Journal of Comparative Physiology B
https://doi.org/10.1007/s00360-020-01323-z

ORIGINAL PAPER

Ontogeny of leukocyte profiles in a wild altricial passerine

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Received: 2 April 2020 / Revised: 8 September 2020 / Accepted: 28 October 2020

Abstract
Ecophysiological studies have highlighted the relevance of the avian immune system in individual fitness prospects in the wild. However, studies on the ontogeny of avian immunity are scarce. We analyse age-related changes in the cellular constitutive immunity throughout nestling development, as well as its relationship with sex and brood size. We found that cellular constitutive immunity could be affected by age, sex, brood size, or daily rhythm. Early-stage nestlings relied more on cells of the innate immunity rather than on cells linked to the adaptive immune system. Cellular immunity may not be fully mature in fledglings, as reflected by differences in phagocytic cell counts with regard to adults. Beyond the age-dependent effects, agranulocyte cell counts were affected by sibling competition while granulocyte cell counts showed a daily rhythm. We also show that the heterophil to lymphocyte ratio was negatively related to body weight when nestlings become more independent. Our study contributes knowledge to the fields of developmental immunology and ecological immunology based on essential components of the cellular immune system.

Keywords Age-specific pattern · Cell-mediated immunity · Daily rhythm · Leukocyte count · Nestling development · *Sturnus unicolor*

Introduction

Hosts develop different immunological mechanisms to protect themselves against pathogens, but their maintenance and effective functioning are costly (Sheldon and Verhulst 1996; Hasselquist and Nilsson 2012). Therefore, from an evolutionary and ecological perspective, we may expect that high investment in immunity must be traded off against investment in other costly traits, such as growth during the nestling period (Van Der Most et al. 2011) or reproductive traits for adults (Ardia 2005; Colominas-Ciuró et al. 2017), which require high energetic demands, in particular during the breeding season (Kulaszewicz et al. 2017). Field studies have used white blood cells (WBC) or leukocyte profiles to assess immune function and environmental physiological stress in wild birds (Davis et al. 2008; Masello et al. 2009). Thus, leukocytes involved with the innate immune system, such as monocytes and granulocytes (heterophils, basophils, and eosinophils), could offer an important measure of the non-specific host immune function and health status in birds (Davis et al. 2008; Masello et al. 2009). Additionally, the acquired immune system, which involves a high metabolic cost due to immunological memory processes, informs us about long-lasting protection, being highly specific and acting more effectively against various pathogens, including ectoparasites, viruses, and bacteria (Masello et al. 2009; Kaiser 2010).

Birds, as with other animal species, can balance resources based on the energy requirements delimited by their trade-offs between reproduction and self-maintenance, which may

Communicated by Philip Withers.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00360-020-01323-z) contains supplementary material, which is available to authorized users.

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Published online: 16 November 2020

vary through different life stages (Lochmiller and Deerenberg 2000), and even between sexes (Klein and Flanagan 2016; Roved et al. 2017). Since immunity varies ontogenetically, younger individuals are more exposed and quite vulnerable to environmental pathogens, as their immune system is not yet fully matured, as well as because they are confined to nests that typically harbor ectoparasites and other pathogens (Fellah et al. 2013; López-Rull and Macías Garcia 2015). Newly hatched birds are rarely exposed to any external pathogen, so it would be expected that their innate immune system is much more developed than the acquired immunity (Bar-Shira and Friedman 2006; but see Killpack and Karasov 2012), which would mature throughout nestling development. Beyond maternally-transferred antibodies produced by their mothers (reviewed in Hasselquist and Nilsson 2009), nestlings start producing their own specific antibodies in response to exposure to foreign antigens over the first few days or weeks after hatching (Staszewski et al. 2007; Hasselquist and Nilsson 2012). Adaptive immune responses are gradually developed and regulated in response to several factors, such as environment, life stage, or season (Evans et al. 2016); although there is evidence that both the acquired and the innate immune systems fully mature post-fledgling (Killpack and Karasov 2012; Stambaugh et al. 2011; respectively). Although the immune systems of adult birds (e.g. Davis et al. 2004; Palacios et al. 2011; Råberg et al. 2003) and nestlings (e.g. Brommer 2004; Soler et al. 2003) have received considerable attention, comparisons between the two ages are more rarely reported (e.g.Stambaugh et al. 2011; Tella et al. 2002). Indeed, these studies mostly focus on measurements taken at a specific age of nestlings, without considering the possible immunological differences that arise throughout nestling development.

Several studies have shown that females and males may differ in both immune responsiveness and parasite load (e.g. Klein and Roberts 2015; Klein 2004). This sexual bias could lie in the effect of sex hormones on the functioning of the immune system (Grossman 1985), where testosterone could have an immunosuppressive effect in males leading to a higher parasite load and a lower immune response than in females (Folstad and Karter 1992; Foo et al. 2017). However, it is barely known whether this sexual-dimorphism in immunocompetence is already present in the first stages of bird development.

Beyond age- and sex-dependent effects, innate and adaptive immune responses could be conditioned by both the nutritional status during the nestling stage (reviewed in Alonso-Alvarez and Tella 2001) and environmental growth conditions (e.g. Ardia 2007; Christe et al. 2001). Among these external factors, some studies have highlighted that chicks raised in large broods, where sibling competition is greater, may show reduced immunocompetence (Ilmonen et al. 2003). For this reason, analysing the impact of body condition and the position occupied by the individual within the size hierarchy of the nest is required to understand the factors affecting the maturation of the cell-mediated immune function in birds.

Here we report a detailed study of the ontogeny of the leukocyte profile, also exploring the effect of some key factors that may influence the maturation of the immune function of developing birds. We used the spotless starling (*Sturnus unicolor*) as a study species, a medium-sized altricial bird. The specific aims of the present study are to analyse: (1) age-related changes in WBC profiles and H/L ratio throughout nestling development, (2) the relationship between WBC profiles and H/L ratio and key factors that may influence immune function (i.e. brood size, body mass, and sex); (3) the differences in both innate and acquired immunity between fledgling and adult individuals. We expect that the proportion of the innate cell types (e.g. heterophils, eosinophils, basophils, and monocytes) would decrease throughout chick's ontogeny while those related to the acquired immunity (e.g. lymphocytes) would increase. Because of the sex-specific energetic, physiological, and behavioural costs of maintaining an effective immune system (Lochmiller and Deerenberg 2000), we expect a difference in immune function between males and females throughout the nestling and independence periods, where probably the larger sex pays higher costs. Finally, we expect a lower immune cell fraction in chicks from nests with a larger brood size (Chin et al. 2005), in particular in the smaller siblings of the brood.

Materials and methods

Study area and study species

This study was conducted during three consecutive breeding seasons (from 2009 to 2011) in a nest-box population of spotless starlings located in central Spain (Soto del Real, Madrid, ca. 40° 45′ N, 3° 48′ W, 920–940 m above sea level). The study area is covered by a deciduous woodland of oak (*Quercus pyrenaica*) and ash (*Fraxinus angustifolius*) with abundant open areas used by grazing cattle. It exhibits a continental Mediterranean climate [Köppen–Geiger climate classification: Csb category (reviewed in Peel et al. 2007)] with hot, dry summers. Thus, breeding conditions become harsher as the season advances, where late breeding conditions are characterized by higher temperatures and scarcer food (Muriel et al. 2015). The spotless starling is a relatively long-lived, colonial, and sedentary passerine species that shows sexually dimorphic characters and exhibits a facultative polygynous breeding system (Cramp et al. 1982–1994; Monclús et al. 2017; Moreno et al. 1999). Females can lay up to two clutches per season, the first one in early April and the second one

about the end of May in our study area (López-Rull et al. 2011). Incubation usually starts before the last egg is laid (modal clutch size is five eggs, López-Rull et al. 2007). The nestling period lasts about 21–22 days (Cramp et al. 1982–1994).

Field protocols and sample collection

During the pre-laying period (from early March until the first egg of the colony was laid), male and female adult starlings were caught by traps placed inside nest-boxes. From every individual captured, we took a blood sample by brachial vein puncture. A drop of blood was smeared on individually marked microscope slides and air-dried. From early April onwards, nest-boxes were inspected each day to determine the laying date. Broods were visited several times a day from the 10th day after the beginning of incubation to determine the exact hatching date (i.e., date first nestling hatches from egg = day 1). Chicks were labelled by distinct down cuttings to carry out an individual identification, and they were measured on different time points: 1, 3, 6, 9, 12, 15, 18 and 21 days post-hatching. At these ages, we recorded body mass with a digital balance (Ohaus Scout II SC2020, China, accuracy = 0.1 g) and tarsus length with digital calipers (Mitutoyo Absolute, Japan, accuracy = 0.01 mm). All chicks were ringed at 6 days old with numbered aluminium bands when their tarsus was large enough for this purpose. A small blood volume (50 µl) was collected from the nestling jugular vein using a 29-ga insulin syringe (U-100, 0.5 ml, TERUMO®, Terumo Corporation, Tokyo, Japan) to perform their molecular sexing and the respective blood smears. All blood samples were collected within 2 min after capture and prior to any further manipulation of the individual to avoid any leukocyte alteration due to handling time (Davis 2005). The solar time at which the blood sampling was carried out was recorded to control for it the statistical analyses. Most of the broods were sampled at consecutive time points. Each chick of a given nest was sampled at its real age. That is, when a brood included nestlings of three days of hatching asynchrony (i.e. nestlings of three different ages), we visited these nests three consecutive days per sampling event to get all the siblings sampled at their own exact age. This allowed us to avoid confounding age and size effects derived from hatching asynchrony in our analyses (Muriel et al. 2019). Overall, we analysed samples of 22 adults captured in 2009 (11 males and 11 females), 60 different chicks sampled in 2010 (30 males and 30 females repeated sampled between 1 and 21 days old), and 34 different chicks sampled in 2011 (16 males and 18 females repeatedly sampled between 1 to 6 days old) (see Supplementary Table 1S). These nestlings belonged to 19 different nests in 2010 and 9 different nests in 2011.

Differential leukocyte count

For identification of white blood cells (WBC), a drop of blood was smeared on one individually marked microscope slide. Once the blood had air-dried, we fixed the slide by 3 min immersion in 100% methanol and stained it using commercial Giemsa diluted with PBS pH 6.8 (1:2). Slides were examined under the microscope using the oil immersion objective (1000 × magnification) to estimate the proportion of different types of leukocytes (Campbell and Ellis 2007). Estimates of the total WBC were calculated per approximately 10,000 erythrocytes. The total count for each type of leukocyte was calculated by multiplying the total leukocyte count by the respective differential WBC proportion, which were classified as heterophils, eosinophils, basophils, lymphocytes, or monocytes. We also took the ratio of heterophils/lymphocytes (H/L ratio) and the total leukocyte count as a measure of physiological stress and immunity in birds (Gross and Siegel 1983; Maxwell and Robertson 1998). One person (JM) conducted all cell counts to eliminate the variation between observers. Counts were done blind to any information from the individual.

DNA extraction and molecular sexing

For sex determination, DNA was extracted from the blood samples using ammonium acetate techniques (Bensch and Åkesson 2003), and diluted to a working DNA concentration of 25 ng/µl. This solution was used in a polymerase chain reaction (PCR; using the primers P2 and P8) to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (Griffiths et al. 1998). PCR products were electrophoresed for 60–90 min at 100 V in 1.5% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, CA) and were visualized under UV light, where one band was scored as male and two bands as female.

Statistical analyses

All calculations were performed in the r language v. 3.5.3 (R Core Team 2019), and the significance level was set at $\alpha = 0.05$ for all tests. We applied Generalized Linear Mixed Models with Poisson distribution using 'glmer' function for count data (for all cellular variables except for the H/L ratio, which was analysed by Negative Binomial models using 'glmer.nb' function) and Linear Mixed Models using 'lmer' function for continuous data (bodyweight) using "*lme4*" package (Bates et al. 2017). These repeated measures analyses were run considering nestling age as a continuous variable (from 1 to 21 days) to allow testing for different trends (age-related increases, decreases or quadratic relationships), taking into account other factors such as sex, time of the day, and brood size by including

them as covariates in the models. To control for non-independence of individuals from the same brood, the nest of origin was defined as a random effect affecting the model intercept, and the identity of the individual was entered as repeated factor. In addition, we created an obs_effect variable (observation-level random effect) with a unique value for each observation to control overdispersion in the model (Harrison 2014). All biologically meaningful double interactions were included in the original model. For thrombocyte and differential WBC counts, for which we had data from 2010 and 2011, we pooled them in the same dataset because we found no significant year effect (all $P > 0.13$). To evaluate the effect of body weight on cell counts but avoiding collinearity problems with age, we carried out generalized linear models where we analyse the effect of body weight on WBC and thrombocyte counts at day 6, when there is an inflection point for the growth curve and nestlings become more independent, and sexual size dimorphism is not detectable yet (see "Results" section). Finally, with the aim of analysing the degree of maturation of the cellular immune system at fledging, we also compared cell counts between fledglings (aged 21 days post hatch; 12 males and 9 females) and adults (11 males and 11 females). To do so, we included ran separate models for each cell type including age as a categorical variable with two levels (fledglings vs. adults), sex, and their interaction, also controlling for sampling time as a covariate. In all cases, initial models were simplified by subsequently following a backward stepwise procedure to remove non-significant terms (i.e. $P > 0.05$).

Results

Within broods, body weight increased throughout nestling development (from 1 to 21 days old) adjusting to a negative quadratic function ($\chi^2 = 591.94$, $P < 0.001$, estimate (SE) $= -0.2556 \pm 0.0105$), where body weight reached an asymptote in the final days before fledging. Male nestlings were heavier than females ($\chi^2 = 3.64$, $P = 0.056$, estimate (SE) $= -1.8068 \pm 0.9463$), although this sexual dimorphism became particularly evident at the end of the phase of linear growth of the nestlings (Fig. 1).

Leukocyte profiles during the nestling period

Age significantly affected all immune variables considered with the exception of monocyte and total leukocyte counts (see Fig. 2, all $P > 0.110$), although the sign of this effect depended on the type of WBC analysed (see Fig. 2, all $P < 0.02$). The relative percentage of each type of leukocyte to total white blood cells counted along the chick ontogeny as well as in the adult stage is detailed in the Electronic Supplementary Material (see Supplementary Fig. 1S). Heterophil levels decreased along the ontogeny (Fig. 2a), while basophil and lymphocyte levels increased (Fig. 2c and d, respectively). In turn, eosinophils respond to a negative quadratic (i.e. inverse U-shaped) effect of age, reaching the highest levels at 9 days old (Fig. 2b). That maximum in eosinophil levels was concomitant with the cross of the opposite trends of heterophils and lymphocytes. In fact, that cross in the trends is responsible for the abrupt fall in the

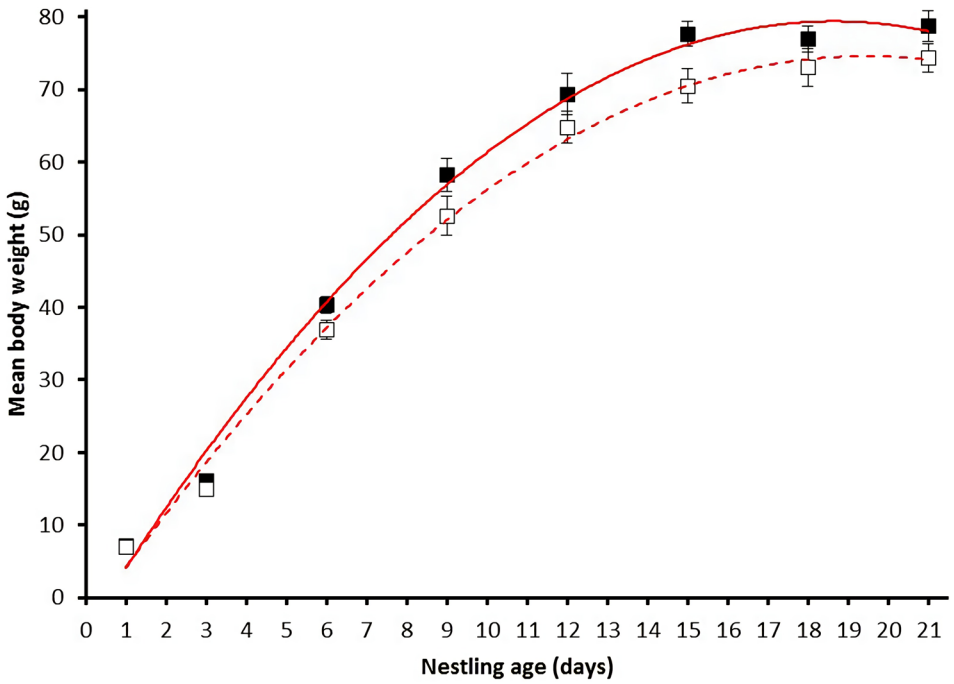


Fig. 1 Variation of body weight (g) throughout the nestling development (age expressed in days). Values represented are means ± SE (white squares: female and black squares: males). The continuous line represents the estimate for males and the dotted line for females

heterophil/lymphocyte (H/L) ratio at the beginning of the nestling period (Fig. 2g).

The results about the effect of age, time and brood size on the total and differential leukocyte count, as well as the H/L ratio, are shown in Table 1. We detected a negative effect of brood size in the interaction with age on both lymphocyte and monocyte counts, where the level of these agranulocytes decreased as sibling competition increased during early development, but increased during late development. Eosinophil level responded to an interaction between age and sex, where males had lower levels than females during the early stage of development (up to approximately 9 days old), a sex-related difference that disappeared after 12 days of age (Fig. 2b). Beyond the age-related effects, the total WBC count, and specifically granulocyte counts (heterophils, eosinophils, and basophils), were affected by the time of day in which the nestlings were sampled throughout their ontogeny. Thus, the levels of these white blood cells increased throughout the day, adjusting to a daily rhythm (see Supplementary Fig. 2S).

Thrombocyte count was similar in both sexes ($\chi^2 = 1.12$, $df = 1$, $P = 0.287$) but it was affected by a positive quadratic (U-shaped) effect of age throughout nestling development ($\chi^2 = 3.971$, $df = 1$, $P = 0.046$, estimate (SE) $= 0.0055 \pm 0.0027$), especially increased number of thrombocytes towards the last phase of the ontogeny (Fig. 2h). Circulating blood thrombocyte levels changed depending on the time of day at which blood samples were taken ($\chi^2 = 9.50$, $df = 1$, $P = 0.002$, estimate (SE) $= -2.8015 \pm 0.9086$). Thus, the presence of these cells decreased as the day advanced.

Effect of body weight on differential cell count

At 6 days-old, we found a negative effect of body weight on H/L ratio (Table 2). This effect seems largely attributable to the negative effect of weight on heterophil levels. We also found a negative impact of body weight on total leukocyte counts, which may be due to the lower levels of heterophiles and, to a lesser extent, the lower lymphocyte levels (Table 2) in the lighter nestlings of each brood as compared to their heavier siblings. Additionally, lighter chicks also had higher thrombocyte levels (Table 2). The rest of the cell types did not show a clear pattern of variation based on body weight, with the exception of a marginally positive effect on basophile levels (Table 2).

Leukocyte comparison between fledglings and adults

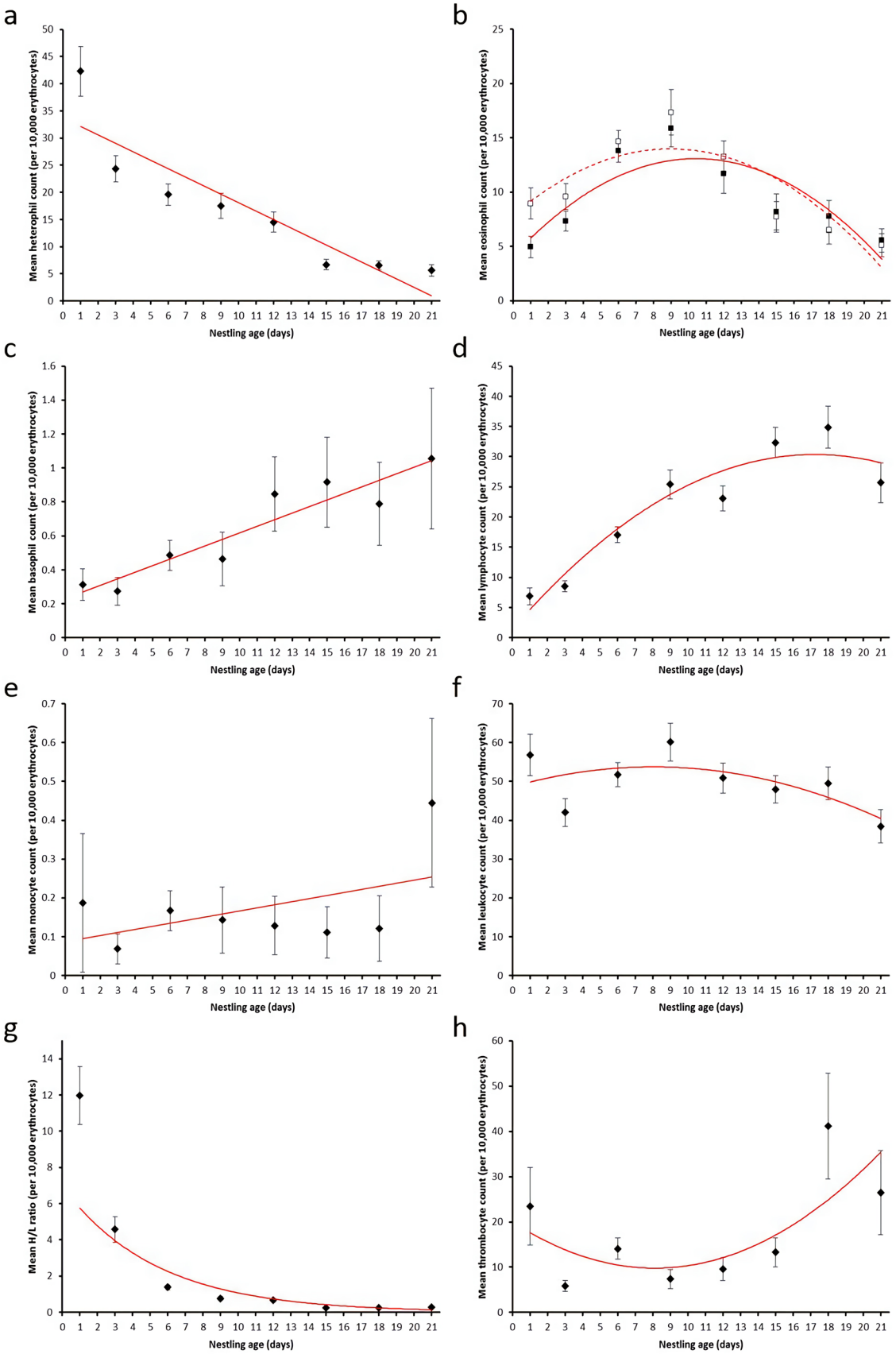
When comparing the cellular counts of fledglings vs adults in order to explore the degree of maturity of the immune function of the former, we found that adults had higher levels of total leukocytes than fledglings. This pattern could result from the differences in heterophils and monocytes (Table 3, Supplementary Fig. 3S-a and 3S-e) between adults and fledglings. However, in the case of eosinophil and lymphocyte counts, the effect of age class showed an interaction with sex (Table 3), showing that adult males had lower levels than the other age-sex combinations (Supplementary Fig. 3S-by 3S-d). For the basophil count, age class also affected interaction with sex (Table 3), but in this case, males maintained intermediate levels irrespective of the age, while female fledglings showed higher basophil levels than adult females (Supplementary Fig. 3S-c). Overall, adults had a higher H/L ratio than fledglings (Table 3). As found before, time of day also had an effect on total WBC count, heterophils, and lymphocytes (Table 3), which showed increasing levels as the day progressed.

Regarding thrombocyte counts, we found similar levels for both sexes ($\chi^2 = 0.22$, $P = 0.633$) and higher counts in adults as compared to fledglings ($\chi^2 = 5.40$, $P = 0.020$, estimate (SE) $= 0.012 \pm 0.005$). We also found a decrease in thrombocyte counts as the day progressed ($\chi^2 = 9.35$, $P = 0.002$, estimate (SE) $= -4.414 \pm 1.442$).

Discussion

In the context of ecoimmunology, the age-specific variation of the immune functions in wild birds has been relatively understudied. However, it is important to understand these variations when evaluating the immune system of nestlings since interpretation errors could arise if early- and late-stage chicks are pooled together, or if comparative analyses are made between adults and chicks of early or late stages. It is known that the immune system develops during ontogeny and thus both relative leukocyte quantifications and H/L ratios are likely to differ between adults and nestlings (Quillfeldt et al. 2008; Palacios et al. 2009). In this regard, our study shows that heterophil numbers decreased significantly along the ontogeny, while the levels of lymphocytes and basophils increased. This age-dependent pattern makes H/L ratios of newly hatched chicks very different from fledgling birds, highlighting a wide range of variation for one of the most used immune activation indicators in field studies on birds (reviewed in Davis et al. 2008). Taking into account this time-dependent effect, we analyse the importance of each cell type based on its relative abundance at specific moments of ontogeny, reducing possible individual variations when considering nestling sex, brood size and day-time (e.g.Chin et al. 2005; Markowska et al. 2017).

Leukocytes are central in effecting innate and acquired immunocompetence in birds. A number of haematological tests, among them differential in leukocyte and thrombocyte profiles, can be used as indirect methods to assess the


a
Mean heterophil count (per 10,000 erythrocytes)
Nestling age (days)
b
Mean eosinophil count (per 10,000 erythrocytes)
Nestling age (days)
c
Mean basophil count (per 10,000 erythrocytes)
Nestling age (days)
d
Mean lymphocyte count (per 10,000 erythrocytes)
Nestling age (days)
e
Mean monocyte count (per 10,000 erythrocytes)
Nestling age (days)
f
Mean leukocyte count (per 10,000 erythrocytes)
Nestling age (days)
g
Mean H/L ratio (per 10,000 erythrocytes)
Nestling age (days)
h
Mean thrombocyte count (per 10,000 erythrocytes)
Nestling age (days)

◂**Fig. 2** Variation of heterophil (**a**), eosinophil (**b**), basophil (**c**), lymphocyte (**d**), monocyte (**e**), and total leukocyte (**f**) count, as well as a variation of H/L ratio (**g**) and thrombocyte (**h**) count (per 10,000 erythrocytes) throughout the nestling development (age expressed in days). Values represented are means ± SE. For the eosinophil count (**b**), values represented are means ± SE (white squares: female and black squares: males), where continuous line represents the estimate for males and the dotted line for females

level of stress, infection, condition and change in several immune responses in birds (Masello et al. 2009). In fact, many authors have studied cellular immunity through total and differential WBC counts, where total WBC has been interpreted as a signal of increased immunocompetence (Gustafsson et al. 1994). However, most of them focus on a single measure (Lobato et al. 2005; D'Amico et al. 2016; Merrill et al. 2019), dismissing potentially relevant effects of the development phase of the individual. By analysing the variation of the thrombocyte and WBC levels every three days since hatching, our results contribute knowledge to the development of the constitutive cellular immunity.

We found an age-related decrease in heterophil levels, whereas the number of lymphocytes increased as chicks got older. Heterophils were highly represented in new-born chicks. This makes sense as heterophils are an essential part of the first line of defence during the initial stages of pathogen infections (Maxwell and Robertson, 1998). In hatchlings, innate immunity is much more developed than the acquired immunity (reviewed in Alkie et al. 2019; Smits and Baos 2005). After hatching, continued exposure to a high diversity of new pathogens could stimulate the chick immune system (Spencer and Garcia 1995; Killpack and Karasov 2012), leading to an increase in lymphocyte levels. This could explain the opposite trend between heterophils and lymphocytes shown in this study. Therefore, newly hatched chicks have much higher H/L ratios than fledglings, mainly due to increased lymphocytes and decreased heterophils throughout the ontogeny. Heterophilic profiles and increased H/L ratios have been interpreted as a symptom of environmental and physiological stress (e.g. ectoparasites, brood reduction, inflammation, etc.) and infection.

Since variations in stress hormone levels (i.e. glucocorticoids) trigger changes in these leukocyte cells (reviewed in Davis et al. 2008), many studies have used the H/L ratio as a measure of stress in birds (Gross and Siegel 1983). However, we cannot conclude from this that younger chicks have higher stress levels than older ones since both the immune system components and the Hypothalamic-Pituitary-Adrenal (HPA) axis tissues are not yet fully mature (Killpack and Karasov 2012; Torres-Medina et al. 2019, respectively), so that these comparisons studying stress could only be made between nestlings of the same age.

We have found a quadratic effect of age on the number of circulating eosinophils, which is coincident with the pattern reported by Burton and Harrison (1969) in domestic chickens. By contrast, Palacios et al. (2009) have shown that eosinophil levels decreased with age in tree swallow nestlings. Males had a lower eosinophil level than females during the first week and a half of development. During this early critical stage of development, trade-offs between growth and immunity could explain this pattern (Van Der Most et al. 2011), as our results showed that males were larger than females from the early stages of ontogeny. No other cell type was affected by chick sex, which is coincident with previous studies (Wilk et al. 2007). The change in patterns in eosinophil, heterophil, and lymphocyte counts (around 9 days old) coincides with a moment in nestling physiology in which eyes are newly open and feathers start to grow (see Fig. 3). Increased mobility and the protective effect of feathers from this age onwards make chicks less susceptible to common blood-sucking ectoparasites of our population, such as the carnid fly *Carnus hemapterus* (López-Rull et al. 2007) or mosquito vectors of blood parasites (Muriel et al. 2018). It is possible that the observed thrombocyte increase, especially high towards the last phase of the nestling ontogeny, is also due to the increase of nest-dwelling ectoparasites and blood-sucking flies, which produce small wounds when feeding on the chicks. This age-related increase in thrombocyte levels is in line with the findings made by Fairbrother and O'Loughlin (1990), who showed that thrombocyte numbers increased from 5 days of age to a peak at 18 days of age. The changes in the composition of WBC could also be due to age-related changes in the microbiota (which changes around day 9 in house sparrows, Kohl et al. 2019) or be a consequence of the catabolism of maternal antibodies that usually disappear from the offspring within 5–14 days old (Staszewski et al. 2007).

Our results also showed that basophil levels increased throughout the ontogeny. This pattern is opposed to data from Burton and Harrison (1969), whereas Palacios et al. (2009) did not find any age-dependent effect on this granulocyte cell. Basophil count could be used as a good biomarker of condition and health (Vinkler et al. 2010). They are one of the main cells of innate immunity (Martin et al. 2006), so we would expect that basophils would be much more represented in the early stages of ontogeny than in the later ones (Bar-Shira and Friedman 2006). However, the age-related increase that we have found suggests that, since they are not precocial species (Burton and Harrison 1969), starling chicks are more exposed to ectoparasites such as *C. hemapterus*, whose abundance increases along with chick growth (Liker et al. 2001). This result would be consistent with an experimental study of relatively higher basophil levels on cliff swallows nestlings exposed to ectoparasites (Chapman and George 1991).

All granulocyte cells showed an increase with the time of day at which blood samples were taken. However, these

Table 1 Summary of final generalized linear mixed models for repeated measures analysis showing the effect of age (days) on total and differential nestling leukocyte counts, taking into account sex, time and brood size. In all cases $df = 1$

Dependent variable	Fixed terms	Estimate ± SE	χ^2	P
Total leukocyte count	Age	0.031 ± 0.019	2.58	0.107
	Age^2	− 0.001 ± 0.001	4.34	0.037
	Time	0.714 ± 0.270	6.97	0.008
Heterophils	Age	− 0.110 ± 0.009	126.03	< 0.001
	Time	1.258 ± 0.444	8.01	0.004
Eosinophils	Age	0.206 ± 0.028	50.63	< 0.001
	Age^2	− 0.009 ± 0.001	61.80	< 0.001
	Sex	0.345 ± 0.144	5.69	0.017
	Age × Sex	− 0.028 ± 0.014	4.14	0.041
	Time	0.848 ± 0.390	4.73	0.029
Basophils	Age	0.060 ± 0.022	7.15	0.007
	Time	2.311 ± 1.036	4.97	0.025
Lymphocytes	Age	0.123 ± 0.046	7.10	0.007
	Age^2	− 0.005 ± 0.001	19.72	< 0.001
	Brood size	− 0.272 ± 0.101	7.22	0.007
	Brood size × Age	0.021 ± 0.008	6.32	0.011
Monocytes	Age	− 0.525 ± 0.219	5.73	0.016
	Brood size	− 1.743 ± 0.739	5.55	0.018
	Brood size × Age	0.169 ± 0.064	7.00	0.008
H/L ratio	Age	− 0.427 ± 0.037	127.6	< 0.001
	Age^2	0.012 ± 0.002	36.61	< 0.001
	Brood size	0.156 ± 0.082	3.60	0.057
	Time	1.740 ± 0.522	11.11	< 0.001

Table 2 Initial generalized linear models of body weight effects on differential leukocyte and thrombocyte counts in 6-day-old nestlings

Dependent variable	Body weight effect		
	Estimate ± SE	χ^2	P
Thrombocytes	− 0.016 ± 0.004	11.7	**< 0.001**
Heterophils	− 0.015 ± 0.004	13.4	**< 0.001**
Eosinophils	0.006 ± 0.004	1.50	0.221
Basophils	0.051 ± 0.029	3.23	0.072
Lymphocytes	− 0.011 ± 0.004	6.78	**0.010**
Monocytes	0.022 ± 0.048	0.22	0.638
H/L ratio	− 0.043 ± 0.016	6.58	**0.010**
Total leukocyte count	− 0.007 ± 0.002	8.25	**0.004**

P values considered significant ($P < 0.05$) are in bold. In all cases $df = 1$

effects were not observed in agranulocyte cells. This diel variation led to an increase in the number of both total leukocytes and heterophils, together with the corresponding rise in H/L ratio. This daily rhythm is supported by previous studies reporting cyclic changes in different components of the immune system (e.g. Markowska et al. 2017; Stinson et al. 1980), which could be mediated by melatonin secretion patterns (Siopes and Underwood 2008). These dynamics could be an adaptive response to a circadian rhythm of parasites (Martin et al. 2001; Navarro et al. 2003; Villanúa et al. 2006) or could be explained by a cumulative effect of sibling competition during the day (Martínez-Padilla 2006). Whatever the cause of this diel variation, our results indicate that sampling hour should be recorded and controlled for in future studies on performing blood cell counts in wild birds.

When comparing adults (older than 1-year-old) with fledglings, we found higher thrombocyte and total leukocyte counts in adults. Although thrombocytes are the most abundant cell (excluding erythrocytes) found in the blood of chickens (St. Paul et al. 2012), their counts are rarely given in the literature because they tend to clump (Campbell and Ellis 2007). However, in addition to their hemostatic effects intervening in blood coagulation, thrombocytes also play a potential role in innate immunity and inflammatory response (Wigley et al. 1999; St. Paul et al. 2012). Previous studies comparing adults vs. young thrombocyte levels have reported contrasting results (Alonso et al. 1991; Howlett et al. 2002; Martin et al. 2006). The fact that the levels of thrombocytes, heterophils, and monocytes are higher in adults than in fledglings in the spotless starling suggests that phagocytic components may require further maturation post-fledgling, as other functions of the immune system do (Killpack and Karasov 2012; Stambaugh et al. 2011).

Regarding the sex-dependent effects on immunity, our data suggest that some components of the cellular immune

Table 3 Summary of final generalized linear mixed models showing the effect of age class (fledglings vs. adults) on total and differential nestling leukocyte counts, taking into account sex and time of the day. In all cases $df = 1$

Dependent variable	Fixed terms	Estimate ± SE	χ^2	P
Total leukocyte count	Age (adults)	0.552 ± 0.196	7.91	0.004
	Sex (females)	0.268 ± 0.131	4.14	0.041
	Time	1.837 ± 0.671	7.49	0.006
Heterophils	Age (adults)	0.023 ± 0.004	32.18	< 0.001
	Time	2.916 ± 1.110	6.90	0.008
Eosinophils	Age (adults)	− 0.025 ± 0.009	7.88	0.004
	Sex (females)	− 0.471 ± 0.395	1.42	0.232
	Age (adults) × Sex (females)	0.012 ± 0.005	5.43	0.019
Basophils	Age (adults)	0.076 ± 0.819	0.008	0.926
	Sex (females)	0.984 ± 0.779	1.59	0.206
	Age (adults) × Sex (females)	− 2.812 ± 1.426	3.88	0.048
Lymphocytes	Age (adults)	− 0.341 ± 0.292	1.35	0.243
	Sex (females)	0.185 ± 0.227	0.66	0.414
	Age (adults) × Sex (females)	0.590 ± 0.313	3.55	0.059
	Time	1.487 ± 0.780	3.63	0.056
Monocytes	Age (adults)	0.024 ± 0.005	22.4	< 0.001
	Sex (females)	0.611 ± 0.296	4.25	0.039
H/L ratio	Age (adults)	0.021 ± 0.006	7.16	0.007

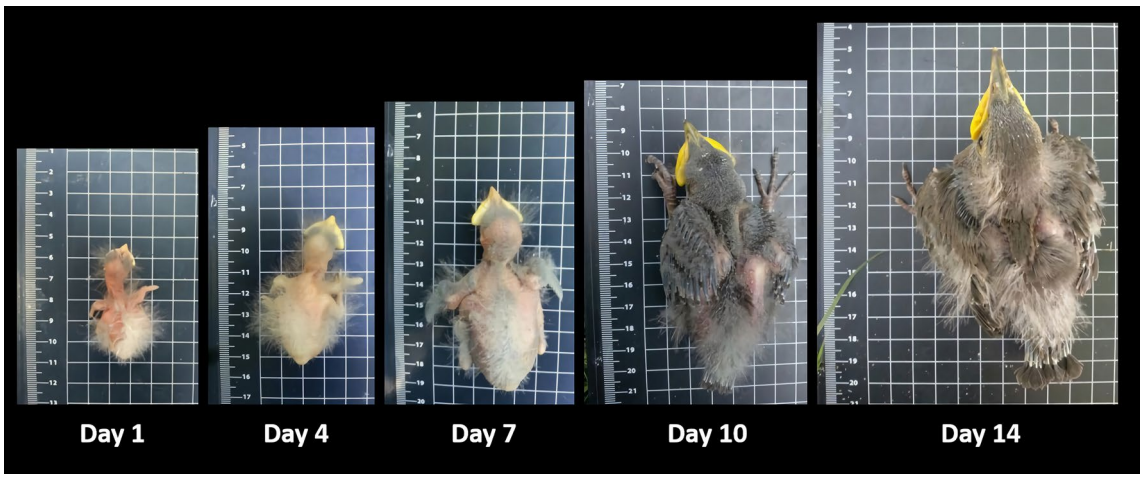


Fig. 3 Different nestling growth stages during the first two weeks of ontogeny. Picture credit: Jaime Muriel

system of males could be less represented than that of females, as previously suggested in our study species (Muriel et al. 2017). In accordance with our results, Tschirren et al. (2003) found a pronounced sexual dimorphism in the cell-mediated immune response with males showing a reduced cellular immunity than females. In fact, according to the immunocompetence handicap hypothesis (Folstad and Karter 1992), a previous study carried out in our starling population showed that the phagocytic function in males was strongly inhibited under moderate physiological concentrations of testosterone (Gil and Culver 2011). This could also explain the differences found between adult and young males, highlighting that the immunological costs derived from the trade-offs between reproduction and immunity may be greater than those between growth and immunity (Lochmiller and Deerenberg 2000).

Conclusion

In conclusion, our study of free-living spotless starlings provides compelling evidence that cellular constitutive immunity throughout early development is associated with key biological factors such as age, sex or degree of sibling competition. In addition, we show that bodyweight is negatively associated with H/L ratio at day 6 post-hatching

when nestlings are in the phase of linear growth. Taking into account that age is essential when evaluating immunity by cell counts, our findings partially support the prediction that early-stage nestlings rely more on cells of the innate immune system rather than on cells linked to the adaptive immune system, as previously shown in other studies (reviewed in Alkie et al. 2019). However, although we have found enormous variability in cell counts throughout ontogeny, it seems that the cellular immune system is still not fully mature days before leaving the nest since we have found differences in phagocytic cell counts between fledglings and adults. Future studies could test whether the variability reflected in our data about the development of the immune system could depend on environmental factors, such as food availability, climatic influences or pressures of endo- and ectoparasites. Since adults can have age-dependent reproductive strategies (Rebke et al. 2010; Oro et al. 2014; Muriel et al. 2019), it would also be interesting to include different classes of adulthood (from one year onwards) to have a wider perspective of the development of the cellular constitutive immunity in wild animal populations.

Acknowledgements We are very grateful to all the volunteers who helped to collect data over the completely breeding season. We also thank one anonymous journal reviewer and Associate Editor for their valuable assistance on the earlier draft of the article. This study is a contribution to the research developed at the "El Ventorrillo" field station.

Author contributions LP-R and DG devised the project and the main conceptual ideas. JM and LP-R carried out fieldwork. JM performed the cell counts. JM and CV analysed and interpreted the data. JM took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Funding The project was funded by projects CGL2011-26318 to DG (Ministerio de Economía y Competitividad) and PGC2018-099596-B-I00 to LP-R (Ministerio de Ciencia, Innovación y Universidades, co-financed by the European Regional Development Fund-ERDF). JM was supported by a postdoctoral grant from the Juan de la Cierva Subprogram (FJCI-2017-34109), with the financial sponsorship of the MICINN. CV was supported by a contract from the program "Contratos Asociados a Proyectos de Investigación", Universidad de Alcalá, Madrid, Spain. LP-R was supported by a postdoctoral contract for accessing the Spanish System of Science, Technology and Innovation (SECTI) from the University of Castilla-La Mancha.

Availability of data and materials All data underlying the findings will be hosted on the Spanish National Research Council (CSIC) digital repository.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Capture and manipulation of birds were authorized by the Consejería de Medio Ambiente (Comunidad de Madrid, Spain) under licence from the Spanish institutional authorities (Consejería de Medio Ambiente and Centro de Migración de Aves de SEO/BirdLife).

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