Ectoparasite activity during incubation increases microbial growth on avian eggs

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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 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)

74 located in Hoya de Guadix (Granada, Southern Spain, 37º18'N, 3º11'W). Cork-made
75 nest-boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole
76 height: 240 mm, hole diameter: 6 mm) were available for starlings, attached to tree
77 trunks or walls at 3-4 m above ground. Nest-boxes were cleaned before each breeding
78 season. Typical clutch sizes are four to five eggs in the population, females lay one per
79 day, and incubation usually starts one day before clutch completion and lasts 12-13 days
80 [35]. Immaculate blue eggs usually become brownish-spotted during incubation as a
81 result of the viscous faeces (and blood remains) that the ectoparasite *Carnus*
82 *hemapterus* (hereafter *Carnus*) deposits all around nests attached to substrates,
83 including bird skin, feathers, and eggs (Fig. 1a; [36-38]). *Carnus* is a 2 mm blood-
84 sucking fly found in nests of an extremely wide diversity of birds. So far, it has been
85 found parasitizing 64 host species from 24 different avian families, from raptors to
86 passerines [39-41]. It has been recorded throughout most of north America and Europe,
87 with more scarce records in Asia and north Africa suggesting that the distribution of this
88 parasitic genus is probably global, but yet undiscovered in most areas [39, 41]. *Carnus*
89 feeds mainly on nestlings, but also on incubating birds [36-38]. After emergence from
90 overwintering pupae inside nests, winged adults may disperse, losing their wings once a
91 suitable nest is found [40-42].
92
93 **Experimental design**
94 Nest-boxes were inspected every 4 days to detect initiation of egg laying by starlings,
95 and eggs were individually numbered with a permanent marker. As a standard protocol,
96 eggs were always handled with new sterile latex gloves further cleaned with 70 %
97 ethanol. Five days after laying of the first egg, we measured length and breadth of all
98 eggs with a digital calliper to the nearest 0.01 mm, we estimated eggshell spottiness,

99 and we sampled eggshell bacteria in one randomly selected egg (see below). Eggshell
100 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
101
102 mm [43]. Then, nests were alternately assigned to the experimental or control treatments
103 (45 nests each; see Table 1 for distribution of nests between years and colonies). In
104 experimental nests, 10 unwinged *Carnus* flies collected from nearby nests were added
105 (which is within the natural infestation level in starling nests; [36, 37]; authors
106 unpublished data), while no flies were added in control nests. At the time of
107 manipulation, abundance of *Carnus* flies in nests was low in both groups, as shown by
108 reduced eggshell spottiness that also did not differ between treatments (see Results).
109
110 **Eggshell spottiness**
111 Estimations of eggshell spottiness (and bacterial sampling, see below) were performed
112 three times during the incubation period. The first one was carried out when incubation
113 had already commenced (on day five after laying of the first egg, immediately before
114 treatment assignment). Incubation is known to reduce eggshell bacterial load [28, 29].
115 Subsequent samplings were performed at middle (day nine) and late (day twelve)
116 incubation period. Egg spots, as indication of ectoparasite abundance [37], were counted
117 in every egg of each nest at the three visits. When egg spots were so abundant that
118 counting all spots became unfeasible, we estimated eggshell spottiness by counting
119 spots within a 1 cm^2 on a random position along the shortest axis of the egg [37], which
120 was extrapolated to eggshell surface. We compared both estimates on a subsample of
121 eggs to confirm that both measures are correlated ($n = 53$, $r^2 = 0.25$, $p < 0.0001$).
122 Within-nest mean eggshell spottiness per egg was used in the analyses, but excluding
123 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 ºC until bacterial culture analyses (within 24 h after collection), and then frozen at -80 ºC for posterior characterization of bacterial communities by ARISA (see below).

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 Faecal 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

149 of bacteria found on eggshells and are related to probability of trans-shell embryo
150 infection [22, 44]. Plates were incubated at 37 ºC for 72 h and, afterwards, number of
151 colonies was counted. Bacterial load was estimated as CFU (Colony Forming Units) per
152 cm^2 of sampled eggshell. For further details on bacterial sampling from eggshells, see
153 [22, 26].

154 Bacterial infections inside unhatched eggs, collected on day 4 after hatching of
155 the first egg, were also estimated by culturing the samples (only for 2011). After
156 disinfection of eggshell surface with disinfectant napkins (Aseptonet, Laboratoires
157 Sarbec, Neuville-en-Ferrain, France), a piece of the eggshell in the blunt end was
158 broken and the yolk and egg white were homogeneously mixed using a sterile
159 inoculation loop. Then, 300 µL of the egg content were diluted in 300 µL of PB, from
160 which 100 µL aliquots were cultured as above to detect internal bacterial infection [47,
161 48]. Presence of bacteria inside unhatched eggs cannot be unequivocally interpreted as
162 these bacteria causing hatching failure, but it reflects a higher probability of trans-shell
163 infection in comparison with eggs without bacteria inside [44, 47].

164

165 **Characterization of bacterial communities**

166 Bacterial communities of the eggshells were also characterized by molecular methods,
167 following the well-established ARISA (Automated rRNA Intergenetic Spacer Analysis)
168 protocol [49], which allows to identify different bacterial strains as operational
169 taxonomic units (OTUs) (see [50, 51] for further details). Bacterial genomic DNA was
170 extracted with Chelex-based DNA extraction protocol [52], and concentrated and
171 purified with centrifugal filter devices (Amicon Ultra-0.5, 100K device, Millipore).
172 ARISA amplifies the Intergenic Transcribed Spacer (ITS) region between the
173 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'-GTCGTAACAAGGTAGCCGTA-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 % 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

274 control nests (rmANOVA: $F_{1,86} = 188.5$, $p < 0.0001$) after controlling for the effect of
275 year ($F_{1,86} = 30.90$, $p < 0.0001$). Eggshell spottiness did not differ between treatments
276 before the experiment, i.e., at early incubation (rmANOVA: post-hoc LSD test: $p =$
277 0.29), and increased during incubation ($F_{2,172} = 211.24$, $p < 0.0001$), but much more
278 markedly in experimental than in control nests (Time * Treatment interaction: $F_{2,172} =$
279 131.91, $p < 0.0001$, Fig. 2).

280 Eggshell bacterial loads along the incubation period are shown in Table 2.
281 Eggshell bacterial loads were explained by experimental treatment (rmMANOVA:
282 Wilks' $\lambda = 0.86$, $F_{4,83} = 3.29$, $p = 0.015$) after controlling for effect of year (Wilks' $\lambda =$
283 0.82, $F_{4,83} = 4.47$, $p = 0.003$) and the positive effect of laying date (Wilks' $\lambda = 0.63$, $F_{4,83}$
284 $= 12.11$, $p < 0.0001$). Bacterial loads did not differ between treatments before the
285 experiment, i.e., at early incubation (rmMANOVA: post-hoc LSD tests: $p > 0.70$).
286 Treatment effect on bacterial loads did not vary significantly along the incubation
287 period (Time * Treatment interaction: Wilks' $\lambda = 0.94$, $F_{8,79} = 0.65$, $p = 0.73$). With the
288 exception of *Staphylococcus* (post-hoc LSD test: $p = 0.12$), all other bacterial types
289 were more abundant in experimental than in control nests (*Enterococcus*: $p = 0.005$;
290 *Enterobacteriaceae*: $p = 0.015$), although not significantly so for mesophilic bacteria (p
291 $= 0.10$). Statistically significant differences were observed for *Enterococcus* at middle
292 (post-hoc LSD test: $p = 0.038$) and late incubation ($p = 0.045$), and for
293 *Enterobacteriaceae* at middle incubation ($p = 0.030$) (Fig. 3).

294 We identified a total of 117 different OTUs in experimental nests and 105 OTUs
295 in control nests. OTU richness varied significantly along the incubation period in
296 relation to treatment (Time * Treatment interaction: $F_{2,86} = 3.47$, $p = 0.036$). Species
297 richness did not differ between treatments before the experiment, i.e., at early
298 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 *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) (χ^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.

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 four 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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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

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 %	45059.6 (28766.2)	0.6–1210672.5
		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)	0–199761.0
	Experimental	Mesophilic	100 %	13110.0 (10615.7)	0.6–472860.5
		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)	0–387988.1
Middle incubation	Control	Mesophilic	100 %	22870.5 (22159.2)	0.6–997607.2
		Enterococci	6.7 %	1751.9 (1722.9)	0–77547.9
		Staphylococci	8.9 %	1724.1 (1723.3)	0–77547.9
		Enterobacteriaceae	13.3 %	1825.4 (1377.2)	0–57925.6
	Experimental	Mesophilic	100 %	3012920.2 (2169043.6)	3.1–86200774.4
		Enterococci	20.0 %	64.6 (38.8)	0–1609.8
		Staphylococci	4.4 %	0.1 (0.1)	0–3.9
		Enterobacteriaceae	31.1 %	699959.2 (647193.4)	0–29138289.9
Late incubation	Control	Mesophilic	97.8 %	12540.4 (7804.2)	0–282480.4
		Enterococci	11.1 %	2170.5 (2085.8)	0–93871.6
		Staphylococci	13.3 %	9.8 (9.3)	0–417.2
		Enterobacteriaceae	24.4 %	216.6 (145.5)	0–4906.8
	Experimental	Mesophilic	97.8 %	311939.0 (290029.6)	0–13056036.3
		Enterococci	24.4 %	283.7 (274.7)	0–12369.0
		Staphylococci	4.4 %	0.0 (0.0)	0–0.7
		Enterobacteriaceae	35.6 %	13066.2 (11124.0)	0–496129.4

Figure legends

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)

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)

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

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

Figure 1

Figure 2

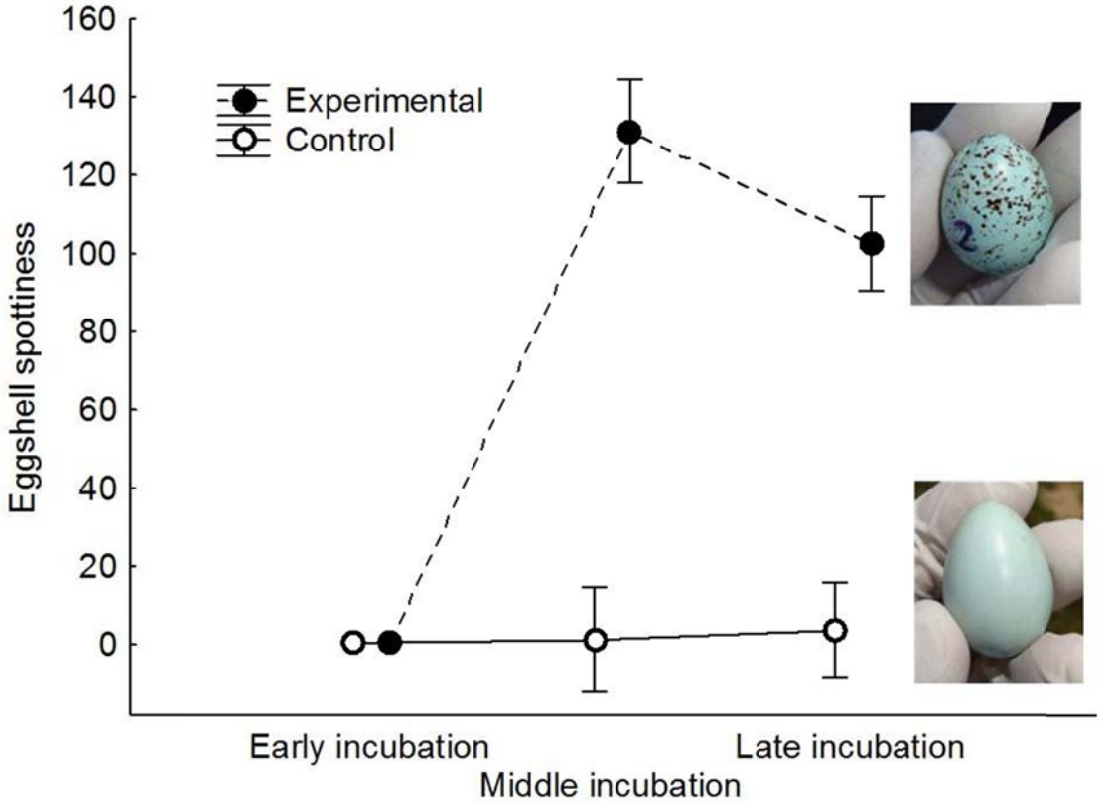

160
140
120
100
80
60
40
20
0
Eggshell spottiness
Experimental
Control
Early incubation
Middle incubation
Late incubation

Figure 3

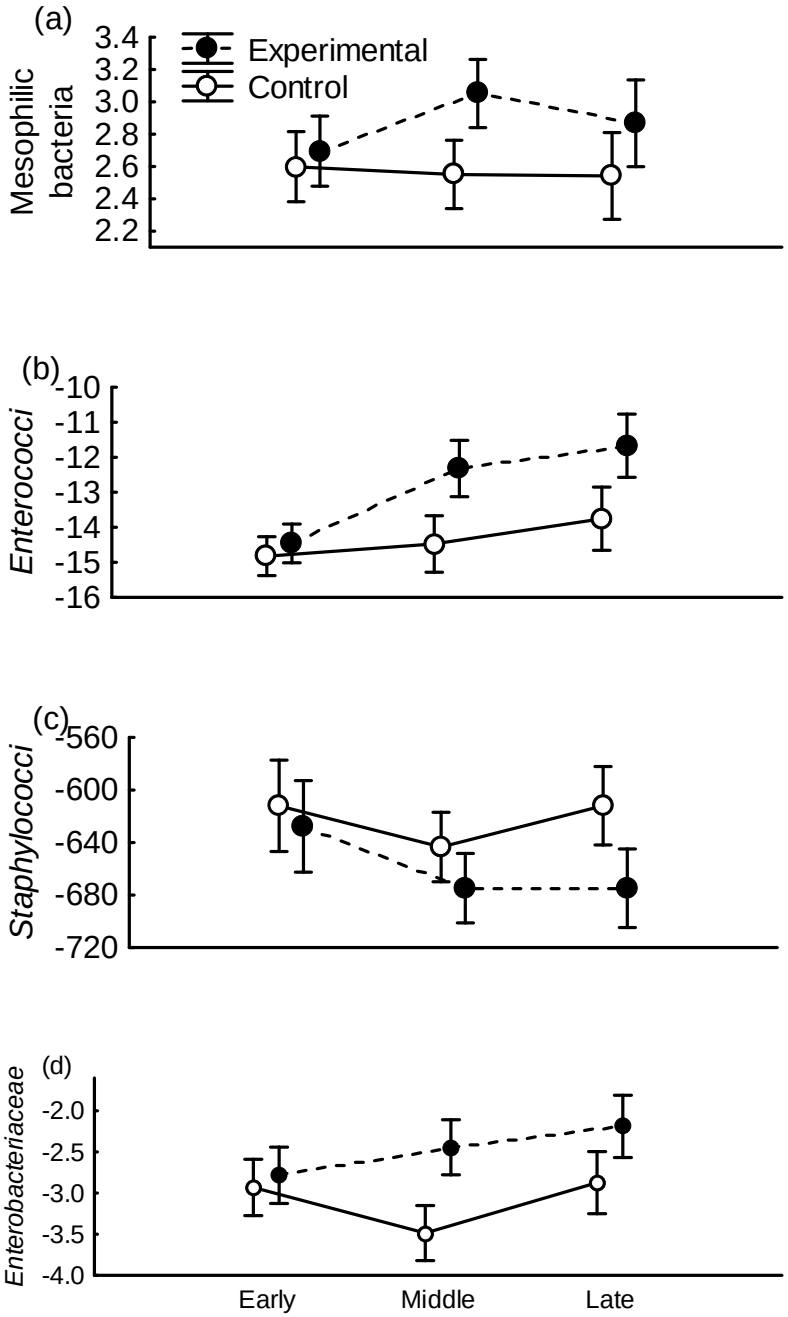
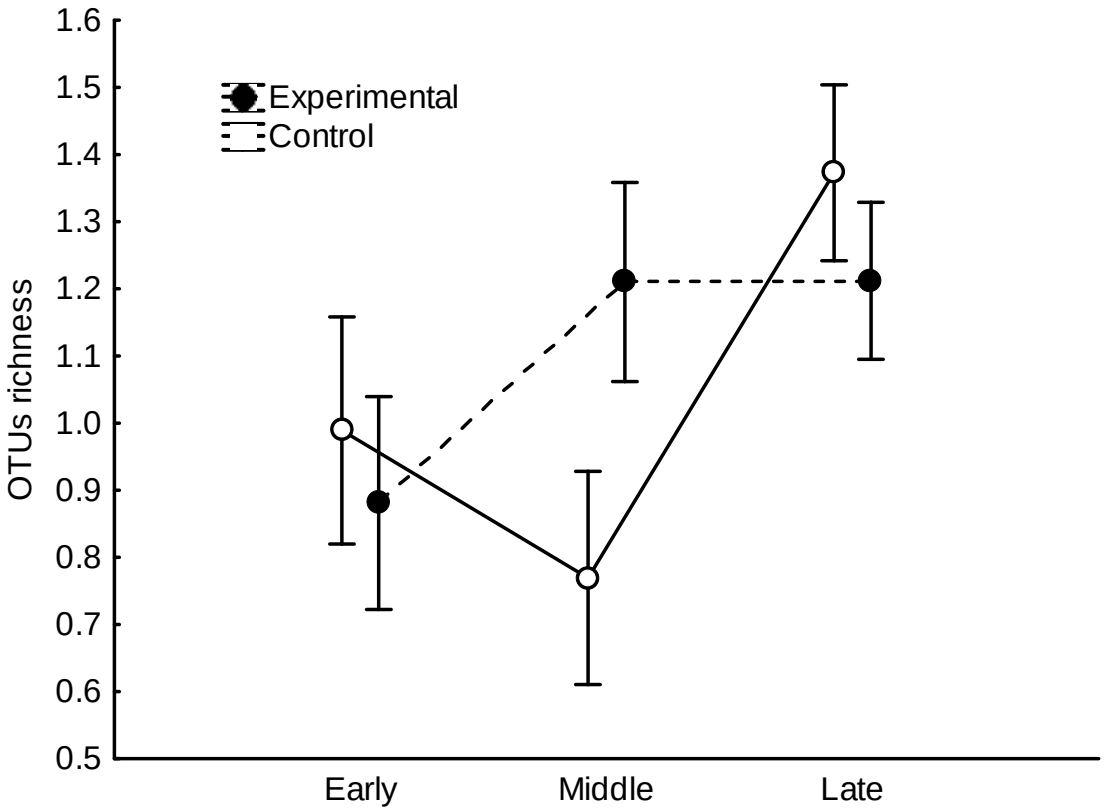

(a)
Mesophilic bacteria
3.4
3.2
3.0
2.8
2.6
2.4
2.2
Experimental
Control
(b)
Enterococci
-10
-11
-12
-13
-14
-15
-16
(c)
Staphylococci
-560
-600
-640
-680
-720
(d)
Enterobacteriaceae
-2.0
-2.5
-3.0
-3.5
-4.0
Early
Middle
Late

Figure 4


1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
OTUs richness
Experimental
Control
Early
Middle
Late