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Article

Antimicrobial capacity is related to body colouration and reproductive success in female spotless starlings

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Pathogenic microorganisms select for a plethora of defensive mechanisms on their hosts. In males of some species, flashy traits might signal antimicrobial capacity and, thus, they might be favoured in scenarios of sexual selection. Antimicrobial capacity of individuals may predict reproductive success in males, and it could be adapted to changing environments. However, evidence for these associations is still scarce in females. Here, we evaluated antimicrobial capacity of spotless starling *Sturnus unicolor* females during the mating and nestling–provisioning phase. We did this by measuring 1) the blood plasma inhibition capacity against 12 bacterial strains (antagonistic index), 2) the constitutive innate humoral immunity (lysis and agglutination capacity, a non-specific first barrier of protection of hosts against microbial parasites) and 3) the uropygial gland size and volume of secretion produced, relevant traits in the protection against, among others, feather-degrading bacteria. We also measured colouration of throat and back feathers, and of leg and beak integuments. This information was collected during the pre-laying and nestling stages to compare values from these two periods. We found an increase in the plasma antagonistic index from the pre-laying to the nestling period, while a decrease on the plasma capacity of agglutination of foreign antigens. Both plasma antagonistic index and humoral immune response measured before breeding were positively related to future female reproductive success. In addition, the level of antimicrobial capacity was related to colouration of leg skin and beak integuments and of back feathers. These associations suggest that, similar to males, females might show through their physical appearance their capacity to fight microbial infections; information that could be evaluated by their potential partners and by female competitors.

Keywords: animal communication, antimicrobial capacity, antimicrobial defences, innate humoral immunity, sexual selection in females, *Sturnus unicolor*

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Introduction

Bacteria are regarded as important drivers of selection in shaping hosts' life history decisions, as evidenced by the steady increase of studies on animal–microorganism interactions in recent years (McFall-Ngai et al. 2013, Rosenberg and Zilber-Rosenberg 2016, Douglas 2018). Amongst all microorganisms, there are numerous pathogenic strains that may cause illness and even host mortality (Gyles et al. 2010). Therefore, animals have developed a wide array of defensive mechanisms to prevent bacterial infections, or to reduce their detrimental effects. The complex immune system is the main antimicrobial barrier (Schmid-Hempel 2003). Specifically, constitutive innate immunity provides a first-line of protection against invading microbes, through different branches that include natural antibodies and complement (Thornton et al. 1994, Carroll and Prodeus 1998, Ochsenbein and Zinkernagel 2000, Matson et al. 2005).

Another defensive line that contributes to the prevention of bacterial infections in animals are exocrine glands, of which a prominent example is the uropygial gland of birds (Jacob and Ziswiler 1982, Shawkey et al. 2003). The uropygium is a holocrine complex situated at the base of the tail. Its relative size varies greatly among species and is associated with their life history traits, probability of infection and production of natural antibodies (Haribal et al. 2005, Vincze et al. 2013). This gland's sebaceous and hydrophobic secretion is mainly composed of aliphatic alcohols, fatty acids and glycerides, some of these chemicals having antibiotic properties (Jacob and Ziswiler 1982, Martín-Vivaldi et al. 2010, Moreno-Rueda 2017). Because of the presence of antibiotic compounds in the secretion (Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2009, 2013, Martín-Vivaldi et al. 2010), the gland is directly implicated in bacterial load regulation of the eggshell (Soler et al. 2012) and feathers (Shawkey et al. 2003, Møller et al. 2009). Indeed, the amount of secretion produced is negatively related to the plumage bacterial load in spotless starlings *Sturnus unicolor* (Ruiz-Rodríguez et al. 2015). Therefore, by considering together estimates on immune response and uropygial gland measurements, we may properly characterize the antimicrobial capacity of individual birds and, thus, explore the possibility that birds signal this defensive capacity, which has been hitherto almost ignored.

Because antimicrobial defences are costly to produce (Habig and Archie 2015), their expression may depend on the parasite context. Indeed, animals modulate their investment on antiparasitic defences according to environmental factors that might reflect probability of infections (Capilla-Lasheras et al. 2017, Ruiz-Rodriguez et al. 2017). Abiotic conditions, such as temperature and humidity, typically influence microbial communities of their avian hosts (Cook et al. 2005a, Peralta-Sánchez et al. 2012) and, hence, are partially responsible for the phenological variation of microbial communities even within the same locality (Bisson et al. 2007). Therefore, individuals should adjust their potential defensive capacities to abiotic and biotic changes that influence probability of bacterial infection. However, immunological responses are expected to be traded-off against others costly activities such as reproduction (Harshman and Zera 2007). In accordance with temporal and/or local variation in immunocompetence, magpie *Pica pica* nestlings from delayed clutches showed lower immune response (Sorci et al. 1997), while those of barn swallows *Hirundo rustica* showed the opposite pattern, likely as a result of adjusting immune response to probability of pathogenic infections (Merino et al. 2000). Moreover, assuming that only animals in prime condition can mount strong immune responses, higher antimicrobial capacities should only be evident in individuals in better condition and overall phenotypic quality (Schmid-Hempel 2003). Such individuals may also experience lower probability of pathogen transmission to offspring, thereby reducing negative effects on embryo (Cook et al. 2005b) and nestling development (Azcárate-García et al. 2019). Parent birds might transmit pathogenic microorganisms to their offspring while incubating, brooding or feeding nestlings. Nevertheless, evidence for the link between microbial defences of birds and reproductive success is still scarce.

Communicating antimicrobial ability and phenotypic condition to conspecifics is important because of its potential to reduce the strength of agonistic interactions among conspecifics, influence mate choice or select for differential investment in reproduction of partners in scenarios of intra- and inter-sexual selection (Andersson 1994, Westneat 2012). Research on the evolution of traits indicating individual quality (including antiparasitic defences) through sexual selection has focused mostly on males (Andersson and Simmons 2006). Specifically in birds, males are typically the extravagantly plumaged and singing sex (Dale et al. 2015, Romano et al. 2017). Although conspicuous female ornamentation was first explained as a consequence of genetic correlation with males (Bonduriansky and Chenoweth 2009, Dale et al. 2015), it is now accepted that elaborate female traits might evolve independently of those of males (de Neve et al. 2007, Soler and Moreno 2012, Price 2015, Webb et al. 2016). Selection pressures acting on females, mainly during mating and reproduction, may differ from those acting on males because of sexual differences in hormone profiles and reproductive duties. Consequently, information provided by equivalent sexual traits in males and females might also differ sexually (Tobias et al. 2012). Therefore, to develop a comprehensive view of social and sexual selection, which conventionally is focused only in male ornamentation, research on female signalling is needed (Clutton-Brock 2007, 2009, Doutrelant et al. 2020), and specifically, studies exploring whether female ornaments may signal antimicrobial capacity.

Female plumage colouration has been shown to indicate status in social interactions (Murphy et al. 2009a, b, Tobias et al. 2012), and to play a role in sexual selection (Amundsen et al. 1997, Soler et al. 2019). Female ornaments, such as the carotenoid-based colouration of blue tits *Cyanistes caeruleus*, may lead to male preferences for more

pronounced partners (Clutton-Brock 2007) because female blue tit colouration is positively associated with reproductive success (clutch size, fledgling success and recruitment), while their UV-blue colouration is positively correlated to survival and laying date (Doutrelant et al. 2008, 2020). Moreover, ornamental plumage of females is also related to immunocompetence and antiparasitic defences (Amundsen 2000, Roulin et al. 2001, Morales et al. 2007). Similarly, some other ornamental structures, as flamboyantly coloured unfeathered bare parts of the body, also contain information about female quality. Well-known examples are the blue foot colouration of blue-footed boobies *Sula nebouxii* (Torres and Velando 2005), or the carotenoids-based beak colouration of mallard ducks *Anas platyrhynchos* (Butler and McGraw 2011) and zebra finches *Taeniopygia guttata* (Alonso-Alvarez et al. 2004). However, specific studies on the relationship between colouration (of both feathers and integuments) and antimicrobial capacity are needed if we are to have a complete view on the evolution of signals of individual quality.

In the present work, we aimed to explore how antimicrobial capacity in spotless starling females is related to colouration of feather and integument structures, and to reproductive success. Spotless starling females show dark, almost black, plumage. During the non-reproductive period, the beak and legs of spotless starlings are also black, but some weeks before reproduction the legs turn a conspicuous red colour in both sexes, the beak base turns blue in males and pink in females, and the beak tip changes from black to yellow in both sexes (Cramp 1998). Interestingly, during the pre-laying period, the beak tip colour intensity reflects the plasma concentration of carotenoids and vitamin A in both sexes (Navarro et al. 2010) and, thus, it could also reflect immune capacity. As traits that could reflect female quality, we measured the colour of different body parts, including throat and back feathers, legs and beak (tip and base). The reproductive parameters that we measured as indicative of female quality were clutch and brood size, as well as hatching and fledging success. As a proxy of antimicrobial capacity, we measured the direct ability of blood plasma to inhibit bacterial growth by confronting the plasma with different standard bacterial strains in antagonistic assays (antagonistic index). In addition, we evaluated two humoral components of the constitutive innate immunity: natural antibodies and complement. Furthermore, we measured the uropygial gland volume and quantified the amount of preen secretion produced, as both variables are correlated in spotless starlings with the inhibition capacity of the oil and are relevant in the protection against, among others, feather-degrading bacteria (Ruiz-Rodríguez et al. 2015). We paid special attention to possible seasonal changes in the strength of the expected association.

We hypothesized that the antimicrobial capacity of spotless starling females is related to their reproductive success, and that they may show this capacity through their colouration. Particularly, we expected 1) that antimicrobial capacity of females would increase from the pre-laying to the nestling period, 2) a positive relationship between antimicrobial capacity and reproductive success and 3) a positive association between the level of antimicrobial capacity with female colouration in the above-mentioned feather and integument structures.

Material and methods

Study area and species

The field work was carried out in 2015 and 2016, in the Hoya de Guadix (Granada, south-eastern Spain, 37°15′N, 3°01′W), where over 100 nest-boxes (internal height × width × depth: 350 × 180 × 210 mm; hole height: 240 mm) were available for breeding spotless starlings (hereafter starlings) (see Soler et al. 2017, for further information). The breeding season starts in early April, and after the first brood, most individuals lay a second clutch during May–June. Females with sporadic help from males incubate eggs for around 14 d. Nestlings fledge after around 21–22 d (Cramp 1998).

Bird sampling and measurements

Before egg laying started, we carried out two trapping sessions in the study area, both during March and with an interval of two weeks. Briefly, one hour before dawn, we closed the entrance of all nest-boxes in the study area, and immediately after dawn, we captured all individuals found roosting inside. Captured birds were kept in clean cotton bags separately, hanging from a stick to keep birds quiet, and after sampling, they were immediately released (Ruiz-Rodríguez et al. 2015). During the nestling period, 4–5 d after hatching, we did other capture attempts both, in first (at the end of April) and second (around mid-June) broods, using nest-box traps. Thus, four trapping events in total were conducted. These procedures do not result in negative effects on breeding performance of captured birds (Soler et al. 2008).

Captured individuals were identified or ringed with a numbered aluminium ring and three plastic rings with a unique combination of colours. We measured their uropygial gland with a digital caliper (height, width and length, ± 0.01 mm) to estimate its volume following Martín-Vivaldi et al. (2009). The uropygial gland secretion was extracted by placing a sterile micro-capillary tube (32 mm 20 μl^{-1}) at the gland opening, and slightly pressing the gland, which was squeezed until no further secretion could be extracted. The length of the capillary filled with secretion was used as proxy of secretion volume. The uropygial gland measurements were obtained only in individuals captured during the pair formation period. We collected blood samples from the brachial vein by filling two–three heparinized capillary tubes (75 mm 75 μl^{-1}) that were emptied into microfuge tubes and kept in portable refrigerators at 4°C. Within 6 h, tubes were centrifuged at 2000 g for 5 min to separate the plasma (which was preserved at −80°C until analyses) from the blood cellular fraction. Blood samples were collected in every capture.

We measured colouration of the beak tip and base, legs (left tarsus, external part) and body feathers (back and breast, the latter being a sexually selected trait in males) of females by means of an Ocean Optics S2000 spectrometer connected to a deuterium-halogen light (D2-W, Mini). These measures were obtained from all captured individuals, before and during reproduction. To standardize ambient light conditions, we used a black bag that wrapped the tip of the probe containing optical fibres and the female body part measured. Before the measurement of each individual, we calibrated the spectrometer using a standard white and black reference. We obtained reflectance spectra at 1 nm intervals between 300 and 700 nm for all measurements. The colour was measured three times on each part, and then the average calculated. We estimated chromatic colouration as the proportion of total reflectance within the ultraviolet (UV) (λ = 300–400 nm), UV-blue (λ = 300–450 nm), blue (λ = 400–475 nm), blue-green (λ = 450–570 nm) and yellow-red (λ = 570–700 nm) ranges of the tetrachromatic avian visual spectra. Achromatic colouration (i.e. brightness) was estimated as average reflectance value across the entire spectrum (λ = 300–700 nm). Prior to analysis, negative values were set to zero and reflectance curves were corrected for noise by using triangular smoothing (Gómez 2006). The use of spectral data covering its visual range has been previously proven to be appropriate to characterize existing variability of interest in this species (Navarro et al. 2010, Azcárate-García et al. 2020) and, although it may have some sources of error and variation, the process is highly rigorous and accurate (Stevens 2011). Since the six colour variables might be related to each other within each of the five measured body parts (i.e. back, breast, leg, beak base and beak tip), we ran a principal component analysis (PCA) to reduce the number of dependent variables and ensure statistical independence among them. PCA factors were rotated (varimax normalized), and their significance established by cross-validation (McGarigal et al. 2000). Only those PCA factors with eigenvalues higher than 1 were considered to summarize colour variance of each body part measured (Supplementary material Appendix 1 Table A1). All the colour variables were summarized in two PCA factors for every body part except for leg colour, in which only one PCA explained 80% of the variance. In all other body parts, both PCA factors together explained around 85% of the variance (Supplementary material Appendix 1 Table A1). In addition, we calculated the importance of each colour variable in the resultant PCAs, which is based on how well it is represented by the PCA model. This is measured by modelling power, defined as the explained standard deviation (Statistica ver. 12 software, Dell-Inc 2015, Electronic Manual). The power coefficient ranges from 0 to 1, with 1 meaning that the variable is completely relevant, and 0 indicating no relevance at all.

Finally, to calculate reproductive success, all nests were visited every third day from the end of March to detect the beginning of egg laying, and then they were periodically visited to count the total number of eggs until the end of laying. Nest-boxes were again visited 12 d after the 4th egg was laid, and daily afterwards until hatching to document number of eggs hatched; hatching success was the number of hatched eggs divided by the clutch size. Then, nests were again visited day 14 after hatching, to count the number of nestlings and estimate the fledging success as number of nestlings that fledged divided by the number of hatched eggs. We assume that nestlings that reached 14 d of age fledge successfully (Azcárate-García et al. 2020).

The individual identification of females from each nest was performed by capturing them at nest-boxes during the nestling phase (see above), or by identifying the unique combinations of colour rings in 1 h video films of nest-box entrances recorded during the 2nd or 3rd day after hatching.

Laboratory procedures

To estimate antimicrobial capacity in blood plasma, we performed antagonistic and immunological tests. Antagonistic tests were performed against 12 indicator bacteria belonging to different taxonomic groups: *Bacillus licheniformis*, *B. megatherium*, *B. thuringiensis*, *Enterococcus faecalis*, *E. faecium*, *Lactobacilus paracasei*, *L. plantarum*, *Lactococcus lactis*, *Listeria inocua*, *L. monocytogenes*, *Proteus* sp. and *Staphylococcus aureus*. Antagonistic plates were prepared as follows: 15 ml of a culture medium previously prepared and sterilized (1.8% of brain–heart infusion (BHI) and 0.8% agar in 0.1 M, pH = 7 phosphate buffer) was melted and then maintained at 50°C for 10 min. Then, 100 µl of a 12 h culture of each indicator bacteria was added to the medium, vigorously vortexed and spread onto a Petri dish. After solidifying about 30 min later, 2 µl of each plasma sample was deposited on the plates and later incubated for 12 h at 28°C, temperature at which the activity can be detected. After incubation, plates were checked for inhibition halos, that is, transparent zones around the plasma in which the growth of the indicator bacterium was inhibited (Supplementary material Appendix 1 Fig. A1). Halos were measured (in mm) from the limit of the plasma drop to the end of the halo (i.e. where growth of the indicator bacteria begins) (for more details see Ruiz-Rodríguez et al. 2012). Antagonistic assays against each bacterium species were made to all the samples in the same randomly selected order. Then, an index of antagonistic activity was calculated as the average activity intensity (i.e. halo radius) against the twelve indicator bacteria tested. For some of the samples, there was insufficient plasma for testing all indicator bacteria, and in those cases, the index was calculated from the available number of tested bacteria. We performed antagonistic tests to 124 plasma samples from 77 females from both study years.

The immune responses mediated by natural antibodies (NAb) and complement were estimated by evaluating lysis and agglutination capacity of female plasma following the procedure described in Matson et al. (2005). The NAbs serve as recognition molecules capable of opsonizing invading microorganisms and initiating the complement enzyme cascade, which ends in cell lysis. On the other hand, levels of

complement are positively correlated with NAb diversity, and the interaction between NAbs and complement is an important link between innate and adaptive immune responses (Matson et al. 2005).

Briefly, 50 µl of plasma were serially diluted in sodium phosphate buffer (PBS) in two consecutive polystyrene 96-well assay plates where 25 µl of 1% rabbit blood cell suspension (Hemostat laboratories, Dixon, CA 95620, USA) in PBS was added. Quantification of lysis and agglutination titres was assessed as the number of titres with the last plasma dilution at which the lysis or agglutination reaction of rabbit blood was observed (Matson et al. 2005) (Supplementary material Appendix 1 Fig. A2). These values were used as indicators of immunocompetence. Agglutination and lysis titre values were not correlated in our samples ($F_{1,76} = 0.06$, $R^2 = 0.0009$, $p = 0.79$, estimate (SE) = 1.61 (0.18)). We analysed 78 plasma samples belonging to 54 females, only from 2015.

Statistical analyses

All analyses were performed with Statistica 12 software (Dell-Inc. 2015). To explore variation in the plasma antimicrobial capacity across the breeding season, we used only the information for females that were captured twice during the same breeding season (Table 1). All females were first captured before egg laying, while the second capture of the same females occurred either before egg laying (two weeks after the first capture), or during reproduction (a few days after hatching of first or second clutches). Thus, to explore differences in immune response between captures, we used Repeated Measures ANOVAs (RMA) with the two subsequent measures of antimicrobial capacity (antagonistic index, agglutination and lysis) as the within factor. The breeding status of females in the second captures (i.e. before egg laying (n = 4), during first (n = 7) or second (n = 9) clutch) was the between factor.

In addition, to explore the variation in the antimicrobial capacity of all captured individuals (i.e. the population average) among different captures, we performed general linear mixed models (GLMM). We considered the antagonistic index, the lysis titre or agglutination titre as dependent variables, while the period of capture (i.e. pre-laying, first clutch or second clutch) and the year (in the case of the antagonistic index) were the fixed categorical predictors, and the individual was the random predictor.

To explore the association between antimicrobial capacity and reproductive success, we performed a GLMM in which the dependent variable was each proxy of reproductive success: clutch size, brood size, hatching success and fledging success. Values of antimicrobial capacity were introduced as continuous predictors (plasma antagonistic index (n = 74), agglutination and lysis capacity (n = 51), uropygial gland volume and uropygial secretion quantity (n = 75)), and study year as the fixed factor (except for analyses on immune responses which were obtained for a single study year). Individual identity was introduced as the random factor. All analyses were separately performed for first and second clutches.

Finally, to explore whether antimicrobial capacity of females was reflected by their colouration, we performed GLMMs in which the dependent variables were the PCA scores on factor 1 that summarized colour scores. As explanatory variables we used the plasma antagonistic index and immune response variables (lysis and agglutination titres), uropygial gland size and uropygial secretion volume as continuous predictors. The effects of each of these continuous predictors on different colour variables were tested in separate models that also included study year and breeding status (i.e. before breeding, first or second clutch) as fixed categorical factors, and individual identity as the random factor.

Since different GLMMs were implemented for each dependent variable, the false-discovery-rate (FDR) correction was applied to establish the appropriate Q values (Pike 2011).

Table 1. Number of female spotless starlings captured before laying started, and during the nestling period of the first or second clutch, every year (2015 and 2016). Total number of females captured in the two years is also reported (note that some individuals were captured at different times and so total number of females are not straight sums of row or column numbers). Number of females captured twice in the same year (before laying (BL1), and then again before laying (BL2) or with nestlings, in the first (C1) or second (C2) clutch), as well as the total number of different individual females captured in these two times, are also included.

		Number of females captured			
		2015	2016	Both years	Total
Before laying		44	31	14	61
First clutch		15	14	1	28
Second clutch		20	6	2	24
Total females		55	47	22	**80**
BL1 and	BL2	5	0		5
	C1	5	1		6
	C2	9	0		9

Results

Changes in immune capacities across the breeding season

The antagonistic index of the female blood plasma increases from first to second captures of the same individual during the breeding season (RMA, $F_{1,17} = 11.75$, $p = 0.003$, Fig. 1A). Interestingly, these between-captures differences did not depend on the breeding status of the second capture (i.e. the interaction between repeated measures and the breeding status of second time captured females; RMA, $F_{2,17} = 1.80$, $p = 0.19$). The detected increase in antagonistic index from first to second capture came from comparison of females that were first captured before egg laying and again during first (post-hoc LSD test, $p = 0.04$) or second ($p = 0.001$) clutches. However, no differences were detected in those females captured twice before laying started ($p = 0.65$) (Fig. 1A). Therefore, the

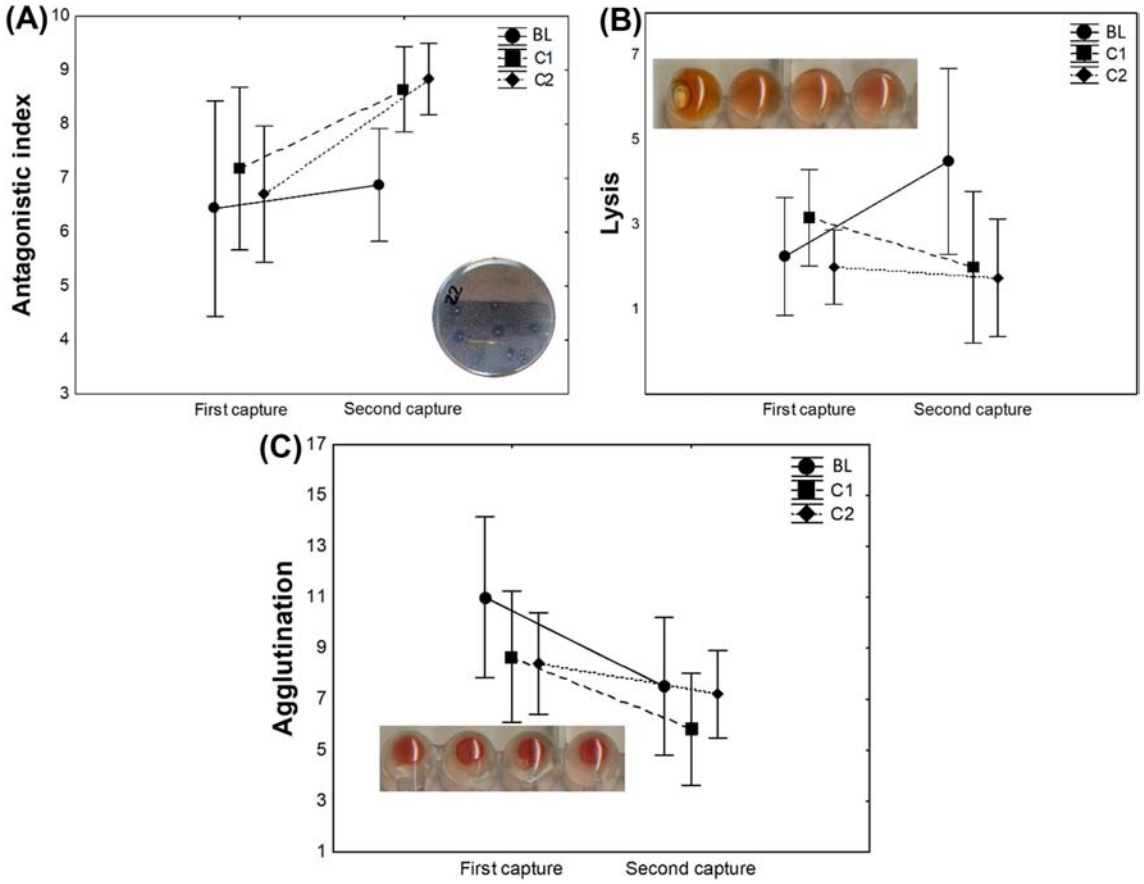

Figure 1. Differences in the plasma (A) antagonistic index, (B) lysis and (C) agglutination variables, in female spotless starlings captured twice, when the second capture was performed before laying started (BL), or with nestlings from the first (C1) or second clutch (C2). Vertical bars denote 0.95 confidence intervals.

antagonistic index of female plasma was significantly higher during the nestling period than before laying started.

GLMM results with all the captured individuals showed similar results, i.e. the population antagonistic index increased from before laying started to the nestling period ($F_{2,44} = 8.73$, $p < 0.001$, Supplementary material Appendix 1 Fig. A3). There were no differences between the nestling periods of first and second clutches (post-hoc LSD test, $p = 0.14$), while there were differences among the pre-laying and both nestling periods ($p < 0.017$ in both cases). We also detected a significant effect of year ($F_{1,44} = 87.64$, $p < 0.001$), but not of individual ($F_{76,44} = 1.10$, $p = 0.36$).

Regarding the immune traits, the lysis capacity of female plasma did not change from first to second captures (RMA, $F_{1,16} = 0.005$, $p = 0.94$, Fig. 1B), independently of whether females were captured before egg laying or with nestlings during the second capture, although there is a non-significant trend (RMA, $F_{2,16} = 3.20$, $p = 0.067$). Finally, the agglutination capacity of female plasma decreased from first to second captures (RMA, $F_{1,16} = 5.47$, $p = 0.032$, Fig. 1C), independently of the reproductive stage at second captures (RMA, $F_{2,16} = 0.66$, $p = 0.52$).

When considering the entire population, we found no effect of capture period or individual in any of the innate immune responses (Lysis: period, $F_{2,22} = 1.27$, $p = 0.30$; individual, $F_{2,22} = 0.91$, $p = 0.61$. Agglutination: period, $F_{2,22} = 1.92$, $p = 0.17$; individual, $F_{2,22} = 1.09$, $p = 0.42$). However, agglutination titres decreased from before egg laying to both nestling periods, which is in agreement with the results obtained for variation within individuals (Supplementary material Appendix 1 Fig. A3).

Immune capacities and reproductive success

The antagonistic index of female blood plasma before laying started was positively related to fledging success of second clutches even after correcting for the significant effect of study year (Table 2). Clutch size of second broods was marginally associated positively with the lysis capacity of female plasma during the mating period, while females with higher agglutination capacity showed higher hatching success in first clutches (Table 2). All the significant relationships were positive, suggesting that females with higher antimicrobial capacity at the time of mating also experienced higher breeding success. However, antimicrobial capacity of females captured during the nestling period of first or second broods did not predict any of the variables related to breeding success (results not shown, all $Q > 0.28$). Finally, after applying the FDR

Table 2. Results of GLMM analyses on the association between variables describing the antimicrobial capacity of female spotless starlings during the pre-laying period and indicators of reproductive success. Statistically significant results after the false-discovery rate correction (Q) are in bold, and marginally-one-tailed significant result are in italics. β are the regression coefficients.

		First clutch						Second clutch					
Dependent variable	Predictor	F	df	p	β	SE (β)	Q	F	df	p	β	SE (β)	Q
Clutch size	Antagonistic index	0.11	1,18	0.74	−0.16	0.5	0.74	0.33	1,10	0.57	0.55	0.96	0.66
	Year	0.16	1,18	0.69	−0.2	0.5	0.75	0.33	1,10	0.57	0.56	0.96	0.57
Hatching success	Antagonistic index	0.33	1,17	0.57	−0.29	0.5	0.74	0.21	1,9	0.66	0.46	1.02	0.66
	Year	0.09	1,17	0.75	−0.16	0.5	0.75	0.34	1,9	0.57	0.6	1.02	0.57
Fledging success	Antagonistic index	1.09	1,15	0.31	−0.56	0.53	0.62	14.5	1,9	0.004	2.11	0.55	**0.016**
	Year	2.15	1,15	0.16	−0.73	0.53	0.32	20.59	1,9	0.001	2.52	0.55	**0.004**
Clutch size	Lysis	0.89	1,11	0.36	0.28	0.29	0.48	10.33	1,5	0.02	0.82	0.25	*0.08*
Hatching success		1.22	1,10	0.29	−0.33	0.29	0.48	1.02	1.5	0.35	−0.41	0.4	0.46
Fledging success		0.04	1,8	0.84	−0.07	0.35	0.84	0.33	1,5	0.58	−0.25	0.43	0.58
Clutch size	Agglutination	1.57	1,11	0.23	−0.35	0.28	0.36	0.1	1,5	0.76	−0.14	0.44	1
Hatching success		24.97	1,10	0.0005	0.84	0.17	**0.002**	1.46	1,5	0.28	0.47	0.39	1
Fledging success		1.41	1,8	0.27	−0.38	0.32	0.36	0.31	1,5	0.6	−0.24	0.43	1

correction, none of the measures of reproductive success were associated with uropygial gland size or the amount of preen secretion (results not shown, all $Q > 0.16$).

Antimicrobial capacity and colouration

Some of the variables describing level of antimicrobial capacity of starling females were related to colourations of different body parts (Table 3). Preen oil volume was positively associated to the PCA1 describing back colouration (mainly related positively to chroma and blue UV, and negatively to yellow-red intensity, see Supplementary material Appendix 1 Table A1). Also, the uropygial gland size was negatively associated to the PCA1 of the base of the beak colouration (i.e. those females with larger glands had a higher yellow-red intensity, and lower UV blue and blue-green colouration in the beak base, see Supplementary material Appendix 1 Table A1). In this case, there was also a significant effect of study year (Table 3 and Supplementary material Appendix 1 Table A2), indicating that in 2016 the yellow-red intensity of the beak base in the population was higher than in 2015. Moreover, the plasma agglutination capacity was positively associated with the PCA1 describing leg colouration (Fig. 2), i.e. it was negatively related to the yellow-red colouration but positively to the rest of the colours, including brightness (Table 3 and Supplementary material Appendix 1 Table A1, A3). In addition, leg colouration was also negatively associated with the breeding status of the captured females (Table 3 and Supplementary material Appendix 1 Table A3), which means that, when the season progresses, the yellow-red leg colouration increases, but other chromatic variables decreases. Finally, no significant relationships were detected between the antagonistic index and the measured colour variables (Supplementary material Appendix 1 Table A4).

Discussion

Our main results indicate that antimicrobial capacity of spotless starling females vary from pre-laying to nestling periods, and the level of antimicrobial capacity before laying is related to reproductive success and colouration of body feathers, beak base or legs. These results therefore suggest an association between antimicrobial capacities of females and their reproductive success. Although different colour measurements had different relationships with the antimicrobial traits, males could potentially use female colouration to infer future female reproductive potential by having a set of different signals.

Females were captured first during the pre-laying period, and most recaptures took place when nestlings were 4–5 d old, either in the first or second broods. Agglutination capacity of females' plasma significantly decreased from first to second captures. These differences in immune response may be due to variation in abiotic (e.g. temperature, humidity) and biotic (e.g. female condition, microbial communities) factors along the nesting period that also determine variation in bacterial

Table 3. Significant results of GLMMs on the associations between the antimicrobial capacity and the body colouration of spotless starling females. Statistically significant results after the false-discovery rate correction (Q) are in bold. Non-significant results between the variables of interest are shown in Supplementary material Appendix 1 Table A2–A4. β are the regression coefficients.

Dependent variable	Predictor	F	df	p	β	SE (β)	Q
Back colouration (PCA1)	Preen oil volume	11.23	1,20	<0.001	1.19	0.36	**0.04**
	Uropygial gland size	5.87	1,20	0.03	0.37	0.15	0.13
	Year	7.82	1,20	0.01	0.56	0.20	0.09
	Individual	1.59	74,20	0.12	−0.08	0.14	0.31
Beak base colouration (PCA1)	Secretion volume	0.93	1,20	0.35	−0.25	0.26	0.62
	Uropygial gland size	30.83	1,20	<0.001	−0.63	0.11	**<0.001**
	Year	18.13	1,20	<0.001	−0.63	0.15	**<0.001**
	Individual	2.48	74,20	0.01	0.09	0.10	0.07
Leg colouration (PCA1)	Lysis	3.25	1,15	0.09	0.22	0.12	0.29
	Agglutination	11.45	1,15	<0.01	0.52	0.15	**<0.01**
	Breeding status	19.40	2,15	<0.001	−0.78	0.13	**<0.001**
	Individual	3.08	50,15	0.01	−0.29	0.11	0.07

environment. For instance, we know that, as the season progresses, temperature typically increases and relative humidity decreases in our population, and that these factors influence risk of hatching failure due to changing nest bacterial environment (Cook et al. 2005b, Peralta-Sánchez et al. 2012) as it has been detected in magpies (Soler et al. 2015). Moreover, breeding activity such as incubation or brooding (Cook et al. 2005b, Peralta-Sánchez et al. 2012, Soler et al. 2015), ectoparasites (Tomás et al. 2018), egg breakage (Soler et al. 2015), or nestling faeces that parents sometimes fail to remove (Ibáñez-Álamo et al. 2014, Azcárate-García et al. 2019), are known to affect bacterial environment of nests. Taken together, this evidence suggests that females experienced higher probability of microbial infection during the nestling period than before laying started. Thus, because immune responses are typically positively associated with risk of infection (Moller and Erritzoe 1996), starling females would increase their immune response during the nestling period under a higher parasite–pressure scenario, a prediction that our results on antagonistic index of plasma fulfilled. In addition to factors associated with climatic conditions, those associated with reproduction are likely also causing the detected patterns. This inference is supported by the fact that between-captures differences in plasma antagonistic index of females that were recaptured during their first or second clutches were similar, in spite of second captures taking place about two months later, with notable abiotic changes between both captures. Likewise, antagonistic index of plasma from those females captured twice before laying started (with a time lapse of two weeks) did not change.

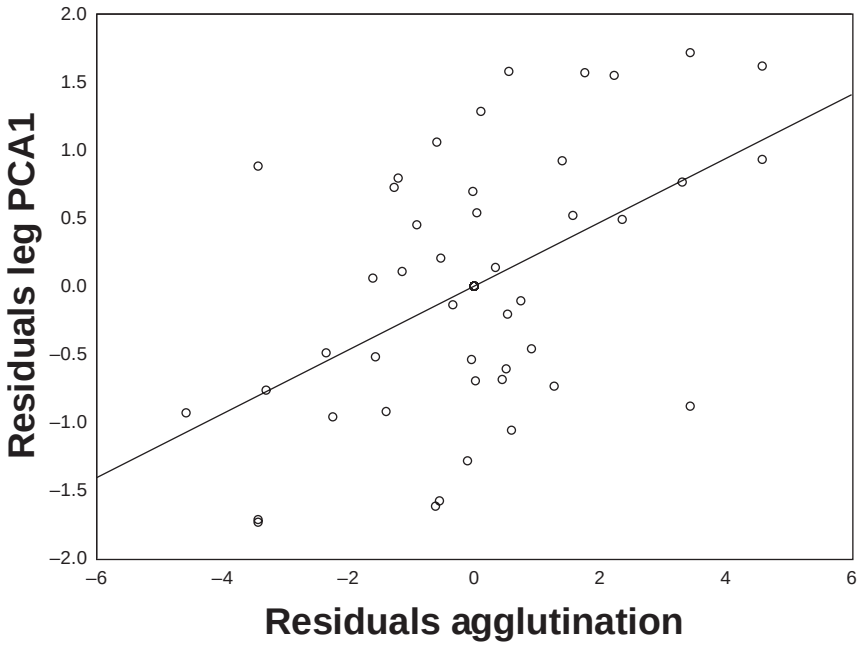

Figure 2. Relationship between residuals of agglutination capacity and of leg colouration (PCA1), after controlling for the effects of lysis (continuous predictor), breeding status (categorical predictor) and individual identity (random factor).

However, different seasonal patterns may occur in different immune parameters, and due to the costs of reproduction, certain immune responses might decrease as a consequence of trade-offs (Sorci et al. 1997). In agreement with this prediction, immunocompetence of female tree swallows *Tachycineta bicolor* tended to decrease along the breeding season (Ardia et al. 2003), a similar pattern observed with the agglutination capacity of foreign antigens in our starling females, which was lower during the nestling period than during the mating period. Thus, depending on the study species and the branch of immune response considered, the expected association between immunity and phenology may be positive, negative or masked by costs associated with reproduction or with different types of immune defences (Schmid-Hempel 2003, Soler et al. 2003, Martin et al. 2008).

An alternative explanation that does not imply an adjustment of immunity to environmental conditions is that immune capacity is the result of historical contacts with antigenic microorganisms. The plasma antibacterial capacity integrates cytological (Keusch et al. 1975) and serological (Merchant et al. 2003) immune components. Previous exposure to microorganisms closely related to the indicator strains assayed could have affected bacterial inhibition capacity (Matson et al. 2006), either directly by the production of specific IgY antibodies (Roitt 1997), or indirectly by increasing nonspecific IgM antibodies (Reid et al. 1997). Infection status might also determine antimicrobial capacity of animals (Millet et al. 2007). To overcome this potential confounding factor, it is advisable to conduct assays with a range of different microorganisms to have a more complete picture of the whole response (Matson et al. 2006), as we did in our study. Our inference comes from interpreting results that integrate multiple components of innate immunity and assumes that in vitro bacteria-killing capacity (i.e. antagonistic index) of a larger number of indicator strains reflects a higher immune capacity of individuals (Matson et al. 2006). Thus, even though some of the detected bacterial inhibition might be due to historical contexts, it would hardly explain the overall detected patterns.

We have also found that the studied immunological variables of females before egg laying, i.e. at the time of mating, had only modest effect at predicting reproductive success. This fact may be related to the existence of trade-offs between immune response and energy/resource expenditure during the nestling period due to offspring provisioning. However, females with higher immunocompetence would at some extent attain direct and indirect fitness benefits (Horváthová et al. 2011, Tobias et al. 2012) if they were able to show their immune capacity to conspecifics since those in better condition may be preferred by males. Furthermore, females with traits showing high immune capacity level would attain direct benefits from the potential increased investment in reproduction by their mates (Burley 1986, Sheldon 2000, Haaland et al. 2017). Here, we explored and found support for the possibility that reflectance spectra of female coloured traits are associated with their immune responses. However, since we did not include the avian colour vision models in our analysis, and due to the correlational nature of the study, the directions of causality in the associations presented cannot be unequivocally elucidated. Further studies exploring the signalling roles of these female traits are urged, as well as studying the mating and reproductive success in consecutive seasons in relation to colour variation.

Starling males show their capacity of fighting against bacterial infections through the integrity of their elongated ornamental throat feathers, which are much shorter in females (Ruiz-Rodríguez et al. 2015). Therefore, different selective pressures on each sex may lead to a diversification on their signalling characteristics (Tobias et al. 2012). Previous research also showed that during the mating period (before egg laying), both female and male starlings reflect their plasma concentration of carotenoids and vitamin A in the colouration intensity of the beak tip (Navarro et al. 2010). Here, the size of the uropygial gland was related to the colouration of the beak base, which is a sexually dimorphic character in this species. In tawny owls *Strix aluco*, changes in the preen oil production cause changes in bill colouration, which may be a result of birds taking the preen oil with the beak (Piault et al. 2008). Therefore in starlings, it could also be a makeup effect of the preen oil on the beak at least during the breeding season, when females exhibit the pink colour, since those females with higher glands also reflected a higher colour intensity. In addition, the agglutination capacity was related to colouration of the legs. Hence, starling females may be reflecting different capabilities through the colouration of different parts of the body. Signalling of individual quality through different ornaments has been a topic usually studied in males (Galván 2010), although females can also signal their immune capacity through multiple signals (Zanollo et al. 2012). Thus, similarly to females, it is possible that males use multiple signalling characters for mate choice (Møller and Pomiankowski 1993, Candolin 2003, Loyau et al. 2005, Robson et al. 2005), a possibility that deserves further investigation.

To summarize, we found that antimicrobial related traits in starling females may change across the breeding season, and those females that are better able to defend themselves from bacterial infections before egg laying (i.e. during the mating period) are those with higher reproductive success. In addition, their antimicrobial capacity is related to reflectance spectra of different body parts, including feathers and integuments. This opens the possibility that sexual selection processes in females explain the evolution of those colourations. Overall, these results support the crucial role of microorganisms in shaping life-histories of their hosts.

Transparent Peer Review

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Data deposition

Data will be available from the Dryad Digital Repository: <http://dx.doi.org/cjsxksn3s> (Ruiz-Rodríguez et al. 2020).

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Author contribution – Conceived and designed the study: GT, JJS, MRR. Field work: CRC, GT, JJS, MAG, MRR, SDL. Laboratory work: CRC, GT, MAG, MRR, SDL. Viewing and taking data from the videos: TPC. Data organization: MAG. Data statistical analysis: MRR. First draft writing: MRR, with substantial help of JJS, and all the authors.
Permits – Blood sampling and nest visiting were done following ethical standards and under the permission of Junta de Andalucía, Environmental Management Agency, which authorized the field protocol.

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Supplementary material (available online as Appendix jav-02425 at <www.avianbiology.org/appendix/jav-02425>). Appendix 1.

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