



## Avian malaria, haematocrit, and body condition in invasive wetland passerines settled in southwestern Spain

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### ABSTRACT

Avian malaria and related haemosporidian parasites can negatively impact fitness in many songbirds. Research on the malaria infection and its physiological costs on their avian hosts is heavily skewed toward native passerines, with exotic species underrepresented. However, introduced species may carry on and spread new pathogens to native species, and play a role on parasite transmission cycle in invaded bird communities as pathogen reservoir. Here, we molecularly assess the prevalence and diversity of haemosporidian parasites in three introduced wetland passerines (the Red Avadavat *Amandava amandava*, the Yellow-crowned Bishop *Euplectes afer*, and the Common Waxbill *Estrilda astrild*) captured during the same season in southwestern Spain. We also explored the relation between parasite infection, body condition, haematocrit, and uropygial gland volume. We detected an overall parasite prevalence of 3.55%, where Common Waxbills showed higher prevalence (6.94%) than Red Avadavats (1.51%). None Yellow-crowned Bishops were infected with haemosporidians. Almost 60% of infections were caused by *Leucocytozoon*, and about 40% by *Plasmodium*. We identified four unique lineages of *Plasmodium* and three of *Leucocytozoon*. Moreover, 91% of the identified host–parasite interactions represented new host records for these haemosporidian parasites. Parasite infection was not related to body condition, haematocrit, and uropygial gland volume of the wetland passerines. Haematocrit values varied seasonally among bird species. Additionally, haematocrit was positively related to body condition in the Yellow-crowned Bishops, but not in the other species. Red Avadavats had higher haematocrit levels than Yellow-crowned Bishops, whereas Common Waxbills showed the lower haematocrit values. The uropygial gland volume was positively correlated with body condition in all bird species. Common Waxbills showed higher uropygial gland volumes related to their body size than birds from other two species. These outcomes highlight the importance of exotic invasive species in the transmission dynamics of haemosporidian parasites.

### 1. Introduction

Invasive alien species (hereinafter IAS) are exotic species able to colonize new areas and to establish flourish populations beyond their natural range (Blackburn and Ewen, 2017). These species usually compete and displace indigenous species (Damas-Moreira et al., 2020; Emiroglu et al., 2020), provoking harmful effects and even the extinction on these native populations (Jeschke, 2014; Tsiamis et al., 2016; Falaschi

et al., 2020). As an example, of the 395 European native species listed as critically endangered by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, 110 are in danger because of IAS (Lipa, 2013). Moreover, invasive species may also impact native species by infecting them with new diseases, serving as vectors for existing diseases, amplifying local pathogens, or causing injuries through bites, allergens, or other toxins (Mazza et al., 2014; Chinchio et al., 2020). For example, several species of invasive crayfish are involved in

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the spread and introduction of Crayfish Plague (*Aphanomyces astaci*) to native European crayfish species, causing a population decline (Nylund and Westman, 2000; Maguire et al., 2016; James et al., 2017). IAS may also carry zoonotic pathogens such as avian influenza or West Nile virus (Crowl et al., 2008; Marzal et al., 2022) affecting not only native species but also human health (Health and Who, 2021), and imposing economic costs (Kettunen et al., 2008). Therefore, the study of IAS, their pathogens and their potential harmful effects on native populations has important implications for both human and animal health, and biodiversity conservation.

A large number of exotic bird species have been intentionally or accidentally introduced beyond their original ranges in last two centuries with critical consequences in native bird communities (Blackburn et al., 2009). Because only a limited number of alien species arriving to new environments become successful invaders (Williamson and Fitter, 1996; Jarić and Cvijanović, 2012), it is important to identify the features favouring an individual to become a fruitful colonizer. In addition to some ecological attributes, such as rapid reproduction and growth, behavioural plasticity and generalist feeding preferences (Sodhi, 2010), the presence of co-transported pathogens carried by alien species (or the lack of them) have been also identified as an important factor explaining the establishment and spread of introduced bird species (Marzal and Garcia-Longoria, 2020). Moreover, there is increasing interest in public and animal health to explore the parasites infecting exotic species as sources of new pathogens leading to emerging diseases through the spillover to local species and humans, as well as its role enhancing local disease risk acting as amplifiers of local pathogens (Chinchio et al., 2020; UNEP, 2020). For example, the high prevalence found of the psittacine beak and feather disease (Pbfd) among the sympatric invasive populations of Rose-ringed Parakeets (*Psittacula krameri*) and Monk Parakeets (*Myiopsitta monachus*) in Spain can seriously affect the fitness and survival of the native bird communities in southern Spain (Morinha et al., 2020).

Avian malaria and related haemosporidian parasites are protozoan organisms able to infect a broad number of bird species worldwide causing severe effects on the survival, body condition and reproductive success of their hosts (Valkiūnas, 2004; Delhaye et al., 2018; McClure et al., 2020; Santiago-Alarcon and Marzal, 2020). These protozoans are transmitted by the bite of blood-sucking dipteran vector (Valkiūnas, 2004; Valkiūnas and Atkinson, 2020), and both sexual and asexual reproductions are required in the vector and vertebrate host, respectively, for the completion of the malaria parasite cycle (Valkiūnas and Iezhova, 2018). The accidental introduction of mosquitoes *Culex quinquefasciatus* and vector-borne avian malaria *Plasmodium relictum* to the Hawaiian Islands is often cited as a prime example of the devastating impact of invasive diseases on native bird communities (van Riper et al., 1986). Co-transported avian malaria parasites by exotic bird species are responsible for economic losses, mass mortality, population declines and even extinctions of many bird species worldwide after their emergence outside their native range (Marzal and Garcia-Longoria, 2020). The tandem invasive bird species-malaria parasite is a crucial key for ecology studies since bird exotic species might be parasite reservoirs for native species (Schoener et al., 2020). As a result, bird population dynamics in native species might suffer the effects of the occurrence of new parasite lineages generated through the interaction exotic bird species-malaria parasites as a spill back to local species (Chinchio et al., 2020). For these reasons, the IUCN considers some avian malaria species within the 100 world's worst invasive species (Lowe et al., 2000). Hence, the study of prevalence and diversity of avian malaria parasites in exotic bird populations is crucial for biodiversity conservation.

More than 375 exotic bird species have been introduced into the wild in Spain and Portugal during the last 100 years, of which up to 32 have been successful colonizers and establish self-sustaining populations (Abellán et al., 2016). Several recent studies have aimed to analyse the volume and origin of introduced bird species in Iberian Peninsula over the last 30 years (Souviron-Priego et al., 2018; Magory Cohen et al.,

2022), showing that the majority of detected invasive bird species are parrots originally from South America, Africa, or Asia. In addition, other studies have explored the ecological effects of exotic passerine bird species might have in the Iberian Peninsula, as well as their present and future distribution (Cardoso and Reino, 2018; Baquero et al., 2021). However, the number of studies focused on invasive passerine species and their effects on parasite dynamics at the community level in Iberian Peninsula are still scarce and mainly focused on Portuguese regions, which may reduce the knowledge on the real distribution range of haemosporidians. For example, Ventim et al. (2012) investigated the prevalence and diversity of haemosporidian parasites in four exotic passerine species (the Common Waxbill *Estrilda astrild*, the Red Avadavat *Amandava amandava*, the Black-headed Weaver *Ploceus melanocephalus*, and the Yellow-crowned Bishop *Euplectes afer*) in four coastal wetlands in Portugal. In addition, Lopes et al. (2018) screened Common Waxbills for haemosporidian infection in 23 sites in Portugal. Furthermore, the number of analysed individuals is still limited for some of these IAS, which could lessen the accuracy of the prevalence estimates (Jovani and Tella, 2006). For example, Ventim et al. (2012) only analysed the malaria infection in four Red Avadavats and two Yellow-crowned Bishops.

Specific host attributes such as morphology, behavioural, or physiological traits could determine susceptibility of individuals or species to malaria infection and hence influence on its role as potential reservoir and amplifier of infection. For example, larger hosts can be more easily located by haemosporidian vectors due to their larger body size and higher secretion of chemical cues, and hence a positive correlation between haemosporidian infection prevalence and body size is expected (Scheuerlein and Ricklefs, 2004; Santiago-Alarcon et al., 2016; Marzal et al., 2022). Moreover, uropygial secretions could act as attractants for natural vectors of haemosporidians (Fallis and Smith, 1964; Russell and Hunter, 2005), although other studies have suggested that uropygial gland secretion may prevent acquiring malaria infection (Magallanes et al., 2016; Marzal et al., 2018, 2022). Also, individuals with worse body condition and poor immunocompetence may be more susceptible to developing acute infection when parasites are inoculated into their bloodstream by blood-sucking vectors (Gonzalez et al., 1999; Navarro et al., 2003). In fact, host nutrition could shape immune defences and host susceptibility to infection (Scrimshaw et al., 1959), ultimately having strong effects on infection dynamics and parasite virulence (Cornet et al., 2014). Thus, those individuals with better body condition or nutritional status would have more resources available to successfully cope with infections (Muriel, 2020). In addition, reduced body mass resulting from lack of intake or consumption of energy resources to compensate for parasite damage could lead to a reduction in host body condition (Dawson and Bortolotti, 2000; Valkiūnas et al., 2006). This deterioration of the host body condition could also be a consequence of haemosporidian-driven hemolytic anemia (de Roode et al., 2005a; White, 2018), leading to low hematocrit levels (ratio of the volume of packed red blood cells to the total blood volume; Booth and Elliott, 2002; Palinauskas et al., 2011; Delhaye et al., 2018).

The Common Waxbill is invasive wetlands passerine originally from sub-Saharan Africa that has successfully established in Iberian Peninsula since 1970's (Silva et al., 2002). The Red Avadavat colonized Spain and Portugal from tropical South Asia in the 1980s (Matias, 2002). The Yellow-crowned Bishop was introduced from Africa to Iberian Peninsula in the 1980s. All these species have arrived to Europe as a result of accidental escapes of caged birds or intentional released into the wild due to the pet trade (Silva et al., 2002), and have successfully established in wetlands and riverine areas (Matias, 2002; Matias et al., 2007; Sullivan and Franco, 2018). However, very little information is available on the role of these invasive bird species in the transmission dynamics of haemosporidians in their new geographic ranges, as well as on which biometric variables might determine different susceptibility to these malarial parasites.

Here we analyse the parasite prevalence and lineage diversity of 507 individuals from three exotic bird species (the Red Avadavat, the

Common Waxbill and the Yellow-crowned Bishop) in southwestern Spain. We also explore the relationship between body condition, haematocrit, and uropygial gland volume with haemosporidian infection in these three bird species. We predict that infected individuals should show poor body condition and lower haematocrit levels. We also expect a negative relationship between the uropygial gland volume and haemosporidian prevalence. Our outcomes could help in better understanding of the ability of exotic bird species to colonise new territories beyond their natural range, as well as providing new information about morphological features of invasive bird species and its association with haemosporidian infection.

## 2. Materials and methods

### 2.1. Study site and study species

Bird sampling was carried out between mid-December 2018 and late March 2019 in the countryside close to Casas Aisladas of G vora (38°55'39" N, 6°57'59" W), 7 km north from Badajoz city (Spain). These exotic bird species group in large flocks during the winter season, hence facilitating their capture (Molina et al., 2022). The area consists of a set of paddy fields (flooded fields of arable land used for growing rice), where they alternate with fallow plots or abandoned stubble, constituting the characteristic agrarian mosaic in the region of Las Vegas Bajas del Guadiana. It exhibits a hot-summer Mediterranean climate [K ppen–Geiger climate classification: Csa category (reviewed in Peel et al., 2007)] with dry summers and mild, wet winters. This anthropogenic habitat is ideal for the settlement of exotic bird species, as is the case with our study species, which have a preference for heterogeneous habitats, including agricultural fields along river systems and tributaries (Sullivan et al., 2015; Sullivan and Franco, 2018). Until the European Wild Bird Trade Ban was adopted (October 2005), the introduction and settlement into Iberian Peninsula of exotic bird species such as the Red Avadavat, the Yellow-crowned Bishop and the Common Waxbill is attributed to the accidental escapes of individuals that were held in captivity as pets by private citizens (Carrete and Tella, 2008). The association of these species with humid environments linked to irrigated agriculture and moderate/high temperatures makes them be exposed to high density of blood-sucking Diptera species typical of semi-aquatic ecosystems, which could act as vectors of certain pathogens, such as avian haemosporidian parasites (Ventim et al., 2012; Muriel et al., 2021).

### 2.2. Field procedure and sampling collection

Five hundred and seven birds belonging to three exotic species ( $N_{A. amandava} = 198$ ;  $N_{E. afer} = 93$ ;  $N_{E. astrild} = 216$ ) were captured using mist-nets (their presence in the area was assured during previous visits). On every capture session, two 12 m long, one 6 m long, and one 4 m long mist nets (12 mm gauge, 2.5 m height) were set up before dawn, and they were open for the next 4 h, checking them every 40 min. From each individual, we recorded body mass with a digital balance (Ohaus Scout II SC2020, China, accuracy = 0.1 g), tarsus length with a digital calliper (Mitutoyo Absolute, Japan, accuracy = 0.01 mm), wing length with an end-stop ruler (accuracy = 1 mm). An index of body condition was estimated for each species, using the residuals from a regression of body mass on tarsus length (Schulte-Hostedde et al., 2005). We also recorded length, height, and width of the uropygial gland with the digital calliper. Uropygial gland volume was estimated as the product of the three dimensions of the uropygial gland (Galv n and Sanz, 2006), measuring them three times to calculate average measures (M ller et al., 2009; Magallanes et al., 2021). To remove the allometric effect of body size on uropygial gland volume, we calculated the residuals from a linear regression of total uropygial gland volume against tarsus length on species-level values, thus obtaining a scaled uropygial gland index to carry out interspecific comparisons. A blood sample was taken from the jugular vein of each bird using a 0.5 mL insulin syringe with a 31-gauge

ultrafine needle (Insured 31G Insulin Syringe 31G × 8 mm; Picsolution, Artsana, Grandate, Italy). The volume of blood extracted depended on the size of the bird but never exceeded 1% of its body mass. Blood samples (ca. 40  $\mu$ L) were immediately added to 500  $\mu$ L of SET buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) and stored at 4  $^{\circ}$ C until DNA extraction in the lab. For a subsample of individuals (128 Common Waxbills, 120 Red Avadavats, and 89 Yellow-crowned Bishops), we used capillary tubes of blood in order to measure haematocrit values in the laboratory. These capillaries were also kept cold on an icebox (approx. 4  $^{\circ}$ C) until the adequate processing in the laboratory within 3 h, and they were subsequently centrifuged at 10,000 r/min for 10 min (Biofuge 13/Haemo, Heraeus Instruments, Osterode, Germany). The haematocrit was measured with a graphical scale (haematocrit reader chart) in each capillary tube as the ratio between the length of the capillary tube occupied by packed red blood cells and the total length of the capillary tube occupied by the whole blood sample.

Based on the Spanish legislation on welfare, invasive and health issues related to alien species, exotic specimens extracted from nature by any procedure should not be returned to the natural environment (Article 7 of the Royal Decree 630/2013). Therefore, captured birds were segregated into small monospecific groups and kept in aviaries with seed mix food and water ad libitum during the next weeks before handing over to regional authorities.

### 2.3. Molecular parasite screening

Haemosporidian infections were detected in blood samples using molecular techniques (Hellgren et al., 2004). Genomic DNA was extracted from all blood samples collected in this study using GeneJET<sup>TM</sup> Genomic DNA Purification Kit (Thermo Scientific Inc., reference #K0722) according to the manufacturer's instructions. The quantity and quality of DNA were determined with a spectrophotometer (NanoDrop 1000, Thermo-Fisher Scientific, Waltham, MA). All samples were diluted to a standard working concentration of 25 ng/ $\mu$ L for PCR analysis. A nested PCR protocol was applied for amplification of a 479 bp fragment of the mitochondrial cytochrome *b* gene (cyt *b*) (Hellgren et al., 2004). For the first PCR, primers HaemNFI (5'-CATATATTAAGAGAANTATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') were used to amplify DNA fragments from *Haemoproteus*, *Plasmodium* and *Leucocytozoon* species. In the second PCR, two new set of primers were used: the primers HaemNF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemNR2 (5'-GCATTATCTGGATGTGATAATGGT-3') for *Haemoproteus* spp. and *Plasmodium* spp. amplification, and HaemNFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemNR2L (5'-CATTATCTGGATGAGATAATGGIGC-3') for *Leucocytozoon* spp. amplification. Negative controls (using nuclease-free water instead of genomic DNA as template). Additionally, one *Plasmodium*, one *Haemoproteus* and one *Leucocytozoon* sample recorded as infected by microscopic examination of blood films were used as positive controls in PCR analyses. The amplification was evaluated by running 2.5  $\mu$ L of the final PCR on a 2% agarose gel stained with SYBR Safe (Invitrogen, Carlsbad, CA) under UV light, looking for bands of the appropriate size (478 bp for *Leucocytozoon* spp. and 480 bp for *Haemoproteus* spp. and *Plasmodium* spp.). Positive amplifications were purified using the GeneJET PCR Purification Kit (Thermo-Fisher Scientific) and then sequenced in both directions on an ABI 3130 genetic analyser. The obtained sequences of 478–480 bp of the cyt *b* were edited, aligned and compared in a sequence identity matrix using the programs BioEdit (Hall, 1999) and Geneious (Kearse et al., 2012). Finally, the aligned sequences were blasted in MalAvi database (version 2.5.2, 14 Dec 2021; Bensch et al., 2009) in order to identify parasite lineage. Lineages previously identified in the MalAvi database (100% pairwise identity compared to known sequences) were named accordingly. Parasites with sequences differing by one nucleotide substitution were considered to represent evolutionary independent lineages (Bensch et al., 2004; Ricklefs et al., 2005).

### 2.4. Statistical analysis

Statistical analyses were performed in R version 3.5.3 (R Core Team, 2019), and the significance level was set at  $\alpha = 0.05$  for all tests. First, we used a Chi-square test to determine whether prevalence of the overall malaria infection, or *Plasmodium*/*Haemoproteus* and *Leucocytozoon* infection separately, varied between different bird species. Secondly, we analysed whether haemosporidian infection status was conditioned by variations in body condition or uropygial gland volume, or whether, on the other hand, these variables were determined by the presence or absence of haemosporidian parasites. To this end, differences on infection status (dependent variable) for each parasite group between different bird species were modelled using binomial generalized linear models (GLMs) with a logit link function using the package MASS (Venables and Ripley, 2002). Tukey's multiple comparisons were carried out using the function "glht" in multcomp package. On the contrary, when body condition, haematocrit level and uropygial gland volume were considered as dependent variables, we applied general linear models with the glm function in the 'lme4' package (Bates et al., 2015), and depending on the data distribution we used Gaussian or Poisson distributions. For these cross-sectional analyses (data at a specific point in time), initial models were controlled for capture date (day of year), haemosporidian infection, and biologically meaningful biometric variables (body condition, haematocrit and uropygial gland volume) except when they were the dependent variable. Non-significant ( $p > 0.05$ ) terms were sequentially removed from the initial models following a backward stepwise procedure, until only the significant explanatory variables were retained in the models. Finally, the last analyses were focused on testing biometric differences (body weight, tarsus length, wing length, body condition, haematocrit, and uropygial gland volume) between avian species, for which species was considered as a fixed factor (predictor variable).

## 3. Results

### 3.1. Prevalence and genetic diversity of haemosporidian parasites

Haemosporidian parasites were detected in 18 out of 507 individuals, with an overall prevalence of 3.55% (Table 1). We found differences among species in overall haemosporidian prevalence ( $\chi^2 = 13.11$ ,  $df = 2$ ,  $p = 0.001$ ). Post-hoc analyses revealed that Common Waxbills showed higher haemosporidian prevalence than Red Avadavats and Yellow-crowned Bishops (both  $p = 0.007$ ); however, we found no differences between Red Avadavats and Yellow-crowned Bishops ( $p = 0.790$ ). No Yellow-crowned Bishops were infected with haemosporidians (Table 1).

*Leucocytozoon* was the most frequent parasite genus, infecting 11 individuals (2.17% of prevalence), whereas eight birds showed *Plasmodium* infection (1.58%). No individuals were infected with *Haemoproteus*. We found significant differences among species when considering *Leucocytozoon* prevalence ( $\chi^2 = 7.39$ ,  $df = 2$ ,  $p = 0.025$ ), but *Plasmodium*

prevalence was similar among the different invasive wetland passerines ( $\chi^2 = 3.91$ ,  $df = 2$ ,  $p = 0.141$ ). Of the 18 birds that tested positive for infection, 61.11% were infected with *Leucocytozoon*, and 44.44% with *Plasmodium*. One Red Avadavat showed a simultaneous co-infection with *Plasmodium* and *Leucocytozoon*. By analysing genetic diversity of haemosporidian parasites, we identified four unique lineages of *Plasmodium*, and three of *Leucocytozoon* (Table 1), based on BLAST queries of cyt b haplotypes against the MalAvi database Version 2.5.2 (Grand Alignment December 14, 2021; Bensch et al., 2009).

### 3.2. Variations on body condition, haematocrit and uropygial gland volume in relation to haemosporidian infection

Results from cross-sectional analyses predicting haemosporidian infection status (both by analysing the parasite genera jointly and when distinguishing between *Plasmodium* and *Leucocytozoon* infection) in Red Avadavats and Common Waxbills did not show significant differences in body condition or uropygial gland volume (all  $p > 0.225$ ).

On the other hand, we analysed whether haemosporidian infection (as an independent variable) could influence body condition, haematocrit levels or uropygial gland volume. Neither overall haemosporidian infection nor date of capture significantly affected body condition in wetland passerines (Table 2). Moreover, our results showed no effect of infection status on body condition of Red Avadavats (all  $p > 0.512$ ) or Common Waxbill (all  $p > 0.561$ ) when considering haemosporidian parasite genera separately (*Plasmodium* or *Leucocytozoon*).

Haematocrit, the percentage of packed red blood cells per blood volume, was not affected by the presence of haemosporidians in any of the bird species sampled, both when considering the overall prevalence (Table 2) or considering each parasite genus separately (all  $p > 0.734$ ). However, haematocrit was affected by the capture date in Red Avadavats and Yellow-crowned Bishops, showing higher values towards the end of winter (February–March); on the contrary, Common Waxbills showed higher haematocrit values during early winter (Table 2). Additionally, the haematocrit values were positively related to body condition in Yellow-crowned Bishops, but not in the other wetland species (Table 2).

Finally, uropygial gland volume was not related to neither overall malaria infection (Table 2) nor *Plasmodium* or *Leucocytozoon* infection when analysed separately in Red Avadavats or Common Waxbills (all  $p > 0.324$ ). The uropygial gland volume was positively correlated with body condition in the three species (all  $p < 0.001$ ; Table 2). In Common Waxbills, the uropygial gland volume was also positively influenced by the capture date (Table 2), where individuals captured towards the end of winter showing higher gland volumes.

### 3.3. Variations in body condition, haematocrit and uropygial gland volume among species

Body mass significant varied among species (Table 3); specifically, post-hoc analyses revealed that Yellow-crowned Bishops had higher body

**Table 1**

Sample size (N) of invasive wetland species and number of infections of each parasite genus.

Bird species	N	No. infected (prevalence)			Lineage names (N)
		<i>Plasmodium</i>	<i>Leucocytozoon</i>	Total	
Red Avadavat ( <i>Amandava amandava</i> )	198	2 (1.01%)	2 (1.01%)	3 (1.51%) *	P_SYAT24 (1), L_SGS1 (1), L_RS4 (2)
Common Waxbill ( <i>Estrilda astrild</i> )	216	6 (2.77%)	9 (4.16%)	15 (6.94%)	P_SYAT24 (1), P_SGS1 (1), P_PADOM02 (1), P_COLL1 (3), L_SYCON06 (2), L_RS4 (4), L_CIAE02 (3)
Yellow-crowned Bishop ( <i>Euplectes afer</i> )	93	0 (0.00%)	0 (0.00%)	0 (0.00%)	–
Total	507	8 (1.57%)	11 (2.17%)	18 (3.55%)	*

Lineage names and number of infections per parasite lineage (N) are also indicated (P\_ = *Plasmodium*, L\_ = *Leucocytozoon*). \*Note that the total number of infected Red Avadavats is only three because one of them showed a *Plasmodium*–*Leucocytozoon* co-infection (P\_SGS1–L\_RS4).

**Table 2**  
Initial linear models showing the effect of overall haemosporidian infection on body condition, haematocrit and uropygial gland volume for each of the invasive avian species considered.

Species	Dependent variable	Predictors	Estimate ± SE	$\chi^2$	df	p
Red Avadavat ( <i>Amandava amandava</i> )	Body condition	Date	0.101 ± 0.091	1.24	1	0.266
		Haemosp. infection	0.248 ± 0.583	0.18	1	0.669
	Haematocrit	Date	0.012 ± 0.004	9.40	1	<b>0.002</b>
		Haemosp. infection	0.005 ± 0.034	0.02	1	0.871
		Body condition	0.002 ± 0.003	0.77	1	0.379
	Uropygial gland vol.	Date	-0.004 ± 0.007	0.34	1	0.557
		Haemosp. infection	-0.066 ± 0.045	2.15	1	0.143
		Body condition	0.017 ± 0.005	10.3	1	<b>0.001</b>
		Haematocrit	-0.139 ± 0.184	0.57	1	0.451
		Date	-0.135 ± 0.088	2.34	1	0.127
Common Waxbill ( <i>Estrilda astrild</i> )	Body condition	Date	-0.135 ± 0.088	2.34	1	0.127
		Haemosp. infection	-0.039 ± 0.270	0.02	1	0.882
	Haematocrit	Date	-0.008 ± 0.003	6.67	1	<b>0.010</b>
		Haemosp. infection	0.003 ± 0.011	0.08	1	0.778
		Body condition	0.0005 ± 0.003	0.02	1	0.867
	Uropygial gland vol.	Date	0.018 ± 0.007	6.31	1	<b>0.012</b>
		Haemosp. infection	0.004 ± 0.021	0.03	1	0.844
		Body condition	0.023 ± 0.005	19.2	1	<
		Haematocrit	-0.076 ± 0.180	0.18	1	0.670
		Date	-0.167 ± 0.117	2.03	1	0.156
Yellow-crowned Bishop ( <i>Euplectes afer</i> )	Body condition	Date	-0.167 ± 0.117	2.03	1	0.156
		Haemosp. infection	-	-	-	-
	Haematocrit	Date	0.021 ± 0.005	18.1	1	<
		Haemosp. infection	-	-	-	-
		Body condition	0.011 ± 0.004	5.49	1	<b>0.021</b>
	Uropygial gland vol.	Date	-0.086 ± 0.041	1.60	1	0.193
		Haemosp. infection	-	-	-	-
		Body condition	0.033 ± 0.008	3.94	1	<
		Haematocrit	0.280 ± 0.190	2.15	1	0.145
		Date	0.021 ± 0.005	18.1	1	<

In the case of factors, estimates refer to the second level of each group (infected individuals in the case of haemosporidian infection). Non-significant variables were removed from the model based on stepwise selection by p-values criterion (p < 0.05, corresponding to the numbers in bold).

weight than Red Avadavats (t = -54.6, p < 0.001) and Common Waxbills (t = -60.7, p < 0.001), whereas Red Avadavats showed higher body mass than Common Waxbills (t = -6.86, p < 0.001). Similarly, tarsus and wing length significant varied among species (Table 3). Yellow-crowned Bishop had larger tarsi than Common Waxbills (t = -39.5, p < 0.001) and Red Avadavats (t = -49.1, p < 0.001), whereas Common Waxbills had larger tarsi than Red Avadavats (t = -12.9, p < 0.001). In the case of wing length, Yellow-crowned Bishop had larger wings than Common Waxbills (t = -43.0, p < 0.001) and Red Avadavats (t = -38.1, p < 0.001), whereas Common Waxbills had shorter wings than Red

Avadavats (t = 5.58, p < 0.001). Our results did not show significant differences among species in body condition (Table 3).

We observed significant differences among bird species in haematocrit values (Table 3). Red Avadavats had higher haematocrit levels than Yellow-crowned Bishops (t = 2.65, p = 0.021) and Common Waxbills (t = 6.55, p < 0.001), whereas haematocrit values from Yellow-crowned Bishops were higher than those from Common Waxbills (t = -3.39, p = 0.002). Similarly, we also detected significant differences among species in the relative uropygial gland volume (Table 3). Common Waxbills had larger uropygial gland volumes than Yellow-crowned Bishops (t = 5.97, p < 0.001) and Red Avadavats (t = -9.52, p < 0.001). However, our results showed no differences in uropygial gland volume between Yellow-crowned Bishops and Red Avadavats (t = -1.56, p = 0.259).

#### 4. Discussion

Malaria and related haemosporidian parasites have been proposed as a main factor that explains the invasion success of their avian hosts (Marzal and Garcia-Longoria, 2020). We found a lower prevalence of infection with haemosporidians in invasive wetland passerine samples from our study (2.17% *Leucocytozoon*, and 1.57% *Plasmodium*) than those found on these species in previous studies on their natural range. For example, Ishtiaq et al. (2007) reported a *Plasmodium* prevalence of 35% in Red Avadavats in India. Also, Lutz et al. (2015) showed an average of 30% of prevalence with haemosporidians when analysing malaria infection in estrildid finches in Africa. Moreover, we compared our sequences to those in MalAvi database (Bensch et al., 2009) and discovered that most of haemosporidian lineages infecting estrildid finches on their natural range were absent in birds from invaded localities. In fact, most of haemosporidian lineages detected in this study are commonly found in Europe infecting birds from different genera and orders. All these outcomes suggest that invasive wetland passerines from our study have left behind their natural haemosporidian parasites when colonizing new areas and have acquired some infections from transmitted parasites in the new communities. These results are in accordance with the Enemy Release Hypothesis, stating that successful colonizers frequently harbour a reduced native grounds parasite fauna compared with their native conspecifics, either because they were absent in the colonizers or because parasites were lost during transit or after arrival (Torchin et al., 2003; MacLeod et al., 2010). Similar results have been reported in House Sparrows (*Passer domesticus*) when analysing the prevalence and genetic diversity of haemosporidians in introduced and native populations (Lima et al., 2010; Marzal et al., 2011, 2018). Also, Ventim et al. (2012) explored the prevalence and diversity of *Haemoproteus* and *Plasmodium* in Red Avadavat, Common Waxbill and Yellow-crowned Bishop in wetlands from Portugal, showing reduced parasite prevalence (<0.1%) in these exotic passerines (indeed, no infection was found in the Red Avadavat). Likewise, Lopes et al. (2018) analysed malaria prevalence in introduced Common Waxbills in 23 sites in Portugal, reporting a lower prevalence of haemosporidians than in native grounds.

Several preventive and management actions on animal IAS (e.g. surveillance protocols, risk and impact assessments) have been developed in the last decade (Roy et al., 2018). However, the majority of these tools have focused on environmental impact, and the possible disease emergence risk in animal populations has received less attention (Essl et al., 2011; Srebalienė et al., 2019). IAS can disrupt local infection dynamics if they are susceptible and able to transmit local parasites. We showed that estrildid finches from our study harboured local parasites commonly infecting European bird communities (Garcia-Longoria et al., 2019). Moreover, as far as we know, this is the first study showing Red Avadavats infected with haemosporidian parasites outside their native range. Furthermore, our outcomes also revealed that more than 90% of the bird-parasite interactions recognized in this study have not been previously reported, thus identifying new host records for these haemosporidian parasites (MalAvi database Version 2.5.2, December 14,

**Table 3**Biometric variables taken from individuals of the three invasive wetland passerines showing the sample size (*N*), mean and 95% confidence intervals (CI).

Trait	Red Avadavat	Common Waxbill	Yellow-crowned Bishop	<i>F</i>	<i>p</i>
<i>N</i>	198	216	93	–	–
Body weight (g)	9.16 [9.1–9.2]	8.67 [8.6–8.7]	14.16 [13.9–14.4]	2001.0	< 0.001
Tarsus length (mm)	13.05 [13.0–13.1]	13.72 [13.7–13.8]	16.29 [16.1–16.4]	1237.3	< 0.001
Wing length (mm)	47.03 [46.8–47.3]	46.11 [45.9–46.4]	56.57 [56.2–57.0]	992.94	< 0.001
Body condition (residuals)	–0.009 [–0.15–0.13]	0.014 [–0.12–0.15]	–0.001 [–0.20–0.20]	0.0037	0.9963
Haematocrit (%)	51.68 [51.0–52.3]	47.96 [47.3–48.7]	49.95 [48.9–51.0]	21.601	< 0.001
Urop. gland vol. (mm <sup>3</sup> )	36.91 [36.0–37.8]	44.64 [43.6–45.7]	41.32 [39.5–43.1]	53.904	< 0.001
Rel. urop. gland vol. (residuals)	–0.40 [–0.51 to –0.29]	0.47 [0.34–0.60]	–0.42 [–0.43–0.00]	48.528	< 0.001

*F* and *p* values are also shown as a result of the comparison of each variable between the three bird species.

2021; Bensch et al., 2009). These outcomes reveal a potential increase of local disease risk because parasites acquired by IAS may be amplified and spill back to local species (Chinchio et al., 2020).

It has been proposed that individuals with better body condition or nutritional status should have a more efficient immune response and more resources available to successfully cope with infections (Gonzalez et al., 1999; Navarro et al., 2003; Muriel, 2020). However, our results showed that body condition did not determine the haemosporidian infection status in Red Avadavats and Common Waxbills. Also, no association was detected in any of the bird species analysed between body condition and haemosporidian infection. Similar results reporting no association between body mass and haemosporidian infection have been shown in previous studies carried out in different passerine species (Krams et al., 2010, 2013; Schultz et al., 2010; Santiago-Alarcon et al., 2013; Granthon and Williams, 2017; Dimitrov et al., 2019), which may suggest that haemosporidian parasites did not impact negatively in the body mass of these species. Alternatively, the lack of negative effects of the haemosporidian infection on the body condition of birds could also be explained by the selective disappearance of lower-quality individuals from the population. Given the detrimental effect of malaria infection on survival of birds (Martínez-de La Puente et al., 2010; Ilgūnas et al., 2016; Marzal et al., 2016), only infected birds in a prime body condition may overcome the malaria infection and survive, whereas individuals in poor condition would not be able to survive once infected. In support of this hypothesis, Navarro et al. (2003) showed that House Sparrows in prime body condition had stronger immune response against pathogens, and lower intensity of haemosporidian infection.

Although haematocrit is considered a good predictor of body condition in birds (Sánchez-Guzmán et al., 2004), we only observed this positive association for Yellow-crowned Bishops. Haematocrit is generally considered a reliable measure of red blood cell levels (Campbell and Ellis, 2007), with values between 35% and 55% being normal for birds (Fair et al., 2007). Several studies have linked the presence of blood parasites with low hematocrit levels (Christe et al., 2002; Ishtiaq and Barve, 2018). In fact, haemosporidian parasites may cause severe hemolytic anemia (de Rooode et al., 2005b; Palinauskas et al., 2011). However, our results did not show any association between the haemosporidian prevalence and hematocrit levels. This could be explained because the majority of haemosporidian infections found in wild birds are low parasitemia chronic infections (Santiago-Alarcon and Marzal, 2020), in which the parasites persist at a low density thanks to the control of the immune system preventing severe hemolytic anemia (Hahn et al., 2018; Muriel, 2020). Contrary to previous studies, hematocrit increased over time in Red Avadavats and Yellow-crowned Bishops, but this could be a consequence of the capture date, since most of those studies were carried out during the breeding season, when there is a decrease in hematocrit associated with the increase in temperature throughout the spring (Hörak et al., 1998; Granthon and Williams, 2017). However, hematocrit decreased over time in Common Waxbills, perhaps because the levels of red blood cells decrease during the reproductive period (Christe et al., 2002), as this species begins its reproductive season at the end of February in the Iberian Peninsula (Norte et al., 2010). Austral spring–summer of Red Avadavats and Yellow-crowned Bishops runs from the end of August to

January in the Iberian Peninsula, although nuptial plumage has been observed during the previous months (Ortiz, J.A., unpublished observations). Common Waxbills only stop reproducing during winter (January–February), and in the months of extreme heat (July–August), being able to reproduce practically throughout the year (Ortiz, J.A., unpublished data).

Several recent studies have reported in different bird species that individuals with a larger uropygial gland were less likely to be infected by haemosporidians (Magallanes et al., 2016, 2021; Marzal et al., 2018). Despite this evidence, our results show no relationship between infection status and uropygial gland volume. A plausible explanation for the lack of this relationship with haemosporidian infection could be due to the low prevalence detected in these three bird species in comparison with those species analysed in previous studies. In accordance with other studies, our results also showed a positive correlation between the uropygial gland volume and body condition in the three studied species (Moreno-Rueda, 2017). Due to the high energy cost involved in the development of the uropygial gland (Pap et al., 2013; Moreno-Rueda, 2015), individuals with larger condition indices may invest more resources in developing larger uropygial gland volumes, leading to increased secretion of preen oil (Sandland and Minchella, 2004; Pap et al., 2010).

Nonetheless, the findings of this study must be seen in light of some potential limitations that should be addressed in further investigations. First, seasonal fluctuations have been reported in birds from temperate zones in uropygial gland volume (Pap et al., 2010), body mass (Wu et al., 2014) and haematocrit levels (Fair et al., 2007), which may limit the generalization of our results from bird sampled during winter. Second, naturally occurring haemosporidian infections in wild birds are frequently chronic and with low parasitemias during winter at high latitudes (Valkiūnas, 2004), and thus our estimates of prevalence could underestimate the true prevalence. However, a recent study in House Sparrows has shown that the probability of being infected by *Plasmodium* parasites in the same area of our study location in South Spain seems to be relatively high during the winter period (Neto et al., 2020). Moreover, PCR based methodologies can accurately detect haemosporidian genetic lineages in low-intensity infections (Valkiūnas and Atkinson, 2020). Finally, amplification of parasite DNA by nested PCR does not differentiate gametocytes from asexual parasite stages (Valkiūnas and Atkinson, 2020), and hence we cannot discard that some of the records on haemosporidian infections in our study could be abortive infections.

## 5. Conclusion

Our outcomes call for a greater attention on the potential impact of IAS on native parasite dynamics, stressing the importance of integrative studies between ecologists, parasitologists and health care sector to design coordinated policies on control and eradication programs. While this study reinforces earlier observations of low prevalence of haemo-parasites in invasive passerines from the Iberian Peninsula, it also highlights its potential role in the transmission dynamics of local pathogens in the invaded areas acting as reservoirs and amplifiers of newly acquired haemosporidians. Although haemosporidian infection apparently had no effect on physiological variables, differences between species could be

explained by differential susceptibility to haemosporidian parasites, where the reproductive phenology of each species could have a main role. Finally, initiatives focused on informing citizens about the ecological impact and health threats of IAS are required to promote responsible behaviours.

### Authors' contributions

AM conceived the idea of the study. JAO, JM, LGL and SM participated in the fieldwork. SM, LGL and JM participated in the morphometric and biological sampling of birds, as well as laboratory work. JM and LGL analysed the data and wrote the manuscript. AM contributed significantly to reviewing and editing the manuscript. All authors read and approved the final manuscript.

### Ethics statement

Bird trapping was carried out with all necessary permits with the current regional and national laws of Spain. Methods were evaluated and approved by the institutional Commission of Bioethics of University of Extremadura (CBUE 49/2019) and by Junta de Extremadura Local Government (87/2019).

### Declaration of competing interest

The authors declare that they have no competing interests.

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