Lichens in the nests of European starling Sturnus vulgaris serve a mate attraction rather than insecticidal function

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Abstract: The European starling Sturnus vulgaris is a hole-nesting bird in which the male builds a voluminous nest using a wide variety of materials such as twigs, grass, leaves, feathers, and lichens. The function of lichens in starling nests has not been assessed until now and we hypothesize that this material is related to a mate attraction function or is used to protect nestlings from parasites due to the presence of secondary compounds with insecticidal effects with the lichens. We aimed to identify the lichen species and frequency of lichen use in European starling nests, and to determine if the presence of this material is correlated with mate attraction or with an insecticidal function. We found lichens in 45% of nests, mainly represented by Ramalina celastri. The lichens were added to the nests mainly before the beginning and at the end of nest building and egg-laying started earlier in nests with lichens than those without lichens. No association was found between the presence of lichen and the intensity of Philornis larvae (Diptera), a parasite that infests starling nestlings. Our results suggest that the addition of lichens could be related to mate attraction and a stimulus for the beginning of egg-laying but did not have an antiparasitic function in European starling nests.

Key words: Egg-laying onset, insecticide, mate attraction, nesting material, Philornis, Ramalina, reproduction

1. Introduction
Nest building is a key process in the life of birds (Hansell, 2000). Materials used to build nests have multiple functions that may have important consequences on a bird's fitness (Mainwaring et al., 2014). Some materials have a clear structural function such as twigs, bark, and mud in many bird species (Hansell, 2000). However, the presence of other nest materials is more intriguing and has attracted the attention of researchers (Hansell, 2000; Mainwaring et al., 2014). For instance, some passerines and raptores use feathers and a nonrandom selection of green plants, most of them rich in secondary compounds (Dubiec et al., 2013). This means that birds must be actively selecting them as nesting materials for some reason. Three main nonexclusive hypotheses have been proposed to explain the inclusion of green plant fragments in bird nests (Tomás et al., 2013). One of them has been related to the bond formation or mate status signaling (courtship hypothesis), when birds add green material into the nest to attract a mate or when this material acts as a signal of their condition or paternal quality (Fauth et al., 1991; Gwinner, 1997; Brouwer and Komdeur, 2004; Veiga et al., 2006). A second hypothesis for the use of green material states that their secondary compounds act as olfactory repellents or toxins (Bennet and Wallsgrove, 1994) and benefit nestlings by decreasing nest parasites and pathogens (nest protection hypothesis) (Gwinner and Berger, 2005; Mennerat et al., 2009a; Tomás et al., 2012; Scott-Baumann and Morgan, 2015). A third hypothesis states that the volatile secondary compounds present in green plants positively influence the development and health of nestlings through the stimulation of their immune system (drug hypothesis) (Gwinner et al., 2000; Gwinner and Berger, 2005; Mennerat et al., 2009b).

The European starling Sturnus vulgaris (hereafter referred to as starling) is native to Europe, Asia, and North Africa (Feare, 1984). In Argentina, it was introduced by the bird traffic and the first record in the wild dates from 1987 in Buenos Aires city (Pérez, 1988). Since then, it has expanded 500 km through Buenos Aires province (LI,
unpublished data) and new population establishments have been recorded in provinces from the north and the center of Argentina (Peris et al., 2005; Zufiaurre et al., 2016). In 1998 it was declared a harmful species by the Secretariat of Environment of the Nation. It is a hole-nesting bird that nests in tree cavities, closed nests of other bird species, and artificial constructions such as nest boxes, holes in light poles, and roofs of houses (Rebolo Ifrán and Fiorini, 2010; Ibañez et al., 2015). Inside the cavity, male starlings build voluminous nests using mainly twigs and grass (Kessel, 1957; Feare, 1984). Additionally, males incorporate green leaves, lichens, flowers, feathers, and artificial objects (Feare, 1984; Soulliere and Rusch, 1996; Gwinner, 1997). Kessel (1957) and Feare (1984) reported that these materials are added to the nest by male starlings at the beginning of the breeding season, before the start of egg-laying, and suggested that these materials have a role during mate attraction. After that, many authors that studied the use of green material in European and also spotless starling (Sturnus unicolor), a closely related species with similar nest building behaviors, found support for the courtship hypothesis (Fauth et al., 1991; Gwinner, 1997; Brouwer and Komdeur, 2004; Veiga et al., 2006). On the other hand, male European starlings are known to choose plants whose volatiles are more likely to inhibit arthropod hatching (Clark and Mason, 1985), thus supporting the nest protection hypothesis for the use of greenery (reviewed by Scott-Baumann and Morgan, 2015). In turn, Gwinner et al. (2000) added aromatic plants to a group of starling nests and observed that nestlings from this group had better development than nestlings in a group of nests without aromatic plants. These differences in development were not related to differences in the number of parasites between groups. Therefore, the authors proposed the ‘drug hypothesis’ that states that the volatile compounds of green material have a direct positive effect on nestlings through some mechanism that could be related to a stimulation of the immune system. Other green materials that starlings add to their nests are lichens (Gwinner, 1997), which are known to possess secondary compounds, such as usnic acid. These compounds are produced by the fungus and secreted to the hyphal surface (Tay et al., 2004), and many of them have insecticidal properties (Dayan and Romagni, 2001). This type of compound was found in Brazil, Chile, and South Africa, in lichen species of the genus Ramalina, which are also present at our study site in Argentina (Kashiwadani and Kalb, 1993). Previous observations in starling nests in our study area showed the use of lichens as part of the greenery (LI, personal observation). Here, we studied two of the main hypotheses about the use of green materials in bird nests, the courtship hypothesis and the nest protection hypothesis, focusing on the use of lichens, one component of the green material whose function in starling nests has not been analyzed until now. Our results are expected to be similar to those found in North America and Europe for the use of green plants as a mate attractor, but the function related to an insecticidal effect may differ due to the presence of compounds with different properties. Therefore, the aims of our study were to: 1) taxonomically identify the lichen species and the frequency with which they were incorporated into starlings nests; 2) analyze the presence of secondary compounds in the lichen species most used by starlings in their nests; 3) determine if the presence of lichens correlates with the beginning of egg-laying (as an indicator of mate attraction); and 4) determine if the presence of those lichens correlates with the parasitism by Philornis sp. (Diptera) larvae on starling nestlings.

2. Materials and methods

2.1. Study area and field work

The study was conducted in the Pereyra Iraola Provincial Park and Forest Reserve, Berazategui, Buenos Aires Province, Argentina (34°50’S, 58°06’W). Fifty nest boxes were located in this area and were checked every 1–3 days from September to December 2013. When newly added lichens were present, a piece was taken for species identification and analysis of secondary compounds. For each nest, the beginning of egg-laying was calculated in terms of Julian days as the number of days between the date of the first egg-laying in that nest and the date of the first egg-laying during the breeding season (7 October). Twenty nests were randomly selected previous to the hatching date to be searched for Philornis larvae, which parasitize starlings in the study area (Ibañez et al., 2015). Philornis larvae are subcutaneous parasites of nestlings that remain under the nestlings’ skin breathing through a hole in the skin until they reach their maximum size. At this time the larvae leave the nestlings to pupate in the nest material (Segura and Reboreda, 2011). Fifty-seven nestlings were born from 17 of the 20 nests selected to be searched for larvae. We carefully examined the body of each of those 57 nestlings for Philornis sp. during each visit. Philornis intensity was determined as the maximum number of larvae that each nestling had.

2.2. Identification of lichen species and presence of secondary compounds

Lichens were identified to the species level on the basis of macro- and microscopic characters of the thallus, spot tests with potassium hydroxide (K) and sodium hypochlorite (C), and reaction under UV light (Wirth, 1995), following Kashiwadani and Kalb (1993).

The presence of secondary compounds was analyzed in Ramalina celastri using thin-layer chromatography (TLC), following Culberson (1972) to determine if the lichens added to the nests had compounds with insecticide properties.
2.3. Statistical analysis

To determine whether the beginning of egg-laying was correlated with the presence of lichens, we applied a generalized linear model (GLM) with negative binomial error distribution and log as link function, using Julian day as the response variable and lichen (presence/absence) as a predictor variable. Only first clutches were considered for this analysis (n = 37) because the start of second clutches is affected by the date of fledging of the first clutches. Due to the fact that the onset of egg-laying could show a clumped spatial distribution, we analyzed if the relationship between the presence of lichens in the nest and the onset of laying resulted from a random spatial coincidence. For this purpose, we drew a map of the studied population depicting both the nests where lichens were present and the 25% of nests where egg-laying started first (Figure 1).

To analyze whether nestling parasitism by *Philornis* correlated with the presence of lichens, we applied a generalized linear mixed model (GLMM) with binomial distribution and a logit link function, using parasitized individual (yes/no) as a response variable. The presence of lichens (presence/absence), the Julian day of the beginning of egg-laying (since the intensity of *Philornis* larvae may vary depending on the time of the breeding season; Ibañez et al., 2015), and the interaction between both variables were included as predictor variables. In addition, we analyzed the effect of lichen, Julian day, and the interaction between both variables on the intensity of *Philornis* larvae per nestling, using a GLMM with negative binomial error distribution and log as link function. Nest was included as a random factor for both GLMMs to take into account the lack of independence among nestlings of the same brood. We used hypothesis testing with the backward elimination method to evaluate the statistical significance of the predictor variables (Crawley, 2007). The analyses were implemented with the packages glmm.admb and MuMin available in R software v. 2.10.1 (R Development Core Team, 2009).

3. Results

Starlings occupied all 50 nest boxes, where 66 clutches were recorded, 37 in a first brood and 29 in a second one. Nests were built primarily from twigs, grass, leaves, and feathers. We found lichens in nests from 30 pairs (45%), 22 first broods and eight second broods. The most frequently used lichen species was *R. celastri*, which was recorded in the nests of 29 pairs (44%) (Figure 2). *R. aspera*, *Xanthoria parietina*, and *Teloschistes chrysophthalmus* were found in one nest each (2%). In first clutches, lichens were added once in 17 nests and twice in five nests, totaling 27 events of addition in 22 nests. Lichens were mainly added before the beginning of nest building, and after the completion of nest building, but before egg-laying (Figure 3).

The presence of secondary metabolites in the specimens of *R. celastri* could not be confirmed by TLC because concentrations were below the lower detection threshold of the method. However, we observed an orange fluorescence at the tips of the laciniae when thalli were exposed to UV light (Huneck and Yoshimura, 1996). The

![Figure 1](image1.jpg)

**Figure 1.** Spatial location of the nests in the study area. Full circles: nests with lichens, empty triangles: nests without lichens, empty circles: the 25% of nests without lichens that started the egg-laying earlier, full triangles: the 25% of nests with lichens that started the egg-laying earlier.

![Figure 2](image2.jpg)

**Figure 2.** Nest box with the lichen *Ramalina celastri* that was used by some of the starlings at the beginning of nest building.
observation of this fluorescence suggests the presence of low levels of a secondary metabolite. Usnic acid is the only metabolite cited for the cortex of this species (Krog and Swinscow, 1976); thus, we assume that this reaction is due to its presence.

We found that egg-laying started earlier in nests with lichens than in nests without lichens (Table). As the distribution of the nests that started their egg-laying first did not show a clumped distribution, we discarded the possibility of a random spatial coincidence as the explanation for the presence of lichens in nests and the onset of egg-laying results. Nestling parasitism by Philornis and intensity of parasitism per nestling were not affected by the presence of lichens in the nest or by the onset of egg-laying (represented by the Julian day) (Table).

4. Discussion
Our observations are consistent with the courtship hypothesis. The incorporation of lichens into the nest boxes prior to nest building suggests that male starlings added lichen to attract females. Additionally, we observed that egg-laying occurred earlier in nests with lichens than without lichens, reinforcing the courtship hypothesis. The addition of lichens at the end of nest building could influence the female investment in the eggs, as was seen in spotless starling in response to an experimental addition of green plants (Polo et al., 2004). Nevertheless, we did not evaluate the female investment on eggs and this idea remains for future research. It is important to clarify that lichens are not seasonal so they can be found at similar abundances at the same site throughout the year (Nash, 2008); thus, the presence of lichens in starling nests is probably not related to the environmental availability of this material. On the other hand, we did not find a relationship between the presence of lichens in nests and the presence of Philornis larvae, suggesting that starlings do not add lichens in the nest as an insect repellent.

Table. Summary of generalized linear model evaluating the effect of the presence of lichens on Julian day and generalized linear mixed models evaluating the effect of the presence of lichens and Julian day (number of days between the date of the first egg-laying in that nest and the date of the first egg-laying during the breeding season) on Philornis parasitism and intensity. Statistically significant values are shown in bold.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Predictor variable</th>
<th>Estimate</th>
<th>SE</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Julian day (n = 37)</td>
<td>Intercept</td>
<td>1.47</td>
<td>0.23</td>
<td>6.27</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Lichens (presence)</td>
<td>−0.62</td>
<td>0.27</td>
<td>−2.33</td>
<td>0.02</td>
</tr>
<tr>
<td>Nestling parasitism (yes/no) (n = 57)</td>
<td>Intercept</td>
<td>0.50</td>
<td>0.72</td>
<td>0.69</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Lichens (presence)</td>
<td>−0.21</td>
<td>1.19</td>
<td>−0.17</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Julian day</td>
<td>0.06</td>
<td>0.06</td>
<td>1.12</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Lichens (presence) × Julian day</td>
<td>0.13</td>
<td>0.20</td>
<td>0.67</td>
<td>0.50</td>
</tr>
<tr>
<td>Intensity of parasitism per nestling (n = 39)</td>
<td>Intercept</td>
<td>1.46</td>
<td>0.36</td>
<td>4.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Lichens (presence)</td>
<td>0.23</td>
<td>0.40</td>
<td>0.56</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Julian day</td>
<td>0.02</td>
<td>0.03</td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>Lichens (presence) × Julian day</td>
<td>−0.07</td>
<td>0.06</td>
<td>−1.16</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Starling nestlings are also parasitized by mites (Lareschi et al., 2017) and larvae of Coleoptera (LI, unpublished data), but we did not analyze the effect of lichens on these groups of ectoparasites. Therefore, it is not possible to discard the possibility that they play a role repelling ectoparasites, but if this were the case, it would be expected that starlings continue adding this material when nestlings are present in the nest.

The use of lichens for mate attraction is consistent with the findings of other authors that studied the use of green material in starling nests (Fauth et al., 1991; Gwinner, 1997; Brouwer and Komdeur, 2004; Veiga et al., 2006). In addition, some authors found intriguing results when studying the function of greenery in starling nests. For instance, the spotless starling adjusted its offspring sex ratio and clutch size in response to experimental enhancement of green nest material (Polo et al., 2004; López-Rull and Gil, 2009). These results suggest that the use of green material by starlings plays a role in sexual selection (Mainwaring et al., 2014). In another cavity-nesting bird, the blue tit (Cyanistes caeruleus), the addition of aromatic plants was associated with higher male risk-taking during the stage of provisioning nestlings (Tomás et al., 2013). Since male risk-taking was also positively related to female fitness, it was suggested that, similar to findings in starlings, the use of aromatic materials acts as a sexually selected trait in this species.

When considering the nest protection hypothesis, Clark and Mason (1985, 1988) found an association between the amount of green material and the abundance of ectoparasites in starling nests. The presence of green material was also associated with a reduction of bacteria (Clark and Mason, 1985; Gwinner and Berger, 2005). Nevertheless, other authors did not report such relationships (Fauth et al., 1991; Gwinner et al., 2000; Brouwer and Komdeur, 2004). The nest protection hypothesis was also studied in blue tits, with one study showing a partial effect of green material on nidicolous parasites (Tomás et al., 2012) and another showing a negative effect on bacteria (Mennerat et al., 2009a). In turn, Mennerat et al. (2008) did not find any effect of aromatic plants on blowfly abundance (nest protection hypothesis reviewed by Scott-Baumann and Morgan, 2015). These results suggest that the use of green material in bird nests as a repellent of ectoparasites and bacteria remains controversial and needs further research.

The drug hypothesis was also studied in European starlings and in blue tit nests, where the presence of aromatic plants was related to higher nestling mass (Gwinner et al., 2000; Gwinner and Berger, 2005), feather growth (Mennerat et al., 2009b), blood hemoglobin (Clark and Mason, 1988), and hematocrit (Gwinner et al., 2000; Gwinner and Berger, 2005; Mennerat et al., 2009b). We found no support for the nest protection hypothesis, although the usnic acid present in lichens has been previously found to have insecticidal properties against different development stages of Diptera, including larvae of Aedes aegypti (Vinayaka et al., 2009), Culex pipiens (Cetin et al., 2008), and adults of Drosophila melanogaster (Uysal et al., 2009). However, we only detected potential evidence of low concentrations of usnic acid in R. celastri samples; thus, this could be not enough to repel Philornis larvae. In any case, there was a lack of correlation between lichen presence and Philornis larvae in the nests. On the other hand, the concentration of secondary compounds in lichens depends on environmental factors such as light, temperature, and humidity (Cocchietto et al., 2002). Consequently, the studied lichen species at other sites under different environmental conditions may contain higher levels of secondary compounds capable of having a negative impact on ectoparasites.

Several species of lichens such as Ramalina peruviana, Teloschistes chrysophthalmus, Xanthoria parietina, Parmotrema conferendum, Teloschistes flavicans, Parmotrema conferendum, Punctelia constantimontium, and Usnea strigosa have been recorded in the Pereyra Izola Park and surrounding areas during the starling breeding season (Osorio, 1979; Calvelo and Liberatore, 2002, LI, personal observation). Nevertheless, the most frequently used lichen by starlings was R. celastri. This could be due to its high availability in the study area or due to a starling preference for this lichen species. Further studies are needed to elucidate this issue.

In conclusion, our results would not add support to the idea that lichens play an insecticidal function in starling nests. Moreover, our observations support the idea that lichens play a role in mate attraction in starlings, as has been shown in other observational and experimental studies of the function of green materials in nests.

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