Ecology of Mosquito Vectors in Relation to Avian Malaria in Zoological Gardens in the United Kingdom

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by

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This thesis is the product of my work and the assistance and advice from others is fully acknowledged.

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Abstract

Avian malaria is one of the most serious diseases in penguins under human care and could become a severe threat to the conservation of vulnerable wild populations. It is caused by the Haemosporidia parasites of the genus *Plasmodium* and needs a mosquito vector for its transmission. We captured mosquitoes during two years in Chester Zoo (Cheshire) and one year in Flamingo Land (Yorkshire); both zoos house Humboldt penguins (*Spheniscus humboldti*). The mosquito temporal and spatial abundance across the seasons and sites were analysed. It was found that *Culex pipiens*, the principal avian malaria vector in Europe, was the most abundant species. There was a peak in the mosquito abundance during the summer as expected, but it was at different months between sites and years. The abundance of mosquitoes also varied among sampling areas; one area in Chester Zoo captured a greater proportion of mosquitoes than the others in both years, and in Flamingo Land, we also found an area with consistent high catches. Blood-fed mosquitoes were captured and analysed to identify the host on which they had fed. Different proportions of blood-fed mosquitoes were captured by areas and months; more were collected during the summer and in certain areas that not in all cases were related to a high abundance of un-fed mosquitoes. Most of these mosquitoes were *Culex pipiens* and *Culiseta annulata*; it was confirmed that the first one prefers to feed on birds and the second one on non-human mammals. However, many *Culex pipiens* fed on humans, which alert us about the possible nuisance for visitors and the potential transmission risk of zoonotic diseases. A partially identified Culicinae mosquito, likely to be *Culex pipiens*, and an *Anopheles maculipennis* s. l. fed on penguins; so, they could be involved in avian malaria transmission. It was found that mosquitoes travel variable distance after feeding and therefore, the control measures against mosquitoes should cover more than the areas of immediate concern. The environmental variables were analysed to understand the drivers of the diverse mosquito captures. The temperature was the most important variable related to mosquito abundance, and the dense vegetation, proximity to mosquito oviposition sites and closeness to animal exhibits were also significant. Therefore, the temperature could guide actions for mosquito control and avian malaria prevention and avoiding those surrounding features near the penguin exhibits could prevent high densities of mosquitoes. Many aspects of avian malaria epidemiology are uncertain so, through an online survey, the knowledge of the staff in zoos and wildlife parks about the disease was gathered. It was found that avian malaria had affected penguins in more than half of the answering institutions, involving mainly Humboldt and African penguins (*Spheniscus demersus*) with high lethality rates; therefore, efforts on preventive actions are encouraged. Avian malaria parasites were found in *Culex pipiens* mosquitoes and their saliva, wild birds and penguins, suggesting that the transmission process happens locally. Mosquito populations are dynamic, and the biosurveillance of their populations is needed to better understand their role as disease vectors and to implement effective control measures at the right time, assisting in this way the prevention of avian malaria in captive penguins.

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Chapter One

General Introduction

1.1 Introduction

In recent decades, globally important epidemiologic events have taken place with severe repercussions for health, wellbeing and economy. Their relevance encompass different fronts, they have been zoonotic diseases (capable of infecting humans), affected animal health and welfare or even threaten wildlife populations (Evans and Leighton, 2014). Many of these events were caused by diseases related to animals and Emerging Infectious Diseases (EID); ergo, those that have been recently discovered, that affect new populations, or that have rapidly increased their incidence and geographic distribution, and, consequently, it is hard to manage or control them (Reed et al., 2003, Bengis et al., 2004). Furthermore, the epidemiologic processes in natural or disturbed systems are complex and usually involve multiple interactions between hosts and pathogens, causing many challenges for the efficient implementation of preventive and control measurements; thus, requiring considerable and adaptable research efforts.

Wild birds are a conspicuous and very diverse group of vertebrates that have occupied niches in a wide range of ecosystems (Stiller and Zhang, 2019). The common proximity of birds to domestic animals and humans is the reason that has allowed the spread of infectious diseases among these groups (Van Hemert et al., 2014). Notorious examples of EID related to wild birds are avian influenza and West Nile virus encephalitis.

In this chapter, a description of basic concepts and background in wildlife diseases, diseases of wild birds and avian malaria is presented, highlighting the impacts on the conservation and wellbeing of wild birds, particularly penguins under human care.

1.2 Diseases of Wildlife

Diseases of wildlife have been long reported but their scientific study has not progressed at the same rate as the investigation of diseases in domestic animals or humans, probably as they are deemed less important from the human perspective. Additionally, they are hard to observe and even harder to control or prevent and the ecological implications, especially in the long term, are difficult to understand and predict. Nevertheless, the study of wildlife diseases is becoming progressively more relevant due to the increasing number of emerging diseases and recognition that many human EIDs originate from wildlife, the use intensification of natural habitats, the changing pressures on the environment and our increased understanding of disease ecology (Daszak et al., 2000).

Diseases in wildlife are usually difficult to address because if they seriously affect their wild host, it is challenging to find ill or dead animals in the wild as they are quickly removed from the environment by predators, scavengers and decomposition (Wobeser, 2006, Ryser-Degiorgis, 2013). Looking for the causative agent directly in the host or in the environment could provide more complete information (Wobeser, 2006), but the size, distribution and movements of populations could imply an extensive evaluation to gather valuable information, and this is particularly challenging to accomplish in remote areas or with limited resources (Ryser-Degiorgis, 2013, Wobeser, 2006). If diseases do not obviously affect the health of their host (maybe because wild animals show few or no signs been reservoirs or incidental hosts) and if their occurrence is not common, then to prove the disease presence and estimate its prevalence could require obtaining a substantial sample size with its implied effort and investment (Wobeser, 2006, Ian Dohoo et al., 2003). Additionally, the lack of complete biological and ecological information, diagnostic limitations, and the analysis and interpretation of the collected data could be challenging (Ryser-Degiorgis, 2013, Wobeser, 2006).

Wildlife diseases could be addressed by their implications to the health of their hosts. They could be relevant for the conservation of vulnerable species, pose a heavy toll on the health and productivity of domestic animals, and therefore in the economy, or represent a risk for human health (Walton et al., 2016). Although a strict classification cannot be done for all diseases as many pathogens and toxins have repercussions in more than one of these groups of hosts or in all of them.

The foregoing highlights the importance and interest in shifting the health management approach for an integrated and multidisciplinary method, centered in prevention and in solving health problems from their origin and not only controlling their effects (Evans and Leighton, 2014). This approach is called One Health and its essential objective is to achieve simultaneously health in these three interdependent but tightly related components: ecosystems, animals and humans (Evans and Leighton, 2014, Black and Butler, 2014). For this, it is necessary to consider the natural events, biodiversity, genetic diversity and human activities. The One Health paradigm can help providing answers for health management problems that arise from global change, it has a solid scientific base and has proved to be effective and with economic benefits (Evans and Leighton, 2014).

1.2.1 Wildlife diseases and public health

Some wildlife diseases are a risk for public health, and domestic animals are commonly the interface between wild animals and humans. More than three fifths of human diseases are thought to be originated from animals (Wardeh et al., 2015). From this vast list, possibly avian influenza (Afanador-Villamizar et al., 2017) and West Nile Virus encephalitis are the most concerning ones related to wild birds (Brugman et al., 2013, Tsiodras et al., 2008).

Influenza type A viruses originate from waterfowl, their natural reservoir are wild birds mainly from the Anseriformes and Charadriiformes orders, and they can infect different groups of hosts, although the mechanism that allows them to do it is not completely understood (Afanador-Villamizar et al., 2017). High pathogenic serotypes frequently cause outbreaks with severe repercussions in the poultry industry and international trade (Chatziprodromidou et al., 2018, Afanador-Villamizar et al., 2017). Occasionally these viruses cause mortality in humans, like the subtype H5N1 (Chatziprodromidou et al., 2018), and get stablished in human populations causing constant health costs. For instance, during the gravest influenza pandemic in 1918, between 20 and 50 million people died (Bengis et al., 2004).

West Nile virus is a vector borne disease that uses mosquitoes for its transmission; it has caused sings and death in several species of wild birds, mainly corvids, it affects horses, and since its introduction in North America, it has caused a serious public health costs (Bengis et al., 2004, Chapman et al., 2017).

The impacts of wildlife diseases have been amplified possibly by the increase of trade globalization, expansion of human populations, intensification of wildlife use, pathogens crossover between domestic and wild animals and climatic changes (Bengis et al., 2004). Therefore, regardless of the complications for the research of wildlife diseases, it is critical to continue with the investigation efforts because the constant changes in the environment are increasing the complexity of the interactions among domestic animals, humans and wild animals, and adaptable responses are constantly needed.

1.2.2 Wildlife diseases and domestic animals

In many occasions, wildlife diseases are a main concern because the wild population is the reservoir in which the pathogens are maintained and transmitted to domestic animals. Controlling the movements of wildlife is complex and usually inefficient to prevent

transmission; hence, alternatives like biosecurity increase, contact prevention and protection against infection of domestic or wild animals are implemented. For example, the contact between domestic and wild ungulates has favoured the transmission of *Brucella*, where *B. abortus* is the most relevant in cattle and *B. melitensis*, in sheep and goats; moreover, these bacteria are zoonotic so serious conflicts between wildlife and farmers arise (Bengis et al., 2004).

Nonetheless, pathogen transmission in the other direction, from domestic to wild animals, is also a concern for conservation as more than 80% of domestic animal pathogens can infect wildlife (Smith et al., 2009). The spillover of canine distemper on diverse wild carnivores in various locations (Smith et al., 2009) and the serologic detection of chicken's infectious bursal disease in penguins illustrates this (Daszak et al., 2000). Therefore, strategies aimed to limit the contact between domesticated and wild animals and to protect the late ones from infection, with vaccination for example or legislation to prevent pathogen pollution may significantly reduce pathogen transmission (Smith et al., 2009, Daszak et al., 2000). For instance, cattle vaccination diminished the declines in wild artiodactyls during the rinderpest epidemic in Africa (Smith et al., 2009).

1.2.3 Wildlife diseases and conservation

At the beginning of the study of diseases in wildlife, it was thought that they were part of an ecological and evolutionary balance and outbreaks occurred after external disturbances or the introduction of exotic diseases. Selective pressures on the host from a given parasite lead to moderate or low virulence levels (Beldomenico and Begon, 2015). Many pathogens share a long co-evolutionary adaptation to their natural host so the pathogen's damage to the host is limited to favour its transmission, and others, after a period of adaptation can promote coevolution, as is suspected in the case of avian malaria, for instance (Grilo et al., 2016). In consequence, diseases could be self-regulated when the susceptible hosts are exhausted due to death, immunity or migration; thus, it was thought that pathogens would not cause extinctions (Joseph et al., 2013, Smith et al., 2009).

From the total number of recent wildlife extinctions, only 3.7% have been related to infectious diseases at least in part, but this could be underestimated mainly due to incomplete data (Smith et al., 2009). Nonetheless, the relevance of infectious diseases for wildlife conservation is progressively more recognised (Joseph et al., 2013, Smith et al., 2009, Daszak et al., 2000). Diseases frequently add a burden to already vulnerable populations or become the main cause of vulnerability, which makes them also a critical

factor for the decline of wildlife and could drive them to extinction (Tompkins et al., 2011). Some diseases could be relevant at different levels; for instance influencing birth and death rates, sometimes without obvious clinical signs, thus affecting their host populations and even influencing the dynamics of entire ecosystems (Tompkins et al., 2011). Some of these particular situations are presented next.

Generalist pathogens (those that can infect a variety of hosts) could spread into communities and seriously affect the populations of threatened susceptible species by using other species as reservoirs, which makes them the greatest threat to disease mediated extinction (Tompkins et al., 2011, Smith et al., 2009). Likewise, the dynamics of vector-borne diseases (those in which the pathogen needs an arthropod to complete its life cycle and be transmitted to a susceptible vertebrate host) are not strongly influenced by the susceptibility of their hosts so they could be highly pathogenic and keep high transmission rates. Cases in which transmission does not depend on host density and by the contrary are frequency-dependent, are particularly propitious to cause conservation threats and extinctions (Joseph et al., 2013, Smith et al., 2009).

When an exotic invasive species is established in a new environment, or a species is artificially introduced or maintained outside its natural distribution, an interaction between its pathogens and the ones from the local community could happen (Tompkins et al., 2011). The natural enemies of exotic species, including pathogens, are not often present in the new habitat, which may provide an advantage over the local competing species that facilitates colonization; this is known as the Enemy release hypothesis (Tompkins et al., 2011, Marzal et al., 2018). Exotic species can also spillover its own pathogens into the local community and they could acquire pathogens from the local community. When the late case happens, the exotic species could be a less competent host, diluting the parasite's impact in the community, but if it is a competent host, it could spillback the pathogen to the native population (Tompkins et al., 2011, Daszak et al., 2000, Smith et al., 2009).

Exotic diseases (those not usually present in a particular area), represent a considerable threat to biodiversity because they can produce severe depopulations of the new naïve hosts during the first contact and local extinctions may occur (Daszak et al., 2000). For instance, the introduction and global spread of chytridiomycosis, produced by the fungi *Batrachochytrium dendrobatidis*, has caused declines in 93 amphibian species worldwide since its global dissemination in the 1960s (Tompkins et al., 2011). Apparently, the sole presence of the fungi is not the only reason for these declines and stressors from human

activities affect the parasite-host interaction (Beldomenico and Begon, 2015). Likewise, endemic diseases such as avian botulism or avian cholera have caused massive outbreaks killing hundreds of thousands of wildfowl in single events over continuous seasons; although, with these birds the long term effects are unknown as they have high reproductive rates that help them to compensate for significant population diminishments (Descamps et al., 2012, Hubalek, 2004).

There are multiple mechanisms that could affect the populations' resilience to be considered and diseases in wildlife should not be conceptualised as isolated elements that are relevant only in certain cases. They are instead related to many other factors that, as a whole, generate the pressures for the subsistence of populations and species. For example, in fragmented habitats the population movements are limited and the contact rates could be higher favouring the transmission of infectious diseases (Smith et al., 2009). Other situations are commented below.

1.2.3.1 Conservation approaches

The current pressures that threaten wildlife conservation have led to the extinction of many species and the population decline of many others. The pressures with the highest known impacts are caused by human activities, such as habitat loss, fragmentation and modification, along with the introduction of exotic invasive species, overexploitation, and the disruption and contamination of the environment (Smith et al., 2009, Beldomenico and Begon, 2015). For example, the habitat loss and fragmentation are particularly relevant in bird ecology as it could reduce biodiversity and cause shifts in species composition (Xu et al., 2018). In most cases, these stressors are permanent and continually growing, having synergistic effects that interact among them and modify the parasite-host interactions in different ways (Beldomenico and Begon, 2015). When the pressures reach a tipping point that overpasses the species population resilience, the decline will be continuous until the population is lost if no intervention is made.

Under the context of multiple menaces for wildlife, the conservation strategies have been classically focused on two approaches, *in situ* and *ex situ*. The first one is represented by natural areas that encompasses part of the species natural distribution range and may have certain category of protection at international, national, regional and local levels. In this case, human intervention is focused on providing all the necessary elements for communities to thrive as close to naturally as is practical. The management of the population of interest is limited, including surveillance and regular check-ups. The actions

towards the conservation of the habitat are more intensive and the species of interest are often used as an emblematic or umbrella species, the protection of which also promotes the conservation of other species and the ecosystem as a whole. Nevertheless, the main challenges of this tactic are negotiating significant actions with the local communities, preventing conflicts between humans and wildlife and ensuring the survival of individuals and populations as the illegal hunting or capture could be a constant threat.

The management of wildlife diseases is closely related to this strategy and it is an increasing practice, mainly motivated by conservation interests (Joseph et al., 2013). It has proved successful in diverse circumstances and could imply the regulation of populations, controlling the interface between domestic and wild animals and even treating wildlife individuals, but the outcomes are difficult to predict and the main limitation is the lack of specific information, particularly for novel pathogens or host-pathogen interactions. Therefore, integrating disease ecology knowledge would improve the management results (Joseph et al., 2013).

The *ex situ* approach is typically represented by zoological gardens, aquariums, and breeding centres, whose aims include the conservation of wildlife, education, public engagement and research (Tribe and Booth, 2003). It has the advantage that diverse interventions can be done in the captive populations, like breeding planning to maintain genetic diversity and recover healthy populations, or behavioural studies that can have implications for wild populations. Their main limitation is that, for some species, it is difficult or impossible to replicate all the conditions of their natural habitat and this in turn poses complications for their health, welfare and breeding success.

Recently, The One Plan Approach (OPA), a new integrative alternative which combines the mentioned conservation strategies, has proved to be highly successful. According to the Conservation Planning Specialist Group (CPSG), part of the International Union for the Conservation of Nature (IUCN), the OPA is the "development of management strategies and conservation actions by all responsible parties for all populations of a species, whether inside or outside their natural range" (CPSG, 2019b). An example is the case of the Central American river turtle (*Dermatemys mawii*) that is distributed in Mexico, Belize and Guatemala and faces an intensive harvesting for human consumption and habitat loss. The stakeholders involved, including a university, the CPSG group, the government and NGOs, developed a management plan for the breeding in captivity that assures a population for

the protection of the species and for legally supplying the turtle meat demand (CPSG, 2019a).

1.2.3.2 Disease ecology in zoos

From the epidemiologic perspective, zoos gather a unique assemblage of potential hosts that belong to all vertebrate classes along with their associated microbiota and parasites (Adler et al., 2011). Many microorganisms that are part of the microbiome of certain species or groups could be highly pathogenic to others if they cross the species barrier. This combination of complex population arrangements makes zoos an exceptional site for the transmission of multispecies pathogens.

Emerging wildlife pathogens like the Elephant Endotheliotropic Herpes Virus (EEHV), are affecting captive populations and due to its recent appearance, the lack of knowledge about its epidemiology, genetics and host interaction, limits the prevention and treatment options making it one of the most devastating infectious diseases for elephants worldwide (Richman et al., 2014). Another example is the bovine spongiform encephalitis in the UK that was present in 58 zoo animals from 17 species which were exposed through contaminated food (Daszak et al., 2000).

Considering the need of joint actions for the conservation of biodiversity, the investigation of wildlife diseases, being in captive or free populations, is crucial to develop efficient strategies and should be done within the context of particular disease dynamics and environmental pressures that threaten the populations; moreover, collaborations among institutions and disciplines are needed to guarantee success in any conservation efforts (Joseph et al., 2013, Daszak et al., 2000).

1.3 Diseases Affecting Wild Birds

1.3.1 Disease ecology of wild birds

The facility to observe, listen to and capture birds makes them attractive subjects for studying biodiversity and disease ecology. They can be indicators of biodiversity changes in the ecosystems because they occupy varied niches and depend on different ecological interactions (Stiller and Zhang, 2019). As top predators they can show issues in the lower levels of the food-web like toxins accumulation, as specialists, they can show particular

environmental features, and as generalists they can interact with a broad scope of other organisms.

An important consideration is that birds have a great mobility in general and with their local movements or migrations, they can carry and introduce pathogens through long distances and initiate new outbreaks (Reed et al., 2003, Van Hemert et al., 2014). For example, migration is considered the cause of the introduction and dispersion of West Nile virus (WNV) in North America (Reed et al., 2003, Pinto et al., 2008) and of avian influenza in certain regions of Europe (Gale et al., 2014). Also, some species congregate in big numbers, like aquatic birds, and these interactions could influence the contact and transmission rates of infectious agents (Van Hemert et al., 2014). Conjunctivitis caused by *Mycoplasma gallisepticum* in house finches (*Carpodacus mexicanus*) and other species, lead to dramatic declines where these birds occurred in high densities and the unusual contagion was attributed to the close contact between individuals in artificial feeders; thus, the adequate management of the feeders was recommended to control the epidemic (Tompkins et al., 2011, Williams et al., 2002). Likewise, the food supply in feeding stations facilitated the infection with *Salmonella typhimurium* DT40 and *Escherichia coli* 086:K61 in the UK (Daszak et al., 2000).

1.3.2 Vector-borne diseases of wild birds

Some vector-borne diseases of wild birds include diseases that are relevant for human health like West Nile virus encephalitis, Japanese encephalitis, Sindbis virus infection and Usutu virus infection (Rizzoli et al., 2015, Hesson et al., 2015b, European Centre for Disease Prevention and Control, 2012). Others, seriously affect the health of wild birds and can pose a conservation threat, such as avian malaria (Lapointe et al., 2012). All these diseases are transmitted by mosquitoes and the same disease could have more than one mosquito vector, or the same mosquito can transmit more than one pathogen, depending on the geographical distribution.

The distribution and prevalence of vector-borne diseases depend on a series of elements and environmental factors. Not only the presence of suitable hosts and the survival of the pathogen during the transmission process are necessary, as in other infectious diseases, but the biology and ecology of the vector are fundamental in the epidemiologic process. The vector needs to survive enough time, be a competent transmitter, and have the appropriate abundance and preference for susceptible hosts. These features are influenced by the environment promoting or limiting the transmission of the pathogens creating a

multidimensional gradient between the optimal and worst conditions (Asigau and Parker, 2018).

1.3.3 Environmental effects on wild bird diseases

It has been established that diseases of wildlife have seasonal patterns and distributions related to environmental variables, including weather and vegetation (de La Rocque et al., 2008). Climate can have a direct impact on infectious diseases that have a development stage outside the final host. Most pathogens do not replicate under a certain temperature threshold and, above it, their growth is strongly modulated; therefore, environmental changes can alter distributions, seasonality and severity of infectious diseases (de La Rocque et al., 2008).

Investigating at deep levels what causes the emergence of infectious diseases reveals that there are interconnected common factors, of which climate change is a critical component (Black and Butler, 2014). The Intergovernmental Panel on Climate Change (IPCC) defines climate change as a modification to climate that persists for a prolonged period (IPCC, 2014). Climate change is additional to natural climate variation and it affects it magnifying some extreme climate events. It is considered a multiplier of threats because it influences direct or indirectly the variables of the complex eco-social system of the planet; it has implications from global to sub-cellular levels (Black and Butler, 2014) and understanding its effects on organisms, in particular microorganisms, is challenging (Cavicchioli et al., 2019). For these reasons, the global climate change has been described as the most important challenge for humankind at the present (Black and Butler, 2014).

Wild birds are one of the vertebrate groups that are most likely to be affected by climate change, because it influences several aspects of their biology and ecology (Crick, 2004). It has caused changes in the population size and distribution, both latitudinal and altitudinal, and in the starting time of migrations. Nevertheless, birds have diverse adaptation capacities depending on the species and the habitats they use (Crick, 2004).

In the case of avian influenza viruses, their reservoir are waterfowl and wetlands, so they need to survive in the environment enough time to be transmitted to a susceptible host and cold temperatures prolong their infectious period. The temperature increase could reduce transmission rates but at the same time, in colder regions, it could find an optimum environment changing their distribution extensively (Gilbert et al., 2008).

The transmission of vector-borne diseases depends on the distribution of the vector, which is strongly associated with temperature and rainfall. Warmer temperatures promote the expansion of *Culex* spp. mosquitoes which are recognised WNV vectors and they have now expanded into northern Canada and Alaska (Van Hemert et al., 2014). Similar situations have been forecasted for human malaria (Chin and Welsby, 2004) and in the case of avian malaria, it is also expected that the climate change will increase the risk for wild birds with stronger effects in Europe and Africa (Garamszegi, 2011). It must be kept in mind as well that the environment affects the pathogens' survival and development in both the vector and the vertebrate host; therefore, predicting these changes poses different challenges.

1.4 Haemosporidians of Wild Birds

Parasites of the order Haemosporida, which belong to the Apicomplexa phylum and the Aconoidasidia class, need a vertebrate host and an arthropod vector to complete their life cycle (Valkiūnas G, 2005). With the advance in molecular and genetic techniques, a large diversity of these parasites has been described and new lineages and morphospecies are constantly described (Bensch et al., 2009, Valkiūnas et al., 2010, Clark et al., 2014, Berthova et al., 2012). Wild birds are commonly found infected by parasites of three main genera from this order, *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. (Valkiūnas G, 2005). The infection effects on the host range from subclinical (without sings of disease) to severe and it could affect reproductive fitness (Knowles et al., 2010) and survival rates (Marzal et al., 2008, la Puente et al., 2010). Nevertheless, the consequences for bird populations and communities in the long term are mainly unknown (Ham-Duenas et al., 2017). The distribution of haemosporidians depends on the abundance and distribution of their vertebrate hosts and vectors, which are influenced by environmental factors such as the landscape, climate and anthropic activities (modification, destruction and fragmentation of natural habitats) (Chasar et al., 2009, Reinoso-Perez et al., 2016, Hernandez-Lara et al., 2017).

Plasmodium spp. is the causative agent of avian malaria and because it can produce severe effects on its host and is the study aim of this thesis, its relationship with wild birds, its vectors and penguins are described in detail in the following sections. *Haemoproteus* spp. and *Leucocytozoon* spp. are commonly found in surveys in wild birds, along with other blood-parasites, with diverse prevalence and infection intensities that depend possibly on the local conditions of the studies; nevertheless, the authors rarely report health issues in

the studied birds (e.g. (Benedikt et al., 2009, Astudillo et al., 2013, Murata, 2002, Chasar et al., 2009)).

In the case of *Haemoproteus* spp., severe anaemia has been reported in some owl species (Atkinson, 2008, Sol et al., 2003). It has been also found that some lineages can reduce the number of fledged offspring, delay the arrival of females to breeding sites, and reproductive success in the great reed warbler (Asghar et al., 2011), and affect negatively body condition in blue tits (Merino et al., 2000). There are some reports of mortalities due to this parasite in captive birds (Ferrell et al., 2007, Olias et al., 2011) and it can reduce the survival probability for feral pigeons (Sol et al., 2003). Sallaberry-Pincheira et al. (2015) reported a low prevalence of *Haemoproteus* spp. in Humboldt penguins (Sallaberry-Pincheira et al., 2015) and Levin et al. (2009) in Galapagos penguins (Levin et al., 2009) but they do not mention sign of disease or epidemiological implications.

On the other hand, clinical signs caused by *Leucocytozoon* spp. are usually unapparent and non-specific, been anaemia the most important one (Atkinson, 2008, Barrow et al., 2019). Ishak et al. described high prevalence of Leucocytozoon in owls from diverse locations and, although they did not assessed health indicators, suggested that there could be implications for the immune system of Spotted owls (Ishak et al., 2008). This parasite has been reported as serious cause of mortality in waterfowl affecting juveniles more severely (Atkinson, 2008). Likewise, it has been found in wild and captive penguins and documented as the cause of death in some cases, which makes it a relevant conservation concern (Vanstreels et al., 2016).

1.4.1 Life cycle of haemosporidians

The haemosporidians' life cycle differs depending on the parasite species, has several stages and involves stages in the bird host and the vector. In general, the cycle starts with an infected mosquito carrying sporozoites, the infectious stage of the parasite; it inoculates the sporozoites while feeding on a susceptible bird; they invade macrophages and fibroblasts, have an asexual reproduction producing merozoites that invade tissues and after another reproduction, they invade erythrocytes where they produce gametocytes (Atkinson, 2008). The gametocytes infect a second mosquito when it feeds on the bird; they undergo through a sexual reproduction and penetrate the intestine of the mosquito and develop as oocyst, which produce sporozoites that migrate to the salivary glands and reach the salivary ducts. Lastly, when the mosquito feeds on a susceptible bird, it inoculates the new sporozoites (Atkinson, 2008, Valkiūnas G, 2005) (Figure 1.1).

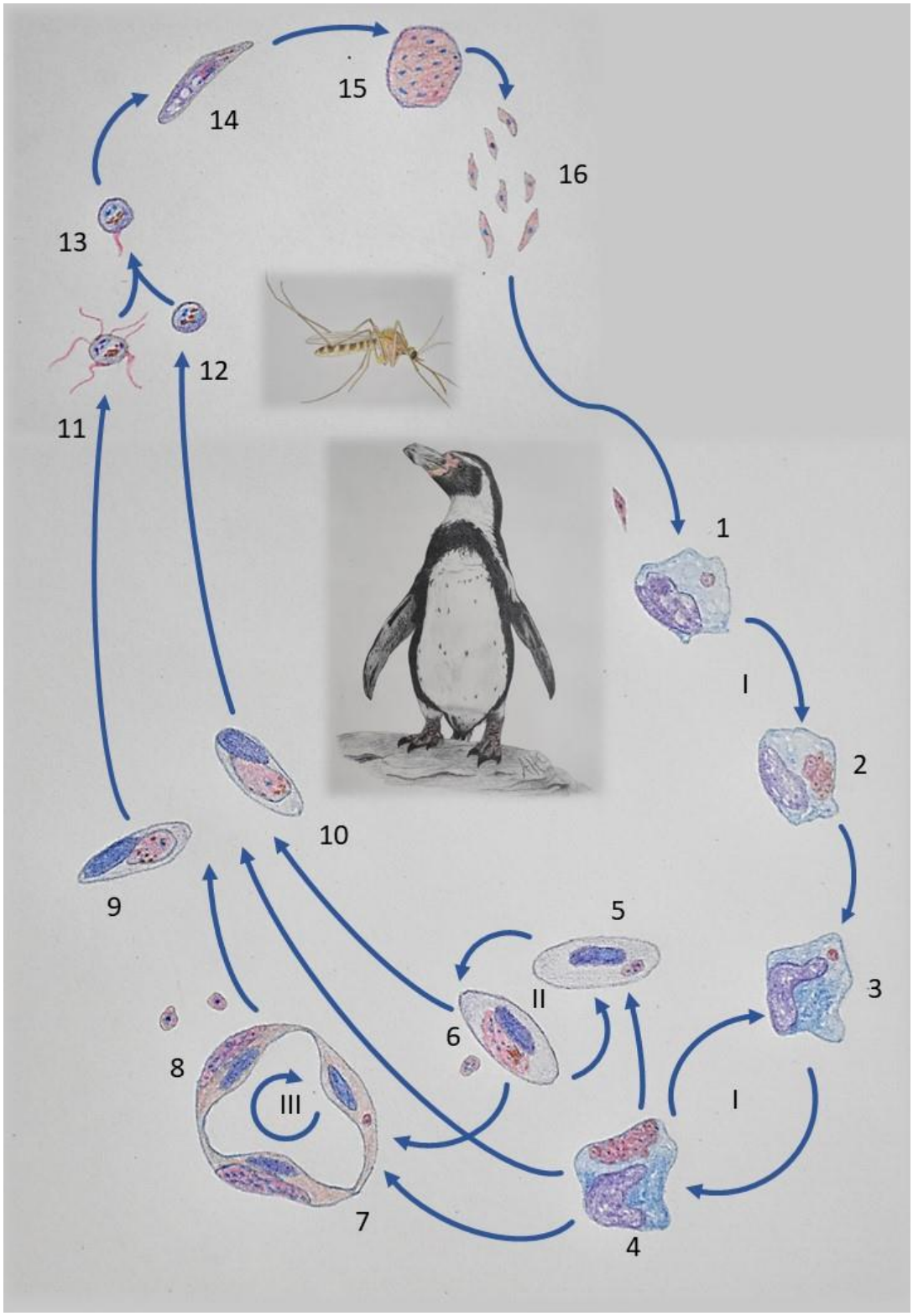

Figure 1.1 Life cycle of avian haemosporidians. Stages: I – primary exoerythrocytic merogony; II – erythrocytic merogony; III – secondary exoerythrocytic merogony; 1 – sporozoite invading a reticuloendothelial cell; 2 – cryptozoite; 3 – merozoite invading a reticuloendothelial cell; 4 – metacryptozoite; 5 – merozoite invading an erythrocyte; 6 – erythrocytic meront; 7 – merozoite invading an endothelial cell; 8 – phanerozoite; 9 – microgametocyte; 10 – macrogametocyte; 11 – exoflagellation of microgametes; 12 – macrogamete; 13 – zygote; 14 – ookinete; 15 – oocyst; 16 – sporozoites. Original illustrations by the author, based on Grilo *et al*. (2016).

1.4.2 Avian malaria in wild birds

Avian malaria is the most common and well-studied disease caused by haemosporidians of wild birds and it was one of the most reported according to the results of Annex Two. Ronald Ross discovered that malaria was transmitted by a mosquito vector studying the infection in birds and during the end of the 19th and the beginning of the 20th century, avian malaria was the study model for human malaria (Huijben et al., 2007), until rodent malaria was discovered and proved to be a more versatile model (Braga et al., 2011). However, avian malaria has resurged as relevant model for ecological and evolutionary studies of host-vector-parasite associations due to the complex life cycle of the parasite and the diverse interactions of the vector with the vertebrate host (Braga et al., 2011, Pigeault et al., 2015) and the mentioned importance for the conservation of wild birds. At the same time, many relevant aspects of its epidemiology and genetics remain unclear due to the diversity of the parasite, the vectors involved in its transmission and the high diversity and mobility of the potential hosts, many of which have never been tested for the parasite.

Avian malaria is caused by protist parasites from the genus *Plasmodium* and the species *P. relictum* and *P. elongatum* are the most frequent ones associated with the disease in wild birds (Sallaberry-Pincheira et al., 2015). This parasite needs a vector for its transmission and the mosquitoes from the genera *Culex* are the most recognised one. Until now, there are more than 60 described species of *Plasmodium* (Vanstreels et al., 2016) that had infected more than 600 species of birds and thanks to the increasing use of molecular techniques, over 900 lineages have been described (Zele et al., 2014).

At the beginning of the study of these parasites, the speciation was done based on the observation of blood smears dyed with the Giemsa technique as a gold standard, looking for particular morphological features such as the number and size of vacuoles, the presence or absence of pigments or the presence of certain stages in the host's blood (Valkiūnas G, 2005). These continue to be useful for the description of new species and when the resources are limited, because after a simple observation the diagnosis can be made (Valkiūnas et al., 2010). The limitations of this approach are that it requires a degree of expertise, it is time consuming, and has its limitations in terms of the actual differences that can be noticed among species and regarding sensitivity.

Plasmodium infection could be unnoticed and the detrimental effects in the long-term are not known with precision on many occasions (Foster et al., 2007); for instance, it can affect

the fitness and survival of some species like Blue Tits (*Cyanistes caeruleus*) (Lachish et al., 2011). In particular cases it can cause death, limit the population distribution and even cause the extinction of certain avian species, as it was observed on some honeycreepers (Drepaniidae) in the Hawaiian Islands (Foster et al., 2007), which along with the penguins (Spheniscidae) are the most susceptible groups of birds affected by *Plasmodium* spp. (Vanstreels et al., 2014).

The previous comment is related to the hypothesis that avian malaria parasites have a long history of co-evolution with their hosts (Huijben et al., 2007). This corresponds with the observed disease effects, as the groups of birds seriously affected are those distributed in areas where the vector and parasite are naturally absent, or that are too dry, cold or windy and thus, unfavourable for the mosquito vector and the parasite is commonly absent; whereas in areas where the mosquito and the parasite are present, the local birds are more commonly infected and they do not present disease signs usually.

After an initial acute infection in which the parasite can be found in blood and cause mild disease signs like anaemia or weight loss, the individuals may recover and the parasite remains dormant in the liver and other organs going into a chronic infection (Atkinson, 2008). Nonetheless, sudden dead has been observed when the parasitaemia decreased or even in the chronic stage, due to blockage of brain capillaries with phanerozoites, leading to cerebral ischaemia (Ilgunas et al., 2016). Moreover, the parasite could return to the systematic circulation in latent infections or accelerate the development of pre-patent infections if the individual faces stressful situations that diminish its immune response which has been observed in House sparrows after been captured (Santiago-Alarcon et al., 2018). Migration and the subsequent breeding season have been also suggested as factors that produce the relapse of latent infections, which would give a transmission advantage to the parasite when potential vectors are abundant and juvenile naïve hosts enter the population (Mendes et al., 2013). Environmental events could also be relevant for the relapse of dormant infections and influence the pathogenicity of the parasite, as suggested in the case of the food limitation for Galapagos penguins caused by the El Niño (Meile et al., 2013). Other sources of stress, like reproduction and moulting, should be also considered in the relapse of the parasite (Meile et al., 2013). It has been also suggested that seasonal relapses of the parasite induced by the increase in sexual hormones and corticosterone, are relevant for the persistence of haemosporidians across multiple seasons (*Leucocytozoon fringillinarum* in White-crowned Sparrow) (Murdock et al., 2013). The hypothesised reason for the presence of chronic infections and their relapse is that it is an

adaptative strategy that allows the parasite to survive during the non-transmission season and maximises the possibilities of later transmission (Cornet et al., 2013).

Changes in the environment can influence the epidemiologic process modifying the pathogen's development and survival, transmission rates and host susceptibility (Smith et al., 2009). Climate change is modifying the distribution of vectors, the duration of transmission season and the replication rate of the pathogen in the vector, and in consequence, the range of potential hosts, the disease transmission efficiency and the incidence of many vector-borne diseases (Cavicchioli et al., 2019). For instance, I It is predicted that dengue fever and human malaria will expand dramatically (Smith et al., 2009, Daszak et al., 2000). This is also expected for avian malaria and there is concern about the possibility of the mosquito vector and the parasite reaching colonies of penguins and other birds that have not been naturally exposed to the parasite; thus adding pressure to the survival of these species in the wild (Garamszegi, 2011). Previous studies concluded that unfavourable conditions for the vector caused a null or rare occurrence of blood parasites in the Arctic; but more recently, evidence of *Plasmodium* transmission was found in Alaska which points out the risk of warmer temperatures for the exposure of naïve species to these parasites (Van Hemert et al., 2014).

1.4.3 Ecology of avian malaria vectors

Several species of mosquitoes have been mentioned as potential vectors for avian malaria, like those belonging to the genera *Culex* spp., *Culiseta* spp. *Mansonia* spp., *Adeomyia* spp., *Aedes* spp., *Anopheles* spp. and *Armigeres* spp., as the parasite has been found in mosquitoes in the wild and it can complete its life cycle in them, at least under laboratory conditions, proving that they are competent vectors (Vanstreels et al., 2016). To confirm if certain mosquito is a competent vector, transmission experiments should be done, the infectious stage of parasite should be found by micro-dissection in the salivary glands or the parasite should be extracted from the salivary glands (Becker et al., 2010).

The mosquitoes from the genus *Culex* spp. are the most widely recognised and distributed vector worldwide. *Cx. quiquefasciatus* is the endemic avian malaria vector in North America and Asia, whereas in Europe, the main avian malaria competent vector is *Cx. pipiens*, the house mosquito (Zele et al., 2014). *Cx. pipiens* is broadly distributed in the UK and has been previously found with *Plasmodium* spp. and being the transmitting vector to wild bird populations such as blue tits (*Parus caeurelus*) (Cosgrove et al., 2008).

1.4.3.1 *Culex pipiens*

Mosquitoes belong to the Diptera order of insects and to the Culicidae family that has two subfamilies, Culicinae and Anophelinae (Becker et al., 2010). There are 34 species of mosquitoes in the UK, 28 belonging to Culicinae and six to Anophelinae (Brugman, 2016, Medlock et al., 2012). The first subfamily includes seven genera and 28 species, *Aedes* (3), *Coquillettidia* (1), *Culex* (4), *Culiseta* (7), *Dahliana* (1), *Ochlerotatus* (11) and *Orthopodomyia* (1) and the second one, contains only *Anopheles* with six species (Medlock et al., 2012).

The *Culex* genus is included in the *Culicinae* subfamily and it has four species in the UK, *Cx. modestus* and *Cx. territans* have limited distributions but *Cx. pipiens* and *Cx. torrentium* are broadly distributed in the UK (Cranston et al., 1987). These species are closely related to each other and although the morphological differences among them are not always conspicuous, they differ by genetic and biological traits; therefore, these mosquitoes are usually referred to as the *Culex* complex which also includes the *Cx. pipiens pipiens* and *Cx. pipiens molestus* biotypes (Becker et al., 2012).

The life cycle of mosquitoes comprises four development stages, egg, larvae, pupae and adult (Figure 1.2). The *Cx. pipiens* female prefers shallow and organic rich water sources such as ponds and any container that could hold a water pocket to lay rafts of 150 to 350 eggs that float on the water surface. After one or two days, the larvae hatch and start feeding on organic matter; there are four larval instars (L1-L4) that are determined by an exoskeleton moulting (Becker et al., 2010, Foster and Walker, 2019). The next stage is the pupae, in which the metamorphosis takes place; the pupa does not feed anymore but it is highly mobile to avoid predation. Although the eggs, larval instars and pupae are entirely aquatic stages, they need to breathe air; the eggs do it by transpiration through their shell, the larvae use a siphon at the end of the abdomen and the pupae has two trumpets on the cephalothorax. The final stage is the adult, both males and females feed on plant's sap and flower's nectar but the females require a blood meal in order to produce eggs (called anautogeny) (Becker et al., 2010). If the female does not get enough blood in a single meal, it can look for another one which could be from a different host. *Cx. pipiens* is ornithophilic, meaning that it prefers to feed on birds; although it has been reported that it can also feed on mammals and humans (Foster and Walker, 2019). A single female can produce up to three egg rafts in its life span which is between two and three months. The time required for each development stage is on average two days in temperate climates but could be up to a week depending on the environmental conditions; therefore, the total time required

between the egg and the adult could be from ten days to few weeks (Becker et al., 2010). Depending on the period of favourable conditions, there could be several generations through a year. This mosquito overwinters as adult by finding shelter in places that are not going to freeze during winter. In nature, it goes inside caves, behind loose tree barks or stone cracks, but in artificial environments it selects sheds, buildings and a variety of structures. The activity season of adult *Cx. pipiens* in the UK typically goes from April to October and starts entering shelters for overwintering in late September; although there are variations depending on the regional and local weather conditions (Brugman, 2016, Vaux and Medlock, 2015).

Culex pipiens is recognised as the vector of several viruses that are transmitted between humans and wild birds including West Nile virus, Sindbis virus, and Usutu virus (Foster and Walker, 2019, Hesson et al., 2015b). However, a main limitation for the study of these diseases, as well as for avian malaria, is the difficulty to have a precise identification of the mosquito. It belongs to a multispecies complex, as noted above, and the identification of many of them can only be done by the dissection of male genitalia or molecular techniques which are not done in many studies. *Cx. pipiens* is the most widely recognised species in Europe (Hesson et al., 2014) but due to the lack of complete identification of the species within its group, there could be a bias in the association between this species and the pathogens that it transmits, including avian malaria. Hence, different members of the complex could be the responsible vectors and have diverse roles in the transmission of pathogens (Fonseca et al., 2004).

Figure 1.2. Life cycle of *Culex* spp. mosquitoes. Original illustrations by the author.

1.4.4 Avian malaria in penguins

Penguins are not constantly exposed to *Plasmodium* spp. but when they are, they could present serious signs and mortality rates. After the Hawaiian honeycreepers, penguins are considered as the second most threatened group of birds by avian malaria which is mentioned as the most important cause of mass mortality in captive penguins (Silveira et al., 2013). From the 18 species of penguins, thirteen have been reported infected with the parasite (Grilo, 2014). Most of the avian malaria adverse effects have been described in zoos and rescue centres possibly because the individuals are under a close and constant examination and the health condition is easier to notice, but it could be also due to the translocation of individuals to areas where the local mosquito and parasites are present and the penguins are incidentally infected as a spillover from local avifauna (Vanstreels et al., 2019). *Plasmodium* spp. has also been found in wild populations of penguins, two yellow-eyed penguins (*Megadyptes antipodes*) and one Fiordland crested penguin (*Eudyptes pachyrhynchus*) died in 2015 due to *P. elongatum* and more than 25 death penguins were infected with the parasite in 2018 ; thus the disease is considered emergent for these penguins in New Zealand (Hunter and Alley, 2019). This represents the main health concern for the conservation of penguins; as the distribution of vector-borne diseases in highly influenced by the environmental conditions, due to climate change, avian malaria could reach the coastal areas that have been historically free from the parasite and the penguin colonies could suffer high mortalities (Garamszegi, 2011).

The suspected reason for the high susceptibility of the penguins to avian malaria is that they have evolved in environments that due to the low temperatures, low humidity or strong wind currents, the vectors are not present and the transmission of the disease does not occur frequently (Grilo et al., 2016). Therefore, they have not gone through a co-evolution process with the parasite as other bird species and their immune system is not able to generate a protective response when first infected (Vanstreels et al., 2016, Grilo et al., 2016). Still, *Plasmodium* spp. has been found in wild populations of penguins but the parasitaemia is low in general and clinical signs have been observed in few cases (Vanstreels et al., 2016, Sallaberry-Pincheira et al., 2015). The prevalence of *Plasmodium* in wild penguins shows a high variability, from 0.7% to 34% detected using blood smears in African penguins, and from 2.1% to 42.9% with molecular techniques in Galapagos penguins, for example (Vanstreels et al., 2016). Nevertheless, when the penguins face stressful situations like injuries or oil spills the prevalence could be raised (Gracyzk et al., 1995).

There has been growing evidence of abortive infections in wild birds, which happen in non-competent hosts when the parasite develops in the initial tissue stages but it is unable to complete its life cycle (Chagas et al., 2017). For instance, mortalities suggestive of abortive infections have been observed in captive parrots in Europe (*Haemoproteus* spp.) (Olias et al., 2011) and other zoo birds (*Haemoproteus* spp. in Montezuma oropendolas and the lesser Flamingos and *Plasmodium* spp. in green jays) (Ferrell et al., 2007). Nonetheless, it is not clear if all the *Plasmodium* spp. infections are abortive in penguins, which is understandable considering the variable susceptibilities of the diverse parasite lineages, penguin species and mosquitoes involved in the transmission. In some cases, the authors find gametocytes in penguin tissues proving a complete development of the parasite (e.g. (Vanstreels et al., 2014, Vanstreels et al., 2019)) but in others, the subclinical state of infection suggest abortive infections (Levin et al., 2009, Levin et al., 2013). In the most pathogenic cases, it seems that vascular occlusion produces vasculitis in lungs, spleen and liver due to the development of meronts in endothelial cells (Vanstreels et al., 2014, Grilo et al., 2016). Although it could be complicated to clarify if the pathogenicity depends on the parasite lineage or the penguin susceptibility or if a particular infection is abortive or not, the comparison with domestic and other wild birds could be helpful (Vanstreels et al., 2015).

Because of the aforementioned, it is recommended to complement the molecular diagnosis of the infection with the observation of blood-smears, where the parasite stages (gametocytes) can be observed to confirm a competent host-parasite relationship; likewise, histological studies and chromogenic in situ hybridization can find evidence of the parasites in tissues (Gonzalez et al., 2015, Chagas et al., 2017, Dinhopl et al., 2011).

1.4.5 Avian malaria prevention and control

The management of avian malaria in wild birds is a huge challenge due to the impossibility of delimiting the populations of birds and mosquitoes, their changing dynamics and the extensive areas that they cover. Nevertheless, some habitat management, in accordance with local conditions, could influence the prevalence of the infection (e.g. removing mosquito oviposition sites) (Reiter and Lapointe, 2009).

In captivity, the birds are under partially controlled circumstances in an artificial environment, thus there are more possible actions. For the prevention, an integrated mosquito management program could be implemented, including eliminating mosquito oviposition sites like artificial and natural water containers, biological control (mosquito

feeding fish or *Bacillus thuringiensis israelensis* bacteria) mosquito refuge sites, protecting the birds with nets or in enclosed facilities, and prophylactic treatment can be dispensed (Adler et al., 2011). When the disease is already present, the treatment becomes the main intervention, although there are not standardised protocols or doses and the results have been inconsistent, some penguin colonies recover quickly and in others the mortality rate cannot be contained (Bueno et al., 2010).

1.5 Objectives

This thesis aims to contribute to the knowledge and management of avian diseases, particularly avian malaria in captive populations of penguins in relation to the mosquito responsible for its transmission in the UK. The results could be applied to the prevention and control of the disease promoting in this way the conservation and welfare of penguins under human care.

Chapter Two presents the general material and procedures that were used for obtaining the results of chapters Three, Four and Five.

The third chapter describes the research done in Chester Zoo, Cheshire, and Flamingo Land, Yorkshire, capturing mosquitoes with the purpose of understanding the local ecology of avian malaria and use this knowledge to implement effective prevention measures.

As we found a considerable number of blood-fed mosquitoes, an additional analysis was developed to explore the mosquitoes feeding preferences and the biting risks in both zoos. This is described in Chapter Four.

The surroundings in the sampling areas and temperature and humidity data were analysed in Chapter Five to find associations between the local environmental and climatic conditions and the abundance of mosquitoes and the parasite prevalence.

There is limited evidence about the epidemiology and management of avian malaria events in zoological gardens and wildlife parks, although the veterinarians and animal keepers could have valuable information. Chapter Six presents the outcomes of an online survey that was used to investigate the status of avian malaria in captive birds from the UK and the analysis of the risk factors detected.

The final chapter integrates the findings of this work presenting final recommendations for the better care of captive birds susceptible to avian malaria and the study of mosquito ecology.

For achieving the aims of the project, we divided it into two main parts: the mosquito analysis and the parasite analysis. It is important to mention that I was responsible for the mosquito analysis, and the parasite analysis, including the diagnosis from mosquitoes and birds and the subsequent genetic investigation, was led by Merit Gonzalez-Olvera. We assisted each other for the fieldwork at the zoos and the post-mortem examinations and sampling from dead birds.

Chapter Two

General Materials and Methods

This methodology was applied to the research presented in chapters Three, Four and Five. Chapter Six followed different methodology that is explained in the corresponding chapter section.

2.1 Study Design

We established collaboration with Chester Zoo and Flamingo Land for the surveillance of mosquitoes and blood parasites in their premises, both housing Humboldt penguins (*Spheniscus humboldti*). We had two sampling seasons in Chester Zoo, in 2017 and in 2018, and one in Flamingo Land in 2017.

The University of Liverpool Veterinary Ethics Committee granted the ethical approval for all the activities of this project during the proposed duration with a further amendment (reference VREC532a). Likewise, the scientific committees of both zoos gave their ethics approval and scientific consent for the project. The Approved Research Proposal Form of Chester Zoo can be found in Appendix 2.1; Flamingo Land does not follow a similar procedure.

2.2 Sampling Techniques

The assessment of the mosquito community consisted of three main parts, the sampling of adult mosquitoes, immature mosquito stages and overwintering mosquitoes.

2.2.1 Adult mosquitoes

The adult mosquitoes were captured using two kinds of traps, the BG-Mosquitaire trap and the CDC-Gravid trap. These traps were selected for their efficiency at capturing our mosquito of interest, *Culex pipiens*. A comparative analysis of traps can be found in section 3.3.3.

The BG-Mosquitaire® trap has a fan inside a plastic container that needs to be plugged into the power; it has a regulator that transforms the electric current from 220 V to 12 V. Before the fan, it has a plastic funnel to which the funnel net and the capture net are attached. The basic attractant of this trap is the BG-Sweetscent®, a sachet that contains a lure based on lactic acid to mimic mammalian's sweat (Figure 2.1). Therefore, this trap captures mosquitoes looking for a blood meal and it was designed particularly for the Asian tiger mosquito (*Aedes albopictus*), nonetheless it is also highly effective for *Cx. pipiens*.

The CDC-Gravid trap model 1712 was designed by the Centre for Disease Control of the United States specifically for capturing *Culex* mosquitoes. It consists of an electric fan located inside a plastic cylinder and powered by a 6 V battery; the fan is placed over a tray that contains four litres of infusion media as attractant and covered with a capture net (John W. Hock Company, 2013) (Figure 2.2). The infusion media is prepared with tap water (40 L), hay (200 gr), brewer's yeast (2 gr) and milk powder (2 gr) following Reiter (1983) recommendation the mix is then rested for at least one week before use (Reiter, 1983). The female mosquitoes that are looking for a suitable place for laying their eggs are attracted by the infusion media, they do an exploratory flight over the water and when they get close to the fan, it sucks them into the capture net. An advantage of this trap is that gravid females have had at least one blood meal before and thus, the possibility of being infected with vector-borne pathogens is higher than mosquitoes captured with other traps.

Figure 2.1. BG-Mosquitaire trap: releases and odour to attract mosquitoes looking for a blood-meal. White arrow: TinyTag® logger, black arrow: sign to prevent public disturbances.

Figure 2.2. CDC-Gravid trap: attract females looking for a place to lay their eggs with an oviposition media. White arrow: battery, black arrow: collection net.

2.2.2 Immature mosquitoes

For the sampling of immature mosquitoes (larvae and pupae), a 500 ml cup with a 1.5 m long pole was used (termed dipper), in the accessible potential oviposition water bodies. In each site, I fully submerged the dipper in the water surface close to the edge and among the vegetation. In some places, it was not possible to fill the cup with a single submersion, so I did as many as needed to obtain approximately 500 ml per dipping. After each

submersion, I emptied the dipper into a plastic tray and, using a plastic pipette, I transferred all the mosquito larvae of any instar and the pupae into a 30 ml universal flask for transportation (Figure 2.3).

2.2.3 Overwintering mosquitoes

During the winter, we explored the inside of buildings and sheds around our sampling areas looking for mosquitoes. We used an Improved CDC-Backpack Aspirator Model 1412 (John W. Hock Company, 2017) powered with a 12 V battery to catch them by screening walls and ceilings systematically. The internal area of the buildings was not estimated as it was highly variable and some parts, like the high ceilings and walls, or animal enclosures were inaccessible (Figure 2.4).

Figure 2.3. Sampling of immature mosquitoes. The samples were taken from water body and emptied into a tray from which the mosquitoes were taken. White arrow: dipper. Picture by M. Gonzalez-Olvera.

Figure 2.4. Sampling of overwintering mosquitoes. A portable aspirator was used inside sheds, staff buildings and animal enclosures. Picture by M. Gonzalez-Olvera.

2.3 Sampling Protocol

The fieldwork was done weekly requiring two days of activities; the BG-Mosquitaire traps were operating constantly and the CDC-Gravid traps were run for 24 hours. In the first day we replaced the collection net of BG-Mosquitaire traps with an empty one and we prepared the CDC-Gravid traps by filling the tray with infusion media, placing the capture net and turning on the trap.

During the second day, we replaced the nets from the BG-Mosquitaire traps, collected the nets from the CDC-Gravid traps, turned the last ones off, poured the infusion media away and covered the trap and battery with the tray. The larva sampling took place on the second day, doing ten submersions per site that were on average two meters apart; therefore, this sampling encompassed an area rather than a single point (Table 2.1).

Table 2.1. Sampling protocol activities.

Trap / Sampling	Activity	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
BG-Mosquitaire trap	Operating							
	Nets collection							
CDC-Gravid trap	Preparation							
	Operating							
	Nets collection							
Immature mosquitoes	Sampling							

The manufacturer recommends changing the attractant of the BG-Mosquitaire traps every six to eight weeks; we renewed it every six weeks to reduce the possible bias due to the diminishing efficiency of the attractants.

In the second sampling year we were assisted by the zoo staff doing the second day activities, picking up and replacing the nets and sending them to Leahurst campus, with the exception of the dipping which we did on the first day.

With this sampling protocol, we had two collections per week; the first one was from the BG-Mosquitaire traps that were operating for six consecutive days and the second one from the 24 hours period for both kinds of traps. Although we identified all mosquitoes and tested all females for the *Plasmodium* parasite, we expected that the BG-Mosquitaire

collections after six days would provide more representative results to assess the mosquito community, whereas the 24 hrs collections would be better preserved for the molecular analyses. Additionally, the results of both types of traps from the 24 hours collections were comparable.

2.4 Processing of Samples

2.4.1 Adult mosquitoes

After collection, the nets of both traps were transported to Leahurst campus and placed in a -20°C freezer for around two hours to kill the captured insects. If the nets were wet after collection, they were hanged to dry before freezing for the better preservation of the specimens. When the insects were dead, the nets were emptied on a petri dish (30cm diameter) and the mosquitoes were separated from other accompanying insects. The mosquitoes were stored at -20°C in Eppendorf tubes inside the corresponding bags per trap and collection until further identification and analysis.

2.4.2 Immature mosquitoes

In the insectary at Leahurst, the content of the flasks was emptied into a plastic pot which then was placed inside an entomological cage (BugDorm®). I fed the mosquito larvae weekly with brewer's yeast and placed a cotton ball damped with a sugar-saturated solution to feed the mosquito adults when they eclosed. When all the larvae from a cage had emerged as adults, I placed them into a -20°C freezer, first to kill them and then, after identification, into Eppendorf tubes for storing. Representative individuals of each species and sex were pinned following entomological techniques for further reference and training purposes, although we did not capture both sexes for all species.

The adult mosquitoes that eclosed from pupae were used for testing and training in the molecular methods. For instance, eight *Culex* spp. were used to test the extraction and PCR protocols for their molecular identification (see section 2.7). Also, the test described in section 2.7.3 used mosquitoes from this source and the students that assisted the project were trained in the molecular identification using eight *Culex* spp. each. Likewise, eight *Culex* spp. females were used for testing the haemosporidian PCR positive controls (see section 2.9.3); two mosquitoes were used as negative controls, 1 µl of penguin blood positive to *Plasmodium* spp. was added to other four mosquitoes before extraction and 1 µl of murine *Plasmodium* (*P. berghei*) control was added to two mosquitoes. The positive

controls produced clear bands in all cases and the negatives, did not. In all these tests, the concentration of DNA was measured using a Nanodrop® spectrophotometer (ND-1000) before performing the PCR to assess the quality of extraction and nucleotides concentration.

2.4.3 Overwintering mosquitoes

The mosquitoes were transported in plastic pots and killed at -20°C. They were separated from accompanying insects and stored at -20°C until identification and analysis.

2.5 Sampling Areas

2.5.1 Choice of sampling areas

We looked for suitable locations for placing the traps considering their proximity to the following features:

- Mosquito oviposition sites, like ponds, artificial containers, and other structures that could hold water for several days.
- Mosquito resting places, such as vegetation and buildings.
- Zoo bird exhibits.
- Priority around the penguins' exhibit.

The sampling areas were established as a 30 m diameter circle in which we included one BG-Mosquitaire, one CDC-Gravid tap and, if possible, an immature sampling area. With the QGIS 3.2© Software, I used the Open Street Map as the base layer and the coordinate reference system: WGS84 - EPSG: 4326, I added the coordinates of each trap and larvae sampling point to create a triangle or a line (in the absence of a larvae sampling point), calculated the centroid and then transformed it into the 30 m circle of the sampling area; in this way, I could confirm that the sampling areas did not overlap and I also measured the distance between the traps inside them and from other sampling areas to confirm that they were at least 10 m apart to prevent interference.

2.5.2 Sampling areas in Chester Zoo

We proposed 12 sampling areas and prioritise them depending on their proximity to zoo birds, vulnerable birds, and resting and oviposition sites (Figure 2.5). The two areas with low priority (A8 and A9) were dismissed due to the difficulty of providing power for the BG-Mosquitaire traps or the high influx of visitors. We also set temperature and humidity

loggers (TinyTag®) programmed to record every hour, at less than one meter from the BG-Mosquitaire traps, to assess the conditions in which the mosquitoes look for a blood meal. After the loss of one of the traps by public disturbance, we placed signs warning about the traps, next to all those that were in public areas (Figure 2.1). The traps were placed where they could remain without interfering with the activities in the zoos, considering staff and visitors, and do not represent a risk for any person or zoo animals. Both types of traps were placed near water bodies or vegetation, but not covered by it for at least one meter above, away from busy areas, protected from direct sunlight, artificial lighting, wind and rain, when possible.

From 2017 to 2018, some areas of Chester Zoo were renovated; A2, A3 and A11 were closer to these building sites but the exact location of traps was unaffected, so we could continue with the sampling as planned; with the exception of the BG-Mosquitaire trap in A3 which had to be moved 15 m away from the original point. For the 2018 sampling season, we cancelled the areas A5, A6, and A7, because they were the less productive in terms of captured mosquitoes. Due to the increasing importance of the project and the avian malaria risk for the penguins, we added a new area, A13, inside the penguins' exhibit to assess the mosquito activity closer to the penguins (Figures 2.6 and 2.7). Likewise, the immature mosquitoes sampling areas were cancelled for the 2018 season. Table 2.2 describes the locations of the traps.

2.5.3 Sampling areas in Flamingo Land

The sampling areas were planned and prioritised as described earlier; we proposed eight areas and four were implemented (Figure 2.8). Due to the particular setting of the zoo and the variability on water sources, there were no immature sampling areas available for our study; the ponds in the zoo proved to be too deep and lacking enough organic matter to attract mosquito females and just few larvae were sporadically observed.

The configuration of the sampling areas was in the same way as before, although this could not be done in A1 as the traps were inside an animals' exhibit and protected with metallic mesh in a limited space, thus they were 8.2 m apart. The sampling points are shown in Figures 2.9 and 2.10. The final location of the traps was done with the same surrounding and safety considerations and the TinyTag® loggers were set equally. There were no changes in the sampling throughout the season and all the traps were in staff areas or animal exhibits so there were no interferences from the public. The description of the sampling areas in Flamingo Land is included in Table 2.3.

Table 2.2. Sampling areas and sampling points for the adult mosquito traps and immature mosquitoes in Chester Zoo.

Sampling Area	Priority	Sampling Point	Location	Kind of Area	Coordinates* Latitude, Longitude
A1	High	M1	Next to flamingo's night enclosure	PA	53.22736, -2.87812
		G1	Bushes next to the bridge	PA	53.22740, -2.87776
		L1	Shores of flamingo's pond	SA	N/A
A2	High	M2	Behind ice-cream shop	SA	53.22666, -2.87753
		G2	Bushes across the footpath next to the tree	PA	53.22657, -2.87760
A3	High	M3	In front of the doors of the birds' enclosures	SA	53.22606, -2.87700
		G3	Bushes between the pond and the wood fence	SA	53.22594, -2.87691
		L3[a]	Pond shores	SA	N/A
A4	Medium	M4	In the corner of the owl's enclosure	SA	53.22524, -2.87818
		G4	Bushes in the corner next to the owl enclosures	SA	53.22515, -2.87823
		L4	Plants' waterbed and round tanks	SA	N/A
A5[a]	Medium	M5	Bushes aside red panda enclosure, close to access gate	PA	53.22426, -2.87900
		G5	Bushes in front red panda enclosure	PA	53.22428, -2.87917
		L5	Ponds in Chinese rock garden	PA	N/A
A6[a]	Medium	M6	Bushes next to dragons' building	PA	53.22700, -2.87924
		G6	Bushes between flamingos and dragons	PA	53.22693, -2.87912
A7[a]	Medium	M7	Bushes aside Andean condor enclosure	PA	53.22709, -2.88149
		G7	Bushes aside Andean condor enclosure, next to the trees	PA	53.22713, -2.88133

Table 2.2. Continued**.**

A8[b]	Low	M8	Bushes in front macaws	PA	N/A
		G8	Bushes in front macaws	PA	N/A
A9 [b]	Low	M9	Bushes aside Chimpanzee breading centre	PA	N/A
		G9	Bushes aside Chimpanzee breading centre	PA	N/A
A10	Medium	M10	Bushes in wetland enclosure, closer to access	AE	53.22551, -2.88489
		G10	Bushes in wetland enclosure	AE	53.22564, -2.88494
		L10[a]	Shores in wetland enclosure	AE	N/A
A11	Medium	M11	Bushes next to conservation golf	PA	53.22519, -2.88269
		G11	Bushes aside conservation golf, opposite side	PA	53.22535, -2.88290
		L11[a]	Ponds in conservation golf	PA	N/A
A12	High	M12	Behind penguin kitchen	AE	53.22691, -2.87739
		G12	Bushes in front of giant otter enclosure	PA	53.22706, -2.87723
A13[c]	High	M13	Inside penguin exhibit, next to filtering system	AE	53.22684, -2.87770
		G13	Inside penguin exhibit, next to filtering system at the opposite side	AE	53.22692, -2.87779

* Coordinate Reference System: WGS84 - EPSG: 4326. M = BG-Mosquitaire trap, G = CDC Gravid trap, L = Immature mosquitoes sampling point, PA = public area, SA = Staff Area, AE = Animal Enclosure, N/A = Not Applicable (the larvae sampling was done in an area rather than a single point). a = these areas and sampling points were active only during the 2017 season, b = these areas were proposed but were cancelled due to logistic complications and low priority, c= this area was active only during the 2018 sampling season.

Table 2.3. Sampling areas and sampling points for the adult mosquito traps and immature mosquito in Flamingo Land.

Sampling Area	Priority	Sampling Point	Location	Kind of Area	Coordinates* Latitude, Longitude
A1	High	M1	Close to the bridge next to Penguins enclosure	AE	54.20568, -0.80614
		G1	Close to the bridge next to Penguins enclosure	SA	54.20566, -0.80620
		L1[b]	Puddles and water pockets surrounding penguin enclosure	AE	N/A
A2	High	M2	In external enclosure (South America exhibit)	AE	54.20506, -0.80518
		G2	In external enclosure (South America exhibit)	AE	54.20514, -0.80511
		L2[b]	Puddles and water pockets in external enclosure	AE	N/A
A3	Medium	M3	Below the trees close to Lemurs exhibit	AE	54.20719, -0.80685
		G3	Below the bushes next to Lemurs exhibit	AE	54.20715, -0.80662
		L3[b]	No suitable place found	AE	N/A
A4	Medium	M4	Next to a shed behind the Camels enclosure	SA	54.20863, -0.80323
		G4	Bushes behind the Camels enclosure	SA	54.20850, -0.80310
		L4[b]	Pond behind the Camels enclosure	SA	N/A
A5[a]	Medium	M5	Bushes in front of Red panda enclosure	PA	N/A
		G5	Bushes in front of Red panda enclosure	PA	N/A
		L5[b]	Ponds in front of Red panda enclosure	PA	N/A
A6[a]	Low	M6	Bushes in front of Camels enclosure	PA	N/A
		G6	Bushes in front of Camels enclosure	PA	N/A
		L6[b]	Ponds in front of Camels enclosure	PA	N/A

Table 2.3. Continued**.**

A7 [a]	High	M7	Below the trees towards hippopotamus enclosure	SA	N/A
		G7	Below the trees towards hippopotamus enclosure	SA	N/A
		L7 [b]	No suitable place found	SA	N/A
A8 [a]	Low	M8	Below bridge in Ibis enclosure	AE	N/A
		G8	Bushes in Ibis enclosure	AE	N/A
		L8 [b]	Pond in Ibis enclosure	AE	N/A

* Coordinate Reference System: WGS84 - EPSG: 4326. M = BG-Mosquitaire trap, G = CDC Gravid trap, L = Immature mosquitoes sampling point, PA = public area, SA = Staff Area, AE = Animal Enclosure, N/A = Not Applicable. a = these areas were proposed but not implemented due to logistic complications, b = we could not find suitable and constant areas for the immature mosquitoes sampling.

Figure 2.5. Sampling areas at Chester Zoo. Red line: perimeter of the zoo; Read area: penguin exhibit; Orange circles: sampling areas; Blue line: area under renewal. Areas A8 and A9 were proposed but not implemented due to logistic implications and low priority. Areas A5, A6 and A7 were active only during the 2017 season and area A13 was added for the 2018 sampling season.

Figure 2.6. Sampling points at Chester Zoo in sampling areas A1, A2, A3, A6, A12 and A13. Red line: perimeter of the zoo; Red area: penguin exhibit; Orange circles: sampling areas; Blue line: area under renewal. The location of the traps is labelled by the letter M and the corresponding area number for the BG-Mosquitaire traps, G for the CDC-Gravid traps and L for the immature mosquitoes sampling areas.

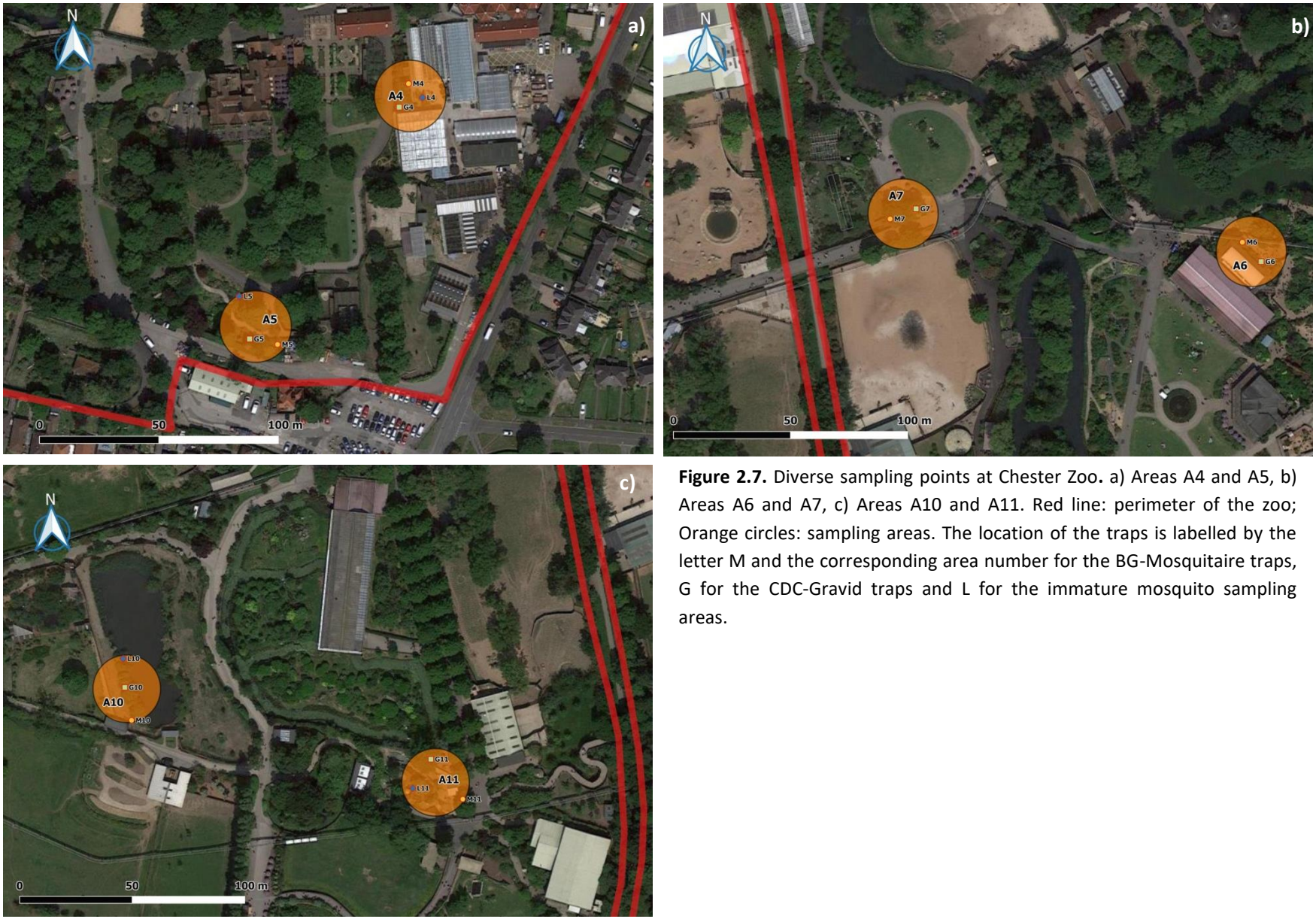

Figure 2.7. Diverse sampling points at Chester Zoo**.** a) Areas A4 and A5, b) Areas A6 and A7, c) Areas A10 and A11. Red line: perimeter of the zoo; Orange circles: sampling areas. The location of the traps is labelled by the letter M and the corresponding area number for the BG-Mosquitaire traps, G for the CDC-Gravid traps and L for the immature mosquito sampling areas.

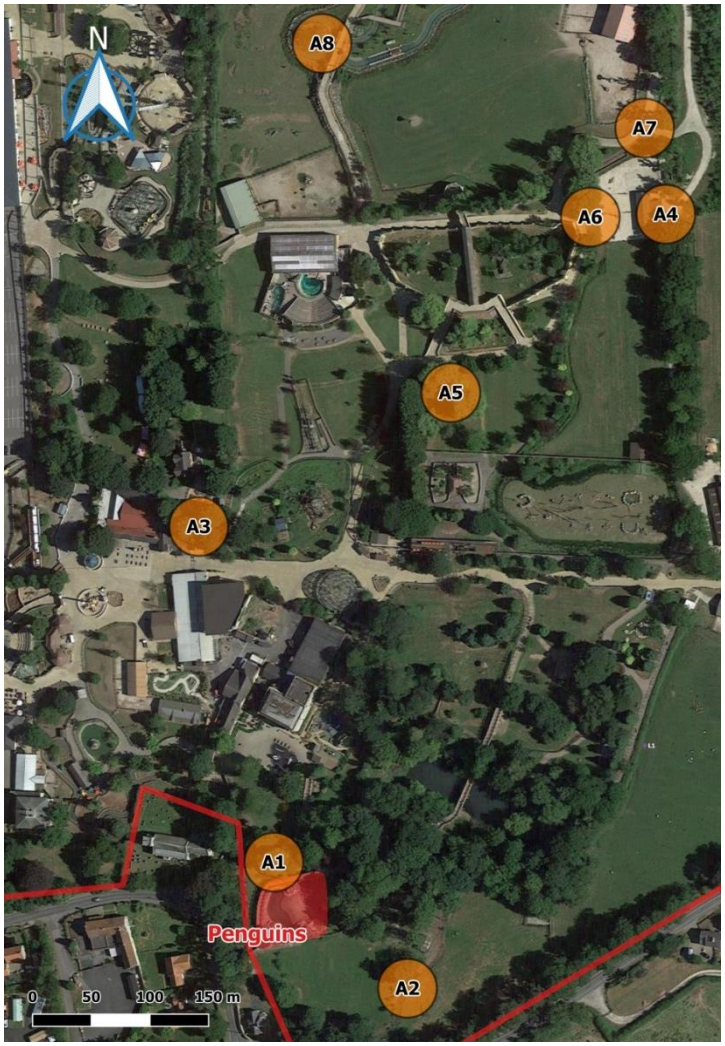

Figure 2.8. Sampling areas at Flamingo Land. Red line: perimeter of the zoo; Read area: penguin exhibit; Orange circles: sampling areas. Areas A5 to A8 were proposed but not implemented due to logistic implications and alternative close areas.

Figure 2.9. Sampling points at Flamingo Land in sampling areas A1 and A2. Red line: perimeter of the zoo; Orange circles: sampling areas. The location of the traps is labelled by the corresponding area number and the letters M for the BG-Mosquitaire traps and G for the CDC-Gravid traps.

Figure 2.10. Sampling points at Flamingo Land in sampling areas A3 and A4. Red line: perimeter of the zoo; Orange circles: sampling areas. The location of the traps is labelled by the letter M and the corresponding area number for the BG-Mosquitaire traps and G for the CDC-Gravid traps.

2.6 Morphological Identification of Mosquitoes

The identification of all the captured mosquitoes was done by individual observation under a stereoscopic microscope and following identification keys (Becker et al., 2010, Cranston et al., 1987). The mosquitoes were placed on a chill table at -16°C during the process. After identification, they were stored in individual tubes with unique labels that identified collections, traps, origin (adults or immature mosquitoes) and zoos. At the same time, the abdomens of the blood-fed mosquitoes were cut and stored in individual tubes.

The list of morphological features of *Cx. pipiens* or *Cx. torrentium* and *Culiseta annulata* used for the identification of adult mosquitoes and for training purposes, is presented in Table 2.4. It was not possible to identify several mosquitoes because they were damaged; in the case of legs loss, we could only identify them to the subfamily or genus level and when the abdomen was missing, damaged or without scales on the tergites, we could identify them only to the genus level.

Table 2.4. Morphological features of the most common species of mosquitoes during our samplings. The numbers correspond to the characteristics illustrated in Figure 2.11.

	Culex pipiens/torrentium		*Culiseta annulata*	
	Female	**Male**	**Female**	**Male**
Head	**1** Pilous antenna	**2** Plumous antenna	**1** Pilous antenna	**2** Plumous antenna
	3 Short palps	**4** Long palps, upturned and with hairs	**3** Short palps	Long palps, straight
Thorax	**5** Scutellum trilobed	**5** Scutellum trilobed	**5** Scutellum trilobed	**5** Scutellum trilobed
	6 No prespiracular hairs	**6** No prespiracular hairs	**7** With prespiracular hairs	**7** With prespiracular hairs
	8 No postspiracular hairs	**8** No postspiracular hairs	**8** No postspiracular hairs	**8** No postspiracular hairs
	May have scales in the prealar area	May have scales in the prealar area	With postspiracular scales	With postspiracular scales
Abdomen	With abundant scales	With abundant scales	With abundant scales	With abundant scales
	9 Parallel sided and rounded at the tip	**9** Parallel sided and rounded at the tip	**9** Parallel sided and rounded at the tip	**9** Parallel sided and rounded at the tip
	10 Cerci short, hardly visible		**10** Cerci short, hardly visible	
	11 With white scales in the front margin of segments forming complete bands	**11** With white scales in the front margin of segments forming complete bands	**11** With white scales in the front margin of segments forming complete bands	**11** With white scales in the front margin of segments forming complete bands
Legs	**12** Without rings on any tarsus	**12** Without rings on any tarsus	**13** With rings in the tarsus	**13** With rings in the tarsus
	14 Tibia and first tarsi almost the same size	**14** Tibia and first tarsi almost the same size	Scales in femur and tibia intermingled black and white	Scales in femur and tibia intermingled black and white
	15 With pulvilli in the last tarsi	**15** With pulvilli in the last tarsi	**16** With sub apical white ring on femur	**16** With sub apical white ring on femur
	17 With simple claws		**18** Broad white rings in tarsus	**18** Broad white rings in tarsus
			19 With central white ring on first tarsi	**19** With central white ring on first tarsi
Wings	**20** With narrow scales	**20** With narrow scales	**20** With narrow scales	**20** With narrow scales
	Without dark spots	Without dark spots	**21** With dark spots	**21** With dark spots
			22 Costa mainly with dark scales	**22** Costa mainly with dark scales

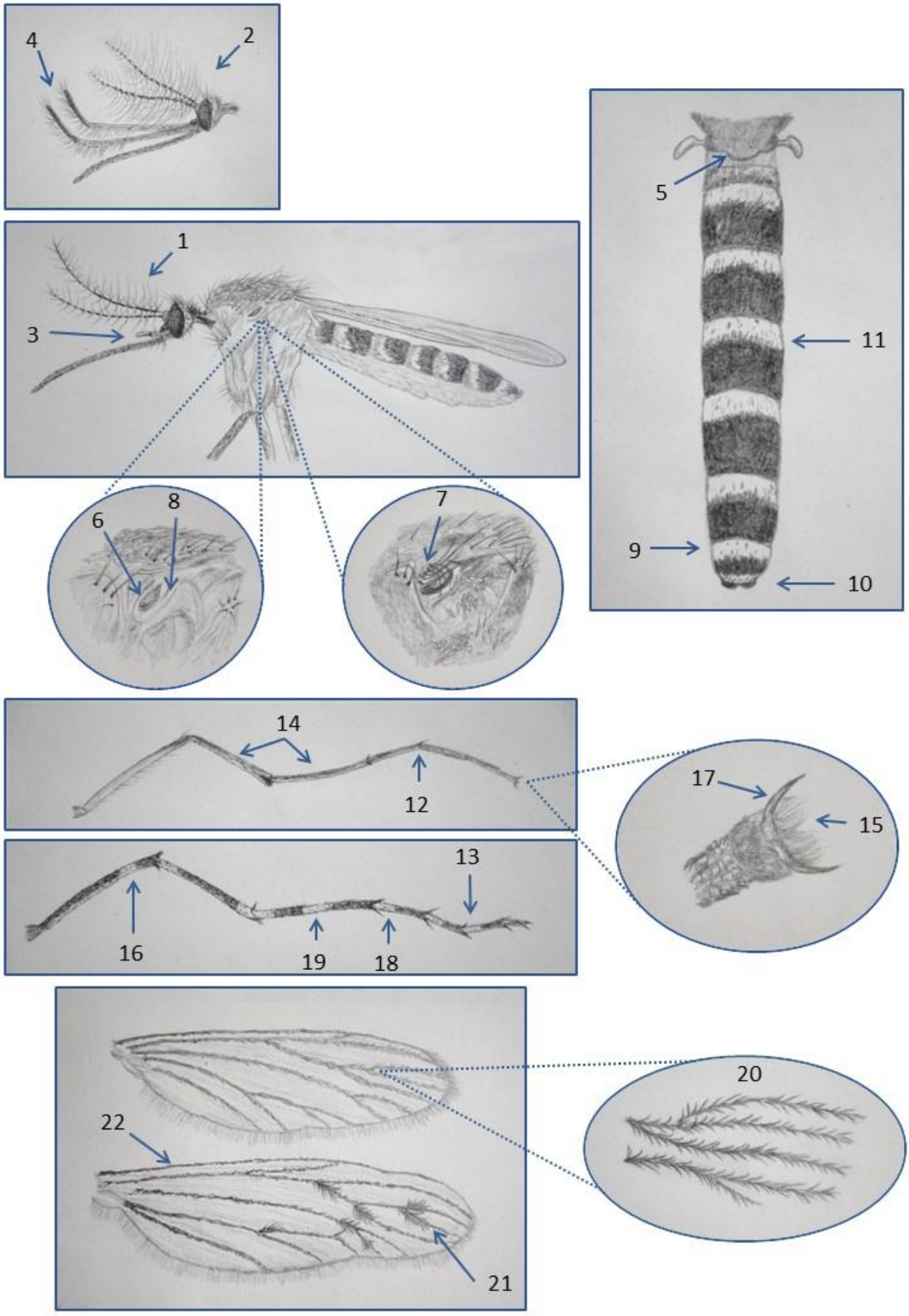

Figure 2.11. Morphological identification features of *Culex pipiens* and *Culiseta annulata*. The numbers correspond to the characteristics in Table 2.4. Original illustrations by the author.

2.7 Molecular Identification of Mosquitoes

It is difficult to distinguish between *Culex pipiens* and *Culex torrentium* as the anatomic features needed are subtle, could be missing and are not consistently presented, and because of this, the specific role of this species in the transmission of pathogens and avian malaria in particular, has not been thoroughly explored as they have not been differentiated in many studies (Hesson et al., 2014). Moreover, these mosquitoes could have different epidemiological roles across their activity season as *Cx. torrentium* is more frequently found earlier than *Cx. pipiens* (Hesson et al., 2014).Thus, we used the enzymatic digestion method developed by Hesson *et al.* (2010) (Hesson et al., 2010) to set apart these two species. Only female mosquitoes fully identified by morphology were identified this way.

2.7.1 DNA extraction

The DNA extraction was done using the OMEGA Bio-Tek E.Z.N.A ® Tissue DNA kit. The manufacturers' instructions were followed with the exclusion of the optional steps for transferring the sample to a new Eppendorf tube after homogenisation (the material amount did not interfere with subsequent steps) and for a second elution step.

The mosquitoes were placed into a 2 ml Eppendorf tube with a 2 mm diameter steel bead, 200 µl of TL Buffer and 20 µl of OB Protease Solution; then, they were homogenised with a QIAGEN© TissueLysser at frequency of 24 Hz per second for 2 minutes, and they were incubated for three hours at 55 °C or at 37 °C overnight. Afterwards, 220 µl of BL Buffer were added, the tubes were vortexed, and incubated at 70 °C for 10 minutes. Then, 220 µl of pure molecular grade ethanol were added, the tubes were vortexed, the content was transferred to a DNA Mini Column placed into a collection tube and centrifuged for one minute. The filtrate was discharged, 500 µl of HBC Buffer were added, the column was centrifuged for 30 seconds, the filtrate was discharged, and the column was placed into a new collection tube. The Wash Buffer was added (700 µl) and the columns were centrifuged; this step was repeated, and the empty columns were dried by centrifuging for 2 minutes. The Mini Column was transferred to a new 1.5 ml Eppendorf tube, 100 µl of Elution Buffer, preheated at 70 °C, were added and the columns rested at room temperature for at least 2 minutes before centrifuging for one minute. The columns were removed, and the extractions were stored at -20 °C. All centrifuging steps were done at maximum speed (1500 rpm) and filtrates were disposed as chemical waste. The quality of

extraction and nucleotides concentration was evaluated in 195 samples from three collections using a Nanodrop® spectrophotometer (ND-1000).

2.7.2 PCR and enzymes protocol

The DNA extract was used for a conventional PCR and an enzyme digestion protocol (Hesson et al., 2010), with some modifications. The primers C1-J-2183: CAACATTTATTTTGATTTTTTGG and TL2-N-3014: TCCAATGCACTAATCTGCCATATTA were used to amplify an 830 bp region of the COI-3' mitochondrial gene. The reaction mix contained per sample, 7.9 µl ddH_2O, 4 µl DNA extract, 2 µl PCR buffer, 2 µl MgCl2, 1 µl deoxynucleoside triphosphate (dNTPs) (0.125 mM each), 1 µl BSA, 0.8 µl of each primer, 0.4 µl of DMSO, and 0.1 µl Taq DNA polymerase native enzyme, for a final volume of 20 µl. The BSA was added to the original protocol and the DNA extract was increased from 2 µl to 4 µl after several samples failed to yield a clear band on the agarose gel.

The PCR reaction was done in a Applied Biosystems 2720 Thermal Cycler with the following conditions: initial denaturation 95 °C for 3 minutes, five cycles of denaturation (94°C for 30 seconds), annealing (47.8°C for 30 seconds) and extension (72°C for one minute); 30 cycles of denaturation (94°C for 30 seconds), annealing (49.8°C for 30 seconds) and extension (72°C for one minute), final extension (72°C for seven minutes) and indefinite storage at 8°C.

The restriction enzymes used were FspBI, which reacts to C'TAG, only present in *Cx. torrentium*, and the SspI enzyme that recognizes AAT'ATT, only present in *Cx. pipiens*; both enzymes cut the PCR product between ~620 and 210 bp. When the product is exposed to an enzyme, a positive reaction is observed by two discernible bands in the agarose gel, one with a weight of around 620 bp and another at 830 bp of the uncut product; sometimes, the cut band of 201bp was observed. A negative reaction consists on one uncut band at 830 bp. The PCR products were exposed to enzymes separately, so the results included a positive and a negative reaction for each sample.

The enzymes were prepared by mixing, for the FspBI enzyme, 1 µl of FastDigest Buffer, 0.04 µl of enzyme, and 8.96 µl of ddH_2O, and for the SspI enzyme, 1 µl of FastDigest Buffer, 0.08 µl of enzyme, and 8.92 µl of ddH_2O. The final volume was 15 µl after adding 5 µl of PCR product for each enzyme. The thermocycler conditions were incubation at 37 °C for 5 minutes and inactivation at 80 °C for 5 minutes. Afterwards, the products were visualised in

a 1% agarose gel stained with peqGreen (3 µl per 150 ml) once the electrophoresis took place at 110 V for 50 minutes in 0.5 X TAE buffer (Tris-acetate-EDTA).

The reagents were supplied by Thermo Fisher Scientific Inc. and the primers, by Sigma-Aldrich Company Ltd.

2.7.3 Identification by individuals and by pools

We identified the mosquitoes by PCR in two ways, individually and by pools, to facilitate the lab work. The proportion of *Cx. pipiens* (95.4%) and *Cx. torrentium* (4.6%) was estimated using 195 mosquitoes from three collections. With this proportions and a binomial distribution approach we estimated the probability of getting a positive result in different pool sizes; for instance, in a pool of five mosquitoes we would expect at least one *Cx. torrentium* mosquito with a probability of 0.21, with pools of ten mosquitoes, it would be 0.38, with pools of 20, 0.61, and with pools of 30, 0.76 (Table 2.5). Although we could have used pools of whole mosquitoes and estimate the minimum infection rate (MIR) (Karki et al., 2016) assuming that at least one mosquito per pool was infected with the parasite or in the case of the species, at least one mosquito of each species was present, we would not have been able to obtain precise data about the parasite, the species of the infected mosquitoes and their relation. Therefore, for the mosquito molecular identification, we used pools of legs. If the result was positive for *Cx. pipiens* and *Cx. torrentium*, we analysed the mosquitoes belonging to the corresponding pool individually.

I prepared a test for assessing the sensitivity of the methods for the leg pools using the mosquitoes from the immature samplings as a source of *Cx. pipiens*, and for a *Cx. torrentium* control, I used specimens from a different project where mosquitoes were captured in North Wales and previously identified at the larvae stage by PCR. Additionally, a single leg and two legs per species, and a proboscis and a pair of wings were tested for considering their use in the case of damaged mosquitoes without enough legs. The identification of all these mosquitoes was confirmed by individual PCRs using their abdomens. The DNA extraction and PCR protocols were as described before. The mosquito legs were organised as presented in Table 2.6.

From these results, it was confirmed that a single leg can be detected individually and in pools of up to 30 legs and when two legs of *Cx. torrentium* were added to the pool, its band was clearer. The test with the proboscis and the pair of wings was negative to both enzymes and did not yield a PCR product. Although using pools of 20 or 30 mosquitoes

would have saved more lab materials, the high likelihood of positive pools means that a bigger number of mosquitoes would have to be tested subsequently for the precise identification. Therefore, we decided to use pools of 10 mosquitoes with two legs per mosquito to have an accurate result and more manageable material during the process, thus expecting to have one third of pools (33.51%) positive to both enzymes.

Table 2.5. Probability of obtaining a positive result for each mosquito species. Calculated with a binomial distribution and a q proportion of 0.046.

	Positive result probability			**Positive result probability**	
Pool size	***Cx. pipiens***	***Cx. torrentium***	**Pool size**	***Cx. pipiens***	***Cx. torrentium***
(x)	**(p)**	**(q)**	**(x)**	**(p)**	**(q)**
1	0.95	0.05	16	0.47	0.53
2	0.91	0.09	17	0.45	0.55
3	0.87	0.13	18	0.43	0.57
4	0.83	0.17	19	0.41	0.59
5	0.79	0.21	20	0.39	0.61
6	0.75	0.25	21	0.37	0.63
7	0.72	0.28	22	0.35	0.65
8	0.69	0.31	23	0.34	0.66
9	0.65	0.35	24	0.32	0.68
10	0.62	0.38	25	0.31	0.69
11	0.60	0.40	26	0.29	0.71
12	0.57	0.43	27	0.28	0.72
13	0.54	0.46	28	0.27	0.73
14	0.52	0.48	29	0.26	0.74
15	0.49	0.51	30	0.24	0.76

Table 2.6. Pools of mosquito legs used to assess the sensitivity of the DNA extraction and PCR methods. A positive result was observed as two bands in the agarose gel, for *Cx. pipiens* when the enzyme SspI was used and for *Cx. torrentium* whit the FspBI enzyme.

Pool/sample number	Number of *Cx. pipiens* legs	Number of *Cx. torrentium* legs	Total	*Cx. pipiens* result	*Cx. torrentium* result
1	1	0	1	+	-
2	2	0	2	+	-
3	0	1	1	-	+
4	0	2	2	-	+
5	9	1	10	+	+
6	8	2	10	+	+
7	19	1	20	+	+
8	18	2	20	+	+
9	29	1	30	+	+
10	28	2	30	+	+

2.8 Analysis of Blood-fed Mosquitoes

2.8.1 DNA extraction

The DNA extraction from abdomens of blood-fed mosquitoes was done using the OMEGA Bio-Tek E.Z.N.A ® Tissue DNA kit following the manufacturer's instructions, with the addition of 200 µl of PBS (phosphate buffered saline) per sample before homogenisation (Brugman et al., 2017). The homogenisation was done using sterile plastic pestles or with a QIAGEN© TissueLysser at frequency of 24 Hz per second for 2 minutes. This procedure was the same as described in section 2.7.1. The DNA extractions were stored at 4 °C until further processing for no more than two weeks and at -20 °C afterwards.

2.8.2 PCR protocol

A nested PCR was used to amplify a 758 bp region of the *cox*1 gene following the procedure of general primers for vertebrates proposed by Alcaide *et al.* (2009) (Alcaide et al., 2009). The first PCR reaction had a final volume of 10 µl containing 4.94 µl ddH_2O, 1 µl PCR buffer, 1 µl $MgCl_2$, 1 µl dNTPs (0.125 mM each), 1 µl DNA extract, 0.5 µl of DMSO, 0.17 µl BSA, 0.16 µl of each primer, M13BC-FW (TGTAAAACGACGGCCAGTHAAYCAYAARGAYATYGG) and BCV-RV1 (GCYCANACYATNCCYATRTA), and 0.07 µl Taq DNA polymerase native enzyme. The thermocycler conditions used were: initial denaturation 94 °C for 4 minutes, 35 cycles of annealing (45°C for 40 seconds), extension (72°C for one minute) and denaturation (94°C for 40 seconds), final extension (72°C for seven minutes) and indefinite storage at 8°C.

The second PCR contained 18.05 µl ddH_2O, 3 µl PCR buffer, 3 µl dNTPs (0.125 mM each), 2.04 µl MgCl2, 1.5 µl of DMSO, 1 µl first PCR product, 0.48 µl of each primer, M13 (GTAAAACGACGGCCAGTG) and BCV-RV2 (ACYATNCCYATRTANCCRAANGG), 0.25 µl BSA, and 0.2 µl of Taq DNA polymerase native enzyme. The reaction was carried out with the following thermocycler conditions, initial denaturation 95 °C for 3 minutes, 16 cycles of annealing with a decreasing temperature from 60 °C to 45 °C (-1°C per cycle) during 40 seconds, extension (72°C for one minute) and denaturation (94°C for 40 seconds); followed by 24 cycles of annealing (45 °C for 40 seconds), extension (72 °C for 40 seconds) and denaturation (94°C for 40 seconds) with a final extension at 72°C for seven minutes and indefinite storage at 8°C.

Negative controls were added every five samples and DNA extract from liver of Black headed gull (*Larus ridibundus*) was used as a positive control. The amplification was verified by electrophoresis at 110 V for 50 minutes in a 1% agarose gel stained with peqGreen (3 µl per 150 ml) inside 0.5 X TAE buffer (Tris-acetate-EDTA) using 10 µl of PCR product. The PCR products of the samples that created a band in the right weight were sent to the company Macrogen Europe for sequencing using the Sanger method with the M13 primer in the forward direction. All the PCR negative samples were tested at least twice.

2.9 Avian Malaria Parasite Analysis

2.9.1 Sample collection

We requested the zoo veterinarians to provide us with surplus blood and blood smears from zoo birds, and carcasses or tissue samples from zoo birds and free wild birds that have been found dead in the zoo grounds or euthanized.

The surplus blood was taken by the zoo veterinarians during routine health checks or for diagnosis purposes. We requested ideally 200 µl per individual, collected into an EDTA-coated microtainer, although the volume depended on the bird size and condition and on the amount used by the veterinarians. At the moment of collection, we also requested the veterinarians to prepare three thin blood smears per bird, air dried for three minutes and fixed with absolute methanol (Ventim et al., 2012b, Valkiūnas G, 2005). The blood smears were stained using Giemsa's solution and screened with a light microscope for parasite detection for 30 minutes according to Valkiunas (2005). These samples were stored and

transported to the University of Liverpool, Leahurst campus, at <4 °C and the analysis of the blood samples were done in no more than three days after collection.

We performed the post-mortem examination (PM) of the birds into a biosafety cabinet. We recorded the birds species, age (juvenile or adult), sex, weight, body condition (scores scale 1-5: 1 = emaciated, 5 = overweight), degradation condition (scores scale 1-4: 1 = death within a few hours, 2 = frozen soon after dead, 3 = evident signs of autolysis and degradation, 4 = severe degradation and loss of organs' integrity) date of death or date founded, PM date, possible cause of death if it was evident and other observations. We took approximately 1 cm^3 of brain, heart or clotted blood, and liver, into 2ml Eppendorf tubes. The PMs of birds that were of diagnostic interest for the zoo veterinarians were done by Julian Chantrey, professor of veterinary pathology at the University of Liverpool, who took the same organ samples in the same way for the parasite testing. The carcasses and organ samples were stored at -20°C.

We also recommended the zoo staff to inform us when they decided to take samples from the zoo birds or if any of the birds presented avian malaria signs like anaemia, lethargy, anorexia or ruffled feathers (Lapointe et al., 2012), in order to process the samples as soon as possible and concentrate our efforts in the most relevant species.

The smears staining and observation as well as the bird organs and mosquitoes testing for the avian malaria parasite were performed by Merit Gonzalez-Olvera.

2.9.2 DNA extraction of bird samples

The DNA from the blood and organ samples was extracted using a Qiagen DNeasy® Blood and Tissue Kit following the manufacturer's instructions (Qiagen, 2006). The blood was centrifuged for 2 minutes at maximum speed and only the cell's pellet was used for the DNA extraction. The organ samples were macerated on a petri dish using disposable sterile scalpels before DNA extraction.

2.9.3 DNA extraction of mosquito samples

After the morphological and molecular identification of the mosquitoes, in the case of the *Culex* species, the well-preserved specimens (with head, thorax and abdomen) were tested individually for blood parasites; whereas the damaged mosquitoes (without abdomen) were pooled by collection, trap and species in up to five mosquitoes per pool.

The mosquito's DNA was extracted individually in two ways; first, using the Omega E.Z.N.A. kit, for the mosquitoes used for analysing the presence or absence of the parasite (see section 2.9) and then, with the method used by Livak (1984) (Livak, 1984) for insects DNA extraction for the rest of the mosquitoes to reduce costs.

The PCR used enables to screen for *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. species from the same samples, following the protocol proposed by Hellgren *et al.* (2004) (Hellgren et al., 2004). It is a nested PCR that amplifies DNA of the mitochondrial cytochrome *b* gene of the parasites from the three genera in the first step and uses different primers in the second one to differentiate between *Plasmodium* spp. and *Haemoproteus* spp. from *Leucocytozoon* spp.; then, the parasite lineages can be identified analysing the sequences.

The primers used for the first PCR were: HaemNFI and HaemNR3; in the second PCR, HaemF and HaemR2 were used for *Plasmodium* spp. and *Haemoproteus* spp. and HaemFL and HaemR2L for *Leucocytozoon* spp. (Table 2.7).

Table 2.7. Primers used for the detection of blood parasites. Hellgren *et al.* (2004).

PCR	Primer	Primer Sequence 5'-3'	Amplicon size	Parasite
First	HaemNFI	CATATATTAAGAGAAITATGGAG	617 bp	All
	HaemNR3	ATAGAAAGATAAGAAATACCATTC		
Second	HaemF	ATGGTGCTTTCGATATATGCATG	480 bp	*Plasmodium*
	HaemR2	GCATTATCTGGATGTGATAATGGT		*Haemoproteus*
Second	HaemFL	ATGGTGTTTTAGATACTTACATT	478 bp	*Leucocytozoon*
	HaemR2L	CATTATCTGGATGAGATAATGGIGC		

The final volume of the first PCR was 25 µl, including 1 µl of DNA extract, 1.25 mM of each dNTP, 1.5 mM $MgCl_2$, 0.6 mM of each primer, and 0.5 units of Taq DNA polymerase. The thermocycler conditions used were an initial denaturation at 94°C for 3 minutes, 22 cycles of 30 sec at 94°C, 30 sec at 50°C and 45 sec at 72°C, a final extension at 70°C for 10 minutes and storage at 8°C; the number of cycles was modified to increase the product yield as the original protocol stated 20 cycles. For the second PCR, the final volume was again 25 µl with the same reagents proportions and including 1µl of the first PCR product; it was done separately for *Plasmodium* spp. and *Haemoproteus* spp. and for *Leucocytozoon* spp. with

the corresponding primers, creating products of 478 bp and 480 bp respectively. The thermocycler conditions were the same but over 35 cycles. The successful amplifications were observed after electrophoresis in a 2% agarose gel.

The positive samples were sent to the company Macrogen Europe for sequencing using the Sanger method. Afterwards, the results were aligned and edited with BioEdit© software and compared in the NCBI Standard Nucleotide BLAST (NCBI, 2018a) and MalAvi (Egerhill M et al., 2016) databases.

2.10 Minimum Sample Size Estimation

We identified most of the mosquitoes as *Culex pipiens* since the first samplings; thus, I estimated the sample size for proving the presence or absence of the parasite and the expected prevalence for the host of interest, wild birds, penguins and mosquitoes. I did a literature review looking for papers reporting the prevalence of *Plasmodium* parasites in the three hosts of interest and with preference for works done in Europe and in zoological collections. I found fourteen suitable papers and organised the data in Table 2.8. From this table, I used, as the minimum expected prevalence, the lowest prevalence reported in wild birds and penguins and I used the two lowest ones reported for *Cx. pipiens* to have a broader estimation.

For estimating the minimum samples size for proving the presence of the infection, I used the formula for infinite populations from Dohoo *et al.* (2003) (Table 2.9):

$n = \ln \alpha / \ln q$

where: n = required sample size

α = 0.05 for 95% confidence or 0.01 for 99% confidence

q = 1 – (expected prevalence)

I used the formula from Thrusfield (2007) for calculating the prevalence of infection in an infinite population:

$n = (1.96^2 P_{exp} (1-P_{exp})) / d^2$

where: n = required sample size

P_{exp} = expected prevalence

d = desired absolute precision

1.96^2 = constant multiplier

In the case of the penguins, which population comprised 42 individuals in Chester Zoo, I used the adjustment for a known size population (Thrusfield M, 2007) (Table 2.10):

$n_{adj} = (N \times n) / (N + n)$

where: n_{adj} = required adjusted sample size

N = study population = 42

n = sample size for an infinite population

Table 2.8. *Plasmodium* spp. prevalence reported in some wild birds, penguins, and mosquitoes.

Host	Species	Parasite	Prevalence	Diagnostic Technique	Mortality	Location	Reference
Wild Birds	Wild birds, non-native	*Plasmodium* spp.	9-26%	PCR	NR	New Zealand	(Lapointe et al., 2012)
	Hawaiian amakihi	*Plasmodium* spp.	55-83%	NR	NR	Hawaii	(Lapointe et al., 2012)
	Blue tit	*Plasmodium* spp. (overall)	42%	(q)PCR	NR	Oxford, UK	(Knowles et al., 2011)
		P. relictum	22.6%	(q)PCR	NR		
		P. circumflexum	19.1%	(q)PCR	NR		
	Exotic zoo birds (not penguins)	*Plasmodium* spp.	11%	PCR	n = 6	Netherlands	(Huijben et al., 2007)
	Wild bird	*Plasmodium* spp.	12.8%	thin blood smears	NR	Baltimore, US	(Beier and Stoskopf, 1980)
Penguins	African penguin	*Plasmodium* spp.		microscopy	n = 6	Netherlands	(Huijben et al., 2007)
	African penguin	*P. relictum* and *P elongatum*	19-24%	thin blood smears	16.3% (juveniles)	Baltimore, US	(Beier and Stoskopf, 1980)
	African penguin	*Plasmodium* spp.	100% (34)	ELISA	NR	Baltimore, US	(Graczyk et al., 1994b)
	African penguin (wild in rehabilitation)	*Plasmodium* spp.	35%	NR	NR	South Africa	(Grilo et al., 2016)
	African penguin (wild in rehabilitation)	*Plasmodium* spp.	17-34%	NR	NR	South America and South Africa	
	Magellanic penguin (wild in rehabilitation)	*Plasmodium* spp.	7-13%	NR	NR		
	African penguin (free life)	*Plasmodium* spp.	<1%	NR	NR	South Africa	
	Captive penguin	*Plasmodium* spp.		NR	50-80% (accumulative)	NR	
	Magellanic penguin	*Plasmodium* spp.		PCR and thin blood smears	60% (n = 3/5)	Brazil	(Bueno et al., 2010)
	African penguin	*P. relictum* and *P. elongatum*	52%	ELISA	NR	South Africa	(Gracyzk et al., 1995)
	Gentoo penguin	*P. relictum* and *P. elongatum*	33%	ELISA	NR	French Sub Antarctic Territories	
	King Penguin	*P. relictum* and *P. elongatum*	58%	ELISA	NR		

Table 2.8 Continued.

Penguins	Yellow-eyed penguin	*P. relictum* and *P. elongatum*	100%	ELISA	NR	New Zealand	(Gracyzk et al., 1995)
	Magellanic penguin	*P. relictum* and *P. elongatum*	43%	ELISA	NR	Antarctica	
	Little penguin	*P. relictum* and *P. elongatum*	92%	ELISA	NR	New Zealand	
	Humboldt penguin	*P. relictum* and *P. elongatum*		in-situ hybridization and PCR	33% (n =11/33)	Austria	(Dinhopl et al., 2011)
	Rockhopper penguin	*P. relictum* and *P. elongatum*		in-situ hybridization and PCR	20% (n = 2/10)		
Mosquitoes	*Cx. pipiens*	*Plasmodium* spp.	1.7%	PCR	NA	Netherlands	(Huijben et al., 2007)
	Cx. pipiens and *Cx. restuans*	*Plasmodium* spp.	0.6%	histology	NA	Baltimore, US	(Beier and Stoskopf, 1980)
	Culex spp.	*Plasmodium* spp.	0.5% 1/188	PCR	NA	Brazil	(Bueno et al., 2010)
	Cx. perexiguus	*Haemoproteus* spp. and *Plasmodium* spp.	0.91% (MIR)	Nested PCR	NA	Portugal	(Ventim et al., 2012a)
	Cx. pipiens	*Haemoproteus* spp. and *Plasmodium* spp.	0.04% (MIR)	Nested PCR	NA		
	Cx. theileri	*Haemoproteus* spp. and *Plasmodium* spp.	0.03% (MIR)	Nested PCR	NA		
	Cx. pipiens	*Plasmodium* spp.	6.6%	Nested PCR	NA	Switzerland	(Glaizot et al., 2012)
	Cx. pipiens group	*Plasmodium* spp.	5.2% (MIR / 1000)	Nested PCR	NA	Japan	(Ejiri et al., 2009)
	Lutzia vorax	*Plasmodium* spp.	51.3% (MIR / 1000)	Nested PCR	NA		
	Cx. pipiens	*Haemoproteus* spp. and *Plasmodium* spp.	9.56% ± 0.82%	Nested PCR	NA	South France	(Zele et al., 2014)
	Culex pipiens pallens	*Plasmodium* spp.	16%	Nested PCR	NA	Japan	(Ejiri et al., 2011)

MIR: minimum infection rate, proportion of positive pools over all captured mosquitoes or over 1000, NR: not reported, NA: not applicable.

Table 2.9. Results of the sample estimation for proving the presence of infection.

Host	Minimum expected prevalence (%)	q	ln α (0.05)	ln α (0.01)	ln q	Sample size (95%)	Sample size (99%)
Wild Birds	9	0.91	-2.99	-4.6	-0.09	32	49
Penguins	7	0.93	-2.99	-4.6	-0.07	41	63
Humboldt penguins	33	0.67	-2.99	-4.6	-0.4	7	11
Cx. pipiens	0.04	0.99	-2.99	-4.6	-0.00	7488	11511
Cx. pipiens	1.7	0.98	-2.99	-4.6	-0.01	175	269

Table 2.10. Results of the sample size calculation for estimating the prevalence of infection.

Host	Minimum expected prevalence (%)	P_{exp}	$1-P_{exp}$	Sample size (95%)	Sample size (99%)	Adjusted sample size (95%)	Adjusted sample size (99%)
Wild Birds	9	0.09	0.91	126	5435	NA	NA
Penguins	7	0.07	0.93	100	4320	30	42
Humboldt penguins	33	0.33	0.67	340	14672	37	42
Cx. pipiens	0.04	0.0004	0.999	1	27	NA	NA
Cx. pipiens	1.7	0.017	0.983	26	1109	NA	NA

The constant multipliers used were 1.96^2 and 2.57^2 for the 95% and 99% confidence, respectively. The d values were 0.05 and 0.01. The study population size (N) used was 42. NA = not applicable.

From the previous calculations, we expected to prove the absence of the parasite with a 99% confidence after analysing at least 49 wild birds, all Humboldt penguins and 269 *Cx. pipiens* mosquitoes. To estimate the prevalence of the infection with a 95% confidence in the local community of wild birds, 126 individuals must be tested; for the penguins also, all of them had to be tested and the mosquito sample was smaller in this case, so it was practical to use the former estimation. This sample sizes and confidence levels were chosen to have a representative and manageable numbers; the other sample sizes were considered as a reference.

2.10.1 Proposed pooling for the estimation of the prevalence

The DNA extraction from all mosquitoes was done individually but considering the big number of mosquitoes captured in 2017, we decided to optimise the lab work by doing the PCR parasite testing by pools. The mosquitoes that we processed individually for comparing the proportion of *Cx. pipiens* and *Cx. torrentium* (see section 2.7.3) were also used to have an insight into the parasite's prevalence. This average prevalence of 8.31% was used to estimate the optimum pools size using a binomial distribution as described before. A reasonable pool size of five samples per reaction was chosen expecting to have at least one positive sample in 35.1% of the pools. Although this estimation assumed of a constant prevalence, we expected that the prevalence could present significant changes thought out the season. Afterwards, the DNA from positive pools were tested individually to detect the actual infected mosquitoes and send that PCR product for sequencing.

2.11 Mosquitoes Saliva Analysis

We found several mosquitoes positive to *Plasmodium* spp. since the first samplings; nevertheless, although they were identified as *Culex pipiens*, the main avian malaria vector, we could not ascertain that they were actively transmitting the parasite as they could have fed on already infected birds and the parasite was in their abdomens. Therefore, I extracted saliva from 59 mosquitoes captured in the 2018 season in June 28th (n=15), July 4th (n=25), September 26th (n=4), and September 27th (n=15); I selected the mosquitoes in the best conditions and most likely to be alive.

The mosquitoes were anesthetised using FlyNap® (Carolina Biological Supply Company, Burlington, NC, USA) which contains triethylamine, ethanol, 2-propanol, methanol, and fragrance; following the procedure suggested by the manufacturer. I dipped the anaesthetic wand into the FlyNap® solution and placed it into a closed plastic bag along with the trap nets. After ten minutes, I emptied the nets on a petri dish to separate the mosquitoes from the accompanying insects and identified the mosquitoes by morphology as described before. I fixed capillary tubes containing immersion oil in approximately 0.5cm of the tip on a petri dish with adhesive tape and placed the whole proboscis of the mosquitoes into the oil (Figure 2.12). After at least five minutes, I took the mosquitoes and stored them as previously described and emptied the oil into 2ml reaction tubes for later DNA extraction and PCR testing for *Plasmodium* following the afore mentioned procedures.

Figure 2.12. Saliva extraction procedure. The mosquitoes were anesthetised with FlyNap® and their proboscis was placed into a capillary tube with mineral oil for 5 minutes; the DNA extract from the oil was tested with the described PCR.

2.12 Database

All the information regarding the samplings and laboratory results was managed using a database developed with the software Microsoft Access 2010©. The main components of the database are the tables in which the information is stored. I used nine tables named: Collections, Mosquitaire collections, Gravid collections, Larvae collections (immature mosquitoes), Morphological ID, Larvae ID, PCR ID, Blood analysis and *Plasmodium* testing.

In the Collections table, I recorded the date, type of collection (day or week periods), the collection number, mosquitoes captured in in the BG-Mosquitaire traps, in the CDC-Gravid traps and in the larvae sampling, and the total of mosquitoes per collection. The Mosquitaire, Gravid and Larvae collections tables included the number of mosquitoes captured by area for the corresponding trap or method and the total for the collection. The Morphological ID table contained an individual record for each mosquito in the whole site or sampling year, along with the source (trap or method), their unique identification label, sex, subfamily (Culicinae or Anophelinae), genus, species, blood-fed condition and if the specimen presented damage in the body sections that could compromise the morphological identification (legs, tarsi, abdomen, abdomen scales, antenna, or wings). The Larvae ID table has the same structure as the Morphological ID table but was used to separate the records of the mosquitoes from the immature stages sampling. The unique identification label, DNA extraction status (extracted or pending), extract location, test kind (by pools, individually or not applicable), pool code, date of the PCR, PCR results by pool and individually (amplification success, enzymes digestion result, and times tested), and complete identification, were included in the PCR ID table. The Blood analysis table

included the unique identification label, complete mosquito identification, extract location, PCR results (amplification success and times tested) and the blood-meal sequencing results by scientific name, common name and origin (zoo animal, wild animal or other). Finally, the *Plasmodium* testing table comprised the complete mosquito identification, DNA extraction status (extracted or pending), extract location, PCR date, results for *Plasmodium* spp. and for other blood parasites, times tested, and sequencing result. All the tables included the collection date, collection number and additional notes.

The shared fields among tables that created the relationships were the key fields and the relevant collective fields, like the collection date, collection number and the unique identification label; the kind of relationship used was “one to many”. This allowed to automatically feed the data from the tables with the higher hierarchy into the dependent tables.

For ease of data input, I designed forms and sub-forms. The Collections form encompassed the information of the Collections table as the main form and the fields of the Mosquitaire collections, Gravid collections, and Larvae collections as sub-forms (Figure 2.13). The Morphological ID, Larvae ID, PCR ID, Blood analysis and Parasite testing tables had their own forms. The forms were linked with tabs in a single window and included drop-down menus for searching records by unique identification label, collection number and collection date, facilitating in this way the work and consultation (Figure 2.14 and 2.15).

I also included queries and reports generated automatically for specific consultations and for tracking the work progress. I used different databases with the same design for each sampling by zoo and year, three in total, to prevent mistakes.

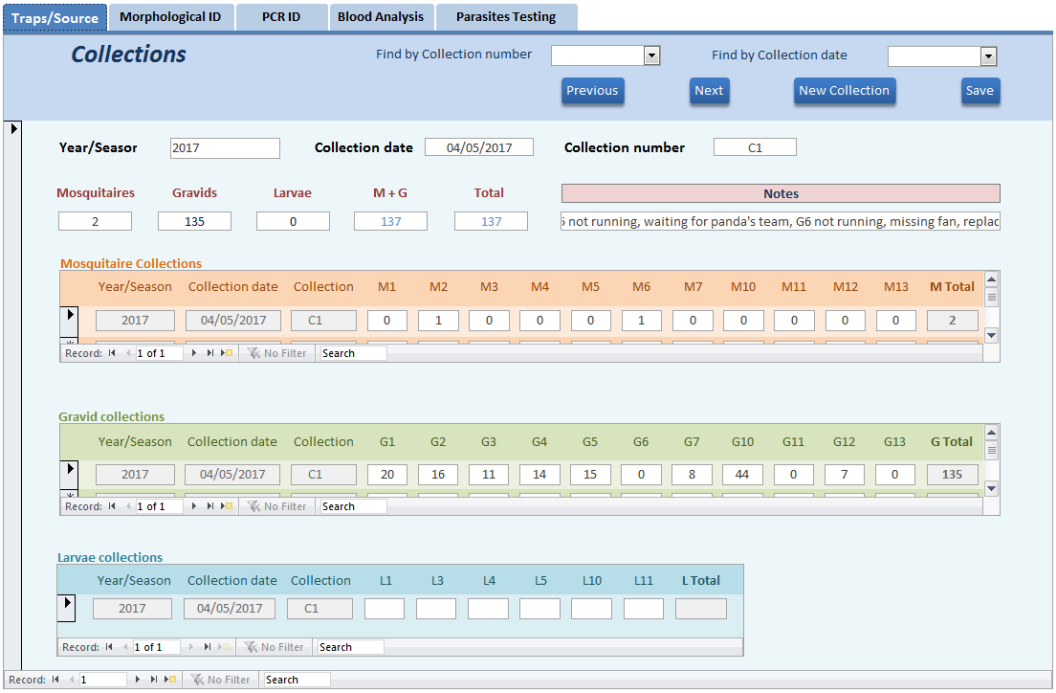

Figure 2.13. Pop-out window of database showing the main form for data input of the collections and the sub-forms of the Mosquitaire, Gravid and Larvae collections.

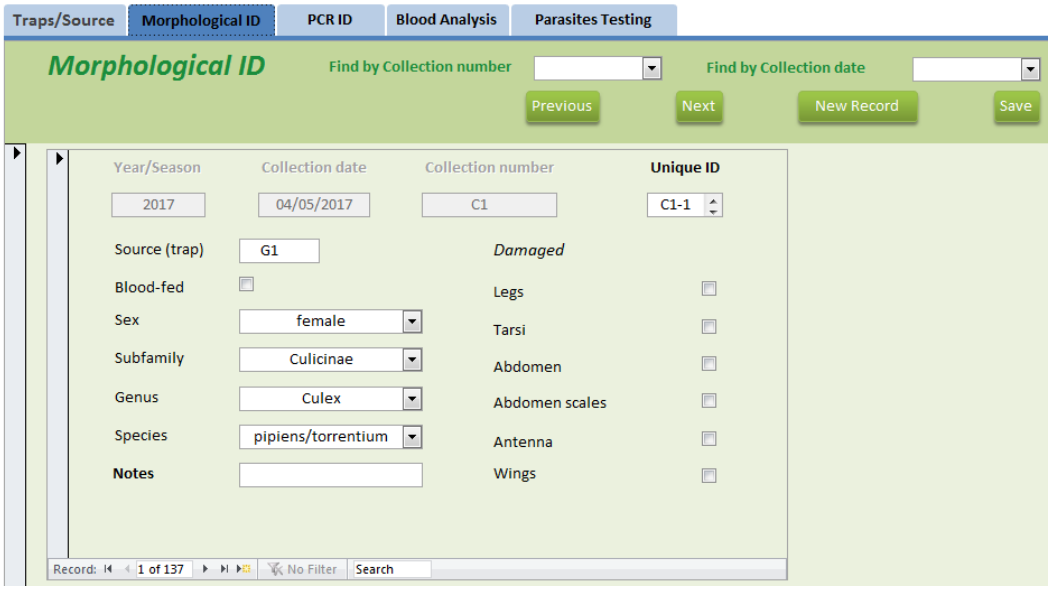

Figure 2.14. Pop-out window of the database showing the form for data input into the Morphological ID table.

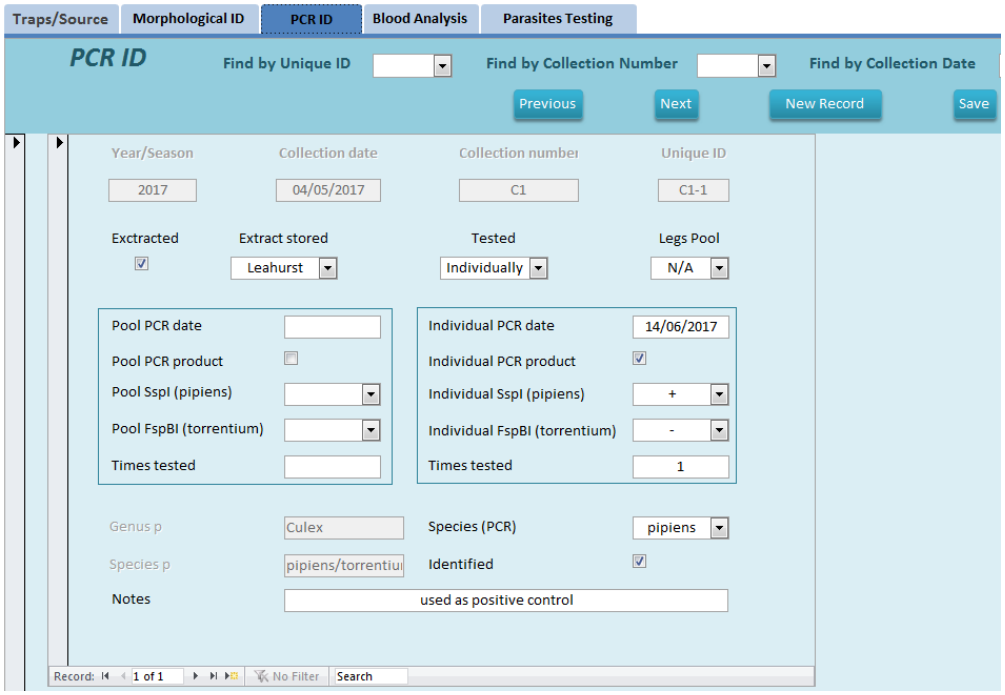

Figure 2.15. Pop-out window of the database showing the form for data input into the PCR ID table.

2.13 Study Bias

The main sources of bias in this work are related to the local weather, the location of the sampling areas, the selection of traps used, the operation time of the traps and the sensitivity of the molecular methods.

During windy or rainy days, it was expected that the mosquito activity would diminish and less specimens would be captured. Nevertheless, as the aim of the sampling was to analyse the mosquito activity across the season to evaluate the mosquito biting and disease transmission risks, all the collected mosquitoes were analysed. The influence of the local environmental conditions is explored in Chapter Five.

It was also considered that if the nets were wet due to a high humidity or rain, the mosquitoes could get damaged and their identification would be inaccurate; therefore, in these cases, the nets were hanged indoors for enough time to dry before freezing, as mentioned before.

2.13.1 Samplings

It is unrealistic to assume that the mosquitoes distribute randomly over certain area, they are attracted to different features that cover their needs, like shelter and overwintering places, oviposition sites and feeding sources; thus, their distribution tends to be aggregated and dynamic. Without pilot studies in the zoo, we could not predict beforehand the mosquito abundance and distribution, so the selection of the sampling areas followed the surroundings features and logistic considerations but not a fixed design, with the exception of the previously mentioned sampling area size and trap distances.

As our main interest was to capture as many avian malaria vectors as possible, we selected traps specifically designed for *Culex* spp. or that proved to be efficient for capturing these species; hence, a complete representation of the mosquito community in terms of abundance and species richness cannot be assumed from our data. The same consideration applies to the sampling of immature stages because, although it was more generic and represented a higher diversity, other mosquito species develop in small pockets of water, for instance *Anopheles plumbeus* uses tree cavities (Becker et al., 2010), and these were not sampled.

The attractants used in the traps vary in their effectiveness. The Sweetscent© used for the BG-Mosquitaire traps could be effective for two months, as the manufacturer specifies, but its lure activity diminishes over time, so it is expected that it would be higher at the beginning and decrease gradually. To prevent a major variance, we replaced the Sweetscent© every six weeks.

The oviposition infusion used for the CDC-Gravid traps is prepared with tap water, hay, brewer's yeast and milk powder and the fermentation that occurs in the mix is influenced by the temperature. We allowed one week for the fermentation before using the infusion but as the containers were kept outdoors, the degree of fermentation could have varied depending on the current temperature.

The design of the CDC-Gravid traps affected the number of mosquitoes that could be identified. As the fan is located before the collection net, the mosquitoes must pass through it and at this moment many specimens were damaged when hit by the fan; most commonly their abdomens were lost. This is a problem faced by other researchers and a different gravid trap, the Frommer Updraft Gravid Trap Model 1719 (John W. Hock Company, 2019), which has the collection net placed before the fan, could be used instead

for future samplings. Many of the damaged mosquitoes could be identified as *Culex* spp. and as no other species from this genus were found, it could be assumed that they were *Cx pipiens* or *Cx. torrentium*; however, for the data analyses they were considered as a separated set.

Regarding the mosquito molecular identification, Hesson J. C. *et al*. (2010) reported in their original description of the method that only one larvae out of 227 adults and larvae used for the evaluation of their enzyme assay, was positive to both enzymes (FspBI and SspI) (Hesson et al., 2010). Other authors that used this protocol reported absolutely clear results (Byriel et al., 2018, Kazlauskiene et al., 2013, Hesson et al., 2014). In the test for defining the optimum pool size in this study (see section 2.7.3), the results were also completely clear. Consequently, this test is very sensitive and reliable, but we could not get a result from 27 mosquitoes possibly due to DNA degradation or PCR inhibitors.

2.13.2 Malaria PCR

Initially, the PCR success was lower using Livak's method than with the extraction kit, possibly due to the presence of contaminants interfering (mainly proteins and ethanol). Thus, a trial with different dilutions was done finding that the best results were achieved with a 1:2 dilution, which was used for all the samples extracted with this method. Nevertheless, the PCR success was compared further; the extraction of 239 samples was done with both methods (Livak's and extraction kits) resulting in 47 and 48 samples positive for the Livak's method and extraction kits respectively; 33 samples were positive to both methods and it was confirmed by sequencing that all samples were true positives. A Fisher's exact test of independence showed that the sensitivity of the methods was not significantly different ($p = 1$) so the results were considered comparable.

2.14 Data Analysis

All the statistical analyses were done using the R software version 3.6.0 (R Core Team, 2012). All the analyses were done excluding the overwintering collections and the aspirating samplings and only the females' data was used. The damaged, and thus not completely identified mosquitoes, were excluded from the species comparisons.

The differences in mosquito abundance by area and month were done using the collections of the BG-Mosquitaire traps merging the catches after six and after one day per week. All the evaluations between kinds of trap, for the mosquito abundance and the source of

blood-fed mosquitoes, were done using the one-day collections from the BG-Mosquitaire traps compared to the CDC-Gravid traps catches.

It was observed that the males did not show the same abundance pattern as the females although their proportion increased towards the end of the sampling. To see if the abundance of females and males was correlated, I did a Spearman rank correlation comparing the number of mosquitoes captured by sex which was not significant (S = 34743, Spearman's rho = 0.081, df = 59, p = 0.533) suggesting that the males were collected randomly, possibly because they flew close to the traps. This was probably due to the females going for overwintering indoors while the males remain in the environment until they die in winter (Becker et al., 2010). The analyses done are described along with the corresponding results.

Appendix 2.1

Approved Research Proposal Form of Chester Zoo

FOR OFFICE USE ONLY

Received:

Research Proposal Form

Section 1 Project Summary

Project Title			
Study of avian malaria epidemiology and genetics in Chester Zoo.			
Brief project summary *(Max. 100 words; please include justification for study, project aims, methods and expected project outcome)*			
The concern about avian malaria has increased after recent reports in zoo birds. Until now, Chester Zoo has not had cases of the disease; therefore, the aim of this proposal is to evaluate the avian malaria risk in Chester Zoo. For this, we will study the mosquito community to identify the main vector, evaluate blood and organs samples of zoo birds and wild birds, and perform genetic analysis of the parasite. This will allow us to assess the epidemiology and genetics of avian malaria and propose explanations for the current situation in the zoo and recommendations for preventing the infection of zoo birds.			
Proposed start date of data collection with Chester Zoo (dd/mm/yyyy)	13/03/2017	**Proposed end date of data collection with Chester Zoo** (dd/mm/yyyy)	13/11/17

Section 2 Researchers involved in the study

Please add as many rows as is required.			
Name and Institution Please include all people involved in the project	**Role(s) in the study** e.g. Main Researcher or Principal Investigator	**Is this contributing to your academic qualification?** If so, please include qualification level (e.g. M.Sc.) and course subject	**Institutional contact details**
Arturo Hernandez-Colina, University of Liverpool	Main researcher	PhD, not related to any course.	**Address:** Leahurst campus, Chester High Road, Neston CH64 7TE **Email:** arturoh@liverpool.ac.uk **Tel:** 07401496983

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Matthew Baylis, University of Liverpool	Supervisor		**Address:** Leahurst campus, Chester High Road, Neston CH64 7TE **Email:** matthew.baylis@liverpool.ac.uk **Tel:** 0151 794 6084
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Section 3 Project Outline

Section 3a Introduction

Background introduction:

Avian malaria is a world-wide distributed disease caused by the infection of blood parasites from the genus *Plasmodium* [1]. From the more than 40 *Plasmodium* morphological species, *P. relictum* is the most relevant since it has been reported in more than 400 bird species and has affected wild birds and caused outbreaks in zoos worldwide [1, 2].

This parasite requires a vector for its transmission and although there is scarce information about the species that it infects, the most recognised one is *Culex* spp. mosquitoes [2]. Only few *Culex* species have been proven to be natural vectors but other mosquito genera in which it can complete its life cycle are *Aedes*, *Anopheles, Coquillettidia* and *Culiseta* [1-4].

The impacts of avian malaria in wild bird populations are poorly understood, varying from subclinical fitness effects to population decline and extinction. It can produce significant long-term effects like reduced reproduction fitness of the avian host [1, 5], but perhaps the most evident case is the population declines, distribution restriction and extinction of several Hawaiian birds [1].

In captive birds the morbidity can be severe and often provokes death, predominantly in penguin populations and young birds [2]. There have been reports of zoo birds affected by avian malaria in Europe [2] and in UK zoos, the disease has affected African black-footed penguins (*Spheniscus demersus*) [3].

Climate change is expected to increase the distribution and intensity of vector-borne diseases like avian malaria [5] and despite there are no reports of penguin mortality due this disease in wild populations, the spread of mosquito species can represent a risk for them [1]; therefore, understanding how this disease affects penguins in zoos can also provide valuable information for the conservation of these birds in their natural habitats.

The study of *Plasmodium* species in the field is limited and the differences of host susceptibility, vector competence and pathogenicity has not been clarified; nevertheless, the analysis of its genetic structure, especially mitochondrial genes, can provide relevant information [1]. Lately, molecular methods are revealing that the prevalence of the disease is higher in non-native birds and that the genetic lineages structure of this parasite is more complex than previously though [1]. Likewise, the highest mortalities have been reported in cases where the hosts present mixed

infections of two or more malaria species, with the exception of the Hawaiian birds' epidemic caused by a single lineage [5]. The genetic analysis of the infected hosts is key to disclose these epidemiological features.

Understanding the vector ecology and the inherent epidemiology of mosquito born-diseases can help to optimize the preventive measures and improve considerably the animal health management [3]. So far, there have not been avian malaria cases in Chester Zoo, despite the sudden and serious outbreaks that have occurred in other zoos under similar conditions in the UK. Therefore, investigating the local epidemiology of the disease encompassing mosquitoes, birds, and genetics of the parasite, can provide insights of the reasons for the current situation and potential risks in Chester Zoo and provide information to suggest measures for preventing the infection of the zoo birds and propose strategies for the surveillance of this disease.

Research aim(s):

Investigate the presence and distribution of avian malaria and avian malaria vectors at Chester Zoo in order to establish baseline data for the recommendation and assessment of preventative and management measures and to inform the disease potential risk. If avian malaria is identified, to analyse the parasite epidemiologic and genetic properties and to compare its genetic lineage with other lineages reported in the UK and Europe.

List individual hypotheses:

The vector of avian malaria is not present in the area.

The vector is present but it is not infected with the parasite.

The vector is present and infected with the parasite but transmission to the wild or zoo birds has not been detected.

The avian malaria parasite has infected wild birds or zoo birds.

The genetic lineage of the parasite belongs to one or more of the previously reported ones.

Section 3b Methods

Does this study involve human subjects?*	☐ Yes ☒ No
Does this study involve non-human animal subjects (hereafter known as 'animals')?	☐ Yes ☒ No
Will the researcher need to use a camera or video camera as part of the study to record animal or human subjects? *If yes please refer to section 2.5 of the Chester Zoo Research Policy*	☐ Yes ☒ No
Will the animals or their environment be manipulated for research purposes during the course of the study? *For example altering feeding practices, adaptation of enclosure or use of contraception, wild animal trapping.**	☒ Yes ☐ No ☐ N/A

**** N.B.** Projects which involve human subjects and projects which involve manipulation of the animal or its environment may be reviewed by Chester Zoo's Ethical Review Committee*

For projects involving animals, please fill in the following information. Alternatively, if the project involves several species please attach a species list on a separate sheet including the information required below.

If you require a Taxon Report in order to fill in this section, please contact research@chesterzoo.org

Species:	Total N:
Control animals (if applicable):	Subjects (non-control):
N (infants):	N (infants):
N (juveniles):	N (juveniles):
N (adolescents):	N (adolescents):
N (adults):	N (adults):

Research design:

Vector screening

Larvae sampling: The method consists on submerging a standard dipper in the potential oviposition sites for mosquitoes to capture larvae which then are filtered and preserved in a plastic container. We will choose ten sampling sites randomly in water bodies in the zoo birds' enclosures and in the zoo facilities from which we will take no more than 1 L of water; these sites will be of different depth, sizes and kinds (artificial, natural, with or without vegetation, with or without shade) and separated at least 50 m from each other. The larvae collected will be identified morphologically.

Adult mosquito sampling: 10 BG-Mosquitaire traps and 10 CDC Gravid traps Model 1712 will be used to capture adult mosquitoes. The traps will be distributed in the zoo birds' enclosures, prioritising the penguins, and near potential oviposition or resting sites for the mosquitoes in the zoo facilities. The traps will be placed where they can remain without interfering with the activities in the zoo, considering staff and visitors, and do not represent a risk for any person or the zoo animals.

Both types of traps will be placed near vegetation, but not covered by it, or water bodies; away from busy areas, protected from direct sunlight, artificial lighting, wind and rain, and at a distance of at least 50 m form each other to avoid interference. Neither the BG-Mosquitaire nor the CDC Gravid traps release any dangerous substance to people or zoo birds. The mosquitoes will be inactivated by refrigerating at 4 °C and preserved until their analysis at -20 °C [4].

BG-Mosquitaire: the BG-Mosquitaire is a 40 cm trap that works by releasing an odour (BG-Sweetscent) to attract mosquitoes that are looking for a blood meal and sucking them with a fan into a capture funnel net; it needs to be plugged to electricity with a cable and it does not require gases to operate like propane or CO^2 [6].

CDC Gravid Trap: the CDC Gravid trap was designed to capture *Culex* mosquitoes attracting females that are looking for a place to lay their eggs. It has a tray that contains an oviposition medium, which is an infusion of hay in tap water (4 L per trap), and a collection bag in which mosquitoes are blown by a fan. It operates using a 6-volt, 12-amp DC battery [7].

Sampling Protocol: The larvae sampling will be performed once a week and will be used for the mosquito community assessment.

The BG Mosquitaire traps will be operating continuously all weekdays and we will collect two sets of samples. The first one will gather the mosquitoes captured during a week, which is consistent with the Project MOSI methodology [3] and will be useful to assess the mosquito community. The second one represents the mosquitoes captured during a 24 hours period, minimizing their mortality, which is ideal for the molecular analysis.

The CDC Gravid traps will capture mosquitoes during one day per week, which are going to be used for the molecular analysis.

We will need to visit Chester Zoo every week in two consecutive days at 9:00 hrs during the sampling period, from March to November 2017; we suggest that these days can be Tuesday and Wednesday.

In the first day we will:

Do the larvae sampling.

Collect the adult mosquitoes from the BG Mosquitaire traps and turn them on.

Prepare the CDC Gravid traps with the oviposition medium and turn them on.

In the second day we will:

Collect the adult mosquitoes from the BG Mosquitaire traps and turn them on.

Collect the adult mosquitoes from the CDC Gravid traps and turn them off.

Pick up the bird samples processed during the week.

Zoo staff assistance will be required where it is necessary to access animal areas.

Mosquito identification: the collected mosquitoes will be counted and identified morphologically using the key from Cranston P. S. (1987) [11]. Mosquitoes from the *Culex* complex will be identified to the species level by extracting the DNA with ammonium acetate [5] and performing a PCR following the procedure used by Hesson J. C. (2015) [12]. We will look for differences and similarities in the mosquito community and trap locations across the sampling period.

Avian malaria screening

Samples required: Surplus blood from routine or diagnostic blood sampling of zoo birds, ideally 200 µl but could be less depending on the bird size and condition. The blood sample should be collected into an EDTA-coated microtainer for capillary collection for immediate dispatch to our laboratory for DNA extraction. At the moment of collection, we also request that three thin blood smears are carried out per bird, air-dried for 3 minutes, and fixed with 100% methanol. [8, 9]. This may be done by Zoo staff or by ourselves as convenient. We recommend that the zoo staff inform us if they decide to take samples for the diagnostic tests if any bird of the collection presents avian malaria symptoms (anaemia, lethargy, anorexia and ruffled feathers [1]).

Tissue samples (liver, spleen, lungs, brain, heart and kidneys) from dead zoo birds and tissues or whole wild birds found dead within the zoo, stored at -20 °C.

Plasmodium* diagnosis*: blood smears will be stained with Giemsa's solution and screened with a light microscope for parasite detection according to Valkiunas G. (2005) [9]. From mosquitoes, DNA extraction will be performed by

standardized methods (phenol extraction and ethanol precipitation) [2] and from zoo birds and wild birds' blood, with ammonium acetate standard protocols [8]. The diagnosis of *Plasmodium* spp. and the characterization of its lineage will be done by nested PCR using specific designed primers for mitochondrial cytochrome B. The positive samples will be precipitated and sequenced to compare the present *Plasmodium* lineage to others using the MalAvi Database [4, 10]

Data Analysis

All samples will be analysed in University of Liverpool after its collection.

Section 3c Additional information required

Is this project being carried out at more than one site?
☐ Yes ☒ No *If yes, please provide details of collaborating institutions including, if applicable, the fate of the animals at the end of the research project:*
Is this project endorsed by the TAG/EEP or BIAZA approved? *For more information about BIAZA research support please click here.*
☐ Yes ☒ No *If yes, please provide details:*
Does any of the work in this project require a Risk Assessment to be carried out? *For example manual handling, hazardous substances, use of equipment or access to behind-the-scenes.*
☒ Yes ☐ No *If yes, please attach completed risk assessments from your institution. Please note that for some research activities, Risk Assessments need to been drawn up by Chester Zoo's Science Team.*
Irrespective of the location of the actual study, would it require a Home Office licence if it was being conducted in the UK? *For guidance, please click here to see The Animals (Scientific Procedures) Act, 1986 and the Performance of Procedures by Veterinary Surgeons. We use the same criteria for projects conducted outside the UK.*
☐ Yes ☒ No *If yes, please provide details:*
Are other licenses or permits required in this project (e.g. for capture and handling of wild animals)?
☐ Yes ☒ No *If yes, please provide details:*

Are there any costs for project activities outside of standard husbandry and/or zoo operational procedures?		
Costs	**Description**	**How are these costs being met?**
		The research cost will be covered by the supervisors' funding.

Section 4 Project Plan and Timetable

Please list the specific project activities that will allow you to achieve your research aims. *Add as many rows as necessary.*			
List of project activities	**Where will these activities take place?**	**Proposed start date for each activity** (dd/mm/yyyy)	**Proposed end date for each activity** (dd/mm/yyyy)
Literature review	University of Liverpool	01/02/2017	28/02/2017
Data collection at Chester Zoo or other study site	Chester Zoo	13/03/2017	13/11/2017
Data analysis	University of Liverpool	13/03/2017	15/01/2018
Report writing	University of Liverpool	13/11/2017	19/02/2018
Submission of written report to Chester Zoo's Science Team	University of Liverpool	12/03/2018	26/03/2018
Expected details of any publications arising from this work, if applicable	University of Liverpool	02/04/2018	28/05/2018

Section 5 Project Ethics, Benefits and Output

N.B. All applicants must complete Section 5a. Please also ensure that you complete each section that applies to your project (5b and/or 5c).

Section 5a Complete for all projects:

Will this project be or has it been submitted to another ethical review committee for approval?
☐ Yes ☒ No *If yes, please provide evidence if approval has already been obtained or please give details of the status of the application:*
How will this project contribute to science, education, welfare, husbandry and/or conservation?
This project will help us to understand the particular epidemiology and genetics of avian malaria in Chester Zoo, providing information about the transmission, prevalence, genetic features and epidemiologic risks. In this way, recommendations for the further disease survey and prevention of the infection could be made, in order to protect the health and welfare of the zoo birds, including those species which conservation is threatened.
How will the results of this project be disseminated?
The outcomes of the project will be presented in a written report to Chester Zoo's Science Team, may be published in a peer-reviewed journal and will constitute part of the degree thesis of the participating Ph.D. students, Merit Gonzalez-Olvera and Arturo Hernandez-Colina.

Section 5b Complete for projects involving animals:

What is the scientific basis for using the number of animals you have stipulated? *Please show sample size calculations or give justification why this is not needed.*
Summarise the potential negative effects to the animals used in this project. *For example, due to animal handling, presence of observer, disease transmission, etc.*
.
If, during the course of the research, the negative effects to the animal(s) rose above that expected, please describe the point at which you would remove the animal from the research. *Please also include any systems in place you have to monitor any deleterious effect and what system you have to adapt the experimental design if required.*

Section 5c Complete for projects involving human participants (e.g. visitors and zoo employees):

Please outline the number of human participants involved in the project and the scientific basis for using the sample size you have stipulated. *Please show sample size calculations or give justification why this is not needed.*
How will potential participants be identified and recruited to take part in this project? How will informed consent be obtained? *Please attach any additional document, e.g. project information sheet for participants, consent form, if applicable.*
Will any of the participants be vulnerable people? *For example, children under 16, adults and children with learning disabilities or mental illnesses.*
☐ Yes ☐ No *If yes, please justify their inclusion:*
Will participants be able to withdraw from the research at anytime?
☐ Yes ☐ No *If no, please explain why:*
Please outline any potential stress, anxiety or other negative consequences, which may be caused by the research and how this will be addressed. *For example, does the research involve discussion of sensitive topics, exposure to upsetting imagery, etc.?*
What steps will be taken to ensure confidentiality and anonymity of participants during data collection, data storage, dissemination or sharing, and publication of the results? *Please include who will have access to the data, and how long will the data be stored.*

Section 6 Bibliography

Please ensure all cited references are listed

1. Lapointe, D.A., C.T. Atkinson, and M.D. Samuel, *Ecology and conservation biology of avian malaria.* Ann N Y Acad Sci, 2012. 1249: p. 211-26.

2. Huijben, S., et al., *Avian malaria in Europe: an emerging infectious disease?*, in *Emerging Pests and Vector-Borne Diseases in Europe*, W. Takken and B.G.J. Knols, Editors. 2007. p. 59-74.

3. Quintavalle Pastorino, G., et al., *Project MOSI: rationale and pilot-study results of an initiative to help protect zoo animals from mosquito-transmitted pathogens and contribute data on mosquito spatio-temporal distribution change.* International Zoo Yearbook, 2015. 49(1): p. 172-188.

4. Ventim, R., et al., *Avian malaria infections in western European mosquitoes.* Parasitol Res, 2012. 111(2): p. 637-45.

5. Marzal, A., *Recent Advances in Studies on Avian Malaria Parasites*, ed. O. Okwa. 2012: InTech.

6. AG, B. *BG-Mosquitaire*. 2016 [cited 2017 03/01/2017]; Available from: http://www.biogents.com/bg-mosquitaire/.

7. Company, J.W.H. *CDC Gravid Trap*. 2017 [cited 2017 03/01/2017]; Available from: http://johnwhock.com/products/mosquito-sandfly-traps/cdc-gravid-trap/.

8. Ventim, R., et al., *Characterization of haemosporidian infections in warblers and sparrows at south-western European reed beds.* Journal of Ornithology, 2012. 153(2): p. 505-512.

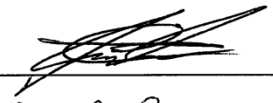
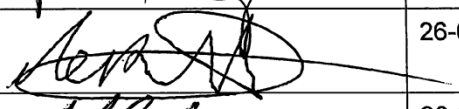
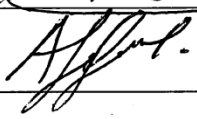
9. Valkiunas, G., *Avian malaria parasites and other haemosporidia*. 2005, United States of America: CRC Press.

10. Egerhill, M., B. Canbäck, and S. Bensch, *MalAvi, Version 2.2.9*. 2016: Lund University, Department of Biology.

Section 7 Acceptance of terms and conditions

All people named in Section 2 must sign this form

By signing this form, applicants/supervisors accept the terms and conditions of Chester Zoo's Research Policy and agree to provide the Science Team with an electronic and hardcopy of the project report.

Name (please print)	Signature	Date
Arturo Hernandez-Colina		26-01-2017
Matthew Baylis		26-01-2017
Merit Gonzalez-Olvera		26-01-2017
Andrew Jackson		26-01-2017

Chapter Three

Avian Malaria Vectors in UK Zoos

3.1 Abstract

Avian malaria is an important cause of mortality in captive penguins and could represent a serious threat for the conservation of wild penguins and other susceptible birds. We collected mosquitoes during 2017 and 2018 in Chester Zoo and during 2017 in Flamingo Land. We established ten sampling areas in Chester Zoo in 2017 and eight in 2018, and four in Flamingo Land. In every sampling area we installed one BG-Mosquitaire trap and one CDC-Gravid trap and, where possible, an area for sampling immature mosquitoes (larvae and pupae). The BG-Mosquitaire traps were operated continuously and their nets were collected after six days and after one day. The CDC-Gravid traps were operated one day per week and the sampling for immature mosquitoes was done also once a week. The mosquitoes were identified by morphology and if adult mosquitoes belonged to the *Culex* spp. genus, they were identified to species by PCR. Afterwards, the mosquitoes were tested for avian malaria parasites with a nested-PCR. In Chester Zoo, we collected 7,938 adult mosquitoes and 1,658 immature mosquitoes in 2017 and 2,962 adult mosquitoes in 2018; in Flamingo Land the collection was of 1,588 mosquitoes. The dominant species in the mosquito communities was *Cx. pipiens*. The abundance of mosquitoes varied across the seasons and there were differences by sampling areas. There was a strong correlation between the number of immature and adult mosquitoes a few weeks later. It is important to consider that the traps used were highly effective at capturing *Culex* spp. but other species could be also involved in avian malaria transmission. The conditions of the oviposition sites determined the species and abundance of immature mosquitoes. We were able to identify critical moments and locations with higher mosquito abundance which could be used for planning mosquito control strategies, although a constant surveillance of the mosquito community is recommended.

3.2 Introduction

Avian malaria is not usually associated with serious illness or mortalities of wild birds, but it has caused mortalities and extinctions in endemic birds (Lapointe et al., 2012) and can affect survival rates (Marzal et al., 2008) and reproductive fitness (Knowles et al., 2010). Moreover, as its effects are been investigated in further detail, increasing negative consequences for the survival of populations have been found (Hunter and Alley, 2019). This disease is also the main infectious cause of mass mortalities in captive penguins and outbreaks in zoos have been reported worldwide (Silveira et al., 2013).

The precise factors that determine the occurrence of avian malaria or its severity are not completely understood (Knowles et al., 2011, Lapointe et al., 2012). Some studies have analysed the prevalence and parasitaemia on free wild birds and the impacts on their individual fitness (e.g. (Asghar et al., 2011)), and other investigations have been done in captive birds (e.g. (Chagas et al., 2017)), but some epidemiological details are still unknown (Lapointe et al., 2012). In many cases, the final diagnosis of the disease is not done, and avian malaria is suspected, based on clinical signs and lesions, which are unspecific, and after excluding other possible pathogens (see section 6.4.3). Therefore, complete diagnosis and outbreak investigation are usually missing despite the recognised importance of the disease. When avian malaria affects a penguin colony, the implementation of preventive measures and treatment have variable outcomes as the colony could recover favourably or succumb to the disease (Vanstreels et al., 2014).

Motivated by the threats of vector-borne diseases and the unique ecological configuration of zoos, the Project MOSI (Mosquito Onset Surveillance Initiative) was established for the permanent monitoring of mosquito vectors in the zoo environment (Quintavalle Pastorino et al., 2015). In this project, the efficiency of four traps to capture mosquitoes was tested with different protocols and the authors recommendations include the use of a convenient trap (BG-Mosquitaire) with the manufacturer's attractant, weekly collection of mosquitoes for identification and if possible, the analysis of host preferences in blood-fed mosquitoes and of pathogen carriage in gravid specimens (Quintavalle Pastorino et al., 2015).

Other aspects of mosquito ecology in zoos have been investigated; Tuten (2011a) conducted a comprehensive examination of the mosquito community in Greenville Zoo and Riverbanks Zoo (South Carolina, USA) describing mosquito diversity, distribution, abundance, and host preferences. Nevertheless, the role of the mosquitoes as avian

malaria vectors in zoo environments has not been studied extensively in other locations and even the investigation of avian malaria events has been mostly descriptive. Therefore, the study of the abundance, species composition, and temporal and spatial variations in the mosquito community is needed for a better understanding of the disease transmission risks.

As described in Chapter 2, the design of this research consisted of studying avian malaria epidemiology in two zoos from the UK, with emphasis on the mosquito ecology. At the beginning of the project, the diseases had not been detected in Chester Zoo, but it caused a serious outbreak in Flamingo Land in 2016. The situation inverted during 2017 when the prophylactic treatment and additional veterinary care prevented the occurrence of the disease in Flamingo Land, but after moving the penguins in Chester Zoo to a temporary enclosure, a multiple cause outbreak affected the colony.

The aim of this chapter was to do a comparative surveillance of avian malaria parasites in mosquitoes and birds from the mentioned zoos. The particular objectives were:

- To define if the mosquito vectors of avian malaria are present in the study sites.
- To describe the abundance of adult and immature mosquitoes in time and spatial scales.
- To test the mosquito and bird samples for Haemosporidians.

3.2.1 Avian malaria outbreak in Flamingo Land

Located in Kirby Misperton, North Yorkshire, Flamingo Land Resort is a popular zoo and theme park that opened to the public in 1959. The whole park has an extension of 152 ha and it houses 140 different species and over 1000 animals, including 17 bird species. It received almost 1.7 million visitors in 2017; the full park opens between April and December and the zoo is constantly open (Flamingo Land Ltd., 2019).

The Humboldt penguins (*Spheniscus humboldti*) are in an open exhibit in the South American section, surrounded by another exhibit that contains mammals like Patagonian mara (*Dolichotis patagonum*), capybara (*Hydrochoerus hydrochaeris*), vicunya (*Vicuna vicuna*), alpaca (*Vicugna pacos*) and Brazilian tapir (*Tapirus terrestris*); along with greater rhea (*Rhea americana*). The visitors can appreciate this area from a wooden elevated footpath (Figure 3.1).

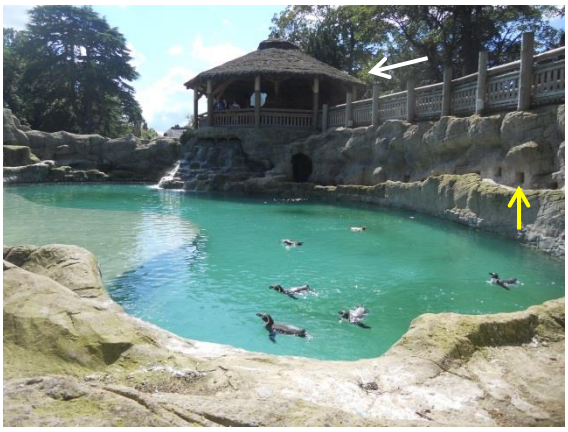


Figure 3.1. Penguin exhibit in Flamingo Land. Yellow arrow: penguin nest boxes; white arrow: visitor footpath and observation point.

During the 2016 breeding season, 16 out of 17 chicks successfully fledged around mid-July. However, the avian malaria outbreak started at this time with the first penguin's death in the 3rd of July and continued with other deaths at irregular times. The first eight penguins and some others died without any signs, but the observed signs were ataxia, lethargy, anorexia, ocular discharges and increased respiratory effect.

Post mortem examinations were done in the first three penguins that died and it was confirmed that one of them was infected with the avian malaria parasite; because no other pathogens were found, it was assumed as the cause of the outbreak.

The veterinary care included treating the penguins with 2.75 mg of primaquine daily from the 8th of July; initially only for the adults, but from the 13th of July also the chicks were receiving a dose of 1.375 mg daily. From the 25th of July until the 7th of August the treatment was complemented with 15 mg of chloroquine per penguin per day. On the 26th of August, the frequency of the treatment was reduced to 2.75 mg once a week until the 10th of October; in this period, three more penguins died. Treatment for the weaker penguins comprised food supplementation with "Critical care formula" given by tubing. Intensive nursing was attempted for one critically ill penguin including antibiotics, antiemetic, intravenous fluids, and stomach tubing, but was unsuccessful and it died after five days. Despite the constant care and efforts, the outbreak lasted for almost eleven weeks and 41 penguins died in total with the last death on the 16th of September; many of them were chicks (n=19).

Many of the penguins in the colony were born there and others were brought from other zoos some years before, so it is likely that they acquired the infection in site. It is suspected that the demanding situation of raising chicks increased the stress in the birds; this

combined with the timing of the outbreak in the summer, when it is expected that mosquitoes are more abundant, and the fact that many of the affected birds were young and possible naïve to the parasite infection, are the probable reasons for the high mortality. Likewise, it was noticed in that year that the constant rain created several small pounds and puddles in the enclosure surrounding the penguin exhibit which could have been potential mosquito developing places (Figure 3.2).

After the avian malaria mortality, Flamingo Land acquired five additional penguins from Marwell Zoo having a current population of 20. As a precautionary measure, the penguins were not allowed to breed in the 2017 season and preventive treatment (3.75 mg of Primaquine per week) was given from April until November. The elimination of water pockets and the regular cleaning of ponds were also implemented. With these measures in place, no more cases of avian malaria have occurred.

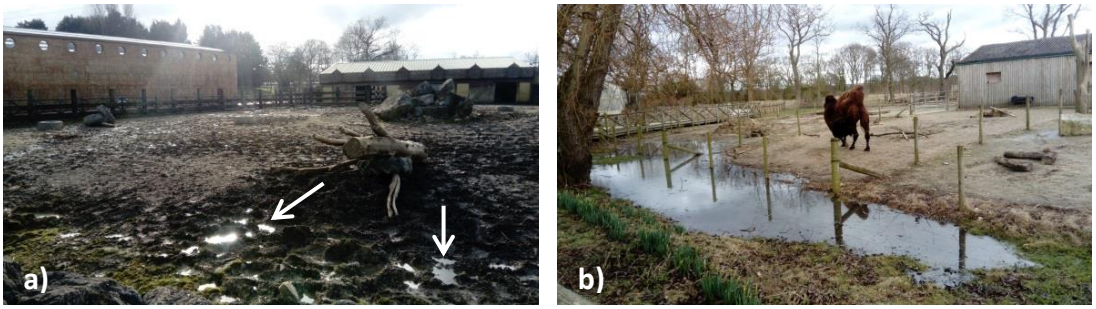


Figure 3.2. Potential mosquito oviposition sites in Flamingo Land. a) Water pockets created in footprints (arrows). b) Puddle in the camel exhibit. Picture by M. Gonzalez-Olvera.

3.2.2 Avian malaria outbreak in Chester Zoo

Chester Zoo is the most popular zoo in the UK receiving 1.9 million visitors in 2018. It was established in 1934 in Upton by Chester, Cheshire, and houses over 21,000 animals that belong to more than 500 species, 1,912 of which are birds from 154 species. The zoological gardens cover an area of 51 ha and it has a natural reserve in its north boundary that extends over 6 ha (TNEZS, 2019).

Like in Flamingo Land, the penguins housed in Chester Zoo are Humboldt penguins (*Spheniscus humboldti*). Their open exhibit is surrounded by a footpath on two of its sides and has a viewing glass in their pool so the visitors can watch them underwater.

In the 4th of September 2017, the penguins were moved to a temporary enclosure while their original exhibit was renovated (Figures 3.3). As we started our sampling in Chester Zoo in May 2017, we were able to follow the penguins moving and provide advice regarding mosquito abundance and avian malaria infection status.

The temporary enclosure was a former orangutan exhibit with a pond of flowing water that is completely closed except for window openings in one of its walls. As high humidity was a concern, the ventilation was improved with fans. We set two CDC-miniature light traps inside the enclosure and four more around it, collecting the nets every week. In the first two collections we got seven mosquitoes from the traps inside the enclosure, so the zoo staff installed fine plastic mesh in the window openings and closed any gaps with spray foam. After this, we did not find more mosquitoes inside the enclosure. The outside traps captured only seven mosquitoes.

When the penguins were moved out, blood samples for PCR testing and smears were taken from nine individuals and smears only from other 21. During the moving back to the original exhibit, another ten blood samples and smears were taken. Nevertheless, none of these samples were positive for Haemosporidians with either of both techniques.

The first ill penguin was detected on the 21st of September and died soon after; more penguins became unwell and died at irregular intervals in the following weeks. In some penguins no signs were noticed but anorexia, ataxia, dyspnoea, lethargy, regurgitation, weight loss and lymphocytosis were observed. Avian malaria was confirmed by PCR in five penguins that died at the beginning of the outbreak and as shortly as the first case was confirmed, the daily treatment with primaquine was instigated, complemented with doxycycline. Some penguins showed improvement with the treatment but died nonetheless, others were able to recover. The last death happened on the 31st of October; thus, the outbreak span was of almost six weeks.

The species of the parasite found in the five positive penguins was *Plasmodium matutinum*, a common species found in wild birds and Humboldt penguins (Valkiūnas et al., 2017), but never reported in association with penguin mortalities before. Other causes of death were also found: five penguins died from aspergillosis, two, due to the ingestion of foreign bodies and the cause of death could not be determined in the rest. From the 45 penguins in the colony at the time, 26 died.

After the penguins were moved back to their original exhibit, no more sick penguins were observed, the ones under treatment completed recovery and no more deaths occurred. It is likely that the stressful situation of the moving compromised the immune system of the penguins and the outbreak was caused by opportunistic infections. As the mosquito abundance was high in the zoo at the moment of the moving and later on it was low in and around the temporary enclosure, it is possible that the avian malaria positive penguins were infected before the moving. Nonetheless, the blood samples were negative; therefore, the *Plasmodium* infection could have been in its early stage or in a dormant stage in organs and the circumstances favoured the virulence or relapse of the infection.

The following year, the zoo acquired 25 penguins to compensate for the losses, preventive treatment was provided from April to October and some measures to control the mosquito population, like the regular cleaning of organic matter in nearby ponds, were performed. However, another penguin was positive to *Plasmodium*, in this case *P. relictum*, but recovered favourably after treatment.

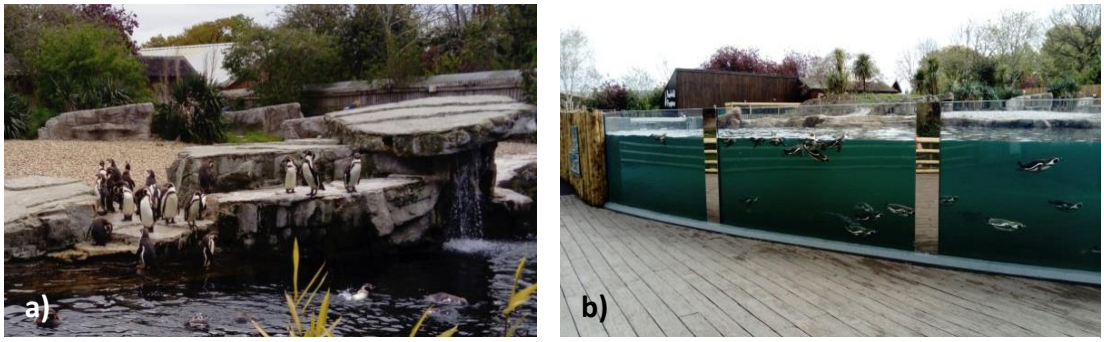


Figure 3.3. Penguins exhibit in Chester Zoo. Exhibit before (a) and after (b) the renovation. Picture by M. Gonzalez-Olvera.

3.3 Materials and Methods

The sampling period was based on the expected activity season of the mosquitoes through the year, described by Brugman (2016) as April to November for *Culex* spp. (Brugman, 2016). We had two sampling seasons in Chester Zoo, from the beginning of May until the beginning of December in 2017 and from the beginning of April until the beginning of November in 2018. In 2017, the mosquitos were already active when we started the sampling; therefore, in 2018 we started earlier to catch the start of the mosquito season. In Flamingo Land we had one sample season from June until November 2017. We did not continue with the analysis of mosquitoes in Flamingo Land in 2018 because, due to the

travelling distance, the sampling was done by the veterinary staff and it proved to be time demanding for them and for us. The end of sampling each year was defined as the absence of female mosquitoes catches for two consecutive weeks in the single day collections.

3.3.1 Sampling of mosquitoes

The mosquito sampling consisted of three parts: adults, immature stages and overwintering mosquitoes. Adult mosquitoes were of especial interest as the females carry the avian malaria parasite. For capturing adult mosquitoes, different kinds of traps could be used but all of them have limitations and a single one cannot provide enough information for the comprehensive assessment of a mosquito community. Therefore, we also performed larvae sampling in potential oviposition sites, as in this way we could get a better representation of the mosquito species and to analyse a possible correlation with the adult mosquitoes in the area. Finally, we were interested on the overwintering places for mosquitoes and the risk that these could represent to the birds at the beginning of the following season.

Sampling areas were defined as 30 m diameter circles in which we had two adult traps and if possible, a sampling area for immature stages (see section 2.5); they were coded with the letter A and a unique number per site. During the first season in Chester Zoo, we established ten sampling areas around the penguin exhibit and near other bird enclosures. The active areas during 2017 were A1-A7 and A10-A12 (areas A8 and A9 were proposed but not implemented due to logistic complications). In 2018 we removed areas A5-A11, due to their low mosquito catches and their location away from the penguin exhibit; we added area A13 inside the penguin exhibit to have better representation of the mosquito activity and biting risks for the penguins (see section 2.5.2). We proposed eight areas in Flamingo Land but again, for logistic implications, only the four most relevant were implemented (A1-A4) (see section 2.5.3).

The adults were captured using two different traps, the BG-Mosquitaire® and the CDC-Gravid trap model 1712. Both traps use an electric fan for sucking the mosquitoes into a collection net. The BG-Mosquitaire attracts females that are looking for a blood meal with a lactic acid compound (BG-Sweetscent®) that mimics mammalians sweat. This trap can be adapted to release CO_2 from a cylinder with a nozzle and a regulator which makes it more effective, but for safety concerns, we used it without CO_2. The CDC-Gravid trap uses an infusion that contains hay, brewer's yeast and milk powder, to attract females that are looking for a suitable breeding site to lay their eggs. The functioning of the traps is detailed in section 2.2.1.

In Chester Zoo, we collected immature mosquitoes in six sampling areas, A1, A3, A4, A5, A10 and A11. The sampling was carried out in an area rather than a point; thus, these areas were coded with an L and the corresponding area number. For L1 the sampling area was the shore of the flamingo pond, for L3, the margins of the off-show aviaries pond, for L4, the water-bed in the greenhouses area, for L5, the ponds and channels of the Chinese garden, for L10, the shores of the wetland's aviary pond, and for L11, the ponds in the mini-golf. The sampling procedure for immature mosquitoes is explained in section 2.2.2.

We did not find a consistent sampling site in Flamingo Land; we did some samplings in the water bodies including a natural pond in one of the exhibits in which we found *Cx. pipiens* larvae. Nevertheless, this pond lasted for few weeks and the access to it was difficult, so we did not analyse these samples beyond the identification of the specimens.

The sampling of overwintering mosquitoes consisted of aspirating inside potential resting places such as sheds, buildings, indoor exhibits and structures that could provide some protection for the mosquitoes like penguin nesting boxes. For this, we used an Improved CDC Backpack Aspirator Model 1412. We did two overwintering samplings in each zoo, on the 6th of December 2017 and the 2nd of February 2018, in Chester Zoo and on the 30th of November 2017 and the 25th of January 2018 in Flamingo Land. We sampled the buildings in the surroundings of our established sampling areas.

3.3.2 Sampling protocol

The sampling protocol comprised of two days of activities per week. We started the sampling seasons setting all the traps in the first day and collecting the nets on the following day. Afterwards, in the first day we prepared the CDC-Gravid traps adding the infusion media and connecting a charged battery and changed the nets of the BG-Mosquitaire traps. In the second day, we collected the nets of the CDC-Gravid traps and switched them off, swapped again the nets of the BG-Mosquitaire traps, which were constantly running, and did the immature mosquitoes sampling. In this way we had collections after six days from the BG-Mosquitaire traps (week collections) and after one day from both traps (day collections).

When the penguins were moved to the temporary enclosure, we installed two CDC-miniature light traps inside it and another five around it. These traps attracted mosquitoes only with UV light and their nets were collected after operating for one night every week from the 27th of September until the 3rd of November 2017. Additionally, we aspirated

mosquitoes in a shed attached to the enclosure twice, on the 23rd of November and the 6th of December 2017, as described for the overwintering samplings. These samplings were used only for assessing the presence of mosquitoes and were not included in the analyses. There were no additional or overwinter samplings during 2018.

The mosquitoes were processed for morphological identification, DNA extraction, molecular identification and parasite testing as described before (sections 2.7 and 2.8). Except for the 2018 *Culex* spp. mosquitoes, which were identified by morphology and only those positive to *Plasmodium* were identified by PCR.

3.3.3 Traps selection

I reviewed ten scientific papers that reported the capture of *Cx. pipiens* in Europe presenting results with enough detail for comparing the traps' efficiency. In these studies, the field work was done at different times, in diverse locations and the sampling protocols were different in each case; thus, a direct comparison was not possible. Therefore, I transformed their results into sampling effort units which are calculated by multiplying the number of traps by the number of nights in which the traps were operational. Then, the capture efficiency of the traps was obtained by dividing the number of caught mosquitoes by the sampling effort units (Table 3.1 and Figure 3.4).

The data was heteroscedastic (Barlett's test, $p < 0.00$), so I did a Welch's Anova to compare the efficiency by trap kind, and a two-way Anova to analyse differences by location. Some authors did not report their results by location; thus, these data were not included in the second analysis. In some cases, the same traps were selected but if the attractant used was different, they were analysed separately.

The most popular trap was the Mosquito Magnet, used in six studies, followed by the CDC-light traps used in five, the BG-Sentinel used in two and the Heavy-Duty Encephalitis Vector Survey trap (EVS), BG-Mosquitaire and CDC-Gravid, used in one each. The traps with data for only one observation (EVS and BG-Mosquitaire) were removed from the comparison. Most of the studies took place in some kind of wetland (six); four were in urban and suburban areas, and one in agricultural and woodland environments.

There was a difference by trap kind ($p = 0.029$); therefore, a Fisher's Least Significance Difference comparison with Bonferroni correction was used to show that the BG-Sentinel trap was the most effective trap for capturing *Cx. pipiens*. The two-way Anova confirmed the difference by traps ($p = 0.015$) but there were no differences by location ($p = 0.306$) nor

a trap by location interaction (p = 0.681), possibly due to the diversity of locations and the low number of observations in each one.

In view of these findings, the zoo staff were also interested in following the sampling protocol proposed by Quintavalle et al. (2015) in the Project MOSI to have comparable results (Quintavalle Pastorino et al., 2015). In this project, the authors recommend the use of the BG-Mosquitaire trap for its versatility and efficiency. The BG-Sentinel and BG-Mosquitaire traps have a similar design and same attractant with the difference that the first one was designed for research and field work and the second one, for domestic use. We also considered that it could be a risk for the zoo animals, visitors and zoo staff, to operate traps that require flammable or compressed gases.

Considering that CDC-Gravid trap was designed for capturing *Culex* spp. mosquitoes and that other authors have found high catches of this genus in comparison with other traps (Cilek et al., 2017, Hesson et al., 2015a), we decided to use it as well to complement our samplings. An additional advantage of this trap is that it attracts gravid mosquitoes meaning that they had fed on a vertebrate host at least once, which could increase the possibility of finding *Plasmodium* spp. in them. The functioning of the traps is described in section 2.2.1.

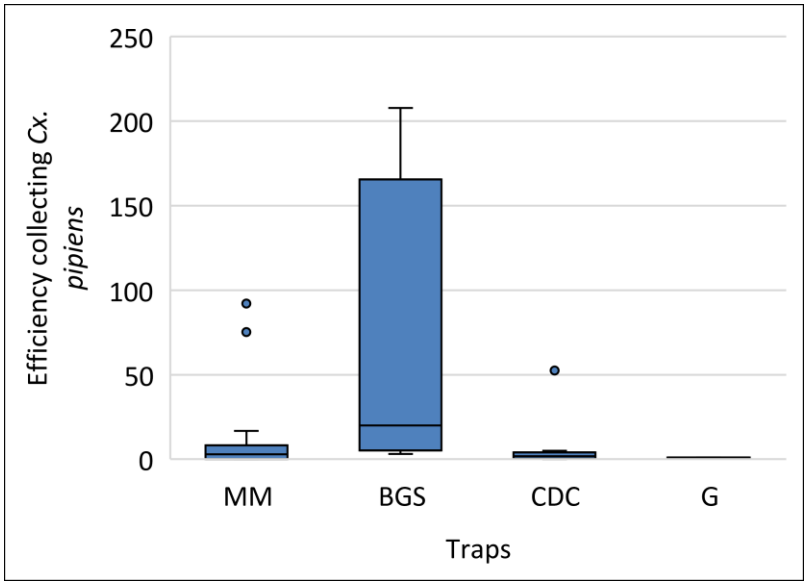


Figure 3.4 Trap efficiency for *Cx. pipiens*. MM: Mosquito Magnet, BGS: BG-Sentinel, CDC: CDC-light trap, G: gravid trap.

Table 3.1. Catch efficiency for *Culex* spp. of traps used in mosquito studies in Europe.

Author	Country	Trap	Attractant	Location	Sampling Effort			*Cx. pipiens* collected	Catch efficiency
					Traps	Active days	Sampling units		
(Byriel et al., 2018)	Denmark	Mosquito Magnet	Octenol, CO2	various	3	609	1827	220	0.12
(Vogels et al., 2016)	Sweden	BG-Sentinel	BG-Lure, CO2	agricultural (farms)	1	6	6	19	3.17
				suburban	1	6	6	56	9.33
				wetland	1	6	6	33	5.50
	Sweden	Mosquito Magnet	Octenol, CO2	agricultural (farms)	1	6	6	29	4.83
				suburban	1	6	6	44	7.33
				wetland	1	6	6	24	4.00
	Netherlands	BG-Sentinel	BG-Lure, CO2	agricultural (farms)	1	6	6	252	42.00
				suburban	1	6	6	1063	177.17
				wetland	1	6	6	969	161.50
	Netherlands	Mosquito Magnet	Octenol, CO2	agricultural (farms)	1	6	6	64	10.67
				suburban	1	6	6	451	75.17
				wetland	1	6	6	101	16.83
	Italy	BG-Sentinel	BG-Lure, CO2	agricultural (farms)	1	6	6	128	21.33
				suburban	1	6	6	111	18.50
				wetland	1	6	6	1247	207.83
	Italy	Mosquito Magnet	Octenol, CO2	agricultural (farms)	1	6	6	21	3.50
				suburban	1	6	6	37	6.17
				wetland	1	6	6	553	92.17
(Hesson et al., 2015b)	Sweden	CDC-miniature light trap	Light, CO2	wetland (floodplains)	35	6	210	367	1.75

Table 3.1. Continued

(Hesson et al., 2015a)	Sweden	CDC-trap	Light, CO2	wetland (floodplains)	1	156	156	20	0.13
		CDC-Gravid trap	Hay infusion	wetland (floodplains)	1	28	28	28	1.00
		CDC-trap	Light, CO2	urban	1	28	28	143	5.10
		CDC-Gravid trap	Hay infusion	urban	1	28	28	6	0.21
(Quintavalle Pastorino et al., 2015)	United Kingdom	Mosquito Magnet	Octenol, CO2	urban (zoo)	10	365	3650	283	0.08
		BG-Mosquitaire	BG-Lure	urban (zoo)	3	365	1095	881	0.80
(Vaux et al., 2015)	United Kingdom	Mosquito Magnet	Octenol, CO2	wetland (marshes)	NR	NR	75	164	2.19
(Luhken et al., 2014)	Germany	BG-Sentinel	BG-Lure, CO2	various	1	332	332	1398	4.21
		CDC-miniature light trap	Light, CO2	various	1	332	332	655	1.97
		Heavy Duty Encephalitis Vector Survey trap	CO2	various	1	332	332	861	2.59
		Mosquito Magnet	Octenol, CO2	various	1	296	296	29	0.10
(Ventim et al., 2012a)	Portugal	CDC-miniature light trap	Light, CO2	wetland (marshes)	1	22	22	1153	52.41
(Hutchinson et al., 2007)	United Kingdom	CDC-trap	Light, CO2	urban	1	56	56	14	0.25
				woodland	1	68	68	12	0.18
				wetland (saltmarsh)	1	68	68	215	3.16
				wetland (freshwater marshland)	1	68	68	114	1.68
	United Kingdom	Mosquito Magnet	Octenol, CO2	urban	1	56	56	23	0.41
				woodland	1	68	68	13	0.19
				wetland (saltmarsh)	1	68	68	0	0.00
				wetland (freshwater marshland)	1	68	68	6	0.09

Table 3.1. Continued

(Huijben et al., 2007)	Netherlands	Mosquito Magnet	Octenol, CO2	urban (zoo)	3	112	336	348	1.04
Hernandez-Colina, A. *et al*. (2017, this study)	United Kingdom	BG-Mosquitaire	BG-Lure	zoo (Chester Zoo)	10	218	2180	3906	1.79
		CDC-Gravid trap	Hay infusion	zoo (Chester Zoo)	10	32	320	3192	9.97
		BG-Mosquitaire	BG-Lure	zoo (Flamingo Land)	4	179	716	937	1.31
		CDC-Gravid trap	Hay infusion	zoo (Flamingo Land)	4	19	76	185	2.43

Other traps that were used with less frequency or that collected small numbers of *Cx. pipiens* were not included in the analysis.

3.4 Results

3.4.1 Adult mosquitoes

3.4.1.1 Chester Zoo

The total number of adult mosquitoes captured in 63 samplings during the first year was 7,938, including the ones caught with BG-Mosquitaire and CDC-Gravid traps (n = 7,888), during overwintering samplings (n = 28), using the CDC-miniature light traps in and around the penguins' temporary enclosure (n = 14), and aspirating mosquitoes in the shed next to the temporary enclosure (n = 8).

The captured mosquitoes belonged to four genera and eight species: *Anopheles claviger*, *An*. *maculipennis* and *An*. *plumbeus* from the Anophelinae subfamily and *Coquillettidia richiardii, Culex pipiens*, *Cx*. *torrentium*, *Culiseta annulata* and *Cs*. *morsitans* from Culicinae. Most of the mosquitoes were females (n=6,920, 87.2%) but there were some males as well (n=1,012, 12.7%). The dominant species was *Culex pipiens* (63.65 %) followed by *Culiseta annulata* (3.49 %). Some mosquitoes were damaged and could not be fully identified, most of them from the *Culex* spp. genus (n = 916) and the Culicinae subfamily (n = 436); in total they constituted 1,380 individuals (Table 3.2).

In 2018, we captured 2,962 mosquitoes from 61 samplings; the species found were the same as in the previous year although their proportions varied slightly. A similar percentage of females and males was observed, 91.46% (n = 2,794) and 8.51% (n = 260) respectively, and the most abundant species were, again, *Cx*. *pipiens* (76.98 %) and *Cs*. *annulata* (4.19 %) (Table 3.2). The average collection of mosquitoes per sampling area and by month/year is shown in Figure 3.5. The totals of mosquitoes captured as adults and immature stages by collections and areas are presented in Appendix 3.1 (Chester Zoo 2017) and Appendix 3.2 (Chester Zoo 2018).

3.4.1.2 Flamingo Land

The 47 samplings using the traps in Flamingo Land yielded 1,588 mosquitoes in total. Both mosquito families were represented but fewer species were collected; from the Anophelinae subfamily only *Anopheles maculipennis* was captured and from Culicinae, we found *Culex pipiens*, *Cx*. *torrentium* and *Culiseta annulata*. A higher proportion of males was found compared to Chester Zoo, the females accounted for 80.0% (n = 1270) and the males for 20.0% (n = 318). The most abundant species was again *Cx. pipiens* (70.78%) and the

other species were found in lower proportions (Table 3.2). In Figure 3.6, the average collections by sampling areas and months are presented and the full collections can be found in Appendix 3.3.

Table 3.2. Adult mosquitoes collected in Chester Zoo and Flamingo Land.

Subfamily	Genus	Species	Chester Zoo 2017 (%)		Chester Zoo 2018 (%)		Flamingo Land 2017 (%)	
Anophelinae	*Anopheles*	*claviger*	2	(0.03)	4	(0.14)	0	(0)
		maculipennis	11	(0.14)	28	(0.95)	11	(0.69)
		plumbeus	2	(0.03)	1	(0.03)	0	(0)
		unknown	3	(0.04)	1	(0.03)	0	(0)
Culicinae	*Coquillettidia*	*richiardii*	5	(0.06)	2	(0.07)	0	(0)
	Culex	*pipiens*	6163	(77.64)	2278[a]	(76.91)	1124	(70.78)
		torrentium	48	(0.60)			60	(3.78)
		unknown	927	(11.68)	126	(4.25)	90	(5.67)
	Culiseta	*annulata*	276	(3.48)	128	(4.32)	31	(1.95)
		morsitans	3	(0.04)	12	(0.41)	0	(0)
		unknown	21	(0.27)	23	(0.78)	1	(0)
	unknown	unknown	477	(6.00	359	(12.12)	271	(17.07)
Total			**7938**		**2962**		**1588**	

[a]: The differentiation between *Cx. pipiens* and *Cx. torrentium* was not done on all 2018 mosquitoes, thus their count is presented together.

3.4.1.3 Analysis

It was observed that the sex proportion varied across the seasons; more males were captured towards the end of both years and in both zoos (Figure 3.7). The sex ratios were analysed for each zoo and year in relation to the regional temperature and rainfall (see section 5.3.2 for data source) constructing generalised linear models (GLM). The number mosquitoes captured in the day and week collections were merged by week. The GLM showed a significant difference in proportions in relation to temperature but not to rain or their interaction in all cases (Table 3.3). This was probably due to the females starting to overwinter indoors while the males remain in the environment until they die in winter (Becker et al., 2010). Therefore, the males were excluded and only the females' data were used in the following analyses to prevent additional variation.

Table 3.3. Parameters of the GLMs for the proportion of mosquito sexes.

Variables	Chester Zoo 2017		Chester Zoo 2018		Flamingo Land	
	t value	Pr (>|t|)	t value	Pr (>|t|)	t value	Pr (>|t|)
Intercept	-4.267	< 0.00	-1.948	0.062	-1.131	0.274
Temperature	5.368	< 0.00	4.325	< 0.00	2.288	0.035
Rain	-0.016	0.988	1.736	0.094	-1.053	0.307
Temperature: Rain	-0.025	0.980	-1.745	0.092	0.942	0.359

Quasibinomial family used to account for overdispersion.

Due to the nature of sampling in a working zoo, some collections could not be done; in Chester Zoo, one trap was lost possibly by public disturbance, the cables of two BG-Mosquitaire traps were cut during gardening works, one sampling area was inaccessible for one week due to contractors on site, the BG-Mosquitaire trap was unplugged during an oil spill in the flamingo exhibit, and it was not possible to collect some nets due to veterinary emergencies. I conducted Kruskal-Wallis tests to compare the data sets by year including and excluding these missed collections, for all sampling areas together and comparing individual sampling areas. There was no significant difference, all p-values > 0.7 for 2017 collections and p > 0.2 for 2018 in Chester Zoo and disturbances did not affect the collections in Flamingo Land. Therefore, subsequent analyses included all the collections.

For all data sets used in the analysis, I did an Anderson-Darling test, a Bartlett test of homogeneity of variances and analysed the histogram of the residuals to test for normality. In all cases, the raw data was non-normally distributed, with heteroscedasticity (non-homogeneity of variances) and were closer to a negative binomial distribution instead. Different approaches were taken depending on the particular analysis.

I did a two-way Anova to test the hypothesis of different captures by months and sampling areas (as categorical variables) and to see if there was an interaction between these factors. I used the data from the BG-Mosquitaire traps consolidating the captures after one day and after six days for the same week to have a better representation of the mosquito abundance. I used a Log10 (n + 1) transformation of the data as this showed a more robust model with residuals closer to normality. Afterwards, I did a least-square means comparisons to find the differences among groups.

There was a strong significance by sampling areas, months and by the interaction between them in both years in Chester Zoo (all p values < 0.000). The multiple comparisons showed that area A1 got significantly more mosquitoes than the other areas in 2017 with the

exception of A3; areas A3, A4 and A7 were not different among each other such as areas A2, A5, A6, A10, A11, and A12 (Figure 3.5 a). Similarly, in 2018 area A1 captured significantly more mosquitoes than the other areas which were grouped together and only areas A2 and A4 were different from area A12 (Figure 3.5c).

In the first year in Chester Zoo, July and August were different from the other months, as well as June and September, and May and November, October was different from all others (Figure 3.5b). In 2018, July, August and September were different from the other months although September was not different from June (Figure 3.5d).

The differences by sampling areas and months were also significant in Flamingo Land ($p < 0.001$) although the significance in the areas and months interaction was lower ($p = 0.028$). The grouping showed more overlapping and area A3 was different from areas A2 and A4 (Figure 3.6a). Regarding the months, September and October were different from June, July and November, which can be seen by the higher catches between August and October (Figure 3.6b).

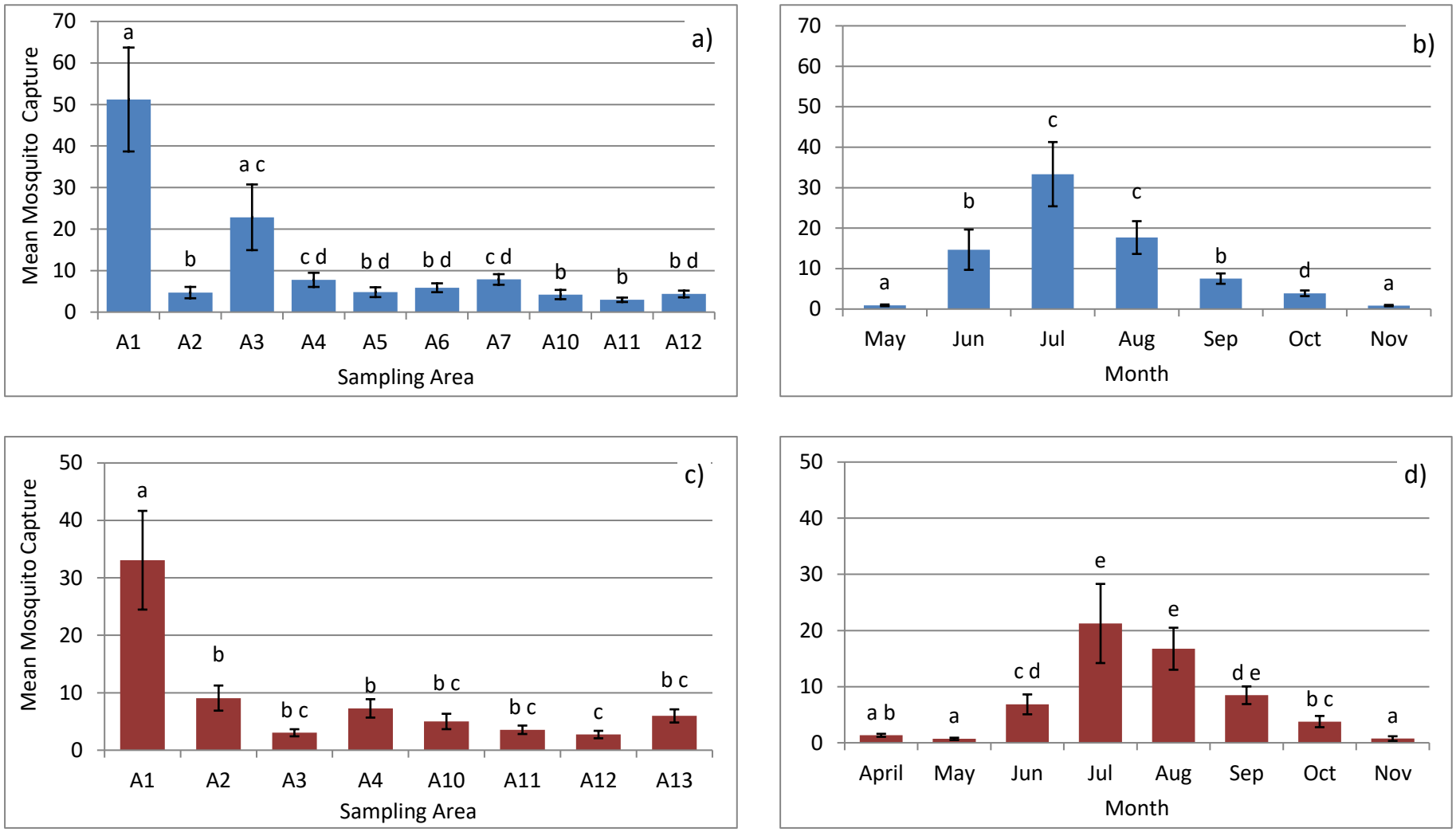


Figure 3.5. Average collection of mosquitoes in Chester Zoo. a) Sampling areas in 2017, b) Months in 2017, c) Sampling areas in 2018 and d) Months in 2018. Error bars: standard error of the mean (SEM). Groups with shared letters were not significantly different among each other.

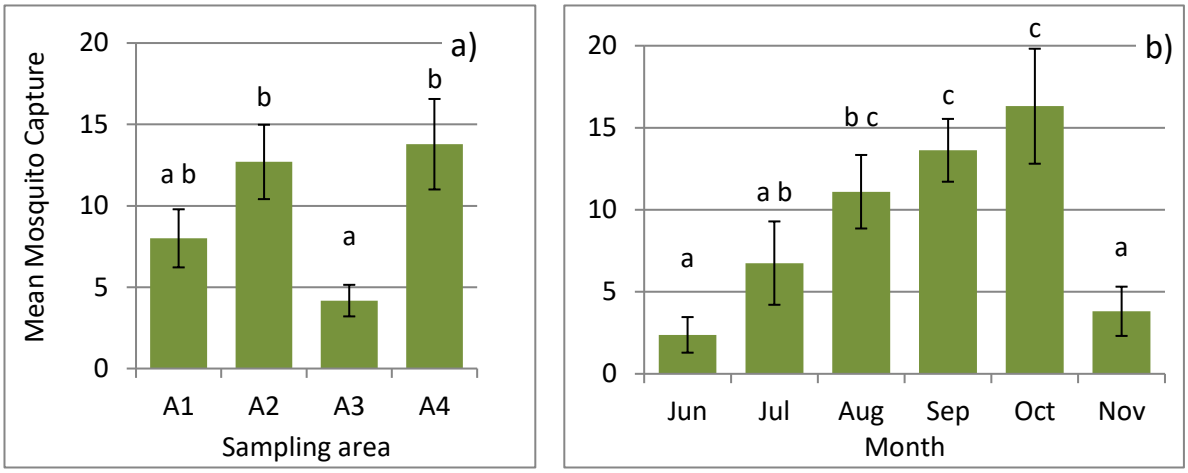


Figure 3.6. Average collection of mosquitoes in Flamingo Land. a) Sampling areas in 2017, b) Sampling months in 2017. Error bars: standard error of the mean (SEM). Groups with shared letters were not significantly different among each other.

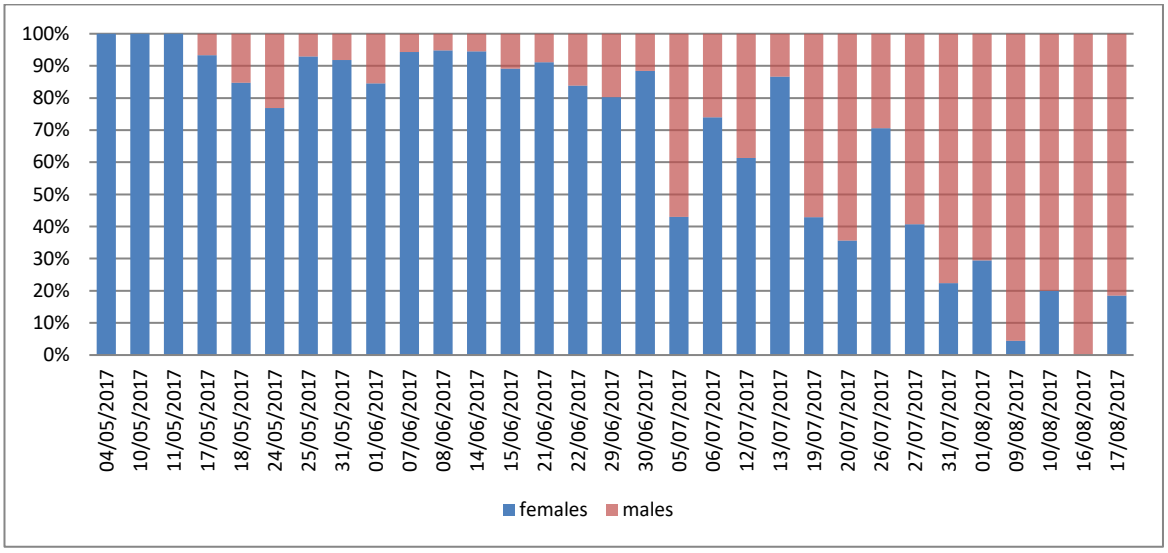


Figure 3.7. Sex proportions of mosquitoes captured in the week collections from Chester Zoo, 2017.

I tested the efficiency of the traps by comparing the mosquitoes captured by trap, using only the one-day collections from the BG-Mosquitaire on the same days as the CDC-Gravid. In this way, in 2017 the BG-Mosquitaire traps captured 572 mosquitoes and the CDC-Gravid traps, 3,371, and in 2018 they captured 371 and 830, respectively. In the first three weeks of sampling in Flamingo Land, the CDC-Gravid traps were not operating so these collections were excluded from the comparison and the total captures analysed were 266 in the BG-Mosquitaire and 248 in the CDC-Gravid traps. The Kruskal-Wallis test provided $p < 0.001$ in both years in Chester Zoo showing that the CDC-Gravid trap is more efficient for capturing mainly *Culex* spp., but in Flamingo Land the difference was not significant ($p = 0.387$). The

data transformation, Log10 (n + 1), did not improved normality, thus the non-transformed data was used organised by sampling area (Table 3.4).

For comparing catches between years in Chester Zoo, I used the data from the BG-Mosquitaire traps consolidated by week and did a Paired t-test with a Log10 (n+1) transformation. In this case, I used the data from the sampling areas that were active in both years, these being A1, A2, A3, A4, A10, A11 and A12, and for the months that were sampled in both years (from May to October). The mosquito abundance was higher in 2017 (p=0.011) (Table 3.5).

Table 3.4. Female mosquitoes captured by trap after one day operating.

Trap Type	Chester Zoo 2017 (%)	Chester Zoo 2018 (%)	Flamingo Land 2017 (%)
BG-Mosquitaire	572 (14.51)	371 (30.89)	266 (51.75)
CDC-Gravid	3371 (85.49)	830 (69.11)	248 (48.25)
Total	3943	1201	514

Table 3.5. Female mosquitoes captured by area in Chester Zoo in the BG-Mosquitaire traps.

Year	Area 1	Area 2	Area 3	Area 4	Area 10	Area 11	Area 12	Total
2017	1536	141	685	233	127	89	131	2942
2018	959	263	88	211	145	103	79	1848
Total	2495	404	773	444	272	192	210	4790

3.4.2 Immature mosquitoes

From the immature stage samplings, we collected 1,658 mosquitoes during 2017 in Chester Zoo. The seven species identified were *Anopheles claviger*, *An*. *Maculipennis, An*. *plumbeus, Aedes detritus, Culex pipiens or Cx. torrentium* and *Culiseta annulata*. The sex proportion was around 50% as expected, and again the most abundant species were *Cx. pipiens* or *Cx torrentium* (73.82%) and *Cs*. *annulata* (21.29%) (Table 3.6). Higher collections were obtained from Areas L1, L4 and L10 and during July and August (Figure 3.8).

Table 3.6. Immature mosquitoes collected in Chester Zoo in 2017.

Subfamily	Genus	Species	Chester Zoo 2017	(%)
Anophelinae	*Anopheles*	*claviger*	29	(1.75)
		maculipennis	11	(0.66)
		plumbeus	15	(0.90)
		unknown	2	(0.12)
Culicinae	*Aedes*	*detritus*	1	(0.06)
	Culex	*pipiens / torrentium* [a]	1224	(73.82)
		unknown	3	(0.18)
	Culiseta	*annulata*	353	(21.29)
		unknown	1	(0.06)
	unknown	unknown	19	(1.15)
Total			**1658**	

[a]: The molecular identification for *Cx. pipiens* and *Cx. torrentium* was not done.

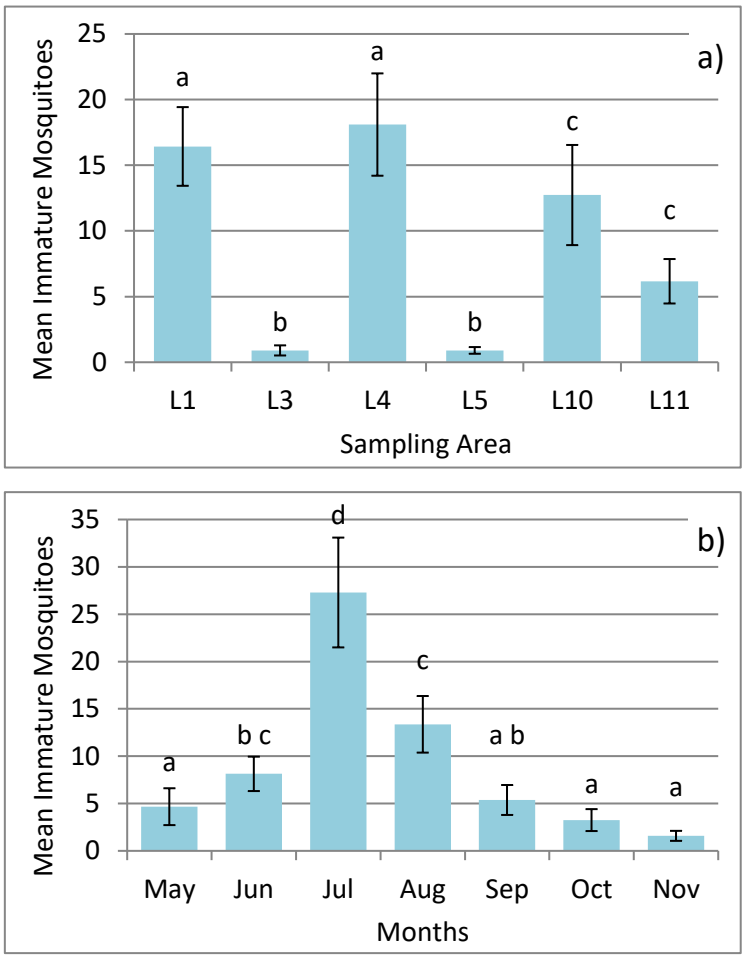


Figure 3.8. Average collection of immature mosquitoes in Chester Zoo, 2017. a) Sampling areas, b) Months. Error bars: standard error of the mean (SEM). Groups with shared letters were not significantly different among each other.

3.4.2.1 Analysis

Using a two-way Anova and least-square means comparisons, I explored the differences in immature mosquitoes collected by sampling areas and months. The months were used as a grouping factor maintaining the individual collections separately, but the collection of immature mosquitoes from December was excluded because it comprised one observation and one specimen and thus, it was not comparable with the other months. The differences were significant for areas, months and the interaction among them (all p values < 0.001). The multiple comparisons showed that July was different from the other months, August was different from the others, but June was not different from September. Regarding the areas, there was no overlap among groups and the areas were grouped as follow, L1 and L4, L10 and L11, and L3 and L5; each group being different from the others (Figure 3.8).

There was a higher abundance of adult mosquitoes and immature stages during July and August in 2017, so I did Spearman rank correlations looking for associations between sampling types. I used only female's data as the sex proportions were different between data sets (it was constantly around 50% in the immature samplings, but the proportion of adult females decreased towards the end of the year). The adult mosquito data was taken from the BG-Mosquitaire collections consolidated by week and the comparisons included the collections of both sets that were done in the same week. As the proportions per species were also different, I separated the analysis by genus and did the comparison for *Culex* spp. and *Culiseta* spp.; the captures of other species were not enough for analysis. I also separated the data by sampling areas as they were not equivalent, and some areas were not analysed due to the low number of mosquitoes per species (L3, L5 and L11 were excluded for *Culex* spp. and L4 and L5 for *Culiseta* spp.); otherwise, a false correlation would have occurred due to the high number of samplings without catches.

The association between immature and adult mosquitoes may not be reflected immediately and could depend on the mosquito development time instead; therefore, I applied a series of lags of one week to perform additional correlations between them. The adult mosquitoes were considered as the dependent variable and the lags were applied to the immature mosquito collections. I continued with the lags until there was clear lack of significance.

I found significant correlations that varied among sampling areas, lags and mosquito genus. For *Culex* spp., the significant lags were negative; this means that the collection of immature mosquitoes was correlated with the capture of adult mosquitoes from later

weeks, suggesting that the abundance of immature mosquitoes was reflected in adult's abundance after some delay. Based on the p and rho values of the Spearman rank correlation, the most significant lags for area L1 were Lag 0, Lag -1 and Lag -2, for area L4, Lag -4, Lag -5 and Lag -6, and for Area L10, Lag -1, Lag -2 and Lag-3 (Table 3.7).

In the case of *Culiseta* spp., significant lags were also negative, although positive lags were observed in two areas; in this case, it could mean that both populations influence each other at different times with some delay. The significant lags for area L1 were Lag -1, Lag -2 and Lag -4, for area L3, Lag -1, Lag -2 and Lag -3, for area L10, Lag 1, Lag 0 and Lag -1, and for area L11, Lag 3, Lag 2 and Lag 0 (Table 3.8). It is interesting to notice that many of the correlations (rho values) were positive for *Culex* spp. and negative for *Culiseta* spp., with the exceptions of area L4 for the first one and L3 for the second one.

Table 3.7. Spearman rank correlation results for different lags and sampling areas for *Culex* spp. in Chester Zoo, 2017.

	Lag -6		Lag- 5		Lag -4		Lag -3		Lag -2		Lag -1		Lag 0		Lag +1		Lag + 2		Lag +3	
Area	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**
L1	0.178	0.306	**0.011**	0.530	**0.001**	0.625	**<0.000**	0.742	**<0.000**	0.777	**<0.000**	0.825	**<0.000**	0.787	**0.001**	0.613	**0.014**	0.485	0.176	0.286
L4	**<0.000**	-0.700	**0.004**	-0.587	**0.010**	-0.514	**0.010**	-0.514	**0.030**	-0.306	**0.034**	-0.417	0.181	-0.265	0.218	-0.250	0.138	-0.306	0.127	-0.321
L10	0.396	-0.237	0.440	-0.208	0.340	0.247	**0.011**	0.585	**0.001**	0.714	**0.002**	0.657	0.063	0.413	0.069	0.414	0.396	0.207	0.588	0.137

Lag 0 was obtained comparing the adult and immature collections from the same week; the change in lag is weekly applied to the immature collections. r: Spearman's rho. Significant values in bold.

Table 3.8. Spearman rank correlation results for different lags and sampling areas for *Culiseta* spp. in Chester Zoo, 2017.

	Lag -6		Lag- 5		Lag -4		Lag -3		Lag -2		Lag -1		Lag 0		Lag +1		Lag + 2		Lag +3	
Area	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**
L1	0.420	-0.209	0.119	-0.381	**0.005**	-0.619	**0.007**	-0.586	**0.001**	-0.678	**0.005**	-0.572	**0.001**	-0.630	**<0.000**	-0.710	**0.041**	-0.449	0.072	-0.411
L3	0.140	0.386	0.100	0.413	0.162	0.344	**0.008**	0.593	**0.006**	0.589	**0.010**	0.550	0.441	0.173	0.846	0.045	0.957	0.013	0.451	0.184
L10	0.779	-0.091	0.648	0.140	0.599	0.154	0.466	-0.204	0.058	-0.483	**0.005**	-0.649	**0.009**	-0.595	**0.035**	-0.513	0.059	-0.482	0.150	-0.391
L11	NA	NA	NA	NA	NA	NA	0.869	0.060	0.732	-0.117	0.980	-0.008	0.263	0.335	0.901	0.040	0.483	-0.237	0.084	-0.572

Lag 0 was obtained comparing the adult and immature collections from the same week; the change in lag is weekly applied to the immature collections. r: Spearman's rho; NA: not applicable (there were not enough data points for those lags). Significant values in bold.

3.4.4 Overwintering mosquitoes

The overwintering samplings in Chester Zoo were done in the buildings in and near the sampling areas. The first sampling produced more mosquitoes (n = 20) than the second one (n = 8). These mosquitoes were identified as *Anopheles maculipennis* (n = 1), *Cx*. *pipiens* / *torrentium* (n = 22) and *Cs*. *annulata* (n = 5) (Table 3.9).

Table 3.9. Overwintering samplings in Chester Zoo.

	Area	6th December 2017 Species (n)	2nd February 2018 Species (n)
A1	Flamingo night enclosure	(0)	(0)
A2	Snack shed	(0)	(0)
A3	Interpretation cottage	(0)	(0)
A4	Greenhouses	(0)	(0)
A5	Tool shed	(0)	(0)
A6	Staff area in Komodo dragon exhibit	*Culex pipiens / torrentium* (1)	*Culex pipiens / torrentium* (4)
A7	Tool shed	(0)	(0)
A10	Wetlands aviary night enclosure	*Anopheles maculipennis* (1) *Culex pipiens / torrentium* (3) *Culiseta annulata* (2)	(0)
A11	Snack shed	(0)	(0)
A12	Penguin kitchen and freezers shed	*Culex pipiens / torrentium* (1) *Culiseta annulata* (1)	*Culex pipiens / torrentium* (2) *Culiseta annulata* (1)
P	Shed outside penguin temporary enclosure	*Culex pipiens / torrentium* (11)	*Culiseta annulata* (1)

In Flamingo Land we found 160 mosquitoes in both overwintering samplings, 138 in the first one and 22 in the second one. Most of them were *Cx*. *pipiens* / *torrentium* (n = 152) but we also found some *Anopheles maculipennis* (n = 4) and one *Culiseta annulata* (Table 3.10).

Table 3.10. Overwintering samplings in Flamingo Land.

	Area	30th November 2017 Species (n)	25th January 2018 Species (n)
A1	Penguin nest boxes and staff area	*Anopheles maculipennis* (3) *Culex pipiens / torrentium* (94) Culicinae (3) *	*Anopheles maculipennis* (2) *Culex pipiens / torrentium* (20)
A2	Tapir house	*Culex pipiens / torrentium* (39) *Culiseta annulata* (1)	(0)
A3	Shed and cover in Lemur exhibit	(0)	(0)
A4	Camel night enclosure and hay shed	(0)	(0)

*Some mosquitoes were damaged and only identification to the subfamily level was possible.

3.4.5 *Plasmodium* testing

The species of Haemosporidia found in mosquitoes from Chester Zoo were *Plasmodium matutinum* (subgenus *Haemamoeba*.), *P. vaughani* (subgenus *Novyella*) and *P. relictum* (subgenus *Haemamoeba*). From the mosquito saliva testing described in section 2.10, out of 59 mosquito samples, two were positive to *P. matutinum*. No positive mosquitoes were found in Flamingo Land.

As described in section 3.2.2, the species of *Plasmodium* found in Chester Zoo penguins in 2017 was *P. matutinum*; next year another penguin was found to be infected, in this case with *P. relictum*, and recovered favourably. In that zoo, the species found in free wild birds were *P. matutinum* and *P. vaughani.* None of the bird samples from Flamingo Land were positive to Haemosporidia.

Some of the lineages found do not correspond clearly to a particular species; thus, genetic analyses, which are ongoing, are needed to clarify their phylogeny. The prevalence of the parasites in the mosquitoes cannot be estimated hitherto because some testing is still in progress.

3.5 Discussion

This is the first study of avian malaria vectors in Chester Zoo and Flamingo Land and we found differences in the mosquito communities regarding abundance, species composition, temporality, distribution, variation between years in the first site, and parasite prevalence. Therefore, the mosquito communities are constantly changing, and regular mosquito monitoring is needed. Many of these features depend on environmental factors, thus their analysis and discussion are detailed in Chapter 6.

With the standardised sampling units for urban and zoo environments, we estimated that using the CDC-Gravid trap for one day a week and the BG-Mosquitaire trap continuously, would give us a minimum mosquito catch of almost 1800 specimens in Chester Zoo and almost 800 in Flamingo Land after seven months. Our total catches surpassed those estimations. This shows that the local environment has a strong influence in mosquito ecology and therefore, doing studies at the local scale is essential to understand drivers of mosquito abundance.

The efficiency of our traps was high, not only due to the long sampling period and the number of sampling areas. Comparing their catch efficiency for *Culex* spp. as described before in section 3.3.3, the BG-Mosquitaire had an efficiency of 1.79 in Chester Zoo and 1.31 in Flamingo Land which was higher than the 0.8 of the Project MOSI (Quintavalle Pastorino et al., 2015). Likewise, the CDC-Gravid traps had an efficiency of 9.97 and 2.43 in the respective zoos and was higher than the reported by Hesson *et al*. (2015a) in wetlands (1) and urban environments (0.21) (Table 3.1). Nevertheless, the surroundings of the traps, the environment, time of the year and the site conditions must be considered when comparing trap efficiency, thus these comparisons should be taken with caution and only as a reference.

It is not clear why the BG-Mosquitaire, designed for mammalophilic mosquitoes, is highly efficient for capturing *Culex pipiens* which is an ornithophilic species. Indeed some studies have shown better catches of this genus by placing CDC-light traps above ground level as these mosquitoes are expected to look for blood-meals among the birds perching in the canopy (Hutchinson et al., 2007). It is likely that the mosquitoes in zoos have developed a more generalist preference of hosts due to the broad offer of alternatives, including an important flow of visitors and staff members. The mosquito host preferences are analysed in Chapter 5.

As the CDC-Gravid trap was designed for *Culex* spp. it is not surprising that it was also very efficient capturing it. Nonetheless, the need for a battery limits its operating time and continuous sampling would require servicing the trap regularly; but at the same time, its portability is an advantage in remote locations. The main disadvantage of its design is that many mosquitoes are cut between the abdomen and thorax while passing through the fan, which complicates identification and pathogen testing, as observed by others (Hesson et al., 2015a). To solve this issue, a design modification has been suggested by Russell and Hunter (2010) (Russell and Hunter, 2010) and alternatively, the Frommer updraft gravid trap could be used instead (John W. Hock Company, 2019). In both cases, the collection net is placed before the fan to better preserve the specimens.

An important consideration is that these traps target our mosquito of interest, *Cx. pipiens*, but for having a better representation of the mosquito community, the samplings must be complemented with other traps and techniques. For example, the Mosquito Magnet can provide a broader range of species (Hutchinson et al., 2007). Sampling for immature mosquitoes can also increase the scope of species, although in our study we found more

species as adults and only one additional species (*Aedes detritus*) from the immature mosquito samplings. Therefore, the sampling for immature stages should consider the species biology; mosquitoes have different preferences for the type of habitats in which they carry out their aquatic development. For example, *Anopheles plumbeus* looks for water pockets in tree cavities, *Aedes detritus* prefers saline marshes and some species are highly specialised, like *Coquillettidia richiardii,* which we only found as adult, and has special siphons as larvae and trumpets as pupae that allow it to obtain oxygen from the arenchyma of aquatic plants (Becker et al., 2010, Cranston et al., 1987); thus dipping in the water surface is not the best method for these species. By contrast, *Culex pipiens* and *Culiseta annulata* can be found in a more general range of permanent or semi-permanent habitats with clean water or with abundant organic matter (Becker et al., 2010, Foster and Walker, 2019).

3.5.1 Adult mosquitoes

The dominance of *Culex* spp. in the mosquito community observed in both zoos and years could be explained in part by the trap bias mentioned above. But if it was the only factor, the BG-Mosquitaire traps would have attracted mammalophilic mosquitoes like *Aedes* spp. or *Anopheles* spp. in higher proportions. This was observed only with *Culiseta annulata*, which prefers to feed on mammals, as 60 - 80% of the mosquitoes from this species were caught in BG-Mosquitaire traps.

The traps in Chester Zoo showed a similar performance in both years, with the highest collection from site A1 and significantly different from all the others in 2018 (except for A3 in 2017); possibly due to its proximity to an oviposition site, the flamingo exhibit pond, which also showed a high abundance of immature mosquitoes.

During 2018, part of the off-show aviaries near area A3 were renewed and expanded; the vegetation in the zone was trimmed and the BG-Mosquitaire was moved 15 m from its original position. These changes in the surroundings certainly influenced the performance of this trap and caused its catches to drop from 22% in 2017 to 5% in 2018 in relation to all BG-Mosquitaire collections. Therefore, reducing the vegetation could be an effective measure to reduce mosquito density.

The difference in collections between 2017 and 2018 in Chester Zoo is possibly related to the particular weather of those years. In 2017, the monthly average temperature was lower, and the rainfall was higher during the summer compared to 2018. Also, the winter

was colder in 2018 with snowfall extending into March. These conditions could have made 2017 more favourable for mosquitoes, with warm temperatures and more humidity. By contrast, in 2018 the higher temperatures and less precipitation reduced the extension and number of mosquito oviposition sites and the colder winter delayed their population increase.

The peak of the mosquito season occurred in July and August during the first year, but in 2018 it was in July, August and September, as these months were not different among each other, but they varied from the rest. This change in the peak between years means that to have a clear idea of the mosquito abundance, the monitoring of the population should be done constantly.

The sampling areas in Flamingo Land showed less variance and only area A3 captured significantly fewer mosquitoes compared to the others. It is likely that the location of area A3 was a determinant in this case; the BG-Mosquitaire trap was next to a building and the CDC-Gravid trap beside some bushes and both were in a zone with scarce vegetation compared to the other areas. Despite the later start of collections in Flamingo Land, the peak of the season was observed from August to October. It is possible that the temperature and humidity conditions were favourable during the late summer and this was followed by an increase in the mosquito abundance or, as the model proposed by Ewing et al. (2016) suggests, lower temperature can delay the exit from overwintering which happens synchronously when mosquito development is not restricted by photoperiod causing a large population growth. Later, the temperature drops in November caused a marked decline in the population.

The dissimilar timing in the peaks of mosquito abundance, mainly influenced by *Culex* spp., between our sampling sites, could be related to the differences in the local environment derived from climatic conditions and latitude. Hesson et al. (2014) predicted a longer length of the growing season for *Culex* spp. in more southern latitudes in Europe (Hesson et al., 2014), which would allow mosquito populations to reach an abundance peak faster in southern locations. Although their results do not show a difference in the region of our sampling sites, the influence of latitude in mosquito seasonality should be studied at smaller scales. Temperature is a main driver of mosquito development as higher temperatures reduce the development time of mosquitoes (Ewing et al., 2016) which could contribute to their faster population growth. Nevertheless, the seasonal fluctuations should be considered as well (Ewing et al., 2016). It is important to consider the timing of the

abundance peaks for it could have implications in the transmission risk and endemic circulation of pathogens.

3.5.2 Immature mosquitoes

Culex spp. and *Culiseta annulata* breed in artificial containers or water bodies with shallow still water, rich in organic matter, and are usually found in association (Becker et al., 2010), as we noticed in our samplings. The higher captures of immature mosquitoes were influenced mainly by the abundance of these species in areas were the optimum conditions were present.

It was observed that in the areas L1, L10 and L11, the presence of organic matter was associated with aquatic plants from the genus *Typha* spp. Hence, this plant could create a microhabitat in which the immature mosquitoes can find refuge from predators and food sources. Similarly, L4 consisted of artificial water containers, water beds in which the gardeners place potted plants that require being constantly wet. Here, the immature mosquitoes could find refuge among the pots and the shallow water promoted the growing of diverse algae and in consequence, the organic matter was also high. On the contrary the water in areas L3 and L5 was cleaner and deeper which is preferred by Anopheline mosquitoes, mainly collected in these areas.

It is expected that the number of immature stages in an oviposition site will influence the number of adult mosquitoes in the same site as soon as they hatch, with a development period delay. In the same way, the number of adult mosquitoes could also influence the number of immature stages as the females are being attracted to lay their eggs in that oviposition area. This was shown by the high abundance of immature mosquitoes and their strong correlation with the adults' population in Chester Zoo for *Culex* spp. and *Culiseta* spp. Although in Flamingo Land it was not possible to prove this association due to the difficulty to find and access permanent oviposition sites, from the few samplings done all the larvae were *Culex* spp. It must be considered that the correlation analysis assumes independence among data points and the most common violation happens with time series data. In this case, because the sampling intervals were shorter than the mosquito generational time, the removal of mosquitoes by the sampling will influence the availability of mosquitoes for the next sampling. To compensate for this, I excluded the first collection, and, in this way, the influence of the sampling was constant across the collections.

The correlation was positive for *Culex* spp., so the populations of immature and adult mosquitoes increase jointly; this is due to the several generations per year with some overlapping that this species has. The exception was found in area L4 in which the best lags were from a negative correlation. In this area, the gardeners manipulated the waterbed adding plant pots and refilling the water when necessary, and also severe evaporation was noticed. It was observed that after these changes, the mosquito abundance declined; thus, the collections in this area were not representative of the natural development of the mosquitoes.

For *Cs. annulata*, more significant correlations were negative. This species has few generations per year and longer development times, so after laying the eggs, the adult's population declines while the immature mosquito population increases. This is the consequence of clearer divisions among generations. Another exception was found in area L11 were most of immature mosquitoes belonged to this species, indicating that it was a very convenient location for it and some generational overlapping could have happened.

By doing sequential correlations with lags of one week, I was able to identify the significant lags and their direction. For both species, the best lags were negative in most areas, showing that the abundance of immature mosquitoes is reflected in the adults' population after a few weeks. The exception was area L11 for *Cs*. *annulata* in which the positive lags were more significant meaning that the abundance of the adult's population is reflected in the number of larvae and pupae. The difference in the lags with the strongest correlations between species could be due to their different development times and number of generations that they may have in the season. The larvae of *Cx. pipiens* hatch after one, three or ten days at 30°C, 20°C and 10°C, respectively (Becker et al., 2010); then, the adults take around 15 days to emerge at 20°C and 10 at 24°C, for field captured mosquitoes (Ciota et al., 2014). *Cs. annulata* can have from one to three generations per year, its eggs hatch after three to five days and the adults emerge after 18 days at 20 – 23°C or after 16 at 24 - 27°C (Becker et al., 2010). It seems that *Cs. annulata* takes longer to develop than *Cx. pipiens* which could reduce the number of generations and the overlapping among them during the developing season and in consequence, more adults would emerge after the initial point of observation (lag 0) producing more significant correlations in positive lags as observed. Nevertheless, this should be tested under completely comparable conditions.

3.5.3 Overwintering mosquitoes

Culex spp. overwinters as adult and *Culiseta* spp., as adult and larvae. The adult females go inside buildings and sheds where they are protected from the wind and freezing, often in dark and humid places. Looking for these mosquitoes is relevant because the identification of overwintering places represents an opportunity for controlling the mosquito population for the next season, although its effectiveness could be hard to prove. The risk of avian malaria transmission that overwintering mosquitoes represent could be low because the females that have a blood meal before overwintering have less chances of survival and these mosquitoes were not found infected with the parasite.

In Chester Zoo, some of the buildings that we aspirated did not have cracks or holes through which mosquitoes could enter and other buildings did not offer much protection against the wind. But the shed next to the penguin temporary enclosure had many access points, was dark, humid and isolated from the wind; therefore, we found more overwintering mosquitoes in it than in the other places. Most of the mosquitoes that we found in Flamingo Land were overwintering inside the staff area behind the penguin nest boxes. This place is humid, dark, and well protected from the environment and the mosquitoes can access through the entrances to the nest boxes and holes. Possibly the removal of overwintering mosquitoes in the first sampling negatively affected the numbers of mosquitoes collected in the second visit, although the colder temperatures could have also increased the mortality of mosquitoes.

3.5.4 Avian malaria parasites

The species of *Plasmodium* found are resident to Europe and have been reported before in wild birds. Nevertheless, this is the first time in which *P. matutinum* is found in penguins in association with mortalities. The parasite testing needs to be completed to analyse the prevalence of the parasite in the mosquito. This information will allow us to see if there are changes in the prevalence by areas or months, which could indicate different risks for the penguins. Meanwhile, it can be mentioned that the same parasite species was found in wild birds, penguins and mosquitoes, and it was confirmed that *Cx. pipiens* is a competent vector; therefore, suggesting that the transmission happens locally. Nevertheless, phylogenetic analysis, which are in progress, are needed define if the species and lineages of the parasite in the three elements of the transmission network are the same and therefore, confirm the local transmission of the parasite and the vector role of *Cx. pipiens*.

3.5.5 Conclusion

Through this project, we proved that the main avian malaria vector is present in both sampling sites, it is the dominant species in the mosquito community and that it is a competent vector for the disease. We found that the mosquito communities are highly variable, not only at the local scales as we found differences between our sampling sites, but also at the regional scale and through the sampling seasons. We found the *Plasmodium* parasite in the three elements of the transmission network, free wild birds, zoo birds and mosquitoes, and in the mosquito saliva, suggesting that the epidemiologic process happens locally, although the pending phylogenetic analysis will define this.

Controlling the vector population could be an effective measure to prevent the transmission of avian malaria to susceptible birds. As the most abundant mosquito was *Cx. pipiens*, control measures should target this species for example, preventing the development of aquatic stages by the management of water bodies (drying, removing organic matter and aquatic vegetation, or increasing the water flow and edges depth). Additionally, reducing the vegetation could decrease the abundance of mosquitoes as perceived in the different catches between years in one area (see also the discussion in Chapter Five). Nevertheless, these activities demand time and resources from the already occupied zoo staff; therefore, considering the high variability in mosquito abundance observed in our samplings, the monitoring of mosquito populations could assist on the planning and effective delivery of such measurements. For instance, when the increase on mosquito abundance is observed (adults or immatures), the population control should be implemented considering a lag in the development of immature mosquitoes of no more than three to four weeks giving time for the activities planning. Likewise, the parasite species may change over time, so its surveillance in mosquitoes could alert about the lineages present and the possible introduction of exotic ones before it affects susceptible birds in the zoos.

Appendix 3.1. Mosquito Collections in Chester Zoo in 2017

		Area 1			Area 2		Area 3			Area 4			Area 5			Area 6		Area 7		Area 10			Area 11			Area 12		Totals		
Collection date	Collection number	G	M	I	G	M	G	M	I	G	M	I	G	M	I	G	M	G	M	G	M	I	G	M	I	G	M	G	M	I
04-May	C1	20	0	1	17	1	10	0	0	14	0	7	16	0	1	0	1	8	0	43	0	0	0	0	0	7	0	135	2	9
10-May	C2	-	0	1	-	0	-	2	0	-	0	15	-	1	0	-	1	-	0	-	0	0	-	1	0	-	1	-	6	16
11-May	C3	1	0	-	2	0	2	0	-	1	0	-	0	0	-	3	0	1	0	1	0	-	1	0	-	0	0	12	0	0
17-May	C4	-	2	2	-	0	-	0	0	-	1	30	-	0	0	-	0	-	0	-	0	0	-	0	8	-	0	-	3	40
18-May	C5	1	0	-	3	0	2	0	-	0	1	-	7	0	-	1	0	2	0	1	0	-	5	0	-	3	0	25	1	0
24-May	C6	-	1	12	-	0	-	2	0	-	4	35	-	0	0	-	0	-	0	-	1	0	-	0	0	-	0	-	8	47
25-May	C7	5	0	-	2	0	4	0	-	10	0	-	0	0	-	16	0	7	0	3	0	-	6	0	-	8	0	61	0	0
31-May	C8	-	2	-	-	0	-	4	-	-	0	-	-	3	-	-	0	-	1	-	3	-	-	2	-	-	1	-	16	0
01-Jun	C9	5	4	13	1	0	6	0	0	9	0	15	1	0	1	8	0	2	1	12	0	0	2	0	1	0	0	46	5	30
07-Jun	C10	-	26	-	-	0	-	8	-	-	1	-	-	1	-	-	3	-	2	-	2	-	-	2	-	-	1	-	46	0
08-Jun	C11	8	2	16	2	0	10	5	0	21	0	7	2	1	1	10	0	17	0	1	1	0	1	0	9	4	0	76	9	33
14-Jun	C12	-	32	-	-	3	-	27	-	-	1	-	-	5	-	-	2	-	3	-	1	-	-	2	-	-	2	-	78	0
15-Jun	C13	9	11	4	50	1	53	2	0	34	1	9	5	0	5	26	0	19	0	63	1	0	73	0	16	23	0	355	16	34
21-Jun	C14	-	104	9	-	1	-	58	0	-	2	29	-	1	2	-	2	-	15	-	3	0	-	2	20	-	9	-	197	60
22-Jun	C15	45	34	-	42	0	108	7	-	107	0	-	89	0	-	84	0	134	4	157	0	-	278	3	-	57	0	1101	48	0
29-Jun	C16	-	119	21	-	1	-	75	2	-	1	30	-	5	2	-	0	-	16	-	5	0	-	7	32	-	3	-	232	87
30-Jun	C17	16	8	-	22	2	76	13	-	35	0	-	16	0	-	12	2	8	0	34	0	-	80	0	-	18	1	317	26	0
05-Jul	C18	-	154	-	-	11	-	204	-	-	6	-	-	14	-	-	10	-	10	-	16	-	-	6	-	-	8	-	439	0
06-Jul	C19	9	32	53	28	0	36	7	0	25	3	57	2	2	0	13	1	4	4	48	5	0	33	4	0	12	1	210	59	110
12-Jul	C20	-	139	-	-	7	-	83	-	-	1	-	-	5	-	-	6	-	10	-	12	-	-	10	-	-	8	-	281	0
13-Jul	C21	19	38	19	54	3	67	7	9	14	0	45	18	1	0	6	1	1	5	5	6	43	13	2	22	53	2	250	65	138
19-Jul	C22	-	165	-	-	27	-	140	-	-	7	-	-	8	-	-	11	-	18	-	21	-	-	7	-	-	21	-	425	0
20-Jul	C23	26	61	57	30	5	81	29	2	62	2	104	18	0	2	25	3	24	4	52	1	57	62	0	21	16	1	396	106	243
26-Jul	C24	-	122	-	-	12	-	6	-	-	5	-	-	3	-	-	16	-	3	-	5	-	-	3	-	-	6	-	181	0
27-Jul	C25	7	14	43	1	0	6	0	7	18	1	19	2	3	4	5	2	4	2	0	3	72	2	1	19	1	0	46	26	164
31-Jul	C26	-	77	-	-	6	-	19	-	-	8	-	-	38	-	-	11	-	13	-	8	-	-	8	-	-	6	-	194	0
01-Aug	C27	7	27	7	4	2	20	5	1	10	3	23	0	1	0	6	4	2	0	0	1	57	1	1	7	5	2	55	46	95
09-Aug	C28	-	117	-	-	23	-	10	-	-	4	-	-	18	-	-	17	-	17	-	23	-	-	16	-	-	4	-	249	0
10-Aug	C29	7	14	36	5	4	20	11	2	5	2	4	5	2	4	6	6	0	1	4	6	29	2	0	2	9	1	63	47	77
16-Aug	C30	-	59	-	-	4	-	11	-	-	3	-	-	11	-	-	10	-	9	-	11	-	-	8	-	-	4	-	130	0
17-Aug	C31	8	16	23	6	1	26	8	0	3	0	15	3	0	0	13	4	2	0	1	0	34	28	1	1	14	2	104	32	73

Appendix 3.1. Continued

		Area 1			Area 2			Area 3			Area 4			Area 5			Area 6			Area 7			Area 10			Area 11			Area 12			Totals	
Collection date	**Collection number**	**G**	**M**	**I**	**G**	**M**	**G**	**M**	**I**	**G**	**M**	**I**	**G**	**M**	**I**	**G**	**M**	**G**	**M**	**G**	**M**	**I**	**G**	**M**	**I**	**G**	**M**	**G**	**M**	**I**
23-Aug	C32	-	82	-	-	1	-	23	-	-	9	-	-	7	-	-	10	-	10	-	1	-	-	6	-	-	8	-	157	0
24-Aug	C33	9	12	42	2	1	32	1	3	18	5	8	2	8	2	1	2	0	3	4	3	31	7	3	0	14	3	89	41	86
30-Aug	C34	-	44	-	-	2	-	11	-	-	30	-	-	10	-	-	12	-	13	-	3	-	-	4	-	-	9	-	138	0
31-Aug	C35	1	4	39	0	1	6	3	0	7	7	0	6	3	2	3	2	2	4	1	0	29	7	0	0	7	1	40	25	70
07-Sep	C36	-	25	-	-	7	-	12	-	-	41	-	-	9	-	-	21	-	34	-	10	-	-	3	-	-	5	-	167	0
08-Sep	C37	2	2	18	1	0	9	2	0	4	5	0	1	1	0	1	2	0	2	0	0	6	1	0	0	1	0	20	14	24
13-Sep	C38	-	5	5	-	1	-	20	0	-	17	10	-	8	0	-	11	-	9	-	3	5	-	2	0	-	4	-	80	20
14-Sep	C39	0	1	-	0	0	1	3	-	1	1	-	0	5	-	0	0	0	1	0	3	-	0	1	-	0	2	2	17	0
20-Sep	C40	-	3	-	-	2	-	30	-	-	20	-	-	8	-	-	1	-	20	-	10	-	-	5	-	-	7	-	106	0
21-Sep	C41	0	0	12	1	0	0	0	1	1	0	8	0	0	0	0	2	1	1	0	2	4	0	0	0	0	0	3	5	25
26-Sep	C42	-	0	-	-	5	-	1	-	-	18	-	-	3	-	-	12	-	9	-	2	-	-	3	-	-	7	-	60	0
27-Sep	C43	0	1	23	0	1	0	2	0	3	3	27	4	2	1	1	0	0	4	0	1	9	0	0	0	0	1	8	15	60
04-Oct	C44	-	0	-	-	2	-	27	-	-	14	-	-	5	-	-	14	-	9	-	2	-	-	9	-	-	2	-	84	0
05-Oct	C45	0	0	3	0	1	0	19	0	0	0	7	1	6	0	2	0	0	3	0	2	1	0	2	0	0	0	3	33	11
11-Oct	C46	-	9	-	-	2	-	36	-	-	20	-	-	5	-	-	1	-	7	-	5	-	-	15	-	-	1	-	101	0
12-Oct	C47	0	1	2	0	1	0	5	0	4	1	12	0	2	0	0	2	1	0	0	0	5	0	3	0	2	1	7	16	19
18-Oct	C48	-	0	-	-	2	-	0	-	-	17	-	-	2	-	-	20	-	16	-	3	-	-	3	-	-	5	-	68	0
19-Oct	C49	0	1	0	0	0	0	1	0	2	0	4	1	1	0	0	2	2	1	0	0	0	0	0	21	0	1	5	7	25
25-Oct	C50	-	1	-	-	1	-	1	-	-	5	-	-	0	-	-	2	-	5	-	1	-	-	0	-	-	11	-	27	0
26-Oct	C51	0	1	16	0	2	1	2	0	2	7	7	0	1	0	1	2	1	1	0	1	0	0	3	0	0	1	5	21	23
02-Nov	C52	-	1	-	-	13	-	5	-	-	21	-	-	3	-	-	9	-	5	-	2	-	-	3	-	-	14	-	76	0
03-Nov	C53	1	2	7	0	1	0	2	0	1	11	4	0	0	0	1	1	0	3	0	2	0	0	2	0	0	0	3	24	11
08-Nov	C54	-	0	-	-	2	-	1	-	-	10	-	-	0	-	-	0	-	1	-	0	-	-	0	-	-	3	-	17	0
09-Nov	C55	0	1	4	0	0	0	3	0	0	6	9	0	2	0	0	0	0	1	0	2	0	0	2	1	0	1	0	18	14
15-Nov	C56	-	1	-	-	6	-	10	-	-	28	-	-	2	-	-	6	-	2	-	11	-	-	1	-	-	1	-	68	0
16-Nov	C57	0	1	4	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	1	0	0	0	5	0	2	0	7	9
22-Nov	C58	-	0	-	-	7	-	10	-	-	8	-	-	1	-	-	3	-	5	-	5	-	-	0	-	-	1	-	40	0
23-Nov	C59	0	0	1	0	1	0	4	0	0	0	3	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	9	4
28-Nov	C60	-	0	-	-	0	-	5	-	-	1	-	-	2	-	-	1	-	0	-	1	-	-	0	-	-	0	-	10	0
29-Nov	C61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
06-Dec	C62	-	0	0	-	2	-	3	0	-	8	1	-	5	0	-	2	-	3	-	2	0	-	0	0	-	2	-	27	1
Total		**206**	**1578**	**493**	**273**	**178**	**576**	**985**	**27**	**411**	**372**	**544**	**199**	**226**	**27**	**244**	**253**	**242**	**311**	**430**	**215**	**382**	**602**	**156**	**185**	**254**	**177**	**3437**	**4451**	**1658**

Counts include both sexes. Overwintering samplings and collections from CDC-miniature light traps not included. G: CDC-Gravid trap, M: BG-Mosquitaire trap, I: immature stages sampling.

Appendix 3.2. Mosquito Collections in Chester Zoo in 2018

		Area 1		Area 2		Area 3		Area 4		Area 10		Area 11		Area 12		Area 13		Totals	
Collection date	Collection number	G	M	G	M	G	M	G	M	G1	M1	G	M	G	M	G	M	G	M
9-Apr	C2	-	-	-	-	-	1	-	-	-	-	-	1	-	-	-	1	0	3
10-Apr	C3	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	0	2
18-Apr	C4	-	-	-	2	-	2	-	2	-	-	-	4	-	-	-	-	0	10
19-Apr	C5	1	-	1	-	-	-	-	-	-	2	-	1	-	1	2	1	4	5
25-Apr	C6	-	3	-	2	-	1	-	-	-	-	-	2	-	3	-	2	0	13
2-May	C8	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	0	2
3-May	C9	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	0
9-May	C10	-	4	-	-	-	2	-	1	-	1	-	-	-	-	-	1	0	9
10-May	C11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	0
16-May	C12	-	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	0	2
23-May	C14	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	3
24-May	C15	-	-	-	-	1	1	-	-	1	-	-	-	-	-	1	-	3	1
30-May	C16	-	1	-	1	-	3	-	2	-	2	-	-	-	-	-	7	0	16
31-May	C17	1	-	-	-	2	1	-	-	1	-	-	-	-	-	28	-	32	1
6-Jun	C18	-	8	-	2	-	3	-	1	-	2	-	4	-	3	-	9	0	32
7-Jun	C19	2	-	-	-	-	-	-	1	3	-	-	-	-	-	8	-	13	1
13-Jun	C20	-	35	-	2	-	5	-	4	-	5	-	1	-	7	-	6	0	65
14-Jun	C21	3	9	1	-	-	-	2	-	-	-	-	1	1	-	4	-	11	10
20-Jun	C22	-	14	-	2	-	-	-	4	-	4	-	1	-	1	-	1	0	27
21-Jun	C23	-	4	2	1	-	-	4	1	-	1	2	-	4	-	9	-	21	7
27-Jun	C24	-	31	-	2	-	3	-	-	-	4	-	4	-	-	-	7	0	51
28-Jun	C25	18	11	1	1	4	4	2	5	7	1	7	7	4	-	41	-	84	29
4-Jul	C26	-	109	-	3	-	16	-	5	-	7	-	21	-	12	-	11	0	184
5-Jul	C27	9	15	2	-	17	-	3	2	7	2	4	3	9	-	19	6	70	28
11-Jul	C28	-	74	-	4	-	3	-	6	-	7	-	12	-	9	-	9	0	124
12-Jul	C29	31	-	4	-	1	6	16	1	20	11	5	16	-	-	20	1	97	35
18-Jul	C30	-	162	-	11	-	-	-	-	-	-	-	-	-	-	-	-	0	173
19-Jul	C31	12	20	4	-	7	-	2	-	3	2	2	2	1	1	16	-	47	25
25-Jul	C32	-	40	-	17	-	3	-	-	-	12	-	4	-	5	-	9	0	90
26-Jul	C33	31	17	13	8	10	1	2	-	19	10	-	2	1	1	20	2	96	41
1-Aug	C34	-	68	-	31	-	9	-	3	-	11	-	-	-	12	-	9	0	143

Appendix 3.2. Continued

		Area 1		Area 2		Area 3		Area 4		Area 10		Area 11		Area 12		Area 13		Totals	
Collection date	**Collection number**	**G**	**M**	**G**	**M**	**G**	**M**	**G**	**M**	**G1**	**M1**	**G**	**M**	**G**	**M**	**G**	**M**	**G**	**M**
2-Aug	C35	1	7	22	10	2	-	3	1	13	5	3	3	8	-	17	3	69	29
8-Aug	C36	-	78	-	19	-	1	-	-	-	25	-	8	-	-	-	18	0	149
9-Aug	C37	7	7	4	2	4	-	2	6	21	2	6	-	4	1	14	-	62	18
15-Aug	C38	-	36	-	4	-	8	-	1	-	6	-	5	-	2	-	9	0	71
16-Aug	C39	12	3	3	5	11	1	1	-	6	2	2	1	5	6	5	1	45	19
22-Aug	C40	-	109	-	46	-	4	-	15	-	12	-	-	-	8	-	20	0	214
23-Aug	C41	6	-	3	3	3	-	2	4	1	-	1	1	-	-	1	-	17	8
29-Aug	C42	-	18	-	23	-	1	-	8	-	-	-	6	-	2	-	6	0	64
30-Aug	C43	5	2	-	-	-	-	3	1	5	1	-	-	1	1	16	-	30	5
5-Sep	C44	-	19	-	10	-	7	-	33	-	6	-	11	-	-	-	7	0	93
6-Sep	C45	1	3	1	2	-	-	-	-	-	-	-	-	-	-	7	-	9	5
12-Sep	C46	-	29	-	13	-	6	-	17	-	3	-	1	-	3	-	1	0	73
13-Sep	C47	3	1	2	3	1	1	2	2	-	-	-	1	-	-	5	-	13	8
19-Sep	C48	-	6	-	19	-	7	-	21	-	-	-	3	-	3	-	2	0	61
20-Sep	C49	-	-	-	2	-	-	1	1	-	1	-	-	-	-	-	-	1	4
26-Sep	C50	-	-	-	6	-	4	-	8	-	-	-	4	-	-	-	5	0	27
27-Sep	C51	-	2	1	4	-	2	1	9	-	-	-	3	1	-	7	1	10	21
3-Oct	C52	-	1	-	5	-	3	-	17	-	-	-	3	-	2	-	4	0	35
4-Oct	C53	-	1	1	4	-	1	-	1	1	-	1	2	1	1	3	3	7	13
10-Oct	C54	-	1	-	8	-	5	-	24	-	-	-	11	-	5	-	6	0	60
11-Oct	C55	4	-	1	3	3	1	1	11	-	-	-	-	1	-	3	2	13	17
17-Oct	C56	-	3	-	5	-	2	-	14	-	-	-	2	-	2	-	3	0	31
18-Oct	C57	-	-	-	-	-	-	2	1	-	-	-	-	1	-	-	-	3	1
24-Oct	C58	-	2	-	-	-	-	-	5	-	1	-	7	-	2	-	5	0	22
25-Oct	C59	-	-	1	-	-	1	2	2	-	-	1	-	-	1	-	-	4	4
1-Nov	C60	-	-	-	3	-	1	-	5	-	1	-	2	-	-	-	-	0	12
2-Nov	C61	-	-	-	-	1	-	2	-	-	-	-	-	-	-	-	-	3	0
	Total	**147**	**957**	**67**	**288**	**67**	**120**	**53**	**245**	**108**	**151**	**35**	**163**	**42**	**94**	**247**	**178**	**766**	**2196**

Counts include both sexes. G: CDC-Gravid trap, M: BG-Mosquitaire trap.

Appendix 3.3. Mosquito Collections in Flamingo Land in 2017.

Collection date	Collection number	Area 1		Area 2		Area 3		Area 4		Totals	
		G	M	G	M	G	M	G	M	G	M
15-Jun	F1	-	0	-	1	-	0	-	0	-	1
26-Jun	F2	-	5	-	3	-	0	-	5	-	13
27-Jun	F3	-	2	-	3	-	0	-	0	-	5
03-Jul	F4	-	0	-	5	-	0	-	4	-	9
04-Jul	F5	-	0	-	0	-	0	-	0	-	0
10-Jul	F6	-	0	-	7	-	0	-	2	-	9
11-Jul	F7	-	0	-	0	-	0	-	0	-	0
17-Jul	F8	-	2	-	5	-	1	-	4	-	12
18-Jul	F9	3	0	0	5	0	0	0	3	3	8
24-Jul	F10	-	32	-	18	-	0	-	6	-	56
25-Jul	F11	9	9	54	16	0	0	2	3	65	28
31-Jul	F12	-	27	-	22	-	10	-	7	-	66
01-Aug	F13	9	5	14	7	3	0	0	1	26	13
07-Aug	F14	-	14	-	21	-	1	-	14	-	50
08-Aug	F15	3	1	7	14	0	1	1	1	11	17
14-Aug	F16	-	1	-	15	-	1	-	2	-	19
15-Aug	F17	10	1	22	8	2	0	1	1	35	10
21-Aug	F18	-	7	-	14	-	1	-	3	-	25
22-Aug	F19	8	4	29	2	0	0	2	2	39	8
28-Aug	F20	-	6	-	17	-	2	-	7	-	32
29-Aug	F21	5	5	28	14	1	2	0	1	34	22
04-Sep	F22	-	17	-	24	-	6	-	9	-	56
05-Sep	F23	0	12	0	24	0	5	0	7	0	48
11-Sep	F24	-	7	-	9	-	28	-	10	-	54
12-Sep	F25	1	4	9	18	0	1	1	4	11	27
18-Sep	F26	-	5	-	13	-	8	-	20	-	46
19-Sep	F27	1	1	3	1	1	0	0	0	5	2
25-Sep	F28	-	13	-	13	-	7	-	23	-	56
26-Sep	F29	0	1	3	4	1	2	0	3	4	10
02-Oct	F30	-	5	-	1	-	9	-	27	-	42
03-Oct	F31	3	8	3	0	0	4	0	8	6	20
09-Oct	F32	-	15	-	18	-	6	-	29	-	68
10-Oct	F33	0	2	1	0	1	0	0	3	2	5
16-Oct	F34	-	9	-	15	-	11	-	58	-	93
17-Oct	F35	1	1	3	3	0	0	0	2	4	6
23-Oct	F36	-	8	-	28	-	6	-	36	-	78
24-Oct	F37	0	0	0	6	0	3	0	17	0	26
30-Oct	F38	-	6	-	21	-	13	-	34	-	74
01-Nov	F39	1	1	1	2	1	6	0	4	3	13
06-Nov	F40	-	2	-	2	-	2	-	14	-	20
07-Nov	F41	0	0	0	0	0	0	0	2	0	2
20-Nov	F42	-	3	-	2	-	15	-	5	-	25
21-Nov	F43	0	0	0	1	0	0	0	0	0	1
27-Nov	F44	-	0	-	1	-	0	-	3	-	4
04-Dec	F45	-	0	-	0	-	1	-	0	-	1
05-Dec	F46	0	0	0	0	0	0	0	0	0	0
11-Dec	F47	-	0	-	0	-	0	-	0	-	0
Total		**54**	**241**	**177**	**403**	**10**	**152**	**7**	**384**	**248**	**1180**

Counts include both sexes. Overwintering samplings not included. G: CDC-Gravid trap, M: BG-Mosquitaire trap. CDC-Gravid traps were not operating until the fourth sampling week.

Chapter Four

Blood Feeding Activity of Mosquitoes in UK Zoos

4.1 Abstract

The vector's host preference is a relevant factor in the study of the pathogen transmission process. In the zoo environment, the mosquitoes can present unique feeding behaviours due to the diversity of potential hosts that cannot be found in nature. In our mosquito samplings we found considerable proportions of blood-fed mosquitoes that we analysed to identify the source of the blood-meal in their abdomens using a nested-PCR and sequencing techniques. We found differences in the proportions of blood-fed mosquitoes compared to unfed mosquitoes by sampling areas and months. More blood-fed mosquitoes were present in areas where the abundance of potential hosts was apparently higher. We captured a significantly higher proportion of blood-fed *Culiseta* spp. than *Culex* spp. In total, 145 vertebrate hosts were identified including 56 birds, 20 mammals and 69 humans. We confirmed that *Culex* spp. prefers to feed on birds and that *Culiseta* spp. prefers mammals. Unexpectedly, we found many *Culex* spp. mosquitoes feeding on humans during the summer months in both zoos, possibly due to the increased number of visitors. Some mosquitoes fed on zoo animals and knowing the location of their exhibits the minimum travelling distance were estimated. The travelling distances where highly variable and there were no differences by mosquito species. We identified two blood meals from Humboldt penguins, one in an *Anopheles maculipennis* s. l. and the other in a partially identified Culicinae mosquito. *Anopheles* spp. mosquitoes could have a relevant role in the transmission of avian malaria to penguins thus complementary samplings are recommended. To improve the analysis of blood-fed mosquitoes in zoos, the parallel study of local wild birds is recommended to complement the understanding of the host availability in the host choice of mosquitoes and disease transmission risks.

4.2 Introduction

One of the most relevant aspects in the study of vector-borne diseases is the ability of the vector to effectively transmit the pathogen to susceptible hosts. The competence of the vector and its interaction with the host constitute the vectorial capacity and includes factors such as the biting behaviour, survival rate of the vector, incubation time of the pathogen and the host preference (Garrett-Jones, 1964a, Garrett-Jones, 1964b). The vector competence is given by the ability of the pathogen to reproduce or amplify and disseminate inside the vector up to the stage of being infective to the host; this happens from the time the vector feeds on an infected vertebrate to the moment the pathogen is ready for transmission, named the extrinsic incubation period (EIP) (Unnasch et al., 2006). The biting rate is the average daily probability of a mosquito feeding on a host; for example, a mosquito that feeds every four days has a biting rate of 0.25 per day (Brugman, 2016). Finally, the host preference is which hosts the vector chooses to feed on and in what proportions (Unnasch et al., 2006). Here, the host preference is determined by the identification of the source of blood meals.

4.2.1 Host preference and pathogen transmission risk

Regarding the host choice, the vectors could be generalists, preferring to feed in different species or classes of vertebrates, or specialists, selecting just certain kind of hosts. The preference for different hosts is what determines the risks of cross-species transmission of pathogens in relation to the mosquito community. Generalist mosquitoes could facilitate pathogen transmission among unrelated species and the local conditions could force specialised mosquitoes to feed on unusual hosts (Abella-Medrano et al., 2018). By contrast, generalist mosquitoes can also have fewer encounters with reservoir hosts and then, a dilution effect in the transmission can be produced (Tuten et al., 2012). Nevertheless, it should be considered that not all the available hosts for the vector may be involved in the transmission of the pathogen because they are not susceptible to the initial infection or if they are, their immune response may prevent the pathogen to reach an infective stage for uninfected vectors to continue the transmission process; which has been suggested in some cases for bird haemosporidians with the observation of abortive infections (Gonzalez et al., 2015, Chagas et al., 2017). Therefore, the interaction between host preference of the vector and the host competence determine if a cross-species transmission occurs and whether a dilution or amplification effect are observed in relation to host diversity (Faust et al., 2017).

Mosquitoes that have strong preferences for certain hosts are important for the enzootic transmission of pathogens and mosquitoes that feed on diverse hosts can transmit a pathogen, like arboviruses, from one vertebrate group to another; thus they are known as bridge vectors (Farajollahi et al., 2011, Egizi et al., 2018). In the case of West Nile virus for instance; the mosquito *Culex pipiens* biotype *pipiens* feeds mostly on birds and maintains the transmission of the virus in that group, whereas *Cx. pipiens* biotype *molestus* feeds on birds and humans so it has the potential of transmitting the virus to the last ones, which are incidental hosts. The virulence of mosquito-borne pathogens can be higher when mosquitoes transmit them to alternative hosts, in comparison to the natural host that may be less susceptible. For these reasons, disentangling the mosquito host preferences in zoos could provide relevant information about the pathogen transmission risks among the animals in the collections and even the health risk and nuisance for visitors.

For understanding the epidemiology of avian malaria in susceptible birds, a better description of the mosquito ecology is needed. As *Plasmodium* is a common parasite in the local resident birds such as those from the Corvidae and Tordidae families, it is assumed that the native mosquitoes are responsible for its transmission. Consequently, susceptible birds such as penguins are exposed to the mosquitoes and incidentally infected with the parasite, sometimes with serious consequences.

4.2.2 Blood feeding of mosquitoes in zoos

In the zoo environment, the study of mosquito host preferences is not only interesting due to the unique composition of potential mosquito hosts, but it is also relevant for the interspecific transmission of pathogens among vertebrate groups that usually cannot happen in natural environments (Adler et al., 2011). Some studies in zoos have shown that mosquitoes feed on a broad range of hosts, sometimes presenting mixed blood-meals (Tuten et al., 2012). This is often not typical of the natural behaviour of the mosquito species. It has been found that some species can have unexpected host shifts; for instance, *Aedes japonicus* that usually feeds on mammals (mammalophilic) has been found to feed on birds in nature (Schonenberger et al., 2016).

Previous studies about the flying distances of blood-fed mosquitoes in zoological parks have proved that they feed on captive animals, suggesting that these mosquitoes do not travel long distances and may be implied in the local transmission of vector-borne diseases like avian malaria (Ejiri et al., 2011, Greenberg et al., 2012).

When female mosquitoes are looking for a blood-meal, they can travel long distances; *Culex* spp. for instance can disperse for several kilometres (Goodman et al., 2018). After feeding, they do not tend to travel long distances (Greenberg et al., 2012) because they are more vulnerable to predation, are not as aerodynamic as when they are empty, and at this point, they are looking for a resting place where they can digest the blood and produce eggs.

4.2.3 Mosquito host identification

The identification of mosquito hosts has been described previously in several vector studies. Some of the first techniques to identify blood from mosquito abdomens were based on serologic tests to detect antibodies to certain classes of vertebrates, but were limited to the general identification of groups or required sequential tests to find a particular species (Tempelis, 1975, Reeves et al., 2016).

Recently, PCR protocols that amplify a section of the cytochrome c oxidase subunit I (CO1) or the cytochrome b (cyt-b) gene have been developed (Reeves et al., 2016). Some techniques consist of multiplex or sequential PCR assays for vertebrate groups using different primers for avian, mammalian, or reptile or amphibian DNA (Goodman et al., 2018, Brugman et al., 2017). This is an inexpensive and quick technique, particularly useful when the potential hosts are known a priori or if there is a particular interest in a certain host (Brugman, 2016). Another approach is to use generic primers that amplify any vertebrate DNA and then compare the sequences to find a match in databases (Alcaide et al., 2009). This method facilitates the precise identification of the host in a single step and is useful for broad screenings of hosts in natural habitats.

A continuous issue with blood-meals analysis is that the digestive system of the mosquito quickly degrades the host's DNA which can be also damaged during storage, affecting the host identification chances (Brugman et al., 2017, Reeves et al., 2016). Reeves *et al*. (2016) compared storage methods and times finding that the use of filter paper and a shorter storage prior to sample processing give the best results (Reeves et al., 2016). Similarly, Brugman *et al*. (2018) and Santos *et al*. (2019) also concluded that mosquitoes with fresh blood-meals provided better results compared to partially digested blood (Brugman et al., 2017, Santos et al., 2019).

Because blood-fed mosquitoes are generally not attracted to common traps, the usual techniques for capturing them include passing a net over vegetation and the aspiration of resting places or resting boxes with a portable aspirator (Egizi et al., 2018). Nonetheless, we

found a considerable number of blood-fed mosquitoes in our collections, thus we decide to analyse them to explore the host preferences in the zoos and get more comprehensive knowledge about the mosquito activity. The particular objectives of this work were:

- To identify the main sources of mosquito blood-meals in the zoos.
- To analyse the temporal and spatial differences in mosquito feeding activity and host preferences.
- To explore the variability of mosquito flying distance after feeding.
- To define potential risks for disease transmission based on the host preferences observed.

4.3 Materials and Methods

The mosquitoes were collected during two years in Chester Zoo (2017 and 2018) and during one in Flamingo Land (2017). In the first site, we set ten sampling areas during the first year and eight in the second one; in the second zoo we used four. In each sampling area we set one BG-Mosquitaire trap operating continuously and we collected the net twice per week, after six days and after one day. We also used one CDC-Gravid traps per area which operated for one day per week. The sampling lasted for 25 weeks in Flamingo Land (June to November), 32 weeks in Chester Zoo in the first year (May to December) and for 31 in the second one (April to November). After collecting the nets, the mosquitoes were stored at -20 °C until morphological identification and further analysis. The full description of the sampling areas and procedures can be found in Chapter 2.

4.3.1 Selection of blood-fed mosquitoes

As field captured mosquitoes have different degrees of blood digestion that affects the host identification, the mosquitoes were selected for analysis if they were engorged with a red, dark red or blackish abdomen indicating blood content. This is equivalent to the stages 2 and 3 of the Sella scale, a classification system developed for the study of *Anopheles* in relation to human malaria that is commonly used to categorise blood-fed mosquitoes (Detinova, 1962, Santos et al., 2019).

During the morphological identification, the abdomens of the blood-fed mosquitoes were cut off using entomological forceps and disposable scalpels; the materials were cleaned with 70% ethanol and DNA Away® between specimens. The abdomens and thoraxes were

stored separately in individual reactions tubes at -20 °C for no more than three weeks until processing.

4.3.2 Molecular analysis

The DNA extraction of the blood-fed abdomens was done with the OMEGA Bio-Tek E.Z.N.A ® Tissue DNA kit auditioning 200 µl of PBS (phosphate buffered saline) per sample before homogenisation. Afterwards, the PCR protocol and the primers proposed by Alcaide *et al.* (2009) (Alcaide et al., 2009) were used to obtain a 758 bp amplification of a region of the *cox*1 gene. The positive samples were sent for sequencing by the Sanger method. The detailed protocols can be found in section 2.8.

The successful sequences were edited and analysed using the BioEdit© software and compared to the reported sequences in the BLAST® database, optimized for the highly similar sequences, and the Barcoding of Life Data System© (BOLD). The most similar sequences were aligned and compared using BioEdit© to find the best match, considering the identity and query covers and excluding wild native species absent in the area and exotic species not included in the zoo's collection. With the same software, we inspected the electropherograms for double peaks in a single base position, which, if they were consistent, indicate the amplification of the *cox*1 locus from two different sources, meaning that the blood-meal was mixed and the mosquito fed on more than one host (Alcaide et al., 2009).

4.3.3 Flying distances

Knowing the identity of the hosts on which the mosquitoes fed, in the case of the zoo animals, I estimated the distance between the animal exhibits and the location of the trap where the blood-fed mosquitoes were captured. The software Species360 ZIMS (Zoological Information Management Software) (Species360, 2019) was used to determine the location of the zoo animals at the time when the mosquitoes were captured.

Afterwards, I added the polygons of the exhibits over the Open Street Map layer in the QGIS 3.2© software. I estimated the centroid of the exhibits, and from them, I drew a straight line to the corresponding trap to represent the minimum distance travelled since feeding. Finally, I measured the distances of the travelling lines.

4.3.4 Statistical analysis

The numbers of blood-fed and empty mosquitoes were analysed by sampling year to compare the collections in Chester Zoo (2017 and 2018), and separately by site and year to analyse differences by sampling areas and by months. Similarly, the host preferences were analysed comparing the results by vertebrate group (birds, mammals and humans) by mosquito species. The efficiency of the traps used to collect blood-fed mosquitoes was also compared using the total numbers of blood-fed and empty mosquitoes separated by year and site. As the data was organised in contingency tables, the Chi-squared test of independence was used for most of the analyses (the Yates continuity correction was applied for two by two tables), and the Fisher's exact test of independence was chosen instead when appropriate (low number of observations). When the Chi-squared test gave significant p values, the contributions to significance were established obtaining the residuals of the test for each cell to define which of the observations in the table were significantly different from the expected values.

The comparisons between blood-fed mosquitoes and empty mosquitoes were done only with females' data and, for the empty mosquito proportions, the specimens with damaged or missing abdomens were excluded as we could not confirm if they were blood-fed or not. These comparisons were done using the data from all blood-fed mosquitoes with a couple of exceptions. The analysis by mosquito species included only the counts of those completely identified by morphology and the host preferences were examined using only the data from *Cx. pipiens* and *Cs. annulata*, as they were collected in sufficient numbers. The blood-meals that gave mixed results for two hosts were counted as a mosquito preference for both. Lastly, the flying distances were compared by mosquito species using a Welch's Anova.

4.4 Results

The proportions of blood-fed mosquitoes that we found were 3.5% in Chester Zoo in 2017, 9.7% in 2018 and 7.2% in Flamingo Land. In 2017 and 2018 we collected and analysed 213 and 245 blood-fed mosquitoes respectively in Chester Zoo, 458 in total. In 2017 all mosquitoes were *Cx. pipiens* (n = 150) and *Cx. annulata* (n = 41) although some did not have legs and were not completely identified (n = 22). In 2018 the vast majority also were from these same species (n = 199 and n = 29 respectively) and 12 could not be fully identified; we also captured one *Anopheles claviger* and four *An. maculipennis*. From the first-year

samples, 95 (44.6%) produced positive bands in the agarose gel and 75 (35.2%) out of these gave sequence matches in the databases. These included 31 bloods from birds, 9 from mammals and 40 positive results for human blood; we found five mixed blood-meals, three of human and wild birds and two of zoo animals and birds. In 2018, 56 (22.8%) samples produced bands and 45 (18.4%) were successfully sequenced. Twenty-four belonged to birds, five to mammals and 17 to humans. Again, we found mixed blood-meals, one mosquito fed on two different wild birds.

The total number of blood-fed mosquitoes in Flamingo Land was 75; 50 from *Cx. pipiens*, seven from *Cs. annulata* and 18 partially identified. Thirty (40%) produced positive PCR results and 19 (25.3%) gave positive sequencing results that included one bird, six mammals and 12 humans. We did not find mixed blood-meals in this sampling.

4.4.1 Temporal and spatial variations of blood-fed mosquitoes

4.4.1.1 Analysis per sampling area

The Chi-squared test of independence revealed a significant difference by areas in Chester Zoo in 2017 (X^2 = 17.556, df = 9, p = 0.041). Areas A1 and A3 showed the highest numbers of blood-fed mosquitoes, (n = 67 and n = 48 respectively) but they were also areas with high number of catches in general. Thus, examining the contributions to the significance, it was clear that A3 had a significant higher number of blood-fed mosquitoes than the other areas as well as A1 but in less degree. Other important contributions came from A4 and A5 but in this case the difference between observed and expected values were negative, meaning that these areas captured fewer blood-fed mosquitoes than expected. More positive differences were found in areas A7 and A10 and negative differences in the remaining areas (Figure 4.1 a).

The next year's sampling again showed significant differences regarding the sampling areas (X^2 = 17.894, df = 7, p = 0.0125). The highest contribution came from area A10 where more blood-fed mosquitoes than expected were captured and by area A4 followed by A13 presenting a negative difference (Figure 4.1 c). Areas A1 and A11 also got more than expected blood-fed catches and the rest of the areas had minor negative differences.

We also found a significant difference in the blood-fed mosquitoes captured by area in Flamingo Land (X^2 = 24.868, df = 3, p < 0.000) due to the positive difference from area A2 and the negative differences from A3 and A4; thus, the captures of blood-fed mosquitoes

were more than expected in A2 and less than expected in A3 and A4; area A1 had a smaller negative difference (Figure 4.2 a).

4.4.1.2 Analysis per month

The data from the first year in Chester Zoo were different by months (X^2 = 50.596, df = 6, p <0.001). Most of the significance was provided by the unexpectedly high catch in June and in minor degree by the less than expected catch of July (Figure 4.1 b). There were more mosquitoes than expected in May and less than expected in the other months.

The 2018 sampling season in Chester Zoo also revealed significant differences by months (X^2 = 54.346, df = 6, p <0.000). In this case, due to the obvious negative differences in September and October but also by the higher than expected catches in July and August. May and June had small positive differences and April, a lesser negative difference (Figure 4.1 d).

Finally, in Flamingo Land, the collections by months presented a significant difference (X^2 = 106.51, df = 5, p < 0.000) manly because we got a higher proportion of blood-fed mosquitoes in July and August than in the other months which showed negative differences, especially September, October and November (Figure 4.2 b).

4.4.2 Methodology analysis

4.4.2.1 Differences by traps

The catches of blood-fed and empty mosquitoes were compared for traps (BG-Mosquitaire trap and CDC-Gravid trap). The data included the females without abdominal damage and only for the one-day collections of the BG-Mosquitaire traps. There were no significant differences either in Chester Zoo in 2017 (X^2 = 0.047, df = 1, p = 0.829) or 2018 (X^2 = 1.373, df = 1, p = 0.241) or Flamingo Land (X^2 = 2.688, df = 1, p = 0.101).

4.4.2.2 Success in molecular methods

Comparing the total proportions of successful and unsuccessful PCR reactions, a significant difference was found among samplings by site and years (X^2 = 25.441, df = 2, p <0.000). This was related to the more than expected successful tests in 2017 and less than expected in 2018 in Chester Zoo. There were no differences when comparing the PCR amplification success by mosquito genus (*Culex* spp. and *Culiseta* spp.) (Fisher's exact test, p = 0.068). The

difference in PCR reaction success by sampling type, week or day-collections, was also analysed and no significant difference was found (X^2 = 0.855, df = 1, p-value = 0.355).

From the positive PCR reactions, comparing the proportion of successful products sequencing did not show significant differences in general (X^2 = 3.697, df = 2, p = 0.156) or by genus (*Culex* spp. and *Culiseta* spp.) (Fisher's exact test, p = 0.312).

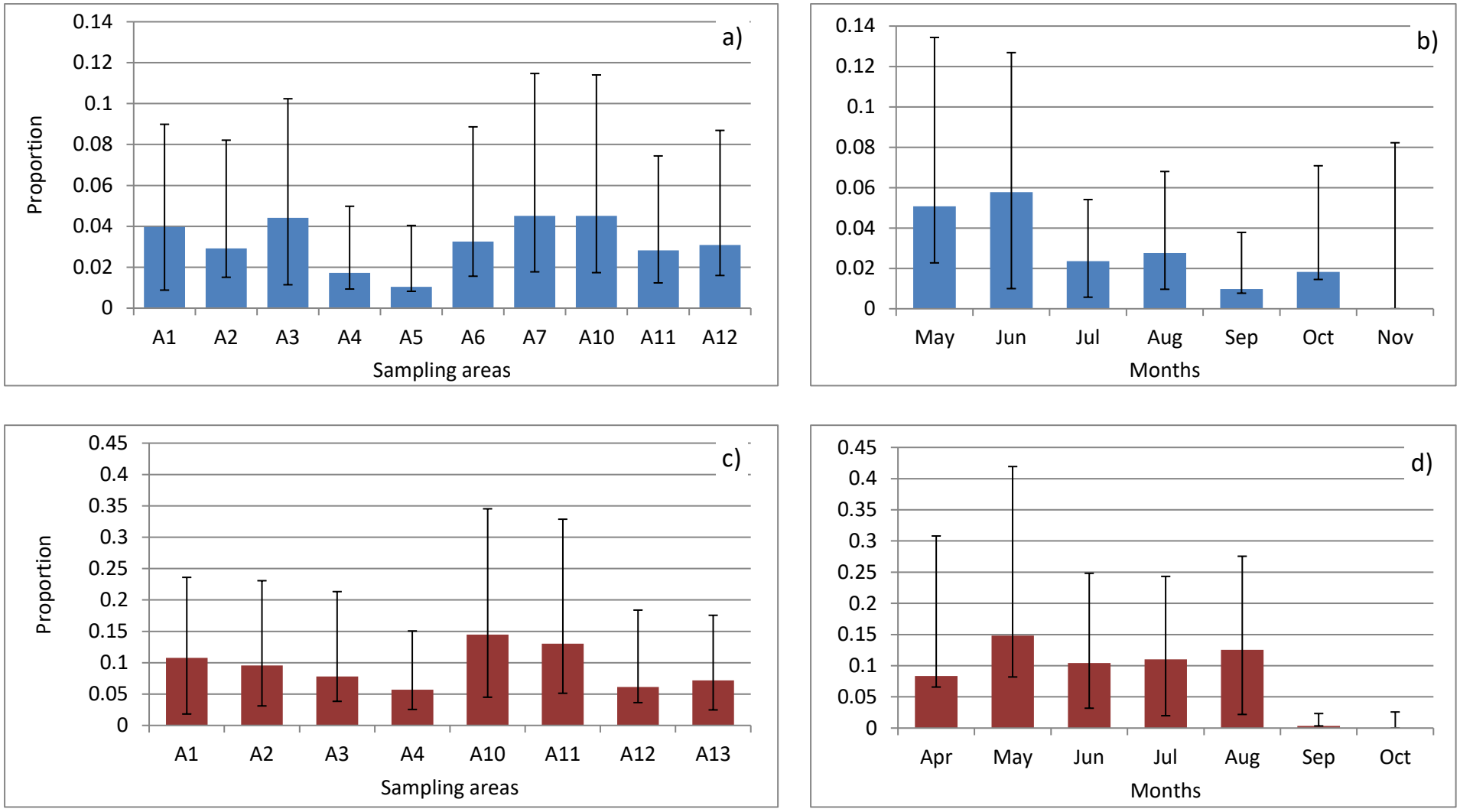


Figure 4.1. Proportion of blood-fed mosquitoes in Chester Zoo. a) Sampling areas in 2017, b) Months in 2017, c) Sampling areas in 2018 and d) Months in 2018. Error bars: 95% confidence intervals.

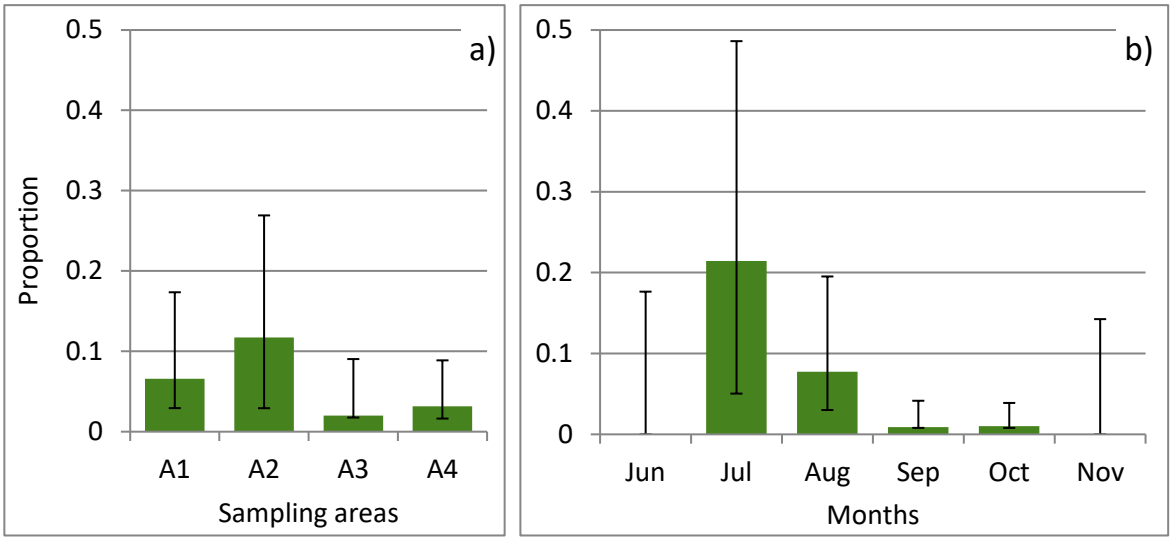


Figure 4.2. Proportion of blood-fed mosquitoes in Flamingo Land. a) Sampling areas in 2017, b) Months in 2017. Error bars: 95% confidence intervals.

4.4.3 Host preferences

The host preferences were analysed for the two genera of mosquitoes that were captured as blood-feds in enough numbers, *Culex* spp. and *Culiseta* spp. The hosts were grouped as birds, mammals and humans.

A significant difference was found in Chester Zoo in 2017 (Fisher's exact test p <0.001). *Culex* spp. preferred to feed equally on humans and birds, more than non-human mammals; *Culiseta* spp. preferred to feed on humans, then on non-human mammals and lastly on birds (Table 4.1). In the following year, a similar preference was observed, *Culex* spp. chose to feed on birds and humans more than non-human mammals and *Culiseta* spp. preferred non-human mammals, avoiding humans and birds (Fisher's exact test p =0.025) (Table 4.2).

Although there was also a significant difference in the host preference in Flamingo Land (Fisher's exact test p = 0.021), the low number of successful sequences can only suggest that *Culex* spp. prefers humans and *Culiseta* spp., non-human mammals (Table 4.3).

To see if the mosquitoes had different host preferences by areas or months in Chester Zoo, the data was analysed in this sense for the main host groups for *Culex* spp., birds and humans. The data was not enough for analysing the results of *Culiseta* spp. or Flamingo Land in this way. In 2017, there was no difference in the host preferences by area (Fisher's exact test p = 0.594) but there was by month (Fisher's exact test p = 0.003), *Culex* spp.

preferred to feed on humans during June and on birds during July (Figure 4.3). In 2018, there were no differences for *Culex* spp. host preferences by month (Fisher's exact test p = 0.513) or areas (Fisher's exact test p = 0.074); but the numbers for comparison were lower in this year.

Of note, more *Cs. annulata* blood-feds were captured than *Cx. pipiens* in all samplings (all p values < 0.000). Figures 4.4, 4.5 and 4.6 illustrate the proportions of hosts identified from the blood-meals per zoo and year.

Table 4.1. Host preferences of blood-fed mosquitoes captured in Chester Zoo, 2017.

Host Group	Scientific Name	Common name	*Culex* spp.	*Culiseta* spp.	Unknown*	Total
Birds (n = 26)	*Anas platyrhynchos*	Mallard	1	0	0	1
	Cissa thalassina	Javan green magpie	2	0	0	2
	Corvus monedula	Eurasian jackdaw	1	0	0	1
	Passer domesticus	House sparrow	3	0	0	3
	Pica pica	Eurasian magpie	4	1	1	6
	Prunella modularis	Dunnock	1	0	0	1
	Pyrrhula pyrrhula	Eurasian bullfinch	1	0	0	1
	Tauraco schalowi	Schalow's turaco	3	0	1	4
	Turdus merula	Eurasian blackbird	5	0	1	6
	Turdus philomelos	Song thrush	1	0	0	1
Mammals (n = 7)	*Bos taurus*	Cattle	0	1	0	1
	Camelus bactrianus	Bactrian camel	0	3	0	3
	Rucervus eldii thamin	Eld's deer	0	2	0	2
	Tragelaphus eurycerus	Bongo	0	1	0	1
Humans (n = 37)	*Homo sapiens*	Human	27	9	1	37
Mixed blood-meals (n = 5)	*Camelus bactrianus* / *Turdus merula*	Bactrian camel / Eurasian blackbird	1	0	0	1
	Giraffa camelopardalis rothschildi / *Tauraco schalowi*	Rothschild's giraffe / Schalow's turaco	1	0	0	1
	Pica pica / *Homo sapiens*	Eurasian magpie / Human	3	0	0	3
		Total	**54**	**17**	**4**	**75**

*These mosquitoes could not be identified beyond the Culicinae family due to the absence of legs.

Table 4.2. Host preferences of blood-fed mosquitoes captured in Chester Zoo, 2018.

Host group	Scientific Name	Common name	*Culex* spp.	*Culiseta* spp.	*Anopheles maculipennis*	Unknown*	Total
Birds (n = 22)	*Anas platyrhynchos*	Mallard	4	2	0	0	6
	Gallus gallus	Chicken	2	1	0	1	4
	Cyanistes caeruleus	Blue tit	3	0	0	0	3
	Passer domesticus	House sparrow	2	0	0	0	2
	Spheniscus humboldti	Humboldt penguin	0	0	1	1	2
	Corvus monedula	Western jackdaw	1	0	0	0	1
	Erithacus rubecula	European Robin	1	0	0	0	1
	Strix leptogrammica	Brown wood-owl	1	0	0	0	1
	Turdus merula	Eurasian blackbird	1	0	0	0	1
	Turdus philomelos	Song thrush	1	0	0	0	1
Mammals (n = 5)	*Bos taurus*	Cattle	1	0	0	1	2
	Camelus bactrianus	Bactrian camel	0	1	0	0	1
	Rucervus eldi	Eld's deer	0	1	0	0	1
	Sus scofra	Pig	1	0	0	0	1
Humans (n = 17)	*Homo sapiens*	Human	17	0	0	0	17
Mixed blood-meals (n = 1)	*Columba palumbus / Streptopelia decaocto*	Wood pigeon / Eurasian collared dove	1	0	0	0	1
		Total	**36**	**5**	**1**	**3**	**45**

*The absence of legs prevented the identification of these mosquitoes beyond the Culicinae family.

Table 4.3. Host preferences of blood-fed mosquitoes captured in Flamingo Land, 2017.

Host Group	Scientific Name	Common name	*Culex* spp.	*Culiseta* spp.	Unknown*	Total
Birds (n = 1)	*Parus major*	Great tit	0	0	1	1
Mammals (n = 6)	*Camelus bactrianus*	Bactrian camel	0	1	0	1
	Canis lupus familiaris	Dog	1	0	0	1
	Hydrochoerus hydrochaeris	Capybara	1	1	0	2
	Oryx dammah	Scimitar-horned Oryx	0	1	1	2
Humans (n = 12)	*Homo sapiens*	Human	10	0	2	12
		Total	**12**	**3**	**4**	**19**

*These mosquitoes could not be identified beyond the Culicinae family due to the absence of legs.

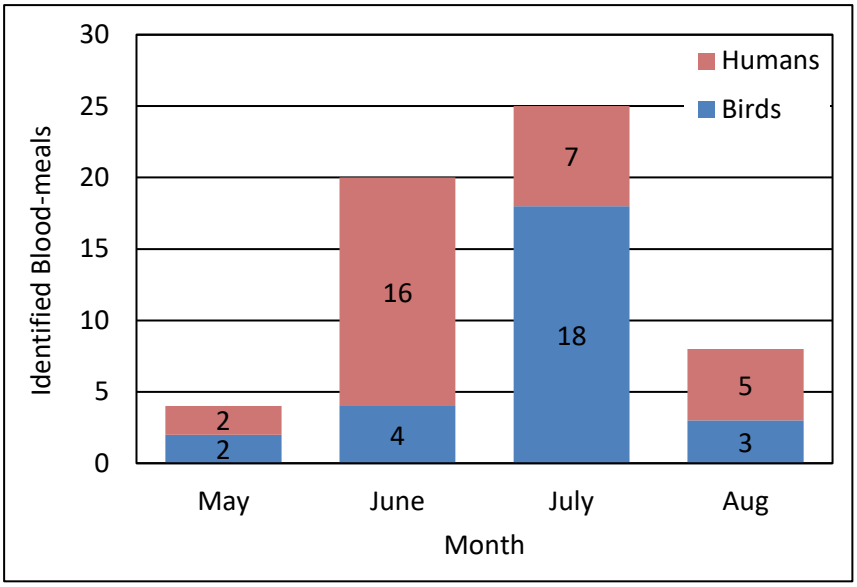


Figure 4.3. Host preferences of blood-fed *Culex pipiens* in Chester Zoo, 2017.

4.4.4 Flying distances

The blood meals from zoo animals identified in the 2017 season from Chester Zoo were 14, whereas they were five in both, 2018 and in Flamingo Land (Table 4.4). Mosquitoes in Chester Zoo in 2017 travelled an average minimum distance after feeding of 142.6 m (minimum = 34.16m, maximum = 336.73 m), in 2018, the average distance was 92.38 m (minimum = 21.34 m, maximum = 185.26 m), and in Flamingo Land, they flew 156.06m on average (minimum = 24.76m, maximum = 366.74 m) (Figures 4.7 and 4.8).

There were no significant differences between mosquito genera in Chester Zoo in terms of flying distances in 2017 (Welch's Anova, p = 0.4) and the sample size from the 2018 sampling and Flamingo Land was too low for analysis.

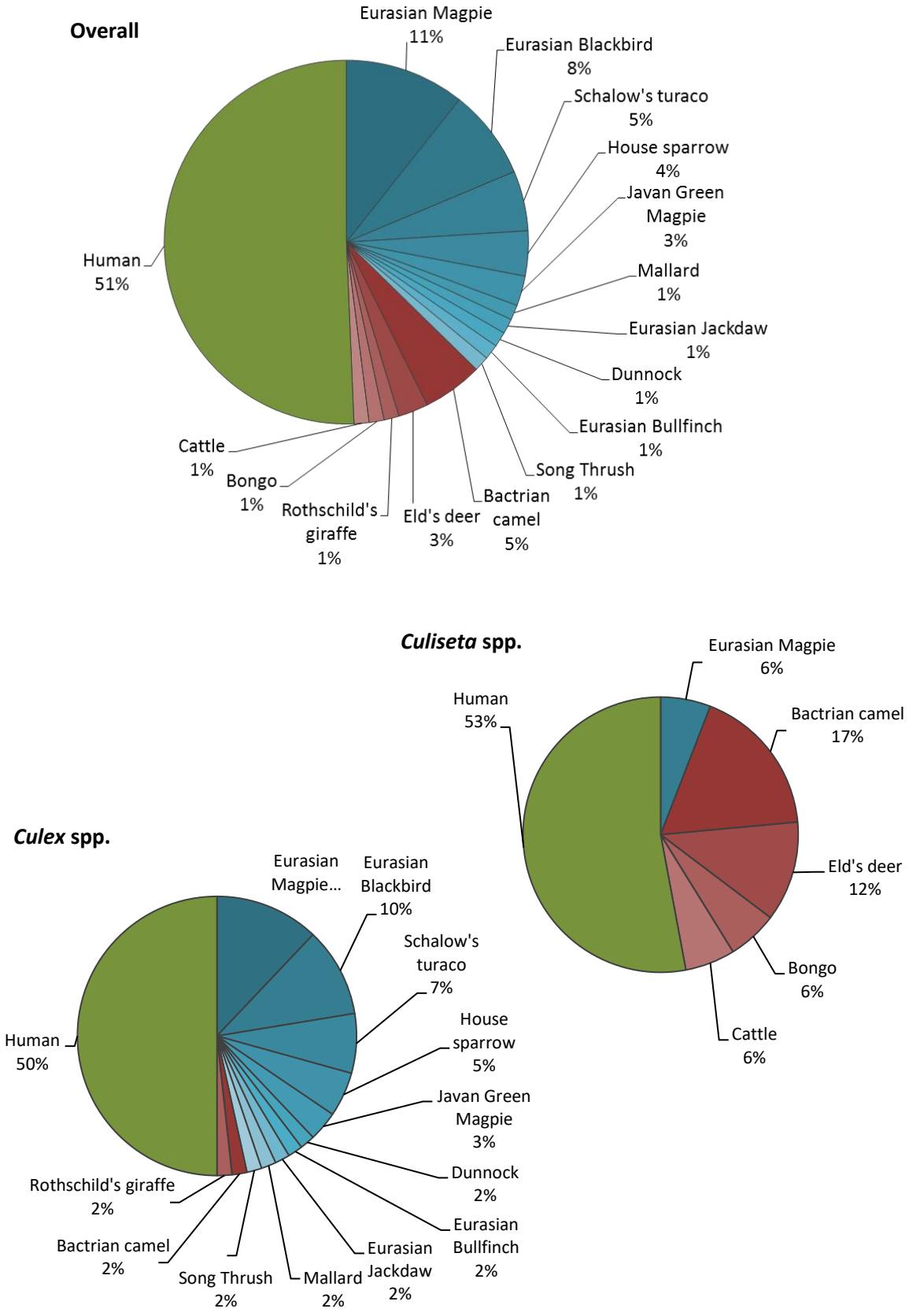


Figure 4.4. Host preferences of blood-fed mosquitoes in Chester Zoo, 2017. Birds in shades of blue, non-human mammals in shades of red.

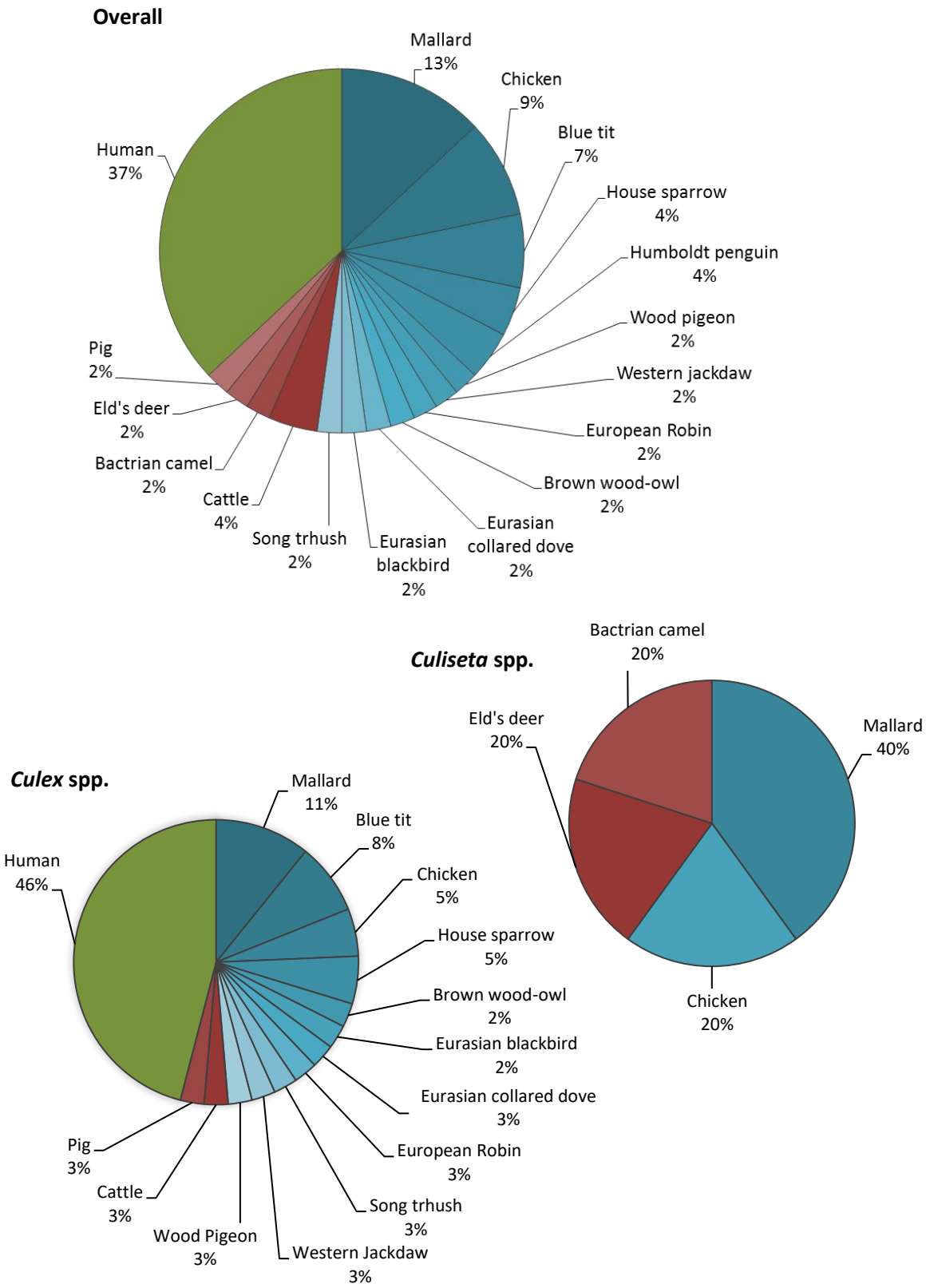


Figure 4.5. Host preferences of blood-fed mosquitoes in Chester Zoo, 2018. Birds in shades of blue, non-human mammals in shades of red. Missing host species in the genus graphs belong to partially identified mosquitoes.

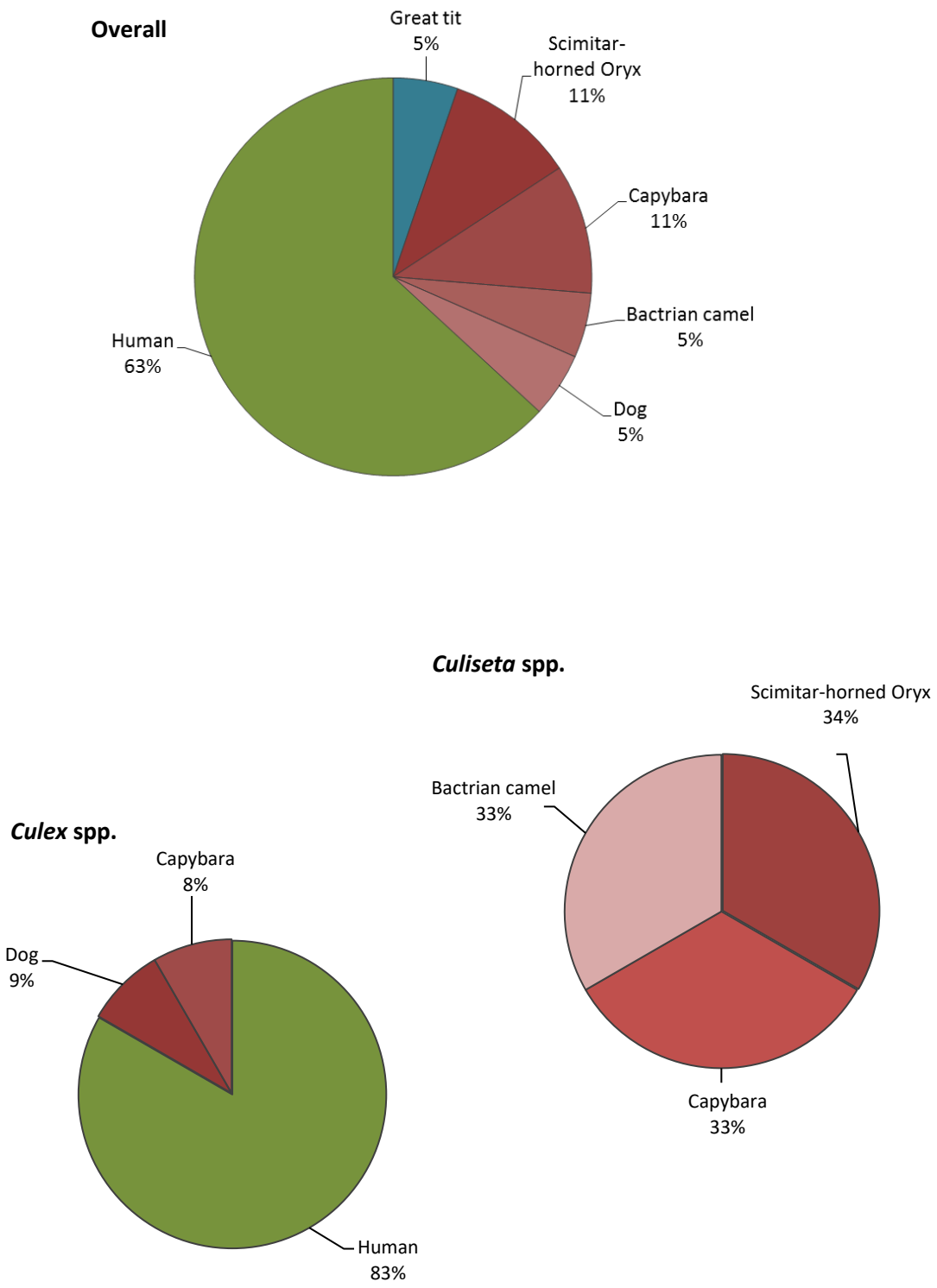


Figure 4.6. Host preferences of blood-fed mosquitoes in Flamingo Land, 2017. Birds in shades of blue, non-human mammals in shades of red. Missing host species in the genus graphs belong to partially identified mosquitoes.

Table 4.4. Minimum flying distances of blood-fed mosquitoes that fed on zoo animals.

Sampling Season	Mosquito genus	Zoo animal Scientific name	Common name	Trap	Minimum flying distance (m)
Chester Zoo 2017	*Culex* spp.	*Camelus bactrianus*	Bactrian camel	M1	234.3
		Cissa thalassina	Javan Green Magpie	G4	58
		Cissa thalassina	Javan Green Magpie	M3	34.16
		Giraffa camelopardalis rothschildi / Tauraco schalowi	Rothschild's giraffe / Schalow's turaco	M1	303.28[a]
		Tauraco schalowi	Schalow's turaco	G1	168.58
		Tauraco schalowi	Schalow's turaco	G1	168.58
		Tauraco schalowi	Schalow's turaco	M7	204.7
	Culiseta spp.	*Camelus bactrianus*	Bactrian camel	M7	56.18
		Camelus bactrianus	Bactrian camel	M7	56.18
		Camelus bactrianus	Bactrian camel	M7	56.18
		Rucervus eldii thamin	Eld's deer	M1	336.73
		Rucervus eldii thamin	Eld's deer	M11	55.88
		Tragelaphus eurycerus	Bongo	M10	107.06
	Unknown*	*Tauraco schalowi*	Schalow's turaco	M1	156.61
Chester Zoo 2018	*Anopheles maculipennis*	*Spheniscus humboldti*	Humboldt penguin	M2	21.34
	Culex spp.	*Strix leptogrammica*	Brown Wood-Owl	G13	185.26
	Culiseta spp.	*Camelus bactrianus*	Bactrian camel	M11	178.07
		Rucervus eldii thamin	Eld's deer	M11	55.88
	Unknown*	*Spheniscus humboldti*	Humboldt penguin	M2	21.34
Flamingo Land	*Culex* spp.	*Hydrochoerus hydrochaeris*	Capybara	M2	46.67
	Culiseta spp.	*Camelus bactrianus*	Bactrian camel	M4	24.76
		Hydrochoerus hydrochaeris	Capybara	M1	49.23
		Oryx dammah	Scimitar-horned Oryx	G1	366.74
	Unknown*	*Oryx dammah*	Scimitar-horned Oryx	M2	292.94

[a] Mixed blood-meals, includes the distance between the exhibits of both animals and the trap. * These mosquitoes were only identified as Culicinae due to damaged legs. M: BG-Mosquitaire trap, G: CDC-Gravid trap.

4.5 Discussion

Analysing the blood-fed mosquitoes in our collections, we noticed that some sampling areas and some months had more blood-fed mosquitoes than expected showing that their feeding activity is influenced by the local conditions and the season. We found significant differences in the host preferences, *Culex* spp. preferred birds over mammals and *Culiseta* spp., mammals over birds, which corresponds to their typical biology; nevertheless, there was a surprisingly high proportion of mosquitoes feeding on humans. Lastly, the analysis of the mosquito flying distances after feeding on zoo animals revealed a high variability.

Figure 4.7. Minimum flying distances of blood-fed mosquitoes in Chester Zoo, 2017. Not all distances are shown. Red area: penguin exhibit; yellow areas: animal exhibits; orange circles: sampling areas; M: BG-Mosquitaire traps, G: CDC-Gravid traps; green dots: exhibit centroids; dotted lines: minimum flying distances.

As blood-fed mosquitoes are looking for a place to rest and digest the blood, we did not expect to find many of them in our collections. Nevertheless, other authors have also found blood-fed mosquitoes incidentally; for instance, Goodman *et al*. (2018) found a proportion of 1.7% for *Culex* spp. using BG-Sentinel traps (Goodman et al., 2018). These could be mosquitoes partially fed that are looking for a second source of blood before producing eggs, but in this case, we would not find them in the CDC-Gravid traps. An alternative is that they were looking for a resting place and were attracted by the dark colour and location of the traps, but we cannot exclude the possibility that they were randomly captured as we did not find significant differences by trap type. Thus, to define what factor attracted them to the traps, other traps like the resting traps should be compared.

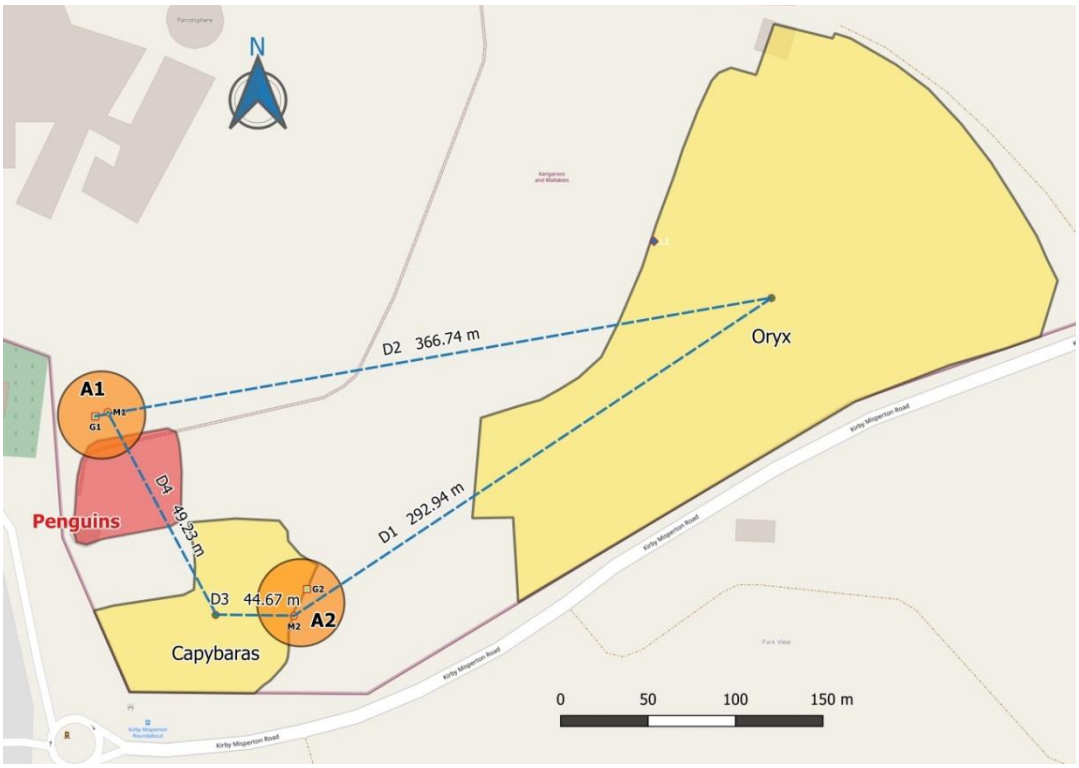


Figure 4.8. Minimum flying distances of blood-fed mosquitoes in Flamingo Land, 2017. Not all distances are shown. Red area: penguin exhibit; yellow areas: animal exhibits; orange circles: sampling areas; M: BG-Mosquitaire traps, G: CDC-Gravid traps; green dots: exhibit centroids; dotted lines: minimum flying distances.

We did find significantly more engorged *Culiseta* spp. than *Culex* spp. mosquitoes. An initial possible explanation was that *Culiseta* spp. mosquitoes, been mammalophilic, were attracted to the lure in the BG-Mosquitaire traps which mimics mammals sweat. But when comparing the proportions of mosquitoes by species and traps, we did not find a significant difference. Thus, other not considered factors attracted more *Culiseta* spp. mosquitoes to both types of traps that need further investigation, like the location and colour of the traps.

4.5.1 Temporal and spatial variations of blood-fed mosquitoes

There is a possible relation between the general abundance of mosquitoes and the proportion of blood-feds, which has been noticed in other studies (Goodman et al., 2018). During the first year in Chester Zoo, we found that areas A1 and A3 collected a high number of mosquitoes and had a high proportion of blood-feds which was observed again for A1 in 2018. In Flamingo Land, we observed a similar situation in area A2 which captured a bigger number of mosquitoes and had a higher proportion of blood-feds. Nevertheless, other areas with lower general catches showed high proportions of blood-fed mosquitoes, like A7 and A10 in Chester Zoo in 2017 and areas A10 and A11 in 2018. Therefore, the mosquito abundance is not the only explanatory factor.

The surroundings of the sampling areas could have a major influence in both the mosquito abundance and their feeding activity. For instance, area A3 captured a significant high number of blood-fed mosquitoes in 2017 possibly due to its proximity to a picnic area and a children's playground, added to the availability of dense vegetation where the mosquitoes could rest. In 2018, the children's playground was renewed, and the off-show aviaries were expanded; as part of these renovations, a considerable portion of the vegetation was reduced, and the playground was closed for a number of weeks. Together, this could have been the reasons for fewer blood-fed captures than expected in the second year in this area.

Similarly, the proximity and abundance of potential hosts could attract the mosquitoes to certain areas. Area A7, which caught more blood-feds than expected in Chester Zoo in 2017, is also near a picnic garden and an area with high transit of visitors; moreover, the camels, on which three mosquitoes captured in this area fed, have their exhibit across a footpath from it. In both years, area A10 also captured a high proportion of blood-feds which matched mainly wild birds; this area is inside the wetland aviary and the abundance of wild passerines could be high as they are attracted to the aquatic birds' food besides the netting of the exhibit.

In Flamingo Land, area A2 showed a higher proportion of blood-feds than expected. The location of this trap is in the boundary of the South American exhibit which contains large mammals and birds like capybara (*Hydrochoerus hydrochaeris*), vicunya (*Vicuna vicuna*), alpaca (*Vicugna pacos*), Brazilian tapir (*Tapirus terrestris*) and greater rhea (*Rhea americana*). Therefore, the constant presence of suitable hosts could be an attractant factor for mosquitoes.

Regarding the differences observed by months, in the first year in Chester Zoo, June had more blood-feds than expected and July, less than expected; mainly due to the significant preference of *Culex* spp. for humans in June and for birds in July. It is possible that the number of visitors had influenced the mosquito host preference; the most crowded months in Chester Zoo are from April to August (Eckley L. personal communication) and all mosquitoes with human blood were captured in this period in both years.

During 2018, the mosquito feeding activity was higher in July and August possibly in relation to the hosts availability but in this case also influenced by the delay in the peak of mosquito abundance compared to the previous year, which took place from July to August in the first year and between July and September in the second one (see section 3.4.1.1).

The difference in blood-feds in Flamingo Land, showing a significantly higher proportion in July and August, could be related to both the peak of the mosquito abundance and the higher number of visitors in the park. Unfortunately, the low number of successfully sequenced blood-meals does not allow a better understanding of mosquito feeding activity drivers in this site.

On the other hand, mosquitoes do not tend to feed before overwintering as this reduces their chances of survival. This is the likely reason for the low proportions or absence of blood-fed mosquitoes in the later months of the season in all samplings.

4.5.2 Success of techniques

The difference observed in the results of PCR assays among sites and years was due to a higher proportion of successful reactions in the 2017 and a lower success rate in 2018 in the Chester Zoo samplings. It was observed that a number of blood-fed mosquitoes from the 2018 season were completely dehydrated and the content of their abdomens was dry despite their evident blood-fed status. Reeves et al. (2016) mention that desiccation is a poor method for preserving blood-fed mosquitoes (Reeves et al., 2016), thus the warmer and dryer conditions of 2018 could have influenced this result.

The other comparisons in the success of the methodology in all samplings, by traps type, mosquito genera, positive sequencing and collection type did not yield significant differences, showing that our methods remained consistent and the results were comparable. We did not find a difference in the proportions of blood-fed mosquitoes by trap type for two possible reasons, they were mosquitoes randomly captured or they were looking for a second blood meal and they were similarly attracted by the lure in the BG-Mosquitaire traps or the CO^2 released from the fermenting infusion in the CDC-Gravid traps. We suspected that a difference per collection type (week or day collection) would be found as it has been reported that fresh mosquitoes yield better rates of host identification (Brugman et al., 2017, Santos et al., 2019, Reeves et al., 2016). Nevertheless, the mosquitoes from our week and one day collections were no different, possibly because we excluded the mosquitoes with partially digested blood that were more probably to be found in the week collections.

The cumulative positive PCR amplifications in our study were 34% and from those, 80% produced sequences that allowed the host identification. Other authors reported better identification rates; for example, Brugman *et al*. (2017) had 77% of positive PCR

amplifications and 93% of successful sequences using a multiplex PCR (Brugman et al., 2017). Our success was possibly affected by the partial storage of samples at -20°C before analysis which is not an ideal method (Reeves et al., 2016). Additionally, the time between collection and analysis could have also decreased the number of successful amplifications.

4.5.3 Host preferences

The host choice of the mosquitoes depends on the natural preference of the mosquito and the availability of host species in the environment (Unnasch et al., 2006, Santos et al., 2019). Most of the birds on which *Culex* spp. fed were local wild birds and some zoo birds. Considering the diversity of birds in the zoos, it is interesting to notice that this mosquito fed repeatedly on only two zoo species, the Schalow's turaco (*Tauraco schalowi*) and the Javan Green Magpie (*Cissa thalassina*). The second one is related to the Eurasian magpie (*Pica pica*), the preferred wild bird host of this mosquito, as both belong to the Corvidae family. Therefore, this mosquito could have an inclination for birds of this family but a broader sampling using bird-baited traps would be needed to clarify this.

Culiseta spp. showed a clear preference for mammals that varied depending on the local availability of potential hosts which corresponds to the opportunistic feeding behaviour of this mosquito. Our results confirm the natural host preferences of *Culex* spp. for birds and *Culiseta* spp. for mammals but the preference for humans requires further analysis.

Culex pipiens is primarily an ornithophilic species (Goodman et al., 2018), but it has been reported feeding on humans in the USA (Goodman et al., 2018) and southern Europe (Martinez-de la Puente et al., 2016). In these studies, the authors reported a low proportion of human feeds and it was therefore considered to be an opportunistic feeding. By contrast, around 50% of the successful sequences from this species in our collections were from humans. We discounted the possibility of major sample contamination as none of the negative controls produced a positive band in the PCR assays, none of the sequenced samples matched the positive control and the human positives showed a pattern. These mosquitoes fed on humans during the summer, when the zoos have more visitors and temporary staff, and no human blood-meals were found at other times, although the occurrence of birds and other mammalian blood-meals continued to be observed. Additionally, the preference for humans was higher in *Culex* spp. than in *Culiseta* spp. which is not expected either as *Culiseta annulata* has been reported as a biting nuisance for people in the UK (Asgharian et al., 2015). These changes in host preference could influence

the transmission dynamics of avian malaria parasites as has been observed before (Kim and Tsuda, 2010).

It is possible then that these mosquitoes belong to the *Cx. pipiens* biotype *molestus* which is known to prefer feeding on mammals and has different biological attributes. Contrary to the *pipiens* biotype, *molestus* thrives in urban environments especially underground where it can breed in confined spaces (stenogamous) and can lay eggs without a blood-meal (autogenous) (Martinez-de la Puente et al., 2016). Martínez-de la Puente *et al*. (2016) found a bigger proportion of the *pipiens* biotype in natural than in urban environments and an opposite trend for *molestus* in Spain; they observed an intermediate situation in rural environments (Martinez-de la Puente et al., 2016). Although it has been reported in several locations in Europe, the *molestus* biotype has not been found in environments such as zoos. These biotypes and their hybrids could be vectors of relevant pathogens for human health due to their mixed feeding preference (Martinez-de la Puente et al., 2016). Hence, it is of particular interest to fully identify the mosquitoes in our collections; for identifying the *Cx. p. molestus* biotype and its hybrids with *Cx. p. pipiens*, a PCR protocol has been proposed (Bahnck and Fonseca, 2006).

To compare host preferences in different settings, a feeding index can be used to estimate the relative host preference for different species (Unnasch et al., 2006). It could be particularly useful because a dominant susceptible host could have a higher probability of been bitten by the mosquito and infected with a pathogen (Abella-Medrano et al., 2018). Nonetheless, it requires an estimation of the host's abundance and although we know the number of zoo animals in each exhibit, we did not monitor the population of wild birds or the flow of visitors in the zoos which would be necessary in future studies for better understanding the relative mosquito host preferences.

4.5.4 Travelling distances

The minimum travelling distances of blood-fed mosquitoes were highly variable. It is more likely to capture them close to their food source as discussed before, but it is also important to notice that some mosquitoes travelled long distances from their hosts' location to the traps. Tuten *et al*. (2012) found in two California zoos that the average minimum and maximum travelling distances of blood engorged mosquitoes was 15.5 m and 327 m, respectively. In a Japan zoo, Ejiri *et al*. (2011) reported an average travelling distance of 40 m and a maximum distance of 350 m, which can be also influenced by the zoo setting (Ejiri et al., 2011) and Greenberg *et al*. (2012) reported a maximum flying distance of 170 m

(Greenberg et al., 2012). Therefore, our maximum travelling distance (367 m) was closer to the findings of first two authors.

It is possible that in some areas flight paths exist aiding the mosquito's movement in a certain direction (Ejiri et al., 2011). Area A1 captured more blood-fed mosquitoes that feed on zoo animals and looking at the origin of these hosts, it can be noticed that they are located from the southwest to the northwest from this area. The wind in the previous day of these collections came from a similar direction in four out of six mosquitoes with zoo animal's blood (data from Chester Weather Station, http://www.chesterweather.org.uk). The lack of difference in the travelling distances between *Culex* spp. and *Culiseta* spp. could be also related to the wind influence; it would be expected that as *Culiseta* spp. is a bigger mosquito, it would avoid travelling far after feeding, but our results do not support this concept. Brugman et al. (2017) reported that the wind diminishes the capture of blood-fed mosquitoes (Brugman et al., 2017), so the influence of the wind in the mosquito movement could be examined using a weather station with anemometer and wind vane in the sampling areas.

It was assumed that the zoo animals would be randomly distributed in their exhibits and the exhibits centroids represent an average of the possible location of the animals when the mosquitoes fed on them. Nevertheless, some animals trend to spend more time in certain parts of their enclosures, like around the feeders during the day or in the night enclosure at night-time. A more precise estimation of the mosquito minimum travelling distances should take this into consideration and delimit areas with different degrees of animal occupancy inside the exhibits.

4.5.5 Risk of interspecific transmissions

The higher risks of interspecific pathogen transmission are usually observed when a generalist vector feeds on different species or groups of hosts and, in the case of zoonotic diseases, when the vector abundance and the human host-use are increased (Goodman et al., 2018). Nevertheless, the susceptibility of the host and its competence are critical factors for the endemic transmission of a pathogen within a community, and quantifying the importance of each host is relevant for targeting control measures and wildlife conservation, despite the challenges to determine it (Fenton et al., 2015).

Multi-host feeding has been reported to be very low (Cornet et al., 2013) as we observed. All mixed blood-meals that we found were from *Culex* spp. mosquitoes and involved at

least one bird host. There are no known pathogens transmitted by *Culex* spp. shared between birds and giraffes or camels but in the case of humans, West Nile virus (WNV), Sindbis virus and Usutu virus are particularly concerning. Furthermore, the mixed blood meals that involved humans were combined with blood of Eurasian magpie, a species that belongs to the Corvidae family; this family includes several species that are suspected or confirmed reservoirs of WNV. Additionally, it has been shown that the temporal and spatial variations in host preferences by *Culex* spp. could influence the timing and severity of WNV infections, possibly in relation to its seasonal shifts between ornithophilic and anthropophilic cycles (Goodman et al., 2018), a preference change that we observed in our 2017 sampling in Chester Zoo. For these reasons, this mosquito is the bridge vector for WNV between wild birds and humans and should be constantly monitored despite the lack of evidence confirming WNV's establishment in the UK (Vaux et al., 2015).

The interspecific transmission of vector borne diseases is also important for the health of the animals in the zoo collections. Besides avian malaria, mosquitoes have been involved in the transmission of Eastern equine encephalitis virus to African penguins (*Spheniscus demersus*), Usutu virus to great grey owls (*Strix nebulosa*) and West Nile virus to birds, which caused the death of exotic animals in about 100 zoos in the United States (Adler et al., 2011, Greenberg et al., 2012).

We found two mosquitoes feeding on Humboldt penguins, one *Anopheles maculipennis* s. l. and an unknown Culicinae. It is very likely that the latter one was a *Culex pipiens* as it had all the corresponding morphological features with the exception of those evaluated on the legs and we did not find any other *Culex* or similar species in our samplings. *Anopheles* spp. mosquitoes are considered as potential vectors for avian *Plasmodium* and have been found susceptible to the parasite infection (Huff, 1965). Therefore, this genus could have a relevant role in the transmission of avian malaria, although *An. maculipennis* s. l. has not been found infected with *Plasmodium* yet (Martinez-de la Puente et al., 2015, Inci et al., 2012). The first step in the further study of this mosquito genus would be the identification of the three species present in the UK is in our sampling site, which could be done with the protocol proposed by (Danabalan et al., 2014). Unfortunately, we did not capture many of mosquitoes from this species.

As we could not confirm that the other mosquito was *Cx. pipiens,* we cannot ascertain that is the responsible vector for avian malaria transmission to the penguins besides the evidence that it carries the parasite (see section 3.4.5). The chances of finding mosquitoes

feeding on particular animals are low due to the variables that affect dispersion after feeding; so, a specific sampling for blood-fed mosquitoes should be attempted.

4.5.6 Improvement on techniques

For the efficient analysis of mosquito host preferences in zoo environments, the sampling protocol should be designed for the prompt collection and processing of samples. The quality of the blood inside the mosquito's abdomen certainly affects the results, as mentioned before; hence, the traps should be emptied as soon as possible, and this would also prevent the mosquitoes getting damaged, facilitating their identification. Likewise, other techniques for the collection of recently fed mosquitoes, like the use of resting boxes and the aspiration in resting places (Egizi et al., 2018, Brugman et al., 2017), could be attempted. After collection, the mosquitoes should be stored at -80 °C which is an ideal preservation method (Santos et al., 2019).

The possibility of capturing blood-fed mosquitoes diminishes as the distance from the host location increases. We observed that more mosquitoes that fed on zoo animals were captured in sampling areas close to their exhibits. We also noticed a tendency for blood-fed mosquitoes to be captured in certain areas, possibly by the influence of wind currents. These variables should be studied in advance and considered for the better sampling of blood-fed mosquitoes around potential hosts of interest.

4.5.7 Conclusion

Blood-fed mosquitoes showed differences in their proportion by sampling area and month, and we also observed a different proportion of blood-fed mosquitoes per species. The success capturing blood-fed mosquitoes could be related to their abundance but a closer examination using specific techniques should be done.

We confirmed that mosquitoes in zoos feed in a wide range of hosts mostly within the expected host preferences; nevertheless, we found many *Culex pipiens* mosquitoes feeding on humans. This could be related to the abundance of potential hosts or to the preferences of mosquito biotype, but we did not differentiate *Cx. pipiens pipiens* from *Cx pipiens molestus*. This could be relevant for the assessment of interspecific transmission risk of zoonotic pathogens like West Nile virus.

The minimum travelling distances of mosquitoes after having a blood-meal were variable and it is likely that landscape features influence their movements; thus, certain features like the wind should be evaluated.

The main period of feeding activity corresponded with the increase in mosquito abundance and although it also was higher in areas with greater number of mosquito catches, there were other areas with high proportions of blood-fed mosquitoes. Therefore, the availability of potential hosts could attract mosquitoes to certain areas becoming a nuisance for visitors and a disease transmission risk for animals (see discussion in Chapter Five); although the host susceptibility and host competence also are relevant in the disease transmission process. Considering this and the variable travelling distances, the control of mosquitoes should be done also in areas that could be attractive for them when feeding. Avoiding the constant aggregation of animals or visitors in certain areas could reduce the number of mosquitoes, if this is practical to implement, and the use of mosquito repellents for visitors and staff could be recommended during the months of high mosquito activity. The effects and efficiency of mosquito repellents for animals or animal exhibits should be evaluated before their regular use.

To better understand the mosquito's feeding activity and the influence of potential hosts in zoos, the study of the community of wild birds and visitor flow, in terms of abundance, density, and distribution could provide valuable information. Likewise, although we found two species of mosquitoes feeding on Humboldt penguin, more analyses are needed to make clear which mosquitoes usually feed on them and in what rate.

Chapter Five

Influence of Environmental Factors on Mosquitoes in UK Zoos

5.1 Abstract

Temperature, humidity and rainfall can influence the geographical distribution and seasonal activity of mosquitoes. At the local scale, other factors can promote or limit mosquito abundance like habitat features that provide sources of food and shelter like the vegetation or the availability of potential hosts. Understanding these factors is essential for the effective control of mosquito populations and the prevention of diseases that they can transmit such as avian malaria. During our samplings in Chester Zoo and Flamingo Land, we monitored the surroundings of the traps and recorded temperature and humidity using loggers and gathered the regional temperature and rainfall data from the E-OBS gridded dataset. The surrounding variables observed were vegetation, distance to oviposition sites, availability of artificial resting areas and distance to zoo animal exhibits. The aggregated data from all the mosquito collections were analysed statistically using generalised linear models (GLM) including both zoos and years and with a GLM for specific zoos and years. We used traps optimised for either mosquitoes searching for a blood-meal and for mosquitoes looking for an oviposition site. The temperature was the most influencing variable in all models showing a strong correlation with the mosquito abundance. Other significant variables were the presence of dense vegetation, proximity to oviposition sites and proximity to zoo animal exhibits. Possible mechanisms of action of these variables are as follows: temperature affects the physiology of mosquitoes and therefore their activity; vegetation provides shelter and food; proximity to oviposition sites is a natural attractant for gravid females and they are the source of recently emerged adult mosquitoes; and finally, the abundance of potential hosts can also attract mosquitoes to a certain area. To plan mosquito control measurements, temperature could alert about the mosquito activity, while avoiding the presence of the other features near susceptible birds could diminish mosquito abundance and therefore, avian malaria transmission risk.

5.2 Introduction

Some environmental variables set thresholds for the species distribution and influence their development, reproductive fitness, activity and survival (Ciota et al., 2014). Mosquitoes are sensitive to differences in the habitat and climate; for instance, microhabitat and availability of oviposition sites and hosts could modify the proportions of mosquito species and biotypes inside communities (Vogels et al., 2016). As human activities modify these environmental factors and in consequence the distribution of mosquitoes and the pathogens they transmit, the environment is of particular importance for the study of mosquito-borne pathogens and vectorial capacity. Moreover, artificial habitats provide conditions for disease transmission that are not usually well described and that differ from the natural processes. Therefore, the study of the regional environment is particularly useful for epidemiologic modelling and forecasting, and the study of the local conditions is relevant for establishing efficient preventive and control measures.

The influence of temperature on mosquito biology has been studied extensively, particularly in relation to the effects of climate change and pathogen transmission risks (Karki et al., 2016, Ewing et al., 2016, Ciota et al., 2014). Warmer weather increases mosquito activity and shortens the development time of aquatic stages and the period between host feeding and oviposition which could increase the efficiency of disease transmission (Karki et al., 2016, Cavicchioli et al., 2019). At higher temperatures, the lifespan of adult mosquitoes decreases, and at lower temperatures, it is extended (Karki et al., 2016, Ewing et al., 2016, Ciota et al., 2014), up to a certain point in which extreme temperatures will decrease survival; showing that mosquitoes have low and high temperature thresholds (Ciota et al., 2014). These temperature values regulate their geographical distribution and their seasonal activity and it is expected that increased temperature would lead to a growth in mosquito numbers (Ewing et al., 2016). However, this is relative to the species and initial climatic conditions; for instance, the median longevity of field derived *Culex* spp. was over 75 days at 16 °C and less than 50 days at 24 °C (Ciota et al., 2014).

Rainfall is related to the abundance, size and duration of suitable places for the development of immature stages (larvae and pupae) (Karki et al., 2016, Ewing et al., 2016); nevertheless, artificial containers in urban settings can become alternatives independently of the rain, as shown for *Cx. pipiens* in the UK (Townroe and Callaghan, 2014). In some environments, the variables that influence the life cycle of mosquitoes are specific, like the

interaction of rainfall and high tides in estuary habitats that could alter the dynamics of aquatic stages (Michael John and Christian, 2011).

Landscape features can also influence mosquito abundance at the regional and local scales. Vegetation for instance, provides shaded resting sites and sugars for mosquitoes to feed (Karki et al., 2016) and could increase the density of mosquitoes and therefore, their capture in traps (Brugman et al., 2017). Furthermore, the interaction among factors is also relevant; for instance, the number of generations that a mosquito can complete depends on temperature and availability of larvae habitats and hosts (Foster and Walker, 2019).

Tuten (2011a) described the habitat characteristics that affect the oviposition behaviour in two zoos in South Carolina finding that the ambient and site temperature, precipitation, dissolved oxygen, presence of natural habitats and absence of aquatic vegetation were associated with larval abundance (Tuten, 2011b). However, the studies of mosquito ecology in zoological gardens are scarce and in them, the habitat features are rarely considered.

During our mosquito samplings to evaluate the avian malaria vectors in Chester Zoo and Flamingo Land, we observed the environmental surroundings of our traps and monitored weather variables. In Chapter 4, the temporal and spatial changes in the mosquito community across the sampling seasons were described. It was found that there were important differences by sampling area and month of the collection. The aim of this chapter is to analyse the influence of environmental conditions that could explain those differences in the mosquito catches. This analysis also provides tools for the control of mosquito populations through the modification of environmental features. The particular objectives were:

- To define the environmental factors that drive mosquito catches in relation to two behaviours: the search for potential hosts and for oviposition sites.
- To compare local and regional weather variables as descriptors of mosquito catch.

5.3 Materials and Methods

We collaborated with Chester Zoo and Flamingo Land for the surveillance of avian malaria parasites in mosquitoes, free wild birds and penguins. We did two samplings in Chester Zoo (in 2017 and 2018) and one in Flamingo Land (2017). For the mosquito trapping, we defined the sampling areas as 30 m diameter circles in which we placed one BG-Mosquitaire trap, one CDC-Gravid trap and there was an immature mosquito sampling area, if possible. In the first zoo we operated ten sampling areas during 2017 and eight in 2018; in the second one, we had four areas. The sampling areas are described in section 2.5. The traps were operated from May to December and from April to November in Chester Zoo and from June to November in Flamingo Land. The collection nets from the BG-Mosquitaire traps were replaced after six days and after one day; the CDC-Gravid trap nets were removed after one day. The nets were transported to Leahurst campus where the insects were killed and stored at -20°C. Afterwards, the mosquitoes were processed for molecular identification, blood-meal analysis and avian malaria testing. The protocol details are presented in section 2.3.

The traps that we used work in different ways; the BG-Mosquitaire trap lures mosquitoes looking for a blood-meal using a scent that mimics mammalians sweat (Sweetscent®) and the CDC-Gravid trap attracts gravid females looking for a place to laid their eggs. Therefore, we were able to analyse the conditions that drive two basic mosquito behaviours, questing for potential hosts and selecting oviposition places, referred hereafter as host search and site choice respectively. For the host search analysis, the data from the one day and six-day mosquito collections of the BG-Mosquitaire traps was aggregated by weeks. For the analysis of the site choice, the mosquito collections of the CDC-Gravid traps were used.

5.3.1 Traps surroundings

The traps were located considering the proximity to mosquito oviposition sites, mosquito resting places and zoo bird exhibits, prioritizing the penguin exhibits. We placed the traps near vegetation but not covered by it at least 2 m above and with some protection from the sun and wind currents if possible.

The immediate surroundings of the traps were observed constantly, and major variations were documented. The variables considered were vegetation, proximity to suitable mosquito oviposition sites, mosquito resting areas, proximity to zoo animal exhibits, regional temperature, regional precipitation, local temperature and local humidity. The

suitable oviposition sites were defined as water bodies with shallow water at least on its edges and rich in organic matter. The weather variables were numerical and the rest, categorical (Table 5.1).

Some important variations in the surrounding conditions of the traps were considered. The foliage decreased during the autumn and was limited towards the end of our samplings; thus, the vegetation value was changed to scarce for all traps from the dates when this was noticed, at the end of October. The conditions of oviposition sites remained relatively constant except for changes in the water level in area A4 in Chester Zoo due to gardening works or evaporation; when the water level was low, the value was changed to intermediate. The proximity to zoo animals was considered only for those in open exhibits and although the distance to the exhibits was constant, it was not practical to measure the actual distance to the animals. The values were assigned to each trap in relation to the others, not in relation to other areas of the zoos (Table 5.2). Examples of the trap values are presented in Figure 5.1 and some oviposition sites are shown in Figure 5.2.

Table 5.1. Variables and values considered for the environmental analysis.

Variable	Values
Vegetation	**Scarce:** few plants no higher than 2m and without trees **Medium:** some plants like bushes 2m high and lower plants with trees **Dense:** abundant plants with dense foliage at all levels including tree cover
Distance to oviposition sites	**Close:** less than 20m from a suitable oviposition site **Intermediate:** between 20 and 50m from a suitable oviposition site **Remote:** more than 50m apart from a suitable oviposition site
Resting areas (not considering vegetation)	**Rare:** few unsuitable resting structures, like fences **Medium:** some resting places available, like walls or containers **Abundant:** diverse resting places available including sheds and accessible buildings
Distance to zoo animal exhibits	**Close:** less than 20m from an open animal exhibit **Intermediate:** between 20 and 50m from an open animal exhibit **Remote:** more than 50m apart from an open animal exhibit
Regional temperature	The temperature for the corresponding zoo region
Precipitation	The rainfall for the corresponding zoo region
Local temperature	The temperature from the individual loggers next to the traps
Humidity	The average humidity from the individual loggers next to the traps

Table 5.2. Values of the categorical variables for each trap.

Zoo	Trap	Vegetation	Oviposition sites	Resting Areas	Animals Exhibits
Chester Zoo	M1	Medium	Close	Abundant	Close
	M2	Scarce	Remote	Medium	Close
	M3	Dense	Intermediate	Rare	Close
	M4	Scarce	Close	Medium	Intermediate
	M5	Dense	Remote	Medium	Remote
	M6	Medium	Intermediate	Medium	Remote
	M7	Scarce	Remote	Medium	Close
	M10	Medium	Intermediate	Medium	Close
	M11	Medium	Intermediate	Rare	Remote
	M12	Scarce	Remote	Abundant	Close
	M13	Scarce	Close	Rare	Close
	G1	Dense	Remote	Medium	Remote
	G2	Dense	Remote	Medium	Intermediate
	G3	Medium	Close	Rare	Intermediate
	G4	Medium	Close	Medium	Intermediate
	G5	Dense	Remote	Rare	Remote
	G6	Medium	Intermediate	Medium	Remote
	G7	Scarce	Remote	Medium	Intermediate
	G10	Medium	Intermediate	Medium	Close
	G11	Dense	Intermediate	Rare	Remote
	G12	Dense	Remote	Medium	Intermediate
	G13	Scarce	Close	Rare	Close
Flamingo Land	M1	Scarce	Remote	Abundant	Close
	M2	Medium	Remote	Rare	Close
	M3	Scarce	Remote	Medium	Intermediate
	M4	Scarce	Remote	Medium	Intermediate
	G1	Scarce	Remote	Abundant	Close
	G2	Medium	Remote	Medium	Close
	G3	Scarce	Close	Medium	Intermediate
	G4	Medium	Remote	Medium	Remote

M: BG-Mosquitaire trap, G: CDC-Gravid trap. The trap numbers correspond to the sampling areas.

5.3.2 Weather variables

Daily temperature and precipitation at the regional scale were obtained for the closest pixel to the zoos location (25 km by 25 km) from the E-OBS gridded data for Europe (Cornes et al., 2018). The temperature was given in Celsius (referred as regional temperature hereafter) and the precipitation in mm per day. The data from the day before the nets of the CDC-Gravid traps were collected was used for the site choice analysis and as an average of the week before the collection of the BG-Mosquitaire trap nets, for the host search analysis.

Figure 5.1. Surrounding characteristics of the mosquito traps in Chester Zoo. a) BG-Mosquitaire trap with scarce vegetation and medium resting areas (area A4); b) CDC-Gravid trap in dense vegetation and medium resting areas (area A1); c) BG-Mosquitaire trap with medium vegetation and abundant resting areas (area A1); d) CDC-Gravid trap surrounded by dense vegetation and medium resting areas (area A12); e) BG-Mosquitaire trap in medium vegetation and rare resting areas (area A11), arrow: TinyTag© logger.

Figure 5.2. Mosquito oviposition sites near the mosquito traps in Chester Zoo. a) Flamingo pond in area A1; b) Pond in Area A3 near the off-show aviaries; c) Water beds for plants in area A4, green houses, arrow: BG-Mosquitaire trap.

We used TinyTag© loggers (Gemini Data Loggers, UK) to record temperature and humidity next to the traps, 13 of the Plus 2 TGP-4500 model and two of the Ultra 2 TGU-4500 model. Before use, the loggers were tested in an incubator model MIR-154, Sanyo, Japan, which was set at a constant temperature of 23°C; the humidity cannot be controlled in this incubator, so the room value was recorded. They were programmed for recording every five minutes for 50 hours. After this period, the average temperature and humidity readings of all loggers presented a minimum variation within a range of 0.5 °C and 9.4% RH. The temperature of the loggers is referred as local temperature henceforth.

As the main interest was to know the environmental conditions that favour the host search, these loggers were placed less than one meter away from the BG-Mosquitaire traps, protected from the rain. The loggers were programmed to record every hour and one of them remained indoors as a control. They provide minimum, maximum and average temperature and humidity, so the average readings were used. As for the regional weather data, the average of the readings was calculated for the day before collecting the CDC-Gravid trap nets and a weekly average for the BG-Mosquitaire traps.

5.3.3 Modelling

Modelling of mosquito abundance was conducted with aggregated data across both zoos and both seasons, to analyse the influence of all factors involved, and to detect influences of the variables in specific settings. Although the use of General Linear Mixed Models is recommended in parasitology, the number of levels recommended for the random effects was not met (Paterson and Lello, 2003, Harrison et al., 2018). Therefore, a generalised linear model (GLM) was developed including the overall mosquito catches from both zoos and sampling years. The explanatory variables used were zoo, year, regional temperature, regional rain, local temperature, local humidity, vegetation, proximity to oviposition sites, abundance of resting areas and distance to zoo animal exhibits. To look for influences, the data were analysed separately for each year of sampling (in Chester Zoo) and sites, and independent GLMs were constructed. In Flamingo Land we did not find a consistent oviposition site, so this variable was excluded for that zoo. In all cases, the analysis was done for the host search (using the BG-Mosquitaire trap data) and site selection (with the CDC-Gravid trap data) independently. The months or areas were not used as explanatory variables because the data was kept separately per collection (weekly aggregated) and area and could have produced collinearity issues in the case of areas; besides, the differences in this sense were explored in section 3.4.1.3.

The response variable, the mosquito collections, was analysed for normality with the Anderson-Darling test and in all cases, distribution was significantly different from normality ($p < 0.005$). The shape of the distribution was examined and, although the Poisson distribution is commonly used with count data, in this case the frequencies were much closer to a negative binomial distribution; besides, the variance over the mean was greater than one (Figure 5.3). Therefore, a negative binomial distribution was used with a log link in all models.

A backwards elimination procedure was applied removing non-significant variables ($p > 0.05$), starting with the least significant, and the best models were selected when the removing of variables caused a significant difference tested with an Analysis of Variance (Anova); it was confirmed that these models had the lowest AIC (Akaike criterion) and residual deviance.

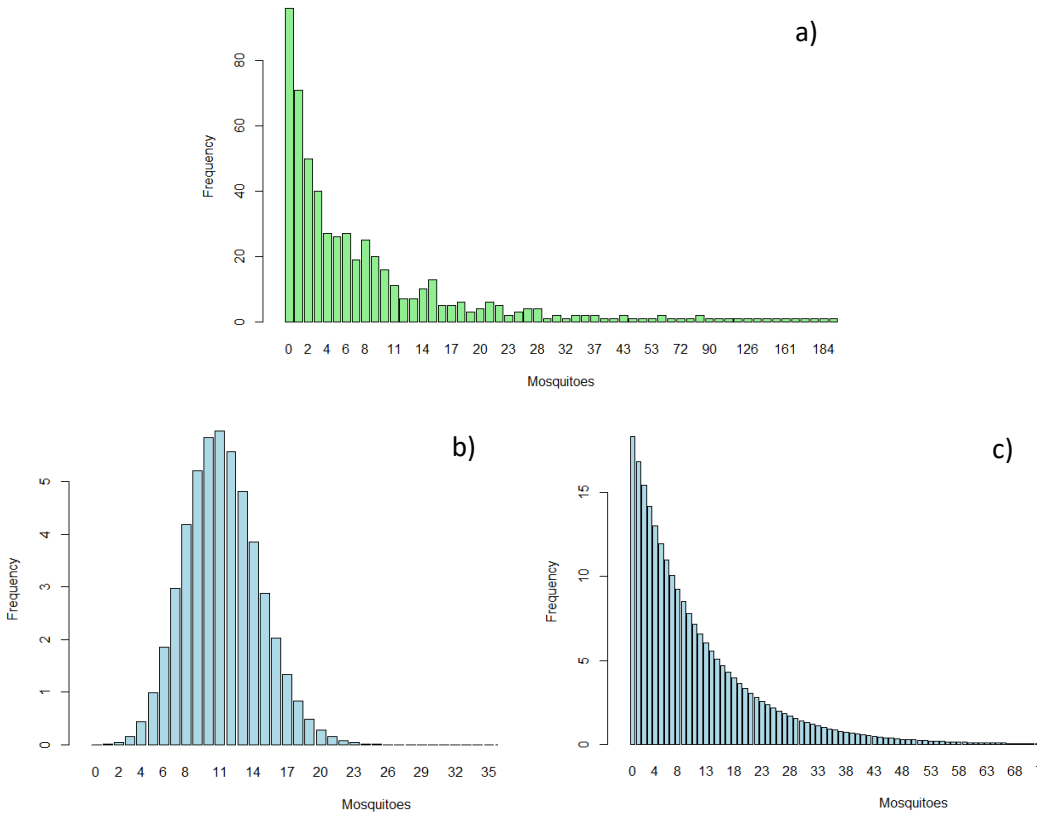


Figure 5.3. Distribution of overall collection of mosquitoes. a) Observed frequency of captured mosquitoes per collections; b) Estimated Poisson distribution; c) Estimated negative binomial distribution; n = 550, mean = 11.22, range = 0:224.

As the regional temperature has a strong correlation with the local temperature, it was decided a priori to test which one provides the best fit of the models and exclude the other one.

It was found that some of the categorical variables were collinear among themselves, so to integrate them in the models, dummy variables were created for each value of the categorical variables and a value of one was assigned if it was present or zero if not. To identify the collinear variables in each data set, a multiple correlation test was done. Then, the better fit of the models was analysed excluding these variables alternatively and including the ones that gave the best model fit; in most cases, the intermediate variable was excluded and the two more extreme ones remained (e.g. for the vegetation variable, medium vegetation was excluded and dense and scarce vegetation remained).All analyses were done using the R software version 3.6.0 (R Core Team, 2012).

5.4 Results

5.4.1 Environmental influences in the mosquito population

The correlation of coefficients was <-0.85 in all models when comparing the local against the regional temperatures. The difference in p values between the models without the local temperature and the full models was lower than when comparing without the regional temperature. Likewise, the AIC criterion and the residual deviance was lower using the regional temperature. Therefore, the regional temperature was used.

5.4.1.1 Overall mosquito capture analysis

The general relation of the variables with the collection of mosquitoes is presented in Table 5.3 for the categorical variables and in Figure 5.4 for the numerical ones.

The GLMs including zoo and year as variables, showed that, for the host search, the zoo, year, humidity, temperature, dense vegetation, close distance to oviposition sites and rare resting areas were the significant variables that explained the mosquito abundance. In the case of the site choice, the significant factors were again the zoo, year, temperature, scarce vegetation, dense vegetation, close oviposition sites, close zoo animal exhibits and remote animal exhibits. The model parameters are presented in Table 5.4 and 5.5.

5.4.1.2 Mosquito capture analysis by zoos and years

The variable that was significant in all the models was the regional temperature; other recurring variables with statistical significance were the presence of dense vegetation, close oviposition sites and close animal exhibits and other variables were significant in particular cases (Table 5.6). The relation between temperature and mosquito collections is illustrated in Figure 5.5.

Table 5.3. Number of mosquitoes captured in each category of the nominal variables.

Variable	Value	All	Chester Zoo 2017	Chester Zoo 2018	Flamingo Land
Vegetation	Dense	5238	3701	1151	386
	Medium	2392	1820	392	180
	Scarce	2590	1071	989	530
Oviposition sites	Close	4148	2590	1558	-
	Intermediate	2734	2311	423	-
	Remote	2242	1691	551	-
Resting Areas	Abundant	2828	1656	946	226
	Medium	4568	2890	914	764
	Rare	2824	2046	672	106
Animal exhibits	Close	5791	3207	1912	672
	Intermediate	2510	1831	352	327
	Remote	1919	1554	268	97

The green shade indicates the higher number of mosquitoes in relation to the other values of the same variable.

Table 5.4. Parameters of the GLM for the host search of mosquitoes overall.

Variable	Estimate	Std. Error	z value	P (>|z|)
Intercept	879.050	230.189	3.819	< 0.00
Zoo	0.756	0.136	5.548	2.89^{-08}
Year	-0.437	0.114	-3.834	< 0.00
Humidity	0.0127	0.004	3.256	0.001
Temperature	0.274	0.0167	16.421	$< 2^{-16}$
Dense Vegetation	1.005	0.118	8.488	$< 2^{-16}$
Close oviposition sites	0.564	0.121	4.675	2.94^{-06}
Rare resting areas	-0.244	0.119	-2.05	0.040
Dispersion parameter for Negative Binomial (1.0678) family taken to be 1				
Residual deviance: 604.67 on 541 degrees of freedom. AIC: 3229.4				

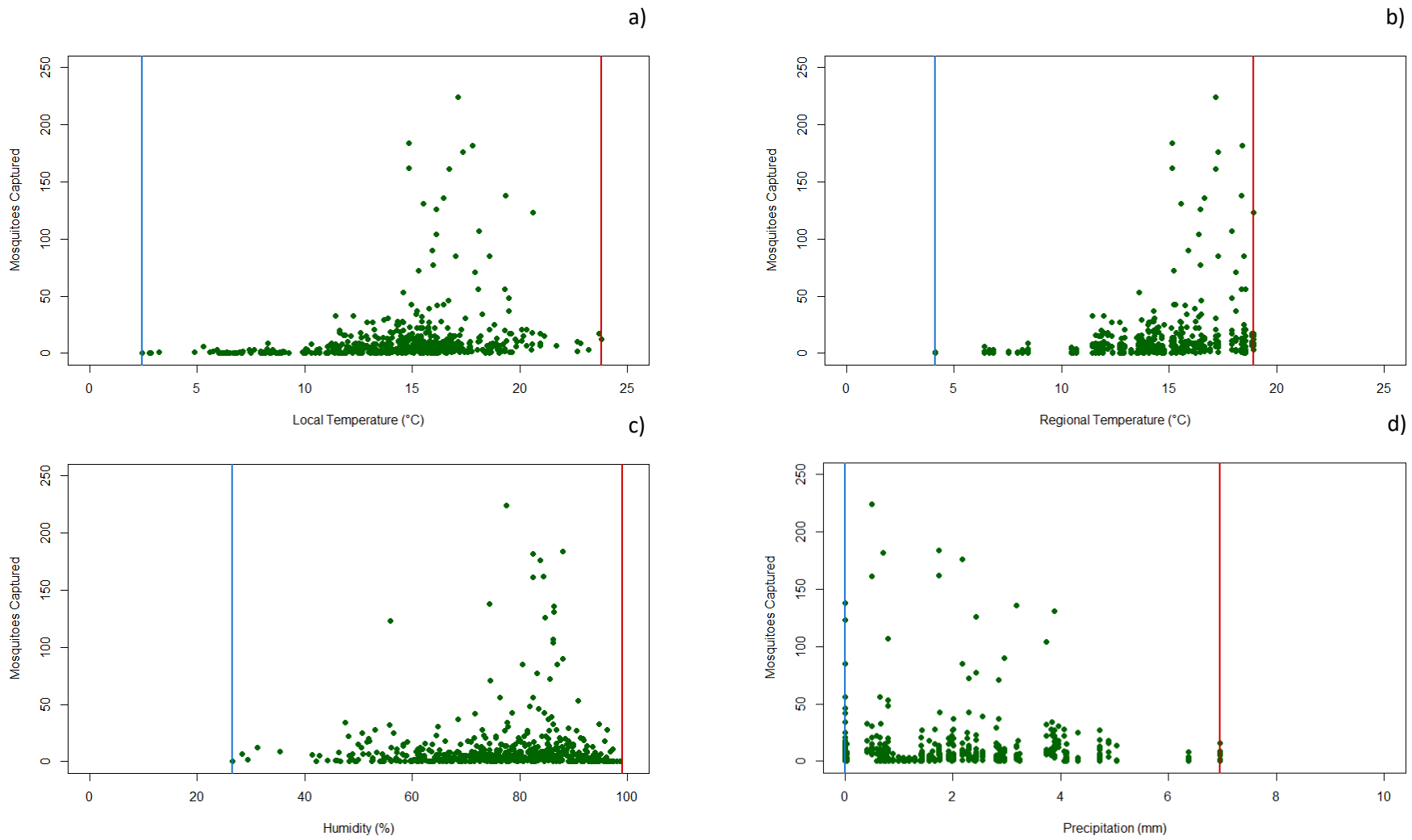


Figure 5.4. Mosquitoes per collection for the feeding behaviour analysis in relation to the weather variables. a) Local temperature, b) Regional temperature, c) Humidity, d) Precipitation. The lines indicate the minimum and maximum values recorded.

Table 5.5. Parameters of the GLM for the site selection of mosquitoes overall.

Variable	Estimate	Std. Error	z value	P (>|z|)
Intercept	4192.802	315.941	13.271	$< 2^{-16}$
Zoo	-1.512	0.257	-5.883	4.03^{-09}
Year	-2.081	0.157	-13.283	$< 2^{-16}$
Temperature	0.379	0.027	14.176	$< 2^{-16}$
Scarce vegetation	-0.895	0.182	-4.917	8.81^{-07}
Dense vegetation	0.841	0.194	4.324	1.53^{-05}
Close oviposition sites	1.39	0.201	6.919	4.53^{-12}
Close animal exhibits	1.577	0.19	8.318	$< 2^{-16}$
Remote animal exhibits	0.361	0.18	1.996	0.046
Dispersion parameter for Negative Binomial (0.5734) family taken to be 1				
Residual deviance: 506.64 on 550 degrees of freedom. AIC: 2382.8				

As the temperature indicated a strong influence in the mosquito abundance, its interaction with the other weather variables was explored further with another GLM that included mosquitoes captured as dependent variable, the temperature, humidity and precipitation as fixed effects and the mosquito collection, sampling area, year and zoo as random effects. The interactions were not significant even after removing the least significant factors.

The data used for the construction of the models were uploaded to the Open Science Framework repository separately by model, behaviour, zoo and year. In appendixes 5.1 and 5.2, the parameters of the models can be found.

Table 5.6. Significant variables from the GLMs

Variable		Host Search Behaviour				Site Selection Behaviour			
		Overall	Chester Zoo 2017	Chester Zoo 2018	Flamingo Land	Overall	Chester Zoo 2017	Chester Zoo 2018	Flamingo Land
Regional Temperature		↑ ($<2^{-16}$)	↑ ($<2^{-16}$)	↑ ($<2^{-16}$)	↑ (1.12^{-6})	↑ ($<2^{-16}$)	↑ ($<2^{-16}$)	↑ ($<2^{-16}$)	↑ (0.05)
Humidity		↑ (<0.00)			↑ (<0.00)			↑ (<0.00)	
Precipitation				↑ (0.03)				↓ (2.33^{-6})	
Vegetation	Dense	↑ ($<2^{-16}$)	↑ (9.54^{-14})	↑ (8.62^{-5})	↑ (0.01)	↑ (1.53^{-05})	↑ (8.42^{-13})		↑ (0.01)
	Medium						↑ (2.49^{-14})		
	Scarce					↓ (8.81^{-07})		↓ (1.35^{-4})	↓ (<0.00)
Oviposition sites	Close	↑ (2.94^{-06})	↑ (4.13^{-8})	↑ (3.74^{-10})		↑ (4.53^{-12})		↑ (2.13^{-14})	
	Medium								
	Remote			↑ (0.01)				↑ (2.42^{-9})	
Resting Areas	Abundant			↓ (<0.00)					
	Medium						↓ (0.04)		
	Rare	↓ (0.04)		↓ (3.94^{-5})	↓ (3.90^{-6})				
Zoo Animals	Close		↑ (<0.00)	↑ (0.034)		↑ ($<2^{-16}$)		↑ ($< 2^{-16}$)	↑ (1.79^{-5})
	Medium								
	Remote			↑ (<0.00)		↑ (0.05)	↓ (0.01)	↑ (3.54^{-8})	

Arrows upwards indicate a positive influence and arrows downwards, a negative one on mosquito abundance; p values in parenthesis. The parameters of the models can be found in Table 5.4 and 5.5, and in Appendix 5.1 and 5.2.

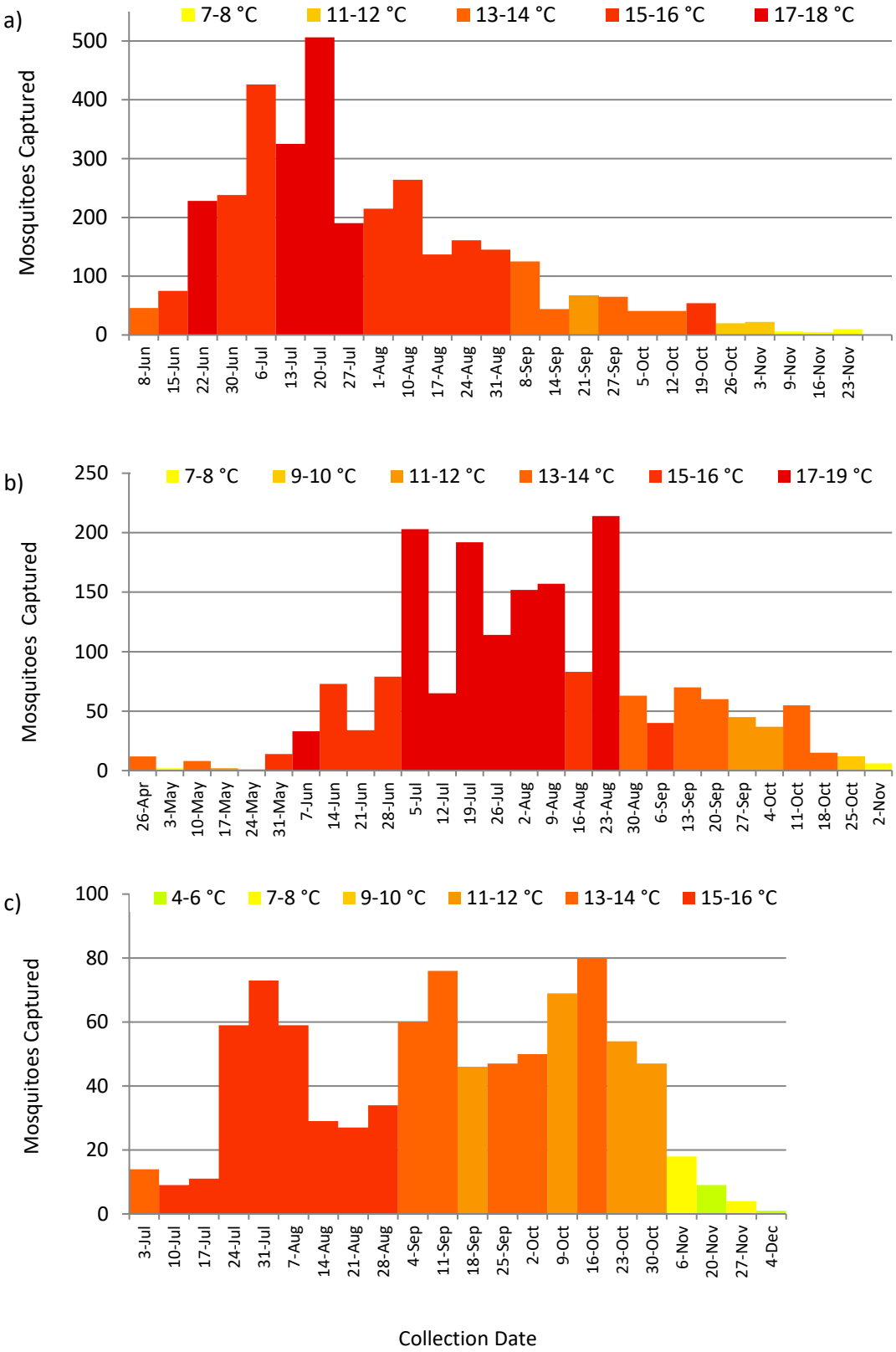


Figure 5.5. Mosquitoes collected in the BG-Mosquitaire traps in relation to the average regional temperature. a) Chester Zoo 2017, b) Chester Zoo 2018, c) Flamingo Land 2017.

5.5 Discussion

During our avian malaria study, the surrounding variables were observed and weather data were obtained, at regional scale as temperature and rain, and at the local scale, using temperature and humidity loggers next to the traps. It was found in the GLMs that the regional temperature had a strong statistical association with mosquito abundance and other recurrent factors significantly related were the presence of dense vegetation, close oviposition sites and close zoo animal exhibits. Nonetheless, no significant interactions were found among the weather variables.

Is important to consider that the mosquito traps used showed differences in their efficiency for capturing *Culex* spp. (see section 3.3.3). The BG-Mosquitaire collections (used for the analyses of host search behaviour) were higher overall, but the ones from the CDC-Gravid traps (used for the site choice behaviour analyses) included more mosquitoes per day. It is unlikely that the particular surroundings of each individual trap have influenced this general result because the environmental variables observed were randomly present for both kinds of traps (i.e. vegetation density, availability of resting areas, proximity to oviposition sites or animal exhibits, temperature and humidity). However, the attractant used in the CDC-Gravid traps (hay infusion) could be perceived by mosquitoes at farther distances as it has been reported as an effective trap by other authors (Cilek et al., 2017, Hesson et al., 2015a). By contrast, the lure of the BG-Mosquitaire traps was designed for mammalophilic mosquitoes and it is unclear why this trap also attracts ornitophilic mosquitoes, but it is possibly related to the preference of *Culex* spp. for feeding on humans. In consequence, the collections of both traps are not entirely comparable, and it was expected to find more precise results in the independent analyses by mosquito behaviour than in the overall analysis, but all models showed similar results.

5.5.1 Environmental influences in the mosquito population

The mosquito catches were different between zoos in the overall analyses, but this was clearly related to the different sampling efforts and latitudes; thus, a comparison would not be entirely valid. Regardless of the difference in sampling effort and considering the results from Chapter Three, the collections between years in Chester Zoo were different (n = 7,938 in 2017 and n = 2,962 in 2018) and it was observed that 2017 was rainier and 2018 was warmer, which could explain the significance in the overall models for the year variable. The local average

temperature from the loggers was 13.6 °C and 15.3 °C and the humidity, 79% and 73.3% respectively for 2017 and 2018 during our sampling periods. It was expected that some interactions among variables would be significant, especially between temperature and humidity or precipitation, giving insights into these catch differences but our tests do not provide evidence for this, possibly due to other variables involved.

As anticipated, the regional and local temperatures were strongly correlated. The regional temperature was the same for all sampling areas in each sampling and the local temperature was recorded next to the traps every hour, providing a detailed measurement. Nevertheless, the fit of the models was unexpectedly better with the first one. This has the practical advantage that the regional temperature could be used for further assessment of the mosquito abundance instead of constantly recording local temperatures by area.

In all the models, some nominal variables were collinear and without assessing this issue, the models provided significant levels for equivalent variables and the direction of their influence on the mosquito abundance was not clear. For instance, if the significance was given only for the medium vegetation value, it was unclear if the vegetation was favouring the capture of mosquitoes or not, as it was not obvious if it was collinear with dense or scarce vegetation. One of the collinear variables was excluded in each case based on the multiple correlation tests, and in most models, it was the intermediate variable.

The observation of the nominal variables could be improved by adapting their values to relevant factors. For example, if the vegetation is cut, booming, dry or moved, it could affect the mosquito abundance, so recording these differences, along with a more detailed description of the vegetation types, could provide valuable information as even different species of vegetation can influence the mosquito population (Karki et al., 2016), which is relevant in zoo habitats where exotic plants are regularly introduced (Tuten, 2011a). By contrast, other variables could be simplified to the presence or absence of a factor, as for artificial resting areas. In all cases, it would be very useful to define the variables and their values based on experimental studies that prove their association with the mosquito population at the local scale.

It should be considered that the variables of the surroundings were not balanced among traps and sites; therefore, some values were not present in the same frequency and this could have

altered the models results. Another consideration is that other unmeasured variables might have influenced the mosquito activity. For instance, wind currents can affect the travelling distance and dispersion of mosquitoes before or after having a blood-meal and strong winds can reduce mosquito captures (Karki et al., 2016). Likewise, the abundance of potential hosts can attract mosquitoes to certain areas; in the case of zoos, the exotic animals and visitors could have an important role. Regarding the aquatic stages, the water temperature can be an important parameter (Spielman, 2001).

5.5.2 Environmental influences in the host search

The temperature was the most influential variable in the abundance of mosquitoes in relation to the host search as it had the lowest p value in all the models with the exception of the site selection in Flamingo Land where the lowest value was for close animal exhibits. The other influencing factors in the aggregated analysis were the presence of dense vegetation and close oviposition sites, which were also significant in the specific analysis for both years in Chester Zoo and Flamingo Land, with the exception of the last variable that was not observed in Flamingo Land.

The temperature influences the physiology of mosquitoes and when it is within the optimum limits, it increases the metabolic process of mosquitoes and in consequence, all mosquito activities. Temperature below or above these limits diminishes the lifespan of mosquitoes in their different development stages (Ciota et al., 2014). The dense vegetation provides shelter to the mosquitoes and as they aggregate in these areas, it is more likely that they are captured in traps. The proximity to oviposition sites could also increase the abundance of mosquitoes that recently emerged as adults increasing the possibility of capture them before they disperse.

The close location of the traps to animal exhibits also increased the collection of mosquitoes in both samplings in Chester Zoo. Zoo animals are potential hosts for diverse species of mosquitoes and attract them constantly (Heym et al., 2019). Mosquitoes can be attracted by the biomass of their vertebrate hosts which is given by their abundance and size (Heym et al., 2019); nonetheless, these features or the effective distance from the traps to the actual location of the animals inside the exhibits were not evaluated during our samplings. Nevertheless, closeness to animal exhibits could also imply closeness to visitors who are attracted the zoo animals; considering the high preference of mosquitoes for human hosts

described in Chapter 4, the congregation and flow of visitors in the zoos should be analysed in relation to mosquito abundance.

Precipitation can limit the flying activity of mosquitoes and subsequently the host search and heavy rain can reduce mosquito survival (Karki et al., 2016).From the models results, it is unclear if this effect could be detected as the precipitation was only significant in the analysis of Chester Zoo in 2018. As the zoos are modified environments, it was expected that the availability of artificial resting areas could increase the abundance of mosquitoes. However, we did not find consistent evidence to support this, but a significant negative influence was observed in three occasions.

5.5.3 Environmental influences in the site choice

As for the host search, the site choice was also strongly influenced by the temperature in the overall and specific analyses. Other authors have also obtained different mosquito captures in relation to the temperature. Karki *et al*. (2016) used gravid and light traps, and observed that catches increased in both of them in warmer weather, so looking for a host or an oviposition site was also related to temperature (Karki et al., 2016). Temperature can also influence the proportion of species in the mosquito communities, even of those that are closely related. The distribution of sibling species like *Culex pipiens* and *Cx. torrentium* can vary with an average difference of only about 1.5°C (Werblow et al., 2014); nevertheless, the latitude also has a significant influence in the distribution of these two species (Hesson et al., 2014).

Other significant variables in the overall analysis include precipitation, vegetation and distance to oviposition sites. Water bodies act as natural attractants for gravid mosquitoes, and it is logical that traps close to them are going to catch more specimens. This was confirmed in the aggregated analysis and in the analysis of the sampling in Chester Zoo in 2018. Rainfall increases the number and size of potential oviposition sites, increasing larvae density and in consequence the mosquito population can be favoured (Karki et al., 2016, Ewing et al., 2016). It should be considered that artificial water containers could influence the dynamics of mosquito development, especially in urban environments and in this case, zoos, because they can provide oviposition alternatives during drier months (Ewing et al., 2016).

The vegetation was again an important factor possibly also providing shelter or shade for the gravid mosquitoes. In the general analysis the dense vegetation was beneficial for the

mosquitoes and the scarce vegetation had a negative influence; this effect was confirmed in the zoo and season specific analyses.

Oviposition sites are very important in mosquito ecology and their variables could be measured in more detail to define their suitability for gravid females and the development of immature mosquitoes. These could include volume or surface area, aquatic vegetation, shade, water temperature and pH (Townroe and Callaghan, 2014, Tuten, 2011a) . Although all this information could be summarised in the systematic count of immature mosquitoes, identifying the influence of particular variables could provide alternative options for the control of mosquito populations or advise in the effective timing of control measures.

Environmental variables do not always have linear impacts on mosquito ecology; therefore, their influence should be considered at different parts of the mosquito life cycle using detailed records to have accurate predictors of mosquito development (Ewing et al., 2016). Likewise, mathematical models could be very beneficial to understand mosquito ecology and improve epidemiological models (Ewing et al., 2016). In this way, the study of environmental influences could improve our understanding of how the vectorial capacity and the patterns of pathogen transmission may change (Ciota et al., 2014).

5.5.4 Conclusion

The temperature was a significant variable in all analyses for the host search and site selection and the most significant one compared to the others in most of them. Thus, it can be used as a main indicator of the mosquito activity in general. The higher catches of mosquitoes were obtained when the temperature was above 13 °C or at 11 °C after a warmer period.

The presence of dense vegetation and the proximity to oviposition sites were consistent variables that also have a strong influence in the mosquito population. Therefore, the general recommendation is to prevent the occurrence of these variables in the proximity of the exhibits of susceptible birds, in particular during the warmer months of the season.

We observed the effect of the vegetation reduction in the decrease of mosquito captures in one of the sampling areas. Therefore, it could be a practical and affordable measure to reduce the abundance of mosquitoes in areas of interest; although more comparisons should be done to evaluate the proportion of mosquito reduction (see the discussions in chapters three and

four). The influence of plant types or species should be analysed as well to understand if they offer a particular benefit for mosquitoes and, if they do, the management of gardens and exhibits could be adjusted accordingly.

It could be more complicated to manage the proximity to animal exhibits, but it should be considered that it was also a recurrent factor and avoiding high densities of potential hosts could prevent attracting mosquitoes to certain areas. Nonetheless, it should be considered that the presence of visitors attracted to the zoo animals, could be the real influence reflected in this variable.

Appendix 5.1. Parameters of the GLMs for the Host Search Behaviour

Chester Zoo 2017

Variables	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-3.082	0.378	-8.164	3.25^{-16}
Regional temperature	0.308	0.025	12.362	$< 2^{-16}$
Dense vegetation	1.185	0.159	7.447	9.54^{-14}
Close oviposition sites	0.868	0.158	5.485	4.13^{-8}
Close animal exhibits	0.396	0.131	3.018	0.003
Dispersion parameter for Negative Binomial (1.2564) family taken to be 1				
Residual deviance: 271.09 on 252 degrees of freedom. AIC: 1508.8				

Chester Zoo 2018

Variables	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-3.853	0.543	-7.09	1.34^{-12}
Regional temperature	0.286	0.027	10.601	$< 2^{-16}$
Precipitation	0.112	0.045	2.243	0.025
Dense vegetation	0.988	0.252	3.926	8.62^{-5}
Close oviposition sites	1.635	0.261	6.265	3.74^{-10}
Remote oviposition sites	0.793	0.281	2.825	0.005
Rare resting places	-1.075	0.262	-4.111	3.94^{-5}
Abundant resting places	-0.918	0.29	-3.171	0.002
Close animal exhibits	0.682	0.331	2.056	0.034
Remote animal exhibits	1.686	0.523	3.225	0.001
Dispersion parameter for Negative Binomial (1.1439) family taken to be 1				
Residual deviance: 229.56 on 195 degrees of freedom. AIC: 1121.2				

Flamingo Land 2017

Variables	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-1.887	0.75	-2.517	0.012
Humidity	0.027	0.008	3.405	0.001
Regional temperature	0.1642	0.034	4.87	1.12^{-6}
Dense vegetation	0.911	0.322	2.83	0.005
Rare resting places	-1.282	0.278	-4.616	3.90^{-6}
Dispersion parameter for Negative Binomial (1.4312) family taken to be 1				
Residual deviance: 101.58 on 83 degrees of freedom. AIC: 560.16				

Appendix 5.2. Parameters of the GLMs for the Site Selection Behaviour

Chester Zoo 2017

Variables	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-3.853	0.553	-6.97	3.16^{-12}
Regional temperature	0.325	0.037	8.71	$< 2^{-16}$
Medium vegetation	2.062	0.271	7.622	2.49^{-14}
Dense vegetation	1.946	0.272	7.154	8.42^{-13}
Medium resting places	-0.465	0.228	-2.044	0.041
Remote animal exhibits	-0.567	0.226	-2.512	0.012
Dispersion parameter for Negative Binomial (0.523) family taken to be 1				
Residual deviance: 251.35 on 261 degrees of freedom. AIC: 1349.9				

Chester Zoo 2018

Variables	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-11.572	1.218	-9.501	$< 2^{-16}$
Humidity	0.026	0.009	3.075	0.002
Regional temperature	0.512	0.045	11.385	$< 2^{-16}$
Precipitation	-0.214	0.045	-4.722	2.33^{-6}
Scarce vegetation	-1.079	0.283	-3.817	1.35^{-4}
Close oviposition sites	2.356	0.308	7.643	2.13^{-14}
Remote oviposition sites	1.788	0.3	5.967	2.42^{-9}
Close animal exhibits	2.733	0.306	9.062	$< 2^{-16}$
Remote animal exhibits	1.399	0.254	5.513	3.54^{-8}
Dispersion parameter for Negative Binomial (1.4894) family taken to be 1				
Residual deviance: 192.42 on 212 degrees of freedom. AIC: 731.39				

Flamingo Land 2017

Variables	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-2.869	1.359	-2.111	0.035
Regional temperature	0.165	0.085	1.938	0.053
Scarce vegetation	-1.572	0.532	-2.955	0.003
Dense vegetation	1.212	0.439	2.762	0.006
Close animal exhibits	1.843	0.43	4.289	1.79^{-5}
(Dispersion parameter for Negative Binomial (1.2379) family taken to be 1)				
Residual deviance: 59.986 on 66 degrees of freedom. AIC: 219.9				

Chapter Six

Past and Current Situation of Avian Malaria in UK Zoos, Aquariums and Wildlife Parks

6.1 Abstract

The experience that zoo veterinarians, keepers and other personnel acquire about the health of animals is highly valuable and could be critical for the improvement of conditions in captivity. Nevertheless, gathering and analysing this collective knowledge is not commonly done and information sharing is limited in some occasions. Here, we used an online questionnaire to collect relevant information about avian malaria in penguins; the period of interest was the last 20 years, to cover relevant events reported in the UK. The questionnaire was divided in two sections, the first one requested contact data, information about the current penguin population in the institution and general information about avian malaria. The second part solicited details about avian malaria outbreaks. In this way, the participants could provide valuable information about the penguin population and avian malaria regardless of the disease background in their institutions. We contacted 42 institutions with penguins in the UK, obtaining 27 complete responses and five partial responses. The most popular species were the Humboldt and African penguins and they are kept almost always in outdoors exhibits. Aspergillosis was perceived as the main health issue for penguins, followed by avian malaria. The avian malaria outbreaks occurred mainly during the summer months and more have been reported in recent years. Most of the outbreaks involved Humboldt and African penguins with low to medium prevalence and mortality but high lethality. Many of the events involved few individuals but the prevalence in some was over 50%. Around half of the outbreaks were suspected but not confirmed, thus regular testing of the penguins is recommended. As the treatment options seem to be ineffective, the implementation of preventive measurements, especially to monitor and control the mosquito population, is recommended. Further efforts to gather information could be done improving the design of this questionnaire for instance, targeting relevant issues like the outcomes of treatment protocols and preventive measures.

6.2 Introduction

Plasmodium infection is the major cause of mass deaths in captive penguins worldwide (Sallaberry-Pincheira et al., 2015). Until now, seven species of *Plasmodium* have been demonstrated to infect penguins (*P. relictum, P. elongatum, P. juxtanucleare, P. tejerai, P. cathemerium, P. unalis,* and *P. nucleophilum*), of which the first two are the most commonly found in penguins (Vanstreels et al., 2016, Grilo et al., 2016). From the eighteen species of penguins, thirteen have been reported to be infected with *Plasmodium* parasites in captivity or in the wild (Vanstreels et al., 2016) and from these, the highest mortality rates have been reported in the Magellanic (*Spheniscus magellanicus*) (Bueno et al., 2010), Humboldt (*Spheniscus humboldti*) (Sallaberry-Pincheira et al., 2015), and African penguins (*Spheniscus demresus*) (Sallaberry-Pincheira et al., 2015, Beier and Stoskopf, 1980).

Risk of *Plasmodium* infection is greater for captive penguins primarily because in zoological gardens or rescue centres they are exposed to the local mosquito populations that may transmit the parasite among the native wild birds, which are the suspected reservoir of the parasite (Beier and Stoskopf, 1980). Alternatively, the parasite could have already infected the penguins and migrated to the endothelium or haematopoietic tissue (Graczyk et al., 1994b) and stressful situations immunosuppress the penguins, boosting an acute phase of the infection (Sallaberry-Pincheira et al., 2015, Grilo et al., 2016). However, the species and lineage of the parasite could also have a major role in the pathogenicity of the disease (Vanstreels et al., 2014).

The seasonality of the disease is strongly marked with most of the outbreaks occurring in the late summer and early autumn in the Northern hemisphere, which correlates with the highest density of local mosquitoes and the high rates of infection in wild birds (Vanstreels et al., 2014, Graczyk et al., 1994b, Beier and Stoskopf, 1980). One of the critical factors is the exposure of the penguins to the mosquito because many of the fatal infections occur after the first exposure; although, if they survive, they can develop some immunity and also transfer maternal antibodies to chicks (Graczyk et al., 1994a, Graczyk et al., 1994b).

Avian malaria produces diverse and unspecific clinical signs commonly including anorexia, weight loss, lethargy, vomiting, and greenish faeces; also, sudden death has been observed without previous signs (Grilo et al., 2016). A common treatment combines the use of

chloroquine and primaquine due to their antiprotozoal effects in the circulating and tissue stages, respectively; nevertheless, in some cases, the mortality rates have diminished and in others they have remained high, suggesting that the efficiency of these drugs is variable (Vanstreels et al., 2014, Grilo et al., 2016).

Preventive measures are focused on reducing the contact risk of the penguins with mosquitoes, which could be achieved by controlling the mosquito populations and using physical barriers. A prophylaxis treatment to decrease the severity of the infection is another option and allowing the penguins to develop an immune response to the infections has been also suggested (Grilo et al., 2016).

The high morbidity and mortality reported in captive penguins also raise concern about wild penguins in breeding areas where mosquitoes have been absent and could be introduced (Grilo et al., 2016). Species of special concern are the African (Gracyzk et al., 1995), Galapagos, and yellow-eyed penguins due to their limited distribution, their already recognised status as endangered species, and the detection of *Plasmodium* spp. in wild populations (Vanstreels et al., 2016).

Several outbreaks have affected penguins in zoos and rehabilitation centres worldwide with rapid mortalities varying from 10 to 83% (Vanstreels et al., 2016, Vanstreels et al., 2014, Bueno et al., 2010, Gracyzk et al., 1995). In the UK, there have been regular press releases and social media posts mentioning the loss of penguins due to the disease and at least seven zoos have been affected, some of them losing their entire colony of penguins (Exmoor Zoo, 2016, The Telegraph, 2000, BBC News, 2019, BBC News, 2018, BBC News, 2016, BBC News, 1999, The Guardian, 2012).

Some works evaluate the situation of avian malaria in the zoo environment, reporting the presence of the parasite in penguins and mosquitoes (Bueno et al., 2010, Ejiri et al., 2009); nevertheless, there is not an updated reference that summarises the historical and current situation of the disease or the effectiveness of the preventive and control measures. Likewise, the information about avian malaria in mainland Europe and UK penguin populations is limited. Therefore, more research is needed to assess the main epidemiological risks and recommend effective preventive and control actions to protect penguins and other birds at risk. We consider that the experience of veterinarians, animal keepers, and assistants in zoological

gardens and wildlife parks is highly valuable and gathering their knowledge about avian malaria in captive penguins is of interest. Here, we implemented an online survey that is easy to distribute, answer and analyse. The aim of this project was to assess the main epidemiologic features of avian malaria in UK zoos and wildlife parks in species that could be highly susceptible, like penguins. The specific objectives were:

- To gather, analyse, and report the information about the most recent avian malaria cases.
- To determine the population currently at risk.
- To analyse the periodicity and distribution of the disease if there are suspected patterns.
- To report and discuss the prevention and control measures.

6.3 Methods

We designed a questionnaire for the persons responsible for the penguins in UK zoological gardens and wildlife parks to collect the relevant information about the epidemiology of avian malaria. The questionnaire consists of two parts, the first one provides a summary of instructions and requests basic information including contact details, information about the current population of penguins, and prevention and surveillance for avian malaria; it ends asking if avian malaria has been suspected or diagnosed at their institution. If the participants respond that their birds had had avian malaria, a logic rule takes them to answer the second part, which requests more details about the avian malaria incidents like species affected, prevalence, diagnosis, control measures, and outcomes. In this way if the zoo or park has not reported the disease, they do not need to spend more than a few minutes completing the basic information, and if they had, they could continue providing more details. The information requested is detailed for the last avian malaria event and general for the previous events to facilitate the participant's response and obtain relevant and updated information.

The questionnaire was designed and managed using SurveyMonkey®, an online survey software that allowed us to create a friendly version easy to use and distribute. A combination of text boxes for open questions and closed questions with ratio buttons and check lists was used. When a checklist was presented, the order of the options was randomised to prevent order bias, except for the list of penguin species which was presented in alphabetic order by common name. Another advantage of this software is that it allows the use of logic structures

depending on the answers. To facilitate the data input, two forms for collecting the information about the most recent event of avian malaria and about previous events were designed.

The period of interest was the last twenty years in order to encompass the most relevant outbreaks reported for the UK and the recent preventive and control actions; that is, from 1999 to 2018. To encourage the replies of the participants and perform the project in a reasonable period, we set a deadline for answering of two months after sending the questionnaire. If the participants had not answered during the first month, we sent a reminder and if they had not answered fifteen days before the deadline, we sent a final reminder.

The content and structure of the questionnaire was evaluated by members of Chester Zoo and Flamingo Land, including a person with broad experience in survey design, and by other volunteers with different backgrounds in order to guarantee clarity and ease of response.

It was clearly stated that no personal data would be disclosed or shared by any means and it would be used only for personal communication. The responses and personal data were handled with confidentiality and kept only in The University of Liverpool computer used for this project, which is protected by antivirus software and is exclusively accessed by the researcher with his username and password.

We did not assess the performance of the zoos and park staff in any way. Most of the information was synthesized in form of frequencies and proportions; therefore, its association with the zoo or park of origin is not be possible, favouring anonymity. Nevertheless, the final section of the questionnaire requests the authorisation of the participants to disclose the name of their institutions, to relate their institutions at a geographical coarse scale (Nomenclature of Territorial Units for Statistics-NUTS 1, of the UK) and to agree if they wanted their institutions included in the acknowledgments.

The final version of the questionnaire was approved by The University of Liverpool Veterinary Research Ethics Committee in the amendment of the ethics for our avian malaria project (reference VREC532a). Therefore, a participant information sheet was also written and sent to the participants to comply with the University requirements. The scientific committee of Chester Zoo also reviewed and approved the questionnaire. Additionally, we obtained a support letter from BIAZA (British and Irish Association of Zoos and Aquariums) encouraging participants to take part in our research.

The participants were contacted by email and the relevant documents were attached. During the response analysis, some participants were contacted to clarify details of their answers or request missing information. The invitation email can be found in Annex 1, the questionnaire including the avian malaria events forms, in Annex2, the BIAZA support letter in Annex 3 and the participant information sheet in Annex 4.

We sent a result report to the participants who answered the questionnaire as a direct benefit for them, which is a summarised version of this chapter.

6.4 Results

We invited 42 institutions in the UK that have or had penguins. From these, 27 (64%) completed the questionnaire, five started to answer but did not finish, and the remaining 10 did not respond to our communications. In the past 20 years, 18 institutions reported the disease, 14 had not have it and we could not determine if 11 had been affected. All institutions but one currently house penguins.

6.4.1 Penguin population

In total, 31 institutions provided information about their penguin populations; 26 have only one species of penguins, four have 2 and one has three. Eight species of penguins were listed, of these the Humboldt penguin (*Spheniscus humboldti*) was the most popular, kept in 21 institutions with a total population of 577 individuals; it was followed by the African Penguin (*Spheniscus demersus*) and the Gentoo Penguin (*Pygoscelis papua*) (Table 6.1).

Regarding the origin of the penguins, in most institutions they were raised on site or brought from other sites (n = 27), in two cases, they were only brought from other sites and also in two institutions their penguins were raised on site, brought from other site or captured from the wild. In relation to this, 29 institutions currently have a breeding program for their penguins and two do not.

Table 6.1. Species and populations of penguins kept in zoos and wildlife parks in the UK.

Penguin Species		Number of Institutions	Total population
Common name	**Scientific Name**		
African penguin	*Spheniscus demersus*	6	215
Gentoo penguin	*Pygoscelis papua*	4	148
Humboldt penguin	*Spheniscus humboldti*	21	577
King penguin	*Aptenodytes patagonicus*	2	19
Little blue penguin	*Eudyptula minor*	1	20
Macaroni penguin	*Eudyptes chrysolophus*	1	13
Magellanic penguin	*Spheniscus magellanicus*	1	16
Northern rockhopper penguin	*Eudyptes moseleyi*	1	22
	Total	**31**	**1030**

In most of the institutions (n = 26), the penguins are kept always outdoors, without considering their nest boxes as an indoors shelter. Three locations that only have Gentoo penguins keep them all the time in an indoor facility. In another two, their Humboldt penguins are kept intermittently indoors and outdoors.

Aspergillosis is perceived as the main health issue in captive penguins as it was selected 24 times and avian malaria is the second one, chosen eight times. Degenerative conditions are also perceived as common in captive penguins as they were selected seven times. Other health issues mentioned include digestive conditions, pododermatitis, respiratory infections, penguin diphtheria in chicks, clostridium infection, overheating and heavy metal intoxication due to a diet based on herrings from the Baltic Sea.

6.4.2 Avian malaria background

Twenty-five institutions (59.5%) reported that they have in place preventive or control measures against avian malaria. The most reported ones were the constant cleaning and maintenance of water bodies to prevent immature mosquitos to develop and the administration of prophylactic or therapeutic treatment for penguins (Table 6.2).

The continuous surveillance of avian malaria is done in 12 institutions (28.5%), most commonly through blood tests that include thin blood smears, haematocrit and biochemistry. Most times, the blood sampling is done along with general health checks or in an opportunistic way, but it was reported that specific samples are taken when avian malaria is suspected. Several

participants mentioned that they do regular health checks of their penguins through observation, physical examination and weighing; post-mortem examinations and histology were also mentioned. The two institutions with whom we collaborate mentioned the active research and mosquito monitoring of our project as part of the surveillance efforts. Interestingly, in one institution the parasite lactate dehydrogenase (pLDH) test was tried and validated but is no longer used in a regular basis.

Table 6.2. Preventive and control measures implemented in zoos and wildlife parks in the UK.

Measures	Times selected
Barriers to prevent mosquito bites	0
Constant cleaning and maintenance of water bodies	18
Disrupting bacteria for mosquito larvae in water bodies	1
Elimination of water pockets	8
Keeping the penguins indoors	3
Mosquito larvae predators in water bodies (fishes, dragonfly larvae)	1
Prophylactic treatment for penguins	16
Quarantine of sick penguins	7
Surveillance of the parasite in mosquitoes	2
Surveillance of the parasite in penguins	4
Treatment for sick penguins	13
Use of fans to increase wind flow	2
Use of mosquito chemical repellent products	1
Use of mosquito repellent plants	7
Use of traps for adult mosquitoes	8

6.4.3 Analysis of avian malaria events

From the 32 participants who answered if they have had avian malaria cases, the majority, 18 (56.3%), responded affirmatively. In the period of interest, 75 events of avian malaria were reported, 38 suspected and 37 confirmed. An event was defined as one or more birds affected in the same period; avian malaria was suspected if the diagnosis was based on signs and lesions, but no diagnostic tests were done and confirmed if a diagnostic test provided a positive result. Most of the institutions have had few suspected or confirmed events of avian malaria (five or fewer) but one had 12 and another, over 20.

The duration of the avian malaria events varied from one to 134 days. It was reported as one day when no signs were observed and few birds were affected; without considering these outbreaks of unknown duration, the minimum extent was 16 days and the median, 73 days. The monthly distribution is shown in Figure 6.1; it does not represent the independent number of outbreaks per month, but their accumulation throughout the months. An aggregation of outbreaks in recent years was observed; out of 28 outbreaks with reported dates, half of them occurred in the last five years and 22 in the last ten years (Figure 6.2).

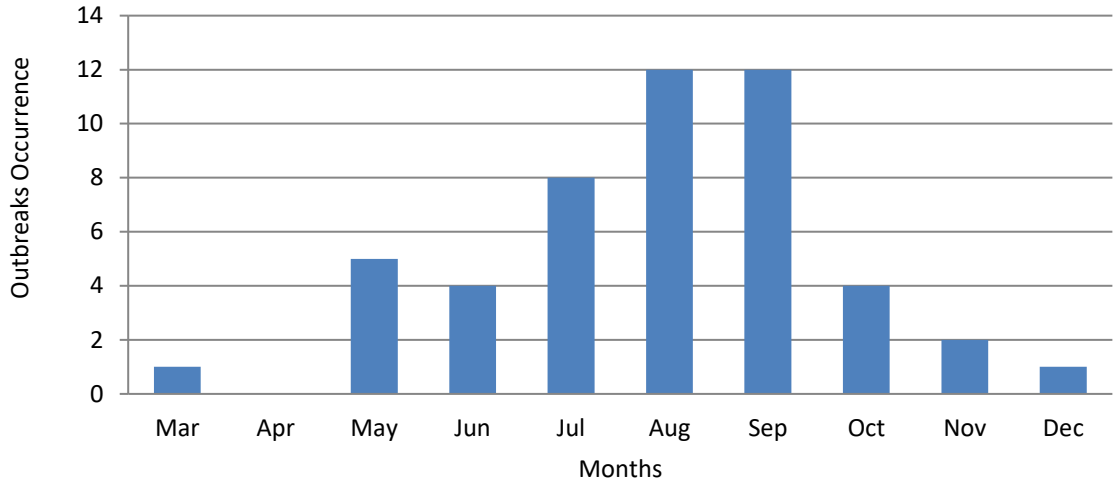


Figure 6.1. Temporal distribution of the avian malaria outbreaks in the UK from 1999 to 2018.

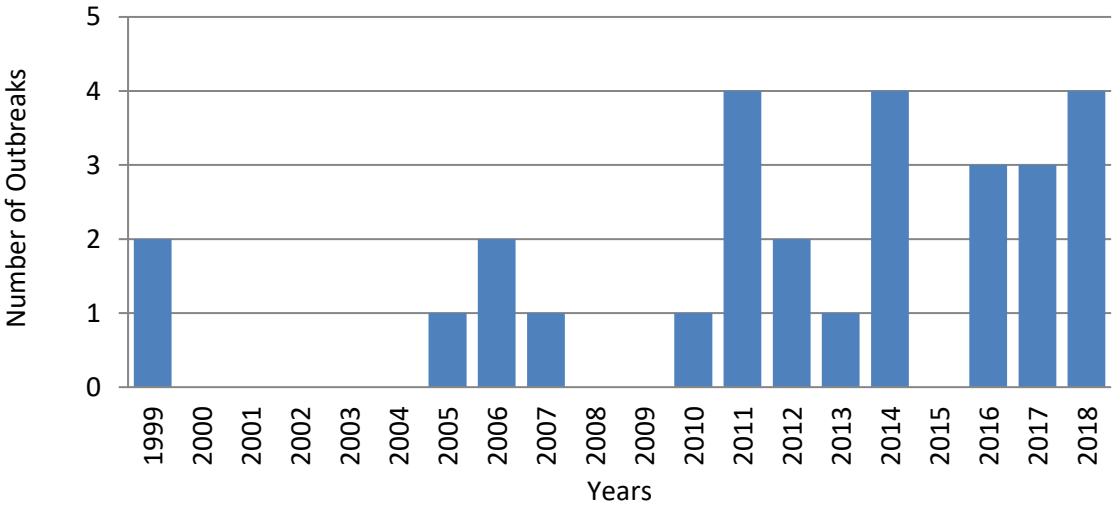


Figure 6.2. Number of avian malaria outbreaks per year in the UK.

A Spearman rank correlation showed that the number of institutions was correlated with the number of avian malaria outbreaks (p=0.001, rho = 0.82) at the geographical scale given by the NUTS1 regions of the UK. Nevertheless, it must be noticed that a disproportionate number of outbreaks was reported in the South East region making the relative risk of avian malaria events more than twice in that region, and in general, the number of institutions and outbreaks is higher in the south of England (Figure 6.3).

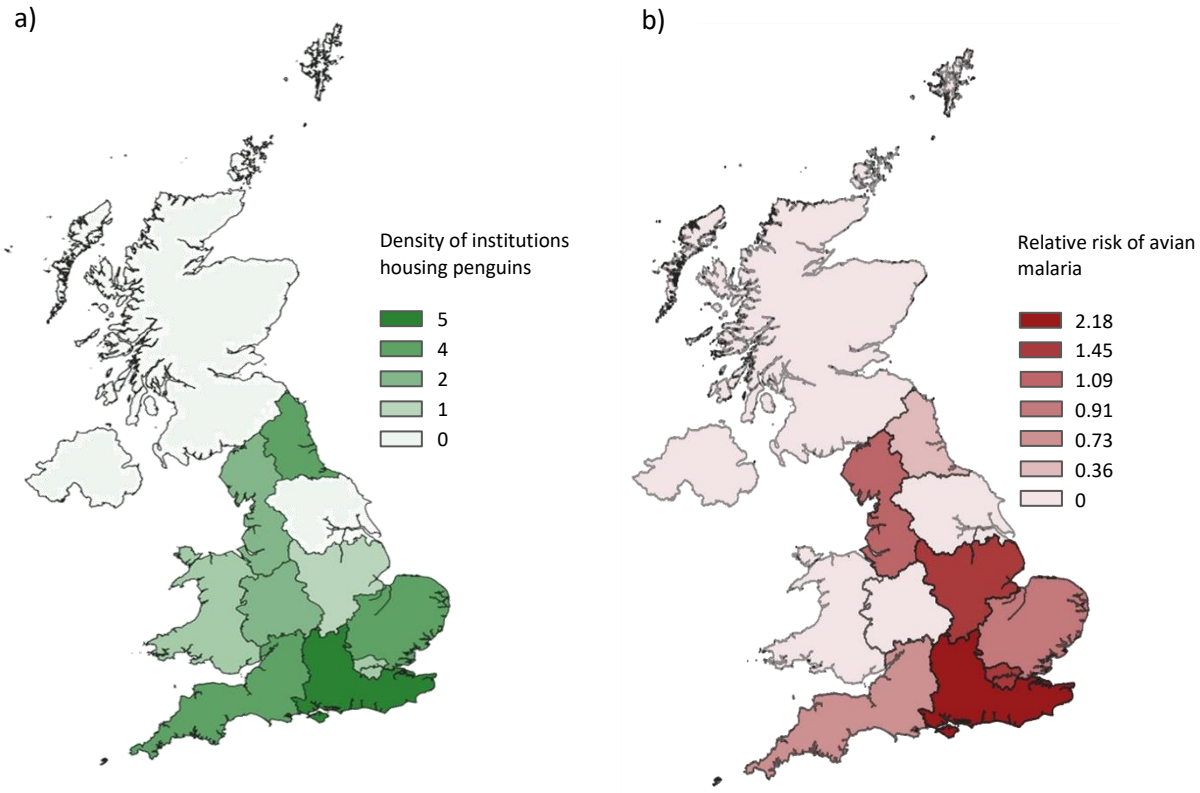


Figure 6.3. Institutions housing penguins and relative risk of avian malaria by NUTS1 regions of the UK. a) Number of institutions per region. b) Relative risk per region. The relative risk was estimated dividing the proportion of avian malaria events by the proportion of institutions per region. Only the institutions that gave consent for the geographical use of information were included.

In the most recent events of avian malaria, the signs and lesions observed were unspecific and from the 14 different ones reported, the most frequent one was lethargy or depression (Figure 6.4). Diagnosis was based mainly on gross lesions detected during the post-mortem examination, histopathology and the observation of thin blood smears (optical microscopy); in six cases each. It was also done with the clinical signs in three cases and by PCR in one. The

blood parasites observed where *Plasmodium* spp. (n = 7), *Leucocytozoon* spp. (n = 2) and *Haemoproteus* spp. (n = 1); although one participant mentioned that *Leucocytozoon* spp. was suspected but not confirmed. The only species identified was *Plasmodium relictum* in two occasions. The available information about the implemented treatments is found in Table 6.3.

Seven participants recorded nine stressful events beyond the routine activities happening before or at the same time as the avian malaria event. These included moulting (n = 4), changes in the exhibit (n = 1), outbreak of a different disease (n = 1), reproduction (n = 1), sampling or health check (n = 1), and addition of new birds (n = 1). In four occasions, it was noted that other diseases affected the penguins simultaneously, in three by aspergillosis and one by bacterial and fungal infections.

The detailed information about 30 avian malaria events revealed that the most commonly affected species were the Humboldt and African penguins, involved in 17 and 10 events respectively; the Gentoo, King and Macaroni penguins were also affected (Figure 6.5). Out of 21 institutions with Humboldt penguins, 11 reported avian malaria events; all the institutions with African penguins, six, reported avian malaria, and from the 10 institutions with other species of penguins, three reported that the disease had affected them. A Fisher's exact test of independence showed a significant difference among these proportions ($p = 0.023$), suggesting that African penguins could be more susceptible to the disease. In general, when the outbreaks affected few birds (< 5), all of them were tested for the parasite but if several individuals were sick or died (>10), only a small proportion (between 2.5% and 10%) were tested.

The prevalence, mortality and lethality were calculated when the information was sufficient to do so (Table 6.4). The prevalence varied from 2.3% to 100% with an average of 20.9% and a median of 6.3%. The mortality ranged from 0% to 100% with an average of 20.3% and a median of 6.3%. It is expected that the prevalence and mortality parameters are similar considering the high lethality that was 100% in 25 cases. Therefore, the recovery rate was low, being recorded in three events, 3/27 (11%), 1/4 (25%) and 1/1 (100%); in two cases the number of sick birds was not mentioned. It must be noticed that these values are based on low numbers; many of these events involved few individuals, in 20 of them only one penguin was affected.

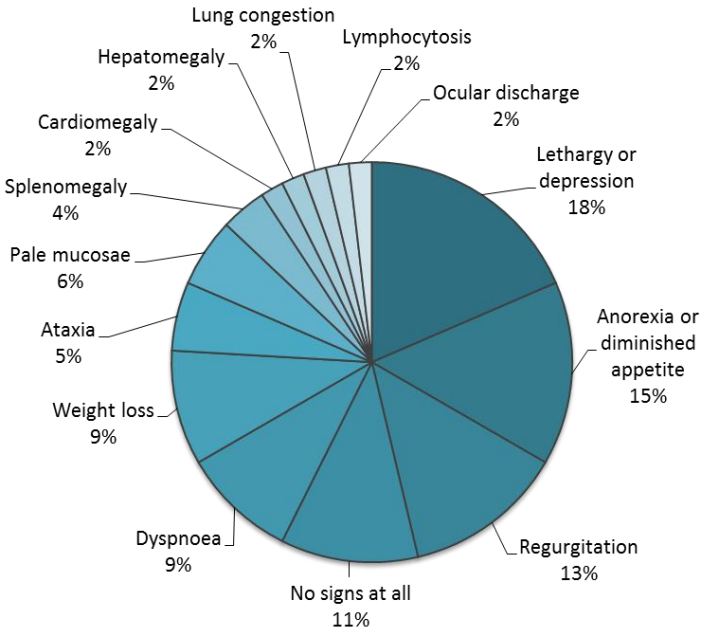


Figure 6.4. Proportion of signs and lesions observed by the participants during the avian malaria outbreaks in the UK.

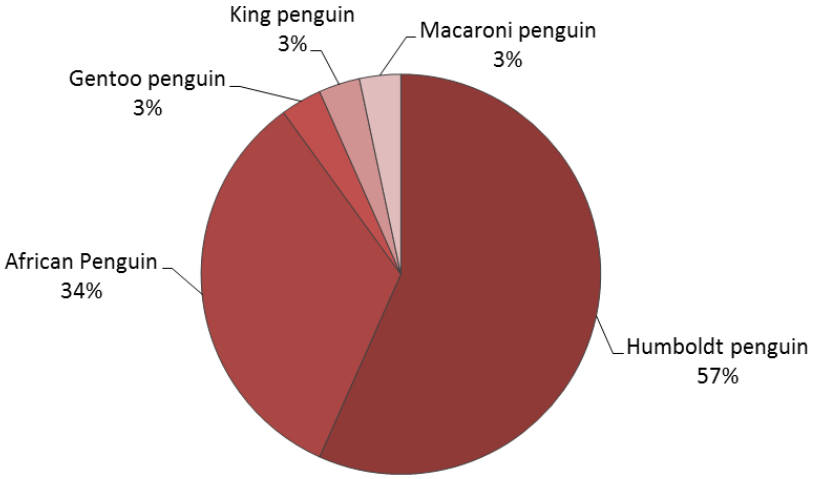


Figure 6.5. Proportion of penguin species affected by avian malaria outbreaks in the UK.

Table 6.3. Treatments described for the most recent avian malaria events by ten participants.

Zoo/ Park	Treatment	Dose	Frequency	Duration	Sick penguins	Outcome	Notes
1	Primaquine	NR	daily	NR	1	recovered	
	Doxycycline	NR	NR	NR			
2	Itraconazole (Sporonox)	NR	NR	NR	10	death	Respiratory disease suspected. Metronidazole instated latter.
	Enrofloxacin (Baytril)	NR	NR	NR			
	Metronidazole	NR	NR	NR			
3	Oxygen therapy	NR	once	once	1	death	Emergency care, penguin found in critical condition and died 2 hours later
	Antibiotics	NR	once	once			
	Antifungals	NR	once	once			
4	Enrofloxacin (Baytril 10%)	0.3 ml	once	once	1	death	Died the same day. The other penguins receive routinely Primaquine (3.75mg tablets) once weekly.
5	Chloroquine	1ml/kg	every 6 hrs	3 doses	NR	six penguins died	Chloroquine dose was 0.5ml/kg after the first dose.
	Primaquine	0.3mg/kg	daily	NR			
6	Doxycycline	20 mg/kg	twice daily	10 days	1	death	Prophylactic treatment in the other penguins afterwards.
7	NR	NR	NR	NR	1	death	Prophylactic treatment: Primaquine 1/2 tablet per bird twice weekly
8	Atovaquone / Proguanil (Malarone)	87.5 mg tablets	1/2 tablet for three days, then 1/4 tablet for 11 days	depending on outcome	4	one recovered, three died	
9	Primaquine	2.75 mg (adults), 1.375 mg (chicks)	daily	depending on outcome	41	death	Intensive nursing was attempted for one penguin but failed.
	Chloroquine	15mg	daily				
10	Primaquine	2mg/kg	twice daily	depending on outcome	1	death	Prophylactic treatment since the outbreak until 2018 with no more cases.
	Chloroquine	10mg/kg	twice daily				

NR: not reported. The zoo or park numbers were assigned randomly.

Table 6.4. Epidemiological features of avian malaria outbreaks in the UK.

Penguin Species		Event number	Population [a]	Sick birds	Dead birds	Recovered birds	Prevalence (%)	Mortality (%)	Lethality (%)
Common name	**Scientific name**								
King penguin	*Aptenodytes patagonicus*	1 [C]	NR	NR	1	NR	-	-	-
Macaroni penguin	*Eudyptes chrysolophus*	2 [C]	5	5	5	0	100	100	100
Gentoo penguin	*Pygoscelis papua*	3 [C]	NR	NR	5	NR	-	-	-
African Penguin	*Spheniscus demersus*	4 [C]	NR	1	1	0	-	-	100
		5 [C]	16	1	1	0	6.3	6.3	100
		6 [C]	27	4	3	1	14.8	11.1	75.0
		7 [S]	15	1	1	0	6.7	6.7	100
		8 [C]	19	1	1	0	5.3	5.3	100
		9 [S]	16	1	1	0	6.3	6.3	100
		10 [S]	5	1	1	0	20	20	100
		11 [S]	18	1	1	0	5.6	5.6	100
		12 [C]	20	20	20	0	100	100	100
		13 [C]	NR	1	1	0	-	-	100
Humboldt penguin	*Spheniscus humboldti*	14 [C]	44	1	0	1	2.3	0	0
		15 [S]	10	10	10	0	100	100	100
		16 [C]	23	1	1	0	4.3	4.3	100
		17 [S]	16	1	1	0	6.3	6.3	100
		18 [C]	27	1	1	0	3.7	3.7	100
		19 [S]	10	1	1	0	10	10	100
		20 [C]	NR	41	41	0	-	-	100
		21 [C]	18	1	1	0	5.6	5.6	100
		22 [C]	45	27	24	3	60	53.3	88.9
		23 [C]	NR	1	1	0	-	-	100
		24 [C]	22	1	1	0	4.5	4.5	100
		25 [S]	15	1	1	0	6.7	6.7	100
		26 [C]	32	1	1	0	3.1	3.1	100

Table 6.4. Continued.

Humboldt penguin	*Spheniscus humboldti*	27 [C]	31	3	3	0	9.7	9.7	100
		28 [C]	42	6	6	0	14.3	14.3	100
		29 [C]	39	1	1	0	2.6	2.6	100
		30 [S]	33	1	1	0	3	3	100

NR: not reported, [C]: avian malaria confirmed, [S]: avian malaria suspected, [a]: population at the beginning of the outbreak.

6.5 Discussion

Using an online questionnaire, we collected relevant information about the occurrence and epidemiology of avian malaria in zoos, wildlife parks and aquariums in the UK. We contacted 42 institutions that have or had penguins and got full responses from about two thirds, as well as some partial responses; only about one quarter did not respond at all. The most popular species are the Humboldt, African and Gentoo penguins. Numerous events of avian malaria were reported, 38 suspected and 37 confirmed; many of them extending throughout the summer months and occurring in recent years. The distribution of the participant institutions and the avian malaria outbreaks was concentrated in the south of England. The signs and lesions observed were unspecific and the diagnosis was based mainly on lesion, histopathology and blood smears. The species most frequently affected was the Humboldt penguin. The reported treatments varied although the outcome was unfavourable in most cases as the prevalence and mortality rates were low to medium, but the lethality was high.

Our survey was divided in two main sections and operated with logical rules, so the participants did not need to read irrelevant questions. This was particularly useful because we gathered information about the penguins' population at risk, risk factors, prevention measurements and additional information, independently of the avian malaria background in the institutions.

In the second section, asking for details of all avian malaria events would have provided more valuable and complete information but may also have led to some participants quitting the survey due to the high time demand. To encourage completion, we asked for detailed information only on the most recent avian malaria events and general information about previous events. We assumed that recent information would be easier to obtain, and more relevant as updated diagnosis techniques and treatments are constantly used, whereas the temporal and spatial patterns of previous events were related for the historical understanding of the disease.

Having the support of BIAZA was certainly an incentive that improved the response rate; thus, institutional support increases the confidence of the participants beyond the good scientific or practical reasons that a researcher could provide. Likewise, following up the communications from the general institution contacts to science committees and then to the vets or curators

allowed us to reach the right persons. The reminders sent also resulted in more responses and the competition of partial answers.

Common problems when doing questionnaires arise from the lack of information. In our case, mainly due to old or incomplete records and the information quality decreased with time. Additionally, some institutions outsource the veterinary services to local practices and do not have all the information themselves, so another communication step was added to the process. Internal communication was also a problem in some cases and the request of answering the questionnaire had to pass through different people in the organization and even in the same team, before reaching the right person.

When the outbreak reports were recent and digitalised and avian malaria affected a low number of penguins, the participants could finish the questionnaire faster with complete answers. But the added complexity of multiple penguins affected, and multiple events apparently made some participants quit after completing the basic information simply due to the time needed.

All these inconveniences created a mix of partial responses that was not easy to compare and that should be considered with caution. Nevertheless, with more accessible digital tools for handling animal data, the completeness and consistency of the records in zoos, aquariums and wildlife parks could be improved. The software Species360 ZIMS (Zoological Information Management Software) is a popular database used worldwide (Species360, 2019); two of its modules, ZIM for Husbandry and ZIM for Medical, could be particularly useful for the retrospective research of diseases.

6.5.1 Population at risk

The most abundant species of penguins kept in UK zoos and wildlife parks, Humboldt and African penguins, are also highly susceptible to avian malaria; hence, the disease could continue to cause outbreaks. The difference in the proportions of institutions with certain species and background of avian malaria suggests that African penguins are more susceptible than other species, although the numbers analysed were low. It is likely that these species are more frequently reported with the disease because they are more common in captivity but not particularly more susceptible to the infection; thus, a re-assessment of their susceptibility should be considered.

Due to the constant movement of penguins among institutions, the penguin populations should not be seen as independent and isolated within particular zoos or parks, instead they should be considered as a metapopulation, reaching even other countries. Therefore, the information about the origin of the penguins, avian malaria records, health checks, prevention and control, is relevant for the better management of penguins under human care. Routine avian malaria testing should be done before moving the penguins and for a period of some months after their arrival to their destination.

6.5.2 Events temporality

The increase of avian malaria outbreaks in recent years is alarming; from the 28 with reported dates, 14 occurred in the last five years. It is possible that changing climatic conditions that are favouring the transmission of the disease in wild birds, as suggested before (*Plasmodium* prevalence has increased over the last 20 years (Garamszegi, 2011)), could also represent an increase in the risk for penguins. Another important consideration is that recent information tends to be more available and complete, which was reflected by the better data quality in recent reports. Thus, the more regular diagnosis of the disease and better record keeping could reveal more cases that before would have been classified as unknown mortality.

The occurrence and duration of the outbreaks showed a clear peak from July to September which corresponds with the abundance peak of the mosquito population (see Chapter Three). This suggests that the penguins that are affected by the disease are those exposed to the pathogen for the first time and develop an acute phase of infection. Alternatively, those penguins could have been infected previously and stressful factors happening at that time triggered the disease, but this would be expected to show a more random pattern throughout the year.

6.5.3 Events distribution

Warmer weather increases the abundance and persistence of the vector for a longer period increasing the risk of avian malaria transmission and thus explaining the higher proportion of outbreaks and relative risk in the south of England. A high concentration of outbreaks in the south east region could be due to better environmental conditions for the mosquito. However, the significant correlation between the distribution of institutions and outbreaks shows that the presence of susceptible birds is a determinant factor; moreover, some outbreaks were also

reported in northern locations. Therefore, a geographical association of outbreak occurrence requires further investigation at a finer scale.

6.5.4 Epidemiology

The most frequently observed sign was lethargy or depression, which along with anorexia and isolation from the group are also the most commonly reported manifestation of illness in penguins; besides, almost all the signs reported for avian malaria are considered general manifestations of illness in penguins (AZA Penguin Taxonomy Advisory Group, 2005). As some participants mentioned, the signs could suggest other diseases like aspergillosis or clostridial infection. This, in addition to the asymptomatic deaths repeatedly reported, makes the early diagnosis of avian malaria very challenging.

Aspergillosis is one of the most common diseases affecting penguins in captivity, possibly because the causative agent, fungi from the genus *Aspergillus* spp., are ubiquitous and the infection happens in stressed or debilitated individuals (AZA Penguin Taxonomy Advisory Group, 2005). This was reflected by the answers of our participants and as some of them mentioned, *Aspergillus* spp. was found during avian malaria events. Its signs are similar to those of avian malaria making the differentiation between these two diseases even harder.

The observation of blood smears requires a considerable amount of training, histopathology can take long time to analyse and the PCR techniques need especial equipment and time to perform. Despite this, more regular diagnosis of avian malaria should be done to confirm suspected outbreaks which were almost half of the ones reported here. For now, there is not a quick and affordable diagnostic technique that can be done immediately, but the mentioned ones should be attempted when possible. This could also clarify if certain parasite species are responsible for the more severe outbreaks and if there are other pathogens involved.

One of the participants mentioned the use of the pLDH test, which was designed as a rapid test to detect the *Plasmodium* lactate dehydrogenase (pLDH) antigen for human malaria diagnosis (Killick et al., 2008). Nevertheless, due to the nucleated red cells of the avian blood, it was found that the samples do not migrate well up the test strip and it was not entirely reliable; hence, they use conventional blood smears instead.

It is well stablished that one of the physiological alteration caused by stress is the diminishment of the immune response, but pathogens are also a source of stress so parasite infection can be a cause and consequence of the stress response (Beldomenico and Begon, 2015). Stressful events could increase the risk and severity of avian malaria outbreaks; this was considered since the analysis of the outbreaks in Chester Zoo and Flamingo Land (see chapter 4). Nevertheless, the evidence from the questionnaire responses is inconclusive; seven participants observed stressful events around avian malaria cases and six did not. Events like moulting, breeding, thermal discomfort or other subclinical conditions could not be seen as stressful factors or be hard to detect. Nonetheless, stress is a natural response to changes in the environment and the effects on the immune system depend on the kind and duration of the stressor (Beldomenico and Begon, 2015).

A participant mentioned that one penguin that was sent to another zoo died a few months later due to avian malaria before an outbreak in their own institution. Therefore, there is a possibility that that penguin was infected in the first zoo and developed the disease in the second one possibly in relation to the stress of the moving. Nonetheless, excluding the possibility of it getting infected in the new site is extremely hard. It is likely that the occurrence and severity of the disease depends on a combination of factors rather than just one.

The prevalence and mortality were low in many outbreaks affecting one or few individuals in the colonies (<10%) but in some occasions it reached 100%. From our results, it is unclear why this was the case as each penguin population was apparently under similar conditions at the moment of the outbreaks. Therefore, it is not only the exposure to the parasite that causes serious mortalities, other factors could be involved such as the pathogenicity of certain *Plasmodium* species or lineages, concomitant diseases or compromise of the immune system. Detailed information about serious avian malaria events, with prevalence over 50%, was provided only in four cases; in three of them a stressful event was observed around the outbreak and there was no information about it in the other. In one of these events, other infectious diseases were noticed but no other factors were reported.

6.5.5 Prevention and control

The awareness about the consequences of avian malaria seems to be broadly shared as most of the participants responded that they have prevention measures against the disease regardless of the occurrence of the disease in their institutions, although the active surveillance was scarce. Some measures for mosquito control could be effective but they also demand effort and time. Therefore, the regular monitoring of mosquito populations could indicate the areas and locations where the control measures are needed. Likewise, it could also direct the most appropriate time for the preventive treatment.

All the institutions with records of avian malaria keep their penguins always in an outdoors exhibit, meaning that the birds are in a constant risk of being bitten by mosquitoes. Preventing the exposure of penguins to mosquitoes could be impractical; none of the participants mentioned the use of nets or other kind of barriers, possibly because their installation and maintenance could be too expensive and would affect the appreciation of the penguins by visitors. Other measures commonly in place, like the maintenance and cleaning of water bodies, could be more practical and its integration with other actions to prevent mosquito development would be an effective strategy. These could include the elimination of mosquito oviposition sites such as water pockets in natural or artificial containers and the use or promotion of predators and disrupting bacteria for mosquito larvae.

It is likely that many institutions started preventive measures against the disease after the first outbreak preventing new ones and where the outbreaks are persistent, unusual factors like the abundance of mosquitoes, presence of reservoirs or adverse issues for the penguins' health could be affecting the colonies.

The effectiveness of other preventive actions has not been systematically evaluated. The use of repellent plants is common but it has not been tested under field conditions and their use is assumed from the repellent effect against arthropods of essential oils (mixtures of volatile compounds) directly applied to the skin to prevent mosquito bites (Choi et al., 2002, Lee, 2018), rather than a proven ambient repellent. Empirical evidence of the effectiveness of these measures would add more options for the prevention of the disease.

The prophylactic and therapeutic treatments for penguins are done constantly but without studies about pharmacokinetic and pharmacodynamics in penguins, the effective dose and

possible secondary effects have not been defined (Grilo et al., 2016). Although different treatment options have been tried and discussed (Grilo et al., 2016), their success in the colonies of our participants seems to be low as a lethality of 100% was noticed in 25 cases. Unfortunately, we did not receive enough detailed information about treatment protocols to present a deep discussion of the outcomes, but the use of traditional anti-malaria drugs, especially primaquine and chloroquine, continues to be widespread. A questionnaire particularly designed to investigate the treatment options and protocols complemented with interviews could provide more valuable information and details about the outcomes.

6.5.6 Conclusion

Avian malaria has occurred regularly across UK zoos and wildlife parks and reports increased in recent years. Most outbreaks have had low to medium prevalence and mortality but high lethality. Events with higher mortalities could be related to stress or other factors but more research is needed to explain it.

The occurrence and severity of avian malaria outbreaks is possibly due to a combination of factors such as the high population of susceptible penguins that are exposed to the transmission, the high abundance of mosquitoes during the summer and the wide distribution of the parasite. Aggravating factors could be the moulting period during which the penguins are more exposed and disease signs are harder to detect, the breeding season that increases the physiological demands and adds susceptible chicks to the population, and other stressful events that compromise the penguin immune system like concomitant diseases.

Considering that avian malaria has unspecific signs, most of the penguins are constantly at risk of infection and that the treatment is seldom successful, preventive measures are the best option against the disease. Health checks and avian malaria diagnosis should be done regularly, in particularly before the mobilisation of individuals because if infected, the stress of the moving could trigger an acute infection.

The monitoring of mosquitoes would provide information to guide the control actions for their population and the prophylactic treatment of penguins. Finally, a great benefit could derive from retrospective studies and the constant recording and sharing of epidemiologic information for the long-term study of avian malaria and other diseases of zoo animals.

Appendix 6.1. Invitation for Participants (Invitation Email)

Dear participant,

We are inviting you to answer a short survey about avian malaria in captive birds. It is not going to take much of your time, but it will provide us with valuable information that we can analyse in order to integrate useful and practical recommendations for the surveillance, control and prevention of the disease.

This project is coordinated by the University of Liverpool and Chester Zoo with the support and approval of BIAZA.

Background:

Avian malaria is an important disease that seriously affects penguins and other species in captivity and could become a major threat to wild populations. We need to investigate more about its epidemiology in zoological gardens and wildlife parks for improving the strategies to protect these vulnerable and emblematic birds.

The experience and knowledge of zoo veterinarians, animal keepers and assistants are indispensable to understand the epidemiology of the disease in captive birds and provide effective measures for its prevention and control.

Benefits:

If you complete this questionnaire before two months after receiving this email, we will send you a report at the end of the research. The report will summarize and analyse the answers of all participants and discuss the most relevant aspects of the prevention and control of the disease. We hope that it will be a useful reference for the management of the penguins at your institution.

Use of the information:

All the information will be handled with strict confidentiality; we won't disclose any personal data and the performance of the staff won't be assessed by any means. We won't disclose the

name of your institution without your consent. The responses will be kept in a PC from the University of Liverpool protected by antivirus and spyware software and accessed only by the main researcher with his username and password.

By completing this questionnaire, you consent to us using your responses (not personal data) for research and publication purposes; to be included in a PhD thesis, scientific papers, and the report delivered to the collaborating institutions. Please read the attached Participant Information file.

Guidelines:

This questionnaire is divided into two main parts. The first is regarding contact information and the penguins currently kept in your institution; it should take less than five minutes to answer. The second part is about the occurrence of avian malaria in your institution and will take about twenty minutes to finish depending on the availability of the information. In this part, if there have been confirmed or suspected cases of avian malaria, you will need to fill the forms attached to this invitation and upload them where requested in the survey (as PDF or Word documents) or send them back to us by email (any format), any option is fine but please do not forget to do so.

We recommend you review the questionnaire first so you can prepare in advance the information that will be requested.

Contact:

If you have any concerns, questions or suggestions, you can send us an email to:

Arturo Hernández-Colina: arturoh@liverpool.ac.uk

To access the survey please follow this link: https://www.surveymonkey.co.uk/r/7JP6JKC

Appendix 6.2. Avian Malaria Questionnaire

Part 1

Instructions

This questionnaire is divided into two main parts. The first is regarding contact information and the penguins currently kept in your institution; it should take less than three minutes to answer. The second part is about the occurrence of avian malaria in your institution; in this part, if there have been confirmed or suspected cases of avian malaria, you will need to fill the forms attached and upload them where requested in the survey. This part could take between fifteen to twenty minutes to finish depending on the availability of the information.

We recommend you review the questionnaire first so you can prepare in advance the information that will be requested about your penguin collection and avian malaria events. The files Most Recent Event and Previous Events attached to the invitation email can be uploaded directly into the questionnaire or sent back to us by email, any option is fine but please do not forget to do so.

If you can't finish the questionnaire in a single time, you can continue later or edit your answers, but in order for your answers to be saved, you need to finish the current section by clicking on the "Next" button, use the same device and browser the next time and agree with the cookies policy if requested.

Please fill the information requested in the corresponding fields or select your preferred options, the fields with an asterisk (*) are mandatory.

After answering a question, you can press the OK button or just scroll down the page to continue.

If you have any questions, please contact us by email: arturoh@liveprool.ac.uk

Contact Information

(Ideally, veterinarian or responsible for penguins or birds)

1. Person to contact:

   Name of the zoo or park: [Text box] *

   Address of the zoo or park: [Text box]

   Email address: [Text box]

   Phone number: [Text box]

Your details will be only used for contact purposes in case we have questions related to the aims of this research; we will keep them in a PC from the University of Liverpool protected by antivirus and spyware software and accessed only by the main researcher with his username and password. If you don't want to be contacted, please leave a blank in the "Person to contact" and "Email address" boxes.

Penguins Information

2. Do you keep penguins in your institution at the present time? [Yes / No ratio button]

   [If yes, a logic rule takes the participant to the question 3; if no, it takes him to question 7]

3. Please list the number of individuals by species.

Common name	Scientific name	Number of Individuals
African Penguin	*Spheniscus demersus*	[Text box]
Gentoo Penguin	*Pygoscelis papua*	(Per species)
Humboldt Penguin	*Spheniscus humboldti*	
King Penguin	*Aptenodytes patagonicus*	
Macaroni Penguin	*Eudyptes chrysolophus*	
Magellanic Penguin	*Spheniscus magellanicus*	
Northern Rockhopper Penguin	*Eudyptes moseleyi*	
Southern Rockhopper Penguin	*Eudyptes chrysocome*	
Others		

4. What is the origin of your penguins? (You can choose more than one option). [Check list with “Other” option for free text]
    a. Raised on site
    b. Brought from another site
    c. Captured from wild
    d. Other (please specify) [Text box]
5. Do you have a breeding program for your penguins? [Yes / No ratio button]
6. How are your penguins kept? [Check list]
    a. Always indoors (entirely closed facility)
    b. Always outdoors (open facility, not considering nest boxes as indoors)
    c. Indoors and outdoors at different times (not considering nest boxes as indoors)
    d. Other (please specify) [Text box]
7. In your experience, what would you consider the main health issue that affects or could affect penguins in your institution (you can select more than one)? [Check list with “Other” option for free text]
    a. Aspergillosis
    b. Avian malaria
    c. Digestive infections
    d. Pododermatitis (bumblefoot)
    e. Degenerative conditions
    f. Respiratory infections
    g. Others (please specify) [Text box]

Avian Malaria Information

8. Do you have prevention or control measures against avian malaria? [Yes / No ratio button]
9. If yes, please select from the following options: [Check list]
    a. Barriers to prevent mosquito bites (nets for example)
    b. Constant cleaning and maintenance of water bodies
    c. Disrupting bacteria for mosquito larvae in water bodies
    d. Elimination of water pockets
    e. Keeping the penguins indoors
    f. Mosquito larvae predators in water bodies (fishes, dragonfly larvae)

g. Prophylactic treatment for penguins
h. Quarantine of sick penguins
i. Surveillance of the parasite in mosquitoes
j. Surveillance of the parasite in penguins
k. Treatment for sick penguins
l. Use of fans to increase wind flow
m. Use of mosquito chemical repellent products
n. Use of mosquito repellent plants
o. Use of traps for adult mosquitoes
p. Others (please specify) [Text box]

10. Do you have a surveillance program for the disease? [Yes / No ratio button]
11. If yes, describe it briefly. [Text box]
12. Has avian malaria ever been suspected or diagnosed in the birds of your institution? [Yes / No ratio button]

    [If the answer is "Yes", a logic rule will take the participant to the next question, otherwise, it will take him to the "Extra information" section.]

Part 2

Avian Malaria Events Information

13. How many events of avian malaria have been suspected in your institution in the last twenty years? (Consider an event as suspected if one or more individuals were affected in the same period and the diagnosis was based on signs and lesions but no diagnostic tests were done). [Text box]
14. How many events of avian malaria have been confirmed in your institution in the last twenty years? (Consider an event as confirmed if there were cases of one or more individuals in the same period and at least one diagnostic test provided a positive result). [Text box]

Only for the most recent event (confirmed or suspected), please provide the following information.

15. Starting and ending dates of the event.
    Start (detection of the first case) [Date input]
    End (recovery or death of the last case) [Date input]
16. For the affected species during the last event, including penguins and other birds, please download the form “Most recent event” attached to the invitation email, fill it providing species names, origin, population number, number of sick, dead, recovered, and tested individuals, and upload it here. [Upload file]
17. What disease signs or lesions did you observe in the penguins? [Check list with “Other” option for free text]
    a. Anaemia
    b. Anorexia or diminished appetite
    c. Ataxia
    d. Cardiomegaly
    e. Dyspnoea
    f. Fever
    g. Green faeces
    h. Hepatomegaly
    i. Hydropericardium
    j. Hyperthermia
    k. Lethargy or depression
    l. Lung congestion
    m. Pale mucosae
    n. Regurgitation
    o. Splenomegaly
    p. Weight loss
    q. No signs at all
    r. Others (please specify) [Text box]
18. What diagnostic technique was used to confirm the disease? (You can select more than one) [Check list with “Other” option for free text]
    a. Clinical signs
    b. ELISA
    c. Gross lesions observed during post-mortem
    d. Histopathology
    e. In-situ hybridization
    f. PCR
    g. Thin blood smears (optical microscopy)
    h. Others (please specify) [Text box]

19. Which blood parasites were found (even if they are not related to avian malaria)? [Check list with “Other” option for free text]
    a. *Babesia*
    b. *Borrelia*
    c. *Haemoproteus*
    d. *Leucocytozoon*
    e. Microfilariae
    f. *Plasmodium*
    g. *Trypanosoma*
    h. Others (please specify) [Text box]
20. If particular species or lineages were identified, please specify which ones. [Text box]
21. Please, mention briefly the treatment protocol that was provided. [Text box]
22. Did you observe a stressful event beyond the routine activities affecting the birds before or at the same time as the avian malaria event? [Yes / No ratio button]
23. If you did, please add the start and end date of the corresponding event. If the stressful event is not listed, please describe it in the “Other” option including the dates.
    a. Changes in the exhibit [Text box]
    b. Moulting [Text box]
    c. Outbreak of a different disease [Text box]
    d. Rehabilitation [Text box]
    e. Reproduction [Text box]
    f. Sampling or health check [Text box]
    g. Transferring the birds to a different enclosure [Text box]
    h. Other [Text box]
24. If you found another disease affecting the birds at the same time as avian malaria, please specify it here including the start and end dates. [Text box]
25. If other avian malaria events (confirmed or suspected) have occurred, please download the from “Previous Events” attached to the invitation email, fill it providing the starting and ending dates, affected species, the population at the beginning of the event and the number of sick and dead individuals, and upload it here. [Upload file]

Extra Information

26. We handle all this information with strict confidentiality; nevertheless, please consider the following options and chose the one that you prefer. [Multiple-choice list]

    a. I agree that the name of my institution is disclosed as part of this study.
    b. I do not want my institution to be disclosed but I am happy for findings from my institution to be used at a coarse geographic scale (see Figure 1).
    c. I do not wish my institution to be disclosed at any geographical level.

Figure 1. Nomenclature of Territorial Units for Statistics, NUTS 1 of the United Kingdom.

27. We would like to acknowledge your participation in this study; therefore, please chose the best way we can do it. [Multiple-choice list]
    a. I agree that my institution is included in the acknowledgements.
    b. I prefer that the name of my institution is not mentioned in any acknowledgements.
28. Do you have any other comments that you would like to add? [Text box]

Thank you very much for taking part in this survey, we appreciate your participation which will contribute to the better understanding and prevention of avian malaria in captive penguins.

[“Finish” button]

Most Recent Event Form

Instructions

For the affected species during the last event, including penguins and other birds, please provide species names, origin, population number, number of sick, dead, recovered, and tested individuals for avian malaria in the following form. You can add as many rows as you need and consult the example in the next sheet.

For the "Origin of the individuals" column, you can write A for Raised on site, B for Brought from another site or C for Captured from wild; if they have any other origin, please specify; you can also write more than one option.

Species		Origin of the individuals	Population at the start of the event	Number of sick birds	Number of dead birds	Number of recovered birds	Number of tested birds
Latin name	Common name						

Example

Species		Origin of the individuals	Population at the start of the event	Number of sick birds	Number of dead birds	Number of recovered birds	Number of tested birds
Latin name	Common name						
Spheniscus humboldti	Humboldt Penguin	A, B	36	12	8	4	12
Pygoscelis papua	Gentoo Penguin	B	15	2	0	2	1
Fratercula artica	Common puffin	Other, rescue centre	24	6	5	1	0

Previous Events Form

Instructions

For the remaining events please provide the starting and ending dates, affected species including penguins and other birds, population at the beginning of the event and number of sick, death and recovered individuals. Please identify each event with a number; you can see the example in the next sheet. You can add as many rows and events as you need. Consider an event as Confirmed if at least one diagnostic test provided a positive result; if the diagnosis was based only on signs and lesions, consider it as Suspected.

Event number	Confirmed or Suspected	Starting date (detection of the first case)	Finishing date (recovery or death of the last case)	Species		Population at the start of the event	Number of sick birds	Number of dead birds	Number of recovered birds
				Latin name	Common name				

Example

Event number	Confirmed or Suspected	Starting date (detection of the first case)	Finishing date (recovery or death of the last case)	Species		Population at the start of the event	Number of sick birds	Number of dead birds	Number of recovered birds
				Latin name	Common name				
1	Confirmed	20/05/2015	13/07/2015	*Spheniscus humboldti*	Humboldt Penguin	36	12	8	4
				Pygoscelis papua	Gentoo Penguin	15	2	0	2
				Fratercula artica	Common puffin	24	6	5	1
2	Suspected	16/06/2013	25/08/2013	*Spheniscus humboldti*	Humboldt Penguin	42	14	14	0
				Fratercula artica	Common puffin	26	3	1	2

Appendix 6.3. BIAZA Support Letter

BIAZA
BRITISH & IRISH ASSOCIATION OF ZOOS & AQUARIUMS

BIAZA Research Committee Letter of Support for Research Project

The BIAZA Research Committee promotes good quality basic and applied research by and within BIAZA's member collections.

Following critical consideration of the research proposal and subsequent satisfactory responses by the researcher, the committee has agreed to give a letter of support for this study by Arturo Hernandez Colina of the University of Liverpool.

In the opinion of the BIAZA Research Committee the methodology proposed by this researcher will provide robust data that will answer their research question

In the interest of scientific training [and the furthering of science], the BIAZA Research Committee encourages BIAZA members to take part in this research project.

The BIAZA Research Committee has recently given letters of support for other similar projects but believes that this research has the potential to provide new and worthwhile information about this subject. We have encouraged all the supported researchers to contact each other and share data as much as possible to reduce their combined impact on collections agreeing to take part.

Please be advised that we would require an update or your completed project report within 1 year from today.

Yours faithfully,

Jessica Harley

Chair, BIAZA Research Committee 24 October 2018

Appendix 6.4. Participant Information Sheet

Version 1 26/03/18

Participant Information Sheet

Past and current situation of avian malaria in the UK zoos and wildlife parks

Dear collaborator,

You are being invited to take part in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and feel free to ask us if you would like more information or if there is anything you do not understand. We would like to stress that you do not have to accept this invitation and should only agree to take part in the study if you wish to.

Purpose of the Study

The study aims to gather and analyse the information about penguins and avian malaria events in UK zoos and wildlife parks to provide useful recommendations for the health care of these birds in captivity. For this, we ask you to complete the questionnaire linked in the invitation email.

Why have I been chosen to take part?

You have been chosen because you have or had penguins in your bird collection. Your participation is voluntary and you will be free to withdraw at any time during the study.

What will happen if I take part?

If you agree to participate, we would be grateful if you could access and respond the questionnaire and upload or send us by email the attached files “Most recent event” and “Previous events” completed.

Are there any risks in taking part?

We only ask you to complete the questionnaire; therefore, there are no risks.

Are there any benefits to taking part?

At the end of the study, we will send you a report detailing the results and the implications for the health of penguins in captivity with suggestions for the disease survey and prevention.

What if I am unhappy or there is a problem?

In this case, please feel free to let us know by contacting Arturo Hernandez Colina on 07401496983, or Professor Matthew Baylis on 01517946084, and we will try to solve the problem. If you remain unhappy or have a complaint which you feel you cannot come to us with, then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide the name or description of the study (so that it can be identified), the names of researchers involved, and the details of the complaint you are making.

Will my participation be kept confidential? What will happen to the results of the study?

All data collected and the results from the sampling will be entered into a secure database at the University of Liverpool and only the research team will have access to it; the computers used are all password and anti-virus protected. The data collected will be stored for 5 years in accordance with University Regulations. The results will be presented in a written report to you, may be presented in scientific meetings, may be published in a peer-reviewed journal and will constitute part of the degree thesis of the participating PhD student, Arturo Hernandez-Colina.

What will happen if I want to stop taking part in the study?

You can withdraw from the study at any time without explanation. If you agree, data up to the period of withdrawal may be used; otherwise, you may request that it be destroyed.

Who can I contact if I have further questions?

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Chapter Seven

General Discussion

7.1 Overview

Avian malaria has caused serious outbreaks in captive penguins worldwide; reports of the disease are numerous in the UK and they may be in augment. Although avian malaria is commonly present in wild bird communities and numerous investigations have been done around its ecology, many features about its epidemiology and mosquito vector remain unknown.

The purpose of this thesis was initially to investigate the ecology of avian malaria vectors in UK zoos to understand why this disease is a devastating problem in some cases and seems to be absent in others. During the course of our investigation, we observed a multi-causal outbreak in a penguin colony which included avian malaria cases. As a result, we decided to expand the scope of our research to investigate other features of mosquito ecology including host preferences and the drivers of their abundance and distribution at a local scale. The final aim was to provide recommendations for the improvement of the health and wellbeing of penguins under human care. After the discussion of results, the main recommendations are presented in this chapter.

7.1.1 Importance of avian malaria in wild birds

The number of emerging diseases has increased in recent years, in part due to the intense interactions of human activities with natural environments but also to the improvement of diagnostic techniques and biosurveillance programs (Daszak et al., 2000, Williams et al., 2002). These diseases represent a great challenge in terms of prevention and control, and they threaten public health, the health of domestic animals and the conservation of wildlife. Moreover, their distribution, ecology and epidemiologic processes are changing as a result of the alteration of natural habitats, environmental pollution and climate change. Consequently, there are many unknown features that are essential for their prevention and control that demand a great research effort.

Many emerging diseases are related to wild birds; some of them, like West Nile Virus encephalitis and Japanese encephalitis, are important zoonotic problems (Brugman et al., 2013). Others are major concerns for the health of domestic animals, for instance avian influenza can cause catastrophic losses in poultry and it is also a serious zoonotic disease (Gale et al., 2014). Finally, some diseases can increase the pressures for the conservation of threatened species and cause their extinction (Smith et al., 2009).

Avian malaria falls in the last group as it has caused the extinction of a number of endemic species and subspecies in the Hawaiian Islands (Foster et al., 2007) and it is also considered the main cause of mass mortalities of penguins in captivity (Vanstreels et al., 2014). Despite the efforts to prevent and control this disease, cases are regularly reported in zoological gardens, aquariums and rescue centres. It is more concerning that the aetiological agent, the *Plasmodium* protozoa, has been detected and associated to outbreaks in wild populations of penguins like the threatened yellow-eyed penguins (Webster T et al., 2019). This urges the investigation of risk factors and ecological traits of the mosquito vector which could expand its distribution due to climate change taking the parasite to regions were the birds have not been exposed before, with unknown but potentially devastating consequences.

7.2 Mosquito Ecology in Zoos

7.2.1 Mosquito species

Our mosquito sampling methods were designed to target *Culex* spp. mosquitoes as *Cx. pipiens* is the most recognised vector of avian malaria in Europe (Zele et al., 2014). We were successful in this sense as the majority of mosquitoes collected belonged to this species. We only found *Plasmodium* spp. in *Cx. pipiens*, but we captured other eight species of mosquitoes that could be involved in the transmission; especially considering that from the two mosquitoes in which we identified penguin blood, one was an *Anopheles maculipennis* s. l. All traps differ in the range of species that they attract; therefore, other sampling techniques and traps could be tried to get a better representation of the mosquito community and the species proportions.

As knowledge about mosquito ecology advances, it is clear that more species are actually complexes that include similar species and biotypes. Species inside these complexes have different biological traits and can be sympatric or produce hybrids with mixed features (Becker et al., 2012). It is important to achieve the complete identification of specimens to elucidate their role in the epidemiological process. We differentiated the almost identical species *Cx. pipiens* and *Cx. torrentium*, finding that the latter one is much less abundant and was negative for the parasite testing; therefore, it is unlikely to have an important role in the transmission of avian malaria to penguins in our sampling sites.

7.2.2 Mosquito abundance

Mosquitoes in temperate regions have delimited activity periods with an abundance peak during the summer months (Ewing et al., 2016). Their distribution is also different depending on the particular habitat that they occupy and many studies have done comparisons in this sense (Hesson et al., 2014, Karki et al., 2016, Vogels et al., 2016). Nevertheless, mosquitoes can also have significant differences in their local distribution inside a particular landscape; this has not been broadly studied but could have relevant implications especially in modified habitats like zoos. We identified periods of the seasons with higher mosquito abundance and found that they vary among sites and years. We also found differences among sampling areas; thus, the temporal and spatial aggregation of mosquitoes is different at the local scale and changes over time.

By recording and modelling a set of important features in each sampling area, we were able to identify common variables that influence how mosquitoes aggregate, explaining the spatial and temporal differences mentioned before. Certain mosquito species are favoured by the presence of suitable places for oviposition; gravid mosquitoes are naturally attracted to this areas and mosquitoes that emerge as adults come from them. Dense vegetation can provide shelter and food (depending on the kind of plants), but apparently, artificial structures that could also be resting areas do not have a relevant influence. Nevertheless, artificial shelters like sheds, buildings and animal enclosures that mosquitoes can access are important refuges during the overwintering period. Mosquitoes are attracted by the presence of potential hosts such as zoo animals and this varies depending on the feeding preferences of mosquito species, but it must be considered that we measured the distance to the animal exhibits and not to the animals themselves.

Temperature was the only weather variable that we found influencing mosquito abundance and it was the most significant overall in the analyses. This was observed using temperature recorded at a regional scale, this is by zoo and sampling season, rather than at the microhabitat level in the sampling areas. The effect of temperature has an important role in mosquito ecology (Ewing et al., 2016); although in some cases, other factors like the latitude and growing season can be more related to this for certain species like *Cx. pipiens* and *Cx. torrentium* (Hesson et al., 2014). The relation between mosquito abundance and temperature is not linear, it is given by a temperature range in which an optimum point maximises mosquito physiology but outside it, mosquito survival is compromised. The study

of this temperature range at the local scale could bring relevant information for the understanding of mosquito populations in relation to disease transmission.

Rainfall and humidity have been also related to mosquito abundance in natural environments (Asigau and Parker, 2018); so it was expected that they could have an influence in our sites as well. But as we did not find a clear effect, it is possible that artificial maintenance of gardens and ponds could have compensated for the natural lack of water sources. Other variables that we did not measure could also influence the distribution and abundance of mosquitoes, like the speed and direction of the wind.

7.2.3 Host feeding

As with the mosquito abundance, the proportion of mosquitoes with blood-meals was significantly different among sampling areas and seasons. We found an increase in the mosquito feeding activity in the warmer months of the season, but this was not entirely associated with mosquito abundance. Some areas with high mosquito catches did not get a high proportion of blood-fed mosquitoes and the opposite was observed in areas with low catches; then, the attractant factors are different for blood-fed and empty mosquitoes. The availability of potential hosts is the most likely variable that lures mosquitoes to certain area; we did not measure the distance from the traps to the zoo animals, the abundance of wild birds or the flow of visitors in the zoos, but doing so could help to identify and predict areas with high feeding activity of mosquitoes.

The host preferences of mosquitoes have been studied in other zoos, finding that the vast diversity of animals is reflected in the blood-meals (Tuten et al., 2012, Heym et al., 2019). We found that two species of mosquitoes, *Cx. pipiens* and *Culiseta annulata*, consistently feed in the zoos. There were differences in their host preferences, *Cx. pipiens* preferred birds over mammals and *Cs. annulata*, mammals over birds, corresponding to their biology. What is interesting to notice is that *Cx. pipiens* preferred humans, a behaviour reported before (Farajollahi et al., 2011) but not in the proportion that we observed. It is possible that this was influenced by the host availability as these human-fed mosquitoes were collected in the months with higher numbers of visitors. Something that needs to be tested further is the biotype of these mosquitoes; they could be *Cx*. *pipiens pipiens* or *Cx*. *pipiens molestus* from which a main difference is that the first one prefers to feed on birds and the second one on humans (Vogels et al., 2016).

The human feeding behaviour is not only related to the potential nuisance for visitors and staff but also to the disease transmission risk. From the six mosquitoes with mixed blood-meals that we identified, three contained human and Eurasian magpie (*Pica pica*) blood. This bird species belongs to the Corvidae family from which some species are important hosts for West Nile virus; which is not established in the UK but if it is ever introduced, it could find a suitable niche and become a serious health problem. Notwithstanding, the medical care that is given to the animals in zoos makes them important biosurveillance targets for important diseases, including exotic vector borne pathogens such as West Nile virus (Adler et al., 2011, Greenberg et al., 2012). Considering the high proportion of mosquitoes feeding on humans that we observed, the possibility, in the opposite sense, that humans may represent a dilution host for the transmission of avian malaria to penguins could be evaluated with a deeper understanding of the transmission process in the community.

The zoo animals on which mosquitoes fed were located and the minimum flying distance of the mosquitoes was estimated. This showed that the dispersion of mosquitoes in the zoos is highly variable and could reach long distances after feeding. To improve these estimations, a precise location of the zoo animals in the exhibits would be needed. In another study, the flying distances of mosquitoes were also distant and it was suggested that there may be a flight path that could direct the mosquitoes in a certain direction (Ejiri et al., 2011). We observed that some mosquitoes fed on animals located at the west from the traps, which in some collections corresponded to the main wind direction of the day before the catch; so the wind could influence the travelling distances and along with the setting of the zoos, it could favour the passive dispersion of mosquitoes in a particular direction (Becker et al., 2010). To prove this effect, the wind direction and speed should be monitored at a regional scale with meteorological data and at a local scale with weather stations; complementarily, release and re-capture experiments could be done. The wind could also reduce the flight and biting activity of mosquitoes (Brugman, 2016), so preventing wind currents with barriers or promoting them with fans would depend on the particular results and objectives per area.

Cornet et al. (2013) found that *Cx. pipiens* prefers to feed on birds that are in the chronic phase of infection, possibly as mechanism of the parasite to manipulate its host behaviour to increase its transmission (Cornet et al., 2013). Therefore, the study of mosquito host preferences, along with the *Plasmodium* testing of those hosts, could be relevant to

establish host-parasite relationships and for the assessment of avian malaria transmission risks locally.

7.3 Avian Malaria Situation

Through our samplings, we were able to find the *Plasmodium* parasite in the three elements of the transmission network, penguins, mosquitoes and wild birds. Additionally, we confirmed that *Cx. pipiens* is a vector for the parasite as we could extract it from the salivary glands of the mosquito. This suggests that the transmission cycle happens locally and that the penguins in open exhibits are at risk of getting infected. Nonetheless, to confirm that the same species and lineages of the parasite are been transmitted locally and are responsible for the infection in penguins, the ongoing phylogenetic analyses must be completed.

From the results of our questionnaire, we found that many events of avian malaria had occurred in the zoos, aquariums and wildlife parks of the UK. The recent increase in avian malaria reports could be due to the facility to recall and access records of recent events, the improvements in the use of databases for clinical records, more accessibility to diagnostic techniques or a real increase in the disease prevalence. Although many institutions have in place control measure for the mosquito population and do health checks and provide preventive treatments, avian malaria is a constant problem for captive penguins.

Most zoos and wildlife parks have breeding programs for their penguins and there is a regular translocation of penguins among institutions; thus, there is a changing and susceptible population that is constantly exposed to different risk factors. Therefore, avian malaria testing should be included in the regular health check and the mobilisations of penguins. Additionally, avian malaria signs and lesions are unspecific, and some cases are detected after a sudden death, making the initial diagnosis particularly challenging. Then, the treatment protocols, primarily based on primaquine and chloroquine, have unpredictable outcomes; in some cases, the birds recover but, in the majority, they die. More research is needed to define therapeutic options, their efficiency and possible secondary effects. In consequence, the combination of susceptible individuals and difficult therapeutic approaches complicate the control response when the disease occurs; therefore, preventive measurements should be promoted.

The pathogenicity of avian malaria is not only related to the susceptibility of the host, it also depends on the species and lineage of the parasite (Lapointe et al., 2012). We found *Plasmodium matutinum,* which is a European species commonly found in local wild birds, infecting penguins and in association to mortality for the first time. To understand the characteristics of this parasite, genetic analyses, which are currently ongoing, are needed.

Stressful events were observed during the avian malaria outbreaks that happened in Chester Zoo and Flamingo Land, as well as others described in the questionnaire responses. Likewise, it is suspected that the stress of captivity in rescue centres can also increase the prevalence of avian malaria (Grilo et al., 2016). Although a direct association cannot be done because, in our case, stress indicators were not monitored simultaneously and in the outbreak of Chester Zoo other infectious diseases were found. Nevertheless, clinical indicators of stress, like blood cell counts, could be done to assess the penguins' wellbeing, keeping in mind that these parameters can also be affected by *Plasmodium* infection.

The community of wild birds is another element of the epizootic process of avian malaria that needs evaluation. Monitoring species richness, abundance and avian malaria status in local wild birds could help define the wildlife reservoir of the parasite and demonstrate if one or more species or groups are involved. Furthermore, this could also improve our understanding of the mosquito host preferences in zoo environments as indicators such as the feeding index, could be calculated. In this way, additional surveillance and control measures could be implemented on the reservoir population.

7.4 Recommendations for the Care of Penguins in Captivity

A biosurveillance plan should be developed in accordance to the capabilities and interests of the institution and the following main points can be considered; nevertheless, it is advised that the discussions in the chapters of this thesis be consulted. These recommendations derived from the results and observations of this thesis, but additional actions could be complementary. Inside each of the following sections, the recommendations are organised in order of priority and feasibility and should be implemented following a precautionary principle; for example, if the mosquitoes cannot be tested for *Plasmodium*, it should be assumed that they carry the parasite and corresponding actions should be taken.

7.4.1 Biosurveillance of mosquito communities

Mosquito communities are dynamic and the role of certain species as vectors for avian malaria is unclear but zoos are ideal for the long term surveillance of mosquitoes (Adler et al., 2011). Therefore, monitoring their populations provides relevant information for their control and avian malaria prevention in penguins.

1. Monitoring the mosquitoes on a regular basis at the local scale is needed to detect when they start to be active after overwintering and when their abundance increases.
2. All mosquito traps have biases towards certain species; it is important to recognise the avian malaria vectors in the area and use the ideal traps for those species. In the UK, the BG-Mosquitaire trap and CDC-Gravid trap are efficient at capturing *Culex pipiens*.
3. The mosquitoes should be tested for the avian malaria parasite to identify which species could act as vectors. This can be done for a proportion of the captured mosquitoes or by pooling specimens if testing all the catches is not feasible.
4. The monitoring of immature stages of mosquitoes (larvae and pupae) in water bodies is a partial indicator of the mosquito abundance and can direct where control measurements are needed especially for *Culex* spp.

7.4.2 Control of mosquito populations

The threshold in mosquito populations under which transmission risk of avian malaria to penguins diminishes is unknown and challenging to investigate. Therefore, the measurements for the mosquito population control need to be evaluated in the long term to establish their efficiency in the prevention of avian malaria.

1. The control should be implemented before the mosquito abundance increases and ideally beyond the immediate area of concern.
2. The areas in the penguin exhibits and their surrounding should be constantly monitored regarding the following features and ideally be designed without them:
    a. Vegetation: prevent the growth of abundant plants with dense vegetation.
    b. Oviposition sites: identify and eliminate suitable oviposition sites (water bodies with shallow water at least on its edges and rich in organic matter); if eliminating them is not possible, they can be modified removing organic matter and aquatic plants (those of the genus *Typha* spp. particularly

favour mosquito density), increasing the depth of their shores and increasing the water flow.

c. Consider that the abundance of potential hosts, like zoo animals or visitors, may attract mosquitoes. Avoid the closeness of potential hosts in high densities if possible.

3. Prevent the access of mosquitoes to potential overwintering shelters like sheds and buildings. Cleaning these places from mosquitoes by aspiration could be beneficial.
4. Specific control strategies can be more effective against certain species, so the identification of the mosquito species in the local community and defining which ones are avian malaria vectors could direct better approaches.

7.4.3 Prevention of avian malaria

A main part of avian malaria prevention depends on the biosurveillance and control of mosquito populations, so the following recommendations focus on penguins.

1. Prolonged stressful events beyond the usual activities could trigger avian malaria events or increase their severity, as well as for other diseases; so, they should be avoided.
2. If something that could cause stress to the penguins, like health checks or translocations, has to be done, avoid doing it during periods of high mosquito abundance or at the same time as other natural events that compromises their physiology, such as breeding or moulting.
3. When health checks of the penguins are done, the diagnosis of avian malaria should be included; for instance, before and after moving them to other institutions.
4. Whenever a case of avian malaria is suspected, the precise diagnosis should be attempted; the observation of thin blood smears and PCR techniques are the choice options for the moment. Diagnosis could be particularly useful for planning preventive measures for other individuals in the colonies.
5. The timing of the preventive treatment can be adjusted depending on the presence of mosquitoes in the area and their abundance throughout the season.
6. The integration of a knowledge network for sharing epidemiologic information that helps to evaluate the outcomes of preventive and control actions would be extremely beneficial in the long term. This network does not need to be exclusively about avian malaria and other existing networks could be used as platforms.

7.5 Conclusions

The work presented in this thesis highlights the importance of the study of vector mosquitoes in relation to avian malaria and penguins in zoos. Some information about the mosquito vector was confirmed, more information was added in support of previous hypotheses and novel information was found, such as the mortality of penguins in relation to *Plasmodium matutinum,* the host-feeding of *Anopheles maculipennis* s. l. on penguins and the high number of *Culex pipiens* feeding on humans.

Mosquito communities are dynamic and biosurveillance strategies are needed for the efficient control of their populations and to define the role of certain species in the transmission process. The feeding behaviour is an important part of the study of mosquito ecology because it provides information about host preferences, emphasising transmission risks, and the distribution and movement of mosquitoes.

Avian malaria transmission happens locally involving mosquitoes, wild birds and penguins; thus, if the penguins are exposed to the mosquitoes, they are at risk of avian malaria. Wild birds are an important element of the transmission network that needs further investigation to define the species that constitute the reservoir. More avian malaria cases and outbreaks have been observed recently and although this could be related to an information availability bias, this disease represents a constant threat to the health of penguins across the UK.

Due to difficulties obtaining prompt diagnoses, the lack of standardised treatment protocols and the unclear outcomes, strong efforts should be put into prevention measures and in this case, they can be focussed on the control of mosquito populations.

The study of mosquito ecology in zoos in relation to avian malaria can provide information and recommendations for the prevention of the disease in penguins under human care and even guide research strategies in wild populations as it is easier to do tests them first in an easily accessible environment.

Annexes

Annex 1. Common and Scientific Names of Wild Birds

Introduction

A common issue when studying a group of organisms is that their classification and nomenclature are constantly changing due to advances in taxonomy, phylogeny, systematics and especially, genetics. The problem arises when publishing scientific results, the organisms' names are fixed and, in many occasions, linking these names with previous or actualized ones is not easy. Likewise, different organizations or specialists have their own classification systems and names. This is common for pathogens and hosts, so when studying their interactions, this could be particularly complex.

On the other hand, the increasing use of databases allows us to explore and understand relationships among organisms at a scale never seen before which is extremely beneficial for the biosurveillance, prevention and control of diseases. When defining the importance of diseases, the information for decision making could be biased due to its quality and accessibility; therefore, the use of databases or other approaches is very convenient (Cox et al., 2016). Nevertheless, if the nomenclature problem is not considered, the searches in databases can lead to many serious unnoticed mistakes having a volume of duplicates or missing valuable information.

Birds are a very diverse group of vertebrates that comprises approximately 10,500 species. The phylogeny of particular bird groups has been an unsolved challenge but thanks to efforts like the Bird Genome 10K (B10K) Consortium that aims to sequence all bird genomes, genetic analysis will clarify the disputed points (Stiller and Zhang, 2019). Nonetheless, due to constant taxonomical changes and multiple ornithological organisations using different names, the species can have several synonyms, both in scientific and common names. Until now there is not a standard nomenclature list that relates all previous and current names of the bird species. Therefore, I integrated a list of synonyms that could be used to update the EID2 database and in that way, improve the results of the searches.

The EID2 database is open access and integrates information about pathogens and their hosts, the interactions among them, their localization and the temporal distributions. Thus, it facilitates research in epidemiology, life sciences and climate change. It also shows the research tendencies, both in publications and in genetic sequences reports; therefore, it is useful for detecting knowledge gaps (Wardeh et al., 2015, McIntyre et al., 2014).

Getting and merging the lists of scientific and common names

A base list of common and scientific names of birds was downloaded from the EID2 database, including the EID2 and NCBI taxonomical identification numbers and abbreviated names. The international ornithological authorities that produce open access lists of names were identified and all their lists versions were downloaded to include old and current names. In total, 15 lists from four main authorities were used:

- Bird Life International (2007-2015): 11 Lists versions (0, 1, 2, 3, 4, 5, 5.1, 6, 6.1, 7, 8)
- IOC World Bird List (2015): Versions: 5.3 and 5.4
- Clements, J. F. et al. (2015)
- Sibley and More (1990-1993)

Elimination of the duplicated names

The lists from the same authority were merged first and then integrated with the others using Microsoft© Excel. The duplicates names were identified with conditioning formulas and eliminated manually to avoid mistakes. The integrated list included 13695 names of species, from which 2200 were detected as synonyms and merged.

The taxonomical identification numbers were also used to detect duplicated names. Of the 288 names with different taxonomical number, 110 have an updated number and were merged. The final number of names is 11385. The minimum and maximum number of names in the last version of the ornithological authority lists were 10425 and 10765 respectively, which, compared to the number of names in the integrated list, gives an error of 5.47 – 8.46 %.

Nomenclature changes

The main taxonomical changes that I found were: the transference of a species to a different genus, modification in the nomenclature, shifts between species and subspecies levels, split of one species into multiple species and lumping of some species into one. Many of the species passed through several of these changes. I also found common names that correspond to different species, mainly due to previous splits or lumps and the species kept the former name. When all the names of certain species have been changed or are entirely different because they come from diverse lists, the synonyms cannot be tracked, producing more names than the maximum expected.

In some cases, certain species passed through multiple splits and lumps and this created false synonyms. When a species was split in two or more and then lumped again as one, the

names were considered as synonyms; but when some species were lumped and split again few of them kept the name of other species as synonym. This situation and all other contradictions were solved following the last version of the Bird Life International list. In Figure A1.1, a representation of the changes in names is presented.

Looking for the missing taxonomical ID numbers

The EID2 Database works by consulting the open access NCBI database and it uses the taxonomical numbers of the organisms in that database. In the integrated list names, 5859 numbers were missing and by tracing the bird names in the NCBI Taxonomy database, I found and updated 1323 numbers. Nevertheless, some names were merged previously leading to 2736 numbers absent which are due to the error in the list of names and because the NCBI database is not perfectly updated taxonomically and it includes 10% of the described life species on the planet (NCBI, 2018b).

To solve the remaining errors and to improve the final lists, it is necessary to do periodic updates and consult sources that integrate different names, like the database Avibase (Lepage D, 2016).

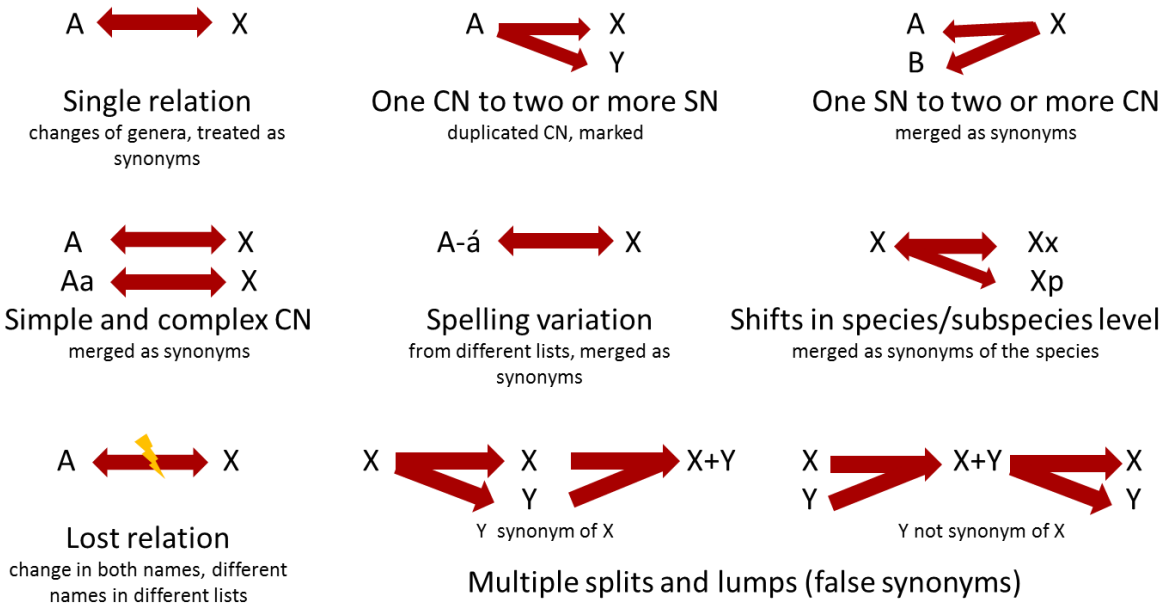

Figure A1.1. Changes in the nomenclature of bird names found in the integrated list. A and B represent common names, X and Y, scientific names.

Annex 2. Systematic Review of Wild Bird Diseases

Introduction

Systematic reviews have an important and increasing role in research and they have been used to address epidemiological and health issues such as disease prevalence and incidence, causal agent, risk factors, diagnostic test efficiency and preventive and therapeutic actions (Bruce N et al., 2008, Sargeant and O'Connor, 2014).

The main characteristics of systematic reviews are that they must have clearly stated objectives, pre-defined inclusion criteria for the information search, an explicit and reproducible methodology, a systematic search to identify most of the relevant sources of information, an assessment of the quality of the information found, and a systematic synthesis of the characteristics and results of the studies (Sargeant and O'Connor, 2014). If systematic reviews fulfil these requirements, they can provide an evaluation and summary of evidence with a reduced bias on its methodology and results that can be replicable, showing the state of knowledge of a certain subject, which is valuable for the decision-making process (Sargeant and O'Connor, 2014). However, if the information is not handled properly, they can also have serious bias and be misleading and detrimental (Yuan and Hunt, 2009).

Some systematic reviews have been done to describe the situation of diseases in wild birds providing valuable information. They were focused on particular topics such as zoonotic diseases related with wild birds (Tsiodras et al., 2008), epidemiologic features (Chatziprodromidou et al., 2018) or specific diseases like mycobacteriosis (Soler et al., 2009) and avian influenza (Afanador-Villamizar et al., 2017). Nonetheless, the description of the general situation of diseases in free wild birds still is missing, so I carried out a systematic review to have an updated reference about it, identifying main groups of pathogens and hosts and additional data.

Methodology

Inclusion criteria

I performed a systematic search on December 4th 2015 of the main bibliographic databases that include medical, biological and ecological journals: Scopus, Web of Science and PubMed. I divided the search in two sets, one regarding wild birds in which the key words were treated as exact phrases and another concerning the diseases. The construction of the queries was the following:

Keywords: ["wild bird" OR "migratory bird" OR "resident bird" OR "terrestrial bird" OR "aquatic bird"] AND [“disease” OR “sickness” OR “illness” OR “syndrome” OR “mortality” OR “morbidity”].

The search fields used were for Scopus, “Article Title, Abstract, Keywords”, for Web of Science, “Topic” and in PubMed, “All Fields”. In all cases the timespan included the last ten years 2005 - 2015. I refined the searches limiting the results to articles and reviews published in English or Spanish and within the “science and technology” domain; excluding the papers belonging to other domains and subdomains such as geography, pharmacology, etc.

Afterwards, I combined the results from the searches and eliminate the duplicated papers using the EndNote X7® software. Still, many duplicated papers remained so I removed them manually. I also eliminated the irrelevant articles, first with base on the title and abstract and then, on the review of the full text. The papers excluded in this step where those referring exclusively to experimental or laboratory research, domestic animals, human health or wild birds in captivity. For species which have wild and domestic populations such as pigeons, ducks and turkeys, only research papers concerning the wild forms where included.

The review articles were not analysed as they do not present the information about hosts, pathogens and locations in a consistent way and they can duplicate the information from original articles also included in the systematic search.

Classification of the information

I made a database using the Microsoft© Access software by creating eleven tables and an interface to add the information from the papers. In this way, each category has its own

table avoiding excessively large tables maintaining their independent fields in a simple way and making it easy to create relationships among tables (Table A2.1).

I established relationships among tables that allow tracking the origin of the data and simplify the data acquisition by filling automatically the relational fields. The type of relationships used is “one to many” which means that a single field in the origin table can have more than one field in the related table. For example, a disease caused by a single agent can have multiple hosts in the Wild bird host table without duplicating the disease and agent records.

I made a form for each table and integrated them to create an interface. I included the fields that need to be filled by the user like the name of the affected individuals, continent, country, year, etc., and some of the relational fields like the paper title, disease name, agent, and wild bird names (Figure A2.1).

To facilitate entering the data and standardise it, I used combo boxes with pre-stablished records in some fields, for instance, in the Disease form for agent type and in the Location form for continent and country. I also added two searching combo boxes to find the papers by their title or ID. I also divided the interface to present basic information separated from advanced information (Figure A2.2).

The forms are also a useful way to consult the information in the database because when an already reviewed paper is searched, the form presents all the introduced information, so it can be accessed or edited by category and field.

Fifty papers were randomly selected for the analysis.

Table A2.1. Tables and fields used in the database.

	Tables									
Fields	**Papers**	**Diseases**	**Wild Bird Host**	**Location**	**Time**	**Other Hosts**	**Environment**	**Effects**	**Importance**	**Management**	**Transmission**
	Title	*Paper Title*	*Paper Title*	*Paper Title*	*Paper Title*	*Paper Title*	*Paper Title*	*Paper Title*	*Paper Title*	*Paper Title*	*Paper Title*
	Authors	*Paper ID*	*Paper ID*	*Paper ID*	*Paper ID*	*Paper ID*	*Paper ID*	*Paper ID*	*Paper ID*	*Paper ID*	*Paper ID*
	Journal	Disease	*Disease*	*Disease*	*Disease*	*Disease*	*Disease*	*Disease*	*Disease*	*Disease*	*Disease*
	Year	Agent type	*Agent name*	*Agent name*	*Agent name*	*Agent name*	*Agent name*	*Agent name*	*Agent name*	*Agent name*	*Agent name*
	Type (original, review)	Agent name	*Disease ID*	*Scientific name*	*Scientific name*	Other wild life names	*Disease ID*	*Disease ID*	*Disease ID*	*Disease ID*	*Disease ID*
	Availability		Scientific name	*Common name*	*Common name*	Wild life animals affected	Temperature	Population decrease	For wild birds	Control actions	Not infectious
	Reviewed status		Common name	*Host ID*	*Host ID*	Domestic animals	Humidity	Local extinction	For domestic animals	Eradication actions	Direct
	Inclusion status		Mortality	Continent	Year	Domestic animals affected	Precipitation	Global extinction	For humans	Prevention actions	Indirect
	Exclusion criteria		Morbidity	Country	Month	Humans affected	Season	Reduced survival	For other wildlife	Epidemiologic situation	Intermediate species
			Asymptomatic	Region	Season		Extreme event	Reduced reproduction			Vector borne
			Negative	Sub-region			Other	Secondary disease			Vector species
			Other diseases					Other			Other

The fields in italics are relational and their data is filled automatically from other tables which helps preventing mistakes. The “ID” fields present a unique identification number of the record on the origin table.

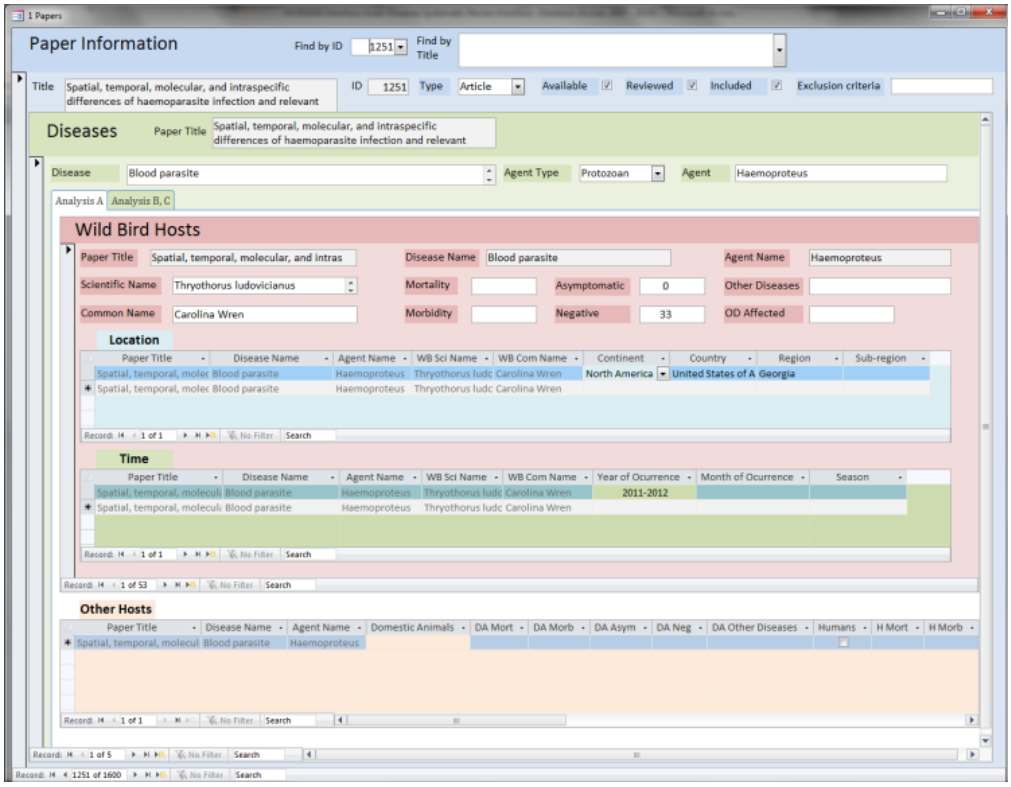

Figure A2.1. Database Interface showing the basic analysis fields.

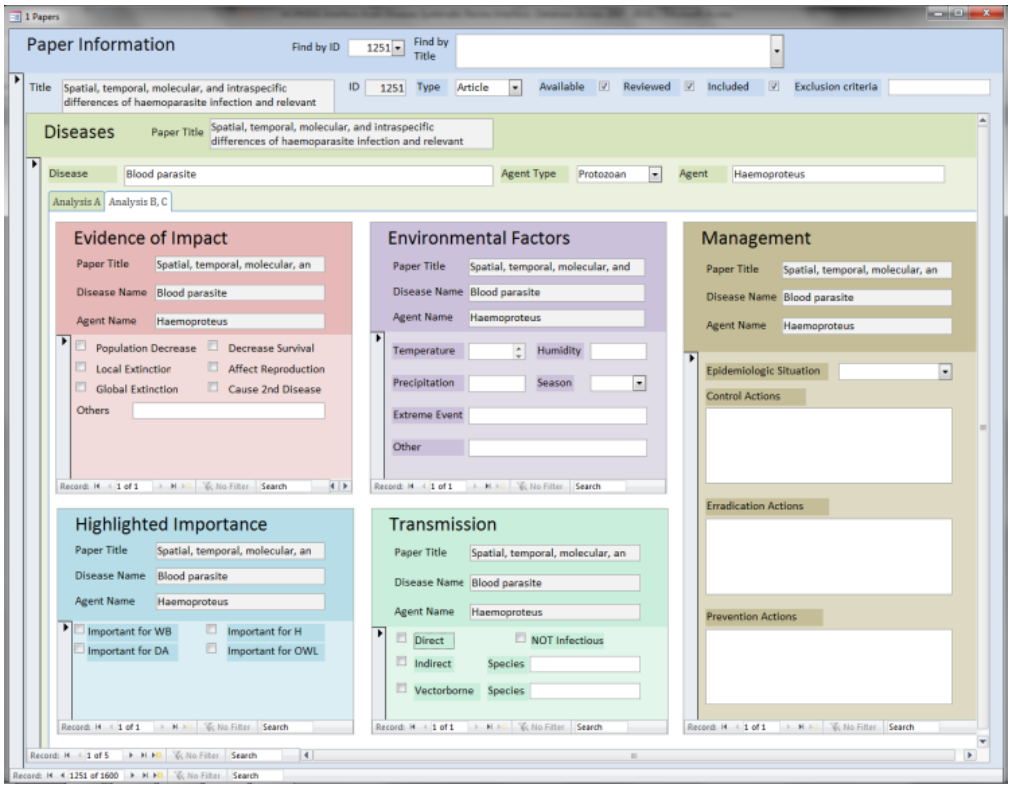

Figure A2.2. Database Interface showing the advance information fields.

Results and Discussion

The outcome of the searches was 1988 papers from Scopus, 1174 from Web of Science and 553 from PubMed. After the elimination of the duplicates and the no related papers, the result was 1126; but for several of them, the full text was not available.

From the 50 papers selected, 44 were original articles, 5 were review articles, and one was excluded because it included information from other sources and was unspecific about the host species. Most of the papers for this trial were published in recent years showing a relative increase in the research of wild bird diseases (Figure A2.3).

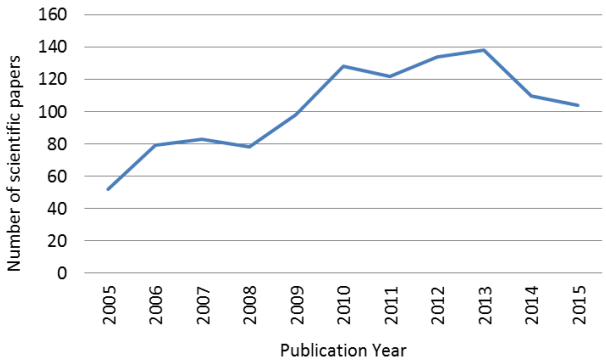

Figure A2.3. Number of scientific papers in the period of interest.

The authors reported 36 different diseases but duplicated in some occasions, representing 71 occurrences. I compared the reports by the occurrence and by the disease to know the proportion of pathogen kinds. By occurrence, the most prevalent diseases were those caused by bacteria (28%), followed by viruses (24%), and unknown pathogens (16%). The proportions by disease name (without duplicate occurrences) behaved similarly with the bacterial diseases being the predominant ones (25%) followed again by viral diseases (22%), and unknown pathogens (17%) (Figure A2.4).

The similar proportions between the total number of diseases by group and the number without duplicated diseases, suggests that there is not a strong bias towards a certain group of diseases and that scientific papers report disease occurrences similarly among groups but wider investigation and analysis is needed to confirm this.

Apparently, the bacterial and viral infections are dominant over other pathogen groups, although it is worth to mention that the unknown agent's group have a considerable proportion, meaning that a deeper investigation of the morbidity and mortality cases in wild birds with complete diagnosis is needed. The most prevalent diseases were blood

parasitism, including avian malaria (five occurrences), avian influenza and West Nile Virus encephalitis (four occurrences each).

Most of the reports were about asymptomatic birds (56.2%), those accounting for mortality and morbidity represented 35.9% and 7.8% respectively. This reflects the researchers' interest in screening for diseases relevant to the conservation of wild birds and to domestic birds and human health over the report of disease cases or outbreaks and it also shows the difficulty of finding ill and death animals in wildlife. There were 12 mentions of the importance of a certain disease for the conservation of wild birds, 10 about the health of domestic birds and nine about human health, which shows no clear tendencies yet.

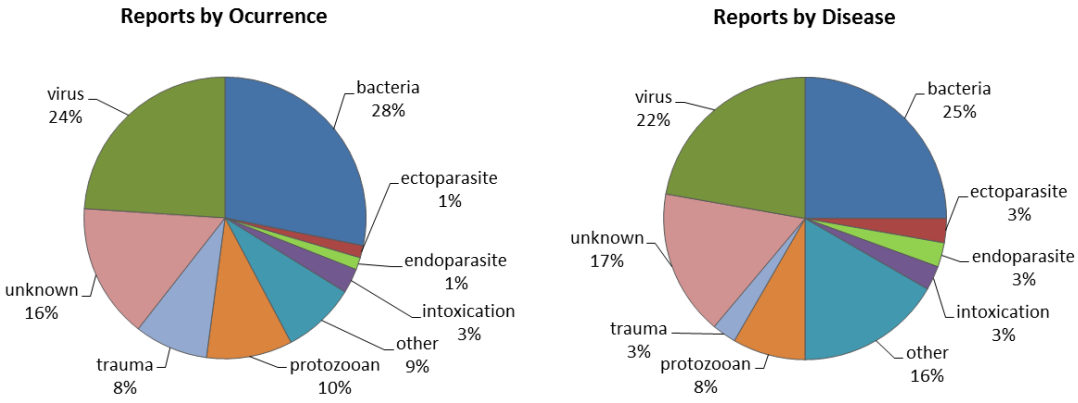

Figure A2.4. Proportions of diseases reported by group of pathogenic agents.

Regarding the diagnostic techniques, the most common ones were the isolation of the agent (n = 17), molecular techniques (n = 16), and serology techniques (n = 14); morphology descriptions (n = 8), pathology (n = 2), other techniques (n = 2), and bioassays (n = 1) were less frequent, and on three occasions the authors did not report the used technique. The diagnostic techniques are for specific pathogens; therefore, their use reflects the kind of pathogen investigated. Nevertheless, the preferred isolation and molecular techniques have a high sensibility and present evidence of the pathogen at the moment of the study, which is relevant from the epidemiologic perspective. On the other hand, serology techniques are usually cheaper than other procedures and perhaps they were chosen for their cost-benefit value.

I classified the reported species taxonomically using the list names of wild birds that was created previously (see Annex 1). The authors analysed 171 species from 11 orders and 35 families. The most frequent orders were Passeriformes (n = 98), Anseriformes (n = 29), Columbiformes (n = 13), and Charadriiformes (n = 12); the rest of the orders were reported

in five or fewer occasions. The more common families were Anatidae (n = 29), Columbidae (n = 13), Fringillidae (n = 11), Corvidae (n = 11) and Turdidae (n = 10), the rest of the families were infrequent and many were reported only on one occasion. The mallard (*Anas platyrhynchos*) and the common dove (*Columba livia*) were reported more often, nine and eight times respectively, among the other species most of which were reported only once.

Comparing these results, it is interesting to note that Passeriformes is the order with more species worldwide (almost six thousand) and as expected, it was the dominant one, which represents the proportion of the bird's diversity. For the family and species levels this was not the case as for example the Anatidae family, which includes the mallard, was the only one reported belonging to the Anseriformes order. This could be explained as a bias towards the study of birds that are easy to capture or spot if they are sick or death in the field. It can also mean that these groups and species are more susceptible to certain diseases of interest or due to their particular biology (population size, gregarious behaviour, flock density, etc.) constitute an important reservoir for several pathogens; likewise, a combination of these factors is also possible. Assessing the relation between host species and particular pathogens in a more extensive manner can help to clarify these tendencies.

The only domestic animals evaluated during these studies were hens, geese, ducks, pigeons, turkeys, and horses; reported in five papers. Other wildlife studied includes opossums, grey foxes, cottontail rabbit, evening bat, pocket mouse, white-footed mouse, cotton rat, Mexican ground squirrel, and wood rat. Only in one paper, the researchers screened humans for Venezuelan equine encephalitis virus with negative results. Most of the papers analysed wild birds exclusively, providing insights into their health status and epidemiology, but for some diseases that have complex interactions and interfaces with domestic animals and can represent a zoonotic risk for humans, like avian influenza, salmonellosis, West Nile virus encephalitis, and other viral encephalitis, a simultaneous and broader exploration of potential hosts and reservoirs (including the environment) could provide important information on the local situation at that particular moment, useful for disease management and decision making.

Many studies were performed in Europe (n = 13), North America (n = 10), and Asia (n = 7), and only one included two locations (Europe and Africa); the rest of them (n = 13) did not present the location of the study clearly. Ten studies were done in the USA, four in Spain, two in Japan, two in New Zealand and the rest in unrepeated countries; two studies took place in more than one country. The majority originated in developed countries, which can

be due to the resource's availability for research, but a higher prevalence of the studied diseases in those countries can be also a possibility.

The information regarding the other aspects of interest was scarce: two papers mentioned an environmental factor influencing the disease or outbreak (hunting pellets for lead intoxication and the season for avian malaria), one pointed out the potential decrease of survival due to hyperplastic goitre in captive black stilts, three declared the epidemiologic situation, and one suggested a treatment as a control measure.

This review was beneficial to examine the scientific literature concerning wild bird diseases and also to establish an efficient methodology that could be useful for doing other more comprehensive systematic reviews. Nevertheless, these assumptions about agent groups, affected hosts, and reported locations have to be evaluated using a bigger set of papers and for specific diseases.

Regardless of the use of strict selection criteria and a methodology for the extraction of information, this process has been performed by one person; so, subjectivity is implicit and human errors could have happened. Ideally, the selection of information sources and the extraction of the information should be done by multiple reviewers independently (Sargeant and O'Connor, 2014). An advantage in this work is that the database that contains the papers and the extracted information is easy to use and someone else could rectify these results.

Doing a systematic review requires a considerable time investment; therefore, setting specific objectives and methodology is essential to obtain reliable information efficiently. The approach of this work was general so a broad picture of wild bird diseases was obtained but for future analysis, the objectives could be prioritised to guide the extraction of the information. For example, a first stage could encompass the information of the pathogen, host, location, and time, which is relevant for evaluating the importance of the diseases and a second phase, could target environmental factors and management, which is necessary for better interpretation of the disease impacts; always keeping in mind that a consistent methodology should be followed.

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