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Sources of variation in enterococci and *Enterobacteriaceae* loads in nestlings of a hole-nesting passerine

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Gut bacterial loads in avian nestlings may be affected by factors such as climate, seasonality and brood size. There is no published information on this subject for wild birds, despite its potential importance for nestling welfare and growth. We studied the associations of these factors with abundances of two common gut bacterial types, enterococci and *Enterobacteriaceae*, in nestling Pied Flycatchers *Ficedula hypoleuca* in central Spain. To that end, we obtained faecal samples from 54 broods (102 nestlings) on day 7 and 13 after hatching, for detection and estimation of bacterial abundance. *Enterobacteriaceae* loads on day 7 were positively correlated with mean temperature during the preceding seven days and negatively correlated with rainfall and hatching date. The negative associations of *Enterobacteriaceae* loads with rainfall were only found for early broods, at low temperatures. Enterococci loads on day 7 were positively associated with mean temperature. On day 13, *Enterobacteriaceae* loads were negatively correlated with hatching date, while enterococci loads were positively correlated with mean temperature. *Enterobacteriaceae* are apparently more sensitive to seasonal changes and climatic variation than enterococci, possibly in relation to variation in diet and nutrition. By contrast, enterococci are only sensitive to thermal variation. The attainment of full thermoregulatory capacity by nestlings reduces climatic effects on *Enterobacteriaceae* loads.

Key words: Pied Flycatchers, nestlings, enterococci, *Enterobacteriaceae*, mean temperature, rainfall, hatching date

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The study of host–parasite interactions has been of primary interest for avian ecologists. However, bacteria have only recently received attention in this respect (Maul & Farris 2005, Benskin *et al.* 2009). Associations between birds and bacteria may involve pathogenic interactions, but also positive symbiotic interactions (Martín-Platero *et al.* 2006, Ruiz-Rodríguez *et al.* 2009). These interactions begin in the nest and may affect the growth and survival of altricial and semi-altricial nestlings (Potti *et al.* 2002, Moreno *et al.* 2003, González-Braojos *et al.* 2012). The early stage of the post-hatching period is critical for establishment of the

gut microbial community. This process starts from a sterile gastrointestinal environment at the moment of hatching and continues towards establishing a relatively stable status as the nestling ages. Thus, Mills *et al.* (1999) reported that microorganisms colonize nestling cloacae shortly after hatching, suggesting the source of microbes to be adults, local food items or their local environment. Understanding the factors modulating bacterial abundances in nestling digestive tracts could improve our understanding of bird–bacteria interactions in the wild.

Several factors including climate, food, age and

health state affect the composition of the gut microbiota of individual birds (Brittingham *et al.* 1988, Lombardo *et al.* 1996). Nutrient richness in the environment, humidity and temperature have been identified as important factors affecting growth in bacterial cultures (Ratkowsky *et al.* 1982, Madigan *et al.* 2006). Nutrient availability for digestive bacteria may change with seasonal variation in diet of both adults and nestling birds (Blanco *et al.* 2006, Novotny *et al.* 2007). Nutritional quality may also affect bacterial growth, especially for nestlings competing for parental food deliveries. Late-breeding pairs tend to offer less or poorer quality food to their nestlings (Naef-Daenzer *et al.* 2000, Rossmanith *et al.* 2007, Wilkin *et al.* 2009). Nestlings in larger broods may also suffer nutritionally from stronger competition with brood mates (Naguib *et al.* 2004, Pichorim & Monteiro 2008). Thus, breeding phenology and brood size could affect the growth of bacteria in digestive tracts through nestling nutrition in terms of quantity and quality. Gut mass declines in conditions of poor nutrition (Brzek & Konarzewski 2001), possibly driving higher competition among bacteria for space. To our knowledge, there is no published information about the associations of environmental factors with growth of bacteria in nestling digestive tracts. The only study, to our knowledge, relating gut bacterial growth to seasonal climate changes did not include nestlings (Janiga *et al.* 2007). Ambient temperature may affect bacterial growth through the thermoregulatory capacity of altricial nestlings, which improves with age until thermal independence from adult brooding behaviour (O'Connor 1984, Starck & Ricklefs 1998). If the body temperature of non-thermally independent offspring fluctuates more when the ambient temperature is low (Starck & Ricklefs 1998, Bize *et al.* 2007), bacterial growth might suffer accordingly. Thermally independent nestlings may offer more stable thermal regimes for gut bacteria. Rainfall may affect the foraging capacity of adults and thereby nestling nutritional condition (Rosa & Murphy 1994, Elliott *et al.* 2005, Spencer 2005, Geiser *et al.* 2008, Morrison *et al.* 2009, Arlettaz *et al.* 2010).

In this study we assessed whether environmental factors modulate the abundance of two types of gut bacteria (enterococci and *Enterobacteriaceae*) at two nestling ages in the Pied Flycatcher *Ficedula hypoleuca*. Enterococci are widely distributed in animal gastrointestinal tracts (Foulquié-Moreno *et al.* 2005) and may exist as commensal organisms of the alimentary tract of chickens (Klein 2003) and wild birds (e.g. Moreno *et al.* 2003). They have probiotic properties and are able to limit the colonization of the digestive tract by patho-

genic bacteria (Mazur-Gonkowska *et al.* 2006). Moreno *et al.* (2003) found a significantly positive association between nestling mass shortly before fledging and the presence of *Enterococcus faecium*. *Enterobacteriaceae* are also common in the intestinal microflora of wild birds. Thus, Winsor *et al.* (1981) showed that the most prevalent intestinal bacteria of this group in Turkey Vultures *Cathartes aura* were *Escherichia coli* and *Proteus mirabilis*. Moreover, *Enterobacteriaceae* contribute to the digestion of food and play an important role in controlling other gut bacteria (Hudault *et al.* 2001, Reid *et al.* 2001). It has been shown that both enterococci and *Enterobacteriaceae* grow best at temperatures between 22 and 45°C (Ron 1975, Martínez *et al.* 2003, Foulquié-Moreno *et al.* 2005), so thermal fluctuations in thermally dependent nestlings during parental absences may affect bacterial growth conditions.

Accordingly, we hypothesized that: (1) later broods will have lower bacterial counts due to poor nestling nutrition, (2) larger broods will have lower bacterial counts due to poor nestling nutrition, (3) lower ambient temperatures will result in lower bacterial growth in non-thermally independent offspring (7 days) while having smaller effects in nestlings about to fledge (13 days), (4) higher rainfall will induce poorer bacterial growth due to restricted parental food deliveries to nestlings. Finally, (5) we looked for synergistic effects of rainfall and temperature in driving bacterial growth in guts of nestlings, as low nutrition may have especially strong effects when the costs of thermoregulation are high.

METHODS

The study was conducted during the 2009 breeding season in a deciduous forest of Pyrenean Oak *Quercus pyrenaica* at an elevation of 1200 m a.s.l. in Valsaín, Segovia province (40°54'N, 4°01'W), Spain. The local population of Pied Flycatchers breeds in nest-boxes and has been under study since 1991 (Sanz *et al.* 2003). Nest-boxes are cleaned every year after the breeding season. For the current study, nest-boxes were checked daily for nest-building activity, and the hatching dates and brood sizes were recorded.

The Pied Flycatcher is a small (12–13 g) passerine bird, which breeds in many forested areas of the Palaearctic region (Lundberg & Alatalo 1992). It only stays in European woodlands for the spring and summer, spending the rest of the year on migration or in the wintering areas in tropical West Africa. It breeds naturally in tree cavities, but if nest-boxes are provided,

these are preferred over natural cavities. Egg-laying in the population under study typically begins in late May, and clutch sizes range from 4 to 7 eggs with a mode of 6 eggs (mean 5.5 ± 0.6). The female incubates alone and receives part of her food from her mate (Lundberg & Alatalo 1992). Young are brooded by the female only up to day 8 (hatching day = day 1) (Sanz & Moreno 1995). Both sexes feed the young. Young fledge within 14–16 days of hatching. This occurs in the second half of June in our study area (Moreno *et al.* 2001).

A sample of 54 broods of four to six chicks was used for this study. Of these nests, we obtained samples from two randomly selected chicks in 43 nests and one randomly selected chick in the remaining nests. Nestlings were measured and weighed at two ages (7 and 13 days).

Bacterial sampling

Bacterial samples were obtained as described in González-Braojos *et al.* (2012). Briefly, we sampled freshly faecal sacs and assumed that most bacteria contained in those were derived from gut cloacal communities. Faecal sacs were collected at 7 and 13 days in sterile eppendorf tubes and were processed in the laboratory 3–6 h after collection. Here, we impregnated one sterile cotton swab per faecal sac with faecal matter, transferred this to transport media Amies (*Sterile R, Meus s.r.l.*, Piove di Sacco, Italy) and conserved the samples at 4°C until processed. All samples were analysed after exactly 20 days to avoid effects of differences in time elapsed between sampling and laboratory processing.

Swabs were transferred into 1 ml of phosphate buffered saline (pH = 7.2, Química Clínica Aplicada, Tarragona, Spain). Optimal bacterial concentration for the quantification (Herbert 1990, Maier *et al.* 2000) was determined by serial dilution in sterile physiological saline (0.85% NaCl). The samples were cultured by plating out 100 μ l of the following dilutions: 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} . Samples were cultured on the following solid selective and differential bacterial media: MacConkey agar (bioMérieux, Madrid, Spain; *Enterobacteriaceae*) and D-Coccosel agar (bioMérieux, Madrid, Spain; enterococci) or Enterococcosel for 20 samples (Difco, Detroit, Michigan, USA; enterococci). There were no significant differences in bacterial counts obtained with the latter two media (both $P > 0.1$), so data were pooled. Plates were incubated for 48 ± 2 h at $37 \pm 1^\circ\text{C}$, after which colonies were counted using a colony counter ‘sensor’ (Suntex Instruments Co., Ltd., Taipei County, Taiwan) by the same observer (SG-B).

Environmental data

Daily environmental mean temperature and rainfall were obtained from the meteorological station “Casa de la Mata”, located 2 km from the study area ($40^\circ54'N$, $4^\circ00'W$, 1150 m a.s.l.). Two temperature averages were obtained for each brood: (1) the average of mean temperatures between hatching date and day 7, and (2) the average of mean temperatures between days 7 and 13. For rainfall, we used the rainfall accumulated (1) between hatching date and day 7, and (2) between days 7 and 13.

Statistical analyses

Bacterial loads were successfully normalized through logarithmic transformation prior to analyses. We first tested for intra-brood repeatability of bacterial loads for the two bacterial types and two nestling ages separately (Statistica 6.0).

Loads of the two types of bacteria and for the two nestling ages were included separately in four different linear mixed models using Satterthwaite’s correction for estimating degrees of freedom. Each model included hatching date, mean temperature, rainfall, brood size and their interactions as fixed effects and nest as a random effect. Model selection was based on the Corrected Akaike Information Criterion (AICc), which is more suitable than AIC at moderate sample sizes. We present only models with a ΔAICc smaller than 2 with respect to the preferred model (having lowest AICc). We also present the sign and strength of the significant effects included in the preferred models. Linear mixed models were performed in SAS 9.2. For graphical representation of significant interactions between variables, we split one of the variables according to the median and plotted these separately.

RESULTS

On day 7 the loads of *Enterobacteriaceae* ($r = 0.433$; $F_{1,26} = 2.530$; $P = 0.011$), but not of enterococci ($r = 0.229$; $F_{1,30} = 1.530$; $P = 0.126$) were significantly correlated within broods. No significant within-brood similarity was found for loads on day 13 either for *Enterobacteriaceae* ($r = 0.159$; $F_{1,30} = 1.380$; $P = 0.192$) or enterococci ($r = 0.014$; $F_{1,33} = 1.027$; $P = 0.468$). Thus, we did not calculate intra-brood average bacterial counts.

For each bacterial type and nestling age, we present only the preferred model, as alternative models showed ΔAICc values higher than 2. The preferred model for *Enterobacteriaceae* on day 7 included hatching date,

mean temperature, rainfall and the interactions between hatching date and rainfall and mean temperature and rainfall during the preceding period (Table 1). All variables and interactions included in the model with lowest AICc were significant (Table 2). There was a negative association of *Enterobacteriaceae* loads on day 7 with rainfall, but only for early broods (Figure 1). Likewise, rainfall showed a negative association with *Enterobacteriaceae* loads on day 7, but less so at low ambient temperatures (Table 2). The preferred model

for *Enterobacteriaceae* on day 13 included only hatching date (Table 1), which showed a negative association with bacterial loads (Table 2, Figure 2).

The preferred model for enterococci on day 7 included only mean temperature (Table 1), which showed a positive association with bacterial loads (Table 2). The model for enterococci on day 13 included only mean temperature (Table 1), which showed a positive association with bacterial loads (Table 2).

Table 1. Linear mixed models of bacterial loads (two bacterial types at two nestling ages) in relation to mean temperature in the preceding week (*Temp*), rainfall (*Rain*), hatching date (*HD*), brood size and their interactions. Nest was included as a random effect. Only the preferred models for each analysis are presented (no alternative models showed Δ AICc lower than 2). K = the number of parameters in each model; Weight = the probability that the model is the preferred model in the model set.

Model	K	AICc	Weight
Enterobacteriaceae on day 7			
<i>HD+Rain+Temp+HD×Rain+Temp×Rain</i>	6	294.6	0.8
Enterobacteriaceae on day 13			
<i>HD</i>	2	254.2	0.7
Enterococci on day 7			
<i>Temp</i>	2	286.7	0.7
Enterococci on day 13			
<i>Temp</i>	2	196.3	0.9

Table 2. Parameter estimates of effects included in the preferred models (see Table 1) of *Enterobacteriaceae* and enterococci loads at two nestling ages. Effects are mean temperature in preceding week (*Temp*), rainfall (*Rain*), hatching date (*HD*) and interactions.

	Coefficient \pm SE	df	F	P
Enterobacteriaceae on day 7				
<i>HD</i>	-1.345 \pm 0.202	1,74	44.1	<0.001
<i>Temp</i>	2.852 \pm 0.497	1,74	32.9	<0.001
<i>Rain</i>	-2.571 \pm 0.792	1,74	10.5	0.001
<i>HD×Rain</i>	0.085 \pm 0.017	1,74	23.7	<0.001
<i>Temp×Rain</i>	-0.194 \pm 0.040	1,74	23.0	<0.001
Enterococci on day 7				
<i>Temp</i>	0.127 \pm 0.046	1,54.6	7.4	0.008
Enterobacteriaceae on day 13				
<i>HD</i>	-0.127 \pm 0.038	1,44.1	10.8	0.002
Enterococci on day 13				
<i>Temp</i>	0.162 \pm 0.071	1,91	5.1	0.025

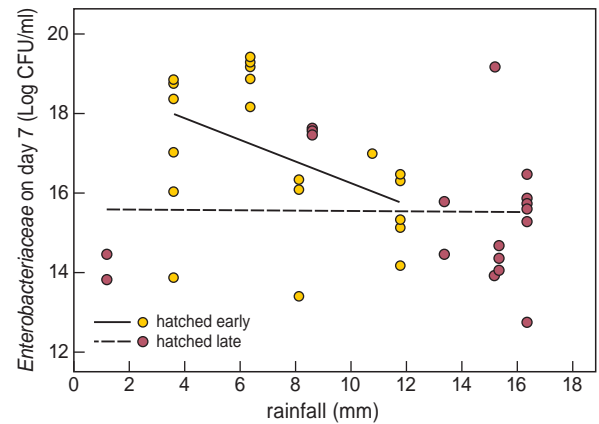


Figure 1. Associations of *Enterobacteriaceae* loads on day 7 with rainfall for broods hatched before or after the median hatching date in the population. Lines represent the simple regression which is not corrected for other significant effects presented in Table 2. A single nestling per nest is presented to avoid pseudoreplication.

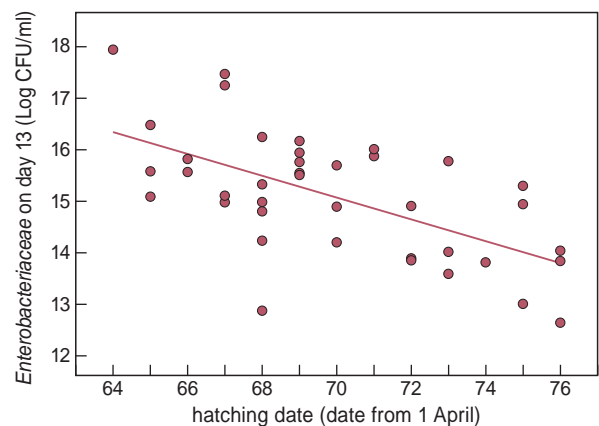


Figure 2. Association of loads of *Enterobacteriaceae* on day 13 with hatching date. A single nestling per nest is presented to avoid pseudoreplication.

DISCUSSION

Our results showed that *Enterobacteriaceae* loads were lower in later-hatching nestlings. We found no association between brood size and loads of either bacterial type. Ambient temperature was positively correlated to *Enterobacteriaceae* loads, but only on day 7. Temperature was positively correlated with enterococci loads at two nestling ages. Higher rainfall resulted in lower *Enterobacteriaceae* loads on day 7.

Nestlings in late-hatched broods have fewer *Enterobacteriaceae* in their guts at both ages than early-hatched nestlings. This effect may be due to seasonal changes in diet. For example, Waldenström *et al.* (2002) found that the prevalence of *Campylobacter* spp. in migrating birds was highly influenced by feeding habits. Also, Lombardo *et al.* (1996) suggested that different feeding habits might explain the greater prevalence of bacteria in insectivorous than in omnivorous birds. Blanco *et al.* (2006) found differences in composition, richness and prevalence of faecal microbiota associated with the diet of adult Red Kite *Milvus milvus*. Furthermore, they found that *Klebsiella* sp. showed a higher prevalence in January than in February, whereas Novotny *et al.* (2007) found that the occurrence of *Yersinia enterocolitica* in adult Alpine Accentors *Prunella collaris* was high in summer, especially during the nestling period. It is possible that late-breeding parents are less efficient at collecting prey, and that late nestlings may therefore be undernourished. Malnourished nestlings may have lower bacterial growth in their guts due to intestinal shrinkage (Brzek & Konarzewski 2001) or to lower nutrient input. However, the non-significant effect of brood size suggests that malnourishment induced by competition among siblings is less important than seasonal changes in diet. In contrast to *Enterobacteriaceae*, enterococci were not responsive to differences in hatching date, which suggests that they are relatively insensitive to nutritional effects.

As expected, loads of *Enterobacteriaceae* and enterococci of chicks at early nestling ages are positively associated with mean temperature, while in grown nestlings this association is only found for enterococci. At low ambient temperatures, poorly thermoregulating chicks (younger than 7 days) may lose relatively more heat, drop their body temperature and reduce their metabolism (Starck & Ricklefs 1998), thereby negatively affecting bacterial growth in the gut. Nestlings about to fledge maintain body temperature within a much smaller range than nestlings of 7 days (O'Connor 1984). The responsiveness of enterococci to tempera-

ture at late nestling ages indicates the extreme thermal sensitivity of these bacteria when compared with other gut bacteria.

Enterobacteriaceae loads showed a negative association with rainfall, but only in young nestlings. The negative effects of rainfall on day 7 on loads of *Enterobacteriaceae* could also be explained by poor thermoregulation as temperatures in the nest drop during rain showers. Rainfall also has a negative effect on the foraging capacity of adults (Radford *et al.* 2001, Geiser *et al.* 2008, Arlettaz *et al.* 2010). This could adversely affect nestling nutritional condition, so we would expect a negative effect of rainfall on the capacity to sustain large bacterial populations. By contrast, enterococci were not affected by rainfall at any nestling age. This suggests that enterococci are less responsive to rainfall-mediated nestling nutritional condition than *Enterobacteriaceae*.

Only early-hatched broods experienced nesting environments conducive to strong predicted effects of high rainfall on bacterial loads in nestling guts. In fact, loads of *Enterobacteriaceae* on day 7 in early-hatched broods, but not in late-hatched broods, showed a negative association with rainfall. This may be related to poor nestling nutrition due to low foraging capacity of adults during periods of high humidity. Finally, low temperatures and high rainfall may interact synergistically as predicted to induce thermoregulatory problems for small nestlings, thereby inducing reduced bacterial growth. In contrast, enterococci showed no response to rainfall at any ambient temperature.

To conclude, growth of important intestinal bacteria appears sensitive to seasonal and climatic factors, presumably mediated by nestling diet, thermoregulatory capacity and nutritional state. Different bacterial types vary in their responsiveness to environmental and seasonal variation.

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SAMENVATTING

Zoals bij alle dieren spelen darmbacteriën ook bij vogels een belangrijke rol bij de vertering van voedsel. In het ei zijn de darmen van kuikens nog vrij van bacteriën. De darmflora komt tot stand in het nest. De periode net na het uitkomen is dus cruciaal voor een gezonde darmflora. In deze periode zijn jonge vogels echter nog niet in staat zelf hun lichaamstemperatuur te reguleren. Het ligt dus voor de hand dat samenstelling van de darmflora bij jonge kuikens varieert met de omgevingstemperatuur en andere weersomstandigheden, wat gevolgen zou kunnen hebben voor de gezondheid van de kuikens. In dit Spaanse onderzoek werd het voorkomen van twee typen darmbacteriën in kuikens van de Bonte Vliegenvanger gekwantificeerd. Bij jonge kuikens waren de aantallen Enterobacteriaceae in de uitwerpselen van kuikens relatief hoog bij hogere temperaturen en relatief laag tijdens regenachtige periodes. Het laatste gold alleen bij vroege broedsels en bij lage temperatuur. Voor oudere kuikens was er alleen een correlatie tussen Enterobacteriaceae en de uitkomstdatum. De hoeveelheden van een ander type darmbacterie (enterococci) waren alleen afhankelijk van de omgevingstemperatuur. Ongeacht hun leeftijd hadden kuikens meer enterococci bij hogere temperaturen. Deze resultaten laten zien dat het weer inderdaad invloed heeft op de samenstelling van de darmflora bij jonge vogels. Hierbij spelen waarschijnlijk de onmacht van jonge kuikens om hun lichaamstemperatuur constant te houden en het vermogen van de ouders om genoeg voedsel aan te dragen een rol. (KK)

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