regression explained less of the variation than the mean values of parasite prevalence and annual temperature. Estimates of parasite prevalence during the dry season (May-November) tended to be higher than expected under the linear model, while wet season (December-April) were lower than expected (Figure 2.2B). I also evaluated the relationship between monthly parasite prevalence and rainfall and found no relationship ($F_{1,10}= 1.43$, $P < 0.02$, $R^2= 0.04$). The multiple regression model including both independent variables (monthly temperature and rainfall) to predict parasite prevalence was significant ($F_{2,9} = 10.238$, $P < 0.005$, $R^2 = 0.69$, Adjusted $R^2 = 0.63$) but again only temperature contributed significantly to the model (temperature Beta = 0.76 P = 0.003; rainfall Beta = -0.32 P > 0.11).

Discussion

I found strong relationships of temperature to overall parasite prevalence. To facilitate the discussion, I used the lowland (0-400 m) and the upland (600-1200 m) distinction of climatic zones, based on forest structure (Siddle et al. 2010). In general, birds inhabiting the lowland areas where temperatures were higher had higher parasite prevalence. In contrast, species distributed in the upland regions with lower temperatures had lower parasite prevalence. However, two subpopulations presented low sample sizes (100CU and 800AU). Nevertheless, 800AU showed very similar parasite prevalence values as 1000CU with very similar mean annual temperature and enough sample sizes. For 100CU, even when the value of parasite prevalence found follow the linear trend imposed by the other subpopulations along the gradient, the results of this locality will be taken with caution. Prevalence within each family and within the two well sampled species showed the same trends along the

gradient as for overall parasite prevalence, showing that the decrease in prevalence with elevation did not reflect a changing composition of host taxa with elevation.

One of the mechanisms that could explain these results is that abundance of vectors is directly related to temperature. Bird haematozoa are transmitted by arthropod vectors (Atkinson 1991), and ecological factors associated with vector abundance can explain differences in the prevalence of parasite species independently of host (Arriero and Moller 2008; Garvin and Remsen 1997; Piersma 1997; Tella et al. 1999). Nevertheless, studies in other regions like the Hawaiian islands have shown a negative correlation between abundance of mosquitoes, the main vector for *Plasmodium spp.*, and elevation (Van Riper et al. 1986). Like most vector-borne diseases, transmission of avian malaria is affected by ambient temperature. The onset, duration, and completion of the parasite's development to the infective stage in the vector are determined by temperature. The development of *Plasmodium relictum* occurs between 16-30^{o}C, temperatures lower than 16^{o}C inhibit parasite development, whereas optimal temperatures fluctuate between 28-30^{o}C (LaPointe et al. 2005). Nevertheless, other studies have found different temperature range tolerances. One study found that temperatures between 15.5 and 17.5 ^{o}C for nine days are lethal for *Plasmodium vivax* and *Plasmodium falciparum.* Other study showed that exposure to 12 ^{o}C for 6 h did not inhibit the development of *Plasmodium relictum.* A third study showed that *Plasmodium vivax* can survive temperatures of 4-5.5 ^{o}C for three weeks. Finally, it has been reported that *Plasmodium relictum* can survive temperatures as low as 4 ^{o}C (reviewed in Santiago-Alarcon et al. 2012). This indicates that *Plasmodium spp.* displayed different tolerances of temperature and even for the same species (*Plasmodium relictum*) distinct tolerances has been described.

The effects of temperature and rainfall on the intensity of infection for other group of parasites are controversial. For example, a global study of current and future habitat suitability for ticks under different climate change scenarios predicts that even though some tick species are likely to undergo range expansions, others may suffer drastic range contractions

worldwide (Cumming and Van Vuuren 2006). Studies on parasitic flies whose larvae infect bird nestlings show the same controversial results. A study of parasitic flies of the genus *Philornis spp.* on Argentinean forest birds found that temperature and rainfall were positively correlated with intensity of infection (Tracey 1982). In contrast, another study on parasitic *Protocalliphora* (blow flies) on swallows showed that the number of blow flies varied in a curvilinear fashion with temperature, with parasite pressure highest in nest around 25^{o}C and decreasing at both higher and lower temperatures (Bensch et al. 2009). The results found in our study suggest that low temperatures of the higher elevations, especially during winter, can help to reduce both the development of avian haematozoa and the abundance of these parasite vectors, leading to low parasite prevalence. In contrast, the high temperatures of the lowland areas provide an excellent environment for the development and transmission of haematozoa. However, further research will be vital to determine both specific vectors for each parasite genus and their trends of distribution along the elevation gradient. It would be also important to do further research in the region to be able to determine trends of parasite lineages found along the gradient.

It was found that other ecological and morphological host traits did not relate significantly to parasite prevalence. Nevertheless, body mass presented marginal values of significance. Shuerlein and Ricklefs (2004) found a significant correlation between body mass and parasite prevalence of *Plasmodium spp.*, *Haemoproteus spp.*, *Leucocytozoon spp.* and *Trypanosoma spp.*in European passerines . Body mass could be related to parasite prevalence because larger birds produce greater quantities of CO_2, which is one of the main cues used by blood sucking vectors to locate their hosts (Sutcliffe 1986).

The AWT are characterized by two marked seasons, the wet and dry. The dry season begins in May and is extended and acute until October or November, when the wet season begins. There was an interesting trend for parasite prevalence during the dry season to be higher and lower during the wet season. However, I found no significant relationship between

monthly parasite prevalence and rainfall. Further research is needed to show the influence of seasonal shifts that include both changes in rainfall and temperature.

Implications for infection dynamics in a warming climate

Average global temperatures increased 0.6^{o}C in the period 1901-2000 (Folland et al. 2001) and they are expected to increase by 1.4^{o}C to 5.8^{o}C by 2100 (Cubasch et al. 2001). In tropical regions, this temperature increase may be accompanied by heightened variability in rainfall with more severe dry seasons(Houghton et al. 2001; Walsh and Ryan 2000). The regression of overall parasite prevalence and temperature documented in this study predicts an increase of about 10% in the prevalence of parasites, for each 1^{o}C increment in temperature (Figure 2.4). Hosts could respond to this in three ways. First, their immune systems could adapt to the higher parasite pressure. However, the life cycles of birds are much longer than of the parasites and rapid adaptation is unlikely. Second, there could be increased mortality rates and/or lower birth rates in host populations, reducing population density. Decreased reproductive success has been associated with high infection of *Haemoproteus spp.* and *Leucocytozoon spp.* in passerine birds (Marzal et al. 2005; Norte et al. 2009). *Haemoproteus spp.* can also cause severe disease and high mortality in avian hosts (Donovan et al. 2008). Third, birds could shift their elevational distributions to hold parasite prevalence constant.

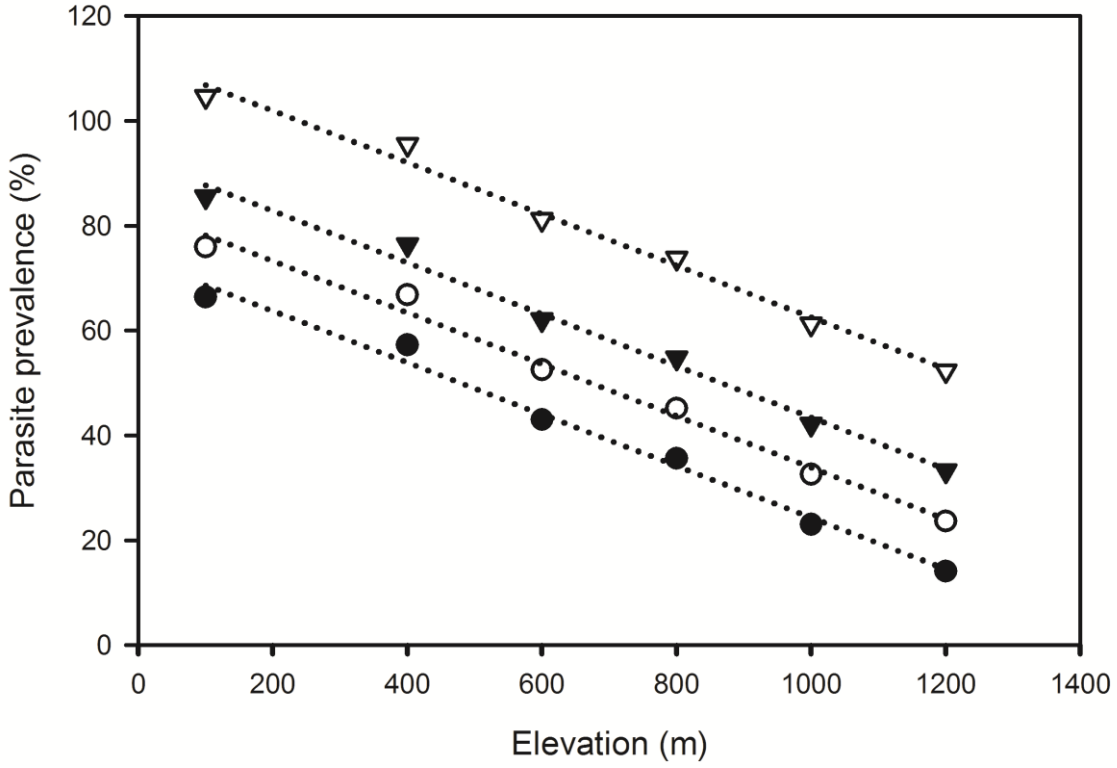

Figure 2.4. Extrapolations of parasite prevalence with increments of temperature.
Parasite prevalence along the elevational gradient with increments of 0°C (●), 1°C (○), 2°C (▲) and 4°C (Δ), using the equation of the linear regression between overall parasite prevalence and mean annual temperature (temperature – 140.62/0.1047 = parasite prevalence). Extrapolations indicated that there will be an increase of about 10% in the prevalence of parasites for each 1°C of increment in temperature.

Figure 2.5 illustrates the shifts of host distribution along the elevation gradient that would be required to hold parasite prevalence to current values. Filled bars represent the predicted distribution of birds with increments of temperature. At 0°C all bars are filled representing the actual distribution of birds along the elevation gradient. For each 1°C increase in temperature, bird distributions would need to ascend 200 m in elevation. Open bars indicate that birds at that site shifted upwards to the next elevation site to avoid an increase in parasite prevalence, leaving that site unoccupied. Given a 4°C temperature increase, only birds that currently live at 400 m or below would be able to offset increases in parasite prevalence by shifting their distributions upwards; therefore for birds currently living above 400 m, some

increase in parasite prevalence would be unavoidable. However, this model have some disadvantages, it analyzed all parasite genera together. Each parasite genus has their own particular vectors and each vector may have their own particular trends of distribution along the gradient and it is very possible that each vector will respond in different ways with increased temperature affecting host parasite prevalence.

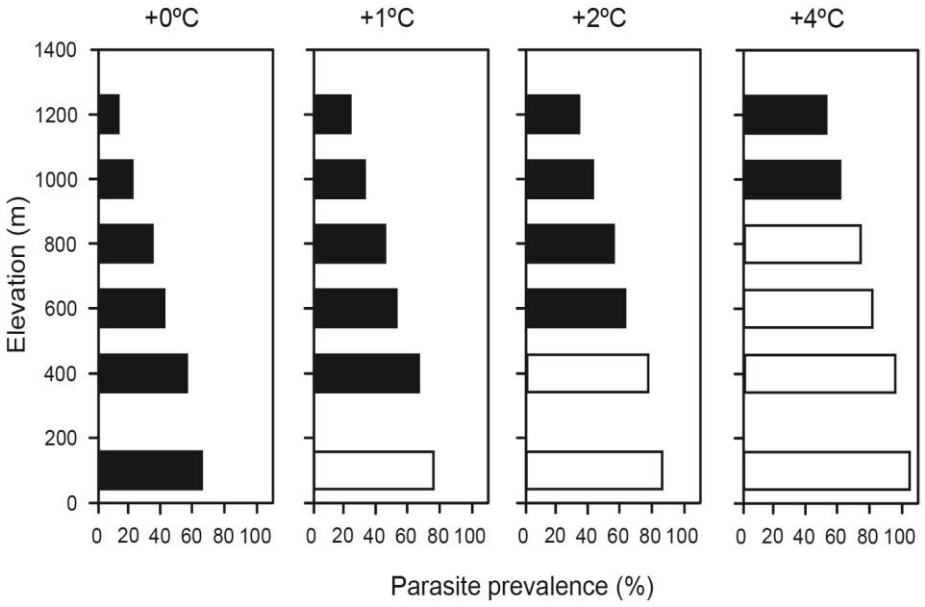

Figure 2.5. Elevational shifts upwards of bird distributions.

One of the mechanisms proposed to compensate increments of parasite prevalence at 0, 1, 2 and 4ºC increase in temperature. Filled bars represent the predicted distribution of birds with increments of temperature. At 0ºC all bars are filled representing the actual distribution of birds along the elevation gradient, with prevalence variation from 64% in the lowlands to 16% at the highest elevations. For each 1ºC increase in temperature, bird distributions need to ascend 200 m in elevation in order to avoid an increase in parasite prevalence. Open bars indicate that birds at that site shifted upwards to the next elevation site to avoid an increase in parasite prevalence, leaving that site unoccupied. Failure to make such a distribution shift would potentially result in higher mortality or reduced reproduction because of elevated blood parasite prevalence. The shifts in parasite prevalence are likely to be very large. At an altitude of 1200 m, for example, a 4ºC temperature rise is predicted to increase parasite prevalence from 16% to 50%. At this higher temperature, only birds that currently live at 400 m or below will be able to offset increases in parasite prevalence by shifting their distributions upwards; for birds currently living above 400 m, some increase in parasite prevalence are unavoidable.

The predicted increase of parasite prevalence due to increased temperature could interact with, and further exacerbate, the projected impacts of decreased range size, increased fragmentation, and decreased population size of birds due to climate change; all these can lead to an increased risk of extinction, especially for species inhabiting the uplands (Shoo et al. 2005a; Williams and Middleton 2008). However this model has some disadvantages and further research is needed to improve it, particularly trends of vector distribution along the gradient. The results of this study suggest that upland areas are currently a low-disease habitat and their conservation must be given high priority in the management plans under climate change.

Summary

- The rising global temperature is predicted to expand the distribution of vector-borne diseases. To understand how host communities could be affected by changing parasite distributions, information on the distribution of parasites in relation to variables like temperature and rainfall that are predicted to be affected by climate change is needed.
- Using PCR screening I investigated the prevalence of four genera of blood parasites (*Plasmodium spp.*, *Haemoproteus spp.*, *Leucocytozoon spp.* and *Trypanosoma spp.*) in 403 birds of the Australian Wet Tropics along elevation gradients.
- Independent of elevation, parasite prevalence was positively and strongly associated with annual temperature. Parasite prevalence showed a tendency to be elevated during the dry season.
- It was shown that rising temperatures are likely to lead to increased prevalence of parasites in birds, and may force shifts of bird distribution to higher elevations.

•It was found that upland tropical areas are currently a low-disease habitat and their conservation should be given high priority in management plans under climate change.

CHAPTER **3**

Host Specificity and Association of Temperature to Avian Haematozoa Diversity along an Elevation Gradient

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Introduction

Parasites can be significant threats to wildlife populations. It is well documented that increasing parasite pressure can have negative effects on the populations of host communities, affecting their growth and fitness, causing higher mortality and/or lower reproductive rates (Donovan et al. 2008; Marzal et al. 2005; Norte et al. 2009; Van Riper et al. 1986). For these reasons, understanding factors influencing the patterns of distribution of parasites in host communities is important for informed management of wild host populations. These studies become more urgent as environmental conditions change due to climate change. One of the predicted effects of a warming climate is the increase in area of optimal habitat for many parasitic diseases, both in altitude and latitude (Benning et al. 2002; Patz and Reisen 2001). Recent studies have demonstrated that increased temperature over the past 30 years has already led to greater prevalence of vector-borne diseases in a highland region of East Africa. In this region records of malaria cases have increased since 1970, at the same time as an increase of 1^{o}C in temperature (Alonso et al. 2011).

Avian haematozoa are transmitted to their avian hosts by arthropod vectors (Hatcher and Dunn 2011). Temperature is one of the most important factors affecting the distribution of these vectors (LaPointe et al. 2005). The duration of the parasite's development to the

infective stage in the vector is determined by temperature (LaPointe et al. 2010) . The development of *Plasmodium spp.*, for example, can occur between 16-30^{o}C, with optimal temperatures around 28-30^{o}C, but is greatly inhibited at temperatures lower than 16^{o}C (LaPointe et al. 2010). Rainfall is another environmental factor that can be important for transmission of vector-borne diseases. Higher precipitation can increase the number and quality of breeding sites for vectors such as mosquitoes (Valkiunas 2005). The spread of malaria was linked to high precipitation during the 1991-1992 El Niño Southern Oscillation in South America (Carter 2008). Recent studies have found that daily temperature variation is an important variable affecting the incubation period of malaria parasites within the mosquito and hence influence malaria transmission rates (Paaijmans et al. 2009). In general, daily fluctuation around cooler temperatures acts to speed up rate processes relative to the mean, fluctuation around warmer temperatures acts to slow them down, and fluctuation around intermediate temperatures tends to have little net effect (Carrington et al. 2013; Paaijmans et al. 2010).

Ecological and morphological traits of hosts are also important factors affecting distribution of infectious diseases (Scheuerlein and Ricklefs 2004). For example, several studies in a wide range of parasite species have shown that greater geographic range size, population density, population size, group size and body mass are associated with increased parasitism in a diverse range of avian and mammal hosts (Côté and Poulin 1995; Davies et al. 1991; Gregory 1990; Hughes and Page 2007; Ortego and Cordero 2010; Ortego and Espada 2007; Poulin and Forbes 2012; Rifkin et al. 2012).

The development of molecular techniques has permitted the detection of parasites more efficiently than more traditional methods, such as blood smears, and also has helped to identify parasite lineages (Bensch et al. 2004; Waldenstrom et al. 2004). Such lineages have been used as biological units at the same hierarchical level as species (Beadell et al. 2009; Beadell et al. 2004; Bensch et al. 2000; Ishtiaq et al. 2008; Krizanaskiene et al. 2006; Poulin

1995; Ricklefs et al. 2004). Nevertheless, this approach has their limitations because a single base-pair difference detected in a sequence is enough evidence to claim it as a new lineage as it is commonly used in MalAvi, the main database for Avian malaria parasites (Bensch et al. 2009). One base-pair difference between sequences could be attributed to methodology errors during PCR or sequencing performance. However, in spite of these limitations, the use of molecular techniques has facilitated the development of phylogeographic research on avian haematozoa and has provide valuable information about parasite host specificity to determine their host-switching potential. Host-switching from closely related primate species may be the source of some diseases that can be lethal in humans, such as malaria (Escalante et al. 1995). *Haemoproteus spp.*, one of the most common genera of avian haematozoans, seems to display high specificity at the family level (Beadell et al. 2004). On the contrary, *Trypanosoma spp.* exhibit low specificity (Sehgal et al. 2001). However, for *Plasmodium spp.* some studies have found low specificity (Beadell et al. 2004; Ishtiaq et al. 2007; Merino et al. 2008) whereas others have shown high specificity (Beadell et al. 2009; Bensch et al. 2000; Ricklefs et al. 2004) It is possible that different species of vectors and *Plasmodium spp*. are playing an important role in the specificity of the parasite. Climate change is predicted to increase the global distribution and prevalence of parasites by providing more favorable conditions for the transmission and development of many pathogens (Kovats et al. 2001; Lindsay and Birley 1996). Some studies have also found range shifts of many infectious diseases, e. g. human malaria has shift to highland areas with increasing temperature in northwest Colombia, central Ethiopia and East Africa (Alonso et al. 2011; Siraj et al. 2014). Under these conditions parasite species with low host specificity will have more chances to undergo host-switching, increasing the parasite pressure on host.

In a previous study on the bird community of the Australian Wet tropics bioregion, it was shown that along an elevation gradient the prevalence of four of the main blood parasites: *Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* (Haemosporida) and *Trypanosoma*

spp. (Kinetoplastida) was positively related to temperature. It seems that the warmer temperature of the lowland areas favors the development and transmission of these parasites. The prevalence decreased with elevation and in the upland areas the number of host individuals infected was very low, presumably because the lower temperatures of these areas inhibited the transmission of diseases and the activity and abundance of vectors. It was also determined that each 1^{o}C increase in temperature corresponded to an approximately 10% increase of parasite prevalence (Zamora-Vilchis, Chapter 2). Here, I used the parasite lineage composition of four of the main parasite genera from birds (*Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* (Haemosporida) and *Trypanosoma spp*. (Kinetoplastida)) in the same bird community, to analyze aspects of the parasite patterns of distribution and host specificity. I also discussed some of the possible effects of climate change on host populations. The main aims of the study were:

i) To determine the relationship between environmental factors (temperature, elevation and rainfall) and parasite lineage richness along the elevation gradient,

ii) To determine if a relationship exists between parasite lineage richness and host ecological and morphological traits (geographic range size, population density, population size, group size and body mass),

iii) To describe the phylogenetic relationships between the parasite lineages and examine the host specificity of each parasite genus to evaluate its host-switching potential.

Methods

Study area and bird community

See study area and bird community in methods in Chapter 2 for details

Sample collection

See sample collection in methods in Chapter 2 for details

Molecular techniques

DNA was extracted from all samples using silica fines (Elphinstone et al. 2003). Two nested-PCR protocols were used to detect four of the main blood parasite genera in all bird samples: *Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* (Haemosporida) and *Trypanosoma spp*. (Kinetoplastida) (Sehgal et al. 2001; Waldenstrom et al. 2004). Parasite screening and identification of lineages were performed using methods described previously (Zamora-Vilchis, Chapter 2). For *Plasmodium spp., Haemoproteus spp.* and *Leucocytozoon spp.* a 478 bp long fragment of the cytochrome b gene was amplified, whereas for *Trypanosoma spp.* a 326 bp section of 18S rRNA gene (18S) was used. To identify parasite lineages, all the positive products were bidirectionally sequenced. Sequences were edited and aligned using the program Sequencher 4.8. Sequences were deposited in both MalAvi database (Bensch et al. 2009) http://mbio-serv4.mbioekol.lu.se/avianmalaria and GenBank (Accession numbers JX021535-JX021582; see Supplement II for details).

Estimates of environmental and ecological variables

Mean annual temperature for each elevation site was estimated using data from weather stations located every 200m of elevation at each locality during 2005 and 2006 (Zamora-Vilchis, Chapter 2). Mean monthly rainfall for each elevational site at each locality was estimated using daily rainfall data extracted from the Australian Water Availability Project http://www.bom.gov.au/jsp/awap/. The total population size of each bird species within the AWT was estimated from its known geographic range size and mean population density within the region (Williams et al. 2010b). I obtained information concerning social group size and body mass for each bird species (Higgins and Peter 2002; Higgins et al. 2006; Higgins et al.

2001). For group size, species that are mainly solitary or occur in pairs were given a value of 0. Species living in groups of 3-5 birds were scored as 1, 6-10 individuals (2) and 11-20 birds (3).

Phylogenetic analysis

To detect host specificity, two phylogenies were reconstructed; the first one using cytochrome b sequences of *Haemoproteus spp.*, *Plasmodium spp.* and *Leucocytozoon spp.* and a second one with the 18S sequences of *Trypanosoma spp.* Cytochrome b sequences were divided into first, second and third codon positions using the program CODONSPLIT (Ingrid Jakobsen, University of Queensland). The program RNAalifold (Bernhart et al. 2008) was used to determine stem and loop regions for 18S sequences. Two loop regions were recognized and the stem regions consisted of 288 bp. A $\chi 2$ stationarity test was performed for each cytochrome b partition and the stem regions of 18S using the program TREEPUZZLE 5.2 (Schmidt et al. 2002) to detect potential variation in sequence composition among sequences in each dataset. The best model of nucleotide substitution was found using the program MrModeltest (Guindon and Gascuel 2003; Posada 2008). The cytochrome b input file to reconstruct the phylogeny consisted of a matrix with nucleotide data partitioned into the three codon positions, whereas the 18S input matrix was a mixture of nucleotide data for the stem regions and the loop regions were re-coded as numbers and used as "standard"/morphological characters. I did not down-weight paired nucleotides from the stem regions, since the data was not concatenated with any other gene region. Phylogenies were reconstructed with a Bayesian approach using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) running two parallel analyses for 10,000,000 Markov Chain Monte Carlo (MCMC) generations, and sampling every 100 generations. Consensus trees were constructed using the final 50,000 trees of both runs.

Samples from three birds captured at the same localities before 2005 that were infected with *Plasmodium spp.* and *Trypanosoma spp.* were donated by the University of

Adelaide and used as complementary information only to reconstruct the two phylogenies. GenBank accession numbers KF811172-KF811174.

Analysis of host specificity

I used the application PACo (Procrustes Application to Cophylogenetic Analysis) on R (v 3.1.0 http://www.r-project.org/) to determine host specificity for each of the four parasite genera used in this study. PACo was runned using 100, 000 permutations for high precision of the P estimate.

Results

Excluding samples collected prior to 2005, a total of 48 unique lineages of parasites (including the 4 genera) were found across 130 infected individuals. All these are new reported lineages and a search in MalAvi data based (Bensch et al. 2009) http://mbio-serv4.mbioekol.lu.se/avianmalaria displayed no morphospecies attached. *Haemoproteus spp.* was the genus exhibiting the highest number of lineages (30) and was also the most abundant genus in this bird community, infecting 80 individuals. In contrast, for *Plasmodium spp.* only 5 unique lineages were detected in 7 infected birds. For *Trypanosoma spp.* and *Leucocytozoon spp.* I detected 28 and 25 infected individuals respectively. For *Trypanosoma spp.*, I only detected 7 lineages in 18 birds and for *Leucocytozoon spp.*, I recovered 6 lineages in 10 infected birds (Appendix II). Only partial sequences were obtained for the rest of the infected birds for both genera due to low PCR amplification or poor-quality sequence; hence these shorter sequences were excluded from all the analyses.

Across well-represented host families (>15 individuals, Table 3.1), Petroicidae displayed the highest lineage richness for *Haemoproteus spp.* (17 lineages in 48 infected individuals) and *Trypanosoma spp.* (4 lineages in 8 infected individuals). Petroicidae (2 lineages in 2 infected individuals) and Meliphagidae (3 lineages in 7 infected individuals)

exhibited the highest levels of lineage richness for *Plasmodium spp.* and *Leucocytozoon spp.* respectively. Lineage richness for the four parasite genera was uniform across different host families (Table 3.2).

Table 3.1 Parasite lineage richness across well represented host families

Number of host species, total number of lineages and number of infected birds, number of lineages by each parasite genus (*Hae: Haemoproteus spp., Pla: Plasmodium spp.*, *Leu: Leucocytozoon spp.* and *Try: Trypanosoma spp.*).

Host family	Number of host species	Total number of lineages/ birds infected	*Hae*	*Pla*	*Leu*	*Try*
1. Petroicidae	2	23/56	17/48	2/2	0	4/8
2. Pachycephalidae	4	4/14	1/10	1/1	0	2/5
3. Dicruridae	4	4/13	2/4	0	0	2/5
4. Meliphagidae	9	8/15	2/3	1/2	3/7	2/3
5. Acanthizidae	7	7/16	4/9	2/1	0	1/3
6. Estrildidae	2	1/4	1/1	0	0	0
7. Others	12	9/12	4/5	1/1	3/8	1/3

Table 3.2 Uniformity across different host families

Uniformity across host families using an ANOVA test for each parasite genera and the overall parasite richness.

Parasite genera	**F**	***p***
Haemoproteus	0.348	0.878
Plasmodium	0.604	0.697
Leucocytozoon	0.380	0.857
Trypanosoma	0.494	0.777
Overall	0.598	0.702

Parasite lineage richness and environmental factors

The overall lineage richness obtained for each subpopulation was positively related to mean annual temperature ($F_{1,4}$ =27.50, P = 0.006, R^2 =0.87; Figure 3.1a) and negatively to elevation ($F_{1,4}$ =27.50, P = 0.017, R^2 =-0.80). An AIC test was applied to determine which of the two independent variables produced a better model. The analysis showed that the model including temperature (AIC value= -15.928) was better than the one including elevation (AIC value= -11.602).

Analysis of the relation of temperature to lineage richness for each genus showed a positive relation with *Haemoproteus spp.* ($F_{1,4}$ =11.66, P = 0.026 , R^2 =0.74; Figure 3.1b) and *Trypanosoma spp.* ($F_{1,4}$ =7.82, P = 0.04, R^2 =0.66; Figure 3.1c). I did not find a relationship neither between lineage richness and *Plasmodium spp.* ($F_{1,4}$ =0.48 , P = 0.52, R^2 =0.11; Figure 3.1d) nor lineage richness and *Leucocytozoon spp.* ($F_{1,4}$ =0.001 , P = 0.97, R^2 =0.0003; Figure 3.1e). The relationship of temperature to lineage richness was also analyzed in each well-sampled family (represented by >15 individuals and sampled from at least 3 elevations). In the five families used for the analysis, positive relations were found; nevertheless they were non-significant.

Mean monthly values of parasite lineage richness was positively related to mean monthly values of temperature ($F_{1,10}$ = 18.495, P = 0.001, R^2 = 0.65), but not to seasonal changes of rainfall ($F_{1,10}$ = 1.558, P = 0.240, R^2 = -0.13). The AIC test applied to determine which of the two independent variables produced a better model showed again that temperature (AIC value= -28.543) produce a better model to explain the variation of parasite lineage richness than monthly rainfall (AIC value= -3.173).

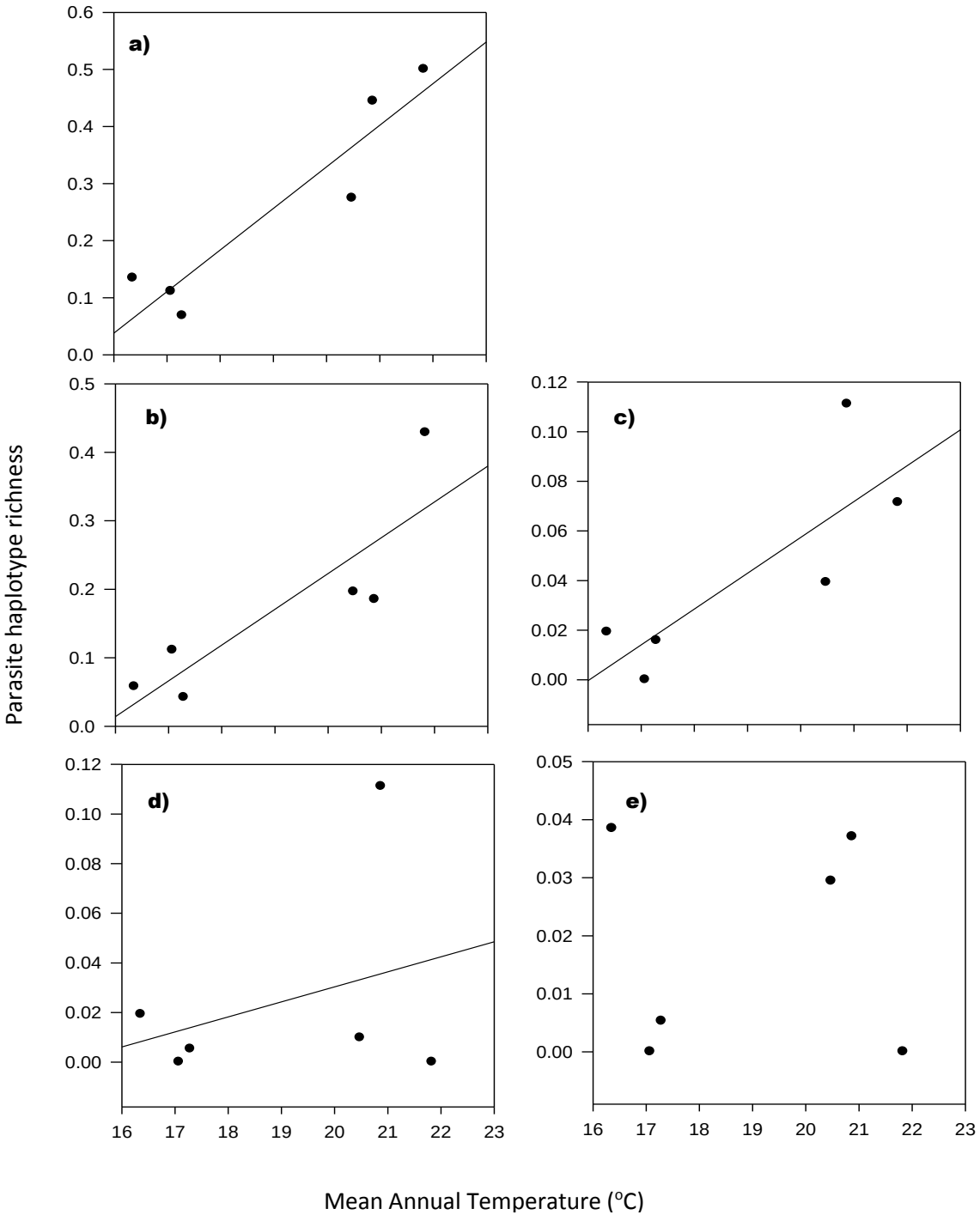

Figure 3.1 Relationship between parasite lineage richness and mean annual temperature

For the a) overall parasite lineage richness, b) *Haemoproteus spp.*, c) *Trypanosoma spp.,* d) *Plasmodium spp.* and e) *Leucocytozoon spp.* The fitted lines showed the positive relationship between mean annual temperature and overall parasite lineage richness, *Haemoproteus spp.* and *Trypanosoma spp.*

Parasite lineage richness along the elevation gradients

The abundance and distribution of each lineage among subpopulations (at each locality) along the elevation gradient are shown in Figures 3.2 and 3.3. Even though each subpopulation exhibited its own particular lineages of each genus, for *Haemoproteus spp.* and *Trypanosoma spp.* there were lineages distributed along the gradient. For example, lineages Hae17, Hae28 and Hae13 (all found in Petroicidae) were the most abundant lineages for *Haemoproteus spp.* and were also distributed along the gradient. Nevertheless, sample sizes of each of these well-distributed lineages are not large enough to determine significant trends of distribution in relation to elevation, temperature or rainfall. In the case of *Trypanosoma spp.*, lineages such as Try01, Try06 and Try08 were found at least in one site of the lowlands and one site of the uplands. In the remaining two genera, most of the lineages were present in only one site along the gradient.

Parasite lineage richness and ecological variables

A multiple regression model was used to determine the relative contribution and significance of morphological host traits (including each species' geographic range size, population density, population size, group size and body mass) in explaining parasite lineage richness (Appendix 3.4). The model explained only 30% of the variance and was non-significant ($F_{5,12} = 1.053$, $P = 0.432$, $R^2 = 0.31$, Adjusted $R^2 = 0.015$; only species with more than 5 individuals were used in the analysis).

Parasite phylogenies and host specificity

I obtained two phylogenies one using cytochrome b for *Haemoproteus spp., Plasmodium spp.* and *Leucocytozoon spp.* and another one using 18S rRNA gene for *Trypanosoma*. The base composition across sequences was uniform for both phylogenies. The substitution model chosen by ModelTest for the cytochrome b dataset was GTR+I+G

(Rodriguez et al. 1990) for the 1st codon position, HKY (Hasegawa et al. 1985) for the 2nd and GRT+G (Rodriguez et al. 1990) for the 3rd,whereas for the 18S rRNA dataset it was F81+I (Felsenstein 1981). The Bayesian phylogeny of Figure 3.2 using cytochrome b, clustered the lineages into three well-supported groups representing the genus to which they belong: *Haemoproteus spp., Plasmodium spp.* and *Leucocytozoon spp.* Lineages of *Haemoproteus spp.* were divided into four clades (I to IV). Clade III had a diverse host family representation; however each lineage (except HAE10) was found in only one family. Clades I and IV were represented by only one family. The large clade I contained only members of Petroicidae and clade IV was represented by only Acanthizidae. Clade II is divided in two well supported small subclades each of them represented by again only one family (subclade HAE06 and HAE09 Paradisaeidae and subclade HAE26 and HAE27, Meliphagidae). In contrast, *Plasmodium spp.* lineages were clustered in only one fully resolved clade, but specificity at any hierarchical level was not displayed, most lineages were found in two or more family groups. Likewise, *Leucocytozoon spp.* lineages were divided into two small clades, V and VI. Each lineage was represented in only one host family (except for LEU06; see Figure 3.2). The Bayesian phylogeny of *Trypanosoma spp.* (Figure 3.3) revealed two small clades (A and B). In the well-supported clade A, each lineage was represented by only one host family, whereas lineages in clade B were represented by several host families.

The PACo analysis confirmed that *Haemoproteus spp.* (m^2_{XY}= 0.271, *P*= 0), *and Leucocytozoon spp.* (m^2_{XY}= 0.0.069, *P*= 0.007) showed high specificity at the family level whereas *Plasmodium spp.* (m^2_{XY}= 0.056, *P*= 0.231) *and Trypanosoma spp.* (m^2_{XY} = 0.149, *P*= 0.276) displayed low specificity.

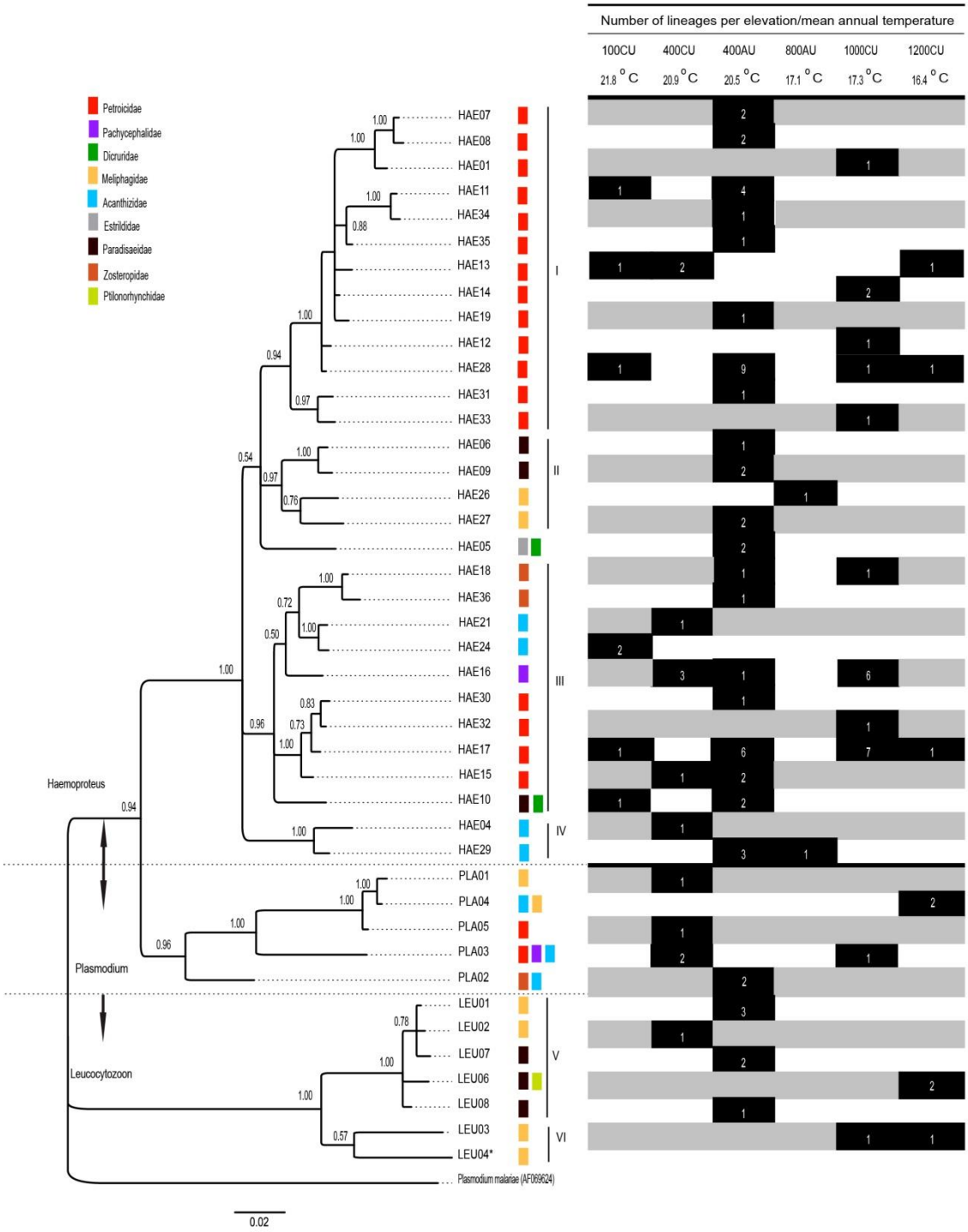

Figure 3.2 Evolutionary relationships among *Haemoproteus spp.*, *Plasmodium spp.* and *Leucocytozoon spp.* cytochrome b

Lineages estimated using a Bayesian approach. Branch lengths and posterior probability values are shown. Color coded squares to the right of the lineage names indicate host family in which a particular lineage was recovered. For each lineage the number of individuals infected in each subpopulation along the gradient and temperature is indicated. Clades are indicated by roman numerals. Parasite lineages from birds sampled before to 2005 used as complementary information are marked with an asterisk (*).

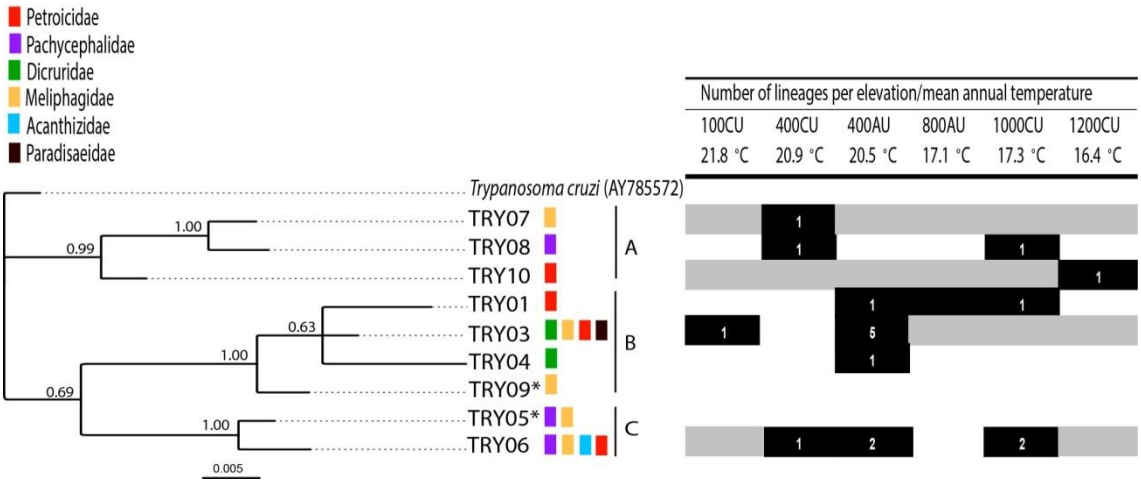

Figure 3.3 Evolutionary relationships among *Trypanosoma spp.* 18S rRNA genes

Lineages estimated using a Bayesian approach. Branch lengths and posterior probability values are shown. Color coded squares to the right of the lineage names indicate host family in which a particular lineage was recovered. For each lineage the number of individuals infected in each subpopulation along the gradient and temperature is indicated. Parasite lineages from birds sampled before to 2005 used as complementary information are marked with an asterisk (*).

Discussion

Similar to what was found for parasite prevalence alone (Zamora-Vilchis, Chapter 2), parasite lineage richness was positively related with temperature (Figure 3.1). However, these results need to be discussed with caution because lineage richness values along the elevation gradients were calculated with only 130 lineages found, which means low sample sizes for many of the sites. To facilitate the discussion, I used a comparison of my data between lowland (0-400 m) and upland (600-1200 m) zones. It seems that the lowland areas favor not only higher parasite prevalence (see Chapter 2) but also higher parasite lineage richness. It appears that higher temperatures may favor both the development of vectors and higher activity levels of vectors. Temperature decreases with elevation and both parasite prevalence and lineage richness decrease. In contrast to the lowlands, the parasite prevalence and lineage richness in the uplands were low and this was related to the lower temperatures which inhibits the development and transmission of diseases. For *Haemoproteus spp.* and *Trypanosoma spp.*

the same pattern of decreasing richness with altitude was found. However, for *Plasmodium spp.* and *Leucocytozoon spp.* no significant pattern was found, perhaps due to the low number of lineages recovered. Other environmental (elevation and rainfall) and ecological and morphological host traits (including each species' geographic range size, population density, population size, group size and body mass) were not good predictors of parasite lineage richness and therefore association with these variables was unlikely to confound the strong relationship between temperature and parasite lineage richness. One of the mechanisms that could explain these results is that abundance of vectors is directly related to temperature. Bird haematozoa are transmitted by arthropod vectors (Atkinson 1991), and ecological factors associated with vector abundance can explain differences in the prevalence of parasite species independently of host (Arriero and Moller 2008; Garvin and Remsen 1997; Piersma 1997; Tella et al. 1999). However, the specific vectors for each parasite genus in this region have been poorly studied and their trends of distribution along gradients remain unstudied. Studies in other regions like Hawaiian islands have shown a negative correlation between abundance of mosquitoes, the main vector for *Plasmodium spp.*, and elevation (Van Riper et al. 1986). Like most vector-borne diseases, transmission of avian malaria is affected by ambient temperature(LaPointe et al. 2005). Nevertheless, the effects of temperature on the distribution of blood parasite vectors are controversial. For example, a global study of current and future habitat suitability for ticks under different climate change scenarios predicts that even though some tick species are likely to undergo range expansions, others may suffer drastic range contractions worldwide (Cumming and Van Vuuren 2006). Studies on parasitic flies whose larvae infect bird nestlings show the same controversial results. A study of parasitic flies of the genus *Philornis spp.* on Argentinean forest birds found that temperature and rainfall were positively correlated with intensity of infection (Tracey 1982). In contrast, another study on parasitic *Protocalliphora* (blow flies) on swallows showed that the number of blow flies varied in a curvilinear fashion with temperature, with parasite pressure highest in nest

around 25°C and decreasing at both higher and lower temperatures (Bensch et al. 2009). The results found in our study suggest that low temperatures of the higher elevations, could be helping to reduce both the development of avian haematozoa and the abundance of these parasite vectors, leading to low parasite prevalence. In contrast, the high temperatures of the lowland areas could be providing an excellent environment for the development of vectors and transmission of haematozoa. However, further research will be vital to determine both specific vectors for each parasite genus and their trends of distribution along elevation gradients in this region.

In the Bayesian phylogenies of the four parasite genera, it was evident that the *Haemoproteus spp.* lineages were clustered in accordance with the host family they belong to. The analysis of host specificity indicates high specificity at family level in this parasite genus. On the contrary, the lineages for *Plasmodium spp.* and *Trypanosoma spp.* did not seem to cluster based on any hierarchical level and most of the lineages were represented by more than one family. The analysis indicates no specificity in these two genera. Finally, for *Leucocytozoon spp.*, even though each lineage was represented in only one family (Except LEU06) and the analysis indicates high specificity at family level, only 10 infected individuals out of 25 were successfully sequenced due to low PCR amplification or poor-quality sequence. Thus, the specificity of this particular genus was unclear until all the lineages can be sequenced. In the study of the host specificity of blood parasites in the Australo-Papuan region, Beadell et al. (2004) found strong host family specificity among the *Haemoproteus spp.* lineages but no specificity in *Plasmodium spp.* lineages. Studies in other regions like Asia, Africa, Europe and South America had also reported *Haemoproteus spp.* as a genus more host specific than *Plasmodium spp.* (Beadell et al. 2009; Ishtiaq et al. 2007; Merino et al. 2008; Waldenstrom et al. 2002). Phylogeographic studies on *Trypanosoma spp.* revealed no specificity in this genus (Sehgal et al. 2001). The results in host specificity indicate that *Plasmodium spp.* and *Trypanosoma spp.* had a higher tendency of host switching than

Haemoproteus spp. For *Plasmodium spp.* and *Trypanosoma spp.* host-switching seems to occur in any species independently of the family it occurs in whereas *Haemoproteus spp.* seems to be more conservative and most of the lineages are constrained to host species of the same family. Differences in the host-switching potential between parasite genera can be at least partially attributed to the feeding habits of their vectors. For example, in the case of *Plasmodium spp.*, generalist feeders such as mosquitoes can potentially infect diverse hosts which can partially explain the low specificity found in this genus (Huff and Coulston 1944; Jansen et al. 2009). Biting midges (Culicoides) is one of the described vectors for *Haemoproteus spp.* and it seems to display both generalist and specialist feeding habits (Lassen et al. 2011; Martinez-de la Puente et al. 2011). For *Leucocytozoon spp.,* blackflies (*Simulium* spp.) have been described as potential vectors (Hatcher and Dunn 2011), whereas the most common vectors for the transmission of *Trypanosoma spp.* in birds are louseflies of the family Hippoboscidae (Olsen 1974). However, for these last two genera, little is known about the feeding habits of their vectors. Further research on the specific vectors and feeding behaviour of each parasite genus is needed to probe the hypothesis that differences in the host-switching potential between parasite genera can be at least partially attributed to the feeding habits of their vectors.

In a recent study, it was found that in the avian community of the same region an average 10% increase in parasite prevalence was predicted for every 1^{o}C increment in temperature. One of the mechanisms I proposed to compensate for this increasing parasite prevalence was elevation shifts upward of host distribution. Every 1^{o}C equates to approximately 200m shift upwards of birds' distribution (Zamora-Vilchis et al. 2012). Under this scenario and according to the results shown here there are two main consequences of climate change for parasite lineage richness. First, the lineage richness could increase with temperature as it was found for parasite prevalence and even though an upward shift in host distributions may help to compensate for the increasing diversity of parasites, bird species

already inhabiting the highest regions will not be able to shift and therefore will experience increasing parasite pressure and diversity (Zamora-Vilchis, Chapter2). Second, *Plasmodium spp.* and *Trypanosoma spp.* which have low specificity may, under these conditions, have a higher potential for host-switching than other more specific parasite genera like *Haemoproteus spp.* (Krizanaskiene et al. 2006; Ricklefs and Fallon 2002; Ricklefs et al. 2004). However, it is possible that climate change will also affect the distribution of vectors and parasite lineages along the gradient (Crowl et al. 2008; Kovats et al. 2001). Vectors and parasite lineages inhabiting specifically upland regions could be also not able to survive due to increases of temperature whereas lowland regions could reach the upper temperature limit for vector development; hence no vectors and parasite lineages in this region could be able to survive. They could also shift upwards to reach optimal temperatures and higher parasite richness could be expected in upland regions (Benning et al. 2002). These results stress both the importance of monitoring the avifauna of the region and the prioritized conservation of the upland areas in the management plans under climate change scenarios.

Summary

- Determination of factors influencing patterns of distribution of parasites is important for correct management of host populations. These studies become even more important as environmental conditions change due to climate change, which is expected to stimulate the expansion of diseases. I also described the phylogenetic relationships among the parasite lineages and examined the host specificity of each parasite genus
- PCR screening was used to investigate the lineage richness and phylogenetic relationships of four genera of blood parasites (*Plasmodium spp.*, *Haemoproteus spp.*, *Leucocytozoon spp.* and *Trypanosoma spp.*) in 130 infected birds of the Australian Wet Tropics.

- There was a positive relationship between parasite lineage richness and temperature. Other environmental and host ecological and morphological traits were not good predictors of parasite lineage richness.
- *Plasmodium spp.* and *Trypanosoma spp.* have low specificity, whereas *Haemoproteus spp.* seems to display specificity at host family level.
- With increased temperature due to climate change, lineage richness is expected to increase and *Plasmodium spp.* and *Trypanosoma spp.* will have greater opportunities for host-switching. This will affect avian host populations, particularly the ones inhabiting the upland areas.

CHAPTER **4**

Parasite-mediated Diversity and Selection of MHC Genes of Birds Distributed along an Altitudinal Gradient: Implications for Disease Impact in a Warming Climate

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Introduction

Parasites are an important selective force in the evolution of host communities (Hatcher and Dunn 2011). They generate evolutionary important elements in host populations, such as genetic diversity (Wakelin 1997). Parasites exert direct selective pressure on host immune systems. These interactions produce a molecular arms race that results in high polymorphisms of host immune genes (Potts and Slev 1995). Genes of the Major Histocompatibility Complex (MHC) have been widely used for studying host-parasite interactions at the molecular level in vertebrate-pathogen systems, due to their role in pathogen recognition and initiation of the immune response. MHC genes show extreme polymorphism within populations (Klein et al. 1993), which provides an opportunity to study the role of balancing selection in the maintenance of genetic variation. Most of the MHC diversity at the population level is thought to arise via interactions of host MHC proteins and parasites (Potts & Wakeland 1990), a phenomenon known as parasite-mediated selection (PMS). There are at least three hypotheses proposing mechanisms by which PMS could

maintain MHC diversity: 1) the "heterozygote advantage" theory, which states that polymorphism at MHC loci increases the range of parasites that can be recognized by the immune system, so that individuals with high heterozygosity have higher fitness (Doherty and Zinkernagel 1975); 2) The "rare allele advantage" or "frequency-dependent" theory, which proposes that in a host population, individuals with rare MHC alleles that are able to defeat new pathogen variants will have higher fitness, and such alleles will increase in frequency as a consequence (Clarke and Kirby 1966); 3) The "fluctuating selection" theory asserts that pathogens can drive MHC diversity by means of fluctuations in space and time of the intensity of selection they exert, which also results in fluctuations of MHC allele frequencies (Hill 1991). It is not clear which of these three selection models is most important or whether the three types of balancing selection may have overlapping roles.

Other PMS studies have also been based on the identification of selection within the MHC sequences using the ratio of nonsynonymous to synonymous substitutions (d_N/d_S) (Jarvi et al. 2004). An excess of nonsynonymous relative to synonymous polymorphism is a clear signal of balancing selection whereas a lack of nonsynonymous relative to synonymous polymorphism is indicative of purifying selection (Kimura 1977; Yang and Bielawski 2000).

Research targeting both genetic diversity and selection supports the idea that selection for MHC diversity is driven by levels of pathogen infection. These levels can vary depending on environmental conditions, in particular temperature (LaPointe et al. 2005). For instance, there are broad latitudinal gradients in which levels of infection increase from the poles to the equator. Such gradients have been observed in many different taxonomic host-parasite systems such as birds and avian haematozoa (Merino et al. 2008), humans and a wide range of diseases (Guernier et al. 2004) and fish and metazoan ectoparasites (Rohde and Heap 1998). Levels of infection can also vary along elevation gradients. In Chapter 2, I described a strong positive relationship between ambient temperature and blood parasite prevalence in the bird community of Australian Wet Tropics along elevation gradients (Zamora-Vilchis et al. 2012).

High temperatures in the lowlands could provide excellent conditions for the development and transmission of blood parasites, while the lower temperatures of the highland areas, particularly in winter, could inhibit the development of these pathogens. This variation is believed to be potentially generated through the direct effects of temperature on parasite metabolism and in the case of vector-borne diseases through an influence on vectors (Brown et al. 1988). Other important environmental factors could be affecting the distribution of vectors and parasites are rainfall, seasonality and large-scale meteorological phenomena such as ENSO or hurricanes. These factors can also alter the quality and quantity of breeding sites for vectors (Carter 2008; Patz et al. 2000; Santiago-Alarcon et al. 2012) The Australian Wet Tropics region is an ideal system for the study of parasite driven selection on the immune systems of birds. It is evident that temperature promotes the existence of different levels of infection along altitudinal gradients which can generate different parasite pressure on quite small spatial scales (Zamora-Vilchis, Chapter 2), and these differences should be reflected in the selection of host immune genes. Here, I test the hypothesis that there is decreasing MHC diversity with increasing altitude caused by the influence of parasites on host immune system in a comparative study of wild populations. To achieve this, I studied fifteen species from two bird families (Acanthizidae and Meliphagidae; Table 4.1) distributed along elevation gradients of the Australian Wet Tropics bioregion. All the species have specific trends of distribution along elevation gradients (Shoo et al. 2005c). Moreover, these species are phylogenetically closely related, which enables the use of Phylogenetic Independent Contrasts analysis (PIC) (Freckleton et al. 2002). The number of MHC class IIβ alleles per bird species was obtained as a measure of immune gene diversity, whereas the average of non-synonymous to synonymous substitutions (d_N/d_S) and number of codons under balancing selection (NCBS) were used as indices of selection. These parameters were compared with parasite prevalence and elevation (measured as the Center of Gravity: CoG; defined as the elevation containing the greatest population density for each bird species and where half of the population is above and half is

below (VanDerWal et al. 2010)) within each bird species. To eliminate the possibility that demographic processes like gene flow and genetic drift influenced the variation of these immune genes, I contrasted the patterns of variation at MHC with those of neutral loci and other important variables including population and range size. I also discussed the implications of climate change on these host-parasite interactions.

Methods

Study area and bird community

See methods in Chapter 2 for details.

Data collection

During 2005 and 2006, 93 birds from fifteen species of the families Acanthizidae and Meliphagidae (Table 4.1) were caught using mist-nets at two localities at different elevation sites within the AWT: South Johnston, Atherton Tablelands (Lat; Long -17.62; 145.72); and Carbine Range (Lat; Long -16.56; 145.28). See Data Collection in Chapter 2 for more details. For each individual bird caught, a volume of 50 to 75 µl of blood was obtained from the brachial vein and stored in Queens lysis buffer.

Table 4.1 The fifteen species of birds used in the study divided by Family

Species abbreviation and sample size for each species is indicated.

Family	Scientific name	Species Abbreviation	Number of samples
Acanthizidae	*Sericornis keri*	Seke	10
	Gerygone mouki	Gemo	4
	Gerygone palpebrosa	Gepa	2
	Oreoscopus gutturalis	Orgu	9
	Sericornis magnirostris	Sema	10
	Acanthiza katherina	Acka	10
	Sericornis citreogularis	Seci	10
Meliphagidae	*Lichenostomus frenatus*	Lifr	5
	Myzomela obscura	Myob	3
	Acanthorhynchus tenuirostris	Acte	5
	Meliphaga gracilis	Megr	3
	Meliphaga lewinii	Mele	5
	Xanthotis macleayana	Xama	6
	Phlidonyris nigra	Phni	5
	Meliphaga notata	Meno	6

Molecular analysis of MHC diversity

DNA was extracted from all samples using either a phenol-chloroform protocol (Friesen et al. 1997) or silica fines (Elphinstone et al. 2003). I amplified a 173 bp fragment of the second exon of the MHC class II β gene using primers HOPE1 and HOPE2 (Vincek et al. 1997). PCR products were cloned using a pGem-T easy vector (Promega) according to the manufacturers' instructions. To avoid the formation of heteroduplexes during PCR reactions, which are expected when amplifying loci of multigene families such as MHC, I modified the PCR and cloning protocol, including time increments in the extension steps of the thermal PCR profiles (Judo et al. 1998) and a variation of a PCR + 1 technique (Borriello and Krauter 1990; Jarvi et al. 2004). A total of 48 non-recombinant clones per individual were screened using SSCP in order to estimate allele diversity per species (see Supplement 1a and 1b for detailed methods). Representative clones from each unique allele were sequenced. Alignments were performed using the program SEQUENCHER 4.8.

Analysis of MHC diversity and selection

1. *Genetic diversity*

The number of "alleles" per bird species was calculated as the average of the ratio between the number of alleles and the number of clones per individual bird (for simplicity the term "MHC alleles" is used; however, given that I found more than two MHC alleles per individual, it is likely that these sequences come from different copies of the MHC class II β locus that has been duplicated in the genome and were amplified simultaneously by the PCR primers used).

2. *Analysis of selection*

The average ratio of nonsynonymous (d_N) to synonymous substitutions (d_S) (d_N/d_S) and the number of codons under balancing selection (NCBS) per bird species were calculated using the program OmegaMap 0.5 (Wilson and McVean 2006), as estimates of the intensity of selection at MHC class II β. The program combines a population genetics approximation to the coalescent and Bayesian methods to infer selection in the presence of recombination. OmegaMap estimates the selection parameter ω that is equivalent to the d_N/d_S ratio. The calculation of ω with OmegaMap does not rely on phylogenic inference as other programs do e.g. PAML (Yang 1997) and HYPHY (Pond et al. 2005), and therefore the use of divergent sequences is not an issue with OmegaMap. The model also takes into account recombination and therefore does not assume identity by descent and mutation as the sole source of allelic variation. Values of ω for each amino-acid site were calculated using a model that assumes independent ω for each codon. Three independent Markov Chain Monte Carlo (MCMC) tests were run for 500,000 iterations, with a burn-in of 5,000 steps and thinning of 10. In order to validate our selected model the ω and ρ parameters were compared for the mean, upper and lower interval against the codon position for a single run and between the three runs, for each bird species. The selection analyses were consistent for all species as indicated by the fact that all three independent runs produced nearly identical results.

Molecular analysis of blood parasite prevalence

To test the association between host MHC diversity and parasite selection pressure, data on parasite prevalence were used for the fifteen bird species of this study (Zamora-Vilchis, Chapter 2). Two nested-PCR protocols were used to detect four of the main blood parasite genera in all bird samples: *Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* (Haemosporida) and *Trypanosoma spp*. (Kinetoplastida) (Sehgal et al. 2001; Waldenstrom et al. 2004). For *Plasmodium spp., Haemoproteus spp.* and *Leucocytozoon spp.* a 478 bp long fragment of the cytochrome b gene was amplified, whereas for *Trypanosoma spp.* 326 bp of the small ribosomal subunit RNA was used.

Other variable estimates

1. *Center of Gravity (elevation), Temperature and total population size*

Species differed in their distribution along the elevation gradient; the point along the elevation gradient that indicates the geographic centre of the spatial distribution of population density was calculated using Center of Gravity (CoG) estimation. Also known as the Centre of Mass, this measure estimates the elevation for which half of the population is above and half is below this point. For the species here, distribution maps of environmental suitability were sourced (Williams et al. 2010b); the environmental suitability is assumed to represent the local abundance (VanDerWal et al. 2009) and the CoG estimate was calculated as the centre of mass weighted by area and environmental suitability using the SDMTools package (VanDerWal et al. 2010) in R (v 2.13 http://www.r-project.org/). CoG was also used to derive an estimate of the temperature associated with each bird species' distribution, using the linear regression of mean annual temperature on elevation in each sub-region (Zamora-Vilchis, Chapter 2):

Temperature = -0.0052(elevation) +22.582 for Carbine Range

Temperature = -0.0056(elevation) +22.159 for South Johnston

The total population size of each bird species in the AWT was estimated from its known geographic range and local density within the region (VanDerWal et al 2010; Williams et al. 2010b; see Appendix IV for variable estimates).

2. *Screening of intron loci variation*

To estimate average allelic variation in neutral markers, three intron sequences were amplified in all the individuals of the fifteen bird species: α-Enolase (ENOL), Laminin (LAM) and Glyceraldehide-3-phosphate dehydrogenase (GAPDH). (See supplement 1c for detailed methods). All the reactions were sequenced. The sequences were aligned by species and analyzed for the presence of double peaks (heterozygous) using the program SEQUENCHER 4.8. Multi-locus allelic variation was estimated as the average of heterozygosity per species (scored 1 for each homozygous sequence and 2 for each heterozygous sequence divided by the number of individuals scored) among the three nuclear intron loci.

Phylogenetically Independent contrasts analysis

I built a phylogeny for the fifteen species of Acanthizidae and Meliphagidae using three mitochondrial genes: cytochrome-b (CYTB), 12S rDNA (12S) and NADH dehydrogenase subunit 2 (ND2), and one nuclear gene, b-fibrogen intron 5 (FIB5). I used available sequences for most of the Meliphagidae species (Driskell and Christidis 2004; Norman et al. 2007) and obtained new sequences for all the Acanthizidae species used in this study and the remaining Meliphagidae (See details of amplification on Supplement 1d and Table 1S). Additionally, I used the consensus sequences per species of the three intron loci obtained for this study (see above) for the phylogeny. All sequences have been submitted to GenBank (see Table 2S for accession numbers). The phylogeny was reconstructed using a Bayesian approach as implemented in the program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) running two

parallel analyses for 10,000,000 Markov Chain Monte Carlo (MCMC) generations, and sampling every 10 generations. Consensus trees were constructed using the final 50,000 trees of both runs (more details for the reconstruction of the phylogeny can be found in Supplement 1d). I re-tested the relationships using phylogenetically independent contrasts as implemented in the PDAP module (Midford 2003) in Mesquite (Maddison and Maddison 2004).

Results

MHC sequences

I found a total 422 unique sequences of 173bp of the MHC class-IIβ gene in 93 different individuals for the 15 bird species. For the analysis only the first 57 codons were used. Thirty-three of these sequences were shared between one or more species. The total number of unique alleles ranged from 12 in *L. frenatus* to 58 in *S. magnirostris*. Based on the maximum number of alleles observed per species, I estimated that the minimum number of loci varied from 4 to 8 (Table 4.2).

In most of the species, I found alleles that contained stop codons, deletions or insertions (47 alleles in total). Such sequences were excluded from further analyses, as it is possible they might correspond to pseudogenes. All the MHC sequences used in this study can be found on Appendix III.

Table 4.2. General results of MHC sequences

Number of unique alleles, range of alleles per individual and estimated minimum number of loci for the 15 bird species. See Table 4.1 for full species names.

Species abbreviation	Number of unique alleles in sample	Range of number of alleles per individual	Estimated minimum number of loci
Seke	54	6 -15	8
Gemo	31	6 -13	7
Gepa	16	7 - 9	5
Orgu	24	2 - 8	4
Sema	58	4 -13	7
Acka	46	1-15	8
Seci	28	3 - 8	4
Lifr	12	3 - 6	3
Myob	23	6 -14	7
Acte	28	5 -10	5
Megr	26	8 -13	7
Mele	32	7 -13	7
Xama	29	6 -9	5
Phni	22	4 -12	6
Meno	30	5 -11	6

Phylogeny of Acanthizidae and Meliphagidae

Figure 4.1 shows the consensus of the 50,000 trees used for the phylogeny reconstruction. The two independent runs showed similar values of likelihood after burn-in, which confirms consistency of results. The bird species were clustered into two well defined groups corresponding to the families Acanthizidae and Meliphagidae. There is good support for most of the internal branches of the tree, as indicated by the posterior probabilities. The molecular phylogeny obtained in this study is in agreement with a previous phylogeny of Acanthizidae based on morphological characters (Williams et al. 2010b) and a previous molecular phylogeny of Meliphagidae based on three mitochondrial and one nuclear markers (with the exception of the position of *A. tenuirostris*) (Driskell and Christidis 2004).

Genetic diversity

Marked differences in MHC allele diversity per species were observed, varying from 0.14 in *O. gutturalis* to 0.33 in *M. notata* (Figure 4.1). The number of positively selected sites per bird species varied from 13 in *O. gutturalis* to 20 in *X. macleayana*, whereas the average of d_N/d_S values varied from 0.81 in *O. gutturalis* to 1.01 in *G. mouki* (Figure 4.1). Table 4.3 showed codons detected under balancing selection ($d_N/d_S > 1$) for the 15 bird species. It was found that not all the codons identified under balancing selection on humans (Brown et al. 1988) corresponded to the codons under balancing selection for the bird species in this study.

Table 4.3. Codons detected Under Balancing Selection ($d_N/d_S > 1$) for the 15 bird species

Codons in dark squares correspond to putative peptide binding region positions in the human DRB1 gene (Brown et al. 1988), amino-acid positions in light squares correspond to positions adjacent to the peptide binding codons of human DRB1 gene. Total Number of Codons under Balancing Selection per bird species (NCBS) is indicated. See Table 4.1 for full species names.

Codon	Seke	Gemo	Gepa	Orgu	Sema	Acka	Seci	Lifr	Myob	Acte	Megr	Mele	Xama	Phni	Meno
6*	-	-	1.32	1.08	1.25	-	1.13	1.14	1.35	1.25	1.66	1.77	1.39	1.65	1.23
7	-	-	-	-	-	-	-	-	1.30	1.16	-	-	-	-	-
8	2.32	1.74	2.03	-	1.32	1.06	1.57	1.11	1.38	2.93	2.07	1.84	2.06	1.90	2.98
10	2.68	2.47	3.89	2.55	4.01	3.73	2.25	2.20	2.19	2.77	2.20	3.69	2.87	2.72	3.16
11	-	-	-	-	-	-	-	-	-	-	-	-	1.06	-	-
12**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	2.30	1.85	1.51	1.96	2.80	1.66	1.70	1.86	1.46	1.97	1.69	2.86		1.64	1.69
18	2.33	2.59	1.65	1.13	1.36	1.25	1.24	1.53	1.90		3.13	1.52	1.92		
19	-	-	-	-	-	-	-	-	-	-	1.06	-	-	-	-
26	-	-	-	-	-	-	1.04	-	1.03	-	-	-	-	-	1.32
27	-	-	-	-	1.29	-	1.88	1.14	1.30	-	1.13	1.14	2.48	1.18	-
33*	2.85	3.00	4.93	2.73	3.77	3.76	4.23	-	3.40	4.03	2.52	2.59	4.48	2.72	3.93
36	-	1.37	-	-	-	-	-	-	1.25	-	-	-	-	1.03	-
37	1.99	3.90		3.11	2.50	1.74	2.65	1.73	3.35	1.37	3.33	3.71	2.56	2.59	4.13
39	-	1.95	1.52	2.07	-	-	-	-	-	-	-	-	1.53		1.15
40	1.54	1.77	1.13	1.68	-	-	-	-	1.50	-	1.95	1.34	1.51	1.69	1.58
41	2.47	3.21	1.94	2.44	2.30	1.76	1.49	-	-	2.43	1.07	1.44	2.99	1.27	1.58
44	2.87	2.42	2.41	1.98	3.51	4.35	1.33	1.39	1.45	-	-	-	2.16	1.11	
45	-	1.50	1.11	-	1.45	1.01	2.23	2.42	1.08	-	1.67	2.18	1.68	2.49	1.62
46	-	1.30	1.26	-	-	1.21	1.04	1.80	2.31	1.35	2.37	1.73	1.12	1.86	3.71
47	2.60	3.16	1.81	3.09	3.14	2.33	3.46	2.47	3.31	4.68	3.28	3.16	4.12	2.81	3.32
48	-		1.22	-	-	-	-	-	1.11	1.04	-	-	-	-	-
50	1.92	2.22	2.15	2.06	2.54	2.06	2.72	2.56	1.56	1.61	2.03	2.44	4.49	1.98	2.21
51	2.29	7.39	3.57	3.88	4.23	3.60	6.35	2.57	3.69	2.81	5.43	3.77	3.87	4.63	6.00
52	-	-	-	-	-	-	-	2.50	-	-	-	-	-	-	-
53	-	1.80	3.01	-	1.03	2.06	2.73	1.37	2.20	1.08	1.90	-	2.96	2.06	1.42
54	2.62	1.58	1.68	-	2.75	1.31	2.39	1.79	1.76	1.09	1.63	1.51	1.37	-	2.72
57	3.28	1.26	1.86	-	4.28	2.26	2.66	1.37	-	1.37	1.41	1.35	1.18	1.44	1.16
NCBS	14	19	19	13	17	16	19	17	21	16	19	17	20	18	19

*Codons under balancing selection in birds but not selected in humans.

**Codons under balancing selection in humans but not selected in birds.

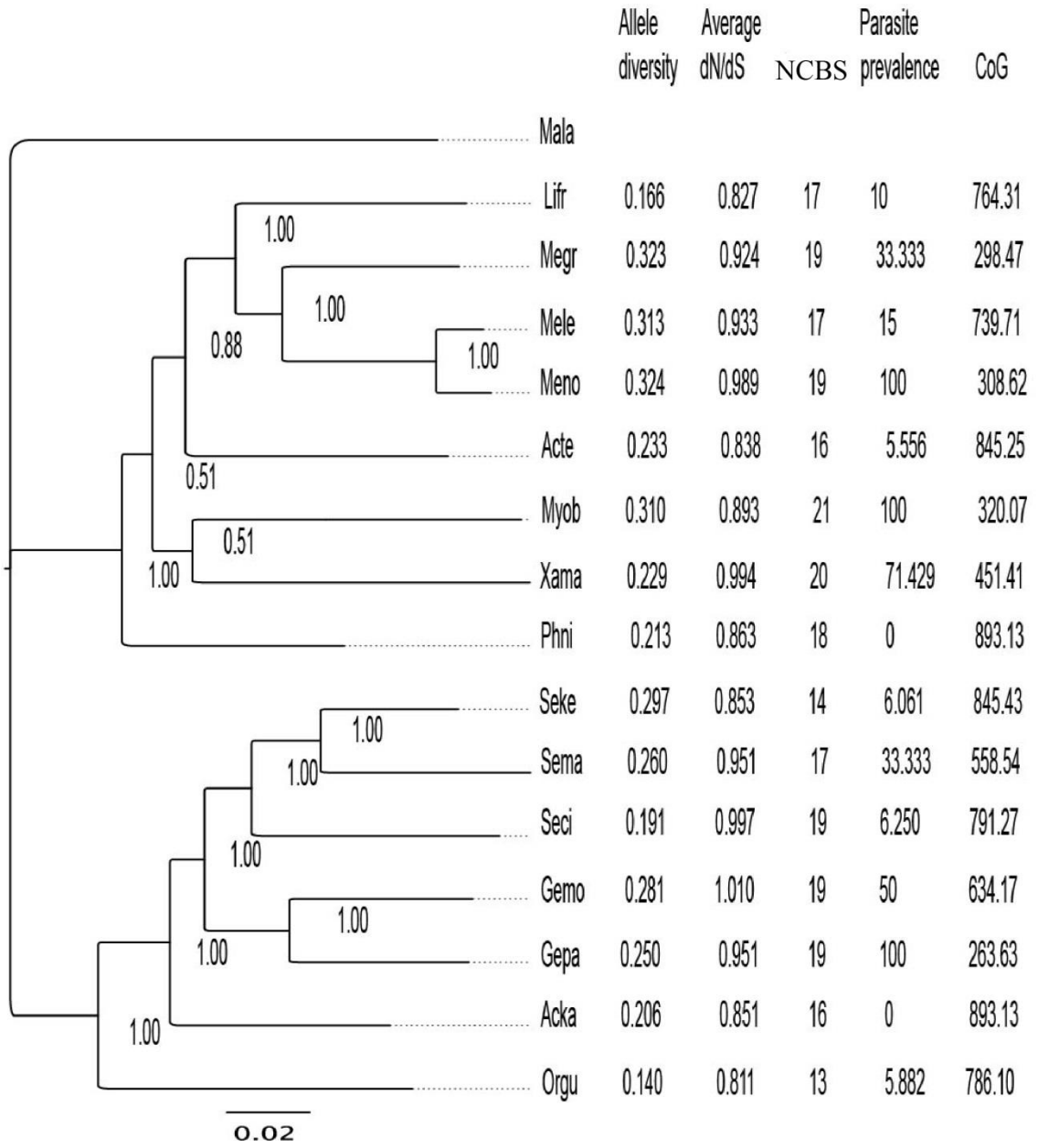

Figure 4.1. Phylogeny of Acanthizidae and Meliphagidae, elevation (CoG) and estimates of MHC diversity and selection

Bayesian consensus tree for Acanthizidae and Meliphagidae and one outgroup *Malurus lamberti* (Mala) using 3 mithocondrial markers (cytochrome-b, 12S rDNA and NADH dehydrogenase subunit 2) and four introns (b-fibrogen intron 5, α-Enolase, Laminin, Glyceraldehide-3-phosphate dehydrogenase) with $1x10^7$ generations. Branch lengths and posterior probability values for internal nodes are shown. MHC diversity and selection values (Allele diversity, Average dN/dS ratio and NCBS; Number of Codons under Balancing Selection), Parasite prevalence and Center of gravity (CoG, elevation) are showed. See Table 4.1 for full species names and Table 2S for Genbank accession numbers.

Relationships of parasite prevalence, temperature and genetic diversity

There was a strong positive correlation between temperature and parasite prevalence (r = 0.92; p = 0.001, Figure 4.2A), which remained significant after PIC (r = -0.94 p = 0.001, Figure 4.2B). In contrast, I found no significant correlation between parasite prevalence and allelic variation of intron loci.

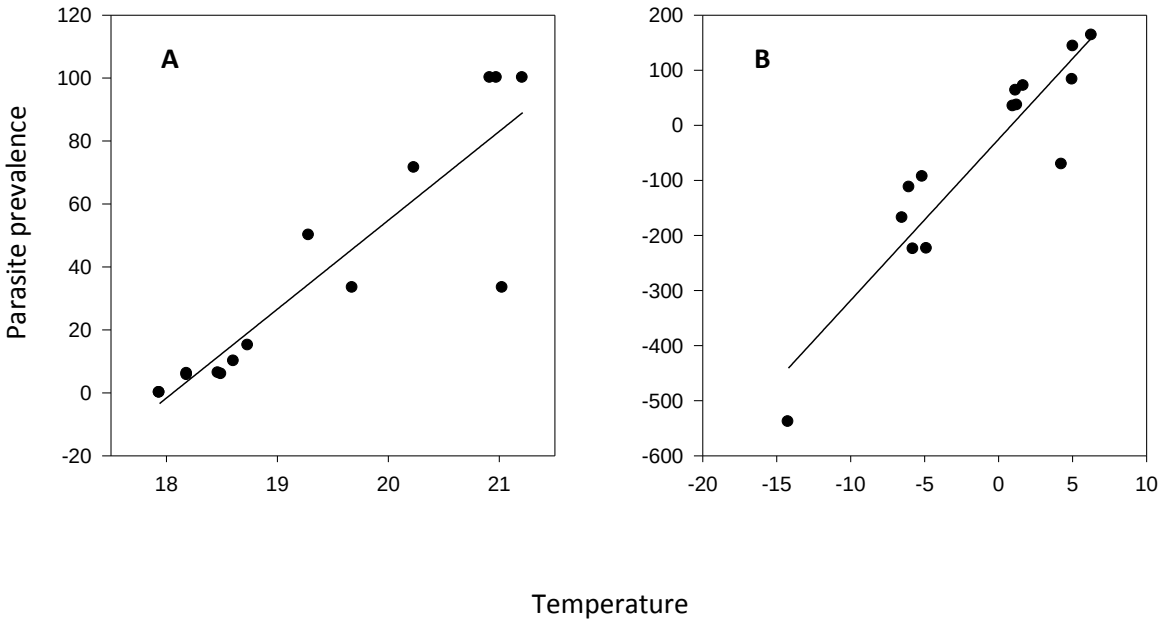

Figure 4.2 Correlation between Temperature and Parasite prevalence

A) Conventional correlation and B) after PIC (Phylogenetically Independent Contrast) analysis.

I found positive correlations between parasite prevalence and the three variables of MHC diversity and selection; all remained significant after PIC (Table 4.4, Figure 4.3). One outlier was found for the PIC data of allele diversity (Fig. 4.3B), when this point was removed I found similar values of significance and correlation with parasite prevalence. Variability at neutral molecular markers did not correlate with any of the MHC diversity and selection parameters, or with parasite prevalence (Table 4.4). The pattern of the three MHC variables

was not driven by other demographic parameters like population size or range size, with the exception of the correlation between NCBS and range size (Table 4.4).

Using PIC data, I also found negative correlations between the three MHC values and elevation (measured by CoG), except that for the allele diversity (without the outlier) the correlation was not significant. To distinguish the importance of parasite prevalence and elevation in the model, partial correlations were run between each of the three MHC variables and parasite prevalence controlling for elevation and the two variables of MHC selection and elevation controlling for parasite prevalence, using only PIC data. I found that only allele diversity (without the outlier) and parasite prevalence correlate significantly ($r = 0.77$ $p = 0.003$). Other partial correlations between any of the other MHC variables and parasite prevalence or elevation were not significant (Table 3S).

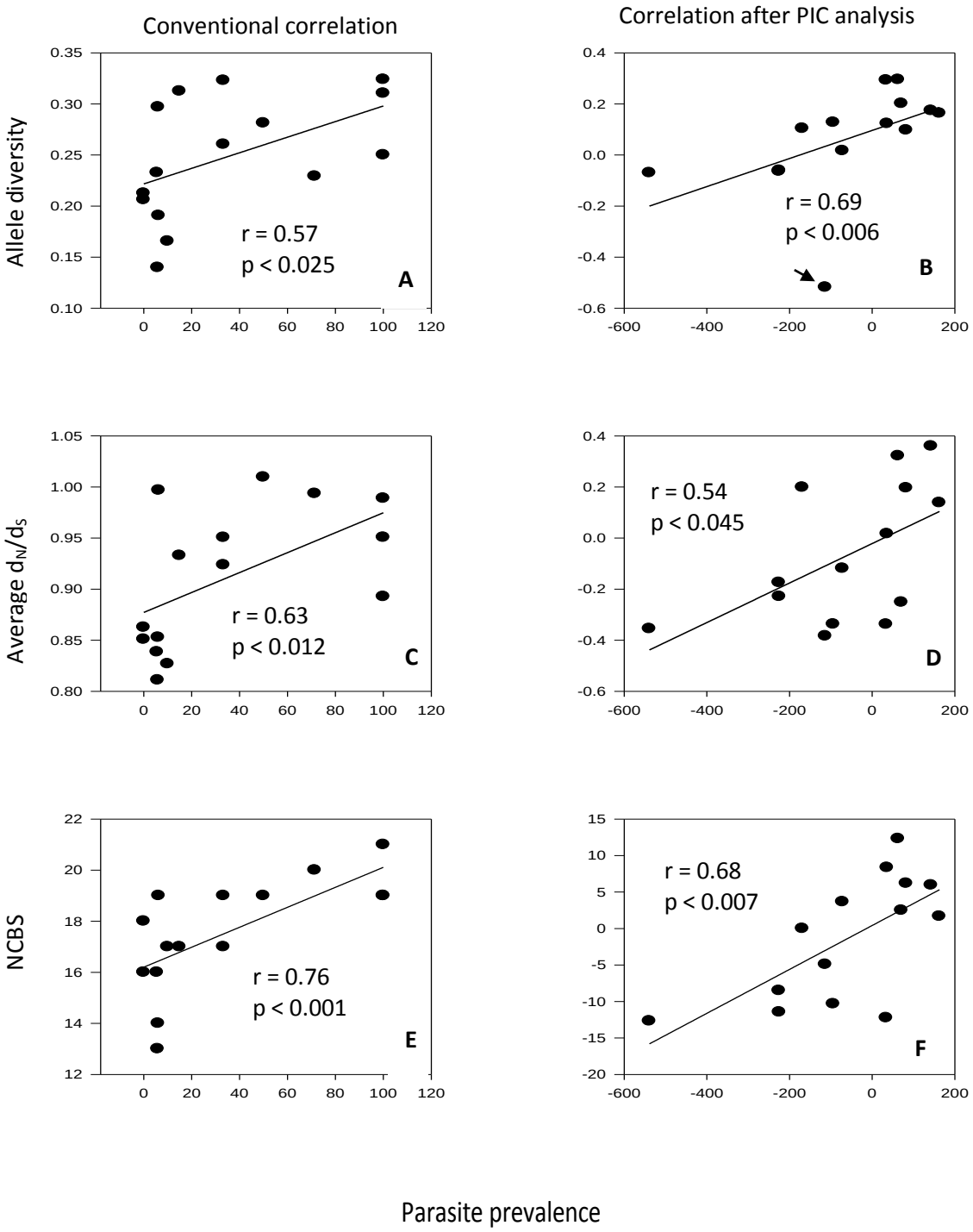

Fig. 4.3. Correlations between Parasite prevalence and MHC diversity and selection

Conventional correlations (left panels) and after PIC analysis (right panels) between parasite prevalence and MHC: Allele diversity (A, B); Average d_N/d_S ratio(C, D) and NCBS (E, F). An outlier for allele diversity is marked with an arrow.

Table 4.4. Correlations between MHC variables and elevation and bird ecological variables

Coefficients and *p*-values of correlations between MHC variables and A) Elevation, B) Population size, C) Range size and D) Multilocus allelic variation of introns (MAVI).

	1) Allele diversity				2) Average d_N/d_S ratio				3) NPSS			
	Conventional		PIC		Conventional		PIC		Conventional		PIC	
	r	*p*	r	*p*	r	*p*	r	*p*	R	*p*	r	*p*
A) Elevation	-0.55	0.03	-0.43	N.S. 0.14	-0.50	N.S. 0.06	-0.54	0.045	-0.67	0.006	-0.61	0.013
B)Population size	0.36	N.S. 0.187	0.05	N.S. 0.873	0.40	N.S. 0.14	0.42	N.S. 0.14	0.30	N.S. 0.272	0.06	N.S. 0.84
C) Range size	0.50	N.S. 0.054	0.48	N.S. 0.094	0.41	N.S. 0.128	0.27	N.S. 0.358	0.79	<0.001	0.65	0.012
D) MAVI	0.06	N.S. 0.829	---	---	0.06	N.S. 0.829	---	---	-0.40	N.S. 0.142	---	---

Discussion

I found a strong positive correlation between temperature and parasite prevalence. This result indicates that parasites increase with temperature. The same positive correlation was found for a larger sample of 40 species from the region (Zamora-Vilchis, Chapter 2). I found positive correlations between blood parasite prevalence and the three parameters of bird MHC diversity and selection with both conventional and PIC analysis. The results suggest that the stronger the parasite pressure the higher the MHC allele diversity, average d_N/d_S ratio and NCBS. The pattern of the three MHC diversity parameters was not driven by other demographic variables like population size or range size (except for NCBS, see further discussion for this below). Additionally, variability at neutral molecular markers did not correlate with any of the MHC diversity parameters, nor with parasite prevalence.

Parasites mediate selection of MHC genes

One of the mechanisms for PMS is the heterozygote advantage theory, according to this theory, heterozygosity at MHC loci increases the range of parasites that can be recognized by the immune system, so that individuals with high heterozygosity have higher fitness (Doherty and Zinkernagel 1975). A classic example supporting this theory is that heterozygosity of a locus in humans confers protection from persistent hepatitis B virus infection (Thursz et al. 1997). In chickens, heterozygosity seems to confer resistance to Rous sarcoma virus (Senseney et al. 2000). In natural populations of yellow-necked mice (*Apodemus flavicollis*), populations with larger numbers of different MHC alleles had lower parasite pressure than populations with few different MHC alleles (Meyer-Lucht and Sommer 2009). In this study, I found that lowland bird species with higher parasite pressure have higher MHC allele diversity than upland bird species with low parasite pressure. These apparent contradictory results arise from the fact that in this study different host species are compared rather than individuals within one host species as many studies do. Immune response is a costly life-history trait that needs to be in trade-off with other fitness characters like reproduction, growth and maintenance (Langand et al. 1998). There are energetic cost associated to evolve immune response (Lochmiller and Deerenberg 2000). In theory, immune diversity needs to evolve proportionally to the parasite pressure that each host species had experience along its evolutionary history. Moreover, models of host-parasite coevolution state that host-parasite fluctuations and cyclic coevolution drives genetic diversity of both hosts and parasites and these models predict more diversity where there are higher parasite pressures (Anderson and May 1982). The high parasite pressure that lowland bird species has exposed along its evolutionary history have decisively contributed to its larger MHC diversity, whereas upland species have cope with less parasite pressure. Other comparative studies have found similar results, e.g. in a study of parasites and MHC diversity of Eurasian kestrels (*Falco tinnunculus* and *Falco naumanni*), lower pathogen pressure and less MHC diversity were found in island

species than mainland species where both parasite pressure and MHC diversity is higher (Alcaide et al. 2010).

d_N/d_S ratio estimates and the use of OmegaMap

The program OmegaMap (Wilson and McVean 2006)offers many advantages for the analysis of MHC genes. The model used for OmegaMap to estimate d_N/d_S ratios do not rely on phylogenetic inference as other commonly used programs do (e.g. PAML (Yang 1997) and HYPHY (Pond et al. 2005)) which allow the analysis of divergent sequences commonly found on MHC genes studies as it was found in this study (Aguilar et al. 2006; Edwards et al. 1995). This program use a model more adjusted to population studies, based in the coalescence theory. The model also takes into account recombination and therefore does not assume identity by descent and mutation as the sole source of allelic variety. In fact, when there has been recombination in the evolutionary history of the sequences, as is the case for most MHC genes (Miller and Lambert 2004), reconstructing a single phylogenetic tree is not appropriate and inference based on programs that use phylogenetic inference can give misleading results. The identification of sites experiencing diversifying selection can suffer from a false-positive rate as high as 90%. OmegaMap is a program that detects variation in the d_N/d_S ratio and does not suffer from high false-positive rate (Wilson and McVean 2006). However, the use of this program in the analysis of MHC genes is not common, even when it provides many advantages (Alcaide et al. 2007; Esparza-Salas 2008).

I calculated the average d_N/d_S ratios and the NCBS as an estimate of selection, even when the approach often used when studying variation of MHC genes in non-model organisms is to calculate the d_N/d_S ratios of only "putative codons under balancing selection" (Dionne et al. 2007; Jarvi et al. 2004). However, such approach assumes that MHC genes across most or all vertebrate species share the same codons under balancing selection as those found in a study in humans (Brown et al. 1988). I found a great variation in the position of codons under

balancing selection like other studies for non-model organisms have found (Esparza-Salas 2008). This suggests it is wrong to assume such a conservation of codons under balancing selection across different taxonomic levels. For this reason, the average of the whole sequence was used and not only the "putative selected codons". The sequence includes codons at balancing, purifying and neutral selection and therefore, the average value can be above 1 even if some of the codons in the sequence set are in fact under balancing selection. The estimates presented in this study allow comparing different average d_N/d_S ratios across different species, which in turn serves as an indication of different selective forces acting in different species.

The NCBS and average d_N/d_S were positively correlated to parasite prevalence, indicating that selection increases with parasite pressure. However, NCBS was also correlated with range size. It is possible that species with higher range sizes are exposed to greater parasites burdens and the NCBS can help to recognize this higher parasite pressure.

Partial correlations of elevation, MHC diversity and parasite prevalence

Parasite prevalence correlated positively with the three MHC diversity variables, and at the same time the two variables of MHC selection correlated negatively with elevation. Partial correlations indicated that only allele diversity (without the outlier) and parasite prevalence correlated significantly when controlling for elevation. This indicated that parasite prevalence is one of the most important variables affecting MHC allele diversity. Other partial correlations between any of the other MHC variables and parasite prevalence or elevation were not significant. It is possible that other related variables are also important influences on MHC selection. For example, other groups of parasites like ectoparasites, nematods, viruses, bacteria, etc. could be also affecting the diversity of MHC genes.

On the whole, all the main results found here suggest an interaction between temperature, parasites and bird MHC diversity. Temperature and parasite pressure are high in

lowlands. This strong parasite pressure on the host immune system could help to promotes higher MHC diversity and selection found in bird species of this region. As elevation increases both temperature and parasite prevalence decreases. The lower temperature of highland areas could help to inhibit the development of parasites, creating a low-parasite environment and hence lower MHC diversity and selection in birds. Life history theory predicts that the immune system is a costly life history trait, it is therefore traded off against other life history characteristics like growth and reproduction (Hart 1997). It seems that at high parasite pressure more energy is allocated to the immune system, while in low-parasite environments, like the upland areas, less energy is invested in this life history trait. Some studies have shown that the reduction of parasites has increased the survival and reproductive rates in birds (Hudson 1986; Marzal et al. 2005). It is possible that lowland birds could be investing less in reproduction or survival than upland birds. Further research on life history traits on these bird species is needed to probe this hypothesis.

Implications of climate change for host-parasite interaction

Rising temperatures due to global climate change are predicted to expand the distribution of vector-borne diseases (Patz and Reisen 2001) . For the bird community of the Australian Wet Tropics, a 10% increment in parasite prevalence has been predicted for every 1^{o}C rise in temperature (Zamora-Vilchis, Chapter 2). How will climate change affect this host-parasite interaction? Even though it will be difficult to predict the consequences of redistribution of host and parasites, it is clear that these changes will affect the global host-parasite dynamics leading to a disruption of adaptive processes (Liang et al. 2002). One of the host mechanisms proposed to avoid higher parasite prevalence is elevation shifts upwards of bird distributions in order to compensate the increments of parasite pressure. However, bird species already inhabiting the upland region might not be able to shift to other elevations or habitats and therefore might experience higher parasite pressure and competition for

resources with other bird species with similar niches, to which they might be maladapted. Are the immune systems of birds in this community prepared for this higher parasite pressure? Upland birds have lower MHC diversity, and rapid adaptation of their immunity could be unlikely due to the long life cycles of birds (Hamilton et al. 1990). Increased parasite pressure is expected to have negative effects on the bird populations of the region, particularly those inhabiting the upland areas and populations unable to shift upwards. Blood parasites have been associated with high mortality rates and/or lower birth rates in host populations, reducing population density (Donovan et al. 2008; Marzal et al. 2005; Norte et al. 2009). Moreover, there is strong evidence that anthropogenic global warming is forcing changes in the distribution of many host species, with shifts to higher latitudes or altitudes (Foufopoulos et al. 2011; Gasner et al. 2010; Hickling et al. 2006). It has been predicted that under impending temperature change many bird species of this region could experience significant range reductions, increased population fragmentation and declines in population size, with upland species particularly susceptible (Shoo et al. 2005a; Williams et al. 2003). The predicted increase of parasite prevalence could interact with, and further exacerbate, the projected impacts of climate change on this bird community, leading to an increased risk of extinction for many bird species. Nevertheless, some studies have shown that infection of the same lineage of *Plasmodium relictum* causes diseases of different severity in different avian host. It is possible that the severity of the infection and the consequences of an increased parasite pressure will vary between species (Palinauskas et al. 2008; Palinauskas et al. 2009). For instance, in Hawaii, many endemic species of birds have gone extinct or become endangered due to the introduction of mosquitoes carrying avian malaria (Van Riper et al. 1982). The high temperatures of the lowland areas favored the development of *Plasmodium spp.* and most of the native populations of birds have gone extinct. Some lowland bird species have survived by shifting their distributions to higher elevations which have not been reached by malaria (Van Riper et al. 1986). However, recent studies has found that some individuals of a Hawaiian bird

(*Hemignatus virens)* have acquired tolerance (withstanding the infection while paying a low fitness cost) against avian malaria while many other remain endanger or are extinct due to the introduction to this parasite to the island (Atkinson et al. 2013).Future projections for the Hawaiian bird community have demonstrated that increases of temperature will allow the invasion of vectors to even higher elevations increasing the range of parasites and restricting even more the distribution of native species (Benning et al. 2002). Conservation of Australian Wet Tropics upland areas must be given a high priority in the management plans of the region. The results in this study showed that the upland areas are a low-disease habitats and birds have low levels of MHC diversity and selection which could make them more susceptible to the negative impacts of increasing parasites infections due to climate change like high mortalities or reductions in population densities (Donovan et al. 2008; Marzal et al. 2005; Norte et al. 2009), unless their immune genes can evolve rapidly in response.

Summary

- Parasites have an important role in shaping the evolution of hosts by imposing selection for genetic variance, especially in genes that underlie the immune response.
- I tested the hypothesis that differences in parasite pressure over small distances can promote selection on Major Histocompatibility Complex genes (MHC). I investigated rainforest birds distributed along elevation gradients, in which prevalence of blood parasites increase with increasing temperature, due to the effect of temperature on parasite transmission.
- I amplified a 173 bp fragment of the second exon of the MHC class II β gene of fifteen species from two bird families (Acanthizidae and Meliphagidae) in order to analyze their allele diversity and selection (average d_N/d_S ratio and number of codons under balancing selection; NCBS).

- MHC diversity and selection were positively correlated to blood parasite prevalence. The results suggest that the stronger the parasite pressure the higher the MHC allele diversity, average and NCBS. It appears that higher parasite prevalence imposed stronger selective pressure in the host immune system, therefore the higher MHC allele diversity and selection allowed them to tolerate higher parasite prevalence.
- On the whole, all the main results found here suggest an interaction between temperature, parasites and bird MHC diversity. Temperature and parasite pressure are high in lowlands. This strong parasite pressure on the host immune system could help to promotes higher MHC diversity and selection found in bird species of this region. As elevation increases both temperature and parasite prevalence decreases. The lower temperature of highland areas could help to inhibit the development of parasites, creating a low-parasite environment and hence lower MHC diversity and selection in birds.
- One of the host mechanisms proposed to avoid higher parasite prevalence is elevation shifts upwards of bird distributions in order to compensate the increments of parasite prevalence. However bird species already inhabiting the upland region might not be able to shift to other elevations or habitats and therefore might experience higher parasite pressure, to which they might be maladapted. Upland birds have lower MHC diversity, and rapid adaptation of their immunity could be unlikely due to the long life cycles of birds
- Upland birds had lower MHC diversity, and there was evidence for more intense selection on MHC genes in lowland birds. Our results suggest that upland birds will be especially susceptible to increased parasitism due to rising temperature unless their immune genes can evolve rapidly in response.

Supplement

Detailed methodology

a) PCR + 1 cloning

The PCR + 1 technique consist of two step reactions. During the first reaction, one of the primers is present in excess of the other, producing an excess of single-stranded non-recombinant products. During the second reaction, a primer complementary to the single strand product, containing a restriction site is added, making it possible to identify non-recombinant clones by using the respective restriction enzyme. The initial PCR reaction was carried out in 50 µl total volume including approximately 50 ng of DNA, 1x PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.08 µM of primer HOPE1 (5'-GAA AGC TCG AGT GTC ACT TCA CGA ACG GC-3), 0.8 µM of primer HOPE2 (5'-GGG TGA CAA TCC GGT AGT TGT GCC GGC AG-3') and 1 unit of Platinum *Taq* polymerase (Invitrogen). The thermal profile included a denaturation at 94 ºC for 4 min, followed by 35 cycles of 94 °C for 1 min, 63 °C for 2 min and 72 °C for 3 min and a final extension at 72 °C for 10 min. The second reaction was performed in 50 µl containing 20 µl of the first PCR reaction, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 µM of primer HOPE1-*Hind* (5'-AAG CTT GAA AGC TCG AGT GTC ACT TCA CGA ACG GC-3') which included a *Hind* III restriction sequence at the 5' end and 1 unit of Platinum *Taq*. The thermal profile for the second reaction was identical to the first PCR, except that it was performed for a single cycle instead of 35.

b) Single-strand polymorphism assays (SSCP)

Cloned alleles were amplified in volumes of 10 µl, containing 2 µl of a solution containing the bacteria colony, 1x GoTaq Green Master Mix (Promega), and 0.5 µM of each of the vector primers F23 (5'-CCC AGT CAC GAC GTT GTA AAA CG-3') and R24 (5'-AGC GGA TAA CAA TTT CAC ACA GGA-3'). The temperature profile consisted of an initial denaturation at 94 °C for three minutes, followed by 25 cycles of 94 °C for 15 seconds; 55 °C for 30 seconds and

72 °C for one minute; and an extension of 72 °C for 10 minutes. Finally, 1 µl of the reaction was digested overnight with *Hind* III restriction endonuclease. After digestion, PCR products were run in a 1.5% agarose gel. Clones were identified as non-recombinant by the presence of shorter size bands on the gel, which indicate the presence of the *Hind* III restriction site. A total of 48 non-recombinant clones per individual were screened using SSCP in order to estimate allele diversity per species. Clones were amplified using primers fluorescent-labeled with HEX at the 5' end. The reactions were performed in 10 µl total volume including 1x GoTaq Flexi PCR buffer (Promega), 0.6 mM $MgCl_2$, 5% Dimethyl sulfoxide (DMSO), 0.2 mM of each dNTP, 4 µM of each primer HOPE1 and HOPE2, 0.25 units of GoTaq Flexi DNA polymerase (Promega) and 50 ng of DNA. The thermal profile included an initial denaturation at 94ºC for 3 minutes followed by 14 touchdown cycles of 92 °C for 15 seconds, annealing at 65 °C to 58 °C for 20 seconds, with 0.5 °C decrement at each cycle and 74 °C for 3 minutes. The touchdown cycle was followed by twenty cycles of 92 °C for 15 seconds; 50 °C for 30 seconds and 74 °C for 3 minutes. A final extension at 72 °C for 60 minutes was applied at the end of the cycles. The resulting PCR products were diluted in a 1:3 ratio with formamide containing bromophenol blue. The mixture was heated for three minutes at 95 °C, and immediately quenched on ice for five minutes. PCR products were run through gels containing 5% Acrylamide: bis-acrylamide 49:1; 2% Glycerol and 0.6x TBE. Gels were run at 1200V and 22°C for 35 min on a GelScan2000 DNA fragment analysis system (Corbett Research). MHC alleles were identified by the pattern of two bands produced by clones with different conformation on SSCP gels.

c) Screening of intron loci variation

I used a general PCR reaction of 25 µl using approximately 50 ng of DNA, 1x GoTaq Green Master Mix (Promega) and 0.3 µM for each primer (Table 1S). The thermal profile was 94^{o}C for 3 min; 35 cycles of 94^{o}C for 30s, 65^{o}C for 30s and 72 C for 45s; with a final extension of 10 min at 72^{o}C. All the reactions were sequenced.

d) Phylogeny of Acanthizidae and Meliphagidae

Conditions for the PCRs varied depending on the gene target. The general PCR reaction was carried out in 50 µl using approximately 50 ng of DNA, 1x GoTaq Green Master Mix (Promega), and 0.3 µM of each primer (Table 1S). The basic amplification protocol was 2 min at 94 °C for initial denaturation; followed by 35 cycles of 30 s at 94 °C, 30 s at 40 to 58 °C (depending on the target locus) and 1min at 72 °C for DNA elongation; and 10min for the final extension at 72 ºC. I aligned the sequences of each locus using the program Se-Al (Rambaut 1996) using the alignment published by (Driskell and Christidis 2004) as a template. The CYTB and ND2 were divided into first, second and third codon positions using the program CODONSPLIT (Ingrid Jakobsen, University of Queensland), whereas the 12S was divided into stem and loop regions. A $\chi 2$ stationarity test was performed using the program TREEPUZZLE 5.2 (Schmidt et al. 2002) to detect potential variation in sequence compositions among each dataset. The best model of nucleotide substitution for each partition was found using the program PAUP* version 4.0b10 (Swofford 1998) with the add-on MrMODELTEST 2.2 (Nylander 2004).

Table 1S. Primers used to amplify loci used to build the Meliphagidae and Acanthizidae phylogeny.

Gene	Primer name	Sequence	Reference
Cytochrome b	L14990	5'-CCATCCAACATCTCAGCATGATGAAA-3'	(Kocher et al. 1989)
	H16065	5'-GGAGTCTTCAGTCTCTGGTTTACAAGAC-3'	(Helm-Bychowski and Cracraft 1993)
12S rDNA	L1276	5'-CACTGAAGATGCCAAGATGG-3'	(Driskell and Christidis 2004)
	H2512	5'-GCAGAGGGTGACGGGCGGTGTG-3'	(Kocher et al. 1989)
ND2	L5206	5'-CTAATAAAGCTTTCGGGCCCATAC-3'	(Kirchman et al. 2001)
	H6313	5'-TTCTACTTAAGGCTTTGAAGGC-3'	
B-fibrinogen intron 5	FIB5	5'-CGCCATACAGAGTATACTGTGACA-3'	(F.K. Barker and S.J. Hackett, unpublished)
	FIB6	5'-GCCATCCTGGCGATTCTGAA-3'	
Enolase	EnolL731	5'-TGGACTTCAAATCCCCCGATGATCCCAGC-3'	(Friesen et al. 1997)
	EnolH912	5'-CCAGGCACCCCAGTCTACCTGGTCAAA-3'	
Lamin	LamL724	5'-CCAAGAAGCAGCTGCAGGATGAGATGC-3'	(Friesen et al. 1997)
	LamH892	5'-CTGCCGCCCGTTGTCGATCTCCACCAG-3'	
Glyceraldehide-3-phosphate dehydrogenase	GapdL890	5'-ACCTTTAATGCGGGTGCTGGCATTGC-3'	(Friesen et al. 1997)
	GapdH950	5'-CATCAAGTCCACAACACGGTTGCTGTA-3'	

Table 2S. Markers used to build the Phylogeny of Acanthizidae and Meliphagidae and Genbank accession numbers. See Table 4.1 for full species names.

Species abbreviation	CYTB	12S	ND2	FIB5	ENOL	LAM	GAPDH
Seke	KC923905[3]	KC923917[3]	KC923924[3]	KC923933[3]	KC923942[3]	KC923957[3]	KC923972[3]
Gemo	KC923906[3]	KC923918[3]	KC923925[3]	KC923934[3]	KC923943[3]	KC923958[3]	KC923973[3]
Gepa	KC923907[3]	KC923919[3]	KC923926[3]	KC923935[3]	KC923944[3]	KC923959[3]	KC923974[3]
Orgu	KC923708[3]	X	KC923927[3]	KC923936[3]	KC923945[3]	KC923960[3]	KC923975[3]
Sema	KC923709[3]	X	KC923928[3]	KC923937[3]	KC923946[3]	KC923961[3]	KC923976[3]
Acka	KC923910[3]	KC923920[3]	KC923929[3]	KC923938[3]	KC923947[3]	KC923962[3]	KC923977[3]
Seci	KC923911[3]	KC923921[3]	KC923930[3]	KC923939[3]	KC923948[3]	KC923963[3]	KC923978[3]
Lifr	KC923912[3]	X	KC923931[3]	KC923940[3]	KC923949[3]	KC923964[3]	KC923979[3]
Myob	AY488366[1]	AY488220[1]	AY488293[1]	AY488447[1]	KC923950[3]	KC923965[3]	KC923980[3]
Acte	AY488332[1]	AY488186[1]	AY488261[1]	AY488412[1]	KC923951[3]	KC923966[3]	KC923981[3]
Megr	AY353241[1]	AY488215[1]	AY488288[1]	AY488441[1]	KC923952[3]	KC923967[3]	KC923982[3]
Mele	KC923913[3]	KC923922[3]	DQ673226[2]	DQ673246[2]	KC923953[3]	KC923968[3]	KC923983[3]
Xama	KC923914[3]	KC923923[3]	KC923932[3]	KC923941[3]	KC923954[3]	KC923969[3]	KC923984[3]
Phni	AY488376[1]	AY488230[1]	AY488302[1]	AY488457[1]	KC923955[3]	KC923970[3]	KC923985[3]
Meno	KC923916[3]	X	DQ673229[2]	DQ673249[2]	KC923956[3]	KC923971[3]	KC923986[3]

1, Driskell and Christidis, 2004
2, Norman, et al, 2007
3, This study
X, Not obtained

Table 3S. Partial correlations between the three MHC variables and Parasite prevalence and elevation (CoG).

A) Parasite prevalence when controlling for CoG and B) CoG when controlling for parasite prevalence.

MHC variables	A) Parasite prevalence controlling for CoG		B) CoG controlling for parasite prevalence	
	r	*p*	*r*	*p*
Allele diversity	0.1	0.749	---	---
dN/dS ratio	0.08	0.780	-0.18	0.558
NCBS	0.13	0.670	-0.24	0.438

General Discussion

Summary of main outcomes

The results of this thesis have demonstrated an interaction between temperature, parasite prevalence and host MHC diversity and selection along elevational gradients of the bird community of the AWT. The four genus of parasite studied here *Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* (Haemosporida) *and Trypanosoma spp*. (Kinetoplastida) are not randomly distributed along elevation gradients, and temperature was one of the main variables explaining patterns of distribution of blood parasites. This interaction creates large and stable differences in parasite pressure over small geographic distances. I found a gradient of host MHC genes diversity and selection that correlate with pressure of parasites. The future of these coevolved interactions in the context of climate change was questioned, given that temperature seems to be an important variable predicting parasite prevalence in this host community and that rising global temperature is predicted to expand the distribution of vector-borne diseases in other regions (Alonso et al. 2011; Caminade et al. 2014; Garamszegi 2011; LaPointe et al. 2005; Loiseau et al. 2013; Siraj et al. 2014)

Temperature, prevalence and lineage richness of bird blood parasites on an elevation gradient

The avian community of the AWT was infected with at least of four of the main blood parasite genera *Haemoproteus spp.*, *Plasmodium spp.*, *Leucocytozoon spp.* and *Trypanosoma spp*. I found that temperature is one of the most important variables driving patterns of distribution of these parasites. The results suggest that the warm temperature of the lowland regions can help to promote both the development of avian haematozoa and the abundance of their vectors, promoting high parasite prevalence/lineage richness. Temperature decreases

with elevation and both parasite prevalence and lineage richness decrease as well. There were similar trends for each genus of parasites surveyed for parasite prevalence, whereas for lineage richness only *Haemoproteus spp.* and *Trypanosoma spp.* followed this trend. Other environmental (elevation and rainfall) and ecological and morphological host traits (including each species' geographic range size, population density, population size, group size and body mass) were not good predictors of parasite prevalence/lineage richness and therefore association with these variables was unlikely to confound the high relationship between temperature and both parasite prevalence and lineage richness. These trends of decreasing parasite prevalence with elevation have also been found in other regions of the world such as the Dominican Republic and Madagascar, but temperature has not been tested as a variable to explain them (Latta and Ricklefs 2010; Savage et al. 2009).

Parasite phylogenies and host specificity

The Bayesian phylogenies of the four parasite genera showed that the *Haemoproteus spp.* lineages were clustered according to host family. Additionally, most of the lineages in this genus were present in only one host family. These findings indicate high specificity at host-family level. On the contrary, the lineages for *Plasmodium spp.* and *Trypanosoma spp.* did not seem to cluster based on any level of host taxonomy and most of the lineages were represented by more than one family. This indicates no specificity in these two genera. The results in host specificity indicate that *Plasmodium spp.* and *Trypanosoma spp.* had a higher tendency of host switching than *Haemoproteus spp.* For *Plasmodium spp.* and *Trypanosoma spp.* host-switching seems to occur in any species independently of the family it occurs in, whereas *Haemoproteus spp.* seems to be more conservative and most of the lineages were constrained to host species of the same family. In the study of the host specificity of blood parasites in the Australo-Papuan region, Beadell et al. (2004) found strong host family specificity among the *Haemoproteus spp.* lineages but no specificity in *Plasmodium spp.*

lineages. Studies in other regions like Asia, Africa, Europe and South America had also reported *Haemoproteus spp.* as a genus more host specific than *Plasmodium spp.* (Beadell et al. 2009; Ishtiaq et al. 2007; Merino et al. 2008; Waldenstrom et al. 2002). Phylogeographic studies on *Trypanosoma spp.* revealed no specificity in this genus (Sehgal et al. 2001). The results in host specificity indicate that *Plasmodium spp.* and *Trypanosoma spp.* had a higher tendency of host switching than *Haemoproteus spp.* For *Plasmodium spp.* and *Trypanosoma spp.* host-switching seems to occur in any species independently of the family it occurs in whereas *Haemoproteus spp.* seems to be more conservative and most of the lineages are constrained to host species of the same family. Differences in the host-switching potential between parasite genera can be at least partially attributed to the feeding habits of their vectors. For example, in the case of *Plasmodium spp.*, generalist feeders such as mosquitoes can potentially infect diverse hosts which can partially explain the low specificity found in this genus (Huff and Coulston 1944; Jansen et al. 2009). Biting midges (Culicoides) is one of the described vectors for *Haemoproteus spp.* and it seems to display both generalist and specialist feeding habits (Lassen et al. 2011; Martinez-de la Puente et al. 2011). For *Leucocytozoon spp.,* blackflies (*Simulium* spp.) have been described as potential vectors (Hatcher and Dunn 2011), whereas the most common vectors for the transmission of *Trypanosoma spp.* in birds are louseflies of the family Hippoboscidae (Olsen 1974). However, for these last two genera, little is known about the feeding habits of their vectors. Further research on the specific vectors and feeding behaviour of each parasite genus is needed to probe the hypothesis that differences in the host-switching potential between parasite genera can be at least partially attributed to the feeding habits of their vectors.

Parasites mediate selection of MHC genes

Host MHC diversity and selection was positively correlated to blood parasite prevalence. The results suggest that the stronger the parasite pressure the higher the MHC

allele diversity, average d_N/d_S ratio and number of codons under balancing selection. These patterns were evidently not influenced by other demographic variables such as population size or geographic range size of host species. Additionally, variability at neutral molecular markers did not correlate with any of the MHC diversity parameters, nor with parasite prevalence. In the specific case of MHC allelic diversity, it appears that higher parasite prevalence imposed stronger selective pressure on the host immune system, therefore the higher MHC allele diversity allowed them to tolerate higher parasite prevalence.

Parasite pressure affects not only the bird MHC at the allelic level, but also the selection of these genes. The number of codons under balancing selection and parasite prevalence were positively correlated, indicating that positive selection increases with parasite pressure. However, the number of codons under balancing selection was also correlated with range size. It is possible that species with higher range sizes are exposed to greater parasites burdens and the NCBS can help to recognize these higher parasite pressures.

On the whole, all the main results found here suggest an interaction between temperature, parasites and bird MHC diversity. Temperature and parasite pressure are high in lowlands. This strong parasite pressure on the host immune system could help to promotes higher MHC diversity and selection found in bird species of this region. As elevation increases both temperature and parasite prevalence decreases. The lower temperature of highland areas could help to inhibit the development of parasites, creating a low-parasite environment and hence lower MHC diversity and selection in birds.

One of the mechanisms for PMS is the heterozygote advantage theory, according to this theory, heterozygosity at MHC loci increases the range of parasites that can be recognized by the immune system, so that individuals with high heterozygosity have higher fitness (Doherty and Zinkernagel 1975). A classic example supporting this theory is that heterozygosity of a locus in humans confers protection from persistent hepatitis B virus infection (Thursz et al. 1997). In chickens, heterozygosity seems to confer resistance to Rous sarcoma virus (Senseney

et al. 2000). In natural populations of yellow-necked mice (*Apodemus flavicollis*), populations with larger numbers of different MHC alleles had lower parasite pressure than populations with few different MHC alleles (Meyer-Lucht and Sommer 2009). In this study, I found that lowland bird species with higher parasite pressure have higher MHC allele diversity than upland bird species with low parasite pressure. These apparent contradictory results arise from the fact that in this study different host species are compared rather than individuals within one host species as many studies do. Immune response is a costly life-history trait that needs to be in trade-off with other fitness characters like reproduction, growth and maintenance (Langand et al. 1998). There are energetic cost associated to evolve immune response (Lochmiller and Deerenberg 2000). In theory, immune diversity needs to evolve proportionally to the parasite pressure that each host species had experience along its evolutionary history. Moreover, models of host-parasite coevolution state that host-parasite fluctuations and cyclic coevolution drives genetic diversity of both hosts and parasites and these models predict more diversity where there are higher parasite pressures (Anderson and May 1982). The high parasite pressure that lowland bird species has exposed along its evolutionary history have decisively contributed to its larger MHC diversity, whereas upland species have cope with less parasite pressure. Other comparative studies have found similar results, e.g. in a study of parasites and MHC diversity of Eurasian kestrels (*Falco tinnunculus* and *Falco naumanni*), lower pathogen pressure and less MHC diversity were found in island species than mainland species where both parasite pressure and MHC diversity is higher (Alcaide et al. 2010).

Other mechanism for PMS is the rare allele advantage theory, which proposes that new rare alleles of hosts that confer resistance against parasites will offer a selective advantage and will be favoured by selection (Clarke and Kirby 1966). This mechanism could be acting on each of the host species in this study, but higher sample sizes per species are needed to probe the hypothesis. Finally, fluctuating selection proposes that pathogens can drive MHC

diversity by fluctuation in the intensity of selection they exert. If the pathogen regime faced by an organism varies in space and time, the intensity of selection at MHC genes will also fluctuate. This means that different subsets of MHC alleles will be selected at different points in space and/or time, which can explain genetic diversity across subpopulations (Hill 1991). To probe this theory in our model, long term studies are needed to predict changes of bird MHC diversity and parasite pressure across time. It is possible that the three selection models (heterozygote advantage, rare allele advantage and fluctuating selection) are important to maintain the diversity of bird MHC, they can be having a role at times or in fact the three types of mechanisms may be overlapping.

Implications for infection dynamics in a warming climate

The regression of overall parasite prevalence and temperature documented in this study predicts an increase of about 10% in the prevalence of parasites, for each 1^{o}C increment in temperature. It was discussed that hosts could respond to this in three ways. First, their immune systems could adapt to the higher parasite pressure. However, the life cycles of birds are much longer than those of parasites and rapid adaptation is unlikely. Second, there could be increased mortality rates and/or lower birth rates in host populations, reducing population density. Third, birds could shift their elevational distributions to hold parasite pressure constant. The shifts of host distribution along the elevation gradient that would be required to hold parasite prevalence to current values were determined using parasite prevalence data of this study. For each 1^{o}C increase in temperature, bird distributions would need to ascend 200 m in elevation. Given a 4^{o}C temperature increase, only birds that currently live at 400 m or below would be able to offset increases in parasite prevalence by shifting their distributions upwards; therefore for birds currently living above 400 m, some increase in parasite prevalence would be unavoidable. Under this scenario and according to the results shown here there could also be two main consequences of climate change for parasite lineage richness.

First, the lineage richness will increase with temperature and even when the shifts upward of host distributions may help to compensate these increments, bird species already inhabiting the highest regions will not be able to shift and therefore will experience higher diversity of parasites. Second, *Plasmodium spp.* and *Trypanosoma spp.* which have low specificity may have higher opportunities of host-switching than other more specific parasite genera like *Haemoproteus spp.* Increased parasite pressures are expected to have negative effects on the bird populations of the region, particularly those inhabiting the upland areas and populations unable to shift upwards. It has been predicted in other studies that under impending temperature change many bird species of this region could experience significant range reductions, increased population fragmentation and declines in population size, with upland species particularly susceptible. The predicted increase of parasite prevalence could interact with, and further exacerbate, the projected impacts of climate change on this bird community, leading to an increased risk of extinction for many bird species. However, it is possible that climate change will also affect the distribution of vectors and parasite lineages along the gradient. Vectors and parasite lineages inhabiting specifically upland regions could be also not able to survive due to increases of temperature whereas lowland regions could reach the upper temperature limit for vector development; hence no vectors and parasite lineages in this region could be able to survive. They could also shift upwards to reach optimal temperatures and higher parasite richness could be expected in upland regions.

Different studies in humans and wildlife have demonstrated or predicted the expansion of infection diseases in different parts of the world. Recent studies have demonstrated that the increment of temperature in the past 30 years has already played an important factor in the exacerbation of vector-borne diseases. In a highland region of East Africa, records of malaria cases in humans and temperature since 1970 showed that there has been an increment of the number of cases of malaria and at the same time an approximately 1^{o}C increment in temperature (Alonso et al. 2010). It is well documented that increments of

parasite pressure could have negative effects on the populations of host communities, affecting their growth and fitness causing higher mortality rates (Donovan et al. 2008; Marzal et al. 2005; Norte et al. 2009; Van Riper et al. 1986). Wildlife studies have shown that avian *Plasmodium spp.* occurrence is predicted to expand to northern areas of France in populations of the house sparrow *Passer domesticus* (Loiseau et al. 2013). Garamszegi (2011) has found that the infection rate by *Plasmodium spp.* is strongly associated with temperature and has been increasing with acceleration tendency during the last 20 years.

Main conclusions

- Temperature is one of the main variables driving patters of distribution of avian haematozoa in this avian community. There is a gradient of parasite prevalence/lineage diversity that decrease with elevation.
- Differences of parasite prevalence and diversity along the altitudinal gradient create a parasite pressure gradient in small geographic distances.
- There is high specificity at family level on *Haemoproteus spp.* lineages, whereas *Plasmodium spp.* and *Trypanosoma spp.* did not showed specificity at any hierarchical level.
- Blood parasites are driving selection and diversity of bird MHC genes. MHC diversity and selection were positively correlated to blood parasite prevalence. The stronger the parasite pressure the higher the MHC allele diversity, average and NCBS. It appears that higher parasite prevalence imposed stronger selective pressure in the host immune system, therefore the higher MHC allele diversity and selection allowed them to tolerate higher parasite prevalence.

- There is an interaction between temperature, parasite prevalence/lineage richness and bird MHC diversity and selection. The higher temperature in the lowland areas helps to promote the development of parasites. This strong parasite pressure on the

host immune system promotes higher diversity and selection of MHC genes. As elevation increases both temperature and parasite prevalence decreases. The lower temperature of highland areas helps to inhibit development of parasites, creating a low-parasite environment and hence lower MHC diversity and selection in birds.

- It was predicted an increase of about 10% in the prevalence of parasites for each 1^{o}C increment in temperature, and for each 1^{o}C increase in temperature, bird distributions would need to ascend 200 m in elevation along the altitudinal gradient to avoid higher parasite pressure.
- Shifts upwards of bird distributions along the elevation gradient can help to reduce the impact of increment of parasite pressure in this community. However upland bird communities and populations unable to shift upwards will be susceptible to the consequences of increment of parasite prevalence.

- Upland birds had lower MHC diversity, and there was evidence for more intense selection on MHC genes in lowland birds. Our results suggest that upland birds will be especially susceptible to increased parasitism due to rising temperature unless their immune genes can evolve rapidly in response.

Future research directions

The model system proposed here including temperature, VBD and host MHC genes along altitudinal gradients seems to be promising to study the effects of climate change on host-parasite interactions. Altitudinal gradients provide ideal conditions because elevation cause large changes in temperature which generate differences in parasite pressure over short spatial distances. These conditions promote differences in the diversity and selection of host MHC genes along the altitudinal gradient which facilitates future projections of host-parasite interactions as demonstrated with the avian community of the AWT. Nevertheless, long term studies of both parasites and host MHC genes are needed to improve the models used in this

study. Other studies on the distribution of avian malaria have used long term information to provide better models and predictions about the future distribution of malaria with increased temperature worldwide. For example, recent research on the relationship of malaria (*Plasmodium relictum*) and environmental factors in house sparrow (*Passer domesticus*) in France showed that prevalence of malaria was highly correlated to temperature. Loiseau et al. (2013) predicted that under climate change scenarios, *Plasmodium spp.* occurrence will spread to regions in northern France, and that prevalence levels are likely to increase in locations where transmission already occurs . In a meta-analysis of *Plasmodium spp.* of more than 3,000 bird species, it was found that the infection rate of malaria has increased in parallel with climate change, especially during the last 20 years (Garamszegi 2011). Other examples include the bird community of the Hawaiian Islands. Future projections of the distribution of malaria and its vectors have suggested that increases in temperature will allow the spread of vectors to even higher elevations, increasing the range of parasites and restricting even more the distribution of native species (Benning et al. 2002).

This model system including temperature, VBD and host MHC genes used in this study could be applied to altitudinal gradients of other regions and/or other vertebrate groups. For example, research on bird Haematozoa distribution along altitudinal gradients in Dominican Republic and Madagascar have proved that parasite prevalence tend to decrease with elevation, but these changes along the gradient were attributed to variation in composition of the avian community and temperature was not incorporated as a variable in their models (Latta and Ricklefs 2010; Savage et al. 2009). The relation between parasite prevalence and temperature and other environmental and ecological variables can be tested and then related to bird MHC diversity. Other interesting regions to probe these hypotheses are the altitudinal gradients of the Andes. This region offers a wider range of altitude (100-5000m) than AWT and the low temperature of high altitudes is predicted to inhibit the existence of vector-borne diseases in these areas.

In order to probe some of the suggested hypothesis about the relation of temperature, parasite prevalence and lineage richness and parasite vectors it would be important to develop studies of vectors for each parasite genus: determine specific species of vectors and feeding behavior, their trends of distribution along the altitudinal gradient and their relation with parasite prevalence/lineage richness. The use of PCR techniques in the detection of parasites in vectors can be very useful in this case. Ishtiaq et al (2008) used mosquito samples to identify vectors infected with bird *Plasmodium spp.* and *Haemoproteus spp.* amplifying cytochrome b. However, PCR methods are just a preliminary step toward determine a competent vector. For this, experimental studies infecting vectors with parasites are needed (Valkiūnas and Iezhova 2004; Valkiunas et al. 2013).

Finally, in this study I have proved that research on host-parasite interactions is important to predict the effects of climate change on ecosystems and to better management of wild populations. It is suggested that more studies like the model system used here are needed to fill this gap in current research.

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Appendix I

The full list of frequency of detection of blood parasites.

Haemoproteus spp. (*Hae*), *Plasmodium spp.* (*Pla*), *Leucocytozoon spp.* (*Leu*) and *Trypanosoma spp.* (*Try*) in all the avian species presented alphabetically by family. Number of infected individuals/number of individuals sampled are shown.

Infected species by host	No. infected/sampled	Parasite				
		Hae	*Pla*	Unknown (*Pla* and/or *Hae*)	*Leu*	*Try*
ACANTHIZIDAE						
Sericornis keri	1/27	0	0	0	1	0
Gerygone mouki	1/2	0	0	0	0	1
Gerygone palpebrosa	2/2	2	0	0	0	0
Oreoscopus gutturalis	1/15	0	1	0	0	0
Sericornis magnirostris	10/25	7	1	1	0	1
Acanthiza katherina	0/11	0	0	0	0	0
Sericornis citreogularis	1/29	0	0	0	0	1
ALCEDINIDAE						
Alcedo azurea	0/2	0	0	0	0	0
CLIMACTERIDAE						
Cormobates leucophaeus	0/2	0	0	0	0	0
COLUMBIDAE						
Chalcophaps indica	0/3	0	0	0	0	0
DICAEIDAE						
Dicaeum hirundinaceum	3/3	0	0	0	3	3
DICRURIDAE						
Rhipidura fuliginosa	1/7	0	0	0	0	1
Rhipidura rufifrons	1/14	0	0	1	0	0
Monarcha trivirgatus	7/18	1	0	0	7	0
Machaerirhynchus flaviventer	4/4	3	0	0	0	4
ESTRILDIDAE						
Erythrura trichroa	1/17	0	0	1	0	0
Neochmia temporalis	3/31	1	0	1	2	0
EUPETIDAE						
Psophodes olivaceus	0/1	0	0	0	0	0

MELIPHAGIDAE						
Lichenostomus frenatus	1/10	1	0	0	0	0
Myzomela obscura	2/2	1	0	0	0	1
Acanthorhynchus tenuirostris	1/18	0	0	1	0	0
Meliphaga gracilis	1/3	0	1	0	0	0
Meliphaga lewinii	2/19	0	0	0	2	0
Xanthotis macleayana	5/7	0	0	3	5	1
Myzomela sanguinolenta	1/1	1	0	0	0	0
Phylidonyris nigra	0/6	0	0	0	0	0
Meliphaga notata	2/2	0	1	0	0	1
MUSCICAPIDAE						
Zoothera lunulata	0/1	0	0	0	0	0
NECTARINIIDAE						
Nectarinia jugularis	1/1	0	0	1	0	0
PACHYCEPHALIDAE						
Colluricincla boweri	6/14	6	0	0	0	1
Pachycephala pectoralis	1/6	6	0	0	0	1
Pachycephala simplex	1/2	0	1	0	0	1
Colluricincla megarhyncha	6/10	4	0	1	0	2
PARADISAEIDAE						
Ptiloris victoriae	3/3	3	0	0	3	1
PETROICIDAE						
Heteromyias albispecularis	39/51	31	1	5	0	7
Tregellasia capito	17/22	17	0	0	0	1
PTILONORHYNCHIDAE						
Ailuroedus melanotis	2/4	0	0	0	2	0
Prionodura newtoniana	0/2	0	0	0	0	0
Scenopoeetes dentirostris	0/1	0	0	0	0	0
ZOSTEROPIDAE						
Zosterops lateralis	3/5	2	1	0	0	0
TOTAL	130/403	80	7	15	25	28

Appendix II

The full list of parasite lineages and host species

MalAvi lineage names (http://mbio-serv4.mbioekol.lu.se/avianmalaria), GenBank accession numbers, Parasite genus, Host Family and Host species are indicated.

	Sequence Name	MalAvi lineage name	GenBank accession number	Parasite genus	Host Family	Host species
1	HAE01	TRECAP02	JX021535	Haemoproteus.	Petroicidae	*Heteromyias albispecularis*
2	HAE04	GERPAL01	JX021536	Haemoproteus	Acanthizidae	*Gerygone palpebrosa*
3	HAE05	NEOTEM01	JX021537	Haemoproteus.	Estrildidae	*Neochmia temporalis*
3	HAE05	NEOTEM01	JX021537	Haemoproteus	Dicruridae	*Monarcha trivirgatus*
4	HAE06	PTIVIC01	JX021538	Haemoproteus.	Paradisaeidae	*Ptiloris victoriae*
5	HAE07	TRECAP03	JX021539	Haemoproteus.	Petroicidae	*Tregellasia capito*
6	HAE08	TRECAP04	JX021540	Haemoproteus.	Petroicidae	*Tregellasia capito*
7	HAE09	PTIMAG01	JX021541	Haemoproteus	Paradisaeidae	*Ptiloris victoriae*
8	HAE10	PTIVIC02	JX021542	Haemoproteus	Paradisaeidae	*Ptiloris victoriae*
8	HAE10	PTIVIC02	JX021542	Haemoproteus	Dicruridae	*Machaerirhynchus flaviventer*
9	HAE11	TRECAP06	JX021543	Haemoproteus	Petroicidae	*Tregellasia capito*
10	HAE12	HETALB04	JX021544	Haemoproteus.	Petroicidae	*Heteromyias albispecularis*
11	HAE13	TRECAP07	JX021545	Haemoproteus.	Petroicidae	*Tregellasia capito*
12	HAE14	HETALB02	JX021546	Haemoproteus	Petroicidae	*Heteromyias albispecularis*
13	HAE15	TRECAP01	JX021547	Haemoproteus	Petroicidae	*Tregellasia capito*
13	HAE15	TRECAP01	JX021547	Haemoproteus	Petroicidae	*Heteromyias albispecularis*
14	HAE16	COLMEG02	JX021548	Haemoproteus	Pachycephalidae	*Colluricincla megarhyncha*
14	HAE16	COLMEG02	JX021548	Haemoproteus	Pachycephalidae	*Colluricincla boweri*
15	HAE17	HETALB01	JX021549	Haemoproteus	Petroicidae	*Heteromyias albispecularis*
16	HAE18	ZOSLAT04	JX021550	Haemoproteus	Zosteropidae	*Zosterops lateralis*
17	HAE19	TRECAP08	JX021551	Haemoproteus	Petroicidae	*Tregellasia capito*
18	HAE21	GERPAL02	JX021552	Haemoproteus	Acanthizidae	*Gerygone palpebrosa*
19	HAE24	SERCIT02	JX021553	Haemoproteus	Acanthizidae	*Sericornis magnirostris*
20	HAE26	LICFRE03	JX021554	Haemoproteus	Meliphagidae	*Lichenostomus frenatus*
21	HAE27	MYZSAN01	JX021555	Haemoproteus	Meliphagidae	*Myzomela sanguinolenta*
21	HAE27	MYZSAN01	JX021555	Haemoproteus	Meliphagidae	*Myzomela obscura*
22	HAE28	HETALB03	JX021556	Haemoproteus	Petroicidae	*Heteromyias albispecularis*
23	HAE29	SERCIT01	JX021557	Haemoproteus	Acanthizidae	*Sericornis magnirostris*
23	HAE29	SERCIT01	JX021557	Haemoproteus	Acanthizidae	*Gerygone mouki*
24	HAE30	HETALB05	JX021558	Haemoproteus	Petroicidae	*Heteromyias albispecularis*
25	HAE31	HETALB06	JX021559	Haemoproteus.	Petroicidae	*Heteromyias albispecularis*
26	HAE32	HETALB07	JX021560	Haemoproteus	Petroicidae	*Heteromyias albispecularis*
27	HAE33	HETALB08	JX021561	Haemoproteus.	Petroicidae	*Heteromyias albispecularis*
28	HAE34	TRECAP09	JX021562	Haemoproteus.	Petroicidae	*Tregellasia capito*
29	HAE35	TRECAP10	JX021563	Haemoproteus	Petroicidae	*Tregellasia capito*
30	HAE36	ZOSLAT05	JX021564	Haemoproteus	Zosteropidae	*Zosterops lateralis*

31	PLA01	MELNOT02	JX021565	Plasmodium	Meliphagidae	*Meliphaga notata*
32	PLA02	ZOSLAT06	JX021566	Plasmodium	Zosteropidae	*Zosterops lateralis*
32	PLA02	ZOSLAT06	JX021566	Plasmodium	Acanthizidae	*Acanthiza katherina*
33	PLA03	FANTAIL01	JX021567	Plasmodium	Petroicidae	*Heteromyias albispecularis*
33	PLA03	FANTAIL01	JX021567	Plasmodium	Pachycephalidae	*Pachycephala simplex*
33	PLA03	FANTAIL01	JX021567	Plasmodium	Acanthizidae	*Sericornis magnirostris*
34	PLA04	OREGUT01	JX021568	Plasmodium	Acanthizidae	*Oreoscopus gutturalis*
34	PLA04	OREGUT01	JX021568	Plasmodium	Meliphagidae	*Meliphaga lewinii*
35	PLA05	MELGRA01	JX021569	Plasmodium	Meliphagidae	*Meliphaga gracilis*
36	LEU01	XANMAC01	JX021570	Leucocytozoon	Meliphagidae	*Xanthotis macleayana*
37	LEU02	XANMAC02	JX021571	Leucocytozoon	Meliphagidae	*Xanthotis macleayana*
38	LEU03	MELLEW02	JX021572	Leucocytozoon	Meliphagidae	*Meliphaga lewinii*
39	LEU06	AILMEL01	JX021573	Leucocytozoon	Ptilonorhynchidae	*Ailuroedus melanotis*
39	LEU06	AILMEL01	JX021573	Leucocytozoon	Paradisaeidae	*Ptiloris victoriae*
40	LEU07	PTIVIC03	JX021574	Leucocytozoon	Paradisaeidae	*Ptiloris victoriae*
41	LEU08	PTIVIC04	JX021575	Leucocytozoon	Paradisaeidae	*Ptiloris victoriae*
42	TRY01	HEAL01	JX021576	Trypanosoma	Petroicidae	*Heteromyias albispecularis*
43	TRY03	HEAL02	JX021577	Trypanosoma	Petroicidae	*Heteromyias albispecularis*
43	TRY03	HEAL02	JX021577	Trypanosoma	Paradisaeidae	*Ptiloris victoriae*
43	TRY03	HEAL02	JX021577	Trypanosoma	Dicruridae	*Machaerirhynchus flaviventer*
44	TRY04	RHFU01	JX021578	Trypanosoma	Dicruridae	*Rhipidura fuliginosa*
45	TRY06	SEMA01	JX021579	Trypanosoma	Acanthizidae	*Sericornis magnirostris*
45	TRY06	SEMA01	JX021579	Trypanosoma	Petroicidae	*Heteromyias albispecularis*
45	TRY06	SEMA01	JX021579	Trypanosoma	Petroicidae	*Tregellasia capito*
45	TRY06	SEMA01	JX021579	Trypanosoma	Pachycephalidae	*Colluricincla megarhyncha*
45	TRY06	SEMA01	JX021579	Trypanosoma	Pachycephalidae	*Colluricincla boweri*
45	TRY06	SEMA01	JX021579	Trypanosoma	Meliphagidae	*Xanthotis macleayana*
46	TRY06	MENO01	JX021580	Trypanosoma	Meliphagidae	*Meliphaga notata*
47	TRY08	PAPE01	JX021581	Trypanosoma	Pachycephalidae	*Pachycephala pectoralis*
47	TRY08	PAPE01	JX021581	Trypanosoma	Pachycephalidae	*Pachycephala simplex*
48	TRY10	HEAL03	JX021582	Trypanosoma	Petroicidae	*Heteromyias albispecularis*
49	LEU04	MELLEU03	KF811172	Leucocytozoon	Meliphagidae	*Meliphaga lewinii*
50	TRY05	COME01	KF811173	Trypanosoma	Pachycephalidae	*Colluricincla megarhyncha*
50	TRY05	COME01	KF811173	Trypanosoma	Pachycephalidae	*Colluricincla boweri*
50	TRY05	COME01	KF811173	Trypanosoma	Meliphagidae	*Meliphaga lewinii*
51	TRY09	MELE01	KF811174	Trypanosoma	Meliphagidae	*Meliphaga lewinii*

Appendix III

MHC genes of Acanthizidae and Meliphagidae

Amino-acid sequence alignment of MHC class II B alleles in A) putative translated sequences and B) putative pseudogenes for the fifteen species of Acanthizidae and Meliphagidae. Identity with the Meno1.5 sequence is shown with dots. Identity with the Meno1.1 and Meno1.5 sequences are shown with dots. Identified codons under balancing selection are indicated with asterisks. Codons under balancing selection in human DRB1 genes (Brown *et al.* 1993) are indicated with crosses.

A)

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                                *** * *     ***       **      *  ** ** *  ***** * ****  *
                                   + +  +    ++         +         +   + +   +  + + +  +
                                     10         20         30         40         50
                                ----|----| ----|----| ----|----| ----|----| ----|----| ----|--
Meno1.1                         TEKVRFLARF IYNQVEFARF DSDVGKYVGL TPYGEKPAQR WNSDPNILEY AQTAVDR
Meno1.3                         .....YME.Q M..R..YM.. ......F..F ..F..RN.E. A..N.SWMD. KRAS...
Meno1.4                         .....YME.Q M..R..YM.. ......F..F ..F..RN.E. A..N.SWVD. KRAS...
Meno1.8                         .....YVQ.Y ...R..YL.. ...M..F..F ..H...N.E. A.....L..N D.....W
Meno1.10                        .....AVD.Y ...R..YL.. .......E.F ......N.E. .....GK... QEASL.T
Meno2.1                         ......VE.Y ...RL.DV.. .........F ......C..D ....EAWM.N TR....W
Meno2.2                         .....VTE.H ...R..YM.. .......... ..F...V..D ...L.DYM.N RR....W
Meno2.3                         ....M.VV.A ...RL.DV.. .......E.F ......N.E. .....GK... QEASL.T
Meno2.7                         .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH ....LEW... RRA....
Meno2.8                         .....L.D.Y ...R..LV.. ......F..F ......V.KY .....TR... L.A...T
Meno2.11                        .K....VE.Y ...RL.DV.. ......H..F ......C..D ....EAWM.N TR....W
Meno2.12                        .......D.Y ...RL.DV.. .......... .........C .......... .......
Meno3.3                         .....YVE.Y ...R..YL.. ......F..F ..A...N.E. A.....L..K D.....W
Meno3.4                         .D...YVH.Y ...R..LV.. .........F ..L...W..D ....EAGM.. RRAQ..T
Meno3.5                         .....YVQ.Y ...RL.DV.. .........F ..L...W..D ....EAGM.. TR....T
Meno4.1                         ....KL.Q.Y ...R..LV.. .......L.F ..L...Q.KY L....TS... L.A...T
Meno4.2                         ......VV.A ...G..YM.. ......F..F ..F..RN.E. A..N.SWMD. KRAS...
Meno4.3                         .....YVH.Y ...R...V.. .........F ..L.V.W.KY ...N.....I K.AE..T
Meno4.5                         ......VE.Y ...RL.DV.. .........F ..F..RN.E. A..N.SWMD. KRAS...
Meno5.4                         ......VQ.L ...R..Y... .....HF.AF ..H...R.RD ...Q.EWM.N LR....W
Meno5.5                         ......VGSA ...R...M.. .........F ..F...N.E. A....D...H .RSL..T
Meno2.5                         .......D.Y ...RL.DV.. .........F ......C..D .....DG... KR.....
Meno6.1                         .......... .....G.... .......... .........R .......... .......
Meno6.2                         .....YME.Q M..R..YM.. ......F..F ..F..RN.E. A..N.SWMD. KRAS..S
Phni1.1                         .......D.Y ...R..W... ......H..F ......N.EY ...LRDYM.S RR....W
Phni1.14                        .....L.Q.L ...RM.Y... .........F .QA...W..D .....D.M.R TRAE..L
Phni1.16                        .....L.Q.Y ...R..W... ......FL.F .....YN.E. .....SWM.. QR.....
Phni1.2                         .....AVQ.Y ...R..W... ......FL.F .....YN.E. .....SWM.. QR.....
Phni1.4                         .......D.Y ...R..W... ..N...F..F ..F..YN.E. ...G.SWM.. KR....T
Phni1.6                         .....LVQ.Y ...R..W... ......H..F ......N.EY ...LRDYM.S RR....W
Phni1.7                         .....L.Q.L ...RM.Y... .....F..F .....YN.E. .......... RRA....
Phni1.8                         .......E.R ...R..Y... .........F ..H..EV.K. L.....R... .......
Phni1.9                         .......D.Y ...R..W... ..N...F..F ..F..YN.E. .....EW... RRA....
Phni2.1                         .......D.Y ...R..DV.. .........F ......V.EN ...QADYMQ. TRA...T
Phni2.2                         ......ME.Q M..R..YL.. ......F..F ..F..YN.E. L.N..SWM.. KR.....
Phni2.3                         .....YME.Q M..RL.HL.. ......F..F ..A...N.E. L...QAWM.. QR....T
Phni2.4                         .....YME.Q M..R..YL.. ......F..F ..F...N.E. L...QARM.I KRAE..T
Phni2.5                         .DG...VK.. ...REQYVH. ......F..F ..F...N.E. L...QAWM.I KRAE..T
Phni3.2                         ......VQ.K ...R..Y... .........F ..F...Q.EY ....EAGM.. KRA....
Phni3.3                         ......VN.L L..R..Y... .....H...F ......Q..Y R..K.DYM.H .R.....
Phni3.4                         ......ME.Y ...R..N... ......F..F .....YN.E. .....D.M.I KRAE...
Xama1.1                         ......V..T ...R..YR.. .........F ......Q.EY G..N.D...N ......W
Xama1.4                         .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. RRA....
Xama1.5                         .....L.D.Y ...R..Y... ......H..F ..A...V.KK ...N.D.... KR....T
Xama1.7                         .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. RRV....
Xama1.8                         .DGA..VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. RRA....
Xama1.9                         .DG...VK.. ...REQYVH. ..V..QF..D ..F...V.RH .....EW.D. RRA....
Xama1.10                        ..R...VK.. ...REQYVH. .....QF..D ..F...V.RY R..N.EWM.. RRA....
Xama2.2                         .DGA..VK.. ...REQYVH. .....QF.RD ..F...V.RH .....EW... RRA....
Xama2.3                         ......V..T ...R..YR.. .........F ......Q.KY R..N.D...N ......W
Xama2.4                         .DGA..VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. RRA....
Xama2.5                         .DGA..VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW... RRA....
Xama2.6                         .......Q.Q ...R..YL.. ......L..F ......M.LY ..R.RDE..H R.....W
Xama3.1                         .....YVQ.Y ...R..EV.. .........F ..F...N.R. L.NN.DR..D Q.AS..T
Xama3.2                         .....Y.E.Q M..R..YL.. ......F..F ..F...N.K. L...LSWM.I KRAE...
Xama3.3                         .DGA..VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. RRA....
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                                  *** * *    ***       **      *  ** ** * *  ***** * **** *
                                    + +  +    ++         +         +   + + +  + + + + +
                                      10         20         30         40         50
                                  ----|----| ----|----| ----|----| ----|----| ----|----| ----|--

Xama3.4                           .......Q.Y ...R..YV.. ......H..F ..F...V.KY F..LSDFM.I KRAE..T
Xama4.2                           .DG...VK.. ...REQYVH. .....QS..D ..F...V.RH .....EW.D. RRV....
Xama4.3                           .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH ...G.EW.D. RRA....
Xama4.4                           .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. GRA....
Xama4.5                           .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW... RRA...G
Xama5.1                           A.....V..T ...R..YR.. .........F ......Q.EY G..N.D...N ......W
Xama5.2                           .DG...VK.. ...REQY.H. .....QF..D ..F...V.RH .....EW.D. RRA....
Xama5.3                           ....TSVD.Y ...R..YL.. .........F ..L...N.R. L....D.... Q..SL.T
Xama6.1                           .DGA..VK.. ...REQCVH. .....QF..D ..F...V.RH .....EW... RRA....
Xama6.2                           .....YVH.C V..R..Y... .....H...F ..G..EA.RY ...V.DYM.G KR....W
Xama6.4                           .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. RRA....
Meno1.11_Mele1.2                  ......V..S ...R..L... .........F ......QGKY ....LDFM.S TR.S..W
Mele1.5                           .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH ....LEW... RRA....
Mele2.10                          .K....VE.Y ...RL.DV.. .........F ......C..D ....EAWM.N TR....W
Mele2.11                          ......ME.H ...R...M.. .........F ..F...N.E. .....DYM.S RR.....
Mele2.12                          .......... ...R...... ....W..... .......T.. .......... .......
Mele2.13                          .......D.Y ...R..HV.. ......F..Y ..F...V.KC ..N.EAWM.N TR....W
Mele2.3                           ..R..YME.Q M..R...V.. ......F..F ..F..RN.E. A..N.SWMD. KRAS..W
Mele2.4                           ......V..S ...R..L... .........F ......WSRD R....DFM.S RR....W
Mele2.5                           .....LVV.N ...R..Y... .........F .L....WSRD R...LDFM.S TR.S..W
Mele2.6                           .....LVV.N ...R..Y... .........F ......WSRD R....DFM.S RR....W
Mele2.7                           ......VQ.A ...R..YV.. ......H..F ......V.KY ....QDFM.S TR.S..W
Mele2.8                           .......D.. ...R..DV.. ......F..F ......V.EY ...L.DFM.G TRAE...
Mele2.9                           ......ME.Y ...R..DM.. ...L.....F ..F...Q.EY ....ETGM.H TR.....
Mele3.1                           ....KL.Q.Y ...R..LV.. ......H..F ......V.KC .....DYM.S RR.S..W
Mele3.2                           ......VQ.A ...R..YV.L .....QF..D ..F...V.RH ....LEW... RRA....
Mele3.4                           ....M.VV.A ...G..GV.. ......S..F ......V.EY ...L.DFM.G TRAE...
Mele3.5                           ......VQ.A ...R..YV.. ......H..F ......V.KC .....DYM.S RR.S..W
Mele3.6                           .......... ...R...... .......... .......... ......... .......
Mele3.7                           ....KL.Q.Y ...R..L... .........F ......QGKY ....LDFM.S TR.S..W
Mele4.1                           ......VE.H ...REQ.VM. .....V.E.F ..LV..V.RH R.NN.ERM.. .R....W
Mele4.2                           .....H.D.Y ...R..Y... .........F ..F...N.EC .........R ..AE..T
Mele4.3                           .....YVH.Y ...R..LTM. .........F ..L.V.W.KY ...N.....V K.AE..T
Mele4.4                           ......VE.Y ...REQ.MM. .....V.E.F ..L..RN.K. F..N.EWM.. .R....N
Mele4.5                           .....YVQ.Q ...R..L... .........F ......C.EY ...LADFM.S IR.S..W
Mele4.6                           K.R..YME.Q M..REQ.VM. .....V.E.F ..LV..V.RH R.NN.ERM.. .R....W
Mele5.1                           ....M.VV.A ...G..GV.. ..N...H..F ......V.KC .....DYM.S RR.S..W
Mele5.10                          ..R..YME.Q M..R..L... .........F ......QGKY ....LDFM.S TR.S..W
Mele5.2                           ......V..S ...R..L... .........F ......QGKY ......L..N D.....W
Mele5.3                           ......V..S ..HR..L... H........F ......QGKY ....LDFM.S TR.S..W
Mele5.5                           .DG....K.. ...REQYVH. H....PF..D ..F...V.RH ....LEW... RRA....
Mele5.7                           ......VE.Q M..R..YL.. .........F ..A...N.E. A.....E... RRAR..T
Meno1.2_Mele5.8                   .....YVT.Y ...R..DL.. .........F ..A...N.E. A.....E... RRAR..T
Megr1.1                           .....L.Q.Y ...R..YL.L .......L.F ..F...L.EY ..TN..L... V..E...
Megr1.2                           .......D.Y ...R..YMK. ......F..F ..F...N.E. A.....V..H ....L.W
Megr1.3                           ......VE.H M..R..HL.. .........F ..F...N.E. A...EARM.. IRAQ..T
Megr1.4                           .....L.Q.Y ...R..YL.L G......L.F ..F...N.E. A.....V..H ....L.W
Megr1.5                           .DG...VK.Y ...REQYVH. .....QF..D ..F...V.RH ....LEW... RRA...S
Megr1.6                           .....YVH.Y ...R..LTM. .........F ..L.V.W.EY ..TN.....I K.AE..T
Megr1.8                           .....L.Q.Y ...R..YL.L .......L.F ..F...L.EY ..TN..L... V..E...
Megr1.9                           .....L.Q.Y ...R..YL.L .......L.F ..F...L.EY ..TN..L... V..E...
Megr2.1                           .....YVH.Y ...R..LTM. .........F ..L.V.W.KY ...N.....M K.AE..T
Megr2.10                          ...E.Y.D.Y ...RL.DV.. .........F ..A...W..D ....EAGM.. TRAV..T
Megr2.2                           .....YVH.Y ...RL..L.. .........F ......W.ED .....TS... L.A...T
Megr2.3                           .......D.H ...R..YL.. .........F ..F...N.E. A.....V..H ....L.W
Megr2.4                           ......VE.Q M..RL.LL.. ......F..F .....EN.KP V....SWM.. KRGE...
Megr2.5                           .....L.Q.Y ...R..YL.L .....QF..D ..F...L.EY ..TN..L... V..E...
Megr2.6                           .....L.Q.Y ...R..YL.L .......L.F ..L...W.KY .....AWM.D QR....T
Megr2.7                           .......D.Y ...R..YV.. .........F ......W.ED .....TS... L.A...T
Megr2.8                           .....YVH.Y ...R..LTM. ......H..F ..L...W.KH .....AWM.D QR....T
Megr2.9                           .....YVH.Y ...R..LTM. .........F ..L.V.W.KY ...N..L... V..E...
Megr3.1                           .....L.Q.Y ...R..YL.L .......L.F ..F...L.EY ..TN..L..N D..E...
Megr3.2                           ..N..LVV.N ...R..Y... .........F ..F...L.EY ..TN..L... V..E...
Megr3.3                           ......VG.Y ..SRLQYLM. .....E...F .....EF.RQ A....EWM.H KRGQ..N
Megr3.4                           .....LVV.N ...R..Y... ......H..F ......Q.EH .....TR... R.AE..T
Megr3.5                           .......D.Y ...R...... .........F ......Q.EY ....EAGM.. TRAE...
Megr3.6                           .....L.Q.Y ...R..YL.L ......N..F ..D...Q.EY .........N D.....W
Acte1.1                           .......E.H ...R..Y... .........F ..P...W.KN L...EARM.. IR....W
Acte1.2                           ......VQKY ...R..Y... .........F ..P...W.KN L...EARM.. IR....W
Acte1.3                           ......VQKY ...R..Y... ......F..F ..F...V.KN ....EAWM.N RRAE...
Acte1.4                           .DR...VK.. ...REQYVY. .....QF..D ..F...V.RH .....EW... RRA....
Acte1.5                           ......VQKY ...R..Y... ......F..F ..F...V.KN ....EAWM.N TRA...A
Acte1.6                           .......D.Y ...R..DV.. ......F..F ......V.NY ...L.DYM.S RR....W
Acte2.1                           .......E.H ...R..HV.. ......F..F ..F...V.KN V...EALM.. KR.....
Acte2.3                           .....YVQKY ...R...... ......F..F ..F..IQ.KY ....EAGM.. RRAE...
Acte2.4                           .....YVQKY ...R...... ......F..F ..F..IQ.KN ....EAQI.. RRAE...
Acte3.2                           .......D.Y ...R..Y... ......F..F ..F...V.KN ....EAWM.N TRA....
Acte3.3                           .......E.R ...R..DV.. .........F ..F...V.NY R..L.DFM.R KR.S..W
Acte3.4                           .......E.R ...R..Y... ......F..F ..F...V.KN ....EAWM.N TRA...T
Acte3.6                           .......D.Y ...R..YV.. ......F..F ..F...V.KN ....EAWM.N TRA....
Acte4.10                          ......AM.S ...R...... .........F ..F...V.KN L...EAWM.. IR.....
Acte4.2                           .....L.D.Y ...R..DV.. .........F ..P...W.KN ....EARM.. TR.....
Acte4.3                           .......D.Y ...R...... .........F ..F...V.KN ....EAWM.N RRAE...
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                                  *** * *     ***       **      *  ** ** *  ***** * **** *
                                    + +  +    ++         +         +   + +   +  + + +  +
                                      10          20          30          40          50
                                  ----|----| ----|----| ----|----| ----|----| ----|----| ----|--
Acte4.4                           .......E.H ...R..HV.. ......F..F ..F...V.KN V...EAQI.. RRAE...
Acte4.5                           .......D.Y ...R...... .........F ......V.KN ....EAGM.. KR....W
Acte4.6                           .......D.Y ...R..Y... ......F.RF .....IQ.KN ....EAGM.. KR....W
Acte4.7                           .......D.Y ...R...... .........F ..F...V.KN L...EAWM.. IR.....
Acte4.9                           .......D.Y ...R...... ......F..F ..F...V.KN V...EAQI.. R.AE...
Acte5.2                           .......E.Y ...R..HV.. .........F ..F..IQ.KY ...L.DVM.S RR....W
Acte5.3                           .......D.Y ...R...... .........F ......V.KN L...EAWM.N TRA...A
Acte5.4                           .....YVQKY ...R...... ......F..F ..F..IQ.KY ...L.DVM.S RR....W
Acte5.5                           .......E.Y ..DR..Y... .........F ..P...W.KN L...EARM.. IR....W
Acte5.8                           .....L.D.R ...R..Y... .........F ..R...V.KN L...EAWM.. IR....W
Acte5.9                           .....YVQKY ...R..HV.. ......F..F ..F..IQ.KN ....EAQI.. RRAE...
Myob1.3                           .....YMQ.Y ...R..Y... ......F..F ......N.KY ...N.....D Q.AS..T
Myob1.4                           .....Y.D.Y ...R..Y... ......H..F ......Q.EY ...N...... KRGQ..T
Myob1.5                           .....YME.Q M..R..YV.. .........F .LH...W.KK .....AWM.F KRAE...
Myob1.6                           .......D.Y ...R..YM.. ......F..F .....MN.K. L...L.R... L.A...T
Myob1.8                           .DG...VK.. V..REQYVH. .....QF..D .LF...V.RH .....EW... RRA....
Myob2.1                           .....YM..A ...R...L.. .N....F..F ..F...V.KN ......H..H ..AS...
Myob2.10                          .DG...VK.. V..REQYVH. G.....F..F .....MN.K. L...L.R... L.A...T
Myob2.11                          .....L.D.H ...R...... ......F..F .....MN.K. L...L.R... L.A...T
Phni1.12_Myob2.12                 .......D.Y ...R..Y... ......F..F ..L...W.EY ...L.DYM.. NRGV..T
Myob2.13                          .......D.Y ...R...V.. .........F ..L...W.KN .....TWMDN RRAQ..T
Myob2.14                          .......D.Y ...R..Y... .N....F..F .....MN.K. .........D E.A...T
Myob2.2                           .....L.Q.Y ...R..L... ......F..F .....IQ.KY ......R... L.A...T
Myob2.3                           .....L.D.H ...R...... .........F ..V...E... L..Q.DYM.R VR....W
Myob2.4                           .......D.Y ...R..Y... ......H..F ......Q.EY ...N...... KRGQ..T
Myob2.6                           ......VD.N ...R..QR.. .........F ..A...W.KN L........N Q.....W
Myob2.8                           .......E.Y ...RM..V.. .........F ..A..IQ.K. L.....R... ......W
Megr1.7_Myob2.9                   ..T..L.QKL ...R..YT.. ...G..F..F ......Q.EY ....QAWV.. L.A...T
Myob3.1                           .......E.R ...R..Y..L .........F ..H...V.K. L.....R... .......
Myob3.2                           .....Y.D.Y ...RM.DV.. ......H..F .....IQ.KY ...N..L..D Q.A....
Myob3.3                           .....L.D.H ...R...... .........F ..F...E... L..Q.DYM.R VR....W
Myob3.4                           .....YM..A ...R...L.. .N....F..F ..F...V.KN ......H..H .RAS...
Myob3.5                           .....YMQ.Y ...RM.DV.. .........F ..A...W..D .....DE.D. RRAQ..T
Lifr1.1                           .DG...VK.. .H.REQYVH. .....QF..D ..F...Q.EY ...LADFM.L TR....W
Lifr1.2                           .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW... RRA....
Lifr1.3                           M.....VV.A ...G..GV.. ..N...H..F ......W..H ...NA.EM.. KMGSGGH
Lifr1.6                           .....L.D.Y ...R..Y... ......H..F ......Q.EY ...LADFM.L TR....W
Lifr2.1                           ......VE.H ...RM.LM.. .........F ......V.EY ...L.DYM.. NRGV..T
Lifr2.2                           .....YVV.Y ...RA.YVH. .....QF..D ..F...V.RH .....EW... RRA....
Lifr2.3                           ......VQ.T ...CM.GV.. ..N......F I.....W.KY L..NA.EM.N RWAQ..T
Lifr3.1                           .....YVV.Y ...RA.YV.. .........F ......V.EY ...L.DYM.. NRGV..T
Lifr4.1                           ......VE.H ...RM..M.. G........F ..F..EQ.EY ...L.DYM.. NRGV..T
Lifr5.1                           ......VE.H ...R...L.. .....H...F ..F.ATQ.RH F..KSDYV.. K.DQ..N
Seci1.1                           ..R...VK.. ...REQYVH. .....YFL.D ..F...V.RY ...H.EWM.. RRA....
Seci2.2                           ......VE.D ...R..Y... .....H...F ..D...N.RY ...L.DYM.D RRAL..T
Seci2.3                           ......VE.H ...R...L.. .....H...F ..F.ATQ.RH F..KSDYV.. K.DQ..N
Seci2.6                           ..R...VK.. ...REQYVH. .....YFL.D ..F...V.RY ...H.EWM.. RRA...W
Xama6.5_Seci4.1                   ..N...V..H T..R..YV.. .....HF..F ..H...T.RY ...N.D.M.. HRGV..T
Seci5.1                           ..N...V..H T..R..YV.. .....HF..F S.F...Q.RY ...L.DYM.D RRAL..T
Seci6.1                           .......D.L ...R...... ......F..F ..R...V.KN L...EAQM.. TR....W
Seci6.2                           ......VE.D ...R..Y... .....H...F ..D..EA.RY ...V.DYM.G KR....W
Seci6.3                           .DG...VK.. ...REQYVH. .....H...F ..D...N.RY ...L.DYM.D RRAL..T
Seci6.4                           ..R...VK.. ...REQYVH. .....YFL.D ..F...V.RH .....EW... RRA....
Seci10.2                          ......VE.Q F..R..YT.. .....HFE.F ......Q..Y R..L.DFM.N RR....W
Lifr1.5_Seci10.3                  .....YVV.Y ...RA.YV.. ......H..F ......Q.EY ...LADFM.L TR....W
Seci11.1                          .....YVV.Y ...RA.YV.. ......H..F ......R.EY ...LADFM.L TR....W
Seci10.1                          .....LVD.Y ...R..QL.. .....HF... ..F...Q.RH F..LQDFM.I KRAE..T
Seci7.1                           .......D.Y ...R..YMK. ......F..F ..F...N.E. A.....V..H ....L.W
Seci7.2                           ..N...V..H T..R..Y... .....H...F ..D...N.RY ...L.DYM.D RRAL..T
Seci7.3                           ..N...V..H T..R..YV.. .....HF..F .SF...V.RY ..NN.EWM.. KRGQ..N
Seci7.4                           ......VE.H ...REQLVM. .....V.E.F ..L...V.RH R.NN.ERM.. .R....W
Seci7.8                           ....K.VE.Y ...RQPY.M. .....VFE.F .SF...V.RY ..NN.EWM.. KRGQ..N
Seci8.1                           ......VE.D ...R..Y... .....HF..F S.F...Q.RY ...N.D.M.. HRGV..T
Lifr1.4_Seci9.1                   .....L.QKL ...R..YT.. ..N...F..F ......Q.KY ....QTWV.V L.A...T
Seci7.6                           .......D.Y ...R..Y... ......F..F .....IQ.KY ....EAGM.. KR....W
Acka10.1                          .....YVE.D ...R..Y... .....H...F ..F...A.RY ...K.DYM.I KRAE..T
Myob1.2_Seci1.3_Acka10.3          .....YVH.C V..R..Y... .....H...F ..D..EA.RY ...V.DYM.G KR....W
Acka11.1                          .A....VYSN ...R..L... .....HF..F ..F...Q.RD ...Q.DFM.. TR....W
Acka11.2                          ..E...VE.H ...R...M.. .....HF..F ..L...N.WN L..Q.EQ... K.GQ..N
Acka11.3                          .......E.H ...R..QVT. .....HF..F ..F...Q..Y ...K.EW..D RRAQ..T
Acka11.5                          .....YVH.C V..R..Y... ..G..H...F ..D..EA.RY ...V.DYM.G KR....W
Acka2.1                           .....YVH.R ...R...... .....H...F ......Q..Y ...N.D.M.I KRAE..T
Acka3.1                           ......VN.L L..R..YT.. .....HF..F ......Q..Y R..L.DFM.N RR....W
Acka3.10                          .....YVH.V T..R..YM.. .....H...F ..H...T..H ..R..DFM.. LR....T
Acka3.11                          ......VY.R M..R..Y... .....H...F ..H...T..H ..R..DFM.. LR....T
Acka3.12                          ......VY.R M..R..Y... .....H...F ......Q..Y ...N.D.M.I KRAE..T
Acka3.15                          ......VE.Q F..R..YT.. .....H...F ......Q..Y R..K.DYM.. .R.....
Acka3.2                           .....LVE.H ...R...L.. .....H...F ......Q..Y R..K.DYM.. .R.....
Acka3.3                           .....LVE.H ...R...L.. .....H...F ..H...Q.RY S..Q.EL... RRAQ..T
Acka3.4                           ......VN.L L..R..Y... .....H...F ......Q..Y R..K.DYM.. .R.....
Acka3.5                           .....YVH.R ...R...... .....H...F ......Q..Y R..L.DFM.N RR....W
Acka3.6                           .....YVH.V T..R..YM.. .....H...F ..H...N..C ......YM.S KR....W
Acka3.7                           ......VY.R ...R...... .....H...F ......Q..Y ...N.D.M.I KRAE..T
Acka3.8                           ......VE.H ...R..VV.. .....HF..F ..F...Q.RY L.NI.GW..N RRAL..T
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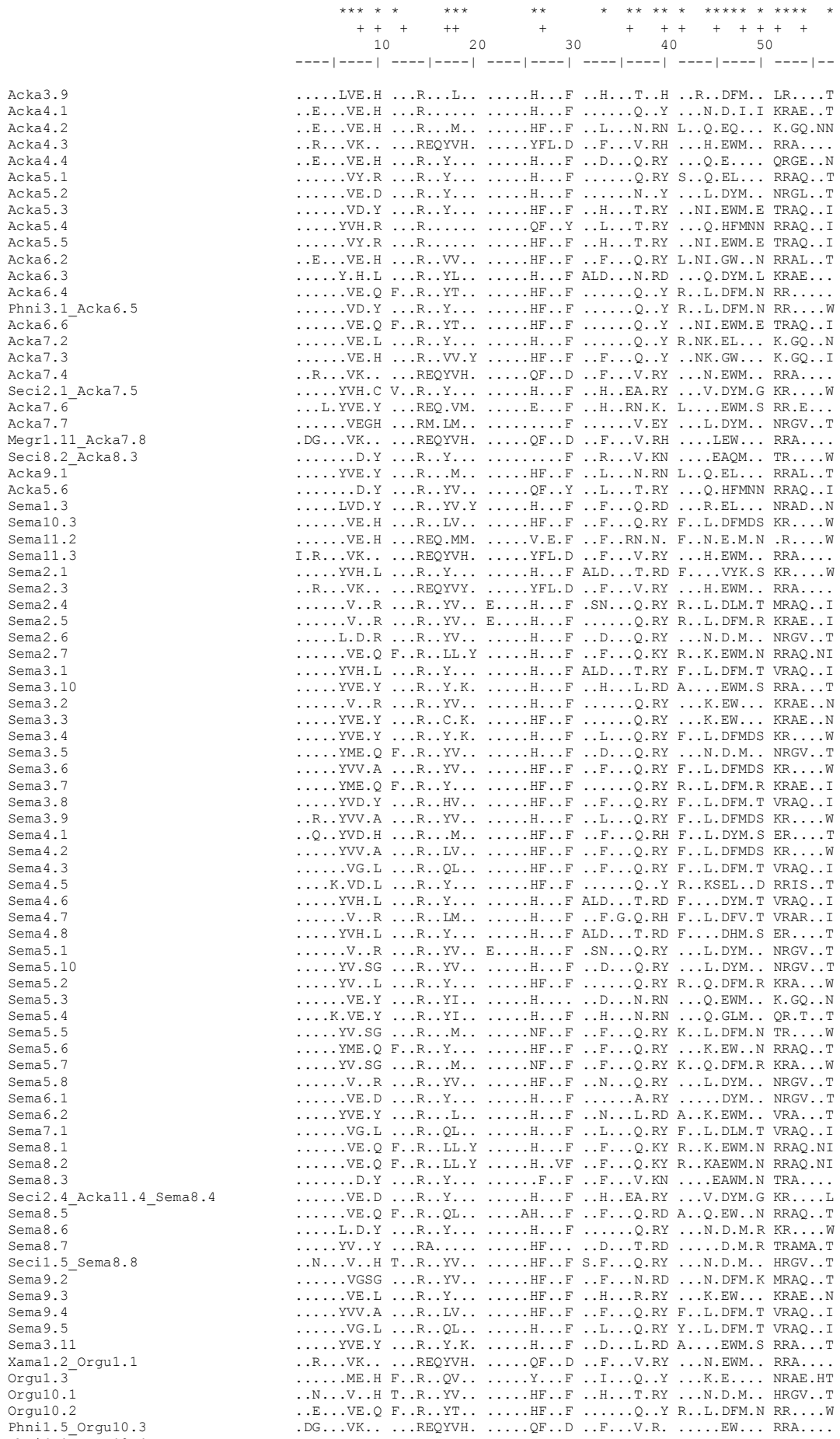
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Acka3.9
Acka4.1
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Acka6.3
Acka6.4
Phni3.1_Acka6.5
Acka6.6
Acka7.2
Acka7.3
Acka7.4
Seci2.1_Acka7.5
Acka7.6
Acka7.7
Megr1.11_Acka7.8
Seci8.2_Acka8.3
Acka9.1
Acka5.6
Sema1.3
Sema10.3
Sema11.2
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Sema5.8
Sema6.1
Sema6.2
Sema7.1
Sema8.1
Sema8.2
Sema8.3
Seci2.4_Acka11.4_Sema8.4
Sema8.5
Sema8.6
Sema8.7
Seci1.5_Sema8.8
Sema9.2
Sema9.3
Sema9.4
Sema9.5
Sema3.11
Xama1.2_Orgu1.1
Orgu1.3
Orgu10.1
Orgu10.2
Phni1.5_Orgu10.3
Phni4.1_Orgu10.4

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                                      *** * *     ***       **      *  ** ** * *  ***** * **** *
                                        + +  +    ++         +         +   + +   +  + + +  +
                                          10         20         30         40         50
                                      ----|----| ----|----| ----|----| ----|----| ----|----| ----|--

Orgu3.1                               .....YVH.C V..R..Y... .....H...F ..D..EA.RY ...V.DYM.G KR..A.W
Sema1.1_Orgu3.3                       .....YVY.D V..R..LV.. .....H...F ......Q.KH R..Q.EL..H RR....W
Meno5.1_Orgu3.4                       ......VE.H ...REQ.MM. .....V.E.F ..L..RN.N. F..N.E.M.N .R....W
Acte2.5_Orgu3.5                       .......E.H ...R..DV.. ......F..F ......V.NY ...L.DVM.S RR....W
Orgu3.6                               ......VE.H ...REQ.MM. .....V.E.F ..L..RN.K. V..N.E.M.N .R....W
Orgu4.1                               ......ME.H F..R..QV.. .....Y...F ..I...Q..Y ...K.E.... NRAE.HT
Orgu9.2                               ......VE.H ...REQIMM. .....V.E.F ..L..RN.K. V.NN.ERM.. .R....W
Orgu5.1                               .....LVD.H ...RLQYVM. .....E...F ......F.RK L....EWM.N IR....W
Orgu7.1                               ......ME.H F.KR..QV.. .....Y...F ..I...Q..Y ...K.E.... NRAE.HT
Orgu8.1                               ..R...VK.. ...REQYVH. .....QF..D ..F...V.RY ...N.EWM.. GRA....
Seci2.5_Orgu9.3                       .....YVH.C V..R..Y... .....H...F ..D...N.RY ...L.DYM.D RRAL..T
Orgu9.4                               ....KLV..G ...R..LI.. .......E.F ..LW..N.E. ......V... ......T
Orgu9.5                               .DG...VK.. ...REQYVH. .....QF..D ..F...V.RH .....EW.D. GR.....
Orgu9.6                               .....YME.Q M..R..YL.. ......F..F ..F...N.E. L...QAWM.I KRAE..T
Orgu9.7                               ....TSVD.Y ...R..YL.. .........F ..P...N.R. L....D.... Q..SL.T
Gepa3.7                               ......VH.R V..R..YM.. .....H...F ..D..NA..Y .....VYM.R KR....W
Gepa2.7                               ......VD.Y ...R..LM.. .....H...F ..F...N.RN A..Q.EL... MR....W
Meno5.7_Gepa1.2                       ......V..E ...RM.QL.. .....DF..F .SL...Q.RY LSRN.DYM.R LRAQ..I
Xama1.3_Gepa1.3                       .....YVE.Y ...R..YM.. .....H...F ..H...N.RN A..K.EWM.. VR....T
Gepa1.6                               .....YVE.H ...R..YM.. .....H...F ..D..NA..Y .....VYM.. NRGV..T
Gepa1.8                               ......VQ.L ...R..Y... .....HF.AF ..H...R.RD ...Q.EWM.N LR....W
Orgu8.2_Gepa2.1                       ......VEGY ...R..Y... .....HF..F ..N...Q.RY ...K.EWV.H KR.....
Gepa2.2                               .....LVRSL ...R..Y... .....HF..F ......Q..Y R..K.EYM.. MRA...T
Gepa2.3                               ......VD.Y ...R..Y... .....HF..F ..D...A.RY ...K.EWV.H KR.....
Gepa2.4                               ......VD.Y ...R..YL.. ...L.H...F ..L...A.RY ..GQSERI.. MRA...T
Gepa2.5                               ..N..YMEKQ F..R..YL.. ...L.H...F ..L...A.RY ..GQSERI.. MRA...T
Gepa2.6                               .....LVRSL ...R..Y... .....HF..F ......Q..Y R..K.EWV.H KR.....
Gepa3.1                               .....YVE.Y ...R..YM.. .....H...F .....EQ.RC R..E.EL.QN RRIS...
Gepa3.2                               .....YVE.H ...R..YM.. .....DF..F .SL...Q.RY LSRN.DYM.R LRAQ..I
Gepa3.3                               ......V..L ...R..YM.. .....H...F ..D..NA..Y .....VYM.. NRGV..T
Gepa3.4                               .....YVE.H ...R..Y... .....HF.AF ..H...R.RD ...Q.EWM.N LR....W
Gemo1.1                               .....YVD.L ...R...MS. .....H...F ..F...Q..H Y...QVYM.R .R....W
Gemo1.2                               .....YVD.L ...R...M.. .....H...F ......Q..H Y...QVYM.R .R....W
Meno5.2_Gemo1.3                       ......V..L ...R..YV.. .....HF..F ..D...Q.RY ...QADYM.. TR....T
Acka10.2_Gemo1.4                      ......VQ.Y ...R..YI.. .....HF.WF ..D...D.RD .....DK... YRAV..T
Gemo1.5                               ......VG.L ...R..YV.. .....H...F ......Q.RY R..K.EW... KRA...T
Gemo1.6                               ......V..L ...R..YV.. .....HF..F ..N...R..Y A..LTDY... TRA...T
Gemo2.1                               ......VD.Y ...R..YV.. .....HF..F ..N...R..Y A..LTDY... TRA...T
Orgu3.2_Gemo2.2                       .....YVE.Y ...REQ.VM. .....V.E.F ..L..RN.N. F..N.E.M.N .R....W
Gemo2.3                               ....K.VE.H ...REQ.MM. .....V.E.F ......V.RH ..NN.ELM.. KRGQ..N
Gemo3.1                               .D....VE.Q F..R..LL.. .....H...F ..F...Q..H Y....GYM.. TR.V..T
Gemo3.10                              .....YVD.L ..DRL..MS. .....H...F ..F...Q..H Y....GYM.. TR.V..T
Gemo3.11                              .....YMEKQ F..R..YL.. ...L.H...F ..L...A.RY ..GQSERI.. MRA...T
Gemo3.12                              .....YVV.. T..R..Y... .....HF..F ..F...Q.RY .....VFM.R KR....W
Gemo3.13                              .....YVE.H ...R..LM.. .....H...F ..L...A.RY .....VFM.I KRAE..T
Gemo3.3                               .....YVE.H ...R..LM.. .....H...F ......Q..H Y....GYM.. KRAE..T
Gemo3.4                               ..R..YVD.L ..DR..YV.. .....H...F ......R..H Y...QVYMDR .R.....
Gemo3.5                               .D....V..R ...R..Y... .....HF..F ..N...Q.RY .....VYM.R KRAE..T
Gemo3.6                               .D....V..R ...R..Y... .....H...F ......Q..H Y...QVYM.R .R....W
Gemo3.7                               ......V..H ...R..QL.. .....H...F ......V.RY ...Q.DK... NRAV..N
Gemo3.8                               .....LVN.L L..R..Y... .....H...F ......Q..Y R..K.DYM.. .R.....
Gemo3.9                               .....YVE.Y ...R...M.. .....H...F ..L...N.RD A..Q.DFM.R .R..E.W
Gemo4.1                               .....YVE.Y ...R...M.. .....HF..F ......Y.RD A....VTM.R RRAV..W
Meno5.3_Sema11.1_Orgu9.1_Gemo4.2      .....YVH.C V..R..Y... ..G..H...F ..D..EA.RY ...V.DYM.G KR....W
Gemo4.3                               ......VE.Y ...R..LM.. .....H...F ..F..IQ.RH Y....EWM.. IRAV..T
Gemo4.4                               .....YME.H ...R..Y... .....H...F ..N..DT.RY ..NL.GW..D ERA...T
Gemo4.5                               ......VY.H L..R..LLT. .....H...F ..F...Q.KH L..Q.EL... RRAE.GN
Gemo4.6                               ..Q...VK.. ...REQYVH. .....YFL.D ..F...V.RY ...H.EWM.. RRA....
Gemo4.7                               .....YVE.Y ...R..YM.. .....H...F ..D.DNT.RD A..K.EWM.. VRA...T
Gemo5.1                               .....YVE.H ...R...L.L ..N..H...F A.F...Q.RY ...Q.DYM.. KRGT...
Gemo5.2                               ......VE.H ...R..LM.. .....H...F ..F..IQ..Y R.NK.EW... NRAE..N
Gemo5.3                               .....YVY.D V..R..LM.. .....H...F ..F...Q.RY R..K.EL... RRA....
Seke1.3                               ..R...VK.. ...REQYVH. .....YFL.D ..F...V.RY ...H.EWM.. RRA....
Seke1.4                               ......VY.H L..R..LLT. .....H...F ..F...Q.KH L..Q.EL... RRAE..N
Seke1.5                               ......VY.D ...R..Y... .....H...F ..F...Q.RY R..K.EL..H RRAQ..T
Seke10.1                              ......V..R ...R..Y... .....HF..F ......Q.RY R..Q.DFM.R KRA...W
Seke10.2                              ......V..R ...R..YV.. .....H...F ......Q.RY R..Q.DFM.R KRA...W
Sema2.2_Seke10.3                      ......V..R ...R..YV.. .....H...F ..D...Q.RY ...N.D.M.. NRGV..T
Seke11.1                              ......VG.L ...R..QL.. .....H...F ..L...Q.RY F..L.DFM.T VRAQ..I
Seke11.2                              ......VE.D ...R..Y... .....H...F ..D...Q.RY ...N.D.M.. NRGV..T
Sema9.1_Seke2.1                       .....YVD.Y ...R..QL.. E....H...F ..N...Q.RY ...Q.DYM.S KR....W
Seke2.10                              .....YVH.N M..R..YM.. .....H..AF ..R.D.T.RY ...K.DF... RR....W
Seke2.11                              .....YVH.N M..R..YV.. .....H...F ..N...R.RY ...Q.DYM.S KR....W
Seke2.12                              .....YVE.H ..SR..YM.. .....H...F ..R.D.T.RY ...K.DF... RR....W
Seke2.3                               ....T.V..Y ...R..LV.. .....NF..F ..F...Q..N ...N.DMM.. HRGL..T
Sema10.1_Seke2.4                      ......VE.H ...R..I... ...L.H...F ......Q.RY R..N.EWM.D RRAQ..I
Seke2.5                               .....YVH.N M..R..YV.. .....H...F ..N...Q.RN ...R.EV... RRAQ..T
Seke2.6                               ......VE.H ...R..V... ...L.H...F ..N...Q.RY ...Q.DYM.S KR....W
Seke2.7                               .....YVH.N M..R..YV.. .....H...F ..N...Q.RY R..K.EL..H RRAQ..T
Seke2.9                               .....YVE.H ...R..YM.. .....H...F ..F...Q.KH L..Q.EL... RRAE..N
Seke3.1                               ......VE.Q F..R..LL.Y .....H...F ..F...Q.KY R..K.EWM.N RRAQ.NI
Seke3.2                               .....YME.Q F..R..... .....H.... ..F...V.RD A..N.EWM.D IRA...T
Seke3.3                               .....LVN.L ...R..Y... .....HF..F ......Q.RY R..K.DFM.R KRAE..T
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                                  *** * *     ***       **      *  ** ** *  ***** * **** *

                                    + +  +    ++         +         +   + +   +  + + + +  +
                                      10         20         30         40         50
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Seke3.4                           .....LVN.L ...R..Y... .....H...F ..D...T..H .....VYM.G KRA...T
Seke3.5                           ..Q..YVD.H ...R...M.. .....HF..F ..F...Q.RD .....DFM.T VRAR..I
Seke3.6                           .....YVH.N M..R..YV.. .....H...F ..D...Q.RY ...N.D.M.. NRGV..T
Seke3.7                           ..Q..YVD.H ...R...M.. .....H...F ..N...Q.RN ...R.EV... RRAQ..T
Seke4.1                           .....YMH.N M..R..YV.. .....H...F ..N...Q.RD ...L.DFMQT VRAR..T
Seke4.10                          ..Q..YVD.H ...R..LM.. .....H..AF ..R.D.T.RY ...K.DF... RR....W
Seke4.11                          ..Q..YVD.H ...R..LM.. .....HF..F .RF...Q.RD ...L.DFMQT VRAR..T
Seke4.2                           .....YVE.H ...R..YM.. .....H..AF ..R.D.T.RY ...K.DF... RR....W
Seke4.3                           .....HVE.D ...R..Y... .....H...F ......N.RY ...L.DYM.R KRAE..T
Seke4.5                           ....K.VD.L ...R..Y... .....H...F ......N.RY ...L.DYM.R KRAEGGH
Seke4.6                           .....YME.Q F..R..Y... .....H...F ALD...T.RY F....VYM.S KR....W
Seke4.7                           .....HVE.D ...R..QL.. E....H...F ..N...Q.RY ...Q.DYM.S KR....W
Seke4.8                           ....K.VD.L ...R..Y... .....H...F ......N.RY ...L.DYM.R KRAE..T
Seke4.9                           ....K.VD.L ...R..Y... .....H...F ..D...T..H .....VYM.G KRA...T
Seke5.1                           ......V..R ...R..YV.. .....H...F ..D...Q.RY ...N.D.M.. NRGV..T
Seke5.3                           ......V... ...R..YK.. .....HF..F ..H...N.RD ...N.D.M.. HRGL..T
Seke5.4                           ......V..R ...R..YV.. .....H...F ALD...T.RY F....VYM.S KR....W
Seke5.6                           ......VY.D ...R...... .....H...F ..F...Q..Y R..K.EL..H RRAQ..T
Seke6.2                           .....YV..L ...R..Y... ....RHF..F ......Q.RY R..N.EWM.D RRAQ..I
Seke6.4                           .....YVE.H ...R...L.L ..N..H...F ..F...Q.RY ...Q.DYM.. KRGT...
Seke6.5                           .....YV..L ...R..Y... .....HF..F ......Q.RY R..Q.DFM.R KRA...W
Seke7.1                           ......V... ..DR..YK.. .....HF..F ..F...Q.RY F..L.DFMDS KR....W
Sema3.13_Seke7.2                  .....YME.Q F..R..Y... .....HF..F ......Q.RY ...K.EW... KRAE..N
Seke8.2                           .....YVH.L ...R..Y... .....HF... ..D...T.RY F....DFM.R KRAE..I
Seke8.3                           .....YVD.Y ...R..Y... .....HF..F ......Q.RY K..L.DFM.R KRAE..I
Seke8.4                           ......V... ...R..YK.. .....H...F ..N...Q.RN ...R.EV... RRAQ..T
Seke8.5                           .....YVH.L ...R..YV.. .....H...F ALD...T.RY F....VYM.S KR....W
Seke8.6                           ....T.V..Y ...R..LVT. .....H...F ..F...Q.KH L..Q.EL... RRAE..N
Seke8.7                           .....YVH.L ...R..Y... .....H...F ALD...T.RD F..N.VYM.R KR.....
Phni1.3_Seke9.2                   .....L.Q.L ...RM.Y... ......F..F .....YN.E. .......... RRA...T
Seke1.1                           .....YVH.L ...R..Y... .....H...F ALD...T.RY F....VYM.S KR....W
Seke1.7                           .....YVE.H ...RE.YM.. .....H..AF ..R.G.T.RY ...K.DF... RR..I.W
Seke3.9                           ......V..R ...R..YV.. .....H..AF ..R.D.T.RY ...K.DF... RR....W
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B)

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                                            10         20         30         40         50
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Meno1.5*                          TEKVSYVHSY IYNRVELTMF DSDVGKYVGF TPLGVKWAKY WNSNPNMPEM KQAEVDT-
Meno1.6*                          .....F.V.A ...G..GVS. ..N....E.. ..Y.E...QH ....T.EM.Y .VGSGGLV
Meno1.7*                          ....MF.V.A ...G..GVS. N.N..... ..Y.E...QH ....T.EM.Y .MGSGGLV
Meno1.9*                          .....FLD.F ......DSS. .......... ..F.E.N.ER F..D..EL*Y SR.S...-
Meno1.12*                         ......ME.* M.....YMS. ......F... ..F.ESN.ER A....SWMDY .R.S..S-
Meno2.4*                          ....MF.V.A ...G..GVS. ..N..... ..Y.E...QH ....A.EM.Y .MGSGGLE
Meno2.6*                          .....*.Q.Q M.....FMS. ......F..L PRM.SSMPST GTVIRITWN. IVGWWTR-
Meno2.9*                          .....*.Q.Q M.....FMS. .T....F... ..Y.E.N.EH ...D.DYM.Y NRGV...-
Meno2.10*                         .....F.V.A ...G..GVS. ..N..... ..Y.E...QH ....T.EM.Y .TGSGGHA
Meno3.1*                          .....F.V.A ...G..GVS. ..N..... ..Y.E...QH ....A.EM.Y .MGSGGLV
Meno3.2*                          .......... .......... .......... .......... .......L.. ....GGHV
Phni.10*                          .......K.. ....L.DVSS TAMW.NSW.S P...SMMPSA GTMTSMNWST DGLSWTG-
Phni.11*                          M....FGA.A ......DVS. N.H...H... .HF.E.V..K ...LRDYM.S SWTS..W-
Phni.17*                          .......V.. ......DVS. .......... .QA.E...QD ...D.D.M.S TR....L-
Xama4.1*                          .....F.V.S ..S...YMS. ..G..... ..D.E.*... *..D...LVN ADCSGLA-
Xama4.6*                          .....F.V.N ..S...YMS. .......... ..D.E.*... *..D...LDN ADCSGLA-
Mele1.2*                          .....F.A.S .......AS. .......... ..Y.E.QG.. ...DLDFM.S TRTS..W-
Mele2.2*                          ....MFMV.A ...S..GVS. ..N..... .LY.E...QH ....A.EM.Y .MGSGGLV
Mele3.3*                          ....MFMV.A ...S..GVS. ..N..... ..Y.E...QH ....T.EM.Y .MGSGGLV
Mele5.4*                          .....F.V.A ...G..GVS. ..N..... ..Y.E..... L...T.EM.Y .TGSGSHV
Mele5.6*                          ....MF.V.A .H.G..GVS. ..N..... .LY.E...QH ....A.EM.Y .MGSGGLV
Mele7.1*                          ....MF.V.A ...S..GVS. ..N..... ..Y.E...QH ....A.EM.Y .MGSGGLV
Megr1.10*                         .....HLD.F .*P...HAS. ......N... ..D.E.Q.E. ...D...L.N D.TA..W-
Megr1.4**                         .....F.V.A ...G..GVS. ..N..... ..Y.E...QH ....T.EM.Y .MGSGGLV
Megr1.9**                         ..S..F.V.A ...G..GVS. ..S..... ..Y.E...QH ....T.EM.Y .MGSGGLV
Megr2.1**                         ..S..F.V.A ...G..GVS. ..N..... ..Y.E...EH ....T.EM.Y .MGSGGLV
Acte1.7*                          .....FLE.H .....DMRGS TAMW.NTW.S PRV.S..PST .TVTSHWWSA DELQWTG-
Acte1.8*                          .....FMM.S ......VQGL TAMW.NSW.S PQMAS.SPST TTASQISWSA DELQWTG-
Acte2.2*                          ....VPGDV. LQPGGVHKVQ QWCGEMRGVI HVW.ETGPAL KQWPKYTGVC TDCSGQA-
Acte2.6*                          .....FLD.. ......YVS. .......... ..R.E.L.*N L..DEARM.Y IRTA..S-
Acte2.7*                          .....FLAMF ......F.S. N........L STY.E.P.QH ...D...L.Y A.TA..S-
Acte5.6*                          .....FMM.S ......VQGL TAMW.NSW.S PQMAS.SPST TTASQISWKA HELHSGLV
Acte5.7*                          .....FAM.S ......VQGL TAMW.NSW.S PQMAS.SPST STASQISWKA HELHSGLV
Myob1.1*                          .....FGA.A ......DVS. N.H..... .HF.E.V.SK ...LRDYM.S SW.S..W-
Myob2.7*                          .....FGA.A ......DVS. N.H.....V. .HF.E.V..K ...LQSLHGE QMNFSGLV
Acka7.1*                          .K..MF.V.A ...G..GVS. ..N..... ..Y.E..... L...A.EM.Y .MGSGGLV
Acka8.1*                          .....FGA.A .....GDVS. N.H..... .HF.E.V..K ...LRDYM.S SWTS..W-
Acka8.2*                          ..R..F.K.F ....EQYVH. .....YFL.G H..WGEGCQA LEQS.G.DGV QTGCGGQA
Orgu1.2*                          .....F.M.R ..SG..*VS. NN...HF... ..H.E.T.Q. ...K.GYM.Y MSTV.NW-
Orgu4.2*                          .DR.HQQPGA APGLRQRCGA LRGGHRVR.D QYRA.EQPTE SAGGVKGTI----------
Orgu5.2*                          .....F.M.R ..SG..*VS. NN...HF... ..H.E.T.Q. ...K.V.WST *GLWLNW-
Orgu7.2*                          ..R..F.K.F ....EQYVH. .....QF..D ..F.E.V.S. .....EWM.Y SR.A.TG-
Orgu7.3*                          A....F.M.R ..SG..*VS. NN...HF... ..H.E.T.Q. ...K.E.L.Y NR...H.-
Orgu8.3*                          .....F.M.R ..SG..*VS. NN...HF... ..H.E.T.Q. ...K.GYM.Y MSTV.ELA
Seke6.1*                          .....F.E.Q F......LSY .....H.... ..F.E.E... R..KAE*M.N SR.Q.NI-
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                                  10         20         30         40         50
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Seke8.1*                     ......ME.Q F.....FAS. .....TMW.S P...SS.PGT RTAIR.GWST YGLLWTR-
Seke9.1*                     .........L ......YVS. .....TMW.S LWM.SSLPGT STATQFTWSA .GLLWTG-
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Appendix IV

Ecological variables of bird species

The table indicates the range size, density and population size for fifteen species of birds studied on Chapter 4

Bird species	*Range size (Km²)*	*Density (number of birds/Km²)*	*Population size (number of birds)*
Sericornis keri	2654.272	7.390	19615.070
Gerygone mouki	7717.8624	199.630	1540716.871
Gerygone palpebrosa	20426.8032	3.400	69451.131
Oreoscopus gutturalis	3739.5712	57.410	214688.783
Sericornis magnirostris	9112.7232	148.420	1352510.377
Acanthiza katherina	2834.8416	52.010	147440.112
Sericornis citreogularis	3962.1696	53.880	213481.698
Lichenostomus frenatus	4593.5488	111.690	513053.465
Myzomela obscura	20652.4992	11.530	238123.316
Acanthorhynchus tenuirostris	4185.408	8.140	34069.221
Meliphaga gracilis	13619.84	61.200	833534.208
Meliphaga lewinii	6169.216	134.670	830808.319
Xanthotis macleayana	9868.992	40.470	399398.106
Phlidonyris nigra	9977.600	19.000	189574.400
Meliphaga notata	10604.5376	43.430	460555.068