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ORIGINAL PAPER

Enemy of the invader: impact of the native ectoparasite *Philornis* spp. on an invasive bird species, the European starling (*Sturnus vulgaris*)

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Abstract Invasive species may be especially susceptible to native parasite infections since invaders have not shared a co-evolutionary history with local parasite species. A recently discovered host-parasite system is the European starling (*Sturnus vulgaris*)—botfly (*Philornis* spp.) larvae. The European starling is one of the most successful invasive bird species in the world and has recently arrived in South America. Botfly larvae from the genus *Philornis* are hematophagous ectoparasites that burrow under the skin of nestlings, or live in the nest material, and can seriously affect host fitness. Most studies regarding *Philornis* spp. parasitism focus on the effects of *Philornis* on native hosts or on naïve hosts when *Philornis* is an invasive parasite. Here, we evaluate the impact of native *Philornis* spp. larvae on cellular and humoral immunity, hematocrit, morphometrics, and survival of nestlings of the invasive European starling in Argentina. Based on evidence from native hosts and on the relatively recent encounter with this new host species, we predicted that *Philornis* spp. infestation would result in considerable sublethal and/or lethal effects on starling nestlings, potentially acting as a biological control on the expansion of this invasive species. When nestlings were 4–8 days old, they were measured, inspected for the presence of *Philornis* spp. larvae, and a blood sample was collected to quantify immune measures and hematocrit. Survival was then monitored until nestlings left the nest. As predicted, parasitized nestlings had lower structural body size and hematocrit levels than non-parasitized ones. In contrast, parasitized and non-parasitized nestlings showed no differences in estimates of cellular and humoral immunity at the age range studied. Furthermore, nestling survival was low and independent of infestation status, suggesting that other sources of mortality are in play. Our results indicate that *Philornis* spp. infestation has sublethal effects on starling nestlings while further studies are needed to understand whether *Philornis* spp. has lethal effects on this species.

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Introduction

The success of invasive species can be partly explained by their liberation from co-evolved natural enemies (Elton 1958; Torchin et al. 2003; Laurimaa et al. 2016). The benefits of enemy release, however, might decrease as exotic species acquire novel generalist parasites from the local fauna (Poulin and Mouillot 2003). The impact of native parasites on exotic hosts has the potential to be severe, since the latter have generally lost genetic diversity and are unlikely to have evolved effective defenses against native parasites, particularly in recently colonized areas (Blackburn et al. 2009). In fact, given the lack of a shared co-evolutionary history, novel parasites could elicit highly costly and damaging non-specific responses from hosts (Sears et al. 2011). Thus, invasive species may be especially vulnerable to parasites to which they are likely naive in their new range (Blackburn et al. 2009; Lee and Klasing 2004). Specific outcomes, however, are difficult to predict and might depend on the parasite and host species involved, stage of the invasion, whether the host population is in the front-wave or already established, if there has been loss of genetic (and/or immunogenetic) diversity during founding, and if there was a single or multiple invasion events, among other factors (White and Perkins 2012).

The European starling (*Sturnus vulgaris,* hereafter starling) is one of the most successful invasive birds in the world (Lowe et al. 2004). Its native range includes Europe, Asia, and North Africa (Lowe et al. 2004) and has become successfully established in New Zealand, Australia, South Africa, the United States, Canada, Mexico, and some Pacific and Caribbean islands (Feare 1984). In Argentina, the invasion by starlings is a secondary introduction resulting from escapes from the pet trade near Buenos Aires city in 1983, of birds imported from the United States (Navas 2002). While starlings from the USA population (i.e., primary introduction in the 1890s) show similar genetic diversity to that of the native population, starlings in Argentina exhibit the lowest haplotype and nucleotide diversity (Fiorini et al. 2022). The starling population has been growing exponentially and spreading over the country. According to initial wildlife records, few individuals were observed together in the city (Pérez 1988, Di Giácomo et al. 1993), but by 2003 groups of up to 950 individuals were registered (Peris et al. 2005). In fact, a study carried out in 2008 estimated a population size of 4600 individuals in parks of Buenos Aires city (Rebolo and Fiorini 2010). To date, numerous flocks of hundreds or thousands of individuals have been observed over the country (Rebolo and Fiorini 2010; Codesido and Drozd 2021; Ojeda et al. 2022; Palacio et al. 2022; Ibañez et al. 2023). During the starling expansion, it was found that 70–90% of nests of this species were parasitized by *Philornis* spp., making the starling a new host for these parasite species (Ibañez et al. 2015).

Parasitic flies in the genus *Philornis* (Diptera: Muscidae) include approximately 50 species that are widespread in the Neotropics (McNew & Clayton 2018). Adult flies are free-living and it is their larvae that are obligate parasites. Depending on the species, larvae can develop in avian nest material or subcutaneously in nestlings; in either case feeding on red blood cells, tissue, and fluid of nestlings (Young 1993) but also adults (LaPergola 2023). As a natural enemy, *Philornis* may cause anemia and poor growth on hosts, often resulting in nestling mortality when infection is severe or begins in the first days after hatching (Arendt 1985; Dudaniec et al. 2006; Fessl et al. 2006a, b; Rabuffetti and Reboreda 2007; Segura and Reboreda 2011; Quiroga and Reboreda 2012). As an exotic enemy, the case of *P. downsi*, an invader parasite in the Galapagos Islands, is well documented. This botfly species, whose larvae live in the nesting material and go up to the surface at night to feed on nestlings, has been implicated in the population declines of some Galapagos native bird species (causing anemia, poor growth, and early nestling death) including Darwin's small ground finch (*Geospiza fuliginosa*), medium ground finch (*G. fortis*), and the mangrove finch (*Camarhynchus heliobates*) (Dvorak et al. 2004; Grant et al. 2005; Dudaniec et al. 2006; Fessl et al. 2006a, b).

The European starling—Neotropical botflies system presents the best opportunity to study host-parasite dynamics in the context of a reverse system: i.e., the impact of a native parasite on an exotic invader. In

particular, we ask whether it is possible that *Philornis* spp. infestation could act as a natural biological control that slows down the starling expansion/dispersal in Argentina. For this, we investigated the effect of native botflies *Philornis* spp. on body size (culmen, wing, and tarsus lengths), hematological and immune measures, and survival of nestlings of the invader European starling. Immune response to subcutaneous fly larvae involves primarily an inflammatory response, with local inflammation at the site of larval establishment and increased number of white blood cells (WBCs) in systemic circulation (Owen et al. 2010; Manzoli et al. 2018). Specific-antibody responses are known to take place in other cases of myiasis (Otranto 2001), but as adaptive responses take some weeks to develop after infection, they are unlikely to be observed in passerine nestlings (Manzoli et al. 2018). On the other hand, innate non-specific antibodies, also known as natural antibodies, are produced constitutively and are present in young nestlings (Palacios et al. 2009; Arriero et al. 2013; Muriel et al. 2017; Aastrup and Hegemann 2021). Natural antibodies are reactive to a broad diversity of antigens, providing early defense to infection by diverse bacteria, viruses, fungi, and protozoans (Ochsenbein and Zinkernagel 2000; Palma et al. 2018). Natural antibodies binding antigens from hematophagous ectoparasites have been described in chickens (Wikel et al. 1989). In free-living birds, natural antibodies have been linked to hematophagous ectoparasite loads (e.g., Whiteman et al. 2006, DeCoster et al. 2010) and have been measured in relation to infection by other parasite types (e.g., avian malaria, Names et al. 2022). Yet, to our knowledge, natural antibodies have not been studied in the context of parasitism by *Philornis* spp. Overall as observed in other host species, and particularly given that the European starling and *Philornis* spp. have not a shared co-evolutionary history, we predicted that starlings would have costs in terms of growth, hematocrit level, and/or survival when parasitized by subcutaneous *Philornis* spp. larvae. In addition, we predicted that *Philornis* would elicit a robust, although not necessarily effective, immune response by the host to the novel parasite, reflected in non-specific cellular (WBC counts) and/or humoral (natural antibodies) immune parameters.

Methods

Study area and species

Our study was conducted in 2013 at the *Estación de Cría de Animales Silvestres* (ECAS), north of La Plata city in the province of Buenos Aires, Argentina (34º 56′ S, 57º573′ W), where starlings were first reported in 1990. Starling nests are found in natural cavities such as those in hollow trees, inside nests of the Rufous hornero (*Furnarius rufus*) and in woodpecker holes, as well as in artificial cavities such as house ceilings and nest boxes (Peris et al. 2005; Rebolo and Fiorini 2010; Rizzo 2010; Turienzo and Di Iorio 2010, Ibañez 2015). Thirty wooden nest boxes are distributed randomly on trees, around 2–3 m above the ground, in the study area (more details in Ibañez et al. 2015). Starlings occupy them regularly to breed from September to January, with two reproductive peaks during the season: one in October and another in November–December (Ibañez et al. 2015). Parasitism by *Philornis* spp. is observed during the whole reproductive season and the species of botflies that parasitize starling nestlings in this population are *P. seguyi* and *P. torquans* (Ibañez et al. 2015). Larvae of these species live subcutaneously feeding on nestling blood for 5–8 days and, once completed their development, they emerge to pupate in the nest material (Ibañez et al. 2015).

Field sampling

During the breeding season, nest boxes were monitored every 1–4 days from the nest building stage until chicks fledged or the nest failed due to predation, abandonment, or nestlings were found dead inside the nest box. At each visit during the nestling stage, chicks were marked individually by coloring one of their tarsi with a non-toxic permanent marker (Sharp Sop 2020) and carefully inspected for presence and number of botfly larvae (Fig. 1). When nestlings were between 4 and 8 days old, they were weighed (spring balance 100 ± 0.01 g), and their "Culmen" (length of the beak), "Wing chord" (from the anterior border of the wrist joint to the tip of the primary feather, non-flattened wing), and "Tarsus" (the right tarsus length) (digital caliper ± 0.1 mm) were measured. A whole blood sample (< 50 µl) was taken from each nestling via brachial venipuncture

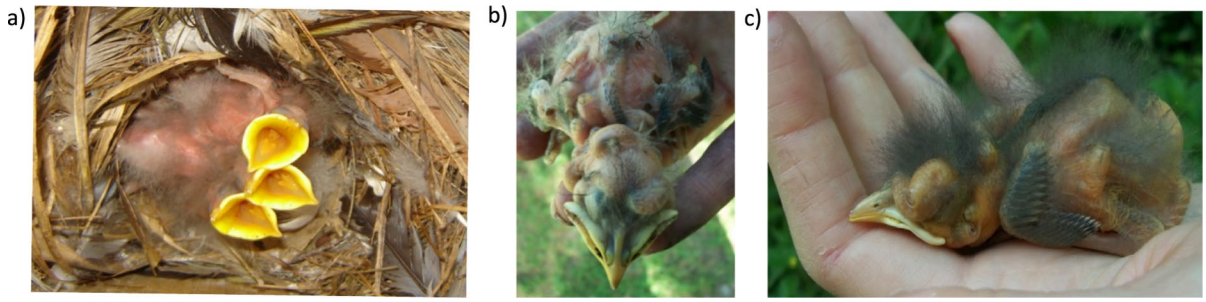

Fig. 1 European starling (*Sturnus vulgaris)* nestlings. **a** non-parasitized brood of three nestlings at 4 days old, **b** dead parasitized nestling with more than 15 *Philornis* spp. botfly larvae, **c** alive parasitized nestling at 8 days old. Photos by Lucia Ibañez

and collected into heparinized microcapillary tubes. A thin blood smear was prepared with a drop of blood and the rest of the sample was stored on wet ice while in the field. Whole blood samples were centrifuged at 14,000 G for 5 min in a microcapillary tube centrifuge within 2 h of collection. From the centrifuged samples, hematocrit (volume of red blood cells/total blood volume) was estimated with a standard hematocrit chart. Plasma was then drawn from the capillary tube using a Hamilton syringe and stored in a microcentrifuge tube at − 20 °C until analysis.

Immunological parameters

The levels of natural antibodies in plasma can be assessed by their ability to agglutinate particulate antigens such as foreign red blood cells (Matson et al. 2005) or bacteria (Sahoo et al. 2008). We assayed the agglutination of *Escherichia coli* (ATCC 8739), a non-pathogenic bacterial strain commonly used in ecoimmunology, following a previously developed protocol (Palacios et al. 2018). Briefly, bacteria were cultured in tryptic soy broth and then fixed in 1% formalin at 4 °C overnight. Fixed bacteria were washed three times with phosphate buffered saline (PBS) and adjusted to ~ 1×10^9 bacteria/ml. Plasma samples (20 µl) were added to the first column of a 96-well plate and serially diluted twofold with PBS. Next, 20 µl of fixed bacteria were added to all wells and plates were vortexed and incubated at room temperature (~ 25 °C) overnight. A negative control (PBS instead of plasma) was included in each plate. Agglutination titers were determined as − log2 of the highest dilution showing bacterial agglutination.

White blood cell (WBC) counts provide information on health status and immunity of individuals, are considered valuable indicators in wild animals (Beldomenico et al. 2008; Palacios et al. 2018), and have been used to study the impact of subcutaneous *Philornis* spp. larvae on avian hosts (Manzoli et al. 2018). We counted at least 100 WBCs, differentiating heterophils (H), lymphocytes (L), eosinophils (E), and monocytes (M), by scanning thin blood smears under a light microscope at 1000× magnification (Palacios et al. 2009). We also counted the number of red blood cells (RBC) in 7–10 fields, calculated the mean RBC/field, and 'Total RBC' as an extrapolation of the mean per field to the total number of fields scanned during the differential WBC count. We calculated two variables for each type of WBC: (1) 'WBC Proportion' as a binomial variable formed by the number of one type of WBC and the rest [e.g., H, (WBC-H)], and (2) WBC total counts as an integer variable of the estimation of each WBC type counted expressed in reference to 10000 RBCs. In addition, the heterophil/lymphocyte ratio (H/L proportion) was calculated as an index of stress in vertebrates (Davis et al. 2008).

Data analysis

We estimated nestling survival as the percentage of nestlings that reached 20 days of age. Nestlings are close to fledging, and thus nests were visited for the last time, at this age. Nestling mortality was estimated as the percentage of nestlings that did not reach the age of 20 days. We evaluated if the number of larvae per nestling was related to brood size using a Generalized Linear Mixed Models (GLMM) with logit

link function and negative binomial error term. The effects of botfly parasitism on structural variables (wing chord, culmen, and tarsus) were tested using Linear Mixed Models (LMMs) with identity link function and gaussian error term as they were normally distributed (Shapiro–Wilk tests: wing chord, $P=0.52$, culmen, $P=0.95$, tarsus $P=0.08$). Models were fitted using lme function and nlme package. To determine the effect of botfly parasitism on hematocrit, immune parameters, and survival of nestlings we performed Generalized Linear Mixed Models (GLMM) with logit link function and binomial error term for the different WBC proportions, Proportion H/L, and individual chick survival and GLMM with log link function and negative Binomial error term for the different WBC total counts. Models were fitted using glmmTMB function and glmmTMB package. Hematocrit and natural antibody titers were analyzed through LMMs with identity link function and normal error term. Nest identity was included as a random effect in all models. The explanatory variable of interest was botfly parasitism (0–1), whereas nestling age (4–8 days old) was included as a covariable to account for age variation across nestlings/nests at the sampling date. We estimated body condition through the residuals of the regression of chick mass on wing chord (Ardia 2005). Body condition was evaluated as covariable in the models for hematological and immune parameters. Sample sizes differ among parameters due to limited blood sample volume or, in a few instances, sample loss. We present the full models in our result tables. Non-significant effects were excluded from final models and we included the corresponding estimated effects in the text. Statistical analyses were carried out using R software, v.3.4.0 (R Core Team 2018). All tests were two-tailed and we considered significant differences at $P<0.05$.

Results

A total of 17 starling nests survived until hatching and were thus included in our present study. The mean (±SE) brood size was 3.5±0.2 (range: 1–5) nestlings per nest. Prevalence of *Philornis* spp. larvae in starling nests (calculated as the percentage of parasitized nests) was 47% (8/17 nests). Eighteen of the 43 nestlings (42%) were parasitized by botfly larvae with an intensity of 3.5±1.1 (mean±S.E, range: 1–22) larvae per nestling. The number of larvae per nestling did not vary with brood size (Intercept: estimate±SE: −0.80±1.06, $P=0.45$; Brood Size: estimate±SE: −0.02±0.25, $P=0.94$, n=45). Parasitized nestlings suffered a mortality rate of seventy eight percent (14 of 18 nestlings) and a survival of twenty two percent (4 of 18 nestlings). Non-parasitized nestlings suffered a mortality of sixty eighth percent (17 of 25 nestlings) and a survival of thirty two percent (8 of 25 nestlings). Thus, chick survival did not differ between parasitized and non-parasitized nestlings (estimate±SE: −1.37±1.61, $P=0.40$), although this result should be viewed with caution because of the low power of the test due to low sample sizes.

Botfly parasitism had a negative association with nestling tarsus, culmen, and wing lengths, which, as expected, were larger in older chicks (Table 1 and Fig. 2). Hematocrit was also negatively associated with botfly parasitism (estimate±SE: −4.49±2.11, $P=0.03$, n=43), but did not vary with nestling age and body condition (Table 2 and Fig. 3). Natural antibodies were not associated with the presence of botfly larvae and did not vary with nestling age and body condition (Table 2 and Fig. 3). Proportions (P) or total counts (T) of the different WBC types did not show association with botfly parasitism except for monocyte total count (MT), which was negatively associated with botfly parasitism (estimate±SE: −0.6±0.2, $P<0.01$, n=34; Table 2 and Fig. 3). Effects of covariables varied among WBC types and depended on whether the proportion or the total count was considered.

Total counts, with the exception of MT, increased with nestling age (LT, estimate±SE: 0.20±0.06, $P=0.002$, n=34; HT, estimate±SE: 0.34±0.09, $P=<0.001$, n=34; ET, estimate±SE: 0.16±0.07, $P=0.01$, n=34). Body condition was positively associated with LP (estimate±SE: 0.14±0.06, $P=0.02$, n=34) and negatively associated with HP (estimate±SE: −0.27±0.12, $P=0.03$, n=34), ET (estimate±SE: −0.20±0.08, $P=0.02$, n=34), and the stress index H/L (estimate±SE: −0.22±0.07, $P=0.002$, n=34).

Discussion

We studied the impact of native botfly *Philornis* spp. larvae on nestlings of the exotic host European starling, a relatively recent invasive species

Table 1 Structural size results of LMMs with response variables culmen, wing, and tarsus

Response variable	Independent variable	Estimate ± SE	Test statistic	*P*-value
Culmen	Intercept	7.8 ± 0.9	8.21	< 0.001
	Botfly Parasitism	− 0.8 ± 0.3	− 2.70	0.012
	Nestling Age	0.7 ± 0.1	4.70	< 0.001
Wing	Intercept	2.1 ± 4.4	0.50	0.624
	Botfly Parasitism	− 1.9 ± 0.9	− 2.06	0.048
	Nestling Age	2.7 ± 0.6	4.20	< 0.001
Tarsus	Intercept	8.3 ± 2.7	3.05	< 0.010
	Botfly Parasitism	− 1.7 ± 0.7	− 2.38	0.025
	Nestling Age	1.6 ± 0.4	3.98	< 0.001

Explanatory variable was botfly parasitism (0–1). Nestling age (4–8 days old) was included as a covariable and nest identity as a random effect (see methods for more details). Values indicate contrast estimates with their standard errors. n = 43 nestlings

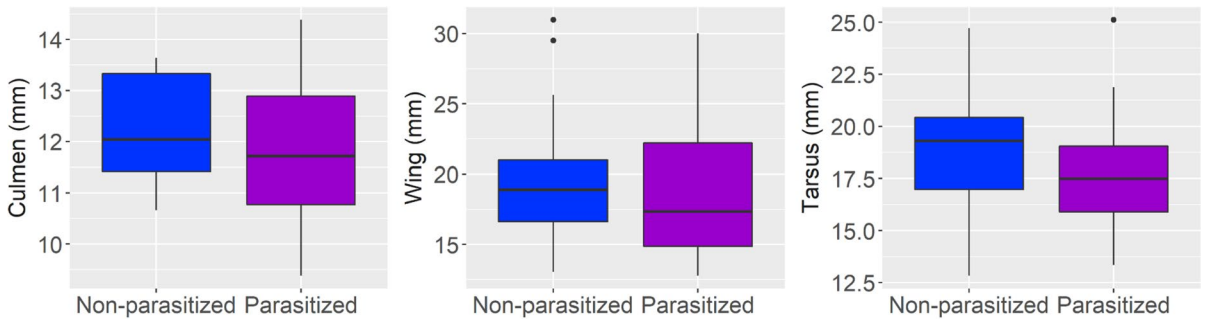

Fig. 2 Boxplots with raw data of structural body size parameters of parasitized and non-parasitized European starling (*Sturnus vulgaris)* nestlings (n = 43) by *Philornis* spp. botflies. Boxes indicate the inter quartile range (IQR), the line within each box indicates the median, and whiskers depict 1.5*IQR. Dots represent outlying data points

in Argentina. This study presents the first description on aspects of cellular and humoral immunity of an exotic invasive host parasitized by *Philornis* spp. larvae, contributing empirical evidence to the study of the ecoimmunology of invasions (White and Perkins 2012). As predicted, parasitized nestlings showed reduced general-health state compared with non-parasitized ones, reflected in a smaller structural body size and lower hematocrit. On the other hand, contrary to our prediction, parasitized and non-parasitized nestlings showed no differences in the measured aspects of immunity. Nestling survival was low overall, independently of whether nestlings were infested or not by *Philornis* spp. larvae. Below we discuss our main findings in the context of previous work in this and other nest parasitic larvae-avian host systems.

The impact of botfly parasitism by *Philornis* spp. on the structural body size of starling nestlings generally agrees with studies on native host species in Argentina reporting lower growth of parasitized than non-parasitized nestlings (Rabuffetti and Reboreda 2007, Segura and Reboreda 2011, Quiroga and Reboreda 2012, Manzoli et al. 2018, Domínguez et al. 2015, but see Ursino et al. 2019). For example, in a House wren (*Troglodytes aedon*) population, infested nestlings that survived until fledging showed lower growth rates of head plus bill, wing length, and body mass than nestlings of non-infested broods (Quiroga and Reboreda 2012). In the same direction, our results of reduced hematocrit in parasitized nestlings are consistent with the impact of botfly parasitism by *Philornis* spp. on hematological indicators of aerobic capacity in other host species. For example, Great kiskadee (*Pitangus sulphuratus*), Greater

Table 2 Hematological and immunological parameter results of GLMMs with response variables hematocrit (n = 43), natural antibodies (n = 43), proportion (P) and total (T) of each leukocyte type (Lymphocytes (L), Heterophils (H), Eosinophils (E), and Monocytes (M)), and the stress index H/L (n = 34)

Response variable	Independent variable	Estimate ± SE	Test statistic	*P*-value
Natural Antibodies	Intercept	1.3 ± 0.9	1.42	0.167
	Botfly Parasitism	− 0.1 ± 0.3	− 0.41	0.682
	Nestling Age	0.2 ± 0.1	1.48	0.151
	Body condition	0.1 ± 0.2	0.50	0.618
Hematocrit	Intercept	31.3 ± 6.5	4.76	< 0.001
	Botfly Parasitism	− 4.5 ± 2.3	− 1.99	0.058
	Nestling Age	− 0.8 ± 1.0	− 0.77	0.445
	Body condition	0.4 ± 1.1	0.37	0.711
LP	Intercept	− 1.0 ± 0.5	− 2.23	0.026
	Botfly Parasitism	− 0.1 ± 0.1	− 0.68	0.499
	Nestling Age	− 0.0 ± 0.1	− 0.27	0.791
	Body condition	0.2 ± 0.1	2.46	0.014
LT	Intercept	1.6 ± 0.4	3.7	< 0.001
	Botfly Parasitism	− 0.3 ± 0.2	− 1.8	0.137
	Nestling Age	0.2 ± 0.1	3.5	< 0.001
	Body condition	− 0.0 ± 0.1	− 0.0	0.990
HP	Intercept	− 0.7 ± 0.5	− 1.67	0.215
	Botfly Parasitism	− 0.0 ± 0.1	− 0.35	0.725
	Nestling Age	0.1 ± 0.1	0.96	0.337
	Body condition	− 0.1 ± 0.1	− 2.37	0.039
HT	Intercept	1.6 ± 0.7	2.3	0.019
	Botfly Parasitism	− 0.2 ± 0.3	− 0.6	0.557
	Nestling Age	0.4 ± 0.1	3.3	< 0.001
	Body condition	− 0.2 ± 0.1	− 1.9	0.051
EP	Intercept	− 0.9 ± 0.6	− 1.72	0.086
	Botfly Parasitism	0.1 ± 0.1	0.74	0.461
	Nestling Age	− 0.1 ± 01	− 0.54	0.592
	Body condition	− 0.0 ± 0.1	− 0.23	0.818
ET	Intercept	2.0 ± 0.4	4.43	< 0.010
	Botfly Parasitism	− 0.1 ± 0.2	− 0.43	0.663
	Nestling Age	0.2 ± 0.1	2.44	0.015
	Body condition	− 0.2 ± 0.1	− 2.26	0.024
MP	Intercept	− 1.4 ± 0.8	− 1.69	0.091
	Botfly Parasitism	0.0 ± 0.2	0.103	0.918
	Nestling Age	− 0.2 ± 0.1	− 1.475	0.140
	Body condition	0.1 ± 0.15	1.046	0.296
MT	Intercept	1.5 ± 0.8	1.8	0.068
	Botfly Parasitism	− 0.5 ± 0.2	− 2.4	0.018
	Nestling Age	0.1 ± 0.1	0.4	0.069
	Body condition	− 0.2 ± 0.1	− 1.3	0.197
P(H/L)	Intercept	0.3 ± 0.5	0.5	0.607
	Botfly Parasitism	0.1 ± 0.1	0.4	0.666
	Nestling Age	0.1 ± 0.1	0.7	0.508
	Body condition	− 0.2 ± 0.1	− 3.12	0.002

Explanatory variable was botfly parasitism (0–1). Nestling age and body condition were included as covariables, and nest identity as a random effect (see methods for more details). Values indicate contrast estimates with their standard errors

thornbird (*Phacellodomus ruber*), and Little thornbird (*Phacellodomus sibilatrix*) nestlings suffered a decrease, although to different extents, in RBC counts when infested by *P. torquans* larvae (Manzoli et al. 2018), one of the botfly species present in our system (Ibañez et al. 2015). Similarly, Dudaniec et al. (2006) found that nestlings of Darwin's small ground finch parasitized by *P. downsi* showed lower levels of

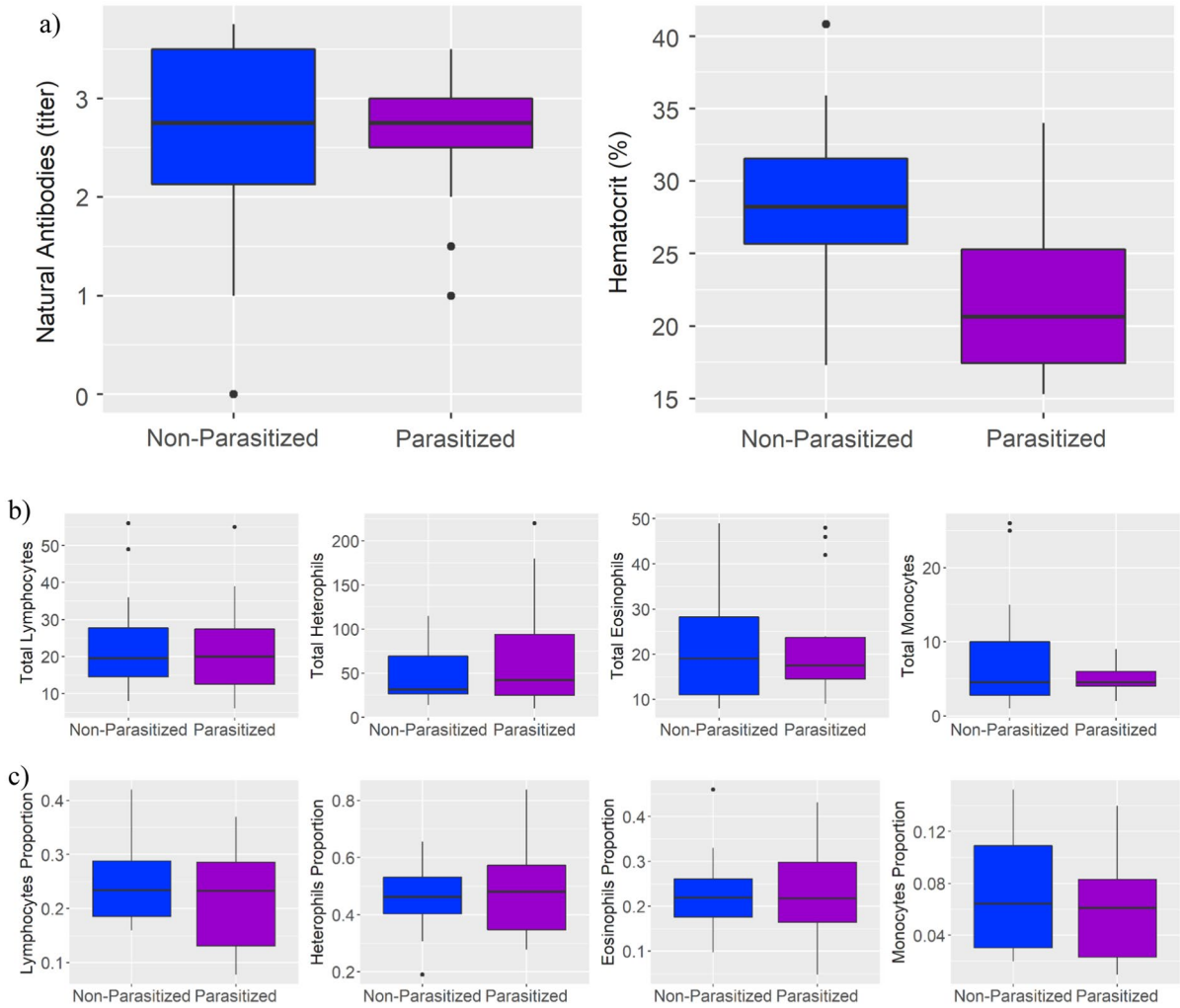

Fig. 3 Boxplots of **a** natural antibodies and hematocrit (n = 43), **b** leukocyte number per 10,000 red blood cells (n = 34), and **c** proportion of the different leukocyte types (n = 34) of parasitized and non-parasitized European starling (*Sturnus vulgaris)* nestlings by *Philornis* spp. Boxes indicate the inter quartile range (IQR), the line within each box indicates the median, and whiskers depict 1.5*IQR. Dots represent outlying data points

hemoglobin in blood and increased number of immature RBC counts. Impact by hematophagous parasites on the aerobic capacity of nestlings might negatively affect energy-demanding activities such as begging (Morrison and Johnson 2002), which could account for, or contribute to, the observed structural size reduction in parasitized starling nestlings.

Contrary to our prediction, botfly parasitism by subcutaneous *Philornis* spp. larvae was not associated with altered indices of non-specific humoral (i.e., natural antibody levels) and cellular (i.e., WBC counts) immunity of starling nestlings at the age-range studied (i.e., 4–8 days old). Regarding antibody responses, our results with natural antibodies in starling nestlings resemble those from studies of infestation by *P. downsi* in two Galapagos avian hosts. Plasma levels of antibodies binding *P. downsi* antigens did not differ between 5 and10 day old nestlings from parasitized and non-parasitized nests of the Galapagos mockingbird (*Mimus parvulus*) and the medium ground finch (Koop et al. 2013, Knutie et al. 2016). This lack of association between *Philornis* spp. parasitism and antibody levels of nestlings is interesting given the several differences between studies:

natural antibodies binding *E. coli* versus antibodies binding parasite antigens, correlational versus experimental parasite-removal studies, subcutaneous versus non-subcutaneous *Philornis* spp. larvae. Together, these results suggest that antibody-mediated defenses are not involved, or might not play a major role, in *Philornis* spp. resistance by young nestlings. Further studies are needed to evaluate this hypothesis.

To our knowledge, only one previous study has investigated the relationship between infestation by skin burrowing larvae of *Philornis* spp. and nestling immune function. Manzoli et al. (2018) used total WBC counts as an index of investment in immune resistance against *P. torquans* identifying different antiparasitic defense strategies across three avian hosts. Nestlings of the main host species, the Great kiskadee, did not mount an immune response (i.e., did not elevate total WBC counts), but rather tolerated the infection (i.e., showing reductions in RBC counts and growth but no decrease in survival). On the other hand, two alternative host species showed lower tolerance and elevated their total WBC counts in response to infection, although with different dynamics and efficiencies (Manzoli et al. 2018). In accordance with those results, the lack of association between infestation status and WBC counts of starling nestlings suggests that they were not mounting a cellular (inflammatory) immune response to resist the parasite. Thus, we found no evidence of either humoral or cellular immune response of starling nestlings to *Philornis* spp. infestation. This is an unexpected result for an exotic species encountering a novel parasite (White and Perkins 2012). While the European starling is naïve for *Philornis* spp., it is parasitized by species of blowflies [*Protocalliphora azurea* (Hicks 1971), *Protocalliphora falcozi* and *Trypocalliphora lindneri* (Grunin 1966)] in its native range. May the shared co-evolutionary history with those parasitic nest flies have afforded starlings immune tolerance to *Philornis* species? Although the pattern of no detectable immune responses together with no evident survival cost in starling nestlings infested with *Philornis* spp. could in principle resemble that described by Manzoli et al. (2018) for Great kiskadees, several unknown factors preclude us from making assertions regarding defense strategies in our system at present. For instance, we cannot rule out that other immune responses (e.g., specific IgY-mediated, local inflammation at the site of larval establishment) and/or the ones we measured but later in the nestling period could be playing a defensive role. Most importantly, however, a better understanding of the causes of high mortality of nestlings, both parasitized and non-parasitized, in our system would be crucial.

In different avian host species of subcutaneous *Philornis* spp. for which the lethal effect of parasitism has been studied, larval infestation generally decreased nestling survival (Antoniazzi et al. 2010, Dominguez et al. 2015, Gonzalez et al. 2021, Quiroga and Reboreda 2012, Rabuffetti and Reboreda 2007, Segura and Reboreda 2011, Manzoli et al. 2018), although exceptions have also been reported (e.g., Young 1993, Nores 1995, Norris et al. 2010, Manzoli et al. 2018 for Great kiskadee; also see Mezquida 2020 for a low nestling mortality). In our study, a high percentage of infested nestlings died, but so did a similar proportion of non-infested nestlings, replicating findings from the 2010 reproductive season in this starling population (Ibañez et al. 2015), and suggesting that the previous finding was not an isolated one-year event. So taken together, these patterns of high nestling mortality irrespective of infestation status by *Philornis* spp. suggest, as discussed in Ibañez et al. (2015), that other environmental factors are likely involved in the reproductive failure of starlings in this population. One possibility is that climatic variables affect nestling survival, as has been shown for several avian species including European starlings in their native range (Bionda and Brambilla 2012, Carey 2009, Dolenec 2009, Elkins 2004). Another possibility is that other ectoparasites could be affecting nestling survival. Starling nestlings of the studied population can be infested by the haematophagous mite *Ornithonyssus bursa* (Lareschi et al. 2017). Although there is scarce information about the effects of this ectoparasite on hosts in Argentina (Santillán et al. 2015), no effect on breeding success of European starlings was reported in New Zealand (Powlesland 1977). Furthermore, during chick sampling and inspection we did not record the presence of mites, which are easily detected if they are in high loads. Therefore, we consider it is unlikely that mite infestation influenced our results on starling survival.

In the light of our present results, is it not possible to determine whether *Philornis* spp. could be acting as a natural biological control slowing the European starling expansion/dispersal in Argentina.

For this, inclusion of other study populations showing the starling-*Philornis* interaction (e.g., from the front-edges of the expansion) would be valuable. In our system, future work should assess potential interactions between botfly parasitism and climate variables in the determination of nestling survival. Moreover, some studies have found that although nestlings parasitized by blow flies evidenced no costs, fledglings suffered reduced survival (Streby et al. 2009). Therefore, future studies should also evaluate whether the costs of infestation by *Philornis* spp. in terms of growth and hematocrit might have impacts later, during the post-fledgling stage. In the context of the ecoimmunology of invasions, future work would benefit from a combination of parasite load manipulation and measurement of immune defenses along the nestling period to accomplish a more complete understanding of the interaction between native *Philornis* spp. and its exotic host, the invasive European starling.

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Author contributions CAU and VDF contributed to the study of conception and design. CAU, LMI and VDF performed data collection and material preparation. MGP performed immunological analyses. CAU and VDF performed statistical analysis. The first draft of the manuscript was written by CAU and VDF with conceptual input from MGP and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Declarations

Competing interest All authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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