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HOST MICROBE INTERACTIONS

Ectoparasite Activity During Incubation Increases Microbial Growth on Avian Eggs

G. Tomás[1] · D. Martín-Gálvez[1,2] · C. Ruiz-Castellano[1] · M. Ruiz-Rodríguez[1] · J. M. Peralta-Sánchez[3] · M. Martín-Vivaldi[3] · J. J. Soler[1]

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Abstract
While direct detrimental effects of parasites on hosts are relatively well documented, other more subtle but potentially important effects of parasitism are yet unexplored. Biological activity of ectoparasites, apart from skin injuries and blood-feeding, often results in blood remains, or parasite faeces that accumulate and modify the host environment. In this way, ectoparasite activities and remains may increase nutrient availability that may favour colonization and growth of microorganisms including potential pathogens. Here, by the experimental addition of hematophagous flies (*Carnus hemapterus*, a common ectoparasite of birds) to nests of spotless starlings *Sturnus unicolor* during incubation, we explore this possible side effect of parasitism which has rarely, if ever, been investigated. Results show that faeces and blood remains from parasitic flies on spotless starling eggshells at the end of incubation were more abundant in experimental than in control nests. Moreover, eggshell bacterial loads of different groups of cultivable bacteria including potential pathogens, as well as species richness of bacteria in terms of Operational Taxonomic Units (OTUs), were also higher in experimental nests. Finally, we also found evidence of a link between eggshell bacterial loads and increased embryo mortality, which provides indirect support for a bacterial-mediated negative effect of ectoparasitism on host offspring. Trans-shell bacterial infection might be one of the main causes of embryo death and, consequently, this hitherto unnoticed indirect effect of ectoparasitism might be widespread in nature and could affect our understanding of ecology and evolution of host-parasite interactions.

Keywords ARISA · Bacterial community · Ectoparasite-host interactions · Hatching success · Niche construction · Trans-shell transmission

Introduction

Parasitism is widely recognized as one of the major selective forces driving the evolution of host organisms [1–4]. Convincing demonstrations of the deleterious effects that parasites impinge on fecundity and survival of their hosts come from a wide range of parasite-host assemblages (e.g., [5–8]). Among them, interactions between ectoparasites and birds have provided many influential and already classical examples of parasite-mediated ecology and evolution of hosts [1, 3]. However, besides these relatively well-documented direct deleterious effects on their hosts' fitness, little is known about the role of ectoparasites as vectors or facilitating infection by microparasites such as protozoa [9], viruses (e.g., [10, 11]), or bacteria (e.g., [12, 13]), which undoubtedly is highly important for disease ecology of avian populations.

Various arthropods, including fleas, adult and larval dipterans, mites, and ticks feed on the blood of adult and nestling birds while in their nests [3, 14]. Actually, many of these nest-dwelling ectoparasites develop, grow, and reproduce in their hosts' nests, thus completing most of their life cycles in close contact with their hosts. As a consequence, side effects of this biological activity, such as skin injuries created by blood-feeding, blood remains, or parasite faeces accumulate and modify nest environmental conditions. This increase in nutrient

✉ G. Tomás
gtomas@eeza.csic.es

[1] Estación Experimental de Zonas Áridas (EEZA-CSIC), Almería, Spain

[2] Present address: European Molecular Biology Laboratory, European Bioinformatics Institute, Wellcome Genome Campus, Hinxton, Cambridge, UK

[3] Facultad de Ciencias, Universidad de Granada, Granada, Spain

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availability may favour colonization and growth of bacteria [15], some of which could be pathogens. As far as we know, this potential role of ectoparasites as mediators of indirect interactions in nest environments has never been explored.

Beyond their roles in disease as infectious agents [16], the importance of bacteria in shaping ecology and evolution of higher organisms has been traditionally neglected. Nevertheless, it has recently started to be subject of in-depth research from an ecological and evolutionary framework (e.g., [17, 18]). Regarding interactions between bacteria and birds, it has been shown that different types of bacteria interact with nestling growth [19, 20], may increase adult predation rates [21], and may be involved for instance in the evolution of uropygial glands [22], plumage colouration or maintenance [23–25], nest material composition [26, 27], incubation behaviours [28, 29], or even cognitive skills of birds [30]. Because of the important and diverse roles that bacteria may play in the ecology and evolution of life histories and behaviour in higher organisms (reviewed in [31–34]), detecting a causal link between ectoparasitism and bacterial infections would contribute to the understanding of parasite-host interactions.

In this study, we aimed to explore experimentally whether ectoparasites can increase bacterial loads, or provoke changes in bacterial diversity and richness on hosts. We manipulated abundance of a common, widespread and generalist ectoparasitic fly of nesting birds (*Carnus hemapterus* Nitzsch) and evaluated subsequent changes in bacterial loads, diversity and richness on eggshells of spotless starlings (*Sturnus unicolor* Temminck). We hypothesized that faeces and blood remains accumulated on eggshells because of the ectoparasite activity while feeding on incubating birds (Fig. 1) would promote microbial colonization and growth on eggshells. In addition, we hypothesized that this increased bacterial load or changes in diversity and/or richness on eggshells may increase the chance of trans-shell bacterial infection, ultimately causing embryo mortality and therefore a lower hatching success.

Materials and Methods

Study Area and Species

The experiment was carried out during 2010–2011 breeding seasons (April–June) on spotless starlings breeding in nest-boxes in two colonies (La Calahorra and Huéneja) located in Hoya de Guadix (Granada, Southern Spain, 37° 18′ N, 3° 11′ W). Cork-made nest-boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm, hole diameter: 6 mm) were available for starlings, attached to tree trunks or walls at 3–4 m above ground. Nest-boxes were cleaned before each breeding season. Typical clutch sizes are four to five eggs in the population, females lay one per day, and incubation usually starts 1 day before clutch completion and lasts 12–13 days [35]. Immaculate blue eggs usually become brownish-spotted during incubation as a result of the viscous faeces (and blood remains) that the ectoparasite *Carnus hemapterus* (hereafter *Carnus*) deposits all around nests attached to substrates, including bird skin, feathers, and eggs (Fig. 1a; [36–38]). *Carnus* is a 2 mm blood-sucking fly found in nests of an extremely wide diversity of birds. So far, it has been found parasitizing 64 host species from 24 different avian families, from raptors to passerines [39–41]. It has been recorded throughout most of north America and Europe, with more scarce records in Asia and north Africa suggesting that the distribution of this parasitic genus is probably global, but yet undiscovered in most areas [39, 41]. *Carnus* feeds mainly on nestlings, but also on incubating birds [36–38]. After emergence from overwintering pupae inside nests, winged adults may disperse, losing their wings once a suitable nest is found [40–42].

Experimental Design

Nest-boxes were inspected every 4 days to detect initiation of egg laying by starlings, and eggs were individually numbered with a permanent marker. As a standard protocol, eggs were always handled with new sterile latex gloves further cleaned with 70% ethanol. Five days after laying of the first egg, we measured length and breadth of all eggs with a digital calliper to the nearest 0.01 mm, we estimated eggshell spottiness, and we sampled eggshell bacteria in one randomly selected egg (see below). Eggshell surface area was estimated according to the formula: $S = (3.155–0.0136 * L + 0.0115 * B) * L * B$; where S is surface in mm^2, L is egg length in mm and B is egg breadth in mm [43]. Then, nests were alternately assigned to the experimental or control treatments (45 nests each; see Table 1 for distribution of nests between years and colonies). In experimental nests, 10 unwinged *Carnus* flies collected from nearby nests were added (which is within the natural infestation level in starling nests; [36, 37]; authors unpublished data), while no flies were added in control nests. At the time of manipulation, abundance of *Carnus* flies in nests was low in both groups, as shown by reduced eggshell spottiness that also did not differ between treatments (see Results).

Eggshell Spottiness

Estimations of eggshell spottiness (and bacterial sampling, see below) were performed three times during the incubation period. The first one was carried out when incubation had already commenced (on day five after laying of the first egg, immediately before treatment assignment). Incubation is known to reduce eggshell bacterial load [28, 29]. Subsequent samplings were performed at middle (day nine) and late (day twelve) incubation period. Egg spots, as indication of ectoparasite abundance [37], were counted in every egg of each nest at

Fig. 1 Clutches of five different bird species from four different families showing natural levels of egg spottiness caused by accumulation of parasite faeces and host blood remains as a result of the activity of *Carnus hemapterus* parasites. **a** Spotless starling (*Sturnus unicolor*, family Sturnidae). **b** Hoopoe (*Upupa epops*, family Upupidae). **c** Eurasian roller (*Coracias garrulus*, family Coracidae). **d** Little owl (*Athene noctua*, family Strigidae). **e** Eurasian scops owl (*Otus scops*, family Strigidae). Note that unparasitized eggs of these species are of uniform, immaculate colours, i.e. blue in starlings and hoopoes (in hoopoes blue at laying and light brown later on) and white in rollers and owls (online version in colour)

the three visits. When egg spots were so abundant that counting all spots became unfeasible, we estimated eggshell spottiness by counting spots within a 1 cm^2 on a random position along the shortest axis of the egg [37], which was extrapolated to eggshell surface. We compared both estimates on a subsample of eggs to confirm that both measures are correlated ($n = 53$, $r^2 = 0.25$, $p < 0.0001$). Within-nest mean eggshell spottiness per egg was used in the analyses, but excluding the eggs that had been swabbed for bacterial sampling in previous visits (see below).

Bacterial Sampling

Eggshell bacteria were sampled by swabbing the whole surface of one egg (a randomly selected egg in each of the three sampling times while avoiding previously sampled eggs) with a sterile swab slightly wet with sterile phosphate buffer (PB, 0.2 M; pH = 7.2). The swab was preserved in a rubber-sealed microfuge tube containing 1.2 mL sterile PB, at 4 °C until bacterial culture analyses (within 24 h after collection), and then frozen at − 80 °C for posterior characterization of bacterial communities by ARISA (see below).

Table 1 Distribution of experimental and control spotless starling (*Sturnus unicolor*) nests between different years and colonies

Year	Colony	Control	Experimental
2010	La Calahorra	7	6
2010	Huéneja	5	6
Subtotal		12	12
2011	La Calahorra	26	27
2011	Huéneja	7	6
Subtotal		33	33
Total		45	45

Estimation of Bacterial Density

Eggshell bacterial density was estimated by mean of culture methods. Briefly, under sterile conditions in the lab, bacterial samples were extracted after shaking the tubes in vortex. Serial decimal dilutions up to 10^{-6} were cultivated by spreading 100 μL of each dilution in plates containing four different sterile solid growth media (Scharlau Chemie S.A., Barcelona, Spain). We used a general medium (Tryptic Soy Agar; TSA) for growing mesophilic bacteria, and three specific media: Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*, Vogel-Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*, and Hecktoen Enteric Agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*. Load of mesophilic bacteria on eggshells is related to probability of trans-shell embryo infection [29, 44]. *Enterococci* are opportunistic pathogens [45] also commonly found inside unhatched eggs [44]. *Staphylococcus* and *Enterobacteriaceae* are saprophytic and opportunistic bacteria commonly found on skin, feathers, and eggs of birds, with known pathogenic effects for avian embryos [44, 46]. Overall, these bacterial groups adequately characterize diversity of bacteria found on eggshells and are related to probability

of trans-shell embryo infection [22, 44]. Plates were incubated at 37 °C for 72 h and, afterwards, number of colonies was counted. Bacterial load was estimated as CFU (Colony Forming Units) per cm^2 of sampled eggshell. For further details on bacterial sampling from eggshells, see [22, 26].

Bacterial infections inside unhatched eggs, collected on day 4 after hatching of the first egg, were also estimated by culturing the samples (only for 2011). After disinfection of eggshell surface with disinfectant napkins (Aseptonet, Laboratoires Sarbec, Neuville-en-Ferrain, France), a piece of the eggshell in the blunt end was broken and the yolk and egg white were homogeneously mixed using a sterile inoculation loop. Then, 300 μL of the egg content were diluted in 300 μL of PB, from which 100 μL aliquots were cultured as above to detect internal bacterial infection [47, 48]. Presence of bacteria inside unhatched eggs cannot be unequivocally interpreted as these bacteria causing hatching failure, but it reflects a higher probability of trans-shell infection in comparison with eggs without bacteria inside [44, 47].

Characterization of Bacterial Communities

Bacterial communities of the eggshells were also characterized by molecular methods, following the well-established ARISA (Automated rRNA Intergenetic Spacer Analysis) protocol [49], which allows to identify different bacterial strains as operational taxonomic units (OTUs) (see [50, 51] for further details). Bacterial genomic DNA was extracted with Chelex-based DNA extraction protocol [52], and concentrated and purified with centrifugal filter devices (Amicon Ultra-0.5, 100 K device, Millipore). ARISA amplifies the Intergenic Transcribed Spacer (ITS) region between the prokaryotic 16S and 23S rDNAs. This region is highly variable both in size and sequence between species and strains [53]. The ITS region was amplified using the primer pair ITSF (5′-GTCG TAACAAGGTAGCCGTA-3′) and ITSReub (5'-GCCAAGGCATCCACC-3′) [54]. The primer ITSReub was labeled fluorescently with 6-FAM. Amplifications were performed in 50 μl reaction volumes containing ultrapure H_2O, 20 μl of 5 PRIME MasterMix (2.5×) including 1.5 mM Mg $(OAC)_2$, 200 μM dNTPs, 1.25 U Taq DNA polymerase, 0.2 μM of primers, and 5 μl of concentrated DNA. PCRs were conducted in the Eppendorf Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94 °C 2 min, followed by 30 cycles with denaturation at 94 °C 45 s, annealing at 52 °C 45 s, and extension at 72 °C 1 min, with a final extension at 72 °C 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in formamide. Fragment lengths were determined by automated fluorescent capillary electrophoresis in a 3130 Genetic Analyzer with GeneScan™ 1200 LIZ dye Size Standard (both Applied Biosystems).

Peak Scanner 1.0 (Applied Biosystems) was used to determine fragment length (in base pairs; bp) of each peak that enables the identification of different OTUs within each sample. For methodological reasons, the estimated length of the same OTU from different samples may differ slightly. Thus, binning DNA fragment lengths from different samples is necessary before comparing bacterial communities. We did so by using available scripts in R environment [http://cran.r-project.org/] at http://www.ecology-research.com [55] with a window size of 3 bp and a distance of two consecutive binning frames (i.e., shift) of 0.1. The algorithm rearranges the data and calculates the relative fluorescence intensity (RFI) of each peak by dividing individual peak areas by the total peak area for the respective sample. All peaks with RFI values of < 0.09%0.09% were not included in further analyses since they consisted of background peaks. Only fragments above a threshold of 50 fluorescence units and ranging between 100 and 1000 bp were taken into consideration so as to include the maximum number of peaks while excluding background fluorescence [55]. We used the presence-absence matrix generated after the binning process for the analyses of bacterial community. Molecular fingerprinting techniques are highly reproducible and robust and have proven useful for comparative analysis of microbial community structure [56, 57].

Estimation of Egg Viability and Hatching Success

Hatching success (proportion of eggs that hatched) was estimated by visiting nests daily around expected hatching date. Egg viability before hatching was also recorded as a complementary estimate of hatching success in 2011. Egg viability was recorded with a device measuring embryo heart rate (Avitronics-Buddy Digital Egg monitor, Avian Biotech International, Tallahassee, FL, USA) at late incubation (i.e., on the third sampling day), and proportion of viable eggs was computed. Unfertile eggs (those with no sign of embryo development) were discarded from subsequent estimations of egg viability and hatching success. In cases where fate of some eggs was unknown, we averaged possible outcomes (e.g., if 3 or 4 eggs out of 5 were known to hatch, then: 3/5 = 0.6; 4/5 = 0.8; hatching success = 0.7).

Statistical Analyses

Eggshell bacterial loads were Box-Cox transformed before analyses. Analyses on log-transformed variables for all bacteria, or on ranked values for *Enterococcus*, *Staphylococcus*, and *Enterobacteriaceae*, provided the same qualitative results (data not shown). To explore the effectiveness of the experiment in increasing ectoparasite abundance, a repeated-measures ANOVA (rmANOVA) was carried out with values of eggshell spottiness (Box-Cox transformed) at early, middle and late incubation as dependent repeated-measures variable, with treatment,

year, and colony as factors, and laying date as continuous predictor. Standardized laying dates relative to the first laying date in each year and colony were used in analyses. To explore differences in bacterial loads between treatments, a similar repeated-measures multivariate analysis of variance (rmMANOVA) was carried out, with the four bacterial loads at early, middle and late incubation as dependent repeated-measures variables, and the same predictors as above (e.g., [26, 47, 58]). Including clutch size in analyses did not change the results.

Bacterial species richness (number of OTUs per sample) was Box-Cox transformed before analyses. Analyses on log-transformed species richness provided the same results. For some nests ($N = 58$), bacterial richness at one or more of the sampling times was not estimated because of failures during DNA extraction and/or ARISA analysis. Thus, trying to use information from all sampled nests while considering the repeated measured nature of the dataset, we used General Linear Mixed Model (GLMM) with nest identity (nested within the interaction between year and treatment) and the interaction between nest identity and sampling event (i.e. Time) as random factors [59]. Study year was considered as a random factor, and Time (early, middle and late incubation) and experimental treatment as fixed effects. All first order interactions that included the study year (random factor) were considered as random factors, while those including only fixed effects were considered as fixed factors. Colony was not included as few data were available for one of the colonies (Huéneja) in 2010.

Beta diversity analyses to compare community composition between samples (i.e. Principal Coordinate Analysis (PCoA) based on the Jaccard similarity matrix) were performed using scripts from the Quantitative Insights Into Microbial Ecology software (QIIME, version 1.9) pipeline [60] and R environment, and the EMPeror software for graphic representations of the PCoA space [61]. We explored the effects of treatment, Time and its interaction by means of Procrustes ANOVA, a non-parametric test that estimates the probability of shape variation attributable to one or more factors in a linear model, via distributions generated from resampling permutations [62, 63]. We also performed Trajectory Analysis in order to evaluate statistically the changes in trajectory shapes in a multidimensional space. The first factor (treatment) defines groups and the second one (Time) defines trajectory landmarks. Trajectory Analysis tests significant changes in attributes of trajectory, as path distance, principal vector angles and trajectory shape [64–67]. This analysis was performed twice, including all samples and including only nests with bacterial data at the three sampling times (see above). As no qualitative differences were found, we only show results that include all samples.

General Linear Models (GLM) were carried out with either proportion of viable eggs or hatching success (both arcsine square-root-transformed) as dependent variable, with treatment and colony as factors, year as random factor, and laying date as continuous predictor, and these analyses were restricted to nests where egg failure was observed. These two models were repeated replacing predictors by load of mesophilic bacteria, prevalence of *Enterococcus*, *Staphylococcus*, and *Enterobacteriaceae*, and bacterial species richness, at late incubation. Except for comparisons of community composition, all other analyses were performed in STATISTICA 8.0, and statistical models simplified by backward removing one by one non-significant terms with the largest p value.

Results

Nests under different experimental treatments did not differ significantly in laying date or clutch size (both $p > 0.50$), with laying date being earlier in 2011 than in 2010 ($F_{1,88} = 4.93$, $p = 0.029$). As expected, eggshell spottiness was higher in experimental than in control nests (rmANOVA: $F_{1,86} = 188.5$, $p < 0.0001$) after controlling for the effect of year ($F_{1,86} = 30.90$, $p < 0.0001$). Eggshell spottiness did not differ between treatments before the experiment, i.e., at early incubation (rmANOVA: post hoc LSD test: $p = 0.29$), and increased during incubation ($F_{2,172} = 211.24$, $p < 0.0001$), but much more markedly in experimental than in control nests (Time * Treatment interaction: $F_{2,172} = 131.91$, $p < 0.0001$, Fig. 2).

Eggshell bacterial loads along the incubation period are shown in Table 2. Eggshell bacterial loads were explained by experimental treatment (rmMANOVA: Wilks' $\lambda = 0.86$, $F_{4,83} = 3.29$, $p = 0.015$) after controlling for the effect of year (Wilks' $\lambda = 0.82$, $F_{4,83} = 4.47$, $p = 0.003$) and the positive effect of laying date (Wilks' $\lambda = 0.63$, $F_{4,83} = 12.11$, $p < 0.0001$). Bacterial loads did not differ between treatments before the

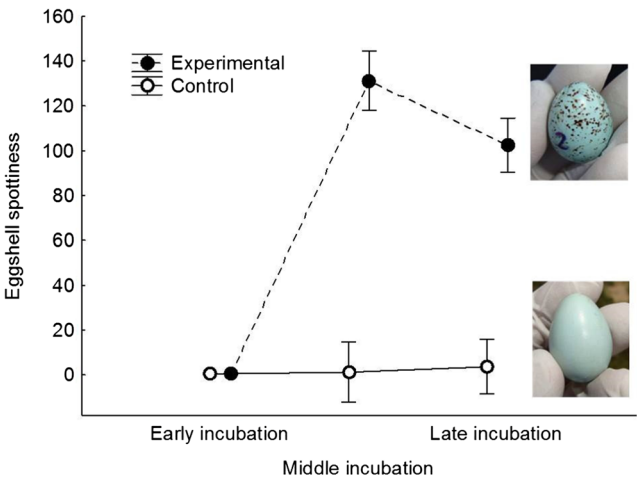

Fig. 2 Effect of experimental addition of *Carnus hemapterus* flies on spottiness (number of spots per egg) of starling eggshells along the incubation period. Mean ± SE values at early (before treatment), middle, and late incubation, for experimental ($n = 45$) and control nests ($n = 45$) are shown. Insets show representative eggs of *Carnus*-infested (above) and control clutches (below) (online version in colour)

Table 2 Eggshell bacterial prevalence (proportion of infected nests) and loads (mean (SE) and range of colony forming units per cm^2) at early, middle, and late incubation in experimental (*Carnus*-infested) and control starling nests

			Prevalence	Mean (SE)	Range
Early incubation	Control	Mesophilic	100%	45,059.6 (28,766.2)	0.6–1,210,672.5
		Enterococci	4.4%	1.2 (1.2)	0–54.5
		Staphylococci	13.3%	0.1 (0.0)	0–1.8
		Enterobacteriaceae	24.4%	6288.2 (4757.1)	0–199,761.0
	Experimental	Mesophilic	100%	13,110.0 (10,615.7)	0.6–472,860.5
		Enterococci	6.7%	12.9 (10.0)	0–424.4
		Staphylococci	11.1%	0.1 (0.1)	0–2.1
		Enterobacteriaceae	26.7%	8668.6 (8621.0)	0–387,988.1
Middle incubation	Control	Mesophilic	100%	22,870.5 (22,159.2)	0.6–997,607.2
		Enterococci	6.7%	1751.9 (1722.9)	0–77,547.9
		Staphylococci	8.9%	1724.1 (1723.3)	0–77,547.9
		Enterobacteriaceae	13.3%	1825.4 (1377.2)	0–57,925.6
	Experimental	Mesophilic	100%	3,012,920.2 (2,169,043.6)	3.1–86,200,774.4
		Enterococci	20.0%	64.6 (38.8)	0–1609.8
		Staphylococci	4.4%	0.1 (0.1)	0–3.9
		Enterobacteriaceae	31.1%	699,959.2 (647,193.4)	0–29,138,289.9
Late incubation	Control	Mesophilic	97.8%	12,540.4 (7804.2)	0–282,480.4
		Enterococci	11.1%	2170.5 (2085.8)	0–93,871.6
		Staphylococci	13.3%	9.8 (9.3)	0–417.2
		Enterobacteriaceae	24.4%	216.6 (145.5)	0–4906.8
	Experimental	Mesophilic	97.8%	311,939.0 (290,029.6)	0–13,056,036.3
		Enterococci	24.4%	283.7 (274.7)	0–12,369.0
		Staphylococci	4.4%	0.0 (0.0)	0–0.7
		Enterobacteriaceae	35.6%	13,066.2 (11,124.0)	0–496,129.4

experiment, i.e., at early incubation (rmMANOVA: post hoc LSD tests: $p > 0.70$). Treatment effect on bacterial loads did not vary significantly along the incubation period (Time * Treatment interaction: Wilks' $\lambda = 0.94$, $F_{8,79} = 0.65$, $p = 0.73$). With the exception of *Staphylococcus* (post hoc LSD test: $p = 0.12$), all other bacterial types were more abundant in experimental than in control nests (*Enterococcus*: $p = 0.005$; *Enterobacteriaceae*: $p = 0.015$), although not significantly so for mesophilic bacteria ($p = 0.10$). Statistically significant differences were observed for *Enterococcus* at middle (post hoc LSD test: $p = 0.038$) and late incubation ($p = 0.045$), and for *Enterobacteriaceae* at middle incubation ($p = 0.030$) (Fig. 3).

We identified a total of 117 different OTUs in experimental nests and 105 OTUs in control nests. OTU richness varied significantly along the incubation period in relation to treatment (Time * Treatment interaction: $F_{2,86} = 3.47$, $p = 0.036$). Species richness did not differ between treatments before the experiment, i.e., at early incubation (post-hoc LSD test: $p > 0.59$) nor at the end of incubation ($p > 0.40$). However, it was significantly higher in experimental than in control nests at middle incubation ($p = 0.023$) (Fig. 4). Changes in bacterial community did not vary between experimental and control nests nor along the incubation period (Procrustes ANOVA: Treatment: $F_{1,168} = 0.83$, $p = 0.714$; Time: $F_{2,168} = 0.11$, $p = 0.227$; Treatment * Time: $F_{2,168} = 0.66$, $p = 0.979$). Moreover, those changes showed similar patterns between control and experimental nests (Trajectory Analysis: Path distances, pairwise absolute differences between path distances = 0.005, $p = 0.92$; Principal Vector Angles, pairwise angles = 74.72, $p = 0.49$; Trajectory Shape differences, pairwise shape differences = 0.17, $p = 0.386$).

Experimental treatments did not explain differences in proportion of viable eggs and hatching success (GLM: $p > 0.3$). However, proportion of viable eggs ($F_{1,7} = 14.67$, $p = 0.006$) and hatching success ($F_{1,25} = 6.05$, $p = 0.021$) were lower in nests where *Enterobacteriaceae* and *Enterococcus*, respectively, were detected at late incubation. In addition, load of mesophilic bacteria at late incubation showed a negative relationship with hatching success in nests with hatching failures ($n = 39$, $r = -0.37$, $p = 0.022$). Finally, no significant associations between presence of bacteria in eggshells at late incubation and inside eggs were found for *Enterococcus* and

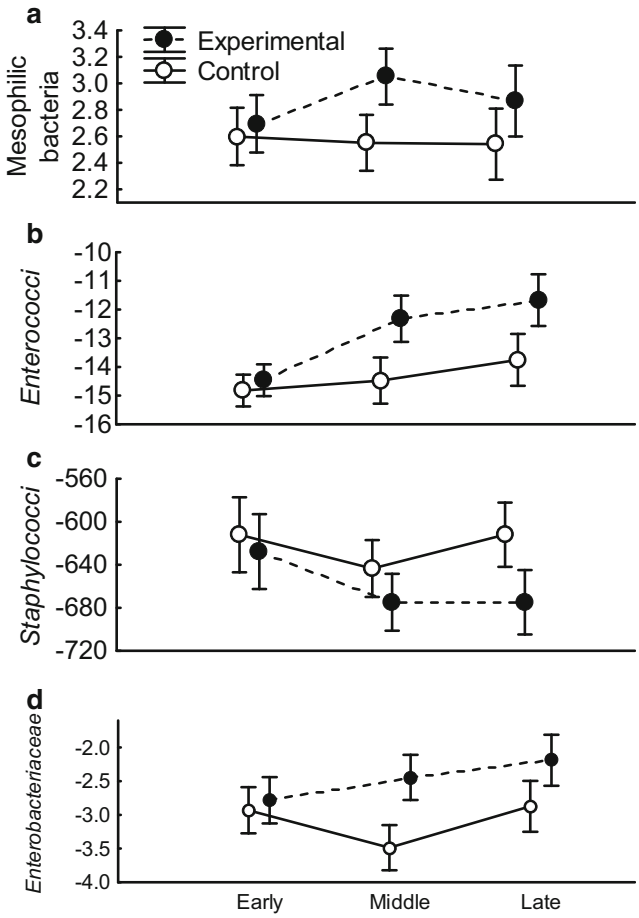

Fig. 3 Effect of experimental addition of *Carnus hemapterus* flies on cultivable bacterial loads of starling eggshells. Mean ± SE Box-Cox transformed counts of **a** mesophilic bacteria, **b** *Enterococci*, **c** *Staphylococci*, and **d** *Enterobacteriaceae*, at early (before treatment), middle, and late incubation, for experimental ($n = 45$) and control nests ($n = 45$), are shown

Enterobacteriaceae ($p > 0.8$). However, trans-shell colonization of unhatched eggs by *Staphylococcus* was more frequent in nests where these bacteria were detected on egg surface at late incubation ($n = 3/3$) than where it was not ($n = 3/14$) ($\chi^2_1 = 6.68$, $p = 0.010$). Taken together, these results suggest that increased eggshell bacterial loads might be related with increased trans-shell infection, which ultimately may cause embryo mortality and reduce hatching success.

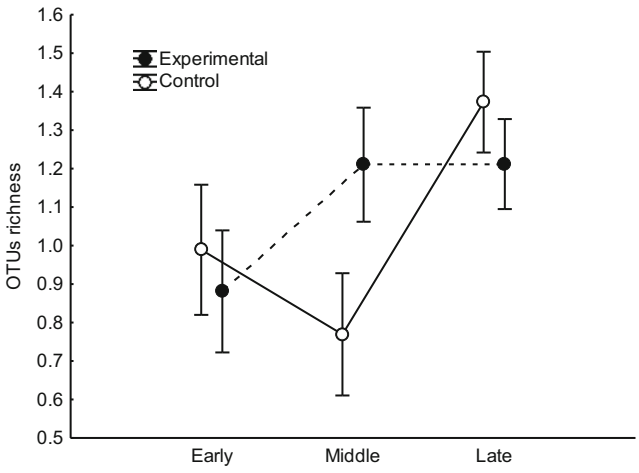

Fig. 4 Effect of experimental addition of *Carnus hemapterus* flies on average number of OTUs (species richness) in starling eggshells. Mean ± SE Box-Cox transformed counts at early (before treatment), middle, and late incubation, for experimental and control nests, are shown

Discussion

This is the first study, to our knowledge, showing that ectoparasitism affects bacterial environment of nests of a wild bird. Our experiment shows that activity during incubation of a common, generalist ectoparasite of a multitude of bird species in a wide geographic range, increases abundance of different bacterial types and overall bacterial species richness on spotless starling eggshells. This effect was detected just 4 days after experimental addition of the *Carnus* ectoparasites and was reduced at late incubation, probably matching a parallel reduction in eggshell spottiness of experimental nests at late incubation due to incubation activity. This reduced effect at the end of incubation may reflect adaptive behavioural or physiological defences by birds to reduce ectoparasite and/or bacterial load during incubation [28, 29, 68, 69]. Nevertheless, no differences in bacterial community composition were detected between treatments, suggesting that ectoparasites may not cause differences in bacterial community of eggshells in general, but in bacterial abundance and species richness. Although we did not detect a direct effect of experimental treatment on egg viability or hatching success, variability in the capacity of incubating birds to reduce eggshell bacterial loads might be the reason. In accordance with this possibility, we detected correlational links between egg viability and hatching success with the presence of *Enterobacteriaceae* and *Enterococcus*, respectively, at late incubation. Moreover, hatching success was also lower in nests with a higher load of mesophilic bacteria, while trans-shell colonization of eggs by *Staphylococcus* was more frequent in nests where these bacteria were detected at late incubation than where it was not. A plausible explanation for such results is that some incubating birds failed to control bacterial growth caused by ectoparasite activity on eggshells, resulting in reduced hatching success. Our experiment affected loads of *Enterococcus*, *Enterobacteriaceae*, and (not significantly so) mesophilic bacteria, at middle incubation, and loads of *Enterococcus* at late incubation. These bacteria are known to reduce embryo viability, according to extensive studies in poultry, and to more limited evidence in wild avian species (see [22, 28, 29, 44, 70]). While culture methods do not characterize the entire microbial community, we selected the cultivation media to detect the most common groups of bacteria inhabiting avian eggs and potentially causing embryo mortality [28]. We also showed experimental effects of ectoparasite infestation on bacterial OTUs richness estimated by molecular techniques. Thus, our experimental results demonstrated an effect of

ectoparasite activity on eggshell bacterial environment and a potential indirect link with egg viability and hatching success.

An alternative explanation to our results could be that the parasites added experimentally directly affected behaviour and/or condition of incubating females and, thus, eggshell bacterial environment. Our experiment does not allow disentangling indirect effects of ectoparasite activity through their faeces from direct effects of ectoparasites on incubating birds. It would require collecting ectoparasite faeces for manipulating eggshell environment without adding ectoparasites to the nests, an experiment that would be logistically challenging. Whatever the mechanism involved, our study ultimately shows that ectoparasites increase abundance and richness of bacteria on eggshells, which might be potentially associated to a reduced hatching success.

Only a handful of studies have suggested that ectoparasites may modify the nest environment in a substantial manner. Heeb et al. [71] showed that fleas can increase nest humidity and modify infestation patterns of other ectoparasites such as *Protocalliphora* blowflies. Mennerat et al. [69] also pointed out that nests highly infested by *Protocalliphora* blowflies are often wetter than usual, and reported a correlation between abundance of this ectoparasite and bacterial density on feathers and skin of nestling birds. However, Mennerat et al. [69] did not manipulate parasite abundance, so a common unmeasured third factor (e.g., nest humidity or temperature; [71, 72]) may be responsible for abundance of both ectoparasite and bacteria. Our study is the first to show experimentally a causal, direct relationship between ectoparasite loads and bacterial loads and richness. This provides indirect support to ectoparasites affecting reproductive success of hosts through indirect interactions mediated by changes in eggshell bacterial environments.

This hitherto overlooked important effect of ectoparasitism may be widespread in bird-ectoparasite systems, as other common and abundant nest-dwelling ectoparasites of birds such as fleas, mites, and blowflies also develop and reproduce within the nest matrix in close contact with eggs and nestlings, thereby likely creating the necessary conditions for successful colonization and growth of potentially pathogenic microorganisms. For example, there are known cases of spottiness on eggs or nest environments apparently caused by fleas [73], bugs [74], mites *Dermanyssus gallinoides* in hens *Gallus gallus* (G. Tomás, pers. obs.), or unidentified parasites [75–77]. Further research involving other host and parasite species is therefore urged to confirm the generality of the results presented in this study. The importance of bacteria, the world's most abundant living beings, shaping ecology and evolution of wild organisms has only recently started to be recognized [31–33, 78] and is changing the way we interpret ecological interactions and animal biology [34]. Our study is an example of how cross-disciplinary research may most benefit a proper comprehension of interactions between parasites and their hosts [18, 79]. The novel observation that ectoparasites can modify bacterial communities living with their hosts may profoundly affect our current understanding of disease transmission patterns and wildlife disease ecology.

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References

1. Clayton DH, Moore J (1997) Host-parasite evolution: general principles and avian models. Oxford University Press, Oxford
2. Combes C (2001) Parasitism: the ecology and evolution of intimate interactions. The University of Chicago Press, Chicago
3. Loye JE, Zuk M (1991) Bird-parasite interactions. Ecology, evolution and behaviour. Oxford University Press, Oxford
4. Poulin R, Morand S, Skorping A (2000) Evolutionary biology of host-parasite relationships: theory meets reality. Elsevier, Amsterdam
5. Barber I, Hoare D, Krause J (2000) Effects of parasites on fish behaviour: a review and evolutionary perspective. Rev Fish Biol Fish 10:131–165
6. Degen AA (2006) Effect of macroparasites on the energy budget of small mammals. In: Morand S, Krasnov BR, Poulin R (eds) Micromammals and macroparasites: from evolutionary ecology to management. Springer-Verlag, Tokyo, pp 371–399
7. Møller AP, Allander K, Dufva R (1990) Fitness effects of parasites on passerine birds: a review. In: Blondel J, Gosler A, Lebreton J, McCleery RH (eds) Population biology of passerine birds. Springer-Verlag, Berlin, pp 269–280
8. Patterson JEH, Neuhaus P, Kutz SJ, Ruckstuhl KE (2013) Parasite removal improves reproductive success of female North American red squirrels (*Tamiasciurus hudsonicus*). PLoS One 8:e55779
9. Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC Press, Boca Ratón
10. Gilbert L, Jones LD, Laurenson MK, Gould EA, Reid HW, Hudson PJ (2004) Ticks need not bite their red grouse hosts to infect them with louping ill virus. Proc R Soc Lond B 271:S202–S205
11. Naugle DE, Aldridge CL, Walker BL, Cornish TE, Moynahan BJ, Holloran MJ, Brown K, Johnson GD, Schmidtmann ET, Mayer RT, Kato CY, Matchett MR, Christiansen TJ, Cook WE, Creekmore T, Falise RD, Rinkes ET, Boyce MS (2004) West Nile virus: pending crisis for greater sage-grouse. Ecol Lett 7:704–713
12. Lobato E, Pearce-Duvet J, Staszewski V, Gómez-Díaz E, González-Solís J, Kitaysky A, McCoy KD, Boulinier T (2011) Seabirds and the circulation of Lyme borreliosis bacteria in the North Pacific. Vector Borne Zoo Dis 11:1521–1527
13. Staszewski V, McCoy KD, Boulinier T (2008) Variable exposure and immunological response to Lyme disease *Borrelia* among North Atlantic seabird species. Proc R Soc Lond B 275:2101–2109

14. López-Rull I, Macías-Garcia C (2015) Control of invertebrate occupants of nests. In: Deeming DC, Reynolds SJ (eds) Nests, eggs, and incubation: new ideas about avian reproduction. Oxford University Press, Oxford, pp 82–96
15. Weiss MR (2006) Defecation behavior and ecology of insects. Annu Rev Entomol 51:635–661
16. Salyers AA, Whitt DD (2002) Bacterial pathogenesis. A molecular approach, 2nd edn. ASM Press, Washington, DC
17. Flórez LV, Biedermann PHW, Engl T, Kaltenpoth M (2015) Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. Nat Prod Rep 32:904
18. Hahn MA, Dheilly NM (2016) Experimental models to study the role of microbes in host-parasite interactions. Front Microbiol 7:1300
19. Moreno J, Briones V, Merino S, Ballesteros C, Sanz JJ, Tomás G (2003) Beneficial effects of cloacal bacteria on growth and fledging size in nestling pied flycatchers (*Ficedula hypoleuca*) in Spain. Auk 120:784–790
20. Potti J, Moreno J, Yorio P, Briones V, García-Borboroglu P, Villar S, Ballesteros C (2002) Bacteria divert resources from growth for magellanic penguin chicks. Ecol Lett 5:709–714
21. Møller AP, Peralta-Sánchez JM, Nielsen JT, López-Hernández E, Soler JJ (2012) Goshawk prey have more bacteria than non-prey. J Anim Ecol 81:403–410
22. Soler JJ, Peralta-Sánchez JM, Martín-Platero AM, Martín-Vivaldi M, Martínez-Bueno M, Møller AP (2012) The evolution of size of the uropygial gland: mutualistic feather mites and uropygial secretion reduce bacterial loads of eggshells and hatching failures of European birds. J Evol Biol 25:1779–1791
23. Ruiz-Rodríguez M, Tomás G, Martín-Gálvez D, Ruiz-Castellano C, Soler JJ (2015) Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. Funct Ecol 29:701–709
24. Ruiz-Rodríguez M, Valdivia E, Soler JJ, Martín-Vivaldi M, Martín-Platero AM, Martínez-Bueno M (2009) Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. J Exp Biol 212:3621–3626
25. Shawkey MD, Pillai SR, Hill GE, Siefferman LM, Roberts SR (2007) Bacteria as an agent for change in structural plumage color: correlational and experimental evidence. Am Nat 169:S112–S117
26. Peralta-Sánchez JM, Møller AP, Martín-Platero AM, Soler JJ (2010) Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study. Funct Ecol 24:426–433
27. Ruiz-Castellano C, Tomás G, Ruiz-Rodríguez M, Martín-Gálvez D, Soler JJ (2016) Nest material shapes eggs bacterial environment. PLoS One 11:e0148894
28. Cook MI, Beissinger SR, Toranzos GA, Arendt WJ (2005) Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. Ecol Lett 8:532–537
29. Cook MI, Beissinger SR, Toranzos GA, Rodriguez RA, Arendt WJ (2003) Trans-shell infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's eggs: a constraint on the onset of incubation? Proc R Soc Lond B 270:2233–2240
30. Soler JJ, Peralta-Sánchez JM, Martín-Vivaldi M, Martín-Platero AM, Flensted-Jensen E, Møller AP (2012) Cognitive skills and bacterial load: comparative evidence of costs of cognitive proficiency in birds. Naturwissenschaften 99:111–122
31. Archie EA, Theis KR (2011) Animal behaviour meets microbial ecology. Anim Behav 82:425–436
32. Benskin CMWH, Wilson K, Jones K, Hartley IR (2009) Bacterial pathogens in wild birds: a review of the frequency and effects of infection. Biol Rev 84:349–373
33. Ezenwa VO, Gerardo NM, Inouye DW, Medina M, Xavier JB (2012) Animal behavior and the microbiome. Science 338:198–199
34. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Loso T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci U S A 110:3229–3236
35. Soler JJ, Navarro C, Pérez-Contreras T, Avilés JM, Cuervo JJ (2008) Sexually selected egg coloration in spotless starlings. Am Nat 171:183–194
36. Avilés JM, Pérez-Contreras T, Navarro C, Soler JJ (2009) Male spotless starlings adjust feeding effort based on egg spots revealing ectoparasite load. Anim Behav 78:993–999
37. López-Rull I, Gil M, Gil D (2007) Spots in starling *Sturnus unicolor* eggs are good indicators of ectoparasite load by *Carnus hemapterus* (Diptera: Carnidae). Ardeola 54:131–134
38. Tomás G, Martín-Gálvez D, Ruiz-Rodríguez M, Soler JJ (2017) Intraspecific avian brood parasites avoid host nests infested by ectoparasites. J Ornithol 158:561–567
39. Brake I (2011) World catalog of the family Carnidae (Diptera, Schizophora). Myia 12:113–169 (updated in http://diptera.myspecies.info/files/Carnidae_catalog_0.pdf/; Accessed 6 June 2013)
40. Calero-Torralbo MA (2011) Factores ecológicos y mecanismos implicados en la variabilidad de la interacción entre un ectoparásito generalista (*Carnus hemapterus*) y sus hospedadores. PhD dissertation, University of Granada, Granada, Spain
41. Grimaldi D (1997) The bird flies, genus *Carnus*: species revision, generic relationships, and a fossil *Meoneura* in amber (Diptera: Carnidae). American Museum of Natural History, New York
42. Mercier L (1928) Contribution à l'étude de la perte de la faculté du vol chez *Carnus hemapterus* Nitzsch, Diptère à ailes caduques. C R Hebd Seances Acad Sci 186:529–531
43. Narushin VG (2005) Egg geometry calculation using the measurements of length and breadth. Poult Sci 84:482–484
44. Bruce J, Drysdale EM (1994) Trans-shell transmission. In: Board RG, Fuller R (eds) Microbiology of the avian egg. Chapman & Hall, London, pp 63–91
45. Byappanahalli NM, Nevers MB, Korajkic A, Staley ZR, Harwood VJ (2012) Enterococci in the environment. Microbiol Mol Biol Rev 76:685–706
46. Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore
47. Soler JJ, Peralta-Sánchez JM, Martínez-Bueno M, Martín-Vivaldi M, Martín-Gálvez D, Vela AI, Briones V, Pérez-Contreras T (2011) Brood parasitism is associated with increased bacterial contamination of host eggs: bacterial loads of host and parasitic eggs. Biol J Linn Soc 103:836–848
48. Wang JM, Firestone MK, Beissinger SR (2011) Microbial and environmental effects on avian egg viability: do tropical mechanisms act in a temperate environment? Ecology 92:1137–1145
49. Fisher MM, Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl Environ Microbiol 65:4630–4636
50. Martínez-García A, Soler JJ, Rodríguez-Ruano SM, Martínez-Bueno M, Martín-Platero AM, Juárez-García N, Martín-Vivaldi M (2015) Preening as a vehicle for key bacteria in hoopoes. Microb Ecol 70:1024–1033
51. Soler JJ, Martínez-García A, Rodríguez-Ruano SM, Martínez-Bueno M, Martín-Platero AM, Peralta-Sánchez JM, Martín-Vivaldi M (2016) Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell. Biol J Linn Soc 118:763–773
52. Martín-Platero AM, Peralta-Sánchez JM, Soler JJ, Martínez-Bueno M (2010) Chelex-based DNA isolation procedure for the

identification of microbial communities of eggshell surfaces. Anal Biochem 397:253–255
53. Danovaro R, Luna GM, Dell'Anno A, Pietrangeli B (2006) Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. Appl Environ Microbiol 72:5982–5989
54. Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia A, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl Environ Microbiol 70:6147–6156
55. Ramette A (2009) Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. Appl Environ Microbiol 75:2495–2505
56. Bent SJ, Forney LJ (2008) The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. ISME J 2: 689–695
57. Loisel P, Harmand J, Zemb O, Latrille E, Lobry C, Delgenès JP, Godon JJ (2006) Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environ Microbiol 8:720–731
58. Møller AP, Flensted-Jensen E, Mardal W, Soler JJ (2013) Host-parasite relationship between colonial terns and bacteria is modified by a mutualism with a plant with antibacterial defenses. Oecologia 173:169–178
59. Quinn GP, Keough MJ (2002) Experimental design and data analysis for biologists. Cambridge University Press, Cambridge
60. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Tumbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336
61. Vázquez-Baeza Y, Pirrung M, Gonzalez A, Knight R (2013) EMPeror: a tool for visualizing high-throughput microbial community data. GigaScience 2:16
62. Collyer ML, Sekora DJ, Adams DC (2015) A method for analysis of phenotypic change for phenotypes described by high-dimensional data. Heredity 115:357–365
63. Goodall C (1991) Procrustes methods in the statistical analysis of shape. J R Stat Soc B-Method 53:285–339
64. Adams DC, Collyer ML (2007) Analysis of character divergence along environmental gradients and other covariates. Evolution 61: 510–515
65. Adams DC, Collyer ML (2009) A general framework for the analysis of phenotypic trajectories in evolutionary studies. Evolution 63:1143–1154
66. Collyer ML, Adams DC (2007) Analysis of two-state multivariate phenotypic change in ecological studies. Ecology 88:683–692
67. Collyer ML, Adams DC (2013) Phenotypic trajectory analysis: comparison of shape change patterns in evolution and ecology. Hystrix-Ital J Mammal 24:75–83
68. Clark L (1991) The nest protection hypothesis: the adaptive use of plant secondary compounds by European starlings. In: Loye JE, Zuk M (eds) Bird–parasite interactions: ecology, evolution and behaviour. Oxford University Press, Oxford, pp 205–221
69. Mennerat A, Mirleau P, Blondel J, Perret P, Lambrechts MM, Heeb P (2009) Aromatic plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria. Oecologia 161:849–855
70. Cook MI, Beissinger SR, Toranzos GA, Rodriguez RA, Arendt WJ (2005) Microbial infection affects egg viability and incubation behavior in a tropical passerine. Behav Ecol 16:30–36
71. Heeb P, Kölliker M, Richner H (2000) Bird-ectoparasite interactions, nest humidity and ectoparasite community structure. Ecology 81:958–968
72. Dawson RD, Hillen KK, Whitworth TL (2005) Effects of experimental variation in temperature on larval densities of parasitic *Protocalliphora* (Diptera: Calliphoridae) in nests of tree swallows (Passeriformes: Hirundinidae). Environ Entomol 34: 563–568
73. McNeil DAC, Clark F (1987) Markings on the eggs of house martins *Delichon urbica*. Bird Study 34:26–27
74. Krinsky WL (2002) True bugs (Hemiptera). In: Mullen G, Durden L (eds) Medical and veterinary entomology. Academic Press, San Diego, pp 67–86
75. Feare CJ, Constantine DAT (1980) Starling eggs with spots. Bird Study 27:119–120
76. Hornsby MAW, Fairn ER, Barber CA (2013) Male European starlings do not use egg spots as a cue to adjust investment in nestlings. Wilson J Ornithol 125:109–115
77. Jackson JA (1970) Spotted eggs in a local population of starlings. Bird Band 41:308–310
78. Soler JJ, Martín-Vivaldi M, Peralta-Sánchez JM, Ruiz-Rodríguez M (2010) Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. Open Ornithol J 3:93–100
79. Tompkins DM, Dunn AM, Smith MJ, Telfer S (2011) Wildlife diseases: from individuals to ecosystems. J Anim Ecol 80:19–38