Nest material preferences by spotless starlings

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Abstract

The avian nest is an essential structure for offspring development. For adults, nest building entails costs in terms of time, energy and exposure to predators and parasites. Amount and diversity of materials used for nest building depend on their availability and functionality in scenarios of sexual selection and parasitism. Green plants and feathers of different colors have been hypothesized to play key roles in offspring protection against pathogens, and we here experimentally assessed spotless starling (*Sturnus unicolor*) preferences for pigmented vs. unpigmented feathers and for different green plants (aromatic vs. non-aromatic plants) as nest materials. We predicted a preferential selection of unpigmented feathers and aromatic plants according to the antimicrobial properties of these materials described in the literature. We evaluated these predictions during nest building and during egg-laying stages. As expected, starlings preferentially selected unpigmented feathers both before and during egg laying, while aromatic plants were preferentially selected only during the egg-laying stage. These results suggest that starlings prefer nest materials that enhance antimicrobial protection of their offspring. We also discuss some other, non-exclusive functions that might explain the observed preference for nest materials, especially with regard to their potential role in sexual signaling.

Keywords: Aromatic plants, Feather pigmentation, Nest building behavior, Nest lining feathers, Nest material preference.
Introduction

The avian nest is an essential structure that protects offspring from a wide diversity of environmental challenges during development (Hansell 2000; Moreno 2012; Mainwaring et al. 2014). Nest building entails costs in terms of, for instance, expended time and energy, or exposure to predators and parasites (Hansell 2000; Mainwaring and Hartley 2013).

Different nest materials have different properties, so birds are expected to carefully adjust nest-building behavior to optimize the balance between costs and benefits while selecting the more appropriate combination of materials to maximize fitness (Soler et al. 1998; Hansell 2000).

Among the several materials employed by birds to build their nests, the use of green plants and feathers has received special attention in evolutionary ecology research (Hansell 2000; Dubiec et al. 2013). Green plants, which are used as structural or lining material, may also have several non-exclusive functions in avian nests that include sexual signaling (Fauth et al. 1991; Brouwer and Komdeur 2004; Polo et al. 2004; Veiga et al. 2006; Moreno 2012; Tomás et al. 2013). Yet, the most studied function of green plants is related to the antimicrobial and antiparasitic properties of their volatile secondary compounds and essential oils that protect offspring from pathogens (Clark and Mason 1985; Lafuma et al. 2001; Gwinner and Berger 2005; Shutler and Campbell 2007; Mennerat et al. 2009a; Dubiec et al. 2013; Scott-Baumann and Morgan 2015). Through different metabolic routes, many of these plants can also have therapeutic effects improving nestling growth, health, or immunocompetence (Gwinner et al. 2000; Gwinner and Berger 2005; Mennerat et al. 2009b). Therefore, the use of green plants in the nests of birds is considered as a form of self-medication (De Roode et al. 2013). Different plants have different compounds, so birds
may adjust their nest building behavior according to the selective pressures exerted by pathogens.

Feathers are another material commonly used in bird nests, mainly for nest cup lining, but also as structural material (Hansell 2000). Although the most advocated function of feathers in nests is related to thermal insulation (Møller 1984; Lombardo et al. 1995; Hilton et al. 2004; Pinowski et al. 2006; Dawson et al. 2011; Windsor et al. 2013), they can also be involved in sexual signaling (Polo and Veiga 2006; Sanz and García-Navas 2011; García-López de Hierro et al. 2013; Mainwaring et al. 2016) and offspring protection from microbial infections (Soler et al. 2010). These two last functions are respectively based on attractiveness of feathers of different color (Veiga and Polo 2005; Avilés et al. 2010, Sanz and García-Navas 2011), and on the antimicrobial properties of the bacterial communities that grow digesting the keratin of feathers (Peralta-Sánchez et al. 2014; Ruiz-Castellano et al. 2016). These two functions are not mutually exclusive, and we here concentrate our predictions on the hypothetical antimicrobial functionality of feathers. Apparently, bacterial growth (Goldstein et al. 2004; Gunderson et al. 2008) and antimicrobial properties are higher for bacterial communities of unpigmented feathers (Peralta-Sánchez et al. 2014). Thus, birds may preferentially select unpigmented feathers for nest building.

Some of these hypothetical effects of feathers and green plants in the nests of birds have been experimentally demonstrated, but evidence of associated adaptive preference is restricted to a few species. For green plants, previous research on European starlings (Sturnus vulgaris) has suggested olfactory discrimination of plant volatiles (Clark and Mason 1987; Gwinner and Berger 2008). Moreover, an observational study concluded that plant species composition of blue tit (Cyanistes caeruleus) nests results from individual preferences of particular species (Mennerat et al. 2009c). With respect to feathers, Peralta-
Sánchez et al. (2011) found that barn swallows (*Hirundo rustica*) preferentially selected white feathers (experimentally offered) for lining their nests. Exploring these preferences in some other species, as well as possible variation depending on environmental conditions, would aid understanding of the functional significance of nests and nest building behaviors.

Selection of nest materials by birds can be tackled by looking at the final assemblage of materials in nests after considering the availability of different materials in the nest surroundings (Petit et al. 2002; Mennerat et al. 2009c; Pires et al. 2012). However, birds could also collect nest materials far away from their territories (Bailey et al. 2016), which challenges a proper statistical control of nest material availability. Therefore, an experimental approach might be the most suitable way to study the preference for certain nest materials, which we accomplished here by manipulating their availability. During nest building stage, unlimited pigmented and unpigmented feathers, as well as different aromatic and non-aromatic plant species, were offered to spotless starlings. Later we recorded its presence and abundance in their nests. We predict that birds would select preferentially unpigmented feathers and aromatic plants if antimicrobial protection of the offspring were one of the functions of these nest materials.

Materials and Methods

*Study species and area*

The spotless starling is a medium-sized, hole-nesting passerine that mostly breeds in colonies (Cramp 1998). Green plants and feathers are commonly used as nest materials, with plants and feathers being carried to the nest by males and females respectively (Polo and Veiga 2006). Both materials are embedded in the nests, forming part of both their structural and lining layers (Veiga and Polo 2016). Starlings in our population usually
commence to build their nests in March, laying eggs at mid-April. Since April 10th nest-boxes were visited every three days until the first egg was laid.

The study was performed during the 2013 breeding season in a population located in the old railway station of La Calahorra (37°18’ N, 3°11’ W) within the Hoya de Guadix, a high-altitude plateau 1000 m a.s.l., with a semi-arid climate. Apart from a few sparse almond trees (*Prunus dulcis*), vegetation in the area is typical of an agro-pastoral steppe, with abandoned fields interspersed within barley (*Hordeum vulgare*) and lettuce (*Lactuca sativa*) cultivated fields. There were 80 cork-made nest-boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm) available for spotless starlings, attached to tree trunks or walls at 3-4 m above ground. Nest-boxes were often less than 1 m apart from each other (as in natural holes).

**Experimental procedures**

Feathers and plants were offered *ad libitum* to starlings during nest building and laying stages. Feathers were provided in 24 * 24 cm broad plastic meshes placed in the ground, and distributed around the study area, so that all breeding pairs could have access to feathers. Twenty meshes were located in adjacent pairs, containing 50 unpigmented and 50 pigmented feathers each. Feathers offered to starlings, of approximately 10 cm long, were from domestic turkeys (*Meleagris gallopavo*) to distinguish them from chicken feathers that were the most abundant in starling nests in our population. In addition, turkey feathers were marked on the quill with a permanent marker. Meshes were checked daily and feathers were replenished whenever there were 15 feathers or less in the mesh.

Plants were provided in plastic containers (height * width * length: 6 * 7 * 9.5 cm) filled with insulating foam and water to maintain them fresh. In each container, four apical
plant fragments of approximately 10 cm length of four aromatic species (*Marrubium vulgare*, *Artemisia barrelieri*, *Lamium amplexicaule* and *Anacyclus clavatus*) and one non-aromatic plant (barley, *Hordeum vulgare*) were provided. Plants were marked in the stem with seal ink. Containers were attached on top of 2/3 of nest-boxes and were replaced every three days to provide fresh plants, when the number of fragments of each plant species collected by starlings was counted. Starlings made use of plants irrespective of whether those were above their own or above adjacent nest-boxes (authors’ personal observation) and, if considering six consecutive containers, plant fragments of all offered species were always available at the time of container replacement.

Starting one week before the expected laying date of the first egg (the onset of egg laying of starling pairs in our population is quite synchronous), plants and feathers were provided to starlings during three weeks. Nest-boxes were checked every three days, from the day plants and feathers were offered until eggs were found in the nest. During each visit, the number of feathers present in nests was counted, distinguishing between pigmented and unpigmented, and between experimental and non-experimental feathers. Plants present in nests were also weighted (± 0.1 g) and fragments of experimental plants found in nests were counted. When experimental nest materials were offered for the first time, none of the nests contained eggs. Some nests (N = 21) were detected with eggs three days later, during the second visit, while for 27 nests, eggs were detected in subsequent visits. Since nest material composition during the pre-laying stage was estimated during the visit just before eggs were detected, only that of the latter group of nests could have been influenced by the experimental *ad libitum* availability of materials. Thus, these two groups of nests serve to test for the influence of the experiment on nest material composition before egg laying.
In addition, as proxies of the selection made by starlings of feathers and plants, the number of times that each experimental mesh containing either pigmented or unpigmented feathers needed to be replenished, and the number of steams of each plant species that had been collected from the containers, were used.

To estimate plant availability around nests, the percentage of a circular area of 20 m-radius around each nest-box that was covered by green plants was recorded (hereafter percentage of vegetation coverage). These values were used for statistically controlling plant availability when analyzing variation in mass of green plants found in starling nests.

**Antimicrobial activity of green plants offered to starlings**

The antimicrobial activity of the five green plants offered to spotless starlings was assessed in the laboratory. First, plants were sterilized using a UV sterilizer chamber (Burdinola BV-100). Second, 1 cm fragments of each species were placed in Brain Heart Agar (BHA, Scharlau Chemie S.A., Barcelona) plates, inoculated with 100 µL of a 24 h culture of the indicator bacteria strain (see below), and then incubated 24 h at 37 ºC. The antimicrobial activity of each plant was revealed by the presence of growth-inhibition halos around the plant fragment. Two kinds of halos were distinguished: a clear halo, where growth of the indicator bacteria was completely inhibited, and a colored halo, presumably due to pigments from plants that do not completely inhibit growth of the indicator bacteria.

Antimicrobial activity of each plant was summarized by an index resulting from the addition of standardized values (i.e., mean = 0 and SD = 1) of three different variables describing (i) halo transparency: 0 (no halo), 1 (not clear halo), 2 (clear halo); (ii) size of halo: 0 (no halo), 1 (1-2 mm), 2 (3-4 mm), 3 (5-6 mm) and 4 (>6 mm), and (iii) number of
indicator bacteria to which the tested plant demonstrated antimicrobial activity (from 0 to 11).

Antimicrobial activity assays were performed against 11 bacterial typified strains from the Spanish Type Culture Collection (CECT) and from our own laboratory collection: *Bacillus licheniformis* D13, *Enterococcus faecalis* MRR10, *Listeria innocua* CECT340, *Listeria monocytogenes* CECT4032, *Staphylococcus aureus* CECT240, *Enterococcus faecalis* F-58, *Enterococcus faecalis* JH2, *Enterococcus faecalis* S47, *Enterococcus faecium* 115, *Lactobacillus paracasei* 11-2 and *Lactococcus lactis*. However, it should be noted that none of the tested plant species showed antimicrobial activity against the last six indicator bacteria.

**Sample size and statistical analyses**

We used information on feathers and plants in 48 nests at the two different times of interest: once eggs were found in the nest for the first time (hereafter laying stage), and in the previous visit three days before (hereafter pre-laying stage). Total and experimental number of pigmented and unpigmented feathers, as well as mass of plants found in starling nests, were log10 transformed to approach Gaussian distributions. Thus, transformed variables and/or the residuals of the statistical models did follow normal distributions.

Due to variability in laying dates, experimental feathers and plants were only available for 27 out of the 48 nests sampled during the pre-laying stage, but for all of them during the laying stage. Thus, to explore differences in number and color composition of feathers in nests due to the presence of *ad libitum* experimental feathers, we performed two different models. First, we explored the effects of availability of feathers (nests with vs without experimental feathers available, fixed factor) and feather pigmentation on number...
of feathers detected in nests during the pre-laying stage (dependent variable). We used a
Repeated Measures ANOVA (RM-ANOVA) with numbers of feathers of each color
detected in the same nest as within effects, and availability of experimental feathers as
between factor. Furthermore, to explore the effects of reproductive stage (i.e., pre-laying vs
laying) and feather composition on total number of feathers in starling nests, we only used
nests that had experimental feathers available both before and during egg-laying
reproductive stages. These effects were explored in a RM-ANOVA with two within factors,
feather color and reproductive stage. The effects of laying date on repeated measures (i.e.,
interactions) were estimated in separate models. In addition, to analyze feather color
preference by starlings, we used the number of times that meshes of different color were
replenished as dependent variable, and feather color as fixed factor in a GLM.
The effect of plant availability on the amount of green plants found in starling nests
was explored in GLM models with availability of experimental plants as the categorical
factor, and the percentage of vegetation coverage as a covariable. The effect of nest stage
on the amount of green plants in starling nests was explored using nests with available
experimental green material during the pre-laying and laying stages. For this, we used a
RM-ANOVA with nest stage as repeated measures variable. The effect of laying date on
the repeated measures variable (i.e., interactions) was estimated in a separate RM-ANOVA.
In addition, we further explored the preference of starlings for any of the experimental
plants offered in a Generalized Linear Model (GLZ) with a binomial distribution and logit-
link function while controlling for overdispersion. We used the presence/absence
information of experimental plants of each species in starling nests (during the pre-laying
and laying stages) as dependent variable, and plant species and nesting stage as categorical
independent factors. Plant preferences were also assessed by analyzing number of plants of
each species that were collected by starlings from the containers. We used the log10 number of stems collected by starlings as dependent variable, and species and container identity as factors in a GLM.

FDR (false discovery rate) procedure was used to adjust P values for multiple comparisons by *p.adjust* function of stats package in R 3.4.1 (http://www.r-project.org/).

All other statistical analyses were performed with Statistica 8.0 (Statsoft Inc. 2011). Values reported are means ± 95% CI.

**Results**

**Feather color preferences**

During the pre-laying stage, unpigmented feathers were significantly more abundant in starling nests than pigmented feathers ($F_{1,46} = 6.73$, $P = 0.029$). Moreover, neither number of feathers ($F_{1,46} = 0.41$, $P = 0.603$), nor feather color composition ($F_{1,46} = 0.04$, $P = 0.604$; Fig 1A) depended on availability of experimental feathers before egg laying started.

Nest stage (laying vs pre-laying) affected total number of feathers in nests. Feathers were more abundant in starling nests at the time of laying (mean (95% CI) = 9.37 (6.27 – 12.47)) than before egg-laying began (mean (95% CI) = 4.89 (2.50 – 7.28)) ($F_{1,26} = 21.84$, $P < 0.001$), with laying date explaining a non-significant proportion of variance ($F_{1,25} = 0.57$, $P = 0.399$). In addition, number of feathers in starling nests depended on feather color. Unpigmented feathers (mean (95% CI) = 9.93 ± (6.69 – 13.16)) were significantly more abundant than pigmented feathers (mean (95% CI) = 4.33 (1.19 – 7.18)) ($F_{1,26} = 18.00$, $P < 0.001$), and this difference tended to be more pronounced during the laying stage ($F_{1,26} = 4.73$, $P = 0.071$, Fig 1B). Laying date affected differences in feather color composition since unpigmented feathers were relatively more abundant than pigmented feathers as the
season progressed \( F_{1,25} = 4.47, P = 0.071 \), but did not affect the detected effects of the interaction between feather pigmentation and reproductive stage \( F_{1,25} = 0.12, P = 0.737 \).

Similar results were achieved when only experimental feathers found in nests were considered. Unpigmented experimental feathers were significantly more abundant (mean (95% CI) = 4.81 (2.94 – 6.69)) than experimental pigmented feathers (mean (95% CI) = 0.02 (-0.01 – 0.38)) \( F_{1,25} = 48.12, P < 0.001 \). These differences were more pronounced during the laying stage (Pre-laying: unpigmented feathers, mean (95% CI) = 1.37 (0.14 – 2.6); pigmented feathers, mean (95% CI) = 0.04 (-0.04 – 0.11); Laying: unpigmented feathers, mean (95% CI) = 3.44 (2.12 – 4.77); pigmented feathers, mean (95% CI) = 0.15 (-0.03 – 0.33)) \( F_{1,25} = 17.99, P < 0.001 \).

Finally, meshes with unpigmented feathers needed to be replenished more frequently than those with pigmented feathers (unpigmented feathers: range: 0-4, mean ± SE = 1.6 ± 0.5; pigmented feathers: range: 0-1, mean ± SE = 0.2 ± 0.1; \( F_{1,18} = 7.35, P = 0.014 \); Fig 1C). Thus, independently of the used variable, all results invariably suggest that starlings preferentially chose unpigmented feathers, mainly during the laying stage.

**Green plant preferences**

In the pre-laying stage, mass of plants in nests with and without plants offered did not differ significantly (with experimental plants available: mean (95% CI) = 0.90 g (0.35 – 1.46); without experimental plants available: mean (95% 5CI) = 0.44 g (0.15 – 1.74); \( F_{1,48} = 0.81, P = 0.557 \). Moreover, nest stage did not affect mass of plants in starling nests (Pre-laying: mean (%95CI) = 0.90 g (0.35 – 1.46); Laying: (95% CI) = 0.67 g (0.67 – 1.16)) \( (F_{1,26} = 0.32, P = 0.579 \), but laying date tended to explain a significant proportion of variance \( F_{1,25} = 6.60, P = 0.051 \). Mass of plants found in nests during the laying stage increased as the
season progressed, while the opposite trend was detected for the pre-laying stage (Fig 2A).

Percentage of vegetation coverage around nests did not significantly affect mass of plants found in nests (mean (95% CI) = 78.93% (71.73 – 86.13); $F_{1,22} = 2.77$, $P = 0.220$).

We analyzed the starling preferences for experimental plants during the two nest stages separately. In the pre-laying stage, prevalence of different experimental plant species in starling nests did not differ ($\chi^2 = 3.13$, $P = 0.579$; Fig 2B). However, starlings showed some preferences during the laying stage ($\chi^2 = 14.79$, $P = 0.030$; Fig 2B). *L. amplexicaule* and *M. vulgare* were the experimental plants most frequently found in the nests, while *A. barrelieri* and *H. vulgare* appeared with the lowest prevalence (paired comparisons: *L. amplexicaule* – *H. vulgare*: $\chi^2 = 6.00$, $P = 0.014$; *M. vulgare* – *H. vulgare*: $\chi^2 = 4.35$, $P = 0.037$; Fig 2B). Similarly, the number of aromatic plant stems collected by starlings from the containers were larger than that of non-aromatic plants ($F_{4,435} = 4.73$, $P = 0.001$; Fig 2C; post-hoc LSD: *L. amplexicaule* – *H. vulgare*: $P = 0.0001$; *M. vulgare* – *H. vulgare*: $P = 0.0002$; *A. clavatus* – *H. vulgare*: $P = 0.004$; *A. barrelieri* – *H. vulgare*: $P = 0.006$; Fig 2C).

Thus, it is during the laying stage when preference for aromatic plants comes out.

Finally, antimicrobial activity assays of green plants offered to starlings showed that *A. barrelieri* was the plant with the highest antimicrobial activity, followed by *A. clavatus*, and *L. amplexicaule*. The lowest antimicrobial activity was shown by *M. vulgare* and *H. vulgare* (Table 1).

Discussion

The experimental results showed that starlings preferred unpigmented over pigmented feathers and aromatic plants over non-aromatic plants for building their nests. Moreover, these preferences were more clearly detected during the laying stage, when the hypothetical
protective function of these nest materials for developing offspring would be more important.

**Feather preferences**

Feathers were more abundant in nests of starlings during the laying stage than during the pre-laying stage. This is in accordance with the common yet often overlooked assumption that nest building in birds does not end with the onset of laying, but continue during posterior stages, likely to accommodate successive selection pressures (Hansell 2000).

Independently of the nesting stage, unpigmented feathers were more abundant than pigmented feathers in starling nests. The main studied function of nest feathers concerns their thermoregulatory properties (Møller 1984; Windsor et al. 2013), which for instance would help to reduce clutch and brood cooling rates (Hilton et al. 2004). It is known that pigmentation partially determines thermal insulation properties of structures in nature (Hochscheid et al. 2002; Hetem et al. 2009; for a similar argument in avian eggs see Lahti and Ardia 2016). However, previous studies dealing with insulation properties of feathers in avian nests have not considered the potential effect of feather pigmentation (Hilton et al. 2004; Dawson et al. 2011; Windsor et al. 2013). This possibility is out of scope of the present study. However, any hypothetical effect of feather pigmentation on nest thermoregulation will mainly apply to open nests since external radiation reaching nest contents inside holes is limited (Kilner 1999; Hunt et al. 2003; Avilés et al. 2008) and, thus, would hardly apply to starling nests.

A second functional explanation for the preference of unpigmented feathers is related to scenarios of sexual selection (Veiga and Polo 2005), with feathers in nests acting as a courtship display affecting mate choice, and/or as a post-mating sexual signal eliciting
differential reproductive investment in mates (Sanz and García-Navas 2011; but see Veiga and Polo (2011)). Spotless starling females carry feathers to the nest in response to green plants carried by males (Polo and Veiga 2006). We also found that feathers were more abundant during the laying stage than during the pre-laying stage. This would be in accordance with a role for feathers as sexual signals used by females to elicit differential male investment because this period is closer to the early nestling stage, when contribution to parental care by males is much needed (Soler et al. 2008). Moreover, amount of feathers in starling nests is related to female experience (Polo and Veiga 2006), and the experimental addition of feathers resulted in reduced nestling mortality (Veiga and Polo 2011). Thus, it is likely that feathers induced differential paternal investment in reproduction (Veiga and Polo 2011).

Even if feather color preference is driven by sexual selection, it may also function in additional scenarios. In this sense, trying to figure out possible scenarios explaining the detected positive effects of experimental feathers reducing nestling mortality, Veiga and Polo (2011) discussed the role of feathers as a possible antiparasitic material that would reduce nestling mortality. Thus, it is even possible that the reason underlying the preference for unpigmented feathers is their higher antimicrobial properties that have been demonstrated in laboratory (Peralta-Sánchez et al. 2014) and in the field in nests of barn swallows (Peralta-Sánchez et al. 2010). An alternative explanation for color composition of nest-lining feathers is that it could mirror the availability or detectability of feathers of different color in the nest surroundings. However, this possibility would not explain feather color composition of nests in our study because pigmented and unpigmented feathers were available *ad libitum* and easily locatable.
Even knowing the starling preference for unpigmented feathers, and the *ad libitum* availability of our experimental approach, very few nests (7 out of 48 nests) harbored only unpigmented feathers, which suggest that starlings tried to incorporate feathers to the nest following a certain combination of colors that they prefer. Interestingly, the detected combination of feather colors changed, with a relative increase of unpigmented feathers as the season progressed. Previous studies found that bacterial colonies from pigmented feathers also have antibacterial activity (Peralta-Sánchez et al. 2014) and that the abundance of pigmented feathers has a positive effect on phenotypic quality of spotless starling nestlings (Ruiz-Castellano et al. unpublished). Moreover, antimicrobial activity of bacterial colonies isolated from unpigmented feathers in nests of barn swallows was higher in nests where experimental pigmented feathers were added (Peralta-Sánchez et al. 2014). Thus, it is possible that certain combination of pigmented and unpigmented feathers in nests of birds maximizes feather-derived antimicrobial activity. Moreover, environmental conditions at the end of the breeding season favor bacterial growth in avian nests (Soler et al. 2015; Møller et al. 2015). Therefore, it is possible that starlings tried to compensate such increase in probability of bacterial infection by carrying to the nest feathers with higher antimicrobial activity as the season progressed (Peralta-Sánchez et al. 2014). Further research on antimicrobial properties of bacteria isolated from unpigmented and pigmented feathers in nests of birds with different combinations of feather color is necessary to explore this possibility.

**Green plant preferences**

Starlings also showed a preference for specific green plants to build their nests. Availability of experimental plants did not affect plant abundance in their nests, but starlings that breed
later used more plants than early breeders did. This positive association between laying date and amount of green plants in the nest has also been found for European starlings (Clark and Mason 1985; Gwinner and Berger 2005; Dubiec et al. 2013). It could be argued that this is just the outcome of a higher accumulation of plants in nests that commenced egg laying later. However, this explanation is unlikely for several reasons. First, because it is possible that starlings, as some other bird species do, remove part of the old dried plants after some days in the nest (Petit et al. 2002), and second, because for a given starling nest, the amount of plants found during the laying stage was smaller than during the pre-laying stage. In addition, it is known that female starlings remove from the nest the green plants carried by males (Veiga and Polo 2012), which reflect male quality (Veiga et al. 2006). This female behavior has been explained as a mean to difficult the assessment of male and nest quality by neighboring rival females (Veiga and Polo 2012), which usually prospect other nests to gather public information (Parejo et al. 2008). Removing green plants would therefore prevent nest usurpation and reduce nest attractiveness for conspecific brood parasites in European and spotless starlings (Sandell and Diemer 1999; Veiga and Polo 2012). Thus, it is possible that the relatively smaller amount of plants detected in starling nests during the laying stage was the consequence of this female behavior. Another explanation could be that males do not incorporate green material to the nest during the laying stage, as has been claimed for European starlings (Gwinner 1997), and for some other populations of spotless starlings (Veiga et al. 2006). However, this explanation is unlikely since spotless starling males in our population continue carrying green material to the nests during the laying and incubation stages (Ruiz-Castellano et al. 2016).

In agreement with a possible antimicrobial function of plants, we also found that starlings did not show a marked preference for any of the offered plant species to be carried...
to their nests during the pre-laying stage, but during the egg-laying, males preferentially selected aromatic plants. It is possible that, before egg laying males attract females to their nests by carrying fresh green material with less emphasis on antimicrobial and antiparasitic properties of plants (Hansell 2000). However, once reproduction has started, selecting aromatic plants would have the additional advantage of protecting the nests against parasites (Clark and Mason 1985) and microorganisms (Mennerat et al. 2009a). We know that European starling males discriminate plant volatiles (Clark and Mason 1997) and that this ability is maximized during the courtship and nest building stage (Clark and Smeraski 1990), when they select plant materials based on volatile compounds. Thus, it is possible that spotless starling males also select plants with antimicrobial properties relying on their volatile compounds. Most of the aromatic plants that we provided to starlings have strong antimicrobial activity (see Table 1). The most preferred plant species appeared to be L. amplexicaule, and not A. barrelieri that showed the highest antimicrobial activity. We estimated antimicrobial activity of these plants by testing inhibitory potential of growth of 11 bacterial strains that include possible avian pathogens. Thus, although antimicrobial activity against different bacterial strains usually covaries (Table 1; see also Al-Bakri and Afifi 2007; Khalil et al. 2009), we cannot rule out the possibility that the most preferred plants were active against some particular bacterial pathogens more abundant in the study area. Additional analyses, ideally with bacteria isolated from nests, would be needed to determine antimicrobial activity of nest plants against bacteria present in starling nests. Independently of the reasons determining preferences for specific aromatic plants, the detected higher amount of green plants during the pre-laying stage, as well as the preferences for aromatic plants during the laying stage, suggest a primary role of this
material in sexual signaling and nest protection against pathogens, which may be more prominent during the egg laying stage (see Rubalcaba et al. 2016).

Conclusion

Irrespective of differential functionality of the diverse nest materials, and of the possible optimization of nest material composition, we have shown that starlings make a selection of nest building materials in a possible adaptive way. We have shown this preference regarding two common nest materials profusely employed by many bird species from different orders and in different habitats to build their nests, i.e., green plants and feathers (Hansell 2000; Dubiec et al. 2013). Furthermore, these two materials play pivotal roles in the study of nest building behaviors, and may aid a better comprehension of self-medication tool use by animals (Hansell and Ruxton 2008; Healy et al. 2008; De Roode et al. 2013). Thus, our study help to better understand the adaptive functionality of nest building processes and their involved behaviors.

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Data Accessibility
Analyses reported in this article can be reproduced using the data provided by Ruiz-Castellano C, Tomás G, Ruiz-Rodríguez M, and Soler JJ. (2017) Data from: Nest material preferences by spotless starlings. Behavioral Ecology. In Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.q6ng9

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Veiga JP, Polo V, Viñuela J. 2006. Nest green plants as a male status signal and courtship display in the spotless starling. Ethology. 112:196-204
Table 1: Antimicrobial activity of the different green plants offered to spotless starlings for nest building. We show the scores for halo transparency, halo size, number of bacteria for which tested plants demonstrated antimicrobial activity, and the index scores for each tested plant.

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<tr>
<th>Plant</th>
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<th>Halo size</th>
<th>Number of inhibited bacteria</th>
<th>Index score</th>
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<td>4</td>
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</table>
Fig 1: (A) Feather color composition (± CI 95%) in spotless starling nests during the pre-laying stage, with or without experimental feathers available. (B) Number of pigmented and unpigmented feathers (± CI 95%) in spotless starling nests during the pre-laying and the egg laying stages. (C) Mean number of times (± CI 95%) that meshes containing the experimental pigmented and unpigmented feathers offered to spotless starlings needed to be replenished.

Fig 2: (A) Relationships between laying date and log10 of plant mass (g) of spotless starling nests recorded both at the pre-laying and at the egg laying stages. Regression lines are shown. (B) Prevalence of different plant species (aromatic: *L. amplexicaule*, *M. vulgare*, *A. clavatus*, *A. barrelieri*; non-aromatic: *H. vulgare*) in spotless starling nests at the pre-laying and egg laying stages. (C) Number of experimental plant fragments (± CI 95%) of the different species collected from containers by spotless starlings during the three weeks that plants were available.
Fig 1:

(A) Number of feathers

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(B) Nest stage

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(C) Number of times replenished

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