Señales químicas, bacterias y ectoparásitos en aves

Mónica Mazorra Alonso

Tesis doctoral

Granada 2023

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CSIC
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TESIS DOCTORAL

2023

Editor: Universidad de Granada. Tesis Doctorales
Autor: Mónica Mazorra Alonso
ISBN: 978-84-1195-109-8
URI: https://hdl.handle.net/10481/85767

Este trabajo ha sido posible gracias a la concesión de una “Ayuda para contratos predoctorales para la formación de profesorado universitario 2015” del Ministerio de Universidades (FPU15/03420), y a la financiación del Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación/y el Fondo Europeo de Desarrollo Regional/10.13039/501100011033 con los proyectos (CGL2017-83103-P, PID2020-117429GB-C21) y por el “Fondo Europeo de Desarrollo Regional, una manera de hacer Europa”. Esta tesis ha sido desarrollada en el Departamento de Ecología Funcional y Evolutiva de la Estación Experimental de Zonas Áridas del CSIC.

Portada

Dibujo: Pilar Antón Martínez

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Memoria presentada por Mónica Mazorra Alonso para optar al Título de Doctor o Doctora en Biología por la Universidad de Granada, dentro del Programa de Doctorado en Biología Fundamental y de Sistemas.

Esta tesis ha sido dirigida por Juan José Soler Cruz, profesor de investigación de la Estación Experimental de Zonas Áridas (EEZA-CSIC), y Juan Manuel Peralta Sánchez, personal investigador del Departamento de Zoología de la Universidad de Sevilla.

En Granada a 5 de septiembre de 2023

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En Granada a 5 de septiembre 2023

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Doctoranda

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AGRADECIMIENTOS:

Por fin ha llegado el momento de sentarme a pensar en estos años. Me ha costado mucho llegar hasta aquí, pero reconozco que ha sido una de las experiencias que más me ha hecho crecer y conocerme.

Mis primeros agradecimientos son para mis dos directores, Juan Soler y Juan Manuel Peralta. Habéis sido la balanza perfecta para guiarme en este camino. Lo que más os agradezco es que durante esta carrera de fondo, he tenido baches que me han hecho dudar mucho si conseguiría acabar, pero de vuestras bocas seguía escuchando que podía hacerlo, que solo debía focalizar todas mis fuerzas en esto, nunca vi ni una pizca de desconfianza en vosotros, así que gracias. Juan, dejas caminar a las personas para que aprendan, pero siempre estás ahí ayudando y reconduciendo para que saquemos cosas en claro. Juanma, gracias por esa humildad tuya y esas palabras que siempre empatizan y animan, además de todas las horas enseñándome a analizar "microbios". Habéis sido dos grandes pilares en esta historia.

Siguiendo mis pensamientos ha llegado a mi recuerdo la sonrisa de Carmen, mi tutora de tesis. Quiero agradecerte precisamente esa sonrisa que me hacía sentir como en casa cuando me quedaba en Granada.

A ti, Benjamín, te dedico unas palabras, porque tú fuiste esa persona que marco mi vida profesional para siempre y me mostró lo fascinantes que eran las bacterias. Nos dejaste un 8 de abril sin mucho aviso, pero nunca has dejado de estar presente. Sigo hablando contigo y oigo fuerte esas palabras que un día me dijiste: "Hagas lo que hagas en la vida, ya seas investigadora, profesora, dependienta en una tienda, lo más importante es que te sientas feliz y realizada, que de eso trata toda esta historia". En su momento no lo entendí, pero creo que ya comienzo a verlo claro ;)

Tampoco hubiera sido este camino igual sin vosotros amigos. Quiero agradecerte Javi todos los ratos que hemos compartido, porque han sido muchos y muy diversos y para mi eres uno de los mayores tesoros que me he encontrado en la vida. Mi querida Laurich, como no acordarme de ti en estos momentos, cuantos ratos increíbles descubriendo mil cosas contigo. Y a ti Rober el norteño que termino en el sur, por esa humildad y esa pasión por las cosas sencillas y bonitas. Ana, me quedo con esas tardes de charla en esa terraza, esos ánimos y esos abrazos tuyos. Y mis dos chicas del norte, Indira y Sandrix, me alegráis los días cuando os veo. Gracias a todos por estar siempre.

También aquellos amigos que ya no están tan cerca pero que siguen vivos en la memoria. David, has hecho que en parte sea quien soy, gracias por apoyarme incluso para decirnos adiós y poder caminar este camino. Sergio, gracias por esas tardes de lluvia ayudándome con el inglés y esas rutas maravillosas para desconectar de todo. Rubén como no agradecerte esos meses de mi parón con la tesis por el accidente del pie, contigo aprendí que podía volver a sonreír y que podría con todo.

No me quiero olvidar tampoco de toda la gente del chumbo y Granada. Creo que escribiría unas cuantas anécdotas bonitas y divertidas con muchos de vosotros. Andrés gracias por siempre echarme una mano con los papelotes y tus dibujos por navidad. Todas las chicas del laboratorio (Pilar, Mª José, Olga) gracias por los momentos de limpieza de nidos con esa alegría. Valera por esa sonrisa y estar siempre dispuesto a ayudar. José Antonio, que te voy a contar, a parte, de la ayuda y enseñanzas de bacterias, tus palabras de ánimos. Héctor, gracias por esa insistencia tuya en irme abriendo camino y por esos cursos del mar tan maravillosos. Miguel y el laboratorio en Granada, gracias por tu ayuda aun cuando estabas hasta arriba de trabajo. Mi compi Ester con la que me hubiera gustado compartir más tiempo en persona y no tanto telemático, gracias por tu ayuda con todo y tu buen ánimo. Pilu y Portugal! Mónica, Juan, Iñaki, Manu, Silvia, Ángel, Cristina, Jordi, Eva, Natalia, Merche, Miguel, Jesús, Almudena, Gustavo, Rafa, Leiva, Sebas, Alberto, Mercedes también quiero agradeceros los buenos ratitos.

Y como no agradecer en mi día a día y con toda mi fuerza a mi familia lo que me han apoyado con esta tesis. A mi madre porque fuera lo que fuese a pasar, es el amor incondicional. A mi padre por su esfuerzo en sacar todo lo positivo y grandioso de esta experiencia cuando me ha visto derrumbarme. Aún recuerdo algún muestreo de abubillas con ellos. A mi hermana que nunca supo muy bien de que iba mi tesis ;) pero que ante todo siempre me ha querido ver feliz. Y quiero dedicarle unas palabras a mi tío Carlos, biólogo de la familia que me metió esa inquietud por conocer el mundo natural, tú me enseñaste por primera vez lo que era un liquen y el concepto de “simbiosis” que aparece tanto en esta tesis.

Y por último a ti Nacho, estos últimos meses gracias por la paciencia porque he sido la noria de las emociones. Y sobre todo gracias por no dudar de mí y repetírmelo sin descanso. Me siento muy afortunada por habernos encontrado en estos últimos años, porque sé que esto de la tesis es cosa mía y para mi futuro, pero lo he luchado mucho más y mejor desde que te conocí.

A mi familia y para ti.

“Si me lleva cien años, será una pena,

pero no dejaré de trabajar mientras viva”

Marie Curie

ÍNDICE

RESUMEN GENERAL

El estudio de las relaciones ecológicas entre las bacterias (i.e. microbiota) y los eucariotas es fundamental para entender la evolución de los animales. Estos microorganismos establecen relaciones en ocasiones muy complejas con los animales, siendo fundamental su papel en procesos patogénicos, simbióticos o mutualistas, así como en la fisiología, el desarrollo, la morfología, e incluso el comportamiento animal. Entre las interacciones y procesos ecológicos en los que pueden estar involucrados los microorganismos, una línea de investigación emergente en biología evolutiva es entender la importancia de la microbiota en los sistemas de comunicación química de sus hospedadores. El metabolismo de las bacterias simbiontes genera productos químicos, algunos de ellos volátiles, que influyen en el perfil de olor de los individuos. Además, la microbiota está íntimamente ligada tanto con el fenotipo como con la actividad fisiológica del individuo y, por tanto, los volátiles de origen bacteriano formarán parte de las características de las señales y pistas emitidas por los animales que quedan a disposición de conespecíficos y heteroespecíficos.

La importancia de los microorganismos en la comunicación química animal fue descrita por primera vez en la hipótesis de la fermentación en la década de 1970. Esta hipótesis originalmente se propuso para explicar los olores de las secreciones de las glándulas de mamíferos, sugiriendo que la variación en la comunidad bacteriana del hospedador genera variaciones en el perfil de olor del individuo que funcionarían como pistas de reconocimiento entre conespecíficos. En la actualidad, esta hipótesis se aplica a cualquier perfil químico (olor) que pueda ser consecuencia del metabolismo bacteriano asociado a un hospedador y que, por tanto, puede operar en escenarios de comunicación química. Se han descrito evidencias del papel bacteriano en la contribución de los olores del hospedador en un amplio rango taxonómico, que incluye no solo a mamíferos, sino también a insectos, anfibios y aves. Por lo tanto, estos olores son información pública que pueden ser usados tanto por conespecíficos como por heteroespecíficos. Especialmente interesante son aquellas pistas que pueden ser usadas por receptores no deseados como depredadores y/o parásitos, ya que estas interacciones conllevan costos para los hospedadores. No solo las bacterias que están íntimamente asociadas al hospedador (microbiota) pueden generar olores, sino también aquellas bacterias asociadas al ambiente donde los animales viven y/o se reproducen; en el caso de las aves, el nido. Las bacterias de los nidos de las aves metabolizan compuestos orgánicos que se acumulan entre los

materiales durante la actividad reproductiva de las aves y, por lo tanto, se han sugerido como responsables de los volátiles asociados con el ambiente de los nidos (es decir, los olores de los nidos). Dado que los ectoparásitos utilizan principalmente el olfato para detectar a sus huéspedes, las bacterias simbiontes asociadas a los nidos de las aves y los volátiles de su metabolismo deberían influir en la probabilidad de que los parásitos detecten dichos nidos y, por tanto, en las presiones de selección del parasitismo que sufren los pollos.

En esta tesis, exploramos esta hipótesis, lo que implica la existencia de dos tipos de asociaciones: (A) que los volátiles del ambiente del nido y las bacterias de los materiales de los nidos, así como los volátiles y las bacterias de las secreciones de las aves deben estar relacionadas entre sí; y (B) que tanto los volátiles como las bacterias deben asociarse con la probabilidad y/o la intensidad de ectoparasitismo que sufren los individuos. Además, en esta tesis prestamos especial interés en el conocimiento de taxones bacterianos y volátiles específicos que se relacionen entre sí, y que puedan tener efectos en la probabilidad de ectoparasitismo sufrido por las aves y/o que afecte a su éxito reproductor. Estas relaciones estudiamos en primer lugar mediante una revisión bibliográfica y experimentalmente a nivel intraespecífico en abubillas y en un estudio interespecífico con varias especies de ave.

Las hembras y pollos de abubilla (*Upupa epops*) albergan una compleja comunidad bacteriana responsable de la emisión de compuestos químicos volátiles producidos por la secreción de su única glándula exocrina, el uropigio. Las abubillas no construyen nidos, prefiriendo reutilizar agujeros o cajas nido que contienen restos de materiales reproducciones anteriores de conespecíficos o de otras especies de aves. El ambiente bacteriano de los materiales del nido de reproducciones previas afecta a la microbiota de la glándula uropigial de las hembras de abubilla. Para testar esta hipótesis, manipulamos experimentalmente el entorno bacteriano del nido de abubillas. El experimento consistió en la instalación de cajas nido nuevas a las que pusimos material de cajas nido antiguas en las que el año anterior se habían reproducido de forma exitosa abubillas, y que previamente autoclavamos (nidos experimentales) o no (nidos controles). En cajas controles y experimentales que las abubillas utilizaron para reproducirse, analizamos la microbiota del material del nido y de las secreciones de la glándula de hembras y pollos, así como el perfil de volátiles del nido y de la secreción. Además, exploramos estas relaciones en dos momentos de la estancia de los pollos en el nido: al

principio, cuando los pollos no tienen desarrollada la glándula y la hembra aún pasa tiempo en el nido; y en el último tercio de dicha estancia, cuando todos los pollos tienen plenamente desarrollada la misma. Estimamos la intensidad de parasitismo tanto de un ectoparásito hematófago (*Carnus hemapterus*) en los pollos, como de piojos de plumas (suborden Mallophaga) en las hembras. *C. hemapterus* es una mosca generalista que busca de forma activa nidos en plena reproducción y, muy probablemente, los volátiles de los nidos les sirvan como pistas para encontrar a sus huéspedes. Por otra parte, los piojos masticadores solo los encontramos en las hembras y no en los pollos. Estos parásitos se alimentan masticando áreas blandas de las plumas y la piel, y succionando sangre cuando provocan un sangrado. En el caso de las abubillas estos ectoparásitos se colocan en las plumas de la cresta donde están más protegidos del acicalamiento que realizan las aves.

Los resultados mostraron que el material de nido recogido durante la reproducción de abubillas en cajas experimentales presentaba menos densidad y diversidad bacteriana, y mayor diversidad de volátiles que el de los nidos controles. Además, la intensidad de parasitismo (i.e., número de picaduras) detectado en los pollos que se desarrollaban en cajas experimentales fue menor que el encontrando en pollos nacidos en cajas controles. De acuerdo con la primera parte de la hipótesis, encontramos que las comunidades microbianas de secreciones uropigiales y de materiales de los nidos covariaron con sus perfiles volátiles, mientras que el perfil volátil de las secreciones explicó el perfil volátil del nido. Apoyando la segunda parte de la hipótesis, encontramos una asociación positiva entre la densidad bacteriana del material del nido durante la fase de pollos y la intensidad del ectoparasitismo que solo fue puesto de manifiesto en las cajas nido experimentales con material autoclavado. Por último, algunos de los volátiles y bacterias detectados en el material y el ambiente del nido, así como en las secreciones se asociaron con la intensidad del ectoparasitismo de las hembras y los pollos, y con el éxito de vuelo. Estos resultados sugieren un vínculo entre la comunidad de microorganismos de los restos de material del nido y la intensidad del ectoparasitismo. Además, respaldan la existencia de una relación estrecha entre las comunidades microbianas y los olores de los animales y del nido. En su conjunto sugieren que, en abubillas, las asociaciones entre las bacterias y tanto el ectoparasitismo como el éxito reproductivo están mediadas por volátiles de origen bacteriano.

También probamos nuestra hipótesis explorando la variabilidad interespecífica en ambientes microbianos y perfiles de volátiles de nidos de diez especies de aves, recogiendo información de bacterias, volátiles y ectoparasitismo al principio y al final del período de estancia de los pollos en el nido. De acuerdo con la hipótesis, (i) la diversidad alfa de las comunidades bacterianas (la diversidad de la comunidad) se asoció con el perfil de volátiles del entorno del nido, aunque esta asociación dependió del índice de diversidad utilizado y del momento de muestreo de los pollos. Además, (ii) la diversidad beta (las diferencias de diversidad entre muestras) basada en las distancias PhILR explicó el perfil de volátiles, pero sólo en la fase tardía de los polluelos. Por otra parte, (iii) la diversidad alfa de los volátiles se asoció con la intensidad de parasitismo en la fase inicial de los pollos, mientras que la diversidad alfa de la comunidad bacteriana y la diversidad beta de los volátiles se relacionaron con la intensidad del parasitismo de los pollos al final de su periodo de estancia en el nido. (iv) Sólo la diversidad alfa de la microbiota del nido al principio del periodo de nidificación explicó el éxito de vuelo (porcentaje de pollos que sobreviven desde el primer al segundo muestreo). Por último, (v) la abundancia de algunas bacterias y volátiles clave aparecieron fuertemente relacionadas entre sí y, algunos de esos elementos resultaron asociados con la intensidad del parasitismo y, en menor medida, con el éxito del vuelo. En conjunto, estos resultados también respaldan los vínculos esperados entre el ambiente microbiano y los olores de los nidos en diferentes especies de aves, y entre ellos y la intensidad del ectoparasitismo y el éxito de vuelo.

Aunque los resultados variaron dependiendo de los índices de diversidad utilizados para la caracterización de los perfiles de volátiles y de las comunidades de bacterias, así como del momento del muestreo (al principio o al final del periodo de estancia de los pollos en el nido), de la especie de estudio y de los elementos (bacterias o volátiles) considerados, los resultados presentados en están tesis apoyan la hipótesis de trabajo. Por tanto, concluimos que las comunidades de bacterias asociadas a los nidos de las aves son parcialmente responsables de los perfiles de volátiles de los mismos y del riesgo de parasitismo y éxito de vuelo de los pollos que crecen en ellos.

GENERAL ABSTRACT

The study of ecological relationships between bacteria (i.e. microbiota) and eukaryotes is essential to understand the evolution of animals. These microorganisms usually establish complex relationships with animals, paying a key role in pathogenic, symbiotic or mutualistic processes, as well as in animal physiology, development, morphology, and even behaviour. Among the ecological processes in which microorganisms are involved, an emerging line of research is directed to understand the importance of the microbiota in the chemical signalling of their hosts. The metabolism of symbiotic bacteria generates chemical volatiles, which should influence the odour profile of individuals. Furthermore, since the microbiota is closely linked to phenotypic traits and physiological activity of their hosts, volatiles of bacterial origin will be part of the chemical signals and cues of animals that inform con- or hetero-specifics of individual and environmental characteristics.

The importance of microorganisms in animal chemical communication was first described by the fermentation hypothesis in the 1970s. This hypothesis was originally proposed to explain the odours of mammalian gland secretions, suggesting that variation in the host bacterial community generates variations in the individual odour profile that would function as recognition cues between conspecifics. Currently, the hypothesis applies to any chemical profile (odour) that operate in scenarios of social communications and that may be a consequence of the metabolism of animals' bacterial symbionts. Evidence for a bacterial role in the contribution of host odours has been described in a wide taxonomic range, including mammals, insects, amphibians and birds. Therefore, these odours are public information that can be used by both conspecifics and heterospecifics including unwanted receptors such as predators and/or parasites. In addition, to bacteria that are closely associated with the host (microbiota), metabolism of those inhabiting the environment where the animals live and/or reproduce would also produce cues for parasites or predators looking for victims. Bacteria growing in bird nests can metabolize organic compounds from bird reproductive activity that accumulate within the nest environment and, thus, those bacteria have been suggested to be partially responsible of the nest-environment volatiles (i.e. nest odours). Since ectoparasites primarily use olfaction to detect their hosts. Consequently, the symbiont bacteria associated with bird nests and the volatiles from their metabolism should influence the

probability parasites detecting these nests and, therefore, the strength of selection pressures of parasitism suffered by nesting birds.

In this thesis, we have explored this hypothesis, which implies the existence of two types of associations: (A) that volatiles of the nest environment and bacteria from nests material, as well as volatiles and bacterial of the secretions of avian must be related to each other; and (B) that both volatiles and bacteria should be associated with the probability and/or intensity of ectoparasitism suffered by nesting individuals. Furthermore, this thesis we pay special attention to detecting particular bacteria and volatiles that were related to each other as possible predictors of probability and intensity of ectoparasitism suffered by nesting birds and/or of fledgling success. I first review literature on these matters and, then, explore experimentally and empirically some predictions of the hypothesis both at the intraspecific level (mainly in hoopoes (*Upupa epops*) and at the interspecific levels.

In hoopoes, the uropygial gland of nesting females and nestlings harbour complex bacterial communities responsible of the emission of some volatile-chemical compounds. Hoopoes do not build nests and prefer to reuse holes or nest boxes that contain remains and materials from previous reproduction. Moreover, the bacterial community of the nest materials from previous reproductions affects the microbiota of the uropygial gland of female hoopoes. To test the hypothetical role of bacteria determining nest odours and parasitism, we manipulated the bacterial environment of the hoopoe nest. The experiment consisted of the installation of new nest boxes in which we included materials from old hoopoe nests. In half of the new nest boxes the added mater were (experimental nests) or were not (control nests) we previously autoclaved. Latter, in control and experimental boxes that hoopoes used to reproduce, we sampled and analysed the microbiota of the nest material and of gland secretions of females and nestlings, as well as the volatile profile of the nest and secretion. Furthermore, we explored these relationships at the beginning, when the nestlings have not developed the uropygial gland and the female still spends most of the time within the nest; and the end of the nestling period, when all the nestlings have fully developed the gland. Moreover, at these nestling stages, we estimated the intensity of parasitism of a hematophagous ectoparasite (*Carnus hemapterus*) in nestlings and of chewing lice (suborder Mallophaga) in females (only at the beginning of the nestling period). *C. hemapterus* is a generalist hematophagous fly that actively searches for avian nests (Calero-Torralbo et al., 2013; Martín-Vivaldi et al., 2006), and

that likely eavesdrop on the volatiles from active nests to find and select their hosts. Chewing lice feed on soft areas of feathers and skin, and when they cause bleeding they also suck blood. In the case of hoopoes, these ectoparasites are only detected in females and are placed in the feathers of their crest where they are more protected from grooming.

Nest material collected during hoopoe reproduction in experimental nest-boxes presented bacterial communities of lower density diversity, and volatile profiles that more divers than that of the control nests. Furthermore, the intensity of parasitism (i.e., number of bites) detected in nestlings that developed in experimental nest-boxes was lower than that found in nestlings hatched in control nest-boxes. Consistent with the first part of the hypothesis, we found that the microbial communities of the uropygial secretions and of nest materials covaried with their volatile profiles, while the volatile profile of the secretions of females and nestlings explained the volatile profile of the nest. Supporting the second part of the hypothesis, we found a positive association between the bacterial density of the nest material during the nestling stage and the intensity of ectoparasitism an effect that was only evident in the experimental nest boxes with autoclaved material. Finally, some of the bacteria and volatiles respectively detected in the nest material and in the environment, as well as those detected in secretions, were associated with the intensity of ectoparasitism of females and nestlings, and with fledging success. These results suggest a link between the community of microorganisms in the nest materials and the intensity of ectoparasitism suffered by nesting birds. Furthermore, they support the existence of a close relationship between microbial communities and odours of animals and nests. Taken together, those results suggest that, in hoopoes, the associations between bacteria and both ectoparasitism and reproductive success are mediated by volatiles of bacterial origin.

We also tested our hypothesis by exploring interspecific variability in microbial environments and volatile profiles of nests of ten bird species. we collected information on bacteria, volatiles and ectoparasitism in nests, at the beginning and at the end of the nestlings stay. In accordance with the hypothesis, microbial alpha diversity (the diversity of the community) associated with alpha diversity of volatiles in interaction with bird species identity, but the results varied depending on the index and the sample (age of nestlings) used in the analyses. In addition, (ii) beta diversity (the differences of diversity between samples) of bacterial community based in PhILR distances explained the beta diversity of volatile profile but only at the late stage of nestlings. Moreover, (iii) alpha

diversity of volatiles associated with intensity of parasitism at the early stage of nestling period, while alpha diversity of bacterial community and beta diversity of volatiles were related to intensity of parasitism at the end of the nestling period. (iv) Only alpha diversity of the microbiota of the nest material at the begging of the nestling period explained the fledging success. Finally, some key bacteria and volatiles that were related to each other also associated with intensity of parasitism and, at lower rate, with fledging success. Taking together, those results support the expected links between microbial environment and nest odours in different bird species, and between them and ectoparasitism intensity and fledging success. Future research should prioritize experimental approaches directed to determine the role of particular bacteria and volatiles in the outcomes of host-ectoparasite interactions.

Results presented in this thesis in general support the working hypothesis. Statistical support however varied depending on the diversity indices used to characterize the volatile profiles and bacterial communities, the time of sampling (at the beginning or at the end of the nestlings stay in the nest), the species under study and the used elements (bacteria or volatiles). Therefore, we conclude that the bacterial communities associated with bird nests are partially responsible for their volatile profiles and the risk of parasitism and fledging success of the nestlings.

INTRODUCCIÓN

1. Relaciones entre bacterias y animales: mediando información social

Las bacterias son microorganismos ubicuos que interaccionan con los animales a nivel fisiológico y comportamental y que son esenciales para entender su evolución (McFall-Ngai et al., 2013). Las interacciones que se establecen entre animales y bacterias van desde el mutualismo hasta el parasitismo (Steinert et al., 2000) y, aunque tradicionalmente se han estudiado por sus efectos negativos, actualmente son muchos los estudios que se focalizan en entender y poner de manifiesto relaciones mutualistas entre bacterias y animales. La microbiota tiene un papel importante en la defensa frente a patógenos; indirectamente mediante la estimulación y entrenamiento del sistema inmune, o, directamente, mediante la síntesis de compuestos defensivos frente a micro- y macro-organismos patógenos (Flórez et al., 2015). La comunidad bacteriana más conocida por sus efectos beneficiosos es la microbiota intestinal, facilitando la absorción de nutrientes y sintetizando ciertos micronutrientes esenciales como vitaminas (Clemente et al., 2012; Douglas, 2009; LeBlanc et al., 2013; Rosenbaum et al., 2015). Más recientemente se ha demostrado que no solo tiene funciones nutritivas para sus hospedadores, sino que también tienen un papel importante en la modulación del comportamiento y en el funcionamiento del sistema nervioso central de los animales (Sherwin et al., 2019). Actualmente se acepta que existe una comunicación directa entre las bacterias del intestino y el cerebro a través de la producción de ciertos metabolitos que activan el nervio vago, o que inhiben otros nervios del sistema gastrointestinal, que indirectamente influyen en la señalización de varios mediadores cerebrales. Por ejemplo, se ha demostrado que la microbiota intestinal puede enviar señales al celebro por diferentes vías de comunicación que, en su conjunto se conoce como el eje microbiota intestinal cerebro. Entre esas vías se incluyen la activación del sistema inmune, la producción de metabolitos y péptidos, la activación del nervio vago y la producción de ciertos neurotransmisores y neuromoduladores (Sherwin et al., 2019). Existen evidencias de que, gracias a la existencia de estos canales de comunicación entre bacterias intestinales y cerebro, la microbiota intestinal afectaría al desarrollo neurológico de los animales y a la programación del comportamiento social en el celebro (Sherwin et al., 2019). Por ejemplo, características de la microbiota intestinal influye en las preferencias de pareja en la mosca *Drosophila melanogaster* (Leftwich et al., 2018; Sharon et al., 2010), mientras que otras comunidades bacterianas son responsables de la producción de señales

sociales (i.e., olores) en una diversa gama de taxones animales (Ezenwa & Williams, 2014). Determinar la importancia de la microbiota en la comunicación animal es sin duda un tema emergente en biología evolutiva (Ezenwa & Williams, 2014).

Todos los organismos adquieren información directa de su entorno, pero también lo pueden hacer mediante la evaluación de las interacciones de otros con el entorno, lo que se denomina información social (IS) (Danchin et al., 2004). Esta información social engloba las señales y la conocida como Información Social no Intencionada (ISI). Las señales son caracteres que han evolucionado para transmitir información intencionadamente a los receptores y ese intercambio de información beneficia tanto al emisor como al receptor (Dawkins & Krebs, 1978; Maynard-Smith & Harper, 2003). Como su nombre indica, ISI proporciona a otros pistas de manera no intencionada mientras realizan sus actividades biológicas (Danchin et al., 2004) – un grupo de gacelas comiendo tranquilamente en una llanura informa a otros individuos de que no hay depredadores cerca. Ambos tipos de caracteres, tanto las señales como cualquier carácter de ISI, van a informar sobre las características del emisor de forma honesta. En el caso de las señales, la fiabilidad de la información transmitida por los emisores está basada en la hipótesis del Hándicap, en la que se plantea que sólo aquellos individuos de buena calidad son capaces de señalizar y cubrir los costos de la señal (Maynard-Smith & Harper, 2003; Searcy & Nowicki, 2010; Zahavi & Zahavi, 1997). Sin embargo, la honestidad de los caracteres de ISI viene como resultado de los propios procesos vitales del individuo (p. ej. lugar de forrajeo, metabolismo, eliminación de desechos) (revisado en Danchin et al., 2004).

Según el canal utilizado para adquirir esta información del entorno, los estímulos se han clasificado en visuales, auditivos o químicos. Entre ellos, el uso de compuestos químicos es la forma más antigua, extendida y utilizada por todos los organismos para evaluar su entorno (Wyatt, 2010). Curiosamente una gran cantidad de compuestos químicos responsables de los olores (i.e., volátiles) de los animales son emitidos por comunidades bacterianas simbiontes (Archie & Theis, 2011; Engl & Kaltenpoth, 2018). Por ello, la microbiota podría influir en la comunicación química de sus hospedadores (Ezenwa & Williams, 2014). Además, debido a que la microbiota influye tanto en el desarrollo como en el crecimiento de su hospedador (McDonald et al., 2012), algunos volátiles producidos por la microbiota podrían estar relacionados con, o informar de, la calidad fenotípica y la actividad fisiológica de su huésped (Leclaire et al., 2017; Theis et

al., 2013;Whittaker et al., 2019). De acuerdo con esta posibilidad, se ha demostrado en distintos organismos que la microbiota y el fenotipo del hospedador están relacionadas. Por ejemplo, en *Drosophila*, diferencias en la comunidad bacteriana incrementa la varianza en el tiempo del desarrollo larvario, y en el peso de las pupas y de los adultos. En plantas también se ha comprobado que la microbiota de *Arabidopsis* tiene efectos sobre la biomasa y el tiempo de floración (revisado en Henry et al., 2019). Por lo tanto, debido a que variaciones de la microbiota deben de implicar variaciones en los productos (volátiles) derivados de su metabolismo, la microbiota podría afectar a, o formar parte de, las señales y de los caracteres de ISI, que a su vez pueden usar tanto conespecíficos como heteroespecíficos.

La hipótesis de la fermentación propuesta en la década de 1970 ya sugería un papel preponderante de las bacterias simbiontes en la comunicación química (Albone et al., 1974, 1978). Aunque esta hipótesis se propuso exclusivamente para explicar los olores de las secreciones de los sacos anales de zorros y leones (Albone et al., 1974, 1978), hoy en día se reconoce una influencia mucho más amplia. Como ya dijimos anteriormente, la microbiota de los animales covaría con características fenotípicas del hospedador y, por tanto, los volátiles generados por las bacterias simbiontes podrían formar parte de la comunicación química intraespecífica (i.e., señales) y, por tanto, de la información social utilizada por con- y heteroespecíficos (señales e ISI). Hasta hace relativamente poco, la producción de señales químicas por microorganismos sólo se había descrito en mamíferos e insectos (Ezenwa & Williams, 2014). Sin embargo, en los últimos años se han ido acumulando estudios que suponen una evidencia sólida sobre el papel de las bacterias simbiontes en la producción de volátiles que contribuyen al olor, o perfil de volátiles asociados, de los individuos en varios taxones de animales, como anfibios (Brunetti et al., 2019), aves (Law-Brown, 2001; Martín-Vivaldi et al., 2010; Whittaker et al., 2019), mamíferos (Leclaire et al., 2017; Theis et al., 2013) e insectos (Engl & Kaltenpoth, 2018; Schmidtberg et al., 2019; Sharon et al., 2010). Además, también se ha demostrado que existen volátiles particulares de origen bacteriano que predicen o se asocian con características del huésped, tales como el sexo, la edad, el estatus social, o incluso el grupo social al que pertenecen (Grieves et al., 2021; Leclaire et al., 2017; Theis et al., 2013; Whittaker et al., 2019). Un aspecto interesante, y que esta tesis pretende dilucidar, es la posibilidad de que esos olores emitidos por las bacterias simbiontes puedan ser detectados por receptores no deseados como son parásitos y depredadores (estado del

tema revisado en el **Capítulo I**). Una descripción general de las interacciones potenciales entre los huéspedes y sus simbiontes bacterianos en escenarios de comunicación se muestra en la **Figura 1**.

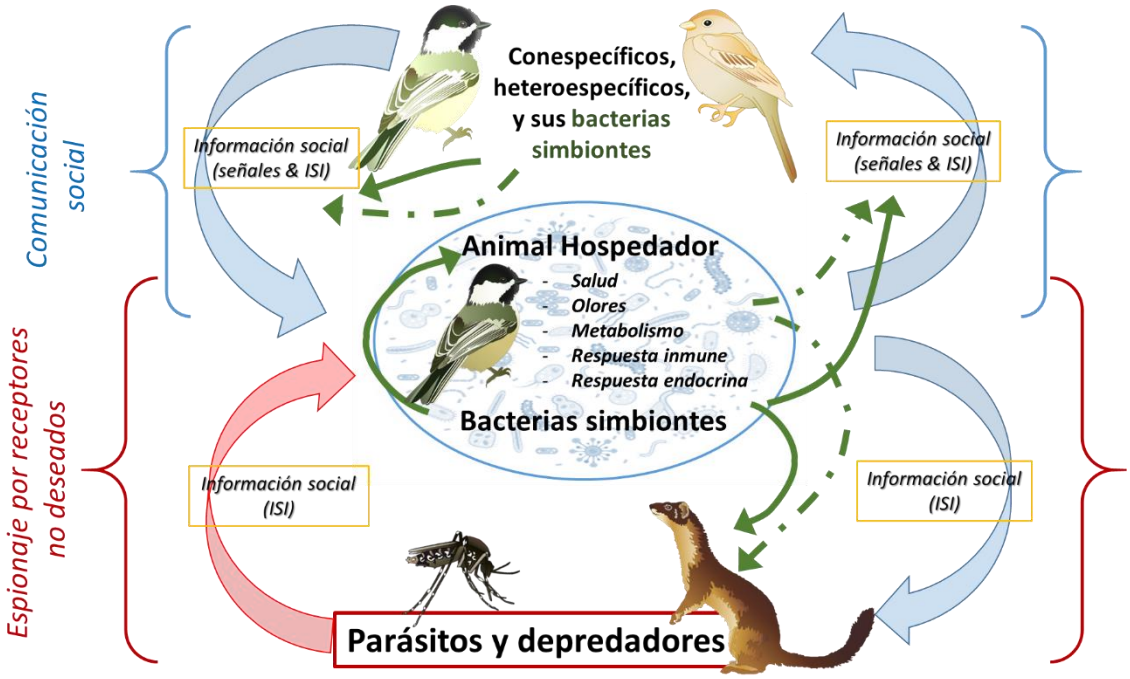

Figura 1. Diagrama en el que se muestra la influencia hipotética de los simbiontes bacterianos (flechas verdes) en escenarios de comunicación social, parasitismo y depredación. Estos efectos podrían deberse directamente a volátiles resultantes del metabolismo bacteriano, o a productos con propiedades antimicrobianas o antidepredatorias (flechas continuas), o, indirectamente, por sus efectos sobre las características del huésped (es decir, salud, aromas, metabolismo, inmunidad y hormonas) (flechas verdes discontinuas). Los simbiontes bacterianos contribuyen a la información social que reciben sus congéneres o heteroespecíficos, incluidos los parásitos y los depredadores. Los efectos negativos de parásitos y depredadores (flecha roja) podrían ser contrarrestados directamente por productos defensivos de origen bacteriano, o indirectamente por rasgos defensivos del huésped que también pueden verse influenciados por bacterias (flechas verdes continuas y discontinuas que conectan al huésped con parásitos y depredadores). Sin embargo, estos efectos negativos se verán potenciados por la utilización de información social involuntaria mediada directa o indirectamente por simbiontes microbianos huéspedes. Por lo tanto, los parásitos y depredadores también influirán en la asociación simbiótica entre animales y microorganismos y afectar indirectamente la comunicación conespecífica. Símbolos cortesía de Integration and Application Network, University of Maryland (ian.umces.edu/symbols/) y freepik.com (consultado el 5 de febrero de 2021).

2. Interacciones parásito-hospedador mediadas por microorganismos: Uso de la información social química

La depredación y el parasitismo se encuentran entre las fuerzas de selección más poderosas que influyen la evolución de los animales en general y de la señalización en particular (Endler, 1986; Futuyma, 2005; Hamilton & Zuk, 1982; Ridley, 1993). Los ejemplos de depredadores y parásitos que detectan señales auditivas o visuales de sus presas u hospedadores se han estudiado ampliamente (Andersson, 1994; Grafen, 1990; Hamilton & Zuk, 1982; Laidre & Johnstone, 2013; Zahavi & Zahavi, 1997; Zuk & Kolluru, 1998). Por ejemplo, los murciélagos que se alimentan de ranas influyen en la evolución de las señales acústicas de las mismas (Akre et al., 2011). La posibilidad de que la microbiota pueda mediar interacciones que los parásitos y depredados tienen con hospedadores y presas respectivamente abre un campo complejo para la evolución de sistemas de comunicación química.

La mayoría de ectoparásitos y algunos depredadores usan el olfato para detectar y seleccionar a sus hospedadores o presas (Bowen, 1991; Poldy, 2020; Reneerkens et al., 2005; Takken & Knols, 1999; Zwiebel & Takken, 2004) y parte de esos olores están producidos por la microbiota (Archie & Theis, 2011; Ezenwa & Williams, 2014; Mazorra-Alonso et al., 2021; McFall-Ngai et al., 2013). Muchos insectos hematófagos tienen un sistema olfativo muy desarrollado, por lo que los volátiles les proporcionan información sobre la ubicación, fenotipo, estado de salud, etc. de sus hospedadores (revisado en Logan & Birkett, 2007). A parte de localizar al huésped, la información sobre particularidades de su fenotipo puede ser importante para el parásito, sobre todo si se trata de caracteres defensivos frente al parasitismo (Milinski, 2006). Existen varios experimentos de laboratorio que revelan una preferencia del mosquito *Anopheles gambiae,* vector de la malaria en humanos, sobre individuos con una gran densidad de bacterias en la piel, y una comunidad poco diversa, y que incluya a *Staphylococcus epidermis* (Verhulst et al., 2011). Por el contrario, los individuos que son poco atractivos para el mosquito poseen una predominancia de bacterias pertenecientes a los géneros *Pseudomonas* y *Variovorax* (Verhulst et al., 2011). Curiosamente cuando Verhulst y colaboradores (Verhulst, et al., 2010) quisieron vincular el perfil de volátiles con seis especies de bacterias de la comunidad de la piel y su influencia en la selección de huésped por parte de *A. gambiae,* descubrieron que este mosquito no se sentía atraído por la mezcla de volátiles que incluía a *Pseudomonas aeruginosa*, mientras sí que sentía atracción por

el perfil de volátiles emitido por *Corynebacterium minutissimum*, *S. epidermis* y *Bacillus subtilis*. Algo similar encontraron con el mosquito que causa la fiebre amarilla en humanos, *Aedes aegypti*, el cual tenía menos preferencia por humanos con perfil de volátiles más diversos (Logan et al., 2008). Esto sugiere que una diversidad alta de volátiles podría enmascarar ciertos volátiles atractivos para los mosquitos.

Como se indica más arriba, la microbiota puede influir en el resultado de las interacciones de su huésped con sus enemigos (p. ej. depredadores y parásitos) de diversas maneras. En primer lugar, los microorganismos pueden determinar el estado de salud de su hospedador (Clemente et al., 2012; McFall-Ngai et al., 2013), encontrándose diferencias en la diversidad microbiana entre individuos sanos y enfermos (Nicholson et al., 2012). Por ejemplo, se conocen trastornos intestinales en humanos como la colitis ulcerosa en el que los brotes de la enfermedad vienen precedidos de cambios en la microbiota intestinal (Schirmer et al., 2018). Obviamente, el estado de salud de un individuo puede afectar a su capacidad para enfrentarse a depredadores y parásitos. En segundo lugar, los efectos de la enfermedad también pueden generar cambios en el perfil de volátiles del individuo que, a su vez, influyan en la probabilidad de depredación y parasitismo. Este es por ejemplo el caso de los volátiles que emiten bacterias que colonizan las heridas de mamíferos que atraen a moscas parásitas y que depositan sus huevos o larvas sobre las heridas infectadas o necrosadas (Hall, 1995). Otros, insectos hematófagos que actúan como vectores de enfermedades humanas y aviares (p. ej. distintas variantes de malaria) se sienten más atraídos por individuos infectados por *Plasmodium* cuando este se encuentra en la fase de gametocitos para su propagación (Díez-Fernández et al., 2020; Lacroix et al., 2005; Verhulst, Takken, et al., 2010). Es posible que *Plasmodium* afecte la microbiota del hospedador, lo cual es determinante en los olores y en la preferencia de los mosquitos por su huésped (Verhulst et al., 2009, 2010, 2011). Recientemente se ha comprobado en la larva de la mariposa de la col (*Pieris brassicae*) que su endoparasitación por parte de una avispa parasitoide (*Clotesia glomerata*) genera cambios en la microbiota del hospedador (Bourne et al., 2023). Curiosamente estos cambios de la microbiota se relacionaron con cambios en el olor de la oruga que afectan la preferencia de un hiperparasitoide (*Barycapus galactopus*) por aquellas orugas parasitadas por *C. glomerata* de aquellas que no lo están (Bourne et al., 2023). Por último, la microbiota también podría generar productos para repeler o matar ectoparásitos como puede ser el olor cítrico del mérgulo empenachado (*Aethia cristatella*)

(Douglas,et al., 2005; Douglas et al., 2004) o los olores de las secreciones de abubillas (*Upupa epops*) (Tomás et al., 2020) en época reproductiva.

Todos estos estudios se revisaron en el **Capítulo I** y apuntan a que ciertos volátiles emitidos por la microbiota pueden afectar la probabilidad de parasitismo que experimentan sus hospedadores. La mayoría de estos trabajos se han llevado a cabo en condiciones de laboratorio y acotándose a ciertos volátiles producidos por la comunidad bacteriana por lo que es necesario realizar estudios experimentales de campo y ampliar el número de especies en el que se exploran esos efectos (ver **Capítulo I**). En esta tesis pretendimos abordar ese tipo de estudios, y exploramos las relaciones entre la diversidad de la comunidad bacteriana de aves y los volátiles mediante experimentos de campo, así como el efecto de ambos en el parasitismo que sufren los pollos y adultos en la época de cría (**Capítulos II**, **III** y **IV**). Además, se pretendia identificar bacterias y volátiles claves que se asocien a la probabilidad de que los pollos y adultos sufran ectoparasitismo (**Capítulos III** y **IV**).

3. La glándula uropigial de las aves y el caso particular de la abubilla

La glándula uropigial es la única glándula exocrina de las aves. La posee la mayoría de las especies y se encuentra en la parte dorsal, en la base de la cola. Aunque existe una gran variabilidad morfológica, en general, está compuesta de dos lóbulos donde se encuentra el tejido glandular, separados por un tabique, que desembocan en una papila donde se almacena la secreción, y de donde el ave la recoge y esparce sobre las plumas y demás tejidos tegumentarios durante el acicalamiento (Jacob and Ziswiler 1982). En general, la secreción uropigial es una sustancia lipídica, y por tanto hidrofóbica, que al aplicarse al plumaje lo impermeabiliza. Los compuestos lipídicos suelen ser ácidos grasos, esteres y en menor medida esteroles como el colesterol, pero también pueden presentar algunos alcoholes e hidrocarburos (Jacob & Ziswiler, 1982; Reneerkens, 2007). La composición química de la secreción varía entre especies y dentro de especies, dependiendo por ejemplo de la época del año (i.e., etapa reproductora), el sexo, calidad fenotípica, etc. (Jacob & Ziswiler, 1982; Martín-Vivaldi et al., 2009; Reneerkens et al., 2002). Como ejemplo de esta variación intraespecífica, en el correlimos gordo (*Calidris canutus*), la secreción uropigial pasa de estar compuesta por monoésteres a una composición de diésteres, una molécula de mayor peso molecular y menos volátil que reduciría la probabilidad de detección por depredadores (Reneerkens et al., 2005).

Las glándulas exocrinas aparecen asociadas a bacterias mutualistas en muchos taxones animales (Douglas, 2020). Este es por ejemplo el caso del órgano de luz de calamares o de algunas glándulas exocrinas de mamíferos (Albone et al., 1974, 1978; Leclaire et al., 2017; Montgomery & McFall-Ngai, 1994) y de insectos (Currie, 2001). En el caso de las aves, fue a principio de siglo cuando se encontraron evidencias de la existencia de bacterias simbiontes en la glándula uropigial de dos especies de aves, la abubilla *U. epops* (Martín-Platero et al., 2006; Martín-Vivaldi et al., 2010; Ruiz-Rodríguez et al., 2013; Soler et al., 2008) y la abubilla arbórea *Phoeniculus purpureus* (Law-Brown, 2001). Sin embargo, en los últimos años se han ido acumulando estudios que ponen de manifiesto la existencia de bacterias en la glándula de otras especies de aves (Braun et al., 2016; Braun, et al., 2018; Braun, et al., 2018b; Braun, et al., 2019; Braun, et al., 2019b; Díaz-Lora, 2020; Whittaker et al., 2019), por lo que se está comenzado a explorar las posibles ventajas adaptativas que estas bacterias simbiontes pueden significar para las aves. Entre ellas, se han explorado posibles funciones determinando los compuestos químicos de la secreción que, en contextos de parasitismo y/o depredación, podrían suponer elementos defensivos para las aves. Existen evidencias de que la secreción uropigial de las aves puede tener efectos antiparasitiarios y depredadores que, en el caso de tener bacterias simbiontes albergadas en ellas, podrían en parte ser un subproducto del metabolismo bacteriano.

La glándula uropigial de las hembras de abubilla (*U. epops*) durante el periodo de estancia en el nido (incubación y primeros días después de la eclosión de los pollos) y la de los pollos se caracteriza por presentar una gran cantidad de bacterias simbiontes, algunas de ellas productoras de compuestos con propiedades antimicrobianas (Martín-Platero et al., 2006; Martín-Vivaldi et al., 2010; Ruiz-Rodríguez et al., 2013; Soler et al., 2008). Las abubillas no limpian sus nidos de excrementos ni de restos de presas, los machos prácticamente no entran a los nidos y, durante la temporada de reproducción, son las hembras y los pollos los que corren un alto riesgo a padecer infecciones bacterianas, de ser ectoparasitados o incluso de ser depredados dentro del nido. Quizás por este motivo, son las glándulas de las hembras y no la de los machos las que cambian durante el periodo de cría. En las hembras, la glándula aumenta considerablemente de tamaño, y la secreción pasa de ser inodora y de un color blanquecino en la época no reproductora, a ser marrón oscuro con un fuerte y desagradable olor durante la época de estancia en el nido (Martín-Vivaldi et al., 2009; Rodríguez-Ruano et al., 2018). Los filos mayoritarios

de bacterias presentes en las glándulas uropigiales corresponden a *Firmicutes*, *Proteobacteria*, *Actinobacteria* y *Bacteroidetes* (Rodríguez-Ruano et al., 2018). Muchos de los *Firmicutes* que aparecen en la glándula son de la clase *Clostridia* (Rodríguez-Ruano et al., 2018) y producen ácido butanoico, el cual es uno de los volátiles más abundantes de las secreciones (Martín-Vivaldi et al., 2010). Este compuesto inhibe ciertas bacterias patógenas (Martín-Vivaldi et al., 2010), y se relaciona con menor intensidad de parasitismo y más éxito de vuelo en abubillas (**Capitulo III**).

Después de la reproducción, cuando los pollos y las hembras abandonan el nido, sus glándulas uropigiales pasan a ser parecidas a las de macho, disminuyendo de tamaño y reduciendo drásticamente la carga bacteriana de su secreción. Por ello, las comunidades han de reestablecerse o de adquirirse en cada evento reproductivo tanto en hembras como en pollos. El establecimiento de esas comunidades tienen un componente vertical (transmisión de padres a hijos) otro horizontal (transmisión desde el ambiente) (Martín-Vivaldi et al., 2018; Rodríguez-Ruano et al., 2015; Ruiz-Rodríguez et al., 2014). Los enterococos presentes en esta secreción (Martín-Platero et al., 2006) son típicos de la cloaca de los vertebrados, por lo que podrían trasmitirse a través de las heces que se acumulan en el nido. La abubilla suele reproducirse en nidos donde anteriormente se han reproducido otras abubillas (Díaz-Lora et al., 2019; Martín-Vivaldi et al., 2014). El material viejo de los nidos reutilizados de abubilla podría actuar como una fuente o reservorio de bacterias potencialmente beneficiosas durante la época de cría. Esta posibilidad podría implicar que la selección del lugar de reproducción (el nido) influya en la comunidad que se establezca en las glándulas de las hembras y de los pollos. Debido a que, como ya hemos mencionado, las características de las comunidades bacterianas de las secreciones deberían afectar a los volátiles u olores de las mismas, las características microbiológicas del lugar de nidificación deberían de afectar, no solo a las características microbianas de la secreción (Díaz-Lora et al., 2019) sino también a los volátiles asociados.

En esta tesis pretendemos demostrar esas relaciones entre nido, bacterias y volátiles. La comunidad bacteriana del nido antes de la reproducción debería de afectar a los volátiles presentes en el nido. Además, si el riesgo de parasitismo depende de los volátiles y/o comunidades bacterianas del nido, y por ende a aquellas de las secreciones, deberíamos de encontrar asociaciones entre volátiles, bacterias y parasitismo. Esas predicciones las estudiamos en los **Capítulos II** y **III** estimando la intensidad de

parasitismo de pollos y de hembras de abubilla, la comunidad bacteriana de la glándula de las hembras y de los pollos y del material del nido, y el perfil de volátiles de las secreciones y de los emitidos por el nido.

4. El Nidobioma como fuente de olores en aves

El ambiente microbiano donde los animales se desarrollan y reproducen influye directamente en la eficacia biológica y, por tanto, en su evolución (McFall-Ngai et al., 2013). En este sentido, las características de los nidos de las aves afectan al desarrollo y a la supervivencia de los pollos (Deeming & Reynolds, 2015; Jacob et al., 2015; Windsor et al., 2013). Por ejemplo, la comunidad bacteriana del nido, denominada Nidobioma, es decisiva para la adquisición de la microbiota intestinal de los pollos (Campos-Cerdá & Bohannan, 2020). El Nidobioma determina la asociación de las bacterias con las plumas (Goodenough et al., 2017; Mennerat et al., 2009), la comunidad intestinal (Hird et al., 2014) o la microbiota de la glándula uropigial (Díaz-Lora et al., 2019; Ruiz-Rodríguez et al., 2014). Algunas de estas bacterias pueden tener efectos negativos, reduciendo el éxito de eclosión de los huevos (Peralta-Sánchez et al., 2018) o degradando la queratina de las plumas (Ramnani et al., 2005). El Nidobioma, por lo tanto, se ha considerado una fuerza evolutiva importante seleccionando adaptaciones que faciliten el establecimiento de comunidades bacterianas óptimas en el nido (Campos-Cerdá & Bohannan, 2020). De acuerdo con esa idea, se ha demostrado que la manipulación de la comunidad bacteriana de los nidos de aves afecta a la probabilidad de supervivencia de los pollos de carbonero común (*Parus major*) (Jacob et al., 2015). También existen evidencias de que el Nidobioma y las plumas utilizadas en la construcción de nidos de carbonero determinaron los volátiles liberados en el nido (Jacob et al., 2018), por lo que el nidobioma también podría influir en la detectabilidad del nido por parte de parásitos y/o depredadores, una posibilidad que hasta la fecha ha sido escasamente explorada (**Capítulo I**).

La hipótesis de trabajo, por tanto, sería que, la asociación entre la comunidad de bacterias del material del nido y el perfil de volátiles del ambiente del nido, y la de estos con la intensidad de parasitismo y depredación podrían estar influida por características de los nidos. Los nidos de las aves son lugares óptimos para el crecimiento de bacterias, debido a la temperatura y humedad óptima que se alcanza durante el periodo de incubación, así como al aporte constante de materia orgánica que las bacterias pueden metabolizar (p. ej. restos de comida, heces etc.) (Mackie et al., 1998; Singleton & Harper, 1998). Características del nido como la orientación y el grado de aislamiento del medio

externo (p. ej. nidos abiertos vs cerrados) determinan condiciones ambientales importantes para el crecimiento bacteriano (Goodenough et al., 2017), tales como la humedad y la temperatura (Cook et al., 2003; D'Alba et al., 2010; Ingala et al., 2021; Peralta-Sánchez et al., 2011; Ruiz-De-Castañeda et al., 2011). Por otra parte, el uso de plantas aromáticas y plumas como materiales de construcción de nidos también tienen un efecto en las comunidades bacterianas de huevos y de pollos (Dubiec et al., 2013; Mennerat et al., 2009; Peralta-Sánchez, et al., 2018; Ruiz-Castellano et al., 2016, 2018) que posiblemente implica el efecto positivo de esos materiales en el éxito de vuelo de los pollos (Soler et al., 2017) que podría ser explicado por sus propiedades antimicrobianas. Además, el comportamiento de saneamiento de algunas especies de aves, eliminando los sacos fecales y restos de alimento de los nidos, reduce la densidad de bacterias en los mismos (Azcárate-García et al., 2019) y, con ello, la probabilidad de ser detectados por depredadores y ectoparásitos (Azcárate-García et al., 2019). Por tanto, la variación interespecífica en las características de los nidos, así como la relacionada con los comportamientos de saneamiento, predice una variación interespecífica en la microbiota y en los volátiles asociados. Además, si la detección de los nidos por parásitos se basa en volátiles de origen bacteriano, la asociación entre bacterias, volátiles y parásitos debería de aparecer incluso después de controlar por las variaciones entre especies en esos componentes. En el **Capítulo IV** se aborda la comprobación de esas predicciones.

OBJETIVOS

El objetivo principal de esta tesis es comprobar la hipótesis de que las bacterias asociadas a aves, tanto las del material del nido como las simbiontes de la glándula uropigial en el caso de la abubilla, emiten volátiles que juegan un papel importante en la interacción parásitos-hospedador. Esta hipótesis por lo tanto implica la existencia de dos tipos de asociaciones. Por un lado, que el perfil de volátiles de las secreciones y del ambiente del nido se deberían de relacionar con características de la comunidad bacteriana de la secreción y del material del nido respectivamente. Y, por otro lado, que (B) tanto volátiles como bacterias se deberían de asociar con la intensidad de ectoparasitismo que sufren los pollos y adultos en la etapa de reproducción y, por lo tanto, con el éxito de vuelo de los pollos.

Se exponen a continuación los objetivos específicos:

1. Revisar el estado del conocimiento sobre (1) el papel de las bacterias en la comunicación química animal entre congéneres y, debido a que la microbiota puede causar efectos beneficiosos y nocivos a sus huéspedes animales, también revisar su papel en la determinación del resultado de las interacciones con parásitos y depredadores; y (2) el papel hipotético de la depredación y el parasitismo como factores determinantes de la evolución del microbioma animal. Finalmente, sugerir predicciones clave que deben probarse para comprender mejor el rol de las bacterias en la biología animal **(Capítulo I)**.

2. Explorar la relación de la carga y diversidad bacteriana del material del nido con los volátiles del nido y la intensidad de ectoparasitismo que sufren los pollos de abubillas. Mediante una aproximación experimental (esterilización y uso de cajas nido nuevas), se pretende poner de manifiesto los efectos del material del nido en la diversidad bacteriana y en el perfil de volátiles del nido, y en la intensidad de parasitismo de los pollos **(Capítulos II y III)**.

3. Investigar a nivel intraespecifico, en nidos de abubilla, la relación entre la composición química de la secreción uropigial y la del ambiente de los nidos, y la de ambas con el grado de ectoparasitismo sufrido por pollos y hembra. Si la secreción uropigial determina el olor del nido, esperamos que el perfil volátil de

la secreción y del nido covaríen. Y, si los ectoparásitos seleccionan los nidos por compuestos del perfil volátil del nido, habrá una asociación entre los perfiles de volátiles del nido y de las secreciones con la carga de ectoparásitos **(Capítulo III)**.

4. Investigar a nivel intraespecifico, en nidos de abubilla, la asociación de la comunidad bacteriana de la secreción uropigial de los pollos y hembras de abubillas y el perfil de volátiles de las mismas, así como la asociación de ambos con el grado de parasitismo. Si las bacterias de la secreción son responsables en parte de sus volátiles, ambos componentes deben de covaríar. Y, si los ectoparásitos detectan compuestos típicos del perfil de volátiles de la secreción, esperamos una asociación entre la comunidad bacteriana de la secreción y del perfil de volátiles con la carga de ectoparásitos que sufren los pollos y hembras de abubillas **(Capítulo III)**.

5. Explorar a nivel interespecífico la asociación entre la comunidad bacteriana de los nidos de distintas especies de aves con el perfil de volátiles de los nidos, así como su relación con la carga de ectoparásitos. Ya que las características de los nidos difieren entres aves, es de esperar que tanto la comunidad bacteriana como los volátiles del nido varíen también entre especies. Por tanto, esperamos que la relación bacteria-volátiles difiera entre aves, así como su asociación con el parasitismo. Dado que nos centraremos en dos especies hematófagas, predecimos que las asociaciones esperadas aparecerán incluso después de controlar por la identidad de la especie **(Capitulo IV).**

6. Detectar bacterias y volátiles particulares del nido, y de las secreciones en el caso de abubillas, que se asocien con la carga de ectoparásitos y con el éxito reproductor. Si las asociaciones entre bacterias, volátiles y parasitismo se deben a bacterias productoras de volátiles particulares, la abundancia de bacterias específicas del material de los nidos, o de las secreciones en el caso de abubillas, que se asocien con volátiles particulares deben de aparecer asociadas con la intensidad de parasitismo **(Capitulo III y IV).**

MATERIAL Y MÉTODOS

1. Especies de estudio

La abubilla (*U. epops*) es un ave de la familia *Upupidae*, que junto con la familia *Phoeniculidae* forman el orden *Upupiformes*. Su distribución abarca Europa, África y Asia, pudiendo existir poblaciones sedentarias, así como migrantes (Reichlin et al., 2009, 2013; van Wijk et al., 2018). Por lo general, prefieren hábitats abiertos, con parches de pastizal de baja altura o terrenos de cultivo con acceso a suelo desnudo donde buscan el alimento. Anida siempre en cavidades (huecos en árboles, muros, entre rocas apiladas, etc.) (Cramp & Brooks, 1992) y en cajas nido (Arlettaz et al., 2010). Durante varios años, suelen reutilizar las mimas cavidades para reproducción, las cuales quedan cubiertas de material compuesto por los restos de anteriores eventos reproductivos, no aportando nuevo material ni construyendo nido (Hoffmann et al., 2015; Martín-Vivaldi et al., 1999). Cuando la hembra selecciona la cavidad, acomoda una pequeña depresión donde pondrá los huevos (Martín-Vivaldi et al., 2014).

En nuestra zona de estudio, las hembras realizan entre una y dos puestas de entre seis y ocho huevos, empezando en febrero y pudiendo llegar a finales de julio (Martín-Vivaldi et al., 1999). La hembra pone un huevo cada día y, normalmente, empieza a incubar con el primer o segundo huevo (Martín-Vivaldi et al., 1999) provocando una considerable asincronía de eclosión y diferencia en tamaño y peso (jerarquía) entre los pollos hermanos (Cramp & Brooks, 1992; Soler et al., 2022). Los pollos con mayor probabilidad de supervivencia son aquellos pollos que eclosionan primero (Martín-Vivaldi et al., 2014). La hembra se ocupa de incubar los huevos, y solo abandona el nido esporádicamente hasta que el primer pollo tenga aproximadamente 8 días. Durante todo el tiempo de incubación, que dura aproximadamente 17 días, y hasta que eclosionan la mayoría de los huevos, el macho se encargará de la alimentación de la hembra y de llevar el alimento al nido para que la hembra lo distribuya entre los pollos. Una vez que la hembra sale del nido, ambos darán alimento a los pollos hasta que estos abandonan el nido entre los 24 y 30 días de edad (Martín-Vivaldi et al., 2014).

La abubilla se caracteriza por presentar una glándula uropigial que, durante el periodo de estancia en el nido (hembras y pollos), aumenta de tamaño y alberga una gran cantidad de bacterias simbiontes (Martín-Vivaldi et al., 2018; Soler et al., 2008). Durante el resto del año las hembras presentan una glándula más parecida a la de los machos y con secreción blanquecina e inodora (Martín-Vivaldi et al., 2014) (**Figura 2)**. Los pollos

comienzan a producir secreción aproximadamente a los 8 días de edad, aumentado el tamaño de la glándula y la producción con la edad. De ahí nuestro interés de hacer dos muestreos en dos etapas de la estancia de los pollos, al inicio y al final en las cuales las características de la glándula (i.e. comunidad de bacterias simbiontes) puede variar.

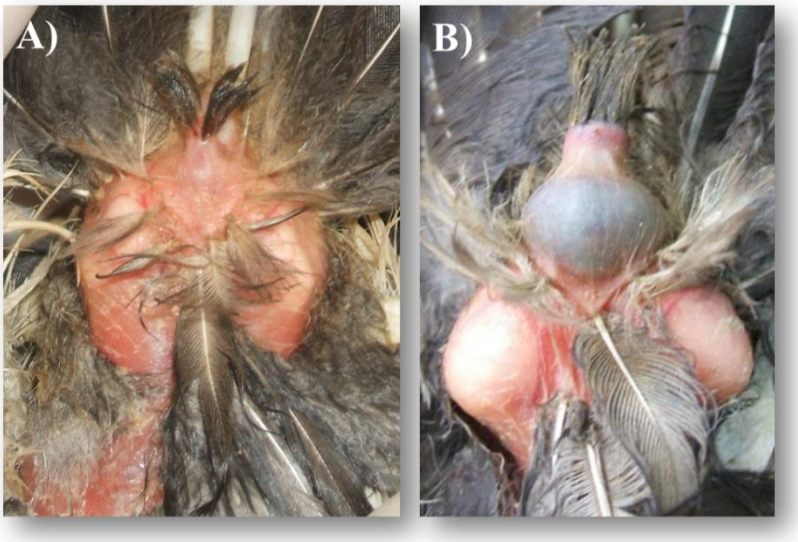

Figura 2. Fotografías de las glándulas uropigiales de un macho (A) y de una hembra (B) de abubilla durante el periodo reproductivo. Se aprecian dos grandes lóbulos de color blanquecino, una ampolla con secreción y un penacho de plumas en el extremo de la ampolla. Puede observarse la variación sexual en color y volumen del contenido de la ampolla (Martín-Vivaldi et al., 2009).

En el área de estudio las cajas nidos las usan no sólo abubillas (**Capítulo II y III**), sino también autillos (*Otus scops*), mochuelos (*Athene noctua*), carracas (*Coracias garrulus*), palomas zuritas (*Columba oenas*), gorriones comunes (*Passer domesticus*), carboneros comunes (*Parus major*), grajillas (*Coleous monedula*) y estorninos (*Sturnus unicolor*). Todas estas especies y la urraca (*Pica pica*), que no anida en cajas nido, se incluyeron en el estudio interespecífico (**Capitulo IV**) (ver **Tabla 1**).

El ectoparásito más abundante en nuestra área de campo es *Carnus hemapterus.* Esta mosca hematófaga de aproximadamente 2 mm de longitud es un parásito generalista de aves (Calero-Torralbo et al., 2013; Martín-Vivaldi et al., 2006) que parasita tanto a adultos como a pollos durante la etapa reproductora (Avilés et al., 2009; López-Rull et al., 2007), y que completa su ciclo biológico en nidos de aves (Valera et al., 2018). Los

adultos ponen los huevos en los mismos nidos que parasitan, las larvas se alimentan de restos de materia orgánica de la reproducción y después pupan en el mismo nido. En primavera, los adultos hematófagos alados emergen sincronizado su ciclo vital con el de su hospedador (Calero-Torralbo et al., 2013; Martín-Vivaldi et al., 2006; Valera et al., 2003). Las moscas adultas pueden permanecer en el mismo nido o dispersarse a otros nidos y, una vez que llegan a los nidos elegidos a parasitar, pierden las alas (Grimaldi, 1997; Roulin, 1998; Veiga et al., 2019). Como ocurre en la mayoría de insectos parásitos (Poldy, 2020), es posible que *C. hemapterus* utilice el olfato (volátiles emitidos desde el nido) para encontrar y elegir a su huésped. El pico de abundancia de *C. hemapterus* en los nidos de aves ocurre justo antes del comienzo del emplume de los polluelos (Liker et al., 2001).

Tabla 1: Clasificación taxonómica de las distintas especies de ave que anidan en cajas nido en nuestra zona de estudio, y que se utilizaron en el estudio interespecífico en el que se caracterizaron la comunidad bacteriana, el perfil de volátiles, la intensidad de ectoparasitismo y éxito de vuelo de las distintas especies. También se muestra información de tipo de material que utiliza cada una de ellas en la construcción de sus nidos.

Orden	**Familia**	**Especie**	**Características del nido**
Upupiformes	Upupidae	*Upupa epops*	Sin aporte de material
Strigiformes	Strigidae	*Otus scops*	Sin aporte de material
Strigiformes	Strigidae	*Athene noctua*	Sin aporte de material
Coraciiformes	Coraciidae	*Coracias garrulus*	Sin aporte de material
Columbiformes	Columbidae	*Columba oenas*	Con escaso aporte de material
Passeriformes	Passeridae	*Passer domesticus*	Con aporte de plantas
Passeriformes	Paridae	*Parus major*	Con aporte de musgos
Passeriformes	Corvidae	*Coloeus monedula*	Con aporte de ramas, plantas aromáticas y lana de oveja
Passeriformes	Corvidae	*Pica pica*	Nido construido de barro y forrado de raíces
Passeriformes	Sturnidae	*Sturnus unicolor*	Con aporte de plantas aromáticas y plumas.

Otro ectoparásito que en nuestra zona de estudio encontramos con bastante frecuencia en las hembras de abubilla durante la época de cría son los malófagos o piojos masticadores (orden Phthiraptera, suborden Mallophaga). Los piojos de aves se alimentan masticando zonas blandas de las plumas y la piel, lo que provoca un sangrado que los parásitos aprovechan para succionar sangre (Agarwal et al., 2011; Mester, 1977). En el

caso de las abubillas, estos ectoparásitos se colocan además de en las alas, en las plumas de la cresta donde están más protegidos.

2. Área de estudio, trabajo de campo y diseño experimental

El área de estudio se localiza en la Hoya de Guadix (Granada, al sur de España, 37°18´N, 38°11´W), con un clima semiárido, y donde se llevan a cabo estudios de ecología evolutiva con aves durante más de 30 años. A lo largo de esos años, se han ido instalado cajas nido de corcho en troncos de árboles y paredes, pero también escondidas entre piedras apiladas (**Figura 3**). Las dimensiones de estas cajas son 35 × 18 × 21 cm (altura interna × ancho × profundidad), 24 cm de altura desde la entrada hasta el fondo de la caja y de un diámetro de entrada de 3,5 cm para especies pequeñas, intermedio de 5,5 para especies intermedias (p.ej., abubillas y estorninos) y de más de 6 cm para especies más grandes como (p.ej. grajillas, mochuelos, etc.). De todas formas, el uso de las cajas por algunos micromamíferos y aves que son capaces de romper el corcho pueden agrandar los agujeros de entrada iniciales y permitir que especies grandes puedan anidar en los distintos tipos de cajas. Las distintas zonas de muestreo se dividen en cuatro hábitats diferenciados que son: 1. Sabana de encina mediterránea, con encinas (*Quercus ilex*) dispersas; 2. Bosque de pinos de las especies *Pinus pinaster* y *P. halepensis* esparcidos por las mesetas áridas; 3. cultivos de regadío donde se cultivan principalmente árboles frutales, así como olivos, hortalizas y verduras; y 4. estepa que son mesetas áridas de gran altitud con escasa vegetación y cultivos principalmente de almendros (*Prunus dulcis*) (**Figura 4**).

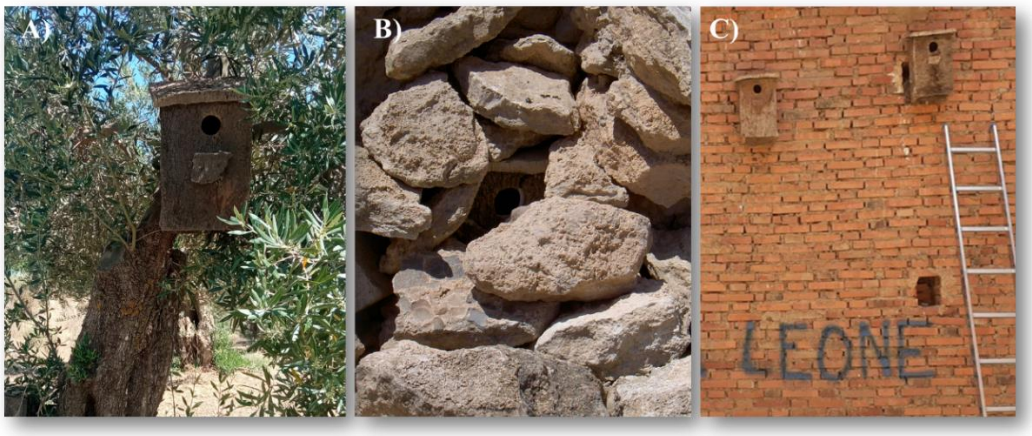

Figura 3. Cajas nido utilizadas por diferentes especies de aves en la Hoya de Guadix. A) Caja colgada en el troco de olivo. B) Caja escondida entre piedras apiladas. C) Cajas en colgadas en muro de ladrillo. (Fotografías cedidas por María Dolores Barón Rodríguez (A), Cristina Ruíz Castellano (B) y Gustavo Tomás (C).

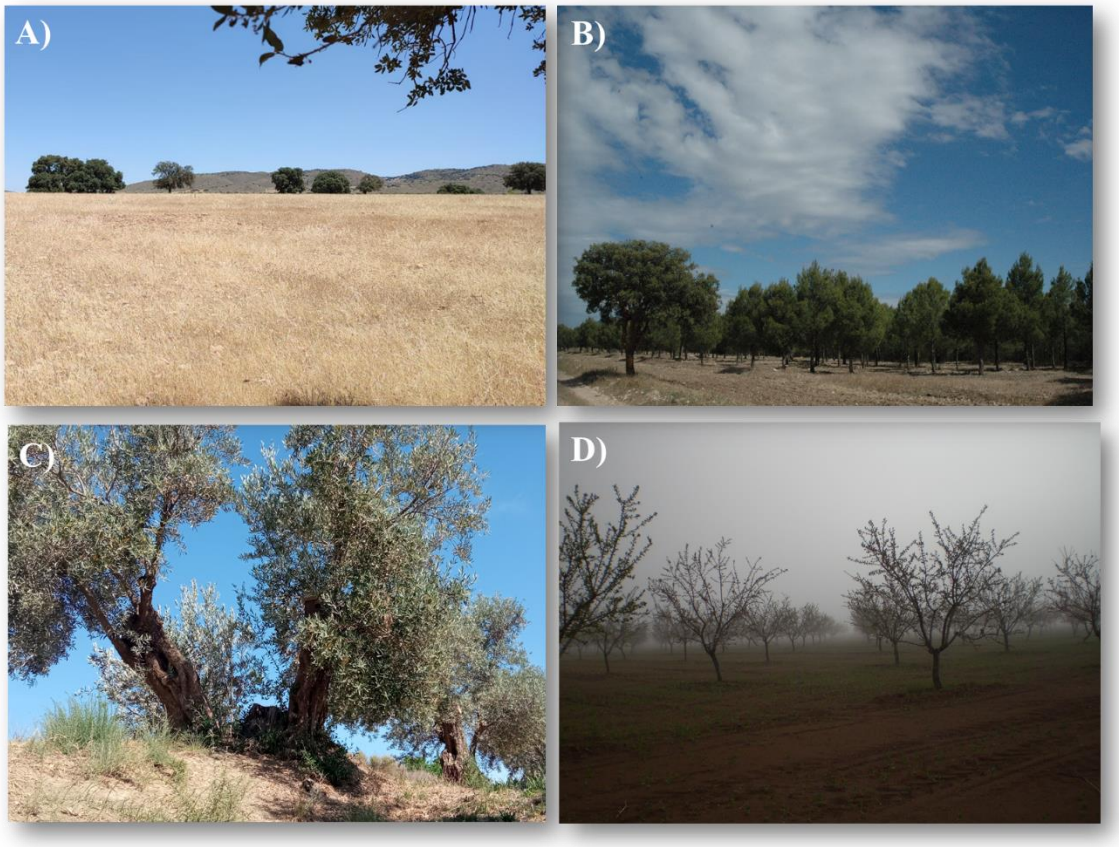

Figura 4. Zonas de muestreo de nuestra área de estudio en la Hoya de Guadix en las que se diferencian cuatro hábitats: A) Sabana de encina mediterránea, con encinas (*Quercus ilex*) dispersas. B) Bosque de pinos (*Pinus pinaster* y *P. halepensis*). C) cultivos de regadío donde se cultivan principalmente árboles frutales, incluidos olivos. D) Estepa con cultivos de almendros (*Prunus dulcis)*. (Fotografías cedidas por María Dolores Barón Rodríguez (A), Silvia Díaz Lora (B), Alejandro de la Concha Maroto (C) y Juan Manuel Peralta Sánchez (D)).

El trabajo de campo lo llevamos a cabo durante las primaveras de 2017 y 2018 (marzo-junio). Durante cada temporada de campo revisamos todas las cajas nidos una vez por semana para detectar inicios de reproducción. Una vez que detectábamos la puesta en una caja nido, identificábamos a qué especie pertenecía para calcular los días que debían de transcurrir para la eclosión del primer huevo; fecha a la que nombrábamos como día 1 en el protocolo de muestreo (**Tabla 2**). Durante la etapa de pollos, los nidos se visitaban varias veces para hacer realizar muestreos (i) del material de nido para el estudio de la comunidad bacteriana, (ii) de los volátiles del ambiente de la caja nido. En distintas visitas también muestreamos tanto hembras como pollos a los que se les recogía (iii) muestras de las secreciones de la glándula uropigial para el estudio de la diversidad de volátiles, y

en el caso de las abubillas también para la comunidad bacteriana, y se estimaba (iv) la intensidad de parasitismo de pollos y hembras.ç

Tabla 2. Protocolo de visitas y muestreos realizados en nidos de distintas especies de aves. Para cada visita (número de la visita) se muestra en el rango de días en los que se realizaba, y que variaba dependiendo de la especie de ave muestreada. Marcado con una X se muestran los tipos de muestras que se recogían en cada visita.

Número de la visita	1	2	3	4	5
Rango de días de muestreo tras la eclosión del primer huevo dependiendo de la especie de ave	4	6 - 7	5 - 8	16 - 22	17- 23
Parasitismo pollos			X		X
Parasitismo hembras	X				
Comunidad bacteriana del material del nido			X		X
Comunidad bacteriana de la secreción de pollos					
Perfil de volátiles de la caja nido		X		X	
Perfil de volátiles de la secreción del a hembra	X				
Perfil de volátiles de la secreción de los pollos			X		X

a. Diseño experimental en abubillas

Al inicio de febrero de 2017 y de 2018, antes de la época de reproducción, recogimos en bolsas de plástico estériles el material de cajas nido donde las abubillas se habían reproducido de forma exitosa el año anterior (47 cajas nido en 2017 y 70 en 2018). El material de nido de las distintas cajas las mezclamos y dividimos en dos mitades. Una de las mitades la autoclavamos y la otra mitad la dejamos sin autoclavar. En 2018, el material de nido no autoclavado lo tamizamos con mallas abiertas de 2, 1 y 0,5 cm de diámetro para eliminar las posibles pupas de los ectoparásitos (p.ej. *Carnus*) (Valera et al., 2018). Además, para evitar posibles influencias de la reproducción anterior, reemplazamos las cajas nido antiguas por nuevas. En las cajas nuevas, añadimos 500 cm^3 de material de nido recolectado mezclado de forma homogénea con 500 cm^3 de serrín (Allspan® Animal bedding, wood shavings). A la mitad de las cajas nuevas se les echaba material autoclavado (tratamiento experimental) y a la otra mitad material no autoclavado (tratamiento control). Los tratamientos los asignamos secuencialmente teniendo en cuenta la distancia entre cajas nido. Un total de 86 y 69 cajas nido nuevas fueron instaladas en el área de estudio en 2017 y 2018 respectivamente. Para todo el proceso de campo, en cada

visita a cada nido utilizamos guantes de látex nuevos a los que les echábamos etanol al 96% para evitar contaminación entre cajas nido.

b. Muestreos de bacterias, volátiles y parasitismo en las especies de ave

Para el estudio de la comunidad bacteriana del nido, al inicio y al final de la estancia de los pollos, recolectamos aproximadamente 10 gramos del material del nido en contacto con los polluelos que se guardaron en dos tubos Falcon de 15 ml (ver **Tabla 2**). Estas muestras las mantuvimos en frío en una nevera portátil hasta llegar al laboratorio donde almacenamos una parte a -20 ºC hasta la extracción de ADN bacteriano y la otra se utilizó para estimar la densidad bacteriana por métodos de cultivo tradicionales, en el mismo día del muestreo. En condiciones de esterilidad, incluimos 1 cm^3 de material del nido en un tubo Falcon estéril con 1 ml de tampón fosfato sódico estéril (PBS, 0.2 molar; pH = 7.2). Los tubos Falcon se agitaron vigorosamente para homogeneizar las muestras. Cultivamos diluciones en serie hasta 10^{-4}, esparciendo 5 microlitros de cada dilución en placas con agar de triptona y soja (TSA), un medio general para el cultivo de bacterias mesófilas aerobias. Las placas las incubamos aeróbicamente a 37ºC durante 24 horas. La estimación de la carga bacteriana de las muestras las estandarizámos al número de unidades formadoras de colonias (UFC) por ml (número de colonias x $10^{\text{factor de dilución}}$) / 0.005 ml de dispersión).

Los volátiles del ambiente de la caja nido los muestreamos dos veces, al principio y al final de la estancia de los pollos (ver **Tabla 2**). En el caso de las urracas no muestreamos los volátiles del nido porque anidan en nidos abiertos (**Tabla 1**). Los volátiles los capturamos en fibras de microextracción en fase sólida (SPME). La fibra la instalamos en una de las paredes de la caja nido con el extremo sensible protegido con una punta de pipeta de vidrio abierta por los dos lados, a unos 7 cm de altura del material del nido (**Figura 5**). El tiempo de exposición de las fibras captadoras de volátiles a los ambientes del nido fue de 24 horas. Posteriormente, recogíamos la fibra del nido y el lado sensible lo introducíamos en un vial de vidrio cerrado que manteníamos en frío (0-4 ºC) en la nevera portátil hasta llegar al laboratorio donde lo almacenábamos a -20 ºC hasta el análisis de cromatografía de gases y espectrometría de masas. El almacenamiento de las fibras nunca excedió una semana. Después de los análisis, las fibras SPME se reacondicionaban, eliminando todos los rastros químicos siguiendo las instrucciones del proveedor, es decir, 1 hora a 270 °C usando el inyector GC. Una vez acondicionadas, las manteníamos a -20 ºC hasta que las reutilizábamos en el campo.

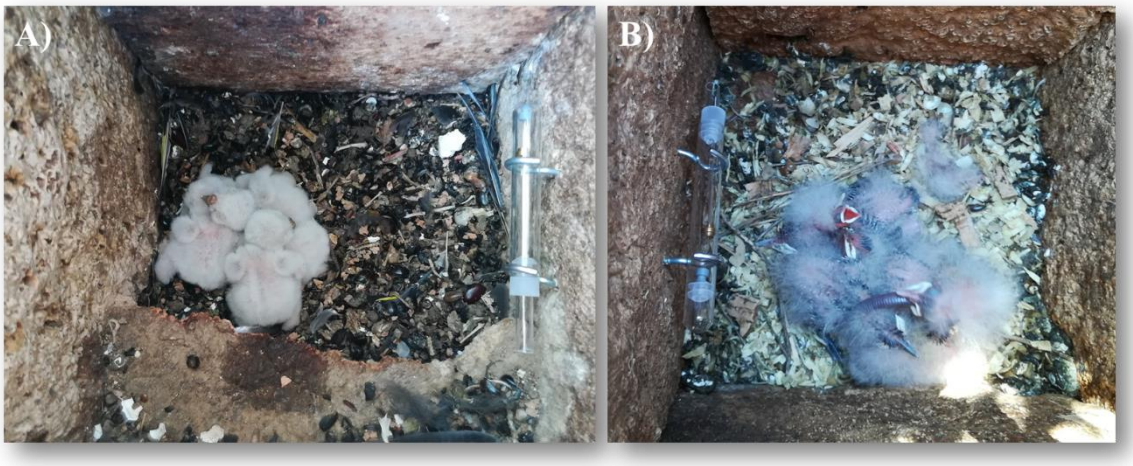

Figura 5. Fotografía que muestra la instalación de las fibras SPME en nidos de mochuelo (*Athene noctua*) (A) y abubilla (*Upupa epops*) en su fase de pollos de entre 8 y 10 días tras la eclosión del primer huevo.

En cuanto a las secreciones uropigiales, a las hembras de abubillas las muestreamos el día 4, y a los pollos los días 8 y 19 (ver **Tabla 2**). Antes del muestreo limpiabamos la glándula uropigial y sus alrededores con un hisopo de algodón empapado en etanol del 96 % y, utilizando una micropipeta automática de 1-10 µL con puntas esterilizadas, que introducíamos suavemente en la papila de la glándula uropigial y pipeteabamos la secreción (**Figura 6**). Para el análisis de ADN bacteriano utilizamos al menos 5 µL de secreción de las hembras o de los pollos de 19 días. Para los análisis de volátiles utilizamos 10 µL de secreción de la hembra o de uno de los pollos del nido, generalmente el de mayor tamaño, y cuando no llegaba a 10 µL de volumen, completamos con la secreción de otros hermanos. Las secreciones para el estudio de volátiles se introducían directamente en viales de vidrio sellados, específicos para los análisis de cromatografía de gases y espectrofotometría de masas. Las muestras las mantuvimos en la nevera portátil (0-4 ºC) hasta llegar al laboratorio donde las almacenábamos a -20 ºC hasta la extracción de ADN y análisis químicos de los volátiles.

Figura 6. Extracción de secreción uropigial a pollo de abubilla de 8 días con micropipeta y punta esterilizada. (Fotografía cedida por Manuel Martín Vivaldi).

La intensidad de ectoparasitismo de los polluelos también la estimamos dos veces, en la etapa temprana y tardía de su estancia en el nido (ver **Tabla 2**). La intensidad de parasitismo la cuantificamos por el número de marcas de sangre y restos de heces de la actividad de moscas *C. hemapterus* en el cuerpo (vientre y alas) de cada pollo siguiendo a Tomás et al. (2018). En los análisis utilizamos valores medios por nido. El éxito de vuelo lo estimamos como el porcentaje de pollos muestreados el día 8 que seguían vivos en el segundo muestreo. El ectoparasitismo de las hembras de abubillas lo estimamos como número de malófagos en las plumas de la cresta (Agarwal et al., 2011; Mester, 1977).

3. Extracción de ADN, secuenciación y análisis de secuencias de las comunidades bacterianas de las secreciones y del material del nido

a. Preparación de muestras y extracción de ADN

Para la extracción del ADN genómico de la comunidad bacteriana de la secreción uropigial de las abubillas se utilizó el kit FavorPrep Blood Genomic DNA Extraction Kit (Favorgen Biotech). Siguiendo las instrucciones del producto, añadimos un tratamiento

previo con lisozima (10 mg/mL de lisozima a 37ºC durante 30 min) asegurando la rotura de la pared celular de bacterias Gram positivas (Rodríguez-Ruano et al., 2018).

Para la extracción de ADN genómico de la comunidad bacteriana del material de nido de distintas especies usamos el protocolo MSOP, propuesto por Martín-Platero et al (2007). Las muestras de material de nido eran sólidas y, para la extracción de ADN, se utilizaron 80 mg. Primero, las muestras se suspendieron con 900 µL de tampón de lisis. Luego, la fase líquida la separamos del contenido sólido y la mantuvimos en diferentes tubos de microcentrífuga de 2 ml (para obtener más detalles sobre el protocolo seguido, consulte Lee et al., 2021). El ADN extraído de las muestras de material del nido la limpiamos utilizando el kit de eliminación de inhibidores de PCR One Step (Zymo Research). También procesamos blancos de laboratorio para detectar posibles contaminaciones durante el proceso.

b. Amplificación y secuenciación de alta resolución (Miseq de Illumina)

En primer lugar, estimamos la concentración de ADN bacteriano del material del nido y de las secreciones uropigiales de abubilla midiendo la absorción a 260 nm y 280 nm con Nanodrop. A continuación, preparamos las placas para PCR con 3 µL de cada muestra, con un control negativo del kit de extracción o de purificación en el caso de muestras de material de nido, y un control positivo de una comunidad conocida a una concentración de 5 ng/µL (ZymoBIOMICS Microbial Community DNA Standard D6306). En el estudio de comunidades bacterianas utilizamos como gen marcador de la subunidad pequeña del ribosoma, el gen 16S rARN. Este gen está formado por unos 1500 pares de bases (pb) y contiene 9 regiones hipervariables, que se intercalan con zonas muy conservadas en todos los procariotas. Por esta razón, la identificación de especies bacterianas se realiza mediante el estudio de estos fragmentos hipervariables y utilizando las partes conservadas para su secuenciación. En nuestro estudio utilizamos los fragmentos hipervariables V6-V8 del gen 16S rRNA que tiene una longitud de aproximadamente 400 pb. En la primera PCR, el gen 16S rRNA fue amplificado usando los cebadores universales B969F (5´ - ACGGGCRGTGWGTRCAA – 3´) y BA1406R (5´ - ACGGGCRGTGWGTRCAA – 3´). En una segunda PCR, las muestras las amplificamos añadiendo *barcodes* específicos para poder identificar a posteriori cada una de las muestras. Las muestras las enviamos a Integrated Microbiome Resource, Centre for Comparative Genomics and Evolutionary Bioinformatics (CGEB), University of

Dalhousie (Canadá), donde se realizaron las correspondientes PCRs y se secuenciaron en la plataforma MiSeq de Illumina.

c. Análisis de secuencias

Las secuencias obtenidas se analizaron mediante QIIME2 2019.10 (**Capítulo III**) y 2021.4 (**Capítulo IV**) (Bolyen et al., 2019). La secuenciación produjo secuencias *forward* y *reverse*. El uso de ambas secuencias una vez unidas, nos permite obtener un largo de secuencia mayor (aprox. 437 pb), lo que permite una mayor precisión a la hora de realizar la asignación taxonómica. Sin embargo, se restringe el número de secuencias con ese tamaño debido a que no todas las secuencias se pueden emparejar. Decidimos utilizar para nuestros análisis solo las secuencias forward debido a dos motivos (**Capítulo III** y **IV**). El primero es que la secuenciación produjo secuencias reverse de baja calidad, dificultando el ensamblaje con las secuencias *forward*. El segundo es que nuestro objetivo era tener una visión de comunidad bacteriana más que una detección más exacta de las especies bacterianas de la misma. Por estos dos motivos, decidimos usar las secuencias *forward* de mayor calidad, lo cual nos permitía largos de secuencia menor (aprox. 200 pb), pero mayor número de secuencias. Esto nos permitió detectar con buena precisión la diversidad de bacterias de nuestras muestras por lo menos hasta el nivel de género.

El primer paso para los análisis fue importar los datos a QIIME2. Después, se cortó el primer (B969F) de las secuencias forward mediante el algoritmo *cutadapt* (Martín, 2011). Las secuencias fueron filtradas con un índice de calidad Phred mayor o igual a 20, que es una medida de la calidad de identificación de los nucleótidos generados en la secuenciación del ADN. Usando el algoritmo de *Deblur* se filtraron y construyeron las tablas de *Amplicon Sequence Variants* (AVS table) a un tamaño de secuencia de 220 pb. Tras el filtrado, construimos el árbol filogenético usando los algoritmos *fragment insertion* (Janssen et al., 2018). La asignación taxonómica las determinamos comparando las secuencias representativas de cada ASV contra la base de datos de Greengenes (versión 13_8) al 97% de similaridad (DeSantis et al., 2006; McDonald et al., 2012). Posteriormente, eliminamos los cloroplastos, las mitocondrias y las secuencias a las que no se les pudo asignar filo alguno de las tablas de ASVs.

En la secuenciación de la comunidad bacteriana del material de nido de abubillas se generaron 11566822 secuencias de las cuales 3349977 fueron retenidas para la tabla de ASVs tras ser filtradas (número de muestras = 161, número promedio de secuencias por muestra (mín, máx) = 20807,31 (2930, 55978)). En el caso de la comunidad

bacteriana de las secreciones de abubillas se generaron 17866248 secuencias de las que 11385161 se retuvieron después del filtrado para la tabla de ASVs (número de muestras = 346, número medio de secuencias por muestra (mín, máx) = 32905,1 (3817, 70 586)). Para calcular la diversidad alfa y beta, controlamos por el esfuerzo de secuenciación rarificando a 5800 secuencias por muestra las tablas de AVS, por lo que se retuvieron 5585 ASVs en las muestras del material del nido y 2 105 ASVs en las de las secreciones (**Capítulo III**).

La secuenciación del material del nido de todas las especies de estudio (incluida la abubilla) generó 22 476 907 secuencias y 7 112 413 fueron retenidas para la tabla de ASVs tras el filtrado. En el caso de los cálculos de la diversidad alfa no se rarificó la tabla de ASVs, y en el caso de la diversidad beta, realizamos una transformación mediante le algoritmo centered log ratio (clr) para poder tener en cuenta la naturaleza composicional de los datos generados por secuenciación masiva (Aitchison et al., 2000) (**Capítulo IV**).

d. Análisis de compuestos químicos volátiles

Los análisis de los perfiles de volátiles, tanto del ambiente del nido, como de las secreciones uropigiales, las llevamos a cabo utilizando cromatografía de gases (GC) acoplado a un espectrofotómetro de masas (MS) Varian 450GC 240MS con un inyector automático Combi Pal con fibra "gris" SPME (50/30 μm DVB/CAR/PDMS, Stableflex 23Ga, Autosampler) con las siguientes especificaciones: Desorción en inyector a 250°C durante 10 minutos en split (20:1) y flujo de He a 2ml/min, recorrido cromatográfico en columna Agilent DB-5MS UI 30m x 0,25mm x 0.25μm o Agilent HP-FFAP 30m x 0.32mm x 0.25μm. La temperatura del horno se inició a 50ºC por 1 min., luego se programó para aumentar 5ºC/min hasta 100ºC, luego a 10ºC/min hasta 200ºC y 50ºC/min hasta 250ºC por 1 min. El análisis por Impacto Electrónico fue en modo TIC Full Scan entre 30 a 500 m/z. La identificación de los compuestos fue establecida por análisis SIM del ion característico y su comparación con la librería de espectros NIST 08. Inyectamos compuestos puros como patrones cuando fue necesario para confirmar el correcto análisis de las muestras. El resumen de los perfiles de volátiles de en el ambiente de las cajas nido y de las secreciones de las glándulas uropigiales de abubilla se muestra en la **Figura 7**. Utilizamos estándares de compuestos puros cuando fue necesario para la confirmación.

La importancia de los distintos compuestos químicos la establecimos como la abundancia relativa (el porcentaje del área de cada compuesto sobre el área total del

cromatograma ajustado al 100%) y la prevalencia (es decir, presencia o ausencia) de un compuesto químico en perfiles volátiles particulares.

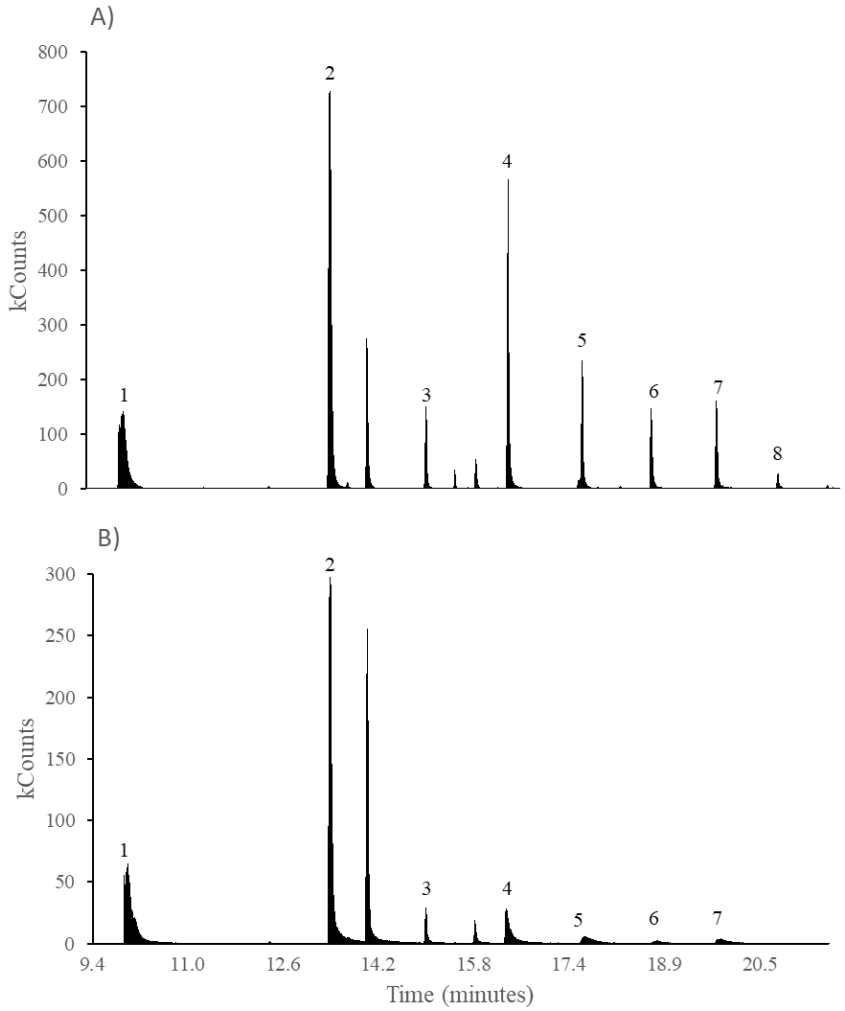

Figure 7. Ejemplos de cromatograma SIM 60m/z de (A) el ambiente de la caja-nido de un nido de abubillas al comienzo de la etapa de cría y de (B) la secreción uropigial de la hembra durante el periodo de incubación. Los picos representan los ácidos detectados en k cuentas (desde menos carbonos hasta carbonos más altos en la molécula: 1. acético, 2. butanoico, 3. pentanoico, 4. hexanoico, 5. heptanoico, 6. octanoico, 7. nonanoico y 8. decanoico).

4. Análisis estadísticos

a. Estimaciones de diversidad alfa y beta para la comunidad bacteriana y el perfil de volátiles

Calculamos los índices de diversidad alfa, que es la diversidad microbiana dentro de una muestra particular, y diversidad beta, que se refiere a la variabilidad en la

composición de la comunidad entre diferentes muestras [matrices de diferencias o distancias (Whittaker, 1972)] para los volátiles y la comunidad bacteriana del ambiente del nido y de las secreciones en el caso de la abubilla. Para la diversidad alfa de volátiles y de bacterias calculamos en QIIME 2 (Bolyen et al., 2019) el índice de Shannon (Shannon, 1948) y, en el caso de las bacterias, también calculamos el índice de diversidad filogenética de Faith [PD-Faith, (Faith & Baker, 2006)], el cual tiene en cuenta la filogenia de la comunidad bacteriana. En el caso de los análisis intraespecíficos con abubillas (**Capitulo III**), para la diversidad beta bacteriana calculamos las matrices de distancias basados en el índice UniFrac ponderado y no ponderado (Lozupone et al., 2005, 2007) en QIIME2 (Bolyen et al., 2019). Para la diversidad beta de los perfiles de volátiles, usamos la abundancia relativa y la prevalencia de los volátiles detectados para estimar respectivamente las matrices basadas en el índice de Bray-Curtis y en el de Jaccard, usando las funciones bcdist y distance en el paquete ecodist (Goslee & Urban, 2007) implementado en R.3.6.1 (R Core Team, 2020).

Para el análisis interespecífico (**Capitulo IV**), la diversidad beta bacteriana y de volátiles, la calculamos con la matriz de distancia transformadas de Aitchison. Los cálculos se realizaron después de aplicar la transformación de la proporción logarítmica centrada (clr) a las abundancias de ASVs en el paquete microbiome 1.18.0 R (Lahti & Shetty, 2012). La transformación clr controla la naturaleza composicional del conjunto de datos de la microbiota y produce valores que no varían en escala (Gloor et al., 2017). La matriz de distancia PhILR (Phylogenetic Isometric Log Ratio) la calculamos utilizando abundancias relativas de ASVs también después de la transformación clr. Este índice tiene en cuenta la información de la relación filogenética de los ASVs (Silverman et al., 2017). Las transformaciones de PhILR la realizamos con el paquete philr 1.22.0 (Silverman et al., 2017) implementado en R.3.6.1 (Team, 2020).

b. Exploración intraespecifica del efecto del tratamiento experimental

El efecto del tratamiento experimental de autoclavar o no autoclavar el material del nido sobre la densidad bacteriana lo evaluamos mediante un modelo lineal general (GLM del inglés *General Linear Models*) que incluía el año de estudio como factor y la fecha de puesta y el número de pollos como covariables. El efecto del tratamiento sobre la diversidad alfa y beta bacteriana y del perfil de volátiles, los evaluamos mediante GLM y PERMANOVAS respectivamente. Los modelos incluían el año de estudio como factor fijo y la fecha de puesta como covariable para la diversidad alfa y el año de estudio y

momento de la reproducción (temprana versus tardía) para la diversidad beta. Las interacciones entre los tratamientos y el año del estudio la probamos en modelos separados que también incluían los efectos principales. Tanto la densidad bacteriana como la diversidad de bacterias y volátiles siguieron una distribución normal (prueba de Kolmogorov-Smirnov para variables continuas, $P > 0,05$).

c. Exploración de las asociaciones entre características de la comunidad bacteriana y del perfil de volátiles, y entre los perfiles de volátiles del nido y de las secreciones

Las asociaciones entre las diversidades beta de las comunidades bacterianas y de los perfiles de volátiles de los distintos tipos de muestras la exploramos mediante la prueba del test de Mantel, que es equivalente a una regresión múltiple, pero utilizando matrices de diferencias entre muestras en un entorno multivariante. En el caso de las abubillas, analizamos las siguientes relaciones:

(1) los volátiles de las secreciones como variable dependiente con las bacterias de las secreciones, tanto de hembras como de pollos al final de la estancia como variables independientes,

(2) los volátiles del material del nido como variable dependiente con las bacterias del material del nido como independiente al inicio y al final de la estancia, y,

(3) los volátiles del nido como variable dependiente con los volátiles de las secreciones de la hembra al inicio de la estancia de los pollos y de las secreciones de los pollos al final de la estancia de los pollos, como variables independientes.

En el caso del estudio interespecífico, estudiamos las asociaciones de la diversidad alfa y beta de bacterias y volátiles del nido en ambos estadios del nido, mediante modelos mixtos lineales (GLMM) y prueba del test de Mantel respectivamente. Analizamos las siguientes relaciones:

(1) volátiles del ambiente del nido como variable dependiente con las bacterias como independiente y la de la identidad de la especie para controlar las relaciones por su efecto aleatorio a la hipótesis que se evalúa.

(2) Dado que estábamos interesados en detectar diferencias interespecíficas en la asociación entre diversidades bacterianas y químicas, también estudiamos la interacción

entre la identidad de la especie de ave y la diversidad bacteriana en un modelo separado que incluía los efectos principales (solo en diversidad alfa)

(3) Y, en el caso de detectar interacciones estadísticamente significativas, exploramos estas relaciones esperadas en cada especie de ave (solo en diversidad alfa). En el caso de beta diversidad, hicimos los análisis por separado para cada especie de ave.

Para explorar las asociaciones entre bacterias específicas y volátiles específicos de la secreción uropigial de las abubillas y del material del nido (**Capítulo III**) utilizamos los ejes (PC) de los resultados de los análisis de componentes principales (PCA) de las abundancias transformadas con log10 de los taxones bacterianos (considerando los seis primeros ejes) y de la abundancia relativa de los volátiles (considerando los cuatro primeros ejes). Las asociaciones entre los valores de cada caso (i.e., *scores*) en cada uno de los PC que describen la abundancia relativa de volátiles particulares (variables dependientes) y aquellos que describen la abundancia bacteriana (factores independientes) las exploramos por medio de modelos lineales generales (GLM). Debido a que realizamos múltiples GLM (uno por cada eje PC de volátiles), los valores de probabilidad que describen la fuerza de las asociaciones parciales se ajustaron por el efecto de múltiples pruebas mediante el algoritmo *False Discovery Rate* (FDR, Benjamini & Hochberg, 1995). De manera similar, realizamos GLMs para explorar la asociación entre los *scores* de PC que describen la abundancia relativa de volátiles de los nidos (variable dependiente) y las de la secreción uropigial de hembras o polluelos (factores independientes). En este caso, las asociaciones se estimaron por separado para hembras y pollos y, por lo tanto, realizamos ocho GLM diferentes. Los valores de probabilidad también se ajustaron siguiendo el algoritmo FDR (Benjamini & Hochberg, 1995).

En el estudio interespecífico (**Capítulo IV**) estábamos interesados en conocer bacterias y volátiles específicos que están relacionados entre ellos y, con ese fin, integramos información de abundancias relativas (transformación clr) de los ASVs y volátiles, y usamos un modelo de mínimos cuadrados parciales o proyección a estructuras latentes (sPLS) con un método de validación cruzada en el paquete mixOmics v6.19.9 (Rohart et al., 2017). Este análisis los hicimos a nivel inter- e intraespecíficos en las dos etapas de la estancia de los pollos en el nido. sPLS ha sido una alternativa a los mínimos cuadrados ordinarios para manejar la multicolinealidad (Chun & Keleş, 2010). Utilizamos el modo de regresión de sPLS, que ajusta una relación lineal entre múltiples respuestas en Y y múltiples predictores en X. Se intenta utilizar los datos microbianos para explicar

los perfiles volátiles. La principal diferencia entre sPLS y otras técnicas de reducción de dimensiones (es decir, PCA) es que maximiza la covarianza entre variables latentes (también denominada componente latente) en lugar de la correlación (Muñoz, 2015). Para visualizar gráficamente la estructura de correlaciones (es decir, la red) entre ASVs y volátiles considerados, usamos la función de red y el paquete igraph v1.3.4 (Csardi & Nepusz, 2006). Esa función dibuja una red con la correlación encontrada en los componentes latentes del modelo sPLS. Sólo representamos elementos del primer componente latente de las variables de respuesta (volátiles), ya que ninguna otra variable superó el valor mínimo establecido de q2 (0,095) para la validación de componentes latentes (ver Wold et al., 2001). Además, el valor de correlación de Pearson entre los componentes latentes fue diferente para cada momento de muestreo (es decir, al principio y al final de la etapa de anidación). Utilizamos para su representación un rango de correlación determinado por el valor máximo de correlación de Pearson y un valor que se corresponde con el 94% del máximo [(Max - 6*Max/100) – (Max)]. Luego exportamos la red a Cytoscape v3.9.9 (Shannon et al., 2003) para su visualización.

d. Exploración de la asociación de características de la comunidad bacteriana y del perfil de volátiles con la intensidad de parasitismo y el éxito de vuelo

En el estudio intraespecífico con abubillas (**Capítulo II y III**) utilizamos un modelo lineal general (GLM) para explorar la relación de la carga del ambiente bacteriano del nido como variable dependiente, la intensidad de parasitismo como covariable, y el efecto del tratamiento como factor (**Capítulo II**). También utilizamos los *scores* en los PC de los análisis de PCA. Para buscar la mejor combinación de ejes de PC de comunidades bacterianas y de perfiles volátiles que explicaran la intensidad del parasitismo de los polluelos y las hembras de abubilla, así como el éxito de vuelo. Además, seleccionamos los mejores modelos en un análisis de regresión general (GRM) mediante el CP de Mallow (Mallows, 1973), que es equivalente al criterio de información de Akaike (AIC) (Boisbunon et al., 2014). Es importante destacar que la información sobre los perfiles bacterianos y químicos no siempre estuvo disponible para el mismo grupo de nidos, por lo que analizamos por separado el efecto de la información química y bacteriana (**Capítulo III**).

Los GRM que exploran predictores de la intensidad del parasitismo en los pollos al inicio de la estancia y en las hembras incluyen información sobre las comunidades bacterianas y sobre los perfiles volátiles de los nidos, de los pollos y de las secreciones

de las hembras que muestreamos al comienzo del período de cría. De manera similar, los GRM que exploran predictores de la intensidad de parasitismo de los pollos al final de la estancia incluyen información sobre comunidades bacterianas y perfil de volátiles del material del nido y de la secreción uropigial de los pollos muestreados al final del período de estancia de los pollos en el nido. Finalmente, las secreciones de la hembra fue el único tipo de muestra utilizado en los análisis GRM con los que se exploraban predictores del nivel de infección por malófagos ya que estos parásitos se transmiten entre individuos y no llegan a los nidos. Los modelos GRM también se utilizaron para buscar factores que expliquen el éxito de los volantones. Con este objetivo, realizamos dos GRM diferentes que consideraron respectivamente información de muestras recolectadas durante las etapas tempranas o tardías de anidamiento.

Las distribuciones de frecuencias de los valores transformados de la raíz cuadrada de la intensidad del ectoparasitismo en los pollos, así como los valores brutos del éxito de volantones no difirieron significativamente de una distribución gaussiana (prueba de Kolmogorov-Smirnov para variables continuas, $p > 0,05$). Los GLM, GRM y PCA se realizaron en el software STATISTICA 12.

En el estudio interespecífico (**Capítulo IV**) la relación entre la intensidad del parasitismo o el éxito del vuelo de los pollos y las características de la comunidad bacteriana del material del nido y de los volátiles del ambiente del nido la exploramos en GLMM o en pruebas de Mantel dependiendo de que se consideren los índices de diversidad alfa o beta como predictores, respectivamente. Las diversidades de volátiles y bacterianas las incluimos en los modelos como factores fijos independientes, y la especie de ave como factor aleatorio en modelos GLMM y como matriz de diferencias en los test de Mantel. Además, como estábamos interesados en detectar diferencias interespecíficas en las asociaciones de interés, también estudiamos en GLMM las interacciones entre la especie de ave y la diversidad bacteriana y entre la especie de ave y la diversidad de volátiles en dos modelos diferentes que incluían los efectos principales del modelo completo. También exploramos a nivel intraespecífico esas asociaciones con el parasitismo y el éxito de vuelo.

Finalmente, dado que estábamos interesados en conocer los efectos de volátiles y de bacterias particulares en la intensidad de parasitismo y en el éxito de vuelo, hicimos nuevos análisis, pero solo con aquellos volátiles y bacterias del primer componente latente que resultaron de la optimización del modelo sPLS y también que se correlacionaron en

el rango del coeficiente de Pearson establecido. La intensidad del parasitismo o el éxito de vuelo se incluyeron como variables dependientes, las abundancias de bacterias (ASVs) como factor fijo y la identidad de la especie de ave como factor aleatorio en GLMMs. En GLMMs similares, estudiamos la relación de la intensidad del parasitismo con la abundancia de volátiles. Realizamos los análisis sPLS y demás modelos estadísticos descritos para cada una de las dos etapas en la que pollos fueron muestreados, al principio y al final de su estancia en el nido.

CAPÍTULOS

Foto: Mónica M.A.

CAPÍTULO I. *Microbially mediated chemical ecology of animals: a review of its role in conspecific communication, parasitism and predation*

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Revista: *Biology*, *10 (4)*, 274

doi: *10.3390/biology10040274*

Year: 2021

Microbially mediated chemical ecology of animals: a review of its role in conspecific communication, parasitism and predation

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Simple Summary

Symbiotic bacteria and fungi facilitate the acquisition of nutrients to their animal hosts, protect them against predators, parasites and diseases, and in some ways modulate complex animal behavior including communication by means of chemical signaling. However, odors of symbiotic bacterial origin would not only inform conspecifics of their animal host, but parasites and/or predators may also use those odors to detect their victims. We here review the role of bacterial symbionts on animal communication, and on interactions of their animal hosts with parasites and predators. Moreover, because microbial symbionts can have negative effects on their hosts facilitating predation and parasitism, these enemies could modulate the microbial community of animals, and we review the available evidence supporting this idea. The inclusion of microorganisms in scenarios of communication, parasitism and predation opens up new avenues of research that will contribute to understanding such interactions. We here elaborate some predictions and provide some guidance for future research.

Abstract

Microbial symbionts are nowadays considered of pivotal importance for animal life. Among the many processes where microorganisms are involved, an emerging research

avenue focuses on their major role in driving the evolution of chemical communication in their hosts. Volatiles of bacterial origin may underlie chemical communication and the transfer of social information through signals as well as inadvertent social information. We review the role of microorganisms in animal communication between conspecifics, and, because the microbiome may cause beneficial as well as deleterious effects on their animal hosts, we also review its role determining the outcome of the interactions with parasites and predators. Finally, we pay special attention to the hypothetical role of predation and parasitism driving the evolution of the animal microbiome. We highlight the novelty of the theoretical framework derived from considering the microbiota of animals in scenarios of communication, parasitism and predation. We aim to encourage research in these areas, suggesting key predictions that need to be tested to better understand what is one of the main roles of bacteria in animal biology.

Keywords: bacteria; chemical communication; ectoparasite-host interaction; microbiome; predator-prey interaction; volatiles

1. Introduction

Interactions between animals and their associated microorganisms (i.e., microbiota) are nowadays considered of pivotal importance to understand the physiology, morphology and behaviour of animals, as well as the outcomes of their interactions with abiotic and biotic environmental conditions (McFall-Ngai et al., 2013). Beyond pathogenesis, the most commonly studied effects of microorganisms on animals link gastrointestinal microbiota with facilitation of nutrient absorption, or even the synthesis of some essential micronutrients (Clemente et al., 2012; A E. Douglas, 2009; Rosenbaum et al., 2015). During the last two decades, the scientific interest has increased up to consider the microbiota as an essential component of living animals, therefore affecting their evolution (Wang et al., 2018). An emerging topic in evolutionary biology deals with the importance of the microbiome in mediating communication in their host organisms (Ezenwa & Williams, 2014).

Animals acquire information from the environment by direct interactions in a trial-and-error-tactics (personal information), or by monitoring the interactions of others with the environment or their outcomes, thereby acquiring what is called social information (SI) (Danchin et al., 2004). Social information can be based on signals, which are traits

that specifically evolved to convey information to receivers (Maynard-Smith & Harper, 2003). Alternatively, social information can also be based on cues provided inadvertently by individuals while engaged in their biological activities (inadvertent social information, ISI) (Danchin et al., 2004). Signals usually inform or advertise receivers on the phenotypic condition and capabilities of the sender, which supposedly benefits both, sender and receiver (Dawkins & Krebs, 1978; Maynard-Smith & Harper, 2003). ISI may inform bystanders for instance about resource location, but also about the quality of the resource, which is revealed by the performance or phenotypic quality of the cue sender (i.e., public information) (Danchin et al., 2004). Importantly, signals, as well as ISI, are supposed to reliably convey information on the phenotypic condition of the sender. Honesty of signalling characters has mainly relied on the hypothesis that only high quality individuals will be able to afford its associated costs (Maynard-Smith & Harper, 2003; Searcy & Nowicki, 2010; Zahavi & Zahavi, 1997). Instead, ISI are supposed to convey information on the phenotypic quality of the sender as a result of individual performance (Danchin et al., 2004).

Depending on the type of the sensitive channel used to transmit the signal or to gather ISI, stimuli have been mainly classified as visual, auditory or chemical. The use of chemicals is the most ancient, widespread, and shared way used by living organisms to evaluate their environment and to communicate with each other (Wyatt, 2010). Remarkably, symbiotic bacteria, or what as a whole is known as microbiota, are largely responsible for animal scents (Archie & Theis, 2011; Engl & Kaltenpoth, 2018). The role of symbiotic bacteria in animal chemical communication is therefore paramount (Ezenwa & Williams, 2014). Moreover, the microbiota is intimately related to the phenotypic quality and physiological activity of their animal hosts (Leclaire et al., 2017; Theis et al., 2013; Whittaker et al., 2019) by influencing their growth and development (McFall-Ngai et al., 2013). This indeed will affect characteristics of signals and ISI that conspecifics and heterospecifics could use. Particularly interesting is the possibility that chemical signals and cues of bacterial origin can be eavesdropped on by unintended receivers such as parasites and predators when locating and selecting hosts and prey.

Yet symbiotic microorganisms may also influence the outcome of the interactions between their hosts and their host enemies (predators and parasites) in other ways. For instance, microorganisms largely determine host health and condition (Clemente et al., 2012; McFall-Ngai et al., 2013), and these effects could be also used by predators and

parasites as inadvertent social information that facilitate host detection and/or selection (Engl & Kaltenpoth, 2018) . Symbiotic microorganisms can also produce metabolites with antimicrobial properties (Riley et al., 2002) that clear or prevent parasitic infections. Some bacterial symbionts are also known to produce metabolites that deter predators or parasites (Feldhaar, 2011). Symbiotic microorganisms might even be related to adaptive hormonal and immunological plastic responses of hosts against stressful environmental conditions, including those related to the risk of parasitism or predation (Sherwin et al., 2019). All these possibilities highlight the hypothetical role of microorganisms driving the interaction between hosts and their parasites and predators, and we here review current knowledge on these matters.

The role of microbial symbionts on animal chemical communication has been reviewed several times during the last decade (Carthey et al., 2018; Engl & Kaltenpoth, 2018; Ezenwa & Williams, 2014) and is not the aim of this essay. Here, we rather explain the rationale behind the social information value of volatiles of microbial origin in scenarios of animal communication. We also formulate key predictions to assess this hypothetical role of bacterial symbionts, and discuss the importance of bacterial symbionts in the evolution of conspecific communication, and in host-parasite and prey-predator interactions. The animal microbiome may cause beneficial as well as detrimental effects to its host. Special attention is paid to the possibility that host enemies might eavesdrop on inadvertent social information mediated by beneficial microbiota from their victims. An overview of the potential interactions between hosts and their bacterial symbionts in scenarios of social communication, parasitism and predation that are dealt with in this essay is shown in Figure 1.

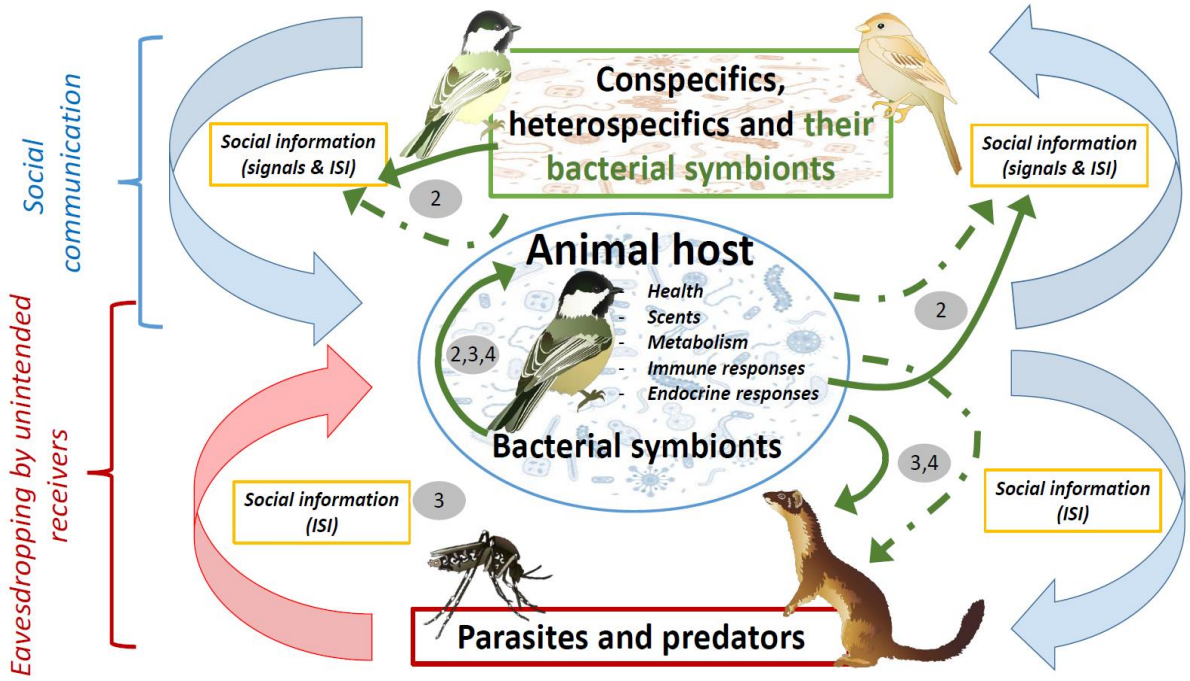

Figure 1. Diagram showing hypothetical influence of bacterial symbionts (green arrows) in scenarios of social communication, parasitism and predation. These influences could be directly due to either, bacterial metabolism or products with antimicrobial or anti-predatory properties (solid arrows), or indirectly through their effects on host characteristics (i.e., health, scents, metabolism, immunity and hormones) (dashed green arrows). Bacterial symbionts contribute to social information that is received by conspecifics or heterospecifics including parasites and predators. The negative effects of parasites and predators (red arrow) would be directly counteracted by defensive products of bacterial origin, or indirectly by host defensive traits that are also influenced by bacteria (continuous and dashed green arrows connecting the host with parasites and predators). These negative effects however will be enhanced by eavesdropping on inadvertent social information directly or indirectly mediated by host microbial symbionts and, thus, parasites and predators will also influence the symbiotic association between animals and microorganisms. Pathogenic parasites could also influence health and, consequently, bacterial symbionts of their victims, and, thus, parasites could indirectly affect conspecific communication. Numbers refer to main sections in the text where that relationships are covered. Symbols courtesy of the Integration and Application Network, University of Maryland (ian.umces.edu/symbols/) and freepik.com.

2. Conspecific chemical communication mediated by bacterial symbionts

The hypothetical role of microorganisms in animal communication is rooted in the "fermentation hypothesis". This hypothesis was originally formulated to explain the odours of anal-sac secretions of cats and foxes in the 70's (Albone et al., 1974, 1978), but is now applied to the general odour profile of animals that could operate in a large variety of scenarios of olfactory communication (Archie & Tung, 2015; Carthey et al., 2018; Ezenwa & Williams, 2014; Maraci et al., 2018). Until very recently, microbial production

of chemical signals had been mainly described in mammals and insects (Ezenwa & Williams, 2014). However, solid evidence for the role of bacterial symbionts producing volatile metabolites that contribute to the host odour profile is rapidly being accumulated for a wider range of animal taxa, including not only mammals (Leclaire et al., 2017; Theis et al., 2013) and insects (Engl & Kaltenpoth, 2018; Schmidtberg et al., 2019; Sharon et al., 2010), but also amphibians (Brunetti et al., 2019) and especially birds (Law-Brown & Meyers, 2003; Martín-Vivaldi et al., 2010; Whittaker et al., 2019).

Evidence supporting the key role of bacterial symbionts in animal communication represents one of the most fascinating and important advances that chemical ecology has experienced during the past few years (Carthey et al., 2018). The role of bacterial symbionts in animal communication is based on the assumption that some genetically and environmentally determined characteristics of animals, like those related to diet and immunity, also determine their microbial symbionts (Bradbury & Vehrencamp, 2011; Lee et al., 2021). Consequently, volatiles of microbial origin would inform on characteristics of their animal hosts (social information), and therefore, these chemicals would contribute to olfactory communication (Ezenwa & Williams, 2014). For instance, because of the many factors determining animal microbiota, and the enormous variability of associated microbial volatiles, the particularities of chemical profiles due to the metabolism of bacterial symbionts may communicate individual identity to conspecifics (i.e. an individual signature) (Caro et al., 2015; Carthey et al., 2018). Microbial volatiles may also aid to an easy targeting toward nest location by parents or offspring due for instance to the particular volatile profile of faeces around nests (Buxton & Jones, 2012; Krause & Caspers, 2012). Also, in scenarios of parent-offspring communication, volatiles from symbiotic bacteria might serve to recognize the own mother nipples by newborn mammals (Arteaga et al., 2013; Logan et al., 2012), while in sexual selection scenarios these volatiles can be used to choose a genetically compatible partner (Howard, 1977; Leclaire et al., 2011).

Previous research in this topic has been mainly focused on the possibility that volatiles derived from metabolism of host bacterial symbionts function to assess host quality by conspecifics. As we mentioned before, the microbiota composition, and thus volatile profiles of bacterial origin, depends on host characteristics, which include components of the host phenotypic quality. Thus, because these volatiles would convey valuable information to conspecifics, selection will favour receivers using such cues of

microbial origin (Bradbury & Vehrencamp, 2011). However, to demonstrate that microorganisms convey information on the phenotypic characteristics of their hosts, linking particular volatiles predicting host characteristics with the microorganisms that produce such volatiles is needed. Exploring associations between microbiotas and odour profiles of the hosts reflecting their physiological characteristics is nowadays a fruitful area of research that will help to unveil general patterns on the role of microbiota in social communication. Metagenomics, together with other omic techniques studying the metabolic production of microbial communities (e.g. proteomics and metabolomics), will help to characterize microorganisms responsible for the production of particular volatile metabolites (Harrison & Cameron, 2020), allowing to fill important gaps in our knowledge on the role of the animal microbiome in chemical ecology and communication.

The evolution of chemical signals mediated by microbial symbionts requires, not only that those odours reliably reflect characteristics of their hosts, but also that receivers use the conveyed information, and that such communication benefits both sender and receiver. By providing microorganisms with substrates in special locations such as the gut or glands, animals of several taxa cultivate bacteria producing substances that are valuable for them in terms of micronutrient provisioning, antimicrobial defences, or even signalling (Flórez et al., 2015; McFall-Ngai et al., 2013; McFall-Ngai, 2002). Scents originated by symbiotic microorganisms that inhabit animal glands are particularly important to gain insight into the evolution of microbially mediated chemical communication. This is mainly because most scents derived from animal glands have been traditionally considered as classical examples of chemical signals. Evidence supporting the existence of microbial symbionts growing within such scent glands are being accumulated in the literature, especially in exocrine glands such as anal glands of mammals (Leclaire et al., 2017; Theis et al., 2013) and the uropygial gland of birds (Martín-Vivaldi et al., 2010; Whittaker et al., 2019). Similarly, scents derived from microorganisms that enhance the survival and reproductive success of their hosts, such as those from gut microbiota, would also signal host phenotypic quality, therefore evolving an associated signalling role. Consequently, in these cases, where scents of microbial origin are part of the animal chemical signalling, the evolution of chemical communication should entail changes, not only in genetically inherited characteristics of hosts, but also in characteristics of their microbial symbiotic communities. However,

changes in animal hosts and in their microbiota should not be seen as completely independent because animal characteristics will largely determine characteristics of their microbiota.

Even though it is generally assumed that fitness of the host is often linked to that of its microbiota (Gerardo & Parker, 2014; Parker et al., 2021) but see (Angela E. Douglas & Werren, 2016) the evolution of symbiotic bacterial communities by means of natural selection acting on hosts entails important theoretical challenges. This is mainly because the characteristics of microbial symbionts are not directly determined by animal genomes and, thus, natural selection acting on host performance or fitness would not be able to directly modulate the bacterial community of symbionts nor their chemical profiles. To overcome this theoretical problem for explaining the evolution of host microbiomes, some authors claimed that microbiome and individual host should be considered together as the unit (holobiont) where natural selection acts (Bordenstein & Theis, 2015; Carthey et al., 2020; Theis et al., 2016). It has been broadly recognized that the characteristics and composition of host associated microbial communities parallel the phylogeny of related host species, and the holobiont concept would *a priori* help to understand the evolution of such phylo-symbiosis (Brooks et al., 2016). This approach, however, may entail some other theoretical problems related to group selection theory (Suárez & Stencel, 2020). A main critique to the holobiont concept is that fitness of hosts and symbionts are not fully linked, and specially not for all members of a host-associated microbiota (Angela E. Douglas & Werren, 2016). Thus, alternative scenarios explaining phylo-symbiosis have been proposed and explored. One possibility is to consider genetically determined traits in animal hosts that allow or favour maintenance of certain microbial communities, driven for instance by host variability in diet or habitat. The allelic variation in genes determining such traits will therefore predict the composition or functional variation in microbiota (Suzuki & Ley, 2020). In this case, host characteristics determining for instance the mode of transmission of bacterial symbionts and the characteristics of the environment where microbes are hosted will also govern the composition of symbiotic bacterial communities and their metabolic activity (Campos-Cerdá & Bohannan, 2020). Therefore, it is possible that natural selection acting on hosts could determine the metabolic activity of the microbiota, including those microorganisms responsible for the production of volatiles with importance in chemical communication. Similarly, for visual traits, we know for instance that the eggshell colouration of hoopoes (*Upupa epops*) is affected by uropygial

secretion rubbed on eggshells by females during incubation, which is indeed mediated by symbiotic bacteria hosted in the uropygial gland (Soler et al., 2014). Eggshell colour functions as a signal of female quality (Díaz-Lora et al., 2020), while the symbiotic bacterial community hosted in the uropygial gland of hoopoes have a significant genetic component (Martínez-García et al., 2016; Ruiz-Rodríguez et al., 2014). Thus, because characteristics of the microbial community of the uropygial secretion are likely mediated by physiological characteristics of hosts (where natural selection can operate), natural selection processes would also be responsible for egg colouration in hoopoes.

Similar processes to those described above for hoopoes can also operate for olfactory traits mediated by symbiotic microorganisms. Host characteristics that enhance the establishment of microbiota with direct beneficial effects for hosts could be identified by characteristic host odours. Moreover, because of the potentially narrow link between those host traits and characteristics of the volatile profile of the associate microbiota, host odour mediated by bacterial symbionts will also reflect hosts characteristics favouring the establishment of particular microbiota. Thus, the effects of natural selection acting of host traits could easily be tracked by following variation in host odour profiles. Interestingly, because mating with individuals with characteristics that enhance growth of beneficial microorganisms would be of selective advantage, sexual selection acting of olfactory traits mediated by bacterial symbionts will also accelerate the evolution of these characteristics. Future research should focus on identifying (i) physiological or morphological host traits enhancing the establishment and growth of beneficial microbiota, (ii) characteristic microbial volatiles narrowly reflecting potential fitness effects for their hosts, and (iii) whether sexual selection favours hosts of particular bacterially-mediated odour profiles. These research will allow to gain insight into the mechanisms underlying the evolution of hosts characteristics that favour particular microbiota.

Microbial symbionts would also contribute to the inadvertent social information provided by their hosts. Interestingly, this information does not necessarily benefit hosts, but reliably informs conspecifics and heterospecifics about host phenotypic characteristics or condition. It may be the case of pathogens, or parasite infections, that influence the host microbiota, which would result in animals displaying particular volatile profiles. Conspecifics could thus use that ISI as a warning chemo-sensory signal to, for instance, avoid close contacts with sick individuals. For example, in humans,

experimental activation of the immune system affected body odour, which were judged by conspecifics as less pleasant, more intense and less healthy (Olsson et al., 2014). Similarly, mice, mandrills and lobsters are able to identify sick conspecifics via chemical cues (Behringer et al., 2006; Boillat et al., 2015; Kavaliers et al., 2006; Poirotte et al., 2017). Thus, the role of bacterial symbionts indirectly mediating communication of health status to conspecifics could be widespread among animals. Importantly, emitting such volatiles might have negative effects on their hosts. Therefore, there may be evolutionary processes selecting host traits that shape microbiota emitting volatiles with less detrimental effects. Future work should also explore the role of bacterial symbionts as producers of inadvertent social information with detrimental effects for their hosts.

3. Negative effects of the microbiome in relation to parasitism and predation

Social information derived from the metabolism of host microbial symbionts can be eavesdropped on by unintended receivers such as parasites and predators. These two actors can substantially mediate some of the negative effects promoted by conspecific social communication, even when this information benefits both senders and conspecific receivers. Predation and parasitism are among the most powerful natural selection forces driving the evolution of animals in general and of animal signalling in particular (Endler, 1986; Futuyma, 2005; Hamilton & Zuk, 1982; Ridley, 1993). Examples of predators and parasites eavesdropping on auditory or visual cues from their victims are relatively well known (Andersson, 1994; Grafen, 1990; Hamilton & Zuk, 1982; Laidre & Johnstone, 2013; Zahavi & Zahavi, 1997; Zuk & Kolluru, 1998). Paramount examples include the adaptive disappearance of song in crickets due to parasitoids eavesdropping on this sexual signal (Zuk et al., 2006), or how frog-eating bats influence the evolution of frog calls (Akre et al., 2011). The possibility that symbiotic microorganisms can also mediate in the interactions that parasites and predators maintain with their victims opens a more complex picture for the evolution of chemical communication systems (Fig. 1). Below we describe some particular scenarios that could exemplify this possibility.

Many microorganisms cause animal disease. Apart from the fact that sick animals typically down-regulate their anti-parasitic and anti-predatory defences (Díez-Fernández et al., 2020; Lacroix et al., 2005; Møller et al., 2012), their microbiome typically differ from that of healthy animals (Nicholson et al., 2012). Thus, the effects of disease on the probability of predation and parasitism could also be mediated by changes in the microbial volatile profiles of sick animals that are detected by parasites and predators.

This is apparently the case of those volatiles emitted by the bacteria that colonize the wounds of hot blooded vertebrates, which attract parasitic flies that lay their eggs or larvae on infected or necrotized wounds (Hall, 1995).

Further examples of symbiotic bacteria indirectly mediating host detection by enemies come from research on host preference by mosquitoes and related ectoparasites (Cozzarolo et al., 2020). Although parasites may directly induce behavioural changes in their hosts aimed to increase parasite transmission (Moore, 2002), preferences by mosquitoes toward odours of already parasitized hosts are likely mediated by changes in host microbiota (Poldy, 2020; Ruiz-López, 2020). Hematophagous insects acting as vectors of human diseases (e.g. malaria, yellow fever, dengue) (Busula et al., 2017; James G. Logan et al., 2008; Verhulst et al., 2009, 2010, 2011) might exemplify this possibility. It has been suggested that mosquitoes may be more attracted to the odour of *Plasmodium* infected humans and birds (Díez-Fernández et al., 2020; Lacroix et al., 2005) but see (Cozzarolo et al., 2020), and some evidence suggests that bacteria play key roles determining host preference by mosquitoes. For example, laboratory experiments have revealed that the mosquito *Anopheles gambiae*, a main malaria vector in humans, is more attracted to individuals whose skin bacterial community is less diverse, but more abundant, and that include *Staphylococcus epidermis* (Verhulst et al., 2011). Similarly, humans whose skin contains more diverse volatile profiles were less susceptible to *Aedes aegypti* bites (James G. Logan et al., 2008). Furthermore, other components of the human skin bacterial community such as *Pseudomonas spp*. and *Variovorax spp.* seem to be the key taxa explaining why some individuals are unattractive to mosquitoes (Verhulst et al., 2011). Interestingly, trying to link the characteristics of the bacterial community with those of the chemical profiles of human skin that influence host selection by mosquitoes, Verhulst and coauthors (Verhulst et al., 2010) tested the effects of volatiles from six species of bacteria obtained from cultivars of human skin on host preference by *A. gambiae*. They found this mosquito was not attracted to *Pseudomonas aeruginosa* and its blend of volatiles, while it was attracted to a blend of volatiles from *Corynebacterium minutissimum, S. epidermis* and *Bacillus subtilis*. Taken together, these results suggest that certain volatiles of bacterial origin can facilitate parasitism, while others can deter enemies (see next section). In addition, outside of blood-sucking ectoparasites, it has been demonstrated that during oviposition and feeding behaviour, *Drosophila melanogaster* and dung beetles show an aversion towards volatiles such as phenol which is produced

by harmful bacteria (Mansourian et al., 2016). Most of this research has been carried out under laboratory conditions, or are focussed on particular groups of bacteria species. Future work should therefore expand on detecting particularities of the complete animal microbiota associated with the production of chemicals affecting host selection by parasites. Furthermore, detecting such associations is necessary to ascertain the role of the whole microbial symbiotic community determining host chemical profiles and host preference by blood sucking ectoparasites.

Field experiments have also revealed that the symbiotic bacteria inhabiting avian nests impinge on the risk of predation and parasitism. For instance, manipulation of the bacterial community of avian nests affected development and survival prospects of great tit (*Parus major*) (Jacob et al., 2015) and spotless starling (*Sturnus unicolor*) nestlings (Soler et al., 2017). Interestingly, the microbiota of great tit nest materials and feathers determined the chemical volatiles released from their nests (Jacob et al., 2018), which therefore could affect the probability of parasitism and predation. In accordance with this possibility, the experimental breakage and delivery of faecal sac contents of spotless starling nestlings increased the bacterial density in their nests, and was related to an increased predation rate and ectoparasite load (Azcárate-García et al., 2019). Similarly, nestling hoopoes developing in nest boxes where microorganisms were experimentally eliminated from nest substrates suffered reduced ectoparasite loads (Mazorra-Alonso et al., 2020). These results, therefore, suggest that selection pressures due to parasitism and predation should influence the evolution of host characteristics allowing the establishment of particular microbiotas that produce volatiles, which reduce the risk of being detected by enemies. This interesting possibility could be tested by performing experiments known to influence both bacterial community of avian nests and risk of parasitism experienced by nestlings (e.g., breakage of nestling faeces, addition of feathers or aromatic plants to the nest, or autoclaving nest material before reproduction (Azcárate-García et al., 2019; Dubiec et al., 2013; Ibáñez-Álamo et al., 2017; Mazorra-Alonso et al., 2020; Ruiz-Castellano et al., 2019)). Differences in risk of parasitism experienced by nests under different experimental treatments should covary with differences in microbiotas and volatile profiles. Moreover, the hypothesis can also be tested by exploring whether prevalence and abundance of particular bacteria and volatiles known to affect detection of potential victims by parasites and/or predators differs in populations under different risk of predation or parasitism. Finally, experiments in laboratory

conditions during several generations of hosts, directed to explore expected evolutionary changes in volatile profiles and microbiota composition in relation to parasitism or predation risk, could be also performed to test the role of animal enemies determining microbiotas and associated chemicals.

All these previous examples support potential negative effects of microbial symbionts of hosts, due to parasites or predators eavesdropping on host volatiles of bacterial origin. However, because parasites and predators also emit volatiles of microbial origin, it is equally plausible that these volatiles have negative effects on predators and parasites because their victims could use this inadvertent social information to reduce parasitism or predation risk. When victims receive ISI of the presence of parasites or predators, they would be able to display antiparasitic or antipredator defences. Especially well known is the case of predators, whose chemical cues can reveal their presence to potential prey of different taxa (Amo et al., 2008; Kats & Dill, 1998; Zidar & Løvlie, 2012). Thus, volatiles produced by microbial symbionts could have negative effects for their hosts independently of the side of the antagonistic interaction where these hosts are (i.e., prey or predator, host or parasite). These negative effects of microbial symbionts in terms of host detectability would occurs independently of whether the symbiotic association qualifies as mutualistic when interactions with undesired receivers of ISI is not considered. Thus, to understand the net cost/benefit balance of the symbiotic associations between microorganisms and their animal hosts, negative effects derived from natural enemies eavesdropping on bacterial derived volatiles should be considered. We are only starting to comprehend the role of volatile-producing microbial symbionts on scenarios of parasitism and predation and more research dealing with possible costs and benefits for either parasites, predators or victims is urged in this matter. Particularly interesting is the possibility that the strength of those ecological interactions affects the fitness outcome of associations between animals and their microbial symbionts.

4. Beneficial effects of the microbiome in relation to parasitism and predation

In this section, we first describe some benefits of microbial symbionts in scenarios of parasitism and predation and, thereafter, we focus in those possibly mediated by volatiles. Beneficial effects of microorganisms on animal health are well established. This is particularly the case for gut microbiota, mainly because nutrient absorption (Clemente et al., 2012), and essential otherwise inaccessible micronutrients (Rosenbaum et al., 2015), including their direct synthesis (A E. Douglas, 2009), entirely depend on bacterial

symbionts. It is also well known that unhealthy animals, or those in suboptimal physical condition, are more heavily parasitized and predated (Packer et al., 2003; Temple, 1987). Thus, because animal health largely depends on their gut microbiota, an indirect general effect of gut microbiota of healthy animals would be a reduced risk of predation or ectoparasitism (Cabreiro & Gems, 2013). As far as we know, the relation between gut microbiota composition and probability of ectoparasitism or predation has never been directly tested.

Moreover, gut microbiota of animals could also partially drive adaptive plastic responses to parasites and predators of their hosts (Liew et al., 2015; van Veelen et al., 2020). These include hormonal (Farzi et al., 2018; Levin & Hammes, 2016; Liu et al., 2020; Noguera et al., 2018) and immunological responses (Belkaid & Hand, 2014; Mao et al., 2018) allowing to face parasitic and pathogen invasions. It is broadly accepted that gut microbiota directly communicate with the animal brain by the production of some metabolites that activate the vagus nerves, or inhibiting other nerves within the gastrointestinal system that indirectly influence signalling of various mediators to the brain (Sherwin et al., 2019). In both cases, gut microbiota would be involved in the mechanisms determining the social behaviour of hosts (Sherwin et al., 2019) and, thus, their exposure to parasites and predators. This theoretical background therefore suggests that, through different pathways, gut microbiota might drive endocrine, immune and behavioural responses to the risk of parasitism and predation. To the best of our knowledge, this possibility has never been tested and, therefore, opens a new interesting research avenue about the role of microbial communities on anti-parasitic and anti-predatory responses of animals.

For defence against predator or parasite enemies, some animals use metabolites synthesized by other organisms with antimicrobial and anti-predatory properties (i.e. self-medication (Clark, 1991; Clayton & Wolfe, 1993; De Roode et al., 2013; Lozano, 1998; Ruiz-Castellano et al., 2019; Tomás et al., 2020)). Animals also use defensive metabolites that are endogenously produced (e.g. uropygial gland secretions of birds (Jacob & Ziswiler, 1982; Møller et al., 2009; Moreno-Rueda, 2017; Ruiz-Rodríguez et al., 2009; Tomás et al., 2020)). Some of these endogenously produced compounds are volatiles, and abundant correlative and experimental evidence supports the role of such metabolites interfering host attractiveness to predators and parasites (Bailey et al., 2006; Douglas, H.D. et al., 2005; H. D. Douglas et al., 2004; J.G. Logan & Birkett, 2007; James G. Logan

et al., 2008; Reneerkens et al., 2005). Interestingly, evidence of bacteria living within exocrine glands of animals is accumulating (Albone et al., 1974, 1978; Law-Brown & Meyers, 2003; Leclaire et al., 2017; Martín-Vivaldi et al., 2010; Theis et al., 2013; Whittaker et al., 2019) and, thus, some of these endogenously produced chemicals may have a bacterial origin, a possibility that has been scarcely explored. The role of bacteria in the production of these chemicals is a recently opened line of research with promising future possibilities, and different studies have provided evidence for these mutualistic associations in different taxa (Flórez et al., 2015; Lopanik, 2014; Molloy & Hertweck, 2017; Torres & Schmidt, 2019; Xie et al., 2019).

Examples of bacteria producing metabolites that defend their hosts against predators or parasites are abundant in invertebrate animals (Feldhaar, 2011). For instance, *Wolbachia* and *Spiroplasma*, two phylogenetically widespread parasitic endosymbionts of insects, enhance resistance of hosts against a variety of viral diseases (Moreira et al., 2009), parasitic nematodes (Jaenike et al., 2010) and parasitic wasps (Xie et al., 2010). Antibiotic producing symbionts are also known for several groups of insects including for instance digger wasps (Kaltenponh et al., 2005), fungus-growing ants (Currie et al., 1999) and pine beetles (Scott et al., 2008). Other insects benefit from the production of toxins or antibiotics by microbial symbionts that reduce palatability or prevent pathogenic infections. For instance, the gut microbiota of coccinellid beetles produces anti-predatory volatiles (i.e., methoxypyrazines) (Schmidtberg et al., 2019). Similarly, endosymbionts of some species of rove beetles of the genus *Paederus* produce toxins that deter wolf spiders (Kellner, 2003). Interestingly, some of these chemicals with protective functions against certain parasites or predators could as well be detected by conspecifics, and inform them on defensive capability of emitters, or by other different parasites or predators, influencing host detectability. Research on these types of three-way interactions (Davis et al., 2013) that included not only the bacterial symbionts and their animal hosts, but also conspecifics and heterospecifics can be approached in the wild by the system composed by birds, their symbiotic bacteria, and their ectoparasites (or predators). Birds possess a unique exocrine gland responsible for most avian odours and for the production of topically applied defensive metabolites, the uropygial gland (Jacob & Ziswiler, 1982), which allows focussing the studies on this special organ. In fact, groundbreaking evidence for this three-way interaction came from birds using their uropygial secretion, which contains microbial symbionts with antimicrobials and/or anti-

predatory properties that also might repel ectoparasites. This is for instance the case of green woodhoopoes (*Phoeniculus purpureus*) hosting symbiotic bacteria in their uropygial gland that produce metabolites which repel predators (Law-Brown, 2001; Law-Brown & Meyers, 2003). Similarly, the uropygial secretion of nestling hoopoes also include bacterial symbionts that produce antimicrobials (Martín-Platero et al., 2006; Ruiz-Rodríguez et al., 2013; Soler et al., 2008) and repellents for mosquitoes and biting midges (Tomás et al., 2020). Associations between characteristics of the uropygial gland and the risk of parasitism (Magallanes et al., 2016, 2017, 2020), as well as symbiotic bacteria living in the uropygial gland (Bodawatta et al., 2020; Braun et al., 2016; Chiale et al., 2017; Whittaker et al., 2019) are now described in phylogenetically distant bird species. Thus, the beneficial effects of uropygial secretions for birds may be mediated by microbial symbionts. This is a field worth to be explored, and that may allow detecting new undescribed mutualistic relationships between bacteria and their avian hosts. In the next section, within the final remarks, we review current knowledge on the possibility that parasites and predators modulate beneficial effects of volatile-producing bacteria for their animal hosts.

5. Final remarks

We have reviewed the state of the art on the role of microbial symbionts on animal communication, paying special attention to chemical communication with conspecifics, and to the interactions between their hosts with parasites and predators. These two issues converge on the possibility that parasites and predators use volatiles of bacterial origin to detect and select their victims, highlighting the possibility that it occurs because, independently of their function, volatiles of symbiotic bacterial origin are in fact host signals or inadvertent social information that can be eavesdropped on by conspecifics and heterospecifics (see Fig. 1).

We have discussed the importance of such interactions in the evolution of conspecific communication and host defensive traits. A wide variety of chemicals are involved in animal communication (Schulte et al., 2016) and, similarly to other signals, may be costly in terms of energy consumed or may increase the risk of parasitism and/or predation (Maynard-Smith & Harper, 2003; Stevens, 2016; Wyatt, 2014). Moreover, inadvertent social information mediated by chemical volatiles, associated for instance to animal respiration (e.g. (Santacroce et al., 2020)) or other biological activities, reliably inform predators and parasites on the location and on the phenotypic condition of their

victims. Most blood sucking ectoparasites and predators have evolved a highly developed olfactory system that, among other functions, allows them to locate potential hosts and/or prey (Conover, 2007; Hughes et al., 2010; J.G. Logan & Birkett, 2007; Takken & Knols, 1999). Thus, similarly to the effects described for auditory cues (e.g., (Balenger & Zuk, 2014; Briskie et al., 1999), both predators and parasites should have shaped the evolution of traits related to volatile production in their victims. That would be the case independently of whether volatiles are partially produced by bacterial symbionts.

Interestingly, some volatiles of bacterial origin that parasites and/or predators eavesdrop on to detect their victims may have beneficial effects for the hosts in scenarios other than parasitism and predation. In these cases, costs imposed by eavesdropping parasites or predators would counteract possible benefits and, thus, would modulate the production of such chemicals, or even the mutualistic symbiotic association between hosts and the bacteria producing such chemicals. Exploring the chemical profiles of animal microbiota, particularly those of bacteria producing antimicrobials or compounds linked to protection against predators is necessary to understand these complex interactions. A possibility for hosts to reduce negative effects of parasites and predators on mutualistic associations is to recruit new bacterial strains somehow able to mask host cues (Carthey et al., 2018), or even to produce volatile compounds that repel parasites and/or predators. Mechanisms to regulate or control bacterial growth rate, and thus the emission of volatile compounds, would also provide selective advantages (Verhulst et al., 2010). Future research focused on exploring these possibilities will clarify the effects of parasites and predators on mutualistic associations between animals and bacteria on the one hand, and how hosts minimize eavesdropping by their enemies on volatiles produced by mutualistic symbionts.

Finally, it is important to highlight that a better understanding of those interactions between hosts and parasites that are mediated by chemicals produced by bacterial symbionts may have ample applications in veterinary and medical research. Most ectoparasites are vectors of important human and animal diseases. Discovering connections between the animal microbiome and the volatiles that attract or deter ectoparasites may aid to the development of new products or methods for protection against ectoparasites and the diseases they transmit (Cozzarolo et al., 2020; Hall, 1995). Besides health concerns (McNair, 2015), parasites cause great economic losses (Carey et al., 2010; Ravikumar et al., 2016). Microbial derived products might substitute or at least

complement the use of insecticides, which produce undesired side effects (Lee & Groth, 1977; Marangi et al., 2012) or induce resistance in ectoparasites due to inappropriate and prolonged use of these drugs (Gordon et al., 2014; Pasay et al., 2006). The possibility of finding natural products that might be applied without side effects is therefore an asset that should encourage further research on the role of bacteria mediating the interaction between parasites and their animal hosts.

DECLARATIONS

Author Contributions

Conceptualization, M.M.A. and J.J.S. developed the idea of performing this review with GT actively participated in the discussion to decide the final focusing. Writing review and editing, M.M.A. performed the preliminary literature review and, together with J.J.S., finished a first draft of the manuscript, on which all three authors worked to build the final version. All authors have read and agreed to the published version of the manuscript."

Funding

This research was funded by Ministerio de Ciencia, Innovación y Universidades and European Regional Development Fund (FEDER) (CGL2017-83103-P, CGL2017-89063-P), by a predoctoral fellowship (MMA, FPU15/03420), and by the Ramón y Cajal programme(GT, RYC-2013-13040).

Conflicts of Interest: "The authors declare no conflict of interest."

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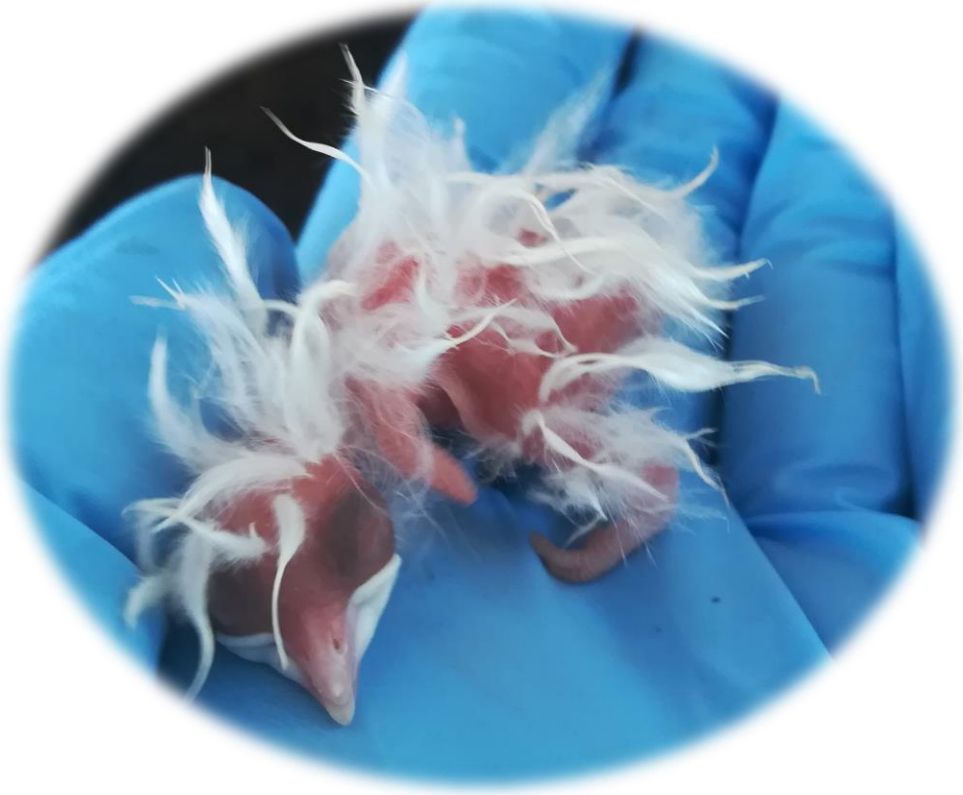
CAPÍTULO II. *Autoclaving nest-material remains influences the probability if ectoparasitism of nestling hoopoes (Upupa epops)*

Autores: *Mazorra-Alonso, M., Martín-Vivaldi, M., Peralta-Sánchez, J.M., & Soler, J.J. (2020)*

Revista: *Biology, 9 (10), 306*

doi: *10.3390/biology9100306*

Year: 2020



Autoclaving nest-material remains influences the probability of ectoparasitism of nestling hoopoes (*Upupa epops*)

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Simple Summary

Microorganisms may have direct negative effects on their animal hosts and cause diseases, but some others provide animals with protection against infections, parasites and even predators. However, parasites or predators might clue on odors produced by bacteria, even on those from protecting microorganisms, which could turn net benefits of *a priori* considered beneficial bacteria to be neutral or even negative. This possibility has scarcely been studied in wildlife populations but we manipulated bacterial community of nest-material of hoopoes and detected a negative effect in terms of intensity of parasitism by blood sucking flies that nestlings suffered. We also detected a positive link between bacterial density of the nest-materials and intensity of ecto-parasitism, which further point at the importance of the bacteria determining parasitism. Blood sucking ecto-parasites are also disease vectors, affecting both human and livestock and considering the role of bacterial environment might help to stablish new transmission control protocols.

Abstract

Nest bacterial environment influences avian reproduction directly, because it might include pathogenic or antibiotic-producing bacteria, or indirectly, because predators or ecto-parasites can use volatile compounds from nest bacterial metabolism to detect nests of their avian hosts. Hoopoes (*Upupa epops*) do not build nests. They rather re-use holes or nest-boxes which contains remains of nest-materials from previous breeding seasons. Interestingly, it has been recently described that the nest bacterial environment partly affects the uropygial gland microbiota of hoopoe females and eggshells. Blood sucking ecto-parasites use chemical cues to find host nests, so we experimentally tested the hypothetical effects of microorganisms inhabiting nest-material remains before reproduction regarding the intensity of ecto-parasitism suffered by 8 days-old nestling hoopoes. In accordance with the hypothesis, nestling hatches in nest-boxes with autoclaved nest-material remains from the previous reproductive season, suffered less from ecto-parasites than those hatched in the control nest-boxes with non-autoclaved nest-material. Moreover, we found a positive association between bacterial density of nest-material during the nestling phase and ecto-parasitism intensity that was only apparent in nest-boxes with autoclaved nest-material. However, contrary to our expectations, nest bacterial load resulted positively associated with fledgling success. These results suggest, a link between the community of microorganisms of nest-material remains and intensity of ecto-parasitism, and, on the other hand, that nest bacterial environment during reproduction is related to fledging success. Here we discuss possible mechanisms explaining experimental and correlative results including the possibility that the experimental autoclaving of nest material affected the microbiota of females and nestlings' secretion and/or nest volatiles that attracted ecto-parasites, therefore indirectly affecting both nest bacterial environment at the nestling stage and fledging success.

Keywords: Bacterial community; chemical volatiles; ectoparasitism; hoopoe; nest-material; uropygial gland

1. Introduction

Microorganisms are a selective force driving the evolution of macroorganisms at different levels. Beyond their well-documented deleterious effects as infectious agents, bacteria are considered as the second genome of host organisms because they are essential

for their correct development and functioning (McFall-Ngai et al., 2013). The protection against pathogens by stimulating the immunological system or directly by synthetizing antimicrobial peptides (e.g. bacteriocins) (Flórez et al., 2015), as well as their role producing essential nutrients for their hosts (LeBlanc et al., 2013; Org et al., 2015), are some classical examples of beneficial effects of bacteria to their hosts. Interestingly, the role of bacteria in the modulation of complex animal behaviors has gained importance (Archie & Theis, 2011; Sherwin et al., 2019). For instance, bacterial are responsible of some volatiles signaling host characteristics (Albone et al., 1978; Ezenwa & Williams, 2014; Sherwin et al., 2019) and, thus, might play an important role in the evolution of social communication. Bacteria metabolism provide their hosts with an odor signature that broadcast information about the sex, social status, kinship, and even group membership of individuals (Leclaire et al., 2017; Theis et al., 2013). Odors for bacterial metabolism might also attract predators and/or parasites with good olfactory system (Braks et al., 1999; Carey et al., 2010; Lindh et al., 2008; Verhulst, Andriessen, et al., 2010; Verhulst, Takken, et al., 2010) . We know for instance that two species of mosquitoes, *Anopheles gambiae* and *Anopheles arabiensis*, have a greater attraction for traps that contains bacterial communities of its natural hosts, humans and birds respectively (Busula et al., 2017). Moreover, *Anopheles gambiae* are even able to distinguish particularities of individual humans depending on skin bacterial richness and diversity through cueing on the volatiles that different bacterial communities produce (Verhulst et al., 2009, 2011; Verhulst, Andriessen, et al., 2010; Verhulst, Takken, et al., 2010). Evidences of the expected association between symbiotic bacteria of hosts and probability of parasitism, however, come from laboratory experiments and human parasites. Studies on natural conditions are therefore necessary to confirm the hypothetical role of bacterial communities of animals, producing volatile compounds that parasites use to detect hosts.

Physiological activity of nestlings and parents, as well as, relatively appropriate climatic conditions of avian nests for bacterial growth (particularly those built in holes) (González-Braojos et al., 2012), make the avian nest environment an ideal niche for testing the hypothetical role of symbiotic bacteria determining the probability of parasitism. Together with incubation and brooding activity, that generate the optimal temperature (Singleton & Harper, 1998), the accumulation of organic materials in nests including feces, feathers, skin, remains of food, etc. would enhance bacterial growth in

these environments. Ecto-parasites or predators might then cue on particular volatiles from the metabolism of bacteria growing in avian nests to detect active hole-nests. In accordance with this hypothesis, Azcárate-Gracía and coauthors detected that the breakage of fecal sacs increased bacterial density of spotless starlings (*Sturnus unicolor*) nests, and that nestling in experimental nests tended to suffer more from ecto-parasites and experienced higher depredation rate than those in control nests (Azcárate-García et al., 2019). However, they did not manipulate bacterial density of nests directly, but added broken fecal sacs to nests of a species in which parents remove nestlings' feces. Although the performed experiment affected nests' bacterial community, a more direct experimental modification of the bacterial nest environment is necessary to check the effects of nest bacterial community on parasitism and predation.

We here performed such experiment in artificial hole-nests used by hoopoes *Upupa epops*. The hoopoe is a particularly relevant biological model for testing the hypothesis that ecto-parasites cue on volatiles from symbiotic bacteria of avian nests. Preen glands of breeding females and nestlings host mutualistic bacteria that produce antibiotic substances (Martín-Platero et al., 2006; Ruiz-Rodríguez et al., 2013, 2009, 2012) and a variety of malodorous volatiles (Martín-Vivaldi et al., 2010) that some ecto-parasites might use to detect active nests. Furthermore, it has been experimentally demonstrated that nest-material remains from previous hoopoe reproductive events affects microbial community of the uropygial secretion of adults and nestlings (Díaz-Lora et al., 2019). Thus, a link between nest bacterial communities and volatiles from symbiotic bacteria likely exists in this species. The expected finding may point out that nest bacterial community and their metabolism would be responsible of volatiles that ecto-parasites might track to detect active hoopoe nests. We evaluated the intensity of ecto-parasitation by *Carnus hemapterus* flies, which parasitizes nestling and incubating adults of several birds species (Capelle & Whitworth, 1973; Tomás et al., 2018; Valera et al., 2003; Veiga et al., 2019). The life cycle of this ecto-parasite fly is synchronized with that of its hosts (Martín-Vivaldi et al., 2006). In spring winged adults emerge from overwintering pupae inside avian nest-materials from previous reproductions. Adult flies stay in the same cavity or disperse looking for holes with avian breeding activity (Jesús Veiga et al., 2019). The peak of *C. hemapterus* abundance occurs before the start of nestling feathering, which in hoopoes occurs 6-8 days after hatching, and decrease thereafter (Liker et al., 2001). Once adults *C. hemapterus* find a nest for parasitism, they

lose their wings and feed on incubating adults and growing nestlings (Grimaldi, 1997; Roulin, 1998).

The hoopoes use artificial nest-boxes for breeding in the study area, and the experiment consisted on installing new nest-boxes filled with old nest-material remains collected from nest boxes where hoopoes bred the previous year. Nest materials from previous years were autoclaved in order to eliminate the microorganisms in comparison to control, non-autoclaved nest materials. We expect that hoopoes breeding in experimental nest boxes, where symbiotic bacteria of added nest-materials were eliminated, experience lower probability of ecto-parasitism than those nesting in control nest-boxes. Moreover, since ecto-parasitism have negative effects on nestling growth, we predicted that nestling in control nests will experience lower fledging success than those growing in experimental nests. This prediction is based on the assumption that experimental nest boxes with autoclaved nest-material should have bacteria at a lower density than control nest-boxes at the time of hoopoes' reproduction.

2. Materials and Methods

a. Study area and species

The fieldwork was carried out during 2017 and 2018 breeding seasons (March-July) on hoopoe nests in a wild population located in the Hoya de Guadix (Granada, Southern Spain, 37°18´N, 38°11´W), a plateau at 1000 m a.s.l. and semiarid climate. In this area, around 300 cork-made nest-boxes are available for hoopoes; most of them attached to tree trunks and walls, but also hidden in piled stones. The dimensions of nest-boxes are 35 × 18 × 21 cm (internal height × width × depth), 24 cm (bottom-to-hole height) and 5.5 cm (entrance diameter). Information about the study area is further described elsewhere (Díaz-Lora et al., 2019; Martín-Vivaldi et al., 1999).

The hoopoe is an Upupiformes, migratory species, distributed throughout Europe, Asia, and Africa (Reichlin et al., 2009, 2013). Hoopoes are hole-nesters that frequently use natural cavities, trees or walls, or artificial nest-boxes for reproduction. They do not build nests, but prefer cavities with remains of soft material from previous reproduction events of conspecifics or heterospecifics where they create a small hole and lay the clutch (Martín-Vivaldi et al., 2014). In our study area, the reproductive season starts in late February and many females lay a second clutch before August.

We assessed ecto-parasitism by *C. hemapterus*, a common generalist hematophagous fly of about 2 mm in length and that is easily detected on the nestling body. *C. hemapterus* is a frequent ecto-parasite in hoopoe nests in our study area (Martín-Vivaldi et al., 2006). Following previously published work (Tomás et al., 2018), the intensity of parasitism was inferred from abundance of traces of blood remains and feces on nestlings' skin (i.e. belly and wing) (see below).

b. Experimental design and fieldwork

The experiment was performed in new nest-boxes installed before the start of hoopoe reproduction in both breeding seasons (2017 and 2018). Before reproduction started, at the beginning of February, we visited nest-boxes where hoopoes successfully bred the year before, and collected old nest-materials that were stored in punched plastic bags (47 nest-boxes in 2017 and 70 in 2018) and maintained at room temperature. Nest-materials from different nest-boxes were pooled, mixed, and divided in two halves: one of them was autoclaved (to be used in experimental nest boxes) and the other was not (to be used in control nest boxes). However, *C. hemapterus* pupae overwinter inside bird nests, and autoclaving material should kill them. Therefore, after treatment, experimental and control material could differ not only in bacterial density but also in probability of *C. hemapterus* flies emerging within the control nest boxes, which might affect experimental outcomes. To evaluate this potential bias, we removed in 2018 all pupae from material of both experimental treatments before autoclaving. We did so by sifting nest-material with opening meshes of 2, 1 and 0.5 cm diameters (Podofillini et al., 2018). Nest-boxes used in this experiment were new and, before installation, each of them were filled with 500 cm^3 of experimental nest-material (autoclaved or non-autoclaved according to the assigned experimental treatment) mixed homogenously with a 500 cm^3 of commercial sawdust (Allspan® Animal bedding, wood shavings). As new nest-boxes replaced old ones, experimental treatments were sequentially alternated and appropriate nest-material added. This procedure was performed wearing new latex gloves for each nest-box to avoid cross-contamination between experimental and control nests materials. A total of 86 and 69 new nest-boxes were arranged in the study area in 2017 and 2018 respectively.

Nest-boxes were inspected every four days, from early March to the end of June, which allowed us to estimate the start of laying when a nest-box was detected with hoopoe eggs. Nest-boxes where visited again 17 days after the start of laying, and daily afterwards, to detect hatching date, which is expected to occur 18 days after the start of

incubation (Martín-Vivaldi et al., 2014). Four days after the first egg hatched, we collected nest-material in contact with chicks for bacterial analyses. Samples were stored in previously sterilized 1.5 ml microfuge tubes and kept in a portable fridge at 4º C until being processed in the laboratory within the following 12 hours. Nest-boxes were again visited when the older nestling were eight and nineteen days old. During these two last visits, we measured body mass of all nestlings with a hanging scale (Pesola 0-50 and 0-100 g, depending of the nestling age, accuracy 1 g), and estimated the intensity of ecto-parasitism by *C. hemapterus* as the number of spots due to blood remains on the skin of the belly and left under-wing of nestlings. We performed all manipulations with new latex gloves cleaned with 70% ethanol to prevent cross-nest contamination.

c. Laboratory procedures

Nest bacterial density was estimated by means of traditional culture methods. Briefly, under sterile conditions, we included 1 cm^3 of nest-material in a sterile Falcon tube with 1 ml of sterile sodium phosphate buffer (PBS, 0.2M; pH = 7.2). The Falcon tubes were vigorously vortexed to homogenize samples. Serial dilutions up to 10^{-4} were cultivated by spreading 5 microliters of each dilution in plates with Tryptone Soya Agar (TSA); a broadly medium used to grow mesophilic bacteria. The plates were incubated aerobically at 37 °C for 24 hours before colony counting. Bacterial counts were performed in plates of the serial dilution with around 30-300 colonies. Estimates of bacterial loads were standardized to number of colony forming units (CFU) per ml (number of colonies x $10^{\text{dilution factor}}$) / 0.005 ml spread).

d. Statistical Analyses

We were interested in detecting expected influences of autoclaved nest-material from previous reproduction on hoopoe nestling parasitism and, thus, we only considered the first breeding attempt detected in each of the experimental nest-boxes.

Nest bacterial density at the early nestling stage (4 days after hatching) approached a normal distribution after log transformation (Kolmogorov-Smirnov test for continuous variables, $P > 0.05$). Intensity of ecto-parasitism of nestlings 8 days after the first egg hatched followed approximately a Gaussian distribution after square root transformation (Kolmogorov-Smirnov test for continuous variables, $P > 0.05$). Fledging success, estimated as the number of alive nestling in the second visit divided by brood size at the

first visit was not transformed before analyses and was used in percentage. Residuals of all statistical models followed normal distributions.

The effect of experimental treatment on nest bacterial density was explored in a general linear model (GLM) that also included study year as fixed discrete factor and laying date and number of nestling as covariables. The experimental effect on ecto-parasitism intensity was explored in a similar GLM that also included nest-material bacterial loads as an additional independent covariable because the hypothesis tested posits that parasitism should be mediated by the nest bacterial environment. Finally, the experimental effect on fledging success was explored in a similar GLM that also included intensity of parasitism as an additional independent covariable. For all these GLMs, we estimated variance explained by the whole model (R^2), and first order interactions between experimental treatments and all others independent factors. These interactions were explored in separate models that also included main effects. In no case brood size explained a significant proportion of variance of dependent variables, either alone, or in interaction with experimental treatment (not showed results). Moreover, considering brood size as additional independent factor did not influence statistical inferences qualitatively (i.e. statistical significance) (results not showed). Thus, we here presented results from models that did not include brood size as independent factor.

All these analyses were performed in R environment (R 3.4.3, https//www.r-project.org/), with library "glm" (Venables & Ripley, 2013).

3. Results

During reproduction, at the early nestling stage, hoopoe nest-boxes with nest-material that was autoclaved before reproduction started tended to harbor lower bacterial densities than hoopoe nest-boxes that were filled with control nest material. That was the case after controlling for the statistically significant effects of laying date, and the study year (Table 1). Interestingly, the detected trend (although non-significant) was consistent between study years (see interaction in Table 1).

With respect to ectoparasitism by *C. hemapterus* flies, hoopoe nestlings developing in nest-boxes with autoclaved material presented lower ecto-parasite loads than those in nest-boxes with control material (Table 1). This experimental effect depended on the study year and nest bacterial loads (Table 1). The effect was stronger in 2018 (Fig. 1), when the expected positive association between ecto-parasitism and nest

bacterial load appeared exclusively in experimental nests (Fig. 1). These patterns were detected after controlling for the positive association between laying date and parasitism intensity (Fig. 1). The expected effects in parasitism intensity were therefore more clearly detected in the year when *C. hemapterus* pupae were removed from nest-materials.

Finally, fledging success of experimental and control nests did not differ significantly (Table 1), but bacterial density of hoopoe nests eight days after hatching resulted positively associated with fledgling success, even after controlling for the non-significant effects of experimental treatment, study year, laying date and intensity of parasitism (Table 1). This positive association was also detected after excluding from the models non-significant variables.

Table 1. Results from General Linear Models exploring the effect of autoclaving nest-material (Treatment) of nest-boxes where hoopoes bred on: log10-transformed bacterial loads of experimental nest-boxes during hoopoes' reproduction, intensity of parasitism (i.e., square root transformed number of bites), fledging success (percentage). We show Least square means (SE) for experimental (Mean 1) and control (Mean 2) nests (first line of each model tested), and for year 2017 (Mean 1) and 2018 (Mean 2) (second line of each model tested). For continuous predictors (laying date, nest-material bacterial load (Bact Load) and intensity of ecto-parasitism (Int parasitism), we show beta (SE) values. The first order interactions between experimental treatment and the other independent factors was tested in separate models that also included main effects, while the main effects were explored in models that do not include interactions. Statistical effects with associated alpha-values lower than 0.1 are highlighted in bold fonts.

				Main effects			Interaction with experimental treatment		
	Mean1 (SE)	**Mean2 (SE)**	β (SE)	F	df	P	F	df	P
Bacterial loads of nest-material (R^2 = 0.49, F = 29.86, df = 3, 94, P < 0.001)									
Treatment	**5.57 (0.18)**	**5.96 (0.17)**		**3.31**	**1, 94**	**0.072**			
Year	**5.05 (0.09)**	**6.68 (0.18)**		**63.35**	**1, 94**	**0.001**	0.23	1, 92	0.630
Laying date			**0.01 (0.01)**	**4.90**	**1, 94**	**0.029**	0.94	1, 92	0.760
Intensity of ecto-parasitism (R^2 = 0.20, F = 4.90, df = 4, 79, P = 0.001)									
Treatment	**2.39 (0.22)**	**3.33 (0.23)**		**4.77**	**1, 79**	**0.031**			
Year	**2.91 (0.18)**	**2.75 (0.29)**		**3.30**	**1, 79**	**0.073**	**8.31**	**1, 76**	**0.005**
Laying date			**0.02 (0.01)**	**6.05**	**1, 79**	**0.016**	0.78	1, 76	0.378
Bact Loads			0.24 (0.17)	2.12	1, 79	0.149	**5.21**	**1, 76**	**0.025**
Fledging success (R^2 = 0.06, F = 2.04, df = 5, 78, P = 0.083)									
Treatment	70.25 (4.45)	59.95 (4.90)		0.55	1, 78	0.462			
Year	59.39 (4.49)	72.92 (4.74)		0.00	1, 78	0.962	0.96	1, 74	0.331
Laying date			-0.20 (0.15)	1.82	1, 78	0.181	1.66	1, 74	0.202
Bact load			**6.01 (2.53)**	**5.63**	**1, 78**	**0.020**	0.03	1, 74	0.855
Int parasitism			-0.89 (1.68)	0.29	1, 78	0.596	0.46	1, 74	0.501

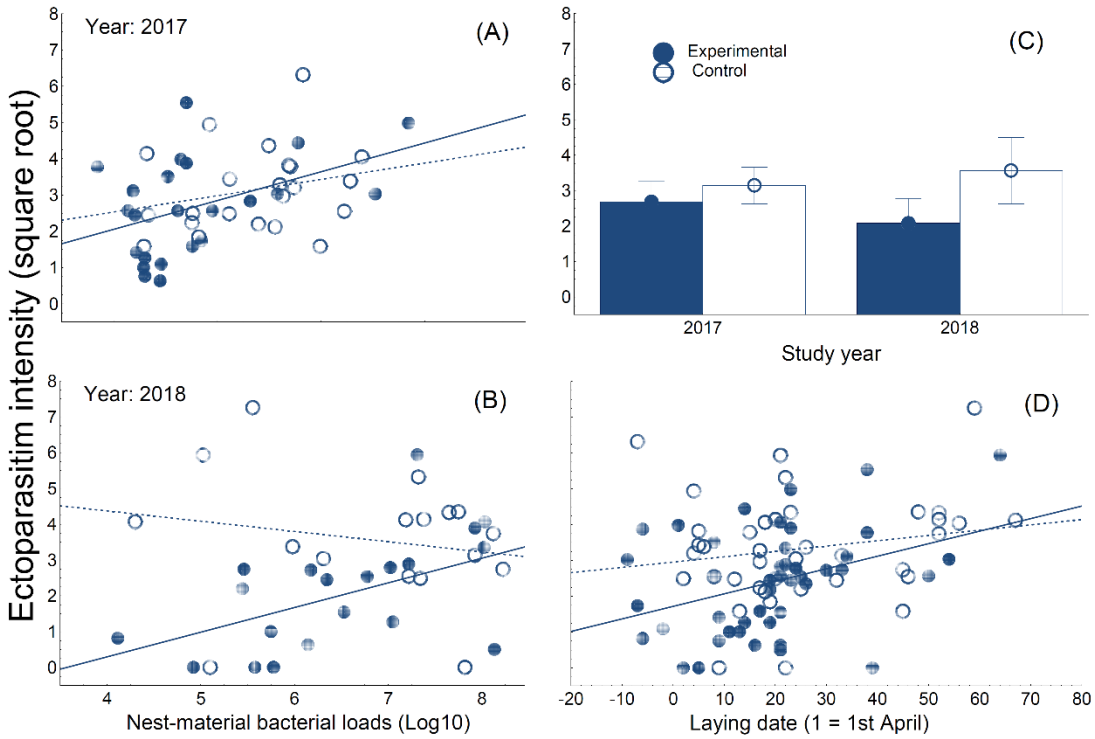

Figure 1. Associations between ecto-parasitism intensity of hoopoe nestlings and bacterial loads of nest-materials that were (filled dots) or were not (open dots) autoclaved before reproduction started in 2017 (A) and 2018 (B) study years. We also show weighted means (± 95% CI) of ecto-parasitism intensity suffered by hoopoe nestlings developing in nest-boxes with nest-material that were or were not autoclaved in 2017 and 2018 breeding seasons (C). Finally, (D), we also show the association between ecto-parasitism intensity and laying date of hoopoe nests with autoclaved or not autoclaved nest-material. Solid and dashed lines are regression lines for experimental and control nest-boxes.

4. Discussion

Our main results show that autoclaving nest-material reduced the intensity of ecto-parasitism suffered by nestling hoopoes. Moreover, in the case of nest-boxes with autoclaved nest-material, but not in the control nest-boxes, ecto-parasitism intensity resulted positively related to bacterial density. In addition, bacterial density of hoopoe nest-material was positively related to fledging success. Finally, we found that nest-boxes with experimental autoclaved nest-material tended to have lower bacterial density than those with control nest-material; although not significantly. All these results, considered together, suggest a role for bacterial environment due to nest-material influencing ecto-parasitism and fledging success in hoopoes. Below, we discuss this inference, alternative hypotheses, and possible mechanisms that could fit our experimental results.

Ecto-parasites might use different sensory channels, such as visual, auditory or olfactory, to detect nests of their avian hosts (Takken & Knols, 1999; Tomás & Soler, 2016). However, *C. hemapterus*, the main blood sucking ecto-parasite of hoopoes in our study area, mainly parasitizes hole-nesting birds and, thus, the most likely is that ecto-parasites detect host nests follow chemical cues. Although we have not measured chemical volatiles associated to experimental hoopoe nests, our results, including the detected experimental effect on intensity of ecto-parasitism, and the association between bacterial density of nest-material remains and intensity of ecto-parasitism, suggest that chemical cues hypothetically used by ecto-parasites should be mediated by bacterial metabolism.

The detected effect of autoclaving nest-material remains on ecto-parasitism intensity might be directly mediated by its effect on bacterial densities of nest-material, or indirectly by the effect of experimental nest-material on the bacterial community of the uropygial gland of hoopoe females. The uropygial gland of incubating or brooding hoopoe females is full of volatile producing bacteria (Martín-Vivaldi et al., 2010) that *C. hemapterus* flies might use to detect active nests. Our experiment might have affected the bacterial community of the uropygial gland and hence the amount and diversity of volatiles in the nest-box and surroundings. However, previously published experimental evidences strongly suggest that the presence of nest-material remains from previous breeding seasons influences the bacterial community of the uropygial secretion of females (Díaz-Lora et al., 2019). Therefore, given the strong association between the bacterial community and the volatile profile of the uropygial secretion (Martín-Vivaldi et al., 2010), it is likely that the detected effect on intensity of ecto-parasitism was mediated by changes produced in the bacterial community of the uropygial gland. The effect of nest bacterial environment on the bacterial community of the uropygial gland surroundings (Whittaker & Theis, 2016) or in the volatile profiles of the secretion (Jacob et al., 2014) has also been detected in dark-eyed juncos (*Junco hyemalis*) and great tits (*Parus major*), respectively. Thus, it is possible that the detected influence of nest-material bacterial community on ecto-parasitism intensity in hoopoes could be extrapolated to other bird species.

Our experimental approach assumed that autoclaving nest-material remains before reproduction started should affect the nest bacterial community at the nestling stage. However, although bacterial density in nest-material of nest-boxes under the

control treatment tended to be higher than that of nest-boxes under the experimental treatment, differences only approached statistical significance. It is worth to mention here that using a similar experimental approach, Díaz-Lora and coauthors (Díaz-Lora et al., 2019) found that bacterial loads on hoopoe eggshells in nest-boxes with nest bacterial remains mixed with sawdust was significantly higher than that of eggshells in nests boxes with only sawdust. Thus, it is possible that the expected experimental effect on nest bacterial density was more easily detected at the egg stage. Contrary to most avian species (Ibáñez-Álamo et al., 2017), hoopoe parents do not remove fresh nestling feces from nests, which could produce an increase in bacterial density after hatching, as has been shown for starlings (Azcárate-García et al., 2019). Bacterial samples were collected 4 days after the egg hatched and, thus, the effects of autoclaving nest-material could have been masked or diluted by nestlings' activity. Information on bacterial communities of nest-material, of uropygial secretion of females and on the associate volatile profiles are however necessary to explore the possibility that it explain the detected experimental effect on ecto-parasitism intensity.

In a similar experimental approach, Podofillini and coworkers (Podofillini et al., 2018) used new nest boxes where they included sand collected from the nest surrounding that were or were not mixed with old nest-material from lesser kestrel (*Falco naumanni*) previous reproduction. Consistent with our results, they found out that nestlings living in nests with materials from previous breeding seasons suffered from *C. hemapterus* more than those living in nest-boxes filled only with sand. However, these results do not necessarily have to be the consequence of bacteria in the experimental nest-material, but rather of the emergence of adult ecto-parasites from overwintering pupae in the used nest material. Trying to differentiate between these two possibilities, we screened nest-material collected during 2018, and eliminated all *C. hemapterus* pupae. In 2017, when we did not eliminate *C. hemapterus* from nest materials, levels of parasitism were similar between experimental and control groups. Not only because of the effects of volatiles from nest-material, but also those from *C. hemapterus* emergence from nest-material that was not autoclaved nor screened for parasites pupae, should influence parasitism positively, differences between control and experimental nests should therefore be higher in 2017. However, contrary to this possibility, the expected experimental effect was clearer in the year in which *C. hemapterus* pupae were removed from nest-materials (i.e. 2018). Thus, it is unlikely that overwintering *C. hemapterus* pupae in nest-material

remains contributed to explain our results. Consequently, the higher intensity of ecto-parasitism of nestlings in control nests in 2018 is the consequence of the experimental treatment.

Interestingly, and consistent with a role of bacteria explaining ecto-parasitism of nestlings, nest-material bacterial loads and ecto-parasitism intensity resulted positively related. Furthermore, although the strength of this association was similar in 2017, the association between nest-material bacterial load and parasitism was absent for control but not for experimental nests in 2018. We do not have a plausible explanation for this result. We only may speculate with the possibility that, *C. hemapterus* clue on volatiles from particular bacterial strains that hoopoe females acquire when breeding in nests-boxes with material remains from previous reproductions (Díaz-Lora et al., 2019). This is a possibility to be explored in future works. An alternative possibility is that parasite activity influences bacterial density of hoopoe nests. We know that *C. hemapterus* parasitize incubating birds and that its activity influences eggshell bacterial loads positively (Tomás et al., 2018). Consequently, higher bacterial density would be expected in nests where *C. hemapterus* feeding on nestlings are more abundant. Another alternative hypothesis explaining our results is related to the possibility that *C. hemapterus* follows chemical cues from flies already present in hoopoe nests. However, for one of the study years, we removed *C. hemapterus* pupae from all collected nest-material and nest-boxes were newly installed, and hence, none of the parasitizing flies in experimental or control nests came from nest material that study year. Thus, because all parasitizing flies in our nest boxes should have come from other nests, chemicals from previous *C. hemapterus* activity would hardly explain the detected experimental effects in ecto-parasitim intensity. Thus, experimental manipulation of *C. hemapterus* flies in hoopoe nests is necessary to further concluding that bacteria are the cause of the detected association with parasitism intensity.

Contrary to our expectation, fledging success and bacterial loads of nest-material resulted positively associated. Nest bacterial loads are usually negatively related to breeding success in birds (Jacob et al., 2015; Peralta-Sánchez et al., 2018; Soler et al., 2017) but not in hoopoes (Díaz-Lora et al., 2019), which might be related to the special life style of this species. Hoopoes do not remove nestling feces and harbor symbiotic bacteria in their uropygial gland at a high density (Soler et al., 2008). Some of the symbionts produced antibiotic substances (Martín-Platero et al., 2006; Ruiz-Rodríguez et

al., 2013, 2009, 2012; Soler et al., 2008) that might protect growing nestlings and, thus, we speculate with the possibility that these antibiotic producing bacteria were more abundant in nests with higher bacterial density and explain the positive detected association. Experimental modification of the uropygial gland bacterial community (e.g. by injecting antibiotics (Martín-Vivaldi et al., 2010)) is however necessary to explore this possibility.

5. Conclusions

In summary, our experimental results linking the nest bacterial communities and ecto-parasitism suggest that *C. hemapterus* flies follow chemical cues to find active nests of their hoopoe hosts, and that these chemical volatiles are produced by bacteria growing in nest-material or in the uropygial gland of females. Characterization of the volatile profiles of hoopoe nests and secretions, as well as those of the associated bacterial communities of experimental and control nests are necessary to further explore mechanisms explaining the detected lower parasitism intensity of nestling developing in nests with autoclaved nest-materials.

DECLARATIONS

Author Contributions: Conceived and designed the experiments: J.J.S. and M.M.V. Fieldwork: M.M.A, J.J.S., and M.M.V. Contributed reagents/materials/analysis tools: M.M.A, J.J.S., J.M.P.S., and M.M.V. M.M.A. wrote the first version with the supervision of J.J.S. All authors substantially contributed to final version.

Funding: "This research was funded by Ministerio de Ciencia, Innovación y Universidades and European Regional Development Fund (FEDER) (CGL2017-83103-P).

Acknowledgments: We thank Natalia Juárez García-Pelayo and Manuel Azcárate-García for assistance in field and laboratory work. The research group benefits from facilities, including an apartment, provided by the city hall of Guadix where a small lab to quickly proceed the samples was installed.

Conflicts of Interest: "The authors declare no conflict of interest."

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CAPÍTULO III. *Volatiles of symbiotic bacterial origin explain ectoparasitism and fledging success of hoopoes*

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Revista: *Enviado a Animal Microbiome* (en segunda revisión)

Volatiles of symbiotic bacterial origin explain ectoparasitism and fledging success of hoopoes

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Abstract

Background: Some parasites use olfactory cues to detect their hosts. Since bacterial symbionts are partially responsible for animal odors, they could also determine parasitism of their hosts. By experimental manipulation of the microbiota of hoopoe (*Upupa epops*) nests before reproduction started, we explored the hypothetical links between symbiotic bacteria and volatiles, and between both of them and parasitism. During the nestling stage, we estimated level of ectoparasitism by chewing lice in females and by *Carnus haemapterus* flies in nestlings, characterized microbial communities (from nest materials and uropygial secretions) and volatile profiles (from nest environments and uropygial secretions).

Results: Nests with autoclaved nest material had less diverse bacterial communities, more diverse volatile profiles, and their occupants experienced lower intensity of

parasitism than those in control nests. The experiment also affected beta diversity of the microbial communities of nest material and of the volatiles of the nestling uropygial secretions. Moreover, microbial communities of uropygial secretions and of nest materials covaried with their volatile profiles, while the volatile profile of secretions explained nest volatile profile. Finally, some of the volatiles and bacteria detected in the nest material and secretions were associated with ectoparasitism intensity of females and nestlings, and with fledging success.

Conclusions: These results support the links between microbial communities and animal odors, and emphasize that the associations between bacteria and both ectoparasitism and reproductive success are partially mediated by volatiles of bacterial origin. Future work should focus on mechanisms underlying the detected patterns.

Keywords: Animal odor, Avian microbiota, *Carnus hemapterus*, Eavesdropping parasites, Fermentation hypothesis, Uropygial secretion

1. Background

Symbiotic microorganisms are essential for understanding the evolution and functioning of their animal hosts (Archie & Theis 2011; McFall-Ngai *et al.* 2013; Wang *et al.* 2018; Sherwin *et al.* 2019). For instance, bacterial symbionts are partly responsible for the emission of volatiles that generate animal odors (Archie & Theis 2011; Engl & Kaltenpoth 2018) . Thus, these microorganisms may play key roles in animal communication, including the use of inadvertent social information by conspecifics and heterospecifics (Ezenwa & Williams 2014; Carthey, Gillings & Blumstein 2018; Maraci, Engel & Caspers 2018; Mazorra-Alonso, Tomás & Soler 2021).

The possible role of bacteria determining animal odors and informing on animal characteristics was first posited within the fermentation hypothesis (Albone *et al.* 1974; Gorman, Nedwell & Smith 1974). This hypothesis was initially restricted to odors derived from mammalian secretions used in chemical communication, but it is currently extended to secretions of other animal taxa (Ezenwa & Williams 2014; Carthey, Gillings & Blumstein 2018). Exocrine glands of animals determine their odor profile (López, Amo & Martín 2006; MacDonald *et al.* 2008; Fraker *et al.* 2009; Barcellos *et al.* 2011; Whittaker *et al.* 2011; Baeckens *et al.* 2018). As exocrine glands provide suitable environments for bacterial growth (Sin *et al.* 2012; Theis *et al.* 2013; Buesching *et al.*

2016; Leclaire *et al.* 2017; Maraci, Engel & Caspers 2018), odor profile could be produced directly by the host, by bacteria that inhabit the gland, or by both. In birds, the only exocrine gland is the uropygium, and its secretion is rich in volatiles (Jacob & Ziswiler 1982) that, at least partially, could be by-products of the metabolism of bacterial symbionts. In accordance, hoopoes (*Upupa epops*) (Martin-Vivaldi *et al.* 2010), dark-eyed juncos (*Junco hyemalis*) (Whittaker & Theis 2016), and song sparrows (*Melospiza melodia*) (Grieves *et al.* 2021) host bacteria in their uropygial glands, that produce key volatiles that are apparently involved in chemical social communication (Whittaker & Theis 2016; Whittaker *et al.* 2019). A relationship between bacterial community and volatile profiles of the uropygial secretion is therefore a key prediction of the hypothesized role of bacteria in animal odors. In spite of the evidence supporting this prediction in different taxa, including mammals (Theis *et al.* 2013; Leclaire *et al.* 2017), insects (Dillon, Vennard & Charnley 2002; Sharon *et al.* 2010; Engl & Kaltenpoth 2018; Schmidtberg *et al.* 2019), amphibians (Brunetti *et al.* 2019), and birds (Law-Brown & Meyers 2003; Martin-Vivaldi *et al.* 2010; Whittaker *et al.* 2019), the role of microorganisms mediating the odors of avian nests or uropygial secretions has rarely been explored (but see Martin-Vivaldi *et al.* 2010; Jacob *et al.* 2018; Grieves *et al.* 2021).

The hypothetical informative value of volatiles of bacterial origin implies that they might be involved in animal communication, either as signals (sensu Maynard-Smith & Harper 2003), or as Inadvertent Social Information (ISI, sensu Danchin *et al.* 2004) that would be of interest for con- and hetero-specifics (Ezenwa & Williams 2014). Communities of symbiotic bacteria, including the gut microbiota, are usually related to phenotypic condition, immune-state, physiology and behavior of their animal hosts (Sherwin *et al.* 2019). Then, particularities of the volatile profiles of bacterial origin would inform on these or other host traits. Accordingly, particular volatiles of bacterial origin are related to host sex, age, social status, or even group membership of individuals in some taxa (Theis *et al.* 2016; Leclaire *et al.* 2017; Whittaker *et al.* 2019; Grieves *et al.* 2021), which is valuable information for interacting conspecifics. In addition, hetero-specifics, as most ectoparasites and some predators, use animal odors as cues to detect and select their victims (Bowen 1991; Takken & Knols 1999; Zwiebel & Takken 2004; Reneerkens, Piersma & Damste 2005; Poldy 2020); some of those odors are possibly produced by bacterial symbionts (Archie & Theis 2011; McFall-Ngai *et al.* 2013; Ezenwa & Williams 2014; Mazorra-Alonso, Tomás & Soler 2021). In accordance with this

scenario, the experimental modification of the microbiota of nest materials determine the volatile profiles (i.e., odors) of great tit (*Parus major*) nests (Jacob *et al.* 2018), the intensity of ecto-parasitism by *Carnus hemapterus* suffered by hoopoe nestlings (Mazorra-Alonso *et al.* 2020), and the probability of nest predation experienced by spotless starling (*Sturnus unicolor*) nestlings (Azcarate-Garcia *et al.* 2019). Volatiles of symbiotic bacterial origin might also act as repellent of ectoparasites and predators, indicating that their effects on host-parasite interactions are complex (Tomás *et al.* 2020). For instance, depending on the considered skin bacteria of humans and their produced volatiles, they can attract or repel mosquitoes (Verhulst *et al.* 2010; Verhulst *et al.* 2011). This scenario leads to the hypothesis that volatiles produced by bacterial communities are partially determining the intensity of parasitism and/or the probability of predation of their animal hosts. Therefore, host traits favoring bacterial symbionts that reduce the strength of selection pressures imposed by predators and parasites on their hosts would be adaptive.

Hoopoe nest environment, the bacterial symbionts in the uropygial glands of females and nestlings, and the microbial communities in the nest materials, is an appropriate biological scenario to test the hypothetical associations between bacterial communities and volatile profiles, and the influence of each of them on risk of parasitism. In fact, the hoopoe is one of the few species where a link between the complex symbiotic bacterial community present in their uropygial gland (Rodriguez-Ruano *et al.* 2015; Martín-Vivaldi *et al.* 2018; Rodriguez-Ruano *et al.* 2018) and the volatile components of their secretion has been experimentally demonstrated (Martin-Vivaldi *et al.* 2010), which support the expected link between symbiotic bacteria and animal odor. Moreover, hoopoes do not build nests and, for reproduction, they prefer cavities with soft-material remains from previous reproductive events of conspecifics or heterospecifics (Diaz-Lora *et al.* 2019). In addition, because the mutualistic association between hoopoes and the antibiotic producing bacteria of their uropygial gland only appeared during the nesting phase (i.e. within nest cavities) (Soler et al. 2008), nest material from previous reproduction may affect bacterial community of the uropygial gland; a possibility that has recently received experimental support (Diaz-Lora *et al.* 2019). This known association, therefore, suggests that microbial communities of uropygial secretion and of old nest-materials are related to each other. Finally, and more importantly, we know that the sterilization of the old-nest material before reproduction started not only affected the

bacterial density of the hoopoe nests, but also the intensity of ectoparasitism suffered nestlings (Mazorra-Alonso *et al.* 2020). Therefore, considering current knowledge on the associations between bacterial communities of hoopoe nest environments and risk of parasitism, demonstrating a link between bacterial communities and volatiles of nests, and between these two components and intensity of ectoparasitism is a strong test of the hypothesis that volatiles produced by bacterial communities are partially determining the intensity of parasitism of their host. In other words, finding evidence of such associations will strongly suggest that the previously detected effects of microbial symbionts on risk of parasitism are mediated by their production of volatile substances.

Our objective is to explore whether the expected links between bacterial communities and volatiles, and between bacterial and volatile profiles and risk of parasitism, in hoopoe nest environments. With this purpose, we experimentally manipulated the bacterial environment of hoopoe nest by installing new nest-boxes added with old nest material that were (experimental) or were not (control) previously autoclaved and exploring its effects on bacterial communities and volatile profiles of nest material and of uropygial secretion of females and nestlings. Non-manipulated old nest-boxes were employed as a second control (i.e., natural) nests. Additionally, we explored (ii) the associations between bacterial communities of the nest material and volatile profiles of nest-box environment and of uropygial secretions of females and nestlings. Similarly, we also explored (iii) the associations between volatiles of nest-box environment and those of the uropygial secretion of females and nestlings. These associations will support the assumed links between bacterial communities and volatiles associated to nest environments. As the preen gland of nestlings usually start to produce secretion sixth-seventh day after hatching (Soler *et al.* 2008), and the secretion harbors volatile producing bacteria at high density (Martín-Vivaldi et al. 2010), we explored the expected associations with information collected in hoopoe nests at the beginning and at the end of nestling stage, when nestlings have develop fully their preen gland.

The second part of the hypothesis is that microbial communities, throughout their effects on volatiles of the nest environments, are partially responsible of the risk of parasitism experienced by nesting birds (e.g. nestling and female hoopoes) and, thus, of reproductive success. To explore these links, we estimated intensity of parasitism of nestling and females by the hematophagous flies *Carnus hemapterus* and by chewing lice, respectively. As *Carnus hemapterus* likely use odors to search and locate active nests

(Calero-Torralbo, Václav & Valera 2013), and its parasitism likely affect fledging success (Hoi *et al.* 2018), we explore the association between characteristics of the bacterial communities and of volatile profile of the nest-box environments and (iv) ectoparasitism and (v) fledging success of hoopoes.

2. Material and Methods

a. Study area and species

The study area was located in the Hoya de Guadix, (Granada, Southern Spain, 37°18´N, 38°11´W), a plateau at 1000 m a.s.l. with semiarid climate, where around 300 cork-made nest-boxes were available for wild birds; most of them attached to tree trunks and walls, but also hidden in piled stones. The dimensions of nest-boxes were 35 × 18 × 21 cm (internal height × width × depth), 24 cm (bottom-to-hole height) and 5.5 cm (entrance diameter). Information about the study area is further described elsewhere (Martín-Vivaldi *et al.* 1999; Diaz-Lora *et al.* 2019).

The hoopoe (Order Upupiformes) is a migratory species, distributed throughout Europe, Asia, and Africa (Reichlin *et al.* 2009; Reichlin *et al.* 2013; van Wijk *et al.* 2018). Hoopoes are hole-nesters that frequently use artificial nest-boxes for reproduction or natural cavities in trees or walls. They do not build nests and prefer cavities with remains of soft material from previous reproductive events of conspecifics or heterospecifics (Diaz-Lora *et al.* 2019), where females create a small hollow and lay the clutch (Martin-Vivaldi *et al.* 2014). Females rarely leave the nest during the incubation and hatching periods. During these periods, females are fed by males, and, afterwards (8-10 days after the first egg hatches) females reduce the time spent inside the nest and, together with males, provide food to their chicks (Martin-Vivaldi *et al.* 2014). In our study area, the reproductive season starts in late February, and extends until late July, with females usually laying two clutches.

The most abundant ectoparasite of hoopoe nestlings in our study area is *Carnus hemapterus*, a common generalist hematophagous parasitic fly of about 2 mm in length (Martín-Vivaldi *et al.* 2006; Calero-Torralbo, Václav & Valera 2013). Chewing lice (Mallophaga) are frequently detected in nesting females, but not in nestlings. These parasites feed by chewing soft areas of the feathers and skin, sometimes causing bleeding at the sucking spots. In hoopoes, chewing lice are common on the feathers of the crest where they are more protected from bird preening (Mester 1977; Agarwal *et al.* 2011).

b. Fieldwork and experimental design

Fieldwork was carried out during 2017 and 2018. Before reproduction started (i.e. beginning of February), we visited nest-boxes where hoopoes successfully bred the previous year. We collected old nest materials in plastic bags with holes plastic bags with holes to maintain nest material in aerobic conditions (47 nest-boxes in 2017 and 70 in 2018) that were maintained at room temperature in the lab. Nest materials from different nest-boxes were pooled, mixed, and divided in two halves. In order to avoid any possible influence of previous reproduction on bacteria communities and volatile profiles, we installed new nest-boxes (86 in 2017 and 69 in 2018) that many times substituted old ones. New nest-boxes were sequentially assigned to one of the two experimental treatments, adding 500 cm^3 of nest material from previous reproduction, that were (experimental) or were not autoclaved (control), mixed with 500 cm^3 of commercial sawdust (Allspan® Animal bedding, wood shavings). This procedure was performed by wearing new latex gloves for each nest-box to avoid cross-contamination between experimental and control nests materials. We also considered a third group of old nest-boxes (i.e., natural nests) that were neither new nor manipulated, and where hoopoes bred the year of sampling (N =13 in 2017 and N =14 in 2018). We only considered first breeding attempts.

During reproduction, from early March to the end of June, both new (i.e., experimental and control) and old (i.e. natural) nest-boxes in the study area were visited every four days until eggs were found. Hoopoes lay one egg per day and clutch size is typically of no less than seven eggs. This nest-box checking schedule allowed us to determine laying date (i.e. that of the first egg). Nest-boxes where inspected again 17 days after the onset of egg laying, and daily afterwards until detecting hatching date (day 1 of the nestling period), which is expected to occur 17 days after the onset of incubation (Martín-Vivaldi *et al.* 2016). During the nestling stage, nests were visited several times to sample nest materials, volatiles of the nest-box environment and uropygial secretions, as well as to record parasitism intensity of adult females and nestlings (ESM Table S1). For collecting each of these samples within a nest, we wore new latex gloves previously cleaned with 96% ethanol to avoid contamination between samples.

c. Bacterial, volatile and ectoparasite sampling

For bacterial community analyses, approximately 10 g of the nest material in contact with nestlings was collected twice in 15 mL Falcon tubes, on days 4 and 15 after the first egg hatched (ESM Table S1). These samples were stored in a portable fridge until being frozen at -20 ºC in the laboratory within the same day of collection. Volatile compounds of the nest-box environment were also sampled twice, on days 7 and 18 after the first egg hatched (ESM Table S1).

Volatiles were captured in Solid Phase Microextraction (SPME) fibers. Each fiber was installed at one side of the nest box, about 7 cm over the nest material, while the sensitive fiber end was protected with a two-side opened glass pipette tip and exposed to the nest-box environment during 24 hours. Afterwards, the fiber was removed from the nest and the fiber end introduced into a sealed glass vial, kept cold (0-4 ºC), and stored within the same day at -20 ºC until gas chromatography-mass spectrometry analysis. Storage of samples never exceeded one week. After the analyses, SPME fibers were re-conditioned (i.e., all chemical trace eliminated) following supplier instructions, i.e. 1hour at 270°C using the GC injector and afterwards, fibers were kept at -20 ºC until they were reused in the field.

Uropygial secretions were collected on days 4 (females), 8 and 19 (nestlings) after the first egg hatched (ESM Table S1). Briefly, before sampling, the uropygial gland and surroundings were cleaned with a cotton swab soaked in 96% ethanol. Afterwards, we used an automatic 1-10 µL micropipette and gently introduced the sterilized tip into the papilla of the uropygial gland and pipetted the secretion. At least 5µL of secretion from females or close-to-fledge nestlings was placed in a sterile 1.5 mL microcentrifuge tube for bacterial DNA analyses. In addition, 10 µL were transferred to 10 ml SPME sealed glass vials for analyses of the volatile profile. Since we were interested on the chemical profile of nests, we sampled a single nestling per nest (usually the largest one) and, when not reaching 10 µL volume, we completed with the secretion of other siblings. Samples were kept cold in a portable fridge and then stored at -20 ºC in the laboratory until DNA extraction and chemical volatile analyses.

Ectoparasitism of females was estimated on day 4 after the first egg hatched by counting the number of chewing lice on the crest feathers, which is a good proxy of parasitism intensity (Agarwal et al., 2011; Mester, 1977). Ectoparasitism of nestlings was estimated twice, when the older nestling was 8 and 19 days old (ESM Table S1). These

estimates consisted of counting the number of spots due to feces and blood remains on the belly skin and left underwing of all nestlings. These spots are traces of *C. hemapterus* parasitism, reflect the abundance of ectoparasites and, thus, the intensity of parasitism in avian nests (López-Rull, Gil & Gil 2007; Tomas *et al.* 2018). We used mean values per nest in the analyses. Finally, fledging success was estimated as the percentage of nestlings that survived from day 8 after hatching of the first egg until day 19.

d. DNA extraction and high-throughput sequencing

Bacterial DNA from secretions and nest material were extracted using the FavorPrep Blood Genomic DNA Extraction Kit (Favorgen Biotech) and MSOP protocol (Martín-Platero *et al.* 2007), respectively. For secretions, and according to manufacturer´s instructions, we added a lysozyme pre-treatment (10 mg/mL of lysozyme at 37ºC for 30 min) to ensure the DNA extraction of Gram positive bacteria (Rodriguez-Ruano *et al.* 2018). Nest material samples were solid and 80 mg were used for DNA extraction. First, samples were suspended with 900 µL of buffer lysis. The liquid phase was then separated from the solid content and kept in different 2 mL microfuge tubes (for further details of the followed protocol see Lee et *al.*, 2021). Extracted DNA from nest material samples was cleaned using the kit One Step PCR Inhibitor Removal Kit (Zymo Research). We also processed laboratory blanks to detect possible contamination during the process.

DNA sequences from nest materials and uropygial secretions were obtained by Illumina high-throughput sequencing of a fragment of approximately 400 bp of the 16S rRNA V6-V8 hypervariable regions. In a first PCR, 16S rRNA gene were amplified using universal primers B969F (5´ - ACGGGCRGTGWGTRCAA - 3´) and BA1406R (5´ - ACGGGCRGTGWGTRCAA - 3´). In a second PCR, samples were amplified adding barcodes for identifying samples. Afterwards, the libraries were sequenced in a single run of Illumina MiSeq sequencer (2 x 300 bp output mode). Sequencing was carried out at the Integrated Microbiome Resource (IMR), University of Dalhousie (Canada). Sequences are available at NCBI under accession numbers: BioProyect ID: PRJNA847390; SUB 11518282 (nest) and BioProyect ID: PRJNA847428; SUB 11582000 (secretion).

Raw sequences were analyzed using QIIME2 2019.10 (Bolyen *et al.* 2019). Briefly, primers were trimmed and, due to low quality of the reverse sequences, analyses were based on forward sequences. Sequences were quality filtered and the Deblur

algorithm was employed to produce an Amplicon Sequence Variants table (ASV table) establishing a sequence size of 220 bp. Afterwards, a phylogenetic tree was built using the fragment insertion algorithm (Janssen et al., 2018). Taxonomic assignation was performed against Greengenes13_8 database at 97% similarity (DeSantis *et al.* 2006; MacDonald *et al.* 2008). Chloroplast, mitochondria and non-phylum assigned ASVs were removed.

The sequencing of the bacterial community of nest material produced 11 566 822 sequences and 3 349 977 were retained in the ASV table after filtering (number of samples = 161, average number of sequences per sample (min, max) = 20 807.31 (2 930, 55 978)). The sequencing of uropygial secretion samples produced 17 866 248 sequences and 11 385 161 were retained in the ASV table after filtering (number of samples = 346, mean average number of sequences per sample (min, max) = 32 905.1 (3 817, 70 586)). The number of collected bacterial samples from experimental, control and natural nests is listed in ESM Table S1.

e. Volatile profiles: Gas chromatography-mass spectrometry (GC-MS)

Analyses were performed on a gas chromatograph coupled to a mass spectrometer Varian 450GC 240MS with an automatic injector Combi Pal to SPME fiber (50/30 μm DVB/CAR/PDMS, Stableflex 23Ga, Autosampler). Injector desorption was performed at 250 ºC for 10 minutes in Split (20: 1) and helium flow at 2mL/min. The capillary column was Aligent HP-FFAP 30m X 0.32mm X 0.25 μm. The oven temperature was initiated at 50 ºC for 1 min, and programmed to increase 5 ºC/min to 100 ºC, then at 10 ºC/min to 200 ºC and 50 ºC/min to 250 ºC for 1 min. The scan range of the mass spectrometer was in mode TIC Full Scan between 30 to 500 m/z. The identification of compounds was established by characteristic ion SIM analysis and the NIST 08 spectrum library (ESM-Figure S1). The summary of volatile profiles of next-box environments and uropygial gland secretions is in the Electronic Supplementary Material (ESM Table S2). Standards of pure compounds were used when necessary for confirmation.

Volatile profiles were measured in two ways: the relative abundance (the percentage of the area of each compound over the total area of the chromatogram adjusted to 100%) and the prevalence (i.e. presence or absence) of a chemical compound in particular volatile profiles. The number of volatile samples collected from experimental, control and natural nest-boxes is listed in ESM Table S1.

f. Statistical procedures

In order to control for the sequencing effort of sampled bacterial communities (i.e., of uropygial secretions and of nest material), the ASV table was rarefied to 5 800 sequences per sample in nest material and uropygial secretion samples. Therefore, 5 585 ASVs were retained in nest material samples and 2 105 ASVs in uropygial secretion samples for subsequent analyses. Based on this rarefied table, we calculated alpha (i.e. the microbial diversity within a particular sample), and beta diversity (i.e., the variability in community composition among different samples) (Whitaker 1972) in QIIME2 (Bolyen *et al.* 2019). As alpha and beta diversity indexes, we respectively used the Shannon index and the weighted and unweighted UniFrac distances (Lozupone & Knight 2005; Lozupone *et al.* 2007), which take into account the phylogenetic relationships among bacterial taxa.

For volatiles of the nest-box environment, we estimated alpha diversity using Shannon index. For beta diversity of volatile profiles, we used the relative abundance and prevalence of detected volatiles to respectively estimate Bray-Curtis and Jaccard matrices, using the functions *bcdist* and *distance* in the package *ecodist* (Goslee & Urban 2007) implemented in R.3.6.1 (R Core Team 2020).

General linear models (GLMs) were performed to study the effect of experimental treatments on alpha diversity estimates. Distribution of alpha diversity values did not differ significantly from a Gaussian distribution (Kolmogorov-Smirnov test for continuous variables, $P > 0.05$). Full models included treatment and study year as fixed factors and laying date as covariable. Interactions between treatments and study year were tested in separate models that also included main effects. These interactions resulted statistically non-significant (i.e. $P > 0.15$), so we present models without them. Residuals of all GLMs approximately followed normal distributions.

The effect of treatment on the beta diversity of bacterial communities and of volatile profiles were explored by means of PERMANOVAs (i.e., nonparametric multivariate analysis of variance) with 10 000 permutations. The models included treatment and study year and early (end of March, April and first half of May) versus late (second half of May and June) reproduction as fixed factors. Interactions between treatment and study year were tested in separated models that also included main effects. These analyses were performed in PRIMER-7.0.17. To visualize segregation of bacterial communities and of volatile profiles due to experimental treatment, we performed

Principal Coordinates Analyses (PCoA) plots, implemented in Emperor 2018.2.0 (Vazquez-Baeza *et al.* 2013). Finally, we used Analyses of Composition of Microbiomes (ANCOM) (Mandal *et al.* 2015), implemented in QIIME2, to determine which particular bacterial genus, or particular volatiles are responsible for the detected differences among experimental, control and natural nests.

The associations between bacterial and volatile profiles of different types of samples were explored by mean of Mantel-test with 10 000 permutations using the function MRM in the *ecodist* package (Goslee & Urban 2007) as implemented in R.3.6.1 (R Core Team 2020). That statistical test is equivalent to a multiple regression but using matrices of differences among samples in a multivariate setting. Importantly, given that for exploring the associations between bacterial and volatile profiles the matrices have to be estimated with the same methodology, we here calculated bacterial matrices based on Bray-Curtis dissimilarities. Matrices were filtered in QIIME2 before Mantel-test analysis to get the same dimensions in each analysis.

To explore the associations between specific bacteria and specific volatiles of uropygial secretion and nest material samples, we used PC-scores (i.e., after varimax rotation) from Principal Component Analyses (PCA) of the log10-transformed abundances of the bacterial taxa (considering the first six axes) and of the relative abundance of the volatile components (considering the first four axes). Different PCA analyses were performed for different types of samples and factor-loading values of each bacterial genus or volatiles in each PC-axis are shown in ESM-Table 3). Each PC-axis was named by a composition of letters that indicate the type of samples. The first letter indicates whether the sample corresponds to bacteria (B) or volatiles (V), the second letter indicates whether the sample is from secretions of females (SF), nestlings (SN) or nest material (M). Finally, samples that were collected at the beginning and at the end of the nestling period finished with a number (1 and 2 respectively). PC-scores were also used to explore the associations between bacteria or volatiles components and intensity of parasitism or fledgling success.

The associations between specific PC-scores describing relative abundance of particular volatiles (dependent variables) and those describing bacterial abundance (independent factors) were explored by mean of General Linear Models (GLM). Because we performed multiple GLMs (one per each PC-axis of volatiles), p-values describing the strength of the partial associations were adjusted for the effect of multiple testing (FDR,

Benjamini & Hochberg 1995). Similarly, we performed GLMs to explore the association between PC-scores describing relative abundance of volatiles of nests (dependent variable) and those of the uropygial secretion of females or nestlings (independent factors). In this case, associations were separately estimated for females and for nestlings, and, thus, we performed eight different GLMs. P-values were also adjusted following the FDR procedure (Benjamini & Hochberg 1995).

To search for the best combination of PC-axes describing bacterial communities and volatile profiles that explained the parasitism intensity of hoopoe nestlings and females, as well as fledging success, we used best subsets General Regression Models (GRM). The best subsets GRM was estimated by means of Mallow's CP (Mallows 1973), which is equivalent to Akaike information criterion (AIC) (Boisbunon *et al.* 2014). Importantly, information on bacterial and chemical profiles were not always available for the same pool of nests, so we separately analysed the effect of chemical and bacterial information. Moreover, we did not successfully collect information for all types of samples within the same nest (e.g., nestling secretion, female secretion or nest material) and, thus, available sample sizes for models that included all types of samples were reduced. When none of the PC-axes describing bacterial (or chemical) variation of one or more types of sample entered in the best model, we again ran the GRM but excluding them from the list of independent factors. In this way, we increased sample size, and thus, statistical power of the model.

GRMs explaining intensity of parasitism in recently hatched hoopoe nestlings and in females included information on the bacterial communities and on volatile profiles of nests, nestling and female secretions that were collected at the beginning of the nestling period. Similarly, GRM explaining intensity of parasitism of nestlings that were close to fledging included information on bacterial communities and volatile profiles of material collected at the end of the nestling period (i.e., nest material and of the uropygial secretion of nestlings that were close to fledge). Finally, female secretions were the only sample type used in the analyses involving chewing lice parasitism of females because we do not expect a major effect of bacteria or bacterial-derived volatiles on lice attraction, whose major mode of transfer is supposed to be through direct host contact (Durden 2019). GRM models were also used to search for factors explaining fledging success. To this aim, we performed two different GRMs that respectively considered information from samples collected during early or late nestling stages.

Frequency distributions of square root transformed values of the intensity of ectoparasitism in nestlings, as well as raw values of fledging success did not differ significantly from Gaussian distribution (Kolmogorov-Smirnov test for continuous variables, $p > 0.05$). GLMs, GRMs and PCAs were performed in STATISTICA 12 software.

3. Results

a. Effects of nest material sterilization in bacterial communities and volatile profiles of the nest environment

Bacterial communities of nest material at the beginning of the nestling stage were less diverse in experimental than in control nests (alpha diversity, GLM: $F_{1,63} = 5.43$, $p = 0.023$, ESM Table S3) and both communities separated to each other after correcting for the effects of study year and laying date (beta diversity, Table 1, ESM-Figure S2). Except for beta diversity estimated from unweighted Unifrac, all these effects disappeared at the end of the nestling stage (Table 1, EMS-Table S1, EMS-Figure S2). Independently of the stage when the samples were collected, microbiota of nest material from unmanipulated nest boxes (i.e., natural nests) showed similar alpha diversity (Shannon index) to control nests at the same nestling stage (GLMs, early stage: $F_{1,51} = 0.99$; $p = 0.334$; late stage: $F_{1,35} = 0.92$; $p = 0.343$), but differed in terms of any beta-diversity index (PERMANOVAs: early stage: *Pseudo* $F_{1,51} > 3.10$; $p < 0.004$; late stage: Pseudo $F_{1,35} > 2.80$; $p < 0.001$). Finally, ANCOM analyses showed differences in abundance of particular bacterial genera among different treatments of the performed experiment. At the beginning of the nestling stage, *Staphylococcus spp.* was more abundant in nest materials of control nests than in those of experimental nests. At the end of the nestling period, the genera *Corynebacterium spp.* and *Rothia spp.* were more abundant in the material collected from control nests (Table 2).

At the early nestling stage, volatile profiles of experimental nests were significantly more diverse than those of control ones (alpha diversity, GLM: $F_{1,55} = 4.61$, $p = 0.036$), even after controlling for the effects of study year and laying date (ESM Table S4). Beta diversity of volatiles also differed between experimental treatments, but only when considering Bray-Curtis dissimilarity matrices (Table 1). However, these differences disappeared at the end of the nestling stage (alpha diversity: GLM: $F_{1,51} = 1.35$, $p = 0.250$, ESM Table S4; beta diversity: Table 1, ESM-Figure S2). Independently of the sampling period, volatile profiles of control and natural nests did not differ

significantly (alpha diversity, early stage GLM: $F_{1,28} = 1.10$; $p = 0.321$; late-stage GLM: $F_{1,26} = 0.400$; $p = 0.532$; beta diversity, early stage PERMANOVAs: Pseudo $F_{1,28} < 0.60$; $p > 0.750$; late-stage PERMANOVAs for nestlings: Pseudo $F_{1,26} < 0.60$; $p > 0.720$). Finally, the abundances of none of the volatiles differ significantly ($p > 0.05$) among experimental treatments (ANCOM analyses).

Table 1. Results from PERMANOVAs exploring the effects of the experimental sterilization of nest materials before reproduction on beta-diversity indexes of bacterial communities (unweighted and weighted Unifrac) and volatile profiles (Jaccard and Bray-Curtis) of different sample types collected at different nestling ages (day). The models included study year (2017 *vs.* 2018) and laying date (early *vs.* late reproduction) as fixed factors. Moreover, the model exploring the effect on the bacterial community of nestling uropygial secretion also included nest identity nested within the interaction between treatment and study year as random factor. P values lower than 0.05 are in bold font.

	Experimental treatment			Study year			Laying date		
	Pseudo F	df	*p*	*Pseudo F*	df	*p*	*Pseudo F*	df	*p*
Bacterial communities									
Nest material (day 4)									
Unweighted	1.90	1,63	**0.004**	2.36	1,63	**0.001**	1.25	1,63	0.121
Weighted	2.11	1,63	**0.036**	2.86	1,63	**0.007**	0.77	1,63	0.623
Nest material (day 15)									
Unweighted	2.22	1,51	**0.003**	2.73	1,51	**<0.001**	1.03	1,51	0.366
Weighted	1.58	1,51	0.133	3.76	1,51	**0.003**	0.78	1,51	0.584
Female secretion (day 4)									
Unweighted	0.94	1,73	0.60	1.93	1,73	**<0.001**	0.97	1,73	0.531
Weighted	0.63	1,73	0.68	3.53	1,73	**0.007**	0.69	1,73	0.631
Nestling secretion (day 19)									
Unweighted	1.24	1,60	0.071	2.30	1,60	**<0.001**	1.30	1,60	0.055
Weighted	1.23	1,60	0.269	3.60	1,60	**0.016**	1.15	1,60	0.298
Volatile profiles									
Nest box (day 7)									
Jaccard	0.52	1,55	0.606	26.26	1,55	**<0.001**	11.66	1,55	**0.000**
Bray-Curtis	2.37	1,55	**0.049**	20.80	1,55	**<0.001**	**4.49**	1,55	**0.004**
Nest box (day 18)									
Jaccard	1.00	1,51	0.416	9.413	1,51	**<0.001**	0.38	1,51	0.720
Bray-Curtis	0.18	1,51	0.976	12.71	1,51	**<0.001**	3.03	1,51	**0.010**
Female secretion (day 4)									
Jaccard	1.59	1,80	0.168	48.70	1,80	**<0.001**	**2.52**	1,80	**0.035**
Bray-Curtis	0.16	1,80	0.944	7.23	1,80	**0.001**	0.56	1,80	0.655
Nestling secretion (day 8)									
Jaccard	1.33	1,58	0.258	0.58	1,58	0.644	1.79	1,58	0.137
Bray-Curtis	0.78	1,58	0.477	0.54	1,58	0.545	0.36	1,58	0.849
Nestling secretion (day 19)									
Jaccard	3.30	1,76	**0.022**	90.49	1,76	**<0.001**	5.91	1,76	**0.001**
Bray-Curtis	1.23	1,76	0.293	13.52	1,76	**<0.001**	2.14	1,76	0.084

Table 2. Pairwise differences in abundance of particular bacterial genus between experimental (E) and control (C) nests, and between each of them and natural (N) nests. Statistical tests were separately performed for each bacterial community. For statistically significant comparisons, we show **W** values from ANCOM analyses, and mean abundance of bacterial genera in each compared group ($\overline{X}$s). Only bacterial taxa for which we found statistically significant differences are shown.

	Nest material (day 4)			**Nest material (day 15)**		
	E - C **W** $\overline{X}$s	**E - N** **W** $\overline{X}$s	**C - N** **W** $\overline{X}$s	**E - C** **W** $\overline{X}$s	**E - N** **W** $\overline{X}$s	**C - N** **W** $\overline{X}$s
Staphylococcus spp.	**434** 12.5-161	-	**442** 161-4.5	-	-	**302** 159-20.5
Pedobacter spp.	-	**339** 4-68	**416** 3-68	-	-	-
Unidentified genus of the order Sphingomonadales	-	**378** 2-23.5	-	-	-	-
Sphingobium spp.	-	**371** 2-22.5	-	-	-	-
Unidentified genus of the family *Sphingomonadaceae*	-	**361** 1-14	-	-	-	-
Unidentified genus of the order Lactobacillales	-	-	**411** 25-1.5	-	-	-
Jeotgalicoccus spp.	-	-	**339** 64-6.5	-	-	-
Nocardia spp.	-	-	**418** 24-1	-	-	-
Corynebacterium spp.	-	-	-	**292** 7-69	-	-
Rothia spp.	-	-	-	**259** 3.5-18	-	-
Sphingomonas spp.	-	-	-	-	**327** 2-14.5	-

b. Effects of autoclaving nest material in bacterial communities and volatile profiles of the uropygial gland of nestling and females

Autoclaving nest material before reproduction did not produce changes on microbiota of the uropygial secretion of nestlings or females. After controlling for the effects of study year and nestling stage, our experiment did not affect alpha (GLM: $F_{1,73}$

= 0.03, p = 0.850, ESM Table S3), beta diversity (Table 1), or the abundance of any of the detected bacterial genera (Table 2) in the uropygial secretion of females. Similarly, the experiment neither affected alpha (GLM: $F_{1,60}$ = 0.03, p = 0.869, ESM Table S4) nor beta diversity of the bacterial community of the uropygial secretion of nestlings after controlling for the effect of study year and laying date (Table 1). Moreover, bacterial community of the uropygial gland secretion of females and nestlings from control and natural nests did not differ in terms of alpha or beta diversity (alpha diversity, GLM for females: $F_{1,53}$ = 0.06; p = 0.819; GLM for nestlings: $F_{1,38}$ = 0.30; p = 0.592; beta diversity, PERMANOVAs for females: Pseudo $F_{1,53}$ < 0.84; p > 0.810; PERMANOVAs for nestlings: Pseudo $F_{1,38}$ < 1.82; p > 0.124). Finally, none of the bacterial abundances differed significantly among experimental treatments (ANCOM analyses).

Beta diversity of volatile profiles of nestling uropygial secretions, but not of female secretions, differed between experimental and control nests when considering Jaccard, but not Bray-Curtis distance matrices (Table 1). Instead, alpha diversity of volatiles did not differ among experimental treatments (ESM Table S4). Volatile profiles of female and nestling secretion did not differ between control and natural nests in terms of alpha (GLM for female: $F_{1,50}$ = 2.50; p = 0.121; GLM for 8 and 19 days old nestlings respectively: $F_{1,37}$ = 0.02; p = 0.903; $F_{1,43}$ = 0.13; p = 0.725) nor beta diversity (PERMANOVAs for females: Pseudo $F_{1,50}$ < 2.17; p > 0.08; PERMANOVAs for 19 days old nestlings respectively: Pseudo $F_{1,42}$ < 39; p > 0.138; Pseudo $F_{1,43}$ < 0.60; p > 0.653). However, abundance of some of the detected volatiles in female secretions differed significantly between experimental, control and natural nests (ANCOM analyses, Table 3).

Table 3. Pairwise differences in relative abundance of chemical volatiles between experimental (E) and control (C) nests, and between each of them and natural (N) nests, in female secretions. For statistically significant comparisons, we show **W** values from ANCOM analyses, and mean relative abundance of volatiles in each compared group ($\overline{X}$s). Only volatiles for which between-groups comparisons reached statistical significance are shown.

	Female secretion (day 4)		
	E-C	**E-N**	**C-N**
	W $\overline{X}$s	**W** $\overline{X}$s	**W** $\overline{X}$s
Esters			
Nonanoic acid methyl ester	-	**15**	**5**
		1.07-1.64	1.09-1.64
Heptanoic acid mehyl ester	-	**13**	**2**
		1.51-1.36	1.10-1.36
Butanoic acid ethil ester	-		**1**
			1.26-1.00
Acids			
Nonanoic acid	-		**5**
			1.04-1.18
Butanoic acid	-		**4**
			12.38-9.16
Pentanoic acid	-		**4**
			1.76-1.42
Isobutyric acid	-		**2**
			2.58-1.90
Octanoic acid	-		**2**
			1.04-1.29
Acetic acid	-		**1**
			13.88-12.40
Isocaproic acid	-		**1**
			10.99-4.96

c. Associations between bacterial communities and volatile profiles

Bacterial community (in this case using Bray-Curtis dissimilarity matrices) of the uropygial gland secretion of females predicted its volatile profile (Mantel tests, R^2 = 0.011, p = 0.027). This relationship was close to statistical significance in nestlings (Mantel tests, R^2 = 0.014, p = 0.060). However, bacterial profiles of nest material at the beginning (Mantel tests, R^2 < 0.001, p = 0.796) or at the end of the nestling stage (Mantel tests, R^2 = 0.006, p = 0.322) did not predict volatile profiles of nest environment at these two nestling stages.

Relative abundance of particular volatiles and bacterial groups from female uropygial secretion summarized by different PC-factors were significantly related (Figure 1). *Bacillus spp.* (negatively related to PC4-BSF, GLM: $F_{1,61}$ = 4.14, p_{adj} = 0.046) and

Syntrophomonas spp. (positively relate to PC6-BSF, GLM: $F_{1,61}$ = 10.61, p_{adj} = 0.007) associated with relative abundance of several acids and one ester (PC2-VSF, Figure 1). Several genera of the phylum Firmicutes (PC4-BSF) associated negatively with the two esters (PC3-VSF) (GLM: $F_{1,61}$ = 6.51, p_{adj} = 0.018; PC3-BSF: GLM: $F_{1,61}$ = 4.76, p_{adj} = 0.033; Figure 1). Some other bacterial genera belonging to phylum Firmicutes (negatively associate with PC3-BSF) also associated negatively with two acids (PC4-VSF) (GLM: $F_{1,61}$ = 8.33, p_{adj} = 0.011, Figure 1).

In the case of nestling uropygial secretions, abundance of several acids (negatively related to PC4-VSN2) associated positively with the abundance of several genera of Actinobacteria, Firmicutes and Proteobacteria (PC3-BSN2) (GLM: $F_{1,55}$ = 8.30, p_{adj} = 0.023), and negatively with other genera of Actinobacteria, Bacteroidetes, Caldithrix, Chlorobi, Chloroflexi, Firmicutes, GN04, NC10, Planctomycetes and Proteobacteria (negatively associated with PC2-BSN2) (GLM: $F_{1,55}$ = 5.96, p_{adj} = 0.036, , Figure 1).

At the beginning of the nestling stage, none of the PC-axes summarizing bacterial abundance in nest materials associated with any of the PC-axes summarizing relative abundance of volatiles in nest environment. However, at the end of the nestling stage, abundances of butanoic acid and two aldehydes (negative and positively related to PC1-VM2, respectively) associated with abundance of genera of Actinobacteria, Bacteriodetes, Chloroflexi and Cyanobacteria (negatively related to PC1-BM2) (GLM: $F_{1,32}$ = 87.16, p_{adj} = 0.047, Figure 1).

For a detailed description of volatiles summarized in different PC-axes see ESM Table S5.

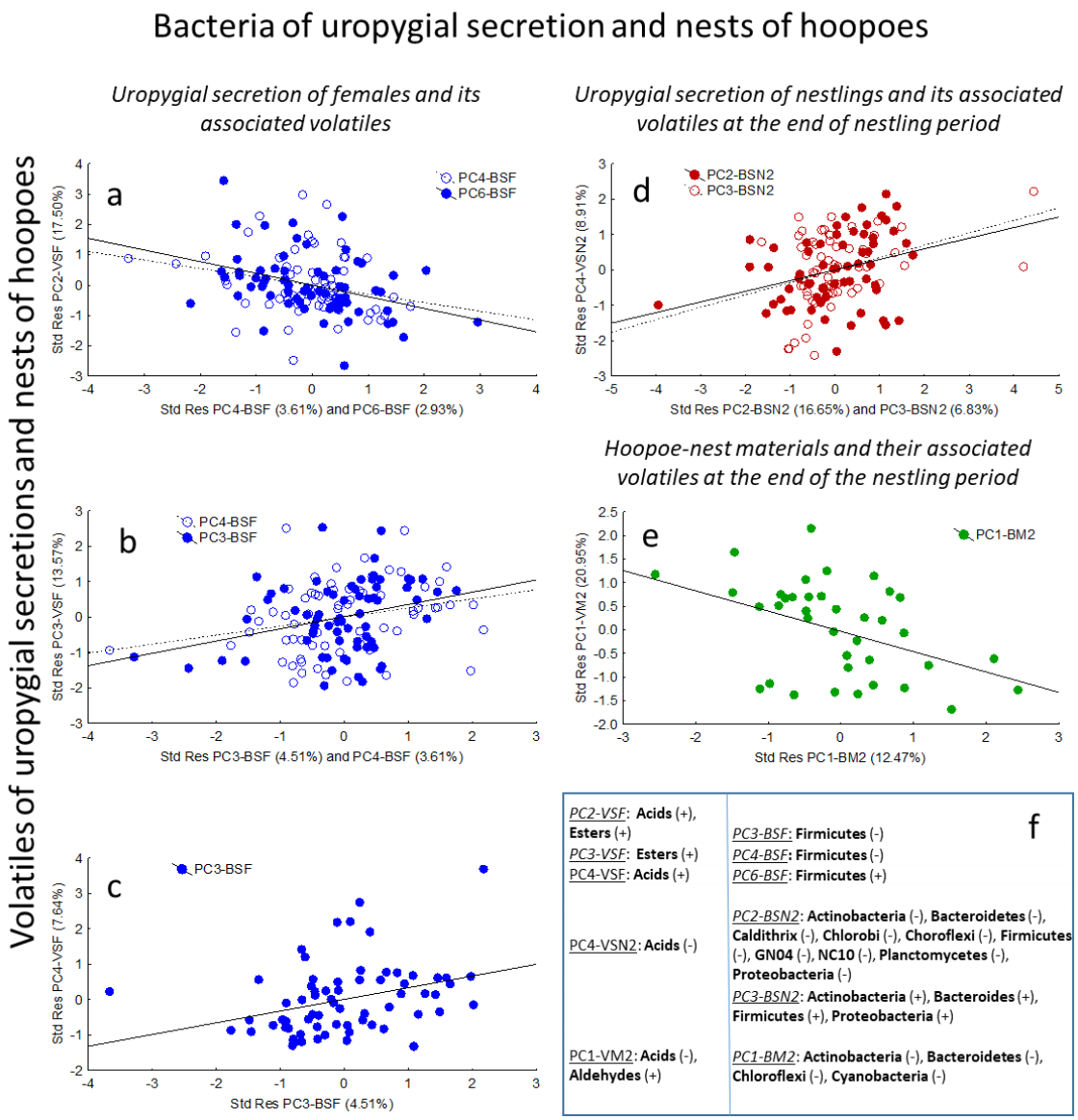

Figure 1. Statistically significant partial associations between scores from Principal Component (PC) axes summarizing volatile profiles (dependent variables) and bacterial communities summarized in bacterial genera (independent factors) of the uropygial secretion of female (blue, BSF: a, b and c) and nestling hoopoes (red, BSN: d), and of their nest materials and nest-boxes (green, BM: e). Each PC-axis was named by a composition of letters that indicate the type of samples. The first letter indicates whether the sample corresponds to bacteria (B) or volatiles (V), the second letter indicates whether the sample is from secretions of females (SF), nestlings (SN) or nest material (M). Finally, for types of samples that were collected at the beginning (1) and at the end (2) of the nestling period, the name finished with a number. To reflect detected partial correlations, we plotted standardized residual values of the dependent and the independent factor of interest after controlling for the rest of independent factors in the model. We also show a list of major groups of volatiles and bacteria (summarized in the phyla to which genera belong) that associate with each of the PC factors in the figures (f). Signs within brackets indicate the direction of the association of different groups of volatiles or bacterial with each PC factor. Percentage of variance explained by each of the PC factors is also showed. Lines are regression lines.

d. Associations between volatile profiles of nest-box environments and of secretions

At the beginning of the nestling stage, volatile profiles of the uropygial secretion of females (Mantel tests, $R^2 = 0.013$, $p = 0.016$) but not that of nestlings (Mantel tests, $R^2 < 0.001$, $p = 0.736$) explained the volatile profiles of nest environment. In accordance, several acids and aldehydes of nest environment (negatively and positively related to PC1-VM1, respectively) were positively related to volatiles of the uropygial secretion of females (PC1-VSF) (negatively related to several acids and aldehydes and positively to Phenol, (GLM: $F_{1,50} = 13.56$, $p_{adj} < 0.001$, Figure 2). These volatiles of nest environment (PC1-VM1) associated negatively with others acids and one ester of female secretions (PC2-VSF) (GLM: $F_{1,50} = 11.61$, $p_{adj} = 0.003$) and in with other two ester (PC3-VSF) (GLM: $F_{1,50} = 23.43$, $p_{adj} < 0.001$; Figure 2). Several acids of the uropygial secretions of 8-days old hoopoe nestlings (negatively related to PC2-VSN1) associated with volatiles of nest environment in PC1-VM1 (GLM: $F_{1,43} = 28.40$, $p_{adj} < 0.001$) and in PC4-VM1 (GLM: $F_{1,43} = 4.69$, $p_{adj} = 0.058$, Figure 2).

At the end of the nestling stage, volatile profiles of nest environment did not associate with those of the uropygial secretion of nestlings (Mantel tests, $R^2 < 0.003$, $p > 0.200$), or females (Mantel tests, $R^2 < 0.003$, $p > 0.280$). However, relative abundance of several acids and aldehydes in nest environment (in PC1-VM2 and PC3-VM2) associated with volatiles of the female secretions. Particularly, scores of PC1-VM2 were predicted by volatiles of the female secretions in PC1-VSF (GLM: $F_{1,47} = 9.73$, $p_{adj} = 0.005$), PC2-VSF (GLM: $F_{1,47} = 10.87$, $p_{adj} = 0.0037$), and PC3-VSF ($F_{1,47} = 22.28$, $p_{adj} > 0.001$), while scores of PC2-VSF (GLM: $F_{1,47} = 4.14$, $p_{adj} = 0.048$), and PC3-VSF (GLM: $F_{1,47} = 5.75$, $p_{adj} = 0.023$) explained those of PC3-VM2 (Figure 2). Volatiles of the uropygial secretion of nestlings in PC1 (GLM: $F_{1,48} = 6.53$, $p_{adj} = 0.018$), in PC2 (GLM: $F_{1,48} = 20.04$, $p_{adj} < 0.001$) and in PC4 (GLM: $F_{1,48} = 11.66$, $p_{adj} = 0.034$) explained the relative abundance of some aldehydes of nest environment (PC1-VM2) at the end of the nestling period (Figure 2).

For a detailed description of volatiles summarized in different PC-axes see ESM Table S6.

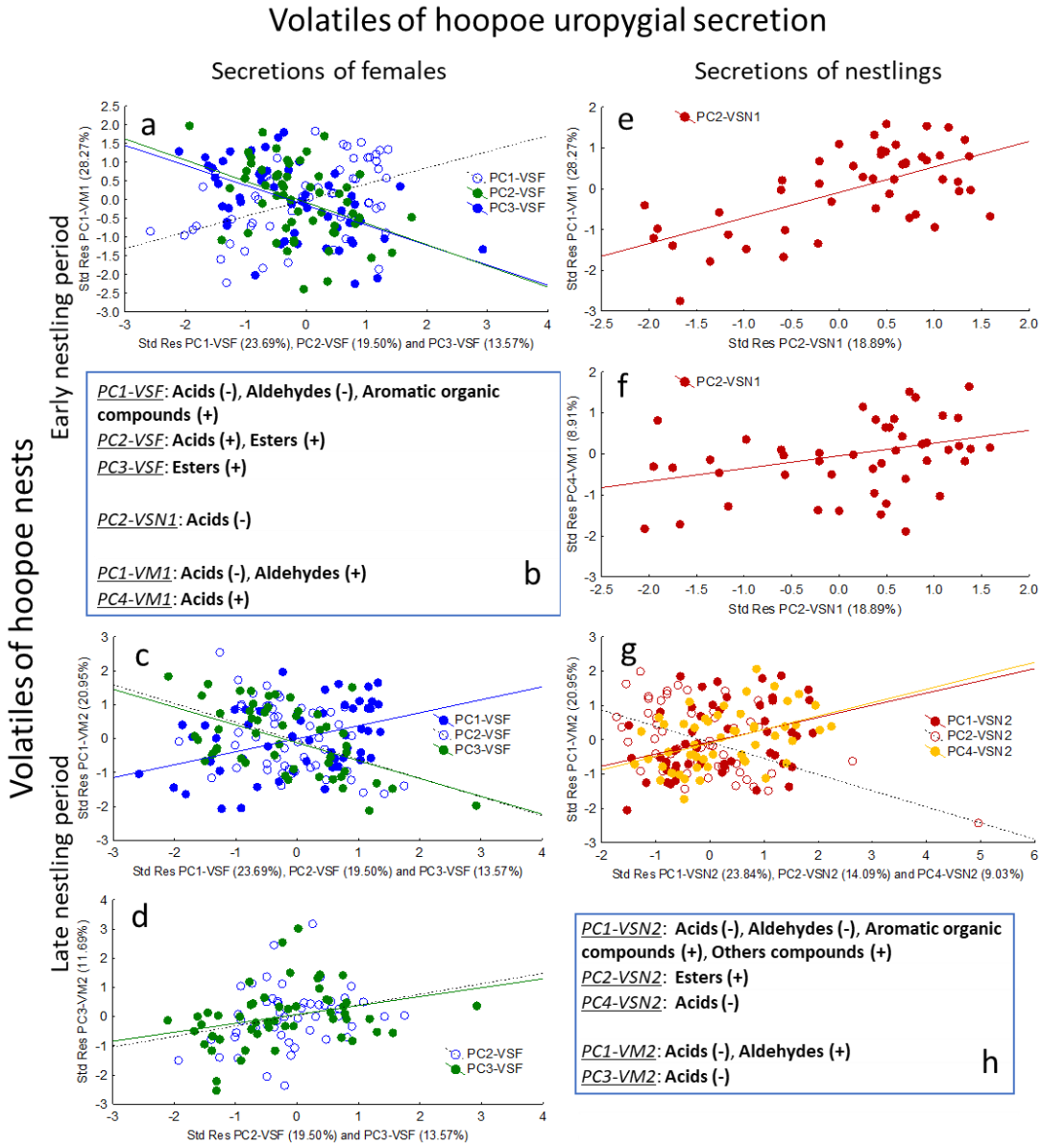

Figure 2. Statistically significant partial associations between scores from Principal Component (PC) axes summarizing volatile profiles of hoopoe nest-box environments (VM1 and VM2) and those summarizing volatiles of the uropygial secretion of females (blue and green, VSF: a, c and d) and of nestlings (red and yelow VSN1: e and f, and VSN2: g). Each PC-axis was named by a composition of letters that indicate the type of samples. The first letter indicates whether the sample corresponds to bacteria (B) or volatiles (V), the second letter indicates whether the sample is from secretions of females (SF), nestlings (SN) or nest material (M). Finally, for types of samples that were collected at the beginning (1) and at the end (2) of the nestling period, the name finished with a number. To reflect detected partial correlations, we plotted standardized residual values of the dependent and the independent factor of interest after controlling for the rest of independent factors in the model. We also show major groups of volatiles that associated with each of the PC factors that appear in the figures (b and h). Signs within brackets indicate the direction of the associations of particular volatiles with each PC factor. Percentage of variance explained by each of the PC factors is also showed. Lines are regression lines.

e. Bacteria and volatile of nest environments affecting the intensity of parasitism

Bacterial and volatile profiles significantly associated with intensity of parasitism of 8-days-old nestlings (see Multiple-R values in Table 4 and Figure 3). On the one hand, parasitism was negatively associated with the abundance of some genera belonging to phyla Actinobacteria, Chloroflexi, and Proteobacteria in the nest (PC5-BM1) (Table 4, Figure 3). On the other hand, intensity of parasitism was associated positively with some acids and negatively to some aldehydes detected in nest environment (PC1-VM1), and negatively to some acids of the uropygial secretion of nestlings (PC2-VSN1) (Table 4, Figure 3).

For a detailed description of volatiles summarized in different PC-axes see ESM-Table S6.

Bacterial and volatile profiles also explained intensity of parasitism of nestlings close to fledge (see Multiple-R values in Table 4 and Figure 3). Nestlings with higher intensity of parasitism were those that grew in nests with nest material that harboured less density of genera belonging to phyla Bacteroidetes (*Larkinella spp.*) and Proteobacteria (*Aquabacterium spp.*) (PC6-BM2) (ESM Table S7) and that had uropygial secretion with less concentration of esters (PC2-VSN2, ESM Table S7) (Table 4, Figure 3).

Finally, intensity of chewing lice parasitism of females also associated positively or negatively with relative abundance of some genera belonging to Firmicutes (PC3-BSF; ESM Table S7), and negatively with volatiles of female secretion in PC2-VSF (ESM Table S7) that include three acids and one ester (Table 4; Figure 3).

Table 4. Results from GRMs looking for best models explaining parasitism by *Carnus* flies in 8 and 19 days old nestlings, and by chewing lice in females. Best models were separately analysed for bacterial (B) and volatiles (V) PCs. As potential variables to explain parasitism of nestlings in booth ages we considered those from the nest material and environment (M) and from the uropygial secretion (S) of females (F) and nestlings (N) collected at the beginning (1) and at the end (2) of the nestling period, respectively. We show multiple R of the best models and highlight partial effects with p-values lower than 0.05 in bold font.

BACTERIAL COMMUNITY						VOLATILE PROFILES					
Parasitism of 8 days old nestlings											
Multiple R = 0.34, F = 4.75, df = 2.63, p = 0.011						***Multiple R = 0.48, F = 4.21, df = 3,44, p = 0.011***					
	Beta	(SE)	F	df	p		Beta	(SE)	F	df	p
PC1-BM1	0.21	0.11	3.44	1,73	0.068	**PC1-VM1**	**-0.61**	**0.17**	**12.33**	**1,44**	**0.001**
PC5-BM1	**-0.29**	**0.11**	**6.74**	**1,73**	**0.011**	**PC2-VSN1**	**0.39**	**0.17**	**5.12**	**1,44**	**0.029**
						PC3-VSN1	0.21	0.14	2.36	1,44	0.132
Parasitism of 19 days old nestlings											
Multiple R = 0.53, F = 3.47, df = 2.35, p = 0.017						***Multiple R = 0.42, F = 5.37, df = 2,49, p = 0.008***					
PC2-BM2	-0.28	0.15	3.41	1,35	0.073	PC2-VM2	-0.19	0.13	2.14	1,49	0.150
PC6-BM2	**-0.32**	**0.15**	**4.55**	**1,35**	**0.040**	**PC2-VSN2**	**-0.36**	**0.13**	**7.46**	**1,49**	**0.009**
PC2-BSN2	-0.27	0.15	3.15	1,35	0.085						
PC6-BSN2	0.27	0.15	3.17	1,35	0.084						
Parasitism of Females											
Multiple R = 0.28, F = 7.69, df = 1.388, p = 0.006						***Multiple R = 0.42, F = 5.37, df = 2,49, p = 0.008***					
PC3-BFS	**0.28**	**0.1**	**7.7**	**1,88**	**0.007**	PC2-VM2	-0.19	0.13	2.14	1,49	0.150
						PC2-VSF	**-0.36**	**0.13**	**7.46**	**1,49**	**0.009**
Fledging success and variables from the early nestling period											
Multiple R = 0.48, F = 3.97, df = 3.40, p = 0.014						***Multiple R = 0.36, F = 4.72, df = 2,62, p = 0.012***					
PC3-BM1	-0.25	0.14	3.21	1,40	0.081	**PC3-VSF**	**-0.31**	**0.12**	**6.51**	**1,62**	**0.013**
PC3-BSF	0.24	0.14	2.79	1,40	0.102	**PC1-VSN1**	**-0.26**	**0.12**	**4.71**	**7,62**	**0.034**
PC6-BSF	**0.34**	**0.14**	**5.89**	**1,40**	**0.020**						
Fledging success and variables from the late nestling period											
Multiple R = 0.38, F = 3.19, df = 2.37, p = 0.053						***Multiple R = 0.24, F = 5.08, df = 1,87, p = 0.026***					
PC1-BM2	**-0.33**	**0.15**	**4.56**	**1,37**	**0.039**	**PC4-VSN2**	**0.24**	**0.10**	**5.10**	**1,87**	**0.026**
PC6-BSF	-0.27	0.15	3.05	1,37	0.089						

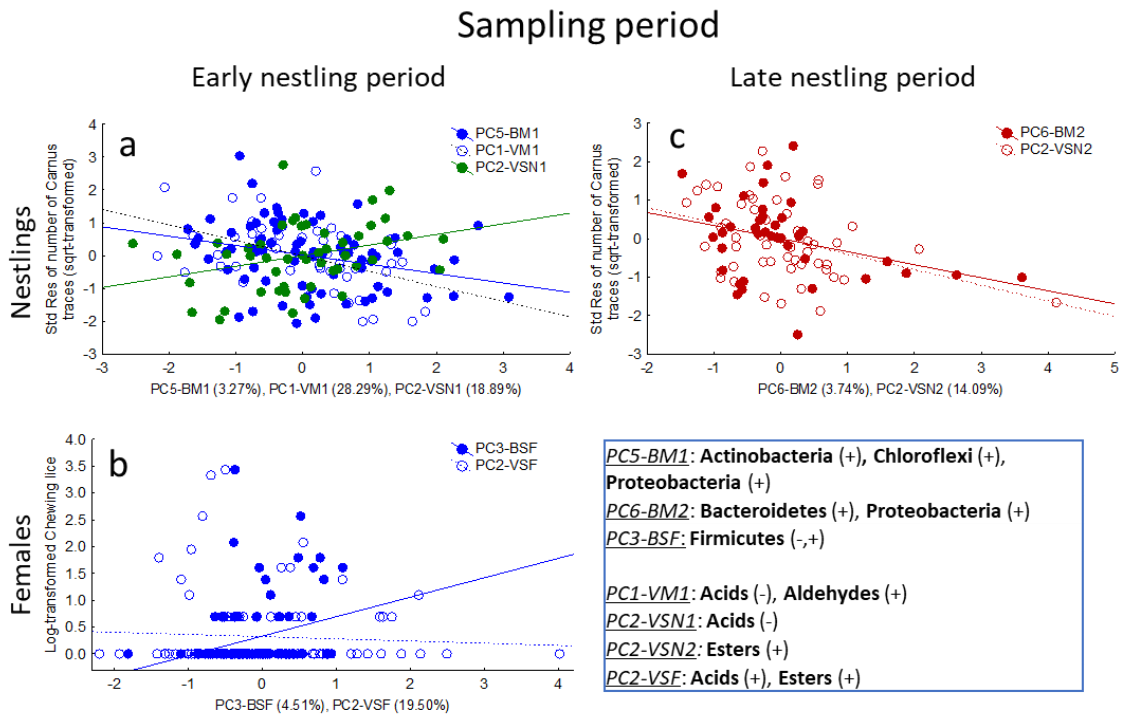

Figure 3. Statistically significant partial associations between intensity of parasitism in nestlings (number of *Carnus* flies traces) (a and c) and females (number of chewing lice - *Mallofaga*) (b) and Principal-Components (PC) scores summarizing bacterial communities (BM1, BM2, BSF) and volatile profiles (VM1, VSN1, VSN2, CSF) of different types of samples, at the early (a and b) and late (red) (c) nestling periods. Each PC-axis was named by a composition of letters that indicate the type of samples. The first letter indicates whether the sample corresponds to bacteria (B) or volatiles (V), the second letter indicates whether the sample is from secretions of females (SF), nestlings (SN) or nest material (M). Finally, for types of samples that were collected at the beginning (1) and at the end (2) of the nestling period, the name finished with a number. To reflect detected partial correlations, we plotted standardized residual values of the dependent and the independent factor of interest after controlling for the rest of independent factors in the model. We also show major groups of volatiles and bacteria (summarized in the phyla to which genera belong) that associate with each of the PC factors in the figures (d). Signs within brackets indicate the direction of the associations of particular volatiles with each PC factor. Percentage of variance explained by each of the PC factors is also showed. Lines are regression lines.

f. Fledging success and bacterial and volatile profiles of hoopoe nests

Fledging success was positively related to the abundance of *Syntrophomonas spp*. (PC6-BSF) in the uropygial secretion of females and with the abundance of several genera belonging to phyla Actinobacteria, Bacteroidetes, Cloroflexi and Cyanobacteria in the

nest material collected at the end of the nestling period (PC1-BM2) (Table 4, Figure 4). In addition, relative abundance of two esters in the uropygial secretion of females (PC3-VSF), three acids of the secretion of young nestlings (PC1-VSN1) and three acids of the secretion of 19 days old (PC4-VSN2) correlated negatively with fledging success (Table 4, Figure 4). Instead, several aldehydes of the secretion of 8 days old nestlings (PC1-VSN1) associated positively with fledging success (Table 4, Figure 4).

For a detailed description of volatiles summarized in different PC-axes see ESM-Table S6).

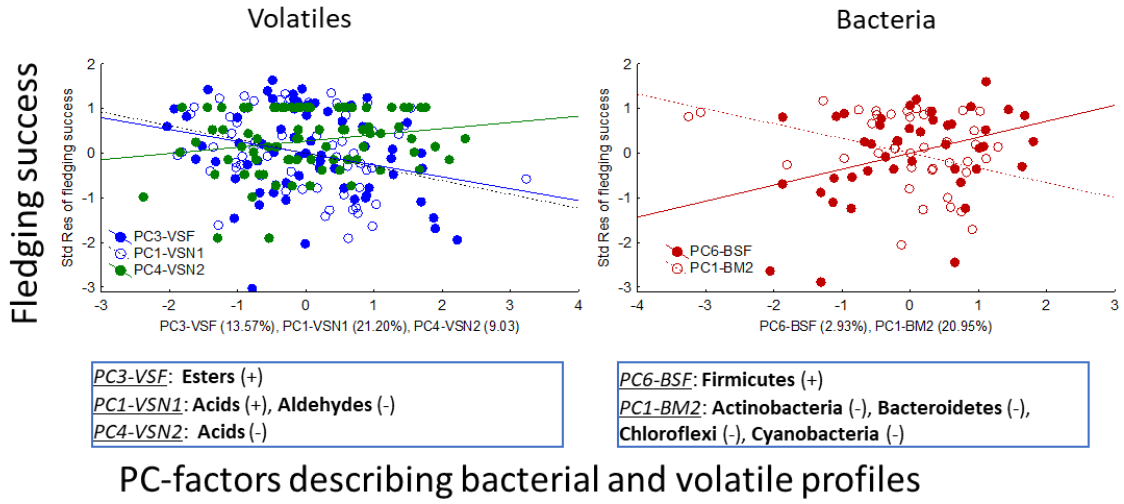

Figure 4. Statistically significant partial associations between fledging success and Principal-Components (PC) scores summarizing bacterial communities [secretion of females (PC6-BSF and nest material collected at the end of the nestling period (PC1-BM2)] and volatile profiles of the uropygial secretion of females (PC3-VSF) and of nestlings [at early (PV1-VSN1) and late nestling period (PC4-VSN2)]. To reflect detected partial correlations, we plotted standardized residual values of the dependent and the independent factor of interest after controlling for the rest of independent factors in the model. We also show major groups of volatiles and bacteria (summarized in the phyla to which the genera belong) that associate with each of the PC factors in the figures. Numbers within brackets indicate particular volatile or bacterial genera associated with each PC factor with an absolute loading-factor value larger than 0.7 in the ESM-Table 3. Percentage of variance explained by each of the PC factors is also showed. Lines are regression lines.

4. Discussion

This is the first study exploring the hypothetical link between bacterial symbionts, animal odors and ectoparasitism within the same study system. By autoclaving nest

materials, we manipulated bacterial communities of hoopoe nests before reproduction started and detected effects on different components of the bacterial communities and volatile profiles of nests during the nestling phase, and on intensity of parasitism in nestlings (see Mazorra-Alonso *et al.* 2020). Volatiles of the nest environment associated with those of secretions along the nestling phase. Moreover, volatiles and bacteria of nests and secretions predicted the intensity of ectoparasitism suffered by brooding females and nestlings. All these results are in accordance with the hypothesis that bacterial communities are partially responsible for the volatile profile of animals, which ultimately affect risk of parasitism and fledging success. Below, we discuss the importance of the detected associations in light of previous results and of the hypothesis tested.

Previous work in the same study system and hoopoe population demonstrated that the presence of unaltered nest material from previous hoopoe breeding attempts increased bacterial density on eggshells (Diaz-Lora *et al.* 2019), and in nest materials during the nestling phase (Mazorra-Alonso *et al.* 2020). In the present work, we have shown that bacterial communities of nest material in experimental nests (autoclaved) during the nestling phase were less diverse and clustered apart from those of control nests, confirming that bacterial communities before reproduction determine the bacterial environment during the nestling phase. Interestingly, we have detected parallel experimental effects on bacterial communities and on the chemical environment of nests during the nestling phase, with experimental nests showing more diverse volatile profiles than control nests. Moreover, characteristics of symbiotic bacterial communities of the uropygial secretion of females and nestlings associated with particularities of their volatile profiles. These results add experimental and correlational support to the expected association between bacterial communities and volatile profiles of birds, which was previously demonstrated by injecting antibiotics in the uropygial gland of hoopoes (Martin-Vivaldi *et al.* 2010) and dark-eyed juncos (*Junco hyemalis*) (Whittaker *et al.* 2019).

Our hypothesis posits that bacterial symbionts in their uropygial glands and the microbial communities in their nests are partially responsible for the general nest odor that conveys inadvertent social information to ectoparasites. In accordance with the first part of the hypothesis, we found that particular bacterial components of nest material associated with particular volatiles captured in the nest environment. Volatile profiles of the uropygial secretion of brooding females, but not those of nestling secretions,

associated with the profile of volatiles captured in nests at the beginning of the nestling period. Furthermore, relative abundance of particular volatiles of the secretion of females and older nestlings predicted volatiles captured in nest-boxes at the end of the nestling period. A previous work demonstrated the association between bacteria and volatiles of secretions (Martin-Vivaldi *et al.* 2010), so our findings further support the hypothesis that symbiotic bacteria of the preen gland are responsible of avian nest odor.

Our hypothesis states that parasites use volatiles of bacterial origin to detect and/or choose the nests of their victims (Mazorra-Alonso, Tomás & Soler 2021). In a previous paper with identical set of nests, we showed that nestlings grown in nest-boxes with experimentally autoclaved nest materials suffered lower intensity of ectoparasitism by *Carnus* flies than those of control nests, while bacterial loads of nest material associated positively with fledging success (Mazorra-Alonso *et al.* 2020). In that paper, we suggested that volatiles from bacteria metabolism could be responsible for the detected experimental effects on intensity of parasitism. That suggestion was based on previous results demonstrating the links (i) between autoclaving the nest material and characteristics of bacterial communities of the uropygial secretions of females and nestlings (Diaz-Lora *et al.* 2019), and (ii) between symbiotic bacteria and the volatiles of the uropygial secretion of hoopoe nestlings (Martin-Vivaldi *et al.* 2010). Here, using information from high-throughput sequencing of bacterial communities and GC-MS chemical analyses of uropygial secretions of nestlings and females, and of nest-environment, we were able to test and find support to those predicted associations. Particular bacterial groups from nest material, as well as particular volatiles of the secretion of nestlings and of nest environment, predicted intensity of parasitism of young nestlings by *Carnus* flies. Similarly, particular bacteria from nest materials collected at the end of the nestling period, as well as volatiles of the uropygial secretion of close-to-fledge nestlings, associated with their intensity of parasitism. Abundance of chewing lice on female feathers associated with particular bacteria and volatiles of their uropygial secretion. Therefore, our results support the hypothetical role of symbiotic bacteria, and of their volatiles on the interactions between hoopoes and their ectoparasites, independently of the identity of bacteria or chemical component responsible of the detected associations with parasitism.

However, correlations do not imply causality and, thus, these results might be interpreted in both directions: volatiles produced by bacteria affect parasitism; or

parasites affect bacterial communities and volatiles of their victims. Parasites, by definition, use host resources for their own and thus influence physical condition and health of their victims (Schmid-Hempel 2021). Since host physical or physiological condition influence characteristics of their microbial symbionts and vice versa (Sherwin *et al.* 2019), parasitism could affect their microbial symbiotic community (reviewed in Knutie 2018; Eleftheriou 2020; Mazorra-Alonso, Tomás & Soler 2021; Rafaluk-Mohr *et al.* 2022). Nutritional condition of hosts also affects their immunological resistance to parasites (Soler *et al.* 2008; Knutie 2018; Schmid-Hempel 2021). Thus, the effects of parasitism on nutritional condition and immune competence of their hosts could indirectly explain the detected association between bacteria and parasitism. Given the complexity of interacting mechanisms potentially explaining associations between parasitism, immunity, and symbiotic bacterial communities of animals (Sherwin *et al.* 2019), the manipulation of bacterial communities is essential to demonstrate their effects on parasitism. Indeed, we manipulated bacterial communities of nests before reproduction, which affected parasitism intensity, volatile profiles, and bacterial communities of nests during the nestling phase. Then, the most likely explanation for the detected associations between particular groups of volatiles and of bacteria with intensity of parasitism is that volatile-producing bacteria affects parasitism, and not the reverse.

Relative abundance of some aldehydes, acids and esters associated with intensity of parasitism in nestlings and females, and with fledging success. Most of these chemicals have been detected in other animals and are known as determining host selection by some arthropod pests (Poldy 2020). Nonanal, for instance, is a typical odor of the volatile profile of some birds (i.e. pigeons and chickens) that attracts mosquitoes of the genus *Culex* (Syed & Leal 2009), and it has been used as sentinel in sighting pest programs (Komar 2001; Deegan *et al.* 2005; Darbro & Harrington 2006). Similarly, relative abundance of different bacterial genera belonging to phyla such as Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Cyanobacteria and Chlorofexi associated with parasitism intensity and fledging success. Some bacteria of these groups, like those belonging to the genus *Enterococcus*, *Pseudomonas* and *Variovorax* have been previously associated with probability of parasitism by blood sucking ectoparasites (Verhulst *et al.* 2010; Tomás *et al.* 2020). Importantly, some groups of bacteria and volatiles of different types of samples associated with fledging success indicating that these chemical and microbiological components may influence host fitness.

5. Conclusions

These results support the links between microbial communities and animal odors, and emphasize that the associations between symbiotic bacteria and both ectoparasitism and reproductive success are partially mediated by volatiles of bacterial origin. Future work should focus on mechanisms underlying the detected patterns.

The detected associations are in any case complex and our results strongly support a central role of volatiles of symbiotic bacterial origin. However, the importance of different bacterial taxa and of different volatile compounds determining risk of parasitism and fledging success urges further experimental approaches.

DECLARATIONS

Ethics Statement

The study was conducted according to relevant Spanish national (Decreto 105/2011, 19 de abril) and regional guidelines. All necessary permits for hoopoe´s manipulations were provided by Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected, but privately owned, and the owners allowed us to work in their properties. The time spent in each hoopoe nest was the minimum necessary for the experiment.

Data Accessibility Statement

Raw sequence reads and their metadata are deposited in the Sequence Read Archive (SRA) in the Genbank - NCBI webpage (https://www.ncbi.nlm.nih.gov/sra/). Nest sequences are available under BioProject ID: PRJNA847390, accession number, SUB11518282 and Secretion sequences under BioProject ID: PRJNA847428, accession number SUB11582000. Volatiles metadata are stored in DataDryad (https://doi.org/10.5061/dryad.8sf7m0csn).

Conflict of Interest

The authors declare no conflict of interest.

Funding

The research group was supported by the projects CGL2017-83103-P, PID2020-117429GB-C21 and PID2020-117429GB-C22, funded by the Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación/10.13039/501100011033 and by “Fondo

Europeo de Desarrollo Regional, a way of making Europe". The research group also benefits from facilities, including accommodation, provided by the City Hall of Guadix, where a small lab to quickly process the samples was installed.

Author Contributions

MMV and JJS designed the study including the experimental approach. MMA, JJS and MMV carried out the fieldwork. MMA with the help of MMB performed all laboratory analyses related to bacterial DNA extraction and amplification. RNG afforded all chemical analyses in determination and quantification of chemicals. MMA and JMPS carried out all bioinformatics and statistical analyses. Together with JJS and JMPS, MMA wrote a first version of the manuscript. All authors contributed to the general discussion of results and to the final version of the manuscript.

Acknowledgments

We thank Natalia Juárez García-Pelayo and Cristina Ruiz Castellano for their help with the fieldwork, and Estefanía López Hernández and Miguel Rabelo Ruiz with the laboratory work. Gustavo Tomás read the manuscript and greatly improved the quality and the understanding of the manuscript. We would like to acknowledge an anonymous reviewer for his/her comments and suggestions that undoubtingly have improved the present manuscript.

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ELECTRONIC SUPPLEMENTARY MATERIAL (ESM)

Table S1. Number of experimental (with autoclaved nest material), control (with non-autoclaved nest material) and natural (with old nest material) hoopoe nests with information of bacterial community, volatile profiles and parasitism at different nestling stages (Day of sampling: days (d) after the first egg hatched). Bacterial and volatile samples were collected from the nest environment [nest material (Nest Mat) or nest air (Nest-box)] and from the uropygial gland (secretion) of females and nestlings.

	Experimental	Control	Natural
Bacterial community			
Nest material d14	34	33	22
Nest material d15	30	25	14
Female secretion d4	38	39	18
Nestling secretion d19	34	30	12
Volatile profile			
Nest-box d7	30	29	3
Nest-box d18	28	27	3
Female secretion d4	43	41	13
Nestling secretion d8	34	23	8
Nestling secretion d19	43	36	11
Parasites			
Females d4	39	40	18
Nestlings d18	36	25	6
Nestlings d19	31	20	4

Table S2. List of chemical compounds detected in both study years and selected in the environment of nest boxes and uropygial secretions of hoopoes in the analysis of GC-MS.

Nest-box environment	Uropygial gland secretions
Volatile profiles	
Acids	
Acetic	Acetic
Butanoic	Butanoic
Decanoic	Decanoic
Heptanoic	Heptanoic
Hexanoic	Hexanoic
-	Hexadecanoic
Isocaproic	Isocaproic
Isobutiric	Isobutiric
Isovaleric	Isovaleric
Nonanoic	Nonanoic
Octanoic	Octanoic
Pentanoic	Pentanoic
Propionic	Propionic
Aldehydes	
Benzaldehyde	Benzaldehyde
Hexanal	Hexanal
Heptanal	Heptanal
Nonanal	Nonanal
Octanal	Octanal
Pentanal	Pentanal
Esters	
-	Butanoic-acid-ethyl-ester
-	Butanoic-acid-methyl-ester
-	Heptanoic-acid-methyl-ester
-	Hexanoic-acid-ethyl-ester
-	Hexadecanoic-acid-methyl-ester
-	Nonanoic-acid-methyl-ester
-	2-methyl-butanoic-acid-ethyl-ester
-	3-mehyl-butanoic-acid-ethyl-ester
-	4-methyl-pentanoic-acid-methyl-ester
Sulfurs compounds	
Dimethyl-disulfide	Dimethyl-disulfide
Dimethyl-trisulfide	Dimethyl-trisulfide
-	Dimethyl Sulfone
Aromatic organic compounds	
-	Phenol
-	Ethanol 2-phenoxy CAS 122-99-6

Table S3. General Lineal Models exploring the effect of autoclaving nest material on Shannon index of bacteria community of nest-material at early and last stages (day 4 and 15 after first egg hatched, respectively) and on uropygial gland secretion of females and nestlings (days 4 and 19 after first egg hatched, respectively). Least square means (SE) for control and experimental (Exp) treatments, and for different study years are shown, as well as beta (SE) values of the associations with laying date.

Experimental treatment					Study year					Laying date			
Exp Mean (SE)	Control Mean (SE)	F	df	P	2017	2018	F	df	P	Beta (SE)	F	df	P
Bacterial community of nest material (day 4)													
4.82 (0.25)	5.55 (0.20)	5.43	1,63	**0.02**	5.41 (0.19)	5.33 (0.22)	0.13	1,63	0.71	-0.11 (0.01)	0.68	1,63	0.41
Bacterial community of nest material (day 15)													
4.56 (0.21)	5.03 (0.22)	1.34	1,51	0.25	4.64 (0.25)	4.87 (0.16)	1.07	1,51	0.31	0.13 (0.01)	0.76	1,51	0.39
Bacterial community of female secretion (day 4)													
3.59 (0.05)	3.61 (0.05)	0.035	1,73	0.85	3.54 (0.05)	3.66 (0.04)	2.74	1,73	0.10	0.05 (0.00)	0.07	1,73	0.69
Bacterial community of nestling secretion (day 19)													
3.38 (0.05)	3.35 (0.05)	0.027	1,60	0.87	3.35 (0.05)	3.39 (0.04)	2.25	1,60	0.14	-0.10 (0.00)	0.50	1,60	0.48

Table S4. General Linear Models exploring the effect of autoclaving nest material on Shannon index of volatile profiles of hoopoe nest environment at early and last stage of the nestling (days 7 and 18 after first egg hatched, respectively) and of uropygial gland secretion of females and nestlings (days 4 and 19 after first egg hatched, respectively). Least square means (SE) for control and experimental (Exp) treatment, and for the study years are shown, as well as beta (SE) values of the associations with laying date.

Experimental treatment					Study year					Laying date			
Exp Mean (SE)	Control Mean (SE)	F	df	P	2017	2018	F	df	P	Beta (SE)	F	df	P
Volatile profiles of hoopoe nest-boxes (day7)													
3.33 (0.05)	3.15 (0.05)	4.61	1,55	**0.036**	3.04 (0.05)	3.37 (0.03)	38.5	1,55	**0.00**	-0.25 (0.00)	5.99	1,55	**0.018**
Volatile profiles of hoopoe nest-boxes (day18)													
3.30 (0.05)	3.18 (0.05)	1.35	1,51	0.25	3.09 (0.36)	3.34 (0.05)	15.4	1,51	**0.00**	-0.21 (0.00)	2.85	1,51	0.09
Volatile profiles of female secretion (day 4)													
2.90 (0.08)	2.92 (0.07)	0.18	1,80	0.67	2.93 (0.08)	2.85 (0.07)	0.01	1,80	0.93	-0.09 (0.00)	0.61	1,80	0.44
Volatile profiles of nestling secretion (day 8)													
2.98 (0.06)	3.02 (0.05)	0.19	1,63	0.66	3.06 (0.05)	2.95 (0.06)	183	1,63	0.18	0.07 (0.00)	0.11	1,63	0.74
Volatile profiles of nestling secretion (day 19)													
2.98 (0.05)	2.99 (0.07)	0.22	1,76	0.64	2.99 (0.05)	2.99 (0.06)	0.08	1,76	0.77	-0.16 (0.00)	1.70	1,76	0.20

Table S5. Results of PCA analyses summarizing bacterial genera (if unknown we use family or order) and volatiles detected in the nest material and the uropygial secretion of female and nestling hoopoes. Values are PC factor loadings after varimax normalized rotated and in their nest material or nest boxes. Each PC-axis was named by a composition of letters that indicate the type of samples. The first letter indicates whether the sample corresponds to bacteria (B) or volatiles (V), the second letter indicates whether the sample is from secretions of females (SF), nestlings (SN) or nest material (M). Finally, for types of samples that were collected at the beginning (1) and at the end (2) of the nestling period, the name finished with a number. Only factors than entered in final models explaining the association between bacterial and volatile profiles are shown.

FEMALE UROPYGIAL SECRETION (MODEL: VOLATILES = BACTERIA)

		PC3	PC4	PC6		PC2	PC3	PC4		
Familia	**Bacteria Taxa**	**BSF**	**BSF**	**BSF**		**VSF**	**VSF**	**VSF**	**Volatile Components**	
Firmicutes	*Bacillus*		-0.89					0.71	Butanoic	*Acids*
Firmicutes	*Trissierellaceae*	-0.86				0.86			Isobutiric	*Acids*
Firmicutes	*F. Erysipelotrichaceae*	-0.7				0.78			Isocaproic	*Acids*
Firmicutes	*Lactococcus*	0.7						0.74	Isovaleric	*Acids*
Firmicutes	*Syntrophomonas*			0.73		0.82			Pentanoic	*Acids*
							0.71		Butanoic-acid-ethyl-ester	*Esters*
						0.7			2-methyl-butanoic-acid-ethyl-ester	*Esters*
							0.7		3-mehyl-butanoic-acid-ethyl-ester	*Esters*
	Explained Variance (%)	**4.51**	**3.61**	**2.93**		**19.5**	**13.57**	**7.64**		

NESTLING UROPYGIAL SECRETION (VOLATILES = BACTERIA)

		PC2	PC3		PC4		
Familia	**Bacteria Taxa**	**BSN2**	**BSN2**		**VSN2**	**Volatile Components**	
Actinobacteria	At425_EubF1	-0.99			-0.78	Isobutiric	*Acids*
Actinobacteria	*C. OPB41*	-0.99			-0.74	Isocaproic	*Acids*
Actinobacteria	*F. Microbacteriaceae*		0.97		-0.82	Pentanoic	*Acids*
Actinobacteria	*F. Nocardioidaceae*		0.77				

Actinobacteria	*Mycobacterium*		0.78
Actinobacteria	O. BPC015	-0.99	
Bacteroidetes	*F. Flammeovirgaceae*	-0.99	
Bacteroidetes	*F. SB-1*	-0.99	
Bacteroidetes	*F. Sphingobacteriaceae*		0.97
Bacteroidetes	*O. Bacteroidales*	-0.93	
Bacteroidetes	*Polaribacter*	-0.9	
Caldithrix	*O. MSB-5B5*	-0.99	
Chlorobi	*F. Ignavibacteriaceae*	-0.99	
Chloroflexi	*C. Ellin6529*	-0.99	
Chloroflexi	*F. Caldilineaceae*	-0.99	
Chloroflexi	*O. SB-34*	-0.99	
Firmicutes	*F. Lachnospiraceae*		0.97
Firmicutes	*O. MBA08*	-0.99	
GN04	*C.GN15*	-0.99	
NC10	*C. wb1-A12*	-0.99	
Planctomycetes	*C.MSBL9*	-0.99	
Planctomycetes	*C.p04 _C01*	-0.99	
Proteobacteria	*Agrobacterium*	-99	
Proteobacteria	*C. Alphaproteobacteria*		0.7
Proteobacteria	*Bradyrhizobium*		0.81
Proteobacteria	*F. Caulobacteraceae*		0.81
Proteobacteria	*F. Cohaesibacteraceae*	-0.99	
Proteobacteria	*F. Cystobacterineae*	-0.85	
Proteobacteria	*F. Desulfobacteraceae*	-0.99	
Proteobacteria	*F. Marinicellaceae*	-0.99	
Proteobacteria	*F. NB1-i*	-0.99	

Proteobacteria	*F. NB1-j*	-0.99				
Proteobacteria	*F. Syntrophobacteraceae*	-0.99				
Proteobacteria	*F. Thiohalorhabdaceae*	-0.99				
Proteobacteria	*Desulfococcus*	-0.99				
Proteobacteria	*Devosia*	0.93				
Proteobacteria	*Dokdonella*	-0.9				
Proteobacteria	*Hyphomicrobium*	-0.99				
Proteobacteria	*Methylobacterium*		0.97			
Proteobacteria	Unknown		0.69			
	Explained Variance (%)	**16.65**	**6.83**		**9.03**	

HOOPOE NESTS (MODEL: VOLATILES = BACTERIA)

		PC1		**PC1**		
Familia	**Bacteria Taxa**	**BM2**		**VM2**	**Volatile components**	
Actinobacteria	*F. Acidobacteriaceae*	-0.99		-0.77	Butanoic	*Acids*
Actinobacteria	*Agrococcus*	-0.7		0.76	Hexanal	*Aldehydes*
Actinobacteria	*F. AKIW874*	-0.99		0.83	Pentanal	*Aldehydes*
Actinobacteria	*O. CCU21*	-0.83				
Actinobacteria	*F. Propionibacteriaceae*	-0.74				
Actinobacteria	*O. Solibacterales*	-0.99				
Actinobacteria	*Solwaraspora*	-0.99				
Actinobacteria	*Streptomyces*	-0.92				
Actinobacteria	*O. Sva0725*	-0.99				
Bacteroidetes	*F. Cytophagaceae*	-0.87				
Bacteroidetes	*Larkinella*					
Chloroflexi	*F. Caldilineaceae*	-0.7				
Chloroflexi	*C. SJA-28*	-0.99				

Cyanobacteria	*Phormidium*	-0.7		
	Explained Variance (%)	**12.5**		**20.95**

Table S6. Results of PCA analyses summarizing volatiles detected in the nest material and the uropygial secretion of female and nestling hoopoes. Values are PC factor loadings after varimax normalized rotated and in their nest material or nest boxes. Each PC-axis was named by a composition of letters that indicate the type of samples. The first letter indicates whether the sample corresponds to bacteria (B) or volatiles (V), the second letter indicates whether the sample is from secretions of females (SF), nestlings (SN) or nest material (M). Finally, for types of samples that were collected at the beginning (1) and at the end (2) of the nestling period, the name finished with a number. Only factors than entered in final models explaining the association between volatile profiles of secretions and nest-box environment are shown.

AT THE BEGINNING OF THE NESTING PERIOD

		FEMALES				NESTLINGS		
		SECRETION			NESTS	SECRETION	NESTS	
		PC1	**PC2**	**PC3**	**PC1**	**PC2**	**PC1**	**PC4**
	Volatile profile	**VSF**	**VSF**	**VSF**	**VM1**	**VSN1**	**VM1**	**VM1**
Acids	Acetic				-0.71		-0.71	
Acids	Butanoic				-0.84		-0.84	
Acids	Heptanoic	-0.78				-0.87		
Acids	Hexanoic	-0.85				-0.87		0.82
Acids	Isobutiric		0.86					
Acids	Octanoic					-0.7		
Acids	Isocaproic		0.78					
Acids	Isovaleric							
Acids	Pentanoic		0.82			-0.72		0.78
Aldehydes	Benzaldehyde							
Aldehydes	Heptanal	-0.88						
Aldehydes	Hexanal	-0.89			0.75		0.75	

Aldehydes	Nonanal	-0.84			0.7					0.7		
Aldehydes	Octanal	-0.9										
Aldehydes	Pentanal	-0.91			0.76					0.76		
Esters	Butanoic-acid-ethyl-ester			**0.71**								
Esters	2-methyl-butanoic-acid-ethyl-ester		0.7									
Esters	3-mehyl-butanoic-acid-ethyl-ester			**0.7**								
Aromatic organic compounds	Phenol	0.73										
	Explained Variance (%)	**23.7**	**19.5**	**13.6**	**28.27**			**18.89**		**28.27**	**8.91**	

AT THE END OF THE NESTING PERIOD

		FEMALES					NESTLINGS				
		SECRETION			NESTS		SECRETION			NESTS	
		PC1	**PC2**	**PC3**	**PC1**	**PC3**	**PC1**	**PC2**	**PC4**	**PC1**	
	Volatile profile	**VSF**	**VSF**	**VSF**	**VM2**	**VM2**	**VSN2**	**VSN2**	**VSN2**	**VM2**	
Acids	Butanoic				-0.77					-0.77	
Acids	Heptanoic	-0.78					-0.7				
Acids	Hexanoic	-0.85					-0.7				
Acids	Isobutiric		0.86						**-0.78**		
Acids	Nonanoic					0.79					
Acids	Isocaproic		0.78						**-0.74**		
Acids	Pentanoic		0.82			-0.7			**-0.82**		
Acids	Propionic					-0.7					
Aldehydes	Heptanal	-0.88					-0.87				
Aldehydes	Hexanal	-0.89			0.76		-0.88			0.76	
Aldehydes	Nonanal	-0.84					-0.8				

Aldehydes	Octanal	-0.9							-0.88				
Aldehydes	Pentanal	-0.91				0.83			-0.8				0.83
Esters	Butanoic-acid-ethyl-ester			**0.71**						0.86			
Esters	Hexanoic-acid-ethyl-ester									0.7			
Esters	2-methyl-butanoic-acid-ethyl-ester		0.7							0.75			
Esters	3-mehyl-butanoic-acid-ethyl-ester			**0.7**						0.8			
Aromatic organic compounds	Phenol	0.73							0.7				
Others compounds	Ethanol2phenoxyCAS122996								0.7				
	Explained Variance (%)	**23.7**	**19.5**	**13.6**		**20.95**	**11.69**		**23.84**	**14.09**	**9.03**		**20.95**

Table S7. Results of PCA analyses summarizing bacterial genera (if unknown we use family or order) and volatiles detected in the nest material and the uropygial secretion of female and nestling hoopoes that explained parasitism and fledging success. Values are PC factor loadings after varimax normalized rotated and in their nest material or nest boxes. Each PC-axis was named by a composition of letters that indicate the type of samples. The first letter indicates whether the sample corresponds to bacteria (B) or volatiles (V), the second letter indicates whether the sample is from secretions of females (SF), nestlings (SN) or nest material (M). Finally, for types of samples that were collected at the beginning (1) and at the end (2) of the nestling period, the name finished with a number. Only factors than entered in final models explaining the intensity of ecto-parasitism in females and nestlings and the fledging success are shown. N MAT refers to nest material and SECR to uropygial secretion.

BACTERIA		N MAT	SECR	VOLATILES		N MAT	SECR
PARASITISM OF 8 DAYS OLD NESTLINGS							
		PC5				**PC1**	**PC2**
Familia	**Bacteria Taxa**	**BM1**			**Volatile profile**	**VM1**	**VSN1**
Actinobacteria	*Conexibacter*	0.9		***Acids***	Acetic	-0.71	
Actinobacteria	*F. AKIW874*	0.87		***Acids***	Butanoic	-0.84	
Actinobacteria	*F. Dietziaceae*	0.75		***Acids***	Heptanoic		-0.87
Actinobacteria	*O. 0319-7L14*	0.9		***Acids***	Hexanoic		-0.87
Actinobacteria	*O. Gaiellales*	0.83		***Acids***	Octanoic		-0.7
Chloroflexi	*C. TK10*	0.7		***Acids***	Pentanoic		-0.72
Chloroflexi	*F. Dolo_23*	0.87		***Aldehydes***	Hexanal	0.75	
Chloroflexi	*O. AKYG885*	0.83		***Aldehydes***	Nonanal	0.7	
Chloroflexi	*O. B07_WMSP1*	0.7		***Aldehydes***	Pentanal	0.76	
Chloroflexi	*O. pLW-97*	0.7					
Proteobacteria	*F. Chromatiaceae*	0.89					
Proteobacteria	*F. Polyangiaceae*	0.8					
	Explained Variance (%)	**3.01**			**Explained Variance (%)**	**28.27**	**18.89**

PARASITISM OF 19 DAYS OLD NESTLINGS

Familia	Bacteria Taxa	PC6 BM2		Volatile profile		PC2 VSN2
Bacteroidetes	*Larkinella*	0.73		***Esters***	Butanoic-acid-ethyl-ester	0.86
Proteobacteria	*Aquabacterium*	0.7		***Esters***	Hexanoic-acid-ethyl-ester	0.7
				Esters	2-methyl-butanoic-acid-ethyl-ester	0.75
				Esters	3-mehyl-butanoic-acid-ethyl-ester	0.8
	Explained Variance (%)	**3.74**			**Explained Variance (%)**	**14.09**

PARASITISM OF FEMALES

Familia	Bacteria Taxa	PC3 BSF		Volatile profile		PC2 VSF
Firmicutes	*F. Erysipelotrichaceae*	-0.7		***Acids***	Isobutiric	0.86
Firmicutes	*Lactococcus*	0.7		***Acids***	Isocaproic	0.78
Firmicutes	*Trissierellaceae*	-0.86		***Acids***	Pentanoic	0.82
				Esters	2-methyl-butanoic-acid-ethyl-ester	0.7
	Explained Variance (%)	**4.51**			**Explained Variance (%)**	**19.5**

FLEDGING SUCCESS AND VARIABLES FROM THE EARLY NESTLING PERIOD

Familia	Bacteria Taxa	PC6 BSF		Volatile profile		PC3 VSF	PC1 VSN1
Firmicutes	*Syntrophomonas*	0.73		***Acids***	Acetic		0.78
				Acids	Butanoic		0.86
				Acids	Isobutiric		0.7
				Aldehydes	Benzaldehyde		-0.73
				Aldehydes	Heptanal		-0.78

				Aldehydes	Hexanal		-0.78
				Aldehydes	Pentanal		-0.82
				Esters	Butanoic-acid-ethyl-ester	0.71	
				Esters	3-mehyl-butanoic-acid-ethyl-ester	0.7	
	Explained Variance (%)	**2.93**			**Explained Variance (%)**	**13.57**	**21.2**

FLEDGING SUCCESS AND VARIABLES FROM THE LATE NESTLING PERIOD

		PC1				**PC4**	
Familia	**Bacteria Taxa**	**BM2**			**Volatile profile**	**VSN2**	
Actinobacteria	*Agrococcus*	-0.7		***Acids***	Isobutiric	-0.78	
Actinobacteria	*F. Acidobacteriaceae*	-0.99		***Acids***	Isocaproic	-0.74	
Actinobacteria	*F. AKIW874*	-0.99		***Acids***	Pentanoic	-0.82	
Actinobacteria	*F. Propionibacteriaceae*	-0.74					
Actinobacteria	*O. CCU21*	-0.83					
Actinobacteria	*O. Solibacterales*	-0.99					
Actinobacteria	*O. Sva0725*	-0.99					
Actinobacteria	*Solwaraspora*	-0.99					
Actinobacteria	*Streptomyces*	-0.92					
Bacteroidetes	*F. Cytophagaceae*	-0.87					
Chloroflexi	*C. SJA-28*	-0.99					
Chloroflexi	*F. Caldilineaceae*	-0.7					
Cyanobacteria	*Phormidium*	-0.7					
	Explained Variance (%)	**12.47**			**Explained Variance (%)**	**9.03**	

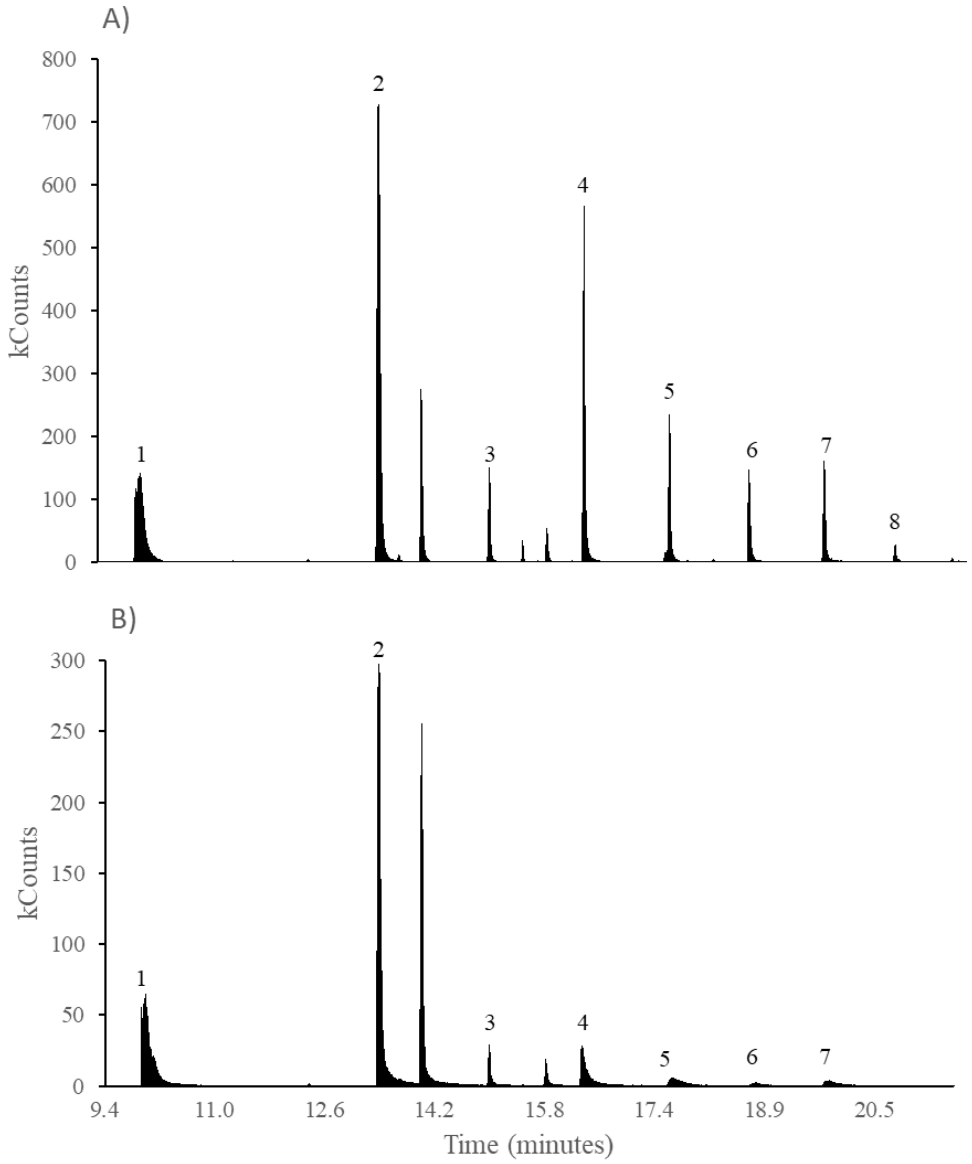

Figure S1: Example of SIM 60m/z chromatograms of A) the nest-box environment of a hoopoe nest at the beginning of the nestling stage and of B) uropygial secretion of the brooding female. Peaks represent the the acids detected in kcounts (from fewer carbons to higher carbons in the molecule:1. acetic, 2. butanoic, 3. pentanoic, 4. hexanoic, 5. heptanoic, 6. octanoic, 7. nonanoic and 8. decanoic).

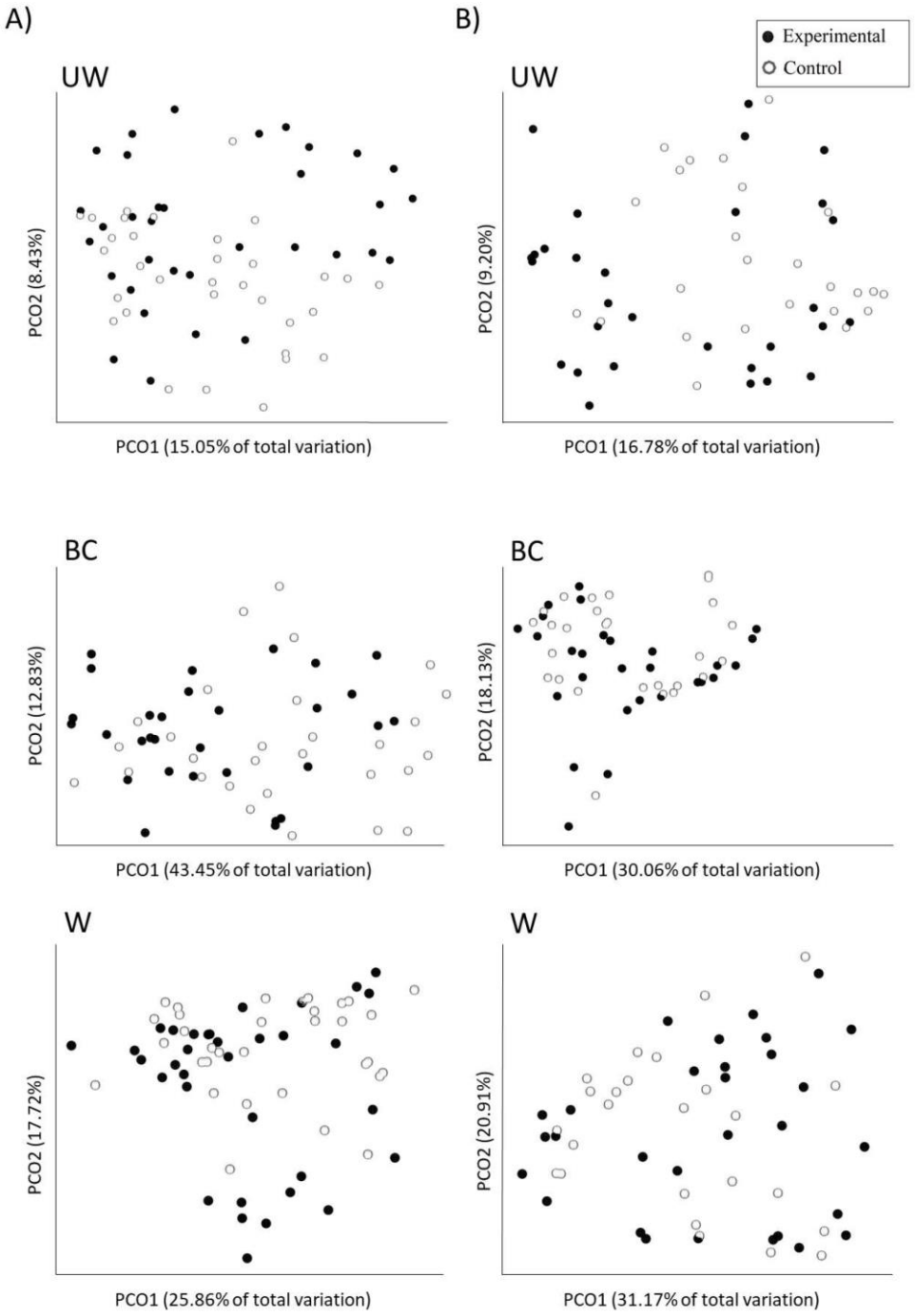

Figure S2. Principal coordinate analyses of the bacterial communities of nest materials and volatile profiles of hoopoe nest-boxes from samples collected A) at the beginning (on the left) and B) at the end of the nestling stage (on the right). PCO scores were based on beta diversity distance matrixes (unweighted (UW) and weighted (W) Unifrac for bacterial communities, and Bray-Curtis (BC) distance for volatile profiles) of samples collected from experimental and control nest.

CAPÍTULO IV. *Microbiota and the volatile profile of avian nest associate with each other and with the risk of parasitism*

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Revista: Manuscrito en preparación

MICROBIOTA AND THE VOLATILE PROFILE OF AVIAN NESTS ASSOCIATE WITH EACH OTHER AND WITH THE RISK OF PARASITISM

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Abstract

Bacteria metabolize organic compounds of nest materials and debris from reproductive activity that accumulate in avian nests during the breeding season, and, thus, have been suggested as responsible of nest environment odours. Since ectoparasites mainly use olfaction to detect their hosts, bacteria located in avian nests (Nidobiome) and volatiles from their metabolism could influence the probability of parasites detecting such nests and, thus, the intensity of selection pressures due to parasites. Here, we tested this hypothesis by exploring intra- and interspecific variability in microbial environments and volatile profiles of avian nests at the beginning and at the end of the nestling period, as

well as the intensity of ectoparasitism by *Carnus haemapterus* flies suffered by nestlings of ten different avian species. As expected (i) microbial alpha diversity associated with alpha diversity of volatiles in interaction with bird species identity, but the results varied depending on the index and the sample (age of nestlings) used in the analyses. In addition, (ii) beta diversity of bacterial community based in PhILR distances explained the beta diversity of volatile profile but only at the late stage of nestlings. Moreover, (iii) alpha diversity of volatiles associated with intensity of parasitism at early stage of nestlings, while alpha diversity of bacterial community and beta diversity of volatiles were related with intensity of parasitism at the end of the nestling period. (iv) Only alpha diversity of the microbiota of the nest at the begging of the nestling period explained the fledging success. Finally, some key bacteria and volatiles that were related to each other also associated with intensity of parasitism and, at lower rate, with fledging success. Taking together, those results support the expected links between microbial environment and nest odours in different bird species, and between them and ectoparasitism intensity and fledging success. Future research should prioritize experimental approaches directed to determine the role of particular bacteria and volatiles in the outcomes of host-ectoparasite interactions.

Keywords: Avian-nest microbiota, avian nests odours, bacteria, ectoparasitism, Nidobiome, volatiles.

1. Introduction

Exploring how the microbiota influences animal behaviour in general and chemical communication in particular is nowadays a cutting edge line of research (Carthey et al., 2018; Ezenwa & Williams, 2014; Maraci et al., 2018; Mazorra-Alonso et al., 2021). The metabolism of symbiotic microorganisms generates volatiles that influences the odour profile of individuals (Archie & Theis, 2011; Engl & Kaltenpoth, 2018), and, thus, bacterial symbionts might play essential roles in animal signalling and chemical communication (Carthey et al., 2018; Ezenwa & Williams, 2014). It is known that characteristics of the bacterial communities reflect the phenotypic and physiological conditions of their animal hosts (Bourne et al., 2023; Leclaire et al., 2017; Theis et al., 2013). Thus, specific volatiles from metabolism of such bacterial symbionts would inform conspecifics and hetero-specifics (i.e., predators or parasites) of characteristics of their animal hosts (Archie & Theis, 2011; Mazorra-Alonso et al., 2021; McDonald et al.,

2012). Because parasites and predators use olfaction to seek and detect their victims (Bowen, 1991; Poldy, 2020; Reneerkens et al., 2005; Takken & Knols, 1999; Zwiebel & Takken, 2004), some of the eavesdropped cues could be of bacteria origin, as it occurs in mosquitoes seeking for human hosts (Verhulst et al., 2009, 2010, 2011).

Odours associated with remains of animal physiological activities such as those of urine, faeces, and decomposing rests of prey or food can act as attractants for ectoparasites and predators (Becker et al., 1995; Hall, 1995; Hassanali et al., 1986; Vale et al., 1986). For instance, the accumulations of faecal waste increase the attraction of mosquitoes towards cages with hamsters (Becker et al., 1995). Moreover, the occupation of avian nest-holes by mammals during winter affected ectoparasitism of hoopoe (*Upupa epops*) nestlings in spring (García-Núñez et al., 2023), and the experimental addition of faeces in nests of spotless starlings (*Sturnus unicolor*) increase probability of predation (Azcárate-García et al., 2019). Faeces and other debris of the biological activity of animals harbour dense bacterial communities that may mediated the associations between those kinds of debris and risk of parasitism and/or predation. Thus, to understand the possible role of volatiles of bacterial origin explaining the association between animal physiological activity and their detectability by parasites or predators, it is necessary to explore the association between symbiotic bacterial communities of animals and volatiles in the environment where the animals live (Mazorra-Alonso et al., 2021). Moreover, finding associations between risk of infection or predation and particularities of the symbiotic bacterial communities of animals or of the volatile profiles of their environment would further suggest a role of the symbiotic bacteria determining the outcomes of the interactions with parasites and predators. Those associations have rarely been explored in natural conditions, and only in particular species (see revision in Mazorra-Alonso et al., 2021).

The avian nest environments, particularly those located in holes, are suitable habitats to explore such associations because it would allow focusing on particular locations where bacterial communities can be characterized, and because holes would allow characterizing volatiles profiles that are mostly derived from animal physiological activity. Most avian species build nests where they lay and incubate the eggs, and where nestlings develop (Hansell, 2000). Location and materials used for nest construction influence temperature and humidity (Deeming & Mainwaring, 2015; Windsor et al., 2013), risk of predation (Mainwaring et al., 2015) and of parasitism (I. López-Rull &

García, 2015), and also bacterial environment where offspring develop (Peralta-Sanchez et al., 2011; Ruiz-Castellano et al., 2016; Soler et al., 2015; West et al., 2015). Nest microbial environment, the Nidobiome (Campos-Cerdá & Bohannan, 2020), includes some other important bacterial communities of birds such as those of the feathers (Goodenough et al., 2017; Mennerat et al., 2009), the faeces (Hird et al., 2014), and the uropygial gland of adult and nestlings (Díaz-Lora et al., 2019; Martín-Vivaldi et al., 2018; Ruiz-Rodríguez et al., 2014). The Nidobiome would also include some bacteria with harmful [e.g., affecting egg viability (Peralta-Sánchez et al., 2018) or feather degradation (Ramnani et al., 2005)], or with beneficial effects [e.g., antibiotic producing bacteria that prevent the establishment of pathogenic ones (Peralta-Sánchez et al., 2010, 2011, 2014; Ruiz-Castellano et al., 2016, 2019; Soler et al., 2010)]. The bacterial environment of avian nests would therefore depend on species-specific particularities of the nest constructions (Peralta-Sánchez et al., 2012) and, thus, the Nidobiome and their associated positive or negative effects (either direct or indirect) on nesting birds should also vary interspecifically (Soler et al., 2011, 2012). Moreover, during the breading season, avian nests accumulate organic compounds that bacteria can metabolize [i.e. remains of food, faeces, dead nestlings, etc. (Mackie et al., 1998)] and, thus, interspecific variation in nest sanitation behaviour (Ibáñez-Álamo et al., 2017) would also explain predicted interspecific variation in characteristics of the Nidobiome.

Volatiles of the nest environment should also vary interspecifically, not only because some of them would be by-products of bacterial metabolism, but also because some species use different aromatic materials (i.e., flowers, green and aromatic plants) to build their nests that also affect the Nidobiome (see above). Then, if parasitism or predation was partially determined by volatiles including those of bacterial origin, their interspecific variation should associate with that of volatile profiles and/or bacterial community of avian nests (Mazorra-Alonso et al., 2021). Several results suggest that volatiles from bacterial metabolism were partially responsible of the incidence of parasitism and predation in different bird species. For instance, autoclaving nest material affected the intensity of parasitism suffered by hoopoe (*Upupa epops*) nestlings (Mazorra-Alonso et al., 2020), indicating that nest material or particular bacteria isolated from hoopoe nests influence ectoparasite attraction (Tomás et al., 2020). In addition, increasing bacterial loads in spotless starling nests increase probability of predation (Azcárate-García et al., 2019). Whether those detected effects of symbiotic bacterial

communities is mediated by volatiles of bacterial origin is however a matter of debate (Mazorra-Alonso et al., 2021). Although some results in laboratory conditions and from animal models suggest that it might be the case, investigations under natural conditions are necessary to reach a firm conclusion (Tomás et al., 2020). Actually, some evidences suggest that animal odours associate with probability of ectoparasitism and/or predation (Bowen, 1991; Poldy, 2020; Reneerkens et al., 2005; Takken & Knols, 1999; Zwiebel & Takken, 2004), but results indicating a link between symbiotic bacterial community and animal odours is scarce (Jacob et al., 2018; Leclaire et al., 2017; Whittaker et al., 2019). More importantly, with few exceptions (e.g., Verhulst et al., 2009, 2011), the effects of bacteria and odours on ectoparasitism has been traditionally explored separately. Thus, exploring the three ways expected associations between symbiotic bacteria, animal odours and parasitism is necessary to test the hypothesis that volatiles from bacterial metabolism mediated the somewhat known effects of volatiles and of microbial symbionts on probability of parasitism. This approach however has rarely been adopted to test the importance of volatiles from bacterial metabolism on parasitism (but see Bourne et al., 2023).

In this study, we explore the hypothesis that bacteria in the avian nests emit volatiles that influence the ectoparasitism of avian nestlings by the hematophagous fly *Carnus hemapterus*, which is the most abundant ectoparasites in our study area. Moreover, we here explore interspecific variation in either the bacterial community of nest materials, nest odours, or parasitism of avian nestlings, as well as the three-ways associations among them. Since parasitism by definition should have negative effects on reproductive success, we also explore the effect of bacterial community and of volatile profiles on fledging success. We collected information from nests environments of 10 species, which frequently use installed next boxes or are abundant in the study area. The hypothesis tested predicts two types of associations. On the one hand, (A) volatile profiles and bacterial community should be related to each other, and, on the other hand, (B) both bacterial and volatile profiles should predict probability and/or intensity of ectoparasitism and of fledging success. We explore those associations at the interspecific and at the intraspecific level. Since ectoparasitism was focused on the same blood sucking species, we predicted that the expected associations appear even after controlling for the species identity.

2. Material and Methods

a. Study area and species

The study area was located in the Hoya the Guadix, (Granada, Southern Spain, 37°18´N, 38°11´W), a plateau at 1000 m a.s.l. with semiarid climate, where around 400 cork-made nest-boxes were available for wild birds; most of them attached to tree trunks and walls, but also hidden in piled stones. The dimensions of the nest-boxes were 35 × 18 × 21 cm (internal height × width × depth), 24 cm (bottom-to-hole height) and 5.5 cm (entrance diameter). Information about the study area is further described elsewhere (Peralta-Sánchez et al., 2018).

We sampled some of the species that frequently breed in this area: hoopoe (*Upupa epops*), scops owl (*Otus scops*), little owl (*Athene noctua*), European roller (*Coracias garrulus*), stock pigeon (*Columba oenas*), house sparrow (*Passer domesticus*), great tit (*Parus major*), jackdaw (*Coleous monedula*), magpie (*Pica pica*) and spotless starling (*Sturnus unicolor*). All of these species use our nest-boxes for reproduction, with the exception of the magpie. Sample sizes for each avian species and type of collected samples are shown in Table 1.

The most abundant ectoparasite in our study area is *Carnus hemapterus*, a common generalist hematophagous fly of about 2 mm in length (Calero-Torralbo et al., 2013; Martín-Vivaldi et al., 2006) that parasitizes several avian species (Capelle & Whitworth, 1973; Tomás et al., 2018; Valera et al., 2003). In spring, winged adults emerge from pupae that developed inside the avian nest materials from previous reproductions. Adults flies can stay in the same nest or disperse, and, once they arrive to the chosen host-nest for parasitism, they lose their wings and feed on nestlings or incubating adults (Avilés et al., 2009; López-Rull et al., 2007). The life cycle of *C. hemapterus* synchronize with reproduction of their hosts (Calero-Torralbo et al., 2013; Valera et al., 2003), and the peak of abundance in avian nests occurs just before the start of nestling feathering (Liker et al., 2001).

Table 1. Sample sizes (number of nests) of ten avian species with information of the bacterial community of nest material (Nest Mat), of the volatile profile of nest boxes ((Nest-Box) and of intensity of ectoparasites by *Carnus hemapterus* (Parasit), both at the beginning and at the end of the nestling period. For species with information of the bacterial community in 10 or more nests that were used to build sPLS models (i.e. those in bold fonts), we show restricted sample sizes and ASVs (Restricted (ASVs)) after eliminating ASVs that represented less than 0.008% of the total ASVs counts and that appeared in less than 4 samples.

	Nest at early stage of nestlings			**Nest at late stage of nestlings**		
Avian species	Bacteria (Nest Mat)	Volatiles (Nest-box)	Parasit	Bacteria (Nest Mat)	Volatiles (Nest-box)	Parasit
Upupa epops	90	62	115	70	58	112
Restricted (ASVs)	45 (599)			40 (466)		
Otus scops	10	13	13	11	13	13
Restricted (ASVs)	11 (301)			12 (295)		
Athene noctua	11	12	11	10	10	10
Restricted (ASVs)	11 (236)			10 (268)		
Coracias garrulus	7	9	9	7	8	8
Columba oenas	5	4	2	3	4	4
Passer domesticus	8	6	6	6	6	6
Parus major	4	4	4	4	4	3
Coloeus monedula	10	10	12	12	10	13
Restricted (ASVs)	8 (509)			10 (373)		
Pica pica	0	0	0	13	0	0
Sturnus unicolor	16	16	15	9	11	12
Restricted (ASVs)	14 (525)			8 (217)		

b. Fieldwork

The fieldwork was carried out during the breeding seasons (March – June) of 2017 and 2018. We checked all nest-boxes once per week to find active nests. Once an active nest was detected, we identified the bird species and, depending on the number of eggs detected in the nests and considering the length of the incubation period of each species, we estimated the expected hatching date of the first eggs (hereafter, day 1 of the sampling protocol). During nestling stage, nests were visited several times to sample the bacterial communities of nest materials, the volatiles of the nest-box environment, and to estimate the intensity of parasitism of nestlings and fledging success. For each nest visit, we wore new latex globes previously washed with 96% ethanol to avoid contaminations between nests and visits.

We collected nest material that were in contact with nestling twice: at the beginning (between the days 4 and 6 depending on the length of nestling period of the sampled bird species) and at the end of the nestling stage (between the days 17 and 23 depending on the bird species). These samples were stored in sterilized 1.5 mL microfuge tubes and kept cold in a portable fridge until arriving to the lab where samples were stored at -20 ºC until bacterial DNA extraction.

We also sampled volatiles of nest-box environments twice: at the two nestling stages described above. In the case of magpies that are non-hole nesters, we did not sample nest volatiles. Volatiles were captured in Solid Phase Microextraction (SPME) fibres. The fibre was installed on one of the walls (around 7 cm high from the nest material) with the sensitive end protected with a two sides opened glass pipette tip. The exposition time of volatiles capturing fibres in the nest environments was 24 hours. Afterwards, we removed the fibre from the nest-box and the sensitive side was introduced into a closed glass vial that was kept cold in portable fridge (0-4 ºC) until arriving to the lab where it was stored at -20 ºC until the gas chromatography-mass spectrometry analysis. Storage of samples in lab conditions never exceeded one week. After the analyses, SPME fibres were re-conditioned (i.e., eliminating any chemical trace) and kept at -20 ºC until they were reused in the field.

Ectoparasitism of nestlings were also estimated twice, at the early (between days 5 and 8 depending on the bird species) and the late stage (between days 17 or 23 depending on the bird species) of the nestling period. As a proxy of parasitism intensity, we counted the number of blood marks and faeces remains from *C. hemapterus* flies activity on the body (belly and wings) of each nestlings following Tomás et al. (2018). Mean values per brood were used in the analyses. We estimated fledging success as the proportion of broodlings that were sampled at the beginning of the nestling period that survive until the end of the nestling period.

c. Volatiles profile: Gas chromatography-mass spectrometry (GC-MS)

Analyses were performed on gas chromatography coupled to a mass spectrometer Varian 450GC 240MS with an automatic injector Combi Pal to SPME fibre (50/30 μm DVB/CAR/PDMS, Stableflex 23Ga, Autosampler). Injector desorption was at 250 ºC for 10 minutes in Split (20: 1) and helium flow at 2 mL/min. The capillary column was Aligent HP-FFAP 30 m X 0.32 mm X 0.25 μm. The oven temperature was initiated at 50 ºC for 1 min, and programmed to increase 5 ºC/min to 100 ºC, then at 10 ºC/min to 200

ºC and 50 ºC to 250 ºC for 1 min. The scan range of the mass spectrometer was in mode TIC Full Scan between 30 to 500 m/z. The identification of compounds was established by ion SIM analysis and the NIST 08 spectrum library. The summary of volatile profiles of next-box environments and uropygial gland secretions can be found in the Electronic Supplementary Material (ESM-Table S1). When necessary, we used standards of pure compounds for confirmation.

As a proxy of volatile abundance, we estimated the relative importance (subarea) in percentage of each compound of the area delimited by the complete chromatogram of each sample.

d. DNA extraction and high-throughput sequencing

Bacterial DNA from nest-material were extracted using the MSOP protocol proposed by (Martín-Platero et al., 2007). Briefly, approximately 80 mg of solid nest material were suspended in 900 µL of buffer lysis and, after centrifugation, supernatant was collected and stored in 2 mL microfuge tubes (for further details of the followed protocol see Lee et *al.*, 2021). Extracted DNA from nest material samples was cleaned using the kit One Step PCR Inhibitor Removal Kit (Zymo Research). We also processed laboratory blanks to detect possible contamination during the process.

DNA sequences from nest materials were obtained by amplification of the V6-V8 hypervariable regions (approximately 400 bp fragment) of the 16S rRNA gene. In a first PCR, this fragment was amplified using the universal primers B969F (5´-ACGGGCRGTGWGTRCAA-3´) and BA1406R (5´-ACGGGCRGTGWGTRCAA–3´). In a second PCR, samples were amplified adding barcodes for identifying samples. Afterwards, the libraries were pooled and sequenced in a single run of Illumina MiSeq sequencer (2 x 300 bp output mode), at the Integrated Microbiome Resource (IMR) in the University of Dalhousie (Canada). Sequences are available under at NCBI under accession numbers: XXXXX-XXXXX.

Raw sequences were analysed using QIIME2 2019.10 (Bolyen et al., 2019). Briefly, primers were trimmed and, due to low quality of the reverse sequences, subsequent analyses were performed based only on forward sequences. Low quality sequences (Phred < 20) were filtered and Deblur algorithm was employed to produce Amplicon Sequence Variants table (ASV table) establishing a sequence size of 220 bp. A phylogenetic tree was built using fragment insertion algorithm (Janssen et al., 2018).

Taxonomic assignation was performed against Greengenes13_8 database at 97% similarity (DeSantis et al., 2006; McDonald et al., 2012). Chloroplast, mitochondria and non-phylum assigned ASVs were removed, as well as positive control samples. ASV diversity reached plateau at approximately 6 000 reads (ESM-Figure S1), and samples with less than 6 000 reads were excluded from downstream analyses (11 samples and all control samples). The sequencing of nest material produced 22 476 907 sequences, and 7 112 413 were retained in the ASV table after filtering (number of samples = 328; number of sequence per sample (min, max) = (6 165, 56 018)). With respect to AVSs, we obtained 14 303 (mean frequency per sample = 21 648.19, SD per sample = 9 955.19).

e. Statistical procedures

Estimating alpha and beta diversity indexes

Based on abundances of ASVs, we calculated alpha (i.e. the microbial diversity within a particular sample) and beta diversity (i.e., the variability in community composition among different types of samples) for each collected bacterial samples (see Whittaker, 1972). For alpha diversity analyses, we calculated Shannon and Faith's phylogenetic diversity (PD) indexes in QIIME2 environment. Shannon index is a quantitative measure of equity that combines the richness and evenness of bacterial species (Shannon, 1948), while PD index is a qualitative measure of diversity that takes into account the length of all branches on the bacterial phylogenetic tree within a sample (Faith & Baker, 2006).

For beta diversity analyses of bacterial samples, we calculated Aitchison's and Phylogenetic Isometric Log Ratio transformed (PhILR) distance matrixes for each sample. Aitchison distance matrix (Aitchison et al., 2000) was calculated after the Centered Log Ratio (CLR) transformation of ASVs abundances in *microbiome 1.18.0 R* package (Lahti & Shetty, 2012). The CLR-transformation controls for the compositional nature of the microbiome dataset and produces values that are scale invariant (Gloor et al., 2017). We also estimated PhILR distance matrix using ASV relative abundances after CLR transformation and accounting for the information of the phylogenetic relationship of ASVs (Silverman et al., 2017). The PhILR transformations were performed as implemented in *philr 1.22.0 R package* (Silverman et al., 2017).

For the volatiles profiles, we estimated alpha and beta diversity indexes based on the relative abundance of each volatile detected in the nest environment. We estimated

the Shannon index for alpha diversity, while for beta diversity we estimated the Aitchison distance matrix after CLR-transformation of relative abundances (for explanations of these indexes and their estimations, see above).

Exploring interspecific differences in alpha and beta diversities and associations between diversities estimated for nest bacterial communities and for volatile profiles of avian nests

Interspecific differences in alpha diversity indexes and beta diversity matrices of the bacterial community of nest materials and of volatile profiles of avian nests were respectively explored in General Lineal Mixed Models (GLMMs) and mixed PERMANOVAs (i.e., nonparametric multivariate analysis of variance) with 9 999 permutations. It is worth to mention here that, for species with information collected during the two study years, we analysed the effect of study year. In all cases, variance of values of different diversity indexes explained by the species identity resulted significantly larger than that explained by the study year (see ESM-Figure S2). Thus, the effect of study year was not included in subsequent statistical models.

The associations between alpha diversity of volatile profiles of avian nests (dependent variable) and that of bacterial communities of nest material (continuous fixed factor) were explored in GLMMs that included bird species identity as random factor. The interaction between species ID and bacterial diversity was studied in a separated model that also included main effects. Intraspecific associations between bacterial (predictors) and volatiles (explanatory) alpha diversities were also analysed in General lineal models (GLM), but only for species with more than seven samples. Residuals of all performed GLMMs and GLMs approximately followed normal distributions.

To analyse the association between beta diversities of bacterial communities of nest material and of volatile profiles of avian nests, we used multivariate Mantel tests with 9 999 permutations. These tests are equivalent to a multiple regression tests but using matrices of differences among samples instead of lineal information. The models included beta diversity of volatiles (based on Aitchison distances) as the dependent matrix, and beta diversity of bacterial communities (based on Aitchison or in PhILR distances) and a matrix of bird species identity (whether each cell in the matrix were (cell value = 1) or were not (cell value = 0) belonged to the same bird species) as independent matrices. In this case, we also explore intraspecific association in species with more than seven samples.

All GLMMs were performed in STATISTICA 12.0, PERMANOVAs in PRIMER-7.0.17, and Mantel-tests in R.3.6.1 (https//www.r-project.org/) environment (function MRM in the *ecodist* package (Goslee & Urban, 2007).

To infer potential relationships between specific bacterial strains and specific volatiles, we integrated information of relative abundances (i.e., CLR-transformed) of the considered ASVs and volatiles, using sparce Partial Least Squares or Projection to Latent Structures (sPLS) models with a leave-one-out cross-validation method as implemented in the package *mixOmics* v6.19.9 (Rohart et al., 2017). sPLS has been used as an alternative method to ordinary least squares for handling multicollinearity (Chun & Keleş, 2010). We used the regression mode of sPLS, which fits a linear relationship between multiple responses in Y and multiple predictors in X. The microbial data is being attempting to be used to explain the volatile profiles. The primary difference between sPLS and other dimension reduction techniques (i.e. PCA), is that it maximizes the covariance between latent variables (also termed latent component) rather than correlation (Burguillo Muñoz, 2015).

At the interspecific levels, we performed separated sPLS analyses for samples collected at the early and at the end of nestling period. The sPLS models are quite sensitive to noise associated with low sample sizes (Arumugam, et al., 2011) and, thus, we only considered ASVs that represented more than 0.008% of the total ASVs counts, and that appeared in at least four samples. These restrictions reduced the number of considered ASVs to 996 and 1 111 in samples of nest material collected at the beginning (N = 110) and at the end (N = 96) of the nestling period, respectively. To graphically show the correlation structure (i.e., the network) among the latent ASVs and volatiles from sPLS models, we used the *network* function and the *igraph* package v1.3.4 (Csardi & Nepusz, 2006). That function uses detected correlations between predictor and response latent components of the sPLS model to draw a network among them. We only represent elements from the first latent component of the response variables (volatiles) since no other variable surpassed de stablished minimum value of q^2 (0.095) for validation of latent components (see Wold et al., 2001). Moreover, the Pearson correlation value between the latent components was different for each sampling moment (i.e. at early and at late of the nestling stage). We use for its representation a correlation range determined by the maximum Pearson correlation value and 6% less than that value [(Max -

6*Max/100) – (Max)]. Then, we exported and visualized the resulting network in Cytoscape v3.9.9 (Shannon et al., 2003).

At the intraspecific level, we performed separate sPLS models for each species with more than seven samples collected either at the beginning or at the end of the nestling period. In this case, we only used ASVs that represented more than 0.01% of the total counts, and that appeared in at least four samples. Sample sizes and number of considered ASVs after sieving information of each species are shown in Table 1.

Exploring specific characteristics of nest bacterial communities and of volatile profiles as predictors of intensity of parasitism and of avian fledging success

The relationship between intensity of parasitism (square-root transformed values) or fledgling success and diversities of volatiles and bacterial communities were explored in General Linear Mixed Models (GLMMs) and Mantel tests for alpha and beta diversities, respectively.

GLMM models included bacterial and volatile alpha diversities as continuous fixed factors, and bird species identity as random factor. Moreover, since we were interested on detecting interspecific differences in the associations of interest, the interactions between species identity and bacterial alpha diversities, and between species identity and volatile alpha diversities, were also studied in two separate models that also included main effects. Mantel-tests included matrices of differences in intensity of parasitism and fledging success between samples as dependent matrices and the beta diversity of volatiles and of bacteria as independent factors. The models also included a matrix with information of the species identity (binary matrix: 1= equal, 0 = different species) as an additional independent factor.

For exploring the associations between particular volatiles or bacteria and intensity of parasitism or fledging success, we only used those elements from sPLS models with correlation coefficients within the considered range ([(Max - 6*Max/100) – (Max)]). The associations were explored in GLMMs. Since considered bacteria and volatiles are the latent components from sPLS models, they are by definition correlated to each other. To avoid problems of collinearity, we explored the effects bacteria and or volatiles in separate GLMM models. These models included species identity as random factor and bacterial or volatile elements as continuous fixed factors. Moreover, in addition to full models, we used Akaike information criterion to look for best models, choosing

that with the lowest AIC value that include the lower number of independent factors. When species identity was included in the final reduced model, its effect was modelled as random effects. The intraspecific associations between parasitism intensity or fledging success and particular bacteria or volatiles from sPLS models were also explored intraspecifically.

3. Results

a. Bacterial communities and volatile profiles of avian nests

Alpha and beta diversity of nest bacterial communities and of volatile profiles differed among species independently of the nestling stage when the samples were collected (ESM Table S2). After controlling for that source of variation, alpha diversity of the nest bacterial community explained alpha diversity of volatiles of nest boxes, but only in interaction with species identity (Table 2). At the early stage of the nestling period, the interaction between species identity and Shannon values of alpha diversity of nest bacterial community explained volatiles detected in the nest boxes (Table 2). That interaction was mainly due to the positive and negative associations that were respectively detected in *Sturnus unicolor* (Beta = 0.56, GLM: $F_{1,12}$ = 5.35, p = 0.039) and *Athene noctua* (Beta = -0.72, GLM: $F_{1,9}$ = 9.39, p = 0.013) (see Figure 1 and ESM Table S3 for results of intraspecific associations). When considering samples from the late stage of nestling period, the interaction with species identity reached statistical significance when considering Faith-PD of nest bacterial community (Table 2). In this case, the effect of the interaction explaining volatiles profiles was mainly due to the non-significant positive and negative associations detected in *Coleus monedula* (Beta = 0.23, GLM: $F_{1,8}$ = 0.43, p = 0.530) and *Otus scops* (Beta = -0.33, GLM: $F_{1,10}$ = 1.19, p = 0.301), respectively (see Fig. 1 and the results of intraspecific associations in ESM Table 3).

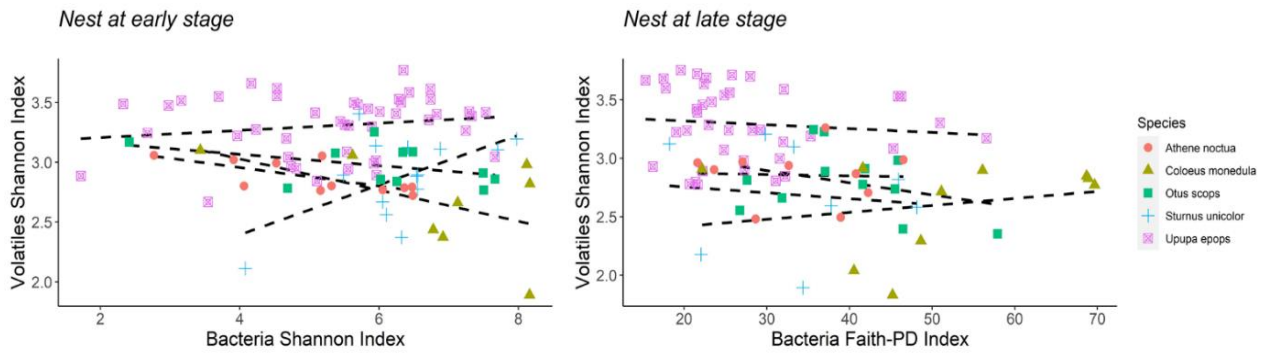

Figure 1. Associations between alpha diversity of volatile profiles (Shannon Index) and the bacterial communities (Shannon Index) of the nest of different species at early stage of nestlings and volatile profiles (Shannon Index) and the bacterial communities (Faith-PD Index) of the nest at late stage of nestlings. Lines are regression lines.

Beta diversity of bacteria of nest material at the early nestling stage did not predict beta diversity of volatiles of nest environments after controlling for the significant effects of species identity (Table 2). Intraspecifically, and independently of the diversity index used to build distance matrices, that association did not reach statistical significance in any of the five analysed species (see results in ESM, Table S3). However, at the end of the nestling stage, beta diversity (i.e., PhILR matrix) of bacterial community of nest material predicted volatiles profiles of avian nests even after controlling for the statistically significant effect of species identity (Table 2). Within species, the positive association reached statistical significance in two of the five species analysed [i.e., *Athene noctua* (PhILR: Mantel tests, $R^2 = 0.40$, $R = 0.30$ $F = 28.86$, $p < 0.002$) and *Otus scops* (Aitchison: Mantel tests, $R^2 = 0.16$, $R = 0.04$, $F = 12.44$, $p < 0.001$; see intraspecific results in ESM Table S3].

Table 2: General Linear Mixed Models and Mantel tests exploring the relationships between alpha and beta diversities of volatiles and of bacterial communities of nest environment during the early and the late nestling stage. To characterize alpha and beta diversity of volatiles, we respectively used Shannon and Aitchison indexes, while Shannon or Faith's Phylogenetic Diversity (PD) (F1), and Aitchison or Phylogenetic-Isometric-Log-Ratio-transformed (PhILR) distance were respectively used to characterize bacterial community. Information of species identity (Sp ID) was included as random effect (R). GLMMs, and as binary matrix (1= equal, 0 = different species) in Mantel tests. In GLMMs, the interactions between bird-species identity and bacteria diversity was tested in separate models that also included main effects, while the main effects were explored in models that do not include interactions. We show partial beta values and associated p-values that for Mantel tests were estimated after 9 999 permutations, and beta values associated to diversities in GLMMs. Variables in bold font are statically significant at the 5% level.

ALPHA DIVERSITY	**Shannon**				**PD**			
	F	**df**	**P**	**Beta**	**F**	**df**	**P**	**Beta**
Early nestling stage								
Sp ID (R)	9.24	8,100	**<0.001**		10.92	8,100	**<0.001**	
Bacteria alpha diversity (F1)	0.00	1,100	0.952	0.005	2.91	1,100	0.091	0.143
F1*Sp ID	2.53	8,92	**0.016**		0.79	8,2	0.616	
Late nestling stage								
Sp ID (R)	8.70	7,87	**<0.001**		8.10	7,87	**<0.001**	
Bacteria alpha diversity (F1)	0.13	1,87	0.719	0.036	0.29	1,87	0.594	0.063
F1*Sp ID	1.49	7,80	0.181		3.31	7,80	**0.004**	

BETA DIVERSITY	**Aitchison**		**PhILR**	
Early nestling stage		**R**		**R**
Sp ID	**<0.001**	0.659	0.647	**<0.001**
Bacteria beta diversity	0.644	-0.002	-0.003	0.730
Late nestling stage				
Sp ID	**<0.001**	0.931	0.709	**<0.001**
Bacteria beta diversity	0.094	-0.009	0.032	**0.014**

Optimization of the sPLS models of samples from the early and the late stage of nestling period resulted in a single latent component in both cases. The latent components from samples of the early nestling period explained 8.52% and 12.49% of the variance of the abundance of bacteria and of volatiles, respectively. The resulting latent components included five bacterial taxa (the taxonomic classification of detected ASVs is shown in the ESM, Table S4, and hereafter we will directly refer to the taxonomic classification (i.e. genus) of each ASV) and one volatile (heptanal). Similarly, the latent components of

samples of the late nestling period explained 9.27% and 11.71% of the variance of the abundance of bacteria and of volatiles, respectively, and included four bacterial taxa and one volatile (heptanal). The strength of the associations between heptanal and bacterial taxa in early and late nestling stage is showed in Figure 2. At the intraspecific level, the identity of bacteria and volatiles that resulted as latent components after the optimization of the sPLS models depended on the considered species and the stage of nestling development (EMS, Table S5). In this case, depending on the considered species, a variety of bacterial taxa associated with different volatiles (i.e. several acids and benzaldehyde) (see ESM, Table S5).

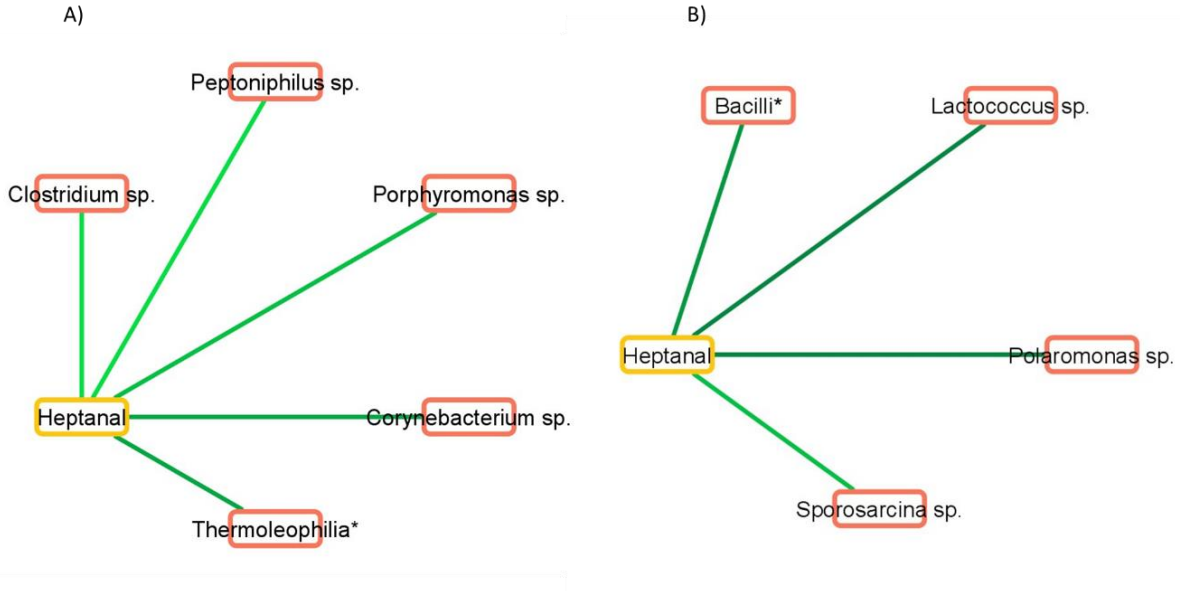

Figure 2: Network representation of sPLS performed on bacteria and volatile profiles data from the nest (A) at the early stage and (B) at the late stage of nestlings, from all bird species. Both networks are bipartite, where each edge links an ASVs to a volatile node, according to a similarity matrix. The correlations shown are between the maximum of the Pearson coefficient correlation found and 6% less than that maximum the degree of green colour of the edges represents coefficients from the lowest (light green colour) to the highest (dark green colour). No correlation is less than 0.60. The ASVs are named with the genus or family to which they belong and the ref. are shown in table ASV in supplementary material. The asterisk (*) indicates the family of bacteria for an unknown genus, as identification at genus level was not possible.

b. Inter- and intraspecific covariation in parasitism and bacterial and volatile profiles of avian nests

Alpha and beta diversities

The intensity of parasitism that nestling suffer at the early stage of development resulted negatively related with alpha diversity of nest volatiles, after controlling for the statistically significant effects of species identity and the non-significant effects of the alpha diversity of the bacterial community of the nest materials (Table 3). Interestingly, that association did not depend on the species identity (see interaction between alpha diversity of volatiles and species identity in Table 3). When considering beta diversities, neither, volatiles nor bacteria of avian nests predicted parasitism suffered by the nestlings at the early state of the nestling period after controlling for the significant effect of species identity (Table 4).

Intensity of parasitism of nestlings at the late stage of development resulted negatively related to the alpha diversity of the bacterial community of nest materials after controlling for the effect of species identity and the non-significant effect of alpha diversity values of nest volatiles (Table 3). The detected association did not vary interspecifically (see interaction between alpha diversity of volatiles and species identity in Table 3). Beta diversity of volatiles, but not that of bacteria from nest environment, explained the intensity of parasitism that nestlings suffer, even after controlling for the significant effect of species identity (Table 4).

Particular bacteria and volatiles from latent components from sPLS models

Final models explaining intensity of parasitism of nestlings at the beginning and at the end of the nestling period included abundance of several bacterial taxa, but heptanal, the only volatiles in latent components of sPLS models, did not enter in any of those final models (Table 5). The abundance of the class Thermoleophilia associated positively with intensity of parasitism at the beginning of the nestling period, while that of the *Porphyromonas sp.* associated negatively (Table 5, Figure 3). Intensity of parasitism at the late nestling period were positively associated with abundance of *Polaromonas sp* (Table 5, Figure 3) (see full models in ESM Table S6). These associations were in all cases controlled by the significant effect of species identity. The subset of bacteria in the final models explained significant proportion of the residual variance of the intensity of parasitism (see statistics of bacterial effects in Table 5).

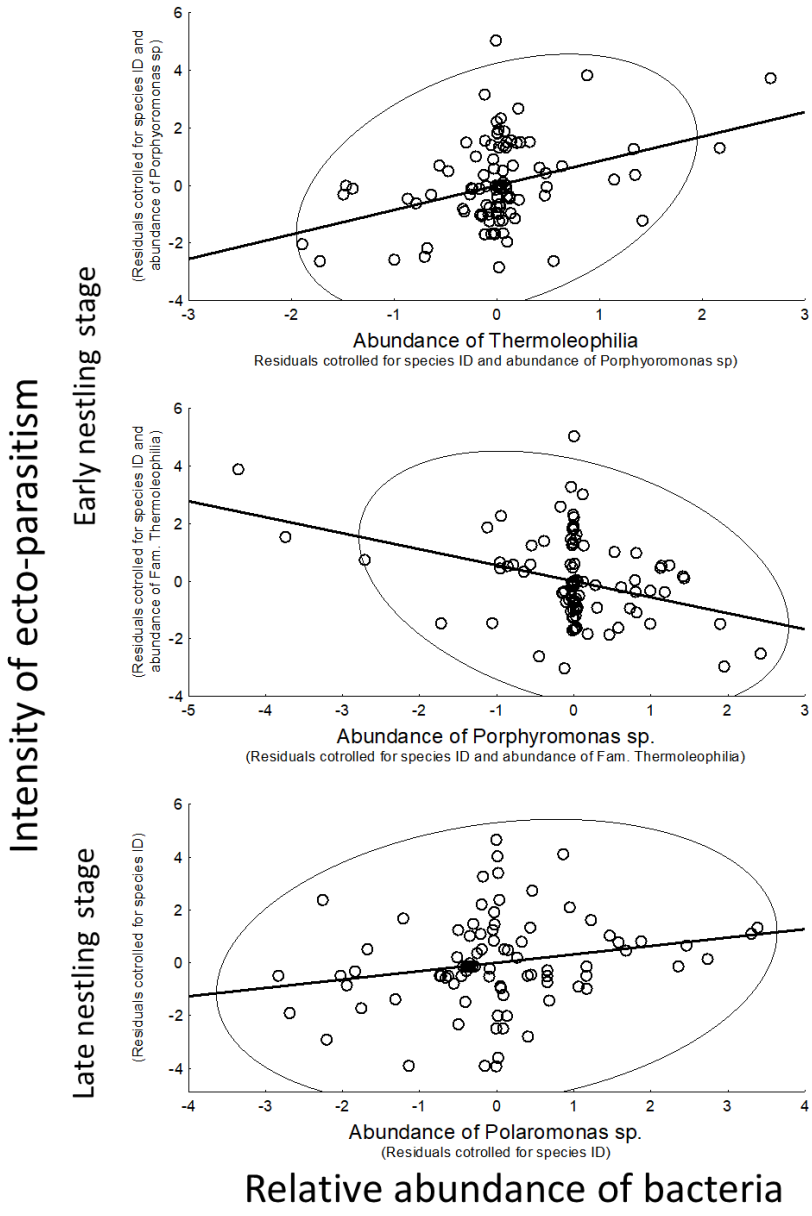

Figure 3: Correlations between intensity of parasitism (square root) with relative abundance of bacteria present in nest material, selected by the first latent component of sPLS analysis in both nestling stage. Lines are regression lines and ellipses represent 99% confidence intervals.

At the intraspecific level, we also found some evidences suggesting significant associations between parasitism intensity and relative abundance of particular bacteria or volatiles (i.e., little owls and hoopoes, See ESM Table S7). However, except for hoopoes, samples size is quite small for reliable statistical inferences. In this species, the relative abundance of the genus *Polaromonas sp.1* (Beta= 0.35, $F_{1,43} = 6.09$, P = 0.018) and of the

acetic acid (Beta= 0.33, $F_{1,43}$ = 5.23, P = 0.027) at the early nestling stage were positively related to the intensity of parasitism of younger nestlings. Intensity of parasitism of older nestlings resulted positively related to abundance of decanoic acid (Beta = 0.44, $F_{1,37}$ = 9.02, P = 0.005) and non-significantly with the abundance *Sporosarcina sp.1* (Beta = 031, $F_{1,35}$ = 3.79, P = 0.059) (Fig 4).

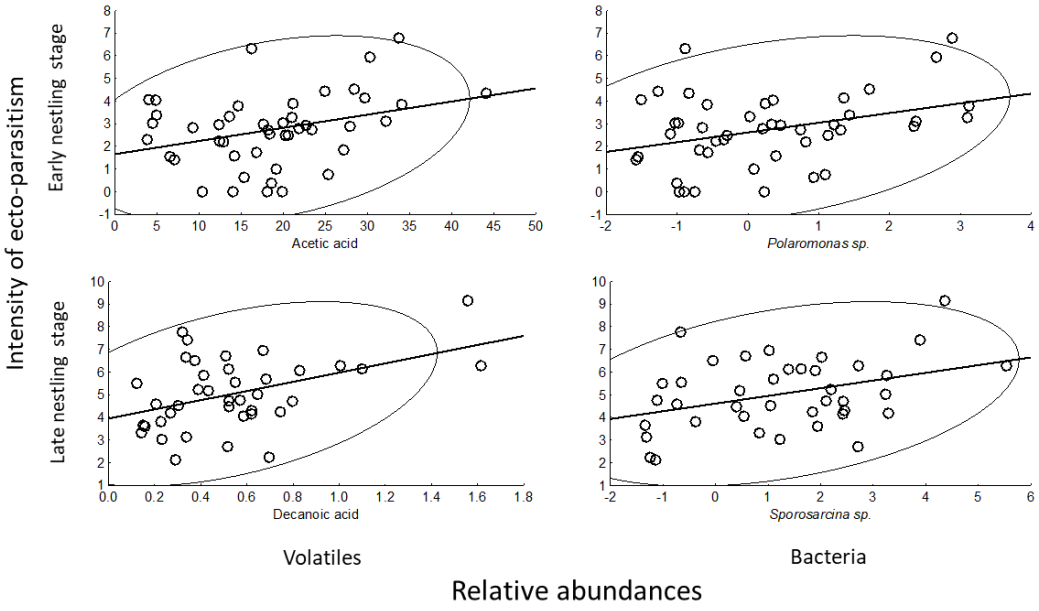

Figure 4: Correlations between intensity of parasitism (square root) with relative abundance of bacteria present in nest material and volatiles selected by the first latent component of sPLS analysis in both nestling stage of the hoopoes nest. Lines are regression lines and ellipses represent 99% confidence intervals.

c. Inter and intraspecific variation in fledging success and in bacterial and volatiles profiles of avian nests

Alpha and beta diversities

Fledging success was negatively related to alpha diversity of the bacterial community of the nest materials at the early, but not at the late stage of nestlings (Table 3). The detected association did not vary interspecifically (see interaction between alpha diversity of volatiles and species identity in (Table 3). Beta diversities of bacterial communities and of volatile profiles estimated at both nestling stages did not explain fledging success (Table 3 and Table 4). All those results we controlled for the non-significant effects of species identity (Table 3 and Table 4).

Table 3: General Linear Mixed Models exploring the relationships between intensity of parasitism or fledging success and alpha diversities of volatiles (Shannon Index) and bacterial communities (Shannon and Faith's phylogenetic diversity (PD) indexes) of nest environments both, at the early and the late developmental stage of nestlings. Species identity (Sp ID) was included as random (R) factor, and alpha diversity of bacterial communities and of volatiles profiles as first (F1) and second (F2) fixed factors respectively. For continuous fixed factors, we show partial beta values. The interaction between bird species identity and bacteria diversity or volatile profiles were tested in separate models that also included main effects, while the main effects were explored in models that do not include interactions. Variables in bold font are statically significant at the 5% level.

	Shannon alpha diversity				**PD alpha diversity**			
	F	**df**	**P**	**Beta**	**F**	**df**	**P**	**Beta**
PARASITISM INTENSITY								
Early nestling stage								
Sp ID (R)	11.72	7,93	**<0.001**		11.54	7,93	**<0.001**	
Bacteria alpha diversity (F1)	0.20	1,93	0.659	-0.037	0.31	1,93	0.574	-0.048
Volatile alpha diversity (F2)	4.74	1,93	**0.032**	-0.221	4.33	1,93	**0.040**	-0.213
F1*Sp ID	0.96	7,86	0.461		0.30	7,86	0.949	
F2*Sp ID	1.76	7,86	0.106		1.67	7,86	0.127	
Late nestling stage								
Species ID (R)	18.90	6,79	**<0.001**		18.40	6,79	**<0.001**	
Bacteria alpha diversity (F1)	4.18	1,79	**0.044**	-0.154	6.25	1,79	**0.015**	-0.217
Volatile alpha diversity (F2)	2.45	1,79	0.121	-0.139	2.47	1,78	0.120	-0.138
F1*Sp ID	0.74	6,73	0.619		0.97	6,73	0.451	
F2*Sp ID	1.64	6,73	0.148		1.58	6,73	0.165	
FLEDGING SUCCESS								
Early nestling stage								
Sp ID (R)	0.45	6,81	0.842		0.71	6,81	0.642	
Bacteria alpha diversity (F1)	0.03	1,81	0.867	-0.019	5.68	1,81	**0.019**	-0.268
Volatile alpha diversity (F2**)**	0.70	1,81	0.791	-0.038	0.00	1,81	0.994	0.001
F1*Sp ID	0.98	6,75	0.445		1.02	6,75	0.419	
F2*Sp ID	0.64	6,75	0.638		0.69	6,75	0.657	
Late nestling stage								
Species ID (R)	0.94	6,79	0.470		0.97	6,79	0.449	
Bacteria alpha diversity (F1)	0.25	1,79	0.615	0.061	0.18	1,79	0.676	-0.059
Volatile alpha diversity (F2)	0.83	1,79	0.365	0.131	0.89	1,79	0.350	0.135
F1*Sp ID	0.82	6,73	0.561		1.75	6,73	0.121	
F2*Sp ID	1.14	6,72	0.221		1.69	6,73	0.135	

Table 4: Multivariate Mantel tests exploring the relationships between matrices of differences in intensity of parasitism and fledging success and beta diversity of volatiles profiles and bacterial communities of nest environments both, at the early and the late developmental stage of nestlings. Beta diversity of volatiles was estimates as Aitchison's distances, while that bacterial beta diversity was estimated by Aitchison or Phylogenetic-Isometric-Log-Ratio-transformed (PhILR) distances. Thus, the partial effects of volatile beta diversity were estimated in models that included bacteria beta diversity estimated by either Aitchison or PhILR distances. The models also included a matrix with information of the species identity (Sp ID) (binary matrix (1= iqual, 0 = different species)). For all independent matrices (species identity (Sp ID) and bacterial and volatiles beta diversities), we show partial correlation coefficients and associated p-values after 9 999 permutations. Variables in bold font are statically significant at the 5% level.

	Intensity of parasitism				**Fledging success**			
	Aitchison		**PhILR**		**Aitchison**		**PhILR**	
	R	**P**	**R**	**P**	**R**	**P**	**R**	**P**
Early nestling stage								
Sp ID	0.570	**<0.001**	0.589	**<0.001**	-2.771	0.249	-1491	0.527
Bacteria beta diversity	0.004	0.625	0.010	0.611	0.177	0.176	0.144	0.626
Volatile beta diversity	-0.701	0.437	-0.071	0.421	-0.583	0.648	-0.587	0.653
Late nestling stage								
Sp ID	1.805	**<0.001**	1.622	**<0.001**	-1.632	0.519	-1.795	0.475
Bacteria beta diversity	-0.010	0.342	0.007	0.761	0.006	0.959	0.105	0.706
Volatile beta diversity	0.261	**0.006**	0.265	**0.008**	0.407	0.683	0.361	0.716

Particular bacteria and volatiles from latent components of sPLS models

Final models explaining fledging success included both volatiles and bacterial components (Table 5). Abundance of *Corynebacterium sp*. (negatively) and of *Peptoniphilus sp.* (positively) at the early stage of nestling period and that of *Polaromonas sp.* (negatively) at the late stage of the nestling period associated with fledging success (Table 5, Fig 5; See full models in ESM Table S6). Abundance of heptanal at both stages of the nestling period tended to be negatively associated with fledging success (Table 5, Fig 5; See full models in ESM Table S6). At the intraspecific level, none of the volatiles or bacteria that were selected in sPLS models associated significantly with fledging success (ESM, Table S7).

Table 5. General Linear Mixed Models exploring the relationships between intensity of parasitism or fledging success and relative abundance of particular bacteria and volatiles from latent components of sPLS models. For each model, only bacterial and volatiles that entered in the final model (following AIC criterion) were included. When species identity (Sp ID) was included in the final model, its effect was modelled as random (R), while relative abundance of particular bacteria and volatiles were included as fixed (F) factor. For continuous fixed factors, we show beta values. Statistics associated (i.e. in the same line of) to bacterial or volatile effects refers to models that included information of the dependent and independent fixed factors after controlling for the effect of species identity. Variables in bold font are those with associate p-values lower than 0.1. The asterisk (*) indicates the lower taxonomic assignation when identification at genus level was not possible.

	Intensity of parasitism				**Fledging success**			
	Beta	**F**	**df**	**P**	**Beta**	**F**	**df**	**P**
Early nestling stage								
Bacterial effects		8.05	2,100	**<0.001**		2.45	2,87	0.092
Sp ID (R)		11.00	7,93	**<0.001**				
Thermoleophilia* (F)	0.657	14.16	1,93	**<0.001**				
***Porphyromonas sp.* (F)**	-0.638	12.38	1,93	**0.001**				
***Corynebacterium sp.* (F)**					-0.463	4.89	1,87	**0.030**
***Peptoniphilus sp.* (F)**					0.412	3.87	1,87	0.052
Volatile effects	-	-	-	-	-0.184	3.07	1,88	0.083
Sp ID (R)		11.15	1,95	**<0.001**				
Heptanal (F)					-0.184	3.07	1,88	0.083
Late nestling stage								
Bacterial effects	0.213	4.09	1,86	**0.046**	-0.210	3.94	1,86	**0.050**
Sp ID (R)		14.90	6,80	**<0.001**				
***Polaromonas sp.* (F)**	0.210	3.35	1,80	0.071	-0.210	3.94	1,86	**0.050**
Volatile effects	-	-	-	-	-0.184	3.01	1,86	**0.086**
Sp ID (R)		21.04	6,81	**<0.001**				
Heptanal (F)					-0.184	3.01	1,86	**0.086**

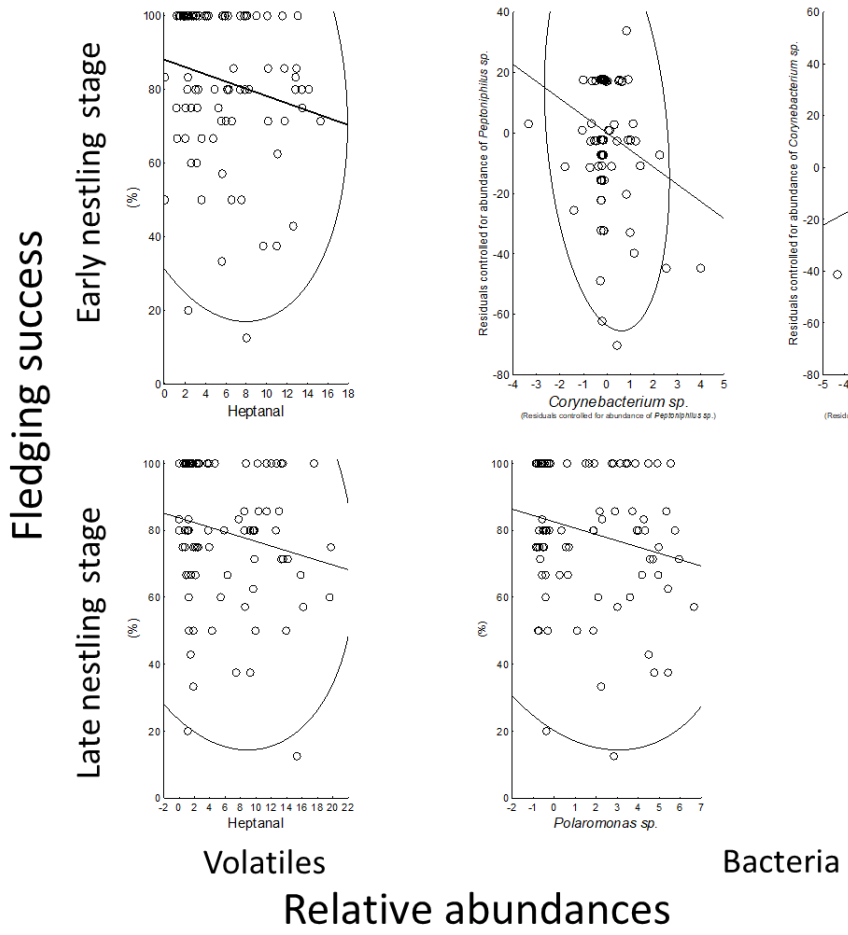

Figure 5: Correlations between fledging success with relative abundance of bacteria present in nest material, selected by the first latent component of sPLS analysis in both nestling stage. Lines are regression lines and ellipses represent 99% confidence intervals.

4. Discussion

The hypothesis tested here is two folds: first, volatiles from bacterial symbionts are partially responsible of the animal odours; and, second, these microorganisms partially determine the probability of parasitism and reproductive success of their animal hosts. To explore this hypothesis, we took advantage of the avian nest environments, which allowed characterizing bacterial environment, volatile profiles, intensity of parasitism and reproductive success in the same focal location. Moreover, we used nests of different species, and explored the expected associations both inter- and intraspecifically. Our main findings are that (i) alpha and beta diversity of bacteria predicted the volatile profile of the nest, although the strength and the sign of the association varied intraspecifically and depended on the nestling stage (i.e., early vs late)

and the index used to estimate the diversity. Although the strength of the associations depended on the diversity index, (ii) alpha and beta diversity of volatiles and/or nest bacterial community predicted parasitism intensity of nestlings either, at the beginning or at the end of the nestling period. Interestingly, (iii) these associations did not depend of the species identity. Furthermore, (iv) particular bacteria and volatiles responsible of the expected associations between bacterial and volatiles environments, and between them and parasitism and fledging success, varied intraspecifically and depended of the nestling stage. Below, we discussed particularities of these results and their importance for the hypothesis tested.

Bacterial communities and volatile profiles of avian nests

Interspecific differences in nests characteristics may explain the detected species-specific differences in the bacterial communities of nest materials, in the volatiles profiles of the nest environment, and in the associations between these two nest environmental components. Avian nests vary not only in the level of isolation from the external environment (e.g., hole vs open nest), location (on the soil, trees, or cliffs) and orientation, but also in the nest structure and the material used for lining the nests (Hassel, 2007). These characteristics would directly affect temperature and humidity (Mertens, 1977) that would also determine the bacterial communities of nest environments (Godard et al., 2007; Goodenough et al., 2017; Martínez-Renau et al., 2022; Peralta-Sánchez et al., 2012; Soler et al., 2015). Moreover, some of the considered species do not build nests (owls, hoopoes and rollers), while some others use aromatic plants with antimicrobial properties (Clark & Mason, 1985; Mennerat et al., 2009), and/or feathers that favour the growth of particular antibiotic producing bacteria (Peralta-Sánchez et al., 2014; Ruiz-Castellano et al., 2019) , which would also determine bacterial communities of nest environment. Our results however do not allow distinguishing particular causes explaining detected interspecific differences or covariations, and experimental manipulations of some the nest materials or of the bacterial communities of nest environment would help to reach further conclusions.

Those species-specific nest characteristics would also determine the volatile profiles of avian nests, either, because of the direct emission of volatiles of some nest lining components (i.e., aromatic plants), or because of volatiles from the metabolism of birds or of the species-specific bacterial communities of avian nests. Thus, the expected associations between characteristics of the bacterial communities and bacterial profiles

should vary interspecifically, a prediction supported by the detected interaction between bacterial community and species identity explaining volatile profiles of avian nests. That interaction is exemplified by the positive association between alpha diversities of volatiles and bacteria detected in the spotless starling, a species that used nest-lining materials with antimicrobial properties (Ruíz-Castellano et al., 2016, 2018, 2019), that turned to be negative in the little owl, a species that do not use nest-lining material in their nests. In addition, we also found interspecific differences in the particular bacterial taxa that associated with particular volatiles, which further support interspecific differences in the associations between bacterial and volatile environments of avian nests.

The expected associations between bacterial communities and volatile profiles sometimes varied depending on the nestling stage, which might be explained by associated variations of environmental condition in the nests. Bacterial communities of avian nests are known to vary along the nesting period (Brandl et al., 2014; Lee et al., 2017; Ruiz-De-Castañeda et al., 2012), which is explained by the accumulation of debris due to rest of food, nestling faeces that parents failed to remove (Azcárate-García et al., 2019) and/or of rests of nestling growth (e.g. feather sheath wrapped). Those sources of variations would also predict variation in volatiles profiles along the nestling period and, thus, should affect characteristics of the association between bacteria and volatiles of avian nests.

Our results do not allow discussing mechanisms explaining detected interspecific differences in the expected associations, which deserve further investigations. However, they confirm the predicted covariation between microbial and volatile profiles of avian nests that had been intraspecifically detected in some other systems including insects (Davis et al., 2013), amphibians (Brunetti et al., 2019) birds (Whittaker et al., 2019), and mammals (Leclaire et al., 2017; Theis et al., 2013).

Inter- and intraspecific covariation in parasitism or fledging success and bacterial and volatile profiles of avian nests

In general, our results support the predictions that related bacterial community with volatiles profile, and both with parasitism intensity and fledgling success. However, the strength of these associations (i.e., statistical significance) depended on the diversity indexes used to characterize bacterial and volatile profiles, besides the breeding stage. For instance, at the beginning of the nestling period, alpha diversity of volatiles explained intensity of parasitism, while, at the end of the nestling period, it was explained by alpha

and beta diversity of the bacterial community of nest materials. Interestingly, detected associations between intensity of parasitism and alpha diversity did not depend on the species identity. Since bacterial and volatiles profiles are related to each other, these results suggest that, to find or choose avian nests for parasitism, the ectoparasite *Carnus hemapterus* follows chemical cues of bacterial origin. However, correlations do not imply causation and, since we know that aftermath of ectoparasites activity (parasite faeces and host blood) may influence bacterial communities in avian nests (Tomás et al., 2018). Therefore, an alternative explanation of the detected associations is that the bacterial community and the volatile profile of avian nests were the consequence of ectoparasite activity. We found that, after correcting for species identity, the relative importance of particular bacteria predicted intensity of parasitism. Future works should then include experiments directed to test the effect of those bacteria attracting or repelling *Carnus* flies, which will allow distinguishing causes and consequences of the detected associations.

We also found partial support to the predicted associations between bacterial community and fledging success. In this case, only alpha diversity of the bacterial community at the beginning of the nestling period associated significantly with fledging success, while the effects of relative abundance of heptanal and of some particular bacteria of avian nests at the beginning and at the end of the nestling period did not reach statistical significance. The prediction was based on the assumption that bacteria and volatiles in avian nests should predict parasitism intensity. Thus, differential selection pressures associated to particular bacterial communities of avian nests will indirectly explain the detected associations between bacteria or volatiles on fledging success. In any case, experiments manipulating the relative importance of particular bacteria and/or heptanal are necessary to infer causation of the detected associations.

Summarising, our results demonstrated interspecific differences in the bacterial communities and volatiles profiles of avian nests and support the hypothesis that both volatile and bacterial profiles covary inter- and intraspecifically. Moreover, our results also suggest that particularities of the bacterial community and of the volatile profile, as well as the abundance of particular elements, predicted intensity of parasitism and fledging success. Since we used parasitism by *Carnus hemapterus* flies as a model system, exploring those associations in some other host-parasite systems is necessary to generalize conclusions. Importantly, we have detected particular bacteria and volatiles that might serve to focus future experiments directed to confirm the role of bacteria

determining risk of parasitism and fledging success.

DECLARATIONS

Acknowledgments

We thank Natalia Juárez García-Pelayo and Cristina Ruiz Castellano for their help with the fieldwork, and Estefanía López Hernández and Miguel Rabelo Ruiz with the laboratory work.

Funding

The research group was supported by the projects CGL2017-83103-P, PID2020-117429GB-C21 and PID2020-117429GB-C22, funded by the Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación/10.13039/501100011033 and by "Fondo Europeo de Desarrollo Regional, a way of making Europe". The research group also benefits from facilities, including accommodation, provided by the City Hall of Guadix, where a small lab to quickly process the samples was installed. Juan M. Peralta-Sánchez enjoyed the stabilization program "Contratos de Acceso al Sistema Español de Ciencia, Tecnología e Innovación para el Desarrollo del Programa Propio de I+D+i de la US (II.4). Anualidad 2022" at University of Seville.

Ethics statement

The study was conducted according to relevant Spanish national (Decreto 105/2011, 19 de abril) and regional guidelines. All necessary permits for bird's manipulations were provided by Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected, but privately owned, and the owners allowed us to work in their properties. The time spent in each nest was the minimum necessary for the experiment.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

JJS and MMV designed the study. MMA and JJS carried out the fieldwork. MMA with the help of MMB performed all laboratory analyses related to bacterial DNA extraction and amplification. RNG afforded all chemical analyses in determination and quantification of volatiles. MMA and JMPS carried out all bioinformatics and, with the

help of OSS and JJS the statistical analyses. Together with JJS and JMPS, MMA wrote a first version of the manuscript. All authors contributed to the general discussion of results and to the final version of the manuscript.

Data Availability Statement

Data used in this paper will be uploaded to our institutional repository before publishing.

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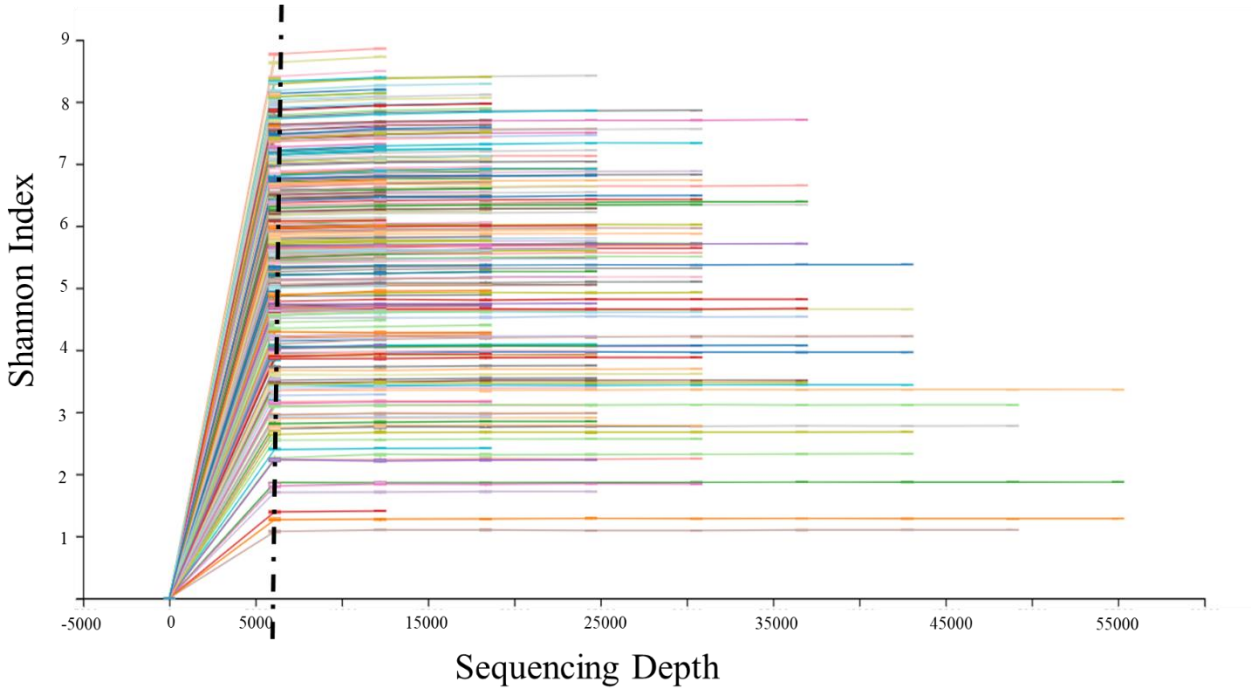

Figure S1: Rarefication curves of bacterial communities of avian nests generates in QIIME2. The lines represent increase in alpha diversity (Shannon diversity index) with the increase in sequencing depth. The dashed line represents the number of reads used as a cut-off for retaining samples in downstream analysis (all samples with fewer than 6 000 reads were removed in subsequent analyses).

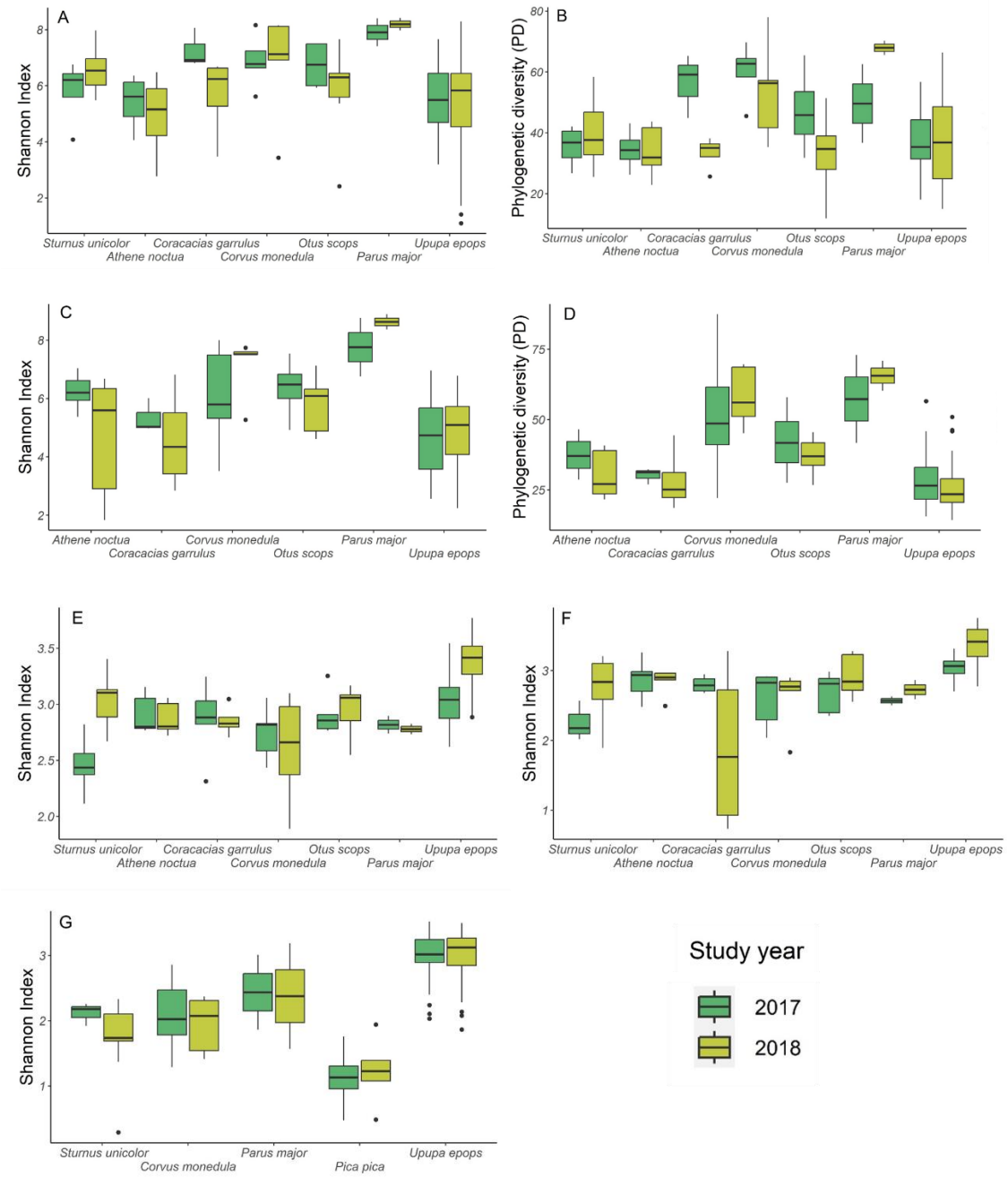

Figure S2: Alpha diversity box-plots showing differences in Shannon diversity and Faith´s phylogenetic diversity (PD) indexes of the bacterial community of the nest material at early stage (A and B) and late stage (C and D); Shannon index of the volatiles profile of the nest-box at early stage (E), late stage (F); and uropygial gland of nestlings (G) in both study years.

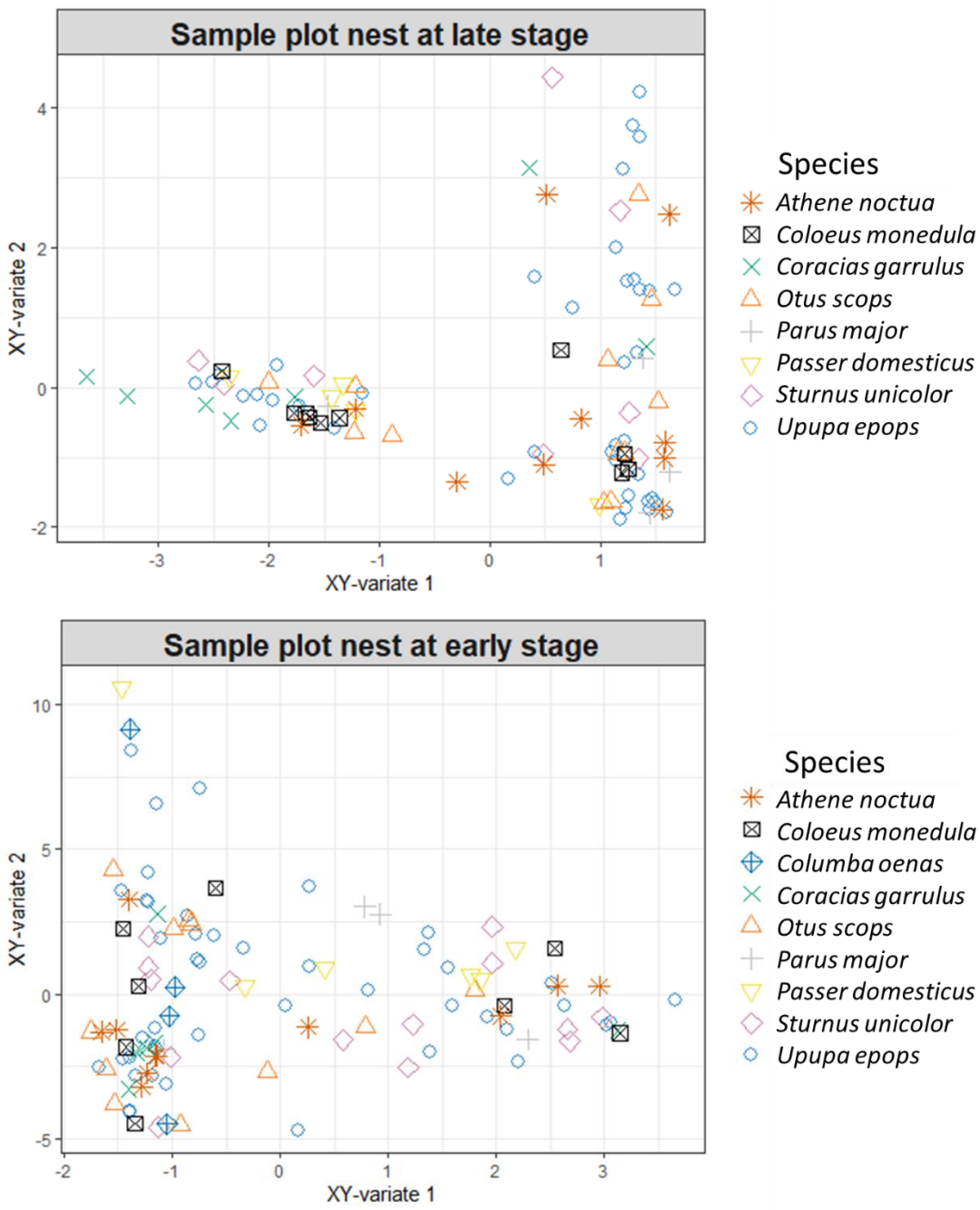

Figure S3: Sample plot for the sPLS (sparce Partial Least Squares or Projection to Latent Structures) performed on bacterial ASVs and volatile profiles from collected avian species. Samples are projected into the space spanned by the average of the first two latent components of both datasets.

Table S1. Average and Standard Error of the relative abundance of the chemical compounds detected in the environment of nest boxes of each of the avian species and in both nest stage of nestlings by mean of Gas Chromatography-Mass Spectrometry (GC-MS).

	Upupa epops Mean (SE)	*Otus scops* Mean (SE)	*Athene noctua* Mean (SE)	*Coleus monerula* Mean (SE)	*Sturnus unicolor* Mean (SE)	*Coracias garrulus* Mean (SE)	*Parus major* Mean (SE)	*Passer domesticus* Mean (SE)	*Columba oenas* Mean (SE)	Total Mean (SE)
Nest-box environment at early stage										
Acids										
Acetic	20.18 (9.73)	17.18 (13.46)	22.18 (12.57)	16.24 (8.75)	25.58 (10.17)	22.63 (8.55)	28.18 (4.72)	19.51 (10.08)	29.84 (7.20)	21.07 (10.40)
Butanoic	10.98 (3.74)	8.78 (3.56)	9.43 (3.89)	5.13 (2.15)	4.43 (3.41)	12.80 (6.20)	4.74 (3.27)	5.84 (2.32)	6.13 (3.54)	9.00 (4.57)
Isovaleric	2.65 (1.27)	1.01 (0.31)	1.46 (1.16)	0.77 (0.41)	1.55 (1.21)	1.26 (1.00)	0.75 (0.35)	1.74 (1.43)	0.65 (0.13)	1.87 (1.31)
Pentanoic	1.76 (0.63)	3.77 (1.14)	2.22 (0.70)	1.23 (0.44)	1.90 (0.65)	2.97 (1.69)	1.99 (1.09)	2.21 (0.39)	2.39 (0.85)	2.09 (1.03)
Isocaproic	1.77 (1.58)	0.01 (0.05)	0.32 (0.54)	0.07 (0.14)	0.06 (0.18)	0.31 (0.54)	0.17 (0.34)	0.39 (0.73)	0.00 (0.00)	0.89 (1.36)
Hexanoic	8.10 (2.72)	11.18 (3.67)	7.99 (3.02)	5.20 (1.70)	9.73 (4.33)	7.13 (2.69)	6.14 (3.26)	9.66 (2.24)	10.69 (3.20)	8.39 (3.30)
Heptanoic	2.31 (0.83)	1.94 (0.39)	1.72 (0.56)	1.71 (1.85)	1.64 (0.50)	1.63 (0.99)	1.41 (0.36)	1.97 (0.75)	1.79 (0.57)	2.00 (0.89)
Octanoic	2.14 (1.17)	1.77 (0.56)	2.37 (1.34)	1.34 (0.39)	1.97 (0.51)	2.24 (1.72)	1.78 (0.41)	2.26 (0.76)	2.22 (0.35)	2.05 (1.05)
Nonanoic	3.30 (2.03)	4.09 (3.91)	5.19 (3.53)	4.34 (2.19)	3.68 (1.86)	5.83 (6.24)	5.39 (2.07)	3.84 (1.66)	3.52 (1.11)	3.92 (2.85)
Decanoic	0.86 (0.69)	1.08 (0.66)	1.40 (1.03)	1.52 (1.40)	1.25 (0.83)	1.57 (1.13)	2.64 (0.91)	1.63 (0.77)	1.61 (0.52)	1.18 (0.90)
Propionic	6.45 (3.75)	16.82 (12.31)	9.49 (5.46)	4.02 (3.80)	5.02 (3.72)	7.73 (7.51)	5.42 (6.58)	7.57 (6.80)	6.45 (6.12)	7.47 (6.53)
Isobutiric	1.69 (0.95)	0.84 (0.43)	0.91 (0.95)	0.72 (0.45)	1.31 (0.97)	0.90 (0.79)	0.32 (0.30)	1.04 (0.91)	0.56 (0.45)	1.27 (0.94)
Aldehydes										
Pentanal	0.72 (0.81)	1.08 (0.66)	0.26 (0.78)	0.27 (0.44)	0.05 (0.19)	0.10 (0.31)	-	0.48 (0.74)	0.49 (0.98)	0.52 (1.07)
Benzaldehyde	5.21 (2.37)	4.33 (2.32)	4.17 (2.37)	6.52 (4.31)	8.23 (9.31)	4.98 (2.99)	11.85 (5.57)	9.11 (6.50)	7.10 (3.41)	5.89 (4.52)
Hexanal	3.90 (3.66)	2.97 (4.36)	4.99 (8.33)	6.15 (9.06)	3.28 (3.89)	1.56 (3.14)	0.82 (1.31)	4.38 (5.39)	4.21 (3.70)	3.78 (4.88)
Heptanal	8.66 (3.01)	2.82 (1.51)	2.47 (1.08)	5.95 (3.29)	2.12 (1.19)	2.50 (2.15)	3.30 (1.26)	2.76 (1.36)	1.76 (1.03)	5.56 (3.83)
Octanal	1.79 (1.47)	2.31 (2.35)	1.14 (0.57)	1.97 (0.44)	4.38 (7.31)	1.35 (0.96)	1.52 (0.49)	2.15 (1.68)	5.01 (4.05)	2.17 (3.00)
Nonanal	17.21 (6.65)	17.91 (8.33)	22.22 (8.43)	36.82 (12.52)	21.88 (11.56)	22.48 (7.50)	23.57 (8.02)	23.37 (7.86)	15.51 (8.19)	20.47 (9.66)
Sulfurs compounds										
Dimethyl-disulfide	0.34 (1.50)	0.08 (0.18)	0.04 (0.06)	0.01 (0.03)	1.65 (6.41)	0.03 (0.10)	-	0.11 (0.11)	0.06 (0.10)	0.37 (2.41)
Dimethyl-trisulfide	0.00 (0.00)	-	0.00 (0.00)	-	0.28 (1.10)	0.00 (0.00)	-	-	-	0.03 (0.38)
Nest-box environment at late stage										
Acids										
Acetic	17.01 (10.62)	11.55 (11.2)	11.88 (8.07)	18.32 (10.38)	22.41 (16.09)	33.39 (31.20)	26.26 (13.51)	25.54 (8.21)	21.42 (15.69)	18.52 (14.04)
Butanoic	11.47 (4.63)	8.28 (3.21)	10.42 (4.48)	8.79 (5.64)	2.86 (2.29)	13.97 (8.80)	5.91 (4.34)	8.03 (2.64)	5.20 (3.36)	9.69 (5.39)
Isovaleric	4.00 (2.10)	1.71 (0.80)	3.36 (4.10)	0.87 (0.76)	2.77 (1.77)	2.40 (2.14)	0.53 (0.15)	1.57 (1.25)	3.81 (4.46)	3.01 (2.41)
Pentanoic	1.81 (0.78)	3.82 (0.96)	3.63 (1.54)	1.86 (1.02)	1.58 (0.88)	4.47 (2.56)	2.07 (1.30)	2.90 (0.77)	2.17 (0.66)	2.40 (1.42)
Isocaproic	3.23 (2.58)	0.05 (0.14)	0.56 (0.77)	0.08 (0.26)	0.04 (0.15)	0.44 (1.04)	-	0.06 (0.14)	-	1.60 (2.36)
Hexanoic	6.76	7.04	6.24	6.88	9.80	6.19	6.49	9.84	9.82	7.23

	(3.28)	(2.50)	(2.93)	(3.07)	(5.15)	(4.97)	(2.99)	(2.77)	(2.05)	(3.54)
Heptanoic	2.44 (1.18)	1.81 (0.84)	1.18 (0.48)	1.11 (0.33)	2.06 (0.79)	1.12 (0.82)	1.35 (0.45)	1.33 (0.58)	1.91 (0.48)	1.94 (1.07)
Octanoic	1.97 (1.34)	2.12 (1.22)	1.63 (1.34)	1.87 (1.46)	2.39 (0.76)	1.22 (0.52)	2.01 (1.10)	1.19 (0.64)	2.23 (0.92)	1.91 (1.22)
Nonanoic	2.89 (1.70)	3.20 (1.78)	4.13 (4.21)	6.33 (6.25)	4.72 (4.29)	2.54 (0.88)	6.01 (4.94)	1.91 (0.68)	2.82 (0.70)	3.49 (3.06)
Decanoic	0.62 (0.49)	0.61 (0.36)	1.00 (1.13)	1.40 (1.11)	0.90 (0.47)	0.81 (0.55)	2.76 (2.14)	0.65 (0.24)	0.77 (0.23)	0.82 (0.80)
Propionic	8.34 (7.10)	32.56 (9.67)	20.08 (9.11)	6.69 (5.73)	5.84 (11.34)	16.02 (13.51)	8.09 (8.74)	13.47 (4.62)	11.10 (10.81)	12.30 (11.44)
Isobutiric	2.72 (1.98)	1.33 (0.52)	1.66 (1.83)	0.41 (0.32)	1.56 (0.91)	1.34 (1.16)	0.29 (0.19)	1.55 (0.77)	1.20 (1.25)	1.93 (1.72)
Aldehydes										
Pentanal	0.35 (0.57)	0.50 (0.67)	0.10 (0.23)	0.16 (0.51)	0.35 (0.87)	0.13 (0.37)	-	0.42 (0.66)	-	0.30 (0.57)
Benzaldehyde	8.42 (7.87)	1.99 (1.13)	1.84 (0.67)	3.96 (3.16)	4.19 (2.84)	1.93 (1.30)	6.94 (1.97)	4.95 (2.57)	5.42 (2.01)	5.75 (6.20)
Hexanal	2.45 (2.62)	5.15 (4.36)	6.28 (11.48)	3.82 (4.06)	5.36 (5.82)	1.14 (3.00)	0.32 (0.38)	4.59 (6.00)	7.45 (6.76)	3.52 (4.97)
Heptanal	10.83 (3.90)	1.55 (0.54)	1.69 (0.81)	2.34 (1.32)	1.79 (0.84)	0.88 (0.52)	2.72 (0.92)	2.25 (1.82)	2.04 (1.41)	6.03 (5.30)
Octanal	1.00 (0.84)	1.73 (1.57)	1.21 (1.07)	1.09 (0.62)	1.57 (0.75)	0.68 (0.93)	2.25 (1.25)	5.51 (11.22)	9.07 (9.56)	1.65 (3.31)
Nonanal	13.01 (5.37)	14.97 (6.19)	20.04 (8.01)	34.02 (15.37)	29.73 (18.99)	11.29 (6.96)	26.01 (19.15)	13.58 (6.28)	13.26 (7.22)	17.30 (11.64)
Sulfurs compounds										
Dimethyl-disulfide	0.62 (1.23)	0.04 (0.05)	3.07 (9.62)	-	0.09 (0.14)	0.03 (0.07)	-	0.66 (1.45)	0.31 (0.33)	0.59 (2.86)
Dimethyl-trisulfide	0.05 (0.11)	0.00 (0.00)	0.00 (0.01)	-	-	0.01 (0.02)	-	-	-	0.03 (0.08)

Table S2: General Linear Mixed Models and PERMANOVAS for respectively exploring interspecific differences (Sp ID) in alpha diversity (Shannon or Faith's Phylogenetic Diversity (PD), and beta (Aitchison or Phylogenetic-Isometric-Log-Ratio-transformed (PhILR)) diversity indexes used to characterize bacterial communities of nest material and volatile profile of nests environment at the early and at the late stage of the nesting period. We show variance explained by the species identity (Sp ID), while models that reached statistical significance are highlighted in bold fonts.

Statistical models	**Shannon**			**PD**		
ALPHA DIVERSITY	**F**	**df**	**P**	**F**	**df**	**P**
Early nestling stage						
Bacteria ~ Sp ID	3.56	8,152	**<0.001**	4.72	8,152	**<0.001**
Volatiles ~ Sp ID	9.33	8,127	**<0.001**			
Late nestling stage						
Bacteria ~ Sp ID	15.48	8,133	**<0.001**	18.30	8,133	**<0.001**
Volatiles ~ Sp ID	8.76	8,115	**<0.001**			
BETA DIVERSITY	**Aitchison**			**PhILR**		
Early nestling stage						
Bacteria ~ Sp ID	3.81	8,152	**<0.001**	6.61	8,152	**<0.001**
Volatiles ~ Sp ID	4.52	8,127	**<0.001**			
Late nestling stage						
Bacteria ~ Sp ID	6.13	8,133	**<0.001**	14.72	8,133	**<0.001**
Volatiles ~ Sp ID	6.84	8,115	**<0.001**			

Table S3: General Linear Models and Mantel tests exploring the relationships between alpha or beta diversities of volatiles and of bacterial communities of nest environment. We show results from models that separately considered samples of different species that were collected at the early or at the late nestling stage. Alpha and beta diversity of volatiles were respectively characterized by the Shannon and Aitchison indexes, while Shannon or Faith's Phylogenetic Diversity (PD), and Aitchison or Phylogenetic-Isometric-Log-Ratio-transformed (PhILR) distances were respectively used to characterize bacterial communities. For the models with alpha diversity at the beginning of the chicken stay, we only used the Shannon index for the bacteria and for the end of the chicken stay we only used the PD index, since these are the indices that were in interaction with the identity of the species in interspecific models. We show partial correlation coefficients (R) and associated p-values that for Mantel tests were estimated after 9 999 permutations, and beta and associated p-values in GLMs. Variables in bold font are statically significant at the 5% level.

	Early nestling stage				*Late nestling stage*			
ALPHA DIVERSITY	**Shannon**				**PD**			
	F	**df**	**P**	**Beta**	**F**	**df**	**P**	**Beta**
Upupa epops	1.26	1,43	0.267	0.169	0.39	1,38	0.536	-0.101
Otus scops	1.99	1,9	0.192	-0.426	1.19	1,10	0.301	-0.326
Athene noctua	**9.39**	**1,9**	**0.013**	**-0.715**	0.01	1,8	0.913	-0.040
Coleus monedula	2.09	1,6	0.199	-0.508	0.43	1,8	0.530	0.226
Sturnus unicolor	**5.35**	**1,12**	**0.039**	**0.555**	0.08	1,6	0.793	-0.112
BETA DIVERSITY	**Aitchison**		**PhILR**		**Aitchison**		**PhILR**	
	P	**R**	**P**	**R**	**P**	**R**	**P**	**R**
Upupa epops	0.710	-0.003	0.307	0.025	0.340	-0.010	0.401	0.018
Otus scops	0.462	-0.019	0.202	0.095	**0.042**	**0.000**	0.404	0.058
Athene noctua	0.693	0.011	0.348	0.075	0.131	0.047	**0.001**	**0.296**
Coleus monedula	0.248	-0.033	0.786	-0.033	0.645	-0.022	0.315	-0.190
Sturnus unicolor	0.665	0.017	0.572	-0.128	0.199	0.050	0.659	0.065

Table S4: The ASVs codes (at 220 pb sequence length) and the class and genus nomenclature and the taxonomic reference (Ref.) used in the text of the article are shown. NA appears for those genera that have not been identified.

ASV	Class	Genus	Ref.
fdded71aa4da950eaee2f4aa8ee1d5c6	Thermoleophilia	NA	Thermoleophilia*
dd71c03f84439e1d39689bc633375492	Actinobacteria	*Corynebacterium*	*Corynebacterium sp.*
cb72bcae0bdca890d5b7027df954538d	Bacteroidia	*Porphyromonas*	*Porphyromonas sp.*
9a1f674bd32a77e061d0311b096d1f4b	Clostridia	*Clostridium*	*Clostridium sp.*
1f9d2e4bb9046bf7ba0a67cd23a27f13	Clostridia	*Peptoniphilus*	*Peptoniphilus sp.*
fef794ea8a5020d73307ab62dce5eb72	Bacilli	NA	Bacilli*
02a8741d8c2a5b4bebf526cd6b8f7fbd	Bacilli	*Lactococcus*	*Lactococcus sp.*
ebf1d36db5872d62694fc31c54019c36	Betaproteobacteria	*Polaromonas*	*Polaromonas sp.*
76e4fe3bc669714b7d419f9a67fe395b	Bacilli	*Sporosarcina*	*Sporosarcina sp.*
88fcd645e82beb658dcefc9fb4bfd82e	Betaproteobacteria	*Polaromonas*	*Polaromonas sp1.*
6e9c0be7a680387f055f9fac5a17eb6e	Bacilli	*Sporosarcina*	*Sporosarcina sp.*
fdfb74cc2c07de18a7d62bc002e5dc54	Actinobacteria	*Leucobacter*	*Leucobacter sp.*
3d54ef9bffe1a9988302b408642ac1ea	Flavobacteriia	NA	Flavobacteriia*
15f9d95170e5ef6d807dc77595f6e1f3	Alphaproteobacteria	NA	Alphaproteobacteria*
8e6b4b55a377c2e70aa0fcc1f104d330	Actinobacteria	NA	Actinobacteria*
1ad6f11f0de75c6103e54cf2b8641535	Alphaproteobacteria	*Sphingomonas*	*Sphingomonas wittichii*
2caa2e6542558cabe391354df2d49351	Flavobacteriia	NA	Flavobacteriia*1
d68abe57f619960eff4e663c5bbe6b22	Chlamydiia	NA	Chlamydiia*
28e52f0e72af52b0dd079a4f2c317aca	Sphingobacteriia	NA	Sphingobacteriia*
2d1cc63824796fc98c917cce5f5ddd8d	Cytophagia	NA	Cytophagia*
b6640be32e3e83c92d3e64e6e03c2aeb	Gammaproteobacteria	*Stenotrophomonas*	*Stenotrophomonas sp.*
414838757f223ac74161b3947d8e528c	Alphaproteobacteria	*Devosia*	*Devosia sp.*
9227fdbe9266976ba908ca382044d67c	Flavobacteriia	NA	Flavobacteriia*2
c4f3b268835586000b96cd88bed31138	Actinobacteria	*Yaniella*	*Yaniella sp.*
9ad177d742dc126cec2d784f2fc88344	Bacilli	*Sporosarcina*	*Sporosarcina sp.*
ccb3bc2dbd963ab0ed2ffadc25f60b80	Betaproteobacteria	*Polaromonas*	*Polaromonas sp.2*
741808fd8fd11d794c309630c05a0c1e	Betaproteobacteria	*Polaromonas*	*Polaromonas sp.3*
921aa629c8002b1dc836b9f39e3505ba	Actinobacteria	*Corynebacterium*	*Corynebacterium sp.2*
cf656040918641894570395 84a1bdc4b	Sphingobacteriia	*Sphingobacterium*	*Sphingobacterium multivorum*
28e52f0e72af52b0dd079a4f2c317aca	Sphingobacteriia	NA	Sphingobacteriia*1
ed8b104383ba3441077ea93f7407ec47	Alphaproteobacteria	*Sphingomonas*	*Sphingomonas sp.*
d12025f37f9e54b9b5dc1356529d1211	Alphaproteobacteria	*Acidocella*	*Acidocella sp.*
ef35ddd25f355edcfc3609ebb41be8e3	Actinobacteria	*Rothia*	*Rothia mucilaginosa*
68813421251a3fc919773a61b7f09b8e	Betaproteobacteria	*Polaromonas*	*Polaromonas sp.4*
c987ce4354dff44472fe754d176fcc8c	Saprospirae	NA	Saprospirae*
180f360d4b33381030a371bc7e2dfe3f	Saprospirae	*Flavisolibacter*	*Flavisolibacter sp.*
c35d43dc005bbe131a8ab4196b560b34	Alphaproteobacteria	*Kaistobacter*	*Kaistobacter sp.*
1964275ba0c9ae34a792fa486fdaab2c	Alphaproteobacteria	NA	Alphaproteobacteria*1
d0c718505df1f240bf5cabe640fbd10d	Deinococci	*Deinococcus*	*Deinococcus sp.*
a07bb5881a836b3f4cc2cd658acda749	Alphaproteobacteria	*Kaistobacter*	*Kaistobacter sp.1*
e0303192ec42bb8f2bbd656ade552631	NA	NA	*Unknown*
0bd2099185f0d595dd6464e283f3ca81	Saprospirae	*Segetibacter*	*Segetibacter sp.*
fe76f5431fef561c9833de71f1ea0aef	Cytophagia	*Hymenobacter*	*Hymenobacter sp.*
97b99177330f6484544445a2795f5c0f	Actinobacteria	NA	Actinobacteria*1
a90076cfdfdfb62b58be15160a20cf7e	Saprospirae	NA	Saprospirae*1
b24c9d8fdcf1633ee0610e448f23995c	Actinobacteria	*Kocuria*	*Kocuria palustris.*
214d48f12c3302c72288f84089d9cd32	Actinobacteria	*Friedmanniella*	*Friedmanniella sp.*

Table S5: Particular bacterial taxa (ASVs) and volatiles from sPLS models (i.e., latent components) separately performed for samples collected at the early and at the late nestling period in nests of five different species. The asterisk (*) indicates the lower taxonomic assignation when identification at genus level was not possible.

EARLY NESTLING STAGE			LATE NESTLING STAGE		
R (range)	**ASVs**	**Volatiles**	**R (range)**	**ASVs**	**Volatiles**
Upupa epops					
0.46-0.49	*Polaromonas sp*1.	Acetic acid	0.53-0.56	*Sporosarcina sp*1 *Leucobacter sp*2 Flavobacteriia*	Decanoic acid
Otus scops					
0.79-0.84	Alphaproteobacteria* Actinobacteria* *Sphingomonas wittichii*	Propionic acid			
Athene noctua					
0.86-0.91	Flavobacteriia* 1 Chlamydiia* Sphingobacteriia* Cytophagia* *Stenotrophomonas sp.* *Devosia sp.*	Nonanoic acid	0.85-0.90	*Yaniella sp.* *Sporosarcina sp.* *Polaromonas sp.*2	Decanoic acid Nonanoic acid
				*Polaromonas sp.*3 *Corynebacterium sp.*2 *Sphingobacterium multivorum* Sphingobacteriia*1	Decanoic acid
	Flavobacteriia*2	Nonanoic acid Decanoic acid			
Sturnus unicolor					
				Sphingomonas sp.	Hexanoic acid
0.79-0.84			0.78-0.83	*Acidocella sp.* *Rothia mucilaginosa* *Polaromonas sp.*4	Hexanoic acid Heptanoic acid
Coloeus monerula					
	Saprospirae*	Butanoic acid	0.82-0.77		
0.74-0.79	*Flavisolibacter sp.* *Kaistobacter sp.* Alphaproteobacteria*1 *Deinococcus sp.*	Butanoic acid, benzaldehyde		Actinobacteria*1 Saprospirae*1 *Kocuria palustris* *Friedmanniella sp.*	Propionic acid
	*Kaistobacter sp.*1 *Unknown sp.* *Segetibacter sp.* *Hymenobacter sp.*1	Butanoic and pentanoic acid, benzaldehyde			

Table S6. General Linear Mixed Modelsexploring the relationships between intensity of parasitism or fledging success and relative abundance of particular bacteria and volatiles from latent components of sPLS models. Species identity (Sp ID) was included in the final as random (R), while relative abundance of particular bacteria and volatiles were included as fixed (F) factors. For continuous fixed factors, we show partial correlation coefficients. Statistics associated (i.e. in the same line of) to bacterial or volatile effects refers to models that included information of the dependent in independent fixed factors after controlling for the effect of species identity. Variables in bold font are those with associate p-values lower than 0.1. The asterisk (*) indicates the lower taxonomic assignation when identification at genus level was not possible.

	Intensity of parasitism				**Fledging success**			
	Beta	**F**	**df**	**P**	**Beta**	**F**	**df**	**P**
Early nestling stage								
Bacterial effects		3.25	5,97	**0.009**		1.48	5,84	0.204
Sp ID (R)		10.74	7,90	**<0.000**		1.11	6,78	0.367
Thermoleophilia* (F)	0.808	8.04	1,90	**0.006**	-0.299	0.56	1,78	0.458
***Porphyromonas sp.* (F)**	-0.607	9.63	1,90	**0.003**	0.076	0.08	1,78	0.778
Corynebacterium sp. (F)	-0.071	0.08	1,90	0.775	-0.284	0.65	1,78	0.423
Clostridium sp. (F)	-0.059	0.16	1,90	0.695	0.184	0.79	1,78	0.375
***Peptoniphilus sp.* (F)**	-0.085	0.23	1,90	0.631	0.589	5.76	1,78	**0.019**
Volatile effects	0.007	001	1,101	0.944	-0.066	0.39	1,88	0.534
Sp ID (R)		10.43	1,94	**<0.001**		0.35	6,82	0.907
Heptanal (F)	0.008	0.01	1,94	0.946	-0.106	0.36	1,82	0.548
Late nestling stage								
Bacterial effects		1.40	4,83	0.24		0.76	4,83	0.557
Sp ID (R)		12.51	6,77	**<0.001**		0.55	6,77	0.769
Bacilli* (F)	-0.007	0.00	1,77	0.965	0.045	0.03	1,77	0.867
Lactococcus sp. (F)	-0.166	1.48	1,77	0.227	-0.005	0.00	1,77	0.981
Polaromonas sp. (F)	0.265	3.73	1,77	0.057	-0.310	1.98	1,77	0.164
Sporosarcina sp. (F)	0.115	0.69	1,77	0.410	0.167	0.58	1,77	0.448
Volatile effects	0.025	0.06	1,86	0.816	-0.184	0.28	1,86	0.086
Sp ID (R)		13.25	6,80	**<0.001**		0.39	6,80	0.882
Heptanal (F)	0.016	0.05	1,80	0.822	-0.109	0.23	1,80	0.612

Table S7: GLMs exploring the relationships between intensity of parasitism or fledging success with bacteria (ASVs) and volatiles that resulted of the first latent component of sPLS analysis explained and with the correlations of Pearson establishment in a range between the maximum of the coefficient correlation found and 6% less than that maximum. The analysis is of each bird species in both nestling stage. Variables in bold font are statically significant at the 5% level. The asterisk (*) indicates the lower taxonomic assignation when identification at genus level was not possible.

	Intensity of parasitism				**Fledging success**			
	F	**d.f**	**P**	**Beta**	**F**	**d.f**	**P**	**Beta**
Nest at early stage								
Upupa epops								
Bacterial community								
Polaromonas sp1.	**6.09**	**1,43**	**0.018**	**0.352**	0.60	1,39	0.442	0.123
Volatiles profiles								
Acetic acid	**5.23**	**1,43**	**0.027**	**0.329**	0.29	1,39	0.595	-0.085
Otus scops								
Bacterial community								
Alphaproteobacteria*	0.50	1,7	0.501	1.599	0.39	1,7	0.554	-1.141
Actinobacteria*	0.70	1,7	0.429	1.013	3.09	1,7	0.122	2.141
Sphingomonas wittichii	1.52	1,7	0.257	-2.286	0.11	1,7	0.753	-0.611
Volatiles profiles								
Propionic acid	1.16	1,9	0.309	0.338	0.60	1,9	0.459	0.250
Athene noctua								
Bacterial community								
Flavobacteriia* 1	0.37	1,2	0.604	-1.089	-	-	-	-
Chlamydiia*	4.41	1,2	0.170	-2.608	-	-	-	-
Sphingobacteriia*	0.15	1,2	0.732	0.831	-	-	-	-
Cytophagia*	1.28	1,2	0.374	-1.837	-	-	-	-
Stenotrophomonas sp.	1.14	1,2	0.397	0.914	-	-	-	-
Devosia sp.	1.37	1,2	0.362	1.808	-	-	-	-
Flavobacteriia*2	1.03	1,2	0.416	1.391	-	-	-	-
Volatiles profiles								
Nonanoic acid	0.65	1,7	0.447	-0.574	0.05	1,5	0.832	0.227
Decanoic acid	0.00	1,7	0.989	0.010	0.00	1,5	0.944	0.075
Coleus monedula								
Bacterial community								
*Saprospirae**	-	-	-	-	-	-	-	-
Flavisolibacter sp.	-	-	-	-	-	-	-	-
Kaistobacter sp.	-	-	-	-	-	-	-	-
*Alphaproteobacteria*1*	-	-	-	-	-	-	-	-
Deinococcus sp.	-	-	-	-	-	-	-	-
Kaistobacter sp.1	-	-	-	-	-	-	-	-
Unknown	-	-	-	-	-	-	-	-
Segetibacter sp.	-	-	-	-	-	-	-	-
Hymenobacter sp.	-	-	-	-	-	-	-	-
Volatiles profiles								
Butanoic acid	2.70	1,4	0.176	0.743	0.57	1,3	0.506	0.864
Benzaldehyde	0.56	1,4	0.496	0.264	0.69	1,3	0.466	-0.584

Pentanoic acid	0.15	1,4	0.722	-0.131	0.17	1,3	0.711	-0.407
Nest at late stage								
Upupa epops								
Bacterial community								
*Leucobacter sp.*2	3.79	1,35	0.862	0.034	0.06	1,35	0.814	0.288
*Sporosarcina sp.*1	0.03	1,35	0.059	0.308	1.93	1,35	0.174	-0.041
Flavobacteriia*	1.49	1,35	0.231	0.232	0.05	1,35	0.822	-0.047
Volatiles profiles								
Decanoic acid	**9.02**	**1,37**	**0.005**	**0.443**	0.00	1,37	0.972	0.006
Athene noctua								
Bacterial community								
Yaniella sp.	0.27	1,2	0.653	-0.919	0.05	1,1	0.856	-2.501
Sporosarcina sp.	0.03	1,2	0.889	0.402	0.00	1,1	0.975	-0.527
Polaromonas sp.2	1.00	1,2	0.422	1.915	0.00	1,1	0.977	0.129
Polaromonas sp.3	0.02	1,2	0.895	-0.466	0.01	1,1	0.936	-0.877
Corynebacterium sp.2	0.60	1,2	0.521	2.205	0.10	1,1	0.805	1.697
Sphingobacterium multivorum	0.97	1,2	0.428	-1.614	0.19	1,1	0.737	1.436
Sphingobacteriia*1	0.07	1,2	0.821	-0.637	0.05	1,1	0.863	1.272
Volatiles profiles								
Decanoic acid	**6.90**	**1,7**	**0.034**	**-2.326**	0.14	1,6	0.725	0.328
Nonanoic acid	**6.57**	**1,7**	**0.037**	**2.250**	0.00	1,6	1.000	0.001
Sturnus unicolor								
Bacterial community								
Rothia mucilaginosa	0.02	1,3	0.898	-0.206	6.68	1,3	0.082	-2.314
Polaromonas sp.4	0.34	1,3	0.603	0.663	2.56	1,3	0.208	-1.105
Acidocella sp.	0.05	1,3	0.837	0.293	**10.32**	**1,3**	**0.048**	**2.537**
Sphingomonas sp.	2.44	1,3	0.216	-1.199	2.48	1,3	0.214	0.730
Volatiles profiles								
Hexanoic acid	0.07	1,5	0.798	-0.339	0.60	1,5	0.473	-0.951
Heptanoic acid	0.02	1,5	0.897	-0.172	0.13	1,5	0.731	0.447
Coleus monedula								
Bacterial community								
Kocuria palustris	0.02	1,4	0.886	-0.074	0.99	1,4	0.376	-0.725
Actinobacteria*1	0.01	1,4	0.912	0.083	0.13	1,4	0.735	0.385
Saprospirae*1	5.19	1,4	0.085	1.594	0.03	1,4	0.870	0.184
Friedmanniella sp.	**8.50**	**1,4**	**0.043**	**-1.348**	0.65	1,4	0.464	0.561
Volatiles profiles								
Propionic acid	0.21	1,7	0.659	0.172	0.18	1,7	0.687	0.157

RESULTADOS GENERALES Y DISCUSIÓN INTEGRADORA

El objetivo principal de esta tesis es comprobar la hipótesis de que las bacterias asociadas a aves, tanto las del material del nido como las simbiontes de la glándula uropigial en el caso de la abubilla, emiten volátiles que juegan un papel importante en la interacción parásitos-hospedador. Esta hipótesis por lo tanto implica la existencia de dos tipos de asociaciones. Por un lado, que el perfil de volátiles de las secreciones y del ambiente del nido se deberían relacionar con características de la comunidad bacteriana de la secreción y del material del nido respectivamente. Y, por otro lado, que (B) tanto volátiles como bacterias se deberían de asociar con la intensidad de ectoparasitismo que sufren los pollos y adultos en la etapa de reproducción y, por lo tanto, con el éxito de vuelo de los pollos.

En un primer capítulo revisamos el estado de conocimiento sobre estas relaciones y concluimos que los parásitos y depredadores pueden utilizar volátiles de origen bacteriano para detectar y seleccionar a sus víctimas. Independientemente de los efectos directos de estos volátiles sobre los hospedadores, en escenarios de comunicación funcionarían como señales del huésped o información social involuntaria que puede ser recibida tanto por conspecíficos como heteroespecíficos (parásitos y/o depredadores) (**Capítulo I**). Los resultados relativos a la descripción de las comunidades bacterianas y de los perfiles de volátiles se presentan en los capítulos individuales y este apartado nos dedicamos a presentar los resultados directamente relacionados con la comprobación de los dos componentes de la hipótesis general de trabajo.

1. Relación comunidades bacterianas y perfiles de volátiles

Encontramos varias evidencias de la existencia de la asociación entre comunidades bacterianas y los perfiles de volátiles asociados. Por un lado, la diversidad beta de la comunidad de bacterias de las secreciones de las hembras de abubilla se asoció significativamente con la diversidad beta de los volátiles de sus secreciones, aunque esta relación no llego a ser estadísticamente significativa en el caso de los pollos de abubilla al final de su estancia en el nido (**Capítulo III**). La diversidad beta bacteriana de los materiales del nido de abubilla tampoco se asoció significativamente con los volátiles recogidos en la caja-nido, ni al inicio ni al final de la estancia de los pollos (**Capítulo III**). Además, al explorar posibles asociaciones entre bacterias y volátiles particulares que se resumían en distintos PC, en la secreción de las hembras de abubilla, la

abundancia relativa de varios géneros del filo Firmicutes se asociaron negativamente con las abundancias relativas de varios ácidos (butanoico, isobutírico, isopropionico, isovalérico, pentanoico) y ésteres (2, 3 metil-éster y butanoico-éster), mientras que la de los géneros *Bacillus*, *Trissierellaceae* y *Erysipelotrichaceae* se relacionaron negativamente con la abundancia de ácidos y ésteres, y *Syntrophomonas* y *Lactoccocus* lo hicieron positivamente (**Capítulo III**). En el caso de secreciones de pollos de abubilla también encontramos varios ácidos relacionados positivamente con géneros bacterianos claves pertenecientes a los filos de Actinobacteria, Firmicutes y Proteobacterias, y negativamente con otros géneros pertenecientes a los filos Actinobacteria, Bacteroidetes, Caldithrix, Chlorobi, Chloroflexi, Firmicutes, GN04, NC10, Planctomycetes y Proteobacteria. También encontramos asociaciones con muestras de bacterias del material del nido y los volátiles del ambiente del nido recogidas al final de la estancia de los pollos.

Algunos resultados dan apoyo a la hipotética relación entre volátiles y bacterias a nivel interespecífico. Detectamos que la relación entre la diversidad alfa de las bacterias del nido y los volátiles captados en la caja nido depende de la especie de ave, tanto al inicio como al final de la estancia de los pollos en el nido. Al inicio de esta estancia, encontramos que la relación era positiva en el estornino negro y negativa en el mochuelo (**Figura 8**) (**Capítulo IV**). Esta variación entre especies podría deberse a características de sus nidos ya que, algunas de las especies que consideramos en el análisis no construyen nido (p. ej. autillo, mochuelo o abubilla), mientras que otras como el estornino negro y el carbonero común utiliza plumas y plantas ricas en volátiles con propiedades antimicrobianas (Pinto et al., 2021; Ruiz-Castellano et al., 2016, 2018). Esta variación entre especies en la construcción de sus nidos podría afectar a las relaciones existentes entre los perfiles de volátiles en el nido y las comunidades bacterianas de los materiales del mismo. Por otra parte, utilizando información de muestras recogidas al final de la estancia de pollos, también encontramos evidencias de la asociación esperada entre volátiles y bacterias en interacción con la identidad de la especie de ave. La diversidad beta de la comunidad bacteriana del material del nido también se relacionó con los volátiles existentes en la cavidad de distintas especies, aunque solo llego a ser significativa para el autillo y el mochuelo. Mientras que en el autillo existe una correlación positiva cuando se tienen en cuenta la presencia o ausencia de bacterias/volátiles para la estima de diversidad alfa, en el mochuelo esa relación se

encuentra cuando para las estimas de alfa diversidad se tiene en cuenta la abundancia relativa de ASVs y volátiles, así como las relaciones filogenéticas de las especies bacterianas que conforman esa comunidad (**Capitulo IV**).

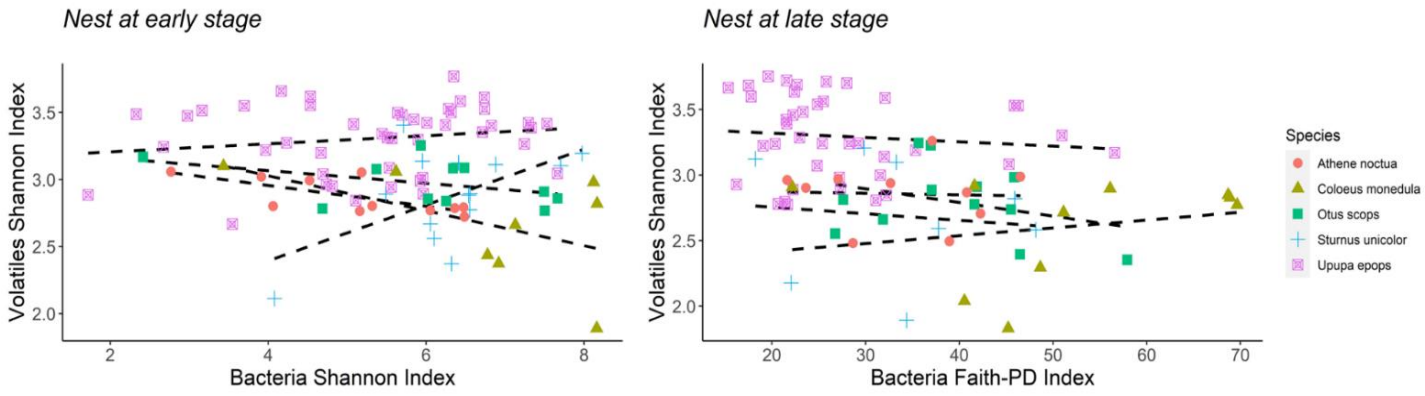

Figura 8: Asociaciones entre la diversidad alfa de los perfiles volátiles (índice de Shannon) y las comunidades bacterianas (índice de Shannon) del nido de diferentes especies en la etapa temprana de los polluelos y los perfiles volátiles (índice de Shannon) y las comunidades bacterianas (índice Faith-PD) del nido en la etapa tardía de los pollos. Las líneas son líneas de regresión.

A nivel interespecífico, también encontramos algunas evidencias que apuntan a que la abundancia de ciertas bacterias y volátiles del entorno del nido están relacionados entre sí. Tanto al inicio como al final de la etapa de pollos, la abundancia de heptanal en el ambiente se relacionó positivamente con la de ciertas bacterias del material del nido. Curiosamente, las bacterias que se asocian con el heptanal variaban dependiendo del momento de muestreo. Al inicio de la etapa de pollos eran las ASVs pertenecientes a los géneros *Clostridium*, *Peptoniphilus*, *Porphyromonas*, *Corynebacterium* y un género desconocido perteneciente a la familia Thermophilia las que se asociaban con el heptanal, mientras que al final del período el heptanal se relacionó positivamente con ASVs pertenecientes al género *Lactococcus*, *Polaromonas*, *Sporosarcina* y a la clase Bacilli (**Figura 9**). También encontramos algunas asociaciones entre volátiles y bacterias particulares dentro de cada especie. Sin embargo, los tamaños limitados de muestras de muchas de las especies no permiten hacer inferencias robustas.

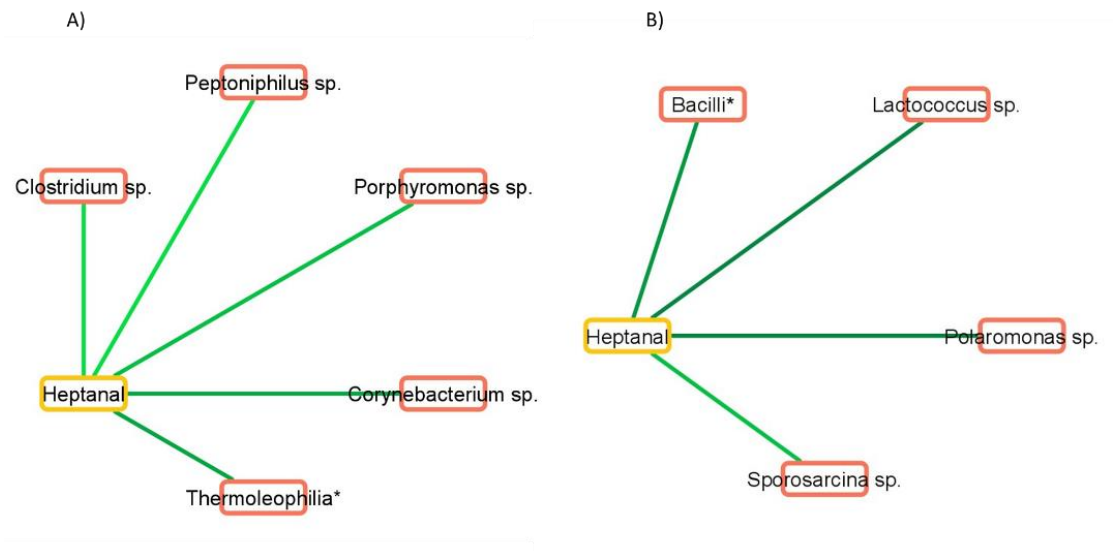

Figura 9: Representación de los resultados de los análisis de redes (sPLS) entre bacterias y volátiles del nido en la etapa temprana (A) y en la etapa tardía (B) de los polluelos de distintas especies de aves. Ambas redes son bipartitas, donde cada borde vincula un ASV a un nodo volátil, según una matriz de similitud. Las correlaciones mostradas están entre el máximo de la correlación del coeficiente de Pearson encontrado y un 6% menos que ese máximo. El grado de color verde de los bordes representa coeficientes desde el más bajo (color verde claro) hasta el más alto (color verde oscuro). Todos los valores de los coeficientes de correlación representados fueron superiores a 0,60. Los ASV se nombran con el género o familia al que pertenecen. El asterisco (*) indica la familia de bacterias de un género desconocido, ya que no fue posible la identificación a nivel de género.

Todos estos resultados apuntan a que, en general, existe una relación entre características de la comunidad bacteria del perfil de volátiles. Sin embargo, el grado de asociación varía dependiendo del índice de diversidad que se use, del tipo de muestra que se analice (secreción, material del nido o volátiles de la cavidad), de la etapa del nido en la que se recoja la muestras, y de la especie de ave en la que se compruebe. Los volátiles que se detectan en las cajas nidos, no solo provienen de los generados por las bacterias del material del nido, sino que los volátiles generados por las aves que lo habitan o por los materiales que contienen los nidos también dejarían su huella. Esas fuentes de variación no se controlan en los análisis y podrían explicar, por ejemplo, los diferentes resultados obtenidos para varias especies. Además, en los nidos existen comunidades bacterianas distintas a las muestreadas (en los materiales de los nidos y, en el caso de abubillas, en la secreción uropigial), asociadas por ejemplo al plumaje o al digestivo de las aves y a excrementos o restos de comida existentes fuera de la taza del nido (i.e. no en contacto directo con los pollos y, por tanto, no muestreados), que

también podrían contribuir al perfil de volátiles detectados en las cajas nidos y que tampoco estaban controlados en nuestros análisis. La importancia relativa de esas fuentes de volátiles podría variar dependiendo del estado de desarrollo de los pollos en el nido y explicar también parte de la variación encontrada en nuestros resultados. Por otro lado, sabemos que el metabolismo bacteriano tiene un componente genético (Pace, 1997) y, por tanto, los resultados obtenidos utilizando índices de diversidad que controlan por un componente filogenético (Faith's PD, Unifrac y PhILR) pueden diferir de los obtenidos utilizando otros índices. El metabolismo bacteriano también puede depender de la presencia de otras bacterias en estos ambientes (Klitgord & Segrè, 2011) y algunos de los índices de diversidad empleados utilizan información de presencia mientras que otros utilizan información de abundancia de bacterias, lo que también podría explicar las diferencias de resultados dependiendo del tipo de índice utilizado. Nuestros diseños de muestreo no permiten aislar el efecto de las comunidades bacterianas del nido sobre el perfil de volátiles de la cavidad, ni predecir el grado y el signo de la relación entre ambos. Sin embargo, dado la gran variedad de fuentes de volátiles y las evidencias encontradas suponen un fuerte apoyo a la hipótesis de que los volátiles del nido están parcialmente determinados por las comunidades bacterianas del mismo.

2. Relación de la comunidad bacteriana y del perfil de volátiles con:

a. Parasitismo

Distintas evidencias apuntan a que la comunidad bacteriana de los nidos determina parcialmente el nivel de parasitismo que van a sufrir los pollos. Por un lado, el experimento de autoclavar el material del nido derivó en una menor intensidad de parasitismo sufrido por los pollos de abubilla (**Capítulos II** y **III**). Además, encontramos una relación positiva entre la densidad bacteriana y la intensidad de ectoparasitismo en las cajas experimentales en uno de los años de estudio (**Figura 10**, **Capitulo II**). En un enfoque experimental similar al nuestro, realizado con nidos de cernícalo primilla (*Falco naumanni*), Podofillini y colaboradores encontraron que, poco después de la eclosión, los pollos sufrieron más ectoparasitismo por *C. hemapterus* en nidos con materiales de la reproducción anterior que en aquellos sin material orgánico del año anterior (Podofillini et al., 2018). Una posible explicación a esos resultados experimentales en los nidos de cernícalo es que los volátiles generados por la comunidad de bacterias manipulada influyen en la detección del nido por esos ectoparásitos. Sin

embargo, también es posible que en el material de nidos antiguos empleado en el experimento hubiera pupas de *Carnus* que parasitaran a los pollos en el mismo nido donde emergen (Valera et al., 2018). No parece que esa posibilidad explique nuestros resultados ya que, en uno de los años de estudio, tamizamos el material de los nidos utilizado en el experimento y eliminamos del mismo todas las pupas de *C. hemapterus* encontradas y, en ese año, los efectos esperados fueron incluso más claros (ver **Figura 10**).

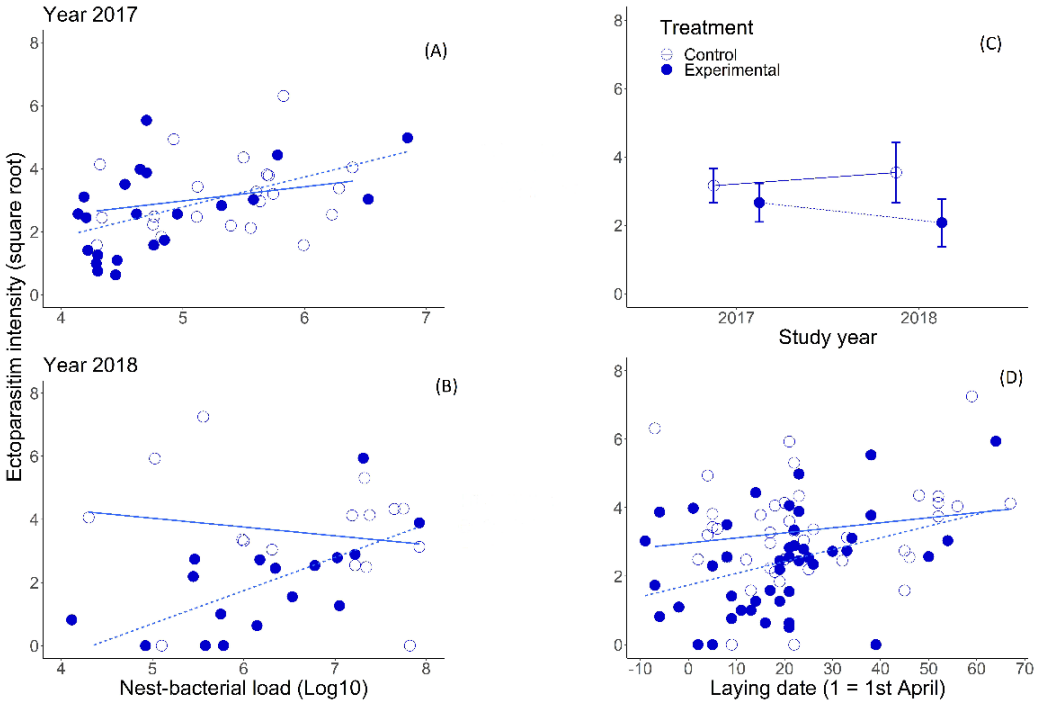

Figura 10. Asociaciones entre la intensidad de parasitismo de los pollos de abubilla y la densidad de bacterias del material del nido que fue (puntos azules) o no fue (círculos azules) autoclavado antes del periodo de reproducción de los dos años de estudio, 2017 (A) y 2018 (B). Mostramos las medias ponderadas (±95% CI) de la intensidad de ectoparasitismo por *Carnus hemapterus* sufrido por los pollos de abubilla desarrollándose en cajas nidos con material de nido que fue y no fue autoclavado en las épocas de cría en 2017 y 2018 (C). Finalmente, (D) También mostramos la asociación entre la intensidad de parasitismo y la fecha de puesta de las abubillas de nidos con material de nido autoclavado o no autoclavado. Las líneas sólidas y discontinuas son líneas de regresión para cajas nido experimentales y control respectivamente.

Para comprobar la hipótesis de que los efectos del experimento de autoclavar el nido sobre la intensidad de parasitismo estuviera mediado por los volátiles producidos por la comunidad de bacterias de los nidos, exploramos las relaciones de características de comunidades bacterianas y/o perfil de volátiles con la intensidad de parasitismo. Varios resultados apoyan esa posibilidad. Por un lado, bacterias y volátiles particulares

que se resumían en distintos ejes de PC de los nidos y secreciones se asoció a la intensidad de parasitismo que sufren los pollos de abubilla a los 8 días tras la eclosión del primer huevo. En concreto, la intensidad de parasitismo se asoció negativamente con la abundancia de algunos géneros pertenecientes a los filos Actinobacteria, Chloroflexi y Proteobacteria del material del nido. También la intensidad de parasitismo se asoció positivamente con algunos ácidos (p.ej. ácido acético y butanoico), negativamente con aldehídos detectados en los nidos (hexanal, nonanal y pentanal), y negativamente con los ácidos detectados en la secreción uropigial de los pollos (pentanoico, hexanoico, heptanoico, octanoico) (**Figura 11**) (**Capitulo III**). Al analizar la intensidad de parasitismo de pollos poco antes de abandonar los nidos, también encontramos asociaciones con característica microbiológicas y químicas de los nidos y/o secreciones. Los pollos que sufrían mayor intensidad de parasitismo fueron los que crecieron en nidos con material de nido que albergaba menor densidad de los géneros *Larkinella* spp. (filo Bacteroidetes) y *Aquabacterium* spp. (filo Proteobacteria) y que presentaban secreción uropigial con menor concentración de ésteres (**Figura 11**) (**Capitulo III**). En cuanto a la abundancia de malófagos en las hembras, también encontramos asociaciones positivas con la abundancia del género *Lactococcus* spp. (filo Firmicutes) y negativa con bacterias pertenecientes a las familias Trissierellacea y Erysipelotrichaceae (filo Firmicutes) y negativas con la abundancia de ácidos y ésteres de la secreción de la hembra (**Figura 11**) (**Capitulo III**).

Independientemente de la identidad de las bacterias o componentes químicos responsables de las asociaciones detectadas con el parasitismo, estos resultados apoyan la hipótesis de que las bacterias simbióticas, y/o de los volátiles que se relacionan con ellas, juegan un papel importante en las interacciones entre las abubillas y sus ectoparásitos. Sin embargo, las correlaciones no implican causalidad y, por lo tanto, estos resultados pueden interpretarse en ambas direcciones: la que propone la hipótesis (volátiles producidos por bacterias que afectan el parasitismo), o parásitos que afectan a las comunidades bacterianas y los volátiles asociados de sus víctimas. Los parásitos, por definición, usan los recursos del huésped para sí mismos y, por lo tanto, influyen en la condición física y la salud de sus víctimas (Schimid-Hempel, 2011). Dado que la condición física o fisiológica del huésped influye en las características de sus simbiontes microbianos (Sherwin et al., 2019), el parasitismo podría afectar la comunidad simbiótica microbiana de sus hospedadores (Eleftheriou, 2020; Knutie, 2018; Mazorra-

Alonso et al., 2021; Rafaluk-Mohr et al., 2022). Sin embargo, nosotros manipulamos las comunidades bacterianas de los nidos de abubillas antes de la reproducción, lo que afectó la intensidad del parasitismo, los perfiles volátiles y las comunidades bacterianas de los nidos de abubillas durante la fase de anidamiento. Por tanto, la explicación más probable para las asociaciones detectadas entre grupos particulares de volátiles y de bacterias con intensidad de parasitismo es que las bacterias productoras de volátiles afectan el parasitismo y no al revés.

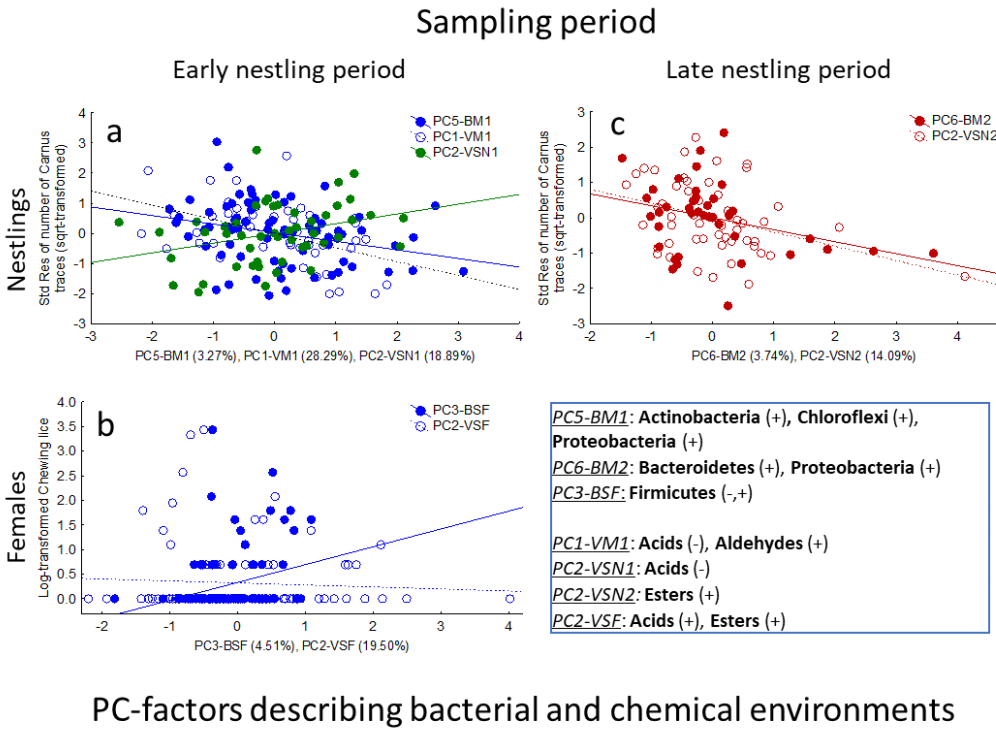

Figura 11. Regresiones parciales estadísticamente significativas entre la intensidad del parasitismo en pollos (número de rastros de moscas *Carnus hemapterus*) (a y c) y hembras (número de malófagos) (b) y valores de componentes principales (PC) que resumen las comunidades bacterianas (BM1, BM2, BSF) y los perfiles de volátiles (VM1, VSN1, VSN2, CSF) de diferentes tipos de muestras, en los períodos de anidamiento temprano (a y b) y tardío (rojo) (c). Cada eje PC se nombra por una composición de letras que indican el tipo de muestras. La primera letra indica si la muestra corresponde a bacterias (B) o volátiles (V), la segunda letra indica si la muestra es de secreciones de hembras (SF), polluelos (SN) o material de nido (M). Finalmente, para los tipos de muestras que se recogieron al inicio (1) y al final (2) del período de cría, el nombre terminó con un número. Para reflejar las correlaciones parciales detectadas, trazamos valores residuales estandarizados del factor de interés dependiente e independiente después de controlar por el resto de factores independientes en el modelo. También mostramos los principales grupos de volátiles y bacterias (nombrados por los filos a los que pertenecen los géneros) que se asocian con cada uno de los factores PC en las figuras (d). Los signos entre paréntesis indican la dirección de las asociaciones de volátiles particulares con cada factor PC. También se muestra el porcentaje de varianza explicado por cada uno de los factores PC. Las líneas son líneas de regresión.

A nivel interespecífico también encontramos evidencias a favor de la relación entre intensidad de parasitismo y características de las comunidades de bacterias y de volátiles del nido. La intensidad de parasitismo que sufren los pollos al inicio de su estancia en el nido se relacionó negativamente con la diversidad alfa de los volátiles del nido, mientras que la intensidad de parasitismo al final de la estancia del nido se relacionó negativamente con la diversidad alfa de bacterias del nido y positivamente con la diversidad beta de los volátiles (p.ej. comunidades bacterianas más parecidas entre ellas experimentaron niveles similares de parasitismo). Además, también encontramos que algunos de los volátiles y bacterias que aparecían relacionados entre sí en modelos sPLS, se relacionaban también con la intensidad de parasitismo nivel intraespecífico, mientras que a nivel interespecífico, solo la abundancia de algunas de las bacterias se asoció con la intensidad de parasitismo después de corregir estadísticamente por los efectos de la especie. La abundancia del género *Porphyromonas sp*., y la de un género desconocido de la familia Thermoleophilia se asociaron positivamente con la intensidad del parasitismo de los pollos más jóvenes. Para los pollos mayores, solo la abundancia del género *Polaromonas sp*. en el material del nido tendió a asociarse con la intensidad del parasitismo (**Figura 12**).

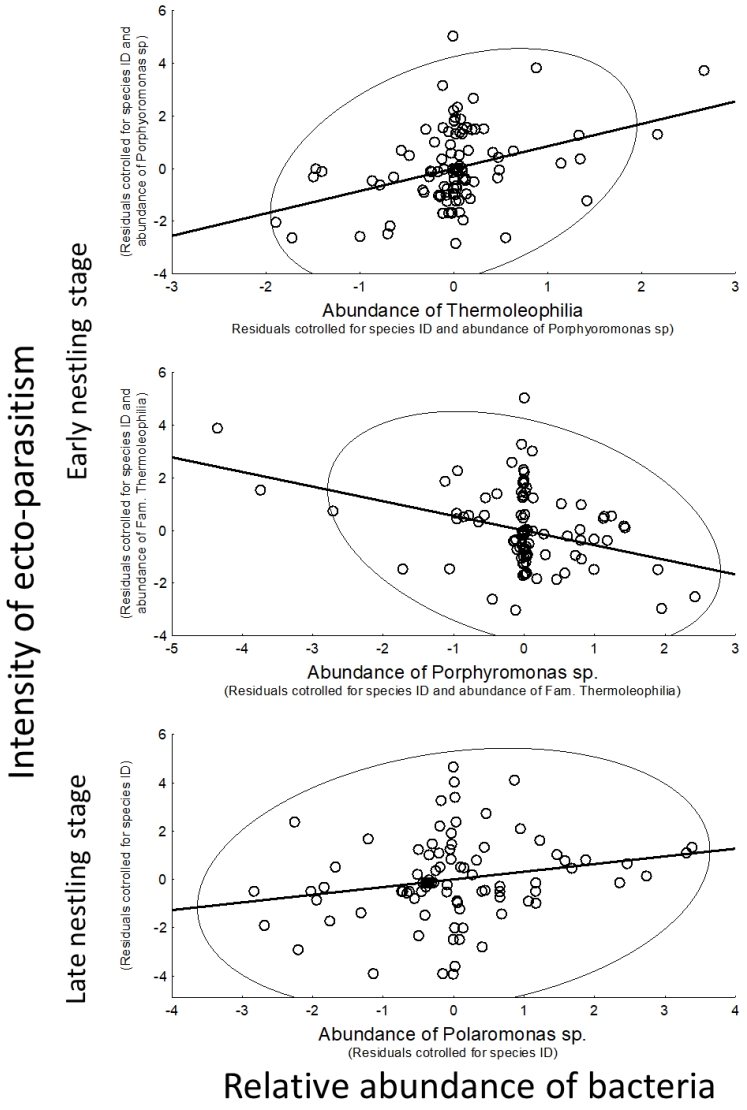

Figura 12: Correlaciones entre la intensidad del parasitismo (raíz cuadrada) con la abundancia relativa de bacterias presentes en el material del nido, seleccionadas por el primer componente latente del análisis sPLS en ambas etapas de polluelo. Las líneas son líneas de regresión y las elipses representan intervalos de confianza del 99%.

Como expusimos para el caso de la abubilla, estos resultados son producto de correlaciones en las que no se puede distinguir entre causa y efecto y, por tanto, no podemos descartar que sea el parasitismo el responsable de las características de las comunidades de bacterias y de los perfiles de volátiles con los que se asocian. Sin embargo, estos resultados están de acuerdo con otros estudios experimentales, realizados en condiciones de laboratorio, en los que se demostró que una mayor densidad, pero menor diversidad de bacterias en la piel, genera una menor atracción al mosquito *A. gambiae* (Verhulst et al., 2011). En otro estudio demostraron que una

diversidad mayor de volátiles en la piel disminuye la probabilidad de atracción del mosquito *A. aegypti* (Logan et al., 2008). Entre la enorme cantidad de volátiles emitidos por los huéspedes, solo unos pocos tienen un efecto sobre el comportamiento de los ectoparásitos (Poldy, 2020). Algunas teorías proponen que ciertas bacterias emiten volátiles que pueden estar enmascarando los volátiles atractivos para los ectoparásitos, o directamente que generen volátiles que los repelen (Verhulst et al., 2011). Sin embargo, más estudios sobre las comunidades bacterianas y los efectos en el comportamiento de los ectoparásitos de los volátiles específicos son necesarios.

b. Éxito de vuelo

De acuerdo también con nuestras predicciones encontramos una asociación entre características de las comunidades bacterianas y los perfiles de volátiles del nido y de secreciones con el éxito de vuelo. En el caso de la abubilla, la densidad bacteriana en el material del nido se relacionó positivamente con el éxito de vuelo tanto en las cajas experimentales como en las cajas control (**Capitulo II**). Este resultado puede parecer contra intuitivo ya que la carga bacteriana del nido suele aparecer negativamente relacionada con el éxito reproductivo de las aves (Jacob et al., 2015; Peralta-Sánchez et al., 2018; Soler et al., 2017). Es posible que esos resultados estén relacionados con el estilo de vida especial de esta especie. Las abubillas no eliminan las heces de los pollos y en su glándula uropigial albergan bacterias simbióticas productoras de sustancias antibióticas de amplio espectro en alta densidad (Martín-Platero et al., 2006; Ruiz-Rodríguez et al., 2009, 2013, 2012; Soler et al., 2008). Esas bacterias que protegen a los pollos durante el crecimiento (Ruiz-Rodríguez et al., 2009), podrían estar presentes en el material de nido a altas densidades explicando la relacion positiva encontrada. Aunque es una explicación especulativa, puede merecer la pena comprobarla en trabajos futuros.

En cuanto a bacterias y volátiles específicos encontramos que el éxito de vuelo en nidos de abubilla se relacionó positivamente con la abundancia relativa del género *Syntrophomonas* spp. en la secreción uropigial de las hembras al inicio de la estancia de los pollos y con la de varios géneros pertenecientes a los filos Actinobacteria, Bacteroidetes, Cloroflexi y Cyanobacteria en el material del nido recolectado al final del período de anidación (**Figura 13**). Además, la abundancia relativa de dos ésteres (ácido butanoico etil-ester y 3-acido metil butanoico etil-ester) en la secreción uropigial de hembras y tres ácidos (acético, butanoico e isobutirico) de la secreción de pollos de

8 días de edad y tres ácidos (isobutirico, isocaproico y pentanoico) de la secreción de 19 días de edad se asociaron negativamente con el éxito de vuelo. Sin embargo, varios aldehídos (benzaldehído, heptanal, hexanal y pentanal) de la secreción de pollos de 8 días de edad se correlacionaron positivamente con éxito de vuelo (**Figura 13**). La detección de estas asociaciones en nidos de abubilla refuerza la idea de las comunidades bacterianas simbiontes de esta especie afectan al éxito reproductor y que este efecto podría estar mediado por volátiles de origen bacteriano.

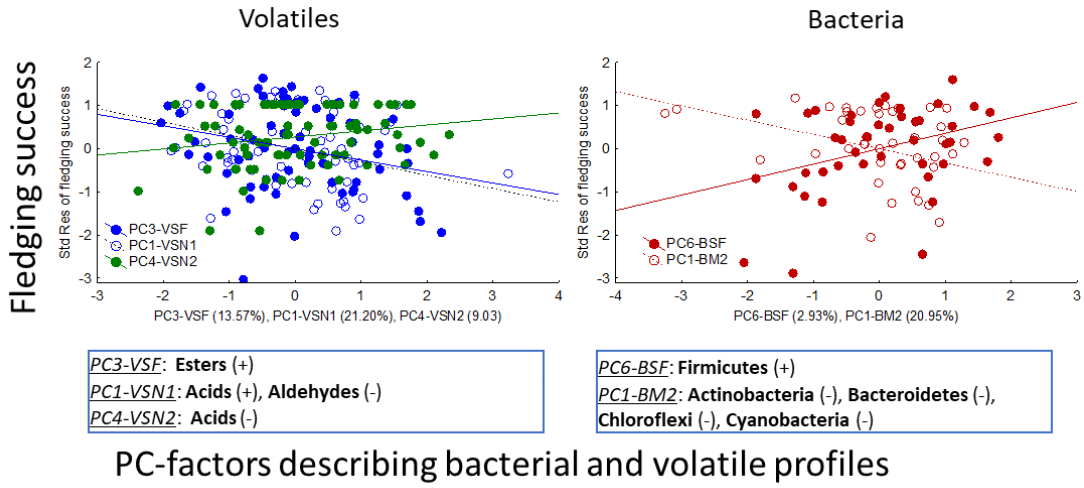

Figura 13: regresiones parciales estadísticamente significativas entre el éxito de vuelo y los valores de los de cada muestra en los ejes PC, donde se resumen las abundancias de las bacterias que componen las comunidades bacterianas [secreción de hembras (PC6-BSF y material del nido recogido al final del período de cría (PC1-BM2)] y perfiles volátiles de la secreción uropigial de las hembras (PC3-VSF) y de los polluelos [en el período temprano (PV1-VSN1) y tardío (PC4-VSN2)]. Para reflejar las correlaciones parciales detectadas, trazamos los valores residuales estandarizados del factor dependiente e independiente de interés después de controlar por el resto de factores independientes en el modelo. También mostramos los principales grupos de volátiles y bacterias (resumidos en los filos a los que pertenecen los géneros) que se asocian con cada uno de los factores PC en las figuras. Los números entre paréntesis indican volátiles o géneros bacterianos particulares asociados con cada factor de PC con un valor de factor de carga absoluto superior a 0,7 en el ESM-Tabla S4 del capítulo 3. También se muestra el porcentaje de varianza explicado por cada uno de los factores de PC. Las líneas son líneas de regresión.

A nivel interespecífico, el éxito de vuelo se relacionó negativamente con la diversidad alfa de las bacterias del material del nido, pero solo con la estimada al inicio de la estancia de los pollos y después de controlar por los efectos de la especie de ave. Esta asociación no varió interespecíficamente. Además, también encontramos que algunos de los volátiles y bacterias que aparecían relacionados entre sí en modelos sPLS, se relacionaban también con el éxito de vuelo. La abundancia relativa de *Corynebacterium* sp. (negativamente) y de *Peptoniphilus* sp. (positivamente) en la etapa

temprana del periodo de cría se asoció con el éxito de vuelo (**Figura 14**) **(Capitulo IV)**. En cuanto a los volátiles, el heptanal tendió a asociarse negativamente con el éxito de vuelo en ambos periodos de la estancia de los pollos (**Figura 14**) **(Capitulo IV)**. Finalmente, a nivel intraespecífico ninguno de los compuestos volátiles y bacterias asociados entre sí en los modelos sPLS se relacionaron significativamente con el éxito de vuelo (**Capítulo IV**).

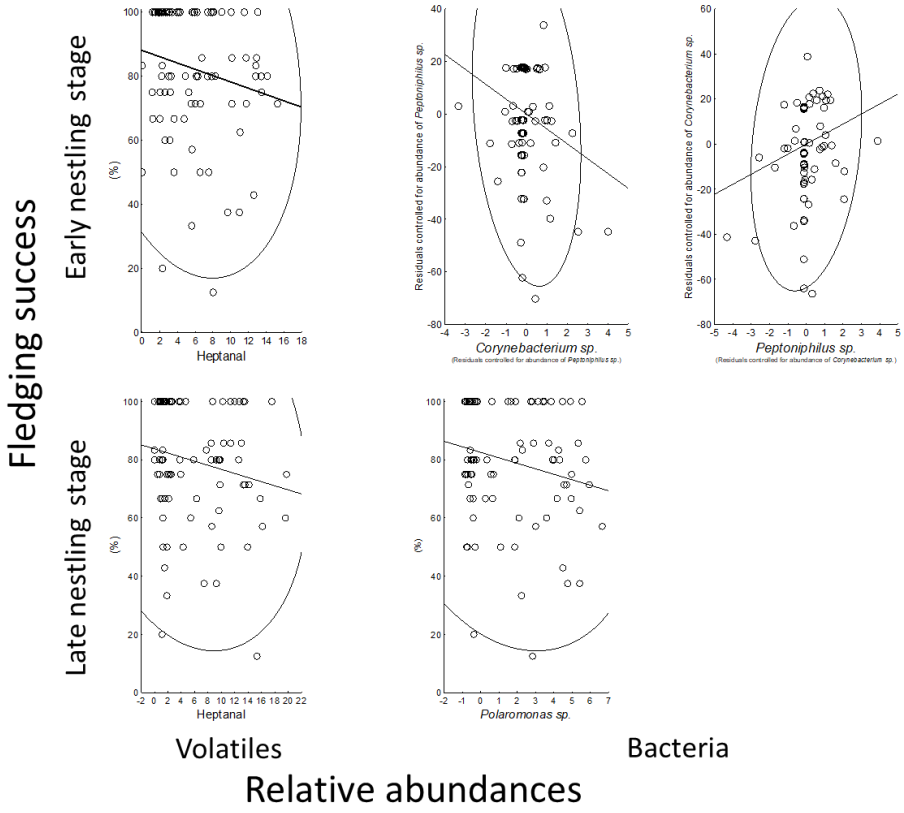

Figura 14: Correlaciones entre el éxito del vuelo (raíz cuadrada) con la abundancia relativa de bacterias presentes en el material del nido, seleccionadas por el primer componente latente del análisis sPLS en ambas etapas de polluelo. Las líneas son líneas de regresión y las elipses representan intervalos de confianza del 99%.

Todos estos resultados en conjunto sugieren que la comunidad bacteriana que se establece en el material del nido en etapas de reproducción previas pueden afectar a la intensidad de parasitismo que sufren los pollos y su éxito de vuelo (**Capitulo II**), mediado por la comunidad bacteriana que se establece y sus volátiles. Estos resultados se ven apoyados por las relaciones que hemos encontrado entre la abundancia relativa de algunos aldehídos, ácidos y ésteres con la intensidad del parasitismo en pollos y

hembras, y con el éxito de volantones (**Capitulo III**). La mayoría de estos productos químicos se han detectado en otros animales y se sabe que determinan la selección del huésped por parte de algunas plagas de artrópodos (Poldy, 2020). Nonanal, por ejemplo, es un olor típico del perfil volátil de algunas aves (p. ej., palomas y pollos de engorde) que atrae a los mosquitos del género *Culex* (Syed & Leal, 2009), y se ha utilizado como centinela en programas de avistamiento de plagas (Dabro et al., 2006; Deegan et al., 2005; Komar, 2001). De manera similar, la abundancia relativa de diferentes géneros de bacterias pertenecientes a filos como Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Cyanobacteria y Chlorofexi se asoció con la intensidad del parasitismo y el éxito de vuelo de los pollos. Algunas bacterias de estos grupos, como las pertenecientes a los géneros *Enterococcus, Pseudomonas* y *Variovorax*, han sido previamente asociadas con probabilidad de parasitismo por ectoparásitos hematófagos (Tomás et al., 2020; Verhulst et al., 2010). Es importante destacar que algunos grupos de bacterias y volátiles de diferentes tipos de muestras se asociaron con el éxito de vuelo, lo que indica que estos componentes químicos y microbiológicos pueden influir en la eficacia biológica del huésped. A nivel interespecífico, nuestros resultados apoyan las predicciones que relacionan la comunidad bacteriana con el perfil de volátiles, y ambos con la intensidad del parasitismo y el éxito de vuelo. Sin embargo, el nivel de asociación dependió de los índices utilizados para caracterizar los perfiles bacterianos y volátiles y de la etapa del nido en la que se muestrearon. Dado que los perfiles bacterianos y volátiles están relacionados entre sí, estos resultados sugieren queel ectoparásito *C. hemapterus* sigue señales químicas de origen bacteriano para localizar y/o seleccionar nidos de sus aves hospedadoras. Sin embargo, las correlaciones no implican causalidad y, dado que sabemos que las consecuencias de la actividad de los ectoparásitos (heces de parásitos y sangre del huésped) pueden influir en las comunidades bacterianas en los nidos de aves (Tomás et al., 2018), una explicación alternativa a las asociaciones detectadas es que sean los ectoparásitos los causantes. Independientemente de la causa, nuestros resultados ponen de manifiesto que, después de corregir por identidad de especie, la importancia relativa de bacterias particulares predecía la intensidad de parasitismo sufrido por los pollos de distintas especies de aves. Las asociaciones detectadas son en cualquier caso complejas y, aunque nuestros resultados respaldan firmemente el papel central de los compuestos volátiles de origen bacteriano para explicarlos, la importancia de diferentes taxones bacterianos y de diferentes compuestos volátiles que determinan el riesgo de parasitismo y el éxito de los volantones insta a

nuevos enfoques experimentales (**Capítulo IV**). Trabajos futuros deberían incluir experimentos dirigidos a probar el efecto de esas bacterias atrayendo o repeliendo a las moscas *Carnus*, lo que permitirá distinguir causas y consecuencias de las asociaciones detectadas.

CONCLUSIONES

1. Una revisión del tema permite concluir que los costos impuestos por parásitos y depredadores que detecten a sus víctimas por el olfato podrían contrarrestar los posibles beneficios asociados a la producción de volátiles. Por tanto, en el caso de que los volátiles sean de origen bacteriano, parásitos y depredadores podrían afectar a las interacciones simbióticas entre bacterias y sus animales hospedadores.

2. La esterilización de material de nido, proveniente de etapas reproductivas anteriores, produjo una disminución en abundancia de bacterias del nido durante la reproducción, y en la intensidad de ectoparasitismo por *Carnus hemapterus* sufrido por los pollos de abubilla. Además, en las cajas experimentales, detectamos una relación positiva entre abundancia de bacterias en el nido y la intensidad de parasitismo sufrido por los pollos de abubilla. Por tanto, el ambiente bacteriano del nido determina el nivel de parasitismo sufrido por los pollos de esta especie.

3. Esta esterilización del material del nido también produjo una reducción en diversidad bacteriana y un aumento en la diversidad de volátiles. Estos resultados apuntan a que las comunidades bacterias de los nidos podrían estar produciendo compuestos químicos que el ectoparásito podría detectar y utilizar para encontrar y seleccionar nidos activos de sus hospedadores.

4. Ciertas bacterias del material del nido se relacionaron con ciertos volátiles del ambiente del nido a nivel intraespecífico y a nivel interespecífico. A nivel interespecífico, el grado y el signo de la relación esperada entre diversidad bacteriana y volátiles del nido dependió de la especie de ave. Estos resultados sugieren que el metabolismo de las bacterias asociadas al material del nido es responsable del olor del nido.

5. Apoyando la relación esperada entre bacteria y volátiles, también encontramos que las comunidades bacterianas simbiontes de las secreciones uropigiales de abubillas se asocian a los perfiles de volátiles de la secreción y, en parte, a los del nido. Por tanto,

las comunidades bacterianas podrían influir en la información química que los parásitos utilizan para detectar los nidos de sus hospedadores.

6. La diversidad bacteriana y/o la de volátiles del ambiente del nido, tanto a nivel intraespecífico (i.e. abubillas) como interespecífico, se asocian con la intensidad de parasitismo de *C. hemapterus*. Tanto la comunidad bacteriana como el perfil de volátiles de las secreciones de hembras y pollos de abubilla ase asociaron con la intensidad de parasitismo por malófagos en las hembras, y de *C. hemapterus* en los pollos. Esto sugiere que la comunidad bacteriana es responsable de los volátiles detectados en el ambiente del nido y que *C. hemapterus* detecta.

7. Además, ciertos volátiles del ambiente del nido y bacterias detectadas en el material del nido, así como los volátiles y bacterias de las secreciones se asociaron con la intensidad del ectoparasitismo de las hembras y de los pollos de abubillas, y con su éxito de vuelo. Entre los volátiles clave encontramos que ciertos aldehídos (i.e., pentanal, hexanal y heptanal) se relacionan negativamente con la intensidad de parasitismo en los pollos, pero positivamente con el éxito de vuelo, y que ciertos ácidos como el acético y el butanoico tuvieron un efecto contrario al de los aldehídos. Esto sugiere que los ectoparásitos detectan los nidos activos mediante ciertos aldehídos y que los ácidos podrían tener un efecto repelente, afectando ambos tipos de compuestos al éxito de vuelo de los pollos.

8. Identificamos en el estudio interespecífico algunas bacterias y volátiles clave que estaban relacionados entre sí y que variaban entre especies. Además, estas bacterias y volátiles resultaron asociados con la intensidad de parasitismo y, en menor medida, con el éxito de vuelo. Esto sugiere la existencia de bacterias y/o volátiles claves, responsables de la detección y parasitación de nidos por parte de *C. hemapterus*, y que podrían ser utilizadas en aproximaciones experimentales futuras dirigidas a comprobar su papel en escenarios de parasismo.

CONCLUSIONS

1. A review of the topic allows us to conclude that the costs imposed by parasites and predators that detect their victims by olfactory cues would counteract possible benefits associated with the production of volatiles. Therefore, if the volatiles are of bacterial origin, parasites and predators could affect the symbiotic interactions between bacteria and their animal hosts.

2. Sterilization of the nest materials from previous reproductive events resulted in a reduced number of bacteria in the nests during nestling growth, and in a reduced number of *C. hemapterus* parasitizing hoopoe nestlings. We also detected the expected positive association between ectoparasitim intensity and bacterial loads in experimental nest. Therefore, the bacterial environment of the nest determines ectoparasitism in this species.

3. The sterilization of nest materials also reduced bacterial diversity and increase diversity of volatiles. These results suggest that the bacterial communities in the nests could produce chemicals that the ectoparasites could eavesdrop to locate and select active nests of their hosts.

4. At the inter and the intraspecific levels we found particular bacteria that associated with particular volatiles of the nest environment. At the interspecific level the strength and the sign of the expected association between bacterial and volatiles diversities varied between species. These results would suggest that the metabolism of bacteria of the nest materials is responsible for the nest odor.

5. We also found that the symbiont bacterial communities of the uropygial secretions of hoopoes are associated with the volatile profiles of the secretion and partly with the nest odor, which further support the expected relationship between bacteria and volatiles the avian nests. Therefore, the bacterial communities of the avian nests could influence the chemical cues that parasites use to detect the nests of their hosts.

6. The bacterial and/or volatile diversities of the nest environment are associated with the intensity of parasitism of *C. hemapterus*, either at the intraspecific (i.e. hoopoe) or at the interspecific level. Both the bacterial community and the volatile profile of the secretions of female and hoopoe nestlings predicted the intensity of parasitism by feather lice in females, and by *C. hemapterus* in nestlings. This suggests that the bacterial community is responsible of the volatiles detected in the nest environment, and that *C. hemapterus* eavesdrop to detect host nests.

7. Furthermore, particular volatiles of the nest environment and particular bacteria of the nest material, as well as volatiles and bacteria from secretions predicted the intensity of ectoparasitism of hoopoe females and nestlings, and the fledging success. Some aldehydes (i.e. pentanal, hexanal and heptanal) are negatively related to the intensity of parasitism in nestlings, but positively with fledging success, while some acids (i.e., acetic and butanoic) had the opposite effects. These results suggest that ectoparasites detect active nests using particular aldehydes, while acids could have a repellent effect, and that both kinds of chemicals affect the fledging success of nestlings.

8. In the interspecific study, we identified some key bacteria and volatiles that were related to each other and that varied between species. Furthermore, these bacteria and volatiles were also associated with intensity of parasitism and, at lower rate, with fledging success. These results point at the existence of key bacteria and/or volatiles, responsible for the detection and parasitization of nests by *C. hemapterus*. These key elements could be used in future experimental approaches aimed at verifying their role in scenarios of parasitism.

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