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El papel de la glándula uropigial en la interacción hospedador-parásito.

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Memoria presentada por el Licenciado en Biología Sergio Magallanes Argany para optar al grado en Doctor en Ciencias Biológicas, dirigida por el Dr. Alfonso Marzal Reynolds del departamento de Anatomía Biología Celular y Zoología y la Dra. Luz García-Longoria Batanete del departamento de biología de la universidad de Lund.

Badajoz, Julio de 2018

A mis padres
A mi hermano
A Luna

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Agradecimientos

Esta tesis ha sido un largo viaje. Un viaje que comenzó antes de licenciarme, cuando Alfonso me dio la oportunidad de trabajar con él, y aceptó mi propuesta de Trabajo Fin de Master. En ese momento, tuve claro que mi futuro tenía que estar ligado a la investigación. Junto con Florentino de Lope, "Tinin" para los amigos, y Alfonso, me dieron la oportunidad de iniciarme en esta aventura gracias a una beca FPI del Ministerio de Economía y Competitividad, y por ello les estaré siempre agradecido.

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INTRODUCTION

Parasites

In nature, living organisms may establish different relationships among them and their ecosystem. Thomas *et al.* (2005) suggested that one of the dominant theory on life is dualism, i.e., the balance between two independent and fundamental principles (good and evil, mind and matter, nature and nurture). This idea can also be found in the Ecology of parasitism. For example, although parasites might provoke the extinction of some species, they may also generate diversity in one ecosystem; they may reduce the reproduction success of their hosts, but may also increase its growth rate; or they may block invasive species, but at the same time they might contribute to the colonization of new environments as invasive species ((Tompkins and Poulin 2006; Despommier 2007). Following up this basic idea, in this thesis we have explored the relation between host, parasite and environment.

Parasites are one the most ubiquitous and abundant organisms in the world living inside or on other organism (animals, plants, fungus, bacteria and virus), including humans (Bush *et al.* 2001). For instance, *Mycobacterium tuberculosis* infects one third of the human population (Cegielski *et al.* 2009), being one of the most ubiquitous organisms on the Earth. Similarly, we could find *Toxoplasma gondii* in all warm-blooded animals (mammals and birds), being present in most areas of the world (Tenter *et al.* 2000). In this sense, it has been suggested that half of the organisms in the world are parasites (Price 1980), thus highlighting the importance of parasites in terms of biodiversity.

Parasites might cause a high range of different negative effects on their hosts. They can affect growth, fecundity and breeding success of the

individuals they infect (Schmid-Hempel 2011). Parasites can also influence the diversity of animal species in the same way that predators do (Mouritsen and Poulin 2002, Morand *et al.* 2015). For this reason, parasitism should be considered as a biotic force capable of determining the biodiversity of communities (Poulin 1999).

Avian malaria parasites

One of the better-known groups of parasites is the order Haemosporidia (Phylum Apicomplexa). This order includes one of the most dangerous organism provoking lethal infection diseases for humans and many other groups of vertebrates: malaria (genus *Plasmodium*). However, the diversity of malaria parasites is huge, where more than 500 morph species of haemosporidians belonging to 15 different genera have been described infecting reptiles, birds and mammals. They are known to be transmitted by at least seven families of dipteran vectors, such as black flies or mosquitoes (Levine 1988, Martinsen *et al.* 2008). Moreover, the diversity of these parasites allow them to be transmitted in different environments. Thus, these parasites are widespread on all continents, except Antarctica (Atkinson *et al.* 2008, Valkiūnas 2005).

Avian malaria and related haemosporidians parasites are the largest group of haemosporidians by number of species. These parasites are excellent model for the study of host-parasite interactions because are widespread, abundant and diverse, and are easily sampled without disrupting their host populations (e.g. García-Longoria *et al.* 2014, Hellgren *et al.* 2015). More than 200 parasite species of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* have been described among the 4.000 bird species investigated worldwide (Valkiūnas 2005). The term “malaria

parasites" has been a controversial issue among parasitologists, ecologists and evolutionary researchers (Perez-Tris *et al.* 2005, Valkiunas *et al.* 2005). The life cycles of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are similar, but they present some differences in vectors, life cycles and epidemiology of these organisms. Thus, the traditional view accepts only *Plasmodium* species as being the true malaria parasites (Valkiūnas et al., 2005). But for many evolutionary ecologists the term malaria also includes species belonging to *Haemorpoteus* and *Leucocytozoon* genera.

The life cycle of these parasites is complex because the presence of a vector is required (Valkiūnas 2005). This life cycle presents two different stages: sexual and asexual. Each of these stages are developed in different hosts organisms: the asexual reproduction takes place inside the vertebrate host, whereas sexual reproduction occurs inside the invertebrate host (vector). These invertebrate hosts are different among the three genera. *Plasmodium* species are mainly transmitted by blood-sucking mosquitoes (e.g. *Culex* sp.), whereas vectors of *Haemoproteus* are biting midges and hippoboscid flies, and simuliid flies transmit *Leucocytozoon* (Atkinson and van Riper III 1991).

Avian malaria and related haemosporidian parasites are one of the most pathogenic species of poultry and wildlife birds. They may provoke negative effects on host fitness causing dramatic reductions in the efficiency of metabolism (Chen *et al.* 2001) and being responsible of economic losses, mass mortality, population declines and even extinctions of many bird species worldwide (Atkinson and van Riper III 1991; Valkiūnas 2005). It should be stressed the extinctions and endangerment of numerous native species provoked by avian malaria invasions in Hawaii, New Zealand and Galápagos Islands (van Riper III *et al.* 1986, Jarvi *et al.* 2001, Tompkins and Poulin 2006). For all these reasons, the International Union for Conservation

of Nature (IUCN) classifies *P. relictum* as one of the 100 of the world's worst invasive alien species (Lowe *et al.* 2000).

Although some researchers suggested that bird malaria parasites were low pathogenic organisms (Weatherhead and Bennett 1992, Bennett *et al.* 1993), some other studies showed the detrimental effects of avian haemosporidians on the life histories of their hosts (Korpimäki *et al.* 1993, 1995, Rätti *et al.* 1993, Allander and Bennett 1994, Dufva 1996). For example, it has been shown that avian malaria parasites can reduce survival (Dawson and Bortolotti 2000, Breman 2001, Valkiūnas 2005), body condition (Valkiūnas *et al.* 2006, Palinauskas *et al.* 2008), clutch size (Marzal *et al.* 2005) and reproductive success in their host (Merino *et al.* 2000, MacDougall - Shackleton *et al.* 2002, Marzal *et al.* 2005).

However, the demonstration of effects of parasites requires an experimental approach, because the experimental manipulation of blood parasite loads may reveal their harmful effects (Keymer and Read 1991, Merino *et al.* 2000, Knowles *et al.* 2010). In this sense, two have been the most successful methodologies employed. Some studies have induced malaria infection on their bird hosts by direct inoculation of a parasite on uninfected individuals. With this methodology Coon *et al.* (2016) demonstrated the the negative effects of blood parasites can be tested trough the experimental removal of parasites in infected birds through medication. For example, Merino *et al.* (2000) reduced the intensity of haemosporidian infection in blue tits *Cyanistes caeruleus* with an anti-malaria treatment, showing that medicated females can invest more resources to breeding care and increased their reproductive success. Also, Martínez de la Puente *et al.* (2010) experimentally reduced, through medication, the intensity of infection by *Haemoproteus* parasites in wild-breeding female blue tits,

experimentally showing long-term direct survival costs of chronic *Haemoproteus* infections in wild birds.

Mechanisms of defence against pathogens

In turn, animals have developed a broad range of mechanisms to reduce probabilities to become infected, or stop the spread once the parasite has overcome the first barriers of defence (Schmid-Hempel 2011, 2017). Prior the activation of immune system, animals have evolved some specific behaviours to avoid parasite infection, or to eliminate parasites once become infected. For example, some fish species might recognize and avoid areas where infective stages are aggregated, thus reducing the chances of become infected (Karvonen *et al.* 2004). Also, some mammalian herbivores ingest some plants with anti-parasitic properties to remove internal parasites using this behaviour as a self-medication (Hutchings *et al.* 2003). But once infected, animals may stop the parasite spread through the immune system. Macrophages may destroy ingested intracellular parasites during infection skipping the parasite infection (Vidal *et al.* 1993, Raper *et al.* 2001, and phagocytes synthesize oxidative products destroying blood parasites (Nordenfelt and Tapper 2011). Additionally, birds own an extra barrier for avoiding pathogens: the uropygial gland secretion (Ruiz-Rodríguez *et al.* 2009, Czirják *et al.* 2013).

Uropygial gland

Uropygial gland is a holocrine gland, exclusive to most species of birds, located on the caudal region (Clark 2004). It is also known as *preen oil* or *coccygeal gland*. This organ is present in all species of birds, except for some

species of the order Struthioniformes, Piciformes, and Psittaciformes (Johnston 1988). The morphology of this gland varies in both size and structural proportion among different species (Vincze *et al.* 2013) (Figure 1).

Figure 1. *Width of the uropygial gland of house sparrow in the aviary.*

The uropygial gland produces a wax or oil (known as *uropygial secretion* or *preen oil*) that is stored in the lobe of the primary sinus (Figure 2). During preening, birds stimulate the uropygial gland with their bill to get the preen oil, and spread the secretion on their feathers with the aim to protect them against degrading agents (Clark 2004; Fülöp *et al.* 2016).

Figure 2. *Collect with a microcapillary of the uropygial secretion of a house sparrow.*

The composition of uropygial secretion is a very complex mixture of lipids, carotenoids and fatty acids esterified with different types of alcohol (Amat *et al.* 2011, Campagna *et al.* 2012, Soini *et al.* 2013). It may vary at both intraspecific (Whittaker *et al.* 2010) and interspecific level (Haribal *et al.* 2005).

It has been proposed that the uropygial secretion benefit bird fitness through several functions, although evidences are still scarce (*see review* in Moreno – Rueda 2017). For example, preen secretion can contribute to plumage maintenance avoiding feather abrasion or reducing feather degradation by keratinophilic organisms with its antimicrobial and antifungal properties (Rodríguez-Ruano *et al.* 2015, Fülöp *et al.* 2016). Also, uropygial secretion improves waterproofing, but the mechanisms is still unknown (Moreno – Rueda 2017). Even, some functions are controversial and studies have shown mixed and inconclusive results. For example, some studies have suggested that uropygial secretion might atract vectors such as

Culex mosquitoes (Russell and Hunter 2005), black flies (Fallis and Smith 1964, Bennett *et al.* 1972) or haematophagous mites (Zeman 1988), which may impair bird fitness.

For this reason, in the present Thesis I present several objectives with the aim to clarify the relationship between uropygial gland and the infection by blood parasites.

In the last decade, the number of studies focused on the mechanisms on host-parasites interactions has increased. However, the knowledge about uropygial gland is still scarce and limited (Moreno – Rueda 2017). For example, results for many of the potential functions have been mixed and without firm conclusions. In this sense, (1) studies analysing the relationship between uropygial secretion and vectors of haemosporidian parasite show inconclusive results (e.g. Shawkey *et al.* 2003, Russell and Hunter 2005, Bernier *et al.* 2008, Martínez de la Puente *et al.* 2011). Consequently, the link between haemosporidian parasites and uropygial secretion remains unclear.

Moreover, there is a lack of evidence concerning fitness consequences of the uropygial gland. For example, (2) whether uropygial secretion may influence survival prospects of birds is still unknown. While some researchers have suggested that the lack of this gland would not affect survival in pigeons and ducks (Elder 1954, Salibian and Montalti 2009), other investigation have suggested that uropygial gland would benefit survival because there is a relationship between size of uropygial glands and probability of capture by aerial predators (Møller *et al.* 2010a).

Additionally, antimicrobial activity of uropygial secretion has been proposed to increase hatching success on birds (Møller *et al.* 2010b). However, there are only few correlational studies analysing the relationship

between reproductive success with uropygial gland size (see review in Moreno – Rueda 2017), and thus further studies are needed to determine the importance of the uropygial gland on breeding success (3).

Moreover, it has been theorised that defensive mechanisms may benefit invasive species in their colonization process (*Invasive Immunity Hypothesis*, Lee & Klasing, 2004). But this hypothesis have been poorly tested, and the possible role or uropygial gland on colonization success of invasive bird species has not been explored yet (4).

Finally, some studies have proposed that the inter-species variation in the size of the uropygial gland and the volume of its secretions have evolved as a consequence of divergent selection by parasites on their hosts (Møller *et al.* 2009, Pap *et al.* 2013). However, this hypothesis has not been widely tested (5), and it deserves further investigation.

These five gaps on the knowledge on the role of uropygial gland on host – parasite interactions constitute the main research core of this thesis. We will now briefly introduce each of these investigations.

CHAPTER I. ***Uropygial gland secretion and avian malaria parasites.***

The uropygial gland secretion has been proposed to play an important role in host-parasite relationships because it may affect the interaction between birds and their ectoparasites. However, the possible role of the uropygial gland in protection against haemosporidian parasites still remains unclear. On one way, the properties of the uropygial secretion can reduce the haemosporidian vector attraction, and thus minimize the likelihood of being infected with these blood parasites (Clayton *et al.* 2010). On the other hand, some other studies have suggested that uropygial secretions may be used by

haemosporidian vectors to locate potential hosts and infect them (Fallis and Smith 1964, Russell and Hunter 2005). In this chapter, we explored the relationship between uropygial gland size, antimicrobial activity of uropygial secretions and malaria infection in house sparrows *Passer domesticus*. We used nested-PCR to identify blood parasite infection (Bensch *et al.* 2000, Waldenström *et al.* 2004) and flow cytometry for detecting absolute cell counting assessing antimicrobial activity of the uropygial secretion.

If the properties of uropygial secretions can decrease the attraction of vectors to birds, then we expect a lower prevalence of malaria infection in sparrows with larger uropygial glands. In contrast, if haemosporidian vectors are attracted to the uropygial secretion, then we should expect that individuals with larger uropygial glands were more prone to infection with malaria than birds with smaller glands.

We also explored the relationship between antimicrobial activity of the uropygial secretion and blood parasite infection. If haemosporidian infection can be mediated by odour stimuli produced by skin and plumage bacteria attracting insect vectors, we should expect that sparrows with higher antibacterial activity of their gland secretions would have a lower probability of being infected with these blood parasites.

CHAPTER II. *Uropygial gland, avian malaria infection and survival in migratory house martins.*

Bacteria, fungi and other pathogens such as malaria and related haemosporidians can regulate populations of their hosts by negatively affecting their growth, body condition, reproductive success and survival (Schmid-Hempel 2011). With the aim to face this parasite challenge, animals have developed a vast variety of defensive mechanisms, allowing them to resist or eliminate parasitic infections, and to reduce their negative fitness costs (Demas and Nelson 2011). The uropygial gland secretion has been proposed to act as defensive barrier of skin and plumage in the fight against bacteria and fungi (Jacob and Ziswiler 1982, Jacob *et al.* 1997, Fülöp *et al.* 2016), and may prevent birds from acquiring haemosporidian infections (Magallanes *et al.* 2016). Thus, uropygial secretion of birds may favour survival of individuals. However, this role of uropygial gland secretion remains unknown.

In this chapter, we explore whether the size of the uropygial gland may influence the survival of house martins, an Afro-Paleartic migratory bird species with global population decline (BirdLife International 2018). This bird species show high breeding site fidelity. This enabled us to build a capture history for each individual during 2013–2016, indicating the survival of individuals. If uropygial gland secretion can influence survival, we predict that house martins with larger uropygial glands to have higher probabilities of survival than birds with smaller glands.

We also used nested- PCR to detect the infection of malaria in house martins to test if haemosporidian infection affects survival in this bird species. Because malaria infection increase mortality of bird hosts, we predict that this parasite infection decrease survival in house martins.

CHAPTER III. ***Reproductive success and uropygial gland volume in the barn swallow.***

Pathogens and parasites provoke detrimental effects on the fitness of their bird hosts, by reducing survival and diminishing their reproductive success (Møller 2005, Schmid-Hempel 2011). To avoid parasite infection and to minimize their negative effects, animals have developed a wide range of defensive mechanisms such as uropygial gland. This gland secretes waxes with antimicrobial and antifungal properties, which may protect birds against microorganisms that may negatively affect reproductive success. However, the numbers of studies exploring benefits of uropygial secretions on breeding success are still scarce and with mixed results.

During 2015-2017, we monitored four breeding colonies of barn swallows in Southwestern Spain to explore if the size of uropygial gland is related to reproductive success in barn swallows. If anti-pathogen properties of uropygial secretion could minimize the negative effects of parasites on reproductive success, then we should expect that barn swallows with larger uropygial glands should have higher breeding success than barn swallows with smaller uropygial glands.

Because the infection incidence increases with the average number of contacts between infected and susceptible individuals (Johnson *et al.* 2011), and the rate of contact is assumed to increase linearly with host density (Anderson and May 1978), we also aim to explore the role of abundance of conspecific may have on the relationship between uropygial gland size and reproductive success. If a higher number of swallows nesting within the same room may determine the abundance and transmission of pathogens (Begon *et al.* 2002), then we should expect that anti-pathogen properties of uropygial secretions may be more important for swallows that

breed close to each other compared to swallows that breed further away one from each other.

CHAPTER IV. *Influence of uropygial gland and malaria parasites in invasion success of house sparrow.*

In last centuries, several bird species have been successfully introduced and become invaders in many parts of the world, both naturally and with human assistance (Blackburn *et al.* 2009). These invasions may represent a serious problem for wildlife, agriculture and even human health. Despite the economic and ecological importance of invasive species and the efforts from scientists to understand biological invasions, the mechanisms that allow one species to become invasive are still poorly understood. Many introduced species fail to establish or to spread significantly, but many others become successful colonizers; hence, we questionnaire what make a species a successful invader.

On one hand, parasites and other pathogens have been proposed to play an important role on the invasive process, facilitating or limiting colonization and spread of their hosts in new continents and islands (Tompkins *et al.* 2011). Malaria and related haemosporidian parasites (genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) have been proposed to play a major role on the successful colonization of exotic bird species. Its broad distribution, its complex cycle life and its lethal effects make malaria parasites one of the most dangerous blood parasites in nature with no preventive treatment. For all these reasons, the International Union for Conservation of Nature (IUCN) classifies some *Plasmodium* species within the 100 world's worst invasive species (Lowe *et al.* 2000).

On the other hand, individuals may face new parasites and pathogens in the new areas during colonization by increasing investment in immune defences, thus allowing colonizers to combat these new pathogens. Therefore, individuals with better immune defences may enjoy better invasion success (*Invasive Immunity Hypothesis*, Lee and Klasing 2004).

In this chapter we test several host – parasite hypotheses accounting for invasion success to analyze whether avian malaria parasites and / or uropygial gland secretion may favored the successful colonization of house sparrows *Passer domesticus* in Peru. We first explore the identity and prevalence of haemosporidian parasites in house sparrows from the colonized area (Peru) and a native range (Spain) to analyse the potential contributions of the Novel Weapon Hypothesis and the Enemy Release Hypothesis to the successful invasion of this bird species. We also examine the size of the uropygial gland and the antimicrobial activity of its secretions to analyse whether uropygial gland secretions may have facilitated the establishment of individual sparrows in these new areas. If the properties of uropygial secretions could help sparrows in their spread to new areas (i.e. facing new pathogens), then we expect larger uropygial glands and / or higher antibacterial activity of the gland secretions of Peruvian house sparrows.

CHAPTER V. ***Analyses of variation of uropygial gland secretion in relation to different pathogen exposure: Tropical vs. Temperate bird species.***

The *Latitudinal diversity gradient* theory suggests increase in species richness or biodiversity from the poles to the tropics. This pattern seems also to be the case for pathogens and parasites, which usually are more diverse

and abundant in regions close to Equator (Nunn *et al.* 2005). Because the negative effects imposed by pathogens to their hosts, it is expected that host defences may differ geographically among hosts according to parasite diversity and abundance (Morand and Krasnov 2010). Following this idea, the *Adjustment to parasite pressure* hypothesis predicts that defensive mechanisms among hosts should vary in relation to the selective pressure imposed by parasites (Hasselquist 2007).

Several studies have shown a wide inter-species variation in the size of the uropygial gland and the volume of its secretions (Johnston 1988, Vincze *et al.* 2013). It has been proposed that this variation may have evolved as a consequence of divergent selection by pathogen on their hosts (Møller *et al.* 2009, Pap *et al.* 2013). However, this hypothesis has so far been poorly explored, and it deserves further investigation. Also, some recent studies have suggested that uropygial secretions may prevent birds from acquiring malaria infections (Magallanes *et al.* 2016, Marzal *et al.* 2018). However, this suggested function is still poorly studied and limited to investigations in two bird species (house sparrows and house martins). Therefore, this potential anti-malarial role should be studies in more bird-malaria systems.

In the last chapter of the thesis we first test for differences in the volume of uropygial gland in bird species living in environments with different pathogen exposure (Neotropical vs. Temperate zones), controlling for phylogenetic differences among bird species. If the uropygial gland has been evolved as a defensive mechanism in the fight against pathogen exposure (as suggested from the *Adjustment to parasite pressure* hypothesis), then we should expect that bird species from the Neotropics should have larger uropygial glands than those from temperate zone. We also analyse the potential role of the uropygial gland in avoiding haemosporidian

infection. If the uropygial gland prevents birds from acquiring haemosporidians, we should expect birds with larger uropygial glands to have lower prevalence of malaria infection.

AIM OF THE THESIS

The general aim of this thesis is to explore the role of the uropygial secretion on host – parasite interactions, mainly drawing towards the role of preen gland as defensive barrier against malaria parasites and its potential contribution on bird fitness. We focused our attention on understanding the importance of the uropygial secretion in life – history traits of wild birds. Specifically, the main objectives of the thesis can be listed as follows:

1. To test the relationship between volume and antimicrobial activity of uropygial secretion and the malaria infection in house sparrows (chapter I).
2. To analyse the effect of the uropygial volume and malaria infection in the survival prospects of house martin (chapter II).
3. To examine the relationship between uropygial gland volume and reproduction success in the barn swallow (chapter III).
4. To identify the role of malaria infection, and the antimicrobial activity and volume of uropygial secretion in the invasion success of house sparrows in Peru (chapter IV).
5. To explore the variation of preen volume as in relation to different pathogen exposure in different species of birds (chapter V).

SUMMARY OF RESULTS AND CONCLUSIONS

Chapter I

Volume and antimicrobial activity of secretions of the uropygial gland are correlated with malaria infection in house sparrows

We found an association between uropygial gland secretion and malaria infection in house sparrows. Specifically, the volume of the uropygial gland was larger in uninfected than in infected house sparrows. (Figure 3A). In addition, antimicrobial activity of uropygial secretion was significantly higher in uninfected than in infected birds (Figure 1B). Our results support the idea that uropygial secretion may reduce the concentration of bacteria in feathers to reduce vector attraction. These findings suggest that uropygial glands may be involved in defensive mechanisms against malaria infections under natural conditions.

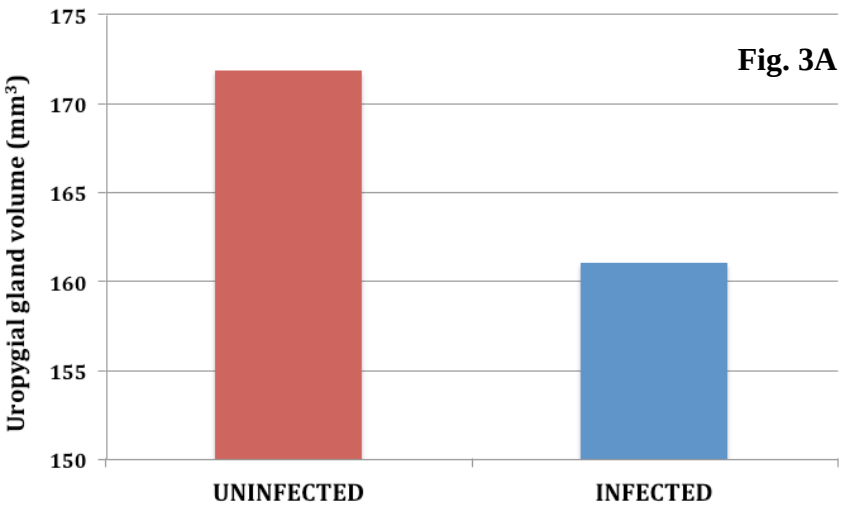


Fig. 3A

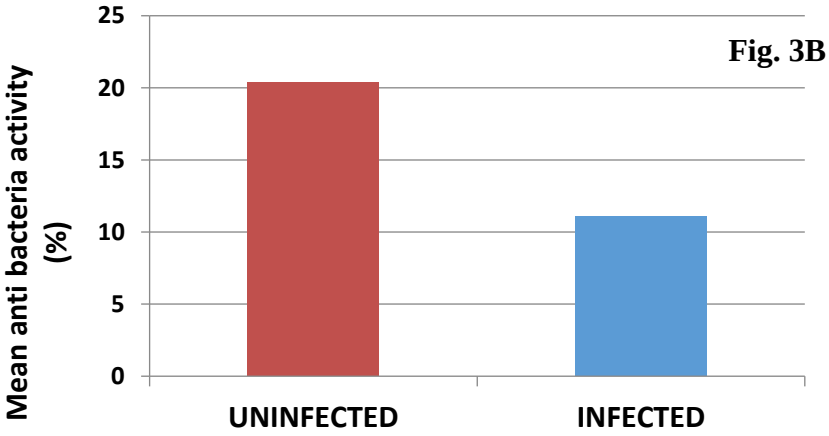


Figure 3. (A) Mean uropygial gland volume and (B) mean antimicrobial antibacteria activity in malaria infected and uninfected house sparrows.

Chapter II

Uropygial gland volume and malaria infection are related to survival in migratory house martins.

We found that the effect of gland size on survival prospects of House martins depended on malaria infection: infected house martins with larger uropygial glands were better able to survive to the next breeding season, while infected birds with small uropygial glands did not survive (Figure 4). These results highlight the importance of the uropygial gland secretion in the life history of wild birds.

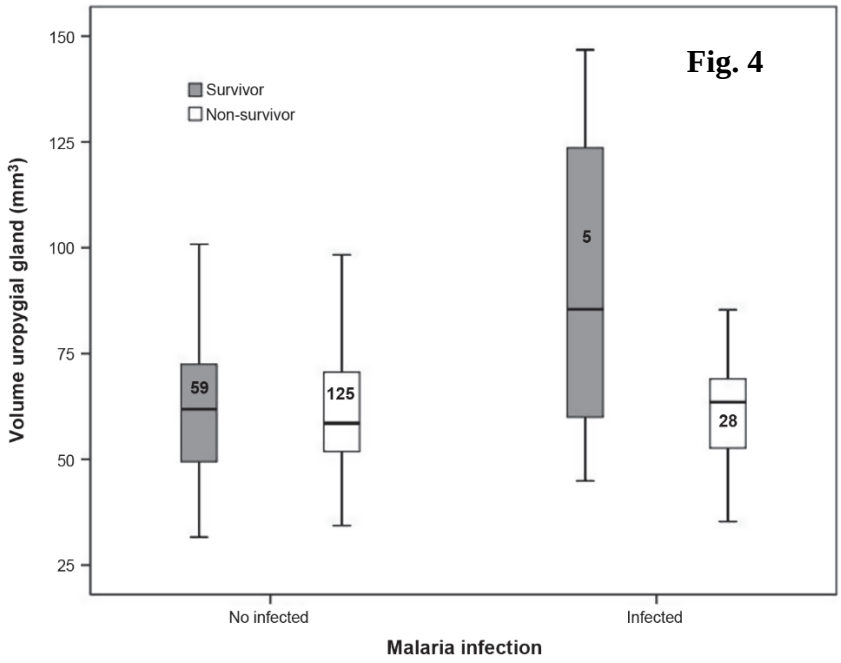


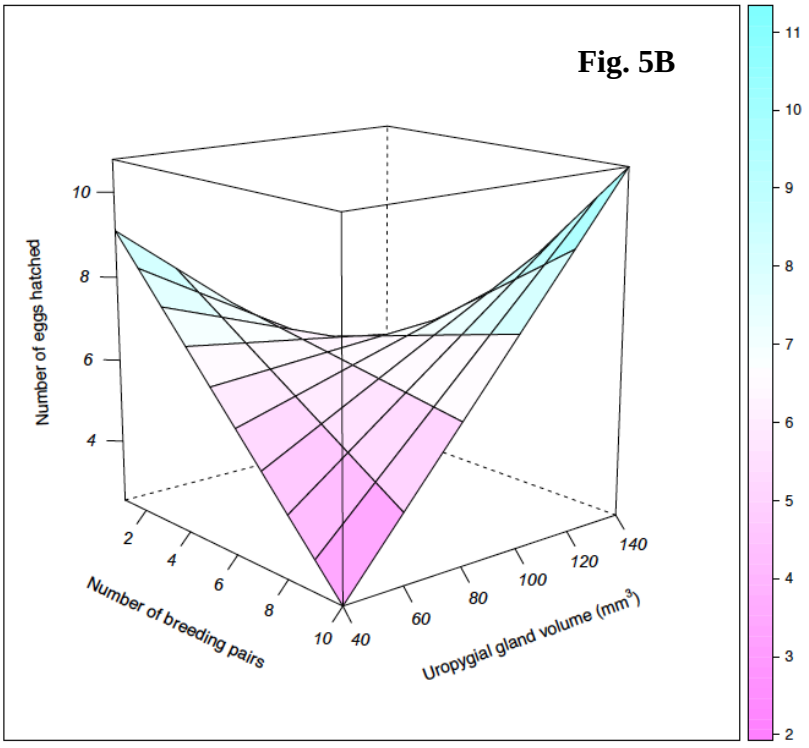
Figure 4. Box plots showing volumes of uropygial gland for malaria infected and no-infected house martins with concern to survival prospects. Values are medians, upper and lower quartiles and extreme observations. Sample sizes of each category are shown inside the box-plots.

Chapter III

Uropygial gland volume is related to reproductive success in the barn swallow Hirundo rustica

In this chapter, we analysed if the size of uropygial gland is related to reproductive success (clutch size, number of hatching eggs and number of fledglings) in barn swallows from four different colonies. We found that reproductive success varied with the interaction term between uropygial gland volume and abundance of conspecifics. Barn swallows with larger uropygial glands had higher breeding success (greater number of eggs

hatched (Figure 5A) and fledglings reared (Figure 5B)) when living in environments with higher abundance of conspecifics. These results may imply a plastic response of uropygial gland volume in barn swallows living in environments with higher pathogen exposure.



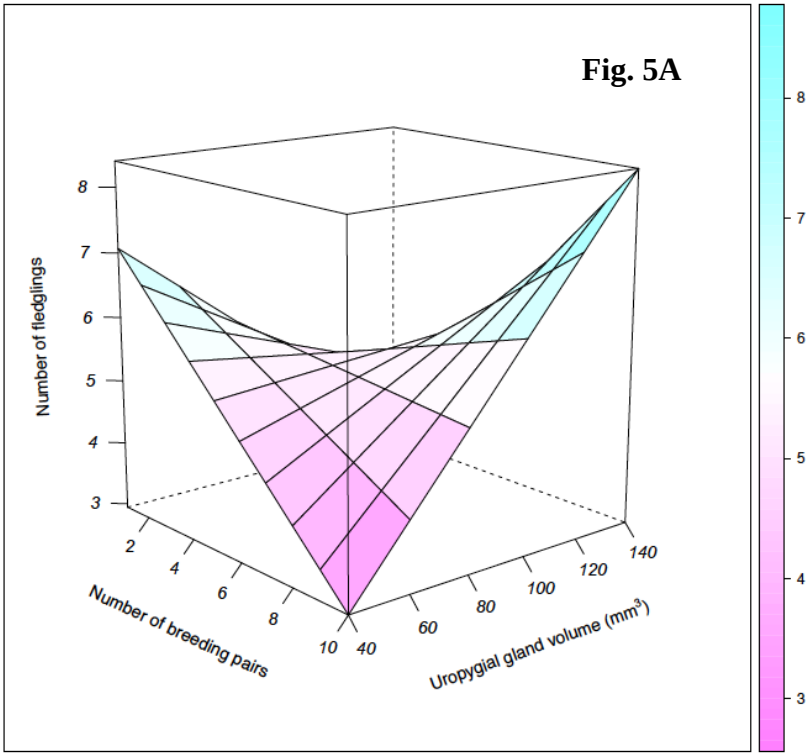


Figure 5. Relationship between uropygial gland volume, number of eggs hatched (5A) or number of fledglings (5B) and number of pairs nesting within the same room. Sample size was 95 barn swallows.

In contrast, barn swallows with larger uropygial glands had lower reproductive success (i.e. less eggs hatched and fledglings reared) when nesting in rooms with lower abundance of conspecifics. These outcomes suggest a trade-off between investment in tow energetically costly traits such as reproduction and defensive mechanisms. The production of larger uropygial secretion volumes in environments with lower pathogen pressure (i.e. few individuals nesting within the same room) may impair resource allocation to other energetically demanding traits such as reproduction.

Chapter IV

Variation in malaria infection and immune defence in invasive and endemic house sparrows.

Prevalence of haemosporidian parasites and locality explained significant variation in size of the uropygial gland. Specifically, Peruvian house sparrows (invasive house sparrows) had larger uropygial gland (Figure 6A) and higher antimicrobial activity of uropygial secretion (Figure 6B) than house sparrows from Spain (endemic house sparrows). Additionally, non-infected house sparrows showed larger volume of preen gland (Figure 6A) and higher antimicrobial activity (Figure 6B) and than infected sparrows, independently of locality.

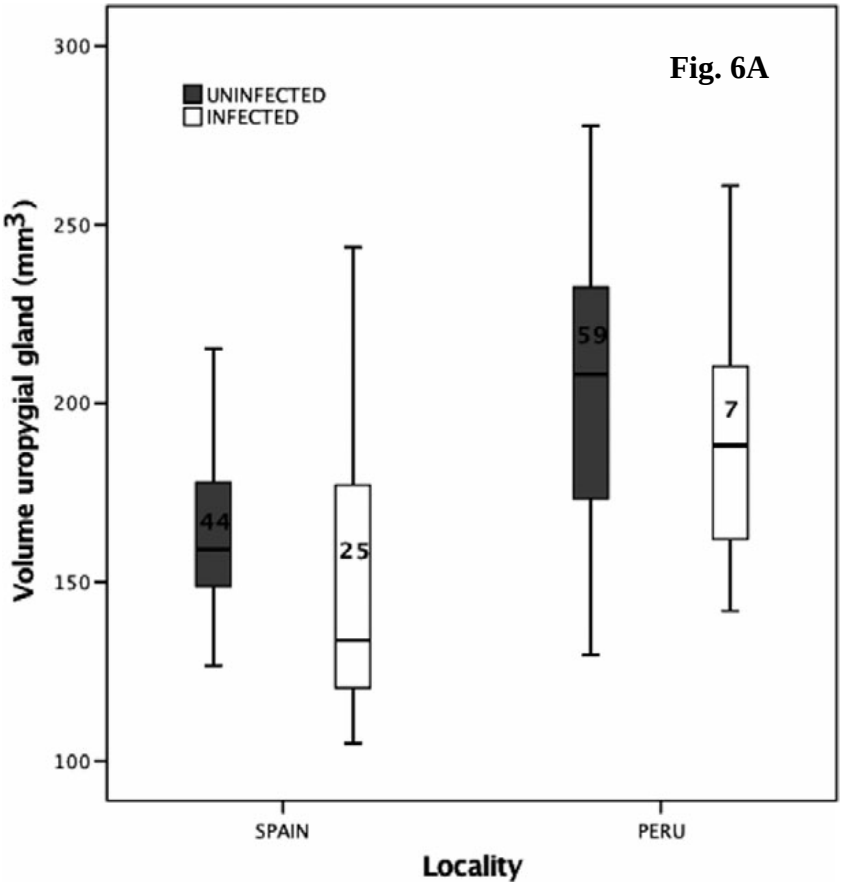


Fig. 6A
UNINFECTED
INFECTED
Volume uropygial gland (mm3)
300
250
200
150
100
44
25
59
7
SPAIN
PERU
Locality

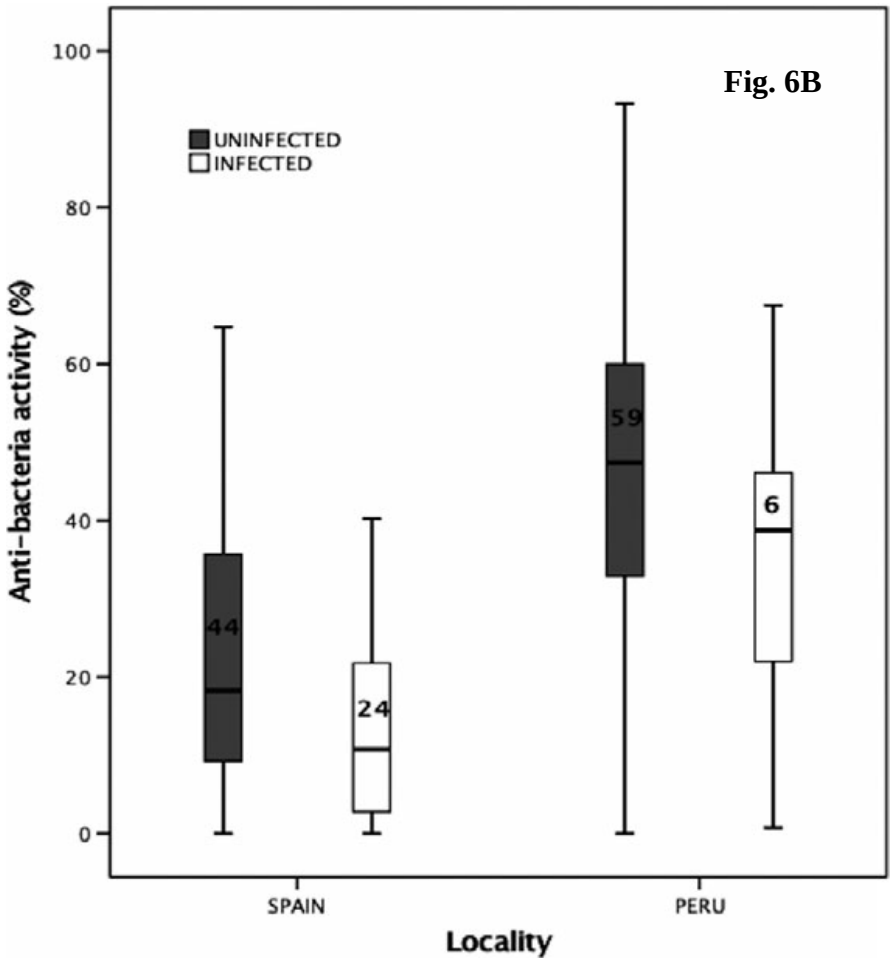


Figure 6. Box plots showing (A) volumes of uropygial gland and (B) anti-bacterial activity (%) of the uropygial gland secretion for malaria infected and non-infected house sparrows with concern to locality. Values are medians, upper and lower quartiles and extreme observations. Sample sizes of each category are shown inside the box-plots.

Our results suggest that the release from their natural haemosporidian parasites and strong defensive traits (e.g. larger uropygial glands and higher

anti-microbial activity in uropygial secretions) may favour individual sparrows in their colonization and spread in Peru. These outcomes provide essential information for identifying potential invaders and designing interventions.

Chapter V

Differences in the uropygial gland size between temperate and neotropical birds

In this study we first analysed the size of the uropygial gland of 1334 individual belonging to 36 bird species from three Neotropical (Peru) and three temperate areas (Spain). We found that mean corrected uropygial gland volume was significantly larger in bird species from the tropics than from temperate areas (Figure 7), which is consistent with the relative size of this defensive organ being driven by selection imposed by parasites.

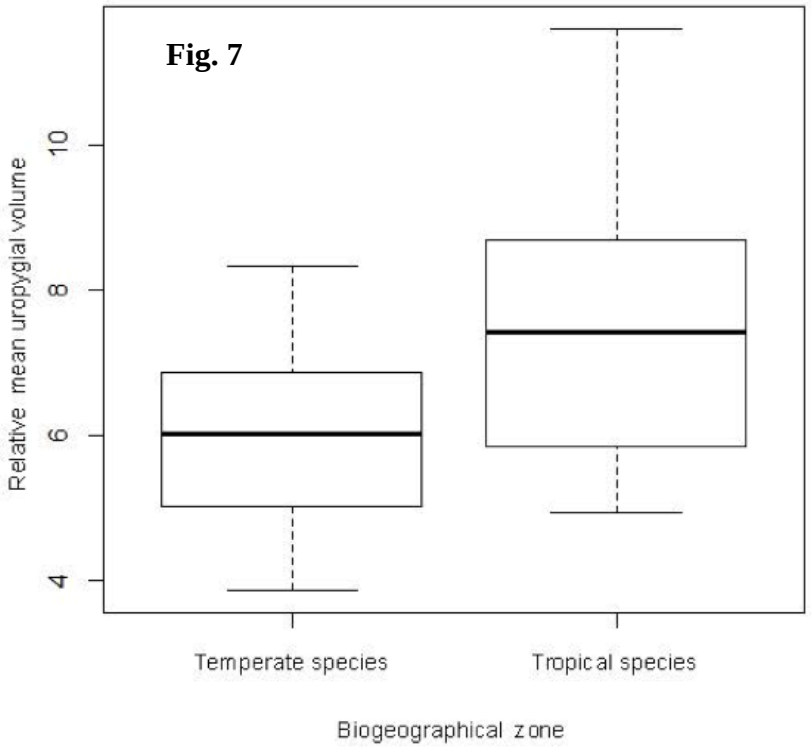


Figure 7. Box plots showing adjusted volume of uropygial gland (mm^3) for tropical species (N = 16) and temperate species (N = 20). Values are medians, upper and lower quartiles, and extreme observations.

We also explored that potential role of the uropygial gland as a means of avoiding haemosporidian infection, showing that individuals with relative larger uropygial glands had lower mean prevalence of haemosporidian infection, regardless of their geographical origin (Figure 8). This result agrees with our previous findings suggesting that secretions from the uropygial gland reduce the likelihood of becoming infected with haemosporidians.

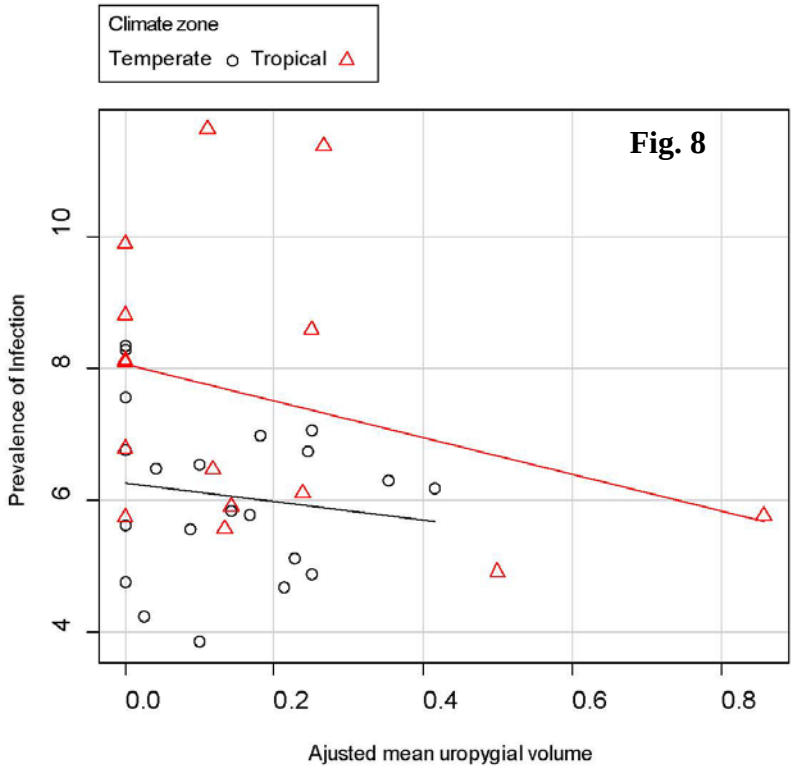


Figure 8. Scatterplot showing the relationship between prevalence of malaria infection and the adjusted volume of the uropygial gland (mm^3) (circles, N = 20) temperate species and (triangles, N = 16) tropical species. The lines are the linear regression lines.

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Chapter I

Volume and antimicrobial activity of secretions of the uropygial gland are correlated with malaria infection in house sparrows.

Autor de la fotografía "Pareja de Gorriones" ***José Ángel Rodríguez***

EL VOLUMEN Y LA ACTIVIDAD ANTIMICROBIANA DE LA SECRECIÓN DE LA GLÁNDULA UROPIGIAL ESTA CORRELACIONADA CON LA INFECCIÓN DE MALARIA EN EL GORRIÓN COMÚN

Resumen

Los animales han desarrollado una amplia gama de mecanismos defensivos contra los parásitos para reducir la probabilidad de infección y los costes fisiológicos que estos provocan. La glándula uropigial es una glándula exocrina que produce una sustancia antimicrobiana y antifúngica que las aves usan como barrera defensiva en piel y plumas. Se ha propuesto que esta secreción puede afectar a la interacción entre el hospedero (el ave) y el ectoparásito (vector). Dado que la secreción uropigial puede constituir un mecanismo de defensa frente a los ectoparásitos, esto puede reducir la prevalencia de infección de parásitos sanguíneos que son trasmitidos por vectores ectoparásitos. Por otra parte, otros estudios señalan que los vectores podían ser atraídos por la secreción uropigial por lo que se incrementaría la probabilidad de infección en los individuos con mayor secreción. En este estudio hemos explorado la relación entre el tamaño de la glándula uropigial y la actividad antimicrobiana de la secreción uropigial y la infección por malaria del gorrión común *Passer domesticus*. Se ha visto que los gorriones no infectados por malaria tenían la glándula uropigial más grandes y además la secreción de esta glándula tenía mayor capacidad antimicrobiana que la de los individuos infectados. Encontramos también una relación positiva entre el tamaño de la glándula uropigial y el índice de masa corporal, pero esta relación estaba solo presente en individuos no infectados. En cuanto a las hembras de gorrión común tenían mayores

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Volume and antimicrobial activity of secretions of the uropygial gland are correlated with malaria infection in house sparrows

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Abstract

Background: Animals have developed a wide range of defensive mechanisms against parasites to reduce the likelihood of infection and its negative fitness costs. The uropygial gland is an exocrine gland that produces antimicrobial and antifungal secretions with properties used as a defensive barrier on skin and plumage. This secretion has been proposed to affect the interaction between avian hosts and their ectoparasites. Because uropygial secretions may constitute a defense mechanism against ectoparasites, this may result in a reduction in prevalence of blood parasites that are transmitted by ectoparasitic vectors. Furthermore, other studies pointed out that vectors could be attracted by uropygial secretions and hence increase the probability of becoming infected. Here we explored the relationship between uropygial gland size, antimicrobial activity of uropygial secretions and malaria infection in house sparrows *Passer domesticus*.

Methods: A nested-PCR was used to identify blood parasites infection. Flow cytometry detecting absolute cell counting assessed antimicrobial activity of the uropygial gland secretion

Results: Uninfected house sparrows had larger uropygial glands and higher antimicrobial activity in uropygial secretions than infected individuals. We found a positive association between uropygial gland size and scaled body mass index, but only in uninfected sparrows. Female house sparrows had larger uropygial glands and higher antimicrobial activity of gland secretions than males.

Conclusion: These findings suggest that uropygial gland secretions may play an important role as a defensive mechanism against malaria infection.

Keywords: Antimicrobial activity, Flow cytometry, Haemosporidian parasites, *Passer domesticus*, Preen gland

Background

Parasites are ubiquitous and the most abundant organisms on Earth [1, 2]. They cause harmful effects on their hosts and negatively influence different host fitness components, such as growth [3], survival [4], fecundity [5] and reproductive output [6, 7]. Because of these effects exerted by parasites, animal hosts have developed a wide range of defensive mechanisms in order to reduce the likelihood of infection and/or its negative effects [8, 9]. These mechanisms include natural resistance to infection such as physical barriers to invading pathogens or high-density lipoproteins in human serum destroying trypanosomes [8, 10], nonspecific immune responses such as generation of oxidative products by the phagocytes to destroy microbes [11], specific immune responses such as the production of a variety of antibodies that bind to specific pathogens [12], or behaviours aiming to control exposure to parasites [13, 14].

Avian malaria and related haemosporidian parasites are among the most pathogenic species of poultry and wild birds [15]. These widespread organisms cause detrimental effects on life history of their avian hosts by reducing

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survival [16, 17], reproductive success [7, 18, 19] and body condition [20, 21]. They show a complex life-cycle [15], in which the presence of a vector (biting midges, black flies, louse flies and a large number of mosquito species) is needed for transmission of the disease [15, 22].

The uropygial gland (also called preen gland) is an exocrine gland of birds secreting waxes with antimicrobial and antifungal properties that is smeared on the plumage during preening and hence acting as defensive barrier of skin and plumage [23–27]. This secretion has been proposed to play an important role in host-parasite relationships because it may affect the interaction between birds and their vectors [28, 29]. However, the possible role of the uropygial gland in protection against haemosporidian parasites still remains unclear.

Uropygial secretions may prevent birds from acquiring blood parasite infection. Mammalophilic and ornithophilic mosquitoes and other ectoparasites act as disease vectors that rely on different cues to locate their potential blood hosts. Host finding by vectors is largely driven by visual stimuli [30], exuded heat resulting from metabolic activity of hosts [31] and odorant and volatile organic chemicals produced by skin and plumage bacteria that are emitted by individual hosts [32–34]. Therefore, the antimicrobial activity of the uropygial secretion from birds can decrease feather and skin microbiota and hence reduce the emission of chemical cues used by haemosporidian vectors. This should minimize the likelihood of being infected with these blood parasites. In addition, compounds isolated from secretions of the uropygial gland have an insecticidal effect [35] and can act as ectoparasite repellents [36]. Therefore, these secretions may also decrease the probabilities of haemosporidian infection.

Alternatively, antiparasite defences may negatively affect hosts when such defences become attractants for parasites or vectors. Following this idea, several studies have indicated that uropygial secretions may be used by haemosporidian vectors to locate potential hosts and infect them. For example, Lowther & Wood reported that some species of black flies were strongly attracted to uropygial gland extracts from common loon (*Gavia immer*) [37]. Similarly, Fallis & Smith pinpointed the uropygial gland as one of the main sources of black fly attraction to common loons [28]. Moreover, Bennett et al. successfully used ether extract of the uropygial gland of the common loon to attract simulids [38]. More recently, Russell & Hunter showed that traps baited with uropygial secretion from American crows *Corvus brachyrhynchos* captured more *Culex* mosquitoes, one of the main avian malaria vectors [15], than unbaited blank control traps [29].

Here we investigate these two hypotheses on the role of uropygial gland secretions on haemosporidian infections in the house sparrow *Passer domesticus*, one of the most ubiquitous hosts for avian malaria [39]. First, we analyse the relationship between haemosporidian infection and the size of the uropygial gland, a reliable measure of the volume of produced secretion [40]. If the properties of uropygial secretions can minimize the attraction of vectors to birds, then we expect a lower prevalence of haemosporidian infection in individual birds with larger uropygial glands. In contrast, if haemosporidian vectors are attracted to the uropygial secretion, then we should expect that sparrows with larger uropygial glands were more prone to infection with haemosporidians than birds with smaller glands. Secondly, we explore the relationship between antimicrobial capacity of secretions from the preen gland and blood parasite infection. If haemosporidian infection can be mediated by odour stimuli produced by skin and plumage bacteria attracting insect vectors, we should expect that sparrows with higher antibacterial activity of their gland secretions would have a lower probability of being infected with these blood parasites.

Methods

Study sites and sample collection

The study was carried out in a rural (38°39'N, 7°13'W) and an urban population (38°53'N, 7°00'W) near Badajoz, southwest Spain between November - December 2014. We captured 222 adult house sparrows (55 sparrows in one urban site, 167 sparrows in one rural site) in five days during the same week with mist-nets and recorded their body mass with a digital balance to the nearest 0.1 g. We measured tarsus length with a digital calliper to the nearest 0.01 mm. We used body mass and tarsus length to calculate scaled body mass index [41], which is a reliable estimate of animal physical condition [42]. Each individual was individually identified with a numbered metal ring and sexed according to Svensson et al. [43]. One microcapillary of blood (70 μl) was obtained from the brachial vein of each individual and stored in 500 μl of SET buffer (0.15 M NaCl, 0.05 Tris, 0.001 M EDTA, pH 8.0) until DNA extraction.

Molecular detection of blood parasite infections

Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.) were detected from blood samples using molecular methods [44, 45]. DNA from the avian blood samples was extracted in the laboratory using a standard chloroform/isoamylalcohol method [46]. Diluted genomic DNA (25 ng/μl) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested-PCR protocols described by Waldenström et al. [45]. The amplification was evaluated by running 2.5 ml of the final PCR on a 2 % agarose gel. All PCR experiments contained one positive control and

one negative control for every eight samples. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all positives were true.

Volume of the uropygial gland secretion

We recorded length, height and width of the uropygial gland with a digital calliper with a precision of 0.01 mm. Uropygial gland volume was estimated as the product of length, height and width [47], which is positively related to the volume of uropygial gland secretions [26, 40, 48]. Because the uropygial gland is a soft tissue [26, 48], we measured the three dimensions of uropygial gland three times to calculate repeatability [26, 49, 50].

We also extracted all the secretion available in the uropygial papilla immediately after capture of 44 individuals from the same location, following the extraction protocol described by Martín-Vivaldi et al. [48]. Briefly, we first washed the uropygial gland and surrounding skin with a cotton swab soaked in ethanol to reduce the risk of contamination of the secretion. After evaporation of the alcohol, the papilla was softly pressed with a finger to expel the secretion and transfer it into a microcapillary tube until the papilla was empty. Immediately after extraction we estimated the volume of the secretion in the filled capillary tube with a digital calliper with an accuracy of 0.01 mm. The extracted secretion was transferred to a sterile Eppendorf vial and kept at about 4 °C in a portable icebox, and stored in the laboratory at -20 °C during the next 4 h until analyses of anti-microbial activity.

Bacterial growth and antimicrobial activity of uropygial gland secretions

The pellet of *Staphylococcus epidermidis* (ATCC ® CRM – 12228™) was re-suspended in 6 ml of Luria - Bertani (LB) media to an OD 0.4–0.6 and incubated at 37 °C with shaking for 24 h. The bacterial suspension was then centrifuged in a Microfuge (Beckman Coulter) for 6 min at 2000 *g*. After discarding the supernatant, the bacterial pellet was re-suspended carefully in 20 ml LB solution. A total of 200 μl per well of bacterial suspension were then dispensed in a 96-well plate. Uropygial secretions were diluted 1:1 in dimethylsulfoxide (DMSO); 1 μl of the uropygial secretion diluted with DMSO was added to the bacteria culture in each well. Four wells on a 96-well plate with bacteria solution were not added with uropygial secretions but with 1 μl of DMSO, as they were used as controls of bacterial growth. After culture incubation for bacterial growth at 37 °C for 24 h, the 96-well plate was covered and centrifuged in a plate centrifuge (Selecta, Spain) for 5 min at 2000 *g* and the pellet were re-suspended in 200 μl of PBS at a final concentration of 0.6 μg/ml. Samples were then incubated at 37 °C for 30 min in the dark with shaking. Flow cytometry detecting absolute cell counting assessed antimicrobial activity of the uropygial gland against *S. epidermidis* secretion. This technique is a rapid, accurate and highly reproducible methodology used in clinical microbiology to monitor antimicrobial activity [51]. A total of 50 μl of the cell suspension from each well was acquired using a MACSQuant® X (Miltenyi Biotec) flow cytometer that allows absolute cell counting. Antimicrobial activity was evaluated by comparison of cell counting (bacterial growth) in wells with presence or absence (controls) of uropygial secretion.

Statistical procedures

Repeatability of uropygial gland measurements was calculated following the approximate Gaussian LMM using REML estimation ($R_{M(REML)}$) described by Nakagawa & Schielzeth [52]. We performed Shapiro-Wilk test for normality of distribution of data and used general linear models (GLM) to investigate the relationship between sex, locality (i.e. environmental variation), scaled body mass index, infection status (uninfected or infected) and the two-way interactions between sex and scaled body mass index, between infection status and scaled body mass index, and between infection and locality, on the uropygial gland volume. We used Pearson correlation test to determine the strength of association between uropygial gland volume and scaled body mass index regarding to sex and infection. We also used a GLM to evaluate the correlation between sex, uropygial gland volume, scaled body mass index and infection status (uninfected or infected) on the antimicrobial activity of the uropygial secretion. All analyses were performed using R version 3.2.2 [53] and JMP [54].

Ethics statement

Methods were evaluated and approved by Institutional Commission of Bioethics of University of Extremadura (CBUE 49/2011). All the experiments comply with the current laws of Spain, where the experiments were performed.

Results

We analysed 222 blood samples from house sparrows in search of blood parasites. A total of 74 % (165 individuals) were uninfected and 26 % (57) were infected with blood parasites (13.5 % of sparrows infected in the rural location, 49.1 % of sparrows infected in the urban location).

The uropygial gland volume and the antimicrobial activity of the uropygial secretion showed a normal distribution (Shapiro-Wilk normality test; Uropygial gland volume: $N = 222$, $W = 0.923$, $P > 0.001$; antimicrobial

activity: $N = 44$, $W = 0.943$, $P = 0.030$). We found a high repeatability between measurements of length, width and height of uropygial gland (all $R > 0.81$ and $P < 0.05$).

Prevalence of haemosporidian parasites, sex and locality explained significant variation in size of the uropygial gland. In contrast, scaled body mass index was not significantly correlated with size of the uropygial gland (Table 1). Specifically, the volume of the uropygial gland was larger in uninfected than in infected house sparrows [mean uropygial gland volume (standard deviation, SD): uninfected = 171.84 mm^3 (43.22); infected = 161.03 mm^3 (34.33)]. Furthermore, the volume of the uropygial gland differed between the sexes, with females having larger glands than males [mean uropygial gland volume (SD): females = 171.57 mm^3 (40.01); males = 167.36 mm^3 (42.26)]. Moreover, the volume of the uropygial gland differed significantly among localities, with house sparrows living in an urban site having larger uropygial glands than sparrows in a rural site [(mean uropygial gland volume (SD): urban sparrows = 184.75 mm^3 (45.28); rural sparrows = 163.90 mm^3 (38.70)].

The relationship between the volume of the uropygial gland and scaled body mass index varied with haemosporidian infection (Table 1; Fig. 1). Specifically, there was a positive relationship between gland size and scaled body mass index in uninfected house sparrows ($r = 0.392$; $P < 0.001$), while there was no significant relationship in individuals infected with haemosporidians ($r = -0.077$; $P = 0.568$). We also found a positive relationship between gland size and scaled body mass index in both males ($r = 0.271$; $P = 0.002$) and females ($r = 0.349$; $P = 0.001$).

In a second GLM we examined if antimicrobial activity of the uropygial secretion varied with sex, haemosporidian infection, scaled body mass index and uropygial gland volume. The estimate of antimicrobial activity of the uropygial gland varied with haemosporidian infection and sex (Table 2). Specifically, antimicrobial activity of uropygial secretion was significantly higher in uninfected than in infected birds [mean antimicrobial activity (SD): infected = 11.08 (9.76); uninfected = 20.39 (15.81)]. Finally, uropygial gland secretions from female sparrows had a slightly higher antimicrobial activity than secretions from male sparrows, although non-significantly so [mean antimicrobial activity (SD): females = 20.79 (16.66); males = 16.18 (13.73)].

Table 1 Factors explaining the variation in volume of the uropygial gland in house sparrows. Scaled body mass index, haemosporidian infection, sex, locality and the two-way interactions between sex and scaled body mass index, between infection status and scaled body mass index and between infection and locality were included in the analysis as predictor variables. Sample size was 222 individuals

Independent variable	Square-sum III	*DF*	*F*	*P*
Scaled body mass index	4695.89	1	0.190	0.7176
Infection	3787.71	1	4.082	0.046
Sex	5574.75	1	4.122	0.036
Locality	14282.25	1	10.464	< 0.001
Sex × Scaled body mass	5061.31	1	4.084	0.045
Infection × Scaled body mass	4695.89	1	4.354	0.027
Infection × Locality	1153.53	1	0.845	0.359

Discussion

Uropygial secretions have been hypothesized to play an important role in the interaction between birds and haemosporidian parasites because it can affect the interaction between hosts and their vectors. Although the results from some studies do not support a potential role of avian uropygial gland secretions in attracting haemosporidian vectors [55], other studies have indicated that preen oil secretions may constitute a defence mechanism against ectoparasites and thus avoidance of blood parasite infections [56]. In contrast, other studies suggest that haemosporidian vectors may be attracted by uropygial secretions [28, 29]. Here we explored the relationship between uropygial gland size, antimicrobial activity of uropygial secretions and malaria infection in house sparrows. The main findings of this study were that (i) uninfected house sparrows had larger uropygial glands and higher antimicrobial activity in uropygial secretions than infected house sparrows; and (ii) female house sparrows had larger uropygial glands and higher antimicrobial activity than males. We briefly discuss these results.

More than 200 blood samples from house sparrows were analysed in search of blood parasites. A total of 26 % of house sparrows were infected with haemosporidian parasites. Similar haemosporidian prevalence during winter has been found in previous studies of house sparrows from the same area [57].

Uninfected house sparrows had larger uropygial glands than infected house sparrows, suggesting that the uropygial gland and its secretions may constitute defences against ectoparasites, and thus against haemosporidian infection. Thus, González [58] have recently shown that ectoparasite richness and ectoparasite burden is negatively related to the mass of the uropygial gland in rock ptarmigan (*Lagopus muta*). Moreover, other studies have shown that individuals with larger glands, and, therefore, able to produce more uropygial secretions, have lower prevalence of ectoparasites [48, 59]. We propose different mechanisms that may explain this protection by preen secretions against ectoparasites. First, uropygial secretions may act as a physical barrier against vectors by reducing their mobility on the bird's plumage or skin [56]. Secondly, uropygial secretions could act as an insecticide and kill ectoparasites by covering the surface of the parasite or blocking their spiracles [35]. Finally,

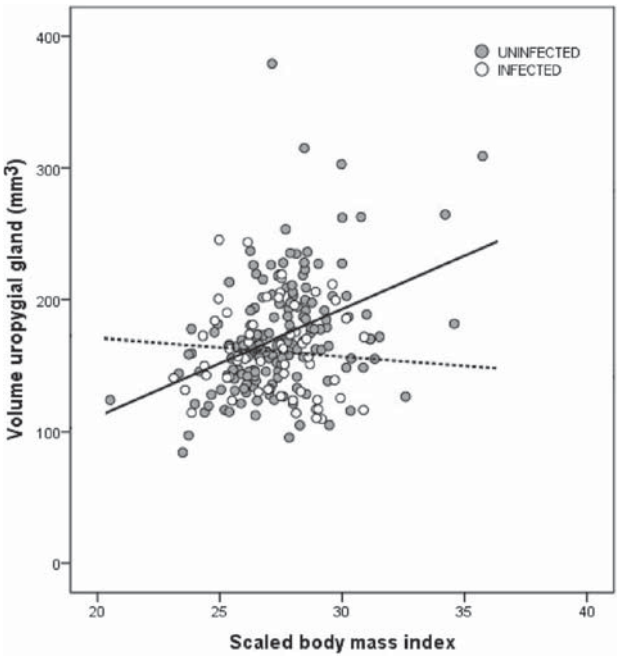

Fig. 1 Scatterplot showing the relationship between the volume of the uropygial gland (mm^3) and scaled body mass index in haemosporidian infected (white circles, dotted line, $N = 57$) and uninfected house sparrows (grey circles, solid line, $N = 165$)

preen secretions may be associated with noxious or repellent odours that possibly affect ectoparasites, as has been shown in some species of birds [60]. We also found a positive and significant relationship between gland size and scaled body mass index in uninfected house sparrows, while the volume of the uropygial gland was not significantly related to scaled body mass index among malaria infected sparrows. These findings suggest that development of the uropygial gland is costly and may impair energetic demands [61, 62]. Thus, only individuals in prime condition (uninfected sparrows) should be able to invest in anti-microbial defence without compromising other fitness-related traits.

Table 2 Factors explaining the variation in antimicrobial activity of the uropygial gland in house sparrows. Scaled body mass index, haemosporidian infection, sex and uropygial gland volume were included in the analysis as predictor variables. Sample size was 44 individuals

Independent variable	Square-sum III	*DF*	*F*	*P*
Scaled body mass index	1.38	1	2.011	0.1756
Infection	-2.43	1	5.850	0.0196
Sex	1.75	1	2.947	0.0872
Gland volume	-5.19	1	0.297	0.4968

The antimicrobial and anti-fungal properties of uropygial secretions may benefit birds by avoiding haemosporidian infection. Consistently, we showed that the antimicrobial activity of the uropygial gland in uninfected house sparrows was higher than that of gland secretions from infected birds. As far as we are aware, this is the first study showing a relationship between antimicrobial activity of uropygial secretions and haemosporidian infection. We hypothesize that this relationship may be mediated by a decrease in olfactory stimuli for insect vectors caused by preen secretions. Accordingly, it has been shown that the odours emanating from gland secretions and the microflora of the skin may play an important role in attraction of malaria vectors in humans [63–65]. Birds harbour a great diversity of microbes on feathers and skin, which may be involved in the production of chemical attractants for haemosporidian vectors like *Culex* spp. and simulids [28, 37, 66]. Hence, the elimination of bacteria and fungi from feathers and skin by uropygial secretions could decrease vector attraction and thus minimize the likelihood of becoming infected with haemosporidians. Following this idea, uropygial secretions may affect different strains

of parasitic bacteria and fungi in different ways. For example, it has been shown that volatile compounds (saturated fatty acids, benzaldehyde and phenol, among others) from uropygial gland secretions provide strong antimicrobial action in birds [67]. Similarly, Jacob et al. showed that uropygial secretions from birds of the order Pelecaniformes had an antagonist effect on fungal dermatophytes and an antibacterial effect on Gram-positive bacteria [68]. Moreover, Law-Brown experimentally showed activity of uropygial secretions against 13 pathogenic bacterial strains from the genera *Salmonella*, *Staphylococcus* and *Streptococcus* [69]. Additionally, it has been proposed that pathogenic bacteria and fungi may be controlled through the synthesis of bacteriocins and other antimicrobial actions of certain symbiotic bacteria that live in the preen gland [23–25, 70].

Female house sparrows have larger uropygial glands than male sparrows. In agreement with our results, previous studies of birds have shown that females have larger glands than males [40, 48, 59]. Moreover, the antimicrobial activity of preen secretions from female sparrows was slightly higher than that of males. Bacteria and other pathogens in the nest are known to be one of the main factors affecting egg survival and hatching success [71–73]. Because in many species of birds females spend more time in nests during incubation and nestling periods than males [74, 75], larger uropygial glands and/or higher antibacterial capacities may lead to a fitness advantage in terms of higher hatching success [76]. Alternatively, several studies have shown that females are more infected with haemosporidian parasites than males [77, 78], suggesting a female bias in exposure to the vectors [79]. Because time spent in the nest can increase the risks of becoming infected with haemosporidian parasites [15], larger uropygial glands and higher antimicrobial activity may provide females with a higher protection against haemosporidian vectors.

Birds are expected to modify their investment in defensive traits (uropygial gland) in response to differences in exposure to microorganisms [80]. Animals living in cities are exposed to more pathogenic diseases than their rural counterparts [81, 82]. In agreement with these expectations, we found a significant difference in size of uropygial glands between populations. However, our results rely on only one single comparison, and hence, we need more replicates of uropygial gland sizes from other urban and rural populations of house sparrows in order to draw firm conclusions.

Conclusion

In conclusion, the size of the uropygial gland and the antimicrobial activity of its secretions varied with haemosporidian infection and sex in house sparrows. These findings suggest that uropygial glands may be involved in defensive mechanisms against malarial infections under natural conditions. Further experimental studies could help improve our understanding of this bird-parasite interaction. They may also help to test if uropygial secretions may have properties that reduce or eliminate the risk of malarial infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SM, APM and AM designed the study. All authors processed and analyzed the data. SM, APM and AM wrote the manuscript. All authors read and approved the final version of the manuscript.

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Chapter II

Uropygial gland volume and malaria infection are related to survival in migratory house martins.

LA GLÁNDULA UROPIGIAL Y LA INFECCION POR MALARIA ESTAN RELACIONADOS CON LA SUPERVIVENCIA EN LA MIGRACION DEL AVIÓN COMÚN

Resumen

Patógenos como las bacterias, hongos, malaria y haemosporidios relacionados pueden provocar efectos negativos en la eficacia biológica y supervivencia de sus hospedadores. Los animales han desarrollado una serie de estrategias para tratar de reducir o eliminar estas infecciones parasitarias y los costes negativos asociados.

Se ha propuesto que la secreción de la glándula uropigial es una barrera defensiva en la lucha contra bacterias y hongos, así como ayudar a prevenir las infecciones haemosporidias. Por ello, la secreción de esta glándula puede favorecer la supervivencia de los individuos. Sin embargo, la relación entre la supervivencia y la secreción permanece aún desconocida. La presente investigación, presenta un estudio a largo plazo, se analiza cómo influyen en la supervivencia de un ave migradora de largas distancias, el avión común *(Delichon urbica)*, la relación entre el volumen de la glándula uropigial y la prevalencia de infección por parásitos maláricos. Encontramos que los individuos infectados por malaria son los que disponen de una menor probabilidad de supervivencia. Por otro lado, el volumen de la glándula está positivamente relacionado con la probabilidad de supervivencia de los individuos infectados de esta especie. Estos resultados ponen de manifiesto la importancia de la glándula uropigial y la parasitemia en la historia biológica de las aves silvestres.

Letters

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Uropygial gland volume and malaria infection are related to survival in migratory house martins

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Pathogens such as bacteria, fungi and malaria and related haemosporidians provoke negative effects on the fitness of their hosts. Animals have developed a range of defensive mechanisms to resist or eliminate these parasitic infections and their negative fitness costs. The uropygial gland secretion has been proposed to act as defensive barrier of skin and plumage in the fight against bacteria and fungi, and may prevent birds from acquiring haemosporidian infections. Thus, the secretion of uropygial glands of birds may favour survival of individuals. However, whether uropygial gland secretion influence survival remains unknown. Here we explore if the size of the uropygial gland and malaria infection influence survival of house martins *Delichon urbica*. We showed, for the first time, that the volume of the uropygial gland positively predicted survival prospects of malaria infected house martins. Malaria infected birds had the lowest probability of survival, with the effect of gland size on survival prospects depending on infection: infected house martins with larger uropygial glands were better able to survive to the next breeding season, while infected birds with small uropygial glands were not. These results highlight the importance of uropygial gland secretion in the life history of wild birds.

Parasites can regulate populations of their hosts by negatively affecting their growth, fecundity and survival (Schmid-Hempel 2011). Malaria and related haemosporidian parasites are diverse and pathogenic parasites affect body condition (Valkiūnas 2005), reproductive success (Marzal et al. 2005, Asghar et al. 2011) and survival (Martínez de la Puente et al. 2010, Asghar et al. 2015, Marzal et al. 2016).

All animals are exposed to a wide range of parasites and thus have evolved a wide range of mechanisms to resist or eliminate the pathogen infections, including innate and adaptive immunity (Demas and Nelson 2011). The uropygial gland is a holocrine gland exclusive to birds secreting waxes that are smeared on the plumage during preening as part of plumage maintenance (Jacob and Ziswiler 1982). Although its role is still debated (see Moreno-Rueda 2017 for a review), this secretion has been proposed to act as defensive barrier of skin and plumage in the fight against bacteria and fungi (Jacob and Ziswiler 1982, Jacob et al. 1997, Fülöp et al. 2016). Even though the results from some studies do not support a potential role of avian uropygial gland secretions avoiding haemosporidian vectors (Fallis and Smith 1964, Russell and Hunter 2005, Martínez de la Puente et al. 2010), it has been recently suggested that uropygial secretion may prevent birds from acquiring haemosporidian infections (Magallanes et al. 2016) because it may affect the interaction between birds and their vectors. In this sense, compounds isolated from secretions of the uropygial gland may have an insecticidal effect and kill ectoparasites by blocking their spiracles or covering the surface of the parasite (Moyer et al. 2003), or may reduce the mobility of the vector on the bird's plumage or skin acting as physical barrier (Clayton et al. 2010). In addition, since microbes on feathers and skin are involved in the production of chemical attractants for haemosporidian vectors like *Culex* spp. and simulids (Fallis and Smith 1964, Syed and Leal 2009), the elimination of bacteria from feathers and skin by uropygial secretions could reduce the emission of chemical cues used by haemosporidian vectors and hence minimize the likelihood to becoming infected with these blood parasites.

Because avian malaria and bacteria are known to increase mortality on their hosts (Hoque et al. 2012, Asghar et al. 2015, Marzal et al. 2016), birds producing higher volumes of uropygial gland secretion could benefit from reducing mortality. Despite the increasing interest in the different functions of uropygial gland, the number of studies examining the relationship between uropygial gland secretion and fitness is still scarce (Moreno-Rueda 2017). For example, it has been proposed that the secretion of uropygial gland may be a reliable predictor of reproductive success (Whittaker et al. 2013). Also, Møller et al. (2010a) analysed the variation in size of the uropygial gland in 212 species of birds, showing that species with relatively large uropygial had

higher hatching success. However, whether uropygial gland secretion influence survival remains unknown. On the one hand, some studies have experimentally tested whether uropygial gland may influence survival in birds, showing that the removal of uropygial gland did not affect survival in pigeons and ducks (Elder 1954, Salibian and Montalti 2009). On the other hand, Møller et al. (2010b) reported that avian prey species with smaller size of their uropygial glands were more prone to predation than prey species with larger uropygial glands.

Here we explore for the first time if the size of the uropygial gland may influence the survival of house martins, an Afro-Paleartic migratory bird species with global population decline (BirdLife International 2017). If uropygial gland secretion can mediate survival, we expect that house martins with larger uropygial glands to have higher probabilities of survival than birds with smaller glands. We also analyse whether malaria infection affect survival in its avian host. Because haemosporidians increase mortality of bird hosts, we predict that malaria infection decrease survival in house martins.

Methods

Our study was conducted at one breeding colony of house martins located under a water tank at southern Spain (38°52′N, 6°58′W) during 2013–2016. Each year we performed one capture session at dawn in the middle of the breeding season (June) resulting in more than 95% of the individuals captured. Adult birds were captured and identified with numbered metal rings. From each individual we took a blood sample (50 µl) from the brachial vain and we recorded length, height and width of the uropygial gland with a digital calliper with a precision of 0.01 mm. Uropygial gland volume was estimated as the product of the three dimensions of uropygial gland (Galván and Sanz 2006), which is positively related to the volume of secretions (Martín-Vivaldi et al. 2009, Pap et al. 2010). Because the uropygial gland is a soft tissue (Martín-Vivaldi et al. 2009, Møller et al. 2009), we measured the three dimensions of uropygial gland three times to calculate repeatability (Møller et al. 2009, Moreno-Rueda 2010, 2015) (Supplementary material Appendix 1 Table A1). House martins show high breeding site philopatry (De Lope and Da Silva 1988). Thus, an individual was assumed to have died if it had not been captured for two consecutive years (Marzal et al. 2016).

Malaria parasites (*Plasmodium* spp. and *Haemoproteus* spp.) were detected from blood samples using molecular methods (Waldenström et al. 2004). DNA from the blood samples was extracted using a standard chloroform/isoamylalcohol method (Sambrook et al. 2002). Diluted genomic DNA (25 ng μl^{-1}) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested-PCR protocols (Waldenström et al. 2004). The amplification was evaluated by running 2.5 ml of the final PCR on a 2% agarose gel.

Logistic regression analysis was used to explore whether malaria infection, year, sex, uropygial gland volume and their interactions (year × malaria infection, year × uropygial gland volume, and malaria infection × uropygial gland volume) influenced survival probability of house martins. Survival was treated as a binary variable (survivor or non-survivor). We used a stepwise linear regression with backward procedures to eliminate all non-significant terms ($p > 0.10$) from our starting maximal model. Sex and year did not enter in the final model, and thus only the final consensus model was presented. All analyses were performed using PASW Statistics 18 statistical package for Windows.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.07k21> (Magallanes et al. 2017).

Results

Of all 217 house martins, 33 (15%) were infected with malaria and 184 (85%) were uninfected. In addition, only 64 (29.5%) of the 217 house martins survived to the next breeding season. Malaria infection, uropygial gland volume and the interaction between malaria infection and uropygial gland volume significantly explained variation in survival (Table 1). Malaria infected birds had the lowest probability of survival: 59 (32%) of uninfected house martins, but only 5 (15%) of infected birds, survived to the next breeding season (Chi-square test: $n = 217$, $\chi^2 = 3.849$, $p < 0.05$). Surviving house martins had larger uropygial glands ($n = 64$; mean (SD) = 64.96 (16.83) mm^3) than non-survivor individuals ($n = 153$; mean (SD) = 62.79 (23.38) mm^3). The relationship between gland size and survival prospects differed with respect to malaria infection. Specifically, there was no difference in gland size between survivors and non-surviving uninfected birds. In contrast, infected house martins that survived to the next breading season had larger uropygial glands than infected birds that did not survive (Fig. 1).

Discussion

During the malaria infection, birds suffer from severe pathology such as anoxia, necrosis of tissues and acute anaemia (Valkiūnas 2005). These pathological changes may impair survival of wild birds. Thus, avian malaria and related haemosporidians are known to decrease survival prospects in their hosts (Marzal et al. 2008, 2016, Martínez de la Puente et al. 2010). Our results agree with these studies, because most infected house martins did not survive to the next breeding season.

Table 1. Factors explaining variation in survival for individual house martins. A stepwise logistic regression with backward procedures was used with sex, malaria infection, year, uropygial gland volume and their interactions (year × malaria infection, year × uropygial gland volume, and malaria infection × uropygial gland volume) as predictor variables. Only independent variables selected by the consensus model are listed. Sample size was 217 individuals.

Variable	B	SE	Wald	df	p	Exp (B)
Infection	–4.321	1.832	5.565	1	0.012	84.702
Uropygial gland volume	–0.045	0.021	4.518	1	0.018	0.013
Infection × u. gl. volume	0.045	0.023	3.857	1	0.050	1.046

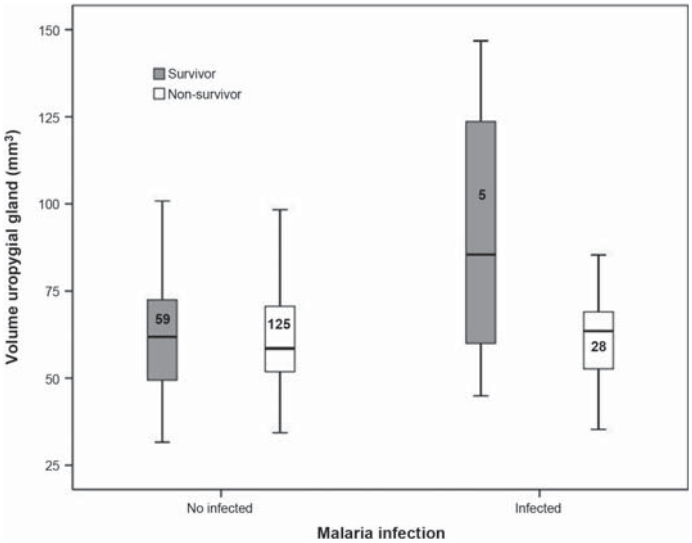

Figure 1. Box plots showing volumes of uropygial gland for malaria infected and no-infected house martins with concern to survival prospects. Values are medians, upper and lower quartiles and extreme observations. Sample sizes of each category are shown inside the box-plots.

We found that only 15% of individuals were infected with malaria. This prevalence is lower than expected from previous studies analysing haemosporidian infections in house martins in neighbour colonies. For example, Marzal et al. (2013) found that 65% of house martins were infected with malaria. Previous studies have also shown differences between years in the prevalence of haemosporidian parasites, suggesting that extrinsic parameters such as environmental conditions affecting vector distribution may explain such fluctuations (Bensch et al. 2007, Wilkinson et al. 2016).

Surviving house martins had larger uropygial glands than non-survivor individuals. Several hypotheses may explain this result. Firstly, it has been recently shown that the uropygial gland and its secretions may constitute a defensive mechanism against malarial infections under natural conditions (Magallanes et al. 2016). Thus, taking into account the mortality linked to haemosporidian infections, larger uropygial glands should prevent malaria infection and therefore increase survival prospects. Secondly, a recent study in more than 150 wild bird species has shown that feather-degrading bacteria may break down keratin and damage plumage (Kent and Burtt Jr 2016). Because feather damage could decrease flight performance, body insulation and fitness (Proctor and Lynch 1993, Barbosa et al. 2003), birds with compromised feather condition should have lower survival prospects (Booth et al. 1993, Clayton et al. 1999, but also see Merilä and Hemborg 2000). Uropygial glands may play an essential role in the regulation of feather-degrading bacteria, thus minimizing the impact of these pathogens on feather quality (Fülöp et al. 2016, but also see Czirják et al. 2013 and Giraudeau et al. 2013 for opposite results). Consequently, birds with larger uropygial glands should have better plumage condition and therefore enjoy higher survival prospects. Thirdly, Czirják et al. (2013) found that the removal of uropygial gland led to higher loads of pathogenic bacteria on bird's skin and feathers, such as *Pseudomonas*, *Staphylococcus* and *Salmonella*, thus implying that the broad antimicrobial spectrum of the uropygial secretion may favor survival of birds. Finally, house martins with larger uropygial glands could also have higher immunocompetence, which could be positively correlated with survival (Norris and Evans 2000). Thus, it has been shown that uropygial gland size is correlated with body condition and immunocompetence in house sparrows *Passer domesticus* (Moreno-Rueda 2010).

We found that the effect of gland size on survival prospects depended on malaria infection. Specifically, there was no difference in gland size between survivors and non-surviving uninfected birds. In contrast, on top of the pathogenic effects of malaria infection, infected house martins with larger uropygial glands were better able to survive to the next breeding seasons than infected birds with smaller uropygial glands did not survive. Several hypotheses may explain these outcomes. First, larger glands also imply better immunocompetence (Moreno-Rueda 2010), which may allow infected hosts to successfully combat the parasitic infection and thus survive. Pap et al. (2013) showed that house sparrows infected with coccidians had reduced uropygial glands, probably because on the absorption of the ester preen waxes and other primary components of the gland secretions (Reneerkens et al. 2002) or by its detrimental effect on the body condition of birds, which may be linked to gland size (Moreno-Rueda 2010). In this sense, a large uropygial gland is related to better body condition in birds (Moreno-Rueda 2010, 2015), which is essential for mounting a strong immune response and control parasite burden (Warburton et al. 2016). Also, it has been experimentally shown that an immune system challenge (LPS injection) impaired the growth of the uropygial gland in tawny owl nestlings (Piault et al. 2008). Thus, only

individuals with large uropygial glands (and hence better body condition) may allocate resources to mount an efficient immune response in the fight against malaria infection. Finally, individuals infected with malaria are at increased risk of invasive bacterial infections (Gómez-Pérez et al. 2014). Although some studies found no effect of avian malaria parasites on adult survival (Bensch et al. 2007), it is known that malaria infection can be highly virulent and provoke severe mortality in birds because of co-infections with other infectious agents (Atkinson and Samuel 2010). In this sense, birds chronically infected with malaria develop more severe clinical disease when co-infected with *Mycoplasma* (Dhondt et al. 2017). Because larger uropygial glands may prevent bacterial infection (Fülöp et al. 2016), infected house martins secreting higher volumes of uropygial oil may prevent deadly co-infections with malaria and bacteria. However, we should be cautious in our conclusions because our results rely on comparisons with low sample size of malaria infected house martins that survived to the next breeding season (n = 5) (Lemoine et al. 2016). Hence, we need more studies analysing the role of uropygial gland on survival of birds infected with haemosporidians to draw firm conclusions.

To conclude, we have shown for the first time that the volume of the uropygial gland is positively related to survival in wild birds infected with malaria. We also found that malaria infection negatively influenced survival. These results highlight the importance of the uropygial gland secretion in the life history of wild birds. Further experimental studies are required to improve our understanding of this bird–parasite interaction.

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Supplementary material (Appendix JAV-01514 at <www.avianbiology.org/appendix/jav-01514>). Appendix 1.

Chapter III

Uropygial gland volume is related to reproductive success in the barn swallow *Hirundo rustica*.

LA GLÁNDULA UROPIGIAL ESTÁ RELACIOANDA CON EL ÉXITO EN LA REPRODUCCIÓN EN LA GOLONDRINA COMÚN *HIRUNDO RUSTICA*

Resumen

Los patógenos provocan diversos efectos negativos en el fitness de sus hospedadores, reduciendo su supervivencia y/o reduciendo su eficacia reproductora. Para enfrentarse a los patógenos, los animales han desarrollado diferentes estrategias con el fin de evitar o minimizar los efectos negativos de las infecciones. La glándula uropigial gracias a su actividad antimicrobiana y fungicida se ha propuesto como un mecanismo eficaz que favorece el fitness de las aves. Sin embargo, la relación que puede existir entre la secreción uropigial y el éxito en la reproducción se mantiene aún desconocida. En este capítulo, exploramos si el tamaño de la glándula uropigial está relacionado con el éxito en la reproducción en la golondrina común *Hirundo rustica*. Nuestros resultados mostraron que la longitud de la rectriz y el tarso explican las variaciones en el tamaño de nidada. Además, la fecha de llegada, así como el peso corporal también afecta positivamente al tamaño de nidada como al éxito en la eclosión de los huevos. También el número de pollos cridados está relacionado con el peso corporal y la edad de los padres. Asimismo, se encontró relación entre la densidad de nidos en los lugares de cría y el tamaño de la glándula uropigial con respecto al éxito de eclosión y a la cría del número de pollos. Por un lado, en zonas de alta densidad de nidos, las golondrinas con mayor glándula uropigial tienen mayor éxito de eclosión y un mayor número de pollos criados. Sin embargo, por otro lado, en las zonas de baja densidad de nidos, las golondrinas con mayor glándula tienen menor éxito en la eclosión de huevos y un menor número de pollos criados con éxito. Estos resultados proponen que los

beneficios de la secreción uropigial pueden ser dependientes de la densidad del conspecíficos, lo que sugiere que los individuos podrían ajustar el volumen de secreción uropigial en respuesta a la exposición al patógeno.

Reproductive success related to uropygial gland volume varies with abundance of conspecifics in barn swallows *Hirundo rustica*

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S. Magallanes et al. Uropygial gland and reproductive success

Abstract

Pathogens provoke negative effects on the fitness of their hosts, reducing survival and/or decreasing their reproductive success. To cope with pathogen challenge, animals have developed a variety of defensive traits to evade parasite infection and minimize their detrimental effects. Uropygial gland secretion has been proposed to have antimicrobial and antifungal properties, what may potentially influence bird fitness. However, whether uropygial gland secretion may affect the breeding success of birds remains unknown. Here, we explore if the size of uropygial gland is related to reproductive success in barn swallows (*Hirundo rustica*), a small colonial migratory hirundine. We predicted that a greater uropygial gland should increase the total number of eggs layed, number of eggs hatched, and number of fledglings reared. We were also interested in the role that sex, age class and abundance of conspecific may have on the relationship between uropygial gland size and reproductive success. We showed that reproductive success varied with the interaction term between uropygial gland volume and abundance of conspecifics. Barn swallows with larger uropygial glands had higher breeding success (greater number of eggs hatched, and fledglings reared) when living in environments with higher abundance of conspecifics. On the contrary, barn swallows with larger uropygial glands had lower reproductive success when breeding in environments with lower abundance of conspecifics. These outcomes imply that benefits of uropygial secretion may be host-density dependent, thus suggesting that individuals may adjust the volume of uropygial secretion in response to pathogen exposure.
Keywords: barn swallow, defensive traits, *Hirundo rustica*, host-pathogen interaction, preen gland, reproductive success.

INTRODUCTION

Pathogens are ubiquitous, diverse and the most abundant organisms on Earth (Dobson et al. 2008). They are known to regulate populations of their natural hosts by reducing their abundance, fecundity and survival (Møller 2005, Schmid-Hempel 2011). For example, avian malaria and related haemosporidians are cosmopolitan and highly virulent parasites negatively affecting the growth (Calero-Riestra and García 2016), body condition (Valkiūnas 2005, Marzal et al. 2008), reproductive success (Merino et al. 2000, Asghar et al. 2011) and survival of their hosts (Martínez-de la Puente et al. 2010, Asghar et al. 2015, Dimitrov et al. 2015, Marzal et al. 2016). To face pathogen challenges, animals have evolved a wide range of barriers and defence mechanisms to avoid parasite infection and/or to minimize negative effects (Wakelin 1996, Poulin 1998). Among others, these mechanisms include non-specific physical and chemical barriers (e.g. epithelial surface and lysozymes), macrophages and neutrophils recognizing and destroying pathogens trespassing the first line of defence, and specific immune responses that involves the recognition of unique molecule structures on pathogens by antigen receptors and the production of antibodies leading to pathogen clearance (Mak et al. 2014).

The uropygial gland (also called *preen g*land) is a holocrine gland exclusive to most bird species, and it produces an oily secretion that is smeared on the plumage during preening as part of plumage maintenance (Jacob and Ziswiler 1982). Although the potential functions of uropygial secretion are still debated (see review *in* Moreno-Rueda 2017), it has been proposed that this secretion has antimicrobial and antifungal properties and hence protect birds against feather-degrading pathogens (Bandyopadhyay and Bhattacharyya 1999, Shawkey et al. 2003, Rodríguez-Ruano et al. 2015). Moreover, it has recently been shown that the volume and

antimicrobial activity of uropygial secretion may play an important role as a defensive mechanism against malaria infection (Magallanes et al. 2016, Marzal et al. 2018), and influence survival in house martins (*Delichon urbica*) (Magallanes et al. 2017).

However, whether uropygial gland secretions may affect the reproductive success of birds remains unknown (see review *in* Moreno-Rueda 2017). In this sense, the numbers of studies exploring benefits of uropygial secretions on breeding success are still scarce and with mixed results. For example, Whittaker et al. (2013) explored the relationship between volatile compounds in avian uropygial secretions and reproductive success in the dark-eyed junco (*Junco hyemalis*), showing that these compounds may reliably predict reproductive success in this songbird species. Moreover, Giraudeau et al. (2010) experimentally blocked access to uropygial gland secretions with a removable mechanism in reproducing female mallards (*Anas platyrhynchos*), provoking a mass loss and reduced egg size, which may negatively impair embryo viability, hatchling success and fledgling survival in birds (Wagner and Williams 2007, Krist 2011). Furthermore, female mallards with restricted access to the preen gland secretions also deposited higher concentrations of carotenoids into egg yolk than control females (Giraudeau et al. 2010), which may boost nestling growth (Muriel et al. 2015) and increase survival prospects in nestlings and fledglings (Saino et al. 2003, Marri and Richner 2014).

Here we test the hypothesis that a larger uropygial gland volume may benefit reproductive success in barn swallows (*Hirundo rustica*), a small semi-colonial migratory hirundine with global population decline (BirdLife International 2018). Several studies have shown that pathogens (e.g. bacteria, ectoparasites and blood parasites) may exert negative effects

on the breeding success of hirundines, by delaying hatching date (Marzal et al. 2013, Fülöp et al. 2017), and by decreasing clutch size (Marzal et al. 2005, 2013) and the number of reared chicks (Williamson and Fitter 1996, Marzal et al. 2005). If anti-pathogen properties of uropygial secretion could minimize these detrimental effects on reproductive success in barn swallows, then we should expect that barn swallows with larger uropygial glands should have higher breeding success than barn swallows with smaller uropygial glands. Furthermore, we hypothesized that the relationship between uropygial gland volume and reproductive success may differ across sexes. Since the female spend more time incubating the eggs, its uropygial secretion could be more important than that of the male to protect the clutch against contamination by microorganisms, which is known to affect hatching success (Baggott and Graeme-Cook 2002). We also hypothesized that the relationship between uropygial gland volume and reproductive success may differ across age class. This is because reproductive traits of birds, including the barn swallow, usually improve during early life and decline during senescence (Forslund and Part 1995; Balbontín et al. 2007; Nussey et al 2013), and also because different immune defense organs regress after accomplishing the first migration (Møller and Erritzøe 2001). Finally, we also hypothesized that the relationship between uropygial gland volume and reproductive success could be determined by the abundance of conspecifics. This is because a higher number of swallows nesting within the same room may determine abundance and transmission of several pathogens such as bacteria or fungi (Begon et al. 2002), and thus anti-pathogen properties of uropygial secretions may be more important for swallows that breed close to each other compared to swallows that breed further away one from each other.

MATERIAL AND METHODS

Field procedures

During 2015-2017, we monitored four breeding colonies of barn swallows in South-western Spain (provinces of Badajoz and Seville): Asesera (38°39'N, 7°13'W), La Alegría (37°29'N, 6°11'W), Las Coladas (37°36'N, 6°14'W) and La Calera (37°34'N, 6°13'W). Our study populations breed in traditional farms surrounded by Mediterranean *Dehesa*. Dominant vegetation was sclerophyllous oak-trees (*Quercus ilex* and *Q. suber*), accompanied by a wide range of bushes (e.g. *Cistus, Genista*, *Retama*, *Pistacia* and *Ulex*) and scattered pine plantations. The study sites had different economic activities such as livestock (ovine, bovine and porcine), wildlife gaming, cork production, tourism and agriculture (cereals, sunflower and olives). Population numbers differed across the study colonies, ranging from eight to thirty breeding pairs. Across our study colonies, barn swallows nest inside buildings such as barns, stables and abandoned rooms of the farms. In this paper, we refer to all these places as "rooms". The number of breeding pairs within each room ranged from one to ten.

From February to July, we captured adult barn swallows with mist nets and identified each individual with both metallic and colour rings. Every year, we trapped birds until 90-100% of the population was captured in each breeding colony. Individuals were sexed by the presence (females) or the absence (males) of brood patch, by the length of the outermost tail feathers, and also by observation of breeding behaviour during courtship and incubation (Svensson 1992, Hermosell et al. 2007). For each captured adult swallow, we measured body mass to the nearest 0.01 g with a digital balance, tarsus length to the nearest 0.01 mm with a digital calliper, and outermost

tail feather length to the nearest 1 mm with a ruler. We also recorded length, height and width of the uropygial gland to the nearest 0.01 mm with a digital calliper. We estimated uropygial gland volume as the product of these three dimensions (Galván and Sanz 2006), which is positively related to the volume of secretions (Dobson et al. 2008, Martín-Vivaldi et al. 2009). Because the uropygial gland is a soft tissue (Martín-Vivaldi et al. 2009, Møller et al. 2009), we measured each dimension of uropygial gland three times to calculate repeatability (Møller et al. 2009, Moreno-Rueda 2010, 2015). Repeatability of uropygial gland measurements was proven high and significant [R = 0.787; SE = 0.016; 95%CI = (0.753, 0.814); p = 0.001], with a sample size of 1386 observations taken from 462 groups.

We categorized barn swallows in two different age-classes: young birds (i.e., yearling individuals that have migrated for the first time that year; n = 87), and experienced birds (i.e., two-years or older individuals that were at least in their second migration year; n = 105). We categorized as young birds individuals ringed as nestlings/fledglings that were recaptured in the next year (n = 12), and also individuals ringed for the first time as adults (n = 75). We categorized as experienced birds individuals ringed as nestlings/fledglings that were recaptured two years or more after their first capture (n = 3), and also individuals ringed for the first time as adults that were recaptured in subsequent years (n = 102). Breeding dispersal is negligible in the study species (Møller 1994a). Therefore, we assumed that adult disappearance from the breeding population indicated mortality rather than dispersal (Saino et al. 2004, Balbontín et al. 2009). Thus, we could assign the age of individuals with accuracy in our study colonies, assuming that un-ringed birds are yearlings at first capture (Møller 1994b, Møller et al. 2005).

To obtain data on reproduction, we visited every breeding colony weekly and we annotated the number of eggs laid (clutch size), the number of eggs hatched and the number of nestlings in each nest. Our study populations laid up to three clutches during the breeding season, and we used for this study the total number of eggs/chicks across all clutches. Assignment of captured adult swallows to their nests was made by visual identification of colour rings during breeding behaviour.

Statistical analyses

To investigate how uropygial gland volume may correlate with reproductive success, we used linear mixed models where total clutch size, total number of eggs hatched, and total number of fledglings were the response variables. We always used normal distribution of errors and the identity link function. This is because our models would not converge when using *Poisson* distribution of errors. Nevertheless, we qualitatively assessed each individual model fit by inspecting "qqplots" and we confirmed that our models were well fitted. In each model, we included together with uropygial gland volume, the covariates body mass, tarsus length, tail length and capture date (day “one” corresponding to first of January). In this way we aimed to statistically control for the relationship between body condition and uropygial gland volume (Magallanes et al. 2016), the importance of tail length on reproductive performance (Møller 1992) and variation in uropygial gland volume throughout the breeding season (Vincze et al. 2013). By including capture date as a covariate, we were also approximately controlling for the arrival date to breeding areas and thus with the effect of arrival date on reproductive success. Individual identity (i.e. metallic ring), breeding colony and year were included as cross-random intercepts in each

model, thus controlling respectively in the dependent variable for repeated measures of the same individual, for specific breeding site variation and for inter-annual effects. We were interested in testing the role of sex, age class and abundance of breeding conspecifics as variables controlling the relationship between uropygial gland volume and fitness. Therefore, we included in our linear mixed models the two-way interaction terms of uropygial gland volume with sex, uropygial gland volume with age class and uropygial gland volume with abundance of conspecific (i.e. the number of pairs nesting within a given room).

We defined a global model that included all these 11 predictors resulting in 2^{11} candidate models that were generated and evaluated following procedures described in Grueber et al. (2011). We first standardized the input variables entering the global model, scaling them by dividing means by two standard deviations, which allowed comparison on the same scale of coefficients of binary factors (e.g. sex) and covariates. Therefore, parameter estimates were standardized effect sizes and were on a comparable scale (Gelman 2008, Grueber et al. 2011). The most parsimonious of all possible candidate models was determined using Akaike information criterion corrected for small sample size (AICc). We calculated Akaike weight (w) for each candidate model ("i") that can be interpreted as the probability that "i" is the best model, given the data and set of candidate models (Burnham and Anderson 2002). We also calculated the Relative Importance (RI) for a given variable as the sum of Akaike weights from candidate models that contained the given variable (Burnham and Anderson 2002). The final model was obtained by averaging the parameter estimates from top models at a cut-off criterion of $\Delta AICc < 6.0$ (Richards 2008). The reference level of the fixed factor sex was "female", and the reference level of the fixed factor age class was "experienced". The confidence intervals

(hereafter 95% CI) were calculated from the final model using the parameter estimates (effect size) and associated standard errors (hereafter SE) obtained after model averaging. We assumed that a predictor term significantly contributed to explain the response variable when the 95%CI for the estimated parameter excluded zero (Grueber et al. 2011).

Repeatability of uropygial gland measurements was calculated following the approximate Gaussian LMM using REML estimation ($R_{M(REML)}$) described by Nakagawa and Schielzeth (2010). To fit the global models, we employed the library "lme4" (Bates et al. 2014), to standardize the input variables we utilized the library "arm" (Gelman and Su 2015), for multi-model selection and model averaging we used the library "MuMIn" (Barton 2015) using R version 3.3.1 (R Development Core Team 2017).

RESULTS

We analysed whether uropygial gland volume may correlate with reproductive success in 192 observations taken from 152 individuals from four different breeding colonies.

In the first model, we analysed factors affecting total clutch size. The first final model showed that clutch size in barn swallows was correlated with capture date, body mass, tarsus length and tail length, as shown by the exclusion of zero from confidence intervals estimated for these variables (Table 1a). More specifically, swallows captured earlier to the breeding areas also laid larger clutches (Supplementary Figure S1). In addition, individuals with larger tarsus, longer outermost tail feathers or higher body mass also laid larger clutches (Supplementary Figures S2-4, respectively). In contrast, none of the two-way interaction terms showed any significant parameter estimate (Table 1a). To test for the main effects of uropygial gland

volume, sex, age class and the number of pairs nesting in the same room, we repeated our first model excluding this time the interaction terms. This reduced model confirmed that the main effect of uropygial gland volume on clutch size was not significant [estimate = 0.43; SE = 0.46; 95% CI = (-0.49, 1.34)], neither the main effect of sex [estimate = 0.10; SE = 0.73; 95% CI = (-1.34, 1.53)], age class [estimate = 0.03; SE = 0.45; 95% CI = (-0.87, 0.92)], nor of the number of pairs nesting in the same room [estimate = -0.73; SE = 0.44; 95% CI = (-1.60, 0.14)].

In the second model, we explored sources of variation in the total number of eggs hatched. The final second model showed that variation in the number of eggs hatched was related to capture date, body mass and the interaction between uropygial gland volume and abundance of conspecifics, as it was shown by the exclusion of zero from confidence intervals for these variables (Table 1b). Specifically, the number of eggs hatched increased for barn swallows captured earlier in the breeding season (Supplementary Figure S5), as well as for barn swallows with higher body mass (Supplementary Figure S6). Also, the significant interaction term indicated that for barn swallows nesting in rooms with higher abundance of conspecifics, the number of eggs hatched increased with the larger uropygial gland volume (Figure 1). In contrast, for barn swallows nesting in rooms with lower abundance of conspecifics, the number of eggs hatched decreased with the larger uropygial gland volume (Figure 1). Neither the interaction term between sex and uropygial gland volume, nor the interaction between age class and uropygial gland volume explained significant variation in the number of eggs hatched (Table 1b). To test for the main effects of uropygial gland volume, sex, age class and the number of pairs nesting in the same room, we repeated our second model excluding this time the interaction terms. This reduced model confirmed that the main effect of uropygial gland

volume on the number of eggs hatched was not significant [estimate = -0.30; SE = 0.63; 95% CI = (-1.56, 0.96)], neither the main effect of sex [estimate = 0.36; SE = 0.70; 95% CI = (-1.04, 1.76)], age class [estimate = -0.70; SE = 0.59; 95% CI = (-1.87, 0.47)], nor of the number of pairs nesting in the same room [estimate = -0.90; SE = 0.63; 95% CI = (-2.14, 0.36)].

In the third model, we tested for factors contributing to variation in the total number of fledglings. The final third model showed that variation in the number of fledglings was correlated with body mass and the interaction between uropygial gland volume and abundance of conspecifics, as it was shown by the exclusion of zero from confidence intervals estimated for these variables (Table 1c). Barn swallows with higher body mass produced more fledglings (Supplementary Figure S8). In addition, the interaction term indicated that for barn swallows nesting in rooms with higher abundance of conspecifics, the number of fledglings reared increased with the larger uropygial gland volume (Figure 2). In contrast, for barn swallows nesting in rooms with lower abundance of conspecifics, the number of fledglings reared decreased with the larger uropygial gland volume (Figure 2). Variation in the number of fledglings reared was not related to capture date, tail length, tarsus length, the interaction between sex and uropygial gland volume, nor to the interaction between age class and uropygial gland volume (Table 1c). To test for the main effects of uropygial gland volume, sex, age class and the number of pairs nesting in the same room, we repeated our third model excluding this time the interaction terms. This reduced model revealed a significant main effect of age class on the number of fledglings [estimate = -1.15; SE = 0.42; 95% CI = (-1.98, -0.32)]. Specifically, experienced barn swallows raised more fledglings (N = 101; mean ± SD = 5.90 ± 3.032 fledglings / year) than younger individuals (N = 78; mean ± SD = 4.59 ± 2.821 fledglings / year) (Supplementary Figure S7).

Additionally, the reduced model confirmed that the main effect of uropygial gland volume on the number of fledglings raised was not significant [estimate = -0.31; SE = 0.45; 95% CI = (-0.60, 1.22)], neither the main effect of sex [estimate = 0.49; SE = 0.51; 95% CI = (-0.52, 1.51)], nor of the number of pairs nesting in the same room [estimate = -0.10; SE = 0.44; 95% CI = (-0.79, 0.96)].

DISCUSION

In this study we have found that the volume of uropygial gland covaries with breeding success depending on the number of conspecific present in the given room used for nesting. Thus, when individuals nested close to each other in the same room, having large uropygial glands reported a fitness benefit, but that was not the case when individuals nested apart one from each other. These results were not cofounded for other traits known to affect breeding success in barn swallow, such as, age, body condition, tail length or capture date.

Secretions from the uropygial gland have been proposed to play an important role as a defensive mechanism against pathogens such as bacteria (Jacob et al. 1997, Law-Brown 2001, Ruiz-Rodríguez et al. 2009), fungi (Jacob et al. 1997) and haemosporidians (Magallanes et al. 2016, 2017, Marzal et al. 2018). For example, it has been suggested that uropygial secretion on the eggshells during incubation likely protect embryos against infections (Martin-Vivaldi et al. 2014). Bacteria and other pathogens on eggshells are responsible of decreased embryo viability, affecting egg survival and hatching success (Cook et al. 2005, Hansen et al. 2015, Peralta-Sánchez et al. 2018). Moreover, nestling mortality has often been associated with pathogenic impact of bacteria, fungi and haemosporidians infection

(Pinowski et al. 1994, Valkiūnas 2005). Therefore, we expected that barn swallows with larger uropygial glands would benefit from higher reproductive success in terms of clutch size, hatching eggs, and number of reared fledglings. Interestingly, we found that barn swallows with larger uropygial glands had higher breeding success (i.e. more eggs hatched, and fledglings reared) when nesting in rooms with greater abundance of conspecifics. The transmission dynamics of many pathogens (e.g. bacteria or fungi) within any given host population depend on the interaction between infectious and susceptible hosts (Altizer et al. 2003, Han et al. 2015). Moreover, infection incidence increases with the average number of contacts between infected and susceptible individuals (Johnson et al. 2011), and the rate of contact is assumed to increase linearly with host density (Anderson and May 1978). Thus, infection risk by pathogens such as virus or bacteria should increase with the number of host living in the same environment. For example, it has been shown that barn swallows living in larger colonies had more feather degrading bacteria than less social conspecifics (Møller et al. 2009). Accordingly, birds are expected to modify their investment in defensive traits (e.g. the production of uropygial gland secretion) in response to differences in exposure to microorganisms. In support of this assumption, Jacob et al. (2014) experimentally showed that male great tits (*Parus major*) modified the size of their uropygial gland when exposed to higher densities of bacteria on feathers. Therefore, barn swallows living in environments with higher risk of pathogen transmission (i.e. many individuals nesting within the same room) that could reduce their reproductive success may benefit by increasing their investment in the uropygial gland size.

Alternatively, barn swallows with larger uropygial glands had lower reproductive success (i.e. less eggs hatched and fledglings reared) when nesting in rooms with lower abundance of conspecifics. Because resources

are limited, individuals maximize their fitness by optimizing their capital investment in different energetically costly traits such as growth, reproduction or defensive traits (Ricklefs 1990). Previous studies have shown that the development of the uropygial gland is costly and may impair energetic demands (Piault et al. 2008, Pap et al. 2013). Therefore, the investment in an energetically costly defensive trait in environments with lower pathogen pressure (i.e. few individuals nesting within the same room) could not be beneficial, because it may impair resource allocation to other energetically demanding traits such as reproduction.

Tail length, body mass and tarsus length were positively correlated with clutch size. In barn swallows, tail length reliably reflects individual quality (Romano et al. 2017). Furthermore, barn swallows in better body condition have longer tail feathers than individuals in worse conditions (Balbontín et al. 2012a). It has also been shown in this species that male tail length is strongly positively related to arrival date, which may influence success in mate acquisition and mating date (Møller 1990), and in the total number of eggs laid per year (Cuervo et al. 1996). These high-quality individuals may have better body condition, which in turn may determine breeding success (Lescroël et al. 2010, Balbontín et al. 2012a). In concordance with our findings, several studies have shown strong positive correlations between body condition and clutch size in birds, showing that individuals in better body condition laid larger clutches than those in worse condition (Gladbach et al. 2010, Balbontín et al. 2012a, Bennett and Murray 2014). Hence, the larger clutches in barn swallows with longer outermost tail feathers and/or higher body condition may be reflecting the benefits in breeding success of higher quality individuals.

In migratory birds, early arrival at the breeding quarters may enhance fitness because it could determine the start of breeding and thus directly influence seasonal reproductive success (Møller 1994b, Smith and Moore 2005, Cooper et al. 2011). These observations are in agreement with our findings, since the advance in capture date was related to an increase in clutch size in barn swallows. This trend has also been shown in other species of hirundines such as house martins (*Delichon urbica*), where individuals that initiated their reproduction earlier also laid larger clutches (Marzal et al. 2013, 2016). These benefits of early arrival on breeding success extended beyond the overall number of eggs laid, since the number of hatched eggs was larger in barn swallows that were captured earlier at the breeding grounds. This could be explained because the pre-laying period (time between arrival to breeding quarters and start of laying the eggs) may be extended in early arrival migrant individuals. Because songbirds need to gather calcium, proteins, lipids and carotenoids on-site shortly before egg laying (Winkler and Allen 1996, Pahl et al. 1997, Nager 2006), early arriving individuals would benefit from extended time available to acquire these essential resources to produce high-quality eggs, and thus increase their hatching success (Kristensen et al. 2015). Alternatively, a long pre-laying period may improve individual body condition and increase hatching success. In this sense, it has been shown that pre-breeding body condition in female snow petrels (*Pagodroma nivea*) had a significant effect on hatching success (Barbraud and Chastel 1999). Also, body condition in males influenced hatching success in blue petrels (*Halobaena caerulea*) (Chastel et al. 1995). Moreover, some studies have shown that the decrease in body mass was the key factor causing clutch abandonment during incubation (Lorentsen and Røv 1995, Tveraa et al. 1997). All these outcomes suggest that only individuals in prime condition (e.g. enough body reserves) are able

to successful incubate the eggs during the energy demanding egg formation period.

Finally, we have shown that age and body mass are correlated with the number of reared fledglings. These findings are in agreement with previous studies in barn swallows. For example, Balbontín et al. (2012b) found an increase in the number of fledglings produced annually with age. Also, males and females in better body condition had a larger number of fledglings than individuals in worse body condition (Balbontín et al. 2012a).

To summarize, our results have also shown density dependence of hosts on the reproductive success associated with volume of uropygial glands, thus suggesting a plastic response of uropygial gland volume in barn swallows living in environments with higher pathogen exposure. Further analyses exploring the modification of this defensive trait under different parasite pressures would be desirable for gaining insights into host-pathogen interactions.

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Table 1. Summary results after model averaging of the effects of uropygial gland volume (i.e. UGV) on subsequent reproductive success of barn swallows breeding in Seville (Spain). Sample size was 182, 95 and 179 individuals respectively for clutch size (a), number of eggs hatched (b) and number of fledglings (c). The reference level of the fixed factor sex was "female", and the reference level of the fixed factor age class was "experienced". Parameters estimated represented comparable effect sizes that have been standardized to two SD following Gelman (2008). Parameters estimated for predictors with confidence intervals that do not include zero are highlighted in bold. RI – Relative Importance.

Parameter	**Estimate**	**SE**	**95% CI**	**RI**
a) Number of eggs laid				
(Intercept)	9.60233	0.72313	(8.18, 11.03)	
Capture date	**-1.15958**	**0.45926**	**(-2.07, -0.25)**	**0.93**
Body mass	**0.94828**	**0.44908**	**(0.06, 1.83)**	**0.79**
Tail length	**0.99889**	**0.49713**	**(0.02, 1.98)**	**0.77**
Tarsus length	**1.34898**	**0.43135**	**(0.50, 2.20)**	**1**
Sex	0.09806	0.72771	(-1.34, 1.53)	0.3
Age class	0.02814	0.45213	(-0.86, 0.92)	0.19
Breeding pairs	-0.72442	0.44085	(-1.59, 0.15)	0.46
UGV	0.417	0.46521	(-0.50, 1.34)	0.35
Sex * UGV	-0.94567	0.88582	(-2.69, 0.80)	0.03
Age class * UGV	-0.08417	0.88213	(-1.83, 1.66)	0.01
Breeding pairs * UGV	-0.61112	0.84062	(-2.27, 1.05)	0.03

Parameter	Estimate	SE	95% CI	RI
b) Number of eggs hatched				
(Intercept)	6.42766	0.56229	(5.31, 7.55)	
Capture date	**-2.24887**	**0.62359**	**(-3.49, -1.01)**	**1**
Body mass	**1.8874**	**0.63413**	**(0.63, 3.15)**	**0.99**
Tail length	-0.00244	0.77829	(-1.55, 1.54)	0.19
Tarsus length	0.46827	0.60518	(-0.74, 1.67)	0.22
Sex	0.38065	0.70904	(-1.03, 1.79)	0.22
Age class	-0.72538	0.56032	(-1.84, 0.39)	0.4
Breeding pairs	-1.11646	0.62307	(-2.35, 0.12)	0.69
UGV	-0.35886	0.61821	(-1.59, 0.87)	0.61
Sex * UGV	-0.79565	1.3233	(-3.43, 1.84)	0.02
Age class * UGV	-0.93972	1.13534	(-3.20, 1.32)	0.06
Breeding pairs * UGV	**3.45545**	**1.19791**	**(1.07, 5.84)**	**0.53**

Parameter	Estimate	SE	95% CI	RI
c) Number of fledglings				
(Intercept)	5.38797	0.28012	(4.84, 5.94)	
Capture date	-0.64914	0.43462	(-1.51, 0.21)	0.48
Body mass	**1.85149**	**0.44734**	**(0.97, 2.73)**	**1**
Tail length	0.42739	0.51994	(-0.60, 1.45)	0.3
Tarsus length	-0.01498	0.42915	(-0.86, 0.83)	0.21
Sex	0.52583	0.50678	(-0.47, 1.53)	0.38
Age class	**-1.16312**	**0.41854**	**(-1.99, -0.34)**	**0.99**
Breeding pairs	-0.02256	0.43782	(-0.89, 0.84)	0.5
UGV	0.28045	0.45988	(-0.63, 1.19)	0.59
Sex * UGV	0.62066	0.89718	(-1.15, 2.39)	0.06
Age class * UGV	0.57038	0.86813	(-1.14, 2.28)	0.14
Breeding pairs * UGV	**2.01091**	**0.82232**	**(0.39, 3.63)**	**0.36**

LEGENDS TO FIGURES

Figure 1. Relationship between uropygial gland volume, number of eggs hatched and number of pairs nesting within the same room. Sample size was 95 barn swallows.

Figure 2. Relationship between uropygial gland volume, number of fledglings reared and number of pairs nesting within the same room. Sample size was 179 barn swallows.

Figure 1

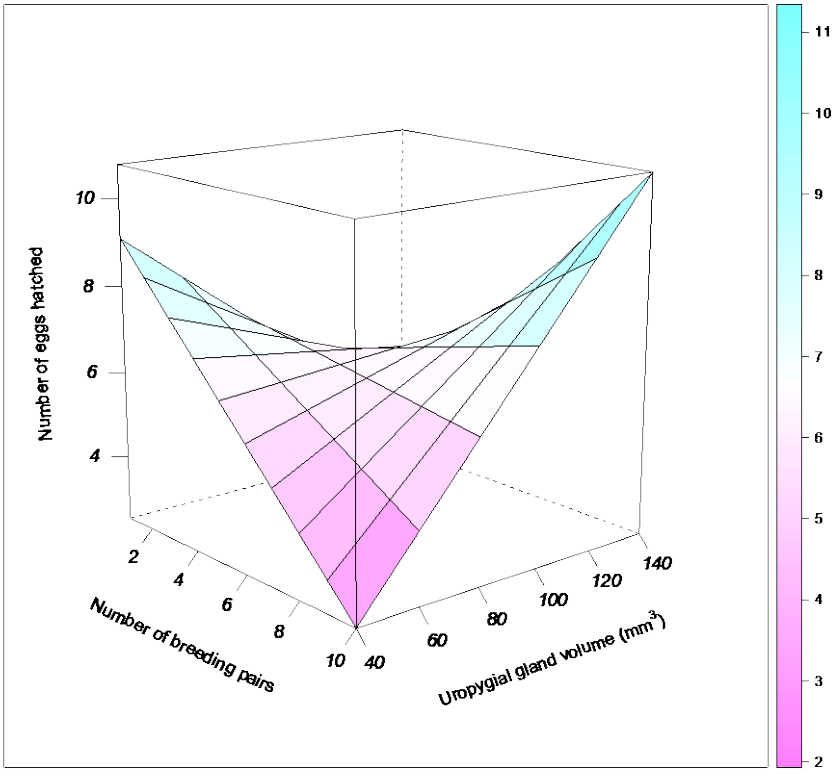
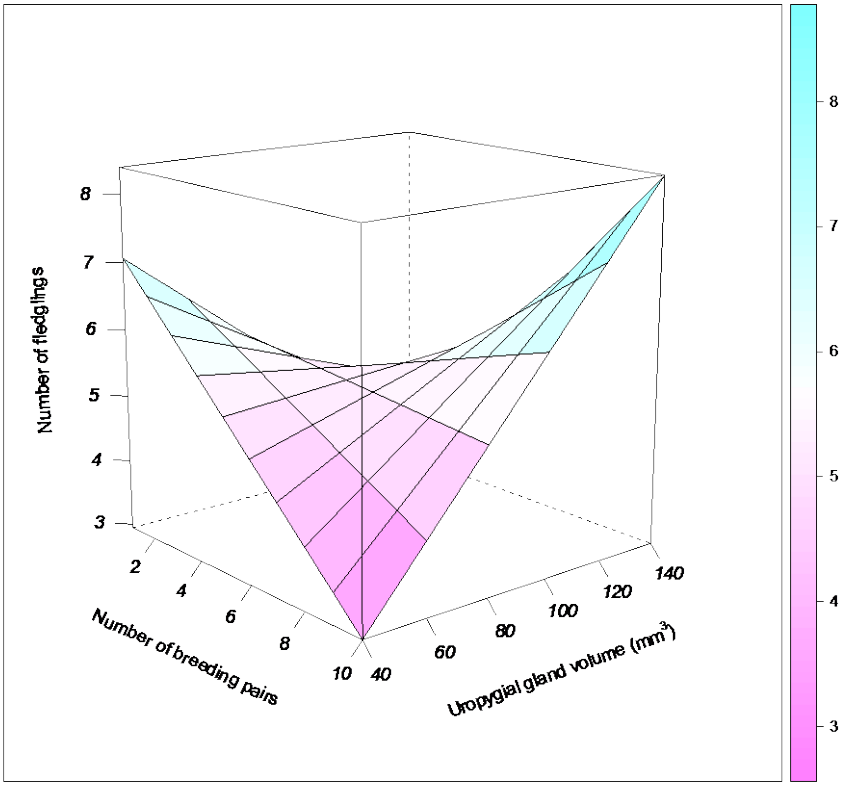


Figure 2


Number of fledglings
8
7
6
5
4
3
2
4
6
8
10
Number of breeding pairs
40
60
80
100
120
140
Uropygial gland volume (mm3)
8
7
6
5
4
3

Supplementary information

Reproductive success related to uropygial gland volume varies with abundance of conspecifics in barn swallows *Hirundo rustica*

Sergio Magallanes, Cosme López-Calderón, Javier Balbontín, Anders P. Møller, Florentino de Lope and Alfonso Marzal

This supplementary information includes: **Figure. S1-S8**

Supplementary material

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure S1. Relationship between clutch size and capture date. Error bars are ± SE. Indents on x-axis are the individual observations. Sample size was 192 barn swallows.

Supplementary Figure S2. Relationship between clutch size and parental body mass. Error bars are ± SE. Indents on x-axis are the individual observations. Sample size was 192 barn swallows.

Supplementary Figure S3. Relationship between clutch size and parental tail length. Error bars are ± SE. Indents on x-axis are the individual observations. Sample size was 192 barn swallows.

Supplementary Figure S4. Relationship between clutch size and parental tarsus length. Error bars are ± SE. Indents on x-axis are the individual observations. Sample size was 192 barn swallows.

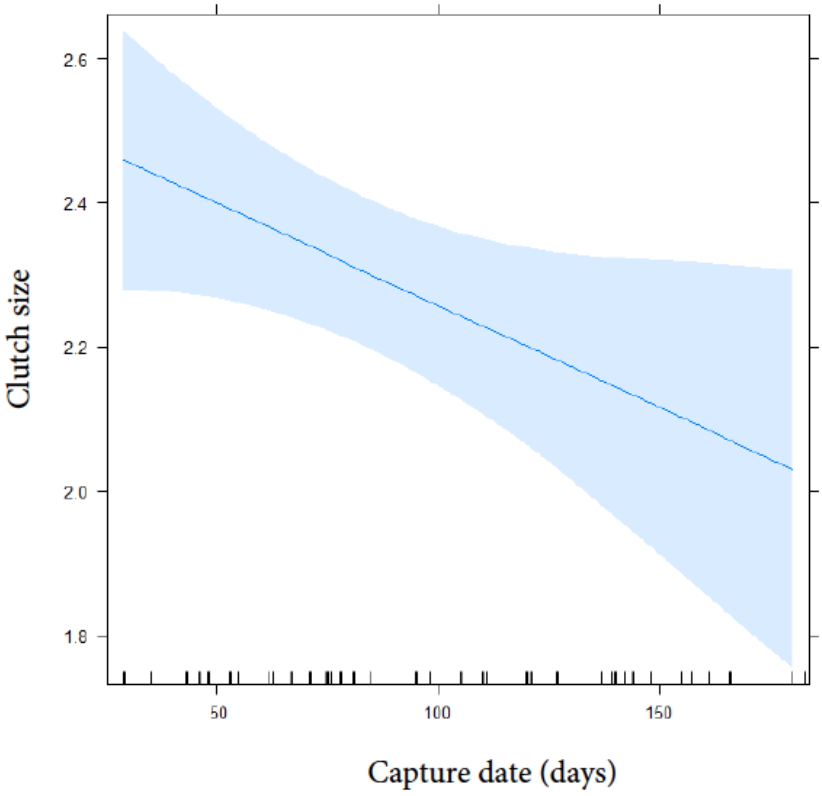
Supplementary Figure S5. Relationship between the number of hatched eggs and capture date. Error bars are ± SE. Indents on x-axis are the individual observations. Sample size was 192 barn swallows.

Supplementary Figure S6. Relationship between the number of hatched eggs and parental body mass (g). Error bars are ± SE. Indents on x-axis are the individual observations. Sample size was 192 barn swallows.

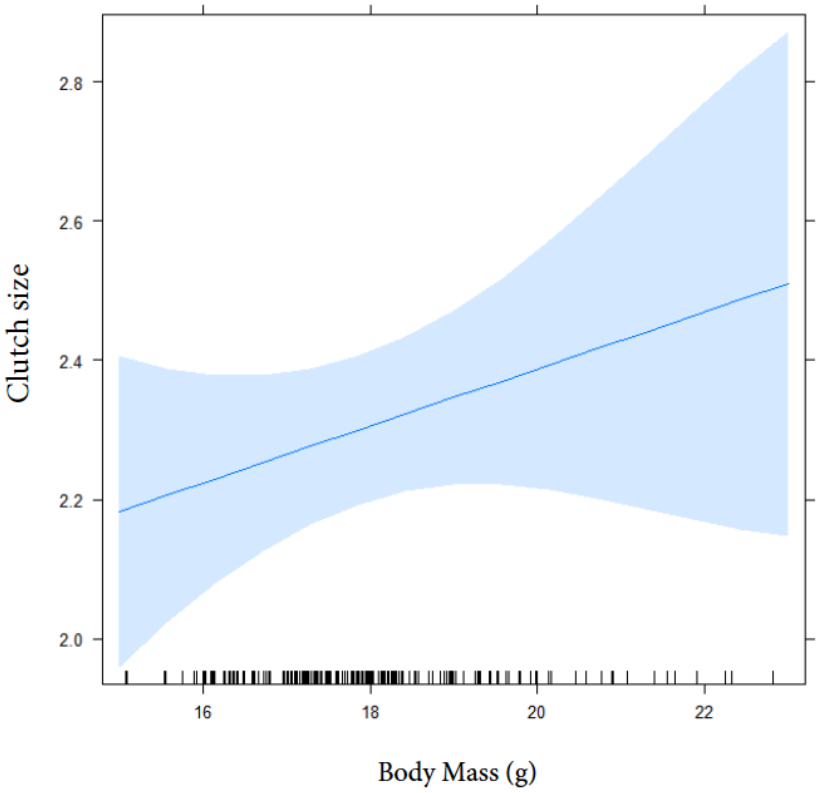
Supplementary Figure S7. Relationship between the number of reared fledglings and parental body mass (g). Error bars are ± SE. Indents on x-axis are the individual observations. Sample size was 192 barn swallows.

Supplementary Figure S8. Box plot showing the number of reared fledglings for experienced (N = 105) and young (N = 87) barn swallows. Values are medians, upper and lower quartiles, and extreme observations.

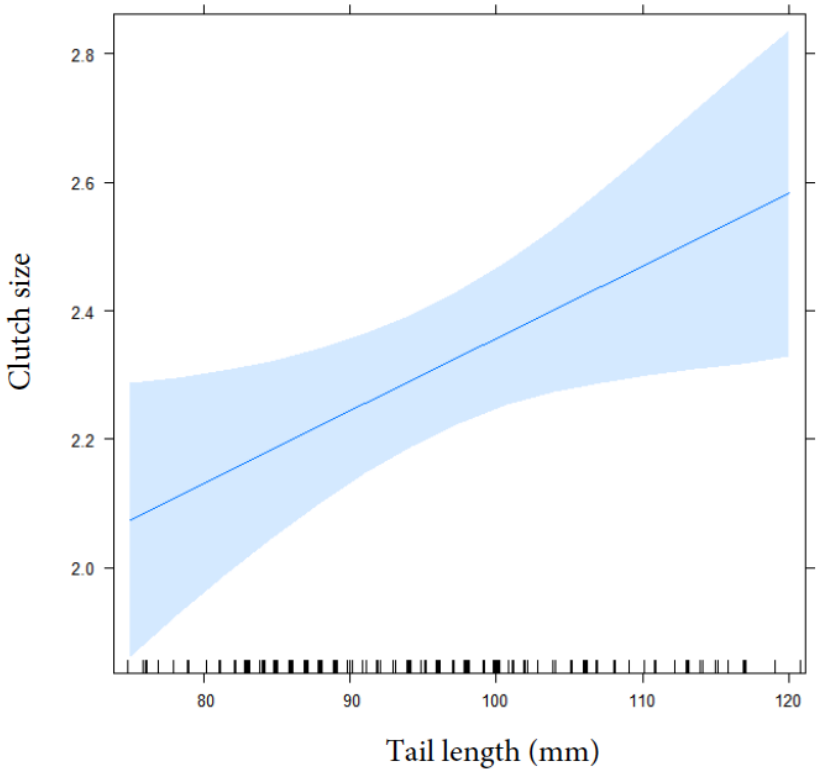
Supplementary Figure S1



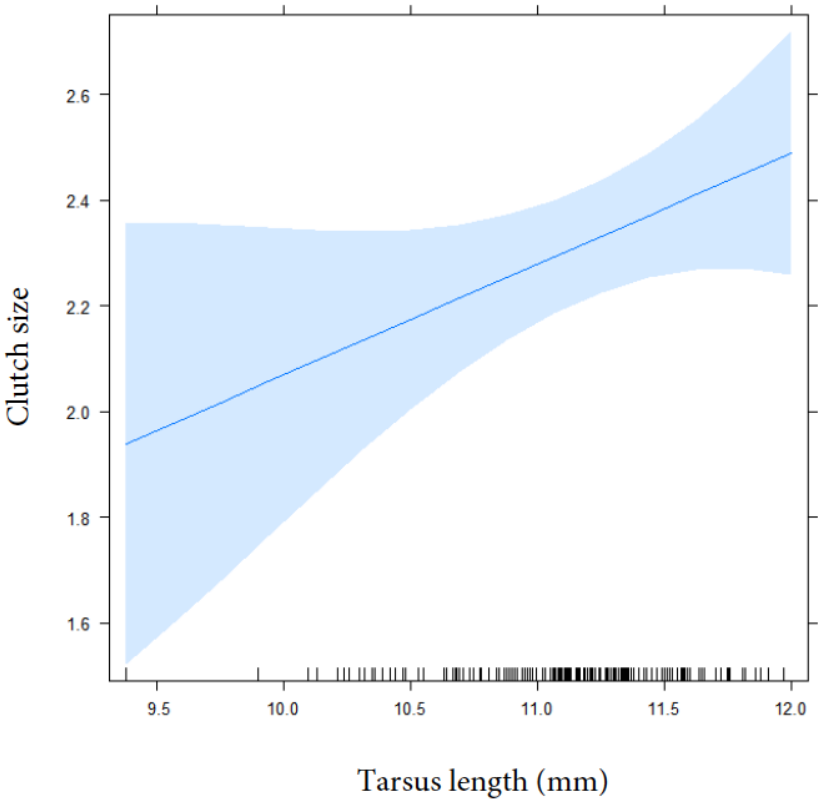
Supplementary Figure S2



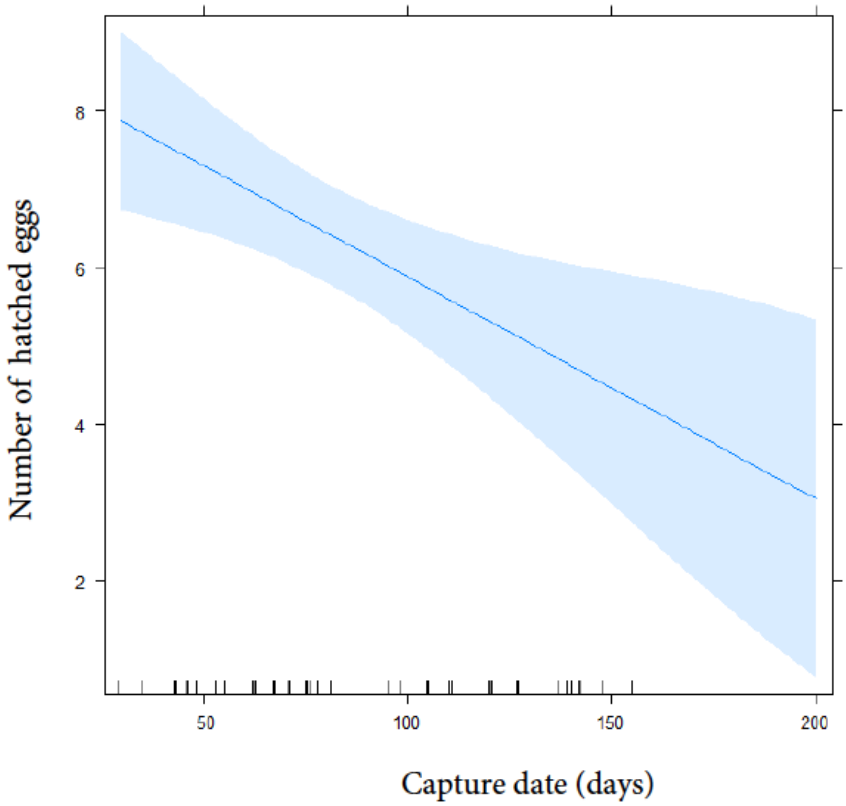
Supplementary Figure S3



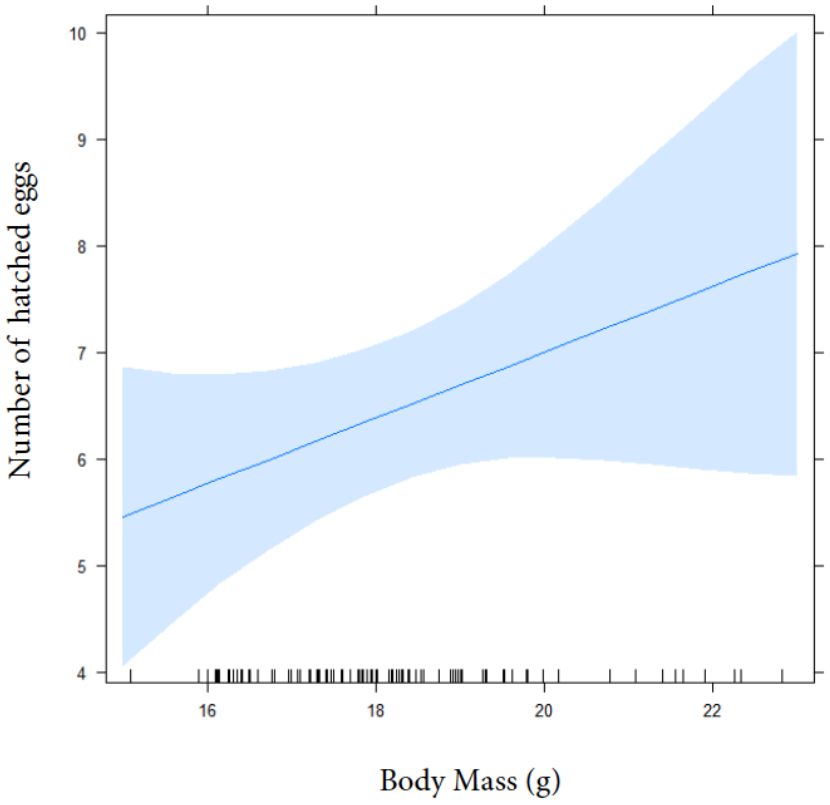
Supplementary Figure S4



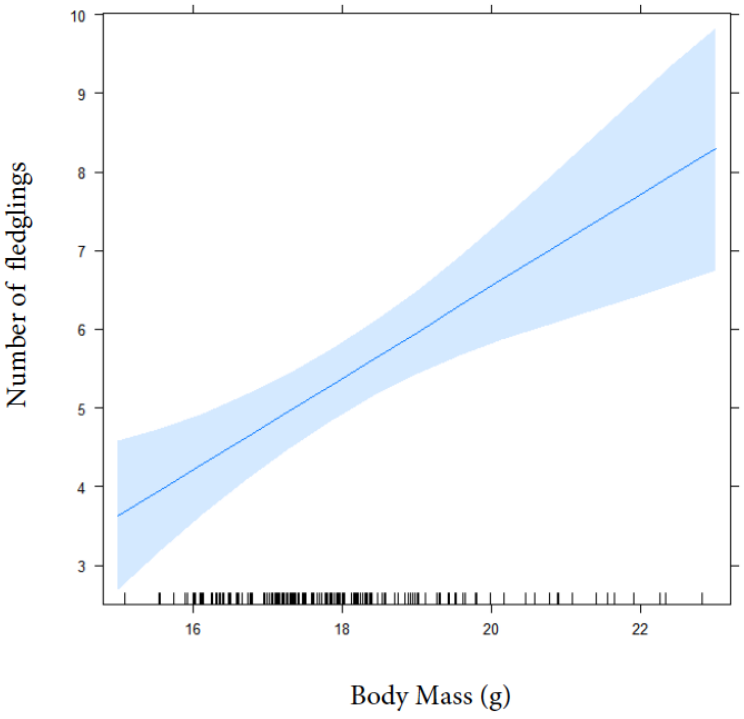
Supplementary Figure S5



Supplementary Figure S6



Supplementary Figure S7



Supplementary Figure S8

Chapter IV

Variation in malaria infection and volume and antimicrobial activity of uropygial gland secretions in invasive house sparrows from Peruvian and Spanish localities.

VARIACIÓN EN LA INFECCIÓN POR MALARIA Y LAS DEFENSAS INMUNES EN LOS GORRIONES COMUNES INVASORES Y ENDÉMICOS.

Resumen

Las invasiones biológicas llevadas a cabo por especies exóticas imponen enormes costes ecológicos, sociales y económicos en todo el mundo. Dado que no todos los individuos introducidos en los nuevos ambientes llegan a ser invasores exitosos, la identificación de los factores que regulan la variación en el éxito de la invasión resulta esencial para evaluar los riesgos de invasión. En este estudio hemos analizado varias hipótesis en el contexto hospedador–parásito para explicar el éxito de la invasión del gorrión común (*Passer domesticus*) en Perú. En línea con la Hipótesis de Liberación de Enemigos, los gorriones de Perú mostraron una menor prevalencia y diversidad genética de parásitos haemosporidios que los gorriones de su área original (España), indicando que la liberación de sus parásitos naturales podría haber favorecido su dispersión a nuevas áreas. También mostramos que los gorriones peruanos tuvieron una mayor glándula uropigial y una mayor actividad bactericida en sus secreciones uropigiales que los gorriones españoles, lo que sugiere una selección mediada por los patógenos en los mecanismos de defensa al colonizar nuevos ambientes. Finalmente, observamos que los gorriones que no estaban infectados por malaria tuvieron una mayor glándula uropigial y una mayor actividad bactericida en sus secreciones que los gorriones infectados con malaria, lo que podría indicar que las secreciones de la glándula uropigial puede actuar como un mecanismo de defensa frente a las infecciones por haemosporidios.

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Variation in malaria infection and immune defence in invasive and endemic house sparrows

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Keywords
invasive species; Enemy Release Hypothesis; *Haemoproteus*; Invasive Immunity Hypothesis; *Passer domesticus*; *Plasmodium*; avian malaria; immune response.

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Abstract

Biological invasions by exotic species impose substantial ecological, economic and social costs worldwide, being a major threat to biodiversity conservation. Because not all individuals introduced in the new environments become successful invaders, the identification of factors underlying variation in invasion success would be essential for evaluating invasion risk. Here, we test several host–parasite hypotheses accounting for invasion success of house sparrows *Passer domesticus* in Peru. According to the *Enemy Release Hypothesis*, invasive house sparrows from Peru showed lower prevalence and genetic diversity of haemosporidian parasites than sparrows from their natural range (Spain), indicating that the release from their natural parasites may have favoured the spread of sparrows in the new area of occurrence. We also showed that Peruvian sparrows had larger uropygial glands and higher anti-bacterial activity in its secretion than sparrows from Spain, suggesting selection in defensive mechanisms driven by pathogens when colonizing new environments. Finally, we showed that uninfected sparrows had larger uropygial glands and higher anti-bacterial activity than malaria-infected house sparrows, implying that uropygial gland secretions may act as a defensive mechanism against haemosporidian infections. Alternatively, a condition-dependent trade-off exists between synthesis of uropygial secretion and immune response. These outcomes provide essential information for identifying potential invaders and designing interventions.

Introduction

Biological invasions by alien species have profound impact in all environments provoking population declines and even extinctions, thus being considered one of the most serious threats for global biodiversity (IUCN, 2000). Moreover, invasive species also have substantial impacts on disease environments for livestock, crops and humans, and impose important economic and social costs worldwide (Simberloff *et al.*, 2013; Jeschke, Bacher & Blackburn, 2014). However, not all individuals arriving to new environments become successful invaders. In this sense, the ecological theory of tens rule predicts that of 100 imported species or individuals appear in the wild (introduced or escaped), about 10 of these introduced become established, and of these only one will become an invader (Williamson & Fitter, 1996; Jarić & Cvijanović, 2012). The question arising is which features cause an individual to become a successful invader. Because the difficulty and costs of control or eradication one species once established outside its natural range, the identification of factors underlying invasion success become crucial for identifying invasion risk and design interventions. While some features have been widely studied to identify factors explaining establishment and spread of introduced bird

species (e.g. availability of resources and abiotic factors in the new environments; see review in Duncan, Blackburn & Sol, 2003), other hypotheses, such as those based on host-parasite interaction traits, have received less attention.

On the one hand, parasites and other pathogens have been proposed to play a major role in biological invasions, facilitating colonization and spread of their hosts in new areas (Tompkins *et al.*, 2011; Blackburn & Ewen, 2017). In this sense, the *Novel Weapon Hypothesis* asserts that invaders may bring parasites with them to the new environments, against which the invader individuals, but not the native ones, have evolved defences. Consequently, individuals and populations in the invaded areas can be seriously damaged by these new parasites (Callaway & Ridenour, 2004; Prenter *et al.*, 2004; Table 1). For example, the parapoxvirus carried by gray squirrel (*Sciurus carolinensis*) has allowed this invasive species to colonize the UK and displace the native red squirrel (*S. vulgaris*) (Collins *et al.*, 2014). Alternatively, the *Enemy Release Hypothesis* poses that some non-native individuals become successfully established because they are freed from their co-evolved pathogens and parasites in the colonization process (Torchin, Lafferty & Kuris, 2002; Colautti *et al.*, 2004; Table 1). For example, it has been shown that seven non-native fish species in England and Wales only retained 8.5% of their native parasite fauna in their novel range, showing evidence of enemy release (Sheath *et al.*, 2015). Because parasites can exert negative effects on their hosts and reduce host population abundance, density and spread (Anderson & May, 1991), a decline in parasite pressure could allow introduced individuals to decrease their investment in defences against parasites. This could lead colonizers to maximize their capital in growth and reproduction, thus increasing their competitive ability and displace native species (Blossey & Nötzold, 1995; Lee & Klasing, 2004). However, the reduction in investment in defence could be self-defeating if it could minimize the success in the fight against novel pathogens in invaded areas. Avian malaria and related haemosporidian parasites are widespread and harmful organisms (Valkiūnas, 2005). Several studies have explored the role of these parasites in invasions in bird-parasite systems, with mixed results (see García-Longoria *et al.*, 2015 for review). For example, Lima *et al.* (2010) found that house sparrows (*Passer domesticus*) native to Europe exhibited significantly higher haemosporidian prevalence than introduced house sparrows from Brazil, suggesting that sparrows from Brazil might have experienced a parasitic release during the process of introduction. In contrast, avian malaria parasites carried by exotic bird species have likely provoked the death of threatened native species of New Zealand (Ewen *et al.*, 2012; Schoener *et al.*, 2014).

On the other hand, individuals may face new parasites and pathogens in the new areas during colonization reducing survival or inducing costly immune responses in naïve hosts. Hence, the parasite fauna in the new environments may reduce the fitness of potential colonizers and thus prevent their spread and establishment (*Biotic Resistance Hypothesis*, Elton, 1958; Ricklefs, 2010). With the aim to successfully counteract the challenge from new pathogens, an increased investment in immune defences may allow colonizers to combat these new pathogens. Therefore, individuals with better immune defences may enjoy better invasion success (*Invasive Immunity Hypothesis*, Lee & Klasing, 2004). This hypothesis has been supported in a study showing a greater efficiency of the immune system of the invasive harlequin ladybird *Harmonia axyridis* than defences from the native model species *Tribolium castaneum*, thus implying that the expansion of AMP gene families and their induction in response to pathogens in *H. axyridis* may play a role in promoting the invasive success of this species (Vilcinskas, Mukherjee & Vogel, 2013). Moreover, Martin *et al.* (2014) have examined spleen size in house sparrows (*Passer domesticus*) from Kenya along their invasive range initiated in Mombasa. They showed that Kenyan house sparrows had larger spleens near the range edge than sparrows from Mombasa. This could mean that birds at the range edge, where they are more exposed to novel parasites, may be more immunocompetent. The uropygial gland (also called preen gland) is an exocrine gland of birds secreting waxes with antimicrobial and antifungal properties acting as a defensive barrier of skin and plumage (Jacob & Ziswiler, 1982; Møller, Czirják & Heeb, 2009; Czirják *et al.*, 2013). Although some studies have pointed out that uropygial secretions may attract haemosporidian vectors and hence increase the probability of becoming infected (Bennett, Fallis & Campbell, 1972; Russell & Hunter, 2005), recently it has also been suggested that uropygial secretion may prevent birds from acquiring haemosporidian infections (Magallanes *et al.*, 2016) and influence survival in house martins *Delichon urbica* (Magallanes *et al.*, 2017). However, whether uropygial gland secretions may assist potential colonizers in their biological invasions remains unknown.

Table 1 Hypotheses accounting for invasion success of hosts.

Hypothesis	Enemy release hypothesis	Biotic resistance hypothesis	Novel weapon hypothesis	Invasive immunity hypothesis
Anti-parasite defences	Reduced investment in anti-parasite defences		Resistance against parasites from the native range	Higher invasion success due to better immune defence
Geographic distribution of parasites	Absence of parasites from the native range among invaders	Native parasites in the new areas prevent establishment of potential colonizers		

Data on historical bird introductions provide a unique opportunity to test hypothesis about biological invasions (Duncan *et al.*, 2003). The house sparrow is native to the Mediterranean region and it has been successfully introduced in America from Europe in the last two centuries, as inferred from recorded releases of imported sparrows (Summers-Smith, 1998; Anderson, 2006) and high genetic similarity of house sparrows in both continents (Schrey *et al.*, 2011). For example, this species was introduced in parks of Lima (Peru) in 1951 (Leck, 1973). Previous studies have shown a release of haemosporidian parasites in house sparrows when colonizing Brazil and Argentina (Lima *et al.*, 2010; Marzal *et al.*, 2011), and also the presence of novel haemosporidian parasites in the native avifauna in Peru (Marzal *et al.*, 2015), which may suggest that several mechanisms facilitating biological invasions may operate simultaneously.

Here, we first characterize the identity and prevalence of haemosporidian parasites in house sparrows from a colonized area (Peru) and a native range (Spain) to analyse the potential contributions of the Novel Weapon Hypothesis and the Enemy Release Hypothesis to the successful invasion of house sparrows in Peru (Table 1). We also examine the size of the uropygial gland and the anti-microbial activity of its secretions to explore if uropygial gland secretions may have facilitated the establishment of individual sparrows in these new areas. If the properties of uropygial secretions could help sparrows in their spread to new areas (i.e. facing new pathogens), then we expect larger uropygial glands and/or higher anti-bacterial activity of the gland secretions of Peruvian house sparrows.

Materials and methods

Study sites and sample collection

The study was carried out in three house sparrow populations in Peru (Manchay: 12°10′S, 76°51′W; Tarapoto 6°29′S, 76°22′W) and Spain (Badajoz: 38°53′N, 7°00′W) in May – June 2016. We captured 135 house sparrows with mist-net (55 in Manchay population, 11 in Tarapoto population and 69 in Badajoz population). Each bird was identified with a numbered metal ring. We measured their tarsus length with a digital calliper to the nearest 0.01 mm. We also recorded their body mass with a digital balance to the nearest 0.1 g. We used body mass and tarsus length to calculate scaled body mass index (Peig & Green, 2009), which is a reliable estimate of animal physical condition (Peig & Green, 2010). One microcapillary of blood (50 μL) was obtained from the brachial vein of each individual and stored in 500 μL of SET buffer until DNA extraction.

Molecular detection of blood parasite infections

Blood samples were examined using molecular methods to determine the presence and genetic diversity of haemosporidian parasite lineages (Waldenström *et al.*, 2004). DNA from the avian blood samples was extracted using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific Inc., reference #K0722). Diluted genomic DNA (25 ng/μl) was used as a template in a polymerase chain reaction (PCR) assay for detection of the parasites using nested-PCR protocols described by Waldenström *et al.* (2004). All PCR experiments contained one positive control and one negative control for every eight samples. The amplification was evaluated by running 2.5 μL of the final PCR on a 2% agarose gel. Parasites detected by a positive amplification were sequenced using the procedures described by Bensch *et al.* (2000). The obtained sequences of 478 bp of the cyt b were edited, aligned and compared in a sequence identity matrix using the program BioEdit (Hall, 1999). Information of the identity of the parasite lineages and their geographic distribution was provided by MalAvi database (Version 2.3.3 November 2017; Bensch, Hellgren & Pérez-Tris, 2009).

Volume of the uropygial gland secretion

We recorded length, height and width of the uropygial gland with a digital calliper with a precision of 0.01 mm. Uropygial gland volume was estimated as the product of the three dimensions of the uropygial gland (Galván & Sanz, 2006), which is positively related to the volume of secretions (Martín-Vivaldi *et al.*, 2009; Pap *et al.*, 2010). Because the uropygial gland is a soft tissue (Martín-Vivaldi *et al.*, 2009; Møller *et al.*, 2009), we measured the three dimensions of the uropygial gland three times and used the average as the best approach to gland volume.

Bacterial growth and anti-microbial activity of uropygial gland secretions

We also extracted all the secretions available in the uropygial papilla immediately after capture of 133 individuals from Peru ($N = 65$) and Spain ($N = 68$), following the extraction protocol described by Martín-Vivaldi *et al.* (2009). We first washed the uropygial gland and surrounding skin with a cotton swab soaked in ethanol to reduce the risk of contamination of the secretion. After evaporation of the alcohol, the papilla was softly pressed with a finger to eject the secretion and transfer it to a micro-capillary tube until the papilla was empty. The extracted secretion was transferred to a sterile Eppendorf vial and kept at about 4°C in a portable icebox, and stored in the laboratory at −20°C during the next 4 h until analyses of anti-microbial activity using *Staphylococcus epidermidis* (ATCC ® CRM – 12228™) as model.

The pellet of *S. epidermidis* was re-suspended in 6 mL of Luria - Bertani (LB) media to an OD 0.4–0.6 and incubated at 37°C with shaking for 24 h. The bacterial suspension was then centrifuged in a Microfuge (BeckmanCoulter) for 6 min at 2000 g. After discarding the supernatant, the bacterial pellet was re-suspended carefully in 20 mL LB solution. A total of 200 μL per well of bacterial suspension were then dispensed in 96-well plate. Uropygial secretions were diluted 1:1 in dimethylsulfoxide (DMSO). One microlitre of the uropygial secretion dilution was added to the bacterial culture in each

well. Four wells on a 96-well plate with bacteria solution were not added with uropygial secretions but with 1 μL of DMSO, as they were used as controls of bacterial growth. After culture incubation for bacterial growth at 37°C for 24 h, the 96-well plate was centrifuged in a plate centrifuge (Selecta, Spain) for 5 min at 2000 g and the pellet was re-suspended in 200 μL of PBS with Propidium Iodine (PI) at a final concentration of 0.6 μg/mL. Samples were then incubated at 37°C for 30 min in the dark with shaking. Flow cytometry detecting absolute cell counts assessed anti-microbial activity of the uropygial gland secretion against *S. epidermidis*. This technique is a rapid, accurate and highly reproducible methodology used in clinical microbiology to monitor anti-microbial activity (Álvarez-Barrientos *et al.*, 2000) and it has been used to assess anti-microbial activity of preen secretions (Magallanes *et al.*, 2016). A total of 50 μL of the cell suspension from each well was acquired using a MACSQuant® X (Miltenyi Biotec) and Cytoflex S (Beckman Coulter) flow cytometers allowing absolute cell counting. Anti-bacterial activity percentage was evaluated by comparison of cell counting (bacterial growth) and percentage of PI + cells (cell death) in wells with presence or absence (controls) of uropygial secretion.

Statistical procedures

We performed contingency-table χ^2 test to determine if the prevalence of different parasite genera varied with locality. We performed log-linear Poisson regression analyses to analyse whether the diversity of parasite lineages infecting house sparrows varied among sites. We used general linear models (GLM) to explore the relationship between scaled body mass index, tarsus length, infection status (uninfected or infected), locality (i.e. environmental variation) and their interaction (malaria infection * locality) on the uropygial gland volume. We also used a GLM to evaluate the correlation between scaled body mass index, uropygial gland volume, infection status (uninfected or infected), locality and their interaction (malaria infection * locality) on the anti-microbial activity of the uropygial secretion. All analyses were performed using R version 3.2.2 (R Development Core Team 2015) and PASW Statistics 18 statistical package for Windows.

Results

Prevalence and genetic diversity of haemosporidian lineages in house sparrows from Peru and Spain

We analysed 135 blood samples from house sparrows from Peru ($N = 66$) and Spain ($N = 69$) in search for blood parasites. The prevalence of avian malaria parasites significantly differed between the two populations (Chi-square test: $N = 135$; $\chi^2 = 10.513$, d.f. = 1, $P = 0.001$). Specifically, house sparrows from Spain showed higher prevalence of infection (36.23%) than sparrows from Peru (10.60%). A total of 23.19% of the Spanish house sparrows were infected with *Plasmodium* lineages, whereas 13.04% carried a *Haemoproteus* infection. In addition, the mean number of haemosporidian lineages infecting house sparrows differed significantly between Peru and Spain (GLM loglineal Poisson: $N = 135$; $\chi^2 = 10.128$, d.f. = 1, $P = 0.001$). Two lineages of *Plasmodium* and 2 lineages from *Haemoproteus* were recovered from Spanish house sparrows, whereas a single different *Plasmodium* lineage was found infecting Peruvian house sparrows (Table S1). The only haemosporidian lineage found infecting Peruvian house sparrows (GRW04) was absent in house sparrows from Spain.

Volume of the uropygial grand

Prevalence of haemosporidian parasites and locality explained significant variation in size of the uropygial gland. In contrast, neither scaled body mass index, tarsus length nor the interaction between locality and haemosporidian infection were significantly correlated with size of the uropygial gland (Table 2). Specifically, the volume of the uropygial gland was larger in uninfected than in infected house sparrows, independently of their locality [mean uropygial gland volume (standard deviation, SD): uninfected = 190.98 mm^3 (41.65); infected = 157.04 mm^3 (41.22)] (Fig. 1). Furthermore, the volume of the uropygial gland differed between localities, with Peruvian house sparrows having larger glands than house sparrows from Spain [mean uropygial gland volume (SD): Peru = 205.67 mm^3 (41.41); Spain = 161.19 mm^3 (34.20)] (Figure 1).

Anti-bacterial activity of the uropygial gland secretion

We also examined if anti-microbial activity of the uropygial secretion varied with scaled body mass index, uropygial gland volume, locality, haemosporidian infection and the interaction between locality and haemosporidian infection. The estimate of anti-microbial activity of the uropygial gland varied with locality and haemosporidian infection (Table 3). Specifically, anti-microbial activity of uropygial secretion was significantly higher in uninfected than in infected birds, independently of their locality [mean percentage anti-microbial activity (SD): uninfected = 34.37 (21.75); infected = 18.51 (17.68)] (Fig. 2). Finally, uropygial gland secretions from Peruvian house sparrows had higher anti-microbial activity than secretions from sparrows from Spain [mean percentage anti-microbial activity (SD): Peru = 41.21 (20.99); Spain = 20.84 (17.76)] (Fig. 2).

Table 2 Factors explaining variation in volume of the uropygial gland in house sparrows. A General Lineal Model was used with scaled body mass index, tarsus length, malaria infection, locality and their interaction (malaria infection * locality) as predictor variables. Sample size was 135 individuals.

Variable	Type III SS	d.f.	*F*	*P*
Scaled Body mass index	573.725	1	0.458	0.500
Malaria infection	9079.970	1	7.254	0.008
Locality	5629.081	1	4.497	0.036
Malaria infection * Locality	363.464	1	0.290	0.591
Tarsus length	4090.562	1	3.268	0.073

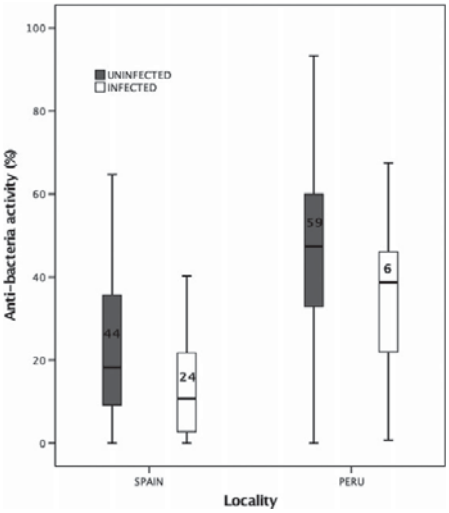

Figure 1 Box plots showing volumes of uropygial gland for malaria infected and non-infected house sparrows with respect to locality. Values are medians, upper and lower quartiles and extreme observations. Sample sizes of each category are shown inside the box-plots.

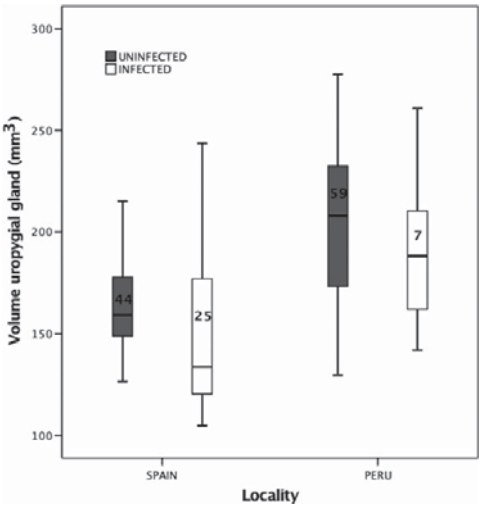

Figure 2 Box plots showing anti-bacterial activity (%) of the uropygial gland secretion for malaria infected and non-infected house sparrows with concern to locality. Values are medians, upper and lower quartiles and extreme observations. Sample sizes of each category are shown inside the box-plots.

Discusion

In last two centuries, many bird species have been transported and introduced to new locations worldwide (Blackburn, Lockwood & Cassey, 2009). Some of these introduced species (e.g. house sparrows and European starlings *Sturnus vulgaris*) have been successfully established in the new environments and become invaders, thus having significant environmental and economic impact. However, not all individuals transported to new locations succeed in establishing and spreading, suggesting that some factors may underlie variation in invasion success among individuals (Duncan *et al.*, 2003). Here, we have analysed if the variation in prevalence and genetic diversity of haemosporidian parasites may explain the invasion of house sparrow in Peru. We have also tested for differences in uropygial gland volume and the antimicrobial properties of its secretion in house sparrows from Peru and Spain. We showed that (1) Peruvian house sparrows showed lower prevalence and genetic diversity of haemosporidians than sparrows from Spain; (2) sparrows from Peru had larger uropygial glands and higher anti-microbial activity on its secretion, and (3) sparrows infected with malaria had smaller uropygial glands and secretions with lower anti-microbial activity than uninfected sparrows, independently from their location. Next we will discuss these outcomes in detail.

Table 3 Factors explaining variation in anti-bacterial activity of the secretion of the uropygial gland in house sparrows. A General Lineal Model was used with scaled body mass index, uropygial gland volume, malaria infection, locality and their interaction (malaria infection * locality) as predictor variables. Sample size was 133 individuals.

Variable	Type III SS	d.f.	*F*	*P*
Scaled Body mass index	1.394	1	0.004	0.951
Uropygial gland volume	107.871	1	0.293	0.589
Malaria infection	1726.956	1	117.329	<0.001
Locality	4259.526	1	23.554	<0.001
Malaria infection * Locality	0.940	1	0.003	0.960

Prevalence and genetic diversity of haemosporidian differed significantly between sparrows from Peru and Spain. Specifically, prevalence of haemosporidian parasites was higher in sparrows from Spain than in individuals from Peru. This pattern is consistent with the spread of invasive populations accompanied by a reduction of parasite prevalence compared to conspecific populations from the native range, as previous studies have shown (Cornell & Hawkins, 1993; Torchin *et al.*, 2003). For example, Marzal *et al.* (2011) compared the malaria prevalence in more than 1100 house sparrows from 23 locations in Europe and 12 locations in South America, showing that the malaria prevalence was higher in sparrows from native populations than in sparrows from invasive populations. Moreover, none of the haemosporidian lineages infecting sparrows from Spain was found in sparrows from Peru. Recent studies have described more than 30 haemosporidian lineages infecting native birds from Peru (Jones, Cheviron & Carling, 2013; Galen & Witt, 2014; Marzal *et al.*, 2015). We only recovered one single

parasite lineage (*Plasmodium relictum* GRW4) in sparrows from Peru. This *Plasmodium* lineage is a wide generalist parasite infecting more than 40 bird species in many continents (MalAvi database version 2.3.3 November 2017; Bensch *et al.*, 2009), but it does not occur in any sparrow from Europe (Marzal *et al.*, 2011; see also MalAvi database version 2.3.3 November 2017; Bensch *et al.*, 2009) and is mainly transmitted out of this region (Hellgren *et al.*, 2015). Hence, in agreement with the Enemy Release Hypothesis, our outcomes showed that house sparrows lost the parasites from their native European range in colonizing Peru, and have assimilated a generalist parasite of the new continent. Our results match with previous studies exploring the role of avian malaria parasites in the spread and colonization of bird species (see review in García-Longoria *et al.*, 2015). Also, Marzal *et al.* (2011) analysed the genetic diversity of haemosporidian parasites in house sparrows from 58 localities on six continents, showing that sparrows did not retain their native parasites in newly colonized regions. These results provide supportive information for the described pattern in the release of their haemosporidian parasites by invasive bird species when colonizing new areas (Lima *et al.*, 2010; Marzal *et al.*, 2011).

The observed decrease in malaria parasite pressure could allow introduced sparrows in Peru to reduce their investment in defences against parasites and maximize their investment in growth and reproduction, thus increasing their competitive ability resulting in displacement of native species (Blossey & Nötzold, 1995; Lee & Klasing, 2004). However, a decline in investment in immunity could have adverse effects on invasive sparrows because it would reduce the success in the fight against novel parasites in recently colonized regions, especially in areas with higher pathogen pressure such as the tropics (Guernier, Hochberg & Guégan, 2004; Møller *et al.*, 2009). Parasites are one of the main selective forces driving the evolution of animals, which selects for host responses to reduce the likelihood of becoming infected and its negative fitness consequences (Price, 1980; Wakelin, 1996; Moore, 2002). Avian malaria parasites (e.g. *P. relictum*) and some bacteria (e.g. *Salmonella* and *Pasteurella*) are known to be deadly microorganisms thus exerting strong selection on their hosts (Hoque *et al.*, 2012; Asghar *et al.*, 2015; Marzal *et al.*, 2016). Uropygial gland secretion is an important defensive mechanism in birds in the fight against bacteria and haemosporidians (Jacob & Ziswiler, 1982; Martín-Vivaldi *et al.*, 2010; Magallanes *et al.*, 2016; *but* see also Bennett *et al.*, 1972; Russell & Hunter, 2005 for evidence of vector attraction by uropygial secretions). It has been shown that the uropygial gland varies enormously in size among species of birds (Soler *et al.*, 2012; Vincze *et al.*, 2013), suggesting that different species can produce a huge diversity in quantity of secretions. Such diversity is likely to have evolved as a consequence of divergent selection by pathogens on their hosts, in the same way as selection due to parasites has caused divergence in the Major Histocompatibility Complex (Wegner, Reusch & Kalbe, 2003; Eizaguirre *et al.*, 2012). Invasive organisms should fence new parasites in the colonized environments that could decrease the fitness (Elton, 1958). Hence, according to Invasive Immunity Hypothesis (Lee & Klasing, 2004), individuals with larger uropygial glands and higher anti-bacterial activity on their secretions should be selected in environments with new pathogens and higher parasite load. In agreement with this hypothesis, we have shown that sparrows from Peru have larger uropygial glands and higher anti-microbial activity on its secretion than sparrows from the original area, thus suggesting that these uropygial gland traits may favour sparrows to colonize and spread in new environments. Alternatively, larger uropygial glands and higher anti-microbial activities of preen secretions from Peruvian house sparrows could also represent a plastic response under a higher parasite pressure in the new area. Because the greater level of virulence and vaster diversity of pathogens in the tropics (Guégan *et al.*, 2001; Guernier *et al.*, 2004; Møller *et al.*, 2009), house sparrows living in Peru would have modified their investment in the defensive trait in response to higher parasite pressure. In this sense, Jacob *et al.* (2014) experimentally showed that Great tits (*Parus major*) modified the size and composition of their uropygial gland when exposed to higher bacterial densities on feathers. However, our results rely on only one single comparison, and thus the observed patterns could be also explained by differences in climatic or environmental conditions between locations. Hence, we need more replicates of the volume of uropygial gland secretion and its anti-microbial activity from other native and introduced populations of house sparrows in order to draw firm conclusions.

Finally, we showed that uninfected house sparrows had larger uropygial glands and higher anti-microbial activity in uropygial secretions than malaria infected house sparrows, independently of the location of study (Peru and Spain). Similar results were reported recently by Magallanes *et al.* (2016) from an independent dataset, suggesting that uropygial gland secretion may affect the interaction between bird and their haemosporidian vectors and prevent birds from acquiring malaria infections under natural conditions. We propose several alternatives to explain these results. First, uropygial secretions could have an insecticide effect and kill ectoparasites by covering the surface of the parasite or blocking their spiracles (Moyer, Rock & Clayton, 2003). Second, preen secretions may also reduce the mobility of the ectoparasites on the feathers or skin of the bird host (Clayton *et al.*, 2010). Third, some constituents of the uropygial secretions may contain chemicals which act as arthropod repellent, as it has been shown in some species of birds (Dumbacher & Pruett-Jones, 1996). Fourth, bacteria from feathers and skin are involved in the production of chemical attractants for haemosporidian vectors like *Culex* spp. and simulids (Fallis & Smith, 1964; Syed & Leal, 2009). Uropygial secretion has a strong antimicrobial action that can eliminate bacteria from skin and plumage (Martín-Vivaldi *et al.*, 2010), hence minimizing the emission of chemical cues used by haemosporidian vectors and thus decreasing the likelihood to becoming infected with these blood parasites. However, we should be cautious in our conclusions based on such small numbers of infected sparrows in Peru. Thus, more studies exploring the relationship between uropygial

gland volumes, anti-microbial activity in uropygial secretions and malaria infection are desirable.

Finally, the negative correlation between malaria infection and uropygial gland size and its anti-bacterial activity may also indicate an energetic trade-off between immune defence and the production of uropygial secretions. In this sense, Moreno-Rueda (2015) showed that the experimental stimulation of the immune system decreased uropygial gland size in house sparrows with reduced body mass, thus suggesting a condition-dependent trade-off between uropygial gland size and immune response. Because avian haemosporidians are known to decrease body condition (Valkiūnas *et al.*, 2006; Marzal *et al.*, 2008), mounting an immune response and the synthesis of uropygial secretion are both energetically costly (Martin, Scheuerlein & Wikelski, 2003; Moreno-Rueda, 2015). Thus, sparrows in better condition (e.g. uninfected sparrows) may allocate more energy to the production of uropygial secretion than sparrows with lower body condition (e.g. infected house sparrows).

Summarizing, we have shown that the release from their natural haemosporidian parasites and strong defensive traits (e.g. larger uropygial glands and higher anti-microbial activity in uropygial secretions) may favour individual sparrows in their colonization and spread in Peru. However, the Enemy Release Hypothesis may not be the sole mechanism facilitating biological invasions, and hence further studies exploring the relative contribution of different mechanisms in diverse ecological contexts are needed. The outcomes of these and the present study would provide important information to identify factors promoting animal colonization and establishment of invaders (e.g. co-transport and/or release from natural parasites, immune defence to fight against novel parasites), and thus design interventions for preventing the spread and impact of invasive alien species.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Prevalence and genetic diversity of haemosporidian lineages infecting house sparrows from Peru and Spain.
Table S2. Mean (SD) values of uropygial gland volume (mm^3) and anti-microbial activity of uropygial secretion in malaria infected and uninfected house sparrows from Spain and Peru.

Chapter V

Uropygial gland volume varies with biogeographical zone and haemosporidian infection.

EL VOLUMEN DE LA GLÁNDULA UROPYGIAL VARÍA ENTRE ZONAS CLIMÁTICAS Y LA INFECCIÓN POR HAEMOSPORIDIOS.

Resumen

La co-evolución puede explicar los diversos procesos adaptativos entre las especies. Se ha propuesto que los parásitos pueden tener un importante papel en los procesos evolutivos de muchas especies en todo el mundo, puesto que estos organismos son los más abundantes del planeta. Los parásitos pueden causar a sus hospedadores multitud de efectos negativos tanto en la reproducción, el crecimiento e incluso en la supervivencia. Debido a esto, los hospedadores han desarrollado una serie de estrategias defensivas, acordes a las diferentes presiones evolutivas ejercidas por los parásitos o el medio ambiente entre otras. La glándula uropigial ha sido propuesta como una eficaz barrera defensiva, tanto para los parásitos como para el correcto mantenimiento de sus plumas, así como una eficaz defensa contra las inclemencias meteorológicas. En este estudio hemos analizado 1334 individuos de 36 especies diferentes de zona templada y tropical, con el fin de determinar si el volumen de la glándula uropigial varía en función a las diferentes presiones entre estos dos ambientes (ej. la diferente exposición a patógenos). Hemos encontrado que aquellas especies con mayor tasa de infección por malaria tienen menor volumen de glándula uropigial. También hemos observado que las especies de zonas tropicales presentan un mayor volumen de glándula uropigial. Nuestros resultados sugieren que la glándula uropigial puede suponer un mecanismo defensivo frente a la infección por malaria en aves. Los resultados obtenidos también apoyan la hipótesis de

una evolución o una plasticidad fenotípica del volumen de la glándula uropigial ante la exposición de vectores.

Uropygial gland volume varies with biogeographical zone and haemosporidian infection

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ABSTRACT

Parasites are globally widespread pathogenic organisms, which impose important selective forces upon the evolutionary ecology of their hosts. Thus, in accordance with the *Adjustment to parasite pressure* hypothesis, it is expected that defences among hosts vary relative to the selective pressure imposed by parasites. According to the latitudinal gradient of diversity, species richness and abundance of parasites are the highest at latitudes near the equator. The uropygial gland is an important defensive exocrine gland that produces biochemical substances against pathogens (e.g. bacteria, fungi and haemosporidians) in birds. The size of the uropygial gland has been proposed to vary among species as a consequence of divergent selection by pathogens on their hosts. Therefore, we should expect that bird species from the tropics should have relatively larger uropygial glands than species from higher latitudes. However, this hypothesis has not yet been explored. In this study we first tested this hypothesis by analysing the size of the uropygial gland of 1334 individual belonging to 36 bird species from three Neotropical (Peru) and three temperate areas (Spain). Mean corrected uropygial gland volume was significantly larger in bird species from the tropics than from temperate areas. This finding is consistent with the relative size of this defensive organ being driven by selection imposed by parasites. We also explored that potential role of the uropygial gland as a means of avoiding haemosporidian infection, showing that individuals with relative larger uropygial glands had lower mean prevalence of haemosporidian infection, regardless of their geographical origin. This result provides additional support for the assumption that secretions from the uropygial gland reduce the likelihood of becoming infected with haemosporidians.

Keywords: *Adjustment to parasite pressure* hypothesis; malaria; Neotropical region; preen oil; temperate region.

INTRODUCTION

Parasites impose important selective forces upon the evolutionary ecology of their hosts (Schmid-Hempel 2011). They cause several negative effects on their hosts, such as reduced fecundity (Abbate et al. 2015), decreased reproductive success (Merino et al. 2000, Marzal et al. 2005) and increased mortality (Martínez-de la Puente et al. 2010a). One of the most recognized ecological patterns is the latitudinal diversity gradient (LDG), which implies an increase in species richness or biodiversity from the poles to the tropics (Pianka 1966, Hillebrand 2004, Morand 2015). Although inverse latitudinal gradients in species diversity have been also observed (Kindlmann et al. 2007), terrestrial biodiversity tends to be the highest near the equator. This seems also to be the case for pathogens and parasites, which usually are more diverse and abundant in tropics and subtropics. For example, Salkeld et al. (2008) compared the blood parasite abundance in seven populations of the lizard eastern water skink (*Eulamprus quoyii*) over a geographical area including both temperate and tropical regions, showing that parasite load was higher in lizard populations in the tropics. Moreover, pathogenic fungi affecting terrestrial mammals and birds are particularly diverse and abundant in the tropics and subtropics (Mueller et al. 2004). Similarly, mosquito vectors transmitting arbovirus provoking dengue, Chikungunya or yellow fever are more abundant in regions around the equator and the tropics (Kraemer et al. 2015). Furthermore, Nunn et al. (2005) analysed data including 330 parasite species reported from 119 primate hosts to survey latitudinal gradients in the diversity of micro- and macro-parasites per primate host species, showing that species richness increased closer to the equator for protozoan parasites, especially for protozoa that are transmitted by arthropod vectors (e.g. *Leihsmania*, *Plasmodium* or *Trypanosoma*).

With the aim to avoid infection by parasites, or counteract their detrimental effects, animals have evolved a wide range of defensive barriers and mechanisms, including recognition and avoidance of infected individuals (Hart 1994), behavioural mechanisms to remove ectoparasites (Moore 2002, Hart 2011), and innate and adaptive immune responses to destroy pathogens (Wakelin 1996). Because pathogens affect host fitness, it is expected that host defences may differ geographically among hosts (e.g. parasite diversity and abundance) (Morand and Krasnov 2010). Thus, the "adjustment to parasite pressure" hypothesis predicts that host species living in parasite-rich areas should invest more in immune function (Hasselquist 2007). For example, several studies of birds have shown that cell-mediated immune response is positively related to parasitism (e.g. parasite prevalence and parasite load) at both interspecific (Møller and Rózsa 2005) and intraspecific levels (Navarro et al. 2003). This suggests that hosts invest in immune function according to parasite pressure.

The uropygial gland (also called preen gland) is a holocrine gland exclusive to birds that has been suggested, among other functions, to be an important defensive mechanism against pathogens influencing survival (Magallanes et al. 2017, Moreno-Rueda 2017). The uropygial gland secretes waxes and other compounds that are spread over the plumage of hosts with the bill during preening for plumage protection (Jacob and Ziswiler 1982). Although its function is still debated (see review in Moreno-Rueda 2017), uropygial secretions have been proposed to have antimicrobial and antifungal properties, thus acting as a defensive barrier of skin and plumage. For example, uropygial secretions can provide defence against feather-degrading bacteria in spotless starlings (*Sturnus unicolor*) (Rodríguez-Ruano et al. 2015) and house sparrows (*Passer domesticus*) (Moreno-Rueda 2014, Fülöp et al. 2016), and it can also prevent infection by other potentially

pathogenic bacteria such as *Pseudomonas*, *Staphylococcus* and *Salmonella* (Czirják et al. 2013). Moreover, uropygial secretions has been experimentally shown to inhibit fungal growth (Bandyopadhyay and Bhattacharyya 1999). Because the size of the uropygial gland and the volume of its secretions varies considerably among species (Johnston 1988, Vincze et al. 2013), it has been proposed that this variation may have evolved as a consequence of divergent selection by pathogen on their hosts (Møller et al. 2009, Pap et al. 2013). However, this hypothesis has so far been poorly explored, and it deserves further investigation.

Avian malaria and related haemosporidian parasites are diverse and widespread provoking negative effects on their avian hosts, such as decreased body condition (Valkiūnas 2005) , reduced reproductive success (Marzal et al. 2005, Asghar et al. 2015) and increased mortality (Martínez-de la Puente et al. 2010b, Asghar et al. 2015, Marzal et al. 2016). Although some studies suggested that uropygial secretions might attract haemosporidian vectors (Fallis and Smith 1964, Russell and Hunter 2005), recent studies have pointed out that uropygial secretions may prevent birds from acquiring haemosporidian infections (Magallanes et al. 2016, Marzal et al. 2018). However, the suggested function of uropygial secretions in the fight against malaria infection is still poorly studied and limited to investigations in one bird species (house sparrows). Hence, the potential anti-malarial role should be studied in more bird-malaria systems.

Here, we first test for differences in uropygial gland size in different bird species from Neotropical and temperate zones. If the uropygial gland has been evolved as a defensive mechanism in the fight against pathogen exposure, then we should expect that bird species from the Neotropics would have larger uropygial glands than those from temperate zone. We also explore the potential role of the uropygial gland in avoiding haemosporidian

infection. If the uropygial gland prevents birds from acquiring haemosporidians, we should expect birds with larger uropygial glands to have lower prevalence of malaria infection.

MATERIAL AND METHODS

Study sites and sample collection

Our study was conducted at six locations from two different biogeographical zones during 2014-2017, Neotropics [Peru: Pantanos de Villa (P.Villa) (12°12'S, 76°59'W), Tarapoto (6°29'S, 76°22'W) and Iquitos (3°44'S 73°15'W)], and the temperate zone [Spain: Badajoz (38°53'N 6°58'W), Cáceres (39°28'N 6°22'W) and Seville (37°22'N 5°59'W)].

A total of 1334 adult birds belonging to 36 different species (N Peru = 16; N Spain = 20) were captured with mist-nets and individually identified with colored or numbered metal rings. From each individual we measured body mass with a digital balance to the nearest 0.1 g, and we also recorded length, height and width of the uropygial gland with a digital caliper with a precision of 0.01 mm. Uropygial gland volume was estimated as the product of the three dimensions of the uropygial gland (Galván and Sanz 2006), which is positively related to the volume of secretions (Martín-Vivaldi et al. 2009, Møller et al. 2009, Pap et al. 2010). Because the uropygial gland is soft tissue (Martín-Vivaldi et al. 2009, Møller et al. 2009), we measured the three dimensions of uropygial gland three times to calculate average measures (Møller et al. 2009, Moreno-Rueda 2010, 2015). We used body mass to estimate the percentage of the uropygial gland size (UGS) in relation to body mass (BM) of birds (100 x UGS/BM) (Johnson 1988). Finally, we took one microcapillary (50 µl) of blood from the brachial vein from each individual and stored it in 500 µl of SET buffer until DNA analyses.

Molecular detection of haemosporidian infection

With the aim to determine the presence of haemosporidian infection, blood samples from each bird were analyzed using molecular methods described in Waldenström et al. (2004). Blood samples were extracted using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific Inc., reference #K0722). Diluted genomic DNA (25 ng/µl) was used as a template in a polymerase chain reaction (PCR). All PCR experiments contained one negative control for every eight samples. We evaluated the amplification using 2.5 µl of final PRC product on a 2% agarose gel. In the very few cases of negative controls showing signs of amplification (never more than faint bands in agarose gels), the whole PCR-batch was run again to make sure that all positives were true.

Statistical procedures

We only included in the analyses those bird species from which we have a minimum sample size of five individuals. We analysed the relationship between relative uropygial gland size as response variable, and mean prevalence of haemosporidian infection, biogeographical area (temperate vs. tropical) and their interaction as predictors. Because uropygial gland size may vary among bird species (Vincze et al. 2013), we controlled for similarity due to common phylogenetic descent in our analyses. With this aim, we used Birdtree.org to create robust phylogenies for comparisons of the different bird species (Rubolini et al. 2015). We created random draws 300 trees, and the software Geneious (version 11.0.4, http://www.geneious.com) was used to make a phylogenetic consensus tree (Supplementary Table S1). To control for the evolutionary relationship among the sampled species we used phylogenetic generalized least square

regression (PGLS) models as implemented in R statistical environment (see Díaz et al. 2013, García-Longoria et al. 2014) for similar approaches). We used the R packages *geiger* (Harmon et al. 2009) and *caper* (Orme et al. 2012) and the function pglm 3.1.r. in R-3.3.3 (R Development Core Team 2017). The strength and type of the phylogenetic signal in the data matrix was accounted for by adjusting branch length transformations (λ) (Freckleton et al. 2002). These transformations were optimized to find the maximum likelihood transformation given the data and the model. With the aim to correct for heterogeneity in sampling effort among species (see Garamszegi and Møller 2010), we weighted each model by the underlying within-species sample size with the aim to make use of all the data relative to the precision of the estimates (Paradis 2011).

RESULTS

We analysed 1334 individual samples from 36 bird species to test for differences in relative size of the uropygial gland between bird species living in tropical (N = 165 individuals from 16 bird species) and temperate (N = 1169 individuals from 20 bird species) biogeographical zones (Table 1). Biogeographical origin of the bird species and mean prevalence of haemosporidian infection significantly explained variation in volume of the uropygial gland (Table 2). Specifically, mean corrected uropygial gland volume was on average 25% larger in bird species from tropical areas than in species from temperate areas [mean uropygial gland volume corrected for body mass (standard deviation): tropical area = 7.612 mm^3 (2.063); temperate area = 6.041 mm^3 (1.249)] (Table 2, Figure 1). Moreover, individuals with larger uropygial glands had lower mean prevalence of

haemosporidian infection, regardless of geographical zone (Table 2, Figure 2).

The value of lambda (λ) in our statistical analyses was -0.068 -0.097, thus indicating that our results were no influenced by phylogenetic relationship among the bird species analysed in this study.

We found no difference in mean haemosporidian prevalence between tropical (mean haemosporidian prevalence = 16.30%) and temperate bird species (15.60%, ANOVA: $F_{1,35} = 0.015$, $P > 0.05$).

DISCUSION

Parasites exert direct selection on host immune defences (Schmid-Hempel 2011). Thus, different components of immunity should be influenced by parasite diversity and complexity. Following this idea, Alcaide et al. (2010) found a larger number of alleles and more divergent Major Histocompatibility Complex (MHC) class I and class II haplotypes in Eurasian kestrels (*Falco tinnunculus*) than in its phylogenetically related lesser kestrel (*Falco naumanni*), as expected from the higher pathogen diversity, richness and prevalence in Eurasian than in lesser kestrels. This conclusion should be made with care since the study was based on a comparison of just two species. The “adjustment to parasite pressure” hypothesis predicts a higher investment in immune function in species living in parasite-rich areas (Møller 1998; Hasselquist 2007). Because parasites abundance and richness is greater in tropical and subtropical areas than at higher latitudes (Nunn et al. 2005, Merino et al. 2008, Salkeld et al. 2008), it is expected that host species living near the equator should invest more in defences against pathogens than host species living in temperate zones. Our results are consistent with this prediction, since birds from tropical areas had

relatively larger uropygial glands than individuals from temperate areas. These outcomes are similar to those reported by previous studies. For example, Møller (1998) conducted a pairwise comparative analysis of host investment in anti-parasite defence in bird species from tropical and temperate zones, showing that the circulating concentration of leukocytes in the blood was consistently higher, and the relative size of the spleen for a given body size was significantly larger in tropical bird species as compared to that of closely related non-tropical species. Moreover, Hasselquist (2007) reported in passerine birds that species breeding closer to the equator showed enhanced humoral immunity, but not cell-mediated immune response. More recently, Marzal et al. (2018) have shown that house sparrows living in a Neotropical area had larger uropygial glands and higher anti-bacteria activity in its secretions than sparrows from the temperate zone. Again, this conclusion should be made cautiously since it is based on a single species. These studies suggest that host species facing high pathogen exposure are likely to experience selection for high investment and maintenance of defensive mechanisms, when compared to host species facing low parasite diversity.

Alternatively, larger uropygial glands from Neotropical bird species could also be result of phenotypic plasticity to higher pathogen pressure at lower latitudes. Thus, Neotropical bird species could modify their investment in size of the uropygial gland in response to increased parasite pressure. In agreement with this idea, Jacob et al. (2014) experimentally showed that the volume of the uropygial gland of male great tits (*Parus major*) increased when exposed to higher bacterial densities on feathers. Furthermore, Giraudeau et al. (2017) studied house finches (*Haemorhous mexicanus*) along a gradient of urbanization, showing a higher abundance of feather-degrading bacteria on the plumage of urban birds. They also reported

an increase in size of the uropygial gland along the same urban gradient, suggesting that birds exposed to higher abundance of microbes coat their feathers with more uropygial secretions.

On further consideration, because humidity increases growth and activity of feather-degrading bacteria (Burtt and Ichida 2004), the larger sizes of uropygial glands of species living close to the equator could also benefit plumage maintenance in these moist environments. Therefore, individuals with larger uropygial glands should be favoured at lower and more humid latitudes, and /or bird species inhabiting humid habitats could increase investment in the production of uropygial secretions to combat feather-degrading bacteria and preserve plumage function.

Two recent studies have shown that the size of the uropygial gland varied with haemosporidian infection in house sparrows, suggesting that uropygial glands may be involved in defensive mechanisms against malarial infections (Magallanes et al. 2016, Marzal et al. 2018). Here, we analysed blood samples from 1134 individuals searching for haemosporidian infection in 36 bird species, showing that individuals with larger uropygial gland volumes have lower prevalence of malaria infection, regardless of the geographical zone. This outcome agrees with our previous findings about malaria infection and uropygial gland volume in house sparrows, providing additional support to the hypothesis that uropygial secretions may interact with haemosporidian vectors and hence minimize the likelihood of becoming infected. We propose different mechanisms to explain these results. First, the antimicrobial properties of uropygial secretions may prevent haemosporidian infection. Bacteria from skin and plumage are responsible of the production of odours and chemical attractants for haemosporidian vectors like *Culex* spp. and simulids (Fallis and Smith 1964, Syed and Leal 2009). Thus, removal of bacteria and fungi from feathers and

skin by antimicrobial activity of uropygial secretions could decrease vector attraction and thus minimize the likelihood of becoming infected with haemosporidians. Second, uropygial secretions may reduce the mobility of vectors on bird feathers and skin by acting as physical barriers (Clayton et al. 2010), thus avoiding mosquito bites. Third, uropygial secretions could act as an insecticide and affect ectoparasites by covering the surface of the vector or blocking their spiracles (Moyer et al. 2003). Finally, several components of uropygial secretions may include chemicals with arthropod-repellent properties, as shown for some bird species (Dumbacher and Pruett-Jones 1996).

To summarize, we have shown variation in relative size of uropygial glands in birds related to latitudinal distribution and malaria infection, consistent with selection driven by exposure to parasites in this defensive trait. Further experimental studies manipulating parasite loads on plumage of birds from different latitudes would provide insights into the evolutionary mechanisms involved in host immunity. Finally, empirical evidence would be desirable to examine the potential role of uropygial secretions for avoidance of malaria infections.

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current laws of Spain and Peru (200-2016-SERFOR/DGGSPFFS), where the experiments were performed.

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Table 1. Mean (SD) uropygial gland volume corrected for body mass (UGV) and mean haemosporidian prevalence of infection for bird species from temperate and tropical biogeographical zones. Study location and sample size for each bird species is shown.

Bird species	Mean (SD) UGV	Mean prevalence	Biogeographical zone	Location	N
Aegithalos caudatus	38.521 (7.053)	0	Temperate	Badajoz	20
Amazilia amazilia	31.621 (10.248)	0	Tropical	P.Villa	5
Amazilia fimbriata	54.159 (37.107)	0.111	Tropical	Iquitos	10
Amazilia lactea	29.639 (12.477)	0.142	Tropical	Tarapoto	8
Carduelis carduelis	109.226 (80.21)	0.181	Temperate	Badajoz	11
Cettia cetti	118.775 (23.716)	0	Temperate	Badajoz	8
Chloroceryle aenea	116.115 (24.969)	0	Tropical	Iquitos	7
Coereba flaveola	74.996 (13.11)	0.25	Tropical	P.Villa	9
Delinchon urbicum	75.594 (19.15)	0.25	Temperate	Badajoz	283
Erithacus rubecula	65.852 (13.786)	0.1	Temperate	Badajoz	25
Fringilla coelebs	144.616 (28.416)	0.25	Temperate	Badajoz	15
Glaucis hirsutus	52.794 (18.657)	0	Tropical	Iquitos	12
Hirundo rustica	98.334 (21.716)	0.099	Temperate	Badajoz and Sevilla	163
Luscinia megarhynchos	105.885 (25.655)	0.214	Temperate	Badajoz	14
Miliaria calandria	372.701 (53.742)	0	Temperate	Badajoz	6
Oryzoborus angolensis	64.831 (14.723)	0.238	Tropical	Iquitos	21

Cyanistes caeruleus	57.804 (11.953)	0.166	Temperate	Badajoz	7
Parus major	110.312 (31.478)	0.352	Temperate	Badajoz	22
Passer domesticus	173.812 (48.381)	0.244	Temperate	Badajoz and Caceres	396
Passer hispaniolensis	175.957 (35.042)	0.041	Temperate	Badajoz	27
Phleocryptes melanops	150.002 (28.685)	0.266	Tropical	P.Villa	15
Phylloscopus collybita	43.524 (6.616)	0	Temperate	Badajoz	5
Ramphocelus carbo	123.334 (34.658)	0.5	Tropical	Iquitos	8
Riparia riparia	93.266 (16.305)	0	Temperate	Badajoz	38
Serinus serinus	73.378 (20.452)	0.142	Temperate	Badajoz	12
Sporophila castaneiventris	56.673 (13.445)	0	Tropical	Tarapoto	7
Sturnus unicolor	358.91 (79.188)	0.025	Temperate	Badajoz	40
Sylvia atricapilla	103.2 (26.418)	0.088	Temperate	Badajoz	39
Sylvia melanocephala	67.792 (13.443)	0.227	Temperate	Badajoz	25
Tachuris rubrigastra	62.711 (11.377)	0	Tropical	P.Villa	12
Thraupis episcopus	210.872 (46.805)	0.117	Tropical	P.Villa and Tarapoto	17
Turdus ignobilis	315.2 (57.95)	0.133	Tropical	Tarapoto	15
Turdus merula	359.296 (62.296)	0.416	Temperate	Badajoz	13
Vireo olivaceus	74.05 (8.332)	0.857	Tropical	Tarapoto	7
Volatinia jacarina	87.31 (17.758)	0	Tropical	P.Villa	5
Zonotrichia capensis	193.805 (46.427)	0	Tropical	Tarapoto	7

Table 2. Relative uropygial gland size adjusted for body mass in relation to Biogeographical area (Temperate or Tropical) and prevalence of infection. Test statistics refer to linear estimates and their standard errors, and the associated P values in phylogenetic analyses weighted by sample size.
λ = -0.068, residual SE = 0.438 λ = -0.097, residual SE = 0.280, df = 36.

Factor	Value	SE	t	P
Climate area	1.340	0.467	2.86	**0.007 ***
Prevalence infection	-3.226	1.221	-2.64	**0.012 ***
Climate area*Prevalence infection	1.325	3.682	0.359	0.721

Legends to figures

Figure 1. Scatterplot showing the relationship between prevalence of malaria infection and the relative volume of the uropygial gland (mm^3) (circles, N = 20) temperate species and (triangles, N = 16) tropical species. The lines are the linear regression lines.

Figure 2. Box plots showing relative volume of uropygial gland (mm^3) for tropical species (N = 16) and temperate species (N = 20). Values are medians, upper and lower quartiles, and extreme observations.

Figure 1.

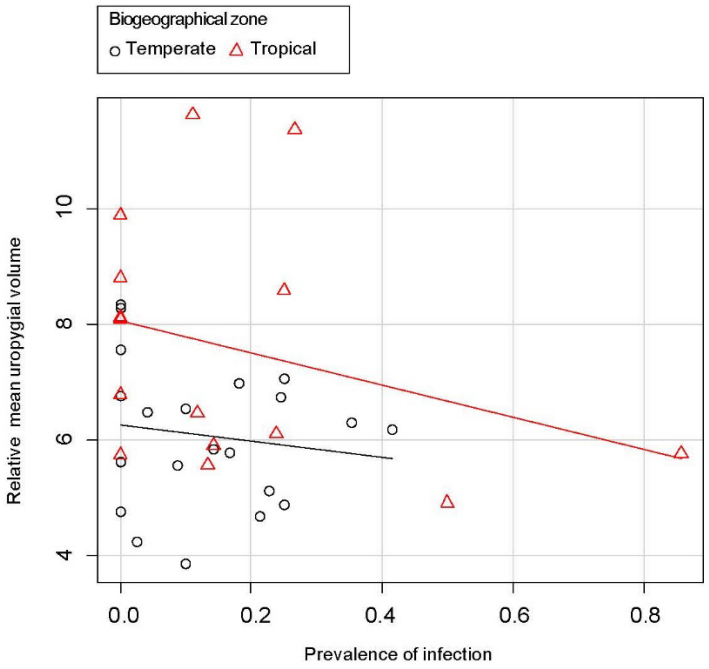
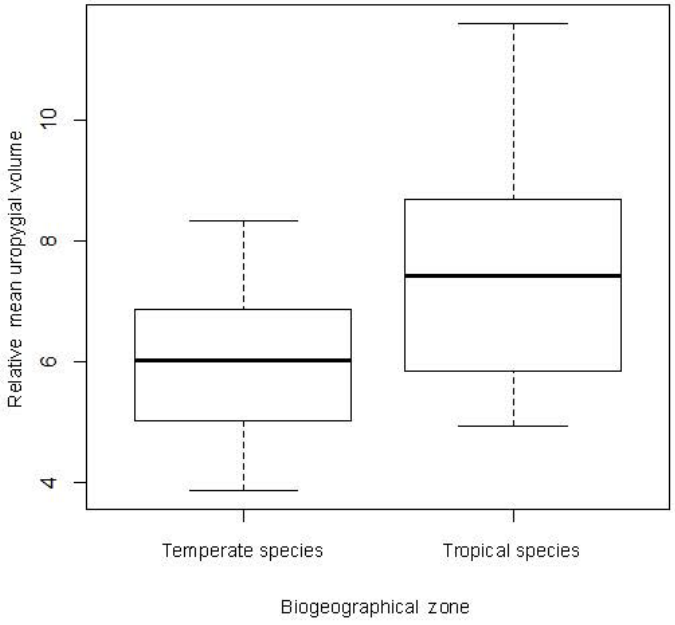


Figure 2.


Relative mean uropygial volume
4
6
8
10
Temperate species
Tropical species
Biogeographical zone

Supplementary information

Uropygial gland volume varies with biogeographical zone and haemosporidian infection.

Sergio Magallanes, Anders P. Møller, Charlene Luján-Vega, Esteban Fong, Daniel Vecco, Luz García-Longoria, Florentino de Lope, José A. Iannacone & Alfonso Marzal.

This supplementary information includes: **Table. S1**

Supplementary Material

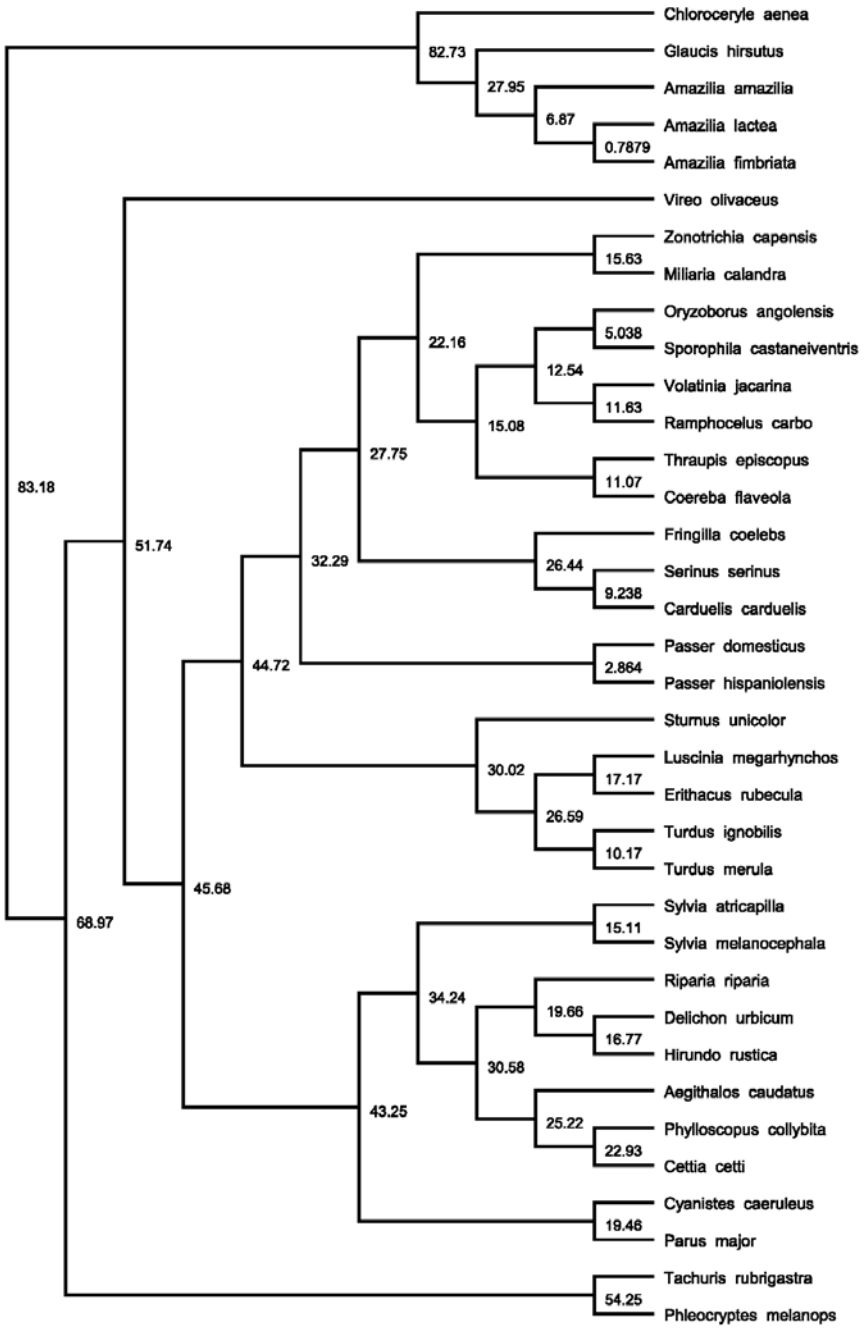


Table S1. Maximum likelihood consensus phylogeny, using Birdtree.org to create robust phylogenies for comparisons of the different bird species (Rubolini et al. 2015) from 36 different bird species. Numbers in branches representbootstrap values based.

CONCLUSIONS

1. Uropygial gland secretion may prevent birds from acquiring malaria infection, as shown by larger uropygial glands and higher antimicrobial activity of malaria uninfected house sparrows.
2. Uropygial gland may enhance survival in malaria-infected house martins. Birds infected with malaria having larger uropygial glands were better able to survive than infected house martins with smaller uropygial glands.
3. Reproductive success of barn swallows can be promoted by uropygial secretion in relation to the abundance of conspecifics, thus suggesting that individuals may adjust the volume of uropygial secretion in response to pathogen exposure.
4. The investment in an energetically costly defensive trait such as uropygial gland secretion in environments with lower pathogen pressure may impair resource allocation to other energetically demanding traits such as reproduction.
5. The release of the natural haemosporidian parasites may have favoured house sparrows when colonizing new areas.
6. Larger volumes of uropygial gland and higher antimicrobial activities of its secretion may have promoted the invasion success of house sparrows when colonizing new environments.
7. Uropygial gland size varies with the latitudinal distribution of the bird species, suggesting a selection driven by parasite exposure in this defensive trait.
8. Preen secretions may reduce the likelihood of becoming infected with haemosporidian, as it has been shown by the lower prevalence of malaria infection of individuals with larger uropygial gland volumes in 36 bird species, regardless its geographical zone.

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II. **Magallanes, S.**, García-Longoria, L., López-Calderón, C., Reviriego, M., de Lope, F., Møller, A. P. and Marzal, A. 2017. Uropygial gland volume and malaria infection are related to survival in migratory house martins. - J. Avian Biol.: 1–5.

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