

Laying date, incubation and egg breakage as determinants of bacterial load on bird eggshells: experimental evidence

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Abstract Exploring factors guiding interactions of bacterial communities with animals has become of primary importance for ecologists and evolutionary biologists during the last years because of their likely central role in the evolution of animal life history traits. We explored the association between laying date and eggshell bacterial load (mesophilic bacteria, *Enterobacteriaceae*, *Staphylococci*, and *Enterococci*) in natural and artificial magpie (*Pica pica*) nests containing fresh commercial quail (*Coturnix coturnix*) eggs. We manipulated hygiene conditions by spilling egg contents on magpie and artificial nests and explored experimental effects during the breeding season. Egg breakage is a common outcome of brood parasitism by great spotted cuckoos (*Clamator glandarius*) on the nests of magpie, one of its main hosts. We found that the treatment increased eggshell bacterial load in artificial nests, but not in magpie nests with incubating females, which suggests that parental activity prevents the proliferation of bacteria on the eggshells in relation to egg breakage. Moreover, laying date was positively related to eggshell bacterial load in active magpie nests, but negatively in artificial nests. The

results suggest that variation in parental characteristics of magpies rather than climatic variation during the breeding season explained the detected positive association. Because the eggshell bacterial load is a proxy of hatching success, the detected positive association between eggshell bacterial loads and laying date in natural, but not in artificial nests, suggests that the generalized negative association between laying date and avian breeding success can be, at least partially, explained by differential bacterial effects.

Keywords Brood parasitism · Climate change · Life history traits · Nest characteristics · Parental activity

Introduction

We live in a bacterial world and exploring factors guiding interactions between bacterial communities and animals has become of primary importance for ecologists and evolutionary biologists during the last years (McFall-Ngai et al. 2013). The bacterial environment has traditionally been considered an important selective force acting on offspring viability in birds (Baggott and Graeme-Cook 2002; Mennerat et al. 2009; Soler et al. 2012), and has likely played a central role in the evolution of many animal life history traits, some of them directed at reducing the probability of bacterial infection (Cook et al. 2005a; Peralta-Sánchez et al. 2012; Møller et al. 2013).

Temperature, humidity and hygiene conditions in nests are known to determine bacterial colonization and growth on the eggshells of birds and hence trans-shell bacterial infection of embryos (Bruce and Drysdale 1991, 1994; Cook et al. 2003; Godard et al. 2007). Particular nest attributes such as nest location or nesting materials protect and insulate developing offspring from climatic environmental

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conditions (Hansell 2000) and can affect the bacterial environment of nests. Thus, green aromatic plants (Clark and Mason 1985; Mennerat et al. 2009; Møller et al. 2013) and/or feathers (Soler et al. 2010; Peralta-Sánchez et al. 2010, 2011, 2014) employed in nest building may confer direct defensive properties against bacterial infection. Egg incubation also contributes to the protection of developing offspring from the environment, given its effect in reducing humidity which otherwise favours eggshell bacterial colonization and may compromise embryo viability (Cook et al. 2003; D'Alba et al. 2010). However, incubation or nest-insulating properties of nest-building material do not fully counteract climatic environmental conditions as shown by comparisons of the influence of incubation on eggshell bacterial loads and/or embryo viability in tropical (Cook et al. 2005a; Shawkey et al. 2009) and temperate areas (Wang et al. 2011; Lee et al. 2014). Thus, variation in climatic conditions likely affects the bacterial environments of avian nests.

In temperate areas, the breeding success of birds typically decreases as the season progresses (Price et al. 1988; Moreno 1998). The association between laying date and breeding success has traditionally been explained as a consequence of the seasonal decline in resource availability for offspring and parents, and/or because parents of poorer phenotypic quality reproduce later (Wardrop and Ydenberg 2003; De Neve et al. 2004; Verhulst and Nilsson 2008). However, because temperature and humidity typically increase and decrease, respectively, as the season progresses, the associated variation in bacterial environment during the breeding season might also contribute to an explanation of the lower reproductive success of late breeders. In addition, the poorer phenotypic quality of late breeders might per se affect the bacterial environment of nests if, for instance, they construct less well-insulated or less defensive nests, or are less efficient in maintaining appropriate hygiene conditions of nests. These two scenarios therefore lead to the prediction that laying date and bacterial environment of nests should be related in nature.

We know that selection pressure due to parasitism increases as the season progresses, affecting development of the immune system of offspring as well as the strength of their immune response (Sorci et al. 1997; Saino et al. 1998; Merino et al. 2000; Soler et al. 2003; Martín-Vivaldi et al. 2006). Here, we argue that breeding time should also affect bacterial environmental conditions of nests, which should contribute to an explanation for the frequently observed seasonal decline in reproductive success of birds. Most bird species have advanced their breeding dates due to climate change (Gordo and Sanz 2006), phenological changes that may affect reproductive success (Visser and Both 2005; Saino et al. 2011) and population trends (Reif et al. 2008) of some species. Thus, support for our

hypothesis may suggest a role for bacteria which explains the reduced breeding success of birds associated with climate change and delayed breeding date (Soler et al. 2014).

As far as we know, this hypothesis has never been previously considered. Trying to fill this gap, we explore the association between laying date and eggshell bacterial load in magpie (*Pica pica*) nests and in artificial nests made of magpie nest-lining material and containing fresh commercial quail (*Coturnix coturnix*) eggs. Moreover, simulating the effects of brood parasitism by great spotted cuckoos (*Clamator glandarius*) (see below) we manipulated the hygiene conditions of magpie and artificial nests by breaking and spilling the contents of quail eggs, and explored possible differential effects of this manipulation on eggshell bacterial loads during the breeding season. As a proxy of nest bacterial environments and risk of embryo infection we estimated the density of mesophilic bacteria on the eggshells of magpies before and after incubation started, and of experimental quail eggs 4 days after the experimental spilling of egg contents on eggs in artificial nests. The prevalence of *Enterobacteriaceae*, *Staphylococcus* sp., and *Enterococcus* sp. in specific culture media was also estimated on eggshells as indicative of the probability of egg contamination. These three groups of bacteria included pathogenic strains; their densities on avian eggshells have been used previously as proxies of the probability of embryo infection (Board and Tranter 1986; Kozłowski et al. 1989; Bruce and Drysdale 1991, 1994; Houston et al. 1997; Cook et al. 2003, 2005a, b; Soler et al. 2008, 2011; Shawkey et al. 2009; Peralta-Sánchez et al. 2010).

Although eggs include abundant antibacterial chemicals (Board et al. 1994; Bonisoli-Alquati et al. 2010; Saino et al. 2002), egg contents are prime nutrients for bacterial growth (Stadelman 1994). Thus, we predicted a positive effect of experimental spilling of egg contents on eggshell bacterial load (prediction 1; P1). Manipulating hygiene conditions by egg breakage and spilling egg contents on eggs in magpie and artificial nests has the additional advantage of enabling the experimental testing of consequences for bacterial environments of magpie nests of the egg-breaking behaviour of the great spotted cuckoo (*Clamator glandarius*), the brood parasite of magpies (Soler et al. 1997). We have previously shown that magpie eggshells harboured higher bacterial density in nests parasitized by cuckoos, and that within the same parasitized nests bacterial density of great spotted cuckoo eggshells was lower than that of magpie eggshells (Soler et al. 2011). These results were interpreted as consequences of poorer hygiene conditions in parasitized nests due to egg breakage and egg-content spilling of magpie eggs, which would select for eggshell characteristics in cuckoos which limit bacterial contamination and growth. The experiment performed here allows one to test the influence of egg-content spilling on the eggshell bacterial load of magpies.

Temperature increase and humidity decrease as the season progresses in temperate areas should affect eggshell bacterial loads in artificial and natural magpie nests. As humidity is a main factor explaining eggshell bacterial proliferation (D'Alba et al. 2010), we should find that eggshell bacterial loads in artificial and natural magpie nests should decrease as the season progresses (prediction 2; P2). Moreover, because the effect of temperature and humidity on the bacterial environment should depend on nutrient availability for bacterial growth, the predicted association between laying date and eggshell bacterial loads should depend on the experimental treatment (i.e. spilling of egg contents). If this were the case, significant interactions between laying date and experimental treatment are predicted both for artificial and natural magpie nests (prediction 3; P3).

If adult phenotypic condition and abilities (i.e. incubation activity and nest sanitation and maintenance) are important determinants of bacterial proliferation in bird nests, the influence of laying date and of experimental treatment on eggshell bacterial loads should vary for artificial (unattended) and natural magpie nests (prediction 4; P4). Magpie incubation activity might ameliorate the effects of climatic conditions on bacterial proliferation on the eggs and, thus, the effects of experimental treatment and laying date should be less obvious in natural magpie nests (P4a). Furthermore, because nest sanitation is designed to combat parasite infections and is an important activity of breeding birds (Christe et al. 1996; Ibáñez-Álamo et al. 2014), the effect of the experimental treatment of egg contents on the bacterial environment, or on the strength of the interaction with laying date, should be reduced in natural magpie nests (P4b). In addition, if the hygiene conditions of magpie nests (i.e. bacterial environment) are determined by the phenotypic quality of adult birds through differences in nest-sanitation ability and/or reproductive investment, we may even find a positive association between laying date and eggshell bacterial loads. Finding evidence of such an association would suggest that the generally lower breeding success of late reproductive attempts may be partially driven by differential bacterial selection pressures mediated by adult quality, rather than by climate-related environmental conditions at the nests.

Materials and methods

Study area

The study was performed during the breeding seasons of 2011–2012 in southeast Spain, in the Hoya de Guadix (37°18'N, 3°11'W), a high-altitude plateau (1000 m a.s.l.), dominated by a semi-arid climate. The typical vegetation in the area is cultivated crops, olive and almond plantations; a

few holm oaks remain from the original Mediterranean forest, there are small shrubs in abandoned fields, and deciduous trees by streams and in villages. The magpie population comprises several subpopulations, some of them in irrigated and others in arid environments (De Neve et al. 2007). We sampled two of these subpopulations, 15–20 km from each other, one in irrigated (Albuñán) and one in an arid environment (Carretera). The probability of brood parasitism of magpie nests by the great spotted cuckoo is quite high in the area, but temporally and spatially variable at the small geographic scale of the study area (Soler et al. 1999, 2013; Soler and Soler 2000; Martín-Gálvez et al. 2007).

Field work

Magpie territories known from previous years were visited once a week from 15 of March to detect new nests. Once we found a new nest, it was visited twice a week, which allowed us to know the laying date of the first egg and to detect brood parasitism. The laying date of sampled nests in 2011 extended from 3 April to 12 May, and in 2012 from 31 March to 12 May (average laying date for both years was 19 April).

For eggshell bacterial sampling, we wore new latex gloves sterilized with 96 % ethanol for each nest to prevent inter-nest contamination. Once gloves were dry, we gently handled and sampled eggs by rubbing the complete eggshell with a sterile rayon swab (EUROTUBO DeltaLab) slightly wetted with sterile sodium phosphate buffer (0.2 M, pH = 7.2). After cleaning the complete egg surface, the swab was introduced into a rubber-sealed microfuge tube with 1.2 ml of sterile phosphate solution and transported in a portable refrigerator at 4–6 °C. Samples were stored at 4 °C until being processed in the laboratory within 24 h after collection. Estimates of bacterial load were standardized to the number of colonies (colony-forming units; CFUs) per square centimetre (i.e. eggshell bacterial density) as previously described (Soler et al. 2011).

Experimental procedures

Natural magpie nests

Each of the magpie nests found before incubation started was randomly assigned to one of the following three experimental treatments:

- (a) Experimental nests, where a broken quail egg was included in the nest. The experimental quail egg was broken inside the magpie nest by making a hole large enough to assure that most of the contents spilled out when we rolled it together with all other eggs in the nest. In this way, the gloves worn when moving the

eggs become smeared with egg contents, which ensures that the entire magpie egg surface comes in contact with the egg contents, either because of direct contact with quail eggshell or because it is gently touched with smudged gloves.

- (b) Control I nests, where a non-broken quail egg was included in the magpie nest and moved around as in the experimental treatment. Quail eggs were cleaned with disinfectant wipes (998077-51EN, Aseptonet; Sarbec) before they were put into magpie nests.
- (c) Control II nests, which were visited and sampled at the same rate as nests in other treatments, but no quail egg was added.

On average, magpies start to incubate when laying the fourth egg, but occasionally with the third egg, or incubation may even be delayed up to the seventh egg (Birkhead 1991). Bacteria from eggshells of experimental and control nests were sampled three times. The first samples were collected 0–5 [mean (SE) = 2.3 (0.04), $n = 236$] days after laying of the first egg (i.e. before incubation started—cool eggs with no sign of incubation); the second samples were collected 4–5 days after the first sampling, i.e. day 5–8 [mean (SE) = 6.2 (0.03), $n = 220$] after laying the first egg (i.e. after incubation started—warm eggs with signs of incubation). The third samples were collected 14–19 days [mean (SE) = 17.1 (0.06), $n = 100$] after the first egg was laid (i.e. before hatching). Magpie eggs which were broken or had traces of egg content spilling were detected in 28.2 % ($n = 220$) of the magpie nests sampled after incubation started, most of them in nests where cuckoo egg(s) were also found (66.1 %, $n = 62$). Bacterial loads of magpie eggshell in parasitized and non-parasitized nests with traces of egg-content spilling were used to explore the effect of natural egg breakage on eggshell bacterial load (see below). During each visit, we numbered all new eggs with an indelible marker and sampled a single egg per nest that had not been sampled in previous visits. Whenever possible, all three sampled eggs per nest in respective visits were within the first four eggs in the laying sequence.

Some of the quail eggs introduced into magpie nests as a control treatment were not rejected by magpies and, thus, we were able to sample incubated quail eggs in natural magpie nests during the second visit. Total eggshell bacterial loads of these eggs did not differ from those of magpie eggs in the same nests (Wilcoxon matched pairs test, $Z = 1.12$, $P = 0.26$, $n = 47$), which supports the use of quail eggs in artificial nests (see below).

Artificial magpie nests

Artificial nests were constructed with nest-lining material (thin roots and grass) collected from eight new magpie nests

before laying, and were assembled in plastic bags and used to cover the bottom of bird cages ($15 \times 30 \times 20$ cm). These cages were used to prevent predation while exposing experimental eggs to environmental climatic conditions. Sixteen bird cages were fastened at a height of 1–2 m in almond and pine trees spread over the study areas of the two magpie sub-populations. Seven of these cages were in the arid zone and nine in the irrigated zone. One hundred and seven pairs of quail eggs, one experimental and one control per pair, were placed on the nest material inside experimental cages during the main egg-laying period of our studied magpie population (from 20 April to 19 May) homogeneously (at least one pair of eggs every second day and an average of 2.4 pairs of eggs per day). Before introduction into the cage, eggs were cleaned with disinfectant wipes. Afterwards, the control egg was gently handled with gloves cleaned with ethanol and laid on the nest material of the experimental cages, whilst the experimental egg was handled with the sample gloves but soiled with the contents of a broken quail egg. Thus, the experimental but not the control egg was coated with egg contents. Experimental and control eggs within the same cage were not in contact with each other. A new pair of eggs was added to the experimental cages every 4 days; the same cage harboured up to four pairs of experimental eggs. None of the eggs were in direct contact with each other.

Each visit we sampled half of the egg surface. Briefly, with an indelible marker, we painted a line from one egg pole to the other, in this way dividing the egg surface into two halves; one of the halves was sampled 4 days after the experiment. The non-sampled surfaces of 53 pairs of eggs were sampled 16 days after the onset of the experiment. We failed to analyse samples from five control and three experimental eggs collected from seven different egg pairs 4 days after the onset of the experiment. These losses were due to the breakage of quail eggs during sampling or because samples disappeared before being analysed in the lab. Thus, we obtained a final sample of 100 pairs of eggs for the analyses.

Laboratory work

Before cultivation, samples stored in microfuge tubes were shaken in a vortex (Boeco V1 Plus) for at least three periods of 5 s. Bacteriology was performed by spreading homogeneously 100 μ l of serially diluted samples onto Petri dishes containing four different solid agar media (ScharlauChemie, Barcelona). We used tryptic soy agar, a broadly used general medium to grow aerobic mesophilic bacteria, and three specific media: Kenner faecal agar for *Enterococcus*; Vogel–Johnson agar for *Staphylococcus*; and Hektoen enteric agar for *Enterobacteriaceae*. The plates were incubated aerobically at 37 °C and colonies were counted 72 h after inoculation. Bacterial density was estimated for each of the four

media as the number of CFUs per square centimetre following a previously described protocol (Peralta-Sánchez et al. 2010; Soler et al. 2011). We estimated eggshell bacterial density for all samples collected during magpie egg laying, at the onset of incubation, and at the end of incubation, and for samples obtained from quail eggs.

Statistical analyses

Log₁₀-transformed density of mesophilic bacteria differed from the normal distribution and we conservatively used ranked values for statistical analyses. Specific groups of bacteria (*Enterobacteriaceae*, *Staphylococcus* and *Enterococcus*) were not detected for many samples (see “Results”) and, thus, frequency distributions were far from Gaussian shaped. Thus, we used information on the prevalence of each bacterial group in the analyses. To statistically account for inter-year variation in laying date, values for each date were standardized by deducting observed from mean values and dividing by the SD. We used these values in subsequent analyses.

The expected effects of having experimental or naturally broken eggs [and/or traces of egg contents (i.e. yolk)] and of laying date on magpie eggshell bacterial loads were analysed by repeated-measures ANOVAs (RMA) with ranked values of mesophilic bacterial loads estimated at different visits (egg laying, onset and end of incubation) as the repeated measures, experimental treatment (having or not having a trace of natural egg breakage), area (irrigated or arid), and year as factors between repeated measures, and standardized laying date as a covariate. Because the association with laying date may depend on experimental treatment (or on egg breakage), we estimated the effect of such interaction in separate models. The effect of experimentally coating commercial quail eggs on eggshell bacterial loads (i.e. the ranked value of mesophilic bacterial density) was explored by RMA with the pair of eggs of the same laying date (experimental and control eggs) as repeated measures, area (irrigated or arid) as discrete between factor, and laying date as a covariable. The prevalence of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus*, and *Enterococcus* in relation to experimental treatment (or natural egg breakage), year (only for natural magpie nests), area, and laying date, were analysed by means of generalized linear models with binomial distribution and logit link functions.

Some of the experimental and natural magpie nests were depredated during incubation or were heavily parasitized by the great spotted cuckoo and, thus, sample size for the third bacterial sampling (i.e. at the end of the incubation) was reduced. However, main effects were detected independently of whether or not information about these third samples was considered. Thus, we report results of models explaining prevalence and bacterial density estimated for

the first and second samplings because of the higher statistical power.

Log₁₀-transformed bacterial densities rather than ranked values were used for figures. All statistical tests were performed in Statistica 10.0 (Statsoft 2011).

Results

Bacterial loads of magpie eggshells

Effects of natural and experimental breakage of eggs in the nest

Contrary to P1, the occurrence of broken eggs in magpie nests due to brood parasitic activity did not affect the density of mesophilic bacteria on magpie eggshells, which was mainly explained by study area (it was higher in the arid subpopulation) (Table 1). Moreover, laying date was positively associated with the density of mesophilic bacteria (Fig. 1), which is contrary to our P2, and the effect of incubation activity depended on the interaction between the study year and the study area (Table 1). In no case did we detect support for the predicted (P3) interaction between experimental treatment and laying date (Table 1).

Results for magpie nests where quail eggs were experimentally broken were quite similar to those with natural eggs broken by brood parasites (Table 1). First, we compared eggshell bacterial load between the two types of control nests, with a non-broken quail egg and without a quail egg, and failed to detect statistically significant differences (identical model to those used in Table 1; effect of treatment, $F = 2.27$, $df = 1,116$, $P = 0.135$). Moreover, the interaction between experimental treatments of the two types of control nests and all other factors did not reach statistical significance ($P > 0.7$). Thus, we considered all control nests together for subsequent analyses. We detected an increase in bacterial density after incubation (Fig. 2), a significantly lower bacterial density of eggs sampled in 2012 and in irrigated areas, and a significant interaction between the study year and the study area (interaction in Table 1; Fig. 2). The only detected effect of experimental treatment was indirect, through its interaction with the year, area, and incubation (Table 1; Fig. 2). These results did not change after removing from the model the non-significant terms (results not shown). Finally, and contrary to P2 and P3, the density of bacteria for this subset of nests did increase as the season progressed (Table 1; Fig. 1) independently of experimental treatments.

Analyses on the prevalence of different groups of bacteria offered similar results. Prevalences of mesophilic bacteria and of *Enterobacteriaceae* were positively associated with laying date (Table 2). For nests with traces of natural

Table 1 Repeated-measures ANOVA explaining variation in density of mesophilic bacteria (ranked values) of magpie eggshells in natural magpie nests before and after incubation started in relation to laying date (standardized values accounting for year variation), study year,

	Incubation	Laying date	Year (1)	Area (2)	Egg breakage (3)	(1) × (2)	(1) × (3)	(2) × (3)	(1) × (2) × (3)	Laying date × (3)
Naturally broken eggs, repeated measures (before vs. after incubation)										
Between effects										
<i>F</i> (1,130)		23.55	2.38	6.02	0.13	0.14	0.20	0.07	0.31	0.51
<i>P</i>		<0.0001	0.125	0.015	0.716	0.711	0.657	0.792	0.579	0.473
Within effects										
<i>F</i> (1,130)	1.39	0.03	1.02	0.32	0.10	4.84	1.61	0.40	0.78	0.07
<i>P</i>	0.241	0.854	0.314	0.570	0.758	0.030	0.206	0.527	0.379	0.793
Experimentally broken eggs, repeated measures (before vs. after incubation)										
Between effects										
<i>F</i> (1,176)		30.40	24.99	26.51	0.82	7.07	0.01	1.90	0.16	0.106
<i>P</i>		<0.0001	<0.0001	<0.0001	0.366	0.009	0.928	0.170	0.690	0.745
Within effects										
<i>F</i> (1,176)	5.47	0.28	0.12	0.50	0.05	1.50	0.96	0.30	4.01	0.904
<i>P</i>	0.020	0.594	0.726	0.480	0.819	0.222	0.328	0.587	0.047	0.343

First- and second-order interactions were included in the models and elimination of non-significant terms did not qualitatively affect results. The interaction between broken eggs and the covariable, laying date, was estimated in separate models

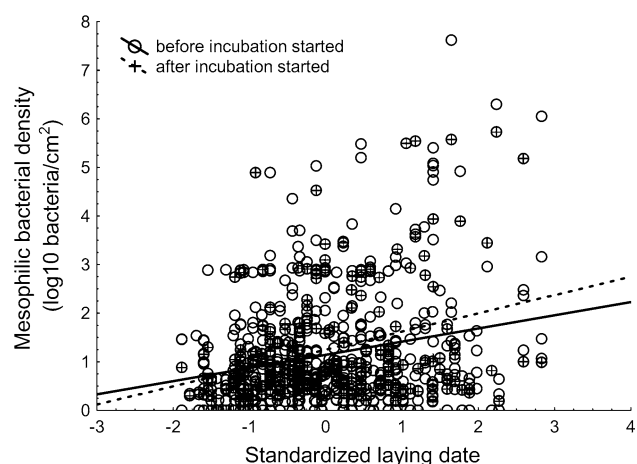


Fig. 1 Relationship between laying date and density of mesophilic bacteria on eggshells estimated before (*open circles and continuous regression line*) and after (*circles with crosses and dotted regression line*) the onset of incubation in magpie nests

egg breakage, the prevalence of *Enterobacteriaceae* varied among the years (Table 2), being more frequent in 2011 (13 out of 59 nests) than in 2012 (three out of 70 nests). Traces of egg breakage on the sampled eggshells increased the probability of *Enterococcus* detection (three out of 14 nests with traces of egg breakage vs. one out of 125 nests with no trace of egg breakage), but did not affect the prevalence of the other considered bacteria (Table 2). In no case did we detect support for the predicted (P3) interaction between experimental treatment and laying date.

study area, and whether or not experimentally or naturally broken eggs, or traces or egg contents due to brood parasitism activity, were detected or experimentally provoked

There were very similar results when we considered natural nests with no detected egg breakage that were subjected to the experimental inclusion of broken quail eggs in the nest (Table 2). The experiment only affected the prevalence of *Enterococcus* positively, while laying date was positively associated with the prevalence of mesophilic bacteria and of *Enterobacteriaceae* (Table 2). Therefore, the former result was in accordance with P1 and the latter was contrary to P2. The loads of *Enterobacteriaceae* on the eggshell of magpies varied for different years. In not one case did we detect support for the predicted (P3) interaction between experimental treatment and laying date.

Taken together, these results suggest limited effects of egg breakage on the bacterial density and prevalence on incubated magpie eggshells. They also indicate that eggshells of late-breeding magpies harboured bacteria at a higher density and prevalence than early-breeding magpies, suggesting that the low environmental humidity of nests is not the main determinant of the seasonal changes in the bacterial load of magpie eggshells.

Bacterial loads of quail eggshells in experimental artificial nests

Eggshell mesophilic bacterial load was higher in the arid than in the irrigated area (RMA, $F = 25.59$, $df = 1,97$, $P < 0.0001$) and, contrary to what we detected for natural magpie nests, but in accordance with P2, eggshell bacterial loads of quail eggs decreased as the season progressed

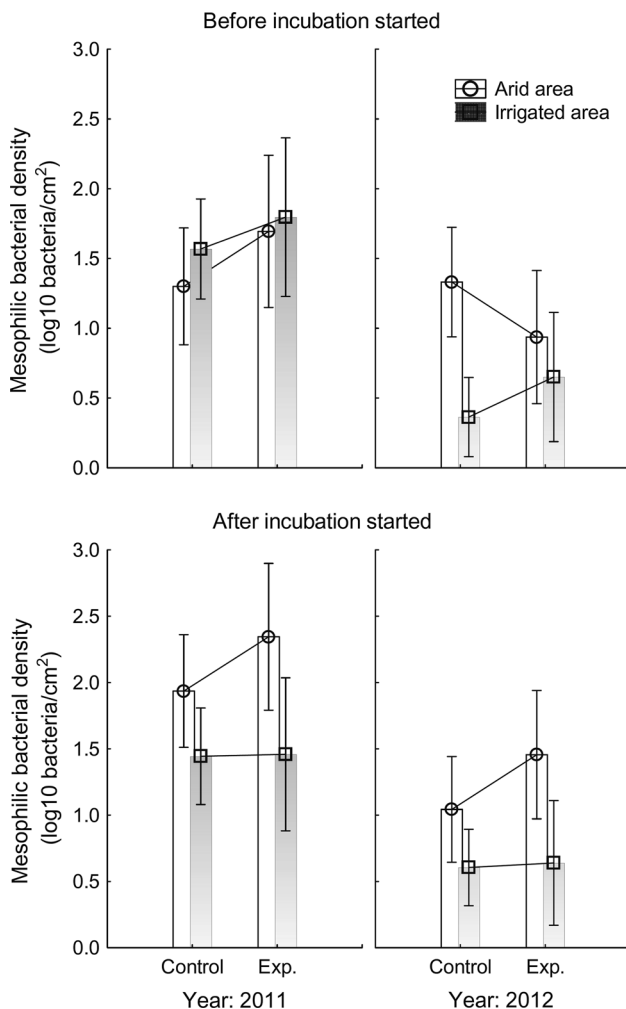


Fig. 2 Average [± 95 % confidence intervals (CI)] mesophilic bacterial loads of magpie eggshells before and after incubation during the 2 study years at the two study areas. Values for magpie nests with (*Exp.*) and without (*Control*) experimental broken quail eggs added are also shown

(RMA, $F = 10.98$, $df = 1,97$, $P = 0.0013$). In accordance with P1, experimental eggs coated with egg contents harboured bacteria at a higher density than control eggs (RMA, $F = 13.65$, $df = 1,97$, $P = 0.0004$) (Fig. 3a). Interestingly, the effect of the experiment on the density of mesophilic bacteria did not depend on the area (RMA, interaction between experimental treatment and area, $F = 1.99$, $df = 97$, $P = 0.162$), but the density of mesophilic bacteria tended to decrease as the season progressed, i.e. mainly on the surface of experimental eggs (RMA, interaction between experimental treatment and laying date, $F = 3.76$, $df = 1,98$, $P = 0.084$) (Fig. 3b); these results do not support P3.

Similar results were obtained when analysing bacterial prevalence. Laying date was significantly associated with the prevalence of mesophilic bacteria (negatively) and

of *Enterococcus* (positively) (Table 3). The prevalence of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus* was higher in experimentally smeared quail eggs than in control eggs (Fig. 4), and this effect did not depend on the area (Table 3). The experimental effects on prevalence of mesophilic bacteria varied depending on laying date (interaction term in Table 3), which supports P3. However, the effects of laying date on the prevalence and density of bacteria on eggshells depended on the considered bacterial group.

Remarks on results from artificial and natural magpie nests

The effects of experimental smearing with the egg contents of quail eggs on eggshell bacterial loads were detected in artificial but not in natural magpie nests, which is in accordance with P4a. The expected negative relationship between eggshell bacterial loads and laying date was only detected in artificial nests; this relationship was positive in natural magpie nests, which agrees with P4b.

Discussion

Our main results are that the experimental besmearing of eggshells with egg contents provokes an increase in eggshell bacterial density and prevalence in experimental non-active nests, but not in nests with magpies incubating eggs. Moreover, laying date was positively related to eggshell bacterial density and prevalence in active magpie nests, but negatively in artificial nests without incubation activity. Quail eggs were used in artificial nests and, thus, detected differences between artificial and natural magpie nests could be explained by differences in eggshell properties between magpies and quail eggshells. However, this possibility is unlikely since magpie and control quail eggs in natural magpie nests harboured a similar bacterial density some days after incubation (see “Materials and methods”).

Therefore, these two results suggest on the one hand that the incubating activity of magpies prevents the proliferation of bacteria on the eggshells in relation to egg breakage and spilling of egg contents. On the other hand, these results imply that the positive association between laying date and eggshell bacterial density or prevalence was due to particularities of nest-attending magpies rather than to climatic environmental conditions (i.e. temperature and humidity) favouring bacterial growth. Below we discuss these and some other possible alternative scenarios explaining our results and their importance for understanding the role of environmental conditions and parental influence as determinants of bacterial environments of nests and the probability of bacterial infection.

Table 2 Results from generalized linear models (GLM) with binomial distribution and logit link function explaining the prevalence of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus*, on magpie eggshells in control nests with and without naturally broken eggs detected (i.e. control magpie nests)

	Control magpie nests ($n = 139$)				Experimental vs. control nests ($n = 185$)			
	Wald statistic	Estimated CI (95 %)	Estimated -CI (95 %)	P	Wald statistic	Estimated CI (95 %)	Estimated -CI (95 %)	P
Mesophilic bacteria^a								
Laying date (1)	8.25	0.620	3.281	0.0041	10.29	0.677	2.803	0.0013
Year	0.94	-0.396	1.168	0.3331	2.31	-0.162	1.282	0.1283
Area								
Broken eggs (2)	2.44 ^a			0.1184	0.62	-1.060	0.451	0.4296
(1) × (2)					0.51	-0.700	1.499	0.4766
<i>Enterobacteriaceae</i>								
Laying date (1)	8.11	0.272	1.471	0.0044	16.22	0.670	1.940	<0.0001
Year	9.34	0.386	1.767	0.0022	14.61	0.616	1.912	0.0001
Area	0.01	-0.577	0.630	0.9321	0.01	-0.511	0.564	0.9237
Broken eggs (2)	0.23	-0.754	1.239	0.6348	0.03	-0.623	0.520	0.8598
(1) × (2)	1.14	-0.334	1.133	0.2860	1.76	-1.274	0.245	0.1845
<i>Staphylococci</i>^b								
Laying date (1)	0.21	-0.811	1.311	0.6441	<0.01	-0.778	0.747	0.9687
Year	0.43	-1.243	0.618	0.5105	0.18	-0.908	0.585	0.6717
Area	2.89	-2.086	0.148	0.0891	4.86	0.133	2.263	0.0274
Broken eggs (2)	1.08			0.2982	<0.01	-0.747	0.757	0.9893
(1) × (2)					0.59	-1.078	0.469	0.4408
<i>Enterococci</i>^c								
Laying date (1)	0.05	-1.270	1.020	0.8306	2.26	-0.187	1.415	0.1330
Year	1.11	-1.918	0.577	0.2924	0.74	-1.299	0.508	0.3909
Area					2.99	-0.130	2.071	0.0839
Broken eggs (2)	4.92	0.160	2.594	0.0266	4.84	-2.315	-0.134	0.0277
(1) × (2)	<0.01	-1.171	1.196	0.9830	0.61	-1.663	0.714	0.4337

Results from comparisons of magpie nests with and without a broken quail egg added (i.e. experimental vs. control nests) are also shown (only nests without traces of natural egg breakage were considered here). The model included laying date (standardized values accounting for year variation) as a covariable and study year, study area, and whether or not broken eggs (i.e. experimental treatment) or traces of egg contents due to brood parasitism activity were detected (*Broken eggs*) as discrete independent factors. Due to the low prevalence of most bacteria we did not test for all of them but only for the interaction between broken eggs and laying date, which was included in the models but estimated separately

^a Mesophilic bacteria were absent in only eight out of 139 non-manipulated magpie nests; all of these were from the same study area and had no remains of broken eggs. Similarly, for nests with experimentally broken quail eggs, mesophilic bacteria were absent in three out of 60 nests, all of them from the same study area. Thus, the effect of study area, and of egg breakage or the interaction between laying date and egg breakage, cannot be estimated in the GLM model. Rather we estimated the effect of egg breakage in separate log-linear models

^b *Staphylococci* bacteria were detected in five natural magpie nests, none of them with remains of broken eggs. Thus the effect of egg breakage or the interaction with laying date cannot be estimated in the GLM model. Rather we estimated the effect of egg breakage in separate log-linear models

^c *Enterococci* were only detected in four natural magpie nests from the same study area. Thus it was not possible to estimate the effect of study area in the GLM model

We knew that brood parasitism by great spotted cuckoos was positively related to bacterial load of magpie eggshells, which among other possibilities, was attributed to the egg-breaking behaviour of cuckoos often resulting in egg-content spilling (Soler et al. 2011). Here, we found no experimental support for this hypothesis. However, experimental coating of quail eggs with egg contents did result in a significant increase in eggshell bacterial loads and prevalence

4 days after the manipulation. Therefore these two main results suggest that egg-breaking behaviour of cuckoos provoking egg-contents spilling should affect eggshell bacterial loads of their magpie hosts, but that the effect is at least partially counteracted by magpie females. The previously detected association between brood parasitism and eggshell bacterial loads of magpie eggs would therefore be the consequence not only of the egg-breaking behaviour

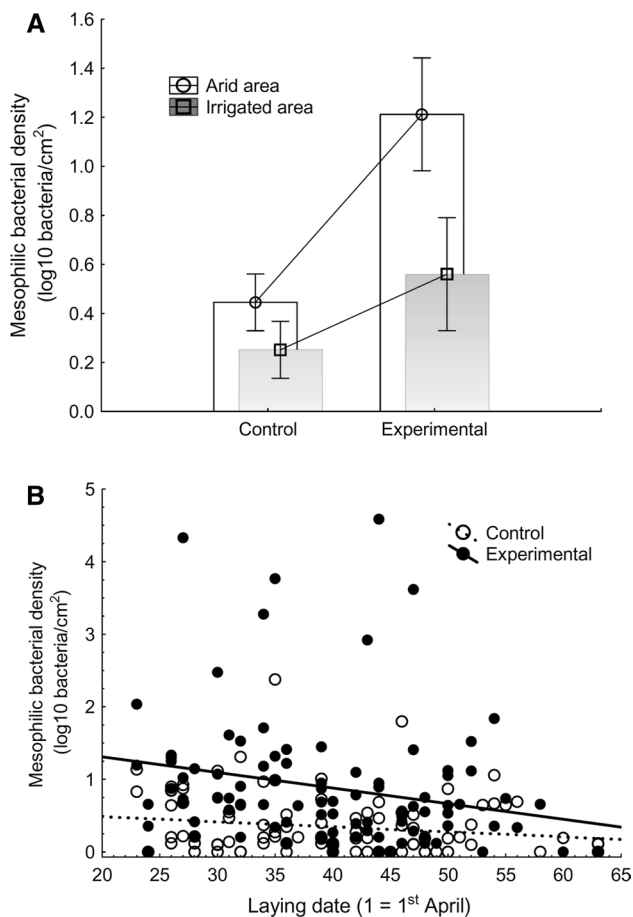


Fig. 3 Density (\pm CI 95 %) of mesophilic bacteria on shells of quail eggs maintained in bird cages in the study areas in relation to experimental treatment (**a**) and laying date (**b**). Experimental eggs were coated with egg contents 4 days before the estimation of bacterial loads. **b** Lines are regression lines

of cuckoos, but also of the input of bacteria from cuckoos onto the parasitic eggs. It can also be due to subsequent visits to magpie nests by the brood parasite (Soler et al. 2011).

Incubation or any other parental behaviour influencing the bacterial environment of nests (Clark and Mason 1985; Cook et al. 2005a; Mennerat et al. 2009; D'Alba et al. 2010; Soler et al. 2010; Lee et al. 2014) is likely a cause of the reduced experimental effects detected in natural magpie nests. Magpies do not use green aromatic plants or feathers in their nests for nest building in our study area and, thus, the antimicrobial properties of these materials (see "Introduction") cannot explain detected differences between artificial and natural magpie nests. However, belly feathers of magpies are unpigmented and, therefore, more degradable by keratinolytic bacteria with important antimicrobial activity (Peralta-Sánchez et al. 2010, 2014). These belly feathers are in contact with the eggshells and may reduce the growth of pathogenic bacteria (Lee et al. 2014). In addition, magpies build a quite apparent mud cup, and we

know of the antimicrobial properties of clays used in mud therapies (Said et al. 1980; Maigetter and Pfister 1975). For our artificial nests we used vegetable nest-lining material (i.e. roots), but not the mud cup of magpie nests. Thus, it is possible that, in addition to incubation activity, mud in the nests of magpies and/or white belly feathers of incubating females might account for the reduced experimental effects detected in natural nests, a hypothesis worth testing in the future.

The second main result is the detected association between laying date and eggshell bacterial load and prevalence. Also in this case the associations detected for magpie nests were contrary to those detected for artificial nests (Figs. 1, 3b), again suggesting an important role of magpie adults determining bacterial environments in nests. While in natural magpie nests the relationship between eggshell bacterial load and laying date was positive, in artificial nests the association was negative. Within the study area temperature increases (2011, $R = 0.774$, $n = 66$, $P < 0.0001$; 2012, $R = 0.497$, $n = 66$, $P < 0.0001$) and humidity decreases [2011, $R = -0.602$, $n = 66$, $P < 0.0001$; but not in 2012, $R = 0.120$, $n = 66$, $P = 0.33$ during the sampling period (average daily temperature and humidity from 1 April to 5 June; data from Consejería de Medio Ambiente y Ordenación del Territorio, <http://www.juntadeandalucia.es/medioambiente/servtc5/sica/Estaciones.jsp>, station: Guadix)]. Thus, our results may indicate a negative influence of temperature and a positive effect of humidity on eggshell bacterial colonization and growth in the absence of incubation. In nests with incubated eggs the association between laying date and eggshell bacterial load was the opposite and, thus, variation of environmental climatic conditions for breeding as the season progresses are unlikely the direct cause of the detected higher risk of bacterial infection experienced in late-breeding attempts of magpies. These results therefore reinforce the importance of parental attendance (including nest building) that protects the offspring from environments influencing bacterial colonization and growth.

Negative associations between laying date and different breeding parameters of birds reflecting breeding success such as clutch size, brood size, and fledging success, are normally found for birds reproducing in temperate areas (see "Introduction"). This association has been traditionally explained by deterioration of environmental conditions (i.e. decreasing and increasing availability of resources and the probability of parasitism, respectively) (Sorci et al. 1997; Siikamäki 1998; Merino et al. 2000; Verhulst and Nilsson 2008) and/or parental quality and adult condition as the season progresses (Hochachka 1990; Christians et al. 2001; Winkler et al. 2014). Our results suggest that the deterioration of the nest bacterial environment as the season progresses would contribute to an explanation for the reduced breeding success of late breeders, a possibility

Table 3 Results from GLMs with binomial distribution and logit link function explaining the prevalence of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus*, on the shells of experimental quail eggs

	Wald statistic	Estimate CI (95 %)	Estimate -CI (95 %)	P
Mesophilic bacteria				
Laying date (1)	4.00	0.001	0.079	0.0455
Area (2)	10.17	-1.255	-0.300	0.0014
Experiment (3)	7.88	0.206	1.163	0.0050
(2) × (3)	0.09	-0.552	0.403	0.7599
(1) × (3)	4.84	-0.098	-0.006	0.0284
Enterobacteriaceae				
Laying date (1)	0.29	-0.038	0.067	0.589
Area (2)	250.90	-5.016	3.911	<0.0001
Experiment (3)	158.13	4.266	5.842	<0.0001
(2) × (3)	— ^a			
(1) × (3)	0.15	-0.091	0.061	0.7000
Staphylococci				
Laying date (1)	2.10	-0.013	0.089	0.1471
Area (2)	2.48	-1.161	0.126	0.1150
Experiment (3)	6.34	0.183	1.466	0.0118
(2) × (3)	0.01	-0.668	0.615	0.9360
(1) × (3)	0.81	-0.039	0.107	0.3689
Enterococci				
Laying date (1)	7.82	-0.096	-0.017	0.0052
Area (2)	0.57	-0.292	0.657	0.4509
Experiment (3)	12.89	0.395	1.344	0.0004
(2) × (3)	1.83	-0.146	0.798	0.1763
(1) × (3)	2.49	-0.089	0.010	0.1144

The model included laying date [1 April (1)] as a covariable and whether or not the eggs were coated with the egg contents of a broken egg. The interaction between experimental treatment and laying date was included in the models, but estimated separately

^a *Enterobacteriaceae* only appeared in one of the study areas and the interaction could not be estimated

that has not been previously suggested. Variations in food availability and/or phenotypic condition of parents (including parasite infection status) would affect parental activity (Winkler and Allen 1996), including nest-building effort (Soler et al. 1995, 1998), incubation attendance (Chastel et al. 1995) and, perhaps, nest hygiene. All these activities potentially determine bacterial communities of nests, at least partially (see “Introduction”). Moreover, birds of poor phenotypic condition would harbour bacteria at a higher density (Møller et al. 2012) and infect nest contents during reproduction. Thus, the extensive theoretical background allowed us to predict positive covariation between the well-known, and widely accepted, seasonal decline in breeding success in temperate areas and nest bacterial environment.

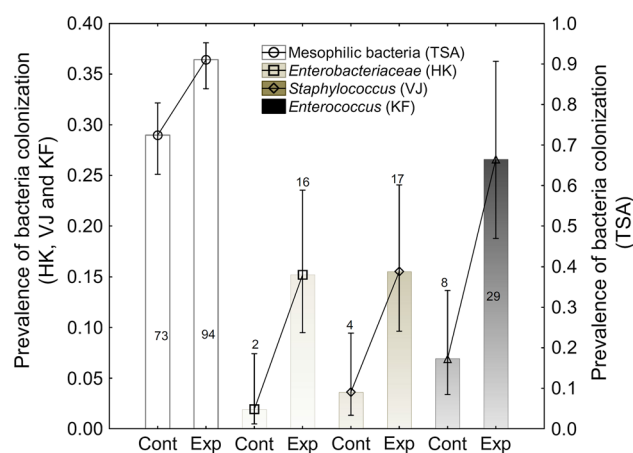


Fig. 4 Prevalence (\pm CI 95 %) of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus*, and *Enterococcus*, on experimental (Exp) and control (Cont) quail eggs. Experimental eggs were coated with egg contents 4 days before estimation of bacterial loads. Numbers of experimental and control quail eggs with bacteria detected are also shown (total control eggs = 102, total experimental eggs = 104)

Our results suggest that the seasonal increase of bacterial density may be caused by a decrease in nest parental attendance, which would suggest a role of bacteria in driving the seasonal decline in breeding success for which we have detected pioneering evidence. Experimental manipulation of factors affecting parental attendance (i.e. incubation) is, however, necessary to reach firm conclusions.

In summary, our experimental approaches allowed us to detect different dynamics in bacterial communities of eggshells in artificial and natural nests in relation to hygiene conditions, incubation activity and laying date. Since laying date was positively associated with bacterial density in natural, but not in artificial nests, we conclude that this association is mediated by parental characteristics, which suggests a central role for bacteria in the generalized negative association between laying date and avian breeding success.

Author contribution statement J. J. S., M. R.-R. and M. M.-V. designed the study; J. J. S., M. R.-R., J. M. P.-S., C. R.-C. and G. T. collected field samples; M. R.-R., C. R.-C., J. M. P.-S., and G. T. performed the laboratory work; J. J. S. performed the statistical analyses and wrote the first draft of the manuscript; all authors contributed substantially to revisions.

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