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Bacterial diversity at the cloaca relates to an immune response in magpie *Pica pica* and to body condition of great spotted cuckoo *Clamator glandarius* nestlings

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Diversity of the gut bacterial community is of prime importance for optimal food digestion and, therefore, for nutritional condition of avian nestlings. Consequently, bacterial community should be considered as a predictor of the future survival and recruitment of young birds. To explore this hypothesis, we studied the cloacal microbiota, by using RISA procedure, in two avian species sharing environmental conditions during growth, the avian brood parasitic great spotted cuckoo *Clamator glandarius*, and their main host in Europe, the magpie *Pica pica*. As estimates of phenotypic condition of nestlings we studied two nutrition-dependent traits, the immune response to an innocuous antigen (phytohemagglutinin), and the residuals of body mass on tarsus and wing length of nestlings. According to the hypothesis, we found significant relationships between microbial diversity and nestling phenotypic traits related to probability of recruitment. Briefly, both magpie and cuckoo nestlings having more similar microbial diversity were also those with similar immune response and body condition index respectively. Our results show a possible association between bacterial communities and variables related to the probability of post-fledging survival and recruitment of birds, as well as possible reasons explaining magpie-cuckoo differences in the nutritionally conditioned variables better associated with their bacterial diversity.

Early development is a crucial determinant of fitness in many animals and, therefore, factors that affect not only offspring growth but also variation in adult phenotypic quality due to environmental experience during growth are of prime importance in evolutionary ecology (Lindström 1999). In birds, in addition to some environmental factors, it is known that body-mass at fledging (e.g. Perrins 1980, Linden et al. 1992), as well as body condition and measures of immunocompetence (e.g. Christe et al. 2001, Møller and Saino 2004), are good predictors of individual survival. In particular, local immune response to the non-pathogenic antigen phytohemagglutinin (hereafter PHA response) of fledglings (Cichon and Dubiec 2005, Moreno et al. 2005) predicts their survival to the next breeding season better than any other simultaneously measured phenotypic trait (i.e. body mass and condition, laying date, etc.). The PHA response is usually positively related to body mass and nutritional condition of nestlings (e.g. Soler et al. 1999, Christe et al. 2001, Moreno et al. 2005), variables that have traditionally been considered to be reliable indexes of survival probability of avian nestlings about to fledge (e.g. Tinbergen and Boerlijst 1990). However, this association with survival could be influenced by the relationship between immunity and body mass (Cichón and Dubiec 2005, Moreno et al. 2005). The PHA response is a nutrition-dependent trait (Alonso-Alvarez and Tella 2001), not only because the development of the immune system is costly in terms of energy and nutrients that otherwise could be used for further development of other phenotypic traits (e.g. Norris and Evans 2000, Bonneaud et al. 2003, Soler et al. 2003), but also because it depends on the availability of essential micronutrients, such as sulphur amino acids and vitamins (see e.g. Grimble and Grimble 1998, Kidd 2004), which are directly acquired from the diet, or after the digestion process.

Gastrointestinal microbiota plays a central role in the digestion of food that parents provide to their nestlings. It is known that bacterial community in the intestinal tract has a

strong environmental component (Lucas and Heeb 2005) and, therefore, varies between individuals of the same species. In addition, intestinal microbiota is related to individual nutritional condition (see for example Glunder 2002, Engberg et al. 2004) and phenotypic quality (Moreno et al. 2003). Moreover, basic research has shown a link between the digestive and immune systems, and nutritional status (see Hammarqvist 2004) and, therefore, gastrointestinal microbiota may be the direct link explaining the relationship between nutritional condition and PHA response (i.e. T-cell-mediated immunity) detected in wild birds.

Pathogenic bacteria may compromise the investment in growth (Potti et al. 2002), reduce probability of survival or even kill their hosts (Nutall 1997). The establishment of pathogenic bacteria in the gut also influences bacterial community, by decreasing diversity of bacterial taxa (Khuel et al. 2005). On the other hand, non-pathogenic bacteria in the digestive tract may have important benefits for hosts, including those directly related to their important function in the digestive process, like the acquisition and storage of nutrients, optimising their use from food intake (e.g. Bäckhed et al. 2004), and the synthesis as well as absorption of essential nutrients (e.g. Stevens and Hume 1998). Moreover, non-pathogenic bacteria may competitively exclude potential pathogens (Hooper et al. 1998), allowing a high and balanced (Davis et al. 2007) bacterial diversity at the gut that, in the absence of pathogenic invasion would be restored even after stress-induced changes (Lan et al. 2004).

Having those precedents, we hypothesized a relationship between intestinal microbiota and nutritional related traits of nestlings. In particular, we predicted similar PHA response and similar body condition for fledglings with more similar bacterial assemblages at the cloaca. Because PHA response and body condition of fledging are good predictors of recruitment probability, data supporting this prediction would evidence the close relatedness of gastrointestinal microbiota and individual fitness.

We explore these predictions by studying bacterial assemblages of nestlings of the parasitic great spotted cuckoos *Clamator glandarius* and those of their magpie *Pica pica* hosts few days before leaving the nests.

Bacterial assemblage was estimated by the analysis of cloacal samplings and using Ribosomal Spacer Analysis (RISA) (García-Martínez et al. 1999) that detects different phylotypes and provides a good approximation of different species of bacteria present in the sample (Stach et al. 2003). Moreover, most sampled nests were from experimental broods that we created with two magpies and two great spotted cuckoos of 2-3 days old and similar weight.

Material and methods

The study area was the Hoya de Guadix (37° 18′N, 3° 11′W), southern Spain, at approximately 1,000 m a.s.l. The vegetation is sparse, with some holm oaks *Quercus rotundifolia* and many orchards of almond trees *Prunus dulcis* in which magpies build their nests (see Soler 1990 for a better description of the study area). Parasitism of magpies by the great spotted cuckoos is quite common in the area (see Soler and Soler 2000).

Field work and experimental procedure

At the beginning of the breeding season of 2003, we identified magpie nests and visited them twice a week to detect laying date, the start of incubation, and parasitism by the great spotted cuckoo. The incubation period of great spotted cuckoo eggs averages 4 d shorter than that for magpie eggs and, thus, in most parasitized nests the great spotted cuckoos hatched earlier than magpie nestlings (Soler 1990). Moreover, magpies preferentially feed the larger nestlings in the nest and, therefore, most magpie nestlings die from starvation in parasitized nests (Soler et al. 1995). To avoid such age related differences we manipulated the nests by exchanging nestlings of different species between magpie nests up to having broods of two cuckoos and two magpies of the same age and similar weight. This approach allows the estimation of cloacal bacterial diversity from the two species growing together in the same environment. Since, on average, more than 2 magpie nestlings hatch in non-parasitized nests, the remaining nestlings were transported and reared in other magpie nests (maximum 7 nestlings per nest) which were not used for sampling.

We got samples of 41 magpies and 26 great spotted cuckoos from 27 nests, from which 19 nests were experimental (i.e. artificial mixing of cuckoos and magpies), and 8 were natural (i.e. non-parasitized magpie nests in which nestlings were not exchanged). From the 19 nests, we collected data for the two species in 11 nests (16 magpies and 17 cuckoos), while samples from a single species were collected in the other 8 nests (9 cuckoos and 2 magpies). Field work and animal manipulations were approved by the Department of Environment of Andalusia (Spain Government).

Bacterial sampling, body condition and T-cell mediated immune response

About four d before fledging, when nestlings were 16-17 d old, we ringed, measured and weighed them. The lengths of right and left tarsi and wings were measured and mean values were used in the analyses. We took bacterial samples from the cloaca of all the nestlings raised in magpie nests, by injecting and repipetting two or three times 500 µl of sterile phosphate buffer (Na_2HPO_4 0.1 M and NaH_2PO_4 0.1 M, pH 7.4) in the cloaca, using sterile tips and automatic pipettes. Afterwards, we immediately lysed the bacterial cells in the field by adding 500 µl of lysis buffer (Tris HCl 50 mM, 1% SDS, EDTA 2 mM, NaCl 100 mM). Samples were kept cool (i.e. 1–3° C) for a few hours and later stored in the lab at −20° C until molecular analyses. Moreover, differences in cloacal samples are assumed to reflect differences in the bacterial community in the intestinal tract (Savage 1977, Vaahtovuo et al. 2001).

Finally, as a variable related to immunocompetence of nestlings, skin swelling elicited by injection of the mitogen phytohemagglutinin (PHA-P, Sigma Chemical Co.) was measured. This is commonly used in evolutionary ecology to estimate T-cell-mediated immunity (Kennedy and Nager 2006), although it also reflects other components of the immune system, both innate and adaptive (e.g. Martin et al. 2006). Briefly, following a well-established protocol, we

injected fledglings subcutaneously in the right wing web with 0.5 mg of PHA dissolved in 0.1 ml of physiological saline solution (Bausch and Lomb Co.). The left wing web was injected with 0.1 ml of saline solution. We measured the thickness of each wing web at the injection site with a digital pressure-sensitive micrometer (Mitutoyo, model ID-CI012 BS; to the nearest 0.01 mm) before injection and 24 h afterwards. The T-cell-mediated immune response or wing-web index was then estimated as the change in thickness of the right wing web (PHA injection) minus the change in thickness of the left wing web (Lochmiller et al. 1993). Measurements of each wing web on each occasion were repeated three times and, because repeatability of all measurements was larger than 95% (data not shown), the mean value was used in subsequent analyses.

Laboratory analysis

To analyse the bacterial community in each nestling, we extracted DNA from 200 µl of each cloacal sample. First, samples were thermally shocked to further lyse the cells, and then DNA was extracted following a slightly modified protocol of Orsini and Romano-Spica (2001). Shortly, after the addition of 400 µl of a buffer prewarmed at 65° C (Tris HCl 10 mM, EDTA 1 mM, sodium acetate 0.3 mM and 1.2% polyvinylpyrrolidone), the DNA was purified by the phenol-chloroform procedure. Finally the DNA was precipitated with isopropanol overnight at −20° C. After washing 3 times with 80% ethanol, the DNA was resuspended in TE buffer pH 8 (Tris HCl 10 mM and EDTA 1 mM). Subsequently, we used the ribosomal intergenic spacer analysis (here after, RISA) method to amplify the spacer regions between the 16S and 23S rRNA genes in the ribosomal operon. These fragments are extremely variable in both sequence and length for the different prokaryotic species, due to the presence of several functional units within them such as tRNA genes (García-Martínez et al. 1999). The primers used were S-D-Bact-1522-b-S-20 and L-D-Bact-132-1-A-18 (Ranjard et al. 2000). The polymerase chain reaction was performed in 50 µl, with 100 ng of DNA, 1 × PCR buffer (QIAGEN), 2 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5 µM of each primer, 150 µM of each dNTP and 1 U Taq polymerase (QIAGEN). The amplification reaction was performed using an initial denaturating at 94° C for 3 min, followed by 25 cycles at 94° C for 1 min, 55° C for 30 s, 72° C for 1 min, and a final extension at 72° C for 5 min. (Ranjard et al. 2000). PCR products were subsequently quantified with a fluorimeter DynaQuant (Hoefler) after staining with Hoechst Dye diluted to 1/10,000. To separate the PCR products (200 ng), we used a 2% Metaphor agarose (FMC Bioproducts) gel electrophoresis for 4 h at 150 V. Each band in the gel corresponds to one Operative Taxonomic Unit (OTU), also called phylotype, which is assumed to be a taxonomic unit (i.e. species, Atlas and Bartha 1997). OTU richness of each sample was estimated by the total number of bands present in one individual.

Statistical analyses

The resulting gels were analysed with GEL COMPARE II (Applied Maths, Kortrijk, Belgium). A similarity matrix was built using pairwise comparisons among RISA profiles (i.e. individuals). The similarities were calculated using the Dice's binary coefficient: $2a/(2a+b+c)$, where a is the number of OTUs in common for the two samples, b is the number of OTUs present only in the first sample, and c is the number of OTUs present only in the second sample. Because of the marked differences in the bacterial diversity among great spotted cuckoos and magpies (Ruiz-Rodríguez et al., in press) we built two different matrices of similarity between individuals, one for each species, and, consequently, great spotted cuckoo and magpies were analysed separately. In addition, other matrices with information of nest of rearing allowed us comparisons both within and across nests.

We also prepared similarity matrices for each variable hypothetically related to the bacterial assemblages (body condition and PHA response), but also for variables that could mask the predicted relationships (nest of rearing, parasitized, or non-parasitized nest, see below). For continuous variables (i.e., PHA response and body condition), similarity matrices were prepared by dividing the values estimated for each pair of nestlings in the matrix. Because the lowest was always divided by the highest value, we obtained square matrices with similarity values varying between one (i.e. identical values) and zero. For discrete variables, binary matrices were built by using 1 when the two nestlings shared identity (e.g. same nest, both parasitized, etc.), and 0 otherwise.

Body condition was independently estimated for great spotted cuckoo and magpies nestlings as residuals of body mass after correcting for tarsus length (magpies: $R=0.76$, $F_{1,\ 39}=54.21$, $P<0.001$; great spotted cuckoos: $R=0.53$, $F_{1,\ 23}=9.23$, $P<0.006$). These residuals were not significantly correlated with wing length (magpies: $R=0.29$, $F_{1,\ 39}=3.72$, $P=0.06$; great spotted cuckoos: $R=0.29$, $F_{1,\ 23}=2.2$, $P=0.15$), indicating that our estimates are free of allometric effects (Green 2001), and thus, we used them as an index of body condition. Although this index has been questioned by some authors, Schulte-Hostedde et al. (2005) showed that it is an index that appropriately estimates individual body condition.

The relationships between matrices were studied by the estimations of partial correlation coefficients using Mantel's test as implemented in FSTAT (Goudet 1995). Statistical significances of correlation coefficients were calculated by Monte Carlo procedure after 10,000 permutations.

Because nestlings within the same nest share the same rearing environment and, thus, the use of nestlings as independent data points may imply a pseudo-replication problem, when significant correlations arose, analyses were run again but including matrices with information of nest of rearing and nest of origin (i.e. pairs of nestlings being of the same or different nests). Matrices of differences in the type of nests from which the nestlings were reared (i.e. experimental or natural nests) were also included in the model.

Given that bacterial richness (i.e. number of OTUs) may influence estimations of similarity coefficients (see above), as a measure of bacterial diversity, we also estimated matrices of absolute differences in the number of OTUs detected at the level of nestlings as well as nests, and included this information as additional independent variables. However, results did not qualitatively differ from those where this

information was not included (results not shown). Thus, here we present results from models that did not include differences in number of OTUs as an additional independent variable.

Results

We detected a total of 45 different OTUs. For magpie nestlings, we found a positive relationship between matrices of similarity in RISA profiles and in PHA response ($N = 41$, Mantel test, $r = 0.18$, $P < 0.001$) (see all the results in Table 1). This relationship was not affected by possible confounding factors such as nest of rearing (Mantel test, Partial correlation coefficients: nest of rearing $= 0.09$, $P = 0.09$; PHA $= 0.17$, $P < 0.001$), nest of origin (Mantel test, Partial correlation coefficients: nest of origin $= 0.09$, $P = 0.01$; PHA $= 0.17$, $P < 0.001$), or type of nest (i.e. only with magpies or both species; Mantel test, Partial correlation coefficients: type of nest $= 0.09$, $P = 0.06$; PHA partial $r = 0.17$, $P < 0.001$). However, the relationship between matrices of similarity in RISA profiles and in body condition of magpie nestlings did not reach statistical significance (Mantel test, $r = -0.07$, $P = 0.052$). In addition, matrices of differences in PHA response and body condition of magpie nestlings were not significant (Mantel test, $r = -0.01$, $P = 0.62$).

In relation to great spotted cuckoos, the results agree with the hypothesis that bacterial assemblage is a good predictor of nestling phenotypic quality, since we found a significant positive relationship between matrices of similarity of bacterial assemblages and body condition index (Mantel test, $r = 0.16$, $P = 0.007$). This relationship was still statistically significant after correcting for the nest of rearing (Mantel test, Partial correlation coefficients: nest of rearing $= 0.10$, $P = 0.17$; body condition $= 0.15$, $P = 0.007$). However, the matrix of similarity in bacterial assemblage did not correlate with that of differences in PHA response ($n = 26$, Mantel test, $r = -0.01$, $P = 0.88$). Finally, the matrices of differences in PHA response and body condition of nestling cuckoos proved significantly related (Mantel test, $r = 0.02$, $P < 0.001$), which did not vary after nest corrections (Mantel test, partial $r = 0.02$, $P < 0.001$).

Discussion

Our results in general suggest that the intestinal bacterial assemblage of both magpies and cuckoos is associated with variables related to probability of survival (i.e. phenotypic quality). While magpies having similar gut bacterial assemblages exhibited similar degrees of PHA-induced swelling, cuckoos having more similar gut bacterial assemblages exhibited similar body conditions.

Given that bacteria in the intestinal tract are vital for digestion in general, and in the synthesis of some important micronutrients as amino acids and vitamins (e.g. Stevens and Hume 1998) in particular, we predicted a positive relationship between bacterial assemblages and phenotypic quality of magpie and great spotted cuckoo nestlings. High bacterial diversity in the gut indicates the existence of a stable bacterial community (Davis et al. 2007), free of pathogenic bacteria (Khuel et al. 2005). The stability and the high bacterial diversity is likely enhanced by some few non-pathogenic bacterial species, as those belonged to the genus *Enterococci*, that are use to enhance individual immune modulation and growth performance in farm animals (Davis et al. 2007). Therefore, although RISA methodology does not allow identification of each bacterial taxon, it is possible to know not only how many OTUs have each individual, but also how many of them have two individuals in common, obtaining a similarity degree concerning to bacterial assemblages between two samples. Consequently, the predicted relationship between bacterial assemblages and nestling's performance in natural condition, independently of bacterial identity, is justified. To our knowledge, this is the first time that the relationship between bacterial assemblages and individual condition is detected in wild animals.

Bacterial sampling at the cloaca is believed to be a good approach for the "in vivo" study of bacterial diversity of animals. Bacterial cells, which are in the intestine walls, fall off and together with faeces abandon the body throughout the cloaca, where OTUs can be detected from the whole intestine. Therefore, the end of the digestive tract contains a larger and more complex microbiota (Mead 1997) than the upper regions.

Bacterial assemblages are directly related with nutrition in two ways: first, they depend on the food ingested by hosts, but also they are responsible of the effectiveness of the digestion process. Second, body condition and PHA response are nutritionally conditioned traits and, thus, bacterial community in the intestinal tract should be directly related to nestling traits that depend on nutritional condition. In addition, both variables are good predictors of nestling survival and recruitment (see Introduction). Consequently, the detection of the expected relationship with characteristics of the bacterial assemblages at the cloaca (i. e., gastrointestinal tract) would suggest an important role

Table 1. Relationship between fitness-related variables and bacterial assemblages for each nestling species.

	Magpies		Great spotted cuckoos	
	r	P	r	P
PHA response	0.18	0.001	−0.01	0.88
PHA (corrected by nest of rearing)	0.17	0.001		
PHA (corrected by nest of origin)	0.17	0.001		
PHA (corrected by type of nest)	0.17	0.001		
Body condition	−0.07	0.052	0.16	0.007
Body condition (corrected by nest of rearing)			0.15	0.007

in nestling's probability of recruitment. Actually, in accordance with the hypothesis, we found a significant relationship between bacterial assemblages in the cloaca with the PHA response of magpie, and with body condition of great spotted cuckoo nestlings.

Because causation cannot be deduced from correlations, we cannot rule out the possibility that, rather than diversity of bacterial assemblages being the responsible for nutritional conditioned traits (i.e. PHA response and body condition), variation in bacterial assemblage was the consequence of variation in the individual phenotypic quality, given that it is known that anatomy, physiology or local immune system of the digestive tract are keys in shaping bacterial gut communities (e.g. Stevens and Hume 1998). A third non-exclusive possibility explaining the detected associations is that, because diet is an important factor influencing both bacterial communities (e.g. Glunder 2002) and body condition of nestlings, variation in parental quality would be responsible for the relationship between gut microbiota and variables related to nestling phenotypic quality. However, because our results were statistically corrected for variation among nests, this possibility is unlikely. In any case, because of the correlational nature of this study, experiments manipulating the bacterial assemblages of nestlings are necessary to explore the causal explanation of the detected relationships.

The variable related to phenotypic quality of nestlings that was found to be associated with their bacterial assemblages at the cloaca differed for great spotted cuckoos and magpies. While the PHA response was the variable most closely related to characteristics of magpie microbiota, residuals of body mass after controlling for tarsus length was the variable that best explained microbiota of cuckoos. Moreover, we did not detect a correlation between these two variables in magpie nestlings, even though this relationship was established in a previous work (Soler et al. 1999), which could be due to the smaller sample size used in the present study. On the contrary, in cuckoos, the two variables were related. In general, it is well demonstrated that body mass and/or condition correlate with nestling PHA response (see Soler et al. 1999, Alonso-Alvarez and Tella 2001), and that a trade-off exists between growth and immunity (e. g. Brommer 2004). Consequently, because natural selection favours the evolution of physiological mechanisms that ensure optimal allocation of limited resources to competing activities (Stearns 1992), it is possible that during development the optimal allocation of resources differs for cuckoos and magpies.

Post-fledging survival is a crucial determinant of future reproductive success in birds (Clutton-Brock 1998). There is strong evidence suggesting that both body condition (e.g. Naef-Daenzer et al. 2001) and immunocompetence at fledging (Cichón and Dubiec 2005, Moreno et al. 2005), are good predictors of post-fledging survival. For instance, body condition and health status predicts probability of predation (e.g. Møller and Erritzøe 2000, Naef-Daenzer et al. 2001) and, together with immunocompetence, predicts the degree of negative effects that parasites and diseases may exert on juveniles (see Møller 1997). During growth, however, it is known that magpies experience larger probability of suffering pathogen attacks than their foster siblings great spotted cuckoos (Soler et al. 1999) and, therefore, it is possible that the optimal allocation of resources in immunity and growth differ for host and parasitic nestlings. Magpie nestlings by adjusting developing immune system to environmental conditions (i.e. risk of parasitism; see Szep and Møller 1999) should, therefore, differentially invest in immunity than their foster siblings.

On the other hand, it is known that the probability of survival during migration is correlated with the body mass and body condition at fledging of chicks (e.g. Schmutz and Ely 1999). Contrary to magpies, great spotted cuckoos are migratory birds and, consequently, optimal allocation of resources in body mass reserves may differ between magpie and cuckoo nestlings. Nestling cuckoos by differentially investing more resources in growth than their foster magpie siblings may maximize probability of survival during migration and consequently improve their probability of recruitment.

To our knowledge, a positive relationship between traits related to phenotypic quality of nestlings and intestinal microbiota has rarely been detected in wild birds. For example, Lombardo et al. (1996) found a positive relationship between bacteria loads (i.e. total number of bacterial colonies that grew in nine different media) and wing length of tree swallows *Tachycineta bicolor* nestlings when 12 d old. Whereas Moreno et al. (2003) found that the presence of *Enterococus faecium*, a bacterium with a well-known beneficial effect in poultry (Foulquié-Moreno et al. 2006), was positively associated with pied flycatcher *Ficedula hypoleuca* nestling's growth rates. This effect was interpreted as the result of physiological benefits for their hosts enhancing host nutrition and the development of host immunocompetence (see, Foulquié-Moreno et al. 2006). In addition symbiotic intestinal microbiota produces compounds that inhibit antagonistic-competing microorganisms (Riley and Wertz 2002) and, therefore, may protect hosts against pathogens and parasites due to bacterial interference (Ji et al. 1997). Here, we are not aware of bacterial identity associated to the detected OTUs and we cannot discuss whether detected association were or were not explained by the presence of particular beneficial species in the gut of great spotted cuckoo and magpies nestlings. However, the presence of beneficial bacteria is closely related to diversity of the bacterial communities (Loreau 2001). Therefore, the detected relationships between the estimations of bacterial diversity (through the RISA analyses and similarity matrices) and nutritional conditioned variables (wich explain post-fledging survival), suggest an important role of gut-bacterial community in avian ecology and evolution.

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