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Haemosporidian infection is related to the expression of female plumage ornamentation in a wild passerine

Enrique González-Bernardo [a, b, *], Gregorio Moreno-Rueda [a], Carlos Camacho [c], Jaime Muriel [d, e], Jesús Martínez-Padilla [f], Jaime Potti [c], David Canal [g]

[a] *Department of Zoology, Faculty of Sciences, University of Granada, Granada, Spain*
[b] *University of Oviedo, San Francisco, Asturias, Spain*
[c] *Department of Ecology and Evolution, Estación Biológica de Doñana (CSIC), Seville, Spain*
[d] *Department of Biology, University of Turku, Turku, Finland*
[e] *Institute for Game and Wildlife Research (CSIC, UCLM, JCCM), Ciudad Real, Spain*
[f] *Department of Biological Conservation and Ecosystem Restoration, Pyrenean Institute of Ecology (CSIC), Jaca, Spain*
[g] *Department of Evolutionary Ecology, National Museum of Natural Sciences (CSIC), Madrid, Spain*

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The role of plumage ornamentation as a signal of parasite infection is a key issue in the evolution of animal behaviour. Despite the undeniable role of host–parasite interactions in sexual selection, it is still unclear how parasites influence the variance in the expression of females displaying multiple ornaments. Here, by taking advantage of a long-term and individual-based, monitored population of pied flycatchers, *Ficedula hypoleuca*, in a Mediterranean area, we investigated the variation in the expression of wing patch (size) and forehead patch (occurrence and size) in relation to infections with haemosporidian parasites in breeding adult females. Haemosporidian infection was related to wing patch size, and this association varied in direction and magnitude depending on the origin of birds (locally born versus immigrant) and the type of breeding habitat (oakwood versus pine plantation). Specifically, differences in wing patch size were most evident among immigrant females breeding in the pine plantation, with noninfected females expressing larger wing patches than infected ones. Regarding the forehead patch, its occurrence was also modulated by haemosporidian infection through two-way interactions with bird origin (lower occurrence in locally born females) and breeding habitat type (lower occurrence among infected than uninfected females in the oakwood, whereas the opposite was true in the pine plantation). Only bird age predicted forehead patch size. Therefore, in addition to haemosporidian infection, the expression of these ornaments was related to extrinsic (breeding habitat) and intrinsic (age, origin) factors, which suggests the existence of trade-offs between physiological responses to infection and the expression of secondary sexual traits. Overall, our results suggest that plumage ornaments provide independent and reliable information on the haemosporidian infection status of female pied flycatchers.

Sexual signals and secondary sexual characteristics can provide valuable information about individual quality and have long been studied in the context of sexual selection (Andersson, 1994). In songbirds, colouration may be correlated with, among other factors, immunocompetence (Faivre et al., 2003; Jaiswal et al., 2021; Zuk & Stoehr, 2002), age-related quality (Bitton & Dawson, 2008; Komdeur et al., 2005; Oficialdegui & Serrano, 2023), reproductive performance (Hernández et al., 2021; Møller, 1993; Webster et al., 2008) and provisioning effort to offspring (García-Navas et al., 2012; Hill, 1991; Senar et al., 2002). In addition, parasites might drive the evolution of secondary characteristics, which would be indicators of genetic resistance against parasites (Hamilton & Zuk, 1982). Specifically, only parasite-resistant individuals could afford the allocation of resources to develop and maintain sexual ornaments, thus serving as honest signals of heritable parasite resistance. Hence, by mating with individuals with elaborate sexual signals, their partners would increase their fitness by having parasite-resistant offspring. However, studies on the relationship between parasitic infection and ornamentation in birds have often yielded mixed results (reviewed in Dougherty et al., 2023).

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* Corresponding author.
E-mail addresses: gonzalezbernardoenrique@gmail.com, inv.egonzalezber@ugr.es (E. González-Bernardo).

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Birds are well known for their elaborate and diverse secondary sexual characteristics, including bright colours and striking plumage patterns, which have often been described as honest signals of exposure to parasites (e.g. Moreno-Rueda & Hoi, 2012; Mougeot et al., 2007; Piersma et al., 2001). This is because parasitic infections could lead to trade-offs in resource allocation against other traits such as condition, reproduction and survival (Zuk & Stoehr, 2002). In fact, the expression of plumage ornamentation has been related to a plethora of parasitic infections such as those by fleas (Fitze & Richner, 2002), dipterans (Roulin et al., 2001), nematodes (Martínez-Padilla et al., 2007), protozoans (Lumpkin et al., 2014), coccidians and the *Mycoplasma* bacterium (Brawner et al., 2000). This is also the case for haemosporidian parasites, which have a significant impact on survival (Atkinson et al., 2009; Ilgunas et al., 2019; Krams et al., 2013; Kulma et al., 2013), senescence rates (Asghar et al., 2015), reproductive strategy (Bosholn et al., 2016; Podmokła et al., 2014; Tomás et al., 2007), reproductive success (Asghar et al., 2015; Marzal et al., 2005; Merino et al., 2000; Sanz et al., 2001), migration (DeGroote & Rodewald, 2010; Emmenegger et al., 2018; Hegemann et al., 2018; Ágh et al., 2022) and population viability (Argilla et al., 2013; Levin et al., 2009). Consequently, the role of secondary sexual characteristics as indicators of resistance to haemosporidian infection has also been extensively studied in birds (e.g. Aguiar De Souza Penha et al., 2022; Henschen et al., 2017; Janas et al., 2018).

The bulk of research on the function of sexual signalling has focused on males (Amundsen, 2000b), whereas its evolutionary role in females has been largely neglected for decades by considering it nonadaptive or genetically correlated with selection on male ornamentation (Amundsen, 2000a; Lande, 1980). However, evidence is accumulating that female ornaments can be adaptive (Doutrelant et al., 2020; Martínez-Padilla et al., 2011; Nolazco et al., 2022) by providing reliable information about body condition and reproductive ability (e.g. Doutrelant et al., 2008; Griggio et al., 2005; Hernández et al., 2021). This may be particularly important in socially monogamous species, where both males and females invest heavily in parental care (Clutton-Brock, 2017). In these species, female resistance to infection might, therefore, be critical for the reproductive success of the pair (see e.g. Merino et al., 2000), so sexual selection is expected to drive the evolution of female ornamentation as an indicator of parasite resistance (Clutton-Brock & Vincent, 1991; Hernández et al., 2021). However, our knowledge of the mechanisms underlying favouring female ornamentation is still far from complete (Amundsen & Pärn, 2006).

Here, we investigated whether two female ornaments provide reliable information on haemosporidian (*Plasmodium*, *Haemoproteus* and *Leucocytozoon* genera) infection in a socially monogamous songbird, the pied flycatcher, *Ficedula hypoleuca*. These ornaments are white, depigmented areas on the forehead (forehead patch) and at the base of the outer vanes of primaries (wing patch), variable in size and/or occurrence within and between sexes (Lundberg & Alatalo, 1992). These signals are costly to produce and/or maintain in this species and the closely related collared flycatcher, *Ficedula albicollis*, so they are good candidates to honestly signal individual quality (e.g. males: Gustafsson et al., 1995; Kilpimaa et al., 2004; females: Moreno et al., 2013). In females, while being less investigated, larger wing patches have been related to increased serum immunoglobulin levels, enhanced reproductive success (Morales et al., 2007) and higher testosterone levels (Cantarero et al., 2017). Similarly, the presence or size of the forehead patch in females has been associated with enhanced survival, fecundity and reproductive success (Morales et al., 2007; Potti et al., 2013), higher aggressiveness (Morales et al., 2014), decreased rates of trypanosome infection (Potti & Merino, 1996) and oxidative status (López-Arrabé et al., 2014; Moreno et al., 2013). Nevertheless, there are also costs associated with the expression of these ornaments by females, such as reduced fledgling success in older ages (Potti et al., 2013), increased oxidative damage (Moreno et al., 2013) and depletion of antioxidant defences (López-Arrabé et al., 2014). It has been suggested that forehead patch and wing patch expression in pied flycatchers are under selection through mutual mate choice, as both sexes would benefit from successful mating or acquisition of parasite resistance in the offspring and thus offset the costs of ornament expression and maintenance (Morales et al., 2007; Potti et al., 2013; Potti & Merino, 1996). Indeed, male flycatchers invest more in the offspring when mated with high-quality females, as mirrored in egg coloration (e.g. Moreno et al., 2004; Moreno et al., 2006; Osorno et al., 2006). In male collared flycatchers, no correlation has been found between the size of these ornaments and haemosporidian infection (Szöllösi et al., 2009). However, the role of both ornaments as putative honest signals of avian malaria and related haemoparasite infections remains largely unknown in females (see Morales et al., 2007), despite the effect they may have on their survival or reproductive investment (Kulma et al., 2013; Sanz et al., 2001). We hypothesized that both the occurrence of the forehead patch and the size of the wing and forehead patches in female pied flycatchers may convey reliable information about female resistance against haemoparasites. Considering that these signals may be costly to product or maintain (see above), we predicted that the size of wing and forehead patches and the occurrence of the forehead patch would be lower in infected females than in noninfected ones.

METHODS

Study Species and Population

The pied flycatcher is a small (11–13 g), hole-nesting, insectivorous passerine with sexual dimorphism in size (females slightly larger than males; Potti, 1999) and plumage, particularly during the breeding season, with females showing a greyish-brown plumage in contrast to the conspicuous black-and-white pattern of males (Potti et al., 2014). It is a trans-Saharan migrant that breeds in the temperate forests of Eurasia and North Africa (Lundberg & Alatalo, 1992; Ouwehand et al., 2016), occupying a wide variety of woodlands, both deciduous and coniferous, with a preference for the former (Mäntylä et al., 2015). The mating system of the species is primarily monogamous, although some males become socially polygamous (Canal et al., 2020) and rates of extrapair young vary between 4 and 24% (Canal et al., 2012, and references therein).

The study population is located in the centre of the Iberian Peninsula near La Hiruela (Madrid, 41° 04′ N, 3° 27′ W) and Colmenar de la Sierra (Castilla La Mancha, 40° 40′ N, 4° 8′ W; Fig. A1). The population occupies two distinct habitat plots that differ markedly in vegetation composition and structure: a mature and structurally complex deciduous oakwood, *Quercus pyrenaica*, of 9.3 ha and a managed and homogeneous coniferous plantation, mostly *Pinus sylvestris*, of 4.8 ha, separated by a 1.1 km wide strip of unsuitable habitat (more details in Camacho et al., 2015, 2018).

Field Data Collection

Data for this study were collected between 2005 and 2014 (see sample sizes in Table A1). Field protocols have been described in detail elsewhere (see Camacho et al., 2016; Canal et al., 2011, 2021). Briefly, during the breeding season, from around the third week of April (when the first males arrive from migration) to the first fortnight of July, all nestboxes were regularly checked to determine their occupation and record laying date (first egg laid), clutch size (typically five to six eggs), hatching date (hatching of the first egg) and number of fledglings (at 13 days old). Adults were captured while feeding nestlings 8–10 days post-hatching. The age of most individuals (ca. 53%) was known because all nestlings are ringed before fledging (Potti & Montalvo, 1991), and local recruitment rate is among the highest reported for the species (up to 22%, on average 14%; Potti et al., 2013, 2014; Potti & Montalvo, 1991). Unringed individuals first caught as breeders were considered immigrants (ca. 49% and 17% of the caught females in the oakwood and pine plantation, respectively) due to infrequent breeding outside our nestboxes (information extracted from unpublished data from occasional surveys of singing males conducted in 2005–2014). Unringed individuals were aged and sexed based on plumage characteristics (Svensson, 1992) and subsequently marked for identification. We took standard morphological measurements for each individual, including tarsus length (±0.01 mm), wing length (±0.5 mm) and body mass (±0.1 g). The wing patch comprises white/cream ‘extensions’ spreading beyond the tip of each primary covert over its corresponding primary feather (Fig. 1a, b). To estimate wing patch size (hereafter, WPS), we measured (±0.01 mm) and summed all the lengths of those white/cream extensions in the primary feathers, from the primary where the white/cream extension first appears to the 10th primary feather (Potti et al., 2016; Török et al., 2003). The forehead patch size (hereafter, FPS; mm^2) was estimated as the product of the largest width and the largest height of the patch (Fig. 1c). Only a fraction of the breeding females (ca. 30%) express the forehead patch throughout their lives (Potti et al., 2013). Thus, we also recorded the occurrence of the forehead patch (hereafter, FPO). Females were considered to lack a forehead patch (Fig. 1d) even if they occasionally had a few (one to two) white feathers in the forehead, but they did not form a measurable, continuous plumage patch (Morales et al., 2007; Potti et al., 2013).

Molecular Methods

Blood samples (10–30 μl) were collected from each female by puncturing the brachial vein with a sterile needle and collecting the blood with a nonheparinized capillary tube. This was carried out each year the females were captured over the study period when the chicks were 8–10 days old (see above). DNA was extracted using a DNeasy Tissue Kit (Qiagen) and, subsequently, DNA concentration was adjusted to 25–35 ng/μl. To detect haemosporidian parasites, a nested PCR protocol was applied for the amplification of a fragment, 479 bp long, of the mitochondrial cytochrome b gene (cyt b; Hellgren et al., 2004; Waldenström et al., 2004). The first PCR was run with the primer pair HaemNFI/HaemNR3 (Hellgren et al., 2004). An aliquot of this PCR product (4 μl) was subsequently used as a DNA template for the second PCR with specific primer pairs HaemF/HaemR2 for *Haemoproteus* and *Plasmodium* cyt b gene amplification (Bensch et al., 2000), and HaemFL/HaemR2L

Figure 1. Photographs of (a) a female pied flycatcher in the nest, showing both forehead and wing patches, (b) an extended wing of a female, showing the white wing patch, (c) a front view of the head of a female expressing a forehead patch and (d) a front view of the head of a female without a forehead patch. Photos: 1a: J. Potti; 1b–d: D. Canal.

for *Leucocytozoon* cyt b amplification (Hellgren et al., 2004). Positive and negative controls (samples from individuals with confirmed infection and ddH2O, respectively) were included among the samples to control for failures during the PCRs and possible contamination, respectively (Bensch et al., 2021). To confirm the absence of infections in sampled individuals, we screened the individuals in triplicate independent PCRs and sexed the birds (following Ellergren, 1996) as a control for DNA quality. The amplification was evaluated by running 2.5 μl of the final PCR on a 2% agarose gel stained with ethidium bromide under UV light. Samples with positive amplification were sequenced in both directions on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The obtained sequences of 478–480 bp of the cyt b were edited, aligned and compared in a sequence identity matrix using BioEdit (Hall, 1999). Finally, the aligned sequences were blasted in the MalAvi database (version 2.5.6, April 2023; Bensch et al., 2009) 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). In total, data on infection status were available for 475 samples from 235 females breeding between 1 and 7 years over the study period.

We assumed that the infection occurred before the spring moult preceding the breeding season analysed and, therefore, that the infection costs are already apparent at the breeding time of the season analysed. This is a reasonable assumption for the following reasons. First, our population has a balanced composition between lineages of African (50% of the lineages and 40% of the positives) and European (50% of the positives) origin. The remaining 10% of the positives correspond to samples for which there was no previous reference in MalAvi, and therefore their origin could not be determined or to samples whose lineage could not be identified (see Table A5). Thus, uncertainty on the origin of infection would affect 50% of the positives. Second, in the pied flycatcher, the prenuptial moult (also affecting the plumage ornaments) occurs before departure on spring migration to Europe, between December and March (Blasco-Zumeta & Heinze, 2022; Jenni & Winkler, 1994). Note that the forehead and wing white patches of the pied flycatcher consist of depigmented areas, and while there are no data in flycatchers, the effect of haemosporidian infection on white ornamentation has been shown to manifest months later, in the following moult, in other species such as the blue tit, *Cyanistes caeruleus* (Badás et al., 2018). Third, the life cycle of the parasites and their vectors, coupled with the prepatent period of haemosporidian infection and our field protocol, makes very unlikely both the infection by haemosporidians in April and May and its detection in the same breeding season. Specifically, the prepatent period of haemosporidian infections, depending on parasite and host species, is about 11–12 days, reaching up to 21 days (see Palinauskas et al., 2008; Valkiūnas & Iezhova, 2004), and the time elapsed between the arrival of the individuals on the breeding areas and their capture is approximately 1 month in the study population (see Methods). Further, while data on the presence of Diptera vectors of avian haemosporidian parasites are not available in our population, the abundance of mosquito vectors peaks between June and August in lowland and warmer areas of the Iberian Peninsula (Roiz et al., 2014). Given the high dependence of these insects on high temperatures (Gangoso et al., 2020; LaPointe et al., 2005; Paaijmans et al., 2009), it is presumable that optimal temperatures for these insects are reached later in the season in our mountainous, high-altitude study area. Finally, low temperatures constrain the sporogonic development of haemosporidian parasites within vectors, precluding haemoparasite transmission (LaPointe et al., 2010). In summary, although we cannot completely rule out that some detected infections could have occurred in the current breeding season, the low probability of infection and detection in the same study season (infections occurring in previous years are indeed detectable, Kulma et al., 2013; Sanz et al., 2001), together with the fact that this uncertainty would only affect a portion of the individuals, supports our assumption.

Statistical Analyses

Haemosporidian infection and the expression of plumage ornaments

To investigate the relationship between haemosporidian infection and the size of female ornaments expressed after infection, we fitted linear mixed models (LMMs, Gaussian distribution, identity link function), in which either WPS or FPS were included as dependent variables (see Tables 1 and 2, respectively), using the lme4 package (Bates et al., 2015) in R (R Core Team, 2022). To investigate the relationship between haemosporidian infection and the probability (yes/no) of expressing the forehead patch (FPO), we ran a generalized linear mixed model (GLMM, binomial distribution and a logit link function; 0 = FP not expressed, 1 = FP expressed;

Table 1
Fixed and random effects of the wing patch size (WPS) general model ($N = 447$)

Fixed effects	β	SE	χ^2	CI
Intercept	25.742	0.811	–	24.153 – 27.330
Infection	1.397	0.967	0.000	−0.500 – 3.293
Bird origin	1.870	1.230	0.101	−0.541 – 4.281
Habitat	1.455	1.078	0.080	−0.658 – 3.568
Age	**1.439**	**0.317**	**22.480**	**0.817 – 2.061**
Age^2	−0.364	0.290		−0.932 – 0.204
Wing length	0.151	0.314	0.224	−0.465 – 0.766
Laying date	0.306	0.306	8.008	−0.294 – 0.906
Infection*Bird origin	−1.648	1.348	6.152	−4.291 – 0.995
Infection*Habitat	−1.146	1.294	4.441	−3.683 – 1.391
Infection*Age	−0.742	0.481	2.530	−1.684 – 0.200
Infection*Age^2	0.083	0.437		−0.774 – 0.940
Infection*Laying date	0.880	0.476	3.531	−0.053 – 1.811
Bird origin*Habitat	0.421	2.633	2.517	−4.740 – 5.582
Infection*Bird origin*Habitat	**−6.026**	**2.813**	**4.770**	**−11.540 – (−0.512)**
Random effects	σ^2		SD	
Female ID	30.062		5.482	
Year	0.020		0.140	

For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI), while, for each random effect, we report variance (σ^2) and standard deviation (SD). The reference levels for factors Infection, Bird origin and Habitat are 'noninfected', 'local' and 'oakwood', respectively. The most important parameters are highlighted in bold.

Table 2
Fixed and random effects of the forehead patch size (FPS) general model ($N = 150$)

Fixed effects	β	SE	χ^2	CI
Intercept	18.655	2.250	–	14.244 − 23.066
Infection	−1.987	4.560	0.083	−10.924 − 6.950
Bird origin	2.851	2.939	0.025	−2.909 − 8.611
Habitat	3.502	3.302	0.779	−2.969 − 9.972
Age	0.4468	1.028	**3.522**	−1.567 − 2.461
Age^2	**−2.047**	**0.961**		**−3.930 − (−0.163)**
Tarsus length	1.1473	0.981	1.586	−0.774 − 3.070
Laying date	−0.3418	1.058	0.165	−2.416 − 1.732
Infection*Bird origin	2.405	5.482	0.023	−8.339 − 13.149
Infection*Habitat	5.429	5.725	0.372	−5.791 − 16.649
Infection*Age	0.9258	1.776	3.421	−2.554 − 4.406
Infection*Age^2	2.910	1.773		−0.565 − 6.384
Infection*Laying date	−0.167	1.858	0.041	−3.808 − 3.474
Bird origin*Habitat	−6.139	7.077	4.626	−20.009 − 7.731
Infection*Bird origin*Habitat	−8.300	9.485	0.935	−26.891 − 10.291
Random effects	σ^2		SD	
Female ID	47.519		6.893	
Year	1.619		1.273	

For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI), while, for each random effect, we report variance (σ^2) and standard deviation (SD). The reference levels for factors Infection, Bird origin and Habitat are ‘non-infected’, ‘local’ and ‘oakwood’, respectively. The most important parameters are highlighted in bold.

Table 3
Fixed and random effects of the forehead patch occurrence (FPO) general model ($N = 452$)

Fixed effects	β	SE	χ^2	CI
Intercept	−0.753	0.777	–	−2.276 − 0.770
Infection	**−3.780**	**1.300**	**2.688**	**−6.327 − (−1.233)**
Bird origin	−0.755	1.150	0.690	−3.008 − 1.498
Habitat	−2.392	1.238	0.202	−4.818 − 0.035
Age	**2.349**	**0.634**	**17.126**	**1.105 − 3.592**
Age^2	**−1.040**	**0.420**		**−1.862 − (−0.216)**
Tarsus length	0.312	0.325	0.921	−0.325 − 0.949
Laying date	−0.036	0.345	0.772	−0.712 − 0.639
Infection*Bird origin	**3.163**	**1.535**	**3.536**	**0.154 − 6.172**
Infection*Habitat	**3.688**	**1.644**	**4.459**	**0.465 − 6.911**
Infection*Age	−0.574	0.582	1.049	−1.714 − 0.566
Infection*Age^2	0.315	0.513		−0.690 − 1.320
Infection*Laying date	0.725	0.518	1.963	−0.289 − 1.740
Bird origin*Habitat	2.652	2.748	0.259	−2.734 − 8.037
Infection*Bird origin*Habitat	−2.607	3.054	0.729	−8.594 − 3.379
Random effects	σ^2		SD	
Female ID	17.139		4.140	
Year	0.072		0.268	

For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI), while, for each random effect, we report variance (σ^2) and standard deviation (SD). The reference levels for factors Infection, Bird origin and Habitat are ‘noninfected’, ‘local’ and ‘oakwood’, respectively. The most important parameters are highlighted in bold.

see Table 3). The models for FPS and FPO contained as predictors age (linear and quadratic terms), structural size (tarsus length), breeding (egg laying) date, haemosporidian infection status (yes/no), bird origin (locally born versus immigrant) and habitat type (oakwood versus pine plantation; see Table A1). The model for WPS had the same predictors except for tarsus length, which was replaced by wing length. Because of the possible combined effects of infection on the other predictors, interaction terms were included in the models along with all fixed factors except for tarsus/wing length, as these factors were included as allometric control variables. To test whether the three haemosporidian genera studied have different effects on female ornaments, we ran additional models using the subset of infected individuals under the same structure as the general models and replacing the presence of infection for the ‘genus’ factor (three levels). No associations were found between any haemosporidian genus and the expression of each ornamental trait (see Tables A2–A4 for more details).

Owing to the low number of birds older than 5 years (14 cases out of 475), we grouped all the individuals aged 5 or more years in the same age category to obtain a more balanced sample size among age groups (see Table A1 for sample sizes and basic statistics of the variables considered in analyses). We included as random intercepts in all models the identity of the female and the year of sampling to account for repeated measures of the same individual and interannual changes in environmental conditions, respectively. We initially considered including the age-specific random slope of individual identity in the model to account for the possible lack of independence of the data points and minimize the likelihood of obtaining overconfident estimates (Schielzeth & Forstmeier, 2009). Thus, we ran each model including both the random intercept and the random slope and only including the random intercept term, and compared the two random structures with the Akaike's information criterion (AIC; Akaike, 1973; Zuur et al., 2009). Additionally, we evaluated the goodness-of-fit using conditional R^2, which estimates the proportion of variance in the response variable explained by fixed and random factors (conditional R^2 and marginal R^2, respectively; Nakagawa & Schielzeth, 2013). AICs were estimated using the R library MuMIn (Barton, 2020), while conditional R^2 was computed with the r.squaredGLMM function of the MuMIn package. In each of the three sets of models, the AIC value was lower for those without a random slope term, and the conditional R^2 was higher (FPS models) or similar ($\Delta R^2c = 0.07\%$, FPO models; $\Delta R^2c = 0.18\%$, WPS models); so, for simplicity, we only considered the random intercept of female identity in the models shown in the results. All continuous variables were standardized to mean = 0 and standard deviation = 1 to make the estimates comparable (Schielzeth, 2010).

Statistical analyses were performed in the R environment, version 4.2.1 (R Core Team, 2022). Before interpreting model outcomes, we systematically performed validations and statistical diagnostics (e.g. normality of residuals, influential data points, collinearity (VIF) among predictors), using DHARMa (Hartig, 2020) and car (Fox & Weisberg, 2011) packages. Based on them, some points were removed from the analyses due to high leverage (11 and 22 points for the FPS and FPO analyses, respectively; see Table A1 for final sample sizes). Analyses were qualitatively similar when considering influential points (results not shown). Aside from influential points, diagnostics showed no obvious deviations from model assumptions. We assessed the significance of fixed effects with verification of the 95% confidence intervals of the estimates (Confint function in the car package; Fox & Weisberg, 2011). Estimates were calculated from restricted maximum likelihood models (Zuur et al., 2009).

Age-related ornament expression and haemosporidian infection

Age-related variation of ornament expression (see Results) may be due to within- and between-subject changes, which may cancel each other out, preventing the detection of age-derived effects in a standard mixed model (van de Pol & Wright, 2009); thus, we assessed their relative contributions and relationship with infection status through the within-subject centring approach (van de Pol & Wright, 2009). This method requires the stepwise construction and interpretation of several models subsequent to each of the three general LMMs/GLMMs explained in the previous section (hereafter, general model). In the first additional model (hereafter, model A), we kept a similar structure of fixed and random factors as in the general model but replaced the term ‘age at sampling’ (x_{ij}) with $\bar{x}_j$ (‘average individual age across all captures’, the between-subject term) and $x_{ij} - \bar{x}_j$ (within-subject term), both in its independent term and in the interaction with haemosporidian infection. This allowed us to identify whether the between- and/or within-subject components are statistically significant (van de Crommenacker et al., 2011; van de Pol & Wright, 2009). Additionally, to test whether both effects are different from each other, a second model (hereafter, model B) included the original term of age at sampling (x_{ij}) and the between-subject term (x_j), in addition to their corresponding interactions with haemosporidian infection. In this model, the between-subject term represents the difference in slopes of the between- and within-subject effects in model A, being nonsignificant when these effects do not differ from each other (see van de Pol & Wright, 2009 for details). Even when detecting between- and within-subject effects with models A and B, it is not possible to discern the age-dependent mechanisms that cause temporary changes in the proportion of phenotypes (selective appearance and disappearance; López-Idiáquez et al., 2016; Hidalgo-Rodríguez et al., 2021). The latter step involves the construction of a final model (hereafter, model C) in which the age is replaced by the ages at the first and last reproduction of each individual (i.e. the first and last measurements of the trait), without varying the other fixed and random factors of the general model (van de Pol & Verhulst, 2006). As in models A and B, infection interaction terms were also included. As we did in the previous subsection for age at sampling, we established five categories for the age at last reproduction, while for age at first reproduction, we grouped the few cases with first reproduction in the 3rd and 4th year (18 cases out of 475) with those captured in their second year (see Table A1).

Ethical Note

Adults were captured when feeding nestlings using a conventional spring trap, which is a harmless design (Friedman et al., 2008). Standardized ringing protocols and measurements were carried out for all captured individuals. Blood samples were taken from the brachial vein with a 30-gauge needle. We have a long experience in this procedure and extracted the smallest amount of blood necessary for laboratory analyses (below 50 μl per individual; less than 1% of the animal's weight), causing the minimum pain to individuals. The time required for field procedures was restricted to a minimum (mostly less than 15 min), and all were carried out as cautiously and efficiently as possible.

All applicable international, national and/or institutional guidelines for the capture and ringing of animals were followed and the study was approved by the Spanish institutional authorities, most recently by licence No. 530293. Doñana Biological Station-CSIC and Autonomous Communities of Madrid and Castilla-La Mancha provided capture and ringing licences. Field procedures were approved by the CSIC Ethical Committee (refs. PAC05-006-2, CGL2006-07481/BOS, CGL2009-10652, CGL2011-29694 and CGL2014-55969-P) and the Andalusian Committee of Animal Experimentation (ref. 2011_03 to J.P.) to comply with Spanish and European legislation on the protection of animals used for scientific purposes.

RESULTS

Haemosporidian Infection and the Expression of Plumage Ornaments

The prevalence of haemosporidian infection was 41.47% ($N = 197$ out of 475 samples) and was quite similar in the oakwood (39.19%; $N = 107$ out of 273 samples) and pine plantation (41.58%; $N = 84$ out of 202 samples). The origin of 178 of the 197 haemosporidian-infected females could be determined by comparison of DNA sequences with the MalAvi database (Bensch et al., 2009). Of the infected females, 40.10% ($N = 79$) had African *Plasmodium* and *Haemoproteus* lineages, while 50.25% ($N = 99$) were infected with European *Plasmodium*, *Haemoproteus* and *Leucocytozoon* lineages (see Table A5). Within individual females, the sizes of both ornaments were not correlated ($r = 0.08$, 95% CI = −0.072 − 0.237).

In the general model constructed to analyse the variation in WPS ($R^2_c = 0.745$, $R^2_m = 0.083$), the most important factors were the age of individuals and the three-way interaction of infection*origin*habitat (Table 1, Figs 2a and 3). WPS increased linearly as females got older (Table 1). Also, the greatest differences in WPS occurred in the pine plantation between infected immigrant females (mean ± SD = 23.24 ± 6.47 mm^2) and noninfected ones (33.65 ± 5.96 mm^2; Fig. 3).

In the general model analysing FPS ($R^2_c = 0.496$, $R^2_m = 0.091$), a negative quadratic relationship of age was the only relevant explanatory variable, meaning that FPS first increased with age and

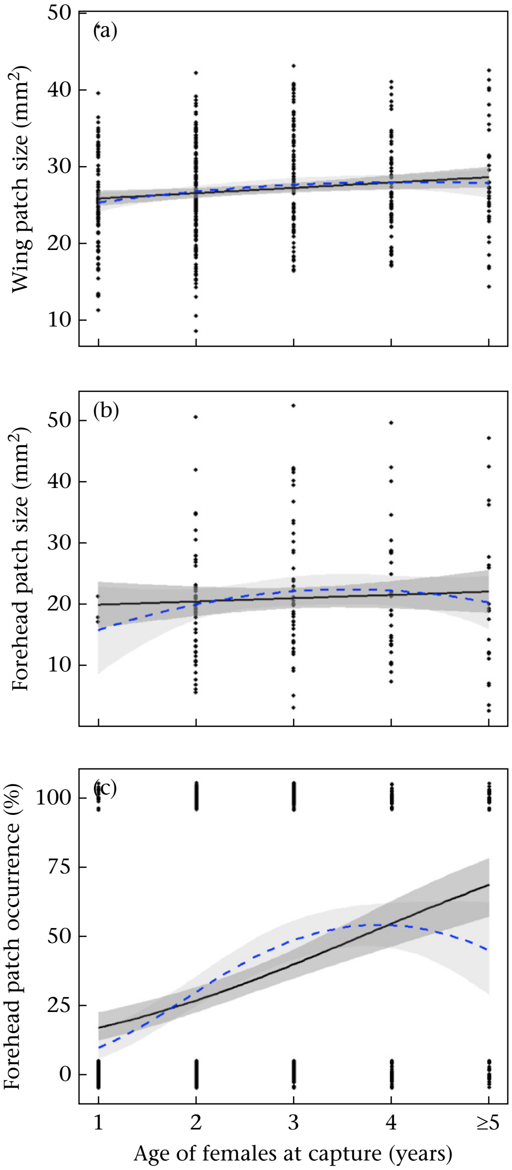

Figure 2. Variation with age of (a) wing patch size, (b) forehead patch size and (c) percentage of female pied flycatchers expressing a forehead patch. The solid grey and dashed blue lines represent linear and quadratic components of age, respectively. Shaded areas around the lines show the confidence intervals (95%).

then decreased at the end of life (Table 2, Fig. 2b). Finally, in the general FPO model ($R^2_c = 0.884$, $R^2_m = 0.275$), in addition to linear (positive) and quadratic (negative) relationships of age (Table 3), the occurrence of the ornament was affected by: (1) the infection*origin interaction (Table 3), as FPO was higher in immigrant individuals (46.67% of noninfected, 41.10% of infected) than in local birds for which the difference was more pronounced (31.21% of noninfected, 19.83% of infected; Fig. 4a); (2) the infection*habitat interaction (Table 3), since differences in FPO between noninfected and infected females were more pronounced in the oakwood (44.30% versus 25.44%, respectively) than in the pine plantation (24.76% versus 32.00%; Fig. 4b).

Age-related Ornament Expression and Haemosporidian Infection

In the case of WPS variation, both the within- and between-subject components showed a positive correlation with WPS, with similar slopes (model A; Table 4). In model B, the terms containing the between-subject components (alone and in interaction with the infection) were not relevant, confirming that the slopes from the previous model (model A) did not differ and derived from the positive relationship of age with WPS. Finally, we did not find age-dependent processes affecting the size of the wing patches (selective appearance and/or disappearance of phenotypes; model C).

As for FPS, only the within-subject component was important (in model A). This difference was confirmed in the next model (model B; between-subject component terms relevant, see Table 5). Consequently, no selective age-dependent processes were detected (model C; AgeFR and AgeLR not relevant).

Concerning FPO, both the within-subject and the interaction infection*between-subject terms were important in model A (Table 6). The slopes of both the between-subject and infection*between-subject terms were not significantly different from those of the within-subject components (model B), showing that within-subject and between-subject components corresponded to a linear and quadratic relationship of age with FPO. However, there was a component of infection in interaction with age (between-subject components), with a smaller increase in FPO with age in infected individuals. We also found selective age-dependent processes involved in the variation of FPO with age (specifically, the age of first reproduction, see model C; Table 6), indicating a potential role of the selective appearance of females expressing the forehead patch in our population.

DISCUSSION

Based on a 10-year data set collected in a wild population of pied flycatchers, we investigated the expression of two female ornaments (wing patch size and forehead patch size and occurrence) in relation to haemosporidian infection. We showed that the expression of the two ornaments was related to different extrinsic (breeding habitat) and intrinsic (age, bird origin) factors, including an ornament-dependent haemosporidian effect. In fact, the occurrence and size of the same ornament (the forehead patch) were not related to the same factors, while only female age maintained a consistent relationship with the three traits studied since their expression increased (although following different functions) with age. The infection by haemosporidians sustained complex interactions with different factors (discussed in detail below) in the expression of the female ornaments. For example, the relationship between haemosporidian infection and wing patch size was jointly related to bird origin and habitat, while the relationship between the infection and forehead patch occurrence depended separately on bird origin and breeding habitat type.

Haemosporidian infection was related to the expression of each ornament differently and often in interactions with aspects such as forest type or dispersal status, suggesting that the effectivity of these signals as indicators of infection status is context-dependent, and that the context relates to each signal in different and complex ways. Several examples of parasite infection-dependent expression of sexual signals have been described in female birds (e.g. Martínez-Padilla et al., 2011, 2012; Piersma et al., 2001; Trigo & Mota, 2016; Zuk et al., 1998). The relationship between sexual

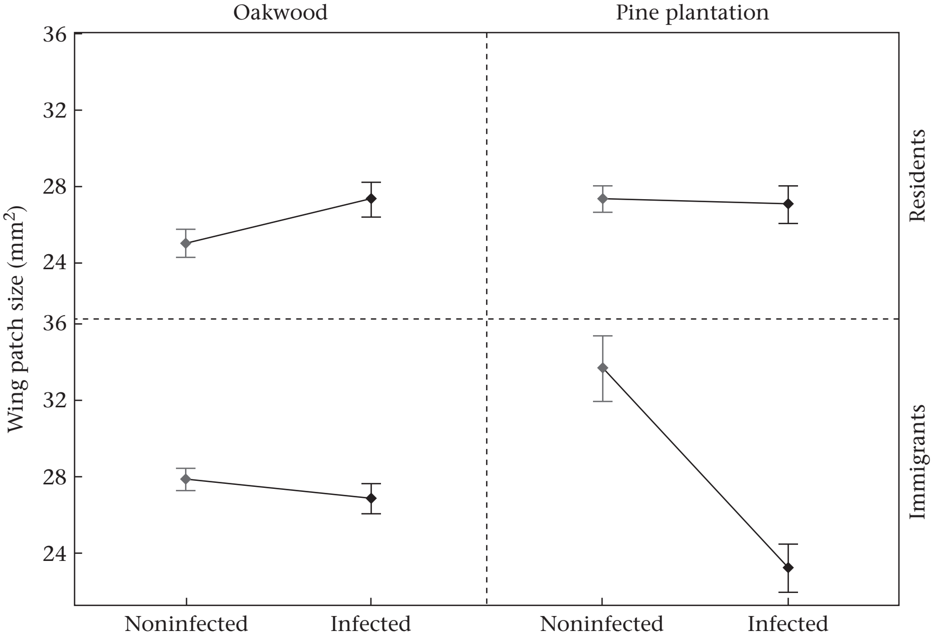

Figure 3. Interaction relationships among wing patch size and haemosporidian infection, bird origin and breeding habitat for female pied flycatchers. Dots represent the mean wing patch size for each subgroup and vertical bars show the corresponding standard errors. Subgroup sample sizes: 137 locally born in the oakwood (76 noninfected, 61 infected); 145 locally born in the pine plantation (93 noninfected, 52 infected); 127 immigrants in the oakwood (79 noninfected, 48 infected); 38 immigrants in the pine plantation (12 noninfected, 26 infected).

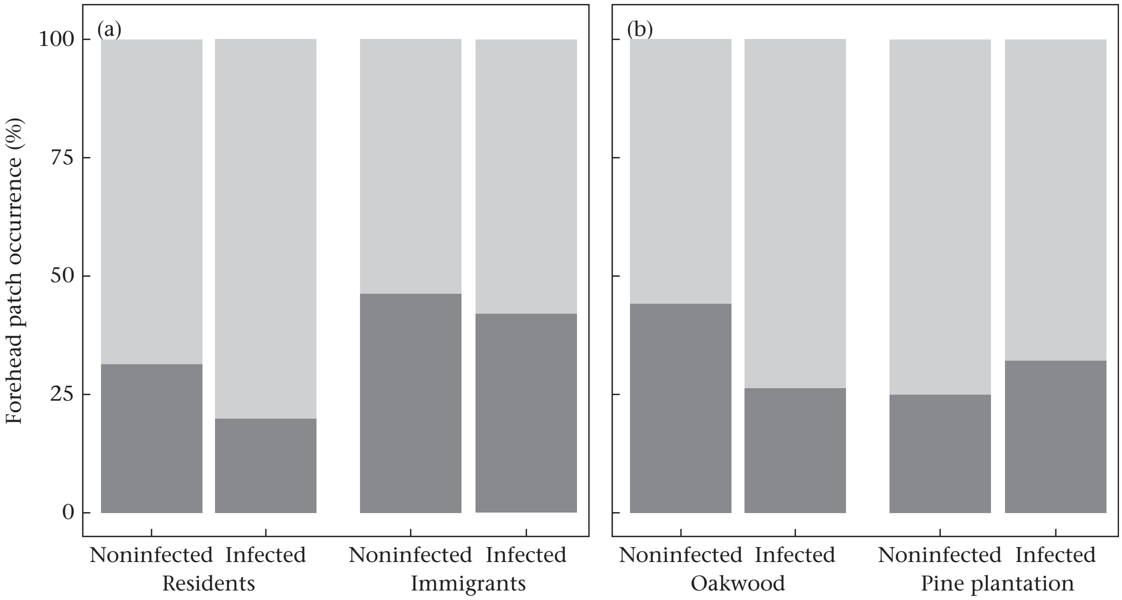

Figure 4. Bar plots showing the percentage of female pied flycatchers expressing a forehead patch according to the interaction between (a) infection status and bird origin and (b) infection status and breeding habitat. The percentage of females expressing a forehead patch is shown in dark grey and the percentage not expressing it in light grey. Subgroup sample sizes (by habitat): 272 females in the oakwood (158 noninfected, 114 infected); 180 females in the pine plantation (105 noninfected, 75 infected). Subgroup sample sizes (by bird origin): 289 locally born females (173 noninfected, 116 infected); 163 immigrant females (90 noninfected, 73 infected).

Table 4
Results of the mixed models built based on the general models for the wing patch size (WPS), following the within-subject centring approach ($N = 447$)

	Fixed effects	β	SE	χ^2	CI
Model A	Intercept	25.814	0.846	–	24.156 – 27.472
	Wth	**1.108**	**0.287**	**16.622**	**0.546 – 1.671**
	Btw	**1.336**	**0.597**	**4.196**	**0.166 – 2.506**
	Infection*Wth	−0.677	0.465	0.078	−1.589 – 0.235
	Infection*Btw	−0.687	0.665	1.083	−1.990 – 0.616
Model B	Intercept	25.757	0.845	–	24.091 – 27.402
	Age	**1.445**	**0.352**	**19.2734**	**0.755 – 2.135**
	Age^2	−0.365	0.297		−0.948 – 0.217
	Btw	<0.001	0.659	0.003	−1.291 – 1.291
	Infection*Age	−0.775	0.585	1.886	−1.921 – 0.372
	Infection*Age^2	0.090	0.443		−0.780 – 0.959
	Infection*Btw	0.077	0.812	0.011	−1.515 – 1.669
Model C	Intercept	25.622	0.969	–	23.723 – 27.522
	AgeFR	0.988	0.560	1.053	−0.109 – 2.086
	AgeLR	0.387	0.592	0.002	−0.773 – 1.547
	AgeFR*AgeLR	0.625	0.529	1.789	−0.411 – 1.661
	Infection*AgeFR	−0.791	0.613	1.924	−1.992 – 0.411
	Infection*AgeLR	−0.410	0.737	0.362	−1.856 – 1.035
	Infection*AgeFR*AgeLR	−0.008	0.639	<0.000	−1.261 – 1.245

Model A assesses within- and between-subject components of age on ornament expression, denoted in the table as Wth and Btw, respectively. Model B examines the effect of the between-subject component of age alone and age on ornament expression (see text for more detailed information). Finally, model C explores the age-dependent processes potentially affecting ornament expression. Only the parameters corresponding to the age-dependent components are shown (see Tables 1, 2 and 3 for the results concerning the rest of the factors). For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI). The most important parameters are highlighted in bold. AgeFR = age at first reproduction; AgeLR = age at last reproduction.

signals and parasites probably emerges from physiological costs associated with the signal and the immune response against parasites (Zuk & Stoehr, 2002). Mounting an immune response is costly in terms of energy, specific nutrients (such as amino acids and vitamins) and generation of oxidative compounds (Amat et al., 2007; Costantini & Møller, 2009; Cotter et al., 2011; Eraud et al., 2005; Hasselquist & Nilsson, 2012; Martin et al., 2017; Sandberg et al., 2007). These costs may preclude or diminish the investment in sexual signals, which may request similar resources or entail a cost in terms of oxidative stress. In the case of white ornaments, as those analysed here, no pigments are involved, but an efficient moult is necessary to produce a signal (Vágási et al., 2012). Once produced, white feathers may be more sensitive to ectoparasites such as chewing lice and feather-degrading bacteria (Gunderson et al., 2008; Kose et al., 1999; Ruiz-de-Castañeda et al., 2012), as well as increased abrasion and breakage (Bonser, 1995; Kose & Møller, 1999; Mackinven & Briskie, 2014). Moreover, white badges may be maintained through the costs associated with the social environment (Qvarnstrom, 1997). Therefore, despite its apparent simplicity, white plumage patches are good candidates to be honest signals of individual quality (Soravia et al., 2020). Not surprisingly, previous studies in this species have reported effects of immune response on FPS in males (Kilpimaa et al., 2004), while both FPS and WPS are associated with oxidative status in both

Table 5
Results of the mixed models built based on the general models for the forehead patch size (FPS), following the within-subject centring approach ($N = 150$)

	Fixed effects	β	SE	χ^2	CI
Model A	Intercept	17.997	2.223	–	13.640 – 22.353
	Wth	**2.518**	**1.201**	**4.769**	**0.164 – 4.872**
	Btw	−1.950	1.528	0.315	−4.944 – 1.044
	Infection*Wth	−1.524	2.166	0.475	−5.770 – 2.721
	Infection*Btw	3.532	2.350	2.509	−1.073 – 8.137
Model B	Intercept	17.666	2.269	–	13.219 – 22.113
	Age	**2.652**	**1.244**	**9.052**	**0.214 – 5.089**
	Age^2	**−2.494**	**0.943**		**−4.344 – (−0.644)**
	Btw	**−5.249**	**1.932**	**3.846**	**−9.036 – (−1.461)**
	Infection*Age	−2.240	2.295	**4.720**	−6.738 – 2.258
	Infection*Age^2	**3.565**	**1.753**		**0.130 – 7.001**
	Infection*Btw	**6.686**	**3.110**	**4.946**	**0.592 – 12.780**
Model C	Intercept	18.171	2.349	–	13.567 – 22.774
	AgeFR	−0.054	1.363	0.001	−2.725 – 2.618
	AgeLR	−1.003	1.271	0.051	−3.493 – 1.488
	AgeFR*AgeLR	−0.767	1.170	0.670	−3.059 – 1.525
	Infection*AgeFR	−0.731	2.156	0.153	−4.957 – 3.495
	Infection*AgeLR	3.922	2.333	3.422	−0.650 – 8.495
	Infection*AgeFR*AgeLR	0.018	2.110	0.002	−4.116 – 4.153

Model A assesses within- and between-subject components of age on ornament expression, denoted in the table as Wth and Btw, respectively. Model B examines the effect of the between-subject component of age alone and age on ornament expression (see text for more detailed information). Finally, model C explores the age-dependent processes potentially affecting ornament expression. Only the parameters corresponding to the age-dependent components are shown (see Tables 1, 2 and 3 for the results concerning the rest of the factors). For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI). The most important parameters are highlighted in bold. AgeFR = age at first reproduction; AgeLR = age at last reproduction.

Table 6
Results of the mixed models built based on the general models for the forehead patch occurrence (FPO), following the within-subject centring approach ($N = 452$)

	Fixed effects	β	SE	χ^2	CI
Model A	Intercept	−0.362	0.731	–	−1.794 – 1.070
	Wth	**1.820**	**0.516**	**17.062**	**0.810 – 2.831**
	Btw	**2.226**	**0.676**	**5.984**	**0.902 – 3.550**
	Infection*Wth	−0.370	0.552	0.451	−1.452 – 0.711
	Infection*Btw	**−1.522**	**0.690**	**4.875**	**−2.873 – (−0.171)**
Model B	Intercept	−0.760	0.799	–	−2.325 – 0.806
	Age	**2.230**	**0.642**	**19.000**	**0.973 – 3.487**
	Age^2	**−1.003**	**0.420**		**−1.826 – (−0.180)**
	Btw	−0.093	0.628	1.150	−1.324 – 1.138
	Infection*Age	0.196	0.726	0.128	−1.228 – 1.620
	Infection*Age^2	0.051	0.523		−0.975 – 1.076
	Infection*Btw	−1.373	0.882	2.424	−3.102 – 0.355
Model C	Intercept	−0.332	0.480	–	−1.272 – 0.609
	AgeFR	**0.572**	**0.288**	**3.337**	**0.008 – 1.136**
	AgeLR	0.430	0.317	3.164	−0.192 – 1.052
	AgeFR*AgeLR	−0.535	0.294	1.639	−1.111 – 0.041
	Infection*AgeFR	−0.434	0.420	0.596	−1.256 – 0.389
	Infection*AgeLR	0.063	0.480	0.065	−0.877 – 1.003
	Infection*AgeFR*AgeLR	0.597	0.439	1.848	−0.264 – 1.458

Model A assesses within- and between-subject components of age on ornament expression, denoted in the table as Wth and Btw, respectively. Model B examines the effect of the between-subject component of age alone and age on ornament expression (see text for more detailed information). Finally, model C explores the age-dependent processes potentially affecting ornament expression. Only the parameters corresponding to the age-dependent components are shown (see Tables 1, 2 and 3 for the results concerning the rest of the factors). For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI). The most important parameters are highlighted in bold. AgeFR = age at first reproduction; AgeLR = age at last reproduction.

males and females (López-Arrabé et al., 2014; see also Moreno et al., 2013). As signals of parasitization, Morales et al. (2007) found no relationship between WPS and FPO and infection by *Trypanosoma* and *Haemoproteus* in females in a nearby population. However, a lower parasitemia of *Trypanosoma* infection in females expressing the forehead patch has been previously described in our population (Potti & Merino, 1996). The results found here, in fact, provide evidence that WPS and especially FPO may act as indicators of the probability of being parasitized in female flycatchers, at least in some circumstances.

We found age-related variation in WPS and FPO at both the within- and between-subject levels, as well as a small association with selective appearance in FPO. An enhanced expression of these ornaments with age has already been reported for *Ficedula* flycatchers, with increased FPO in older females in our study population (Potti, 1993; Potti et al., 2013) and an age-related increase in WPS in *F. albicollis* (Hegyi, Garamszegi, et al., 2008; Hegyi, Rosivall, et al., 2008). Moreover, we found a relationship between FPO and haemosporidian infection in interaction with the between-individual component of age. Like most haemosporidians, the three genera of haemoparasites studied here may cause latent or chronic infections that can reactivate under certain stressors, with young individuals being more vulnerable to haemosporidian infection and suffering increased mortality than older ones (Atkinson & van Riper, 1991; Valkiūnas, 2004). In line with this, the greater likelihood of expression of FPO with increasing age in noninfected females (between-subject component), as well as the negative relationship between FPO and the presence of haemosporidians, is consistent with previous results for female *Trypanosoma* infections in the same population (Potti & Merino, 1996).

FPO was related separately to the interactions of haemosporidian infection with bird origin and breeding habitat type. Regarding the interaction between the infection and the bird origin, the greatest differences in FPO between infected and noninfected birds occurred between the locally born birds (noninfected > infected). If, as we assume, the influence of the infection on FPO occurs before spring moult and migration, the different occurrence of one or the other phenotype between locally born and immigrant individuals would be due to the decisions taken by females according to the information conveyed by the ornament. The forehead patch has been suggested to function as a badge of status in females, which is physiologically costly to express but confers advantages in access to breeding resources (Moreno et al., 2013). Moreover, higher individual quality and breeding performance have been reported in immigrant female kestrels, *Falco tinnunculus*, despite their inexperience in unfamiliar places with pre-existing social structures, compared to locally born females (Fargallo & López-Rull, 2022). Considering the disadvantage associated with being an immigrant (Krebs, 1982) and the fact that we only considered breeding females, the higher FPO in immigrants relative to local birds could be explained if the presence of this ornamental plumage indicates higher individual quality and aggressiveness, as only then would immigrants have access to territories and/or mates. Thus, competitive exclusion would explain the differences in FPO between locally born and immigrant individuals subjected to the trade-offs involved in mounting an immune response and ornament expression. Otherwise, for the locally born individuals that did not experience the added stressor of joining a new population, the expression of this ornament would only be conditioned by the presence of infection.

FPO was also conditional on the interaction between haemosporidian infection and the type of breeding habitat (oakwood versus pine plantation). Pine forests are often considered suboptimal habitats for pied flycatchers (Lundberg & Alatalo, 1992), and an overall preference for the oakwood over the pine plantation has previously been documented in this species (Lundberg & Alatalo, 1992), including our population (Camacho et al., 2019). Thus, in the oakwood, breeding individuals, as well as immigrant males that manage to settle in this habitat, are larger than those in the pine plantation (Camacho et al., 2019). Given the social status or aggressiveness signalling function of the forehead patch, we might expect differences between breeding habitats in the occurrence of phenotypes expressing the forehead patch or not. In fact, the greatest difference in FPO between infected and noninfected females occurred in the oakwood, where females expressing a forehead patch, suggestive of high quality and dominance, would have a higher probability of occupying a territory in a preferred habitat. In contrast, in infected individuals, the expected trade-offs between resistance to infection and ornamentation would come into play (Muriel, 2020). These differences were smaller in the pine plantation, suggesting that the advantages of not being infected (and expressing the forehead patch) are less evident in this habitat.

The relationship between haemosporidian infection and WPS was most evident in a priori unfavourable conditions, in terms of both origin and habitat type, as infected individuals had markedly smaller ornament size if they were immigrants in the suboptimal or nonpreferred habitat (pine plantation) relative to locally born females or to females breeding in the oakwood. Thus, as outlined above, the additive effect of the lack of local familiarity and experience in nonlocal females could trigger the costs of haemosporidian infection and its impact on ornament expression in low-quality females, which could be forced to the less preferred habitat.

We are aware of some inferential limitations on the interpretation of the potential effects of haemosporidian infection on ornament expression. We do not have information about the parasitic load of infected individuals, which could influence the effect of infection on the expression of the ornaments. Furthermore, due to the unequal representation among the infected individuals of the Haemosporida genera studied (e.g. only 11 *Leucocytozoon*-infected females) our ability to analyse the effects of infection by each genus and lineage on ornamentation was limited. Despite these limitations, we were able to detect associations with ornament expression considering only the presence/absence of haemosporidian infection, so our results are probably conservative.

Sexual traits with environment-dependent expression are reliable indicators of the status of individuals under adverse conditions (Vergara et al., 2012). Such seems to be the case for WPS and FPO in female pied flycatchers, as both traits depend on different environmental and parasitic infection conditions. Indeed, this dependence on environmental conditions appears to be more direct in FPO, which could indicate that this trait is more sensitive than WPS to the determinants considered here. Although in this study, we have not included reproductive parameters to evaluate their specific role in the trade-offs between ornamentation and response to infection, it has been suggested that fecundity and survival play a role in the display of forehead patches in females, given the cost of ornamentation (Potti et al., 2013). The different results across traits suggest that they might signal different aspects of quality and/or that different factors influence their expression (Morales et al., 2007; Potti et al., 2013). Moreover, it has been proposed that different age-related processes acting on different plumage ornaments, as is the case here, convey different information (Bitton & Dawson, 2008), which, together with their different expression pattern (wing patch in all individuals, forehead patch of facultative appearance in females; Potti, 1993; Potti et al., 2016), supports the idea of the independence of both sexual traits (Morales et al., 2007).

The signalling role of the haemosporidian infection status in the expression of female plumage ornaments has long been debated due to limited and controversial findings (Hernández et al., 2021). However, there is currently sufficient evidence supporting its signalling function for parasitic infections, even though it depends on the types of ornamentation and parasitic taxa (see the recent review by Dougherty et al., 2023). This appears to be the case for sexual traits in female pied flycatchers, and our results fit into the honest signalling paradigm (Grafen, 1990; Hamilton & Zuk, 1982; Zahavi, 1975). Only high-quality individuals could simultaneously cope with the physiological costs of resistance against parasite infections and enhanced expression of ornaments (Folstad & Karter, 1992), conditioned to environmentally or socially unfavourable situations. Nevertheless, more research is needed to elucidate the differences between males and females on the role of sexual ornaments as indicators of parasite infection.

Author Contributions

Enrique González-Bernardo: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Software. **Gregorio Moreno-Rueda**: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing. **Carlos Camacho**: Investigation, Validation, Writing – review & editing. **Jaime Muriel**: Methodology, Software, Writing – review & editing. **Jesús Martínez-Padilla**: Investigation, Validation, Writing – review & editing. **Jaime Potti**: Funding acquisition, Investigation, Project administration, Validation, Writing – review & editing. **David Canal**: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing.

Data Availability

Data are available in the Supplementary material.

Declaration of Interest

The authors have no conflicts of interest to declare.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.anbehav.2024.07.004.

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Appendix

Table A1
List of variables included in the models and their codes (if any)

		Wing patch size ($N = 447$)				Forehead patch size ($N = 150$)				Forehead patch occurrence ($N = 452$)			
Numerical variables													
Variable (code)	Units	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Forehead patch size (FPS)	mm^2	–	–	–	–	20.99	10.56	2.53	52.26	–	–	–	–
Wing patch size (WPS)	mm^2	26.90	6.54	8.48	48.22	–	–	–	–	–	–	–	–
Tarsus length	mm	–	–	–	–	19.50	0.56	17.74	20.69	19.49	0.56	17.74	20.84
Wing length	mm	77.74	1.66	72.00	82.00	–	–	–	–	–	–	–	–
Laying date	days	21.05	7.44	5	50	19.53	5.37	8	33	20.90	7.05	5	47
Non-numerical variables													
Variable (code)	Levels	*n* (% total)				*n* (% total)				*n* (% total)			
Forehead patch occurrence (FPO)	Nonexpressed	–				–				303 (67.04)			
	Expressed	–				–				149 (32.96)			
Presence of avian malaria infection (Infection)	Noninfected	261 (58.39)				96 (64)				263 (58.19)			
	Infected	186 (41.61)				54 (36)				189 (41.81)			
Bird origin	Resident	282 (63.09)				77 (51.33)				289 (63.94)			
	Immigrant	165 (36.91)				73 (48.67)				163 (36.06)			
Breeding habitat (Habitat)	Oakwood	264 (59.06)				94 (62.67)				272 (60.18)			
	Pine plantation	183 (40.94)				56 (37.33)				180 (39.82)			
Age of individual at capture (Age)	1	92 (20.58)				3 (2.00)				113 (25.00)			
	2	151 (33.78)				51 (34.00)				153 (33.85)			
	3	107 (23.94)				47 (31.33)				105 (23.23)			
	4	58 (12.98)				29 (19.33)				58 (12.83)			
	≥ 5	39 (8.72)				20 (13.33)				23 (5.09)			
Age of first reproduction (AgeFR)	1	200 (44.74)				49 (32.67)				216 (47.79)			
	≥ 2	247 (55.26)				101 (67.33)				236 (52.21)			
Age of last reproduction (AgeLR)	1	44 (9.84)				1 (0.67)				52 (11.50)			
	2	78 (17.45)				22 (14.67)				86 (19.03)			
	3	94 (21.03)				31 (20.67)				95 (21.02)			
	4	96 (21.48)				35 (23.33)				97 (21.46)			
	≥ 5	135 (30.20)				61 (40.67)				122 (26.99)			
Year of capture (Year)	2005	0 (0)				1 (0.67)				25 (5.53)			
	2006	47 (10.51)				12 (8.00)				48 (10.62)			
	2007	59 (13.20)				16 (10.67)				60 (13.27)			
	2008	51 (11.41)				16 (10.67)				49 (10.84)			
	2009	69 (15.44)				30 (20.00)				64 (14.16)			
	2010	55 (12.30)				21 (14.00)				52 (11.50)			
	2011	52 (11.63)				17 (11.33)				47 (10.40)			
	2012	60 (13.42)				22 (14.67)				56 (12.39)			
	2013	32 (7.16)				8 (5.33)				29 (6.42)			
	2014	22 (4.92)				7 (4.67)				22 (4.87)			

Means, standard deviations (SD), minimum (min) and maximum (max) values are provided for numerical variables whereas counts by levels (*n*) and percentages (%) are provided for non-numerical variables. Separate information is provided for the three sets of analyses performed as variables and sample sizes vary between models.

Table A2

Results of the mixed models built to test the existence of differential relationships of each avian malaria genus studied (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) with wing patch size ($N = 187$)

Fixed effects	β	SE	χ^2	CI
Intercept	29.331	1.869	–	25.667 – 32.994
Genus *Haemoproteus*	−3.183	1.998	6.817	−7.099 – 0.733
Genus *Leucocytozoon*	−4.469	4.593		−13.471 – 4.533
Bird origin	−1.816	2.897	1.994	−7.493 – 3.862
Habitat	0.185	2.546	1.321	−4.804 – 5.175
Age	1.146	0.656	3.513	−0.139 – 2.431
Age^2	−0.513	0.548		−1.588 – 0.563
Wing length	0.369	0.522	0.590	−0.655 – 1.392
Laying date	2.283	0.836	8.277	0.644 – 3.922
Genus *Haemoproteus**Bird origin	2.197	3.103	0.511	−3.885 – 8.279
Genus *Leucocytozoon**Bird origin	2.081	4.539		−6.818 – 10.975
Genus *Haemoproteus**Habitat	−0.320	2.914	0.144	−6.030 – 5.391
Genus *Haemoproteus**Age	−0.824	0.831	3.239	−2.452 – 0.803
Genus *Haemoproteus**Age^2	0.138	0.724		−1.281 – 1.557
Genus *Leucocytozoon**Age	−1.008	3.781		−8.419 – 6.404
Genus *Leucocytozoon**Age^2	−4.996	4.710		−14.227 – 4.235
Genus Haemoproteus**Laying date*	−1.702	0.975	5.740	−3.613 – 0.209
Genus *Leucocytozoon**Laying date	1.742	2.081		−2.336 – 5.820
Status*Habitat	−4.574	3.997	4.029	−12.408 – 3.261
Genus *Haemoproteus**Status*Habitat	−1.087	4.375	0.074	−9.662 – 7.488
Random effects	σ^2		SD	
Female ID	30.877		5.557	
Year	1.317		1.148	

The reference levels for factors Genus, Bird origin and Habitat are '*Plasmodium*', 'resident' and 'oakwood', respectively. All individuals infected by *Leucocytozoon* (11) were captured in the oakwood habitat; therefore no model parameters are provided for the interactions 'Genus *Leucocytozoon**Habitat' and 'Genus *Leucocytozoon**Habitat*Bird origin'. For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI). For each random effect, we report variance (σ^2) and standard deviation (SD).

Table A3

Results of the mixed models built to test the existence of differential relationships of each avian malaria genus studied (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) with forehead patch size ($N = 54$)[1]

Fixed effects	β	SE	χ^2	SD	CI
Intercept	16.834	12.014	–		−6.713 – 40.381
Genus *Haemoproteus*	1.464	12.941	9.097		−23.900 – 26.828
Bird origin	12.776	16.837	0.406		−20.224 – 45.776
Habitat	14.858	12.720	0.021		−10.073 – 39.789
Age	5.217	3.158	3.058		−0.972 – 11.405
Age^2	6.474	2.999			0.597 – 12.351
Tarsus length	1.787	1.821	1.458		−1.783 – 5.357
Laying date	1.117	3.142	0.049		−5.041 – 7.275
Genus *Haemoproteus**Bird origin	−9.931	17.718	0.058		−44.658 – 24.796
Genus *Haemoproteus**Habitat	−10.406	14.234	0.088		−38.304 – 17.493
Genus Haemoproteus*Age	−6.350	3.438	11.952		−13.089 – 0.390
Genus *Haemoproteus**Age^2	−5.733	3.343			−12.285 – 0.819
Genus *Haemoproteus**Laying date	−1.201	3.555	0.079		−8.168 – 5.766
Status*Habitat	−24.852	18.909	2.751		−61.913 – 12.210
Genus *Haemoproteus**Status*Habitat	15.751	20.503	0.689		−24.435 – 55.937
Random effects	σ^2			SD	
Female ID	96.360			9.816	
Year	–			–	

The reference levels for factors Genus, Bird origin and Habitat are '*Plasmodium*', 'resident' and 'oakwood', respectively. All individuals infected by *Leucocytozoon* (11) were captured in the oakwood habitat; therefore no model parameters are provided for the interactions 'Genus *Leucocytozoon**Habitat' and 'Genus *Leucocytozoon**Habitat*Bird origin'. For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI). For each random effect, we report variance (σ^2) and standard deviation (SD).

[1] Because of the lack of convergence of the models in their original structure, four influential points, including the only three cases of infection by the genus *Leucocytozoon*, were removed in this analysis. As a result, only parameters corresponding to the genus *Haemoproteus* are provided.

Table A4

Results of the mixed models built to test the existence of differential relationships of each avian malaria genus studied (_Plasmodium_, _Haemoproteus_ and _Leucocytozoon_) with forehead patch occurrence ($N = 197$)[†]

Fixed effects	β	SE	χ^2	CI
Intercept	−8.032	7.009	–	−21.770 − 5.706
Genus _Haemoproteus_	0.015	7.774	0.178	−15.251 − 15.222
Genus _Leucocytozoon_	−3.306	32.200		−63.119 − 63.112
Bird origin	−3.703	12.530	2.314	−28.254 − 20.848
Habitat	1.200	8.997	0.227	−16.433 − 18.833
Age	2.592	1.876	8.076	−1.085 − 6.269
Age^2	−1.895	1.901		−5.620 − 1.831
Tarsus length	0.207	1.191	0.030	−2.128 − 2.542
Laying date	1.754	0.827	4.499	0.133 − 3.375
Genus _Haemoproteus_*Bird origin	8.588	13.130	0.088	−17.146 − 34.322
Genus _Leucocytozoon_*Bird origin	51.674	40.200		−78.743 − 78.847
Genus _Haemoproteus_*Habitat	−1.362	10.320	0.524	−21.587 − 18.864
Genus _Haemoproteus_*Age	0.671	2.350	1.091	−3.935 − 5.277
Genus _Haemoproteus_*Age^2	1.943	2.166		−2.303 − 6.189
Genus Leucocytozoon*Age	−14.635	20.590		−40.376 − 40.347
Genus _Leucocytozoon_*Age^2	40.234	21.650		−42.384 − 42.464
Status*Habitat	11.490	14.660	0.301	−17.238 − 40.217
Genus _Haemoproteus_*Status*Habitat	−9.285	15.800	0.345	−40.249 − 21.679
Random effects	σ^2		SD	
Female ID	128.400		11.330	
Year	0.000		0.000	

The reference levels for factors Genus, Bird origin and Habitat are '_Plasmodium_', 'resident' and 'oakwood', respectively. All individuals infected by _Leucocytozoon_ (11) were captured in the oakwood habitat; therefore no model parameters are provided for the interactions 'Genus _Leucocytozoon_*Habitat' and 'Genus _Leucocytozoon_*Habitat*Bird origin'. For each fixed effect, we report estimate (β), standard error (SE), type II Wald chi-square (χ^2) and 95% confidence interval (CI). For each random effect, we report variance (σ^2) and standard deviation (SD).

[†] Because of the lack of convergence of this model with its original structure when including the avian malaria genus, it was necessary to simplify the model by replacing the interaction 'Genus*Laying date' with the term 'Laying date' alone, thus retaining all predictors in the analysis.

Table A5

Counts of haemosporidian-infected females ($N = 197$) according to genus, lineages and geographical origin of the parasites

Haemosporidian genus	Lineage code	Origin	_N_
Plasmodium	AEMO01	Africa	1
$N = 62$ (31.47%)	COLL7	Africa	7
	GRW09	Africa	9
	LK06	Africa	1
	PLOPRI01	Africa	18
	RTSR1	Africa	11
	WW3	Africa	7
	GRW11	Europe	1
	SGS1	Europe	2
	SYAT05	Europe	3
	New_P1	Unknown	2
Haemoproteus	COLL2	Africa	25
$N = 119$ (60.41%)	COLL3	Europe	10
	PFC1	Europe	54
	PHSIB1	Europe	18
	TURINF01	Unknown	1
	New_H1	Unknown	10
	New_H1	Unknown	1
Leucocytozoon	ROBIN2	Europe	2
$N = 11$ (5.58%)	SFC8	Europe	9
Undetermined	–	Unknown	5

Lineages are listed with the codes as they appear in the MalAvi Haemosporidia database (Bensch, et al., 2009), from which their geographical origin was determined. Lineages designated as 'New_' correspond to samples for which there was no previous reference in MalAvi, and therefore their origin could not be determined (origin = 'unknown'). For parasites infecting some individuals, the lineage could not be identified ('undetermined'). In one case, the genus and lineage of the haemosporidium could be identified ('TURINF01'), but its geographical origin could not be determined with certainty, so it was assigned as unknown. The number of samples infected with each haemosporidian genus and the percentage they represent with respect to the total of 197 positive samples are indicated below the name of each genus.

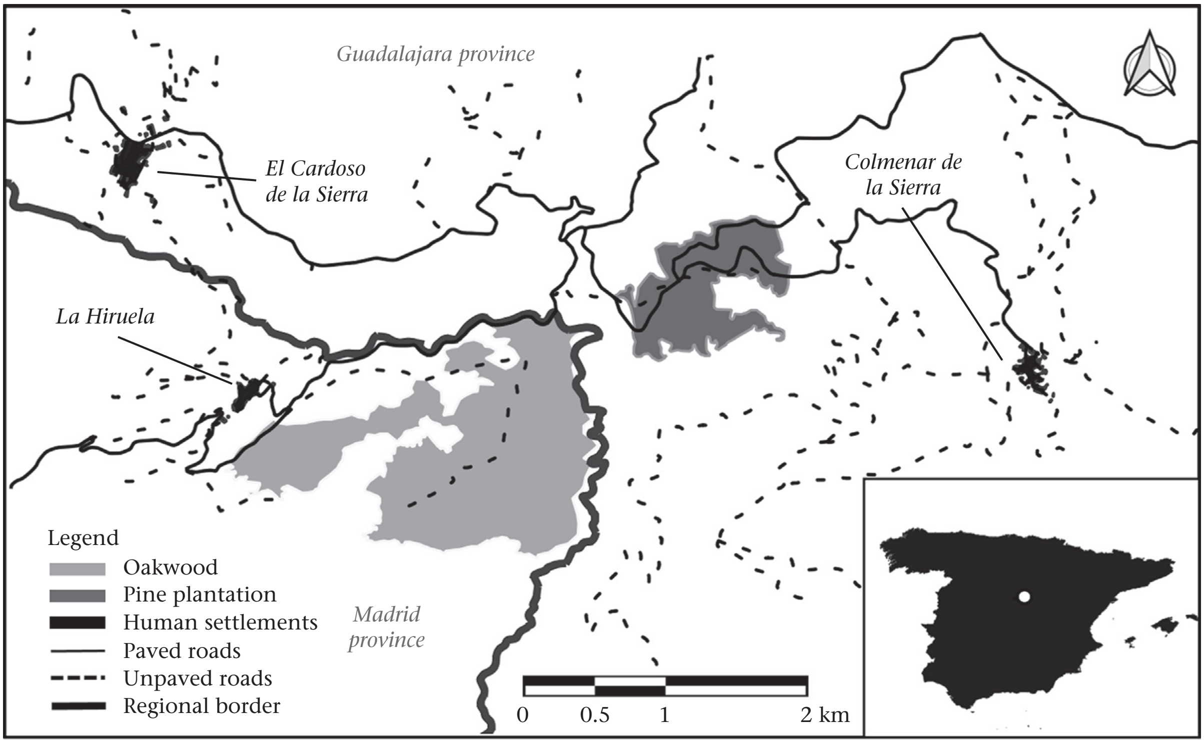

Figure A1. Map showing the study area in central Spain, between the provinces of Guadalajara (Autonomous Region of Castilla-La Mancha region) and Madrid (Autonomous Region of Madrid), showing the two forest stands that comprise the two habitats where the females were captured (oakwood and pine plantation), as well as the urban centres and the surrounding road network.