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Brood parasitism is associated with increased bacterial contamination of host eggs: bacterial loads of host and parasitic eggs

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Factors related to bacterial environment of nests are of primary interest for understanding the causes of embryo infection and the evolution of antimicrobial defensive traits in birds. Nest visitors such as parasites could act as vectors for bacteria and/or affect the hygienic conditions of nests and hence influence the nest bacterial environment. In the present study, we explored some predictions of this hypothetical scenario in the great spotted cuckoo (*Clamator glandarius*)–magpie (*Pica pica*) system of brood parasitism. Great spotted cuckoos visit the nests of their magpie hosts and frequently damage some of the host eggs when laying eggs or on subsequent visits. Therefore, it represents a good system for testing the effect of nest visitors on the bacterial environment of nests. In accordance with this hypothesis, we found that the bacterial load of magpie eggshells was greater in parasitized nests, which may suggest that brood parasitism increases the probability of bacterial infection of magpie eggs. Moreover, comparisons of bacterial loads of cuckoo and magpie eggs revealed that: (1) cuckoo eggshells harboured lower bacterial densities than those of their magpie hosts in the same nests and (2) the prevalence of bacteria inside unhatched eggs was higher for magpies than for great spotted cuckoos. These interspecific differences were predicted because brood parasitic eggs (but not host eggs) always experience the bacterial environments of parasitized nests. Therefore, the results obtained in the present study suggest that parasitic eggs are better adapted to environments with a high risk of bacterial contamination than those of their magpie hosts. *Biological Journal of the Linnean Society*, 2011, **103**, 836–848.

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INTRODUCTION

The bacterial environment of avian nests is traditionally considered to be an important selective agent force acting on embryo viability (Baggott & Graeme-Cook, 2002). Therefore, the factors related to bacterial environment of nests are of primary interest for understanding the causes underlying the probability of embryo infection and the evolution of antimicrobial defensive traits of birds. Temperature, humidity, and hygienic conditions in the nest are known to determine bacterial growth on the eggshells and hence the trans-shell bacterial infection of embryos (Bruce & Drysdale, 1994). Moreover, apart from physical and chemical antimicrobial barriers of avian eggs (Board *et al*., 1994), behaviours such as those related to nest cleaning (Bruce & Drysdale, 1991) or the use of nest materials with antimicrobial properties (Clark & Mason, 1985, 1988; Mennerat *et al*., 2009) also affect the bacterial environment of nests. In addition, others behaviours such as nest site (Godard *et al*., 2007) or when the incubation starts (Cook *et al*., 2003, 2005a; Shawkey *et al*., 2009) may not only affect the temperature and humidity of eggshells, but also the activation of antimicrobial defences, and therefore eggshell bacterial load, which is considered to be a good predictor of trans-shell embryo infection (Bruce & Drysdale, 1994; Cook *et al*., 2003, 2005b). The activity of nest visitors such as ecto- and brood parasites might also influence the bacterial environment. For example, they could directly act as vectors of some potentially pathogenic bacterial strains. Otherwise, some behaviours such as blood sucking and defecation by blood parasites (Avilés *et al*., 2009) or the breakage of host eggs by brood parasites (Soler, Soler & Martínez, 1997) could affect the hygienic conditions of nests and enhance bacterial growth within the nest environment. This hypothetical influence of nest visitors on the bacterial environmental conditions of nests could imply a possible additional cost of parasitism for host species, which, as far we are aware, has never been investigated and would have important implications for the evolutionary relationship with parasites.

Avian brood parasitism is a reproductive strategy by which parasites lay their eggs in the nests of other species, the hosts, which subsequently incubate and take care of parasitic offspring. Brood parasitism often drastically reduces the breeding success of their hosts, and selects for adaptive host responses. When effective defences against brood parasites spread in the host population, counter-defences will rapidly be selected in the brood parasite population, which again selects for more refined host defences in a coevolutionary arms race between brood parasites and their hosts (Rothstein, 1990; Davies, 2000; Soler & Soler, 2000). Brood parasites inflict usually severe fitness costs on their hosts by, for example, reducing the number of (or even eliminating) host offspring in parasitized nests. This reduction can be the result of direct adult behaviour when female parasites eat or break some of the host eggs when laying eggs and/or in possible subsequent visits, or a result of parasite nestlings evicting or outcompeting host nestlings (Davies, 2000). Interestingly, host-egg damage, either as result of rapid laying from the rim of the nest, or from active pecking of host eggs by brood parasites, could lead to a deterioration of hygienic conditions in host nests. This is because nest lining material and eggs could become smudgy with yolk and egg white from damaged eggs, which increase the nutrient availability for bacterial growth on eggshells (Stadelman, 1994). In addition, even without egg destruction, parasitic eggs or visits to the nest by adult parasites could result in new bacteria from the brood parasite species colonizing host nests (Ruiz-Rodriguez *et al*., 2009) and influencing the bacterial community of host nests. Great spotted cuckoos (*Clamator glandarius*) usually break some of the magpie (*Pica pica*) eggs in the nests (Soler *et al*., 1997); multiparasitism is relatively common in this system (Martínez *et al*., 1998) and we have evidence that cuckoos can visit magpie nests in several occasions during egg incubation (Soler *et al*., 1995b). Consequently, there are good reasons to predict that brood parasitism by the great spotted cuckoos would affect the bacterial environment of magpie nests. There is evidence to suggest a relationship between eggshell bacterial loads and the probability of embryo infection (Bruce & Drysdale, 1994; Cook *et al*., 2003, 2005b) and, thus, the hypothetical effect of brood parasitism on the bacterial environment of magpie eggs would imply additional costs for magpie hosts that would affect the evolutionary relationship with great spotted cuckoos.

In the present study, we explored several predictions of the hypothetical influence of brood parasitism on bacterial environments of host nests by estimating eggshell bacterial loads of magpie and great spotted cuckoo eggs. If brood parasitism affects the bacterial environment of magpie nests, we should find that bacterial loads of magpie eggs would be higher in parasitized nests than magpie eggs in unparasitized nests (Prediction 1).

Interestingly, selection pressures as a result of such hypothetical changes in environmental conditions caused by brood parasitism should be asymmetric for brood parasites and hosts (i.e. rare enemy effect; Dawkins & Krebs, 1979). That is, although brood parasitic eggs will frequently experience contaminated nest environments (i.e. with broken eggs or with bacteria from both host and brood

parasitic species), this will not be the case for host eggs (i.e. nonparasitized nests). Because the probability of brood parasitism greatly varies in space and time (Brooke, Davies & Noble, 1998; Soler *et al*., 2001; Stokke *et al*., 2008), parasitic eggs, in comparison with host eggs, should have been selected for developing in nest environments with a relatively higher risk of infection by microorganisms. This scenario predicts that when brood parasitic and host eggs share an identical bacterial environment, the probability of embryo infection (i.e. prevalence of bacteria in eggs) would be lower for parasite than for host eggs (Prediction 2). We explore this possibility by analyzing interspecific differences in eggshell bacterial loads and in the probability of trans-shell bacterial contamination of magpie and great spotted cuckoo eggs.

The hypotheses tested in the present study deal with the exploration of factors that may affect a specific symbiotic interaction between bacteria and birds. Thus, for quantifying bacterial load, we used traditional culture-based techniques for the detection of the most common groups of bacteria known from avian eggshells.

MATERIAL AND METHODS

Study area

The study was performed during the breeding seasons of 2006–2007 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 seasonal streams and villages. The probability of brood parasitism of magpie nests by the great spotted cuckoo is quite high, although temporally and spatially variable at the small geographical scale of the study area (Soler *et al*., 1999; Soler & Soler, 2000; Martin-Galvez *et al*., 2007).

Field work

Magpie territories from previous years were visited once a week after 15 March to detect new nests. Once a new nest was found, it was visited twice a week, which allowed us to know the laying date and to detect brood parasitism. During the incubation period, bacteria from eggshells were sampled twice. First samples were taken 2–4 days after clutch completion, which assured that all sampled eggs were incubated. Second samples were collected 2–3 days before hatching. In accordance with previous studies (Soler *et al*., 1995a), magpie clutch sizes of parasitized and nonparasitized nests did not differ [unparasitized: 6.8(0.12), parasitized: 6.4(0.16); $t = 1.78$, d.f. = 83, $P = 0.08$] and thus the number of days that sampled eggs stayed in the nests before first sampling did not differ between groups of magpie nests. Samples were taken in the field from eggshells, attempting to keep the conditions as aseptic as possible. New latex gloves sterilized with 96% ethanol were used for each nest to prevent internest contamination. Once the gloves were dry, we gently handled and sampled eggs by rubbing the complete eggshell with a sterile rayon swab (Eurotubo® DeltaLab) made slightly wet with sterile sodium phosphate buffer (0.2 M; pH 7.2). We sampled all the eggs of the same species in the nest with a single swab, which, after cleaning the complete egg surface, was introduced into a rubber-sealed microfuge tube with 1.2 mL of sterile phosphate solution and transported in a portable refrigerator at 4–6 °C. Crushed eggs in the nests were not sampled. Samples were stored at 4 °C until processed in the laboratory. Estimates of bacterial load were standardized to number of colonies [colony-forming units (CFUs)] per cm^2 (i.e. eggshell bacterial density; see below) by taking into account total eggshell surface and number of eggs sampled in each nest. Eggshell surface was estimated according to a formula proposed by Narushin (1997):

$$S = 3 \times L^{0.771} \times W^{1.229}$$

where S is the surface in cm^2, L is the length of the egg, and W is the width of the egg. The length and width of each sampled egg were measured with a caliper (accuracy of 0.02 mm).

Unhatched eggs were visually and carefully inspected for fissures or narrow cracks on the eggshells, and we only collected those without such traces. These eggs were stored in individual and sterile tubes at 4 °C until processing in the laboratory approximately 20 days later. Finding bacteria inside eggs that failed to hatch cannot be interpreted as these bacteria being the cause of hatching failure because, for example, trans-shell infection by bacteria may have occurred after the death of embryos. Thus, in accordance with previous studies, we used these unhatched eggs for exploring interspecific differences in the probability of trans-shell contamination of eggs (Bruce & Drysdale, 1994).

Laboratory work

Samples stored in microfuge tubes were shaken in a vortex (Boeco V1 Plus!) for at least three periods of 5 s. Subsequently, the solution containing bacteria was used for cultivation. Bacteriology was performed by spreading homogenously 100 μL of sample of each serial dilution onto Petri dishes of four different agar media (Scharlau Chemie S. A. Barcelona). We

used Tryptic Soy Agar (TSA), a broadly used general medium to grow aerobic mesophilic bacteria, and three specific media: Kenner Fecal Agar (KF) for *Enterococcus*; Vogel–Johnsson Agar (VJ) for *Staphylococcus*; and Hektoen Enteric Agar (HK) for *Enterobacteriaceae*. The plates were incubated aerobically at 32 °C and colonies were counted 72 h after inoculation. Bacterial density was estimated as the CFU cm^{-2}. We estimated eggshell bacterial density for first samples (soon after laying) and second samples (a few days before hatching) for each growth bacterial medium used.

Unhatched eggs were sampled and cultured to detect both internal and external bacterial contamination. The eggshell of each egg was also entirely swabbed using a sterile swab, which was aseptically transferred to a sterile tube with 10 mL of phosphate-buffered saline; from this tube, 1 mL was extracted and used for serial dilutions. Total aerobic mesophilic bacteria were enumerated by duplicate plating of 100-μL aliquots onto TSA (bioMérieux España, S. A.). Plates were incubated at 32 °C for 48 h. Subsequently, colonies were counted and the eggshell bacterial density was estimated. After disinfection of the eggshell surface with ethanol (70%), unhatched eggs were broken and the yolk and egg white were homogeneously mixed using a sterile inoculation loop (single-use). Embryos were separated before mixture and were no longer than 5 mm (i.e. embryos that died during the first few days of incubation). An aliquot of the content was surface plated onto MacConkey agar and Columbia blood agar (bioMérieux España, S. A.). MacConkey agar is a selective medium for growth of Gram-negative bacteria, and Columbia blood agar is a media used to isolate pathogenic organisms and detect hemolytic activity. Plates were incubated for 48 h at 37 °C under aerobic conditions and one additional plate of Columbia blood agar under anaerobic conditions. The most abundant CFU of each sample was selected as the representative isolate and subcultured for further identification.

The bacteria were biochemically identified by using commercial systems (bioMérieux España S. A.). Bacterial analyses were also performed for detection of *Salmonella* contamination. External and internal samples were pre-enriched with buffered peptone water (bioMérieux España, S. A.). After 16–20 h at 37 °C, two methods of enrichment were used for 18–24 h. The first method consisted of inoculation of three drops of sample in a circle close to the periphery of a modified semi-solid Rappaport–Vassiliadis plate (Difco, BD Diagnostic Systems). We verified the presence of the halos of growth after 24 h of incubation at 42 °C. Suspected positive growth were surface plated onto Xylose lysine deoxycholate (XLD) and *Salmonella* detection and identification (SMID) agars (bioMérieux España, S. A.), followed by incubation at 37 °C for 24 h. The second method was a selective enrichment in Muller Kauffmann with tetrathionate and novobiocin broth (bioMérieux España, S. A.), which was incubated at 37 °C for 24 h and plated on the above selective solid mediums. A portion of the suspected colonies from XLD and SMID agars were confirmed by the Enterotube system (Difco).

Culture-based techniques do not characterize microbial communities as well as molecular techniques because only approximately 1% of micro-organisms are cultivable (Amann, Ludwig & Schleifer, 1995). However, culture-independent methods also have limitations of bias and errors (Qiu *et al*., 2001; Speksnijder *et al*., 2001; Shawkey *et al*., 2005). Interestingly, both methodologies yielded similar conclusions when exploring the effects of incubation on eggshell bacterial growth (Cook *et al*., 2005a; Shawkey *et al*., 2009). Moreover, apart from the general medium for aerobic mesophiles, we have selected specific media for the most common groups of bacteria known to live in avian eggshells and known to reduce embryo viability based on extensive studies of bacteria on domestic and wild bird eggs (Board & Tranter, 1986; Kozlowski *et al*., 1989; Bruce & Drysdale, 1991, 1994; Houston, Saunders & Crawford, 1997; Cook *et al*., 2003; 2005a, b; Soler *et al*., 2008; Shawkey *et al*., 2009; Peralta-Sánchez *et al*., 2010). Used together, these media should adequately characterize the relative load of bacterial groups living on the avian eggshell that are known to produce pathogenic infection of embryos.

Sample sizes and statistical analysis

Bacterial loads did not approach normal distributions even after $\log_{10}$-transformation (Kolmogorov–Smirnov tests for continuous variables, $P < 0.05$). Thus, we used rank values rather than $\log_{10}$-transformed bacterial counts in our analyses. To provide information on eggshell bacterial density, we show $\log_{10}$-transformed data.

The year or its interaction with species identity did not significantly explain eggshell bacterial load either at the beginning [multivariate analysis of variance (MANOVA); dependent variables: TSA, KF, VJ, and HK culture media; independent variables: species identity and year of sample: effect of year: Wilks = 0.92, $F = 2.27$, d.f. = 4, 109, $P = 0.07$; univariate results: $F < 2.76$, d.f. = 1, 112, $P > 0.10$; year and species identity interaction: Wilks = 0.97, $F = 0.97$, d.f. = 4, 109, $P = 0.43$; univariate results: $F < 1.23$, d.f. = 1, 109, $P > 0.27$], or at the end of the incubation period (identical MANOVA model: effect of year: Wilks = 0.96, $F = 0.67$, d.f. = 4, 68, $P = 0.61$; univariate results: $F < 1.97$, d.f. = 1, 71, $P > 0.16$; year and

species identity interaction: Wilks = 0.99, $F = 0.21$, d.f. = 4, 68, $P = 0.93$; univariate results: $F < 0.24$, d.f. = 1, 71, $P > 0.62$). Therefore, we did not include year and its interaction with species in the subsequent analyses.

Time of storage of samples at 4 °C (mean ± SE = 9.9 ± 0.7, $N = 191$) would affect our estimates of eggshell bacterial loads. Attempting to take into account this source of variation in our statistical analyses, we included this information in the statistical models described below. Time of storage did not explain a significant proportion of variance of the variation of eggshell bacterial loads along the incubation period (results not shown) and it was removed from the statistical model. We also explored the reliability of estimates of eggshell bacterial loads by estimating the repeatability of bacterial counts in TSA medium from eggs of magpie and great spotted cuckoo that failed to hatch. For eggs of both species, repeatability was very high (great spotted cuckoo eggs: $H = 96.9\%$, $F = 63.5$, d.f. = 8, 9, $P < 0.0001$; magpie eggs: $H = 99.4\%$, $F = 657.8$., d.f. = 12,13, $P < 0.0001$). Thus, we did not duplicate plating for all samples.

MANOVAs with eggshell bacterial densities in TSA, KF, VJ, and HK as dependent variables and nest status (parasitized versus nonparasitized) or species identity (magpie and great spotted cuckoo in parasitized nests) as independent factors were used respectively to test for the effect of parasitism and species on eggshell bacterial loads. These MANOVAs were performed separately for samples collected at the beginning and at the end of the incubation period. For within-nest comparisons, we used repeated measures MANOVAs with media for bacterial growth as dependent variables and species identity (magpie versus great spotted cuckoo eggs) or stage of incubation (at the beginning or at the end of incubation) as within factors.

We collected information from 33 parasitized and 51 nonparasitized magpie nests, although sample sizes differ for first and second sampling of parasitized and nonparasitized nests (Table 1) for several reasons. Some nests that were found after clutch completion were sampled but only swabs from those that hatched 2–3 days after swabbing the eggs were used in the analyses (second samples). Furthermore, we lost a first sample of a great spotted cuckoo egg, and some of the nests sampled at the beginning of incubation were depredated before the second sampling.

Unhatched but incubated eggs of great spotted cuckoos ($N = 9$, from three different nests) and magpies ($N = 13$, from 11 different nests) (only from one nest did we collect eggs from both species) were collected during field work in 2008 and 2009, 5–7 days after the last hatching event in the nests.

Table 1. Number of parasitized and unparasitized nests sampled at the beginning and at the end of the incubation period

Species	Early incubation	Late incubation	Both times
Parasitized			
Magpie	33	19	18
Great spotted cuckoo	32	11	9
Both species	32	10	9
Unparasitized			
Magpie	51	45	36

The number of sampled nests containing eggs of both species, as well as those sampled twice during the incubation period, are also shown.

Within-nest variation in eggshell bacterial counts was smaller than the among nests variation for magpie [one-way analysis of variance (ANOVA): $F = 58.06$, d.f. = 10,2, $P = 0.017$] but not for cuckoo unhatched-eggs (one-way ANOVA: $F = 0.37$, d.f. = 2,6, $P = 0.70$). Thus, we used two types of analyses, with either eggs or nests as independent data points.

All the analyses were two-tailed and conducted with STATISTICA, version 9.0 (StatSoft, Inc.).

RESULTS

Eggshell bacterial loads and brood parasitism

When comparing bacterial loads of magpie eggshells in parasitized and nonparasitized nests, we found statistically significant differences for load estimates at the beginning of the incubation period, with a greater bacterial load on eggs of parasitized nests, but not at the end of the incubation period (Fig. 1, Table 2). In parasitized nests, magpie eggs harboured higher bacterial density on their shells than eggs of the great spotted cuckoo, although this difference completely disappeared at the end of the incubation period (among nests comparisons; Fig. 1, Table 2). Results from repeated measures ANOVAs (i.e. within-nest comparisons; Table 2) again resulted in significant differences between cuckoo and magpie eggs at the beginning of the incubation period, although, at the end of incubation, shells of magpies eggs showed a nonsignificant trend to harbour *Staphylococcus* (i.e. VJ) at a higher density than great spotted cuckoo eggs in the same nests (Fig. 1, Table 2).

Finally, from unhatched eggs (i.e. those collected to study the bacterial load inside the eggs), estimates of density of total aerobic mesophiles were higher on magpie eggshells than on those of great spotted cuckoos, both when considering nests as independent

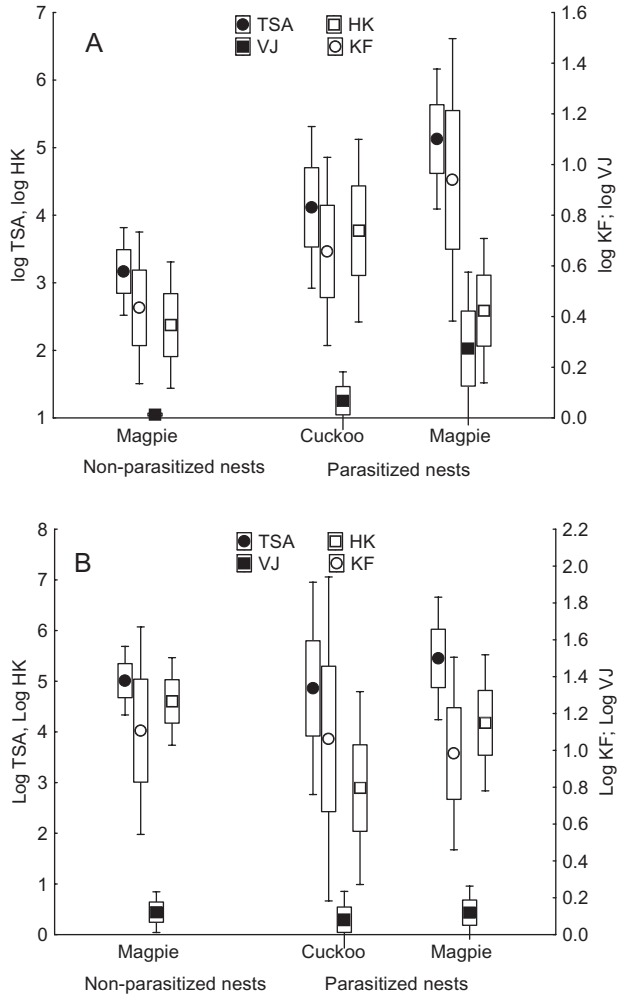

Figure 1. Mean ± SE (bars) and confidence intervals (whiskers) of $\log_{10}$-transformed estimates of eggshells bacterial loads of great spotted cuckoos and magpies in parasitized and nonparasitized nests at the beginning (A) and the end (B) of the incubation period. Estimates were performed from cultures in nonspecific medium (Tryptic Soy Agar, TSA), as well as in specific media for *Enterococcus* sp. (Kenner Fecal Agar, KF), *Staphylococcus* sp. (Vogel–Johnsson Agar, VJ), and *Enterobacteriaceae* (Hektoen Enteric Agar, HK).

data points (Mann–Whitney *U*-test: magpies: $N = 11$, rank sum = 96; cuckoo: $N = 3$, rank sum = 9; $Z = 2.10$, $P = 0.038$; Table 3) and when using eggs as independent data points (Mann-Whitney *U*-test: magpies: $N = 13$, rank sum = 204; cuckoo: $N = 9$, rank sum = 49; $\underline{Z} = 3.64$, $P = 0.0003$; Table 3).

Variation of eggshell bacterial loads along the incubation period

In nonparasitized nests, bacterial load of magpie eggshells increased throughout the incubation period [repeated measures MANOVA, time of sampling (i.e. beginning or end of incubation) as within factor: Wilks = 0.67, $F = 4.02$, d.f. = 4, 32, $P = 0.009$; univariate results: total aerobic mesophilic bacteria (TSA) ($F = 6.77$, $P = 0.01$); *Enteroccoccus* (KF) ($F = 5.59$, $P = 0.023$); *Staphylococcus* (VJ) ($F = 2.03$, $P = 0.16$); and *Enterobacteriaceae* (HK) ($F = 7.07$, $P = 0.01$); all d.f. = 1,35]. This tendency was not detected in parasitized nests (repeated measures MANOVA, time of sampling as within factor: for cuckoo eggs, Wilks = 0.25, $F = 3.65$, d.f. = 4, 5, $P = 0.09$; for magpie eggs, Wilks = 0.63, $F = 2.10$, d.f. = 4, 14, $P = 0.14$) (Fig. 1). However, when looking at the univariate results, *Enterococcus* (repeated measures ANOVAs: magpie eggs in nonparasitized nests: $F = 6.72$, d.f. = 1, 17, $P = 0.019$) and *Enterobacteriaceae* (repeated measures ANOVAs: $F = 5.61$, d.f. = 1, 17, $P = 0.03$) increased during incubation in magpie eggs in parasitized nests. Throughout the incubation period, eggshell bacterial load of cuckoo eggs did change with the exception of *Enterococcus* (repeated measures ANOVAs: $F = 13.16$, d.f. = 1, 8, $P = 0.006$; Fig. 1).

Bacterial loads inside eggs

We detected bacteria inside unhatched eggs of both great spotted cuckoos and magpies (Table 3). However, magpie and great spotted cuckoo eggs differed in probability of trans-eggshell infection (12 infected out of 13 magpie eggs and three infected out of nine cuckoo eggs, Fisher's exact test, $P = 0.007$). Bacterial diversity inside of infected magpie eggs was also higher than that of cuckoo eggs: 11 bacterial species were characterized for magpie eggs, whereas only three bacteria species were detected in unhatched eggs of great spotted cuckoos (Table 3). Furthermore, although a single bacterial species was detected per infected cuckoo eggs ($N = 3$), half of the infected magpie eggs harboured more than a single bacterial species (average = 1.5 species per infected egg; $N = 12$). As noted above, the presence of bacteria inside unhatched eggs should not be interpreted as evidence of bacteria being the cause of embryo death but, instead, in terms of eggshell permeability to different bacteria. Accordingly, these results suggest that eggshells of magpie eggs were apparently permeable to more bacterial species than shells of cuckoo eggs.

DISCUSSION

We quantified the density of *Enterobacteriaceae*, *Staphylococcus*, and *Enterococcus* on the eggshells of magpies and their brood parasite, great spotted cuckoos, at the beginning and at the end of the incubation period. *Enterobacteriaceae* and *Staphylo-*

Table 2. Multivariate comparisons of eggshell bacterial loads of magpie and great spotted cuckoo eggs estimated in parasitized and nonparasitized nests, at the beginning (early incub.) and the end (late incub.) of the incubation period

			Multivariate analysis of variance				Univariate results								
							TSA			KF		VJ		HK	
			Wilks	*F*	d.f.	*P*	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Among nests comparisons															
Parasitized versus	Early	Egg type	0.82	4.27	4,78	0.004	1,81	14.29	0.0003	3.02	0.09	1.68	0.20	0.01	0.92
nonparasitized nests	Incub.	Storage	0.89	2.51	7,78	0.048	1,81	2.53	0.12	6.29	0.014	0.53	0.47	6.62	0.01
	Late	Egg type	0.92	1.23	4,58	0.31	1,61	0.04	0.84	1.64	0.21	0.72	0.40	3.01	0.09
	Incub.	Storage	0.90	1.63	4,58	0.18	1,61	4.95	0.03	3.30	0.07	0.56	0.46	1.18	0.28
Magpie versus cuckoo	Early	Egg type	0.81	3.53	4,59	0.012	1,62	1.10	0.30	0.24	0.63	1.63	0.21	1.93	0.17
eggs	Incub.	Storage	0.85	2.67	4,59	0.04	1,62	2.67	0.11	3.17	0.08	7.39	0.008	5.77	0.019
	Late	Egg type	0.91	0.63	4,24	0.65	1,27	0.18	0.67	0.01	0.97	0.04	0.84	1.86	0.18
	Incub.	Storage	0.98	0.12	4,24	0.97	1,27	0.47	0.50	0.11	0.74	0.02	0.89	0.30	0.59
Within-nests comparisons															
Magpie versus cuckoo	Early	Egg type	0.68	3.18	4,27	0.029	1,30	1.60	0.22	0.61	0.44	1.60	0.22	3.09	0.09
eggs	Incub.	Storage	0.91	0.66	4,27	0.63	1,30	3.42	0.07	1.28	0.27	0.60	0.44	1.97	0.17
	Late	Egg type	0.30	2.91	4,5	0.14	1,8	1.36	0.28	6.35	0.036	4.71	0.06	1.77	0.22
	Incub.	Storage	0.40	1.90	4,5	0.25	1,8	0.39	0.55	17.1	0.003	0.94	0.36	0.01	0.92

Estimates from the four different culture media (TSA, KF, VJ, and HK) were included as dependent variables and parasitism status (parasitized versus nonparasitized), or species identity (magpie versus cuckoo eggs) as between or within (i.e. repeated measures) independent factors. Time of storage of samples at 4 °C before the lab analyses (Storage) was also included as an independent continuous factor. Univariate results are also shown.

Table 3. Log_{10}-transformed eggshell bacterial loads [number of CFU (Colony Forming Units) per cm^2] of unhatched magpies and great spotted cuckoo eggs

Species	Nest (egg)	Eggshell bacterial loads	Species of bacteria detected within eggs
Magpie			
	1	6.696	*Enterococcus faecium*, *Enterococcus faecalis*
	2	7.112	*Staphylococcus auricularis*, *Enterococcus faecium*
	3	7.604	*Pseudomonas fluorescens*
	4 (1)	6.088	*Enterobacter cancerogenus*
	4 (2)	7.424	*Enterococcus faecium*, *Enterococcus faecalis*
	5	6.792	*Enterococcus faecium*
	6 (1)	7.394	*Enterococcus casseliflavus*, *Enterococcus faecium*
	6 (2)	7.119	*Enterococcus faecium*, *Serratia marcescens*
	7	8.548	*Enterococcus faecium*
	8	8.035	None
	9	6.148	*Aerococcus viridans*, *Staphylococcus xylosus*
	10	3.760	*Stomacoccus mucilaginosus*
	11	5.720	*Salmonella* spp.
Great spotted cuckoo			
	11	4.681	*Salmonella* spp.
	12 (1)	4.886	None
	12 (2)	3.942	None
	12 (3)	2.845	None
	13 (1)	2.763	*Staphylococcus xylosus*
	13 (2)	2.767	None
	13 (3)	4.869	*Enterococcus faecium*
	13 (4)	3.658	None
	13 (5)	2.839	None

Bacteria species detected inside each analyzed egg are also shown.

coccus sp. are saprophytic and opportunistic bacteria (Houston *et al*., 1997; Singleton & Harper, 1998; Cook *et al*., 2005a) that live in skin, hair, and feathers of mammals and birds (Krieg & Holt, 1984). They commonly appear on avian eggshells and are known to be pathogenic for avian embryos (Bruce & Drysdale, 1994). Enterococci, the third analyzed group of bacteria, are also frequently found inside unhatched eggs (Bruce & Drysdale, 1994), including those of magpie and great spotted cuckoo (Table 3). Although Enteroccocci are opportunistic pathogens (Franz, Holzapfel & Stiles, 1999), they might also have beneficial effects for embryos (Soler *et al*., 2008). Most of these bacteria are able to penetrate eggshells (Board *et al*., 1994; Cook *et al*., 2003) and, accordingly, we identified some of them inside unhatched eggs (see Results). In addition, we also quantified the total load of eggshell bacteria able to grow in an aerobic heterotrophic medium, which is positively related to the probability of embryo infection (Bruce & Drysdale, 1994; Cook *et al*., 2003, 2005b). We therefore assume that our estimations of eggshell bacterial load of great spotted cuckoo and magpie eggs likely reflect the probability of embryo infection experienced by eggs of both species.

Our analyses showed that, at the beginning of incubation, magpie eggshells in parasitized nests harboured a higher bacterial density than those of nonparasitized nests, and that these differences disappeared at the end of incubation. A second group of results showed that bacterial density on eggshells of great spotted cuckoo eggs was lower than that estimated for magpie eggs, even when considering within-nest variation. Therefore, these results suggest that brood parasitism could increase the probability of bacterial infection of magpie eggs, and that parasitic eggs may be better adapted to environments with a high risk of bacterial contamination than host eggs. This interpretation is supported from bacteriological analyses of eggs that failed to hatch because trans-shell colonization of eggs was more frequent for magpie than for cuckoo eggs. Below, we discuss these interpretations and the alternative scenarios that could explain our results.

Most embryo mortality occurs at the beginning of incubation (Beissinger, Cook & Arendt, 2005), and bacterial loads at that stage appears to be the key factor predicting embryo bacterial infections (Bruce & Drysdale, 1994; Shawkey *et al*., 2009). Therefore, the higher eggshell bacterial loads detected in parasitized magpie nests at the beginning of incubation may result in a lower hatching success and imply an extra cost of parasitism by the great spotted cuckoo. Hatching success in parasitized magpie nests is known to be lower than that in nonparasitized nests (Soler, Martínez & Soler, 1996), which has been interpreted as a consequence of host eggs breakage because of brood parasitism. The number of magpie eggs broken as a result of brood parasitism in the area of the present study was quantified previously at 1.49 per clutch (Soler *et al*., 1997) and, estimates of hatching success of intact eggs in parasitized nests (32.2%) were still lower than those in nonparasitized nests (71.8%) (Soler *et al*., 1996, 1997). Thus, it is possible that variation in hatching success between parasitized and nonparasitized magpie nests was at least partly explained by differences in eggshell bacterial load detected in the present study. This hypothesis is difficult to test with empirical data in host populations heavily parasitized by great spotted cuckoos because parasitism might result in undetected damage of host eggs that could affect hatching failures.

Causes explaining the detected differences in the bacterial load of magpie eggshells could be related to particular environmental conditions related to the activity of brood parasites at host nests that would enhance bacterial growth. For example, at the time of parasitism, and also during subsequent nest visits, great spotted cuckoos can damage some magpie eggs (Soler *et al*., 1997; Soler & Martínez, 2000), and sometimes magpie (and cuckoo) eggshells became partially covered with yolk and egg white from damaged eggs (Soler *et al*., 1997). Such remains of damaged eggs are prime nutrients for bacterial growth (Stadelman, 1994), and we consider that they could be the cause of the detected higher bacterial load in parasitized nests. However, so far, no experimental data are available to test this hypothesis. An alternative hypothesis to explain the higher bacterial load of magpie eggshells in parasitized nests would be a result of bacteria living in the oviduct and/or cloacae of great spotted cuckoos colonizing the host eggshells in parasitized nests. Intestinal microbiota of great spotted cuckoos and magpies likely differ (Ruiz-Rodriguez *et al*., 2009), and eggshells in parasitized (but not in nonparasitized nests) can harbour bacteria from the intestinal tract (i.e. cloacae) of both parasitic and host females.

Our data are non-experimental and, consequently, we cannot exclude any of these alternative explanations, which otherwise should be considered as complementary causes underlying the hypothesis that nest visiting by brood parasites would affect the bacterial environment of nests. Because of the correlative nature of our results, an alternative explanation would imply that great spotted cuckoos selectively parasitize nests with a high bacterial load. However, great spotted cuckoos select foster parents of a higher than random phenotypic quality (Soler *et al*., 1995a) and, as far as we know, a positive relationship between eggshell bacterial load and parental phenotypic quality appears improbable. Thus, assuming a positive relationship between eggshell bacterial load and probability of embryo infection (Bruce & Drysdale, 1994; Cook *et al*., 2003, 2005b), our results suggest an additional direct cost of brood parasitism for magpies that may not only affect the coevolutionary processes between great spotted cuckoos and magpies in particular, but also those between other host species and their brood parasites.

Egg incubation reduces eggshell bacterial load and therefore the probability of embryo infection (Cook *et al*., 2005a; Shawkey *et al*., 2009). Thus, although the mechanism is not clear (Cook *et al*., 2005a), incubation should reduce the effect of brood parasitism on the bacterial load of magpie eggs. In accordance with this prediction, we found no difference in the bacterial load of magpie eggs in parasitized and nonparasitized nests at the end of incubation. However, the bacterial load of magpie eggshells increases rather than decreases during incubation. This increase in bacterial load was mainly detected in nonparasitized nests (i.e. those with lower eggshell bacterial density at the beginning of incubation), suggesting that the effect of incubation on bacterial growth depended on the initial load of bacteria on eggshells. These results would also indicate that magpies (through incubation behaviour) are able to control a runaway growth of bacteria established at a high density on the eggshell of parasitized nests. Another alternative explanation is that the bacterial carrying capacity of eggshells in parasitized nests was close to maximum at the beginning of incubation, and that bacterial density could only increase in eggs that, at the beginning of incubation, harboured a low bacterial density (i.e. those from nonparasitized nests). Our non-experimental results, however, do not distinguish between these alternative explanations and, thus, further experimental approaches are needed.

The detected higher eggshell bacterial load in parasitized nests should have consequences for the evolution of host and parasite strategies that reduce the probability of bacterial colonization and growth on the eggshell if it affects hatching failures as a result of embryo bacterial infection (Bruce & Drysdale, 1994; Beissinger *et al*., 2005; Shawkey *et al*., 2009). We have

found a positive relationship between bacterial load of eggshells in unparasitized magpie nests and the probability of hatching failure. Thus, if we assume a similar relationship for parasitized nests, where bacterial densities are higher, all parasitic eggs (but only some host eggs) will develop in nests with a high probability of eggshell bacterial contamination (i.e. parasitized nests) and, consequently, the former will be under stronger selection than the latter. Therefore, this evolutionary scenario predicts that parasitic eggs should be better adapted to develop in nest environments with an elevated probability of bacterial colonization and/or penetration of the eggshell. Several findings suggest that certain characteristics of the eggshells of the great spotted cuckoo may function to reduce eggshell bacterial load and growth during the incubation period. First, we have found that, at the beginning of incubation, eggshell bacterial density of great spotted cuckoos is lower than that of magpie eggshells. This was the case even when comparing bacterial load of parasitic and host eggs within the same nest (see Results). However, interspecific differences tended to disappear at the end of incubation, and therefore it could be argued that the detected interspecific differences in the bacterial community of eggshells were a result of species-specific traits (i.e. variation in the cloacae bacterial community of great spotted cuckoo and magpie females). Several reasons make this explanation unlikely. First, the eggshells of both species were sampled several days after egg-laying and therefore the identical environmental conditions that the eggs of both species shared for several days should help to dilute any hypothetical initial interspecific differences. Second, at the end of incubation, we found that density of *Staphylococcus* isolates from magpie eggshells was higher than that of great spotted cuckoo eggs. Finally, we found interspecific differences in eggshell bacterial load of eggs that failed to hatch and that were kept in the nests for five-seven days after incubation finished, which cannot be explained by interspecific differences in the bacterial community of adult females.

The second group of results, suggesting that great spotted cuckoo eggshells are better adapted than those of magpies to an environment with a high probability of bacterial infection of eggs, came from the analyses of the bacterial community inside unhatched eggs of both species. Trans-shell bacterial contamination occurs in nature at a quite low rate, and is considered to be consequence of the very efficient antibacterial defences of eggs (Board *et al*., 1994). Therefore, the sample sizes necessary to detect interspecific differences of embryo infection in natural conditions with viable eggs would be enormous, as well as ethically unacceptable. Bacteria are more frequently found inside eggs that fail to hatch independently of whether or not embryo death was a result of bacterial infection. This is not only because physical and chemical barriers deteriorate with time (Stadelman, 1994), but also because eggshell bacterial load should increase when incubation ceases (see above). Thus, by assuming a depreciable rate of bacterial infection during egg formation (Baggott & Graeme-Cook, 2002), unhatched eggs are commonly used for exploring bacteria that are able to penetrate the eggshells or for detecting the differential probability of trans-shell contamination of eggs in relation to different environmental conditions (Bruce & Drysdale, 1994). Accordingly, we tested the null hypothesis of no interspecific differences in probability of trans-shell bacterial infection with cuckoo and magpie eggs that failed to hatch. We found higher bacterial prevalence and a more diverse bacterial community in eggs of magpies than in those of the great spotted cuckoo, which suggests that specific characteristics of eggs of great spotted cuckoos restrict bacterial infection.

It is known that brood parasitic species lay eggs with shells that are thicker, denser, more rounded, and hence stronger than those of both their hosts and their nonparasitic relatives (Rahn, CurranEverett & Booth, 1988; Brooker & Brooker, 1991; Picman & Pribil, 1997). There are four different hypotheses explaining the evolution of the exaggerated strength of parasitic eggs, most of them related to a reduced probability of suffering breakage. Eggs with thick shells would resist breakage if the egg is laid from a distance into the nest, as occurs in cuckoos (Lack, 1968), or protect the eggs from accidental damage during incubation (Blankespoor, Oolman & Uthe, 1982). Another possible advantage of an exaggerated thick shell of parasitic eggs is to increase resistance to puncture ejector hosts that are too small to grasp whole parasitic eggs for ejection (Spaw & Rohwer, 1987), or simply to protect the eggs from damage provoked by other parasitic eggs when multiparasitism occurs (Brooker & Brooker, 1991). In the present study, we suggest that eggshell traits of brood parasitic species might also function as a suitable physical barrier that protects embryos against microorganisms because they penetrate eggshells through eggshell pores and (1) shell thickness is negatively related to porosity (Rahn & Ar, 1974; Vleck & Bucher, 1998) and (2) smoother and less porous eggshells might limit the growth of microorganisms on the eggshell and therefore trans-shell embryo infection (Bruce & Drysdale, 1994; Cook *et al*., 2003, 2005b).

Detected differences in eggshell bacterial-load estimates for cuckoo and magpie eggs within parasitized nests cannot be explained by differences in the bacterial environment experienced by eggs of the two species. Interspecific differences in eggshell properties, such as availability of appropriate space for

bacterial growth (i.e. porosity) and the antibacterial properties of cuticle, etc., should therefore explain differences in bacterial density of cuckoo and magpie eggs sharing environmental conditions of parasitized magpie eggs. Support for this hypothesis would need further research, including detailed microscopy of eggshells of these species and laboratory experiments exploring bacterial permeability to bacteria of eggshells varying in thickness, pore density, and pore size. It should be noted that this suggestion is based on results obtained with a low sample size and that, even after carefull inspections, collected unhatched eggs may have undetected microfissures that increase bacterial permeability. Therefore, the results should be interpreted with caution.

To summarize, the results obtained in the present study suggest that changes in magpie nest environments associated with brood parasitism by great spotted cuckoos elevate bacterial density on eggshells. Furthermore, because parasitic eggs more frequently experience environments with an elevated bacterial density than host eggs, we predicted and found support for the hypothesis that great spotted cuckoo eggs should be better adapted to contaminated environments. Further work is necessary to determine the causes explaining the association between parasitism and eggshell bacterial load, as well as to determine what cuckoo egg traits reduce the bacterial load of their eggshells. These findings may have important consequences for an understanding of coevolutionary patterns between brood parasites and their hosts. For example, additional costs of brood parasitism that increase hatching failure as a result of the associated bacterial environment would affect the evolution of host defences against brood parasites (cuckoo egg recognition and rejection) because the benefits of defensive phenotypes would be greatly reduced. On the other hand, cuckoos might benefits from this situation because lowering the hatching success of their hosts would reduce competition of their offspring with foster siblings. Nonetheless, the production of eggs that resist bacterial penetration may imply extra costs for brood parasitic females if, for example, they need larger amount of calcium, which is limited under natural conditions (Graveland & Drent, 1997) and therefore may limit the clutch size of brood parasites. All these hypothetical consequences remain to be explored, and we hope that the findings of the present study will encourage further research.

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