Aromatic plants in blue tit *Cyanistes caeruleus* nests: no negative effect on blood-sucking *Protocalliphora* blow fly larvae

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Nesting birds use several behavioural or physiological defence mechanisms against parasites. On Corsica, female blue tits *Cyanistes caeruleus* incorporate fresh fragments of a limited number of aromatic plants in the nest cup, from the end of nest construction until fledging. Some of these plants negatively affect bacterial growth and host location by blood-sucking mosquitoes in laboratory conditions. In natural populations, Corsican blue tit chicks are exposed to the highest levels of blood-sucking ectoparasitic blow flies *Protocalliphora* spp. reported in Europe. These ectoparasites can have severe negative effects on chick development and survival probabilities, especially when food constraints are elevated. Here we investigated in several natural Corsican blue tit populations the hypothesis that aromatic plants brought to the nest have anti-blow fly effects during the chick-rearing stage. We predicted that: 1) the amount of aromatic plants should be negatively related to blow fly infestation intensity across nests, 2) experimental addition of aromatic plants in nests should reduce blow fly infestation intensity, and 3) nestlings should be in better physical condition in nests where aromatic plants were experimentally added. No significant relation was found between amount of aromatic plants in nests and blow fly infestation intensity. Experimental addition of aromatic plants did not reduce blow fly infestation intensity and did not affect the chick phenotypic parameters we measured. We conclude that aromatic plants in blue tit nests are not used as a defence against ectoparasitic *Protocalliphora* blow flies in our study population.

Some bird species bring to their nests green plants which are rich in volatile secondary compounds (Wimberger 1984, Clark and Mason 1985). 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). Three, non-mutually exclusive, main hypotheses on the functional significance of the use of greenery by birds have been proposed in the literature. First, breeding birds may exploit the anti-parasite properties of plant secondary compounds to repel, kill or impair the development of nest ectoparasites, and therefore affect host-parasite interactions (the nest protection hypothesis, e.g. Wimberger 1984 Clark and Mason 1985). Second, the chemical compounds of fresh plant fragments may stimulate the host immune system independently from potential detrimental effects on ectoparasites, and perhaps reduce endoparasite loads or pathogen infection probabilities (the drug hypothesis, Gwinner et al. 2000). Finally, the use of green plants by males during nest building may play a role in mate attraction, for instance when the ability to find particular plants reflects aspects related to territory and/or individual quality beneficial to chicks (the courtship hypothesis, Fauth et al. 1991, Gwinner 1997, Brouwer and Komdeur 2004, Polo et al. 2004, Veiga et al. 2005).

Field investigations experimentally demonstrating influences of fresh plant material on nest ectoparasite loads or offspring characteristics are rare. Clark and Mason (1988) first demonstrated a positive effect of green plant addition in nests of the European starling *Sturnus vulgaris*. Experimental addition of green plant fragments from day 10 before-hatching until day 13 post-hatching reduced the number of hematophagous mites in nests relative to control nests. Adding green material to the nest did not influence chick mass or feather development, but resulted in higher levels of blood haemoglobin in nestlings. Clark and Mason’s experimental design did not simulate starling natural behaviour because males usually stop bringing greenery to the nest before the start of incubation (Gwinner 1997, Brouwer and Komdeur 2004). In another starling study, Fauth et al. (1991) experimentally removed green nest material after the end of clutch completion, whereas control nests contained greenery added by males during courtship and nest building. The two treatments did not
differ in ectoparasite loads, breeding success, chick condition or post-fledging survival. In a third experiment comparing herb and grass nests, herbs selected by starlings did not reduce ectoparasite loads, but affected both chick body mass and blood parameters (hematocrit, lymphocyte and basophil counts, Gwinner et al. 2000, Gwinner and Berger 2005). Most investigations in bird species other than starlings also failed to demonstrate a significant negative influence of fresh plants on nest-dwelling ectoparasites. In the wood stork M.c. americana, green plant material in nests had no repelling effect on dermestid beetle larvae Dermentis nidi (Rogders et al. 1988). Dawson (2004) found that experimentally-added yarrow Achillea millefolium in nests of the non greenery-using tree swallow Tachycineta bicolor had no negative effect on blow fly or flea infestation levels. An exception is Shutler and Campbell’s (2007) recent study in tree swallow nests showing negative effects of yarrow on flea number, although not on blow fly pupae numbers.

On Corsica, hole-nesting female blue tits Cyanistes caeruleus actively incorporate fresh fragments of aromatic plants in the nest cup (e.g. Lavandula stoechas, Achillea ligustica, Helichrysum italicum, Mentha suaveolens). They daily add fresh aromatic plant fragments in the nest between the end of nest construction until fledging, and 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 pers. obs.). The plant species found in blue tit nests represent only a small fraction of the plants available in the habitat (Petit et al. 2002). A noticeable difference between starling and blue tit plant-adding behaviours is that blue tits keep adding aromatic plants during the whole nesting period, which suggests that these plants have a little role in mate attraction. Some of the selected plants possess antiseptic, fungicidal or insecticidal properties (Petit et al. 2002, Rossi et al. 2007), and repel blood-sucking mosquitoes in laboratory conditions (Lafuma et al. 2001). In free-ranging blue tit populations on Corsica, nests are infested with very high numbers of Proctolophora blood- sucking blow fly larvae attacking nestlings (Hurtrez-Boussès et al. 1999). Adult female blow flies visit bird nests and lay eggs in the nest material just after the chicks hatch, and keep visiting bird nests throughout the whole nesting period (Bennett and Whitworth 1991, Hurtrez-Boussès et al. 1999). Blow fly larvae grow into three successive, blood-sucking larval stages, and remain in the nest material until they emerge as adults after their pupal phase (Bennett and Whitworth, 1991, Heeb et al. 2000). In Corsican blue tit populations, blow fly larvae have pronounced detrimental effects on nestling development, fledgling mass, hematocrit and post- fledging survival. They also influence aspects related to chick behaviour and parental care, including investment in nest sanitation (Hurtrez-Boussès et al. 1997, 1998, 2000, Banbura et al. 2004, Charmantier et al. 2004, Simon et al. 2004, 2005). Other nest ectoparasites, such as fleas, ticks, lice or mites, are rarely observed in these populations (prevalences <3%, Hurtrez-Boussès et al. 1997, A. Mennerat pers. obs.).

Here we investigate whether fresh aromatic plant fragments incorporated in Corsican blue tit nests have significant negative effects on blow fly infestation, as predicted by the nest protection hypothesis. We used both an observational approach to examine the relationship between aromatic plants and blow fly infestation intensity in non-manipulated nests, and an experimental test of the effects of aromatic plants on blow fly infestation intensity and nestling body condition.

### Materials and methods

#### Study sites and field protocols

The study was carried out in 2005 and 2006 in three Corsican blue tit populations accepting nestboxes for breeding ("Muro-deciduous", 42° 33’ N, 08° 55’ E, broad-leaved deciduous oakwood Quercus humilis, “Muro-evergreen”, 42° 3’6” N, 08° 58’ E, evergreen oakwood Q. ilex, “Pirio”, 42° 31’ N, 08° 46’ E, evergreen oakwood Q. ilex, see Blondel 1985 and Lambrechts et al. 1997, 2004 for a detailed description of the sites). All nestboxes were monitored throughout the breeding season to determine the onset of egg laying (March 1st = day 1), clutch size, hatching date, the number of hatchlings, and the number of chicks fledged (for a description of the field protocols, see e.g. Blondel 1985, Lambrechts et al. 1997). Nestlings were weighed at day 9–11 post-hatching when chick demands are considered to be the highest, and measured and weighted again when chicks had reached their asymptotic mass at day 14–15 post-hatching. Body mass was measured to the nearest 0.1 g with a Pesola spring balance and tarsus length to the nearest 0.1 mm with a digital calliper. Chick body mass and tarsus length are often associated with the probability to be recruited in the local breeding population (Blondel et al. 1998, Heeb et al. 1999). Together with body mass, hematocrit determines chick aerobic capacity, which is related to post-fledging survival in Corsican blue tit populations (Thomas et al. 2007). We therefore used hematocrit as a measure of physiological condition in nestlings from Pirio. For each chick, around 20 μl of blood was collected from the brachial vein into a heparinised microcapillary tube, and centrifugated for 3 min at 13,000 rpm. The hematocrit value was defined as the percentage volume of erythrocytes in the total blood sample.

#### Blow fly quantification procedures

To avoid loss of blow fly larvae during nest removal, we placed each monitored nest in a tissue bag, following the method applied by Hurtrez-Boussès (1996). A bag was inserted under the nest 2 to 4 d before hatching. When chicks were 2–3 d old, the edge of the bag was pulled up to reach the same level as the top of the nest cup. At day 14–16 post-hatching, after measuring the chicks, nests were collected, enclosed in hermetic plastic bags 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. First-stage larvae are particularly difficult to detect. Therefore, 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).

Quantification of aromatic plants in non-manipulated nests

A hundred nests were sampled to examine the relationship between the total amount of aromatic plants and blow fly infestation intensity at day 14–15 post-hatching (Muroevergreen: 2005, 35 nests and 2006, 25 nests; Murodeciduous: 2005, 25 nests and 2006, 15 nests). To avoid damages to plant fragments caused by e.g. mites or microorganisms before sampling of aromatic plant fragments, all nests were microwave-disinfected after blow fly quantification. During sampling, aromatic plant fragments were separated from the rest of the nest material, stored in paper bags and allowed to dry at ambient temperature for several weeks. Dried samples were weighed with a precision balance (Acculab Pocket Pro C/50) to the nearest 0.002 g to obtain the total dry mass of aromatic plants per nest. Aromatic plant fragments were easily identified using morphological characteristics that clearly differed from moss and twigs. A herbarium of local plants identified by a botanical specialist was also used as reference.

Experimental protocol

In Pirio 2005, two experimental groups of 21 nests were monitored. In nests randomly assigned to a “treated” group, we added 1 g of fresh leaves of two locally abundant aromatic plants often found in nests (0.5 g of Lavandula stoechas and 0.5 g of Helichrysum italicum), first 3 d before hatching, then daily from day 2–3 post-hatching until day 14–15 post-hatching. Before adding fresh aromatic plants, we carefully removed all detectable aromatic plant fragments added by female blue tits or by the experimenter during previous days. Therefore the quantity of fresh plant fragments in nests remained constant through time and across nests. Nests assigned to the “control” group received the same treatment as treated nests, but fresh moss (1 g) was added instead of aromatic plants. After drying, 1 g of the aromatic plants used in this experiment weighed approximately 0.3 g, which is within the natural range of fresh aromatic plant fragments daily added in nests by Corsican blue tits (0.03–0.31 g dry mass per nest per day, A. Mennerrat unpubl. data.). Nests were collected at day 14–15 post-hatching, when chicks were measured and blow fly infestation intensity was quantified as described below. The two groups did not differ in egg laying date (mean ± SD, treated nests: 65.95 ± 2.31, control nests: 65.19 × 2.58, t-test, df = 40, t = -1.01, P = 0.32) and clutch size (mean ± SD, treated nests: 7.19 ± 0.81, control nests: 7.43 × 1.60, t-test, df = 40, t = 0.61, P = 0.55), so we assume we controlled for aspects related to the quality of the parents or the territory.

Statistical analyses

Blow fly infestation intensity was square-root transformed prior to analyses. The relation between blow fly infestation intensity and amount of aromatic plants in non-manipulated nests was investigated using a generalised linear model (type 3 GLM procedure, SAS 9.1). We included year and habitat type (deciduous vs evergreen) as fixed factors and brood size as covariate in the model because they may influence blow fly infestation intensity (Hurtrez-Boussès et al. 1999). We also tested for the interaction between year and amount of aromatic plants because the relation between aromatic plants and blow fly larvae infestation may vary according to yearly environmental fluctuations. A mixed-effects model was performed with year as a random factor, habitat type (deciduous vs evergreen) as a fixed factor and brood size as a covariate (MIXED procedure, SAS 9.1). Since the results were approximately the same, we will only present the results from the GLM analysis, which allows to test for the interaction between year and aromatic plants.

To investigate whether adding aromatic plants had an effect on blow fly infestation and nestling survival, blow fly infestation intensity and brood size at days 14–15 post-hatching in the two experimental groups were compared with t-tests. As nestling mass at days 9–11 post-hatching and nestling wing length at days 14–15 post-hatching were positively correlated with chick age (df = 1, t = 19.6, P < 0.001 and df = 1, t = 20.89, P < 0.001, respectively), we considered residuals of the regression of both variables against chick age as corrected variables. We used the first axis of a principal component analysis (PCA) from the correlation matrix of the three measurements of nestling mass, tarsus length and wing length (corrected for age) as an estimate of nestling body condition at day 14–15 post-hatching. The first axis (PC1) accounted for 53% of the total variance. We investigated the effects of treatment, brood size and blow fly infestation intensity on nestling corrected mass at days 9–11, nestling size (PC1) and nestling hematocrit with mixed-effects models considering chick as statistical unit and nest as random factor (MIXED procedure, SAS 9.1). The interaction between treatment and blow fly infestation intensity was also included because effects of treatment may be more pronounced under high infestation intensities.

Results

Blow fly infestation intensity was not significantly related to the total dry mass of aromatic plant fragments found in non-manipulated nests (df = 1, F = 1.90, P = 0.41, Fig. 1). In addition, it was not significantly related to the interaction between year and total dry mass of aromatic plant fragments (df = 1, F = 0.99, P = 0.32), nor to habitat type (df = 1, F = 0.16, P = 0.69). However, nests with larger broods contained more blow fly larvae (df = 1, F = 13.85, P < 0.001), and blow fly infestation intensity tended to be higher in 2006 than in 2005 (mean ± SD, 2005: 35.9 ± 26.34, 2006: 44.39 ± 27.84, df = 1, F = 3.55, P = 0.06, Table 1).

Experimentally adding aromatic plants in nests did not affect blow fly infestation intensity: treated and control groups did not significantly differ in blow fly infestation intensity (mean ± SD number of blow fly larvae, treated nests: 64.00 ± 36.29, control nests: 55.67 ± 31.11, t-test on
Table 1. Relation between (square-root transformed) blow fly infestation intensity and total dry mass of aromatic plants, brood size, habitat type, year and the interaction between year and total dry mass of aromatic plants, as tested by a type 3 GLM (see Materials and methods).

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<th>Df</th>
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<td>Brood size</td>
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<td>Habitat type</td>
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<td>0.69</td>
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<tr>
<td>Year</td>
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<tr>
<td>Year × Aromatic plants</td>
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<td>0.99</td>
<td>0.32</td>
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Discussion

We initially hypothesized that aromatic plant fragments in blue tit nests could alter nest detection capacities in adult blow flies, repel adults or larvae, or contain compounds toxic for adults and/or larvae (e.g. Lambrechts and Dos Santos 2000, Lafuma et al. 2001). Because blow flies were exposed to aromatic plants at the time they lay eggs in blue tit nests, we predicted lower numbers of blow fly larvae in aromatic-treated nests, which was not the case. In addition, our two-year study involving one hundred sampled nests did not reveal any significant correlation between the total dry mass of aromatic plant fragments and blow fly infestation intensities across blue tit nests in two oak habitat types. We therefore conclude that the aromatic plant fragments sampled and used in our experiment have no anti-blow fly effects and therefore could not protect chicks against blow fly attack. This is also supported by our analyses that chick traits influenced by blow fly attack (e.g. nestling hematocrit, see Introduction), were not related to the amount of aromatic plant fragments added in the nest.

Although we are confident that the aromatic plants sampled do not protect blue tit chicks against blow fly attack, significant correlations between aromatic plants and offspring characteristics or breeding success may have been masked by unidentified confounding variables, the particular environmental conditions during the study, or the offspring traits investigated. For instance, inconsistent with former investigations in the same study population, we did not find a significant relationship between blow fly infestation intensity and chick size or mass, perhaps because our study was carried out under highly favourable environmental conditions (own observations, see also Thomas et al. 2007). Gwinner et al. (2000) found that the positive effect of green nesting material on nestling body mass was greater in highly mite-infested nests. Gwinner and Berger (2005) also found that the positive effect of herbs on nestling body mass was particularly revealed under unfavourable conditions (high mite load, low ambient temperature, prolonged rainfall). Also, aromatic plants may have positive effects on post-fledging survival that we were not able to estimate with our measures of nestling condition, e.g. via repellent effects on blood-sucking flying insects that can be vectors of avian endoparasites (Lafuma et al. 2001), or anti-bacterial effects reducing the risk of pathogen infection of nestlings (Rossi et al. 2007). More work on potential parasites other than Protocalliphora blow flies is therefore required to better understand parasite-aromatic plant interactions in blue tit nests, their underlying mechanisms and their potential consequences on chick survival in wild populations.

Fig. 1. Number of blow fly larvae plotted against dry mass of aromatic plants in nests (n = 100 nests).

Fig. 2. Number of blow fly larvae (± SE) in control vs aromatic-treated nests. (t-test: df = 40, t = −0.64, P = 0.53, see Results.)
Table 2. Effects of experimental treatment (aromatic vs control nests), blow fly infestation intensity, brood size and the treatment × infestation intensity interaction on nestling mass at days 9–11 post-hatching. Mass1, corrected for age, nestling body condition (PC1) and nestling hematocrit, as tested by mixed-effects models with nest as random factor (see Materials and methods).

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<th>Mass1</th>
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<td>Nest</td>
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