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Aromatic plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria

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Abstract Several bird species add fresh fragments of plants which are rich in volatile secondary compounds to their nests. It has been suggested, although never tested, that birds use fresh plants to limit the growth of nest micro-organisms. On Corsica, blue tits (*Cyanistes caeruleus*) incorporate fresh fragments of aromatic plants into their nests. These plants do not reduce infestation by nest ecto-parasites, but have been shown to improve growth and condition of chicks at fledging. To understand the mechanisms underlying such benefits, we experimentally tested the effects of these plants on the bacteria living on blue tits. Aromatic plants significantly affected the structure of bacterial communities, in particular reducing bacterial richness on nestlings. In addition, in this population where there is a strong association between bacterial density and infestation by blood-sucking *Protocalliphora* blow fly larvae, these plants reduced bacterial density on the most infested chicks. Aromatic plants had no significant effect on the bacteria living on adult blue tits. This study provides the first evidence that fresh plants brought to the nests by adult birds limit bacterial richness and density on their chicks.

Keywords Nest greenery · Aromatic plants · Bacterial communities · *Cyanistes caeruleus* · *Protocalliphora* sp.

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Introduction

Several bird species add fresh fragments of plants which are rich in volatile secondary compounds to their nests (Clark and Mason 1985; Wimberger 1984). The selected plant species often represent a small, non-random fraction of the available species in the habitat (Gwinner 1997; Gwinner et al. 2000; Lambrechts and Dos Santos 2000; Mennerat et al. 2009b). Among the various hypotheses so far proposed to explain this behaviour, the most frequently invoked is that breeding birds exploit the anti-parasite properties of plant secondary compounds to repel, kill or impair the development of nest ectoparasites (e.g. Clark and Mason 1985; Wimberger 1984). Yet field investigations experimentally demonstrating the influences of fresh plants on nest ectoparasites are rare. Clark and Mason (1988) demonstrated a negative effect of nest greenery on ectoparasites by adding fresh plants to nests of the European starling *Sturnus vulgaris* during the whole breeding period. However, their experimental design did not simulate

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starling natural behaviour because, in this species, males usually stop bringing greenery to the nest before the start of incubation (Brouwer and Komdeur 2004; Gwinner 1997). Subsequent studies in the European starling failed to demonstrate a significant effect of green plants on nest ectoparasite loads (Fauth et al. 1991; Gwinner and Berger 2005; Gwinner et al. 2000), as well as investigations in other bird species (Mennerat et al. 2008; Rodgers et al. 1988). Only one recent study in the non-greenery-adding tree swallow *Tachycineta bicolor* demonstrated negative effects of yarrow *Achillea millefolium* on flea numbers, but not on numbers of blow fly pupae (Shutler and Campbell 2007). As a whole, the protective effect of nest greenery against nest ectoparasites remains unclear.

One hypothesis that has received little attention is that fresh plants could protect chicks from infection by pathogenic microorganisms. Infection by pathogens represents a significant risk, especially when energy is limited because nestlings have to balance growth against immune function (Sheldon and Verhulst 1996; Soler et al. 2003; Tschirren and Richner 2006). Limiting the abundance and diversity of bacteria living on the chicks, hence reducing the probability of pathogen infection, would therefore be beneficial, especially under high environmental constraints.

In a Corsican population of hole-nesting blue tits *Cyanistes caeruleus*, nests are infested by very high numbers of *Protocalliphora* blood-sucking blow fly larvae that have detrimental effects on nestling development, fledging mass, hematocrit and post-fledging survival; they also affect nestling behaviour and parental effort (Banbura et al. 2004; Blondel et al. 1998; Charmantier et al. 2004; Hurtrez-Boussès et al. 1997, 2000; Simon et al. 2004, 2005). These ectoparasites, in addition to other severe environmental constraints (e.g. low food abundance; Blondel et al. 2006), represent a source of stress to both nestlings and adults.

In this population, female blue tits actively incorporate fresh fragments of aromatic plants into the nest cup (e.g. *Lavandula stoechas*, *Achillea ligustica*, *Helichrysum italicum*, *Mentha suaveolens*) during the whole breeding process, i.e. from the end of nest construction until fledging. They replenish the nest with fresh fragments of the same plant species quickly after experimental removal (Lambrechts and Dos Santos 2000; Petit et al. 2002; Mennerat et al. 2009b). All females in this population add aromatic plants to their nests, but in variable amounts. In particular, females add fewer plant fragments when environmental conditions (temperature, food abundance) are less favourable, which suggests that this behaviour may be costly (A. Mennerat, unpublished data). Recent field experiments showed that these plants significantly improve chick growth and condition at fledging (Mennerat et al. 2009a), although they have no significant effect on blow fly infestation (Mennerat et al. 2008). Many of the plant species used by blue tits are already known to have in vitro antibacterial properties (e.g. inhibition of bacterial growth; Rossi et al. 2007), but their efficiency in natural ecosystems such as bird nests remained untested so far. The aim of this study was to investigate the mechanisms underlying the benefits of aromatic plants to blue tits. For this purpose, we experimentally investigated the effects of these plants on the bacteria living on blue tits by testing: (1) the overall effect of aromatic plants on bacterial communities sampled on the birds, and (2) their effect on the abundance and diversity of these bacteria.

Materials and methods

Experimental treatment

This study was carried out in 2005 at the Pirio site in Corsica (evergreen oak *Quercus ilex* forest, 42°31′N, 08°46′E), where blue tits use nest boxes for breeding (see e.g. Blondel 1985 for a description of the site and field protocols). During the whole nestling period, each day we removed fresh plants brought by the birds to all nests under study before adding 0.5 g *Lavandula stoechas* and 0.5 g *Helichrysum italicum* (two plant species often found in blue tit nests) to 20 aromatic-treated nests and 1 g fresh moss (basic nest material) to 20 control nests. After drying, 1 g of the aromatic plants used in this experiment weighed 0.3 g, which is within the natural range of aromatic plants added daily to nests by Corsican blue tits (0.03–0.31 g dry mass per nest per day; A Mennerat unpublished data). The two experimental groups did not differ in egg-laying date (*t*-test, $df = 40$, $t = -1.01$, $P = 0.32$) nor in clutch size (*t*-test, $df = 40$, $t = 0.61$, $P = 0.55$).

Estimation of blow fly infestation intensity

To avoid loss of blow fly larvae during nest inspections, a tissue bag was inserted under the nest 2–4 days before the chicks hatched. When chicks were 2–3 days old, the edge of the bag was pulled up to reach the same level as the top of the nest cup. At day 14–15 post-hatching, after bacterial sampling (see below), nests were collected and replaced by the same amount of fresh moss. In the laboratory, blow fly larvae and pupae were sorted out of the nest material and counted. Blow fly larvae develop into three successive larval stages before pupating (Bennett and Whitworth 1991). The tiny first-stage larvae are particularly difficult to detect so that our estimate of blow fly infestation intensity only included the total number of second-stage larvae, third-stage larvae and pupae, following the protocols applied by Hurtrez-Boussès (1996) and Heeb et al. (2000).

Bacterial sampling

Since the beginning of the breeding season, experimenters systematically washed their hands with ethanol before manipulating birds or nests. At days 14–15 post-hatching, bacterial communities were sampled on both parents and three nestlings per nest by pressing Whole flora agar slides (Hygi-PLUS) for 10 s onto the birds' flanks (including feathers). The slides were stored in cool bags until reaching the lab and then incubated for 48 h at 37°C. The bacterial densities were estimated as colony forming units (CFU) per square centimetre of medium by averaging three counts in the area of the slide where colonies were homogeneously distributed. The cultivable bacterial communities (CBC) were then cropped from the surface of the slides, suspended in 1 ml sterile phosphate buffered saline and stored at −20°C.

Bacterial DNA fingerprinting

Total bacterial DNA was extracted from 200 µl of the CBC samples using the DNeasy blood & tissue kit according to the manufacturer's protocol for Gram-positive bacteria (Qiagen). We used the automated ribosomal intergenic spacer analysis (ARISA) following the method described by Ranjard et al. (2001), with FAM-labelled S-D-Bact-1522-b-S-20 primer (5′-[6FAM] TGCGGCTGGATCCC CTCCTT-3′) and L-D-Bact-132-a-A-18 primer (5′-CCGG GTTTCCCCATTCGG-3′) to amplify the intergenic spacer (IGS) lying between the16S an the 23S rRNA bacterial genes by polymerase chain reaction (PCR). The PCR products were separated using an ABI 3730 capillary sequencer and analysed with the GeneMapper software (Applied Biosystems). The output series of peaks (operative taxonomic units; OTUs), ranging from 150 to 1,200 bp, and their corresponding peak-height ranging from 1 to 40,000 raw fluorescent units, were gathered in a taxa (OTU)—abundance (peak-height) matrix. For each sample, we considered the number of OTUs that contributed to 90% of the total abundance as an index of cultivable bacterial richness of the most dominant species. This is a way to control for random sampling of rare OTUs.

Statistical analysis

To test the overall effect of aromatic plants on bacterial communities, we made a similarity matrix using pairwise comparisons among individual ARISA profiles. Similarity was calculated using the Bray–Curtis index (Legendre and Legendre 1998) on log-transformed relative abundances of bacterial taxa (OTUs). In a first step, we investigated how bacterial communities differed among individuals by testing: (1) whether similarity in bacterial communities was higher within than among nests, (2) whether it was higher within than among age classes (nestlings or adults), and (3) whether similarity in bacterial communities on adults was higher within than among sexes. In a second step, to test the effect of aromatic plants, we tested whether similarity in bacterial communities was higher within than between treatment groups (aromatic-treated nests or control moss-treated nests), for both adults and nestlings. All analyses of similarity were performed using the ANOSIM procedure, which is an approximate analogue of standard ANOVA but based on similarity matrices (Clarke and Gorley 2006). Sample statistics (Global R) were calculated from 999 random pairwise permutations. Similarity analyses were done with the Primer v6.1.6 software (Primer-E).

We also tested the effect of aromatic plants on the density (CFU cm^{-2}) and richness (number of OTUs that contributed to 90% of the total abundance) of bacteria living on the birds. Bacterial densities were log-transformed prior to analysis. Bacterial densities on nestlings and on adults were compared using a mixed-effects model with nest as random factor and age class (adult or nestling) as a fixed factor. Brood size and egg-laying date were included as covariates in all following analyses. For adults, we tested the effects of treatment on cultivable bacterial density and cultivable bacterial richness with mixed-effects models including nest as random factor, treatment, sex as fixed factors and blow fly infestation intensity as covariate. Separate analyses in adult males and females gave similar results and will therefore not be presented here. We tested the effects of treatment on cultivable bacterial richness on nestlings using a mixed-effects model including nest as random factor, treatment as fixed factor and blow fly infestation intensity as covariate. For bacterial density measured on nestlings, the treatment × blow fly infestation interaction was significant ($P < 0.05$). Therefore, mixed-effects models were done separately in control and treated nests, with nest as random factor and blow fly infestation intensity as covariate. The effect of treatment on nestling bacterial densities was tested separately in nests with low and high blow fly infestation intensities (according to the median value, 54 blow flies per nest, range 7–155 blow flies per nest) using a mixed-effects model with nest as random factor and treatment as fixed factor. In all models, two-term interactions were also tested but none of them was significant. For the sake of clarity they will not be presented here. All mixed-effects models were fitted by maximum log-likelihood with the R version 2.6.0 software.

Results

Bacterial communities were significantly more similar within than among nests [analysis of similarities (ANOSIM), global $R = 0.26$, $P = 0.001$]. Within nests, they were

also more similar within than among age classes (i.e. nestlings or adults; nested ANOSIM, global $R = 0.33$, $P = 0.001$). Bacterial communities on adults were not significantly more similar within than among sexes (ANOSIM, global $R < 0.05$, $P = 0.55$). Finally, similarity in bacterial communities was significantly higher within than between treatment groups, on nestlings (ANOSIM, global $R = 0.03$, $P = 0.03$) but not on adults (ANOSIM, global $R = 0.01$, $P = 0.18$).

Aromatic plants reduced cultivable bacterial richness on nestlings ($n = 95$, $P < 0.01$), whereas for the adults we only found a tendency ($n = 54$, $P = 0.09$; Tables 1, 2; Fig. 1). Cultivable bacterial density on nestlings was strongly positively related to blow fly infestation intensity, in control nests but not in aromatic-treated nests (control nests, $n = 48$, $P < 0.001$; treated nests, $n = 46$, $P = 0.26$; Table 1; Fig. 2a). In other words, aromatic plants significantly reduced bacterial densities on nestlings under high blow fly infestation, although not under low infestation (high infestation, $n = 46$, $P < 0.05$; low infestation, $n = 48$, $P = 0.29$; Table 1). Interestingly, cultivable bacterial densities on parents were lower than on their nestlings ($n = 146$, LR = 11.36, $P < 10^{-3}$) and were neither affected by treatment nor related to blow fly infestation ($n = 52$; treatment, $P = 0.78$; blow fly infestation, $P = 0.51$; Table 2; Fig. 2b). Brood size and egg-laying date were not significantly related to bacterial density and richness, neither on nestlings nor on adults (all $P > 0.23$; Tables 1, 2).

Discussion

This study provides evidence for a previously unknown function of fresh aromatic plants brought by birds to their nests. Aromatic plants in blue tit nests significantly affect bacterial communities on nestlings. In particular, they reduce cultivable bacterial richness on nestlings. They also limit the increase in cultivable bacterial density related to ectoparasite infestation of nestlings. Aromatic plants had no significant effect on cultivable bacteria living on adults in terms of bacterial communities, bacterial densities or richness.

Blue tits in our study population face high environmental constraints, including high blow fly infestation intensities—the highest recorded in Europe—and low food availability (Blondel et al. 2006). Under such levels of stress, the trade-off between nestling growth and immune defence is exacerbated (Sheldon and Verhulst 1996; Soler et al. 2003; Tschirren and Richner 2006), so that any immune challenge, even non-lethal, may be detrimental to nestlings by slowing down growth. By reducing nestling bacterial richness and density especially in highly blow fly-infested nests, plants may thus reduce the risk of bacterial infection, hence providing benefits in terms of chick growth. In this study, we did not find any significant effect of aromatic plants on nestling body mass, tarsus length or haematocrit at fledging (see statistical analyses and results in Mennerat et al. 2008). This may be explained by the fact that, in the year this experiment was conducted, environmental conditions were highly favourable (e.g. high food abundance, warm spring temperatures). As a consequence, there was little phenotypic variation among chicks, all of them being of good condition at fledging. In contrast, in a subsequent field study where environmental conditions were experimentally altered, aromatic plants were found to improve several parameters of chick growth as well as hematocrit at fledging, which is related to post-fledging survival in this study population (Mennerat et al. 2009a). These results support our hypothesis that the anti-bacterial effects of plants may be especially beneficial to those nestlings that are facing high environmental constraints.

The strong positive association, observed in control nests, between ectoparasite infestation intensity and bacterial density on nestlings had not been previously described.

Table 1 Effects of addition of aromatic plant to nests (treatment) on bacterial density and richness on nestlings. Since the treatment × blow fly infestation interaction was significant, bacterial density was analysed separately in control versus treated nests (respectively in nests with low vs. high blow fly infestation)

	df	Bacterial richness (95)		Bacterial density							
				Treatment				Blow fly infestation			
				Control (48)		Treated (46)		Low (48)		High (46)	
		LR	*P*	LR	*P*	LR	*P*	LR	*P*	LR	*P*
Treatment	1	6.73	0.009	–	–	–	–	1.13	0.29	4.41	0.04
Blow fly infestation	1	<0.01	0.97	10.58	<0.001	2.19	0.14	–	–	–	–
Brood size	1	1.43	0.23	0.01	0.92	0.13	0.71	<0.01	0.95	2.29	0.13
Laying date	1	0.05	0.82	0.31	0.58	0.65	0.42	0.69	0.41	0.41	0.52

Results are from mixed-effects models (see "Materials and methods"). All models include nest as a random factor. Sample sizes are indicated *in parentheses*. *LR* likelihood ratio

Table 2 Effects of addition of aromatic plant (treatment) on bacterial density and richness on adults

	df	Bacterial richness (54)		Bacterial density (52)	
		LR	*P*	LR	P
Treatment	1	2.84	0.09	0.08	0.78
Sex	1	0.19	0.66	1.33	0.25
Blow fly infestation	1	0.07	0.74	0.44	0.51
Brood size	1	0.11	0.40	0.06	0.80
Laying date	1	0.72	0.80	0.44	0.51

Results are from mixed-effects models (see "Materials and methods"). All models include nest as a random factor. Samples sizes are indicated *in parentheses*

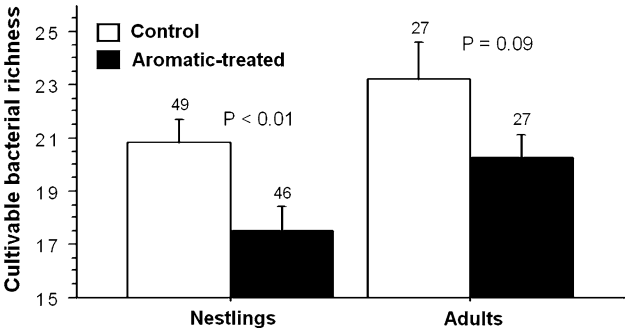

Fig. 1 Effects of addition of aromatic plants to nests (treatment) on cultivable bacterial richness (mean + SE) on nestling and adults. *P*-values are from mixed-effects models (see "Materials and methods")

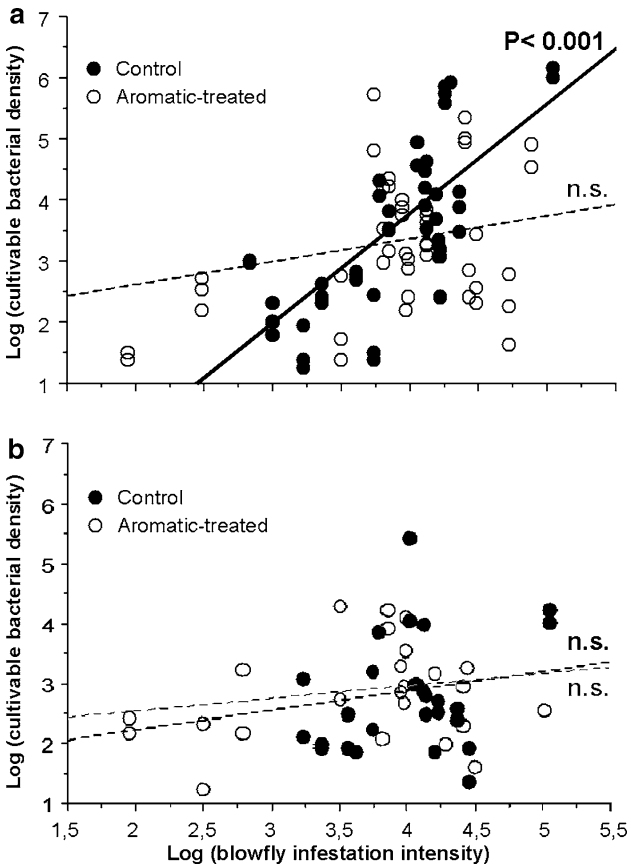

Fig. 2 Effect of addition of aromatic plants to nests (treatment) on the density of cultivable bacteria on **a** nestlings and **b** adults, in relation to blow fly infestation intensity. *P*-values are from mixed-effects models (see "Materials and methods"). *n.s.* Non-significant

In highly blow fly-infested nests, the nest matrix is especially compact, because of the high number of larvae dwelling in them (up to 150 larvae). As a consequence, these nests are often wetter than usual (A. Mennerat, personal observation). High blow fly infestation intensity therefore probably enhances bacterial growth, especially on nestlings, where temperatures lie within the optimal range for most bacteria (25–40°C; Staley et al. 2007). Other factors, such as ambient temperature, may also favour both bacterial growth and the development of blow fly larvae (Dawson et al. 2005). More studies are needed to understand this association between nest ectoparasites and bacteria, and in particular whether infestation by ectoparasites increases the risk of bacterial infection of nestlings (e.g. due to the multiple bites by ectoparasites on their skin).

The structure of bacterial communities carried by adults significantly differed from that of their nestlings, although bacterial richness did not. This fact illustrates the necessity of considering more than one parameter when studying bacterial communities. Differences in bacterial communities between adults and nestlings can be related to the very different environments in which they live (e.g. Bisson et al. 2007). This may also explain why densities of cultivable bacteria were higher on nestlings than on adults. Adults spend most of their time foraging away from the nest, whereas nestlings live in the warm and wet atmosphere of the nest cavity, where bacteria probably are different and grow to higher densities than outside the cavity.

Our measures of bacterial density and bacterial richness rely on cultivable bacteria, which constitute only a fraction of whole bacterial communities. There is no objective reason to believe that our sub-sampling could be biased in such a way that the strong observed effects of plants would disappear when considering whole bacterial communities. Still, identifying which bacteria are most affected by aromatic plants cannot be achieved by such sub-sampling. Therefore, extending our results to whole bacterial communities would be a useful next step towards a better understanding of the effects of aromatic plants on bacteria in bird nests.

A large variety of animals, ranging from insects to humans, exploit plant secondary compounds to protect themselves against parasites or microorganisms (Chapuisat et al. 2007; Hemmes et al. 2002; Huffman 2001; Sherman and Hash 2001). Surprisingly, since Wimberger's (1984) review of the widespread use of fresh plants in bird nests,

few studies—most of them focusing on nest ectoparasites—have experimentally tried to elucidate the use of fresh plants in birds. To our knowledge, the only study of the effects of fresh plants on bacteria in bird nests focused on cultivable bacterial density, but did not investigate the effects of plants on bacterial richness (Gwinner and Berger 2005). Here we demonstrate that the plants brought by female blue tits to their nests, while not being directly effective against nest ectoparasites (Mennerat et al. 2008), reduced both bacterial richness and bacterial density on nestlings, especially under high ectoparasite infestation. These effects may be the mechanism mediating the positive effects of aromatic plants on nestling growth (Mennerat et al. 2009a). Identifying the bacteria that are most affected by these plants and investigating further their effects on nestling growth and survival would certainly be promising steps towards an understanding of the function of aromatic plants in bird nests.

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