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Mamma knows best: why a generalist hummingbird selects the less abundant moss for nest building

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Many birds around the world use plant material to build nests that allow for hatching eggs and keeps chicks safe (Healy et al. 2015). Despite being a common phenomenon in nature, our knowledge of the mechanisms underlying how and why specific plants are selected for nest building is somewhat limited. Hummingbirds (Trochilidae) in particular depend heavily on plant materials to build their nests (Calvelo et al. 2006, Torres-Dowdall et al. 2007). Hummingbirds commonly use mosses as nesting material because mosses can retain moisture for long periods and thereby prevent eggs from drying out (Breil and Moyle 1976, Blem and Blem 1994). Also, mosses may have antimicrobial properties that may enhance nestling survival (Basile et al. 1999, Alabrundzinska et al. 2003).

Recently, we documented that the Green-backed Firecrown (*Sephanoides sephaniodes* Trochilidae), the southernmost hummingbird and main vertebrate pollinator of austral South America, actively selects particular materials during nest building (Osorio-Zuñiga et al. 2014). This hummingbird uses two kinds of plant materials to build their nests: scales or hairs of the fronds of the fern *Lophosoria quadripinnata* to make the nest lining, and mosses to make the nest structure (Fig. 1). Among those mosses, three species are the most common: *Ancistrodes genuflexa* (present in 100% of the nests), *Weymouthia mollis* (present in 27% of the nests), and *Weymouthia cochlearifolia* (present in 17% of the nests), whereas the remaining species are present occasionally, in low proportions, and usually in nests >1 yr old (Osorio-Zuñiga et al. 2014). An intriguing pattern emerges when we examine those nests in detail: *A. genuflexa* represents up to 97% of moss biomass in the nests, but it is particularly scarce in the environment (constituting only 0.1% of the total moss biomass in the forest). Contrarily, *W. mollis* and *W. cochlearifolia* constitute only 3% of moss biomass in the nests despite being the most abundant species by far in the environment (constituting 94% of the total moss biomass in the forest). Therefore, an obvious question arises: why does *S. sephaniodes* actively select of one of the least abundant moss species for nest building and not the most abundant mosses? Active selection of material for nest building has been associated with the presence of secondary compounds in plants that acts as biocides of pathogens and parasites (Clark and Mason 1985, 1988, Pires et al. 2012) and these generally represent a small, non-random proportion of plant species available in the environment (Clark and Mason 1985, 1988, Pires et al. 2012, López-Rull and Macías-Garcia 2015). To elucidate a potential functional role of those three bryophytes as anti-pathogenic agents in nests, we assessed its chemical composition and antimicrobial activity.

We performed chemical analyses on those three moss species (details are available in Appendix S1), finding 65 compounds in total (43 of them extracted with methanol and 22 extracted with dichloromethane; Appendix S1: Fig. S1). From those, 14 compounds were found in *A. genuflexa*, 42 compounds in *W. cochlearifolia*, and 20 compounds in *W. mollis* (Appendix S1: Table S1). Of the compounds present, 64% were polar compounds in *A. genuflexa*, 45% in *W. cochlearifolia*, and 65% in *W. mollis.* The two *Weymouthia* species shared eight compounds, whereas *A. genuflexa* shared only one compound with *W. cochlearifolia* and two compounds with *W. mollis.* Regarding the functions of those components (Appendix S1: Table S2), we found eight compounds in *A. genuflexa* with known functions (Asawaka et al. 2013). Five of those compounds have antibacterial properties (benzothiophene, undecyl acetate, farnesol, oleic acid, and (E,Z)-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol), one has antifungal properties (tetradecenol), and one is known to repel insects (8-methyl-6-nonenamide). It is noteworthy the presence of phthalic acid, known to be toxic for mammals, which might be preventing nest predation by small mammals, and particularly by the arboreal marsupial *Dromiciops gliroides* that feed on eggs of many native birds (Fontúrbel et al. 2012). Except for 8-methyl-6-nonenamide (an insect repellent), none of

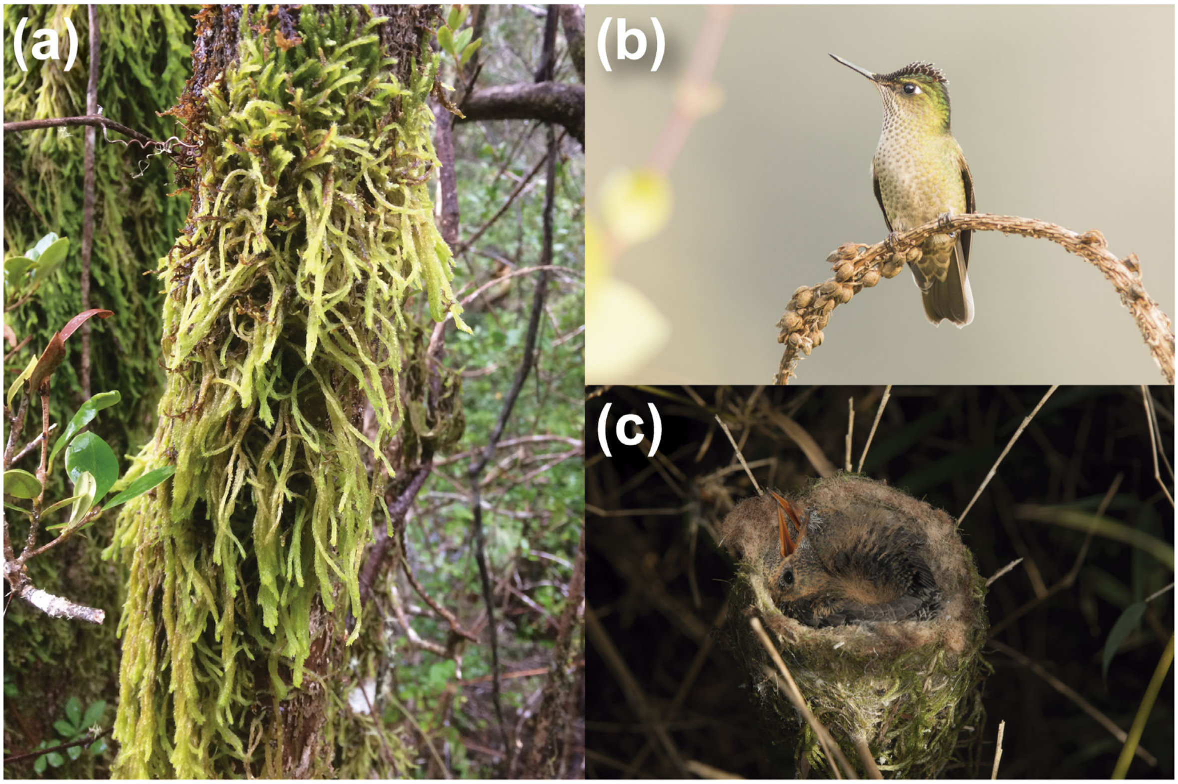

Fig. 1. (a) The moss *Ancistrodes genuflexa* growing attached to a native tree (Oncol Park, October 2017; photo credit: Felipe Osorio), (b) the hummingbird *Sephanoides sephaniodes* (Viña del Mar botanical garden, June 2018; photo credit: Diego Reyes), and (c) *S. sephaniodes* nest with chicks inside (Senda Darwin scientific station, Chiloé Island, June 2018; photo credit: Andrés Charrier).

these compounds are present in any of the *Weymouthia* species.

Weymouthia cochlearifolia had 21 compounds with known properties, 18 of them with antimicrobial properties, one with antiviral properties (only one of them is also present in *A. genuflexa*), one known to be insect repellent and one known to be irritant to humans. In turn, *W. mollis* had nine compounds with known properties, seven with antimicrobial properties and two with antifungal properties (none of them present in *A. genuflexa*). While we performed these analyses, we had spare moss material stored at 4°C in the laboratory; both *Weymouthia* samples rot (attacked by fungi) after six months while *A. genuflexa* samples remained intact for over a year.

Then, we used the moss extracts, obtained from the three species using methanol and dichloromethane, to assess antimicrobial activity in laboratory conditions, against five common pathogenic bacteria. We performed the same procedure used only the solvents as controls. Those bacteria strains included some Enterobacteriaceae (*Escherichia coli* and *Salmonella enterica*) and other common bacteria species (*Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Staphylococcus aureus*). Our trials show that *A. genuflexa* has significant antimicrobial activity over the five bacteria strains tested (Fig. 2). The extracts of *A. genuflexa* were particularly effective against *E. coli* as it presents the widest inhibition halo, and its growth is inhibited with the lowest extract concentration (Appendix S1: Tables S3 and S4). On the other hand, *W. mollis* and *W. cochlearifolia* showed no antimicrobial activity in any case (Fig. 2, Appendix S1: Tables S3 and S4). These results show that, despite the presence of some compounds with a known antimicrobial activity present in both *Weymouthia* species, they are little effective against these common pathogens. In contrast, those compounds present in *A. genuflexa* are highly effective instead. Interestingly, there are a few reports of diseased hummingbirds that have been associated with bacteria of the family Enterobacteriaceae (Godoy et al. 2014), suggesting that they could be infected by these type of pathogens.

Due to its wide distribution range along southern South America and its generalist behavior as a pollinator, responsible for pollinating ~20% of the native woody

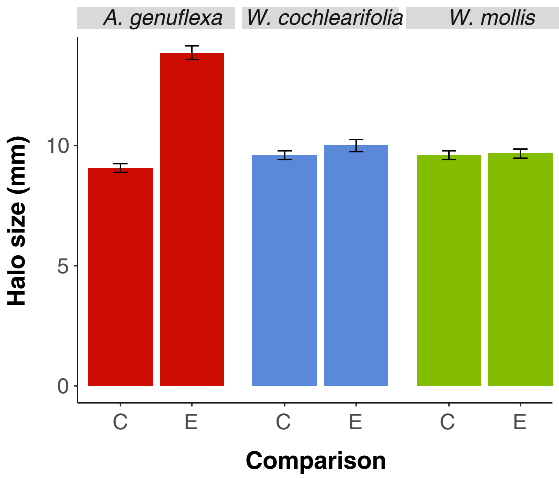

FIG. 2. Summary of the antimicrobial activity of moss extracts (from *Ancistrodes genuflexa*, *Weymouthia mollis*, and *Weymouthia cochlearifolia*) over five common bacteria strands. We compared halo size between control (C) and extract (E) essays in the laboratory. Error bars represent standard error.

flora of the temperate Patagonian rainforests, we first expected *S. sephaniodes* to have a generalist-opportunistic behavior related to the use of nest-building material. Later, we realized that material selection was far from random, showing clear preferences towards a moss species that is particularly scarce (Osorio-Zuñiga et al. 2014), but the reasons for such preference were unknown. Our chemical and microbial analyses provide a potential explanation for that: using *A. genuflexa* instead of the abundant *Weymouthia* species confers more durability to the nest (which is highly relevant considering that *S. sephaniodes* may reuse nests for multiple seasons). Therefore, *A. genuflexa* presence in the nest might benefit the hummingbirds increasing their egg and chick survival by reducing the nest pathogens. Even when the three moss species are structurally similar to the naked (human) eye, they differ greatly in chemical composition. As *A. genuflexa* contains mainly polar compounds with antimicrobial properties, they are soluble in water. They can be active in the nesting material at wet conditions, predominant in the temperate rainforests of South America. Such a particular association between *S. sephaniodes* and *A. genuflexa* could be the result of the natural selection process, in which selective hummingbirds actively searching for this moss have more and healthier offspring than those that use nesting materials according to their availability in the environment. The large cognitive abilities of *S. sephaniodes* and hummingbirds in general (Gonzalez-Gomez et al. 2015) could be responsible for developing such fine nesting material selection behavior.

We tend to label species as generalists or specialists, assuming that they should act as is in every aspect of their lives. However, our results showed that a generalist pollinator such as *S. sephaniodes* can otherwise be very selective and act as a specialist in other aspects of their life history. Among those aspects, nest-building behavior plays an important role, but remains little understood. Based on our results, future studies may explore specialization patterns in nest building in other hummingbird species, or even more generally in birds. We expect that careful nesting material selection will be favored by natural selection, as it may substantially increase offspring survival. Also, we expect these selection patterns to show significant geographic variations, as a result of changes in the nesting materials available.

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