Urbanisation and avian fitness: an investigation of avian malaria prevalence and feather corticosterone level of blue tit Cyanistes caeruleus nestlings across two breeding seasons

Bedur Faleh A Albalawi

M.Sc., B.Sc.

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy
Institute of Biodiversity, Animal Health and Comparative Medicine
College of Medical, Veterinary and Life Sciences

September 2019
University of Glasgow

I. Abstract

Urbanised landscapes vary significantly from natural habitats because of their different and unique ecological features. These features can affect the ability of both animals and plants to occupy urban habitats. Ecological studies on avian species have often reported a reduction in several breeding parameters of urban populations compared to their rural counterparts. However, how the urban environment can influence the breeding success of these species remains largely unknown. One hypothesis is that urban-specific factors alter key physiological traits modulated by stress hormone levels in birds. Indeed, long-term exposure to high levels of stress can lead to sustained elevation of basal glucocorticoid levels and consequent detrimental effects, such as impaired immunity, inhibited growth and reduced survival. For instance, alterations to the microclimate, pollution, and limitations of food resources may act as strong stressors, resulting in an increased likelihood of parasite infection and related fitness costs, affecting the capacity of hosts to occupy urban areas.

Environmental conditions can fluctuate from year to year, affecting birds directly or indirectly by limiting food availability, especially for carnivorous birds of which the main food (insects, e.g. caterpillars) is highly dependent on weather conditions. As the urban conditions are already harsh, fluctuations in the other environmental factors, like weather conditions, have the potential to severely affect urban birds. Conversely, the more favourable conditions in rural habitats may allow the birds to buffer against other potentially negative environmental factors. Therefore, in order to understand the impact of urbanization on bird stress and fitness across fluctuating environmental conditions, the first aim of my thesis was to explore and compare the fitness of an urban and a rural population of blue tit *Cyanistes caeruleus* across two breeding seasons (2016 and 2017) in relation to the stress levels they experience. Previous studies have suggested the use of corticosterone (CORT, the main avian glucocorticoid) levels as a biomarker of stress experienced by animals. In birds, long term or chronic exposure to stressors can be measured from feather corticosterone (fCORT). This non-invasive method can measure the level of CORT that has been metabolised and deposited in feathers during feather growth. Thus, I compared two populations of blue tits in relation to the level of stress experienced by nestlings, throughout their first thirteen days of life, that may influence their fitness in urban and rural habitats. CORT levels in nestlings can also be directly influenced by parents before oviposition (e.g. by maternal deposition of CORT in yolk). Therefore, I also tested the role

of origin of the bird on their fCORT levels, experimentally, to assess whether the pre-oviposition environment has a role in nestling's fitness (Chapter 2). Then, as parasite infections can play a major role in fitness differences between urban and rural birds, I decided to measure the prevalence of avian malaria (haemosporidian parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*), which are widespread parasites in birds, infecting blood cells. Specifically, I compared avian malaria prevalence in nestlings from the two populations across the two breeding seasons, as well as tested the role of parental origin (based on a cross-fostering experiment) on their susceptibility to infection (Chapter 4). However, to detect haemosporidian parasite acute infection and identify the parasite genera in the studied blue tit populations, I developed a new molecular method, as current tools did not allow these investigations (Chapter 3). Using this new approach, I explored my next aim, where I tested the effect of *Leucocytozoon* infection prevalence on fitness-related traits (body weight and survival) of nestling blue tits from the two populations during the two breeding seasons. I also tested the potential synergistic impact of infection and urban-related stress on blue tit fitness by examining the relationship between the two factors – fCORT level and *Leucocytozoon* infection prevalence (Chapter 5). I accomplished these aims using both experimental and correlational approaches, the former involving a cross-fostering experiment and a vector-manipulation experiment. In 2016, I cross-fostered some clutches between and within sites to test for any effects that may be derived from inherited or maternal traits from parents to their offspring. Additionally, as both populations showed high malaria prevalence in 2016, in the 2017 breeding season, I conducted a vector-repellent experiment to experimentally reduce infections in nestlings; this was done with the goal of better understanding the impact of parasites on bird's fitness.

The key findings of my thesis are as follows: First, monitoring the two populations of blue tit over two breeding seasons revealed that most breeding parameters are significantly different between the urban and rural blue tits. In both seasons, urban birds showed a significantly lower clutch size, hatching and fledging success compared to the rural ones. Additionally, fledging success at both sites was considerably lower in one breeding season (2017), during which nestling body weight was significantly lower in urban than rural birds; however, in 2016, when fledging success was higher, the two populations barely differed in their weight. Second, mirroring nestling body weight, during the 2017 breeding season, fCORT levels in nestlings were significantly higher in the urban nestlings compared to their rural counterparts, but not in 2016, further suggesting that 2017 was a

more challenging year. However, I found no association between the reduction in fitness-related traits (body weight and fledging success) and fCORT. This lack of association between fCORT levels and nestlings' fitness-related traits could have been missed as I pooled the feather samples per nest and did not measure individual nestlings for fCORT level, thus losing the inter-individual variation in fCORT levels that could be associated with fitness-related traits. Third, mirroring the fCORT variations, *Leucocytozoon* parasite infections varied in the two populations across seasons, showing that urban populations had lower or higher prevalence compared to rural birds depending on the year (2016 and 2017, respectively). I found a strong association between infection with *Leucocytozoon* prevalence and lower weight of urban nestlings just before fledging (day 13 of age) as well as a reduction in urban nestling survival, which instead was not observed in rural birds. I found no association between infection prevalence and fCORT level, measured per nest, not individuals. Finally, experimentally tested in the field, the origin of the bird did not influence the infection susceptibility to *Leucocytozoon*, nor did it influence fCORT levels.

In summary, my thesis highlighted the importance of year to year variation between the two populations (i.e. the urban and the rural populations of blue tits), that could be influenced by fluctuating environmental factors such as weather and food availability. Urban and rural populations that show similarities in certain traits during one year of study may be different during another year (e.g. fCORT level and body weight). Prevalence of vector-borne pathogens like *Leucocytozoon* parasites in a given population may also differ between populations, which can vary from year to year. The extent of the fitness effect of parasite infection also depends on various factors fluctuating from one year to another. This emphasises the need for longitudinal studies monitoring individuals and populations over multiple years and across a wide range of habitats that differ in quality and features.

Table of Contents

II. List of Tables

III. List of Figures

IV. Acknowledgements

Firstly, I would like to thank my supervisors Barbara Helm, Ruedi Nager and Francesco Baldini for their encouragement, guidance, insight and support. I am grateful for Ruedi and Francesco for their kindness, and instilling me with the confidence I needed to complete this work - for tirelessly being there whenever I was struggling. Thanks to Barbara for her great support during her time in Glasgow and via emails after she left. Thanks for always offering time to discuss my work in person during her visits to Glasgow.

My malaria lab work would not have been possible without the invaluable help and moral support of Francesco. Thanks to Winnifred Boner for allowing the use of the qPCR machine in their lab. I am also grateful to the support from Callum Buchanan on the feather corticosterone lab work and for his supervisor Neil Evans for allowing the use of their lab equipment and software analysis. Thanks to Leo Truglia and his supervisor Michelle Bellingham for conducting the yolk corticosterone lab analysis as part of his internship project. I am also grateful to the lab technician Elizabeth Kilbride, Zara Nelson and her supervisor Barbara Mable for the molecular identification of the sex of the birds in this thesis as part of her honours project. Thanks also to the lab technicians, Dorothy Armstrong at the main campus lab, Lynne Fleming and Ana Monteiro at Garscube campus Lab, for their excellent assistance whenever I needed. Thanks to my first lab mate at Garscube, Magdalena Mladenova for the lovely time I spent and for being a good friend since.

My fieldwork, during the two seasons, heavily relied on Barbara and everyone in her 'Birds and Clocks' group - the tireless and highly organised team. For the first year of study, I received profoundly generous help with the blood sampling and cross-fostering experiment between SCENE and Kelvingrove park from Barbara, for which I am enormously grateful. I am especially thankful to those of you who helped within SCENE fieldwork with all the midges around. I'm indebted to the field assistants Darren Talbot and Crinan Jarrett. I also would like to thank everyone in the 'Birds and Clocks' group. Thanks to Stewart White, Paul Jerem, Luke Powell, Chris Polloch, Joris Wiethase, Heather McDevitt, John Gibson, Rachel Steenson, Christina O'Flaherty, and all the people who had any contribution to the existence of my project. I want also to thank Stephen Larcombe for his advice and directions on designing the vector-manipulation experiment. I would like to give special thanks to Kate Griffith for her kindness and patiently supervising me on my

first attempts in bird blood sampling. Also, to Davide Dominoni for his always meaningful comments on my talks and data analysis, and my PhD mate, Robyn Womack for the pleasant moments we spent together in the lab, office or during break times. I am grateful to Jane Robinson for her kindness and encouraging words whenever I saw her at Garscube Campus.

I am very grateful to the Saudi ministry of education, especially for the University of Tabuk, who funded this work and gave me this opportunity. Thanks to the University of Glasgow, particularly the Institute of Biodiversity Animal Health and Comparative Medicine for offering me a chance to be part of such an inspirational school. Thanks for the PhD convenor team, especially Louise Matthews for helping under challenging times. Thanks to my assessors Dorothy McKeegan, David Eckersall and Peter O'Shaughnessy for their guidance and bits of advice each year. I am very grateful to the great scientists for the knowledge and contribution to the field of ecology and conservation biology, as well as to the internet by which we quickly access this treasure.

Finally, I thank my wonderful family and friends back at home for their unfailing support and unconditional love. Thanks to my mother and her prayers. Thanks to my sisters (Shahad, Albandari and Esmahan) and their joyful chats and video calls, making me feel a lot closer to them. And, of course, thanks to my three little angels (Lamar, Faisal and Meshal) who made this journey more challenging, but full of pleasure. Thanks to my husband (Hani), who is also another PhD student at the University of Glasgow, for being always around – I really could not have wished for a better, or more inspirational and supportive colleague and life partner.

V. Author's Declaration

I declare that, except where explicit reference is made in the Acknowledgements to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or at any other institution.

Bedur Faleh A Albalawi
September 2019

Chapter 1 General introduction

1.1 Abstract

Urbanised areas differ significantly from natural areas in terms of their different ecological features. These features play a crucial role in shaping the properties of both the animal and plant communities that colonise urban habitats. In general, although some wild species thrive in urban areas, urbanisation has led to a loss of natural resources (such as food and habitats). It has also led to alterations in energy flux, hydrology and temperature balance, as well as to high levels of pollution. Urbanisation has affected species in a variety of ways, resulting in populations with reduced reproductive performance. However, the ways by which the urban environment has influenced the breeding success of urban dwelling species remain unclear. Factors such as alteration in microclimate, pollution, and limitation in food resources have negatively affected the health of some vertebrates, including many bird species. Such stresses may increase the likelihood of parasite infestations, affecting the capacity of hosts to occupy urban areas. Birds are the most common, and most studied, taxon in urban areas and are regularly used as model organisms in the field of urban ecology. In this chapter, I will define the basis of my thesis by overviewing the current knowledge of the following topics. First, the stressors in the urban environment that may affect bird species including disease prevalence. I will focus on haemosporidian parasite prevalence as a possible factor influencing the fitness of birds in urban landscapes. Second, corticosterone hormone (CORT) level as a biomarker of stress. Next, my thesis aims will be given, followed by a summary of my study sites and study species, before ending with an outline of my thesis structure.

1.2 A framework for understanding the relationship between urban environment, parasite load and avian fitness

Urban-related stressors such as light, noise, and chemical pollution can impact on the health of some vertebrates, including birds (e.g. Burger, 1991; Partecke et al., 2006; French et al., 2008). Such stresses may increase the likelihood of parasite infestations, affecting the capacity of hosts to occupy urban areas. Therefore, parasite load should be linked with measures of health and stress in birds. This is because parasites that do not influence fitness are unlikely to be factors in determining whether birds live in a given environment (e.g. urban area). An integrative approach among parasitism, immunology and physiology remains to be developed in urban areas (Marzluff, 2017).

Parasite infection may directly affect bird fitness via damage of blood cells or other tissues, but it could also indirectly affect their host by diverting resources away from important processes and self-maintenance, especially in harsh and resource-limited environment like urban areas (Figure 1-1). Nestlings are more likely to be affected by infection because during this early life stages they depend on their immature innate immunity and may need to trade-off between two energetically demanding process; immune defence and self-maintenance (growth and survival).

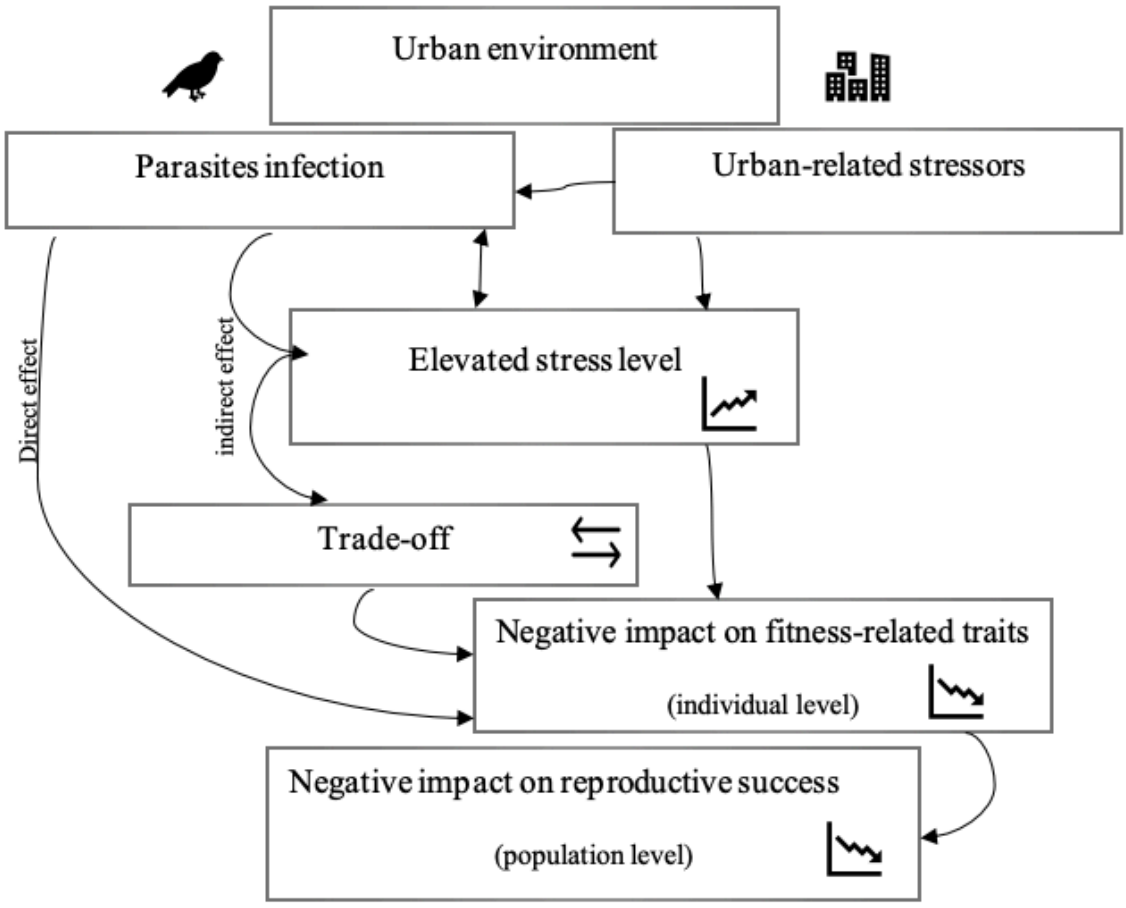


Figure 1-1 A framework for the effect of urban environment and parasite load through stress axis or synergistic effect of both factors on avian fitness and overall reproductive success

1.3 What is an urban landscape?

Urban landscapes are those areas characterised by noticeable levels of human density, buildings and roads. According to the British Office of National Statistics, urban areas are those districts known as villages, towns or cities, which in turn are defined by land use. Any built-up land (e.g. roads and buildings) comprising a minimum of 20 hectares (200,000 square metres) is defined as an urban area (British Office of National Statistics, 2011). Another definition of an urban area is provided by the United States Geological Survey (USGS); here, urban areas comprise a minimum of 30% of areas covered by constructed components, such as roads and buildings.

1.4 Environmental features of urban areas and their impact on wildlife

Urbanised areas are characterised by complex environmental features, some of which are unique to urban landscapes, such as light at night, roads and buildings, while others can be found in other areas far from human habitats. However, the complexity and combination of environmental features in urban areas makes them different from other types of landscapes (Faeth et al., 2011). Urban environment negatively altered several biological traits of its wild species, including their diet (Penick et al., 2015; Murray et al., 2015; Becker et al., 2015; Pollock et al., 2017), behaviour (Sol et al., 2013), physiology (Dominoni et al., 2013; Salmón et al., 2016; Watson et al., 2017) immunity (Bailly et al., 2016; Capilla-Lasheras et al., 2017) and disease transmission (Brearley et al., 2012; Martin and Boruta, 2013; Hassell et al., 2017).

1.4.1 Urbanised areas as ecological traps

While urban-dwelling species have been often reported to be negatively affected by urban environment, some species tend to thrive in cities reaching high abundance in such environments (Seress and Liker, 2015; Meyrier et al., 2017). This could be because of some benefits urban environments provide to these species such as availability of food and nesting sites. In urban areas, humans provide food for animals either intentionally, such as by way of seed feeders, or accidentally, such as by scattering leftovers or food waste (Tryjanowski et al., 2015). Although, such factors of urban habitats can improve the health status and survival of urban birds (Robb et al., 2008; Suri et al., 2017), especially during winter (Batten, 1978), they could represent ecological traps for birds due to mismatches

between attractive sites for breeding and the cost imposed on fitness (Meyrier et al., 2017; Pollock et al., 2017). The greater availability of supplemental food in urban areas may encourage females to anticipate the breeding season, as they might take the abundance of food as an indication of appropriate conditions in which to rear their nestlings (the anticipation hypothesis; (Lack, 1954)). According to this hypothesis, birds evaluate the quality of their environment using information such as food availability. So, availability of winter food may serve as a misleading cue for birds, which predict the timing for abundant food in the breeding season, creating an ecological trap. Birds may start breeding early in the season and lay more eggs but of poor-quality. Rapid environmental change causes organisms to prematurely settle in habitats that turn out to be of poor quality for chick rearing. This early breeding may lead to a mismatch between the demands of nestlings for food and the peak of natural resources of food, which in turn results in reduced rates of nestling survival.

Supporting the anticipation hypothesis, supplementary food experiments have frequently found early laying dates (reviewed by (Robb et al., 2008), and yet have rarely reported any notably larger clutch sizes (such as in blue tit *Parus caeruleus* (Ramsay and Houston, 1997) and great tit *Parus major* (Nager et al., 1997). Rather, Harrison et al., (2010) reported reduced clutch size as well as a reduced number of fledged chicks of both blue tit *Cyanistes caeruleus* and great tit populations at their food supplemented sites. Increased clutch size has been reported with some supplemented birds (e.g, (Schoech, 1996), but with low quality eggs (Nager, 2006).

Supplemented food may influence different species differently and may depend on whether it is provided prior to or throughout the breeding season. For instance, a landscape-scale experiment on blue tits suggests that the provision of food supplements prior to egg-laying is highly likely to improve the condition of the parents, which leads to the enhancement of parental care for the nestling and, hence, higher rates of nestling survival (Robb et al., 2008). Another study of blue tits also shed light on the importance of the quality of the food provided over winter. The provision of fat-rich food negatively affected the quality of eggs and, in turn, nestlings' body mass in the following breeding season, and yet this impact was mitigated when vitamin E was added to the fat (Plummer et al., 2013a; Plummer et al., 2013b). Interestingly, when a group of Florida scrub-jays *Aphelocma californica* were provided with protein- and fat-rich food, they laid their eggs earlier, and

laid larger eggs and clutches, compared to the group provided with fat-rich food only (Reynolds et al., 2003).

1.4.2 The quality of anthropogenic food

The nutritional condition has been proved to have a strong link to immunity; a growing number of studies suggest that food resource limitations are often associated with slower development, decreased immune activity and reduced body mass (e.g., Alonso-Alvarez and Tella, 2000; Killpack et al., 2015). The quality of food provided for birds in urban areas is crucial for their health. It has been reported that abundant but poor-quality anthropogenic food (high in fat and low in protein) has a detrimental effect on animal immune systems, especially antibody-mediated defences (van Heugten et al., 2007; Maggini et al., 2007). The quality of anthropogenic food provided by humans is unlikely to be appropriate for birds both as adults and nestlings. Anthropogenic food is fat-rich and protein-poor compared to food in their natural habitats (Heiss et al., 2009; Murray et al., 2015). The abundance of this suboptimal anthropogenic food has the potential to play a central role in birds physiological pattern and furthermore may culminate in other effects such as their immunity.

Furthermore, bird feeders or waste points play an important role in disease transmission between birds, associated with certain behavioural adjustments (Jones and Reynolds, 2008). Bird feeders or waste points work as a contact point between birds and vectors of a variety of infectious diseases. The human provisioning of animals with food may be the strongest driving factor of disease prevalence in urban areas. An increased number of studies have illustrated that, depending on the nature of provisioning and the particular host-pathogen interaction, anthropogenic resources play a role in the alteration of host-pathogen interactions, resulting in either increased or decreased infection risks for both animals and humans (Becker et al., 2015). For example, some ticks, which are vectors of certain parasites, seem to be more prevalent in natural habitats compared to urban habitats (Evans et al., 2009). This pattern is expected because ectoparasites (including ticks) often have strict microhabitat needs (Logiudice et al., 2003). As a result, Lyme disease, which is caused by *Borrelia burgdorferi* (the tick-borne Ixodes sp. pathogen), is less common in urban areas in comparison with surrounding woodlands in the north eastern United States (Keesing et al., 2009). However, other types of parasites, such as haemosporidian parasites

(avian malaria) that are vectored by flying insects, showed mixed results (review Sehgal, 2015).

In addition, bird feeders works as a point of social conflicts (aggregation) for birds. Aggregations increase the level of stress hormones, and particularly corticosterone, which is the dominant steroid hormone of avian species (Hawley et al., 2006). Animals dwelling in urban habitats and occurring in high densities are vulnerable to disease because this hormone is believed to negatively affect immune functions (Moller and Saino, 2004).

In nestlings, the high amount of anthropogenic food provided for birds during the rearing period may shift their diet towards a higher ratio of sub-optimal food, especially with the reduction of natural food availability (e.g., Sauter et al., 2006; Mennechez and Clergeau, 2006). Since nestlings need protein-rich food, which is often scarce in urban areas (Van Nuland and Whitlow, 2014; Murray et al., 2015; Pollock et al., 2017), a high proportion of anthropogenic diet may result in a reduction in nestling body mass (e.g., Hõrak et al., 1999; Heiss et al., 2009). Moreover, any reduction in urban nestling body mass might be associated with an increased rate of mortality (e.g., Peach et al., 2008; Seress et al., 2012). In addition, nestling body mass is believed to be a good indicator of their potential success as breeding adults in many avian species (Schwagmeyer and Mock, 2008).

1.4.3 Urban microclimate

It is well-known that urbanised environments have significantly modified local meteorological conditions (Niemelä, J, Breuste, JH, Elmqvist, T, Guntenspergen, G, James, P and McIntyre, 2011), including air and ground temperatures, as well as the hydrology of cities and their surroundings. For instance, the high concentration of particulates in urbanised environments acts as condensation, often resulting in increased precipitation (Pickett et al., 2001).

The pattern of urban heat islands is a well-documented feature of urban ecosystems. Urban areas often have higher temperatures compared with their surroundings (Collins et al., 2000; Kalnay and Cai, 2003). This pattern shows the temperature variation between urban and non-urban areas, directly resulting from human activity and land cover. This variation in temperature is most notable after sunset; it is a few degrees on average but, depending on the area, can differ by as much as 10^{o}C (Pickett et al., 2001). Consequently, the life

cycles of insects are affected, which in turn alters the availability of arthropod food for avian species. The earlier occurrence of arthropods may cause asynchrony between the peak abundance of prey (such as caterpillars) and the onset of breeding of insectivorous birds (Visser et al., 2006). However, the warmer climate of cities may play a significant role in the survival of bird species during winter, resulting in elevated breeding populations (Chace and Walsh, 2006). It is indeed challenging to examine the role of the warmer climate in itself as it cannot be separated from other factors, such as food availability.

1.4.4 Urban structures and pollution

Roads are one of the principal features to shape urbanised areas; they can alter hydrological systems (Coffin, 2007), and are responsible for the elevation of avian mortality rates due to collisions (Spellerberg, 1998). Bird populations have experienced a reduction in density due to the elevated proximity of roads (Benítez-López et al., 2010). Birds display behavioural adaptations to roads. For instance, some common European species make adjustments to their flight initiation distance (FID), depending on the speed limit of the road and escape earlier when the speed limit of the road is high (Legagneux and Ducatez, 2013). More importantly, many species avoid noise pollution, which is mainly related to traffic on roads in urbanised landscapes (Forman et al., 2002; Peris and Pescador, 2004). This type of anthropogenic pollution is related to the changed acoustic environments of urban areas and transportation networks, which affects the transmission of acoustic signals used by animals in their communication and behaviour. These signals and behaviour are very important in relation to certain processes, such as territorial defence, attracting mates and begging calls (Warren et al., 2006). For instance, a study of the house sparrow *Passer domesticus* demonstrates that chronic noise affects parent-offspring communication by masking the begging calls of nestlings and, as a consequence, reduces provisioning rate which then reduces their reproductive success (Schroeder et al., 2012).

It has also been proven that noise pollution causes physiological stress and influences other factors of behaviour, for example by interfering with crucial sounds made during predator-prey interactions (Barber et al., 2010). It is noteworthy that several avian species have been able to overcome the consequences of noise pollution by modifying particular characteristics of their singing, such as amplitude and frequency (e.g, Francis et al., 2011).

The considerable amount of artificial light present in urban landscapes is the main source of light pollution. This type of pollution mainly affects the behaviour of animals. For example, it can affect animal foraging behaviour, migration, orientation, reproduction and even communication (review by Longcore and Rich, 2004). The singing behaviour of birds is typically initiated by light; as such, the presence of artificial light at night also has an impact on courtship behaviour (Miller, 2006; Kempenaers et al., 2010).

Artificial light at night also alters interactions between competitors (Petren and Case, 1996), and between predators and their prey (Perry et al., 2008). It affects timing of reproductive behaviour (Kempenaers et al., 2010; Dominoni et al., 2013). Artificial light at night has a detrimental effect on a great variety of organisms, from flying insects (Eisenbeis and Hänel, 2009) to vertebrates such as birds (Miller, 2006). Many migrant birds use artificial light at night as a visual guide instead of natural cues on the horizon, especially when it is foggy or cloudy. Some burrowing seabirds (e.g. puffins, petrels and shearwaters) are attracted to light when fledging. In a natural environment this is the sea where they head for. But increasingly they get attracted to city lights where they are exposed to many threats (Longcore and Rich, 2004).

A recent study on the captive Eurasian blackbird *Turdus merula* reported differences in the development of the reproductive system due to the amount of light to which individual birds were exposed. Birds exposed to low levels of light at night tend to develop their reproductive systems earlier compared to those kept under forest-like night conditions (Dominoni et al., 2013). In investigating the physiological drivers behind this difference, they conducted a comparison between urban and rural populations of the same species and found differences in their chronotypes (the propensity for the individual to sleep at a particular time during a 24-hour period) and circadian clocks (the central mechanisms that drive circadian rhythms). The urban birds were active for longer during the day and had shorter circadian clocks compared with the rural birds, as the former woke before dawn, whilst the latter followed the natural light when starting and ending their daily activities (Dominoni et al., 2013). A similar study of the same species found that birds in urban areas that are exposed to higher levels of light at night forage for longer after dusk, and this pattern was more pronounced when the period of daylight was shorter, such as is the case in early spring (Russ et al., 2014).

Concentrations of chemical pollution in urban areas are believed to be much higher than the global average. Emissions from traffic, factories and heating, as well as other chemical pollutants in the city, all contribute to soil, air and water pollution, which in turn leads to alterations in both bio-geochemical and nutrient cycles, as well as in primary production (Grimm et al., 2008) These negative effects of pollution during the breeding season would affect not only adults but also their offspring, either directly or via maternal transfer from mother to offspring during the egg formation process, for example (Ardia, 2005).

The impact of pollutants may extend beyond the borders of cities and enter the food chain, posing real threats to many species, including birds (Eeva et al., 2005; Eeva and Lehikoinen, 2013). Since they occupy high trophic levels and have a high metabolic rate, small insectivorous songbirds are often used in studies as indicators of the impact of chemical pollution in urban areas. Bioaccumulation of heavy metals has detrimental effects on birds' physiology (e.g. Hofer et al., 2010). Elevated levels of the bioaccumulation of heavy metals have been observed in a number of urban bird species, for example the blue tit (Dauwe et al., 2000; Brahmia et al., 2013), the American robin *Turdus migratorius* (Hofer et al., 2010) and the house sparrow (Swaileh and Sansur, 2006; Kekkonen, 2011; Bichet et al., 2013). The negative effects of this type of pollution on birds' physiology have also been demonstrated by several studies (e.g., Ware, 1993; Eeva et al., 2003; Eeva et al., 2005; Eeva et al., 2014). In general, young birds are believed to suffer the most from chemical pollution (Scheuhammer, 1987) particularly in terms of reduced body mass and higher mortality rates (Janssens et al., 2003).

1.4.5 Disease prevalence

Urban meteorological factors may make urban areas suitable for some diseases, and yet may reduce the prevalence of others (Martin and Boruta, 2013). One of these factors is the higher and less variable ambient temperatures in urban areas compared to their surrounding areas (Grimm et al., 2008; Pickett et al., 2011). Another factor might be the precipitation and relatively impermeable ground surfaces typical in urban areas, which fosters parasite transmission as well as the breeding of vectors (Githeko et al., 2000). In addition, the presence of permanent water bodies (such as ponds or fountains) throughout the year, which can be a limiting factor for vector reproduction during summer in some southern countries (Santiago-Alarcon et al., 2019).

Studies provided contrasting results of the impact of urbanisation on pathogen's prevalence depending on the identity of species involved in the interaction (i.e. pathogen and host species) (Lachish et al., 2012; Renner et al., 2016). While a number of studies have found higher prevalence of disease in urban populations compared to rural populations of wild animals [trichomonoiasis in Cooper's hawks *Accipiter cooperii* (Boal et al., 1998), unknown disease in the common racoon *Procyon lotor* (Prange et al., 2003), chronic wasting disease in mule deer *Odocoileus hemionus* (Farnsworth et al., 2005), West Nile virus in wild birds (Gibbs et al., 2006)], others reported the opposite where the rural wildlife populations have higher parasitism than their paired urban counterparts (Ixodes ticks in the common blackbird *Turdus merula* (Gregoire et al., 2002), dixenous helminths in the red fox *Vulpes vulpes* (Reperant et al., 2007), intestinal roundworm *Baylisascaris procyonis* in the common raccoon (Page et al., 2008)).

1.4.5.1 Haemosporidian parasites prevalence

Haemosporidian parasites (phylum Apicomplexa, order Haemosporida), are parasites commonly found in avian blood. Haemosporidian parasites from the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are often referred to as avian malaria parasites. They are transmitted by vectors (insects belonging to the order Diptera) including mosquitoes *Culicidae* in the case of *Plasmodium* species, biting midges *Culicoides* for *Haemoproteus* species and *Leucocytozoon caulleryi*, and blackflies *Simulium* for other *Leucocytozoon* species (Valkiūnas, 2005).

Avian malaria parasites have been of central interest in the ecological and conservational studies of host-parasite interactions over recent decades (Bensch et al., 2009). These parasites are diverse, and mixed infection, when a bird is infected with more than one parasite genus, is thought to be common in birds (Bensch et al., 2009; Clark et al., 2014). Studies have shown that infection with these parasites is associated with costs on life-history traits. Infections can affect host body condition (Merino et al., 2000; Valkiūnas et al., 2006), result in anaemia (Valkiūnas, 2005) and mortality (Cardona et al., 2002; Ilgūnas et al., 2016; Palinauskas et al., 2016). These parasites infect hosts' blood cells (mainly red cells, but in case of *Leucocytozoon* also the white blood cells), tissues and other major organs such as liver, brain, heart and lungs (Bray, 1957; Garnham, 1966; Frevert et al., 2008).

The parasite life cycle of infection starts by a bite from an infected vector (blood-sucking insects), allowing parasite sporozoites to enter the body and make their way to the liver infecting the hepatocytes and developing into schizonts; each schizont contains multiple numbers of merozoites. Development of schizonts sometimes occurs in the endothelial cells instead of the hepatocytes. Parasites at this stage enter their prepatent period, when the host is infected but show no symptoms of infection, then the merozoites are released into the bloodstream and infect, depending on parasite genera, and sometimes species, either erythrocytes, leukocytes, macrophages or endothelial cells. Each merozoite infects one single host cell and develops into a schizont, which is formed by multiple merozoites again. Merozoites infecting erythrocytes or leukocytes can develop into the sexual stage (gametocytes). The gametocytes are the transmissible stage that will infect and sexually reproduce in the insect vector, when the parasite life cycle starts again (Valkiūnas, 2005).

Infection with haemosporidians found to be associated with reductions in a number of fitness-related traits in adult birds including host body condition, parental investment, reproductive success and survival (Dawson and Bortolotti, 2000; Merino et al., 2000; Marzal et al., 2005; Marzal et al., 2008; Lachish et al., 2011; Sudyka et al., 2019; Dadam et al., 2019). In contrast, some studies found that chronic infection with these parasites has no significant effect on their avian host's overall condition and breeding success (Bennett et al., 1988; Ots and Hõrak, 1998; Kilpatrick et al., 2006; Podmokła et al., 2014). However, most of these studies are observational or correlational studies. Therefore, experimental studies would be essential to provide evidence of harmful effects of malaria parasites on their bird hosts. The effect of haemosporidian infection on their host is complex and has been suggested to be influenced by various factors including host trait (such as age, sex and health condition) as well as host's rearing and nutritional conditions, in addition to the type of both the parasite and the host species (see example studies in Table 1-1).

These parasites present a convenient study system because of their widespread among avian species and over wide geographical area (in every continent except Antarctica) (Valkiūnas, 2005). Studies of the impact of urbanisation on the prevalence of haemosporidian parasites showed mixed results (review Sehgal, 2015). For example, in the UK, eight out of 11 cities showed lower avian malaria (*Haemoproteus*) prevalence in adult blackbird *Turdus merula* compared to their surrounding woodlands (Evans et al., 2009). Similarly, Chasar et al. (2009) found higher prevalence of both *Haemoproteus* and *Leucocytozoon* parasites in the undisturbed areas in two bird species (adult yellow-

whiskered greenbul *Andropadus latirostris* and olive sunbird *Cyanomitra olivacea* in 9-paired natural habitat (disturbed verse undisturbed locations) in Cameroon. Likewise a very recent study on house sparrows (adults and yearlings) along urban gradients in Spain found higher prevalence of *Leucocytozoon* in natural habitat compared to urbanised areas (Jiménez-Peñuela et al., 2019). A study on five songbird species in a desert habitat showed that urban-adapter adult birds generally exhibited lower *Haemoproteus* parasites prevalence in urban compared to rural areas (Fokidis et al., 2008). In contrast, in Brazil, malaria prevalence (*Haemoproteus and Plasmodium)* in wild birds was higher in urban areas than in rural areas (Belo et al., 2011). Ferraguti et al. (2018) found no significant differences on *Plasmodium* prevalence or richness between habitat categories (urban, rural, natural).

1.4.5.2 Need for better tools for avian malaria parasite detection

Detection of haemosporidian parasites in host blood is relatively easy using molecular techniques and/or microscopy (Godfrey et al., 1987; Hellgren et al., 2004). The nested PCR protocol (nPCR) that has been developed by Bensch et al. (2000) and further modified by Waldenström et al. (2004) and Hellgren et al. (2004) has been widely used to detect the three genera of Haemosporidia (Clark et al., 2014). This protocol has been often used to assess the reliability and sensitivity of newly developed protocols by comparing their results with results of Haemosporidian parasite prevalence obtained from the nPCR method (Ishtiaq et al., 2017). Nevertheless, the nPCR and other PCR-based protocols often underestimate mixed infection because similar or equal amplicon sizes of different genera are expected (Valkiūnas, Bensch, et al., 2006). Additionally, cross-reactivity in the nPCR is another issue that adds to the shortcomings of these protocols. Previously, in nine out of 12 samples that were positive for both *Leucocytozoon* and *Haemoproteus*/*Plasmodium* by nPCR, sequencing revealed that only one of the two parasites – *Leucocytozoon* or *Haemoproteus* – was present (Capilla-Lasheras et al., 2017). Thus, the field is in demand of a new sensitive and specific molecular technique to quantify and more importantly distinguish between parasites genera in avian blood.

Table 1-1 Example studies investigating the effect of haemosporidian infection on adult host's fitness traits

Host	Parasite	Host trait	Infection effect	Observational/experimental	Reference
american kestrels *Falco sparverius* (wild)	*Haemoproteus*	body condition during breeding	• in female; poorer condition during incubation, but not prior to egg-laying, and reduction of return to study area • In male; poorer condition during incubation in one year but not the other	observational	(Dawson and Bortolotti, 2000)
blue tit *Parus Caeruleus*	*Haemoproteus* *Leucocytozoon*	breeding performance	• cost of infection paid by offspring body weight • Reduced parental care	medication experiment in the field	(Merino et al., 2000)
house martin *Delichon urbica*	*Haemoproteus*	breeding performance	• reduced clutch size, hatching and fledging success • no effect of infection on offspring quality (tarsus length, body mass, haematocrit and T-cell immune response	medication experiment in the field	(Marzal et al., 2005)
house martin *Delichon urbica* (wild)	*Haemoproteus* *Plasmodium*	breeding performance	• reduced survival. But have a different consequence on the breeding performance of single and double-infected birds	observational	(Marzal et al., 2008)
blue tit *Cyanistes caeruleus* (wild, data from 9 years)	*Haemoproteus* *Plasmodium*	survival and recapture rate	• reduced survival and recapture rate depending on parasite clade, host trait and local risk of infection	observational	(Lachish et al., 2011)
blue tit *Cyanistes caeruleus* (wild)	*Haemoproteus* *Plasmodium*	Telomere length (TL)	• male only infected with *Plasmodium had shorter TL compared to those infected with Haemoproteus*	observational	(Sudyka et al., 2019)
red-winged blackbird *Agelaius phoeniceus*	*Plasmodium*	red cell production, haemoglobin level, body weight, immune system and stress physiology	• treatment decreased parasitaemia, reduced red blood cell production and increased haemoglobin level. However, no effect of	medication experiment in the field	(Schoenle et al., 2017)

			treatment on the rest of the physiological traits tested		
great tit *Parus major* (wild, data from 3 years, from an urban and a rural site (8km between the two sites)	*Haemoproteus*	• cell-mediated and humoral immune response (lymphocyte haemoconcentration and plasma gamma-globulin levels) • inflammatory response (Heterophile haemoconcentration and plasma albumin levels) • anaemia (haematocrit values) body weight	• infected males only had elevated lymphocyte haemoconcentration and plasma gamma-globulin levels, and among these, the magnitude of the effect was more significant in old individuals than yearlings. • heterophile haemoconcentration and plasma albumin levels were not affected by infection status, suggesting that blood stages of *Haemoproteus* infection do not cause a severe inflammatory response. • parasitism was not related to haematocrit values, indicating that *Haemoproteus* infection does not cause anaemia • in two years, infected individuals were heavier than uninfected ones in the urban but not in the rural study area	observational	(Ots and Hõrak, 1998)
native Hawaiian honeycreeper *Hemignathus virens* (wild)	*Plasmodium*	breeding performance	• infected parents did not significantly reduce their reproductive performance (clutch size, hatching and fledging success, daily nestling survival rate)	observational	(Kilpatrick et al., 2006)
blue tit *Cyanistes caeruleus*	*Haemoproteus* *Plasmodium*	breeding performance	• infected birds produced heavier offspring and breed later in the season, but the infection did not affect clutch size • the infection had a stronger positive effect among birds with experimentally enlarged broods.	brood enlargement experiment in the field	(Podmokła et al., 2014)
3739 birds from 15 species of passeriform	*Haemoproteus* *Leucocytozoon*	body mass	• no effect of parasite prevalence on bird's body mass	observational	(Bennett et al., 1988)

(wild)					
house sparrow *Passer domesticus* (wild, 11 suburban sites)	*Plasmodium*	population growth decline	• survival rate of juvenile and adult birds and population growth were negatively related to *Plasmodium relictum* infection intensity	observational	(Dadam et al., 2019)

1.5 Physiological stress response to urban environment

Birds, like other vertebrates, can tackle environmental challenges by activating the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky et al., 2000; Bonier, 2012). The HPA axis is comprised of the hypothalamic paraventricular nucleus (PVN), the anterior pituitary gland, and the adrenal cortex. The activation of HPA axis lead to secretion of glucocorticoids (GCs) for several minutes to hours (Wingfield and Romero, 2011). The process starts when an organism encounters a stressor, the PVN is stimulated, causing the parvocellular neurons to release corticotrophin-releasing hormone (CRH) and other secretagogues, such as arginine vasopressin (AVP) into the hypophyseal portal system that connects the hypothalamus and the anterior pituitary. CRH and AVP stimulate the anterior pituitary to synthesize and cleave the precursor molecule proopiomelanocortin into adrenocorticotropin hormone (ACTH). ACTH is released into the blood stream and stimulates the adrenal cortex to secrete GCs above basal levels (Figure 1-2).

HPA activation triggers an emergency response prioritising energetic expenses for survival over immunity and some crucial behaviours (e.g. escape behaviour or begging behaviour) (McEwen, 1998; Sapolsky et al., 2000). However, long term or chronic exposure to stressors and elevation of glucocorticoid levels may have detrimental effects (Boonstra et al., 1998; Blas et al., 2007). It has often been found that the amplitude and duration of the stress response negatively correlate with the overall health and well-being of animals (Sapolsky, 1983; Boonstra et al., 1998). Thus, GC levels have been used a proxy of animal well-being in ecological and conservational studies (Busch and Hayward, 2009).

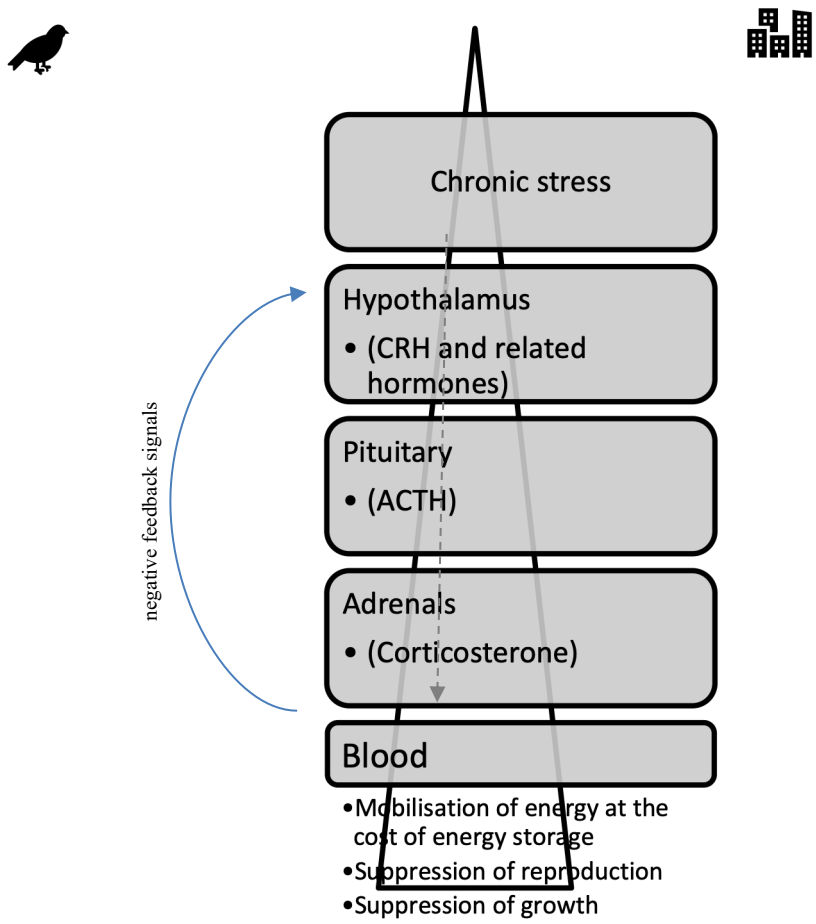


Figure 1-2 The hypothalamic–pituitary–adrenal (HPA) axis, the response of birds to chronic stress due to urban-related stressors, and the main impacts stressors have on host's biological and fitness traits. Figure adapted from (Sheriff et al., 2011)**.**

CORT is the main glucocorticoid for avian species, which has been found elevated in nestlings as a result of a variety of environmental stressors such as lack of food availability (Kitaysky et al., 2007; Alexis P Will et al., 2014; Patterson et al., 2015) and heavy metal pollution (Meillère et al., 2016). The detrimental effects of elevated glucocorticoid levels include impaired immunity (Saino et al., 2003; Eeva et al., 2005; French et al., 2008), inhibited growth (Belden et al., n.d.; Eeva et al., 2014; Alexis P Will et al., 2014; Rodríguez and Barba, 2016), and reduced reproduction (Wingfield and Sapolsky, 2003; Lendvai et al., 2007). Impairing immunity would mean that individuals are more susceptible to and affected by potential diseases in their environment.

Usually, CORT is measured in plasma which provides a measure at a single point in time, giving information about the current physiological state. CORT can also be measured in

metabolites present deposited in feathers during their growth. The feather CORT (fCORT) provides a historical record of an individual's CORT release during the period of feather growth. However, how CORT is deposited in feathers still an open question (Jenni-Eiermann et al., 2015).

It is costly for an organism to mount an immune response to fight off pathogens, thus they should be in a good condition to develop more effective response (Navarro et al., 2003). For instance, according to life history theory and the energetic trade-off hypothesis, during reproduction, there is a high demand for energy and immune functions may be reduced in order to redirect energy to allow an individual to invest in their reproductive effort and, hence, increase the likelihood of offspring survival (Norris and Evans, 2000). However, this process increases potential susceptibility to disease. A number of experimental studies have demonstrated that elevated immune activity can affect other key traits; for example, developing sexual ornamentation (Zuk, 2000), chick-feeding (Ilmonen et al., 2000), growth rates of offspring (Brommer, 2004) and clutch size (Martin et al., 2001).

To conclude, my review of the literature shows that birds' fitness can be affected by different aspects of the urban environment. Furthermore, birds are hosts to a variety of parasites and living in such an environment is likely to increase the susceptibility of birds to parasite infection in urban populations. However, the effect of parasite load on the fitness-related traits of species living in the urbanised areas has received little attention, and efforts are needed in this area. In general, the review shows a gap in evaluating fitness-related traits of wild birds within urban environment. Parasite infections such as with haemosporidian parasites, and urban-related stressors that elevate stress hormone levels in urban-dwelling birds have the potential to form a synergistic impact that could affect the fitness of host living in such a harsh condition.

1.6 Thesis aims

Urbanisation has negatively affected different fitness-related traits of urban species resulting in an overall loss of biodiversity and populations with reduced reproductive performance. However, the mechanisms behind this phenomenon remain unclear. Urban-related stressors such as light, noise and chemical pollution, negatively affect the health of some vertebrates, including birds. These stressors may lead to a chronically elevated level of corticosterone hormone in birds, which may have detrimental consequences on avian body condition and fitness. Such stresses may increase the severity of parasite infestations, affecting the capacity of hosts to occupy urban areas.

The aim of the thesis was to explore two factors that may be linked to differential success in urban and rural populations – stress and parasitism – and their relationship with reproductive fitness. By studying this relationship in both an urban and a rural population of blue tit across two breeding seasons, I aim to gain preliminary insights into whether these specific factors might contribute to reduced fitness in urban areas. I used both correlational and experimental approaches to study the potential link between haemosporidian parasite infection and stress hormone levels and their relationship with blue tit fitness. This thesis also presents a new molecular approach to quantify and identify haemosporidian parasite genera in avian blood.

1.7 My study system

1.7.1 Study sites

I used existing nest box study systems used to monitor annual blue tit breeding success (woodcrete boxes: 260 × 170 × 180 mm, entrance = 32 mm, Schwegler, Germany in one urban and one rural site (Figure 1-3). Blue tits commonly breed in both urban and rural sites, allowing for a comparison of birds between the two habitats. The two populations of blue tit have been previously studied and lower reproductive success was observed in the urban population compared to the rural population (Capilla-Lasheras et al., 2017; Pollock et al., 2017), as has been shown by other studies on this and other passerine species (Chamberlain et al., 2009). Diet (i.e. availability of arthropods) had also been studied in the previous year at these exact study sites and the urban site showed lower quality and quantity of natural food (e.g. caterpillars) compared to the rural site (Pollock et al., 2017).

The rural site is a natural woodland park located on the eastern shores of the largest lake in Britain (Loch Lomond). The woodland has been recognised as an outstanding example of old sessile oak *Quercus petraea* wood with Ilex and Blechnum habitat. Atlantic oak woodland is found on acidic soil and is very rich in lower plants. Associated with this vegetation, under leaves and on the ground, are a myriad of insects providing a feast for birds and as a result, the woodland has high densities of breeding birds (up to 1500 pairs per km^2).

The urban site is a park characterised by intensive urban structures around the park, such as roads and buildings. The park is 34 hectares in size, sitting on the banks of the River Kelvin and located a few kilometres from the city centre of Glasgow. The park is in a central location linking the university of Glasgow from the west to other popular residential areas, making the surrounding of the park a popular route for commuters to the city centre. The park has playgrounds and memorial statues and is heavily visited by people. It is popular with dog-walkers, students, visitors and tourists. One of the largest museums and art galleries in the city is just next to the park. Summer outdoor entertainment events are often held in the park, just during the breeding season of most residential birds including my study species – the blue tit.

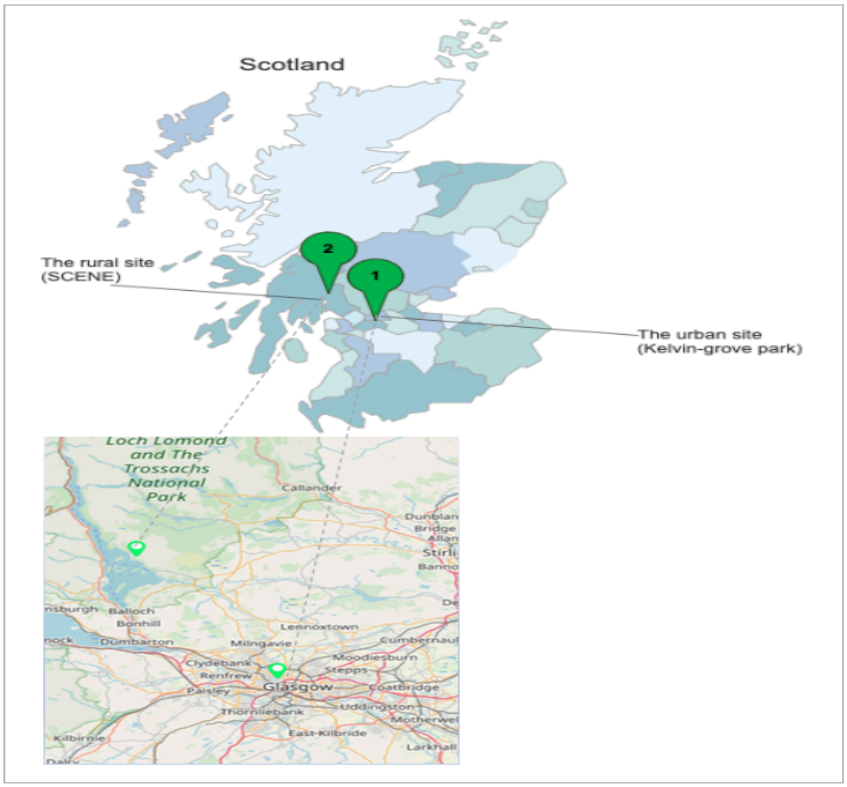


Figure 1-3 The urban and rural sites of blue tit populations under the studies of my thesis

1.7.2 Blue tit *Cyanistes caeruleus*

The blue tit is a small hole-nesting passerine belonging to the Paridae family. The species is widely distributed in temperate regions. In the UK, the blue tit is a common passerine in lowland deciduous woodland, and also common in gardens and parks in urbanised areas. In research, it is an attractive study species because of its large population size. According to the British Trust for Ornithology (2016), there are 3.4 million breeding pairs each year in Britain and it is one of the 24 most common bird species in the country. Blue tits commonly take advantage of artificial nest boxes and use them to breed, which allows easy access to monitor their reproductive performance and conduct some experiments in nest boxes for research purposes. In Scotland, blue tits breed between April and June, start nest building in March and egg laying of a single brood takes place during April or May. Only females incubate the eggs, while the male brings food during incubation (Perrins et al., 1965). After the eggs have hatched, both males and females start to feed the nestlings and continue to do so after fledging for a few days.

1.8 Thesis structure

In Chapter 2 ("Year-dependent variation in feather corticosterone hormone between an urban and a rural population of blue tit *Cyanistes caeruleus*"), I use an integrative measure of the level of corticosterone (the main avian glucocorticoid) metabolised and deposited in feathers (fCORT) of nestling blue tits inhabiting urban and rural sites over two breeding seasons. I aim to determine whether there are variations in the level of fCORT between nestlings from the two populations and whether this variation is consistent over time. In addition, I compare nestling survival and nestling body weight between sites and years and explore the association between these traits and fCORT level. In order to test for any variation in the level of nestlings' fCORT that could be derived from mothers via hormone deposition in the egg, I cross-fostered clutches between urban and rural sites and, in addition, measured yolk corticosterone (yCORT) levels from eggs from both sites. The finding of this chapter gives a piece of overview information regarding the stress level, the fitness and overall breeding success of both urban and rural population. Next, I will investigate the association between these traits (fitness and stress level) and haemosporidian parasite infections. However, before testing this association, I needed to establish an adequate method to detect and accurately identify the parasites in blue tit nestlings in my study populations.

In Chapter 3 ("Molecular quantification of haemosporidian parasites (*Leucocytozoon* and *Haemoproteus*)"), I describe the development steps of a new quantitative Polymerase Chain Reaction (qPCR) approach for detecting haemosporidian parasites, specifically of the *Leucocytozoon* and *Haemoproteus* genera. I then compare the results from this new method with the results of a nested-PCR method (Hellgren et al., 2004). I assess the effectiveness of the qPCR method in detecting a very low intensity of malaria parasites which could not be detected with the nested-PCR method. This new approach also helps to detect co-infection more accurately, as in the nested PCR approach cross-reactivity is not excluded and co-infection might be falsely detected in some cases. Using this new approach, in Chapter 4 I go on to compare the prevalence of *Leucocytozoon* parasite, the predominant infection in my study population, between the two populations. Then, I go on to test the association between infection prevalence and fitness-related traits of blue tits.

In Chapter 4 ("*Leucocytozoon* prevalence in blue tit *Cyanistes caeruleus* populations at an urban versus a rural site across two breeding seasons"), I test whether haemosporidian parasite prevalence differs between urban and rural sites by comparing *Leucocytozoon* infections and intensity of urban and rural populations of blue tits across two breeding seasons. To test if the susceptibility of the bird to the infection is driven by the origin of the bird (i.e. by traits inherited from parents to their offspring), I cross-foster some clutches between the sites in 2016, as described above. In order to test if vector abundance in the nest is influencing the prevalence of infection, I treat some nests with vector-repellent at both sites in 2017.

In Chapter 5 ("*Leucocytozoon* infection prevalence is associated with reduced body weight and fledging success of urban wild blue tit nestlings"), I test the association of *Leucocytozoon* infection with fitness-related traits (body weight and survival) of nestling blue tits dwelling in an urban and a rural site during two breeding seasons.

In Chapter 6 ("General discussion"), I bring the findings of my thesis together and evaluate the main points, and I shed light on possible future directions based on my findings.

In this thesis, I address the knowledge gap regarding the impact of urbanization on bird fitness. I use an urban and a rural population of blue tit *Cyanistes caeruleus* across two breeding seasons (2016 and 2017) to test my hypotheses. I use corticosterone levels in

feathers as a biomarker of stress and test the prevalence of avia malaria parasites in these two populations.

Chapter 2 Year-dependent variation in feather corticosterone hormone between an urban and a rural population of blue tit *Cyanistes caeruleus*

2.1 Abstract

In order to cope and thrive in an urban environment, urban dwelling birds need to respond to the challenges in such an environment that differ from those in their natural habitat. It has often been reported that urban species show lower breeding success and lower body condition compared to their rural counterparts. However, the mechanisms behind this phenomenon remain poorly understood. One of the possible reasons for such an effect may be related to differences in urban species' glucocorticoid secretion between environments. Glucocorticoid levels can modulate a number of physiological and behavioural processes to enable animals to meet the challenges of the urban environment, which can affect breeding success and body condition. Here, I used an integrative measure of the level of corticosterone (the main avian glucocorticoid) metabolised and deposited in feathers (fCORT) of nestling blue tit *Cyanistes caeruleus* inhabiting urban and rural sites over two breeding seasons. It was aimed to determine whether there are variations in the level of fCORT between nestlings from the two populations and whether this variation is consistent over the years. In order to test for any variation in the level of nestlings' fCORT that could be derived from inherited traits from their parents such as from mothers via hormone deposition in the egg, I cross-fostered clutches between urban and rural sites and, in addition, measured yolk corticosterone (yCORT) levels from eggs collected from both sites. I found that fCORT levels differed between the sites depending on the year. I found a significant interaction between site and year, suggesting that annual differences in fCORT levels and nestling's weight are inconsistent between the two populations. In the year where fCORT did not differ between habitats I also found no differences in yCORT levels between urban and rural sites nor did I find differences in fCORT between nestlings reared in a different environment than their environment of origin. In addition, I compared some fitness-related traits of the two populations (e.g. nestling survival (fledging success) and nestling body weight) over the two years. I found no relationship between fCORT and nestling body weight or fledging success. Overall, these results suggest that fCORT levels and blue tit nestling weight differ between years and between the foster parent but not the biological parent's environment. This study emphasises the importance of multi-year monitoring and comparison between urban and rural avian populations in order to understand the mechanisms behind the negative impact of urbanisation on avian physiology and reproductive success.

Key words: blue tit, *Cyanistes caeruleus*, stress, urban, corticosterone, feather, maternal effect, yolk

2.2 Introduction

Human populations living in urban areas are increasing rapidly worldwide; currently, more than 50% of the world's population resides in urbanised areas (UN, 2014). This rapid change will inevitably mean more anthropogenic structures and alterations to the natural environment, affecting wildlife. Although some urban-dwelling birds may benefit from the warmer microclimate during winter and the increased availability of food throughout the year in cities, the urban landscape also presents them with a number of stressors, including human disturbance (Fernández-Juricic and Tellería, 2000; Rebolo-Ifrán et al., 2015; Lamb et al., 2017), traffic (Forman et al., 2002), increased temperature or heat islands (Rodríguez and Barba, 2016), and different types of pollution such as noise (Peris and Pescador, 2004; Schroeder et al., 2012; Meillere et al., 2015), artificial light (Longcore and Rich, 2004; Eisenbeis and Hänel, 2009; Dominoni et al., 2013), chemical pollutants (Bichet et al., 2013; Meillère et al., 2016), and disease burden (Hassell et al., 2017).

Corticosterone (CORT, the main avian glucocorticoid) levels can be used as a biomarker to assess how urban environment, through multiple stressors, affects the stress physiology of urban dwelling birds. To cope with environmental challenges, the hypothalamic-pituitary-adrenal (HPA) axis in vertebrates including birds (Sapolsky et al., 2000; Bonier, 2012) regulates the use of resources by adjusting plasma levels of glucocorticoids, for example the mobilization of glucose to enable high metabolic expenditure (Jimeno et al., 2017). Thus, the activation of the HPA axis triggers an emergency response, prioritising energetic expenses for immediate survival over self-maintenance, growth and immunity (McEwen, 1998; Sapolsky et al., 2000). However, long term or chronic exposure to stressors leading to sustained elevation of basal glucocorticoid levels can have detrimental effects, such as impairing nestlings immunity (Moller and Saino, 2004; Eeva et al., 2005) and reduces their survival, e.g. vulnerability to West Nile virus (Owen et al., 2012), inhibiting growth (A. P. Will et al., 2014; Eeva et al., 2014; Rodríguez and Barba, 2016), and reducing reproduction (Wingfield and Sapolsky, 2003; Lendvai et al., 2007). CORT has been found elevated in nestlings as a result of environmental stressors such as lack of food availability (Kitaysky et al., 2007; A. P. Will et al., 2014; Patterson et al., 2015) and heavy metal pollution (Meillère et al., 2016).

However, no consistent patterns have been revealed and baseline CORT levels (the most frequently used biomarker) have been found to be similar, higher and lower in urban versus rural birds (reviewed in Bonier, 2012). Therefore, how and in which direction the urban environment affects CORT levels in birds is still poorly understood. In order to answer key questions concerning the impact of anthropogenic stressors on stress physiology of wild animals, which consequently affect their ability to thrive in cities, more experimental and comparative field studies are needed (Marzluff, 2017). However, experimental studies in the field, in addition to comparisons across multiple years, are rare in the literature.

The level of glucocorticoid secretion, that is regulated by the HPA axis, can be driven by environmental factors directly affecting an individual throughout its lifetime. Prolonged levels of glucocorticoid secretion can induce alterations in gene expression profiles, through for example methylation of the DNA or histone tail modifications (e.g. Joëls et al., 2007; Goerlich et al., 2012). Lastly, adjusting the level of glucocorticoid secretion may be inherited non-genetically through the mother, directly transferring hormones to the offspring during egg formation (e.g. Hayward and Wingfield, 2004; Saino et al., 2005; Almasi et al., 2012).

CORT levels are usually measured from blood plasma which gives a snapshot of the bird's stress experience at that time point. Although, the technique is relatively new and needs more validation, CORT can also be measured in feathers (fCORT). A number of studies found no correlation between fCORT and parameters of individual birds' condition, indicating that fCORT might not be recording all the ecologically relevant stressors experienced by individuals (Fischer et al., 2017; Studholme et al., 2018; Beaugeard et al., 2019). For instance, a food restriction experimental study on wild caught European starlings *Sturnus vulgaris* found no variation in fCORT levels between birds with unpredictable or continuous food access (Fischer et al., 2017). Similarly, in juvenile house sparrows *Passer domesticus*, fCORT levels were not corelated with individuals' body mass or body condition. However, fCORT levels were significantly and possitively correlated with the degree of urbanisation (Beaugeard et al., 2019). Studholme et al. (2018) reported no relationship between fCORT levels and egg size of Cassin's auklets *Ptychoramphus aleuticus*, but this lack of relationship may be because this study had been conducted under good enviromental conditions when birds do not face trade-offs (elevation of CORT) and have enough recources to allocate for foraging effort and reproduction (egg size), for

example. Neverteless, Bortolotti et al. (2008) and subsequent validation studies (e.g. Bortolotti et al., 2009; Jenni-Eiermann et al., 2015; Ganz et al., 2018) support using fCORT as a non-invasive way of measuring chronic stress experienced by birds over the period of feather growth (e.g. the whole nestling period). In addition, I am interested in an integrative measure of CORT, which can be accomplished by measuring the fCORT levels (e.g. Jenni-Eiermann et al., 2015).

Nestling altricial birds (that need parental care for a length of time after hatching) present a good opportunity to investigate the relationship between habitat stressors, growth and CORT physiology, because of the relatively long period of nestling stage during which the majority of their developmental process occurs (Starck and Ricklefs, 1998), and this may reflect the quality of the surrounding environment and affect their survival and growth. To date, this crucial stage of bird life (i.e. nestling stage), however, has been understudied in research concerning the effect of urban environments on stress physiology. To my knowledge, only a few studies have used nestlings or early-fledglings to test variation between urban and rural populations in terms of CORT release, for example Meillère et al. (2016) on blackbirds *Turdus merula* and Meillère et al. (2015) on house sparrows *Passer domesticus*. The former study showed non-essential trace element burden in birds' feathers is positively correlated with the degree of urbanisation and this increase in trace elements is positively associated with CORT level (Meillère et al., 2016). The latter concluded that urban house sparrows showed lower body size and mass compared to their rural counterparts, but the two groups did not differ in terms of their CORT level, indicating that urban house sparrows did not face stress. However, in a similar context, (Lodjak et al., 2015) compared the level of fCORT in nestling great tit *Parus major* reared in different habitats (coniferous versus deciduous forests) after a brood size manipulation experiment. Coniferous forest is typically a poorer habitat for great tits than deciduous forest, thus brood manipulation is a stronger stressor there than in deciduous habitat. They showed that fCORT increased after enlargement of brood size only in coniferous forests where growth rate tends to be poorer compared to deciduous forests.

The aim of this study was to compare the level of fCORT and the breeding success of blue tits in an urban and a rural site across two breeding seasons. I compared several breeding parameters (clutch size, egg weight, nestlings' weight at day 13 and fledging success (nestlings survival) in urban and rural populations throughout the two breeding seasons of 2016 and 2017. In vertebrates, body condition (based on relating body weight to linear

measures of body size, e.g. tarsus length, or wing length) is assumed to influence an animal's health and fitness. However, I preferred to use nestling body weight instead, aiming to avoid bias or inter-individual variation on morphological measurements (i.e. tarsus length) because different researchers were involved in taking body measurements. I hypothesised that fCORT variation between urban and rural nestlings will be consistent across the years. The second aim of this study was to test if nestlings differ in fCORT, whether the level of fCORT has been derived from parents and passed on to their offspring. Because CORT is deposited in the egg and can affect plasma CORT levels in nestlings in their early stage of life (Hayward and Wingfield, 2004), I also compared CORT levels in the egg yolk (yCORT) between sites. In order to experimentally test whether fCORT are determined epigenetically or environmentally, I conducted a cross-fostering experiment by exchanging clutches between sites. I hypothesised that if the differences in fCORT were environmentally induced during nestling rearing and did not originate from differences at the egg stage (i.e epigenetically), I would expect similar fCORT levels in control and cross-fostered nestlings within the same site, irrespective of their origin. If there are effects of the laying environment, I hypothesised that fCORT of nestlings in the same rearing environment would differ depending on their origin. The third aim of this study was to test whether this possible spatial (from site to site) and temporal (from year to year) impact of urbanisation on fCORT levels is associated with lower nestling body weight at day-13 (just before they leave the nest) and lower nestling survival by looking at within-habitat relationships between these fitness-related traits.

2.3 Materials and methods

2.3.1 Ethical Statement

All egg and feather sampling was conducted under licence of UK Home Office, Animals Scientific Procedures Act 1986, and individual ringing under licence from the British Trust for Ornithology. Egg collection and cross-fostering was carried out under licence from Scottish Natural Heritage (SNH).

2.3.2 Field Protocol and sampling

Work was carried out in one urban and one rural site in and around Glasgow, Scotland, between April and June in 2016 and 2017 (see detailed description in Chapter 1). At the beginning of the season nest boxes were visited once a week to monitor nest building

stages, laying date and the start of incubation. After the tenth day of incubation (first day of incubation was defined as when the last egg was laid), nests were visited every other day and it was estimated whether hatchlings had hatched the same day (day 0) or the day before (day 1) and a hatch date was assigned for every nest (Table 2-1). On day 13 after the first egg hatched (referred to as sampling date), three to four nestlings were weighed, and two to four body feathers (chest feathers cut from the base) from each of these nestlings were collected and kept in Eppendorf® tubes in a dark, dry place until lab analysis. Nest boxes were checked after fledging to search for dead nestlings. The number of hatchlings divided by the number of eggs laid represented hatching success, however since clutch size was reduced to six eggs in the experimental nests in 2016 (see below 2.3.2.2), hatching success for these nests only was calculated as the number of hatchlings divided by six. The number of fledglings divided by the number of hatchlings represented fledging success (nestling survival). Sample sizes are presented in Table 2-2 under the statistical model for each response variable.

2.3.2.1 Weather data

The information of weather condition for the two breeding seasons 2016 and 2017 (April-June), particularly daily mean temperature (ºC) and daily rainfall (mm), was retrieved from the *Met office* (https://www.metoffice.gov.uk/) (Table 2-1). The weather information is for the nearest point to each site (i.e. the urban and the rural site) with complete weather data. These points are Bishopton station which is 12.3 miles away from the urban site, and Gartocharn portnellan farm which is 14.5 miles away from the rural site.

Table 2-1 The timing of different breeding events and the mean temperature (°C) during the breeding season of blue tit s in the year 2016 and 2017: data are presented for both urban and rural sites.

	year	Urban site	Rural site
Date of first egg laid	**2016**	**22 April**	**22 April**
	2017	**20 April**	**17 April**
Mean of laying date	**2016**	**4 May**	**30 April**
	2017	**1 May**	**2 May**
Date of first hatching	**2016**	**11 May**	**16 May**
	2017	**12 May**	**8 May**
Mean of hatching date	**2016**	**21 May**	**23 May**
	2017	**17 May**	**15 May**
***Daily mean temperature (ºC) (maximum- minimum)**	**2016**	**14 (27.3-4.8)**	**13.6 (26.5-4.5)**
	2017	**13.1 (26.2-3.4)**	**13.0 (26.5-3.1)**
***Daily total rainfall (mm)**	**2016**	**1.9**	**3.1**

(maximum- minimum)		**(18.8-0)**	**(20.7-0)**
	2017	**2.7**	**3.1**
		(12.4-0)	**(17.5-0)**

***from https://www.metoffice.gov.uk/**

2.3.2.2 Cross-fostering experiment and egg collection (2016 breeding season)

Eighteen blue tit clutches in the urban and 22 in the rural site were manipulated prior to clutch completion: 8 urban nests and 12 rural nests were swapped with each other within sites, representing control nests; and 10 nests were swapped across sites, representing experimentally cross-fostered nests (in total 40 manipulated nests) (Figure 2-1). Clutches within and across sites were matched based on the date the sixth egg was laid. Before swapping clutches, every egg involved in the experiment was individually marked, weighed, and temporarily stored at 4°C. Clutch size was reduced to six viable eggs in both sites in order to control for possible inter-site differences in clutch size. After swapping clutches, nests were checked every other day and newly laid eggs were replaced with clay eggs.

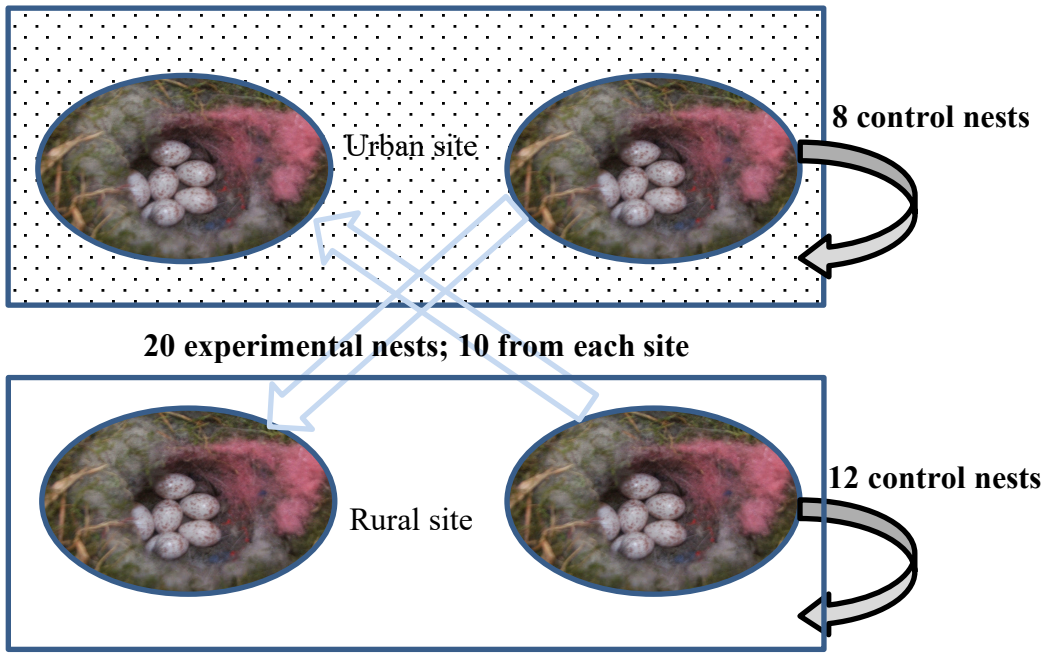


Figure 2-1 Cross-fostering experimental design

All collected eggs were weighed and put into a -80° freezer within three days of collection for later yolk corticosterone analysis. Using clay eggs, the original number of eggs laid by females (each nest contained six real eggs plus a variable number of clay eggs depending

on the number of eggs that females actually laid) was maintained. The clay eggs were removed from nests after hatching. From the eggs collected during the experiment, I used one to three eggs per nest from urban nests and one to five eggs from rural nests to analyse yolk corticosterone (yCORT). Analysing yCORT only from eggs laid late in the laying sequence is still representative for the entire clutch as excepted for the first-laid egg, previous research found no differences in the yCORT level deposited in great tit (a species related to blue tit) eggs depending on egg order (Lessells et al., 2016). When there was more than one egg used from a nest I took the mean of the yCORT level to represent the level of yCORT for this nest.

2.3.3 Corticosterone Extraction and Measurement

2.3.3.1 Feather Samples

In order to extract corticosterone from feathers, it has been suggested that it is best to maximise the surface area by cutting it into pieces < 5mm (e.g. Bortolotti et al., 2008; Lattin et al., 2011). To establish the best treatment of blue tit feathers that maximises surface area and thus the CORT extraction, pilot studies were conducted. I pooled the samples collected from additional adult blue tits and pooled other samples collected from blue tit nestlings. For each group (adult and nestling), I first removed the calamus, then powdered the pooled feathers using a ball mill after freezing the feathers in liquid nitrogen. The results of an ELISA corticosterone assay showed that powdering and cutting the feathers to < 5mm gave a similar amount of fCORT (10.7 and 12.3 pg/mg of feathers, respectively). So, I decided to continue with cutting the feathers and skipping the powdering step in order to reduce the steps involved and lower the risk of possible loss of my small samples (two to four feathers from each nestling).

fCORT was measured following Bortolotti et al. (2008), with minor modifications, by weighing each sample to the nearest 0.01 mg (Mettler AE160 digital analytical balance 162g by 0.01mg) – all samples weighed > 1 mg (1-5 mg). I kept the samples in a 3ml glass vial, washed them with 20% methanol and shook them on a plate shaker for 10 mins before washing them twice with water in order to remove any possible contamination. I air-dried the samples by leaving the vials partially open at room temperature until totally dry.

The day prior to running the ELISA assay for measuring the concentration of fCORT, I added 1ml HPLC-grade methanol to the feather samples and incubated the feather samples

overnight at 52°C, 120 rpm in Gallenkamp environmental shaker (model 10X 400). After 19 hours of incubation, I transferred each sample into a borosilicate glass tube (10-15 ml) via a syringe with a filter to separate the liquid from the feathers. Then I evaporated the extract in a sample concentrator, medium temperature, volatile solvent setting. The sample was then dissolved in 300 µl immunoassay buffer and vortexed for 10 mins, and the assay run according to the manufacturer's instructions (Cayman Chemical, corticosterone ELISA kit, item No. 501320). Each ELISA plate contained two blank wells (background absorbance), two non-specific binding wells, and an eight-point standard curve run in duplicate. All samples were run in duplicate; samples of 2016 were run in two plates, while samples of 2017 were run in a single plate. The average intra-assay coefficients of variation were 6%, and the inter-assay coefficients of variation were 14%. Optical density was measured at wavelength between 405nm and 420nm using a plate reader (Labtech/ LT-4500) and Assay Zap computer software was used to calculate fCORT concentration from optical density. I then corrected the results against the weight of the feathers by dividing the fCORT values by the weight of feathers and also standardised against the dilution factor, which is 3.33 (1 ml of HPLC-grade methanol/300 µl assay buffer).

2.3.3.2 Yolk Samples

Eggs that were stored in the freezer at -80°C were manually separated into yolk and albumen at a very low temperature (on ice) to minimise thawing of the egg and the mixing between yolk and albumen. The yolk was then dissolved in 5 ml 1% formic acid, vortexed for 30 s and centrifuged for 10 min. Then, 2.5 ml aliquot of the supernatant was transferred to another tube, and the lipid was defatted from the corticosterone and other yolk components by adding 2.5 ml of a solvent (hexane). The sample then was centrifuged, and the hexane phase was removed while the lower phase was transferred to another tube to be evaporated using a sample concentrator, medium temperature, volatile solvent setting. The dry residue was reconstituted in 100 µl methanol, vortexed for 10 s, and diluted with 900 µl water. The sample was further purified using C18 SPE (Solid-phase extraction) column (HyperSep^{TM} C18, Thermo Fisher, UK) after conditioning with 1 ml water and 1 ml methanol. Finally, samples were evaporated in a sample concentrator, medium temperature, volatile solvent setting. The samples were then dissolved in 300 µl immunoassay buffer and vortexed for 10 mins in preparation for the ELISA assay. The yCORT levels were then quantified following the instruction of the kit (Cayman Chemical, corticosterone Enzyme Immunoassay (EIA) Kit, item No. 500655). The plate design and reading are as mentioned above for fCORT plates. All samples (65 eggs from 20 rural

nests and 31 eggs from 14 urban nests) were run in duplicate in three separate plates. The intra-assay and inter-assay coefficients of variation were 6% and 53%, respectively.

2.3.4 Determining sexing of nestling

Sex of the nestlings was determined using DNA extracted from blood samples (see Chapter 3 for DNA extraction protocol) and following the molecular approach by (Griffiths et al., 1998). In brief, amplification of the target genes (CHD-W and CHD-Z) from sex chromosomes were obtained using the following PCR cycling profile; initial denaturation 94°C/2 min, denaturation 49°C/45 s, annealing 72°C/45 s, 94°C/30 s template extension 49°C/1 min, and a final extension 72°C/5 min. PCR was performed in 10µl of reaction mixture containing 5-10mg/µl DNA template, 2 mM MgCl2, 0.16 mM dNTP, 0.8 mM of primers P2 and P8, 0.375 U/µl Taq polymerase (Promega) and 1 µl buffer (Promega). PCR products were separated by electrophoresis at 5V/cm for 60 minutes on 2% agarose gel stained with ethidium bromide. Nestlings were sexed according to the presence of two bands for females and one band for males.

2.3.5 Acknowledgements

I am grateful to Leo Truglia under Michelle Bellingham's supervision for conducting the yolk corticosterone lab analysis as part of his internship project at the university of Glasgow. I am also grateful to Zara Nelson under Barbara Mable's supervision, and the lab technician Elizabeth Kilbride for the molecular identification of the sex of the birds as part of Zara's honour project.

2.3.6 Statistical analysis

For every response variable, data analysis started with a global general and generalised linear models including all predictors assumed to be biologically important. Models were diagnosed for multicollinearity, when two or more of the predictors in a regression model are highly correlated, which is common in ecological data (Graham, 2003) using the variance inflation factor test (VIF). Hatching date and brood size were standardised within sites as they showed collinearity with other predictors (e.g. site or year). Hatching date was not normally distributed, so I standardised by subtracting the median date from each date value and divided by the interquartile range from that site. Brood size was normally distributed, and I standardised it by subtracting the mean brood size from each brood size value and divided by the brood size's standard deviation from that site.

Starting from the most complex (global) model, stepwise simplification was applied using likelihood ratio tests (LRTs) of fully nested models until a final model was found. Statistical significance for each term was calculated by assessing the reduction in explanatory power after dropping the factor from the model. Statistical analyses used packages 'car' (Fox and Weisberg, 2011), 'nlme' (Pinheiro et al., 2009) and 'lme4' (Bates et al., 2014) in R v. 3.3.3 (R Development Core Team, 2017).

A number of fitness-related traits were examined for both sites during each breeding season in 2016 and 2017. A linear model (LM), with normal distribution, was used to model **clutch size**, while generalised linear models (GLMs), with binomial distributions and logit link functions, were used to model **hatching success** and overall **nestling survival** (fledging success). In order to account for spatial or/and temporal trends, I included year, site, and interaction between year and site as predictors. In order to account for any variation in each response variable that could have been a result from inter-site or inter-year variation in date (laying date for clutch size and hatching success and hatch date for nestling survival) I included the interaction between year and date, and the interaction between date and site as explanatory factors in each model. I examined **the relationship between fCORT level and nestling survival** by modelling the nestling survival in a different model including only the nests that have been feather-sampled and I included the interaction between fCORT and year and interaction between fCORT and site as covariances in addition to the other variables mentioned above (the overall nestling survival analysis).

Individual egg weight and **yCORT** level (only for 2016) were modelled using linear mixed models (LMMs) with nest ID as a random factor. For egg weight analysis, along with laying date I included the quadratic term of laying date because visual inspection of the data suggested that the relationship could be non-linear (egg weight might increase or decrease with increasing laying date depending whether it is early or late in the season). In order to test for any inter-site variation in relation to clutch size and laying date, interaction between laying date and site as well as interaction between site and clutch size were included as explanatory variables.

Nestling weight (13-day-old) was analysed using LMM, with nest ID as a random factor and the following factors as explanatory factors: sex of nestling, the interaction between year and site, sampling date (day 13 since hatching), brood size on day 13, the interaction

between brood size and site, the interaction between brood size and year, the interaction between sampling date and year and the interaction between sampling date and site. The interaction terms were included to test for inter-site or inter-year variation that could influence the response variable. The sex of nestling was included to control for sex-dependent variation in body weight due to physiological and behavioural variation between male and female nestlings. In a separate model, I examined **the relationship between fCORT level and nestling weight** using LM. I took the mean value of nestling weight from each nest included in the feather sampling to represent the nestlings' weight of that nest. The following variables were included in the model as explanatory variables: site, year, fCORT, the interaction between site and year, the interaction between fCORT and site, the interaction between fCORT and year and brood size.

Feather corticosterone levels were modelled using (LM). I first analysed the samples collected from the cross-fostering experiment in 2016 in order to test for any variation in fCORT that are related to the origin of the birds. This was done with the following variables as explanatory factors: site, treatment (control or cross-fostered), interaction between site and treatment, brood size at day 13 and date of sampling. I found no effect of the treatment on fCORT (see Results), so I started a new global model for all the samples from both years including the cross-fostered nests. In order to test for spatial and temporal variation in level of fCORT, year, site, date of sampling, the interaction between site and year, the interaction between date and year and the interaction between date and site were included as explanatory variables. The interaction between brood size at day 13 and site were also included as explanatory variables in order to test for a possible site-specific variation on the impact of brood size on fCORT level.

2.4 Results

2.4.1 Breeding parameters and nestlings' survival

In comparison with the rural blue tit population, the urban population laid on average 1.63±0.32 fewer eggs, across the two breeding seasons (Table 2-2a). There was no significant variation between the two breeding seasons in relation to clutch size. Similarly, laying date had no significant variation on clutch size. In 2016, urban eggs weighed on average 0.07g less than rural eggs regardless of laying date and clutch size (Table 2-2b).

In both years, urban birds showed significantly lower hatching success compared to their rural counterparts (Table 2-2c). Eggs in urban nests had a 65% probability to hatch, while eggs in rural nests had a probability of 85% to hatch. Regarding nestling survival, the urban population also had reduced survival rate compared to their corresponding rural site in both years. Irrespective of the site, nestling survival was significantly lower during 2017 breeding season compared to 2016 (Table 2-2d). The probability of urban nestlings to survive until fledging was 67%, while it was 72% for the rural birds. The probability of nestlings to survive during 2017 season at both sites was 2% lower compared to the 2016 season (Table 2-2d). I found no relationship between fCORT level and nestling survival. For the subset of nests that were feather sampled, nestling survival was statistically lower in late hatched nests compared to earlier nests at both sites and years, although the coefficients suggest a small variation (Table 2-2e). I found no influence of site or year on the survival of these nests (i.e. feather-sampled nests).

2.4.2 Nestling body weight

Mean nestling body weight decreased with increased brood size at both sites (Table 2-2f). However, there was no relationship between fCORT and mean nestling weight (for nests that were included in the feather sampling). I found a similar pattern when analysing all nests irrespective of whether fCORT was measured or not. For the latter analysis, I used individual birds body weight data, and the effect of the brood size was not significant anymore (Table 2-2g, Figure 2-2b). Adding the sex of nestlings to the model revealed a significant effect of sex on the body weight. Males were on average 0.30±0.26g heavier than females, and this was statistically significant (Table 2-2g, Figure 2-2a). In the urban site only, late-hatched nestlings had lower body weight compared to early-hatched nestlings (Table 2-2g). Rural nestlings were significantly heavier than urban nestlings in 2017 but not in 2016 as there was a significant interaction between year and site (Table 2-2f and 2-2g, Figure 2-2a). The average weight of rural nestlings during the 2016 breeding season was 11.34±0.29g, while for urban nestlings it was 9.03±0.26g during the 2017 breeding season.

Table 2-2 Results from statistical models investigating how life history traits and body weight varied between urban and rural populations of blue tits across two breeding seasons (except egg weight that was measured only in 2016). Estimated coefficients and standard error (s.e.) for the models were selected using likelihood ratio tests (LRT). The reported p-values are from LRTs. Interaction terms are only shown when statistically significant. I converted logit-estimates to probability (presented between brackets next to logit-estimates). Probability = odds/ (1+ odds), odds= exp(logit).

a) Clutch size~ site (N= 289; 76 urban nests and 213 rural nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	7.50	0.14	
site (urban)	-1.63	0.32	<0.001*
year	-0.16	0.26	0.52
Laying date	-0.03	0.01	0.12
b) Egg weight~ site (N= 240; 108 from 18 urban nests and 132 from 22 rural nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	1.22	0.02	
site (urban)	-0.07	0.02	<0.001*
Clutch size	-0.02	0.006	0.25
laying date	0.002	0.003	0.57
laying date^2	-0.002	0.001	0.54
c) Hatching success~ site (N= 289; 76 urban nests and 213 rural nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	1.74 (0.85)	0.19	
site (urban)	-1.12 (0.65)	0.30	<0.001*
Year	0.13	0.30	0.20
Laying date	0.005	0.01	0.46
d) Nestling survival~ site + year (N= 289; 76 urban nests and 213 rural nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	0.94 (0.72)	0.02	
site (urban)	-0.22 (0.67)	0.04	<0.001*
Year (2017)	-0.07 (0.70)	0.03	0.04*
Hatching date	-0.0003	0.008	0.96
e) Nestling survival ~ date (when accounting for fCORT effect) (N= 25 urban nests and 35 rural nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	0.70 (0.67)	0.03	
Site (urban)	-0.02	0.08	0.85
year (2017)	0.11	0.06	0.08
date	-0.13 (0.64)	0.03	<0.001*
fCORT	-0.0007	0.003	0.21

f) Nestling weight (mean weight per nest)~ site*year + brood size (when accounting for fCORT effect) (N= 25 urban nests and 35 rural nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
(Intercept)	11.77	0.18	
year (2017)	-0.24	0.28	
site (urban)	-0.79	0.27	
date	0.07	0.11	0.47
fCORT	-0.01	0.01	0.31
brood size	-0.74	0.42	0.06*
year*site	-1.95	0.41	<0.001*
g) Nestling weight (individuals)~ site*year + site*date+ sex (N= 273; 37 urban nests and 44 rural nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	11.34	0.29	
site (urban)	-0.92	0.26	
year (2017)	-0.47	0.35	
date	-0.45	0.44	
brood size	-0.08	0.07	0.14
sex (male)	0.30	0.26	0.01*
site(urban)*date	0.59	0.26	0.03*
site(urban)*year(2017)	-1.28	0.45	<0.001*

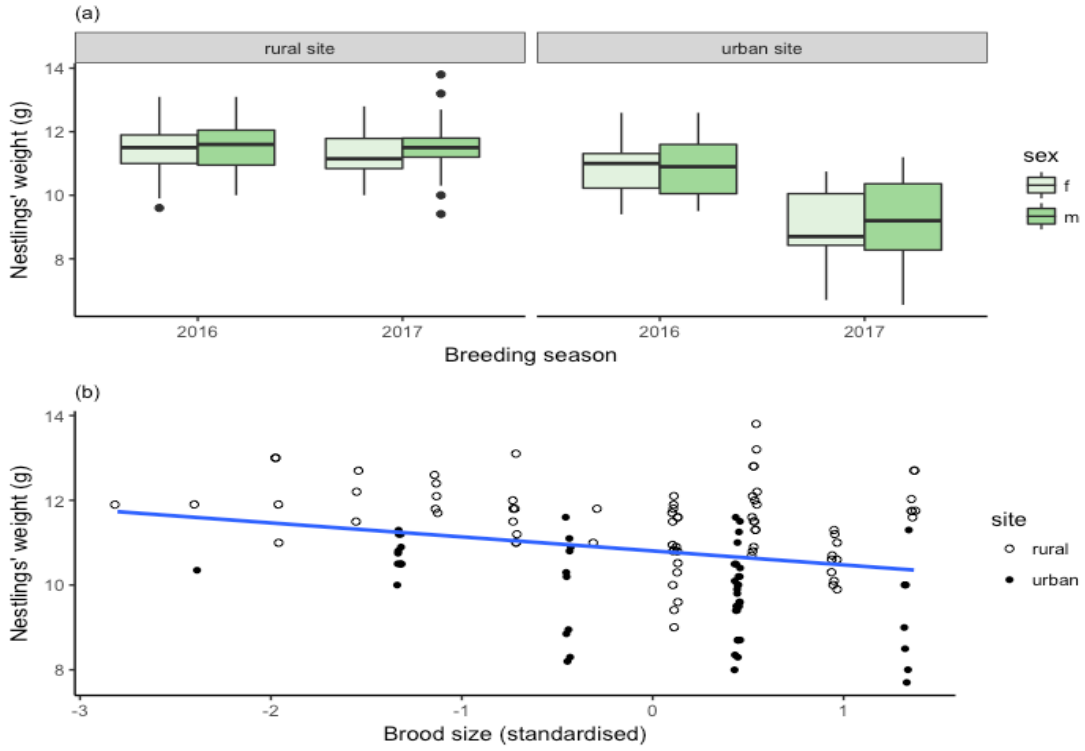


Figure 2-2 Nestlings' body weight at day 13, a) year-specific variation in nestlings' body weight. Male nestlings "m" are heavier than female "f", b) general negative relationship between nestlings' body weight and brood size.

2.4.3 fCORT and yCORT levels

In the cross-fostering experiment in 2016, there was no effect of the origin of the nestling on the fCORT levels (Table 2-3a, Figure 2-3a). When ignoring the treatment of eggs and combining results of both years, nestlings that grew up in the rural site had lower fCORT levels compared to those in the urban site in 2017 but not in 2016; there was a significant interaction between year and site (Table 2-3b, Figure 2-3b). The yCORT levels (2016 only) also did not differ significantly between eggs laid by urban and the rural blue tits. The only factor that was found correlating with yCORT level, as suggested by the statistical model, was the laying date; yCORT levels decreased in late-laid eggs compared to early-laid ones (Table 2-3c).

Table 2-3 Results from statistical models investigating; a) how the treatment (origin of the bird) can affect the level of fCORT (results from cross-fostering experiment), b) how feather corticosterone (fCORT) level varied between urban and rural populations of blue tit across two breeding seasons, c) how yolk corticosterone (yCORT) level varied between urban and rural populations of blue tit. Estimated coefficients and test statistics for the final models selected using likelihood ratio tests (LRT). The reported p-values are from LRTs. Interaction terms are only showed when statistically significant.

a) fCORT (2016)~ site+origin			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	5.86	1.41	
rearing habitat (urban)	0.64	1.87	0.66
Habitat of origin(urban)	0.82	2.06	0.66
Brood size	1.16	0.78	0.10
date	0.21	0.36	0.52
b) fCORT~ site*year			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	6.08	1.95	0.01
site (urban)	0.21	2.90	
year (2017)	2.19	2.90	
Brood size	1.41	4.67	0.75
date	-0.15	1.21	0.44
year (2017)* site(urban)	**19.96**	**4.45**	**<0.001***
c) yCORT~ laying date			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	4.89	0.61	<0.001
site	-0.15	0.42	0.58
laying date	**-0.17**	**0.07**	**0.01***
Laying date^2	0.002	0.03	0.94
Clutch size	0.07	0.12	0.53

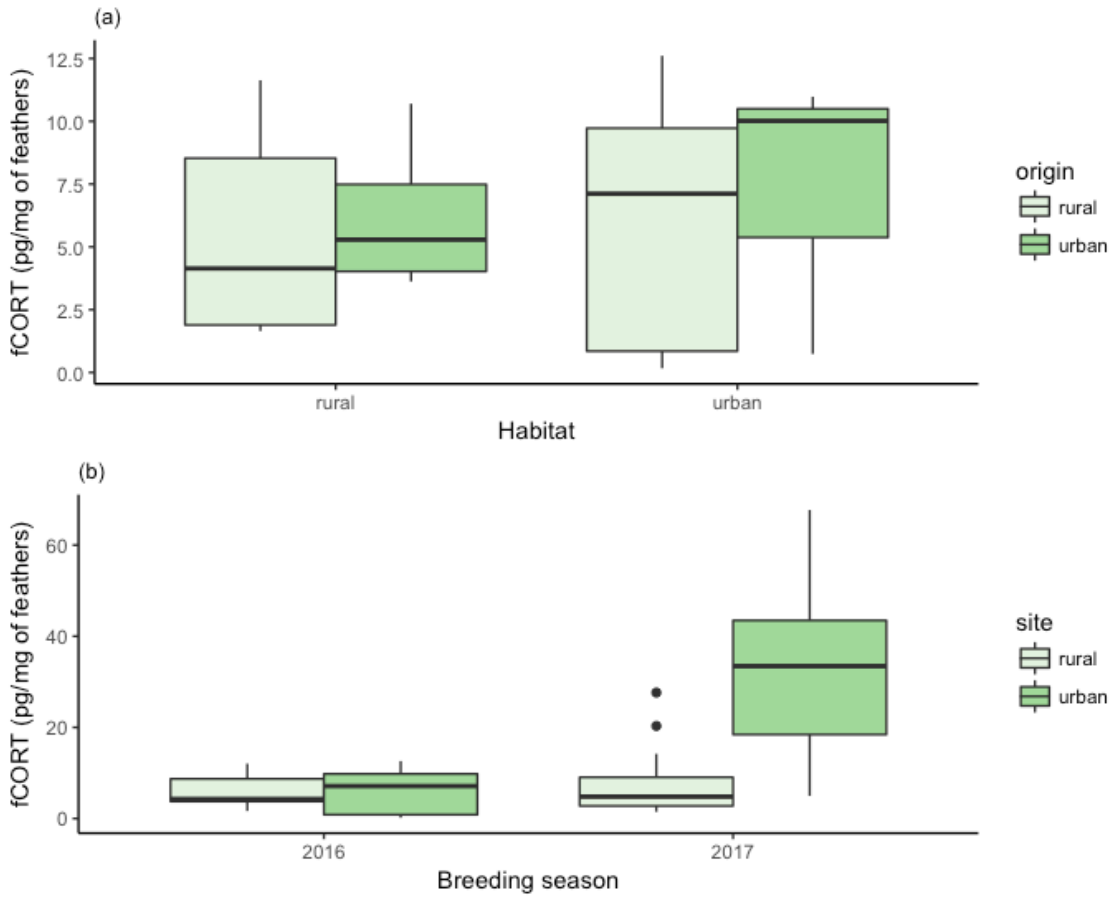


Figure 2-3 (a) no variation in the level of fCORT between nestlings reared in their original site and nestlings originated in one site and reared in the other site for both urban and rural blue tit populations. (b) Variation in the level of blue tit nestlings' fCORT between urban and rural populations that differed from year to year.

2.5 Discussion

This study was conducted with the aim of determining whether there are variations in the level of fCORT between nestlings from the urban and rural populations and whether this variation is consistent over time. The other aim was to compare some breeding parameters of these two populations and determine whether fCORT is associated with fitness-related traits (i.e. nestling survival and body weight). To test for any variation in the level of nestlings' fCORT that could be derived genetically from the parents or directly from mothers via hormone deposition in the egg, I cross-fostered clutches between urban and rural sites and, in addition, measured yolk corticosterone (yCORT) levels from eggs collected from both sites.

I found no influence of the origin of the nestlings on their fCORT level and this was supported by the finding of similar yCORT levels between the urban and rural population under this study in 2016. However, this study revealed three main findings. First, as

expected, urban population had lower breeding parameters compared to their rural counterparts. These parameters included lower clutch size, lower hatching success and lower nestling survival. This reduction on key fitness-related traits in the urban population was consistent between the two breeding seasons. Second, nestling body weight at day 13 (just before they fledged) was lower at the urban site only during 2017 breeding season compared to nestling in the rural site and no significant variation in body weight was observed between the two sites during the 2016 breeding season. Compared to 2016 breeding season, nestling survival was significantly lower in 2017 at both sites. Third, feather corticosterone (fCORT) levels varied between years at both sites, and the extent of differences in fCORT levels between the two sites differed from year to year. I found no evidence that differences in fCORT were a result of maternal effects, neither post-hatching nor arising during the egg formation stage. The level of fCORT had no direct relationship with mean nestling body weight nor nestling survival in both sites during both years. Overall, the results suggest that glucocorticoids and weight of urban versus rural blue tit nestlings differ substantially from year to year. However, other fitness-related traits (hatching success, nestling survival and clutch size) were always lower in the urban compared to the rural site.

2.5.1 Urbanisation and breeding parameters

The poorer breeding success at the urban site is in agreement with the often-reported result that the urban environment is associated with a reduction in fitness of wild populations (Chamberlain et al., 2009; Lutz et al., 2015; Murray et al., 2015; Glądalski et al., 2016; Bailly et al., 2016). Previous studies on the same populations of blue tits during a recent breeding season (2015) also found a lower breeding success in the urban site (Capilla-Lasheras et al., 2017; Pollock et al., 2017). However, my findings suggest that urban nestlings have lower body weight than their rural counterparts in one year, but not in another. The variation in urban nestling body weight between years may be related to food availability or weather conditions that can affect birds directly or indirectly by reducing the abundance of arthropods (the main food source for blue tits) which could be also affected by weather. The potential reduction in natural food availability in some years could be the reason for lower body weight of urban nestlings regardless of the availability of poor-quality urban food (e.g. feeders) as an extra food resource. Table 2-1 shows that the two sites have similar timing of laying or hatching at each year. However, while the rural site had a relatively stable ambient temperature and daily total rainfall in both years, the urban

site was slightly colder and had heavier rain during 2017 breeding season compared to the 2016 breeding season (Table 2-1).

Thus, fluctuating weather conditions in the urban site may also explain why the urban birds show some variation in nestling body weight from year to year, while rural nestling body weight hardly varied across the years. Local conditions that fluctuate from year to year are believed to affect nestlings either directly or indirectly by changes in parental provisioning and brooding behaviour (McCarty, 2001; Greño et al., 2008; Henderson et al., 2017). Specifically, rainfall, was found to negatively affect the growth of blue tit nestling (Morganti, 2017). Rainfall can affect nestling indirectly by affecting the abundance of arthropods available as the most important source of protein-rich food for passerine nestlings (White, 2008), and particularly in urban environments which are known to have a lower abundance of natural food compared to rural areas (e.g. Vergnes et al., 2014; Pollock et al., 2017).

2.5.2 Urbanisation and fCORT level

My finding of higher fCORT at the urban site in 2017 is supported by other studies using plasma to measure stress-induced and/or baseline corticosterone in breeding adults (e.g. Fokidis et al., 2009; Zhang et al., 2011; Meillère et al., 2015; Meillère et al., 2016). Likewise, my finding of no site differences in fCORT during 2016 breeding seasons is also consistent with other studies on the endocrine ecology of a number of species in urban landscapes (e.g. adult dark-eyed junco *Junco hyemalis* (Atwell et al., 2012); adult and juvenile house sparrow (Meillère et al., 2015). Even for the same species (blackbirds) but in different locations and different years, there was no variation in baseline CORT between urban and rural birds (Partecke et al., 2006), while Meillère et al. (2016) found higher CORT levels in nestling blackbirds from more urbanised areas. Possibly the conflicting results could be due to different age, sampling methods or CORT measurements; however, I found the same fluctuation in my system even though I used the same methodology by measuring CORT in feathers throughout the two years and always took measurements from 13-day-old nestlings.

In general, the findings suggest the important influence of year on the level of corticosterone when comparing urban versus rural populations of avian species. Even when I controlled for life history stage (i.e. only sampling nestlings) and sampling method (i.e.

fCORT), I still see a strong interaction between site and year, indicating that urban and rural blue tit nestlings differ in the level of corticosterone in one year but not in another. This finding is supported by a recent study over a four-year period on adult song sparrows *Melospiza melodia* which compared levels of corticosterone between urban and rural birds that also found a strong interaction between year and site, indicating that glucocorticoid differs between sites in some years but not in others (Foltz et al., 2015).

Although the information of weather condition was not measured in exact local sites under this study (retrieved from the *Met office* (https://www.metoffice.gov.uk/) for the nearest point to each site), it could at least partly explain the finding of elevated fCORT observed in my urban blue tits during 2017 season compared to their rural counterparts. Similar to the effect of year on nestling body weight, the influence of year on the variation between urban versus rural blue tit nestlings in fCORT levels may be related to local weather conditions, for example rainfall, wind speed, and temperature during the breeding season. For instance, a study on an altricial alpine swifts *Tachymarptis melba* found that nestlings' baseline CORT level was elevated as a result of cold and rainy conditions with strong winds (Bize et al., 2010). In adult blue tits, Henderson et al. (2017) found that elevated CORT levels were associated with lower temperatures, heavier rainfall and lower territory-scale oak density. In the year with heaviest rain and lowest temperature (~1ºC lower than other years and more than 10 days of rain), adult blue tits showed higher baseline CORT level (Henderson *et al*., 2017), which supports my finding of elevated fCORT during colder and wetter weather (2017) in the urban site compared to 2016 breeding season.

Growing nestling with their high energy demand (Lack, 1968; Ricklefs, 1983) and poor thermoregulatory abilities as the plumage is not fully developed (Howell, 1964), might be particularly harshly affected by unfavourable local environmental conditions. Elevation of glucocorticoid for a long period of time, which has detrimental consequences, can happen when energetic demands exceed the physiological state modulated by glucocorticoid hormones (McEwen and Wingfield, 2003; McEwen and Wingfield, 2010). A recent study on ducklings confirmed that stress conditions early in life temporarily supressed growth, and this stress is reflected by elevated fCORT levels (Johns et al., 2018). Similarly, a study on nestling black kites *Milvis migrans* showed that fCORT level is negatively associated with body condition and ambient temperature (López-Jiménez et al., 2016).

The observed higher fCORT in the urban nestling in 2017 might be at least partly related to poorer local rearing conditions. Even though urban site in 2017 had similar mean temperatures and similar daily rainfall as the usual conditions in the rural site (Table 2-1), urban populations may have been more vulnerable to such weather condition because of all the additional stressors they may experiences in the urban site including poor food (Pollock et al., 2017).

Table 2-1 shows that the daily total rainfall is higher in 2017 compared to 2016 which could be related to the observed lower fledging weight in my urban population. Elevated CORT levels are often reported to be associated with food restriction (Saino et al., 2003; Herring et al., 2011; Boonstra, 2013), specifically for altricial nestlings like blue tits when starvation is believed to be the main cause of mortality at the nestling stage (Martin, 1987). Food limitation can be an important selective pressure in urban sites compared to rural sites for nestling blackbirds (Ibáñez-álamo and Soler, 2010). This could at least partly explain poor nestling weights and the reduction in nestling survival I observed in my system that was mirrored with elevated fCORT in the urban site in 2017 compared to the rural site. Thus, differences between urban and rural nestlings in the level of fCORT may be driven by weather or nutritional stress, but my data don't allow distinguishing between the two. Elevated fCORT levels in blue tit nestlings in the urban site during 2017 may also reflect nestlings' need for trade-offs between important physiological demands. For instance, such a trade-off can be between mounting an immune defence against parasite infection and growth, and both immunity and growth are energetically demanding processes. This idea will be tested in chapter 5 when I will be testing the effect of avian malaria infection on blue tit fitness.

2.5.3 fCORT level and maternal or genetic effect

The lack of an impact of origin on fCORT levels was further supported by the result that there was no difference in the mean of the yolk corticosterone measured in eggs collected from both sites. Even though, it has been found that the level of yolk corticosterone in eggs varies between clutches depending on the mother's stress and level of plasma corticosterone, and eventually has a detrimental effect on nestling development, behaviour, immunity and growth (Hayward and Wingfield, 2004; Saino et al., 2005; Love et al., 2008; Almasi et al., 2012). This lack of an effect of origin on fCORT levels in nestlings suggests a direct impact of the surrounding environment and its related factors, such as limited food

or poor weather conditions after eggs were swapped. However, I collected eggs and conducted the cross-fostering experiment during the 2016 breeding season only, when there was no difference in fCORT levels between sites either (Table 2-2a). Thus, I cannot be certain whether this similarity between the level of yolk corticosterone or no-origin impact on fCORT is valid across all years or if it varies from year to year.

2.5.4 fCORT, nestlings' body weight, nestlings' survival and brood size

Although I found elevated feather CORT levels in the urban site compared to the rural site during 2017 breeding season, I unexpectedly did not find any significant relationship between feather CORT levels and mean nestling body weight or nestling survival, although feather and structural growth occur simultaneously in the nest. Previous studies have found negative correlation between CORT levels and body size and mass in pigeon and house sparrow nestlings (Jenni-Eiermann et al., 2015; Grace et al., 2017). In adult passerines, a study showed an association between high CORT and lower survival (Koren et al., 2012). The reason behind a lack of relationship in my study could be because I pooled feathers per nest which reduced sample size and prevented observing individual variation in such relationships. Taking the mean of body weight for several nestlings that have been feather sampled may be not the ideal procedures. However, ethically, I avoided collecting a sufficient number of feathers to run the assay from each nestling at this critical age (13 days post-hatching). Furthermore, the high mortality rate observed in the urban nestlings may present a selective disappearance of nestlings with low body weight and potentially masking any possible relationship.

Some studies reported a positive relationship between fCORT and brood size. For example, in experimentally enlarged clutches, Saino et al. (2003) found that barn swallow *Hirundo rustica* nestlings show higher level of baseline corticosterone in nests with a larger number of nestlings. Similarly, another study on nestling Florida scrub-jays *Aphelocoma coerulescens* found that baseline corticosterone was positively correlated with brood size (Rensel et al., 2011). More interestingly, a recent study on wild jackdaws *Coloeus monedula* found that this relationship seemed to impact all nestlings within a nest similarly as they found no relationship between baseline CORT and the individual nestlings body condition (Greggor et al., 2017). However, I found no relationship between fCORT level per nest and the number of nestlings in the nest at the day of sampling. This effect seems to vary from species to species because a number of studies with natural

brood variation or experimentally manipulated clutches support my findings and did not find any impact of brood size on fCORT (alpine swift (Bize et al., 2010); black-legged kittiwake *Rissa tridactyla* (Brewer et al., 2010); Eurasian kestrel *Falco tinnunculus* (Müller et al., 2010).

2.6 Conclusion

To conclude, the relationship between urban environment, breeding success and the stress physiology of avian species is complex and influenced by many factors that are difficult to disentangle. In addition, the fact that in my study fCORT levels were measured at nest level while body weight and survival were measured at individual level may limit the ability to test such a relationship. Differences in stress levels between populations were found to vary annually, highlighting the value of collecting longitudinal data to uncover broader trends. Some urban factors, such as food availability can readily vary between years, whereas other urban factors are relatively stable over time such as light, noise and chemical pollution. Given these different types of urban factors, I can conclude that investigating the impact of urban environment on a specific trait needs to be conducted over several years in order to draw an informative conclusion. Future studies on the relationship between urban environment, avian stress physiology and overall breeding success may need to concentrate on fixed factors such as light or pollution rather than making a general comparison between urban and rural populations. This has to be done experimentally and may help to better understand the effect of the urban environment on avian species in the long term.

Chapter 3 Molecular quantification of haemosporidian parasites (*Leucocytozoon* and *Haemoproteus*)

3.1 Abstract

Haemosporidian parasites are common in birds and considered as model systems to study host-parasite interactions and evolution. Historically, they have been detected by microscopic screening of blood smears, but this needs considerable training and expertise. Currently, several molecular detection methods are combined with microscopy to increase the sensitivity and accuracy of detection. However, recent studies show that the available molecular protocols cannot quantify the parasite intensity (parasitaemia) for genus-level. Besides, until a very recent time (during the writing time of this chapter) there was a need for an accurate approach to discriminate between different parasite genera when more than one is present in a sample (mixed infection). Here, I established a new method to quantify Haemosporidian parasites intensity, particularly *Leucocytozoon* and *Haemoproteus* genera, in qPCR reactions. I designed genus-specific primers targeting a region of the mitochondrial genome (intergenic space between *cytochrome B* (*cytB)* and *cytochrome c oxidase subunit* 1 (*cox1)* genes). This protocol was applied to serial dilutions of a known number of specific targets (cloned into plasmids) for each genus. These serial dilutions allow testing the amplification efficiency of each primer set over different numbers of parasite gene copies. I quantified a bird house keeping gene (*GAPDH*) for each sample to calculate the absolute parasite intensity (parasitaemia) as a ratio of parasite gene copies to the bird gene copies. Through this new approach, I tested infection prevalence and intensity of 230 samples collected from wild nestlings and compared with the nPCR approach by Hellgren *et al.* (2004). The new qPCR protocol is significantly more sensitive in detecting infections compared to the nPCR. This protocol presents a sensitive and time effective method for detection and quantification of haemosporidian parasite of the two genera *Leucocytozoon* and *Haemoproteus*.

Key words: avian malaria, passerine birds, nestling, real-time qPCR, *Plasmodium*, nPCR

3.2 Introduction

Quantification of parasite intensity and accurate identification of parasite genera are critical points to understand several aspects of parasite-host interactions, cost of infection on host fitness, and the association between specific parasite species with avian pathology (i.e. the epidemiology of infection) (e.g. (Crofton, 1971)). Haemosporidian parasites (phylum Apicomplexa, order haemosporida), are parasites commonly found in avian blood. These parasites are diverse, and mixed infection – when a bird is infected with more than one parasite genus – is thought to be common in birds (Bensch et al., 2009; Clark et al., 2014). Despite the increased interest in avian malaria studies in the last two decades, progress in this field has been limited because it is still difficult to ascertain the prevalence of these parasites, particularly to accurately identify and quantify avian malaria intensity of infection (Marzal, 2012; Levin et al., 2013; Bernotienė et al., 2016; Ishtiaq et al., 2017). Parasite species associated with severe avian diseases are often difficult to detect under the microscope (low intensity), or they are in the initial exoerythrocytic (schizonts) stage of development and not yet circulated in the blood (Valkiūnas and Iezhova, 2017).

Currently, there are two main methods used to detect and estimate avian malaria parasite intensity: microscopic screening of blood smears, and molecular techniques using polymerase chain reaction (PCR) protocols. Each method has its advantages and limitations (see Table 3-1). There are some molecular-based protocols that have been developed to detect the three closely related genera of haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*), targeting highly conserved mitochondrial gene regions among genera (Table 3-2). These protocols often include primers designed to amplify the target gene of one genus (e.g. Ciloglu et al., 2019) or all the three genera at once (e.g. Bell et al., 2015); or primers targeting specific lineages (e.g. Biedrzycka et al., 2014). The current qPCR protocols have been used only to determine the level of parasitaemia (screened by microscopy). Thus, no absolute quantification of the parasite intensity is accomplished by such qPCR, especially for *Leucocytozoon* genus.

The nested PCR protocol (nPCR) that has been developed by (Bensch et al. (2000) and further modified by Waldenström et al. (2004) and Hellgren et al. (2004) has been widely used to detect the three genera of haemosporidia (Clark et al., 2014). Thus, this protocol has been often used to assess the reliability and sensitivity of newly developed protocols by comparing their results with results of haemosporidian parasite prevalence obtained from

the nPCR method (Ishtiaq et al., 2017). Nevertheless, the nPCR and other PCR-based protocols often underestimate mixed infection because similar or equal amplicon sizes of different genera are expected (Valkiūnas et al., 2006). Additionally, cross-reactivity in the nPCR is another issue that adds to the shortcomings of these protocols. Previously, in nine out of 12 samples that were positive for both *Leucocytozoon* and *Haemoproteus*/*Plasmodium* by nPCR, sequencing revealed that only one of the two parasites – *Leucocytozoon* or *Haemoproteus* – was present (Capilla-Lasheras et al., 2017).

Two very recent works have been published (during the time of writing this chapter) and partly attempted to tackle the mixed infection diagnostic challenge. The first protocol is by Pacheco et al. (2018) which presents a sensitive nested multiplex PCR protocol that can detect *Plasmodium*/*Haemoproteus* mixed infection, however, this protocol does not include *Leucocytozoon*. The second approach is by Ciloglu et al. (2019) which presented a one-step multiplex PCR to detect the three genera (*Leucocytozoon*, *Plasmodium* and *Haemoproteus*) in one single PCR reaction using multiple primers. However, these protocols do not provide a quantitative approach that allows quantifying the parasite intensity in a given amount of host DNA (parasitaemia).

For accurate identification of the parasite by the traditional microscopic screening, the main developmental stages of the parasite are required to be present, which is not always the case especially in low-intensity samples. The relative ease of identification of the *Haemoproteus* and *Plasmodium* species' developmental stages by microscopic screening led to them being reported more frequently in the literature compared to *Leucocytozoon* species, whose main developmental stages are detectable in the peripheral blood for a short time (Valkiūnas, 2005).

Molecular diagnostic methods can overcome limitations associated with the microscopic screening of blood smears while microscopic screening of blood smears is needed for estimating parasite intensity. The qPCR technology has increased accuracy and sensitivity of target DNA detection in human malaria (Hermsen et al., 2001; Farcas et al., 2004; Perandin et al., 2004; Malhotra et al., 2005; Mangold et al., 2005). Thus, I suggest developing a real-time qPCR approach that will add to the effort built previously to increase the sensitivity and specificity of molecular detection of haemosporidian parasites and more importantly provide a tool for quantifying parasite intensity (parasitaemia).

Table 3-1 Comparison between microscopy screening and molecular detection of haemosporidian parasites

	Molecular identification	Microscopic screening
Sample preparation/ time and experience required	**Can be performed on hundreds of samples in a relatively short time** **Archived for long term storage under different conditions** **Requires basic molecular lab skills** **If the DNA is degraded, it could lead to false negatives due to a weak template preventing the amplification of large DNA fragments (Freed and Cann, 2006; Beadell et al., 2006)**	**Screening of blood smears requires much time** **Good quality slides can be kept for long term storage** **Taxonomic expertise is essential (Valkiūnas et al., 2008)** **Preparation of good quality slides is crucial, and the number of microscope fields analysed is critical**
Sensitivity and limit of detection	**Sensitive and can increase the yield of amplification of the target DNA** **limit of detection is protocol-specific issue. As an example, PCR reaches its limit of detection if parasitaemia falls below 0.5 infected red blood cells per microliter of blood (equal to 0.0125 parasite per 104 erythrocytes) (Zimmerman et al., 2004).** **Can underestimate mixed infections in birds (Valkiūnas, Bensch, et al., 2006).**	**Could miss parasite infection of low intensity** **Reaches its limit of detection if parasitaemia falls below 40 infected red blood cells per microliter of blood (equal to one parasite per 104 erythrocytes) (Zimmerman et al., 2004)** **An expert observer can accurately distinguish between different genera**
Identifying parasite stages	**Does not differentiate among the developmental stages within infected cells**	**An expert observer can differentiate among the developmental stages within infected blood cells**
	Not all the PCR amplifications and sequencing of haemosporidian parasites from a blood sample reveals a true/complete parasite in one host, since it can include sporozoites and abortive stages of the parasite, which may not complete the reproduction	**It allows the identification of suitable hosts (both vector and vertebrate hosts) in which parasites complete sexual /asexual reproduction inside the host**

Although most DNA is packaged in chromosomes within the nucleus, mitochondria also have a small amount of their own DNA. This genetic material is known as mitochondrial DNA or mtDNA. Using the outer primers (292F/630R) of Fallon et al. (2003), I can amplify the parasite's gene region of the mitochondrial genome (intergenic space between *cytB* and *cox1* genes). By studying the characteristics of this gene region, different sets of primers can be designed to distinguish between avian malaria genera (i.e. primers are

different between genera, but each set of primers is identical across species within each genus. My qPCR protocol will be assessed by comparing my results on parasite prevalence with results from nPCR by Hellgren et al., (2004). The efficiency and reproducibility of each qPCR reaction will be evaluated by the standard curve method. Therefore, this study, aimed to develop a new qPCR method for avian malaria, presents a specific and time-effective protocol for quantifying *Leucocytozoon* and *Haemoproteus* parasites in bird DNA samples.

Table 3-2 Examples of previously used molecular methods for avian malaria detection and their ability to differentiate DNA amplification of the three genera; H= *Haemoproteus*, P= Plasmodium and L= *Leucocytozoon*. (+) means the parasite' gene is amplified and (-) means it is not amplified. (+) for both genera means that primers cannot distinguish between two genera.

PCR	Primers code	Target gene (genera)	H/P amplification	L amplification	References
Standard PCR	**HAEMF/HAEMR2**	**cytB (H/P)**	+	–	**(Bensch et al., 2000)**
Standard PCR	**HAEMNF/HAEMNR2**	**cytB (H/P)**	+	–	**(Waldenström et al., 2004)**
Nested PCR	**First; (HAEMNF1/HAEMNR3)**	**cytB**			**(Hellgren et al., 2004; Capilla-Lasheras et al., 2017)**
	HAEMF/HAEMR2	**(H/P)**	+	+	
	HAEMFL/HAEMR2L	**(L)**	+	+	
Nested multiplex PCR	**First; (AE298/AE299)**	**cytB**			**(Pacheco et al., 2018)**
	AE980/982	**(H)**	+ **(H) only**	–	
	AE983/985	**(P)**	+ **(P) only**	–	
One-step multiplex PCR	**PMF/PMR**	**Non-coding region of the mtDNA (P)**	+ **(P) only**	–	**(Ciloglu et al., 2019)**
	HMF/HMR	**Between the 5` end of cytB and a non-coding region of the mtDNA (H)**	+ **(H) only**	–	
	LMF/LMR	**COX1 (L)**	–	+	
Standard PCR	**343F/496R**	**SSU and LSU rRNA (H/P)**	+	–	**(Fallon et al. 2003)**
qPCR	**Lpri6-19/Rpri19 (P) Lpri6/Rpri12 (H) CY3- CytB-BHQ2 (TaqMan probe)**	**cytB (P and H)**	+	–	**(Bentz et al. 2006)** **(Christe et al. 2012)** **(Rooyen et al. 2013)**

	18sPlasm7 18sPlasm8	**18s rDNA (P)**	**+ P(specific lineage)**	**–**	**(Cellier-Holzem et al. 2010; Larcombe et al. 2013)**
	SW1F/SW1R SW3F/SW3R	**cytB (H)**	**+ H (specific lineages)**		**(Biedrzycka et al. 2014)**
	R330F/R480RL	**cytB (H/P/L)**	**+**	**+**	**(Bell et al. 2015)**

3.3 Materials and methods

3.3.1 Sampling

Samples were collected from nestling blue tits *Cyanistes caeruleus* breeding in nest-boxes in two locations (May-June), an urban and a rural location in 2016 and 2017 (see Chapter 1). All blood sampling was conducted under licence of the UK Home Office, Animals Scientific Procedures Act, and individual ringing under licence from the British Trust for Ornithology. A total of 259 blood samples (20-50 µl) from the brachial vein were collected, using heparinised capillaries and directly stored in RNA-later in the field or kept in an ice-box for less than 3 hours before storing them in absolute ethanol. Samples then were kept in a refrigerator for up to a week, and subsequently at -40^{o}C until lab analysis.

In the lab, I extracted DNA from the blood samples. The extracted DNA samples were tested for the presence of the parasite DNA following the nPCR protocol by Hellgren et al. (2004). Then, in order to establish a real time qPCR protocol for quantifying *Leucocytozoon* and *Haemoproteus* parasites in bird DNA, I amplified the parasite target gene from several samples. I studied the characteristics of the target gene to design genus-specific primers for my qPCR protocol that allows quantifying the parasite intensity from each genus.

3.3.2 DNA extraction/confirmation

I extracted DNA from blood using a commercial kit (DNeasy whole-blood extraction kit, Qiagen) and eluted with 80µl of water. Successful DNA extraction was confirmed using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE). DNA was further checked by testing against the presence of a bird reference gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), following the protocol by Atema et

al. (2013). Briefly, I used the primer pair: forward (5'-TGTGATTTCAATGGTGACAGC-3') and reverse (5'-AGCTTGACAAAATGGTCGTTC-3') to amplify a 80 bp fragment of the reference gene *GAPDH* (Atema et al., 2013). The thermal cycling profile was as the following: initial denaturation at 95°C for 10 seconds, 40 cycles at 95°C for 1 minute, annealing for 1 minute at 60°C and extension for 1 minute at 72°C, and then a final extension of 5 minutes at 72°C. The PCR products were then run out on a 1% agarose gel (using 0.5xTBE; 54g Tris Base, 27.5g boric acid and 4.65g EDTA in 1L distilled water), stained with SYBR™ Safe DNA Gel Stain (ThermoFisher Scientific, California, USA) and visualised under ultraviolet light.

3.3.3 Parasite detection using nested PCR (nPCR) approach

DNA extracted from the bird blood samples was tested for the presence of the genes of specific parasite genera following the nPCR protocol by Hellgren *et al.* (2004). For the first PCR, I used HaemNF1/HaemNR3 primers. After initial denaturation at 94°C for 3 min, eluted DNA was amplified by 20 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, and then a final extension at 72°C for 10 min. A 2 µl aliquot from the result of this reaction was further amplified using the second primer set (HaemF/HaemR) for *Haemoproteus* and (HaemFL/HaemR$_2$L) for *Leucocytozoon*. The same cycling profile of the first PCR was used for the second PCR, but performed over 35 cycles instead of 20 cycles. Both PCRs were performed in 20µl of reaction mixture containing a range of 15ng to 961ng DNA template, 0.48µM of each primer using 1x GoTaq® G2 hot start green master mix (Promega, USA– a premixed ready-to-use solution containing GoTaq® G2 hot start polymerase, dNTPs, $MgCl_2$ and salts at optimal concentrations for efficient amplification of DNA templates by PCR). To check if the PCRs had been amplified successfully, I used positive controls for each run. The positive controls are samples with known avian malaria infections confirmed by sequencing results (Capilla-Lasheras et al., 2017). I used two types of negative controls in each run: 1) bird DNA samples showing negative amplification (no bands) and 2) nuclease-free water in place of DNA template in order to detect any possible contamination during the run. In addition, to avoid contamination during lab work, I considered working in dedicated spaces, cleaning thoroughly between rounds and careful opening of tubes. Products of the second PCR reaction were run out on a 1% agarose gel and visualised under ultraviolet light in order to check for the presence or absence of the parasite gene.

In order to test for any possible effect of the storing buffer (RNAlater® or absolute ethanol) on the nPCR result, I compared the results of samples collected from 12 individuals and stored in two separate tubes; one containing RNAlater® and the other containing absolute ethanol). Results were identical for each pair of samples stored in the two different buffers.

3.3.4 Establishing a qPCR approach for parasite quantification

In order to establish a real time qPCR protocol for quantifying *Leucocytozoon* and *Haemoproteus* parasites in bird DNA, it was necessary to amplify the parasite target gene from some DNA samples. After sequencing the parasite's gene region of the mitochondrial genome (between *cytB* and *cox1* genes) and studying the characteristic of the gene, I then designed genus-specific primers. Primers were designed based on two criteria: primers are different between the two genera, but they are identical in the species within the same genus. In order to have a known amount of each gene of interest, the target gene was cloned for each genus as well as a bird reference gene (*GAPDH*) was cloned. This design allows 1) test the primers; 2) test reaction efficiency of each qPCR assays by using the standard curve method.

Amplifying and sequencing some DNA samples - As a precautionary step to avoid contamination in the lab, I decided to use a gene region that is different than the standard 478 base-pair fragment of the *cytB* gene (Waldenström et al., 2004; Hellgren et al., 2004) because it is often amplified in our lab. Instead I targeted the conserved region of the mitochondrial genome that Fallon et al. (2003) have used to detect avian haemopsporidian infections because it is quite conserved in avian haemosporida, but still shows some variation between genera. I ran eleven DNA samples that nPCR indicated to be infected with different parasite genera (six infected *with Leucocytozoon*, one infected with *Haemoproteus* and four with mixed-infection with both genera) in standard PCR following Fallon et al.'s (2003) protocol with some modification of the thermal cycling profile (30 seconds instead of one minute for the denaturation, annealing and elongation steps). I used the outer set of primers 292F/631R to amplify the parasite target gene. PCRs were performed in 20 µl reaction mixture using 1xGoTaq® G2 hot start green master mix (Promega, USA) and 0.32µM of each primer. I used two microliters of the eluted DNA for amplification. Cycling conditions were as follows: 5 min initial denaturation step at 94°C, followed by 35 cycles with 30 s denaturation at 94°C, 30 s annealing at 52°C and elongation at 72°C for 30 seconds. After the 35 cycles, a final elongation step followed at

72°C for 10 seconds. Products of the PCR reaction were examined on a 1% agarose gel. In order to amplify a fragment of a bird reference gene *GAPDH*, I ran two DNA samples in PCR following Atema et al.'s (2013) protocol.

I cut and purified PCR product gels (positives for the parasites and for the *GAPDH* genes) following the manufacturer's instructions from the available commercial kit (wizard-SV gel and PCR clean up system (Promega)). For each of the purified products (5 ng/µl), I prepared two sets – one for each primer (292F and 631R in the case of the parasite gene, and the forward and reverse primers for the avian *GAPDH* gene). I then sent purified products with corresponding primers for sequencing services by (Eurofins Genomics, UK) to obtain the full sequence from each sample. DNA sequences chromatograms were visualised and annotated using '4peaks' software (https://nucleobytes.com/4peaks), then I checked them for similarity with available sequences in the GenBank (BLAST server https://blast.ncbi.nlm.nih.gov/Blast.cgi) for the parasite or the bird gene identity confirmation, accordingly.

Alignment of DNA sequences and primers design - The obtained DNA sequences were aligned against the available haemosporidian mitochondrial genome sequences from GenBank for some species commonly found in passerines (Table 3-3). For alignment analysis I used ClustalW in multilabel sequence alignment tool [Molecular Evolutionary Genetics Analysis (mega7 software)] (Kumar et al., 2016). In total, the sequences (obtained from the samples) included in the alignment were as follows: ten *Leucocytozoon*, one *Haemoproteus*. From the alignment of the obtained gene and in silico analysis, I designed primers manually to be genus-specific, but identical among several species within each genus.

I checked primers' features using the online tool 'Net *Primer'* (http://www.premierbiosoft.com/netprimer), taking into account four main requirements: 1) the length of the primers should range from 18 to 24 bp to allow for adequate specificity but avoid excessive annealing; 2) adequate guanine-cytosine content (GC% content) to increase annealing stability; 3) because of the stronger hydrogen bonding of G and C bases (the GC clamp) compared to T/A bonding, primers should have 1-2 G and/or C bases within the last bases from the 3` end of primers which helps promote specific binding at the 3` end and contributes to the stability of the primer and template; and 4) annealing temperature (Ta) to be in the range of 50-60ºC and within 1ºC between each pair of

primers, in order to produce the best result. Additionally, primers' secondary structures were taken into account such as dimers and hairpins produced by intermolecular or intramolecular interactions because they can affect primer-template annealing, generating poor or no yield of PCR product; self- and cross-dimers were avoided (lowest value for dimer were accepted).

Table 3-3 Parasite species sequences used for the alignment and analysis of the intergenetic space between cytB and cox1 genes prior to primer design. Resource; NCBI https://www.ncbi.nlm.nih.gov/gene

Species	Accession number
Leucocytozoon fringilinarum	KY653765.1
Leucocytozoon majoris	FJ168563.1
Leucocytozoon dubreuili	KY653795.1
Haemoroteus coatneyi	KT698210.1
Haemoproteus sacharovi	KY653811.1
Haemoproteus tartakovski	KY653810.1
Haemoproteus.sp	KY653805.1
Haemoproteus belopolskyi	KY653790.1
Plasmodium relictum	KY653773.1
Plasmodium elongatum	KY653801.1
Plasmodium lutzi	KY653816.1
Plasmodium unalis	KY653814.1
Plasmodium vaughani	KY653792.1

Cloning PCR products - In order to test the efficiency of primers and to have different amount of plasmids from each target gene, I prepared three purified PCR products (PCR run and purification as mentioned above under 'Amplifying and sequencing some DNA samples' to insert into a plasmid vector. These products are as the follows Haemo (part of mtDNA *Haemoprotues)*, Leuco (part of mtDNA *Leucocytozoon)* and *GAPDH*-BT (*GAPDH Cyanistes caeruleus* (blue tit)). I used T/A cloning methods following the manufacturer's instructions in the commercial kit (Invitrogen – the original TA cloning kit). Taq polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearised vector supplied in this kit has single 3′ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

I ran all three samples in a total volume of 10 µl mix (5X T4 DNA ligase reaction buffer, 25ng/µl pCR2.1 vector, 5X ExpressLink™ T4 DNA ligase and 50ng/µl DNA). The cloned samples were labelled as follows pCR2.1-Haemo for *Haemoproteus*, pCR2.1-Leuco for *Leucocytozoon,* and pCR2.1-*GAPDH*-BT for the bird reference gene. I left the samples at room temperature for a minimum of 15 minutes to ensure efficient ligation. After ligating the inserts into pCR2.1, samples were placed on ice before I took them next to the Bunsen burner to add 2 µl from each reaction to a labelled tube of competent *E.coli* then stirring the mix gently and placing the tubes on ice for 30 minutes. In order to complete transformation, samples were heat shocked for 30 seconds in an incubator at 42°C before I returned them into the ice to cool down. This sudden increase in temperature creates pores in the plasma membrane of the bacteria and allows plasmid DNA to enter the bacterial cell. I added 250 µl of super optimal broth with catabolite repression (S.O.C), glucose-rich

bacterial growth medium, to each tube. The glucose will help the cells recover and grow faster after the heat shock. Tubes were then left in a shaking incubator at 200 rpm under 37°C for an hour. This time of incubation is a critical step because it allows both the E. coli to recover from the heat shock and for them to express the antibiotic (kanamycin) resistance genes. Next to the Bunsen, I plated 100 µl of each transformation tube on a separate agar plate and incubated them overnight at 37°C to allow for colony growth. The following day, next to the Bunsen, I picked a clone from each part of each plate and let them grow overnight in Luria broth(LB), bacterial growth medium, containing 50 µg/mL of kanamycin (X100). Additionally, I plated each colony in a plate divided into five separate areas for long-term storage.

Then, I ran the plasmid DNA in PCR reactions in order to amplify the parasite gene or the bird reference gene respectively. The successfully amplified samples were chosen for plasmid DNA isolation. I purified plasmid DNA using a commercial kit (QIAprep Spin Miniprep, Qiagen) following the manufacturer's instructions and sequenced using the primers (T7/M13) (Eurofins Genomics, UK). I used '*4peaks'* software (https://nucleobytes.com/) to visualise and annotate plasmids DNA sequence chromatograms, then checked them against the GenBank (BLAST) to confirm the gene's identity.

Quantification of plasmid - I calculated the molecular weight (MW) of each plasmid knowing the bp length and average MW per bp. I calculated the number of moles in a given ng quantified by nanodrop, then using Avogadro's number to obtain the number of molecules per ng.

Giving that pCR2.1 size is 3929 bp long; the mtDNA cloned sequence is 286 bp long; total gene region cloned plasmid (pCR2.1-Haemo or pCR2.1-Leuco) is 4215 bp long and *GAPDH* cloned gene is 151 bp long; total pCR2.1-*GAPDH*-BT gene is 4080 bp long, plasmids' sizes (number of genes) were calculated using the equation below (Elliott and Elliott, 2009). In 1ng of the plasmid (pCR2.1-Haemo or pCR2.1-Leuco) there are ~219.8* 10^6 plasmids and in 1ng of the pCR2.1-*GAPDH*-BT there are 227.1 * 10^6 plasmids.

$$\text{Number of molecules} = \text{gram} \times (6.022 \times 10^{23}) / \text{length} \times 650\text{g/mol}$$

- 650 g/mol is the average molecular weight of a base pair (MW)
- Avogadro's number indicates that there are (6.022×10^{23}) molecules/mol
- MOL = grams/MW
- ng = 10^{-9}g

Given that the blue tit genome is ~1Gbp and I am planning to quantify 60ng (or 12ng/µl*5µl) per PCR run, I calculated the number of bird genome copies (or copies of *GAPDH*) using the equation above and the result was 55587.69 gene copies. Assuming the parasitaemia to be ~1%, in 60ng of an infected bird I would have 555,87 copies of the parasite gene (i.e. 55587.69 copies of bird gene/100). I decided to set a standard for pCR2.1-*GAPDH*-BT and for pCR2.1-Haemo or pCR2.1-Leuco that was 1 or 2 orders of magnitude higher, respectively, in order to have the highest standard that is higher than what was expected to be measured. So, the initial pCR2.1-*GAPDH*-BT amount was $10^5/227.073906*10^6 = 0.00044$ng and the initial amount of pCR2.1-Haemo or pCR2.1-Leuco was $10^4/219.801077*10^6 = 0.0000455$ng. From this calculation I diluted the plasmid so that I had 37.5ng/µl, 17.3ng/µl and 23.7ng/µl for pCR2.1-*GAPDH*-BT, pCR2.1-Leuco and pCR2.1-Haemo, respectively. To calculate the initial volumes needed from each plasmid, to prepare 1 ml of the starting amount of each plasmid, I used the following equation (Elliott and Elliott, 2009):

$$vi \times ci = cf \times vf$$

- vi = initial volume
- ci = initial concentration
- cf = final concentration (ng/µl) (= starting amount of plasmid/5)
- vf = final volume (=1000µl each)

In order to test the amplification efficiency of the primers, I prepared serial dilutions of each plasmid (pCR2.1-Haemo, pCR2.1-Leuco and pCR2.1-*GAPDH*-BT) starting with 10^4 copies in the case of parasite gene standards and with 10^5 in the case of bird *GAPDH* standards, and then four dilutions of 1:10 each time.

Selecting primers - In order to evaluate the different primer concentrations tested below, I specified three features that the primers should present to be selected as an efficient set of primers for amplifying the target gene: firstly, primers that show no cross-reactivity with the DNA of other genera; secondly, primers that amplify only one product

(the target gene) as tested by the dissociation curve; and lastly, primers that amplify earlier are considered to be more efficient than those amplifying later.

I tested nine different combinations of three common concentrations (300nM, 600nM and 900nM) of each pair of primers (L146F/L146R and H160F/H160R) on three types of plasmid containing the same amount of the parasite target gene copies: pCR2.1-Haemo, pCR2.1-Leuco and mix of pCR2.1-Haemo+pCR2.1-Leuco. Each plate design included two negative controls: uninfected bird sample (to ensure that primers are not amplifying bird genes) and distilled nuclease-free water to check for any possible contamination during the run. In qPCR, the DNA amplification is detected when the fluorescent signal cross the detection threshold and the number of cycles required for this to occur is known as the cycle threshold (Ct) (Livak and Schmittgen, 2001). I ran each sample or control in two technical replicate (accepted only if no more than 0.5 Ct difference between the replicates) in order to control for the validity of the result. For *GAPDH*, the same primer concentration (100nM) suggested by Atema et al. (2013) was used. Reactions were run in MicroAmp Optical 96-well plates (0.1 ml) on Stratagene Mx3000. Reaction contained 1X SYBR Select Master Mix (X2) (applied biosystems) and the abovementioned concentrations from each set of primers (i.e. 300 nM, 600 nM and 900 nM). The total volume of the reaction was 15 µl, with 5 µl (12ng/µl) of DNA.

Standards evaluation - I ran different amounts of each plasmid, in three qPCR assays using the corresponding primers concentration and T_a for each target gene (Table 3-4). The resulting Ct values of each serial dilution were plotted by the machine on a logarithmic scale along with corresponding concentrations. Then, the slope of a linear regression (standard curve) of the Ct on the initial quantity (copies) was calculated. As during each PCR cycle the DNA doubles, it requires 3.32 cycles to amplify a target by ten-fold dilution series. Thus, for an assay efficiency of 100% the resulting slope of the plot of log target concentration against Ct is -3.32 (Book et al., 2009). The assay efficiency is given through the equation: $E= -1+ 10^{(-1/SLOPE)}$ (Kubista et al., 2006; Book et al., 2009) with E = 1 representing 100% efficiency and values between 0.9-1.1 representing an acceptable range. In addition, and to indicate the reproducibility of the assays, R^2 value, which is a measure of the closeness of the replicate data points to the best-fit line (the slope), were calculated for each assay.

3.3.5 Parasite detection and quantification using the qPCR approach

After testing the primers on the standards, I ran the DNA samples collected from wild blue tit nestlings in real-time reactions. I ran each sample in three different plates for the three target genes *Haemoproteus* (part of mtDNA), *Leucocytozoon* (part of mtDNA) for parasite gene quantification and *GAPDH* for normalising the parasite quantities result and calculating the absolute parasitaemia (parasite intensity). Each plate contained a standard run for assay evaluation (serial dilutions of plasmids and one distilled nuclease-free water as a negative control). All samples, standard and control were run in duplicate. Any replicate with >0.5 cycle difference was repeated in additional triplicates in order to control for the validity of the results. Results were accepted as positives only when they showed amplification in at least two out of three runs. In *Leucocytozoon* plates, the amplifications of the parasite gene tend to be imprecise when amplifying after 39 cycles (below 1 gene copy); in total 28 samples (<1 copy) were repeated and 60% of them were included as positives. I calculated absolute parasitaemia by dividing the quantity of parasite gene copies by the quantity of the bird gene (*GAPDH*) copies.

3.3.6 Statistical analysis

Statistical analyses were conducted in R v. 3.3.1 (R Development Core Team, 2011). To compare the detection of malaria infection between the two methods (qPCR versus nPCR protocol) I used a chi-square test. In order to examine the relationship between the parasite intensity in a given DNA sample (as quantified by qPCR) and the sensitivity of nPCR to detect the infection status (as tested by nPCR), I used a generalised linear model (GLM) with binomial distribution (package 'lme4') (Bates et al. 2014). The infection status (yes or no) was set as response variable and the parasite intensity was set as a predictable variable in the model. From the model coefficients and using different values of parasite intensity, I calculated the probability of nPCR to detect infection (Probability= parasite intensity * parasitaemia estimate coefficient + intercept estimate coefficient).

3.4 Results

3.4.1 DNA extraction/confirmation

DNA was extracted from 259 blood samples collected in the field and diluted in 80µl of water resulting in an average of 150 ng/ µl (range: 7.5ng/µl - 480.5ng/µl). DNA purities, estimated as the ratio of the intensity of absorption at 260/280 nm and 260/230 nm, were all close to the acceptable range of 1.8-2 and >2.0, respectively (Glasel, 1995). Out of 259 DNA samples, I excluded samples with DNA concentrations <20 ng/µl (n = 10) because they all scored as negatives (no bands) when tested for the presence of the bird reference gene *GAPDH*.

3.4.2 Parasites detection using nested PCR (nPCR) approach

I tested a total of 249 DNA samples for the presence of avian malaria parasites (*Leucocytozoon* and *Haemoproteus* genera) using the nPCR protocol (Hellgren et al., 2004). Of these 21% (N= 52) were found positive, showing a strong band with the expected size (478 bp for *Leucocytozoon* and 480 for *Haemoproteus*/ *Plasmodium*) in agarose gel as visualised under the UV light. 34.6% of these positives were scored as infected with *Leucocytozoon* alone, 3.85% as infected with *Haemoproteus* alone, and 61.53% were scored as a mixed infection with both genera.

3.4.3 Establishing a qPCR approach for parasite quantification

Amplifying and sequencing some DNA samples - The sequences I obtained were similar to a part of the mitochondrial genome between the *cytB* and *cox1* genes (available on GenBank from species of each corresponding genus commonly found in passerine species).

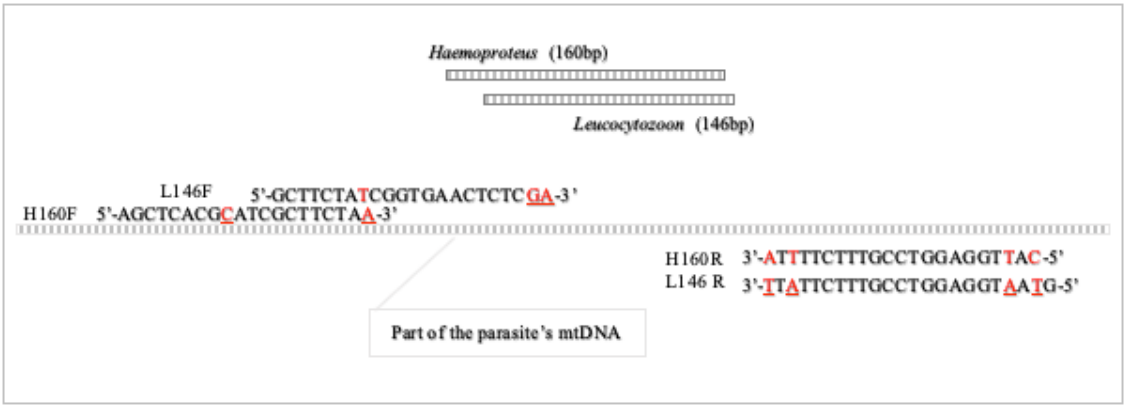


Figure 3-1 Schematic illustration of the directions and combinations of the different primers. L146F/L146R primer combination for amplification of a fragment (146bp) of the target gene region of *Leucocytozoon* genera. H160F/H160R primer combination for amplification of a fragment (160bp) of the target gene region of the *Haemoproteus* genera. Note that red-underlined letters indicate the primer sequences not shared between the two genera.

Alignment of DNA sequences and primer design - The alignment showed that sequences obtained from my samples and sequences available in the GenBank for parasite species of each genus are similar but varied slightly between genera (Appendices, Figure S1). According to the phylogenetic tree of this alignment (Appendices, Figure S2), the samples that had been identified as *Leucocytozoon* clustered around two specific species; most of them around (*L. majoris*) and two samples around (*L. fringilinarum*). However, the single *Haemoproteus* sample I have sequenced showed similarity to a number of *Haemoproteus* as well as *Plasmodium* species, indicating that my *Haemoproteus* primers (below) can also amplify *Plasmodium* species. I will report on my primers for both *Haemoproteus* and *Plasmodium* as *Haemoproteus* only because *Plasmodium* has never been detected in my study population (Capilla-Lasheras et al., 2017).

Following the criteria set out in the methods section, I designed primers manually by selecting part of the sequences that were identical among species of one genus but differed from species of the other genera (Figure 3-1). Both sets of primers (Table 3-4) were suitable to amplify their target gene: 1) of acceptable length 20/22 base pair (bp) and 22/23bp, respectively; 2) CG% content were between 40-50%; and 3) annealing temperature were 58 and 57ºC, respectively.

Table 3-4 qPCR primer sequences, concentrations and annealing temperatures for amplified targets.

primer	Target	Sequence (5'-> 3')	Product size (bp)	Annealing temperature	Concentration
L146F	*Leucocytozoon* (between *cytB* and *cox1* genes)	GCTTCTATCGGTGAACTCTCGA	146	58ºC	300/300nM
L146R		TTATTCTTTGCCTGGAGGTAATG			
H160F	*Haemoproteus* (between *cytB* and *cox1* genes)	AGCTCACGCATCGCTTCTAA	160	57ºC	600/600nM
H160R		ATTTTCTTTGCTGGAGGTTAC			
F	Blue Tit (*GAPDH*)*	TGTGATTTCAATGGTGACAGC	80	60ºC	100/100nM
R		AGCTTGACAAAATGGTCGTTC			

* The set of primers for GAPDH was designed by Atema et al. (2013)

Selecting primers - I found that the optimal conditions for each set of primers were those listed in (Table 3-4). The chosen concentrations for each set of primers showed negligible amplification with the other untargeted gene, amplified only one product as shown by dissociation curves (one peak) and amplified earlier than the other concentrations (Figure 3-3).

Standards evaluation - I tested primer efficiency on different amounts of plasmid and they successfully amplified each target gene in five different dilutions, allowing the testing of efficiency of each reaction run using the standard curve method. However, I excluded pCR2.1-*GAPDH*-BT standard with 10^5 copies because it amplified later than expected or showed an irregular amplification curve compared to the other standards. Figures 2A, B, and C show positive reactions (cycle threshold (Ct) value indicating at which fluorescence signals cross the threshold) for serial dilutions of pCR2.1.Haemo, pCR2.1.Leuco and pCR2.1-*GAPDH*-BT, respectively. The amplification efficiency of each standard assay was in the acceptable range for PCR amplification efficiency (90-112%) for each of the three standard assays.

When testing the primers with higher amounts of plasmid (10^6-10^7 copies), the primers targeting one genus target gene also amplified the other genera target. However, this unspecific amplification was observed ~20 cycles after the expected Ct for the specific target. Therefore, the specificity of these primer sets still allow to determine real mixed infection in case that parasite intensity for both genera were similar (Figure 3-3).

I found a significant correlation coefficient for the mean Ct values and value of each gene quantity (copies) which confirms specific amplification in the gene region of the DNA,

independent of DNA concentration with $R^2 > 0.97$ (Figure 3-2). This confirms the reproducibility of the three assays.

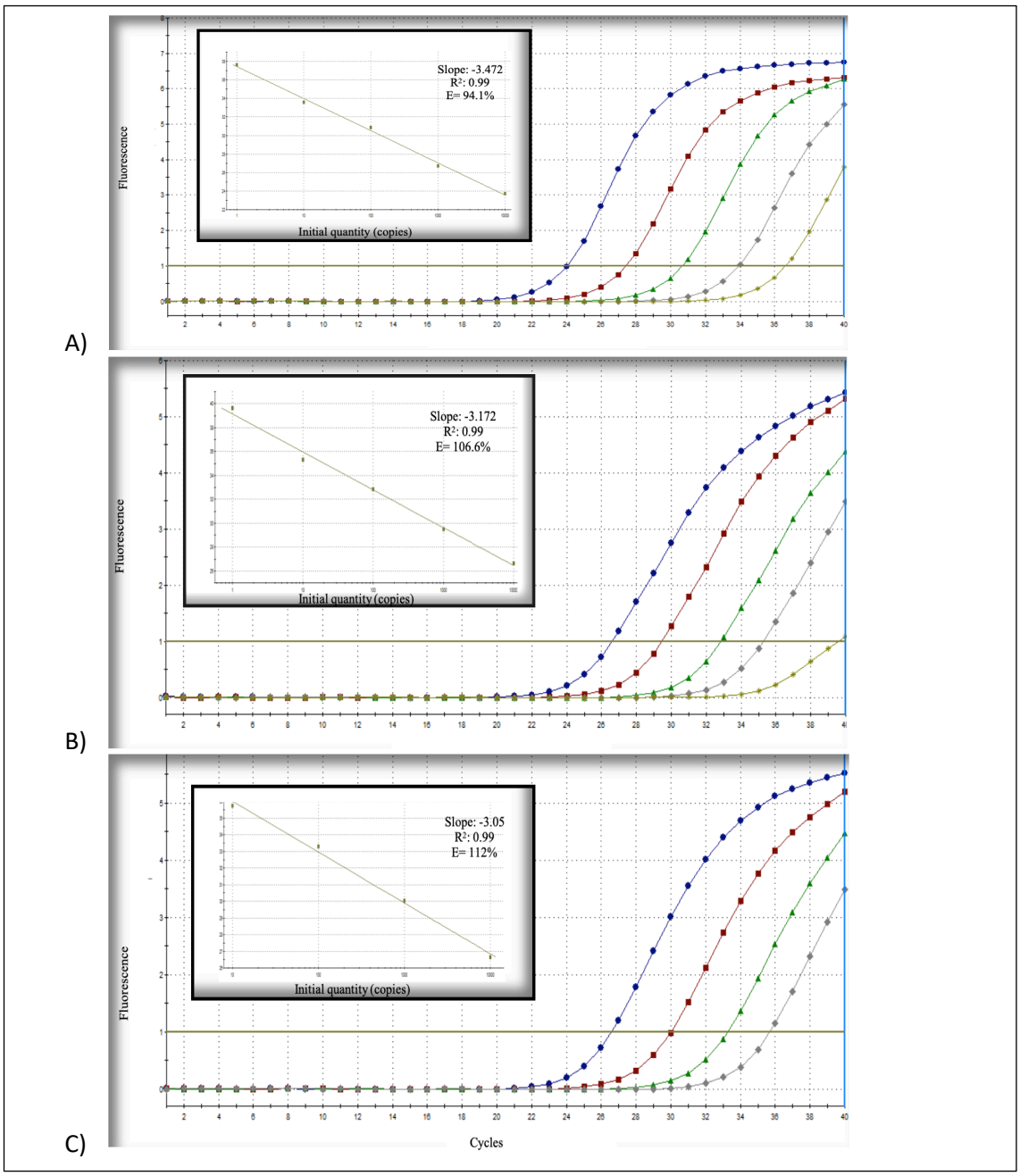


Figure 3-2 Sensitivity and linearity of pCR2.1.Haemo (A), pCR2.1.Leuco (B) and pCR2.1-GAPDH-BT (C) qPCR standard assays. Amplification presented as mean of duplicate. Plot of mean Ct values from standards replicates tested against the corresponding DNA inputs are shown in the inset. Different amount of plasmids are shown in different colours; blue= 10^4 copies; red=10^3; green= 10^2 copies; grey= 10^1 copies and yellow-green= 10^0 copy. The horizontal line at Fluorescence = 1 is the detection threshold line.

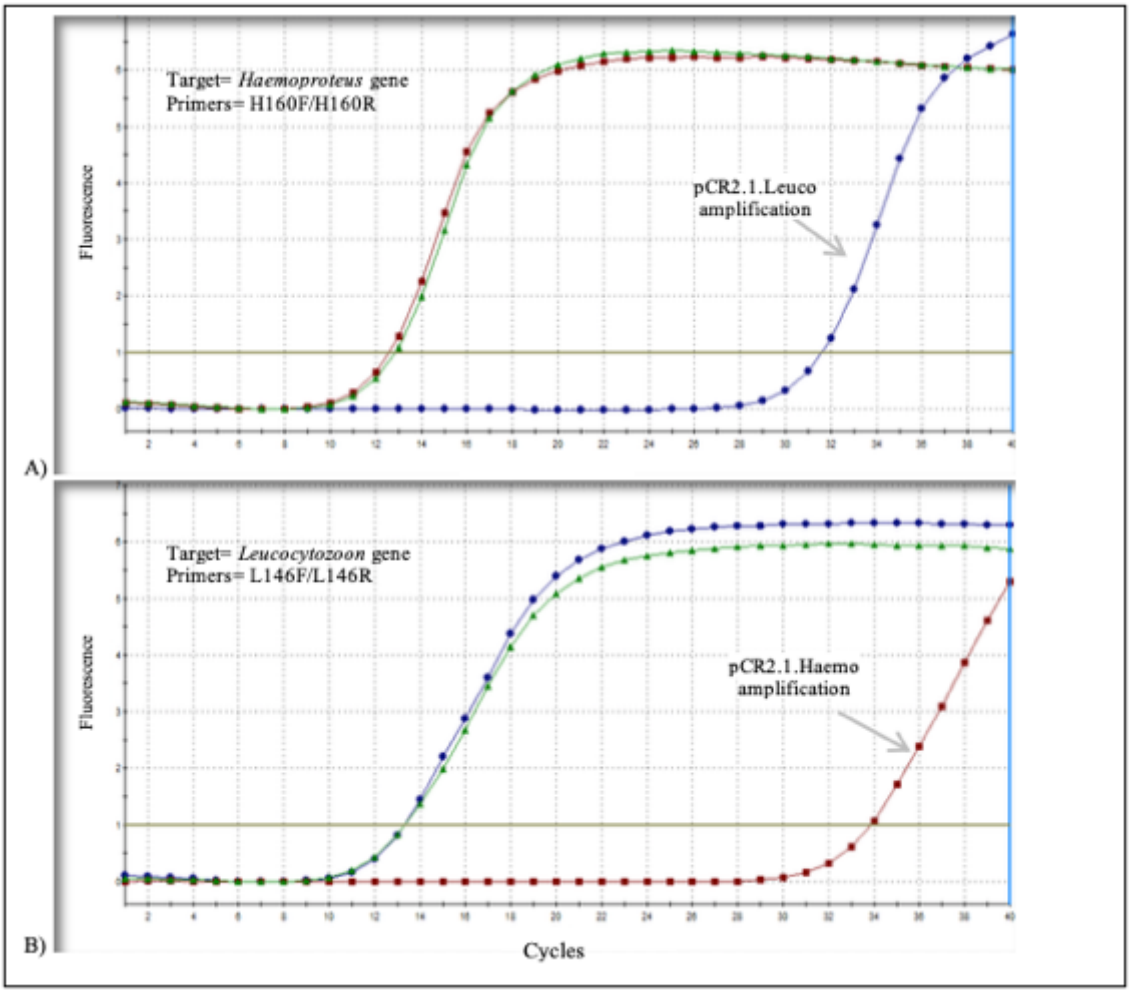


Figure 3-3 qPCR amplifications of three different plasmids (10^7 copies) (green line with triangles: mix of (pCR2.1.Haemo+ pCR2.1.Leuco), red line with squares: pCR2.1.Haemo, and blue line with dots: pCR2.1.Leuco). A) shows amplifications by using *Haemoproteus* primers while B) shows amplification by using *Leucocytozoon* primers. Note that the primers specific for one genus target sequence also amplified the other genera target; this nonspecific amplification appears ~20 cycles after the expected Ct for the specific target, allowing to distinguish such "nonspecific" amplification from real mixed infection cases, where expected parasite amplification is in the range of (10^4-10^0 gene copies). The horizontal line in the amplification plots is a threshold, which is the level of detection or the point at which a reaction reaches a fluorescent intensity above background levels. It was set at (1) for both assays.

3.4.4 Parasite detection and quantification using the qPCR approach

The real-time qPCR protocol identified all 52 samples as infected that had been identified as infected by the nPCR protocol. However, in the qPCR test all 52 infections were identified as *Leucocytozoon* infection and there were no mixed infection or *Haemoproteus* infection as the nPCR result suggested (Figure 3-4). The real-time qPCR protocol identified additional samples as infected that scored as negatives by the nPCR (n = 62 for *Leucocytozoon* and n = 4, for *Haemoproteus*, Table 3-5).

Table 3-5 Comparison of results from 230 samples generated from nPCR and qPCR . The first and fourth rows (in bold) show total samples with identical results from both nPCR and qPCR. The last three rows present the identification of the 52 samples found positive with both methods; nPCR and qPCR.

Samples (N)	*nPCR*	*qPCR*
112	**negative**	**negative**
62	negative	*Leucocytozoon*
4	negative	*Haemoproteus*
52	**positive**	***positive***
32	mixed-infection	*Leucocytozoon*
18	*Leucocytozoon*	*Leucocytozoon*
2	*Haemoproteus*	*Leucocytozoon*

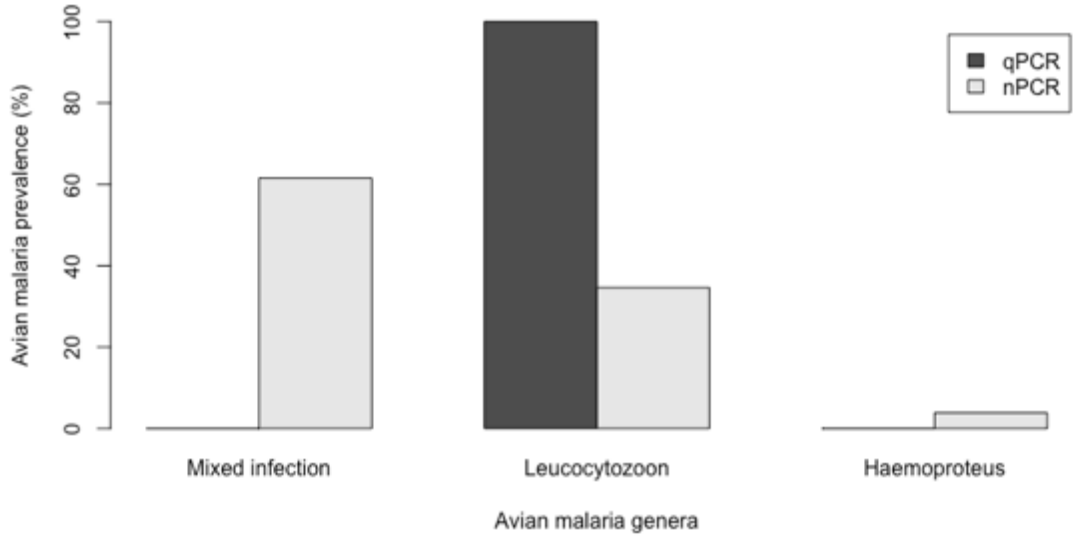


Figure 3-4 Prevalence of avian malaria parasites (*Leucocytozoon* and *Haemoproteus*) in nestling blue tit blood as tested with nPCR approach vs qPCR approach (N = 52)

Some of the samples showed low quantities of the bird reference gene (amplified at 29 Ct). Such low amount of bird DNA could be too low for samples with low parasitaemia (low parasitaemia samples usually amplified 10Ct after the bird gene) to be detected given that the maximum PCR cycle is 40 Ct. Therefore, a cut-off threshold (the minimum amount of

bird gene copies) was set under which parasite-negative samples were excluded and scored as ‘undetermined’, otherwise these samples could be potentially included as false negatives. However, any positive sample under the cut-off threshold, showing amplification of parasite gene, were included as a positive (N = 10) (Figure 3-5). In total, 19 samples were scored as undetermined and excluded from the statistical analysis.

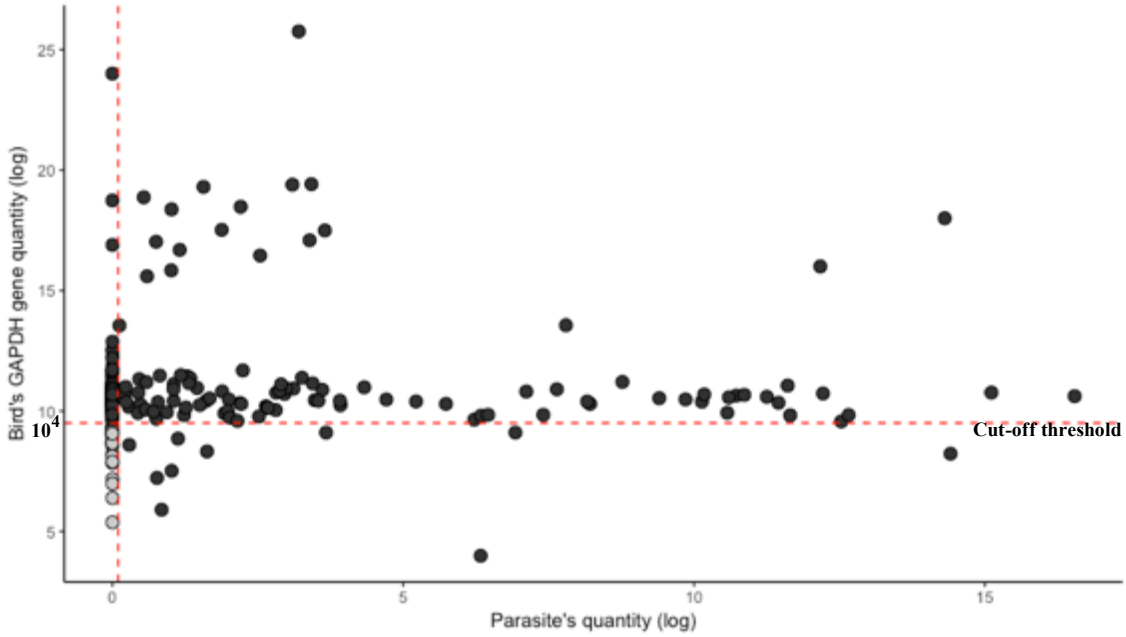


Figure 3-5 A scatter plot shows the parasite gene quantity against the bird GAPDH gene quantity. Log10 of gene quantity was used to linearise this relationship. A cut-off threshold was set at 10^4 copies of the bird genes. All the parasite-negative samples (grey) below it were excluded and were treated as ‘undetermined’ in order to exclude potential false negatives. However, all the positve samples (black) that were under this threshold for the bird gene were included.

3.4.5 Comparison between nPCR and qPCR results

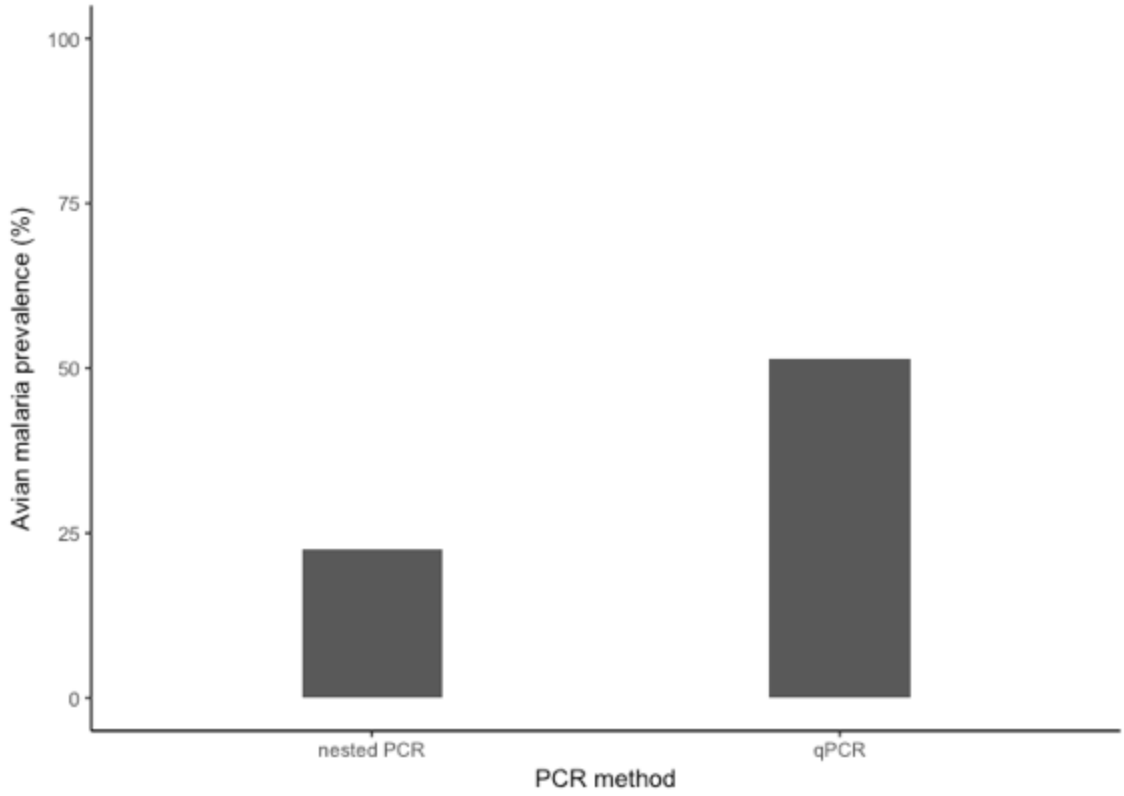


Figure 3-6 Overall prevalence of malaria in (230 samples) is 51.3% when tested by qPCR, while it is only 22.6% when tested with the nPCR approach. The new qPCR method successfully quantified parasite target gene in several samples that were negative when tested by nPCR (N = 66)

Statistical analysis suggests that there was a significant difference between the nPCR and the real-time qPCR in terms of detecting avian malaria infection (n=230, χ^2= 40.64, df = 1, p <0.001) (Figure 3-6). Statistically supported, I found a positive association between the parasite gene copies quantified by qPCR in DNA samples ("parasites") and the infection status (present/absent based on nPCR result); an infection is 73.5 times more likely to be detected with nPCR protocol when the parasite quantity increased by one copy (glm; parasite intensity, estimate coefficients = 75.27 (P<0.001), intercept, estimate coefficients = -1.7948 (P<0.001).When parasite intensity is above 0.1 parasite/blood cell (10% parasitaemia), both methods can detect infection (detection probability= 100%). However, below 10% parasitaemia nPCR probability to detect infection decreased (detection probability is only 60% when parasite intensity is 0.03 parasite/blood cell).

3.5 Discussion

In this chapter, I present the development of a new quantitative protocol, using real-time qPCR for identifying, and most importantly quantifying avian malaria parasite infection by the two genera *Leucocytozoon* and *Haemoproteus* commonly found in passerine species. This approach allows quantification of the intensity of parasite infection in avian blood as well as estimation of the infection prevalence in bird populations. In addition, this approach attempts to tackle issues of existing screening methods for avian malaria. Several factors (which could affect the detection sensitivity and specificity of protocols) were considered when developing this qPCR protocol. These were DNA quality/quantity, parasite intensity and parasite diversity in a sample (Freed and Cann, 2006).

The protocol shows high sensitivity of parasite detection as tested by the standard curve method. It is sensitive and successfully detects parasite intensity of $\geq 10^{-5}$ parasite gene copy per blood cell. The protocol shows generality to detect and quantify some *Leucocytozoon* species commonly found in passerines' blood in the local studied population. *Leucocytozoon* was found to be the most common species in some bird populations including my study population. It is generally believed that *Leucocytozoon* and its main vector (black fly) are common in northern temperate areas (Valkiūnas, 2005; Deviche et al., 2010). Nevertheless, it has been often neglected by studies concerning the effect of these parasites on host fitness. Moreover, wild birds are often reported to be infected with a mix of theses parasites, and interaction between them should not be neglected if effects on host fitness would be examined. My protocol presents a tool to discriminate between *Leucocytozoon* and *Haemoproteus* infection. In order for all the screened samples to be comparable across plates, the protocol allows to test the efficiency of each reaction run (PCR plate) by using the serial dilutions of plasmids (standard curve method).

The presented protocol enables validation of the total DNA in order to avoid failure of detection because of insufficient DNA quality and quantity. PCRs can show failure of detection by either high or low amount DNA used (Altshuler, 2006). Certain concentration of DNA template (according to spectrophotometer readings) are often used in haemosporidian parasite diagnostic protocols. However, degraded DNA is included in the total DNA concentration value (Freed and Cann, 2006). In addition, testing the exitance of host's house-keeping gene in samples by PCR is a good practise to validate the sample for

the presence of sufficient DNA. The extracted DNA from the host blood consists mostly from host DNA, and potentially a given ratio of parasite DNA (or the parasites in case of mixed infection). Thus, quantifying the amount of parasites DNA and the amount of host DNA is a key factor for accurate detection of infection. This allowed setting a cut-off threshold under which I excluded samples which possibly have very low amount of target (parasite) DNA to non-target (host) DNA, but give no amplification of the parasite DNA. Otherwise, such samples could be falsely included as negatives, biasing the overall results. In my protocol, a bird reference gene (*GAPDH*) was quantified, allowing for calculation of the parasitaemia (parasite intensity) as the ratio of parasite gene copies/bird gene copy. Then a cut-off threshold (the minimum amount of bird DNA) was set and negative samples with lower values than this threshold were excluded as false negatives could not be ruled out (Figure 3-5).

The intensity of infections (the parasitaemia) is an important factor that may led to a failure of detection by PCR protocols; the lower the intensity the lower the probability that the PCR will detect infection. This was evidenced in the nPCR protocol I used on my samples (N= 230) to compare its results with the new qPCR. nPCR probability to detect infection decreased when parasite intensity was under 0.1parasite/blood cell (10% parasitaemia). The qPCR protocol found significantly more infected individuals than the nPCR. About 56% of infected samples were missed by the nPCR. Similar to my result, although not statistically significant, Ciloglu *et al.* (2019) found that their multiplex PCR was better able to detect infections in a bird population (N= 180). The nPCR protocol missed 3.9% infected birds detected by their multiplex protocol. Bell et al. (2015) found a similar low infection prevalence that the nPCR protocol missed compared to their real-time protocol (2 out of 42 birds, 4.8%). This could be because nPCR needs to amplify large fragments of the target (parasite) DNA and in case of low parasitaemia the amount of parasite DNA is low and would be more likely to be unable to amplify the required large fragment of the target DNA (Freed and Cann, 2006).

The diversity of parasites in a sample is another factor to consider that could led to failure or inaccurate detection by PCR protocol. In a comparative analysis of five different PCR protocols for detection of mixed infection (*Plasmodium*/ *Haemoproteus*) including the protocol by (Hellgren et al., 2004) that I used, (Bernotienė et al., 2016) concluded that these broadly used molecular assays in haemosporidian research had low to moderate ability to detect mixed infection with these specific genera (7-53% of known mixed

infection). Similarly, Ciloglu et al. (2019) reported that their multiplex PCR detected more mixed infection cases (20%) than the nPCR (14%). However, my protocol shows that nPCR overestimated the mixed infection of *Leucocytozoon*/*Haemoproteus* by more than 50% when used to test 52 samples found to be infected with *Leucocytozoon* only by qPCR. This supports the finding by (Capilla-Lasheras et al., 2017), on my population, who reported a possible cross-reactivity of nPCR where the parasite identity was confirmed by sequencing. The controversial results between my findings and others may be related to the type of parasites combination in mixed infection cases (i.e. in the former studies they were referring to *Plasmodium*/ *Haemoproteus* combination, while in my population I am referring to *Leucocytozoon*/*Haemoproteus* combination.

Nevertheless, my methodology is able to detect with accuracy inter-genera mixed infections with *Haemoproteus* and *Leucocytozoon* in blue tits. However, some studies (e.g. Marzal et al. 2008) have shown that intra-genus mixed infections are more abundant. Thus, one limitation of my methodology is that still could not detect many mixed-infections (intra-genus) and hence sub-estimate the number of mixed infections in one population.

In attempts to solve the issue of inaccurate detection of mixed infection cases, Pacheco et al. (2018) designed genus-specific primers that could discriminate between *Plasmodium*/ *Haemoproteus*, but *Leucocytozoon* is not included. Ciloglu et al. (2019) presented a one-step multiplex protocol that could be used globally to discriminate between *Leucocytozoon*, *Haemoproteus* and *Plasmodium.* However, multiplex PCRs are believed to be associated with a number of issues that affect the sensitivity and specificity of detecting parasite DNA (Han, 2006); 1) each target in a multiplex reaction requires its own optimal conditions which often vary between targets, 2) several primers in one reaction reduce amplification efficiency, 3) differences in amplification efficiency may lead to variations in amplicon yields. Additionally, these PCR-based protocols often require performing of multiple PCR assays for each sample (i.e protocol by Pacheco et al. 2018) and may need further analysis of PCR products (i.e protocols by Pacheco et al. 2018 and by Ciloglu et al. 2019). Furthermore, both protocols did not account for the quality and quantity of the host DNA, which is crucial especially for low parasitaemia cases where parasite DNA is very low and can be missed.

In diagnosis, test sensitivity is the ability of a methodology to correctly identify infected individuals (true positives), whereas specificity is the ability of the method to correctly

identify those without the infection (true negatives). There are a number of advantages of using the presented real-time qPCR protocol as a diagnostic method for haemosporidians. The main advantage is its ability to reliably and quickly identify and quantify haemosporidian infections. In real-time qPCR, there is no need for a gel electrophoresis step, thus, the results for full 96 or even 384 well PCR plates are available in 3 hours. While standard PCR requires more than 4 hours only for cycling time and needs extra time for gel electrophoresis of samples before results are ready. Although my protocol leaves a small proportion of samples undetected (below the cut-off threshold) (Figure 3-5), these are only a very small proportion of the total samples. In that there is a lower risk of biasing the overall prevalence results comparing with the choice of inclusion possible false negatives.

There are some points that need to be considered before using the presented qPCR protocol as a standard method to detect and quantify avian malaria parasites. First, the protocol was developed to detect the common parasites in our local population - *Haemoproteus and Leucocytozoon* - based on the parasite sequences I obtained from several infected bird samples. However, I included some other parasite species under each genus that are commonly found in passerines in order to generalize my primers to some extent. Secondly, my H146F/H146R primers that were designed to amplify *Haemoproteus* may also be used for some *Plasmodium* species (according to the similarity of the primer sequences to some *Plasmodium* species sequences tested by alignment *in silico*). Therefore, I recommend using an extra step for discrimination between the two genera, for example using the primers of Ciloglu et al. (2019). Thirdly, while it could be counted as advantageous that I tested my protocol on samples collected from wild birds in the field reflecting a real-life situation, it can be also seen as a limitation because collecting an exact amount of blood from each bird is a challenge in the field. In addition, DNA extraction procedures may also affect the amount of DNA extracted, especially the parasite DNA which is extracted less efficiently compared to the host DNA, thus affecting the estimation of infection result (Freed and Cann, 2006). Fourth, I collected blood samples from nestlings (<14 days old), which means parasitaemia was expected to be low which limits the possibility for microscopic screening of blood smears to be used as an additional validation step and also as an additional confirmation of the parasite identity and quantity as suggested by (Valkiūnas et al., 2008). The presented protocol is valid for detection of avian malaria at the level of genus, if further information on sub-genus is needed then I recommend using species-specific primers and sequencing the PCR product. Finally, this protocol estimates

the parasite intensity (parasitaemia) in avian blood by quantifying the parasite mitochondrial gene copies. Therefore, the result does not necessarily reflect the actual number of parasites, rather it reflects the number of mitochondrial gene copies. This is because each parasite is likely to have multiple mitochondria depending on their developmental stage. For instance, in the human malaria parasite *Plasmodium falciparum* the early ring stage has one mitochondrial organelle which contains 20 copies of the gene, while gametocytes have up to eight mitochondrial organelles (Preiser et al., 1996; Krungkrai, 2004).This method successfully quantified a few *Leucocytozoon* species, which provides an opportunity to better understand and study the effect of these specific parasites on their host.

3.6 Conclusion

Given the prominence of haemosporidian parasites as a model system to study host-parasite interactions and evolution in parallel with a demand for an accurate and sensitive protocols to detect these parasites, I established a method to quantify parasite intensity of *Leucocytozoon* and *Haemoproteus/Plasmodium* using a qPCR reaction. Because sequencing of some samples (n= 24) obtained previously from birds in my study populations of blue tits showed that birds are infected only with *Leucocytozoo* or *Haemoproteus* with no *Plasmodium* detected (Capilla-Lasheras et al., 2017), my method focused on *Leucocytozoon* and *Haemoproteus* only, although the sequence alignment showed that the newly designed *Haemoproteus* primers could also be useful for some *Plasmodium* species. This new method successfully tackled the issue of cross-reactivity in nPCR protocol by Hellgren et al. (2004), that may lead to false identification of the parasite and/or false mixed infection diagnosis. The protocol presents a criteria of avoiding bias in prevalence result by excluding possible false negative results. In addition, this method presents an accurate and time-effective protocol for quantifying *Leucocytozoon* and *Haemoproteus* parasites' gene copies in bird DNA samples.

Chapter 4 *Leucocytozoon* prevalence in blue tit *Cyanistes caeruleus* populations at an urban versus a rural site: an opposite trend of variation depending on the year

4.1 Abstract

Urban ecological studies have focused on describing how urbanisation alters wildlife breeding performance but have rarely investigated potential mechanisms behind these alterations. Wild species living in urbanised areas are not only exposed to urban-related stressors compared to their rural counterparts, but the prevalence of wildlife disease may also be affected. In this chapter, I tested whether haemosporidian parasite prevalence differs between urban and rural sites by comparing prevalence and intensity of *Leucocytozoon* infections in urban and rural populations of blue tit *Cyanistes caeruleus* across two breeding seasons. To test if the susceptibility of the birds to the infection is driven by the origin of the bird (i.e. by traits inherited from parents to their offspring), I cross-fostered some clutches between the two populations in 2016. In order to better understand the impact of parasites on bird's fitness, I conducted a vector-repellent experiment to experimentally reduce infections in nestlings at both sites in 2017. I found that the origin of the bird does not influence the susceptibility of birds to the infection. Treatment with a vector-repellent did not significantly reduce infection prevalence in treated nests compared to control nests. Furthermore, age affected prevalence. I found that parasite infection prevalence was higher at day-13 of age compared to day-8 post-hatching as tested in 2017. Out of 211 13-day-old blue tits (urban = 75 from 33 nests, and rural = 136 from 45 nests), I found that overall *Leucocytozoon* prevalence was 53.9% during the 2016 breeding season and 38.5% during the 2017 breeding season. While the intensity of the parasites in 13-day-old nestlings' blood was generally similar in both years and between the two populations, I found an inconsistent pattern of infection prevalence. Infection prevalence showed the opposite trend of variation between the sites depending on the year. While it was 60% more likely for a nestling to be infected in the urban site compared to the rural site during the 2017 breeding season, *Leucocytozoon* infection probability was 74% lower in the urban site compared to the rural site during the 2016 breeding season. My findings suggest that monitoring haemosporidian prevalence across multiple years is needed to better explore the spatial pattern of infection prevalence in urban versus rural bird populations.

Keywords: haemosporidian, susceptibility, nestling, blue tit, urbanisation, prevalence, intensity

4.2 Introduction

Urbanisation is often associated with overall loss of biodiversity (McKinney, 2008). A number of urban-related stressors are found to affect several life-history traits of wild species living in urban areas, such as survival, body condition, and stress level (Sepp et al., 2017). Urbanisation may also alter pathogen epidemiology and host susceptibility to infectious diseases (Bradley and Altizer, 2007; Giraudeau and McGraw, 2014; Kernbach et al., 2018). Alteration of landscape is expected to affect the dynamics of vector-borne parasites (e.g. Calegaro-Marques and Amato, 2014; Jiménez-Peñuela et al., 2019) and human activities are often blamed for the emergence of wildlife diseases (Jones and Reynolds, 2008).

Haemosporidian parasites are one of the most popular models that scientists use to examine how parasites influence different aspects of host fitness. However studies of the impact of urbanisation on these parasites are rare (e.g. review Sehgal, 2015). Indeed, only a few studies have compared the prevalence of haemosporidian parasites in avian populations at natural versus urbanised habitats, reporting opposite trends depending on the parasite and the host species (see Chapter 1 under "1.4.5. disease prevalence").

The majority of current studies on wild avian hosts focus mostly on the influence of haemosporidians on adult birds, and overlook the nestling stage. Studies comparing birds of different ages reported contradictory results. While most studies reported significantly lower prevalence of infection in nestlings compared to adults, others found the opposite or no difference (Valkiūnas, 2005; Wilkinson et al., 2016). The low prevalence of infection among nestling in previous work may have been due to two reasons. First, nestlings may have been exposed to vectors for a very short period or not at all, and therefore may be less likely to be infected compared to adults. Second, nestlings might have been infected, but the parasite did not yet circulate in the peripheral blood stream (e.g. *Plasmodium*, *Haemoproteus* in blue tits (Cosgrove et al., 2006); and *Plasmodium* in skylarks *Alauda arvensis* (Zehtindjiev et al. 2012)). Moreover, the sensitivity of malaria detection might be influenced by the host species as well as the parasite genus and its prepatent period. However, the duration of the prepatent period following sporozoite-induced infection of these parasites remains largely undetermined (Valkiūnas et al., 2018).

Infection with *Leucocytozoon* and *Haemoproteus* parasites has been commonly detected in the populations in this study with high prevalence in nestlings since 2014 (unpublished data, 2014; Chapter 3; Capilla-Lasheras et al., 2017). Likewise, recently, Calero-Riestra et al. (2016) found 45% *Plasmodium* and *Haemoproteus* prevalence in seven- to 11-day-old wild tawny pipits *Anthus campestris* (n=90). They found that males are more likely to be infected than females. Another study on columbid species showed a high rate of infection in 14-day-old nestlings (68%) compared to 32% in seven-day-old nestlings (Dunn et al., 2017).

In disease epidemiology, the age of the first infection is a major factor (e.g. Hall et al., 2002) and previous results from my study populations suggested a high prevalence of infection in nestling blue tits (Capilla-Lasheras et al., 2017). In addition, studying nestlings has some advantages over studying adult birds in that they have naïve immune systems, so prevalence (and fitness effects) are not confounded by previous exposures (adaptive immunity) which is the case in adult birds (Norris and Evans, 2000). Furthermore, during the early stage of life, acute infections may lead to selective disappearance from the population of severely infected individuals via death from the population (Atkinson et al., 2000; Salmón et al., 2016), therefore biasing the overall results.

In order to determine the ecological patterns of infection prevalence and intensity in a given avian population, there are two key points to consider. These points are a) the features of surrounding environment, b) the biological traits of the host (i.e. age, sex, body condition, immunity and genetic traits that are inherited from parents to their offspring). This is because the former (environmental features) can influence the overlap between vector abundance and host availability (hatching date in case of nestlings), while the latter (host traits) can influence the susceptibility of individuals to infection.

In the case of vector-borne diseases, parasite prevalence depends largely on environmental conditions such as temperature, humidity and rainfall. These environmental conditions directly or indirectly affect the development, movement, and abundance of vectors (Githeko et al., 2000; Martinez-De la Puente et al., 2009). Furthermore, the absence or disturbance of suitable breeding sites for vectors, e.g. in arid or semi-arid areas, has been reported as a possible cause of low infection prevalence in some avian populations (Valera et al., 2003). Once vectors have entered the nest, infection prevalence could be influenced by the number of nestlings in the nest (brood size) via the encounter-dilution effect. Such

an effect arises when the probability of a nestling to be infected decreases proportionally with brood size and the insect does not feed proportionally on more nestlings in larger broods. In this case, it is very likely that a nestling living in a larger brood is safer from attacks by the blood-sucking insects and the parasites that they vector (Cote and Poulinb, 1995; Patterson and Ruckstuhl, 2013).

In the populations under this study, the predominant haemosporidian genus found to be prevalent is *Leucocytozoon.* According to qPCR results (Chapter 3), out of 249 blood samples, most of the infection was found to be with the genus *Leucocytozoon* (n=246) while only three nestlings were found to be infected by *Haemoproteus. Leucocytozoon* has been found to be more common in cavity-nesting birds (like blue tits) compared to open-cup or closed-cup species (Lutz et al., 2015). *Leucocytozoon* is transmitted by biting blackflies *Simuliidae* which breed in river ecosystems and can transmit the infection from an infected host to another after several days (Valkiūnas, 2005). Disturbance to blackfly habitat by urbanisation via water pollution was found to affect their abundance and overall breeding success (Docile et al., 2015). Other weather-related features, that are likely to vary between urban and the rural sites, have also been suggested to affect the dynamic of blackflies, such as temperature, humidity, and wind speed (Lachish et al., 2012; Renner et al., 2016).

Leucocytozoon can exploit the nestling periods of their altricial hosts for transmitting infection (Ashford et al., 1991; Valkiūnas, 2005). At such an early stage of host life, nestlings have only their naive immune system to respond to and fight off the infection, which is a costly process (Navarro et al., 2003). Allocation of resources to such a costly process may be challenging when nestlings are reared in a stressful environment (i.e. urban) that lacks a plentiful, good quality diet (e.g. Pollock et al., 2017). With poor diet, nestlings are expected to have increased susceptibility to infectious disease (Cornet et al., 2014; Becker et al., 2015).

Furthermore, urban birds seem to have a different gene-expression profile for some immune-related genes when compared to their rural counterparts (Watson et al., 2017). Gene-expression can be influenced by environment (e.g. poor diet post-hatching) or epigenetically from parents to their offspring. If the differential gene expression is triggered by genetic or maternal effects during embryonic development, the origin of

nestlings may also play a role in their susceptibility to infection regardless of their rearing environment or availability of high-quality diet.

The inherited variation between populations can also result in variation in their respond to infection (infection intensity level). Hosts have two ways to respond to infection; 1) resist: they can directly fight off the parasites and protect themselves from subsequent harm (reduced parasite intensity); 2) tolerate: they can limit the harmful effect of parasites without reducing the intensity (Aidoo et al., 2002). Thus, hosts that are able to reduce parasite intensity are not necessarily the healthiest, rather they can sometimes be healthy regardless of parasite intensity or they may die with parasite intensity that other hosts can cope with (Råberg et al., 2009).

Variation in response to infection (resistance versus tolerance) can be also mediated by glucocorticoid hormone concentrations, which often change in response to unstable environmental conditions and mediate changes in immune functions (Schoenle et al., 2019). Glucocorticoids influence immunity through diverse mechanisms, such as limiting inflammation through their influence on immune signalling pathways, regulating adaptive immunity via effects on lymphocyte activation and apoptosis, and influencing cytokine activity (Cain and Cidlowski, 2017).

In vertebrates, there is often variation between males and females in terms of parasite prevalence (Zuk and McKean, 1996). This variation between sexes has been largely interpreted as variation in sex-specific host traits, mainly interaction between endocrine and immune functions. Studies often show that sex hormones influence the immune system. While testosterone can suppress cell-mediated and humoral immunity in males, oestrogen can suppress cell-mediated immunity but boost humoral immunity in female (Schuurs and Verheul, 1990). During the early stage of life, sexes may also differ in their competitive ability and developmental strategies that influence the development of their immune system which is highly dependent on nutrition (e.g. Lochmiller and Deerenberg, 2000). In experimentally enlarged broods (as poor rearing condition) of blue tit nestlings, Dubiec et al. (2006) found that males' cellular immune response was more negatively affected than their female nest-mates. Similarly, pre-fledging survival of male but not female lesser black-backed gulls *Larus fuscus* was reduced as a result of experimentally manipulated parental condition (Nager et al., 2000).

It is clear that there is a lack of knowledge on the impact of urbanisation on haemosporidian infection prevalence in bird populations (Brearley et al., 2012; Sehgal, 2015; Hassell et al., 2017; Santiago-Alarcon et al., 2018). There is a need to advance our ecological understanding of how urbanisation is altering host-parasite interactions at the landscape scale (urban versus rural). Because determining the ecological patterns of infection prevalence in a given avian population is complex and there are several biotic and abiotic factors that can directly or indirectly influence the overall pattern of infection prevalence (as mentioned above), I will be focusing on the following factors using nestling stage and one parasite genus; habitat of rearing (site), habitat of origin (origin of the bird), exposure to vectors, brood size, age, sex of the nestling, hatching date and breeding season (year). The aim of this study is to provide information on avian haemosporidian infection prevalence (particularly *Leucocytozoon*) in blue tit populations inhabiting an urban and a rural site across two breeding seasons using the nestling stage. In order to examine the role of the origin of the bird on infection susceptibility, I cross-fostered some clutches between the two sites (urban and rural) and sampled the nestlings at both sites at day 13 of age. In order to test how infection intensity is changed with age, I sampled nestlings during the 2017 breeding season at two time points: at day 8 and day 13 post-hatching. I sampled each individual at day 8 then at day 13 of age. If intensity increases with age, intensity may be considered a proxy of the longevity of acute infection. Indeed, relatively early infection would lead to higher intensity at a defined older nestling age as the parasite will have had more time to replicate and increase its levels. Because the infection prevalence in the urban and rural populations of blue tits during the past breeding seasons (2015 and 2016) was very high, I decided to conduct a vector-repellent experiment during the 2017 breeding season to reduce the number of vectors visiting the nests and potentially reduce infection prevalence. This manipulation was intended to experimentally test the effect of *Leucocytozoon* infection on their hosts and disentangle, to some extent, the impact of infection from the impact of other urban-related stressors at the urban site (this will be discussed in chapter 5).

I hypothesised that the urban and rural blue tit populations will differ in *Leucocytozoon* prevalence at the nestling stage across the two breeding seasons (2016 and 2017). The pattern of infection prevalence between the two sites may also vary from year to year depending on fluctuating environmental conditions, such as food availability or weather conditions, that can affect the vector dynamic. Two alternative scenarios are possible and expected: 1) urban nestlings may be more susceptible to infection and this will be true

regardless of their rearing environment (*Leucocytozoon* infection prevalence and intensity will be higher in the urban populations); 2) alternatively, vector abundance is likely to be higher at the rural site, and if so I expect higher infection prevalence of *Leucocytozoon* in the rural population of blue tits compared to their urban counterparts. The vector-repellent treatment during 2017 breeding season is expected to reduce infection prevalence in treatment nests compared to control nests.

It is difficult to predict how individual nestlings from each population may respond to infection (resistant or tolerant), and this question is beyond the scope of this study, but I expected to find variation between the urban and the rural populations in terms of *Leucocytozoon* intensity. I also expected intensity to increase with age from day 8 to day 13 post-hatching in both sites. In addition, I expected to find variation between male and female nestlings in terms of *Leucocytozoon* infection prevalence and intensity; male nestlings in both sites are expected to show higher prevalence and intensity of *Leucocytozoon* infection. Vector-repellent is expected to reduce the number of vectors visiting the nests, hence reducing infection prevalence (Krams et al., 2013). Nest-related factors like hatching date and brood size are also expected to influence the overall pattern of *Leucocytozoon* infection prevalence.

4.3 Materials and Methods

4.3.1 Ethical statement

Blood sampling was conducted under licence of the UK Home Office, Animals Scientific Procedures Act, and individual ringing under licence from the British Trust for Ornithology (see Chapter 2).

4.3.2 Field protocol

4.3.2.1 Sampling

Work was carried out in one urban (Kelvingrove park) and one rural site (SCENE) in and around Glasgow, Scotland, between April and June in 2016 and 2017. In both sites, existing nest box study systems were used (see Chapter 1). Samples were collected from 13-day old nestling blue tits (8- day old; only in 2017) for haemosporidian parasite detection. Sample size is presented in the tables and figures below (see results). Blood

samples (20-50 µl) from the brachial vein of the wing were collected, using heparinised capillaries and stored in absolute ethanol or RNAater® until lab analysis (for more details, see Chapter 3).

4.3.2.2 Cross-fostering experimental design

In 2016, 18 blue tit clutches in the urban and 22 in the rural site were manipulated prior to clutch completion: 8 urban nests and 12 rural nests were swapped with each other within sites, representing control nests; and 10 nests were swapped across sites, representing experimentally cross-fostered nests (in total, 40 manipulated nests). Clutches within and across sites were matched based on the date the sixth egg was laid. After females laid the sixth egg, clutches were swapped. At day 13 post-hatching, blood samples for avian malaria detection were collected from nestlings (for details see Chapter 2).

4.3.2.3 Vector manipulation experimental design (2017 breeding season)

For this experiment, at both sites (urban and rural), I used nest boxes that were all located within 0.5 km of a water stream, where most avian malaria vectors reproduce. I randomly allocated each pair of neighbouring blue tit nest boxes to one of two groups: one to the control group, and the other to the experimental group. I treated some nests with insect repellent following a protocol that showed 80% reduction of the number of insects entering the nest due to the treatment by (Krams et al., 2013). The repellent consisted of 0.5 ml (citronella oil, “ASDA”, UK), 200 mg (carrageenan kappa, “special ingredients”, UK), and 0.5 ml water. One day prior to the estimated hatching date a member of the field team installed tubes filled with insect repellent into the experimental nest boxes and empty tubes into the control nest boxes. I filled four Eppendorf tubes, with a hole at the top (3 mm), with insect repellent. The tubes were attached 3 cm away from the box entrance to the inner wall of the box. The repellent was changed once during the nestling stage, during the sampling process of nestlings at day-8 to reduce disturbance of the nests. The hatching date of all the nests involved in this experiment at each site were observed within eight days, and of all the nests of both sites were observed within 10 days.

4.3.3 Lab analysis

4.3.3.1 Determining *Leucocytozoon* prevalence and intensity

A new quantitative approach was used to detect and identify avian malaria parasites in blue tit nestlings (see Chapter 3).

4.3.3.2 Determining sex of nestlings

Sex of the nestlings was determined using DNA extracted from blood samples (see Chapter 3 for DNA extraction protocol), following the molecular approach by (Griffiths et al., 1998) (see Chapter 2).

4.3.4 Statistical analysis

For every response variable, data analysis started with global general and generalised linear models including all predictors assumed to be biologically important. Models were diagnosed for multicollinearity, when two or more of the predictors in a regression model are highly correlated, which is common in ecological data (Graham, 2003) using the variance inflation factor test (VIF) (Kutner et al., 2004). Hatching date and brood size (number of nestlings at day of sampling) were standardised within sites as they showed collinearity with other predictors (e.g. site or year). Hatching date was not normally distributed, so I standardised by subtracting the median date from each date value and divided by the interquartile range from that site. Brood size was normally distributed, and I standardised it by subtracting the mean brood size from each brood size value and divided by the brood size's standard deviation from that site.

Starting from the most complex model (global model), stepwise simplification was applied using likelihood ratio tests (LRTs) of fully nested models until a minimal adequate model was found. Statistical significance ($P \leq 0.05$) for each term was calculated by assessing the reduction in explanatory power after dropping the factor from the model. Statistical analyses used packages 'car' (Fox and Weisberg, 2011), 'nlme' (Pinheiro et al., 2009) and 'lme4' (Bates et al., 2014) in R v. 3.3.3 (R Development Core Team, 2017).

4.3.4.1 Leucocytozoon prevalence

With infection prevalence at day 13 (yes/no) as a response variable, I analysed the data using "glmer" with binomial distribution and nest ID as a random factor. I included the site in order to test for spatial variation between urban and rural populations in infection prevalence. I included brood size, to test for possible encounter-dilution effect, and hatching date, to account for possible differences in overlap with vector availability, as predictors in the model. I also included the interactions between site and brood size, and between hatching date and site, to test if relationships varied between the sites. I included the year to test for year to year variation of infection prevalence. I included the interaction between the year and the site to test if the site difference is consistent between the years. I also included the interaction between the year and brood size as predictor factors of infection prevalence to test for any variation from year to year in the relationship between brood size and infection prevalence. I included sex of the bird as a predictor in order to test if one sex is more susceptible to infection than another due to physiological or behavioural trait variation. For infection prevalence at day 8, I built a similar model as above but with no year as a predictor variable as I collected blood samples at this age only during the 2017 breeding season.

For analysing the 2016 **cross-fostering experiment,** I included site (nestling-rearing habitat), origin (nestling-origin habitat) and their interaction in the "glmer" model as predictors in order to test for any influence of the origin of the bird on infection prevalence and whether this relationship is valid in both sites. I also included hatching date, brood size, the interaction between site and brood size, the interaction between site and hatching date and the sex of the bird as predictors for the same reasons mentioned above (control data analysis).

For analysing the 2017 **vector manipulation experiment**, I tested the effect of the treatment (vector-repellent) on the infection prevalence in blue tit nestlings at both sites during the nestling phase in 2017, with infection prevalence as a response variable and treatment as predictor variable in "glmer" model. Because of the very low sample size in the urban site due to high nestling mortality rate during this season, I analysed the data without including hatching date, brood size and sex, and the interaction between site and treatment.

4.3.4.2 *Leucocytozoon* intensity

With parasite intensity as a response variable, I analysed the data using "lmer" with nest ID as a random factor. I included the same predictor variables and interactions as the ones included above for (*Leucocytozoon* prevalence analysis).

In a separate "lmer" model, I tested the relationship between *Leucocytozoon* intensity and age of nestling using a subset of 2017 samples (samples that were tested for *Leucocytozoon* intensity at both ages (8 and 13 days old). With parasite intensity as a response variable, I analysed the data using "lmer". To control for non-independence of repeated observations from the same nestling and the same nest, I included nest ID and nestling ID as random factors in the "lmer" model.

4.4 Results

4.4.1 Cross-fostering and vector repellent experiments

I found no statistically significant effect of either treatment (cross-fostering or vector repellent) on infection prevalence during the blue tit nestling phase (Table 4-1). Thus, to ensure a robust sample size, I decided to analyse the two years data with treatment nests from both years pooled with the control data. Thereafter, the presented result (Table 4-2) is from pooled data (including experimental data).

Table 4-1 Results from models investigating: a) whether variation in Leucocytozoon prevalence of cross-fostered day-13-old nestling between urban and rural populations of blue tit depends on their origin during 2016 breeding season, b) the relationship between infection prevalence and treatment with repellent during 2017 breeding season. The first column represents all the predictor variables that were included in the initial model (global) while the final model only contained the intercept and any predictors that were statistically significant (shown in bold). Interactions between the predictor variables were reported only if they were significant. Estimated coefficients and standard error (s.e.) for the models were selected using likelihood ratio tests (LRT). The reported p-values are from LRTs. I converted logit-estimates to probability (presented in bold, between brackets, next to logit estimates) only for the significant predictors. Probability = odds / (1 + odds). odds = exp(logit).

a) Leucocytozoon prevelance (2016 data, n= 102 individuals from 47 nests)			
Predictors	Coefficient (probability)	s.e.	p-value (LRT)
Intercept	1.06 (0.74)	0.42	0.01
site (urban)	**-1.87 (0.69)**	**0.64**	**0.001***
origin (urban)	-0.16	0.59	0.77
brood size	-0.17	0.12	0.15
hatching date	-0.38	0.29	0.19
sex (male)	-0.49	1.07	0.77
b) Leucocytozoon prevelance (2017 data, n= 118 individuals from 35 nests)			
Predictors	Coefficient (probability)	s.e.	p-value (LRT)
Intercept	0.14 (0.53)	0.27	0.58
treatment (repellent)	-0.72 (0.64)	0.40	0.10

4.4.2 *Leucocytozoon* prevalence and intensity in an urban versus rural population of blue tit

Out of 211 13-day-old blue tits (urban = 75 from 33 nests, and rural = 136 from 45 nests), I found that overall *Leucocytozoon* prevalence was 46.2%. To specify, the infection prevalence was 53.9% during the 2016 breeding season and 38.5% during the 2017 breeding season. At an earlier age (i.e. 8-day-old nestlings), during the 2017 breeding season I found that the overall *Leucocytozoon* prevalence was 19.5% (total = 118

individuals; urban = 42, rural = 76). As testing took place during the 2017 breeding season only, *Leucocytozoon* prevalence was significantly higher in the urban population of 8-day-old nestlings. The probability of a nestling being infected was 68% more likely in the urban site compared to the rural site (Table 4-2a, Figure 4-1a). However, the infection prevalence in 13-day-old nestlings, which was tested during the two breeding seasons, showed the opposing trends of variation between the sites depending on the year. While it was 60% more likely for a nestling to be infected in the urban site compared to the rural site during the 2017 breeding season, *Leucocytozoon* infection probability was 74% lower in the urban site compared to the rural site during the 2016 breeding season (Table 4-2b, Figure 4-1b). I found no influence of sex, brood size, nor hatching date on *Leucocytozoon* prevalence at both sites (Table 4-2, Figure 4-1).

Regarding the intensity of infection in 13-day-old nestlings, none of the predictor variables seemed to explain the intensity. There was no significant relationship between intensity and site, year, hatching date, brood size or the sex of the bird (Table 4-2c, Figure 4-1c). *Leucocytozoon* intensity increased in all tested nestlings from both sites (n = 38 during the 2017 breeding season) from the age of day 8 to the age of day 13, however, this increase was not significant. The parasite intensity in 8-day-old nestlings was on average (0.23 parasites/blood cell) lower than in 13-day-old nestlings (Table 4-2d, Figure 4-1d).

Table 4-2 Results from models investigating: a) how *Leucocytozoon* prevalence at day-8-old nestling varied between urban and rural populations of blue tit during 2017 breeding season, b) how *Leucocytozoon* prevalence at day-13-old nestling varied between urban and rural populations of blue tit across two breeding seasons, c) how *Leucocytozoon* intensity at day-13-old nestling varied between urban and rural populations of blue tit across two breeding seasons and, d) how *Leucocytozoon* intensity changed with the age of nestling. The first column represents all the predictor variables that were included in the initial model (global) while the final model only contained the intercept and any predictors that were statistically significant (shown in bold). Interactions between the predictor variables were reported only if they were significant. Estimated coefficients and standard error (s.e.) for the models were selected using likelihood ratio tests (LRT). The reported p-values are from LRTs. In the "glm" models (a) and (b), I converted logit-estimates to probability, in models (c) and (d), I anti-log transformed the estimates (presented in bold, between brackets, next to logit estimates) only for the significant predictors. Probability = odds / (1 + odds). odds = exp(logit).

a) *Leucocytozoon* prevalence (at day-8) (2017 data, n= 118 individuals from 35 nests)			
Predictors	Coefficient (probability)	s.e.	p-value (LRT)
Intercept	-2.28 (0.09)	0.54	
site (urban)	**1.35 (0.68)**	**0.66**	**0.04***
brood size	0.05	0.21	0.83
sex (male)	0.04	0.54	0.98
hatching date	0.03	0.10	0.75
b) *Leucocytozoon* prevalence (at day-13) ~ site*year (2016 & 2017 data, n= 211 individuals from 78 nests)			
Predictors	Coefficient (probability)	s.e.	p-value (LRT)
Intercept	1.04 (0.74)	0.40	
site (urban)	-1.88	0.62	
year (2017)	-1.68	0.55	
brood size	-0.06	0.16	0.73
hatching date	-0.21	0.25	0.40
sex (male)	0.61	1.08	0.37
site*year	**2.29 (0.60)**	**0.86**	**0.008***
c) *Leucocytozoon* intensity (at day-13) ~ site (2016 & 2017 data, n=97 individuals from 57 nests)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	0.24239 (0.7473)	0.05	
site (urban)	-0.24226 (0.7468)	0.10	0.15
year (2017)	-0.0758 (0.1906)	0.09	0.14
brood size	-0.03	0.04	0.19
hatching date	0.01	0.25	0.16
sex (male)	-0.12	0.24	0.36
d) *Leucocytozoon* intensity ~ age (2017 data, n= 38 from 23 nests, including uninfected nestlings at day 8)			
Predictors	Coefficient	s.e.	p-value (LRT)
Intercept	0.11992 (0.32)	0.06	
age (8-day-old)	-0.11876 (0.23)	0.08	0.33
site (urban)	-0.09568 (0.19)	0.08	0.18

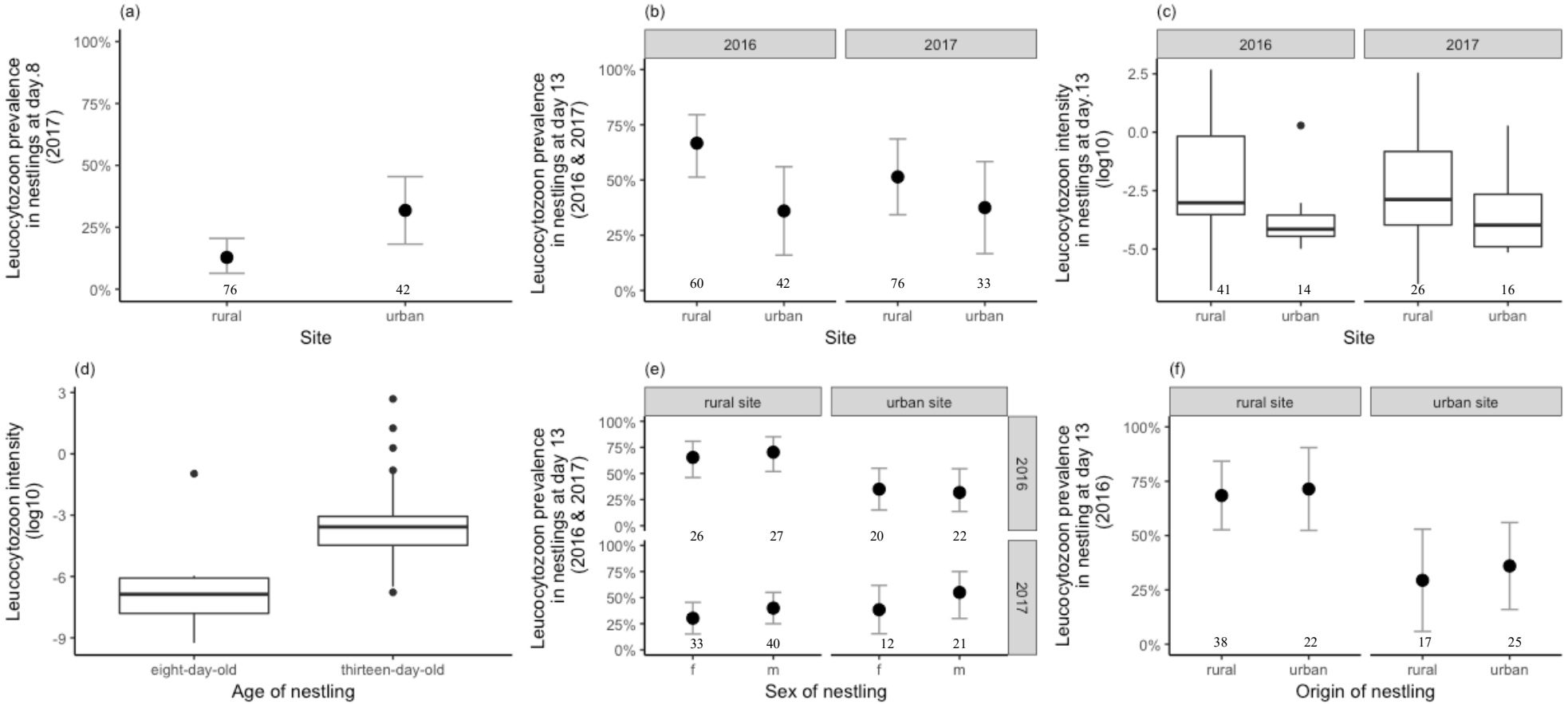


Figure 4-1 *Leucocytozoon* prevalence at 8- day-old and 13-day-old blue tit nestlings from an urban versus a rural population across two breeding seasons (2016 & 2017). Sample size for each group is presented in barallel of the x-axis. The error bars in plots (a), (b) and (d) are boot-strapped 95% confidence intervals;

a) *Leucocytozoon* prevalence in 8-day-old blue tit nestlings is higher in the urban site compared to the rural site as tested during 2017 breeding season only. Samples of day-8-old nestlings is not necessarily always from the exact individuals as at day-13 of age (plot b). Nine day-8-old nestlings died before being sampled at day 13 and 8 nestlings have missing data at one point either at day-8 or at day-13.

b) *Leucocytozoon* prevalence in 13-day-old blue tit nestlings shows opposite pattern between the sites and the years.

c) *Leucocytozoon* intensity in 13-day-old blue tit nestlings shows no significant variation between the sites depending on the year.

d) *Leucocytozoon* intensity increases with age of nestling from day 8 to day 13 post-hatching during 2017 breeding season in both sites.

e) There is no significant difference between male and female nestlings in terms of *Leucocytozoon* prevalence. f= female and m= male.

f) *Leucocytozoon* prevalence at day-13-old blue tit nestlings from an urban versus a rural populations is not influenced by the origin of the nestling during 2016 breeding season.

4.5 Discussion

The aim of the study was to examine the influence of urban environment on disease prevalence; specifically, I tested the prevalence of avian malaria parasites, of the genus *Leucocytozoon*, in two populations of blue tit – one from an urban site and the other from a rural site – during the nestling stage. I cross-fostered some birds between and within sites in order to test for any differences that may be driven from inherited traits from parents to their offspring. In contrast to my predictions, there was no significant effect of four factors: origin, sex, standardized hatching date, and standardized brood size on the susceptibility of infection as measured by the prevalence of *Leucocytozoon* parasites in 13-day-old nestlings. The insect-repellent treatment during the nestling stage in the 2017 breeding season reduced infection probability by 64%, but was not statistically significant. This lack of significant effect may be due to the fact that the citronella is not a strong repellent for insects (Lee, 2018). In addition, the high mortality rate of urban nestlings during this season also affected the sample size. This reduced sample size and consequently the power of the analysis.

However, the statistical analysis of the data revealed the following two key findings. First, *Leucocytozoon* prevalence varied between the urban and the rural populations, but with a contrasting pattern depending on the year, while infection intensity showed no significant variation between the sites and the years. Second, *Leucocytozoon* intensity increases slightly with age. Although the increase of parasite intensity with the age was not statistically significant, it was always in the same direction in each individual; higher at day 13 compared to day eight of age. However, I found no significant variation in parasite intensity between the two populations. This may be because infections were still at the early stage of development when parasites established themselves in the host tissue and started reproduction (e.g .Ilgūnas et al., 2016).

The origin of the nestling may have no important effect on their susceptibility to infection, however the sample size of this experiment (number of cross-fostered nestlings = 39, during a single season) could be too small to detect an effect (underpower). However, similar to my finding, in a recent cross-fostering experiment of the same populations as our system, Capilla-Lasheras et al. (2017) found no evidence of differences in transcript levels of immune-related genes due to the origin of the bird, which could mean that the differential gene expression is a result of the differences in rearing environment (Capilla-

Lasheras et al., 2017). Similarly, for genes related to life expectancy, another cross-fostering of a very close species, great tit *Parus major*, showed no effects of genetic or maternal factors on the observed shortened telomeres in the urban site (Salmón et al., 2016). Furthermore, recent evidence has also shown gene expression profiles affected by urban environment in wild birds (Watson et al., 2017).

The sex of the bird also seems to be not an important predictor of infection, possibly because male and female nestlings have a similar chance of becoming infected because of the limited ability to avoid vectors. Another possible reason for the lack of differences between male and female nestlings could be related to a small variation in physiological embryonic hormones between both sexes in altricial birds like blue tit (Carere and Balthazart, 2007). However, sex steroids can actually be quite high in juvenile males due to different growth strategies between male and female, while for example males develop their song system. Similarly, standardized hatching date does not seem to be an important predictor of infection prevalence nor intensity in nestlings; this could be a result of the standardization, and of relatively short period of time during the nestling stage when vector abundance is stable. Likewise, standardized brood size had no effect on infection, possibly because blue tit nestlings in their hole-nests (nest-boxes) have the same chance of being infected unlike those from open-nests, or adults that have more mobility to avoid vectors compared to nestlings (e.g. (Cote and Poulinb, 1995; Patterson and Ruckstuhl, 2013).

The year-dependent pattern I observed in *Leucocytozoon* prevalence in the urban and the rural populations may be explained by factors related to other environmental factors that can fluctuate from year to year, such as weather and food availability. In chapter 2, I found that the 2017 season seems to be not a favourable season for the urban blue tit nestlings. The results showed a reduction in urban nestlings fitness-related traits and increased in fCORT levels in the 2017 compared to the 2016 season. In addition, higher prevalence in the urban site in 2017 can be also explained by vector-related factors, that can fluctuate from year to year, including distribution of parasite-transmitting vectors and subsequently host exposure (Atkinson and Charles van Riper, 1991; Shurulinkov and Chakarov, 2006) and spatial overlap between host and vectors (Atkinson and van Riper, 1991). Changes in factors such as temperature, rainfall, and humidity have been associated with the dynamics of malaria vector populations and therefore with the spread of the disease (e.g. White, 2008; Lei et al., 2013). Furthermore, other host-related factors can also influence the patterns of infection between sites and years, including physiological variation in the host's

condition such as the host's ability to resist infection (Lindström and Lundström, 2000; Råberg et al., 2009) or differences in host mortality rate (Merino et al., 2000).

Several environmental factors can make the urban site in my study a source of parasite vectors and subsequently lead to elevated prevalence of parasites in host populations. The availability of a water body (the river) in the urban site (Kelvingrove Park) and abundance and diversity of vegetation in the park make it a good breeding site for the biting insects (Murdock et al., 2013). The predominant genus of avian malaria in our system is *Leucocytozoon* (Chapter 3), which is transmitted by blackflies *simuliids* (Woodford et al., 2018). Blackflies are known to be more abundant in habitats with fast flowing streams for their larva stages (Sehgal et al., 2005; Valkiūnas 2005).

The prevalence of infection in the urban site in the current study was high (always > 30%), this finding supports the results of previous work focusing on the prevalence of other types of infection in urban sites. These studies linked this high prevalence of infection mainly to the artificial changes to the land cover and growing energy consumption, resulting in the 'urban heat island' (e.g. lyme disease (Logiudice et al., 2003), nipah virus (Chua et al., 1999) and human malaria (Tadei et al., 1998; Vittor et al., 2006).

4.6 Conclusion

It seems that there is no consistent pattern of variation between urban and rural sites in terms of *Leucocytozoon* parasite prevalence. From year to year, the number of biotic and abiotic factors can change between the sites, therefore affecting the overall pattern of variation in the pathogen prevalence. For understanding urban ecology, it is indeed essential to know which of these spatial patterns of disease is predominant by monitoring the prevalence across multiple years. If there is a spatial pattern in disease risk, then such a pattern may play a role in the likelihood of a given host species to inhabit urban habitats (Shea and Chesson, 2002) and their subsequent reproductive output, longevity, and population density (Moore, 2002).

Chapter 5 Leucocytozoon infection prevalence is associated with reduced body weight and fledging success of urban wild blue tit nestlings

5.1 Abstract

Landscape transformation due to urbanisation strongly affects ecosystems worldwide. Both urban-related stressors and parasite prevalence have detrimental consequences on organism health, especially in their first stage of life when their immune system is still developing. However, the potential synergistic impact of those two factors has been rarely explored. I tested the effect of *Leucocytozoon* infection prevalence on fitness-related traits (body weight and survival) of nestling blue tit *Cyanistes caeruleus* dwelling at an urban and a rural site during two breeding seasons. In addition, I tested the relationship between *Leucocytozoon* infection and the levels of fCORT in nestling blue tits. Regardless of the sex of the bird, *Leucocytozoon* infection prevalence was significantly associated with a reduction in nestling survival and body weight at day 13 post-hatching of the urban but not the rural population of blue tit, significantly in the 2017 breeding season. However, I found no relationship between *Leucocytozoon* infection and the levels of fCORT in nestling blue tits. My findings suggest that urban environment and *Leucocytozoon* infection may have a synergistic effect on blue tit fitness-related traits, which have the potential to affect the breeding success of birds in urbanised landscapes.

Keywords: haemosporidian, fitness, prevalence, survival, urbanisation

5.2 Introduction

Parasites that negatively affect the fitness of their hosts have the potential to form important evolutionary pressures in the wild because they can act as a selective force in natural populations (Poulin, 2007). Such parasites can play an important role in the likelihood of given host populations to inhabit given habitats (Shea and Chesson, 2002). Furthermore, they can influence the subsequent reproductive output, longevity, and population density of their host (Moore, 2002).The effect of parasites on their host is particularly important in the context of the host's age and rearing condition prospects. According to life-history theory, individuals need to divide restricted resources between different processes, resulting in trade-offs between competing functions (e.g. Stearns, 1989). Self-maintenance (i.e growth, tissue repair and survival) and immunity are both energetically costly processes (Lochmiller and Deerenberg, 2000).

Therefore, growing nestlings, particularly those living in stressful environments, may have to trade-off between two primary needs: growth and immune functions to fight off pathogens. Because nestlings still need to developing their immune system, fighting an infection in addition to develop their immune system may come at the expense of investment in growth (Moller, 2009). This would affect their maintenance and survival during the early stage of life. Alternatively, birds may invest their limited resources in growth over the immune defence. This would result in severe effects from the parasite infection on their health and survival, in addition to resources used by the parasites themselves.

Infection with haemosporidians, like *Leucocytozoon* and its closely related genera, *Plasmodium* and *Haemoproteus*, is widespread among wild birds. The developmental stages of these parasites are generally poorly known and can vary slightly from one genus to another or even from subgenus species to another, but they are likely to be broadly similar in having three main stages of development (Valkiūnas, 2005). These developmental stages are necessary for the parasite to complete their life cycle; 1) exoerythrocytic stage, 2) gametocyte production, and 3) sexual production in dipteran insects (see details in Chapter 1). The two main developmental stages in bird (the host) are: 1) exoerythrocytic merogony (schizogony), which occurs before parasitaemia (intensity of parasites in the blood) but also continues after and during parasitaemia; 2) development in blood cells and production of gametocytes (parasitaemia). During the exoerythrocytic stage, extensive development of parasites all over the body can damage the major organs of the host (Valkiūnas and Iezhova, 2017).

Haemosporidian parasites have often been studied in the context of host-parasite interactions and their impact on host fitness traits (Ricklefs et al., 2004; Valkiūnas, 2005). Despite comprehensive research, the effect of these parasites on host fitness in the wild is still poorly understood. A severe negative effect was reported for naïve host populations (Atkinson and Riper, 1991; Atkinson and Samuel, 2010). However, for other populations that have coevolved with these parasites, the impact of infection on the host is unclear (see Chapter 1). A very recent study, in suburban London, found that house sparrows juvenile and adult survival rate and population growth were negatively related to *Plasmodium relictum* infection intensity (Dadam et al., 2019). Conversely, several studies found that chronic infection with avian malaria parasites has no significant effect on their avian host's overall condition and breeding success (e.g. Bennett, Caines and Bishop, 1988; Ots and

Hõrak, 1998; Kilpatrick et al., 2006). This lack of effect of parasites on their host may be because birds which were able to survive the acute phase of infection will be able to tolerate the infection and reduce their negative impact.

Thus, determining the initiation of infection is a critical point to assess the effect of parasites on their hosts fitness. It is difficult to accurately determine for how long a host has been infected in the wild. However, sampling of nestlings will help to estimate the timing of infection because of the relatively short period of time they have been exposed to infection. While intensity of infection can sometimes help estimating how long the host has been infected, it is not always a usable proxy of timing of infection. Indeed, during an acute phase of infection, the parasite intensity fluctuated depending on the parasite cycle. When sampling a bird at one point (e.g. day 13 post-hatching only), the exact part of the parasite cycle during which a bird is sampled is known. All the birds in my study are presumably still in the acute phase of infection. I expects this phase of infection to take more than 9 days in my study system (i.e. blue tit-*Leucocytozoon*). This is because in the previous chapter, I found that the infection intensity, in each bird sampled at two points, increased from day eight to day thirteen of age. Therefore, infected nestlings at day eight typically had the infection for, at least, four days post-hatching (Valkiūnas, 2005). In this case, I cannot reliably use the intensity of the parasite to test the effect of intensity on fitness-related traits of the blue tit nestlings under my study, rather infection prevalence (infected/uninfected) may be more able to answer my questions.

Haemosporidian's effect on wild nestlings has been poorly investigated because most studies reported either no infection or very low infection prevalence. In my study system, blue tit nestlings, breeding in nest-boxes at an urban and a rural site are often found infected with *Leucocytozoon* parasite (see results in Chapter 3). In contrast to the high rate of infection we found in blue tit nestlings, other studies have tended to detect lower infection prevalence at this early stage of life, especially among passerine species (e.g. (Cosgrove et al., 2006; Zehtindjiev et al. 2012). However, recently, Calero-Riestra et al. (2016) found 45% *Plasmodium* and *Haemoproteus* prevalence in seven- to 11-day-old wild tawny pipits *Anthus campestris* (n=90). They found that males are more likely to be infected than females, and infected males have lower daily mass gain than infected females (Calero-Riestra et al., 2016). Thus, haemosporidian infection can have consequences on fitness-related traits of the host, but they may vary between male and female nestlings due to behavioural or physiological differences between the sexes.

Leucocytozoon chronic infection, in poultry and domestic birds, is often linked to a weakened immune system and reduction in reproduction. Severe infection causes death due to dehydration, starvation, and convulsions. Major organs like the liver and spleen are enlarged due to severe infection (e.g. Adler and McCreadie, 2019). The severity of tissue damage during the *Leucocytozoon* exoerythrocytic stage seems to lead to the death of birds, but not necessarily the levels of parasitaemia. Indeed, there was no sign of disease observed in some birds with very high levels of parasitaemia (Bennett et al., 1993).

While *Leucocytozoon* is known to cause death in poultry, its effect on wild hosts is generally not apparent. However, in some populations of Canada geese *Branta canadensis, Leucocytozoon* has been evident to be fatal in nestlings (Herman et al., 1975). Similarly, a high overall prevalence of *Leucocytozoon* spp. in association with a high incidence of chick mortality was observed in the endangered yellow-eyed penguin *Megadyptes antipodes* (Argilla et al., 2013). It has been also suggested that *Leucocytozoon* can cause mortality in nestling raptors (Remple, 2004).

In my study system, across the two years (2016 and 2017), the prevalence of *Leucocytozoon* infection in the urban and the rural site was 53.9% and 38.5%, respectively. During the 2017 breeding season, I found that the urban site had higher *Leucocytozoon* prevalence compared to the rural site, while the opposite pattern was observed during the 2016 season (see Chapter 4, Figure 4-1b). Also, during the 2017 breeding season I found that urban nestlings had higher CORT levels compared to their rural counterparts, as measured from nestlings' feather samples (fCORT). However, the two populations had similar fCORT levels during the 2016 season (see Chapter 2, Figure 2-3b).

Leucocytozoon infection may directly affect blue tit nestlings and show association with reductions in nestling body weight and survival due to blood pathogen or due to tissue damage during parasites development in birds' main organs. Alternatively, *Leucocytozoon* infection may indirectly affect nestlings because the infection could divert resources away from growth. Male and female nestlings may be affected differently by infection. This is because, in blue tits, it has been suggested that males are heavier than females due to differences in growth strategies and competition for food in the nest (Dubiec et al., 2006; Mainwaring et al., 2011). Moreover, immune response to fight an infection may be supressed by high levels of corticosterone hormone (e.g. (Sheldon and Verhulst, 1996)). Thus, I expect to find a positive relationship between infection prevalence and the fCORT

level. The effect of infection on nestling body weight and survival may be more pronounced in urban nestlings, because urban nestlings are living in a stressful urban environments and are likely more limited in resources than rural birds (e.g. Chamberlain et al., 2008; Pollock et al., 2017). Trade-off between growth and immune defence to fight off the parasites might be more pronounced in urban birds than rural ones. The three following hypotheses will be tested. First, there is a negative relationship between fitness-related traits (nestling body weight at day eight, body weight at day 13, and nestling survival) and *Leucocytozoon* infection in nestling blue bit across the two breeding seasons. Within individuals, I expect the same pattern for body weight gain. Second, the negative effect of *Leucocytozoon* infection on the fitness-related traits above will be more significant in urban nestlings compared to their rural counterparts and in male nestlings compared to females. Finally, there is a positive interaction between fCORT level and *Leucocytozoon* infection prevalence.

5.3 Materials and Methods

5.3.1 Ethical statement

Blood sampling was conducted under licence of the UK Home Office, Animals Scientific Procedures Act, and individual ringing under licence from the British Trust for Ornithology (see Chapter 2).

5.3.2 Field protocol

Work was carried out in one urban and one rural site in and around Glasgow, Scotland, between April and June in 2016 and 2017 (see Chapter 1). In both sites, blood and feather samples were collected from nestling blue tit *Cyanistes caeruleus* breeding in existing nest box study systems (see Chapter 2).

Nestlings' fitness-related traits parameters – During the 2016 breeding season, on day 13 after the first egg hatched, three randomly chosen nestlings were weighed representing weight at day 13. During the 2017 breeding season, on day 8 after the first egg hatched, three to four randomly chosen nestlings were weighed representing weight at day 8. Then, after 5 days later, when possible, the same nestlings that were weighed at day 8 were weighed again representing weight at day 13. Individuals were recognised by their ring number. Body weight gain for each nestling was then calculated by subtracting weight at

day 8 from weight at day 13. During nest visits all dead chicks were recorded and collected to be kept in the freezer for possible future analysis. Nest boxes were checked after fledging to search for further dead nestlings, then individual nestlings were recorded as survived or not survived, representing fledging success. Sex of the birds was determined molecularly using the protocol by (Griffiths et al., 1998), (see Chapter 2).

Nestlings' feather and blood sampling - For measuring corticosterone hormone from feathers (fCORT), two to four body feathers (chest feathers cut at the base) from each of these nestlings were collected on day 13 post-hatching and kept in Eppendorf® tubes in a dark, dry place until lab analysis. For haemosporidian parasite detection, blood samples (20-50 µl) from the brachial vein of the wing were collected, using heparinised capillaries and stored in absolute ethanol or RNAater® until lab analysis. Blood sampling took place during ringing and weighing of nestlings at day 8 and day 13 post-hatching as mentioned above. Feathers were collected first, then blood taken, from each nestling. In 2016 I carried out a cross-fostering experiment and in 2017 a vector-repellent experiment (described in Chapter 4). Because neither cross-fostering nor vector-repellent had a significant effect on infection prevalence, I pooled treatment and control nests in my analyses to ensure a robust sample size.

5.3.3 Lab analysis

A new quantitative approach was used to detect and identify avian malaria parasites in blue tit nestlings. After DNA extraction from blood samples, qPCR reactions were run. I used the set of primers (L146F/ L146R) to identify and quantify *Leucocytozoon* parasites in blue tit blood (see Chapter 3 for more details). The fCORT level was measured following Bortollotti et al. (2008) with some modifications. I pooled feather samples per nest to have a sufficient amount of feather for the ELISA assay (see Chapter 2 for more details).

5.3.4 Statistical analysis

In order to test the hypotheses outlined in the introduction, I started an analysis of each response variable with a global model including all the biologically meaningful predictor factors. The sample size for each response variable is mentioned in Table 5-1. Model selection, diagnosis for multicollinearity and packages used are mentioned in the "Statistical analysis" section of the previous chapter (Chapter 4).

To test the relationship between **fCORT concentration** and the prevalence of *Leucocytozoon* infection at day 13, I modelled the infection prevalence (percentage of infected nestlings per nest) as the response variable using "glm" on data from feather-sampled nestlings only. I included mean fCORT per nest, site, and the interaction between fCORT and site to test for any variation in this relationship from site to site. I included year and the interaction between fCORT and year to test for any variation in this relationship between years. I included the interaction between site and year to test if variation in *Leucocytozoon* infection between the two sites still show variation from year to year, for this subset of samples, following the pattern I found in the previous chapter for the full data set (Table 4-2b and Figure1b in Chapter 4).

Table 5-1 Sample size used for the statistical analysis. Samples were collected from blue tit populations breeding in nest-boxes at an urban and a rural site during 2016 and 2017 breeding seasons.

	Urban site				**Rural site**			
	2016		**2017**		**2016**		**2017**	
	individuals	**nests**	**individuals**	**nests**	**individuals**	**nests**	**individuals**	**nests**
***Leucocytozoon* infection (per nest)**	**-**	**13**	**-**	**9**	**-**	**16**	**-**	**13**
Weight at day 8	**-**	**-**	**42**	**15**	**-**	**-**	**76**	**19**
Weight gain	**-**	**-**	**32**	**12**	**-**	**-**	**63**	**19**
Nestling survival (fledging success)	**42**	**21**	**44**	**15**	**60**	**26**	**79**	**19**
Weight at day 13	**42**	**21**	**36**	**12**	**60**	**26**	**73**	**19**

In order to examine the relationship between *Leucocytozoon* infection prevalence and fitness-related traits of nestlings (i.e. nestling body weight at day eight, weight gain from day eight to day 13, nestling survival, and nestling body weight at day 13), I analysed data for individual nestlings with each trait as a response variable and the *Leucocytozoon* prevalence (infection status for individual nestlings) in addition to other biologically meaningful factors as predictors (see below). Nest ID was always kept in models as a random factor. Nestling survival was analysed using "glmer" with binomial distribution. The remaining response variables were analysed using "lmer". For "weight at day 8" and "weight gain" models, I used the available data (2017 data only). For modelling "weight at day 13" and "nestling survival" I used data from both years 2016 and 2017.

5.3.4.1 2017 breeding season data

When **weight at day eight** is a response variable, I included site as a predictor to control for between-site variation in body weight. I included infection prevalence (infected/ uninfected) as a predictor to test for the possible negative effect of infection on body weight. I also included the interaction between site and infection prevalence to test for site-to-site variation in the effect of infection on body weight. I included sex of nestling to control for natural variation between male and female nestlings in body weight at this age. I included the interaction between infection prevalence and sex of nestlings to test for any possible sex-specific effect of infection on body weight. In addition, I also included brood size (number of hatchlings) because increased brood size could mean less food received by each individual, and this may affect their weight. I included hatching date because seasonality of food availability may influence nestling body weight. For **weight-gain** analysis, I used a similar model as mentioned above, and included weight at day 8 as a covariate.

5.3.4.2 2016 and 2017 breeding season data

For **weight at day 13** analysis, I used the predictors mentioned above (i.e. weight at day eight model), and also included the year and the interaction between year and infection as predictor variables because I used data from two breeding seasons with possible year-to-year variation. I included the interaction between site and year to test for any year-to-year differences in body weight at day 13 between sites. I included the interaction between year and hatch date and the interaction between site and hatch date to test for year-to-year or site-to-site variation in the effect of hatching date on body weight at day 13, respectively. I included the interaction between infection prevalence and brood size to test whether the body weight of infected nestlings is more affected by the infection when brood size is larger. For **nestling survival** (survived/not survived) during the nestling phase, the model did not allow testing of the relationship between infection prevalence and survival for both site due to “complete separation” issue. This issue occurs because there is some linear combination of parameters that perfectly separates the binary response variable (Albert and Anderson, 1984). In my data case, all rural infected birds survived, and only two uninfected nestlings died, both were from the same nest in the 2017 breeding season. Thus, I used urban data only from both years. I included infection prevalence as a predictor to test for the possible negative effect of infection on nestling survival. I included hatching date and brood size to check for any influence of these factors on nestling survival due to

resource availability. I included sex of nestling and the interaction between sex and infection prevalence to test for a sex-specific effect of infection on nestling survival.

5.4 Results

5.4.1 *Leucocytozoon* infection prevalence per nest at day 13 and fCORT concentration

I found no relationship between *Leucocytozoon* infection prevalence per nest at day 13 and fCORT concentration (for the feather-sampled birds) (estimate coefficient = -0.02, s.e. = 0.02, and P = 0.23). The only variable that statistically explained infection prevalence per nest was the interaction between site and year (Table 5-2a). Urban blue tit nestlings had higher *Leucocytozoon* infection prevalence during 2017 compared to their rural counterparts, and the opposite trend was observed during 2016 breeding season. This finding matches the overall pattern of *Leucocytozoon* infection prevalence I found in the previous chapter for the full data set (see Table 4-2b and Figure 4-1b in Chapter 4).

5.4.2 *Leucocytozoon* infection and nestling growth

During the 2017 breeding season, I found that *Leucocytozoon* infection had no significant effect on nestling's body weight at day 8 and body weight gain from day 8 to day 13 (Table 5-2b and Table 5-2c, respectively). Model coefficients suggested that there was a small negative, but not statistically significant effect of infection on body weight at day 8 in addition to a significant effect of the urban site. Urban day-8-old nestlings were on average 1.26±0.26 g lighter than their rural counterparts. I found a small but statistically significant, positive association between weight gain and brood size (Table 5-2c).

5.4.3 *Leucocytozoon* infection and nestling survival

Across both 2016 and 2017, the survival rate of urban nestlings was 69.2% if they were infected with *Leucocytozoon*, while it was 91.5% if they were uninfected, while the survival rate of rural nestlings showed no variation due to infection (Figure 5-1d). Survival rate in the rural site was always high, to the extent that I couldn't include data from this site in my statistical model. But I take this fact to indicate that infection had no effect on the rural site. In contrast, in the urban site, I found a significant negative association between *Leucocytozoon* infection and urban nestling survival (Table 5-2d).

5.4.4 *Leucocytozoon* infection and nestling body weight

Regarding nestling body weight at day 13, there was a significant effect of *Leucocytozoon* infection on nestling's body weight at day 13 depending on the site and the year. However, the effect of both interactions involving infection (with site and year) on nestling body weight was marginally significant (P = 0.05). Irrespective of infection, urban nestlings body weight at day 13 was significantly lower compared to their rural counterpart during 2017 season, while nestlings from both sites showed similar body weight during 2016 season (Figure 5-1c, Table 5-2e). Only in the urban site, body weight of late-hatched nestlings was on average 0.58±0.28g heavier than early hatched ones. Males were heavier at day 13 than females (Table 5-2e).

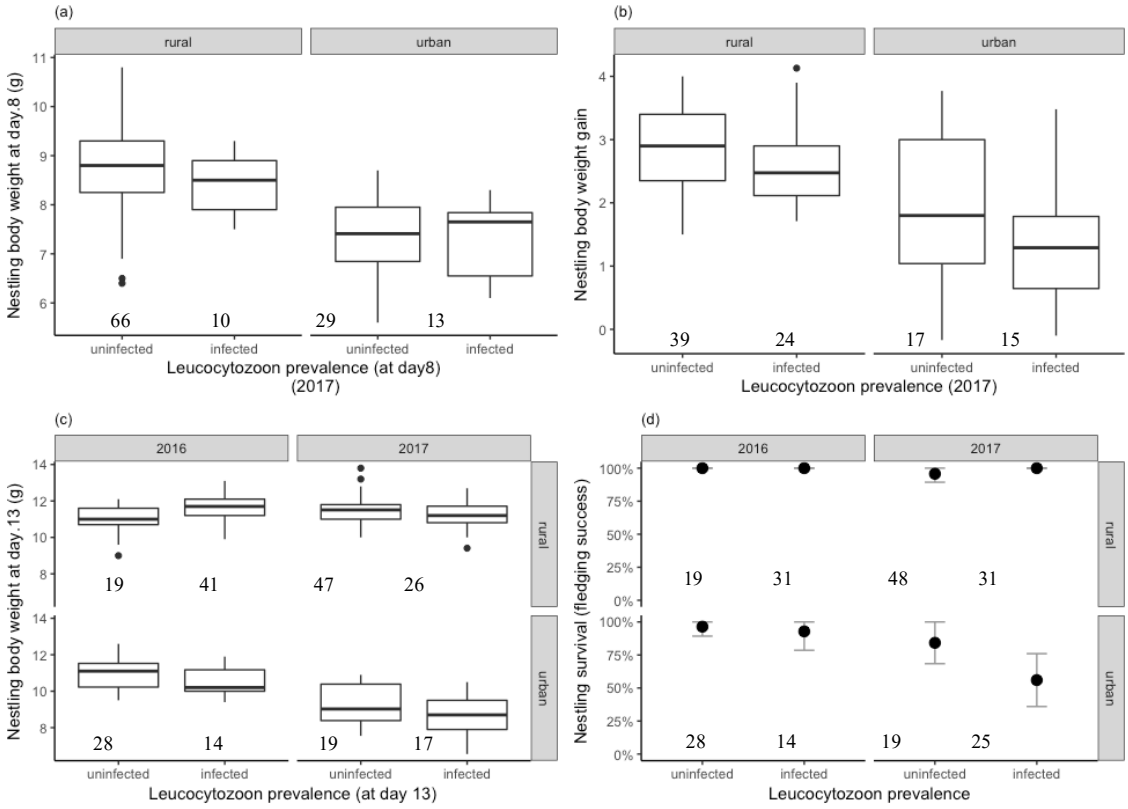


Figure 5-1 Impact of *Leucocytozoon* infection prevalence on fitness-related traits of day-8-old and day-13-old Blue Tit nestlings from an urban versus a rural population across two breeding seasons (2016 &2017). The sample size for each group is presented in parallel of the x-axis. In the boxplots, the horizontal line represents the median of weight, the top part of the box represents the third quartile (Q3) and the bottom part of the box represents the first quartile (Q1). The error bars in (d) are bootstrapped 95% confidence itervals.

Table 5-2 Results from models examining a) the relationship between the proportion of infected nestlings included in the fCORT analysis and the fCORT level. b-c) the impact of *Leucocytozoon* infection on nestlings' body weight at day-8 and weight gain, and how this relationship differs between urban and rural populations of blue tit during 2017 breeding seasons.d) the impact of *Leucocytozoon* infection prevalence on blue tit nestling survival during nestling phase, and how this relationship differ across two breeding seasons. e) the impact of *Leucocytozoon* infection prevalence on the nestlings' weight at day-13 and how this relationship differs between urban and rural populations of blue tit across two breeding seasons. The first column included the response variables (a-e). Under each response variable, all the predictor variables that were included in the corresponding initial (global) model are represented, while the final model only contained the intercept and any predictors that were statistically significant (shown in bold). Infection and any interaction with infection are underlined because they were the main predictor variables tested in the analysis. Estimated coefficients and standard error (s.e.) for the models were selected using likelihood ratio tests LRT. The reported p-values are from LRTs. In (a & d "glm" models), I converted logit-estimates to probability only for the main tested or significant predictors (presented between brackets next to logit-estimates. Probability = odds / (1 + odds). odds = exp(logit).

a) Infection (% per nest) (both years data, n (2016)= 29 nests, n (2017)= 22)	**Coefficient**	**s.e.**	**p-value (LRT)**
Intercept	0.63 (0.65)	0.07	
year **(**2017**)**	-0.28	0.11	
site **(**urban**)**	-0.33	0.11	
fCORT **(**per nest**)**	-0.02	0.02	0.23
site ***** fCORT	0.004	0.01	0.69
year * fCORT	0.01	0.02	0.34
site * year	**0.55 (0.71)**	**0.17**	**<0.001***
b) Weight at day 8 (2017, n=118 from 34 nests)	Coefficient	s.e.	p-value (LRT)
Intercept	8.67	0.26	
site (urban)	**-1.26**	**0.26**	**<0.001***
infection (infected)	-0.22	0.15	0.15
sex (male)	0.09	0.11	0.16
hatching date	-0.005	0.04	0.80
brood size (hatchlings)	-0.11	0.08	0.27
infection* site (urban)	0.29	0.31	0.54
infection *sex (male)	0.23	0.31	0.89
c) Weight gain (day 8 to 13) (2017, n=95 from 31 nests)	Coefficient	s.e.	p-value (LRT)
Intercept	0.86	0.33	
weight at day 8	-0.06	0.03	
site (urban)	**-0.33**	**0.09**	**0.005***
infection (infected)	-0.06	0.04	0.14
sex (male)	-0.02	0.04	0.06
infection* site (urban)	-0.14	0.09	0.29
infection*sex	0.06	0.08	0.11
brood size (hatchlings)	**0.01**	**0.02**	**0.02***
d) Survival (urban site, 2016 & 2017, n=86 from 36 nests)	Coefficient	s.e.	p-value (LRT)
Intercept	9.726 (0.99)	2.72	
infection (infected)	**-2.765 (0.97)**	**1.49**	**0.03***
sex (male)	-0.12	1.00	0.90
year (2017)	-3.75	1.95	0.14
hatchlings	0.51	0.89	0.55
hatching date	-0.10	0.23	0.64

infection*sex (male)	0.75	1.89	0.54
infection*year (2017)	-1.75	2.57	0.49
e) Weight at day 13 (2017 &2016, n= 211 from 79 nests)	Coefficient	s.e.	p-value (LRT)
Intercept	11.13	0.32	
site (urban)	-0.67	0.28	
year (2017)	-0.22	0.35	
infection (infected)	0.25	0.17	
brood size	-0.07	0.07	0.11
hatching date	-0.37	0.25	
sex (male)	**0.30**	**0.25**	**0.01***
infection*brood size	-0.09	0.08	0.17
infection*site (urban)	**-0.44**	**0.20**	**0.05***
infection*sex (male)	-0.07	0.59	0.96
infection*year (2017)	**-0.43**	**0.20**	**0.05***
site (urban)* hatching date	**0.58**	**0.28**	**0.04***
year (2017)*hatching date	0.17	0.23	0.43
site (urban) *year (2017)	**-1.29**	**0.44**	**0.004***

5.5 Discussion

The presented study was conducted with the aim to explore the effect of *Leucocytozoon* infection prevalence on the fitness-related traits of blue tit nestlings dwelling in an urban site and rural site. Because I am sampling wild birds, it was impossible to know for certain how long the birds were infected for; nor was it possible to distinguish between birds resisting the infection so that intensity was reduced, or birds tolerating the infection so that intensity is increased with no sign of disease. This shortcoming prevents detailed quantitative examination of the impact of the intensity of the parasites on their host's fitness. However, the following key findings are related to *Leucocytozoon* acute infection prevalence in blue tit nestlings aged eight and 13 days old. First, regardless of sex, *Leucocytozoon* infection was significantly associated with a reduction in urban but not rural nestling body weight at day 13 in 2017 but not in the 2016 season. Second, *Leucocytozoon* infection during the nestling phase had a significant effect on urban nestling survival in both years. This was tested at day eight and day 13 of age during 2017, and at day 13 of age only during the 2016 breeding season. Third, irrespective of sex or site, *Leucocytozoon* infection at day eight showed no significant association with nestlings' body weight at day eight and body weight gain between days 8 and 13. The small negative effect of infection on nestlings' body weight at day 8 may be because parasites have been in their host body at a very early stage of development and did not yet show significant effects on body weight. Alternatively, the small negative effect of infection might also be due to the small sample size, because I tested this only during the 2017 breeding season with a total of 76 birds from 19 rural nests, and 42 birds from 15 urban nests. Finally, in contrast to my expectation, there was no significant association between nestlings fCORT level and *Leucocytozoon* infection prevalence.

Blue tit nestlings infected with *Leucocytozoon* parasites showed a reduction in body weight at day 13 at the urban but not the rural site (Figure 5-1c). This negative effect of infection at an early stage of life is likely to have negative consequences on the birds' fitness in the longer-term scale. The negative association between *Leucocytozoon* infection and weight at day 13 might be because the effect of infection with these parasites is observed in stressed nestlings like the urban nestlings.

The higher availability of caterpillars, the main natural food for blue tits; at the rural site (Pollock et al., 2017) is likely to help nestlings to allocate sufficient resources to two

demanding processes: growth, and mounting immune defence against parasitic infection. However, *Leucocytozoon* infection in the urban nestlings that have access to fewer caterpillars, thus suffering from nutritional stress (Pollock et al. 2017) may prevent them from allocating sufficient resources to both growth and immunity (Blount et al., 2003; Isaksson, 2015; Nettle et al., 2017). In support of this hypothesis, Pollock and others (2017) found clear evidence of food limitations and nutritional stress in the urban site, when comparing the food availability and provisioning behaviour between these two sites (urban vs rural). Caterpillars were the predominant food provided to the rural nestlings, while urban nestlings received a lower proportions of such a high-quality diet, and instead received some anthropogenic food available in their environment (Pollock et al., 2017). Studies on blue tit populations in Switzerland and Poland showed that resource availability can differ from year to year, and these inter-annual differences in food availability can affect the birds' condition and survival (Naef-Daenzer and Keller, 1999; Marciniak et al., 2007). From the latter study's population, researchers found that haemoglobin levels in nestling blue tits are higher in years and habitats that are rich in ideal food availability (caterpillars) compared to years and habitats that are poor in caterpillar availability (Bańbura et al., 2007). Thus, these studies also support the observed variation in nestling body weight from year to year in my study populations, regardless of infection.

The lack of significant effect of infection on body weight gain in my population could be due to selective disappearance of severely affected nestlings via death before sampling them. Death of birds can occur during the early stages of the parasite development, the exoerythrocytic stage, that cause tissue damage in main organs such as brain kidney and liver (Valkiūnas and Iezhova, 2017). Reduced infection prevalence with *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* in nestling great tits led to greater survival rates during the nestling stage and a few weeks after fledging (Krams et al. 2013). An observational study suggests that the mortality of eight nestling great horned owls (*Bubo virginianus*) was likely associated with *Leucocytozoon* infection (Niedringhaus et al., 2018). *Leucocytozoon* infection has led to mortality of nestling Canada geese (Herman et al., 1975). A recent study in captive cranes in Beijing zoo suggests that *Leucocytozoon* abortive infection at the juvenile stage is most likely responsible for mortality, while infection after a few months has no fatal effect on birds (Jia et al., 2018).

In my study case, I found a significant association between *Leucocytozoon* infection and blue tit nestling survival, which indicates that blue tit nestlings are likely to be severely

affected by this infection but other species may not show such an effect. However, the mode of exoerythrocytic development can be host species-specific, resulting in species-specific mortality. For instance, an exploratory pilot study of the pathology and virulence of *Plasmodium* in juvenile wild passerine birds (one individual each; Eurasian siskin *Carduelis spinus*, common crossbill *Loxia curvirostra*, and common starling *Sturnus vulgaris*) found that all birds died because of infection. Death of birds was observed when parasitaemia reached a very low level (i.e. reflecting the chronic stage) and not during parasitaemia at high levels (Ilgūnas et al., 2016). In a subsequent experiment using eight individuals from each species (house sparrows *Passer domesticus*, common chaffinches *Fringilla coelebs*, common crossbills and common starlings) researchers found differences in mortality rates depending on host species. Infection with *Plasmodium* causes mortality in chaffinches, house sparrows and crossbills (seven, five, and three individuals died respectively), but not in starlings (Ilgūnas et al., 2019). Similarly, a study investigating the developmental stages of *Leucocytozoon* and other haemosporidian parasites in several bird species, including passerines (all histologically tested birds were the result of road casualties or unexplained morbidity) suggests that there is evidence that when many schizonts are present, tissue damage may be fatal in some host species but not others (Peirce et al., 2004). The reason for these species differences is not clear.

In my study populations, feather corticosterone level (as a possible biomarker of chronic stress) was significantly higher in the urban nestlings during 2017 compared to the 2016 breeding season, while at the rural site there were no differences. This suggests that variation in fCORT over years is dependent on environmental conditions (see Chapter 2). Interestingly, *Leucocytozoon* prevalence was also higher in the urban site during the 2017 breeding season, but no direct relationship was found between infection and fCORT levels in these birds. Presumably, in the urban site, birds are challenged by different urban-associated stressors that are likely to increase stress hormone (circulating glucocorticoids) (Partecke et al. 2006; Bonier 2012), which is known to suppress immune function and increase susceptibility to disease. Similar to my finding, a recent study on wild blue-crowned manakin *Lepidothrix coronate* demonstrated the lack of a relationship between fCORT levels and infection prevalence (Bosholn et al., 2019). This may indicate that immunity is not suppressed by corticosterone (if fCORT levels accurately reflect variation of corticosterone hormone). Corticosterone hormone levels have been suggested as a biomarker of allostatic load (McEwen, 1998). Thus, the level of fCORT may indicate that

urban nestlings had to trade-off between growth and immunity, resulting in indirect effects on the consequences of infection on nestlings' body weight and survival.

In the wild, most studies have investigated the effect of erythrocytic stages on fitness, while the effects of exoerythrocytic stages have often been neglected. This may explain why moderate or no signs of avian malaria fitness effects have been observed in wild birds (e.g. Bennett, Caines and Bishop, 1988; Ots and Hõrak, 1998; Kilpatrick et al., 2006). Most of the studies of the exoerythrocytic stage of infection are from old literature using traditional microscopic techniques (reviewed in Valkiūnas and Iezhova, 2017). However, few recent works used sensitive molecular techniques to understand the severity of infection, especially during the early stage of parasite development. All of these studies indicate a link between haemosporidian infection and death of birds (e.g. (Peirce et al., 2004; Krams et al., 2013; Ilgūnas et al., 2016; Niedringhaus et al., 2018; Jia et al., 2018; Ilgūnas et al., 2019). There is a demand for more experimental studies to understand how haemosporidian parasites can affect their host's fitness-traits, as most of the current evidence is correlational; experimental evidence would help our understanding.

5.6 Conclusion

The results of the present study suggested that altricial nestlings living in a harsh environment, like the urban site, that is characterised by a lack in the availability of an abundant and nutritious food, and maybe additionally other urban-related stressors, present an easy target to avian malaria vectors and the parasites they vector. *Leucocytozoon* infection showed a significant association with a reduction of urban nestling body weight and survival compared to their rural counterparts. Future studies could monitor the birds from early infection (during nestling phase) until adulthood in order to test the long-term effects of infection alongside stressful environment with poor food availability (such as in urban areas) on the hosts' overall health and condition. Blue tits, however, might not be the ideal species for such long-term monitoring given its short life expectancy and its low ring recovery rate. In the UK, it has been reported that the survival rate of yearling blue tits is 38% and adults' annual survival rate is 53% (Siriwardena et al., 1998). My findings suggested that *Leucocytozoon* infection under certain conditions, like urban environment, can be related to reduction in their host's fitness.

Chapter 6 General discussion

My thesis has aimed to investigate aspects of the mechanisms behind the often-reported negative impact of urbanisation on avian fitness and reproductive success. Urban environment may affect directly, or indirectly via elevated stress levels, fitness-related traits and malaria acute infection in blue tits (see Figure 1-1 in Chapter 1). Nestling altricial birds present an excellent opportunity to investigate the relationship between habitat stressors, pathogen burden, corticosterone level and fitness-related traits, because of the relatively long period of the nestling stage during which the majority of their developmental process occurs (Starck and Ricklefs, 1998), and this may reflect the quality of the surrounding environment and its effect on their survival, growth and fitness.

Although the urban environment can negatively affect wild species living in urbanised areas (e.g. McKinney, 2008; Kempenaers et al., 2010; Faeth et al., 2011; Aronson et al., 2014; Meyrier et al., 2017), the extent of the impact of such a harsh environment depends on other environmental factors such as weather conditions that fluctuate from year to year. Because the urban conditions are already harsh, fluctuations in the other environmental factors are more likely to show an effect on the urban birds. Conversely, the better conditions at the rural site may allow the birds to buffer against those other environmental factors. If environmental quality and weather are not adverse, then additional urban-related stressors such as light at night, chemical pollution, or noise pollution might be the reason behind the observed pattern of a general reduction in breeding success at the urban site (clutch size, hatching success and fledging success). In my study sites, it seems that, compared to the 2016 season, the 2017 season was not a favourable season for blue tits for several possible inter-related factors. These factors are summarised from the findings across my chapters (presented in Table 6-1). Generally, the urban site in 2017 was colder and wetter than in the 2016 season. During the 2017 season, compared to the rural population, the urban population showed lower nestling body weight and survival, higher fCORT levels and higher *Leucocytozoon* prevalence compared to their rural conspecifics (Table 6-1). These findings have highlighted the importance of having included multiple years of sampling.

For the purpose of my thesis in particular, studying nestlings has some advantages over studying adult birds in that they have naïve immune systems, so parasite infection and its fitness effect on the host are not confounded by previous exposures, which is the case in

adult birds (Norris and Evans, 2000). Furthermore, acute infections may lead to selective disappearance of severely infected individuals via death from the population (Atkinson et al., 2000; Salmón et al., 2016), therefore sampling adult birds only (as the majority of studies do) would not be representative of the entire population, because it would miss early infected birds via death. Ideally, future studies could monitor the birds from early infection (during nestling phase) to adulthood in order to test the long-term effects of infection on hosts' overall health and fitness in stressful environments and/or poor food availability (such as in urban areas) on hosts' overall health and fitness.

Collecting blood samples from nestlings (<14 days old) means parasitaemia is expected, and eventually confirmed, to be low (see Chapter 3). It was not possible to detect all the acute infection with haemosporidian parasites in my study system with the widely used nested PCR methods by Hellgren et al. (2004), nor was it possible to accurately identify the parasite genus using this method (see Chapter 3). Thus, there was a need for a more sensitive assay and I developed a new qPCR protocol to detect low parasitaemia and distinguish between haemosporidian parasite genera encountered in blue tit nestlings in my study sites. However, the very low parasitaemia in the blood of nestlings has limited my ability to accurately conduct a microscopic screening of blood smears to use them as a validation step and also as an additional confirmation of the parasite identity and quantity, as suggested by Valkiūnas et al. (2008). Validation for the protocol in the future can be done with lab infected birds or adult birds for which parasitaemia is expected to be higher.

To cope with environmental challenges, birds need to regulate their use of resources to a different physiological process by secretion of CORT hormone via HPA axis (Crespi et al., 2012). However, chronic exposure to a high level of CORT can have a detrimental effect on birds, including impairing immunity and reduced fitness-related traits such as body weight and survival. If immunity is impaired due to high levels of infection, birds may be severely affected by parasite infection, particularly in an area or during a time of high prevalence of infection. In turn, an infection may also have a synergistic impact with a high CORT level on avian fitness-related traits, eventually resulting in reduced breeding success for the entire population.

From Chapter 2, it became clear that there is a year to year variation in the effect of urban environment on blue tit fitness. The fCORT level in urban birds was only elevated significantly in one year. The prevalence of *Leucocytozoon* infection in both populations

also showed variation from year to year (Chapter 4). In the year of high level of fCORT in the urban population (i.e. 2017), the prevalence of *Leucocytozoon* was higher at the urban site compared to the rural site, while in the year of no significant variation in the level of fCORT between the two populations (i.e. 2016) the prevalence of *Leucocytozoon* was higher at the rural compared to the urban site (see Chapter 4). Nevertheless, I found no direct relationship between the proportion of infected birds included in the feather sampling and the level of fCORT (see Chapter 5). This year to year variation emphasises the need for long-term monitoring and examining the impact of urbanisation on avian physiological and fitness-related traits.

Table 6-1: Comparison of the two sites in relation to number of environmental and fitness-related parameters (the first columns) over the two breeding seasons (lower or higher= means in relation to the other site in that year). = the urban site, = the rural site

	2016	2017	2016	2017
• Daily rainfall	1.9 mm	2.7 mm	3.1 mm	3.1 mm
• Daily temperature	14°C	13.1°C	13.6°C	13°C
• fCORT level	insignificantly higher	higher	insignificantly lower	lower
• Parasite prevalence	lower	higher	higher	lower
• Date of first hatching	11 May	12 May	16 May	8 May
• Nestling body weight	insignificantly lower	lower	insignificantly higher	higher
• Fledging success (survival)	lower	lower	higher	higher
• Clutch size	lower	lower	higher	higher
• Hatching success	lower	lower	higher	higher

For altricial nestlings like blue tits, starvation is believed to be the main cause of mortality at the nestling stage (Martin, 1987). Thus, the overlap between hatching period and the abundance of food is a key factor for nestling survival (Drent and Daan, 1980; Noordwijk et al., 1995). The weather condition factors are very likely to affect the food availability and provision rate for nestlings (Marciniak et al., 2007; White, 2008), thus affecting their body conditions and survival (Bize et al., 2010; Mainwaring and Hartley, 2016). The 2017 season showed colder and rainier conditions compared to the 2016 season, particularly at

the urban site. Foraging patterns and availability of food for birds, particularly insectivores like blue tits, may be affected by changing patterns of weather conditions and rainfall because their foraging behaviour is restricted, and their main prey are less active during adverse weather (Avery and Krebs, 1984). At the rural site, the structure of the forest may work as shelter that allows birds to forage for food provisions for their offspring, while the urban site (the park) lacks this natural structure of the forest, and therefore urban birds might be more restricted in their foraging for food and provide less food to their offspring compared to the rural birds. Furthermore, food limitation can be an important selective pressure at urban sites compared to rural sites for nestlings (Ibáñez-álamo and Soler, 2010). This might be because the abundance of good quality food for rural birds may help parents to compensate for the non-feeding period (e.g. heavy rain) and provide good quality food to their offspring, while the urban nestlings are provided with limited and poor-quality food (Pollock et al., 2017).

Studies have proven that availability of food for certain populations fluctuates from year to year (e.g. Marciniak et al., 2007). Quantification of food availability was done in my study population in previous years (Pollock et al., 2017) but not during my study seasons (2016 and 2017). This is a point that should be taken into consideration for future monitoring of birds' breeding performance in order to precisely evaluate each breeding season's conditions. Although I cannot tell whether or not there was an overlap between the peak of caterpillars (the main food for blue tit) and hatching date of most of the birds during my study period, nestling weight can reflect food availability. Hatching later in one season or at one site compared to another would be considered beneficial only if it was reflected by heavier nestling body weight during that season or at that site. Nestling weight can reflect food availability, thus can also be used as a biomarker of the favourable season or environment (unlimited resource) versus unfavourable season or environment (limited resources). The first hatching date at the urban site was five days earlier than the rural site in 2016, and the average hatching date was two days earlier at the urban site. In 2017, the hatching date at the rural site was on average eight days earlier than their 2016 breeding season. During the 2017 breeding season, the first hatching date at the urban site was a day later than their 2016 hatching date, but on average two days later than their rural counterparts. Therefore, late hatching in 2017 at both sites may contribute to the observed reduced nestling body weight and survival compared to the 2016 season (see Chapter 2). In other words, maybe there was a mismatch between the peak of food and hatching date of most of the birds during the 2017 season, but there was an overlap between the hatchling

period and food abundance in the 2016 season. This again stresses the importance of quantifying food availability at each site, every season, in order to better understand the pattern of breeding performance of these birds.

Differences between urban and rural nestlings in the level of fCORT may be driven by weather or nutritional stress. Other urban-related stressors may have also contributed to the observed high fCORT levels, for instance chemical pollution (e.g. Meillère et al., 2016). Elevated CORT levels are often reported to be associated with food restriction (Saino et al., 2003; Herring et al., 2011; Boonstra, 2013). Thus, this could at least partly explain poor nestling weights and the reduction in nestling survival I observed in my system that was mirrored with elevated fCORT and higher *Leucocytozoon* infection prevalence at the urban site in 2017 compared to the rural site (see Chapter 2 and Chapter 4). The high level of fCORT in the urban blue tit nestling in 2017 may have a detrimental effect on nestling fitness-related traits and may suppress their immunity, which can lead to severe impact of infection with *Leucocytozoon* parasites.

However, I found no direct relationship between fCORT level and birds' fitness-related traits, nor did I find an association between fCROT level and the proportion of *Leucocytozoon* infected nestlings. Pooling the feather samples and measuring the proportion of infected nestlings per nest may have led to limited power to detect significant differences. Thus, instead of pooling feather samples per nest, within-individual sampling could help tease apart how physiological and fitness-related traits interact, affecting the ability to see possible relationships between such traits. In addition, future studies could also measure biomarkers of immunity for nestlings in order to confirm or reject the hypothesis that immunity can be suppressed by a high level of CORT and therefore birds are severely affected.

Both immunity and growth are energetically-demanding processes (Lochmiller and Deerenberg, 2000). Trade-offs between immune defence (against *Leucocytozoon* infection) and self-maintenance (e.g. growth and tissue repair) of nestlings are probably the mechanisms behind the observed year-specific variation between urban and rural populations of blue tit in relation to their body weight and survival, which was found to be negatively associated with *Leucocytozoon* infection prevalence, but only in the urban environment and more significantly in 2017. Nestlings' resource allocation trade-offs are very likely to be during unfavourable seasons when birds face limited resources and higher

infection prevalence (e.g. urban birds during the 2017 breeding season). Urban and rural sites often exhibit variation in resource availability (e.g. Chamberlain et al., 2009), and resource availability tends to fluctuate from year to year (e.g. Marciniak et al., 2007). The unfavourable conditions can lead to the inability of birds to allocate resources to competing physiological needs, like immune defence and self-maintenance or growth.

My thesis has highlighted that the inter-relationship between *Leucocytozoon* infection and blue tit fitness differences between the two sites. Under certain circumstances (in my study case, the urban environment), *Leucocytozoon* infection can be associated with a lower nestling body weight and survival, while under rural environments *Leucocytozoon* seemed to have no effect on nestling blue tit (see Chapter 5). This may explain the contradictory results of studies concerning the impact on haemosporidian parasite on host fitness. While negative effects of other avian malaria parasites have been reported for adult birds and nestlings (e.g. Dawson and Bortolotti, 2000; Merino et al., 2000; Marzal et al., 2005; Marzal et al., 2008; Lachish et al., 2011; Sudyka et al., 2019), other studies reported moderate or no sign of avian malaria fitness effects in wild birds (e.g. Bennett et al., 1988; Ots and Hõrak, 1998; Kilpatrick et al., 2006). Thus, future studies should consider the environmental and nutritional condition of a given population before concluding whether or not parasite prevalence negatively affects host fitness-related traits.

The reason behind the observed negative association between *Leucocytozoon* infection and the urban nestlings' body weight and survival is not clear. I do not know whether this effect is due to blood pathogens or because of tissue damage during the exoerythrocytic stage of parasite development. It has also been suggested that blood stages of the parasites do not cause severe inflammatory response in adult great tit, but infection affects their health status via activation of T-cell and humoral immunity (Ots and Hõrak, 1998). Conversely, activation of the immune system at an early stage of life (i.e. nestling) may be critical because it could drive energy away from growth, which has detrimental consequences on birds' fitness-related traits. A future direction could be to test the exoerythrocytic stage of infection using tissue from the main organs of dead birds that we have already been collecting during both breeding seasons from nests. Tissue damage of main organs may be another reason behind the negative effect of acute infection on hosts' body condition and survival (Valkiūnas and Iezhova, 2017). Sampling dead birds for the exoerythrocytic stage of infection may also reveal more infected birds than we believed, if

infected in the prepatent period or parasites not yet circulated in the blood in a datable level of intensity.

In conclusion, my thesis has highlighted the complexities of the urban environment and the difficulty of examining the mechanisms behind the negative effect of urbanisation on avian fitness, either via correlational or experimental approaches in the field. My thesis has also presented a powerful tool to detect and identify acute infection of avian malaria parasites as well as pointed out a number of potential areas for future research. All in all, my results showed different patterns from year to year in the variation between the urban and rural populations of blue tit in a number of parameters I used to test my hypotheses, such as fCORT level, *Leucocytozoon* prevalence, and nestling body weight. As predicted, the inter-relationship between *Leucocytozoon* infection and blue tit fitness is evident in the urban but not the rural environment. However, in contrast to my prediction, I did not find a direct relationship between infection prevalence and fCORT level. The reason behind this lack of relationship might be because of the way I tested this relationship. In the statistical model, I used fCORT level per nest as a variable (feather samples per nest were pooled), and the proportion of infected birds included in the feather sampling per nest (depending on how many nestlings were included) as another variable. Interindividual variation in fCORT should be used in future studies to test the potential correlation between fCORT level and infection better. More importantly, before generalising the effect of urban-related stressors as well as acute *Leucocytozoon* infection on blue tit fitness, data from more years and sites per habitat is needed. I used two years' data only, and I focused on a specific geographical area and considered urbanisation as a binomial factor, which may sometimes mask the gradual effect of urbanisation on some environmental or biological traits of hosts. Future studies should precisely quantify the degree of urbanisation in order to test whether the effect of urbanisation on patterns of tested traits (e.g. parasite prevalence or/and CORT levels) vary according to the degree of urbanisation.

List of References

Adler, P.H. and McCreadie, J.W. 2019. Black Flies (Simuliidae) *In*: D. Gary, Mullen and Lance, ed. *Medical and Veterinary Entomology* [Online]. Academic Press, pp.237–259. [Accessed 24 May 2019]. Available from: https://www.sciencedirect.com/science/article/pii/B9780128140437000145.

Aidoo, M., Terlouw, D.J., Kolczak, M.S., McElroy, P.D., ter Kuile, F.O., Kariuki, S., Nahlen, B.L., Lal, A.A. and Udhayakumar, V. 2002. Protective effects of the sickle cell gene against malaria morbidity and mortality. *Lancet (London, England)*. **359**(9314), pp.1311–2.

Albert, A. and Anderson, J.A. 1984. On the Existence of Maximum Likelihood Estimates in Logistic Regression Models. *Biometrika*. **71**(1), pp.1–10.

Almasi, B., Rettenbacher, S., Müller, C., Brill, S., Wagner, H. and Jenni, L. 2012. Maternal corticosterone is transferred into the egg yolk. *General and Comparative Endocrinology*. **178**(1), pp.139–144.

Alonso-Alvarez, C. and Tella, J.L. 2000. Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. *Canadian Journal of Zoology*. **79**(1), pp.101–105.

Altshuler, M.L. 2006. *PCR troubleshooting : the essential guide*. Caister Academic Press, UK.

Ardia, D.R. 2005. Tree swallows trade off immune function and reproductive effort differently across their range. *Ecology*. **86**(8), pp.2040–2046.

Argilla, L.S., Howe, L., Gartrell, B.D. and Alley, M.R. 2013. High prevalence of Leucocytozoon spp. in the endangered yellow-eyed penguin (Megadyptes antipodes) in the sub-Antarctic regions of New Zealand. *Parasitology*. **140**(5), pp.672–682.

Aronson, M.F.J., Sorte, F. a La, Nilon, C.H., Katti, M., Goddard, M. a, Lepczyk, C. a, Warren, P.S., Williams, N.S.G., Cilliers, S., Clarkson, B., Dobbs, C., Dolan, R., Hedblom, M., Klotz, S., Kooijmans, J.L., Macgregor-fors, I., Mcdonnell, M., Mörtberg, U., Pysek, P., Siebert, S., Werner, P. and Winter, M. 2014. A global analysis of the impacts of urbanization on bird and plant diversity reveals key anthropogenic drivers A global analysis of the impacts of urbanization on bird and plant diversity reveals key anthropogenic drivers. *Royal Society*. **281**(20133330), pp.1–8.

Ashford, R.W., Green, E.E., Holmes, P.R. and Lucas, A.J. 1991. Leucocytozoon toddi in British sparrowhawks Accipiter nisus : patterns of infection in nestlings. *Journal of Natural History*. **25**(2), pp.269–277.

Atema, E., Oers, K. van and Verhulst, S. 2013. GAPDH as a Control Gene to Estimate Genome Copy Number in Great Tits, with Cross-Amplification in Blue Tits. *Ardea*. **101**(1), pp.49–54.

Atkinson, C.T. and Charles van Riper, I. 1991. Pathogenicity and epizootiology of avian haematozoa: Plasmodium, Haemoproteus, and Leucocytozoon *In*: *Bird-parasite interactions: Ecology, evolution, and behavior* [Online]. London: Oxford University Press, pp.19–48. [Accessed 7 July 2019]. Available from: https://pubs.er.usgs.gov/publication/70126458.

Atkinson, C.T., Dusek, R.J., Woods, K.L. and Iko, W.M. 2000. Pathogenicity of avian malaria in experimentally-infected hawaii amakihi. *Journal of Wildlife Diseases*. **36**(2), pp.197–201.

Atkinson, C.T. and Samuel, M.D. 2010. Avian malaria Plasmodium relictum in native Hawaiian forest birds: epizootiology and demographic impacts on ʻapapane Himatione sanguinea. *Journal of Avian Biology*. **41**(4), pp.357–366.

Atwell, J.W., Cardoso, G.C., Whittaker, D.J., Campbell-Nelson, S., Robertson, K.W. and Ketterson, E.D. 2012. Boldness behavior and stress physiology in a novel urban environment suggest rapid correlated evolutionary adaptation. *Behavioral Ecology*. **23**(5), pp.960–969.

Avery, M.I. and Krebs, J.R. 1984. Temperature and foraging success of Great Tits Parus major hunting for spiders. *Ibis*. **126**(1), pp.33–38.

Badás, E.P., Martínez, J., Rivero-de Aguilar, J., Ponce, C., Stevens, M. and Merino, S. 2018. Colour change in a structural ornament is related to individual quality, parasites and mating patterns in the blue tit. *The Science of Nature*. **105**(17), pp.1–12.

Bailly, J., Scheifler, R., Belvalette, M., Garnier, S., Boissier, E., Clément-Demange, V.-A., Gète, M., Leblond, M., Pasteur, B., Piget, Q., Sage, M. and Faivre, B. 2016. Negative impact of urban habitat on immunity in the great tit Parus major. *Oecologia*. **182**(4), pp.1053–1062.

Bańbura, J., Bańbura, M., Kaliński, A., Skwarska, J., Słomczyński, R., Wawrzyniak, J. and

Zieliński, P. 2007. Habitat and year-to-year variation in haemoglobin concentration in nestling blue tits Cyanistes caeruleus. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. **148**(3), pp.572–577.

Barber, J.R., Crooks, K.R. and Fristrup, K.M. 2010. The costs of chronic noise exposure for terrestrial organisms. *Trends in ecology & evolution*. **25**(3), pp.180–9.

Bates, D., Mächler, M., Bolker, B. and Walker, S. 2014. Fitting Linear Mixed-Effects Models using lme4. *Journal of Statistical Software*. **76**(1), pp.1–51.

Batten, L.A. 1978. The seasonal distribution of recoveries and causes of Blackbird mortality. *Bird Study*. **25**(1), pp.23–31.

Beadell, J.S., Ishtiaq, F., Covas, R., Melo, M., Warren, B.H., Atkinson, C.T., Bensch, S., Graves, G.R., Jhala, Y. V, Peirce, M.A., Rahmani, A.R., Fonseca, D.M. and Fleischer, R.C. 2006. Global phylogeographic limits of Hawaii's avian malaria. *Proceedings. Biological sciences*. **273**(1604), pp.2935–44.

Beaugeard, E., Brischoux, F., Henry, P.-Y., Parenteau, C., Trouvé, C. and Angelier, F. 2019. Does urbanization cause stress in wild birds during development? Insights from feather corticosterone levels in juvenile house sparrows (*Passer domesticus*). *Ecology and Evolution*. **9**(1), pp.640–652.

Becker, D.J., Streicker, D.G. and Altizer, S. 2015. Linking anthropogenic resources to wildlife-pathogen dynamics: a review and meta-analysis. *Ecology Letters*. **18**(5), pp.483–495.

Belden, L.K., Moore, I.T., Wingfield, J.C. and Blaustein, A.R. n.d. Corticosterone and Growth in Pacific Treefrog (Hyla regilla) Tadpoles. *Copeia*. **2005**, pp.424–430.

Bell, J.A., Weckstein, J.D., Fecchio, A. and Tkach, V. V. 2015. A new real-time PCR protocol for detection of avian haemosporidians. *Parasites & Vectors*. **8**(1), p.383.

Benítez-López, A., Alkemade, R. and Verweij, P.A. 2010. The impacts of roads and other infrastructure on mammal and bird populations: A meta-analysis. *Biological Conservation*. **143**(6), pp.1307–1316.

Bennett, G., Peirce, M., Ashford, R., Bennett, G.F., Peircet, M.A. and Ashford, R.W. 1993. Avian Haematozoa: mortality and pathogenicity. *Journal of Natural History*. **27**(5), pp.993–1001.

Bennett, G.F., Caines, J.R. and Bishop, M.A. 1988. Influence of blood parasites on the body mass of passeriform birds. *Journal of Wildlife Diseases*. **24**(2), pp.339–343.

Bensch, S., Hellgren, O. and Perez-Tris, J. 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Molecular Ecology Resources*. **9**(5), pp.1353–1358.

Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H. and Pinheiro, R.T. 2000. Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. *Proceedings. Biological sciences*. **267**(1452), pp.1583–9.

Bentz, S., RigaudI, T., Barroca, M., Martin-laurent, F., Bru, D., Moreau, J. and Faiver, B. 2006. Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (Turdus merula) populations: value of PCR-RFLP and quantitative PCR. *Parasitology*. **133**(06), p.685.

Bernotienė, R., Palinauskas, V., Iezhova, T., Murauskaitė, D. and Valkiūnas, G. 2016. Avian haemosporidian parasites (Haemosporida): A comparative analysis of different polymerase chain reaction assays in detection of mixed infections. *Experimental Parasitology*. **163**, pp.31–37.

Bichet, C., Scheifler, R., Cœurdassier, M., Julliard, R., Sorci, G. and Loiseau, C. 2013. Urbanization, trace metal pollution, and malaria prevalence in the house sparrow. *PloS one*. **8**(1), p.e53866.

Biedrzycka, A., Migalska, M., Bielański, W., Biela, W. and Bielański, W. 2015. A quantitative PCR protocol for detecting specific Haemoproteus lineages: molecular characterization of blood parasites in a Sedge Warbler population from southern Poland. *Journal of Ornithology*. **156**(1), pp.201–208.

Bize, P., Stocker, A., Jenni-Eiermann, S., Gasparini, J. and Roulin, A. 2010. Sudden weather deterioration but not brood size affects baseline corticosterone levels in nestling Alpine swifts. *Hormones and Behavior*. **58**(4), pp.591–598.

Blas, J., Bortolotti, G.R., Tella, J.L., Baos, R. and Marchant, T.A. 2007. Stress response during development predicts fitness in a wild, long lived vertebrate. *Proc Natl Acad Sci USA*. **104**,

pp.8880–8884.
Blount, J., NB, M., TR, B. and PF, S. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science*. **300**, pp.125–127.
Boal, C.W., Mannan, R.W. and Hudelson, K.S. 1998. Trichomoniasis in cooper's hawks from arizona. *Journal of Wildlife Diseases*. **34**(3), pp.590–593.
Bonier, F. 2012. Hormones in the city: Endocrine ecology of urban birds. *Hormones and Behavior*. **61**(5), pp.763–772.
Book, M., Lehmann, L.E., Zhang, X.H. and Stueber, F. 2009. Real-Time PCR in Microbiology: From Diagnosis to Characterization *In*: *Management of Sepsis: The PIRO Approach* [Online]. Berlin, Heidelberg: Springer Berlin Heidelberg, pp.65–85. [Accessed 14 November 2018]. Available from: http://link.springer.com/10.1007/978-3-642-00479-7_6.
Boonstra, R. 2013. Reality as the leading cause of stress: rethinking the impact of chronic stress in nature C. Fox, ed. *Functional Ecology*. **27**(1), pp.11–23.
Boonstra, R., Hik, D., Singleton, G.R. and Tinnikov, A. 1998. The Impact of Predator-Induced Stress on the Snowshoe Hare Cycle. *Ecological Monographs*. **68**(3), p.371.
Bortolotti, G.R., Marchant, T., Blas, J. and Cabezas, S. 2009. Tracking stress: localisation, deposition and stability of corticosterone in feathers. *Journal of Experimental Biology*. **212**(10), pp.1477–1482.
Bortolotti, G.R., Marchant, T.A., Blas, J. and German, T. 2008. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. *Functional Ecology*. **22**(3), pp.494–500.
Bosholn, M., Anciães, M., Gil, D., Weckstein, J.D., Dispoto, J.H. and Fecchio, A. 2019. Individual variation in feather corticosterone levels and its influence on haemosporidian infection in a Neotropical bird. *Ibis*., doi: 10.1111/ibi.12709.
Bradley, C.A. and Altizer, S. 2007. Urbanization and the ecology of wildlife diseases. *Trends in Ecology & Evolution*. **22**(2), pp.95–102.
Brahmia, Z., Scheifler, R., Crini, N., Maas, S., Giraudoux, P. and Benyacoub, S. 2013. Breeding performance of blue tits (Cyanistes cæruleus ultramarinus) in relation to lead pollution and nest failure rates in rural, intermediate, and urban sites in Algeria. *Environmental pollution (Barking, Essex : 1987)*. **174**, pp.171–8.
Bray, R.S. 1957. Studies on the exo-erythrocytic cycle in the genus Plasmodium. *The American journal of tropical medicine and hygiene*. **7**(5), pp.575–576.
Brearley, G., Rhodes, J., Bradley, A., Baxter, G., Seabrook, L., Lunney, D., Liu, Y. and Mcalpine, C. 2012. Wildlife disease prevalence in human-modified landscapes. *Biological Reviews*. **88**, pp.427–442.
Brewer, J.H., O'Reilly, K.M. and Buck, C.L. 2010. Effect of nestling status and brood size on concentration of corticosterone of free-living kittiwake chicks. *General and Comparative Endocrinology*. **166**(1), pp.19–24.
Brommer, J.E. 2004. Immunocompetence and its costs during development: an experimental study in blue tit nestlings. *Proceedings. Biological sciences / The Royal Society*. **271 Suppl**, pp.S110–S113.
de Bruijn, R., Reed, J.M. and Romero, L.M. 2017. Chronic repeated exposure to weather-related stimuli elicits few symptoms of chronic stress in captive molting and non-molting European starlings (*Sturnus vulgaris*). *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*. **327**(8), pp.493–503.
Burger, J. 1991. Human activity influence and diurnal and nocturnal foraging of Sanderlings (*Calidris alba*). *The Condor*. **93**(April 1990), pp.259–265.
Busch, D.S. and Hayward, L.S. 2009. Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. *Biological Conservation*. **142**(12), pp.2844–2853.
Cain, D.W. and Cidlowski, J.A. 2017. Immune regulation by glucocorticoids. *Nature Reviews Immunology*. **17**(4), pp.233–247.
Calegaro-Marques, C. and Amato, S.B. 2014. Urbanization Breaks Up Host-Parasite Interactions: A Case Study on Parasite Community Ecology of Rufous-Bellied Thrushes (Turdus rufiventris) along a Rural-Urban Gradient P. Quillfeldt, ed. *PLoS ONE*. **9**(7), p.e103144.
Calero-Riestra, M., García, J.T., Calero-riestra, M. and García, J.T. 2016. Sex-dependent differences in avian malaria prevalence and consequences of infections on nestling growth

and adult condition in the Tawny pipit, Anthus campestris. *Malaria Journal*. **15**(178), pp.1–11.

Capilla-Lasheras, P., Dominoni, D.M., Babayan, S.A., O'Shaughnessy, P.J., Mladenova, M., Woodford, L., Pollock, C.J., Barr, T., Baldini, F. and Helm, B. 2017. Elevated Immune Gene Expression Is Associated with Poor Reproductive Success of Urban Blue Tits. *Frontiers in Ecology and Evolution*. **5**(64), doi.org/10.3389/fevo.2017.00064.

Cardona, C.J., Ihejirika, A. and McClellan, L. 2002. Haemoproteus lophortyx infection in bobwhite quail. *Avian diseases*. **46**(1), pp.249–55.

Carere, C. and Balthazart, J. 2007. Sexual versus individual differentiation: the controversial role of avian maternal hormones. *Trends in Endocrinology & Metabolism*. **18**(2), pp.73–80.

Cellier-Holzem, E., Esparza-Salas, R., Garnier, S. and Sorci, G. 2010. Effect of repeated exposure to Plasmodium relictum (lineage SGS1) on infection dynamics in domestic canaries. *International Journal for Parasitology*. **40**(12), pp.1447–1453.

Chace, J.F. and Walsh, J.J. 2006. Urban effects on native avifauna: a review. *Landscape and Urban Planning*. **74**(1), pp.46–69.

Chamberlain, D.E., Cannon, A.R., Toms, M.P., Leech, D.I., Hatchwell, B.J. and Gaston, K.J. 2009. Avian productivity in urban landscapes: a review and meta-analysis. *Ibis*. **151**(1), pp.1–18.

Chamberlain, D.E., Robb, G.N., McDonald, R.A., Chamberlain, D.E. and Bearhop, S. 2008. Food for thought: supplementary feeding as a driver of ecological change in avian populations. *Frontiers in Ecology and the Environment*. **6**(9), pp.476–484.

Christe, P., Glaizot, O., Strepparava, N., Devevey, G. and Fumagalli, L. 2012. Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. *Proceedings of the Royal Society B: Biological Sciences*. **279**(1731), pp.1142–1149.

Chua, K.B., Goh, K.J., Wong, K.T., Kamarulzaman, A., Tan, P.S.K., Ksiazek, T.G., Zaki, S.R., Paul, G., Lam, S.K. and Tan, C.T. 1999. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. *The Lancet*. **354**(9186), pp.1257–1259.

Ciloglu, A., Ellis, V.A., Bernotienė, R., Valkiūnas, G. and Bensch, S. 2019. A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites. *Parasitology Research*. **118**(1), pp.191–201.

Clark, N.J., Clegg, S.M. and Lima, M.R. 2014. A review of global diversity in avian haemosporidians (Plasmodium and Haemoproteus: Haemosporida): new insights from molecular data. *International Journal for Parasitology*. **44**(5), pp.329–338.

Clergeau, M. and 2001. Settlement of breeding European Starlings in urban areas_ Importance of lawns vs.

Coffin, A.W. 2007. From roadkill to road ecology: A review of the ecological effects of roads. *Journal of Transport Geography*. **15**(5), pp.396–406.

Collins, J.P., Kinzig, A., Grimm, N.B., Fagan, W.F., Hope, D., Wu, J. and Borer, E.T. 2000. A New Urban Ecology: Modeling human communities as integral parts of ecosystems poses special problems for the development and testing of ecological theory. *American Scientist*. **88**, pp.416–425.

Cornet, S., Bichet, C., Larcombe, S., Faivre, B. and Sorci, G. 2014. Impact of host nutritional status on infection dynamics and parasite virulence in a bird-malaria system. D. Ardia, ed. *The Journal of animal ecology*. **83**(1), pp.256–65.

Cosgrove, C.L., Knowles, S.C.L., Day, K.P. and Sheldon, B.C. 2006. No Evidence for Avian Malaria Infection during the Nestling Phase in a Passerine Bird. *Source: The Journal of Parasitology*. **92**(926), pp.1302–1304.

Cote, I.M. and Poulinb, R. 1995. Parasitism and group size in social animals: a meta-analysis. *Behavioral Ecology*. **6**(2), pp.159–165.

Crespi, E.J., Williams, T.D., Jessop, T.S. and Delehanty, B. 2012. The ecology of stress life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals? *Functional Ecology*. **27**, pp.93–106.

Crofton, H.D. 1971. A quantitative approach to parasitism. *Parasitology*. **62**(2), pp.179–193.

Dadam, D., Robinson, R.A., Clements, A., Peach, W.J., Bennett, M., Rowcliffe, J.M. and Cunningham, A.A. 2019. Avian malaria-mediated population decline of a widespread iconic bird species. *Royal Society Open Science*. **6**(7), c4566323.

Dauwe, T., Bervoets, L., Blust, R., Pinxten, R. and Eens, M. 2000. Can excrement and feathers of

nestling songbirds be used as biomonitors for heavy metal pollution? *Archives of environmental contamination and toxicology*. **39**(4), pp.541–6.

Dawson, R.D. and Bortolotti, G.R. 2000. Effects of hematozoan parasites on condition and return rates of american kestrels. *The Auk*. **117**(2), pp.373–380.

Deviche, P., Fokidis, H.B., Lerbour, B. and Greiner, E. 2010. Blood parasitaemia in a high latitude flexible breeder, the white-winged crossbill, Loxia leucoptera: contribution of seasonal relapse versus new inoculations. *Parasitology*. **137**(2), pp.261–273.

Docile, T.N., Figueiró, R., Gil-Azevedo, L.H. and Nessimian, J.L. 2015. Water pollution and distribution of the black fly (Diptera: Simuliidae) in the Atlantic Forest, Brazil. *Revista de biologia tropical*. **63**(3), pp.683–93.

Dominoni, D., Quetting, M. and Partecke, J. 2013. Artificial light at night advances avian reproductive physiology. *Proceedings. Biological sciences / The Royal Society*. **280**(1756), pp.1–8.

Drent, R.H. and Daan, S. 1980. The Prudent Parent: Energetic Adjustments in Avian Breeding 1). *Ardea*. **38**–**90**(1–2), pp.225–252.

Dubiec, A., Cichoń, M. and Deptuch, K. 2006. Sex-specific development of cell-mediated immunity under experimentally altered rearing conditions in blue tit nestlings. *Proceedings. Biological sciences*. **273**(1595), pp.1759–64.

Dunn, J.C., Stockdale, J.E., Bradford, E.L., Mccubbin, A., Morris, A.J., Grice, P. V, Goodman, S.J. and Hamer, K.C. 2017. High rates of infection by blood parasites during the nestling phase in UK Columbids with notes on ecological associations. *Parasitology*. **144**, pp.622–628.

Eeva, T., Hasselquist, D., Langefors, Å., Tummeleht, L., Nikinmaa, M. and Ilmonen, P. 2005. Pollution related effects on immune function and stress in a free-living population of pied flycatcher Ficedula hypoleuca. *Journal of Avian Biology*. **36**(5), pp.405–412.

Eeva, T. and Lehikoinen, E. 2013. Density effect on great tit (Parus major) clutch size intensifies in a polluted environment. *Oecologia*. **173**(4), pp.1661–1668.

Eeva, T., Lehikoinen, E. and Nikinmaa, M. 2003. Pollution-induced nutritional stress in birds: an experimental study of direct and indirect effects. *Ecological Applications*. **13**(5), pp.1242–1249.

Eeva, T., Rainio, M., Berglund, Å., Kanerva, M., Stauffer, J., Stöwe, M. and Ruuskanen, S. 2014. Experimental manipulation of dietary lead levels in great tit nestlings: limited effects on growth, physiology and survival. *Ecotoxicology*. **23**(5), pp.914–28.

Eisenbeis, G. and Hänel, A. 2009. Light pollution and the impact of artificial night lighting on insects *In*: J. H. B. Mark J. McDonnell, Amy K. Hahs, ed. *Ecology of cities and Towns* [Online]. Cambridge University Press, pp.243–263. Available from: http://wikinight.free.fr/wp-content/uploads/anpcen/Spectre lampes vs Environnement nocturne v2/Impact biodiversite/Chapter 15 Eisenbeis and Hanel with pictures_final2.pdf.

Elliott, W.H. and Elliott, D.C. 2009. *Biochemistry and molecular biology* fourth. Oxford University Press.

Evans, Karl L., Gaston, K.J., Sharp, S.P., McGowan, A., Simeoni, M., Hatchwell, B.J., Evans, K.L., Gaston, K.J., Sharp, S.P., McGowan, A., Simeoni, M. and Hatchwell, B.J. 2009. Effects of urbanisation on disease prevalence and age structure in blackbird Turdus merula populations. *Oikos*. **118**(5), pp.774–782.

Evans, KARL L., Newson, S.E. and Gaston, K.J. 2009. Habitat influences on urban avian assemblages. *Ibis*. **151**(1), pp.19–39.

Faeth, S.H., Bang, C. and Saari, S. 2011. Urban biodiversity: patterns and mechanisms. *Annals of the New York Academy of Sciences*. **1223**(1), pp.69–81.

Fallon, S.M., Ricklefs, R.E., Swanson, B.L. and Bermingham, E. 2003. Detecting avian malaria: an improved polymerase chain reaction. *Journal of Parasitology*. **89**(5), pp.1044–1047.

Farcas, G.A., Zhong, K.J.Y., Mazzulli, T. and Kain, K.C. 2004. Evaluation of the RealArt Malaria LC Real-Time PCR Assay for Malaria Diagnosis. *Journal of clinical microbiology*. **42**(2), pp.636–638.

Farnsworth, M.L., Wolfe, L.L., Hobbs, N.T., Burnham, K.P., Williams, E.S., Theobald, D.M., Conner, M.M. and Miller, M.W. 2005. Dna quality and accuracy of avian malaria pcr diagnostics: a review. *Ecological Applications*. **15**(1), pp.119–126.

Fernández-Juricic, E. and Tellería, J.L. 2000. Effects of human disturbance on spatial and temporal feeding patterns of Blackbird Turdus merula in urban parks in Madrid, Spain. *Bird Study*.

47(1), pp.13–21.
Fischer, C.P., Rao, R. and Romero, L.M. 2017. Exogenous and endogenous corticosterone in feathers. *Journal of Avian Biology*. **48**, pp.1301–1309.
Fokidis, H., Greiner, E.C. and Deviche, P. 2008. Interspecific variation in avian blood parasites and haematology associated with urbanization in a desert habitat. *Journal of Avian Biology*. **39**(3), pp.300–310.
Fokidis, H.B., Orchinik, M. and Deviche, P. 2009. Corticosterone and corticosteroid binding globulin in birds: relation to urbanization in a desert city. *General and comparative endocrinology*. **160**(3), pp.259–70.
Foltz, S.L., Davis, J.E., Battle, K.E., Greene, V.W., Laing, B.T., Rock, R.P., Ross, A.E., Tallant, J.A., Vega, R.C. and Moore, I.T. 2015. Across time and space: Effects of urbanization on corticosterone and body condition vary over multiple years in song sparrows (Melospiza melodia). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*. **323**(2), pp.109–120.
Forman, R.T.T., Reineking, B. and Hersperger, A.M. 2002. Road traffic and nearby grassland bird patterns in a suburbanizing landscape. *Environmental Management*. **29**(6), pp.782–800.
Fox, J. and Weisberg, S. 2011. *An {R} Companion to Applied Regression* [Online] Second Edi. Sage. [Accessed 25 March 2019]. Available from: https://cran.r-project.org/web/packages/car/citation.html.
Francis, C.D., Ortega, C.P. and Cruz, A. 2011. Vocal frequency change reflects different responses to anthropogenic noise in two suboscine tyrant flycatchers. *Proceedings. Biological sciences / The Royal Society*. **278**(1714), pp.2025–2031.
Freed, L.A. and Cann, R.L. 2006. Dna quality and accuracy of avian malaria pcr diagnostics: a review. *Condor*. **108**(2), pp.459–473.
French, S.S., Fokidis, H.B. and Moore, M.C. 2008. Variation in stress and innate immunity in the tree lizard (Urosaurus ornatus) across an urban-rural gradient. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology*. **178**(8), pp.997–1005.
Frevert, U., Späth, G.F. and Yee, H. 2008. Exoerythrocytic development of Plasmodium gallinaceum in the White Leghorn chicken. *International journal for parasitology*. **38**(6), pp.655–72.
Ganz, K., Jenny, D., Kraemer, T., Jenni, L. and Jenni-Eiermann, S. 2018. Prospects and pitfalls of using feathers as a temporal archive of stress events and environmental pollutants: a review and case study. *Journal of Ornithology*., pp.1–13.
Garnham, P.C.C. 1966. Malaria Parasites and other Haemosporidia. *The American Journal of Tropical Medicine and Hygiene*. **16**(4), pp.561–563.
Gibbs, S.E.J., Wimberly, M.C., Madden, M., Masour, J., Yabsley, M.J. and Stallknecht, D.E. 2006. Factors Affecting the Geographic Distribution of West Nile Virus in Georgia, USA: 2002–2004. *Vector-Borne and Zoonotic Diseases*. **6**(1), pp.73–82.
Giraudeau, M. and McGraw, K.J. 2014. Physiological correlates of urbanization in a desert songbird. *Integrative and comparative biology*. **54**(4), pp.622–32.
Githeko, a K., Lindsay, S.W., Confalonieri, U.E. and Patz, J. a 2000. Climate change and vector-borne diseases: a regional analysis. *Bulletin of the World Health Organization*. **78**(9), pp.1136–1147.
Glądalski, M., Bańbura, M., Kaliński, A., Markowski, M., Skwarska, J., Wawrzyniak, J., Zieliński, P. and Bańbura, J. 2016. Spatial variation in haemoglobin concentration of nestling Blue Tits (Cyanistes caeruleus): a long-term perspective. *Journal of Ornithology*. **157**(2), pp.591–598.
Glasel, J.A. 1995. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *BioTechniques*. **18**(1), pp.62–3.
Godfrey, R.D., Fedynich, A.M. and Pence, D.B. 1987. Quantification of hematozoa in blood smears. *Journal of wildlife diseases*. **23**(4), pp.558–65.
Goerlich, V.C., Nätt, D., Elfwing, M., Macdonald, B. and Jensen, P. 2012. Transgenerational effects of early experience on behavioral, hormonal and gene expression responses to acute stress in the precocial chicken. *Hormones and Behavior*. **61**(5), pp.711–718.
Grace, J.K., Froud, L., Meillère, A. and Angelier, F. 2017. House sparrows mitigate growth effects of post-natal glucocorticoid exposure at the expense of longevity. *General and comparative endocrinology*. **253**, pp.1–12.
Graham, M.H. 2003. Confronting Multicollinearity in Ecological Multiple Regression. *Source:*

Ecology. **84**(11), pp.2809–2815.
Greggor, A.L., Spencer, K.A., Clayton, N.S. and Thornton, A. 2017. Wild jackdaws' reproductive success and their offspring's stress hormones are connected to provisioning rate and brood size, not to parental neophobia. *General and Comparative Endocrinology*. **243**, pp.70–77.
Gregoire, A., Faivre, B., Heeb, P. and Cezilly, F. 2002. A comparison of infestation patterns by Ixodes ticks in urban and rural populations of the Common Blackbird Turdus merula. *Ibis*. **144**(4), pp.640–645.
Greño, J.L., Belda, E.J. and Barba, E. 2008. Influence of temperatures during the nestling period on post-fledging survival of great tit Parus major in a Mediterranean habitat. *Journal of Avian Biology*. **39**(1), pp.41–49.
Griffiths, R., Double, M.C., Orr, K. and Dawson, R.J. 1998. A DNA test to sex most birds. *Molecular ecology*. **7**(8), pp.1071–5.
Grimm, N.B., Faeth, S.H., Golubiewski, N.E., Redman, C.L., Wu, J., Bai, X. and Briggs, J.M. 2008. Global change and the ecology of cities. *Science (New York, N.Y.)*. **319**(5864), pp.756–60.
Hall, A.J., Yee, L.J. and Thomas, S.L. 2002. Life course epidemiology and infectious diseases. *International Journal of Epidemiology*. **31**(2), pp.300–301.
Han, J. 2006. Molecular Differential Diagnoses of Infectious Diseases: Is the Future Now? *In*: *Advanced Techniques in Diagnostic Microbiology* [Online]. Boston, MA: Springer US, pp.472–504. [Accessed 29 August 2019]. Available from: http://link.springer.com/10.1007/0-387-32892-0_27.
Harrison, T.J.E., Smith, J.A., Martin, G.R., Chamberlain, D.E., Bearhop, S., Robb, G.N. and Reynolds, S.J. 2010. Does food supplementation really enhance productivity of breeding birds? *Oecologia*. **164**(2), pp.311–20.
Hassell, J.M., Begon, M., Ward, M.J. and Fèvre, E.M. 2017. Urbanization and Disease Emergence: Dynamics at the Wildlife–Livestock–Human Interface. *Trends in Ecology & Evolution*. **32**(1), pp.55–67.
Hawley, D.M., Lindström, K. and Wikelski, M. 2006. Experimentally increased social competition compromises humoral immune responses in house finches. *Hormones and behavior*. **49**(4), pp.417–24.
Hayward, L.S. and Wingfield, J.C. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. *General and Comparative Endocrinology*. **135**(3), pp.365–371.
Heiss, R.S., Clark, A.B. and McGowan, K.J. 2009. Growth and nutritional state of American Crow nestlings vary between urban and rural habitats. *Ecological Applications*. **19**(4), pp.829–839.
Hellgren, O., Waldenström, J. and Bensch, S. 2004. A new pcr assay for simultaneous studies of leucocytozoon, plasmodium, and haemoproteus from avian blood. *Journal of Parasitology*. **90**(4), pp.797–802.
Henderson, L.J., Evans, N.P., Heidinger, B.J., Herborn, K.A. and Arnold, K.E. 2017. Do glucocorticoids predict fitness? Linking environmental conditions, corticosterone and reproductive success in the blue tit, Cyanistes caeruleus. *Royal Society Open Science.* **4**(170875), pp.1–11.
Herman, C.M., Barrow, J.H. and Tarshis, I.B. 1975. Leucocytozoonosis in Canada geese at the Seney National Wildlife Refuge. *Journal of wildlife diseases*. **11**(3), pp.404–11.
Hermsen, C.C., Telgt, D.S.C., Linders, E.H.P., Van De Locht, L.A.T.F., Eling, W.M.C., Mensink, J.B.M. and Sauerwein, R.W. 2001. Detection of Plasmodium falciparum malaria parasites in vivo by real-time quantitative PCR. *Molecular & Biochemical Parasitology*. **118**, pp.247–251.
Herring, G., Cook, M.I., Gawlik, D.E. and Call, E.M. 2011. Food availability is expressed through physiological stress indicators in nestling white ibis: a food supplementation experiment. *Functional Ecology*. **25**, pp.682–690.
van Heugten, E., Coffey, M.T. and Spears, J.W. 2007. Effects of immune challenge, dietary energy density, and source of energy on performance and immunity in weanling pigs. *Journal of animal science*. **74**(10), pp.2431–2440.
Hofer, C., Gallagher, F.J. and Holzapfel, C. 2010. Metal accumulation and performance of nestlings of passerine bird species at an urban brownfield site. *Environmental pollution (Barking, Essex : 1987)*. **158**(5), pp.1207–13.

Hõrak, P., Tegelmann, L., Ots, I. and Møller, A.P. 1999. Immune function and survival of great tit nestlings in relation to growth conditions. *Oecologia*. **121**(3), pp.316–322.

Howell, T.R. 1964. Notes on incubation and nestling temperatures and behavior of captive owls. *Wilson Bulletin*. **76**(1), pp.28–36.

Ibáñez-álamo, J.D. and Soler, M. 2010. Does urbanization affect selective pressures and life-history strategies in the common blackbird (Turdus merula L.)? *Biological Journal of the Linnean Society*. **101**(4), pp.759–766.

Ilgūnas, M., Bukauskaitė, D., Palinauskas, V., Iezhova, T., Fragner, K., Platonova, E., Weissenböck, H. and Valkiūnas, G. 2019. Patterns of Plasmodium homocircumflexum virulence in experimentally infected passerine birds. *Malaria Journal*. **18**(1), pp.1–14.

Ilgūnas, M., Bukauskaitė, D., Palinauskas, V., Iezhova, T.A., Dinhopl, N., Nedorost, N., Weissenbacher-Lang, C., Weissenböck, H. and Valkiūnas, G. 2016. Mortality and pathology in birds due to Plasmodium (Giovannolaia) homocircumflexum infection, with emphasis on the exoerythrocytic development of avian malaria parasites. *Malaria journal*. **15**(1), p.256.

Ilmonen, P., Taarna, T. and Hasselquist, D. 2000. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proceedings. Biological sciences / The Royal Society*. **267**(January), pp.665–670.

Isaksson, C. 2015. Urbanization, oxidative stress and inflammation: a question of evolving, acclimatizing or coping with urban environmental stress A. Hahs, ed. *Functional Ecology*. **29**(7), pp.913–923.

Ishtiaq, F., Rao, M., Huang, X. and Bensch, S. 2017. Estimating prevalence of avian haemosporidians in natural populations: a comparative study on screening protocols. *Parasites & Vectors*. **10**(127), pp.1–10.

Jacquin, L., Récapet, C., Prévot-Julliard, a. C., Leboucher, G., Lenouvel, P., Erin, N., Corbel, H., Frantz, a. and Gasparini, J. 2013. A potential role for parasites in the maintenance of color polymorphism in urban birds. *Oecologia*. **173**(3), pp.1089–1099.

Janssens, E., Dauwe, T., Pinxten, R., Bervoets, L., Blust, R. and Eens, M. 2003. Effects of heavy metal exposure on the condition and health of nestlings of the great tit (Parus major), a small songbird species. *Environmental Pollution*. **126**(2), pp.267–274.

Jenni-Eiermann, S., Helfenstein, F., Vallat, A., Glauser, G. and Jenni, L. 2015. Corticosterone: effects on feather quality and deposition into feathers D. Fisher, ed. *Methods in Ecology and Evolution*. **6**(2), pp.237–246.

Jia, T., Huang, X., Valkiūnas, G., Yang, M., Zheng, C., Pu, T., Zhang, Y., Dong, L., Suo, X. and Zhang, C. 2018. Malaria parasites and related haemosporidians cause mortality in cranes: a study on the parasites diversity, prevalence and distribution in Beijing Zoo. *Malaria Journal*. **17**(1), p.234.

Jiménez-Peñuela, J., Ferraguti, M., Martínez-de la Puente, J., Soriguer, R. and Figuerola, J. 2019. Urbanization and blood parasite infections affect the body condition of wild birds. *Science of The Total Environment*. **651**(Pt 2), pp.3015–3022.

Jimeno, B., Hau, M. and Verhulst, S. 2017. Strong association between corticosterone levels and temperature-dependent metabolic rate in individual zebra finches. *The Journal of Experimental Biology*. **220**(23), pp.4426–4431.

Joëls, M., Karst, H., Krugers, H.J. and Lucassen, P.J. 2007. Chronic stress: Implications for neuronal morphology, function and neurogenesis. *Frontiers in Neuroendocrinology*. **28**(2–3), pp.72–96.

Johns, D.W., Marchant, T.A., Fairhurst, G.D., Speakman, J.R. and Clark, R.G. 2018. Biomarker of burden: Feather corticosterone reflects energetic expenditure and allostatic overload in captive waterfowl T. Williams, ed. *Functional Ecology*. **32**(2), pp.345–357.

Jones, D.N. and Reynolds, S. 2008. Feeding birds in our towns and cities: a global research opportunity. *Journal of Avian Biology*. **39**(3), pp.265–271.

Kalnay, E. and Cai, M. 2003. Impact of urbanization and land-use change on climate. *Nature*. **423**(6939), pp.528–531.

Keesing, F., Brunner, J., Duerr, S., Killilea, M., Logiudice, K., Schmidt, K., Vuong, H. and Ostfeld, R.S. 2009. Hosts as ecological traps for the vector of Lyme disease. *Proceedings. Biological sciences / The Royal Society*. **276**(1675), pp.3911–9.

Kekkonen, J. 2011. Evolutionary and conservation biology of the Finnish house sparrow.

Kempenaers, B., Borgström, P., Loës, P., Schlicht, E. and Valcu, M. 2010. Artificial Night

Lighting Affects Dawn Song, Extra-Pair Siring Success, and Lay Date in Songbirds. *Current Biology*. **20**(19), pp.1735–1739.

Kernbach, M.E., Hall, R.J., Burkett-Cadena, N.D., Unnasch, T.R. and Martin, L.B. 2018. Dim light at night: physiological effects and ecological consequences for infectious disease. *Integrative and Comparative Biology*. **58**(5), pp.995–1007.

Killpack, T.L., Carrel Elijah and Karasov, W.H. 2015. Impacts of Short-Term Food Restriction on Immune Development in Altricial House Sparrow Nestlings. *Physiological and Biochemical Zoology*. **88**(2), pp.195–207.

Kilpatrick, A.M., LaPointe, D.A., Atkinson, C.T., Woodworth, B.L., Lease, J.K., Reiter, M.E. and Gross, K. 2006. Effects of Chronic Avian Malaria (Plasmodium Relictum) Infection on Reproductive Success of Hawaii Amakihi (Hemignathus Virens). *The Auk: Ornithological Advances*. **123**(3), pp.764–774.

Kitaysky, A., Piatt, J. and Wingfield, J. 2007. Stress hormones link food availability and population processes in seabirds. *Marine Ecology Progress Series*. **352**, pp.245–258.

Koren, L., Nakagawa, S., Burke, T., Soma, K.K., Wynne-Edwards, K.E. and Geffen, E. 2012. Non-breeding feather concentrations of testosterone, corticosterone and cortisol are associated with subsequent survival in wild house sparrows. *Proceedings. Biological sciences*. **279**(1733), pp.1560–6.

Krams, I.A., Suraka, V., Rantala, M.J., Sepp, T., Mierauskas, P., Vrublevska, J. and Krama, T. 2013. Acute infection of avian malaria impairs concentration of haemoglobin and survival in juvenile altricial birds. *Journal of Zoology*. **291**(1), pp.34–41.

Krungkrai, J. 2004. The multiple roles of the mitochondrion of the malarial parasite. *Parasitology*. **129**(Pt 5), pp.511–24.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. and Zoric, N. 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine*. **27**(2–3), pp.95–125.

Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*. **33**(7), pp.1870–1874.

Kutner, M.H., Nachtsheim, C. and Neter, J. 2004. *Applied linear regression models.* [Online]. McGraw-Hill/Irwin. [Accessed 26 April 2019]. Available from: https://biblio.co.uk/applied-linear-regression-by-michael-kutner-christopher/work/887181.

Lachish, S., Knowles, S.C.L., Alves, R., Sepil, I., Davies, A., Lee, S., Wood, M.J. and Sheldon, B.C. 2012. Spatial determinants of infection risk in a multi-species avian malaria system. *Ecography*. (36), pp.587–598.

Lachish, S., Knowles, S.C.L., Alves, R., Wood, M.J. and Sheldon, B.C. 2011. Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. *Journal of Animal Ecology*. **80**(6), pp.1196–1206.

Lack 1968. Ecological Adaptations Breeding Birds - AbeBooks. [Accessed 2 May 2018]. Available from: https://www.abebooks.co.uk/book-search/title/ecological-adaptations-breeding-birds/.

Lack, D.L. 1954. *The natural regulation of animal numbers* [Online]. Clarendon Press. [Accessed 30 December 2015]. Available from: https://books.google.co.uk/books/about/The_natural_regulation_of_animal_numbers.html?id=GiAcAAAAMAAJ&pgis=1.

Lamb, C.T., Mowat, G., McLellan, B.N., Nielsen, S.E. and Boutin, S. 2017. Forbidden fruit: human settlement and abundant fruit create an ecological trap for an apex omnivore J.-M. Gaillard, ed. *Journal of Animal Ecology*. **86**(1), pp.55–65.

Larcombe, S., Bichet, C., Cornet, S., Faivre, B. and Sorci, G. 2013. Food availability and competition do not modulate the costs of Plasmodium infection in dominant male canaries. *Experimental Parasitology*. **135**(4), pp.708–714.

Lattin, Christine R, Reed, J Michael, Desrochers, D.W., Romero, L Michael, Lattin, C R, Reed, J M and Romero, L M 2011. Elevated corticosterone in feathers correlates with corticosterone-induced decreased feather quality: a validation study. *J. Avian Biol. J. Avian Biol. J. Avian Biol*. **42**, pp.247–252.

Lee, M.Y. 2018. Essential Oils as Repellents against Arthropods. *BioMed Research International*. **2018**, pp.1–9.

Legagneux, P. and Ducatez, S. 2013. European birds adjust their flight initiation distance to road speed limits. *Biology Letters*. **9**(August), p.20130417.

Lei, B., Amar, A., Koeslag, A., Gous, T. a. and Tate, G.J. 2013. Differential haemoparasite intensity between black sparrowhawk (Accipiter melanoleucus) morphs suggests an adaptive function for polymorphism. *PLoS ONE*. **8**(12), pp.2–10.

Lendvai, Á.Z., Giraudeau, M. and Chastel, O. 2007. Reproduction and modulation of the stress response: an experimental test in the house sparrow. *Proceedings of the Royal Society B: Biological Sciences*. **274**(1608), p.391.

Lessells, C.M., Ruuskanen, S. and Schwabl, H. 2016. Yolk steroids in great tit Parus major eggs: variation and covariation between hormones and with environmental and parental factors. *Behavioral ecology and sociobiology*. **70**, pp.843–856.

Levin, I.I., Zwiers, P., Deem, S.L., Geest, E.A., Higashiguchi, J.M., Iezhova, T.A., Jimenez-uzcategui, G., Kim, D.H., Morton, J.P., Perlut, N.G., Renfrew, R.B., Sari, E.H.R., Valkiunas, G. and Parker, P.G. 2013. Multiple Lineages of Avian Malaria Parasites (Plasmodium) in the Galapagos Islands and Evidence for Arrival via Migratory Birds. *Conservation Biology*. **27**(6), pp.1366–1377.

Lindström, K. and Lundström, J. 2000. Male greenfinches (Carduelis chloris) with brighter ornaments have higher virus infection clearance rate. *Behavioral Ecology and Sociobiology*. **48**(1), pp.44–51.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 C T Method. *Methods*. **25**, pp.402–408.

Lochmiller, R.L. and Deerenberg, C. 2000. Trade-Offs in Evolutionary Immunology: Just What Is the Cost of Immunity? *Oikos*. **88**(1), pp.87–98.

Lodjak, J., Mägi, M., Rooni, U. and Tilgar, V. 2015. Context-dependent effects of feather corticosterone on growth rate and fledging success of wild passerine nestlings in heterogeneous habitat. *Oecologia*. **179**(4), pp.937–946.

Logiudice, K., Ostfeld, R.S., Schmidt, K.A. and Keesing, F. 2003. The ecology of infectious disease: Effects of host diversity and community composition on Lyme disease risk. *Proceedings of the National Academy of Sciences*. **100**(2), pp.567–571.

Longcore, T. and Rich, C. 2004. Ecological light pollution. *Frontiers in Ecology and the Environment*. **2**(4), pp.191–198.

López-Jiménez, L., Blas, J., Tanferna, A., Cabezas, S., Marchant, T., Hiraldo, F. and Sergio, F. 2016. Ambient temperature, body condition and sibling rivalry explain feather corticosterone levels in developing black kites S. Portugal, ed. *Functional Ecology*. **30**(4), pp.605–613.

Love, O.P., Wynne-Edwards, K.E., Bond, L. and Williams, T.D. 2008. Determinants of within- and among-clutch variation in yolk corticosterone in the European starling. *Hormones and Behavior*. **53**(1), pp.104–111.

Lutz, H.L., Hochachka, W.M., Engel, J.I., Bell, J.A., Tkach, V. V., Bates, J.M., Hackett, S.J. and Weckstein, J.D. 2015. Parasite Prevalence Corresponds to Host Life History in a Diverse Assemblage of Afrotropical Birds and Haemosporidian Parasites T. Spielmann, ed. *PLOS ONE*. **10**(4), p.e0121254.

Maggini, S., Wintergerst, E.S., Beveridge, S. and Hornig, D.H. 2007. Selected vitamins and trace elements support immune function by strengthening epithelial barriers and cellular and humoral immune responses. *The British journal of nutrition*. **98 Suppl 1**, pp.S29-35.

Mainwaring, M.C., Dickens, M. and Hartley, I.R. 2011. Sexual dimorphism and growth trade-offs in Blue Tit Cyanistes caeruleus nestlings. *Ibis*. **153**(1), pp.175–179.

Mainwaring, M.C. and Hartley, I.R. 2016. Local weather conditions have complex effects on the growth of blue tit nestlings. *Journal of Thermal Biology*. (60), pp.12–19.

Malhotra, I., Dent, A., Mungai, P., Muchiri, E. and King, C.L. 2005. Real-Time Quantitative PCR for Determining the Burden of Plasmodium falciparum Parasites during Pregnancy and Infancy. *Journal of clinical microbiology*. **43**(8), pp.3630–3635.

Mangold, K.A., Manson, R.U., Koay, E.S.C., Stephens, L., Regner, M., Thomson, R.B., Peterson, L.R. and Kaul, K.L. 2005. Real-Time PCR for Detection and Identification of Plasmodium spp. *Journal of clinical microbiology*. **43**(5), pp.2435–2440.

Marciniak, B., Nadolski, J., Nowakowska, M., Loga, B. and Bańbura, J. 2007. Habitat and Annual Variation in Arthropod Abundance Affects Blue Tit *Cyanistes caeruleus* Reproduction. *Acta Ornithologica*. **42**(1), pp.53–62.

Martin, L.B. and Boruta, M. 2013. *The impacts of urbanization on avian disease transmission and emergence* [Online] (D. Gil & H. Brumm, eds.). Oxford University Press. [Accessed 31

December 2015]. Available from: https://www.researchgate.net/publication/268974968_The_impacts_of_urbanization_on_avian_disease_transmission_and_emergence.

Martin, T.E. 1987. Food as a Limit on Breeding Birds: A Life-History Perspective. *Annual Review of Ecology and Systematics*. **18**(1), pp.453–487.

Martin, T.E., Møller, a P., Merino, S. and Clobert, J. 2001. Does clutch size evolve in response to parasites and immunocompetence? *Proceedings of the National Academy of Sciences of the United States of America*. **98**(4), pp.2071–2076.

Marti-nez-De la Puente, J., Merino, S., Lobato, E., Rivero-de Aguilar, J., del Cerro, S., Ruiz-de-CastaÃeda, R. and Moreno, J. 2009. Does weather affect biting fly abundance in avian nests? *Journal of Avian Biology*. **40**(6), pp.653–657.

Marzal, A. 2012. *Recent Advances in Studies on Avian Malaria Parasites* [Online]. InTech. [Accessed 21 June 2018]. Available from: https://pdfs.semanticscholar.org/8e8e/ea146a95169e218aac5fe27db8075153b8ce.pdf.

Marzal, A., Bensch, S., Reviriego, M., Balbontin, J. and De lope, F. 2008. Effects of malaria double infection in birds: one plus one is not two. *Journal of Evolutionary Biology*. **21**(4), pp.979–987.

Marzal, A., Lope, F. de, Navarro, C. and M□ller, A.P. 2005. Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia*. **142**(4), pp.541–545.

Marzluff, J.M. 2017. A decadal review of urban ornithology and a prospectus for the future. *Ibis*. **159**(1), pp.1–13.

McCarty, J.P. 2001. Variation in Growth of Nestling Tree Swallows across Multiple Temporal and Spatial Scales. *The Auk*. **118**(1), pp.176–190.

McEwen, B.S. 1998. Stress, adaptation, and disease. Allostasis and allostatic load. *Annals of the New York Academy of Sciences*. **840**, pp.33–44.

McEwen, B.S. and Wingfield, J.C. 2003. The concept of allostasis in biology and biomedicine. *Hormones and Behavior*. **43**(1), pp.2–15.

McEwen, B.S. and Wingfield, J.C. 2010. What is in a name? Integrating homeostasis, allostasis and stress. *Hormones and Behavior*. **57**(2), pp.105–111.

McKinney, M.L. 2008. Effects of urbanization on species richness: A review of plants and animals. *Urban Ecosystems*. **11**, pp.161–176.

Meillere, A., Brischoux, F. and Angelier, F. 2015. Impact of chronic noise exposure on antipredator behavior: an experiment in breeding house sparrows. *Behavioral Ecology*. **26**(2), pp.569–577.

Meillère, A., Brischoux, F., Bustamante, P., Michaud, B., Parenteau, C., Marciau, C. and Angelier, F. 2016. Corticosterone levels in relation to trace element contamination along an urbanization gradient in the common blackbird (Turdus merula). *Science of The Total Environment*. **566**, pp.93–101.

Meillère, A., Brischoux, F., Parenteau, C. and Angelier, F. 2015. Influence of Urbanization on Body Size, Condition, and Physiology in an Urban Exploiter: A Multi-Component Approach C. Carere, ed. *Plos One*. **10**(8), p.e0135685.

Mennechez, G. and Clergeau, P. 2006. Effect of urbanisation on habitat generalists: starlings not so flexible? *Acta Oecologica*. **30**(2), pp.182–191.

Merino, S., Moreno, J., Sanz, J.J. and Arriero, E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (Parus caeruleus). *Proceedings. Biological sciences*. **267**(1461), pp.2507–10.

Meyrier, E., Jenni, L., Bötsch, Y., Strebel, S., Erne, B. and Tablado, Z. 2017. Happy to breed in the city? Urban food resources limit reproductive output in Western Jackdaws. *Ecology and Evolution*. **7**(5), pp.1363–1374.

Miller, M.W. 2006. Apparent Effects of Light Pollution on Singing Behavior of American Robins. *The Condor*. **108**(1), pp.130–139.

Moller, A. and Saino, N. 2004. Immune response and survival. *OIKOS*. **2**(July 2003), pp.299–304.

Moller, A.P. 2009. Successful city dwellers: a comparative study of the ecological characteristics of urban birds in the Western Palearctic. *Oecologia*. **159**(4), pp.849–58.

Moore, J. 2002. Parasites and the behavior of animals. *The Journal of Parasitology*. **88**(6), p.1214.

Morganti, M. et al 2017. Rainfall, but not temperature, negatively affects the growth of Blue Tit Cyanistes caeruleus nestlings. *Bird study*. **64**(2), pp.159–167.

Müller, C., Jenni-Eiermann, S. and Jenni, L. 2010. Development of the adrenocortical response to stress in Eurasian kestrel nestlings: Defence ability, age, brood hierarchy and condition. *General and Comparative Endocrinology*. **168**(3), pp.474–483.

Murdock, C.C., Foufopoulos, J. and Simon, C.P. 2013. A Transmission Model for the Ecology of an Avian Blood Parasite in a Temperate Ecosystem R. H. Clarke, ed. *PLoS ONE*. **8**(9), p.e76126.

Murray, M., Edwards, M.A., Abercrombie, B., Cassady, C., Clair, S. and St. Clair, C.C. 2015. Poor health is associated with use of anthropogenic resources in an urban carnivore. *Proceedings of the Royal Society B: Biological Sciences*. **282**(1806), p.20150009.

Naef-Daenzer, B. and Keller, L.F. 1999. The foraging performance of great and blue tits (Parus major and P. caeruleus) in relation to caterpillar development, and its consequences for nestling growth and fledging weight. *Journal of Animal Ecology*. **68**(4), pp.708–718.

Nager, R.G. 2006. The challenges of making eggs. *Ardea*. **94**(3), pp.323–346.

Nager, R.G., Monaghan, P., Houston, D.C. and Genovart, M. 2000. Parental condition, brood sex ratio and differential young survival: an experimental study in gulls (Larus fuscus). *Behavioral Ecology and Sociobiology*. **48**(6), pp.452–457.

Nager, R.G., Ruegger, C. and Noordwijk, A.J. Van 1997. Nutrient or Energy Limitation on Egg Formation: A Feeding Experiment in Great Tits. *The Journal of Animal Ecology*. **66**(4), p.495.

Navarro, C., Marzal, A., de Lope, F. and Moller, A.P. 2003. Dynamics of an immune response in house sparrows Passer domesticus in relation to time of day, body condition and blood parasite infection. *Oikos*. **101**(2), pp.291–298.

Nettle, D., Andrews, C., Reichert, S., Bedford, T., Kolenda, C., Parker, C., Martin-Ruiz, C., Monaghan, P. and Bateson, M. 2017. Early-life adversity accelerates cellular ageing and affects adult inflammation: Experimental evidence from the European starling. *Scientific Reports*. **7**, p.e40794.

Niedringhaus, K.D., Fenton, H.M.A., Cleveland, C.A., Anderson, A.N., Schwartz, D., Alex, C.E., Rogers, K.H., Mete, A. and Yabsley, M.J. 2018. Case Series: Virulent hemosporidiosis infections in juvenile great horned owls (Bubo virginianus) from Louisiana and California, USA. *Veterinary Parasitology: Regional Studies and Reports*. **12**, pp.49–54.

Niemelä, J, Breuste, JH, Elmqvist, T, Guntenspergen, G, James, P and McIntyre, N. (ed.). 2011. *Urban ecology : patterns, processes, and applications*. Oxford: Oxford University Press.

Noordwijk, A.J. Van, McCleery, R.H. and Perrins, C.M. 1995. Selection for the Timing of Great Tit Breeding in Relation to Caterpillar Growth and Temperature. *The Journal of Animal Ecology*. **64**(4), p.451.

Norris, K. and Evans, M.R. 2000. Ecological immunology : life history trade-offs and immune defense in birds. *Behavioral Ecology*. **11**(1), pp.19–26.

Van Nuland, M.E. and Whitlow, W.L. 2014. Temporal effects on biodiversity and composition of arthropod communities along an urban–rural gradient. *Urban Ecosystems*. **17**(4), pp.1047–1060.

Ots, I. and Hõrak, P. 1998. Health impact of blood parasites in breeding great tits. *Oecologia*. **116**(4), pp.441–448.

Owen, J.C., Nakamura, A., Coon, C.A.C. and Martin, L.B. 2012. The effect of exogenous corticosterone on West Nile virus infection in Northern Cardinals (Cardinalis cardinalis). *Veterinary research*. **43**(1), p.34.

Pacheco, M.A., Cepeda, A.S., Bernotienė, R., Lotta, I.A., Matta, N.E., Valkiūnas, G. and Escalante, A.A. 2018. Primers targeting mitochondrial genes of avian haemosporidians: PCR detection and differential DNA amplification of parasites belonging to different genera. *International Journal for Parasitology*. **48**(8), pp.657–670.

Page, L.K., Gehrt, S.D. and Robinson, N.P. 2008. Land-use effects on prevalence of raccoon roundworm (baylisascaris procyonis). *Journal of Wildlife Diseases*. **44**(3), pp.594–599.

Palinauskas, V., Žiegytė, R., Iezhova, T.A., Ilgūnas, M., Bernotienė, R. and Valkiūnas, G. 2016. Description, molecular characterisation, diagnostics and life cycle of Plasmodium elongatum (lineage pERIRUB01), the virulent avian malaria parasite. *International Journal for Parasitology*. **46**(11), pp.697–707.

Partecke, J., Schwabl, I., Gwinner, E., Author, B., Partecke, J., Schwabl, I. and Gwinner, E. 2006. Stress and the city: Urbanization and its effects on the stress physiology in European

Blackbirds. *Ecology*. **87**(8), pp.1945–1952.
Patterson, A.G.L., Kitaysky, A.S., Lyons, D.E. and Roby, D.D. 2015. Nutritional stress affects corticosterone deposition in feathers of Caspian tern chicks. *Journal of Avian Biology*. **46**(1), pp.18–24.
Patterson, J.E.H. and Ruckstuhl, K.E. 2013. Parasite infection and host group size: a meta-analytical review. *Parasitology*. **140**(7), pp.803–13.
Peach, W.J., Vincent, K.E., Fowler, J.A. and Grice, P. V. 2008. Reproductive success of house sparrows along an urban gradient. *Animal Conservation*. **11**(6), pp.493–503.
Peirce, M.A., Lederer, R., Adlard, R.D. and O'Donoghue, P.J. 2004. Pathology associated with endogenous development of haematozoa in birds from southeast Queensland. *Avian Pathology*. **33**(4), pp.445–450.
Penick, C.A., Savage, A.M. and Dunn, R.R. 2015. Stable isotopes reveal links between human food inputs and urban ant diets. *Proceedings. Biological sciences / The Royal Society*. **282**(1806), p.20142608.
Perandin, F., Manca, N., Calderaro, A., Piccolo, G., Galati, L., Ricci, L., Medici, M.C., Arcangeletti, M.C., Snounou, G., Dettori, G. and Chezzi, C. 2004. Development of a real-time PCR assay for detection of Plasmodium falciparum, Plasmodium vivax, and Plasmodium ovale for routine clinical diagnosis. *Journal of clinical microbiology*. **42**(3), pp.1214–9.
Peris, S.. and Pescador, M. 2004. Effects of traffic noise on paserine populations in Mediterranean wooded pastures. *Applied Acoustics*. **65**(4), pp.357–366.
Perrins, A.C.M., Tit, G., L, P.M. and Perrins, B.Y.C.M. 1965. Population Fluctuations and Clutch-Size in the. *Journal of Animal Ecology*. **34**(3), pp.601–647.
Perry, G., Buchanan, B.W., Fisher, R.N., Salmon, M. and Wise, S.E. 2008. Effects of Artificial Night Lighting on Amphibians and Reptiles in Urban Environment. *Urban Herpetology*. **239**, pp.239–256.
Petren, K. and Case, T.J. 1996. An Experimental Demonstration of Exploitation Competition in an Ongoing Invasion. *Ecology*. **77**(1), p.118.
Pickett, S.T.A., Cadenasso, M.L., Grove, J.M., Boone, C.G., Groffman, P.M., Irwin, E., Kaushal, S.S., Marshall, V., McGrath, B.P., Nilon, C.H., Pouyat, R.V., Szlavecz, K., Troy, A. and Warren, P. 2011. Urban ecological systems: Scientific foundations and a decade of progress. *Journal of Environmental Management*. **92**(3), pp.331–362.
Pickett, S.T.A., Cadenasso, M.L., Grove, J.M., Nilon, C.H., Pouyat, R. V., Zipperer, W.C. and Costanza, R. 2001. Urban Ecological Systems: Linking Terrestrial Ecological, Physical, and Socioeconomic Components of Metropolitan Areas 1. *Annual Review of Ecology and Systematics*. **32**(1), pp.127–157.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and Team, R.C. 2009. nlme: Linear and nonlinear mixed effects models. R package version 3.1-93.
Plummer, K., Bearhop, Stuart, Leech, D.I., Chamberlain, D.E. and Blount, J.D. 2013. Fat provisioning in winter impairs egg production during the following spring: a landscape-scale study of blue tits. *The Journal of animal ecology*. **82**(3), pp.673–82.
Plummer, K., Bearhop, S., Leech, D.I., Chamberlain, D.E. and Blount, J.D. 2013. Winter food provisioning reduces future breeding performance in a wild bird. *Scientific reports*. **3**, p.2002.
Podmokła, E., Dubiec, A., Drobniak, S.M., Arct, A., Gustafsson, L. and Cichoń, M. 2014. Avian malaria is associated with increased reproductive investment in the blue tit. *Journal of Avian Biology*. **45**(3), pp.219–224.
Pollock, C.J., Capilla-Lasheras, P., McGill, R.A.R., Helm, B. and Dominoni, D.M. 2017. Integrated behavioural and stable isotope data reveal altered diet linked to low breeding success in urban-dwelling blue tits (Cyanistes caeruleus). *Scientific Reports*. **7**(1), p.5014.
Poulin, R. 2007. *Evolutionary ecology of parasites* [Online]. Princeton University Press. [Accessed 22 July 2019]. Available from: https://press.princeton.edu/titles/8336.html.
Prange, S., Gehrt, S.D. and Wiggers, E.P. 2003. Demographic Factors Contributing to High Raccoon Densities in Urban Landscapes. *The Journal of Wildlife Management*. **67**(2), p.324.
Preiser, P.R., Wilson, R.J., Moore, P.W., McCready, S., Hajibagheri, M.A., Blight, K.J., Strath, M. and Williamson, D.H. 1996. Recombination associated with replication of malarial mitochondrial DNA. *The EMBO journal*. **15**(3), pp.684–93.
la Puente, J.M., Merino, S., Tomás, G., Moreno, J., Morales, J., Lobato, E., García-Fraile, S., Belda, E.J., Puente, J.M. la, Merino, S., Tomás, G., Moreno, J., Morales, J., Lobato, E.,

García-Fraile, S. and Belda, E.J. 2010. The blood parasite Haemoproteus reduces survival in a wild bird: a medication experiment. *Biology letters*. **6**(5), pp.663–5.

Råberg, L., Graham, A.L., Read, A.F., Raberg, L., Graham, A.L. and Read, A.F. 2009. Decomposing health: tolerance and resistance to parasites in animals. *Philosophical Transactions of the Royal Society B: Biological Sciences*. **364**(1513), pp.37–49.

Ramsay, S.L. and Houston, D.C. 1997. Nutritional Constraints on Egg Production in the Blue Tit: A Supplementary Feeding Study. *The Journal of Animal Ecology*. **66**(5), p.649.

Rebolo-Ifrán, N., Carrete, M., Sanz-Aguilar, A., Rodríguez-Martínez, S., Cabezas, S., Marchant, T.A., Bortolotti, G.R. and Tella, J.L. 2015. Links between fear of humans, stress and survival support a non-random distribution of birds among urban and rural habitats. *Scientific Reports*. **5**(13723).

Remple, J.D. 2004. Intracellular Hematozoa of Raptors: A Review and Update. *Journal of Avian Medicine and Surgery*. **18**(2), pp.75–88.

Renner, S.C., Lüdtke, B., Kaiser, S., Kienle, J., Schaefer, H.M., Segelbacher, G., Tschapka, M. and Santiago-Alarcon, D. 2016. Forests of opportunities and mischief: disentangling the interactions between forests, parasites and immune responses. *International Journal for Parasitology*. **46**(9), pp.571–579.

Rensel, M.A., Wilcoxen, T.E. and Schoech, S.J. 2011. Corticosterone, brood size, and hatch order in free-living Florida scrub-jay (Aphelocoma coerulescens) nestlings. *General and Comparative Endocrinology*. **171**(2), pp.197–202.

Reperant, L.A., Hegglin, D., Fischer, C., Kohler, L., Weber, J.-M. and Deplazes, P. 2007. Influence of urbanization on the epidemiology of intestinal helminths of the red fox (Vulpes vulpes) in Geneva, Switzerland. *Parasitology Research*. **101**(3), pp.605–611.

Reynolds, S.J., Schoech, S.J. and Bowman, R. 2003. Diet quality during pre-laying and nestling periods influences growth and survival of Florida scrub-jay (Aphelocoma coerulescens) chicks. *Journal of Zoology*. **261**(3), pp.217–226.

Ricklefs, R.E. 1983. Patterns of growth in birds. *Ibis*. **110**(4), pp.419–451.

Ricklefs, R.E., Fallon, S.M. and Bermingham, E. 2004. Evolutionary Relationships, Cospeciation, and Host Switching in Avian Malaria Parasites K. Johnson, ed. *Systematic Biology*. **53**(1), pp.111–119.

Robb, G.N., McDonald, R.A., Chamberlain, D.E., Reynolds, S.J., Harrison, T.J.. and Bearhop, S. 2008. Winter feeding of birds increases productivity in the subsequent breeding season. *Biology Letters*. **4**(2), pp.220–223.

Rodríguez, S. and Barba, E. 2016. Nestling Growth is Impaired by Heat Stress: an Experimental Study in a Mediterranean Great Tit Population. *Zoological Studies*. **55**, pp.1–32.

Rooyen, J. van, Lalubin, F., Glaizot, O. and Christe, P. 2013. Avian haemosporidian persistence and co-infection in great tits at the individual level. *Malaria Journal*. **12**(1), p.40.

Russ, A., Rüger, A. and Klenke, R. 2014. Seize the night: European Blackbirds (Turdus merula) extend their foraging activity under artificial illumination. *Journal of Ornithology*. **156**(1), pp.123–131.

Saino, N., Romano, M., Ferrari, R.P., Martinelli, R. and Møller, A.P. 2005. Stressed mothers lay eggs with high corticosterone levels which produce low-quality offspring. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*. **303A**(11), pp.998–1006.

Saino, N., Suffritti, C., Martinelli, R., Rubolini, D. and Møller, A.P. 2003. Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (Hirundo rustica). *Behavioral Ecology*. **14**(3), pp.318–325.

Salmón, P., Nilsson, J.F., Nord, A., Bensch, S. and Isaksson, C. 2016. Urban environment shortens telomere length in nestling great tits, *Parus major*. *Biology Letters*. **12**(6), p.20160155.

Santiago-Alarcon, D., MacGregor-Fors, I., Falfán, I., Lüdtke, B., Segelbacher, G., Schaefer, H.M. and Renner, S. 2019. Parasites in space and time: a case study of haemosporidian spatiotemporal prevalence in urban birds. *International Journal for Parasitology*.

Santiago-Alarcon, D., Carbó-Ramírez, P., Macgregor-Fors, I., Chávez-Zichinelli, C.A. and Yeh, P.J. 2018. The prevalence of avian haemosporidian parasites in an invasive bird is lower in urban than non-urban environments. *Ibis*. ((in press)), p.12699.

Sapolsky, R.M. 1983. Individual Differences in Cortisol Secretory Patterns in the Wild Baboon: Role of Negative Feedback Sensitivity. *Endocrinology*. **113**(6), pp.2263–2267.

Sapolsky, R.M., Romero, L.M. and Munck, A.U. 2000. How Do Glucocorticoids Influence Stress

Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions [1]. *Endocrine Reviews*. **21**(1), pp.55–89.
Sauter, A., Bowman, R., Schoech, S.J. and Pasinelli, G. 2006. Does optimal foraging theory explain why suburban Florida scrub-jays (Aphelocoma coerulescens) feed their young human-provided food? *Behavioral Ecology and Sociobiology*. **60**(4), pp.465–474.
Scheuhammer, A.M. 1987. The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: A review. *Environmental Pollution*. **46**(4), pp.263–295.
Schoech, S.J. 1996. The effect of supplemental food on body condition and the timing of reproduction in a cooperative breeder, the Florida scrub-jay. *Condor*. **98**(2), pp.234–244.
Schoenle, L.A., Kernbach, | Meredith and Moore, I.T. 2017. An experimental test of the physiological consequences of avian malaria infection. *J Anim Ecol*. **86**, pp.1483–1496.
Schoenle, L.A., Moore, I.T., Dudek, A.M., Garcia, E.B., Mays, M., Haussmann, M.F., Cimini, D. and Bonier, F. 2019. Exogenous glucocorticoids amplify the costs of infection by reducing resistance and tolerance, but effects are mitigated by co-infection. *Proceedings of the Royal Society B: Biological Sciences*. **286**(1900), p.20182913.
Schroeder, J., Nakagawa, S., Cleasby, I.R. and Burke, T. 2012. Passerine birds breeding under chronic noise experience reduced fitness. *PloS one*. **7**(7), p.e39200.
Schuurs, A.H. and Verheul, H.A. 1990. Effects of gender and sex steroids on the immune response. *Journal of steroid biochemistry*. **35**(2), pp.157–72.
Schwagmeyer, P.L. and Mock, D.W. 2008. Parental provisioning and offspring fitness: size matters. *Animal Behaviour*. **75**(1), pp.291–298.
Sehgal, R.N.M. 2015. Manifold habitat effects on the prevalence and diversity of avian blood parasites. *International journal for parasitology. Parasites and wildlife*. **4**(3), pp.421–30.
Sepp, T., McGraw, K.J., Kaasik, A. and Giraudeau, M. 2017. A review of urban impacts on avian life-history evolution: Does city living lead to slower pace of life? *Global Change Biology*. **24**(4), p.13969.
Seress, G., Bókony, V., Pipoly, I., Szép, T., Nagy, K. and Liker, A. 2012. Urbanization, nestling growth and reproductive success in a moderately declining house sparrow population. *Journal of Avian Biology*. **43**(5), pp.403–414.
Seress, G. and Liker, A. 2015. Habitat urbanization and its effects on birds. *Acta Zoologica Academiae Scientiarum Hungaricae*. **61**(4), pp.373–408.
Shea, K. and Chesson, P. 2002. Community ecology theory as a framework for biological invasions. *Trends in Ecology & Evolution*. **17**(4), pp.170–176.
Sheldon, B.C. and Verhulst, S. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in ecology & evolution*. **11**(8), pp.317–21.
Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R. and Boonstra, R. 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia*. **166**(4), pp.869–887.
Shurulinkov, P. and Chakarov, N. 2006. Prevalence of blood parasites in different local populations of reed warbler (Acrocephalus scirpaceus) and great reed warbler (Acrocephalus arundinaceus). *Parasitology Research*. **99**(5), pp.588–592.
Siriwardena, G.M., Baillie, S.R. and Wilson, J.D. 1998. Variation in the survival rates of some British passerines with respect to their population trends on farmland. *Bird Study*. **45**(3), pp.276–292.
Sol, D., Lapiedra, O. and González-Lagos, C. 2013. Behavioural adjustments for a life in the city. *Animal Behaviour*. **85**(5), pp.1101–1112.
Spellerberg, I.F. 1998. Ecological Effects of Roads and Traffic: A Literature Review. *Global Ecology and Biogeography Letters*. **7**(5), pp.317–333.
Starck, J.M. and Ricklefs, R.E. 1998. *Avian growth and development : evolution within the altricial-precocial spectrum* [Online]. Oxford University Press. [Accessed 29 May 2018]. Available from: https://global.oup.com/academic/product/avian-growth-and-development-9780195106084?cc=gb&lang=en&.
Stearns, S.C. 1989. Trade-Offs in Life-History Evolution. *Functional Ecology*. **3**(3), p.259.
Studholme, K.R., Hipfner, J.M., Romero, L.M., Gormally, B.M., Iverson, S.J. and Crossin, G.T. 2018. Egg size is independent of variation in pre-breeding feather corticosterone in Cassin's auklets during favorable oceanographic conditions. *General and Comparative Endocrinology*. **268**, pp.64–70.
Sudyka, J., Podmokła, E., Drobniak, S.M., Dubiec, A., Arct, A., Gustafsson, L. and Cichoń, M.

2019. Sex-specific effects of parasites on telomere dynamics in a short-lived passerine—the blue tit. *The Science of Nature*. **106**(1–2), p.6.

Suri, J., Sumasgutner, P., Hellard, É., Koeslag, A. and Amar, A. 2017. Stability in prey abundance may buffer Black Sparrowhawks *Accipiter melanoleucus* from health impacts of urbanization. *Ibis*. **159**(1), pp.38–54.

Swaileh, K.M. and Sansur, R. 2006. Monitoring urban heavy metal pollution using the House Sparrow (Passer domesticus). *Journal of environmental monitoring : JEM*. **8**(1), pp.209–13.

Tadei, W.P., Thatcher, B.D., Santos, J.M., Scarpassa, V.M., Rodrigues, I.B. and Rafael, M.S. 1998. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. *The American journal of tropical medicine and hygiene*. **59**(2), pp.325–35.

Tryjanowski, P., Skórka, P., Sparks, T.H., Biaduń, W., Brauze, T., Hetmański, T., Martyka, R., Indykiewicz, P., Myczko, Ł., Kunysz, P., Kawa, P., Czyż, S., Czechowski, P., Polakowski, M., Zduniak, P., Jerzak, L., Janiszewski, T., Goławski, A., Duduś, L., Nowakowski, J.J., Wuczyński, A. and Wysocki, D. 2015. Urban and rural habitats differ in number and type of bird feeders and in bird species consuming supplementary food. *Environmental science and pollution research international*. **22**(19), pp.15097–103.

Valera, F., Carrillo, C.M., Barbosa, A. and Moreno Estaci, E. 2003. Low prevalence of haematozoa in Trumpeter finches Bucanetes githagineus from south-eastern Spain: additional support for a restricted distribution of blood parasites in arid lands. *Journal of Arid Environments*. **55**, pp.209–213.

Valkiūnas, G. (Gediminas) 2005. *Avian malaria parasites and other haemosporidia* [Online]. CRC Press. [Accessed 27 August 2017]. Available from: https://www.crcpress.com/Avian-Malaria-Parasites-and-other-Haemosporidia/Valkiunas/p/book/9780415300971.

Valkiūnas, G., Bensch, S., Iezhova, T.A., Križanauskienė, A., Hellgren, O. and Bolshakov, C. V. 2006. Nested Cytochrome *B* Polymerase Chain Reaction Diagnostics Underestimate Mixed Infections of Avian Blood Haemosporidian Parasites: Microscopy is Still Essential. *Journal of Parasitology*. **92**(2), pp.418–422.

Valkiūnas, G. and Iezhova, T.A. 2017. Exo-erythrocytic development of avian malaria and related haemosporidian parasites. *Malaria Journal*. **16**(1), p.101.

Valkiūnas, G., Iezhova, T.A., Križanauskienė, A., Palinauskas, V., Sehgal, R.N.M. and Bensch, S. 2008. A Comparative Analysis of Microscopy and PCR-Based Detection Methods for Blood Parasites. *Journal of Parasitology*. **94**(6), pp.1395–1401.

Valkiūnas, G., Žičkus, T., Shapoval, A.P. and Iezhova, T.A. 2006. Effect of Haemoproteus belopolskyi (Haemosporida: Haemoproteidae) on Body Mass of the Blackcap Sylvia atricapilla. *Journal of Parasitology*. **92**(5), pp.1123–1125.

Vergnes, A., Pellissier, V., Lemperiere, G., Rollard, C. and Clergeau, P. 2014. Urban densification causes the decline of ground-dwelling arthropods. *Biodiversity and Conservation*. **23**(8), pp.1859–1877.

Visser, M.E., Holleman, L.J.M. and Gienapp, P. 2006. Shifts in caterpillar biomass phenology due to climate change and its impact on the breeding biology of an insectivorous bird. *Oecologia*. **147**(1), pp.164–172.

Vittor, A.Y., Gilman, R.H., Tielsch, J., Glass, G., Shields, T., Lozano, W.S., Pinedo-Cancino, V. and Patz, J.A. 2006. The effect of deforestation on the human-biting rate of Anopheles darlingi, the primary vector of Falciparum malaria in the Peruvian Amazon. *The American journal of tropical medicine and hygiene*. **74**(1), pp.3–11.

Waldenström, J., Bensch, S., Hasselquist, D. and Östman, Ö. 2004. A New Nested Polymerase Chain Reaction Method Very Efficient in Detecting Plasmodium and Haemoproteus Infections From Avian Blood. *Journal of Parasitology*. **90**(1), pp.191–194.

Ware, G.W. (ed.). 1993. *Reviews of Environmental Contamination and Toxicology* [Online]. New York, NY: Springer New York. [Accessed 30 December 2015]. Available from: http://www.springerlink.com/index/10.1007/978-1-4613-9763-2.

Warren, P.S., Katti, M., Ermann, M. and Brazel, A. 2006. Urban bioacoustics: it's not just noise. *Animal Behaviour*. **71**(3), pp.491–502.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M. and Barton, G.J. 2009. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics*. **25**(9), pp.1189–1191.

Watson, H., Videvall, E., Andersson, M.N., Isaksson, C. and Beedle, A.S. 2017. Transcriptome

analysis of a wild bird reveals physiological responses to the urban environment. *Scientific Reports*. **7**, p.44180.

White, T.C.R. 2008. The role of food, weather and climate in limiting the abundance of animals. *Biological Reviews*. **83**(3), pp.227–248.

Wilkinson, L.C., Handel, C.M., Van Hemert, C., Loiseau, C. and Sehgal, R.N.M. 2016. Avian malaria in a boreal resident species: long-term temporal variability, and increased prevalence in birds with avian keratin disorder. *International Journal for Parasitology*. **46**(4), pp.281–290.

Will, Alexis P, Suzuki, Y., Elliott, K.H., Hatch, S.A., Watanuki, Y. and Kitaysky, A.S. 2014. Feather corticosterone reveals developmental stress in seabirds. *The Journal of experimental biology*. **217**(Pt 13), pp.2371–6.

Will, A. P., Suzuki, Y., Elliott, K.H., Hatch, S.A., Watanuki, Y. and Kitaysky, A.S. 2014. Feather corticosterone reveals developmental stress in seabirds. *Journal of Experimental Biology*.

Wingfield, J.C. and Romero, L.M. 2011. Adrenocortical Responses to Stress and Their Modulation in Free-Living Vertebrates *In*: *Comprehensive Physiology* [Online]. Hoboken, NJ, USA: John Wiley & Sons, Inc., pp.211–234. [Accessed 17 July 2019]. Available from: http://doi.wiley.com/10.1002/cphy.cp070411.

Wingfield, J.C. and Sapolsky, R.M. 2003. Reproduction and Resistance to Stress: When and How. *Journal of Neuroendocrinology*. **15**(8), pp.711–724.

Woodford, L., Bianco, G., Ivanova, Y., Dale, M., Elmer, K., Rae, F., Larcombe, S.D., Helm, B., Ferguson, H.M. and Baldini, F. 2018. Vector species-specific association between natural Wolbachia infections and avian malaria in black fly populations. *Scientific Reports*. **8**(1), p.4188.

Zhang, S., Lei, F., Liu, S., Li, D., Chen, C. and Wang, P. 2011. Variation in baseline corticosterone levels of Tree Sparrow (Passer montanus) populations along an urban gradient in Beijing, China. *Journal of Ornithology*. **152**(3), pp.801–806.

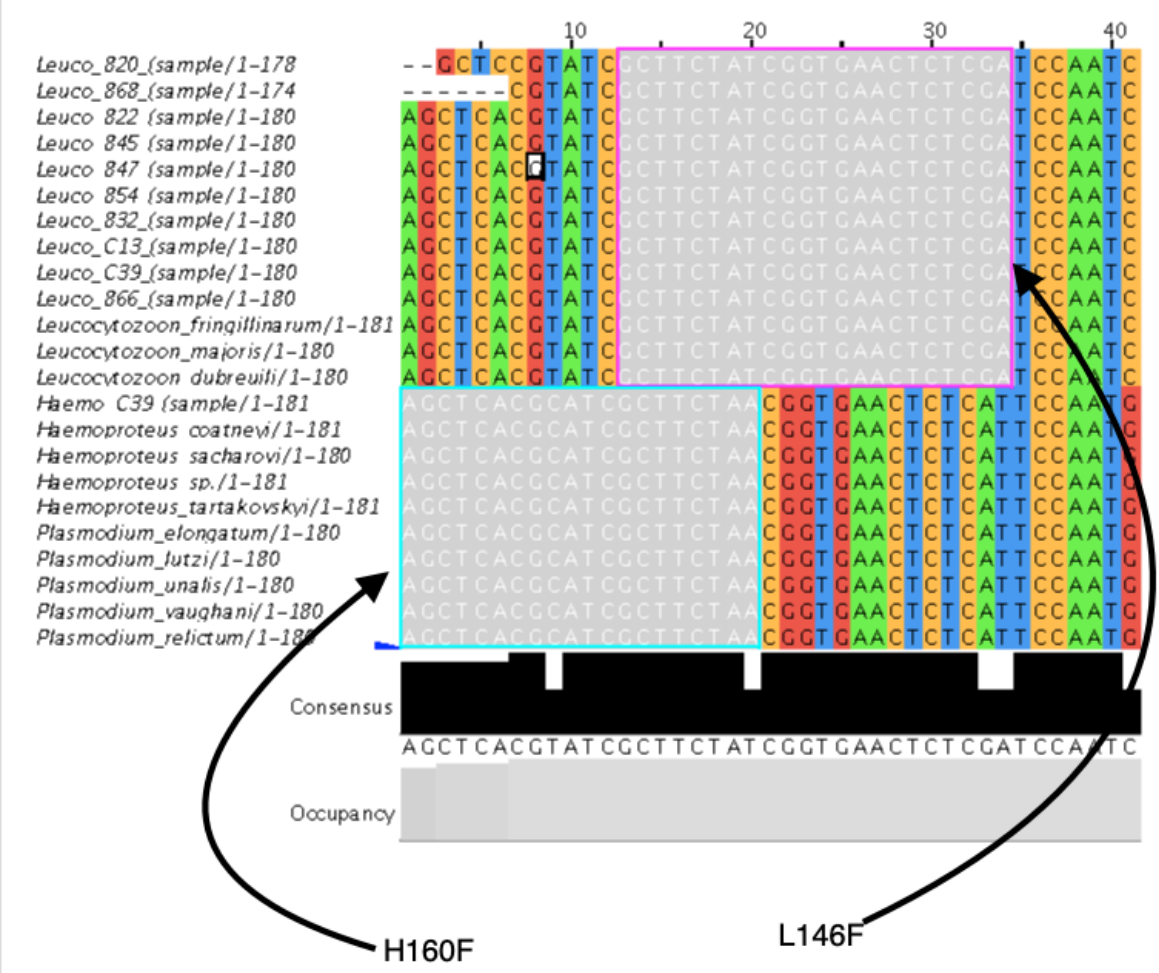
Zimmerman, P.A., Mehlotra, R.K., Kasehagen, L.J. and Kazura, J.W. 2004. Why do we need to know more about mixed Plasmodium species infections in humans? *Trends in parasitology*. **20**(9), pp.440–7.

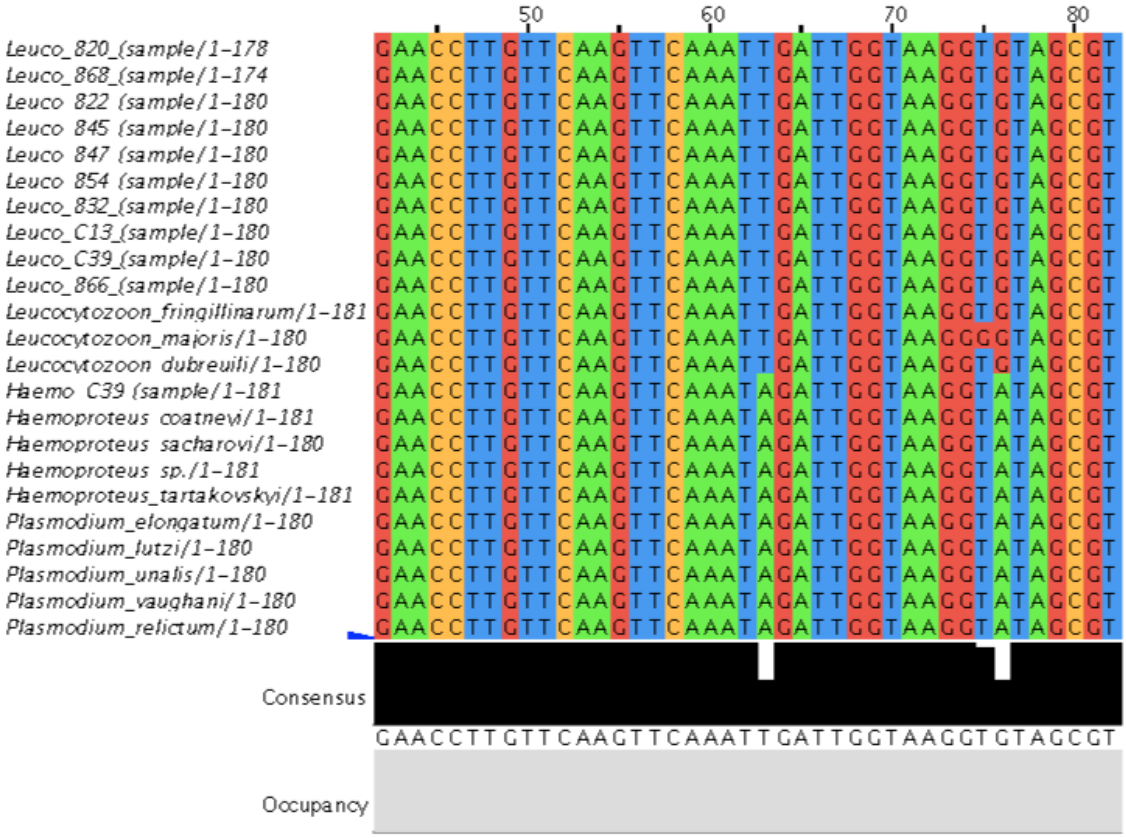
Zuk, M. 2000. Social environment and immunity in male red jungle fowl. *Behavioral Ecology*. **11**(2), pp.146–153.

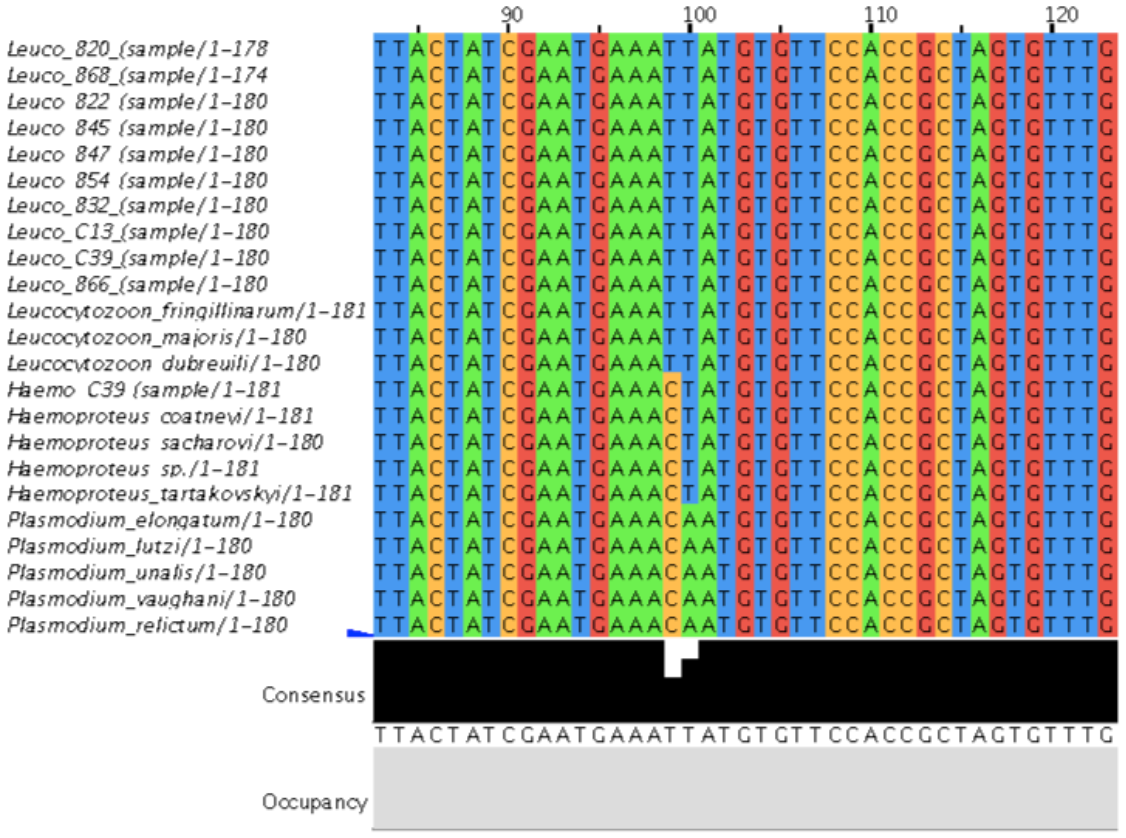
Zuk, M. and McKean, K.A. 1996. Sex differences in parasite infections: patterns and processes. *International journal for parasitology*. **26**(10), pp.1009–23.

Appendices

Supplementary figures for Chapter 3




50
60
70
80
Leuco_820_(sample/1-178
Leuco_868_(sample/1-174
Leuco 822 (sample/1-180
Leuco 845 (sample/1-180
Leuco 847 (sample/1-180
Leuco 854 (sample/1-180
Leuco_832_(sample/1-180
Leuco_C13_(sample/1-180
Leuco_C39_(sample/1-180
Leuco_866_(sample/1-180
Leucocytozoon_fringillinarum/1-181
Leucocytozoon_majoris/1-180
Leucocytozoon dubreuili/1-180
Haemo C39 (sample/1-181
Haemoproteus coatneyi/1-181
Haemoproteus sacharovi/1-180
Haemoproteus sp./1-181
Haemoproteus_tartakovskyi/1-181
Plasmodium_elongatum/1-180
Plasmodium_lutzi/1-180
Plasmodium_unalis/1-180
Plasmodium_vaughani/1-180
Plasmodium_relictum/1-180
Consensus
GAACCTTGTTCAAGTTCAAATTGATTGGTAAGGTGTAGCGT
Occupancy


90
100
110
120
Leuco_820_(sample/1-178
Leuco_868_(sample/1-174
Leuco 822 (sample/1-180
Leuco 845 (sample/1-180
Leuco 847 (sample/1-180
Leuco 854 (sample/1-180
Leuco_832_(sample/1-180
Leuco_C13_(sample/1-180
Leuco_C39_(sample/1-180
Leuco_866_(sample/1-180
Leucocytozoon_fringillinarum/1-181
Leucocytozoon_majoris/1-180
Leucocytozoon dubreuili/1-180
Haemo C39 (sample/1-181
Haemoproteus coatneyi/1-181
Haemoproteus sacharovi/1-180
Haemoproteus sp./1-181
Haemoproteus_tartakovskyi/1-181
Plasmodium_elongatum/1-180
Plasmodium_lutzi/1-180
Plasmodium_unalis/1-180
Plasmodium_vaughani/1-180
Plasmodium_relictum/1-180
Consensus
TTACTATCGAATGAAATTATGTGTTCCACCGCTAGTGTTTG
Occupancy

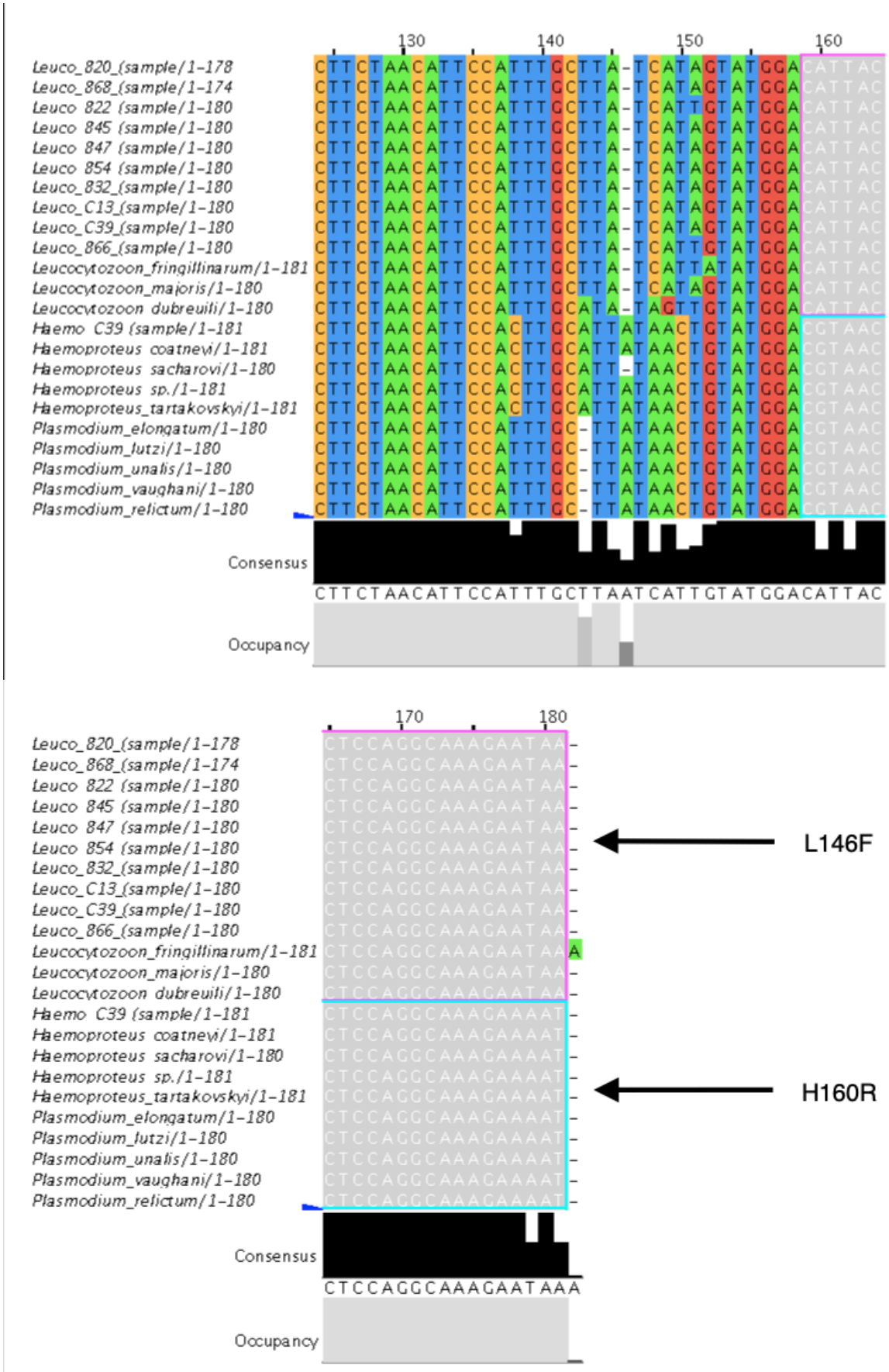


Figure S1 Illustration of sequences alignment shows the position of each set of primers (L146F/L146R) and (H160F/H160R) on the parasite target gene. illustration were built using jalview software version 2.10.5 (Waterhouse et al., 2009).

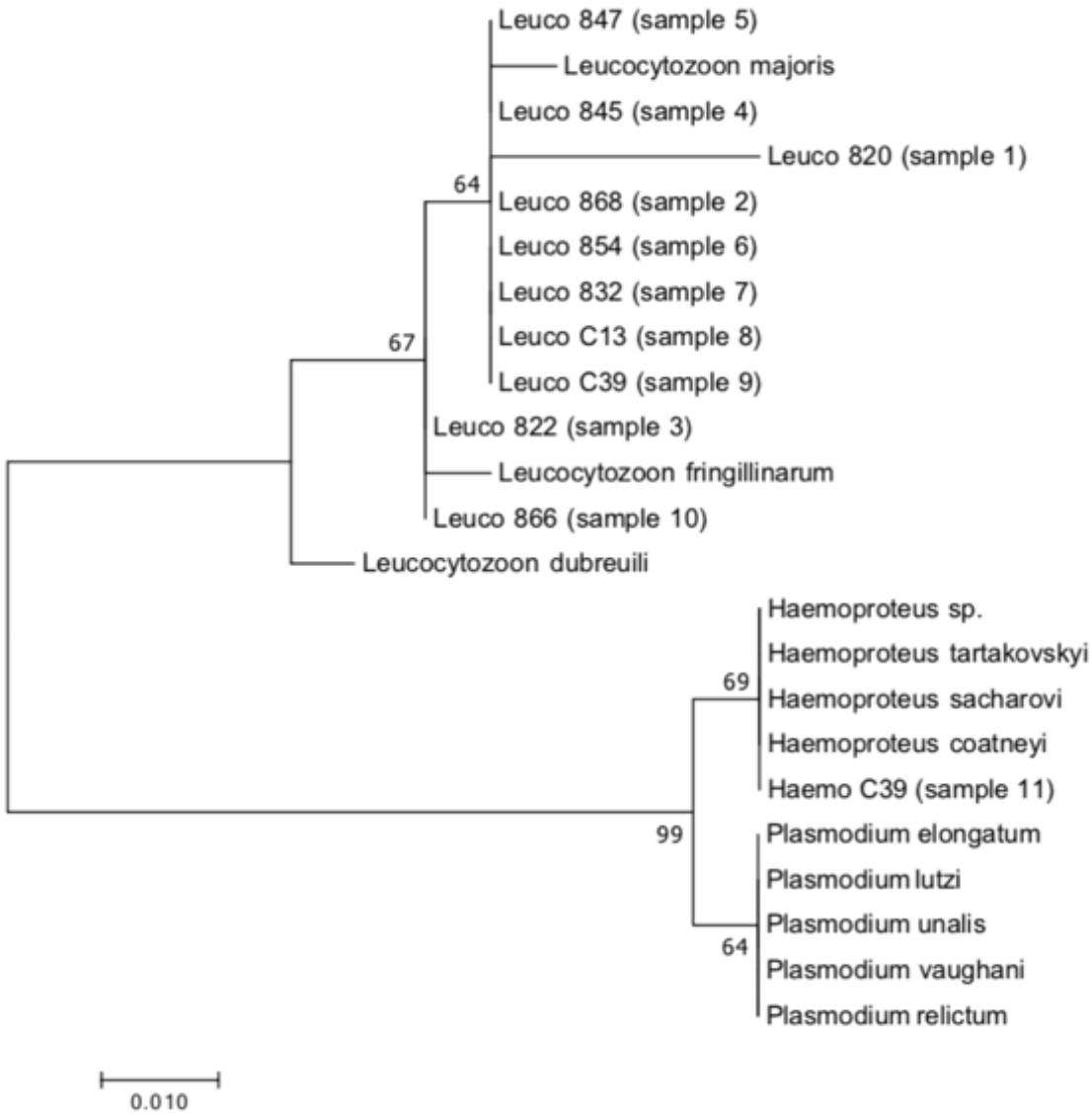


Figure S2 Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-388.56) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 200.0000)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences – 11 sequences from samples collected in my populations (samples 1-11) and 12 sequences obtained from the GenBank (see Table 3-3). There were a total of 182 positions in the final dataset. Evolutionary analyses and explaination of the phelogenetic tree were obtained from MEG