

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

3

4 Short title: Eggshell bacterial load, laying date and incubation

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23 and GT collected field samples, MRR, CRC, JMPS and GT performed the laboratory  
24 work, JJS performed the statistical analyses and wrote the first draft of the manuscript,  
25 and all authors contributed substantially to revisions.

26 **Abstract**

27 Introduction: Exploring factors guiding interactions of bacterial communities with  
28 animals has become of primary importance for ecologists and evolutionary biologists  
29 during the last years because of their likely central role in the evolution of animal life  
30 history traits.

31 Hypothesis/objectives: Here we explored the association between laying date and  
32 eggshell bacterial load (mesophilic bacteria, *Enterobacteriaceae*, *Staphylococci*, and  
33 *Enterococci*) in natural and artificial magpie (*Pica pica*) nests containing fresh-  
34 commercial quail (*Coturnix coturnix*) eggs.

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

39 Results: We found that the experiment did increase eggshell bacterial load in artificial,  
40 but not in magpie nests with incubating females, which suggests that parental activity  
41 prevent the proliferation of bacteria on the eggshells in relation with egg breakage.

42 Moreover, laying date was positively related with eggshell bacterial load in active  
43 magpie nests, but negatively in artificial nests.

44 Conclusions and significance: Results suggest that variation in parental characteristics  
45 of magpies rather than climatic variation along the breeding season explained the  
46 detected positive association. Because eggshell bacterial load is a proxy of hatching  
47 success, the detected positive association between eggshell bacterial loads and laying  
48 date in natural, but not in artificial nests, suggests that the generalized negative  
49 association between laying date and avian breeding success can be, at least partially,  
50 explained by differential bacterial effects.

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

## 53 **Introduction**

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

62       Temperature, humidity and hygienic condition in nests are known to determine  
63 bacterial colonization and growth on the eggshells of birds and hence trans-shell  
64 bacterial infection of embryos (Bruce & Drysdale 1994; Bruce & Drysdale 1991; Cook  
65 et al. 2003; Godard et al. 2007). Particular nest attributes such as nest location or  
66 nesting materials protect and insulate developing offspring from climatic environmental  
67 conditions (Hansell 2000) and can affect bacterial environment of nests. Thus, green-  
68 aromatic plants (Clark & Mason 1985; Mennerat et al. 2009; Møller et al. 2013) and/or  
69 feathers (Soler et al. 2010; Peralta-Sánchez et al. 2011; Peralta-Sánchez et al. 2010;  
70 Peralta-Sánchez et al. 2014) employed in nest building may confer direct defensive  
71 properties against bacterial infection. Egg incubation also contributes to protect  
72 developing offspring from the environment, given its effect reducing humidity which  
73 otherwise favours eggshell bacterial colonization and may compromise embryo viability  
74 (Cook et al. 2003; D'Alba et al. 2010). However, incubation or nest insulating properties  
75 of nest building material do not fully counteract for climatic environmental conditions  
76 as shown by comparisons of incubation influence on eggshell bacterial loads and/or  
77 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

102 our hypothesis may suggest a role for bacteria explaining deteriorated breeding success  
103 of birds associated to climate change and delayed breeding date (Soler et al. 2014).

104 As far as we know, this hypothesis has never been previously considered. Trying  
105 to fill this gap, we explore the association between laying date and eggshell bacterial  
106 load in magpie (*Pica pica*) nests and in artificial nests made with magpie nest lining  
107 material and containing fresh-commercial quail (*Coturnix coturnix*) eggs. Moreover,  
108 simulating the effects of brood parasitism by great spotted cuckoos (see below) we  
109 manipulated hygienic conditions of magpie and artificial nests by breaking and spilling  
110 contents of quail eggs, and explored possible differential effects of this manipulation on  
111 eggshell bacterial loads along the breeding season. As proxy of nest bacterial  
112 environments and risk of embryo infection we estimated density of mesophilic bacteria  
113 on the eggshells of magpies before and after incubation started, and of experimental  
114 quail eggs four days after the experimental spilling of egg contents on eggs in artificial  
115 nests. Prevalence of *Enterobacteriaceae*, *Staphylococcus sp.*, and *Enterococcus sp.* in  
116 specific culture media were also estimated on eggshells as indicative of the probability  
117 of egg contamination. These three groups of bacteria included pathogenic strains and  
118 their density on avian eggshells have been used previously as proxies of probability of  
119 embryo infection (Board & Tranter 1986; Kozłowski et al. 1989; Bruce & Drysdale  
120 1991; 1994; Houston et al. 1997; Cook et al. 2003; 2005a; 2005b; Soler et al. 2008;  
121 Shawkey et al. 2009; Peralta-Sánchez et al. 2010; Soler et al. 2011).

122 Although eggs include abundant antibacterial chemicals (Board et al. 1994;  
123 Bonisoli-Alquati et al. 2010; Saino et al. 2002), egg contents are prime nutrients for  
124 bacterial growth (Stadelman 1994). Thus, we predicted a positive effect of experimental  
125 spilling of egg contents on eggshell bacterial load (Prediction 1, **P1**). Manipulating  
126 hygienic conditions by egg breakage and spilling egg contents on eggs in magpie and

127 artificial nests have the additional interest of experimentally testing consequences for  
128 bacterial environments of magpie nests of the egg breaking behaviour of the great  
129 spotted cuckoo (*Clamator glandarius*), the brood parasite of magpies (Soler et al. 1997).  
130 We have previously shown that magpie eggshells harboured higher bacterial density in  
131 nests parasitized by cuckoos, and that within the same parasitized nests bacterial density  
132 of great spotted cuckoo eggshells was lower than that of magpie eggshells (Soler et al.  
133 2011). These results were interpreted as consequence of poorer hygienic conditions in  
134 parasitized nests due to egg breakage and egg content spilling of magpie eggs which  
135 would select for eggshell characteristics in cuckoos limiting bacterial contamination and  
136 growth. The experiment performed here allows testing the influence of egg-content  
137 spilling on eggshell bacterial load of magpies.

138         Temperature increase and humidity decrease as the season progresses in  
139 temperate areas should affect eggshell bacterial loads in artificial and natural magpie  
140 nests. As humidity is a main factor explaining eggshell bacterial proliferation (D'Alba et  
141 al. 2010), we should find that eggshell bacterial loads in artificial and natural magpie  
142 nests should decrease as the season progresses (Prediction 2, **P2**). Moreover, because  
143 the effect of temperature and humidity on bacterial environment should depend on  
144 nutrient availability for bacterial growth, the predicted association between laying date  
145 and eggshell bacterial loads should depend on experimental treatment (i.e. spilling of  
146 eggs contents). If that was the case, significant interactions between laying date and  
147 experimental treatment are predicted both for artificial and natural magpie nests  
148 (Prediction 3, **P3**).

149         If adult phenotypic condition and abilities (i.e. incubation activity and nest  
150 sanitation and maintenance) are important determinants of bacterial proliferation in bird  
151 nests, influences of laying date and of experimental treatment on eggshell bacterial

152 loads should vary for artificial (unattended) and natural magpie nests (Prediction 4, **P4**).  
153 Magpie incubation activity might ameliorate the effects of climatic conditions on  
154 bacterial proliferation on the eggs and, thus, the effects of experimental treatment and  
155 laying date should be less obvious in natural magpie nests (**P4a**). Furthermore, because  
156 nest sanitation aimed to combat parasite infections is an important activity of breeding  
157 birds (Christe et al. 1996; Ibáñez-Álamo et al. 2014), the effect of experimental  
158 treatment of egg contents on bacterial environment, or the strength of the interaction  
159 with laying date, should be reduced in natural magpie nests (**P4b**). Even more, if the  
160 hygienic conditions of magpie nests (i.e. bacterial environment) are determined by  
161 phenotypic quality of adult birds through differences in nest sanitation ability and/or  
162 reproductive investment, we could even found a positive association between laying  
163 date and eggshell bacterial loads. Finding evidence of such an association would  
164 suggest that the general lower breeding success of late reproductive attempts may be  
165 partially driven by differential bacterial selection pressures mediated by adults, rather  
166 than by climatic-related environmental conditions, at the nests of birds.

167

## 168 **Material and Methods**

### 169 Study area

170 The study was performed during the breeding seasons of 2011-2012 in southeast Spain,  
171 in the Hoya de Guadix (37°18'N, 3°11'W), a high altitude plateau (1000 m a. s. l.),  
172 dominated by a semi-arid climate. The typical vegetation in the area is cultivated crops,  
173 olive and almond plantations, sparse holm oaks remaining from the original  
174 Mediterranean forest, small shrubs in abandoned fields, and deciduous trees in streams  
175 and villages. The magpie population is comprised of several subpopulations, some of  
176 them in irrigated and some others in arid environments (De Neve et al. 2007). We



177 sampled two of these subpopulations, 15-20 km apart from each other, one in irrigated  
178 (Albuñan) and another one in arid environment (Carretera). Probability of brood  
179 parasitism of magpie nests by the great spotted cuckoo is quite high in the area, but  
180 temporally and spatially variable at the small geographic scale of the study area (Soler  
181 et al. 1999; Soler & Soler 2000; Martín-Gálvez et al. 2007; Soler et al. 2013).

182

183 Field work

184 Magpie territories known from previous years were visited once a week since the 15<sup>th</sup> of  
185 March to detect new nests. Once we found a new nest, it was visited twice a week,  
186 which allowed us to know laying date of the first egg and to detect brood parasitism.  
187 Laying date of sampled nests in 2011 extended from the 3<sup>rd</sup> of April to the 12<sup>th</sup> of May  
188 and in 2012 from the 31<sup>st</sup> of March to the 12<sup>th</sup> of May (average laying date for both  
189 years was the 19<sup>th</sup> of April).

190 For eggshell bacterial sampling, we wore new latex gloves sterilized with 96%  
191 ethanol for each nest to prevent inter-nest contamination. Once gloves were dry, we  
192 gently handled and sampled eggs by rubbing the complete eggshell with a sterile rayon  
193 swab (EUROTUBO® DeltaLab) slightly wet with sterile sodium phosphate buffer (0.2  
194 M; pH = 7.2). After cleaning the complete egg surface, the swab was introduced in a  
195 rubber-sealed microfuge tube with 1.2 ml of sterile phosphate solution and transported  
196 in a portable refrigerator at 4-6°C. Samples were stored at 4°C until being processed in  
197 the laboratory within 24 h after collection. Estimates of bacterial load were standardized  
198 to number of colonies (CFU's, Colonies Forming Units) per cm<sup>2</sup> (i.e. eggshell bacterial  
199 density) as previously described elsewhere (Soler et al. 2011).

200

201 Experimental procedures

202            *Natural magpie nests*

203    Each of the magpie nests found before incubation started was randomly assigned to one  
204    of the following three experimental treatments: (1) Experimental nests: we included a  
205    broken quail egg in the nest. The experimental quail egg was broken inside magpie nest,  
206    making a hole of enough size to assure that most content spilled off when we moved it  
207    together with all other eggs in the nest. In that way, wore gloves used for moving the  
208    eggs come to be besmeared with egg contents, which assure that most magpie egg  
209    surface became in contact with egg contents either, because of direct contact with quail  
210    eggshell or because gently touched with smudged gloves. (2) Control I nests: we  
211    included a non-broken quail egg in the magpie nest and moved it as we did with the  
212    broken egg for the experimental treatment. Quail eggs were cleaned with disinfectant  
213    wipes (Aseptonet, LaboratoiresSarbec, Cod.998077-51EN) before using in magpie  
214    nests. (3) Control II nests: we visited and sampled these nests at the same rate as nests  
215    in other treatments, but no quail egg was added.

216            On average, magpies start to incubate when laying the fourth egg, but  
217    occasionally it may occur with the third, or be delayed up to the 7th egg (Birkhead  
218    1991). Bacteria from eggshells of experimental and control nests were sampled three  
219    times. First samples were collected 0-5 (mean (SE) = 2.3 (0.04), N = 236) days after  
220    laying of the first egg (i.e. before incubation started – not warm eggs with no sign of  
221    incubation), second samples were collected 4-5 days after the first sampling, i.e. day 5-8  
222    (mean (SE) = 6.2 (0.03), N = 220) after laying of the first egg (i.e. after incubation  
223    started – warm eggs with sign of incubation). Third samples were collected 14-19  
224    (mean (SE) = 17.1 (0.06), N = 100) days after the first eggs was laid (i.e. before  
225    hatching). Broken magpie eggs or with traces of egg content spilling were detected in  
226    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).

240

#### 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 20<sup>th</sup> of April to the 19<sup>th</sup> of May)  
251 homogeneously (at least one pair of eggs every second day and an average of 2.4 pair of

252 eggs per day). Before introduction in the cage, eggs were cleaned with disinfectant  
253 wipes. Afterwards, the control egg was gently handled with gloves cleaned with ethanol  
254 and laid on the nest material of experimental cages, whilst the experimental egg was  
255 handled with the sample gloves but soiled with the content of a broken quail egg. Thus,  
256 the experimental but not the control egg was coated with egg contents. Experimental  
257 and control eggs within the same cage were not in contact to each other. A new pair of  
258 eggs was added to the experimental cages every four days, and the same cage harboured  
259 up to 4 pairs of experimental eggs. None of the eggs was in direct contact to each other.

260 With an indelible marker, we painted a line throughout the egg poles dividing  
261 egg surface in two halves; one of them was sampled 4 days after the experiment. The  
262 non-sampled surface of fifty-three pairs of eggs was sampled 16 days after the onset of  
263 the experiment. We failed to analyse samples from five control and three experimental  
264 eggs collected from 7 different egg pairs 4 days after the onset of the experiment. These  
265 losses were due to breakage of quail eggs during sampling or because samples  
266 disappeared before being analysed in the lab. Thus, we obtained a final sample of 100  
267 pairs of eggs for the analyses.

268

#### 269 Laboratory work

270 Before cultivation, samples stored in microfuge tubes were shaken in a vortex (Boeco  
271 V1 Plus) for at least three periods of 5 seconds. Bacteriology was performed by  
272 spreading homogenously 100 µl of serially diluted samples onto Petri dishes containing  
273 four different solid agar media (ScharlauChemie S.A. Barcelona). We used Tryptic Soy  
274 Agar, a broadly used general medium to grow aerobic mesophilic bacteria, and three  
275 specific media: Kenner Fecal Agar for *Enterococcus*; Vogel-Johnsson Agar for  
276 *Staphylococcus*; and Hektoen Enteric Agar for *Enterobacteriaceae*. The plates were

277 incubated aerobically at 37°C and colonies were counted 72h after inoculation. Bacterial  
278 density was estimated for each of the four media as number of Colony Forming Units  
279 per cm<sup>2</sup> following previously described protocol (Peralta-Sánchez et al. 2010; Soler et  
280 al. 2011). We estimated eggshell bacterial density for all samples collected during  
281 magpie egg laying, onset of incubation, and end of incubation, and for samples obtained  
282 from quail eggs.

283

#### 284 Statistical Analyses

285 Log<sub>10</sub> transformed density of mesophilic bacteria differed from normal distribution and  
286 we conservatively used ranked values for statistical analyses. Specific group of bacteria  
287 (*Enterobacteriaceae*, *Staphylococcus* and *Enterococcus*) were not detected for many  
288 samples (see results) and, thus, frequency distributions were far from Gaussian shape.  
289 Thus, we used information on prevalence of each bacterial group in the analyses. To  
290 statistically account for inter-year variation in laying date, values for each date were  
291 standardized by deducting observed to mean values and dividing by standard deviation.  
292 We used these values in subsequent analyses.

293         The expected effects of having experimental or natural broken eggs (and/or trace  
294 of egg contents (i.e. yolk)) and of laying date in magpie eggshell bacterial loads were  
295 analysed in Repeated Measures ANOVAS (RMA) with ranked values of mesophilic  
296 bacterial loads estimated at different visits (egg laying, onset and end of incubation) as  
297 within factor, experimental treatment (or having or not trace of natural egg breakage),  
298 area (irrigated or arid) and year as between factor, and standardized laying date as  
299 covariable. Because the association with laying date may depend on experimental  
300 treatment (or on egg breakage), we estimated the effect of such interaction in separate  
301 models. The effect of experimental coating commercial quail eggs on eggshell bacterial

302 loads (i.e. ranked valued of mesophilic bacterial density) was explored by RMA with  
303 pair of eggs of the same laying date (experimental and control eggs) as repeated  
304 measures, area (irrigated or arid) as discrete between factor, and laying date as  
305 covariable. Prevalence of mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus*, and  
306 *Enterococcus* in relation to experimental treatment (or natural egg breakage), year (only  
307 for natural magpie nests), area, and laying date were analysed by mean of Generalized  
308 Linear Models with binomial distribution and logic link functions.

309         Some of the experimental and natural magpie nests were depredated during  
310 incubation or were heavily parasitized by the great spotted cuckoo and, thus, sample  
311 size for third bacterial sampling (i.e., at the end of incubation) was reduced. However,  
312 main effects were detected independently of whether or not information of these third  
313 samples was considered. Thus, we report results of models explaining prevalence and  
314 bacterial density estimated for the first and second samplings because of the higher  
315 statistical power.

316         Log10 transformed bacterial density rather than ranked values were used for  
317 figures. All statistical tests were performed with Statistica 10.0 (Statsoft Inc. 2011).

318

## 319 **Results**

320

321 Bacterial loads of magpie eggshells. Effect of natural and experimental breakage of  
322 eggs in the nest.

323 Contrary to **P1**, the occurrence of broken eggs in magpie nests due to brood parasitic  
324 activity did not affect density of mesophilic bacteria on magpie eggshells, which were  
325 mainly explained by study area (higher in the arid subpopulation) (Table 1). Moreover,  
326 laying date was positively associated with density of mesophilic bacteria (Fig. 1), which

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

330         When considering magpie nests where quail eggs were experimentally broken,  
331 results were quite similar to those with natural broken eggs by brood parasites (Table 1).  
332 First, we compared eggshell bacterial load between the two types of control nests, with  
333 a non-broken quail egg and without quail egg, and failed to detect statistically  
334 significant differences (identical model that those in Table 1, effect of treatment,  $F =$   
335  $2.27$ ,  $df = 1,116$ ,  $P = 0.135$ ). Moreover, the interaction between experimental treatments  
336 of the two types of control nests and all other factors did not reach statistical  
337 significance ( $P > 0.7$ ). Thus, we considered all control nests together for subsequent  
338 analyses. We detected an increase in bacterial density after incubation (Fig. 2), a  
339 significant lower bacterial density of eggs sampled in 2012 and in irrigated areas, and a  
340 significant interaction between study year and area (interaction in Table 1, Fig. 2). The  
341 only detected effect of experimental treatment was indirect, through its interaction with  
342 year, area, and incubation (Table 1, Fig. 2). These results did not change after removing  
343 from the model the non-significant terms (results not shown). Finally, and contrary to  
344 **P2** and **P3**, density of bacteria for this subset of nests did increase as the season progress  
345 (Table 1, Fig. 1) independently of experimental treatments.

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

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

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

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

370

371 Bacterial loads of quail eggshells in experimental artificial nests.

372 Eggshell mesophilic bacterial load was higher in the arid than in the irrigated area  
373 (RMA,  $F = 25.59$ ,  $df = 1,97$ ,  $P < 0.0001$ ) and, contrary to what we detected for natural  
374 magpie nests, but in accordance with **P2**, eggshell bacterial loads of quail eggs  
375 decreased as the season progressed (RMA,  $F = 10.98$ ,  $df = 1,97$ ,  $P = 0.0013$ ). In  
376 accordance with **P1**, experimental eggs coated with egg contents harboured bacteria at a



377 higher density than control eggs (RMA,  $F = 13.65$ ,  $df = 1,97$ ,  $P = 0.0004$ ) (Fig. 3A).  
378 Interestingly, the effect of the experiment on density of mesophilic bacteria did not  
379 depend on the area (RMA, interaction between experimental treatment and area,  $F =$   
380  $1.99$ ,  $df = 97$ ,  $P = 0.162$ ), but density of mesophilic bacteria tended to decrease as the  
381 season progressed mainly in experimental eggs (RMA, interaction between  
382 experimental treatment and laying date,  $F = 3.76$ ,  $df = 1,98$ ,  $P = 0.084$ ) (Fig. 3B), which  
383 do not support **P3**.

384         Similar results were obtained when analysing bacterial prevalence. Laying date  
385 did significantly associate with prevalence of mesophilic bacteria (negatively) and of  
386 *Enterococcus* (positively) (Table 3). Prevalence of mesophilic bacteria,  
387 *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus* was higher in experimental  
388 smeared quail eggs than in control eggs (Fig. 4), and this effect did not depend on the  
389 area (Table 3). The experimental effects on prevalence of mesophilic bacteria did vary  
390 depending on laying date (interaction term in Table 3), which support **P3**. However, the  
391 effects of laying date on prevalence and density of bacteria on the eggshell depended of  
392 the considered bacterial group.

393

394 Remarks on results from artificial and natural magpie nests.

395 Effects of experimental smearing with egg contents of quail eggs on eggshell bacterial  
396 loads were detected in artificial but not in natural magpie nests, which is in accordance  
397 with our prediction number four (**P4a**). The expected negative relationship between  
398 eggshell bacterial loads and laying date was only detected in artificial nests, but turned  
399 to be positive in natural magpie nests, which agrees with **P4b**.

400

401 **Discussion**

402 Our main results are that the experimental besmearing of eggshells with eggs contents  
403 provokes an increase in eggshell bacterial density and prevalence in experimental-non-  
404 active nests, but not in nests with incubating magpies. Moreover, laying date was  
405 positively related with eggshell bacterial density and prevalence in active magpie nests,  
406 but negatively in artificial nests without incubation activity. Quail eggs were used in  
407 artificial nests and, thus, detected differences between artificial and natural magpie nests  
408 could be explained by differences in eggshell properties between magpies and quail  
409 eggshells. This possibility is however unlikely since magpie and control quail eggs in  
410 natural magpie nests harboured similar bacterial density some days after incubation (see  
411 Material and Methods).

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

422 We knew that brood parasitism by great spotted cuckoos was positively related  
423 to bacterial load of magpie eggshells which, among other possibilities, was attributed to  
424 the egg-breaking behaviour of cuckoos resulting many times in egg-content spilling  
425 (Soler et al. 2011). Here, we found no experimental support for this hypothesis in  
426 magpie nests. However, experimental coating of quail eggs with egg-contents did result

427 in significant increases in eggshell bacterial loads and prevalence four days after the  
428 manipulation. These two results therefore suggest that egg-breaking behaviour of  
429 cuckoos provoking egg-contents spilling should affect eggshell bacterial loads of their  
430 magpie hosts, but that the effect is at least partially counteracted by magpie females.  
431 The previously detected association between brood parasitism and eggshell bacterial  
432 loads of magpie eggs would therefore be the consequence, not only of egg breaking  
433 behaviour of cuckoos, but also of input of bacteria from cuckoos on the parasitic eggs or  
434 due to subsequent visits to magpie nests by the brood parasite (Soler et al. 2011).

435 Incubation or any other parental behaviour influencing bacterial environment of  
436 nests (Clark & Mason 1985; Cook et al. 2005a; Mennerat et al. 2009; D'Alba et al.  
437 2010; Soler et al. 2010; Lee et al. 2014) is likely the cause of the reduced experimental  
438 effects detected in natural magpie nests. Magpies do not use green-aromatic plants or  
439 feathers in their nests for nest building in our study area and, thus, the antimicrobial  
440 properties of these materials (see Introduction) cannot explain detected differences  
441 between artificial and natural magpie nests. However, belly feathers of magpies are  
442 unpigmented and therefore more easily degradable by queratinolitic bacteria with  
443 important antimicrobial activity (Peralta-Sánchez et al. 2010; Peralta-Sánchez et al.  
444 2014), that are in contact with the eggshells and may reduce growth of pathogenic  
445 bacteria (Lee et al. 2014). In addition, magpies build a quite apparent mud cup, and we  
446 know of the use of mud therapies because of the antimicrobial properties of clays (Said  
447 et al. 1980; Maigetter & Pfister 1975). For our artificial nests we used vegetable nest  
448 lining material (i.e. roots), but not the mud cup of magpie nests. Thus, it is possible that,  
449 in addition to incubation activity, mud in the nests of magpies and/or white belly  
450 feathers of incubating females might account for the reduced experimental effects  
451 detected in natural nests, a hypothesis worth to be tested in the future.

452           The second main result is the detected associations between laying date and  
453 eggshell bacterial load and prevalence. Also in this case the associations detected for  
454 magpie nests were contrary to those detected for artificial nests (Fig. 1 and Fig. 3B),  
455 again suggesting an important role of magpie adults determining bacterial environments  
456 in nests. While in natural magpie nests the relationship between eggshell bacterial load  
457 and laying date was positive, in artificial nests the association turned to be negative.  
458 Within the study area temperature increases (2011:  $R = 0.774$ ,  $N = 66$ ,  $P < 0.0001$ ;  
459 2012:  $R = 0.497$ ,  $N = 66$ ,  $P < 0.0001$ ) and humidity decreases (2011:  $R = -0.602$ ,  $N =$   
460  $66$ ,  $P < 0.0001$ , but not in 2012:  $R = 0.120$ ,  $N = 66$ ,  $P = 0.33$  along the sampling period  
461 (average daily temperature and humidity from 1<sup>st</sup> of April to 5<sup>th</sup> of June; data from  
462 Consejería de Medio Ambiente y Ordenación del Territorio,  
463 <http://www.juntadeandalucia.es/medioambiente/servtc5/sica/Estaciones.jsp>, station:  
464 Guadix). Thus, our results may indicate a negative influence of temperature and a  
465 positive effect of humidity on eggshell bacterial colonization and growth in the absence  
466 of incubation. In nests with incubated eggs the association between laying date and  
467 eggshell bacterial load was the opposite and, thus, variation of environmental climatic  
468 conditions for breeding as the season progresses are unlikely the direct cause of the  
469 detected higher risk of bacterial infection experienced in late breeding attempts of  
470 magpies. These results therefore reinforce the importance of parental attendance  
471 (including nest building) that protects offspring from environments influencing bacterial  
472 colonization and growth.

473           Negative associations between laying date and different breeding parameters of  
474 birds reflecting breeding success such as clutch size, brood size, and fledging success,  
475 are normally found for birds reproducing in temperate areas (see Introduction). This  
476 association has been traditionally explained by deterioration of environmental

477 conditions (i.e., decreasing and increasing availability of resources and probability of  
478 parasitism respectively) (Sorci et al. 1997; Siikamäki 1998; Merino et al. 2000; Verhulst  
479 & Nilsson 2008) and/or parental quality and adult condition as the season progresses  
480 (Hochachka 1990; Christians et al. 2001; Winkler et al. 2014). Our results suggest that  
481 deterioration of nest bacterial environments as the season progresses would contribute  
482 to explain the reduced breeding success of late breeders, a possibility never suggested.  
483 Variation in food availability and/or phenotypic condition of parents (including parasite  
484 infection status) would affect parental activity (Winkler & Allen 1996), including nest  
485 building effort (Soler et al. 1995; Soler et al. 1998), incubation attendance (Chastel et al.  
486 1995) and, perhaps, nest sanitation. All these activities potentially determine bacterial  
487 communities of nests, at least partially (see Introduction). Moreover, birds of poor  
488 phenotypic condition would harbour bacteria at a higher density (Møller et al. 2012) and  
489 infect nest contents during reproduction. Thus, extensive theoretical background  
490 allowed predicting positive covariation between the well-known, and widely accepted,  
491 seasonal decline in breeding success in temperate areas and nest bacterial environment.  
492 Our results suggest that the seasonal increase of bacterial density may be caused by a  
493 decrease in nest parental attendance, which would suggest a role of bacteria driving the  
494 seasonal decline in breeding success for which we have detected pioneering evidence.  
495 Experimental manipulation of factors affecting parental attendance (i.e. incubation) are  
496 however necessary to reach firm conclusions.

497         Summarizing, our experimental approaches allowed us to detect different  
498 dynamics in bacterial communities of eggshells in artificial and natural nests in relation  
499 to hygienic conditions, incubation activity and laying date. Since laying date resulted  
500 positively associate to bacterial density in natural, but not in artificial nests, we  
501 conclude that this association is mediated by parental characteristics which suggests a

502 central role for bacteria explaining the generalized negative association between laying  
503 date and avian breeding success.

504

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513

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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<br>breakage<br>(3) | (1)x(2)      | (1)x(3) | (2)x(3) | (1)x(2)x(3)  | Laying<br>date x (3) |
| Between effects                                |              |                    |                    |                    |                        |              |         |         |              |                      |
| F(1,130)                                       |              | <b>23.55</b>       | 2.38               | <b>6.02</b>        | 0.13                   | 0.14         | 0.20    | 0.07    | 0.31         | 0.51                 |
| P  |              | <b>&lt; 0.0001</b> | 0.125              | <b>0.015</b>       | 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                   | <b>4.84</b>  | 1.61    | 0.40    | 0.78         | 0.07                 |
| P  | 0.241        | 0.854              | 0.314              | 0.570              | 0.758                  | <b>0.030</b> | 0.206   | 0.527   | 0.379        | 0.793                |
| Experimentally broken eggs                     |              |                    |                    |                    |                        |              |         |         |              |                      |
| Repeated measures (before vs after incubation) |              |                    |                    |                    |                        |              |         |         |              |                      |
| Between effects                                |              |                    |                    |                    |                        |              |         |         |              |                      |
| F(1,176)                                       |              | <b>30.40</b>       | <b>24.99</b>       | <b>26.51</b>       | 0.82                   | <b>7.07</b>  | 0.01    | 1.90    | 0.16         | 0.106                |
| P  |              | <b>&lt; 0.0001</b> | <b>&lt; 0.0001</b> | <b>&lt; 0.0001</b> | 0.366                  | <b>0.009</b> | 0.928   | 0.170   | 0.690        | 0.745                |
| Within effects                                 |              |                    |                    |                    |                        |              |         |         |              |                      |
| F(1,176)                                       | <b>5.47</b>  | 0.28               | 0.12               | 0.50               | 0.05                   | 1.50         | 0.96    | 0.30    | <b>4.01</b>  | 0.904                |
| P  | <b>0.020</b> | 0.594              | 0.726              | 0.480              | 0.819                  | 0.222        | 0.328   | 0.587   | <b>0.047</b> | 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<br>(N = 139) |                         |                           |               | Experimental vs control nests<br>(N = 185) |                         |                           |                   |
|-----------------------------|-----------------------------------|-------------------------|---------------------------|---------------|--|-------------------------|---------------------------|-------------------|
|                             | Wald<br>Statistic                 | Estimate<br>CI<br>(95%) | Estimate<br>- CI<br>(95%) | P             | Wald<br>Statistic                          | Estimate<br>CI<br>(95%) | Estimate<br>- CI<br>(95%) | P                 |
| <i>Mesophilic bacteria*</i> |                                   |                         |                           |               |  |                         |                           |                   |
| LAYING DATE (1)             | <b>8.25</b>                       | <b>0.620</b>            | <b>3.281</b>              | <b>0.0041</b> | <b>10.29</b>                               | <b>0.677</b>            | <b>2.803</b>              | <b>0.0013</b>     |
| 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            |
| <i>Enterobacteriaceae</i>   |                                   |                         |                           |               |  |                         |                           |                   |
| LAYING DATE (1)             | <b>8.11</b>                       | <b>0.272</b>            | <b>1.471</b>              | <b>0.0044</b> | <b>16.22</b>                               | <b>0.670</b>            | <b>1.940</b>              | <b>&lt;0.0001</b> |
| YEAR                        | <b>9.34</b>                       | <b>0.386</b>            | <b>1.767</b>              | <b>0.0022</b> | <b>14.61</b>                               | <b>0.616</b>            | <b>1.912</b>              | <b>0.0001</b>     |
| 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            |
| <i>Staphylococci**</i>      |                                   |                         |                           |               |  |                         |                           |                   |
| 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        | <b>4.86</b> | <b>0.133</b>  | <b>2.263</b>  | <b>0.0274</b> |  |
| 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        |  |
| <i>Enterococci***</i>  |             |              |              |               |             |               |               |               |  |
| 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        |  |
| <b>BROKEN EGGS (2)</b> | <b>4.92</b> | <b>0.160</b> | <b>2.594</b> | <b>0.0266</b> | <b>4.84</b> | <b>-2.315</b> | <b>-0.134</b> | <b>0.0277</b> |  |
| (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 = 1<sup>st</sup> 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<br>Statistic | Estimate<br>CI (95%) | Estimate<br>- CI (95%) | P                 |
|----------------------------|-------------------|----------------------|------------------------|-------------------|
| <i>Mesophilic bacteria</i> |                   |                      |                        |                   |
| <b>LAYING DATE (1)</b>     | <b>4.00</b>       | <b>0.001</b>         | <b>0.079</b>           | <b>0.0455</b>     |
| <b>AREA (2)</b>            | <b>10.17</b>      | <b>-1.255</b>        | <b>-0.300</b>          | <b>0.0014</b>     |
| <b>EXPERIMENT (3)</b>      | <b>7.88</b>       | <b>0.206</b>         | <b>1.163</b>           | <b>0.0050</b>     |
| (2) X (3)                  | 0.09              | -0.552               | 0.403                  | 0.7599            |
| <b>(1) X (3)</b>           | <b>4.84</b>       | <b>-0.098</b>        | <b>-0.006</b>          | <b>0.0284</b>     |
| <i>Enterobacteriaceae</i>  |                   |                      |                        |                   |
| LAYING DATE (1)            | 0.29              | -0.038               | 0.067                  | 0.589             |
| <b>AREA (2)</b>            | <b>250.90</b>     | <b>-5.016</b>        | <b>3.911</b>           | <b>&lt;0.0001</b> |
| <b>EXPERIMENT (3)</b>      | <b>158.13</b>     | <b>4.266</b>         | <b>5.842</b>           | <b>&lt;0.0001</b> |
| (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            |
| <b>EXPERIMENT (3)</b>      | <b>6.34</b>       | <b>0.183</b>         | <b>1.466</b>           | <b>0.0118</b>     |
| (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>         |                   |                      |                        |                   |
| <b>LAYING DATE (1)</b>     | <b>7.82</b>       | <b>-0.096</b>        | <b>-0.017</b>          | <b>0.0052</b>     |
| <b>AREA (2)</b>            | <b>0.57</b>       | <b>-0.292</b>        | <b>0.657</b>           | <b>0.4509</b>     |
| <b>EXPERIMENT (3)</b>      | 12.89             | 0.395                | 1.344                  | 0.0004            |
| <b>(2) X (3)</b>           | 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

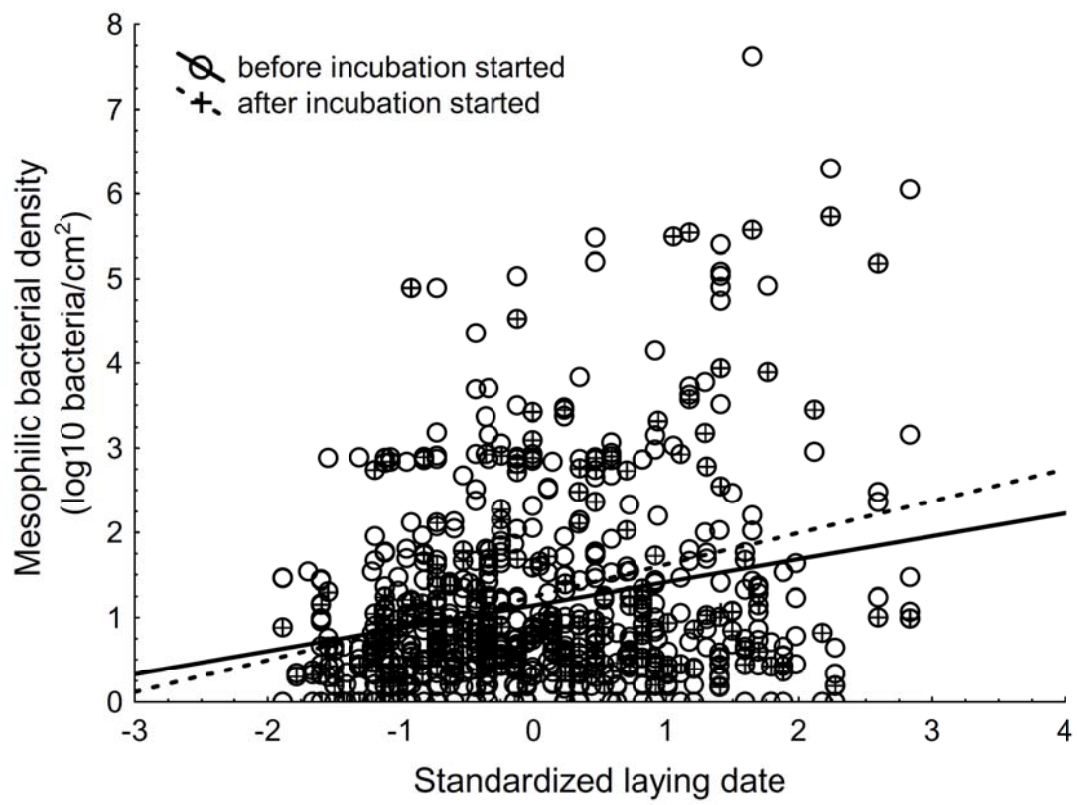


Fig. 2

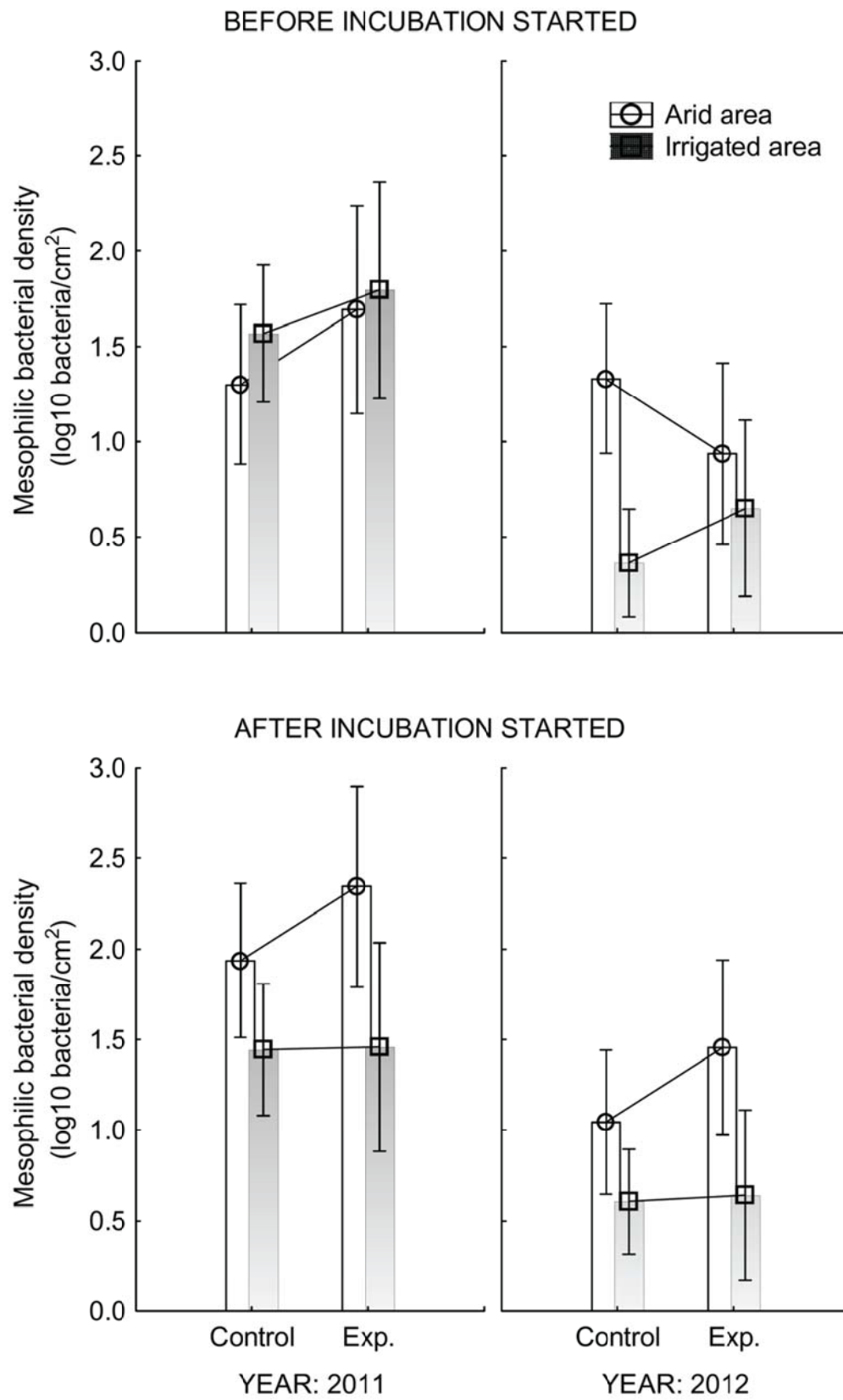


Fig. 3

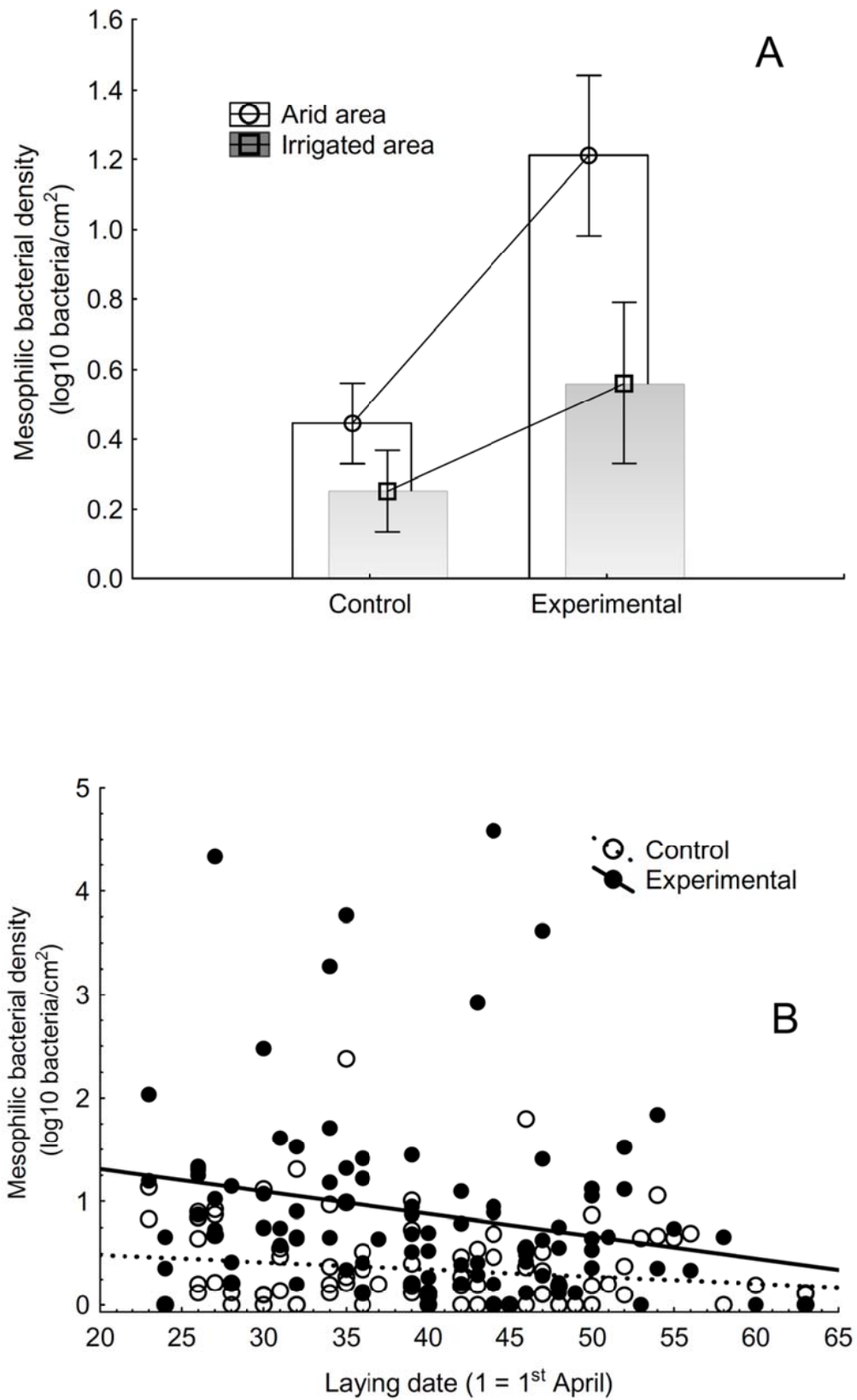


Fig. 4

