Laying date, incubation and egg breakage as determinants of bacterial load on bird eggshells. Experimental evidences

Short title: Eggshell bacterial load, laying date and incubation

Juan José Soler^{1,2}, Magdalena Ruiz-Rodríguez¹, Manuel Martín-Vivaldi^{3,4}, Juan Manuel Peralta-Sánchez^{2,4}, Cristina Ruiz-Castellano¹, Gustavo Tomás¹

¹ Departamento de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas (CSIC), Ctra. Sacramento s/n, La Cañada de San Urbano, E-04120 Almería, Spain. ² Grupo Coevolución, Unidad asociada al CSIC, Universidad de Granada, Granada, Spain. ³ Departamento. Zoología. Universidad de Granada. 18071 Granada, Spain. ⁴ Departamento de Microbiología, Universidad de Granada, 18071 Granada, Spain

Word count: 8704

* To whom correspondence should be addressed: e-mail jsoler@eeza.csic.es, Tf: 34 950281045, Fax: 34 950277100

Author contributions: JJS, MRR and MMV designed the study, JJS, MRR, JMPS, CRC and GT collected field samples, MRR, CRC, JMPS and GT performed the laboratory work, JJS performed the statistical analyses and wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

Abstract

Introduction: 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.

Hypothesis/objectives: Here 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.

Methods: We manipulated hygienic conditions by spilling egg contents on magpie and artificial nests and explored experimental effects along the breeding season. Egg breakage is a common outcome of brood parasitism by great spotted cuckoos (*Clamator glandarius*) on magpie nests, one of its main hosts.

Results: We found that the experiment did increase eggshell bacterial load in artificial, but not in magpie nests with incubating females, which suggests that parental activity prevent the proliferation of bacteria on the eggshells in relation with egg breakage. Moreover, laying date was positively related with eggshell bacterial load in active magpie nests, but negatively in artificial nests.

Conclusions and significance: Results suggest that variation in parental characteristics of magpies rather than climatic variation along the breeding season explained the detected positive association. Because 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.

- 51 **Key words:** brood parasitism, climate change, eggshell bacterial loads, Magpie,
52 parental activity, life history traits, nest characteristics

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). Bacterial environment has traditionally been considered an important selective force acting on offspring viability in birds (Baggott & Graeme-Cook 2002; Mennerat et al. 2009; Soler et al. 2012), and have likely played a central role in the evolution of many animal life history traits, some of them directed to reduce probability of bacterial infection (Cook et al. 2005a; Peralta-Sánchez et al. 2012; Møller et al. 2013).

Temperature, humidity and hygienic condition in nests are known to determine bacterial colonization and growth on the eggshells of birds and hence trans-shell bacterial infection of embryos (Bruce & Drysdale 1994; Bruce & Drysdale 1991; 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 conditions (Hansell 2000) and can affect bacterial environment of nests. Thus, green-aromatic plants (Clark & Mason 1985; Mennerat et al. 2009; Møller et al. 2013) and/or feathers (Soler et al. 2010; Peralta-Sánchez et al. 2011; Peralta-Sánchez et al. 2010; Peralta-Sánchez et al. 2014) employed in nest building may confer direct defensive properties against bacterial infection. Egg incubation also contributes to protect developing offspring from the environment, given its effect 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 for climatic environmental conditions as shown by comparisons of incubation influence on eggshell bacterial loads and/or embryo viability in tropical (Cook et al. 2005a; Shawkey et al. 2009) and temperate

78 areas (Wang et al. 2011; Lee et al. 2014). Thus, variation in climatic conditions is still
79 likely affecting bacterial environments of avian nests.

80 In temperate areas, breeding success of birds typically decreases as the season
81 progresses (Price et al. 1988; Moreno 1998). The association between laying date and
82 breeding success has traditionally been explained as a consequence of the seasonal
83 decline in resource availability for offspring and parents, and/or because parents of
84 poorer phenotypic quality reproduce later (Wardrop & Ydenberg 2003; De Neve et al.
85 2004; Verhulst & Nilsson 2008). However, because temperature and humidity typically
86 increase and decrease respectively as the season progresses, the associated variation in
87 bacterial environment along the breeding season might also contribute to explain the
88 lower reproductive success of late breeders. In addition, the poorer phenotypic quality
89 of late breeders might *per se* affect bacterial environment of nests if, for instance, they
90 construct poorer insulated or defensive nests, or are less efficient in maintaining
91 appropriate hygienic conditions of nests. These two scenarios therefore predict that
92 laying date and bacterial environment of nests should be related in nature.

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

our hypothesis may suggest a role for bacteria explaining deteriorated breeding success of birds associated to 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 with magpie nest lining material and containing fresh-commercial quail (*Coturnix coturnix*) eggs. Moreover, simulating the effects of brood parasitism by great spotted cuckoos (see below) we manipulated hygienic conditions of magpie and artificial nests by breaking and spilling contents of quail eggs, and explored possible differential effects of this manipulation on eggshell bacterial loads along the breeding season. As proxy of nest bacterial environments and risk of embryo infection we estimated density of mesophilic bacteria on the eggshells of magpies before and after incubation started, and of experimental quail eggs four days after the experimental spilling of egg contents on eggs in artificial nests. Prevalence of *Enterobacteriaceae*, *Staphylococcus sp.*, and *Enterococcus sp.* in specific culture media were also estimated on eggshells as indicative of the probability of egg contamination. These three groups of bacteria included pathogenic strains and their density on avian eggshells have been used previously as proxies of probability of embryo infection (Board & Tranter 1986; Kozłowski et al. 1989; Bruce & Drysdale 1991; 1994; Houston et al. 1997; Cook et al. 2003; 2005a; 2005b; Soler et al. 2008; Shawkey et al. 2009; Peralta-Sánchez et al. 2010; Soler et al. 2011).

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 hygienic conditions by egg breakage and spilling egg contents on eggs in magpie and

artificial nests have the additional interest of experimentally testing 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 consequence of poorer hygienic conditions in parasitized nests due to egg breakage and egg content spilling of magpie eggs which would select for eggshell characteristics in cuckoos limiting bacterial contamination and growth. The experiment performed here allows testing the influence of egg-content spilling on 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 bacterial environment should depend on nutrient availability for bacterial growth, the predicted association between laying date and eggshell bacterial loads should depend on experimental treatment (i.e. spilling of eggs contents). If that was 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, influences 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 aimed to combat parasite infections is an important activity of breeding birds (Christe et al. 1996; Ibáñez-Álamo et al. 2014), the effect of experimental treatment of egg contents on bacterial environment, or the strength of the interaction with laying date, should be reduced in natural magpie nests (**P4b**). Even more, if the hygienic conditions of magpie nests (i.e. bacterial environment) are determined by phenotypic quality of adult birds through differences in nest sanitation ability and/or reproductive investment, we could even find a positive association between laying date and eggshell bacterial loads. Finding evidence of such an association would suggest that the general lower breeding success of late reproductive attempts may be partially driven by differential bacterial selection pressures mediated by adults, rather than by climatic-related environmental conditions, at the nests of birds.

Material 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, sparse holm oaks remaining from the original Mediterranean forest, small shrubs in abandoned fields, and deciduous trees in streams and villages. The magpie population is comprised of several subpopulations, some of them in irrigated and some others in arid environments (De Neve et al. 2007). We

sampled two of these subpopulations, 15-20 km apart from each other, one in irrigated (Albuñan) and another one in arid environment (Carretera). 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; Soler & Soler 2000; Martín-Gálvez et al. 2007; Soler et al. 2013).

Field work

Magpie territories known from previous years were visited once a week since the 15th of March to detect new nests. Once we found a new nest, it was visited twice a week, which allowed us to know laying date of the first egg and to detect brood parasitism. Laying date of sampled nests in 2011 extended from the 3rd of April to the 12th of May and in 2012 from the 31st of March to the 12th of May (average laying date for both years was the 19th of 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 wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). After cleaning the complete egg surface, the swab was introduced in 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 number of colonies (CFU's, Colonies Forming Units) per cm² (i.e. eggshell bacterial density) as previously described elsewhere (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: (1) Experimental nests: we included a broken quail egg in the nest. The experimental quail egg was broken inside magpie nest, making a hole of enough size to assure that most content spilled off when we moved it together with all other eggs in the nest. In that way, wore gloves used for moving the eggs come to be besmeared with egg contents, which assure that most magpie egg surface became in contact with egg contents either, because of direct contact with quail eggshell or because gently touched with smudged gloves. (2) Control I nests: we included a non-broken quail egg in the magpie nest and moved it as we did with the broken egg for the experimental treatment. Quail eggs were cleaned with disinfectant wipes (Aseptonet, LaboratoiresSarbec, Cod.998077-51EN) before using in magpie nests. (3) Control II nests: we visited and sampled these nests 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 it may occur with the third, or be delayed up to the 7th egg (Birkhead 1991). Bacteria from eggshells of experimental and control nests were sampled three times. 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 – not warm eggs with no sign of incubation), 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 of the first egg (i.e. after incubation started – warm eggs with sign of incubation). Third samples were collected 14-19 (mean (SE) = 17.1 (0.06), N = 100) days after the first eggs was laid (i.e. before hatching). Broken magpie eggs or with traces of egg content spilling were detected in 28.2 % (N = 220) of the magpie nests sampled after incubation started, most of them in

227 nests where cuckoo egg(s) was also found (66.1%, N = 62). Bacterial loads of magpie
 228 eggshell in parasitized and non-parasitized nests with traces of egg content spilling were
 229 used to explore the effect of natural egg-breakage on eggshell bacterial load (see
 230 below). During each visit, we numbered all new eggs with indelible marker and
 231 sampled a single egg per nest that had not been sampled in previous visits. Whenever
 232 possible all three sampled eggs per nest in respective visits were within the first four
 233 eggs in the laying sequence.

234 Some of the quail eggs introduced in magpie nests as a control treatment were
 235 not rejected by magpies and we were thus able to sample incubated quail eggs in natural
 236 magpie nests during the second visit. Total eggshell bacterial loads of these eggs did not
 237 differ from those of magpie eggs in the same nests (Wilcoxon Matched Pairs test, $Z =$
 238 1.12, $P = 0.26$, $N = 47$), which support the use of quail eggs in artificial nests (see
 239 below).

241 *Artificial magpie nests*

242 Artificial nests were constructed with nest lining material (thin roots and grass)
 243 collected from 8 new magpie nests before laying, that were assembled in plastic bags
 244 and used for cover the bottom inside bird cages (15x30x20 cm) to prevent predation
 245 while exposing experimental eggs to environmental climatic conditions. Sixteen bird
 246 cages were fastened 1-2 meters high to almond and pine trees spread over the study
 247 areas of the two magpie subpopulations. Seven of these cages were in the arid zone and
 248 nine in the irrigated zone. 107 pairs of quail eggs, one experimental and one control,
 249 were placed on nest material inside experimental cages along the main egg laying
 250 period of our studied magpie population (from the 20th of April to the 19th of May)
 251 homogeneously (at least one pair of eggs every second day and an average of 2.4 pair of

eggs per day). Before introduction in 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 experimental cages, whilst the experimental egg was handled with the sample gloves but soiled with the content 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 to each other. A new pair of eggs was added to the experimental cages every four days, and the same cage harboured up to 4 pairs of experimental eggs. None of the eggs was in direct contact to each other.

With an indelible marker, we painted a line throughout the egg poles dividing egg surface in two halves; one of them was sampled 4 days after the experiment. The non-sampled surface of fifty-three pairs of eggs was sampled 16 days after the onset of the experiment. We failed to analyse samples from five control and three experimental eggs collected from 7 different egg pairs 4 days after the onset of the experiment. These losses were due to 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 seconds. Bacteriology was performed by spreading homogenously 100 µl of serially diluted samples onto Petri dishes containing four different solid agar media (ScharlauChemie S.A. Barcelona). We used Tryptic Soy Agar, a broadly used general medium to grow aerobic mesophilic bacteria, and three specific media: Kenner Fecal Agar for *Enterococcus*; Vogel-Johnsson Agar for *Staphylococcus*; and Hektoen Enteric Agar for *Enterobacteriaceae*. The plates were

incubated aerobically at 37°C and colonies were counted 72h after inoculation. Bacterial density was estimated for each of the four media as number of Colony Forming Units per cm² following 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, onset of incubation, and end of incubation, and for samples obtained from quail eggs.

Statistical Analyses

Log10 transformed density of mesophilic bacteria differed from normal distribution and we conservatively used ranked values for statistical analyses. Specific group of bacteria (Enterobacteriaceae, *Staphylococcus* and *Enterococcus*) were not detected for many samples (see results) and, thus, frequency distributions were far from Gaussian shape. Thus, we used information on 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 to mean values and dividing by standard deviation. We used these values in subsequent analyses.

The expected effects of having experimental or natural broken eggs (and/or trace of egg contents (i.e. yolk)) and of laying date in magpie eggshell bacterial loads were analysed in Repeated Measures ANOVAS (RMA) with ranked values of mesophilic bacterial loads estimated at different visits (egg laying, onset and end of incubation) as within factor, experimental treatment (or having or not trace of natural egg breakage), area (irrigated or arid) and year as between factor, and standardized laying date as covariable. 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 experimental coating commercial quail eggs on eggshell bacterial

loads (i.e. ranked valued of mesophilic bacterial density) was explored by RMA with 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 covariable. 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 mean of Generalized Linear Models with binomial distribution and logic 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 third bacterial sampling (i.e., at the end of incubation) was reduced. However, main effects were detected independently of whether or not information of 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 density rather than ranked values were used for figures. All statistical tests were performed with Statistica 10.0 (Statsoft Inc. 2011).

Results

Bacterial loads of magpie eggshells. Effect 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 density of mesophilic bacteria on magpie eggshells, which were mainly explained by study area (higher in the arid subpopulation) (Table 1). Moreover, laying date was positively associated with density of mesophilic bacteria (Fig. 1), which

is contrary to our **P2**, and the effect of incubation activity did depend on the interaction between study year and area (Table 1). In no case we detected support for the predicted (**P3**) interaction between experimental treatment and laying date (Table 1).

When considering magpie nests where quail eggs were experimentally broken, results were quite similar to those with natural broken eggs 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 quail egg, and failed to detect statistically significant differences (identical model that those 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 significant lower bacterial density of eggs sampled in 2012 and in irrigated areas, and a significant interaction between study year and area (interaction in Table 1, Fig. 2). The only detected effect of experimental treatment was indirect, through its interaction with 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**, density of bacteria for this subset of nests did increase as the season progress (Table 1, Fig. 1) independently of experimental treatments.

Analyses on prevalence of different groups of bacteria offered similar results. Prevalences of mesophilic bacteria and of *Enterobacteriaceae* were positively associated with laying date (Table 2). When considering nests with traces of natural egg-breakage, prevalence of *Enterobacteriaceae* did varied among years (Table 2), being more frequent in 2011 (13 out of 59 nests) than in 2012 (3 out of 70 nests). Traces of egg breakage on the sampled eggshells did result positively related with probability

of *Enterococcus* detection (3 out of 14 nests with traces of egg breakage vs 1 out of 125 nests with no traces of egg breakage), but did not affect prevalence of other considered bacteria (Table 2). In no case we detected support for the predicted (**P3**) interaction between experimental treatment and laying date.

Very similar results came out when considering natural nests with no detected egg breakage that were subjected to experimental inclusion of broken quail eggs into the nest (Table 2). The experiment only affected prevalence of *Enterococcus* positively, while laying date were positively associated with prevalence of mesophilic bacteria and of *Enterobacteriaceae* (Table 2). The former result was therefore in accordance with **P1** and the later was contrary to **P2**. Load of *Enterobacteriaceae* on the eggshell of magpies did vary for different years. In no case we detected support for the predicted (**P3**) interaction between experimental treatment and laying date.

Taken together, all these results suggest limited effects of egg breakage on the bacterial density and prevalence of 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 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 (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 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 density of mesophilic bacteria tended to decrease as the season progressed mainly in experimental eggs (RMA, interaction between experimental treatment and laying date, $F = 3.76$, $df = 1,98$, $P = 0.084$) (Fig. 3B), which do not support **P3**.

Similar results were obtained when analysing bacterial prevalence. Laying date did significantly associate with prevalence of mesophilic bacteria (negatively) and of *Enterococcus* (positively) (Table 3). Prevalence of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus* was higher in experimental 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 did vary depending on laying date (interaction term in Table 3), which support **P3**. However, the effects of laying date on prevalence and density of bacteria on the eggshell depended of the considered bacterial group.

Remarks on results from artificial and natural magpie nests.

Effects of experimental smearing with egg contents of quail eggs on eggshell bacterial loads were detected in artificial but not in natural magpie nests, which is in accordance with our prediction number four (**P4a**). The expected negative relationship between eggshell bacterial loads and laying date was only detected in artificial nests, but turned to be 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 incubating magpies. Moreover, laying date was positively related with 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. This possibility is however unlikely since magpie and control quail eggs in natural magpie nests harboured similar bacterial density some days after incubation (see Material and Methods).

Therefore, these two results suggest on the one hand that incubating activity of magpies prevent the proliferation of bacteria on the eggshells in relation with 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 its importance for understanding of the role of environmental conditions and parental influence as determinants of bacterial environments of nests and thus probability of bacterial infection.

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 resulting many times in egg-content spilling (Soler et al. 2011). Here, we found no experimental support for this hypothesis in magpie nests. However, experimental coating of quail eggs with egg-contents did result

in significant increases in eggshell bacterial loads and prevalence four days after the manipulation. These two results therefore 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 egg breaking behaviour of cuckoos, but also of input of bacteria from cuckoos on the parasitic eggs or due to subsequent visits to magpie nests by the brood parasite (Soler et al. 2011).

Incubation or any other parental behaviour influencing bacterial environment of nests (Clark & 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 the 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 easily degradable by queratinolitic bacteria with important antimicrobial activity (Peralta-Sánchez et al. 2010; Peralta-Sánchez et al. 2014), that are in contact with the eggshells and may reduce growth of pathogenic bacteria (Lee et al. 2014). In addition, magpies build a quite apparent mud cup, and we know of the use of mud therapies because of the antimicrobial properties of clays (Said et al. 1980; Maigetter & 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 to be tested in the future.

The second main result is the detected associations 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 (Fig. 1 and Fig. 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 turned to be 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: $R = 0.120$, $N = 66$, $P = 0.33$ along the sampling period (average daily temperature and humidity from 1st of April to 5th of 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 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 probability of parasitism respectively) (Sorci et al. 1997; Siikamäki 1998; Merino et al. 2000; Verhulst & 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 deterioration of nest bacterial environments as the season progresses would contribute to explain the reduced breeding success of late breeders, a possibility never suggested. Variation in food availability and/or phenotypic condition of parents (including parasite infection status) would affect parental activity (Winkler & Allen 1996), including nest building effort (Soler et al. 1995; Soler et al. 1998), incubation attendance (Chastel et al. 1995) and, perhaps, nest sanitation. 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, extensive theoretical background allowed predicting positive covariation between the well-known, and widely accepted, seasonal decline in breeding success in temperate areas and nest bacterial environment. 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 driving the seasonal decline in breeding success for which we have detected pioneering evidence. Experimental manipulation of factors affecting parental attendance (i.e. incubation) are however necessary to reach firm conclusions.

Summarizing, our experimental approaches allowed us to detect different dynamics in bacterial communities of eggshells in artificial and natural nests in relation to hygienic conditions, incubation activity and laying date. Since laying date resulted positively associate to 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 explaining the generalized negative association between laying date and avian breeding success.

Acknowledgements

E. Campanario and E. López-Hernández performed all laboratory work. This work was financed by Spanish Ministerio de Ciencia e Innovación, European funds (FEDER) (CGL2010-19233-C03-01, CGL2010-19233-C03-03, CGL2013-48193-C3-1-P, CGL2013-48193-C3-3-P). MRR and GT respectively received a postdoc from the programmes “JAE-Doc” and “Juan de la Cierva”, and CRC had a pre-doctoral grant from the Spanish Government. We obtained the permissions for sampling eggs and visiting nests of magpies from the Consejería de Medio Ambiente (Junta de Andalucía).

References

- Baggott GK, Graeme-Cook K (2002) Microbiology of natural incubation. In: Deeming DC (ed) Avian incubation behaviour, environment and evolution. Oxford University Press, Oxford, pp 179-191
- Birkhead TR (1991) The Magpies. The ecology and behaviour of black-billed and yellow-billed magpies. T & A D Poyser, London
- Board RG, Clay C, Lock J, Dolman J (1994) The egg: a compartmentalized, aseptically packaged food. In: Board RG, Fuller R (eds) Microbiology of the avian egg. Chapman & Hall, London, pp 43-62
- Board RG, Tranter HS (1986) The microbiology of eggs. In: Stadelman WJ, Cotterill OJ (eds) Egg science and technology. AVI publishing Co., Westport, CT, pp 75-96
- Bonisoli-Alquati A, Rubolini D, Romano M, Cucco M, Fasola M, Caprioli M, Saino N (2010) Egg antimicrobials, embryo sex and chick phenotype in the yellow-legged gull. Behav. Ecol. Sociobiol. 64:845-855
- Bruce J, Drysdale EM (1991) Egg hygiene: route of infection. In: Tullett SG (ed) Avian incubation. Butterworth Heinemann, Northampton, pp 257-276
- Bruce J, Drysdale EM (1994) Trans-shell transmission. In: Board RG, Fuller R (eds) Microbiology of avian eggs. Chapman & Hall, London, pp 63-91

- 533 Chastel O, Weimerskirch H, Jouventin P (1995) Influence of body condition on
534 reproductive decision and reproductive success in the Blue Petrel. *Auk* 112:964-972
- 535 Christe P, Richner H, Oppliger A (1996) Of great tits and fleas: Sleep baby sleep ..
536 *Anim. Behav.* 52:1087-1092
- 537 Christians JK, Evanson M, Aiken JJ (2001) Seasonal decline in clutch size in European
538 starlings: a novel randomization test to distinguish between the timing and quality
539 hypotheses. *J. Anim. Ecol.* 70:1080-1087
- 540 Clark L, Mason JR (1985) Use of nest material as insecticidal and anti-pathogenic
541 agents by the European starling. *Oecologia* 67:169-176
- 542 Cook MI, Beissinger SR, Toranzos GA, Arendt WJ (2005a) Incubation reduces
543 microbial growth on eggshells and the opportunity for trans-shell infection. *Ecol. Lett.*
544 8:532-537
- 545 Cook MI, Beissinger SR, Toranzos GA, Rodriguez RA, Arendt WJ (2003) Trans-shell
546 infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's
547 eggs: a constraint on the onset of incubation? *Proc. R. Soc. Lond. B* 270:2233-2240
- 548 Cook MI, Beissinger SR, Toranzos GA, Rodriguez RA, Arendt WJ (2005b) Microbial
549 infection affects egg viability and incubation behavior in a tropical passerine. *Behav.*
550 *Ecol.* 16:30-36
- 551 D'Alba L, Oborn A, Shawkey MD (2010) Experimental evidence that keeping eggs dry
552 is a mechanism for the antimicrobial effects of avian incubation. *Naturwissenschaften*
553 97:1089-1095
- 554 De Neve L, Soler JJ, Ruiz-Rodríguez M, Martín-Gálvez D, Pérez-Contreras T, Soler M
555 (2007) Habitat-specific effects of a food supplementation experiment on
556 immunocompetence in Eurasian Magpie *Pica pica* nestlings. *Ibis* 149:763-773
- 557 De Neve L, Soler JJ, Soler M, Pérez-Contreras T (2004) Differential maternal
558 investment counteracts for late breeding in magpies *Pica pica*: an experimental study. *J.*
559 *Avian Biol.* 35:237-245
- 560 Godard RD, Morgan Wilson C, Frick JW, Siegel PB, Bowers BB (2007) The effects of
561 exposure and microbes on hatchability of eggs in open-cup and cavity nests. *J. Avian*
562 *Biol.* 38:709-716
- 563 Gordo O, Sanz JJ (2006) Climate change and bird phenology: a long-term study in the
564 Iberian Peninsula. *Global Change Biology* 12:1993-2004
- 565 Hansell M (2000) Bird nests and construction behaviour. Cambridge University Press,
566 Cambridge
- 567 Hochachka W (1990) Seasonal decline in reproductive performance of song sparrows.
568 *Ecology* 71:1279-1288
- 569 Houston CS, Saunders JR, Crawford RD (1997) Aerobic bacterial flora of addled raptor
570 eggs in Saskatchewan. *J. Wildl. Dis.* 33:328-331

- 571 Ibáñez-Álamo JD, Ruiz-Rodríguez M, Soler JJ (2014) The mucous covering of fecal
572 sacs prevents birds from infection with enteric bacteria. *J. Avian Biol.* 45:354-358
- 573 Kozłowski S, Malysko E, Pinowski J, Kruszewicz A (1989) The effect of
574 microorganisms on the mortality of house sparrow (*Passer domesticus*) and tree
575 sparrow (*Passer montanus*) embryos. In: Pinowski J, Kavanagh BP, Gorski W (eds)
576 Proc. Int. Symp. Working Group on Granivorous Birds. Intecol, Slupsk, Poland, pp 121-
577 128
- 578 Lee WY, Kim M, Jablonski PG, Choe JC, Lee Si (2014) Effect of incubation on
579 bacterial communities of eggshells in a temperate bird, the Eurasian magpie (*Pica pica*).
580 PLoS ONE 9:e103959
- 581 Maigetter RZ, Pfister RM (1975) A mixed bacterial population in a continuous culture
582 with and without kaolinite. *Can. J. Microbiol.* 21:173-180
- 583 Martín-Gálvez D, Soler JJ, Martínez JG, Krupa AP, Soler M, Burke T (2007) Cuckoo
584 parasitism and productivity in different magpie subpopulations predict frequencies of
585 the 457bp allele: a mosaic of coevolution at a small geographic scale. *Evolution*
586 61:2340-2348
- 587 Martín-Vivaldi M, Ruiz-Rodríguez M, Mendez M, Soler JJ (2006) Relative importance
588 of factors affecting nestling immune response differs between junior and senior
589 nestlings within broods of hoopoes *Upupa epops*. *J. Avian Biol.* 37:467-476
- 590 McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lo+ió T, Douglas AE,
591 Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll
592 AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A,
593 Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ (2013) Animals in a
594 bacterial world, a new imperative for the life sciences. *Proceedings of the National*
595 *Academy of Sciences* 110:3229-3236
- 596 Mennerat A, Mirleau P, Blondel J, Perret P, Lambrechts M, Heeb P (2009) Aromatic
597 plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria.
598 *Oecologia* 161:849-855
- 599 Merino S, Møller AP, de Lope F (2000) Seasonal changes in cell-mediated
600 immunocompetence and mass gain in nestling barn swallows: a parasite-mediated
601 effect? *Oikos* 90:327-332
- 602 Møller AP, Flensted-Jensen E, Mardal W, Soler JJ (2013) Host-parasite relationship
603 between colonial terns and bacteria is modified by a mutualism with a plant with
604 antibacterial defenses. *Oecologia* 173:169-178
- 605 Møller AP, Peralta-Sánchez JM, Nielsen JT, López-Hernández E, Soler JJ (2012)
606 Goshawk prey have more bacteria than non-prey. *J. Anim. Ecol.* 81:403-410
- 607 Moreno J (1998) The determination of seasonal declines in breeding success in seabirds.
608 *Etologia* 6:17-31

- 609 Peralta-Sánchez JM, Møller AP, Martín-Platero AM, Soler JJ (2010) Number and
610 colour composition of nest lining feathers predict eggshell bacterial community in barn
611 swallow nests: an experimental study. *Funct. Ecol.* 24:426-433
- 612 Peralta-Sánchez JM, Møller AP, Soler JJ (2011) Colour composition of nest lining
613 feathers affects hatching success of barn swallows, *Hirundo rustica* (Passeriformes:
614 Hirundinidae). *Biol. J. Linn. Soc.* 102:67-74
- 615 Peralta-Sánchez JM, Martín-Vivaldi M, Martín-Platero AM, Martínez-Bueno M, Oñate
616 M, Ruiz-Rodríguez M, Soler JJ (2012) Avian life history traits influence eggshell
617 bacterial loads: a comparative analysis. *Ibis* 154:725-737
- 618 Peralta-Sánchez JM, Soler JJ, Martín-Platero AM, Knight R, Martínez-Bueno M,
619 Møller AP (2014) Eggshell bacterial load is related to antimicrobial properties of
620 feathers lining barn swallow nests. *Microb. Ecol* 67:480-487
- 621 Price T, Kirkpatrick M, Arnold SJ (1988) Directional selection and the evolution of
622 breeding date in birds. *Science* 240:798-799
- 623 Reif J, Vorisek P, St'astny K, Koschova M, Bejcek V (2008) The impact of climate
624 change on long-term population trends of birds in a central European country. *Animal*
625 *Conservation* 11:412-421
- 626 Said SA, Shibl AM, Abdullah ME (1980) Influence of various agents on adsorption
627 capacity of kaolin for pseudomonas aeruginosa toxin. *J. Pharm. Sci.* 69:1238-1239
- 628 Saino N, Calza S, Møller AP (1998) Effects of a dipteran ectoparasite on immune
629 response and growth trade-offs in barn swallow, *Hirundo rustica*, nestlings. *Oikos*
630 81:217-228
- 631 Saino N, Dall'ara P, Martinelli R, Møller AP (2002) Early maternal effects and
632 antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. *J.*
633 *Evol. Biol.* 15:735-743
- 634 Saino N, Ambrosini R, Rubolini D, von Hardenberg J, Provenzale A, Hueppop K,
635 Hueppop O, Lehikoinen A, Lehikoinen E, Rainio K, Romano M, Sokolov L (2011)
636 Climate warming, ecological mismatch at arrival and population decline in migratory
637 birds. *Proc. R. Soc. Lond. B* 278:835-842
- 638 Shawkey MD, Firestone MK, Brodie EL, Beissinger SR (2009) Avian incubation
639 inhibits growth and diversification of bacterial assemblages on eggs. *PLoS ONE*
640 4:e4522
- 641 Siikamäki P (1998) Limitation of reproductive success by food availability and breeding
642 time in pied flycatchers. *Ecology* 79:1789-1796
- 643 Soler JJ, De Neve L, Martin-Galvez D, Molina-Morales M, Perez-Contreras T, Ruiz-
644 Rodriguez M (2014) Do climatic conditions affect host and parasite phenotypes
645 differentially? A case study of magpies and great spotted cuckoos. *Oecologia* 174:327-
646 338

- 647 Soler JJ, De Neve L, Pérez-Contreras T, Soler M, Sorci G (2003) Trade-off between
648 immunocompetence and growth in magpies: an experimental study. *Proc. R. Soc. Lond.*
649 *B* 270:241-248
- 650 Soler JJ, Martín-Gálvez D, De Neve L, Soler M (2013) Brood parasitism correlates with
651 the strength of spatial autocorrelation of life history and defensive traits in Magpies.
652 *Ecology* 94:1338-1346
- 653 Soler JJ, Martín-Vivaldi M, Peralta-Sánchez JM, Ruiz-Rodríguez M (2010) Antibiotic-
654 producing bacteria as a possible defence of birds against pathogenic microorganisms.
655 *Open Ornithol. J.* 3:93-100
- 656 Soler JJ, Martín-Vivaldi M, Ruiz-Rodríguez M, Valdivia E, Martín-Platero AM,
657 Martínez-Bueno M, Peralta-Sánchez JM, Méndez M (2008) Symbiotic association
658 between hoopoes and antibiotic-producing bacteria that live in their uropygial gland.
659 *Funct. Ecol.* 22:864-871
- 660 Soler JJ, Martínez JG, Soler M, Møller AP (1999) Genetic and geographic variation in
661 rejection behavior of cuckoo eggs by European magpie populations: An experimental
662 test of rejecter-gene flow. *Evolution* 53:947-956
- 663 Soler JJ, Møller AP, Soler M (1998) Nest building, sexual selection and parental
664 investment. *Evol. Ecol.* 12:427-441
- 665 Soler JJ, Peralta-Sánchez JM, Martín-Platero AM, Martín-Vivaldi M, Martínez-Bueno
666 M, Møller AP (2012) The evolution of size of the uropygial gland: mutualistic feather
667 mites and uropygial secretion reduce bacterial loads of eggshells and hatching failures
668 of European birds. *J. Evol. Biol.* 25:1779-1791
- 669 Soler JJ, Peralta-Sánchez JM, Martínez Bueno M, Martín-Vivaldi M, Martín-Gálvez D,
670 Vela AI, Briones V, Pérez-Contreras T (2011) Brood parasitism is associated with
671 increased bacterial contamination of host eggs: bacterial loads of host and parasitic
672 eggs. *Biol. J. Linn. Soc.* 103:836-848
- 673 Soler JJ, Soler M (2000) Brood-parasite interactions between great spotted cuckoos and
674 magpies: a model system for studying coevolutionary relationships. *Oecologia* 125:309-
675 320
- 676 Soler JJ, Soler M, Møller AP, Martínez JG (1995) Does the great spotted cuckoo choose
677 magpie hosts according to their parenting ability? *Behav. Ecol. Sociobiol.* 36:201-206
- 678 Soler M, Soler JJ, Martínez JG (1997) Great spotted cuckoos improve their reproductive
679 success by damaging magpie host eggs. *Anim. Behav.* 54:1227-1233
- 680 Sorci G, Soler JJ, Møller AP (1997) Reduced immunocompetence of nestlings in
681 replacement clutches of the european magpie (*Pica pica*). *Proc. R. Soc. Lond. B*
682 264:1593-1598
- 683 Stadelman WJ (1994) Contaminants of liquid eggs products. In: Board RG, Fuller R
684 (eds) *Microbiology of the avian egg*. Chapman & Hall, London, pp 139-151

- 685 Statsoft Inc. (2011) STATISTICA (data analysis software system), version 10.
 686 www.statsoft.com,
- 687 Verhulst S, Nilsson JA (2008) The timing of birds' breeding seasons: a review of
 688 experiments that manipulated timing of breeding. *Phil. Trans. R. Soc. Lond. B* 363:399-
 689 410
- 690 Visser ME, Both C (2005) Shifts in phenology due to global climate change: the need
 691 for a yardstick. *Proceedings of the Royal Society B: Biological Sciences* 272:2561-2569
- 692 Wang JM, Firestone MK, Beissinger SR (2011) Microbial and environmental effects on
 693 avian egg viability: Do tropical mechanisms act in a temperate environment? *Ecology*
 694 92:1137-1145
- 695 Wardrop SL, Ydenberg RC (2003) Date and parental quality effects in the seasonal
 696 decline in reproductive performance of the Tree Swallow *Tachycineta bicolor*:
 697 interpreting results in light of potential experimental bias. *Ibis* 145:439-447
- 698 Winkler DW, Allen PE (1996) The seasonal decline in tree swallow clutch size:
 699 Physiological constraint or strategic adjustment? *Ecology* 77:922-932
- 700 Winkler DW, Ringelman KM, Dunn PO, Whittingham L, Hussell DJT, Clark RG,
 701 Dawson RD, Johnson LS, Rose A, Austin SH, Robinson WD, Lombardo MP, Thorpe
 702 PA, Shutler D, Robertson RJ, Stager M, Leonard M, Horn AG, Dickinson J, Ferretti V,
 703 Massoni V, Bulit F, Reboresda JC, Liljeström M, Quiroga Mn, Rakhimberdiev E,
 704 Ardia DR (2014) Latitudinal variation in clutch size – lay date regressions in
 705 *Tachycineta* swallows: effects of food supply or demography? *Ecography*
 706
 707

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, 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. 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 separated models.

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

Table 2: Results from Generalized Linear Models with binomial distribution and logit link function explaining 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). 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 but only for the interaction between broken eggs and laying date, which were included in the models but estimated separately.

Control magpie nests (N = 139)						Experimental vs control nests (N = 185)			
	Wald Statistic	Estimate CI (95%)	Estimate - CI (95%)	P		Wald Statistic	Estimate CI (95%)	Estimate - CI (95%)	P
Mesophilic bacteria*									
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*			0.1184		0.62	-1.060	0.451	0.4296
(1) X (2)						0.51	-0.700	1.499	0.4766
Enterobacteriaceae									
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) X (2)	1.14	-0.334	1.133	0.2860		1.76	-1.274	0.245	0.1845
Staphylococci**									
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

<i>Enterococci</i> ***	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) X (2)						0.59	-1.078	0.469	0.4408
	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) X (2)	<0.01	-1.171	1.196	0.9830		0.61	-1.663	0.714	0.4337

* Mesophilic bacteria were absent in only eight out of 139 non-manipulated magpie nests and all of them were from the same study area and with 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 GLZ model. Rather we estimated the effect of egg breakage in separate log-linear models.

** Staphylococci bacteria were detected in 5 natural magpie nests, none of them with rests of broken eggs. Thus the effect of egg breakage or the interaction with laying date cannot be estimated in the GLZ model. Rather we estimated the effect of egg breakage in separate log-linear models

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

Table 3: Results from Generalized Linear Models with binomial distribution and logit link function explaining prevalence of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus* on the shells of experimental quail eggs. The model included laying date (1 = 1st of April) as a covariable and whether or not the eggs were coated with egg contents of a broken egg. The interaction between experimental treatment and laying date was included in the models, but estimated separately.

	Wald Statistic	Estimate CI (95%)	Estimate - CI (95%)	P
<i>Mesophilic bacteria</i>				
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) X (3)	0.09	-0.552	0.403	0.7599
(1) X (3)	4.84	-0.098	-0.006	0.0284
<i>Enterobacteriaceae</i>				
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) X (3)	*			
(1) X (3)	0.15	-0.091	0.061	0.7000
<i>Staphylococci</i>				
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) X (3)	0.01	-0.668	0.615	0.9360
(1) X (3)	0.81	-0.039	0.107	0.3689
<i>Enterococci</i>				
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) X (3)	1.83	-0.146	0.798	0.1763
(1) X (3)	2.49	-0.089	0.010	0.1144

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

Fig. 1. Relationship between laying date and density of mesophilic bacteria on eggshells estimated before (empty circles and continuous regression line) and after (cross marks and dotted regression line) onset of incubation in magpie nests.

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

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

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 four days before estimation of bacterial loads. Number of experimental and control quail eggs with bacteria detected are also shown (total control eggs = 102, total experimental eggs = 104).

Fig. 1

Fig. 2

Fig. 3

Fig. 4