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A Comprehensive Study of the Avian Preen Wax' Adaptive Role in Feather Microbial Ecology

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A Comprehensive Study of the Avian Preen Wax' Adaptive Role in Feather Microbial Ecology

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par

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A comprehensive study of the avian preen wax' adaptive role in feather microbial ecology

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pour le Doyen
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Prof. Jean-Yves Roignant

Table of contents

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Abstract

Organisms employ diverse adaptive strategies to ensure their protection against environmental microorganisms. Birds, in addition to other strategies common to vertebrates, stand out with two specific integumentary adaptations: feathers and the preen gland. Feathers, crucial for flight, insulation, camouflage and communication, not only benefit from structural protection but are also coated with preen wax, an oily substance produced by the preen gland, and host communities of beneficial (yet potentially pathogenic) microorganisms known as *feather microbiota*. Considering the negative impact of insufficient feather protection, our study combined lipidomic and proteomic analyses and high-throughput 16S rRNA gene sequencing to investigate the role of both preen gland and feather microbiota in protecting barn owl (*Tyto alba*) plumage during reproduction. In the first two chapters, we explored the influence of individual (sex, body condition, plumage coloration) and environmental (weather conditions, brood size) factors on preen gland and feather microbiota. Our results revealed that incubating females secreted more preen wax and exhibited lower feather bacterial diversity compared to rearing females and males. Increasing preen wax secretion may have indirectly reduced feather bacterial diversity in incubating females. However, no direct association was observed between preen wax amount and feather bacterial diversity or composition in the second chapter. In the third chapter, we explored the influence of preen wax' lipid composition on feather microbiota, and contrary to the previous chapter, our results revealed a slight association between preen wax (lipid) composition and feather bacterial composition. This slight association prompted our exploration of preen wax' proteomic composition in the fourth chapter, whose results revealed the presence of immune-related peptides and proteins in barn owl preen wax. This thesis enhances our understanding of preen gland's complex functioning in birds. Not only does it partly support the protective function of preen gland but also suggests its involvement in other functions such as signaling and communication.

Résumé

Les organismes emploient diverses stratégies adaptatives pour se protéger contre les microorganismes environnementaux. Les oiseaux, en plus d'autres stratégies communes aux vertébrés, se distinguent par deux adaptations tégumentaires spécifiques : les plumes et la glande uropygienne. Les plumes, essentielles pour le vol, l'isolation thermique, le camouflage et la communication, bénéficient non seulement d'une protection structurelle mais sont également recouvertes de cire uropygienne, une substance huileuse produite par la glande uropygienne, et hébergent des communautés de microorganismes bénéfiques (mais aussi pathogènes) connues sous le nom de *microbiote des plumes*. Compte tenu de l'impact négatif d'une protection insuffisante du plumage sur les oiseaux, notre étude a combiné des analyses lipidomiques et protéomiques ainsi que du séquençage à haut débit de l'ARNr 16S pour étudier le rôle de la glande uropygienne et du microbiote des plumes dans la protection du plumage de l'Effraie des clochers (*Tyto alba*) pendant la reproduction. Dans les deux premiers chapitres, nous avons exploré l'influence de facteurs individuels (sexe, condition physique, coloration du plumage) et environnementaux (conditions météo, taille de la nichée) sur la glande uropygienne et le microbiote des plumes. Nos résultats ont révélé que les femelles incubatrices sécrétaient davantage de cire uropygienne et présentaient une diversité bactérienne du plumage moindre par rapport aux autres femelles et aux mâles. Cette sécrétion accrue de cire uropygienne pourrait avoir indirectement contribué à la réduction de la diversité bactérienne du plumage chez les femelles incubatrices. Cependant, nous n'avons pas observé d'association directe entre la quantité de cire uropygienne et la diversité ou la composition bactérienne du plumage dans le deuxième chapitre. Dans le troisième chapitre, nous avons exploré l'influence de la composition lipidique de la cire uropygienne sur le microbiote des plumes, et contrairement au chapitre précédent, nos résultats ont révélé une légère association entre la composition lipidique de la cire uropygienne et la composition bactérienne du plumage. Cette légère association nous a incités à explorer la composition protéomique de la cire uropygienne dans le quatrième chapitre, dont les résultats ont révélé la présence de peptides et protéines liés au système immunitaire dans la cire uropygienne de l'Effraie des clochers. Cette thèse améliore ainsi notre compréhension du fonctionnement complexe de la glande uropygienne chez les oiseaux. Elle soutient non seulement partiellement la fonction protectrice de la glande uropygienne, mais suggère également son implication dans d'autres fonctions telles que la communication.

General introduction

Organisms across taxonomic levels have developed diverse adaptive strategies, encompassing biological, physiological and behavioral mechanisms, to thrive in diverse environments, ensure their protection and enhance their fitness. Microorganisms, notably, stand out as key drivers in this adaptive process (McFall-Ngai *et al.*, 2013). Accordingly, vertebrates commonly employ avoidance behaviors to minimize exposure and protect themselves from pathogens, examples of which include reproductive site selection, social distancing, grooming behavior, or release of chemical defenses (Curtis, 2014; Hart & Hart, 2018; Sarabian *et al.*, 2018). In addition to avoidance behaviors, vertebrates also rely on integumentary barriers, *e.g.* scales, feathers, fur, hair, skin and mucous membranes, to prevent pathogens from entering and spreading within their bodies. Indeed, the entire integumentary system provides a range of antimicrobial mechanisms (Akat *et al.*, 2022; Riera Romo *et al.*, 2016; Wilson & Cotter, 2013).

Firstly, integumentary barriers consist of specialized molecules such as keratin and melanin forming resilient structures. Melanin not only contributes to pigmentation but also enhances the resilience of integumentary structures (Bonser, 1995, 1996; Moses *et al.*, 2006). Furthermore, melanocytes and melanosomes carry out enzymatic, phagocytic and antigen-presenting activities, as well as lysosome-related functions (Mackintosh, 2001). Adding to their multifaceted roles, the melanocortin receptor genes exhibit pleiotropic effects impacting the immune response through the *genetic link hypothesis* (Baeckens & Van Damme, 2018; Jacquin *et al.*, 2011). Secondly, integumentary barriers can produce and release antimicrobial secretions. Sweat and sebum, produced in mammalian skin, contain antimicrobial peptides such as dermcidin, lysozyme, lactoferrin, cathelicidins and defensins, create a slightly acidic environment and form a protective oily barrier against pathogens. Mucus, produced in bird and mammalian respiratory and digestive systems, and in gastropod, fish and amphibian skin, also contains antimicrobial peptides, forms a protective viscous barrier and traps/expels pathogens (Akat *et al.*, 2022). Similarly, preen wax, produced in the avian preen gland, is thought to function as sweat, sebum and mucus (J. Jacob & Ziswiler, 1982). Thirdly, the integumentary barriers host a community of beneficial (yet potentially pathogenic) microorganisms, referred to as *microbiota*. Mutualists and commensals help protect from pathogens through bacterial interference (Hooper *et al.*, 2012; Sassone-Corsi & Raffatellu, 2015). Bacterial interference consists in hindering the growth or establishment of pathogens through the production of antibiotics, alteration of the environment, competition for trophic resources and ecological niches, or stimulation of the immune system (Soler *et al.*, 2010). Pathogens can indeed lead to dysbioses, diseases, and even death. Although the integumentary system provides an effective range of antimicrobial mechanisms, it is not infallible. Despite its resilient

structures, antimicrobial secretions and protective microbiota, pathogens can still damage it and/or find entry points. In such cases, the immune system comes into play (Akat *et al.*, 2022; Riera Romo *et al.*, 2016; Wilson & Cotter, 2013).

Birds distinguish themselves from other vertebrates through specific integumentary adaptations requiring maintenance and protection: the feathers. Feathers stand as such integumentary adaptations serving essential functions such as flight, insulation, camouflage, protection, and communication. Feather protection firstly relies on their keratin-based structure and melanin-based coloration (Galván & Solano, 2016; Stettenheim, 2000). For instance, melanized feathers showed a greater resilience to wear and physical abrasion (Bonser, 1995, 1996), and melanized feathers and feather areas to feather-degrading microorganisms compared to unmelanized ones (Gunderson *et al.*, 2008; Justyn *et al.*, 2017; Ruiz-De-Castañeda *et al.*, 2012; though it is crucial to acknowledge potential experimental limitations in those studies). Melanized individuals also showed a lower susceptibility to parasites compared to their unmelanized counterparts (*genetic link hypothesis*; Jacquin *et al.*, 2011). Melanin's protective function, along with variations in protection among its different chemical forms (eumelanin and pheomelanin), presents an intriguing yet incompletely understood research area.

Secondly, feathers also receive protection from preen wax, an oily substance produced through another integumentary adaptation specific to birds: the preen gland. Preen wax mainly consists of lipids (*e.g.* triglycerides, mono- and diester waxes of fatty acids and alcohols), fatty acids, alcohols and hydrocarbons (Haribal *et al.*, 2005; J. Jacob & Ziswiler, 1982) thought to maintain feather integrity, enhance waterproofing, protect from pathogens, and contribute to visual/olfactory communication (Grieves *et al.*, 2022; Moreno-Rueda, 2017). Yet, preen wax' protective function has yielded controversial results in previous experimental studies. For instance, preen wax was found to inhibit a wide range of microorganisms *in vitro* (Alt *et al.*, 2020; Reneerkens *et al.*, 2008; Shawkey *et al.*, 2003). Blocking or removing the preen gland also led to feather damage in rock doves (*Columba livia*) and mallards (*Anas platyrhynchos*) *in vivo* (Giraudeau *et al.*, 2010; Moyer *et al.*, 2003), but did not clearly affect feather bacterial load in house sparrows (*Passer domesticus*) and mallards (Czirják *et al.*, 2013; Giraudeau *et al.*, 2013). In the same contrasting line, preen gland size was found to negatively correlate with feather-degrading bacterial load in barn swallows (*Hirundo rustica*; Møller *et al.*, 2009), but positively in house sparrows (Fülöp *et al.*, 2016). Preen wax' protective function, along with its mechanisms of action and chemical compounds, presents also a promising research area. At least four key mechanisms of action have been proposed to support the preen wax's protective function (Gunderson, 2008). At first, hydrophobic wax compounds may form a protective oily barrier on

eggs and feathers (S. Jacob *et al.*, 2018; Reneerkens *et al.*, 2008; Verea *et al.*, 2017). Lipidic wax compounds may also serve as energy stores promoting mutualists and commensals capable of competing with pathogens for trophic resources and ecological niches (Soler *et al.*, 2010). Alternatively, lipids, acids, alcohols, proteins and peptides, such as 3,7-dimethyloctan-1-ol, lysozymes and immunoglobulins Y, may exert a direct antimicrobial action against pathogens in certain avian species (Braun *et al.*, 2018; Carneiro *et al.*, 2020; J. Jacob *et al.*, 1997; Soini *et al.*, 2007). Bacteriocins and other antimicrobial substances originating from symbiotic bacteria living in preen gland have a similar antimicrobial action in Eurasian hoopoes (*Upupa epops*) and green woodhoopoes (*Phoeniculus purpureus*) only (Martín-Vivaldi *et al.*, 2009, 2010; Soler *et al.*, 2008, 2010).

Thirdly, feathers not only receive protection from preen wax but also host beneficial (yet potentially pathogenic) microorganisms, called *feather microbiota*. On the one hand, mutualists and commensals help protect feathers from pathogens through bacterial interference (Soler *et al.*, 2010). On the other hand, feather-degrading microorganisms can enzymatically break down feather β-keratin, compromising feathers' integrity and functions and adversely affecting birds' health and fitness (Burtt Jr. & Ichida, 1999; Gunderson, 2008). Identifying the intrinsic and extrinsic factors which positively or negatively influence feather microbial communities is then crucial. From an intrinsic perspective, both feather melanin and preen wax may shape feather microbiota (see above for mechanisms of action). Al Rubaiee *et al.* (2021) indeed showed a greater microbial abundance and diversity on melanized feather areas compared to unmelanized ones in white storks (*Ciconia ciconia*). S. Jacob *et al.* (2018) also found one group of preen wax chemicals to negatively correlate with feather bacterial richness in great tits (*Parus major*). From an extrinsic perspective, behavioral and physiological factors, such as preening behavior, daily activities/reproductive duties and sex hormone levels, may also influence microbial exposure and susceptibility, and hence shape feather microbiota. Several studies indeed showed a sex- and breeding stage-specific feather microbiota in pied flycatchers (*Ficedula hypoleuca*) and great tits (Goodenough *et al.*, 2017; Kilgas *et al.*, 2012; Saag, Mänd, *et al.*, 2011; Saag, Tilgar, *et al.*, 2011). Environmental factors, such as nest material, climate (ambient temperature and humidity) and brood size, may ultimately shape feather microbiota as they can provide favorable conditions for microbial growth, proliferation and transmission (Burtt Jr. & Ichida, 2004). Not only identifying, but also understanding how the intrinsic and extrinsic factors collectively influence feather microbial communities, is just as crucial.

This thesis coupled lipidomic and proteomic analyses and high-throughput 16S rRNA gene sequencing from field-collected samples to investigate whether and how a wide range of individual and environmental factors

– including preen gland traits – influences the barn owl's (*Tyto alba*) feather microbiota. Barn owl is a worldwide-distributed color-polymorphic raptor. In Switzerland, barn owls reproduce up to twice between March and August. Females lay about six eggs two or three days apart and incubate as soon as the first is laid, leading to a pronounced within-brood asynchrony. Barn owls exhibit variation in two melanin-based plumage traits. Ventral plumage varies in coloration from white to dark reddish-brown (pheomelanic trait) and in spottiness from immaculate to strongly marked with dark spots (eumelanic trait). These sexually dimorphic traits have already been linked to diverse morphological, physiological and behavioral phenotypes until then (summarized in Roulin, 2020). Barn owl stands out as an ideal model species for this thesis owing to its ecology/environment. During reproduction, females incubate the eggs for about 30 days and then care for the chicks for a few weeks in the nest, while males forage outside the nest. Chicks leave the nest at around 55-60 days old (Roulin, 2020). Barn owl nests, formed from prey cadavers, droppings and pellets, create favorable conditions for microbial growth, proliferation and transmission, making it essential for barn owls to have effective feather protection.

In order to investigate whether and how preen gland traits influence the barn owl's feather microbiota, we first studied how individual and environmental (climatic and social) factors influenced preen gland's morphology and physiology, as well as feather microbiota's diversity and composition through **Chapters 1 and 2**. In Chapter 1, we analyzed preen gland's morphology and physiology measured in fledglings and adults in the field. In Chapter 2, we analyzed breast, belly and back feathers collected from nestlings, fledglings and adults in the field using high-throughput 16S rRNA gene sequencing. We then combined individual, biometric, phenotypic, physiological and nest environment data with these preen gland- and feather microbiota-related data before conducting our statistical analyses. Chick and adult barn owls engage in age-specific daily activities exposing them differently to environmental microorganisms. Males and females engage in sex-specific breeding duties exposing males to environmental microorganisms and females to nest microorganisms. Chicks and adults, and males and females, also experience sex hormone variations throughout breeding. Additionally, fledgling and adult barn owls exhibit variation in melanin-based plumage traits affecting feather susceptibility to microbial degradation in response to ambient temperature, humidity and brood size (Roulin, 2020). Knowing this, we thus expected both individual and environmental factors to account for variation in preen gland and feather microbiota traits in the barn owl.

We then studied whether and how/through which mechanism(s) of action preen wax influences the barn owl's feather microbiota through **Chapter 3**. In Chapter 3, we selected fledglings and adults from which we analyzed body feathers using high-throughput 16S rRNA gene sequencing (Chapter 2) and preen wax using

Gas Chromatography-Mass Spectrometry (GC-MS), and on which we conducted two types of statistical analyses. Considering controversial results in previous experimental studies (Alt *et al.*, 2020; Czirják *et al.*, 2013; Fülöp *et al.*, 2016; Giraudeau *et al.*, 2010, 2013; Møller *et al.*, 2009; Moyer *et al.*, 2003; Reneerkens *et al.*, 2008; Shawkey *et al.*, 2003), preen wax may not only protect feathers from pathogens, but also maintain feather integrity, enhance waterproofing, and contribute to visual/olfactory communication at the same time (Grieves *et al.*, 2022; Moreno-Rueda, 2017). Knowing this, we expected preen wax' lipid composition to influence the barn owl's feather microbiota to a given extent. More specifically, we expected co-occurrences or co-exclusions between some lipid and microbial compounds (based on S. Jacob *et al.*, 2018 study). However, preen wax' other functions may also limit its importance in influencing feather microbiota.

Chapter 3 having nonetheless highlighted a slight association between preen wax (lipid) and feather microbiota, we finally studied the proteome as another possible mechanism of action not only for conferring but also for adjusting the preen wax' protective function to reproduction timing through **Chapter 4**. In Chapter 4, we analyzed preen wax collected from incubating and rearing female barn owls in the field using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). We identified/quantified the peptides and proteins detected, extracted the Gene Ontology (including Biological Process, Molecular Function and Cellular Component) of their related genes, and tested for their differential enrichment across breeding stages. Female barn owls engage in breeding duties, such as egg incubation and nestling rearing, exposing them constantly to nest microorganisms (Roulin, 2020). Reproductive females have thus developed adaptations to protect their eggs, offspring and themselves to this end (Clayton *et al.*, 2010). In light of Braun *et al.* (2018) and Carneiro *et al.* (2020), we proposed the proteome as a possible mechanism of action for conferring the preen wax' protective function, expecting to identify immune-related peptides and proteins in barn owl's preen wax. Additionally, we expected female barn owls to adjust preen wax proteome to reproduction timing and the selective pressures they experience at that time (*e.g.* preening needs, exposure to nest/environmental microorganisms).

In summary, this thesis coupled a large sample size with different analytical techniques, such as lipidomic and proteomic analyses, as well as high-throughput 16S rRNA gene sequencing, to investigate how preen gland and feather microbiota traits interplay – a little-explored research area – in the barn owl.

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Chapter 1

Individual and environmental factors influencing preen gland's morphology and physiology in the barn owl (*Tyto alba*)

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Author Contributions

L.A.: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. S.N.: Conceptualization, Writing – review & editing. L.M.S-J.: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. A.R.: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing – review & editing

Data Availability

Datasets are deposited to Zenodo at doi: 10.5281/zenodo.10619046

Abstract

Avian preen gland helps birds cope with their environment, although its overall functioning remains unclear. We shed light on the complexity of the preen gland's functioning by studying how multiple factors associate with gland morphology (size and shape) and physiology (wax secreted) in barn owls (*Tyto alba*). Individual factors (sex, breeding stage, body condition) were more important predictors of preen gland than environmental factors (temperature, humidity, brood size). Sex, depending on breeding stage in adults, influenced preen gland traits, pointing to the preen gland's regulation by sex hormones and a greater pressure on females to protect their eggs, offspring and themselves throughout reproduction. Adults and fledglings in better condition had larger glands, pointing to the existence of physiological costs. Temperature and humidity, in interaction with plumage coloration, also influenced but to a lesser extent preen gland traits, suggesting that melanin pigmentation and preen gland act as superseding mechanisms when protecting plumage against microorganisms. Finally, fledglings living in larger broods had larger glands, suggesting a role for the social environment in preen gland's functioning. Overall, our study supports the idea that preen gland functions in diverse biological contexts within the same species and is thus subject to multiple selective pressures.

Keywords: air temperature, body condition, brood size, melanin-based plumage traits, preen wax, relative humidity

Introduction

Organisms across taxonomic levels have evolved diverse behavioral, morphological and physiological strategies to adapt to their environment and maximize fitness. Integuments and integumentary appendages, as primary interfaces with the environment, play key roles in many of these strategies. For instance, they offer mechanical support and protection, and aid in metabolic regulation, sensory perception and communication (Bereiter-Hahn *et al.*, 1984, 1986). Similarly to other taxa, birds have developed such behavioral, morphological and physiological strategies. Birds are covered with feathers whose performance in flight, protection and communication depends in particular on morphological (structure, pigmentation), physiological (secretion; Clayton *et al.*, 2010) and environmental aspects (weather conditions, microorganisms). Bird feathers are also regularly preened, *i.e.* coated with preen wax, a substance produced by the preen gland (J. Jacob & Ziswiler, 1982; Moreno-Rueda, 2017). Preen gland traits are associated with a wide range of factors, reflecting the diverse biological functions the preen gland may fulfill. For instance, preen gland size and composition are usually associated with individual (*e.g.* sex, age, body size and condition; Díez-Fernández *et al.*, 2021; Grieves *et al.*, 2019; Moreno-Rueda, 2010; Whittaker & Hagelin, 2021) and environmental factors (*e.g.* breeding season, habitat; Golüke & Caspers, 2017; Vincze *et al.*, 2013), helping protect and waterproof feathers or facilitating visual and olfactory communication (reviewed in Grieves *et al.*, 2022; Moreno-Rueda, 2017). However, little is known about the relative importance of each of these factors on preen gland traits.

To date, only certain functions attributed to the preen gland have been examined (Grieves *et al.*, 2022; Moreno-Rueda, 2017). The *antiparasitic defense hypothesis* was initially tested by removing or blocking the preen gland of rock doves and mallards (*Columba livia* in Moyer *et al.*, 2003; *Anas platyrhynchos* in Giraudeau *et al.*, 2010), leading to feather damage due to mechanical abrasion or bacterial degradation (but see Czirják *et al.*, 2013; Giraudeau *et al.*, 2013). In the same line, other experimental studies tested the *in vitro* antimicrobial activity of preen wax against a wide range of bacterial isolates (Shawkey *et al.*, 2003). Both findings support the idea that the preen gland may help protect the eggs, offspring and parental plumage from life-threatening pathogens and parasites (*e.g.* eggshell or feather-degrading bacteria; Moreno-Rueda, 2017) in the wild. Preen wax reportedly forms a protective barrier on eggs and feathers (S. Jacob *et al.*, 2018; Reneerkens *et al.*, 2008; Verea *et al.*, 2017) or produces antimicrobial compounds (Braun *et al.*, 2018; Carneiro *et al.*, 2020; Martín-Vivaldi *et al.*, 2010; Soler *et al.*, 2008, 2010). Yet, not all studies testing this hypothesis – especially *in vivo* studies – have confirmed it (Czirják *et al.*, 2013; Giraudeau *et al.*, 2013).

In addition to the *antiparasitic defense hypothesis*, Grieves *et al.* (2022) posited two odor-based hypotheses to better understand the frequent sexual dimorphism in preen gland traits (reviewed in Whittaker & Hagelin, 2021). The *olfactory crypsis hypothesis* proposes that the incubating sex(es) of ground-nesting species might alter the preen wax composition during breeding – thereby reducing its scent – to protect their brood and themselves from olfactorily searching nest predators (Reneerkens *et al.*, 2002, 2005). The *sex semiochemical hypothesis* states that this change in preen wax composition might also allow for olfactory recognition and communication among conspecifics or mates (Caro *et al.*, 2015; Whittaker & Hagelin, 2021). Gaining a better understanding of how individual and environmental factors are associated with each preen gland trait would help understand the biological functions and general functioning of avian preen gland.

Our study aimed to shed light on the complexity of preen gland's functioning in birds. We used adult and fledgling barn owls (*Tyto alba*) to study how individual and environmental (climatic and social) factors are associated with morphological (here, preen gland size and shape) and physiological (here, preen wax secretion) aspects of the preen gland, and to assess the relative importance of such associations. Unlike previous studies in the field, we decided to differentiate these two aspects for biologically relevant reasons (see *Discussion*). We measured preen gland and sampled preen wax from individuals studied throughout the breeding process. We also collected other biometric and phenotypic data (body size and condition, plumage color traits), as well as nest-related environmental data (weather conditions, brood size). While we predicted associations of preen gland traits with different predictor types, we could not anticipate their strength and importance. We first expected sex (depending on breeding stage) to be the main predictor of preen gland traits. Sex-specific differences might reflect both behavioral and hormonal variations between sexes (Whelan *et al.*, 2010; Whittaker *et al.*, 2011). Barn owl pairs indeed share reproductive duties, with females caring for the eggs, offspring and themselves in constant contact with nest microorganisms (selective pressure) while males engage in outdoor activities. We expected body condition to be another important predictor of preen gland traits. Since preen gland's functioning seems to be costly to some extent (Magallanes *et al.*, 2016; Moreno-Rueda, 2015; Piault *et al.*, 2008), birds in poorer condition may potentially be unable to afford the energy or nutrient resources demanded for its proper functioning. We finally expected weather conditions (temperature and humidity) depending on plumage color traits to predict preen gland traits. Melanin pigments can strengthen and help protect plumage from microbial degradation (Bonser, 1995; Ruiz-De-Castañeda *et al.*, 2012); the protective roles of both melanin pigmentation and preen wax against environmental microorganisms were thus suggested to supersede each other in warmer and more humid environments. Such interaction should also play a role because the Gloger's rule predicts that

animals in warmer and more humid environments tend to have darker pigmentation than those in colder and drier ones (Delhey, 2017, 2019).

Materials & Methods

Study area and barn owl monitoring

Our study was conducted from March to November 2004-2021 on a wild population of barn owls breeding in Western Switzerland (see Frey *et al.*, 2011 for a detailed description of the study area). We monitored adults from egg incubation to nestling rearing, whereas nestlings were followed until approx. 55 days of age (*i.e.* fledging stage). All birds captured for the first time were fitted with a uniquely numbered ring for identification. The age of nestlings was calculated based on their left-wing length upon ringing (Roulin, 2004b), while that of adults on the basis of their molt pattern unless ringed as nestlings (Taylor, 1993). Adults were classified as first-year adults if they lacked molt or multiple generations of feathers, as barn owls typically do no molt during their first reproductive year (Roulin, 2020); otherwise, they were classified as older adults. The sex of birds was determined from a blood sample using sex-specific molecular markers (Py *et al.*, 2006). Biometric, physiological and phenotypic data (see below for details) were collected on each monitored bird.

Preen gland morphology

At each field visit, we measured the length, width and height of the preen gland of adult (since 2004; N = 2743 observations from 1226 individuals) and fledgling (since 2007; N = 2837 individuals measured once) barn owls using a caliper to the nearest 0.1 mm (Fig. 1). We estimated the inter-observer repeatability by comparing the three preen gland measurements taken by two to four different observers on 64 barn owls in 2021 (N = 178 measurements on 17 adults and 47 nestlings/fledglings) according to Lessells & Boag (1987) and Nakagawa & Schielzeth (2010). Preen gland measurements showed significant repeatability among observers (gland length: R (SE) = 0.81 (0.04), $F_{70,107}$ = 11.76, P < 0.01; gland width: R (SE) = 0.56 (0.07), $F_{70,107}$ = 4.17, P < 0.01; gland height: R (SE) = 0.86 (0.03), $F_{70,107}$ = 16.32, P < 0.01). We reduced the three preen gland measurements by conducting principal component analyses (PCAs; *princomp* function, *stats* R package) on adults and fledglings separately to differentiate gland size from gland shape. The first two principal components explained 81.1 % (in adults) and 83.2 % (in fledglings) of the cumulative variance (Suppl. Table 1). For both adults and fledglings, PC1 was considered as a proxy for gland size given that preen gland length, width and height had similar positive loadings on PC1. PC2 was considered as a proxy for gland shape given that preen gland width and height were respectively positively and negatively correlated with PC2.

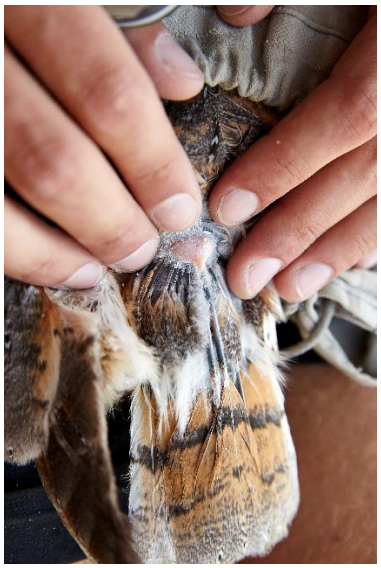

Figure 1. Preen gland in a barn owl. (Credit: Jeremy Bierer)

Preen wax collection

We sampled preen wax on a subset of adult (*N* = 472 observations from 261 individuals) and fledgling (*N* = 400 individuals measured once) barn owls captured between 2018 and 2021. Wearing clean gloves, we lightly pressed on the preen gland with the thumb until emptying it. Preen wax was directly discharged into 50-µL glass capillaries (Brand GmbH & Co. KG, Wertheim, DE). Immediately after sampling, we measured the amount of preen wax collected in the capillary(ies) using a caliper to the nearest 0.1 mm, then converted it into volume (*range*, *mean* ± *standard error* [*SE*]: from 0.0 to 150.0 µL, 28.5 ± 1.4 µL in adults; from 0.0 to 45.7 µL, 10.3 ± 0.4 µL in fledglings). We considered the amount of preen wax obtained to reflect the total amount present in the preen gland at the time of sampling.

Previous studies mainly focused on gland morphology (calculated as the product of the three measurements; Galván & Sanz, 2006) as a proxy for its secretory activity owing to an existing positive correlation with gland physiology (Martín-Vivaldi *et al.*, 2009; Møller *et al.*, 2009; Pap *et al.*, 2010). In our study, we detected no correlation between preen gland volume and the amount of preen wax secreted in adults (Spearman's rank correlation test: $N = 462$, $\rho = 0.02$, $P = 0.71$), and a significant albeit weak correlation in fledglings (Spearman's rank correlation test: $N = 400$, $\rho = 0.14$, $P < 0.01$). We therefore decided to consider preen gland size, shape and preen wax amount secreted independently, assuming that these parameters may have different biological meanings.

Biometric data

We measured the left-wing length using a metallic ruler to the nearest 1 mm, the left-tarsus length using a caliper to the nearest 0.1 mm, and we weighed adults and fledglings using a dynamometer to the nearest 5 g. Tarsus length is known to provide a reliable predictor of bird size, and the residuals from (sex-specific in adults) regressions of body mass on left-tarsus length were computed as an index of body condition (Green, 2001; Schulte-Hostedde *et al.*, 2005).

Plumage color traits

Barn owl ventral body parts differ in the expression of two melanin-based plumage traits which were assessed once a year on the breast of adults and fledglings. Pheomelanin-based coloration varies from white to reddish-brown, and eumelanin-based spottiness from immaculate to strongly marked with dark spots of varying size (Fig. 2; Roulin, 2020). In the field, we visually scored the plumage coloration using a scale from -8 for white to -1 for dark-reddish body parts, a method known to strongly correlate with spectrophotometric reflectance measurements in the brown part of the visible spectrum (Pearson's correlation test: $N = 1107$, $r = -0.78$, $P < 0.0001$; see Dreiss & Roulin, 2010 for details). We then measured the diameter of ten representative dark spots at the feather tips within a 60 x 40 mm-frame using a caliper to the nearest 0.1 mm (Roulin, 2004a).

Figure 2. Barn owl ventral body parts differ in the expression of two melanin-based plumage traits. Pheomelanin-based coloration varies from white to reddish-brown, and eumelanin-based spottiness from immaculate to strongly marked with dark spots of varying size. (Credit: Paul Béziers)

Nest environment

We counted the number of nestlings and/or fledglings present in the nest as the brood size (*range*: from 0 to 10 chicks). We extracted air temperature (°C) and relative humidity (%) data recorded at a ten-minute granularity from the climate database *IDAweb* provided by the Federal Office of Meteorology and Climatology MeteoSwiss (*Swiss Meteorological Institute IDAweb*). We calculated air temperature and relative humidity averages 24 hours before each sampling time from the MeteoSwiss weather station nearest to each occupied nest (between 400 and 800 m alt., up to 15 km distance). In total, we considered 21 MeteoSwiss weather stations recording temperature data to which between 1 and 386 broods were assigned (*mean*: 66.7 broods; *median*: 34 broods), and 20 MeteoSwiss weather stations recording humidity data to which between 1 and 484 broods were assigned (*mean*: 69.5 broods; *median*: 23.5 broods). Some broods had to be associated with different weather stations within the same year since not all of them recorded data continuously throughout the year (177 broods for temperature data, 166 broods for humidity data). Average distance between a nest and a weather station was 6636 m for temperature data, and 7627 m for humidity data.

Ethics

Barn owl monitoring was performed according to a strict animal handling protocol approved by the 'Service de la consommation et des affaires vétérinaires', Switzerland (authorization numbers: VD 3213, 3462 and 3571).

Statistical analyses

All statistical analyses were performed using R Statistical Software (R version 4.1.0; R Core Team, Vienna, Austria). In order to study how predictor types contributed to explaining preen gland size (PC1), shape (PC2) and preen wax amount (log-transformed) in barn owls, we used an information-theoretic approach (Burnham *et al.*, 2011; Grueber *et al.*, 2011; Symonds & Moussalli, 2011). We first built a set of candidate linear mixed-effects models per preen gland trait per age class (*lmer* function, *lme4* R package; Bates *et al.*, 2015) from any combination of our predictors of interest. We created separate models for adults and fledglings due to the considerable differences in morphology, physiology and behavior, including in preen gland traits (LMM for PC: N = 5580 observations from 3827 individuals, $F_{1,3690}$ = 883.15 for PC1, $F_{1,3303}$ = 391.24 for PC2, all $P < 0.01$; LMM for wax amount: N = 872 observations from 645 individuals, $F_{1,592}$ = 112.08, $P < 0.01$), between those age classes. We included the age (class) and sex in each saturated model, and the breeding stage (egg incubation *vs* nestling rearing) in adult models only. Sex was also considered in

interaction with the breeding stage to account for potential sex-specific changes throughout the breeding season. We included the body size and condition as biometric indicators, and the plumage color score (pheomelanin-based trait) and spot diameter (eumelanin-based trait) as phenotypic indicators. We included the (Julian) sampling date, the temperature and humidity averages 24 hours before sampling, and the brood size at the time of sampling to take into account the socio-environmental context. Two-way interactions between each of the two plumage color traits and each of the two environmental variables were considered, as well as sex in interaction with the brood size in adult models only. Sampling year (4 levels) was added as a categorical predictor in preen wax models to account for the interannual variability in collecting data in the field. Bird ID (in adult models), clutch ID (in fledgling models) and sampling year (in PC models) were finally added as random factors to control for repeated measurements taken on the same individuals, individuals sharing the same nest and between-year differences, respectively. All numeric (mean = 0, *SD* = 0.5) and binary predictors (mean = 0, difference = 1) were previously standardized (*standardize* function, *arm* R package; Gelman, 2011). We checked for the assumptions of collinearity of saturated linear mixed-effects models by computing the variance inflation factor (VIF) among predictors, as well as of normality, linearity and homoscedasticity of residuals by plot diagnosis. We also calculated the Cook's distance to detect any influential observations (all Cook's distances < 1; Quinn & Keough, 2002). Due to a too high collinearity between the breeding stage and brood size in adult models (VIF ≥ 3 in PC models, VIF ≥ 7 in preen wax model), we decided to remove the brood size and its interaction with sex in the simplified linear mixed-effects models.

We then compared each candidate linear mixed-effects model using the Akaike Information Criterion (AIC) corrected for small sample size (rank = AICc, *dredge* function, *MuMIn* R package; Barton, 2022) to identify those that best explained variation in gland morphology and physiology. We considered only candidate models having a *ΔAICc* ≤ 2 as the *confidence set* of best models and calculated the evidence ratio and (accumulative) model weights within it (Burnham *et al.*, 2011; Grueber *et al.*, 2011; Symonds & Moussalli, 2011). We finally averaged the *confidence set* using the full-model averaging method (*model.avg* function, *MuMIn* R package; Barton, 2022) to correct for model selection uncertainty and obtain standardized predictor estimates. To discern the relative importance of each predictor, we combined information on a predictor's effect size (standardized estimates from the full-model averaging method), summed weights (sum of Akaike model weights in which a predictor appeared within all candidate models and within the *confidence set* weighed by the full-model averaging method) and the number of consecutive candidate models (ranked by their AICc) in which a predictor was included. Finally, we applied pairwise comparisons

on the best model from the *confidence set* to assess differences between predictor levels (*i.e.* age class, sampling year, sex × breeding stage, plumage color traits × environmental variables), and used the Benjamini-Hochberg procedure for correction (Benjamini & Hochberg, 1995).

Results

In adult barn owls, the resulting *confidence set* of best models included two models for gland size, 19 for gland shape and five for wax amount (Suppl. Tables 2 & 4). Twelve predictors were found in each best model on gland size, four on gland shape, and six on wax amount (Suppl. Tables 3 & 5). In fledglings, the *confidence set* included five models for gland size, 11 for gland shape and seven for wax amount (Suppl. Tables 6 & 8). Seven predictors were found in each best model on gland size, and three on both gland shape and wax amount (Suppl. Tables 7 & 9).

Sex

Sex was the only predictor present in each of the best models. In adults, sex was the second most important predictor of gland size and wax amount, and the most important predictor of gland shape (Suppl. Tables 3 & 5). On average, females had a larger and higher-than-wide preen gland, and they secreted more preen wax compared to males (Fig. 3), although these effects may change when considering the breeding stage (see below). In fledglings, sex was the second most important predictor of gland size, the most important predictor of gland shape, and the third and last most important predictor of wax amount (Suppl. Tables 7 & 9). As in adults, females had a larger preen gland and secreted more preen wax compared to males, but they had a wider-than-high preen gland (Fig. 4).

Breeding stage, and interaction between sex and breeding stage

In adults, breeding stage (egg incubation *vs* nestling rearing) was the most influential predictor of gland size and wax amount, and the fourth and last most influential predictor of gland shape (Suppl. Tables 3 & 5). On average, the preen gland was larger and wider-than-high during nestling rearing, whereas more preen wax was secreted during egg incubation (Fig. 3). When considering both sex and breeding stage, the interaction was the third most influential predictor of gland size and the fifth most influential predictor of wax amount but did not explain gland shape (Suppl. Tables 3 & 5). Rearing females had a significantly larger preen gland compared to incubating females (*est.* ± *SE*: 0.92 ± 0.07, t_{2508} = 12.48, P < 0.01) and to males at any stage (*est.* ± *SE*: 0.90 ± 0.10, t_{2483} = 8.81, P < 0.01 for egg incubation; *est.* ± *SE*: 0.89 ± 0.08, t_{1783} = 11.27, P < 0.01 for nestling rearing). Incubating females showed no significant difference in preen gland size with males at any stage (*est.* ± *SE*: -0.02 ± 0.09, t_{2218} = -0.24, P = 0.93 for egg incubation; *est.* ± *SE*: -0.03 ± 0.08, t_{1861} = -0.35, P = 0.93 for nestling rearing). Males showed no significant difference in preen gland size between egg incubation and nestling rearing (*est.* ± *SE*: -0.01 ± 0.09, t_{2701} = -0.09, P = 0.93; Fig. 5a). Also, incubating females secreted significantly more preen wax than rearing females (*est.* ± *SE*: 0.93 ± 0.07, t_{417} = 13.42, P < 0.01) and

than males at any stage (*est.* ± *SE*: 0.64 ± 0.09, t_{418} = 7.47, P < 0.01 for egg incubation; *est.* ± *SE*: 0.98 ± 0.08, t_{357} = 12.00, P < 0.01 for nestling rearing). Rearing females and rearing males secreted significantly less preen wax than males during egg incubation (*est.* ± *SE*: -0.29 ± 0.10, t_{433} = -3.01, P < 0.01 for females; *est.* ± *SE*: -0.33 ± 0.10, t_{462} = -3.28, P < 0.01 for males). No significant difference was shown in preen wax amount between rearing females and rearing males (*est.* ± *SE*: 0.05 ± 0.08, t_{351} = 0.58, P = 0.56; Fig. 5b).

Age

Age class in adults (first-year, presumed first-year *vs* older) was the twelfth and last best predictor of gland size but explained neither gland shape nor wax amount (Suppl. Tables 3 & 5). First-year adults had a significantly larger preen gland than older adults (*est.* ± *SE*: 0.18 ± 0.06, t_{2306} = 2.88, P = 0.01). First-year adults showed no significant difference in preen gland size with presumed first-year adults (*est.* ± *SE*: 0.08 ± 0.10, t_{1931} = 0.83, P = 0.41), and presumed first-year adults with older adults (*est.* ± *SE*: 0.10 ± 0.09, t_{1808} = 1.12, P = 0.39; Fig. 3). Age in fledglings was the fourth best predictor of gland size but explained neither gland shape nor wax amount (Suppl. Tables 7 & 9). Older fledglings had a larger preen gland (Fig. 4).

Body size

In adults, body size was the fifth most important predictor of gland size but was neither an important predictor of gland shape nor wax amount (Suppl. Tables 3 & 5). Larger adults had a larger preen gland (Fig. 3). In fledglings, body size was the third most important predictor of gland size, the second most important predictor of gland shape, and the most important predictor of wax amount (Suppl. Tables 7 & 9). Larger fledglings had a larger and higher-than-wide preen gland, and they secreted more preen wax (Fig. 4).

Body condition

In adults, body condition was the fourth most influential predictor of gland size, the second most influential predictor of gland shape but was not an influential predictor of wax amount (Suppl. Tables 3 & 5). Adults in better condition had a larger and higher-than-wide preen gland (Fig. 3). In fledglings, body condition was the most influential predictor of gland size but was neither an influential predictor of gland shape nor wax amount (Suppl. Tables 7 & 9). Similarly to adults, fledglings in better condition had a larger preen gland (Fig. 4).

Sampling date

In adults, (Julian) sampling date was the tenth best predictor of gland size and the fourth best predictor of wax amount. Sampling date did not explain gland shape (Suppl. Tables 3 & 5). Preen gland size and wax

amount secreted increased as the breeding season progressed (Fig. 3). In fledglings, (Julian) sampling date was the fifth best predictor of gland size, the third and last best predictor of gland shape but did not explain wax amount (Suppl. Tables 7 & 9). Preen gland increased in size and in width at the expense of height over the breeding season (Fig. 4).

Sampling year

Sampling year – only included in preen wax models – was the third most important predictor of wax amount in adults (Suppl. Table 5) but was not an important predictor in fledglings (Suppl. Table 9). We sampled significantly more preen wax each year compared to 2018 (*est.* ± *SE*: 0.26 ± 0.09, t_{450} = 3.06, P < 0.01 for 2019; *est.* ± *SE*: 0.55 ± 0.09, t_{395} = 6.33, P < 0.01 for 2020; *est.* ± *SE*: 0.56 ± 0.09, t_{422} = 6.07, P < 0.01 for 2021), and each year (except 2018) compared to 2019 (*est.* ± *SE*: 0.29 ± 0.07, t_{388} = 3.92, P < 0.01 for 2020; *est.* ± *SE*: 0.30 ± 0.08, t_{416} = 3.71, P < 0.01 for 2021). No significant difference was shown in preen wax amount between 2020 and 2021 in adults (*est.* ± *SE*: < -0.01 ± 0.07, t_{442} = -0.07, P = 0.95; Fig. 3).

Plumage color traits

In adults, plumage coloration (pheomelanin-based trait) was the sixth most influential predictor of gland size but explained neither gland shape nor wax amount (Suppl. Tables 3 & 5). On average, darker red adults had a larger preen gland (Fig. 3). In fledglings, plumage coloration explained neither gland size, gland shape nor wax amount (Suppl. Tables 7 & 9). In adults, plumage spottiness (eumelanin-based trait) explained neither gland size, gland shape nor wax amount (Suppl. Tables 3 & 5). In fledglings, plumage spottiness was the seventh and last most influential predictor of gland size but explained neither gland shape nor wax amount (Suppl. Tables 7 & 9). On average, spottier fledglings had a larger preen gland (Fig. 4). Plumage color effects were found to be dependent on weather conditions (see below).

Weather conditions

In adults, humidity was the seventh best predictor of gland size, the third best predictor of gland shape, and the sixth and last best predictor of wax amount (Suppl. Tables 3 & 5) but was not among the best predictors of gland size, gland shape nor wax amount in fledglings (Suppl. Tables 7 & 9). On average, the adult gland size increased, gland width was favored over gland height, and wax amount decreased with humidity (Fig. 3). In adults, temperature was the eighth best predictor of gland size but explained neither gland shape nor wax amount (Suppl. Tables 3 & 5). On average, the adult gland size decreased with temperature (Fig. 3). In fledglings, temperature explained neither gland size nor gland shape but was the second-best predictor of

wax amount (Suppl. Tables 7 & 9). On average, the fledgling wax amount increased with temperature (Fig. 4). Weather condition effects were found to change depending on plumage color traits (see below).

Interactions between plumage color traits and weather conditions

In adults, the interaction between plumage coloration and humidity was the eleventh most important predictor of gland size but was neither an important predictor of gland shape nor wax amount (Suppl. Tables 3 & 5). At low humidity, darker red adults had a significantly larger preen gland than paler adults (*est.* ± *SE*: 0.48 ± 0.15, t_{2712} = 3.25, P < 0.01), while a significantly smaller preen gland at high humidity (*est.* ± *SE*: -0.25 ± 0.12, t_{2717} = -2.09, P = 0.04). From low to high humidity, preen gland size increased significantly in paler adults (*est.* ± *SE*: 0.39 ± 0.10, t_{2441} = 4.04, P < 0.01), while showed no significant difference in darker red adults (*est.* ± *SE*: -0.11 ± 0.09, t_{2573} = -1.23, P = 0.22). In adults, the interaction between plumage coloration and temperature was the ninth most important predictor of gland size but was neither an important predictor of gland shape nor wax amount (Suppl. Tables 3 & 5). At low temperature, darker red adults had a significantly larger preen gland than paler adults (*est.* ± *SE*: 0.66 ± 0.17, t_{2710} = 3.98, P < 0.01), while a significantly smaller preen gland at high temperature (*est.* ± *SE*: -0.44 ± 0.14, t_{2717} = -3.09, P < 0.01). From low to high temperature, preen gland size increased significantly in paler adults (*est.* ± *SE*: 0.32 ± 0.11, t_{2579} = 3.02, P < 0.01), while decreased significantly in darker red adults (*est.* ± *SE*: -0.33 ± 0.10, t_{2589} = -3.27, P < 0.01). None of these interactions explained gland size, gland shape nor wax amount in fledglings (Suppl. Tables 7 & 9). None of the interactions between plumage spottiness and humidity, and between plumage spottiness and temperature, explained gland size, gland shape nor wax amount in adults (Suppl. Tables 3 & 5) and fledglings (Suppl. Tables 7 & 9).

Brood size

In fledglings, brood size was the sixth most influential predictor of gland size but explained neither gland shape nor wax amount (Suppl. Tables 7 & 9). Fledglings living in larger broods had a larger preen gland (Fig. 4).

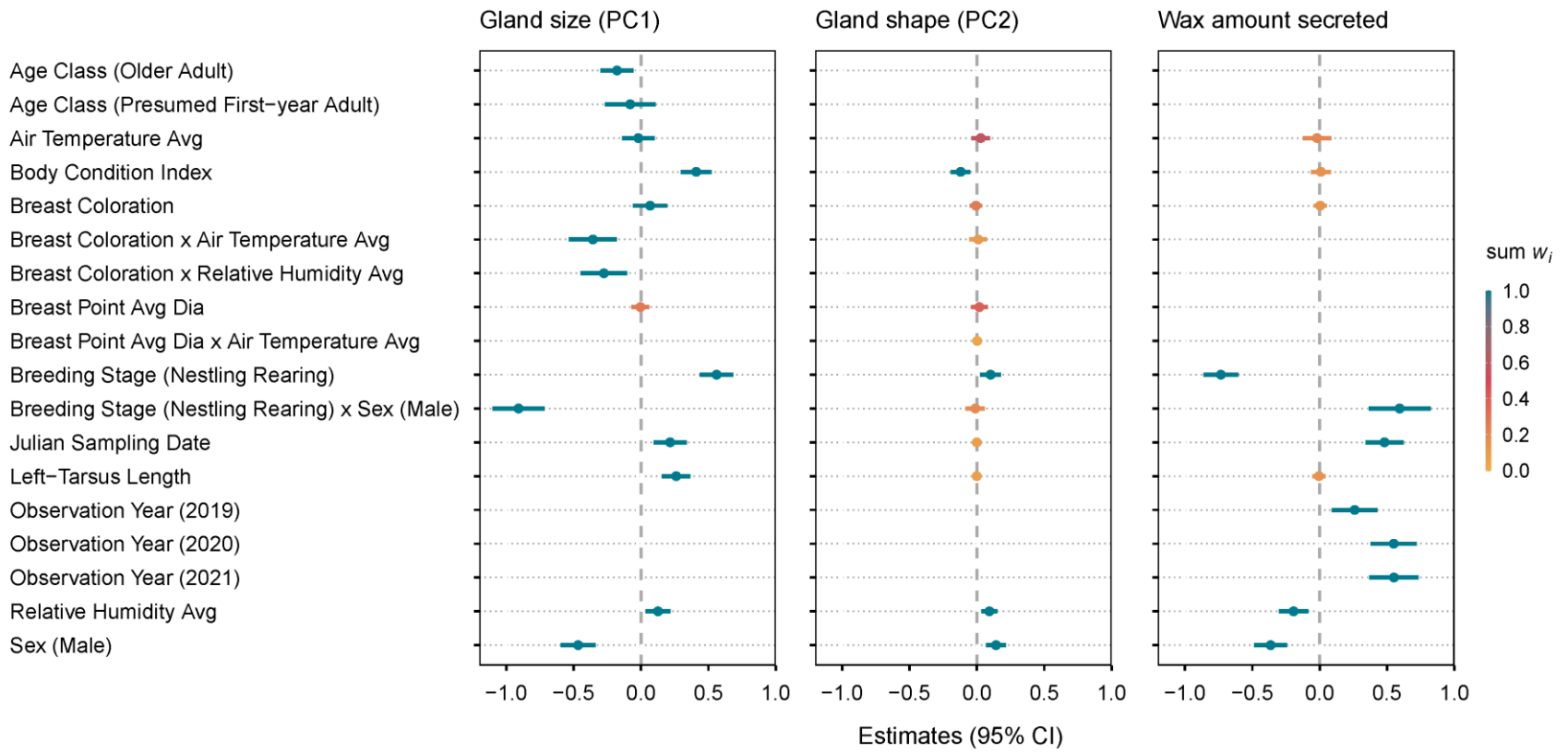

Figure 3. Standardized predictor estimates and 95 % confidence intervals from the full-model averaging method for preen gland size, shape and preen wax amount in adult barn owls. Predictors are colored according to their summed weights (sum of Akaike model weights in which a predictor appeared within the *confidence set* of best models [models with $\Delta AICc \leq 2$]). Air Temperature Avg = Air Temperature Average; Breast Point Avg Dia = Breast Point Average Diameter; Relative Humidity Avg = Relative Humidity Average.

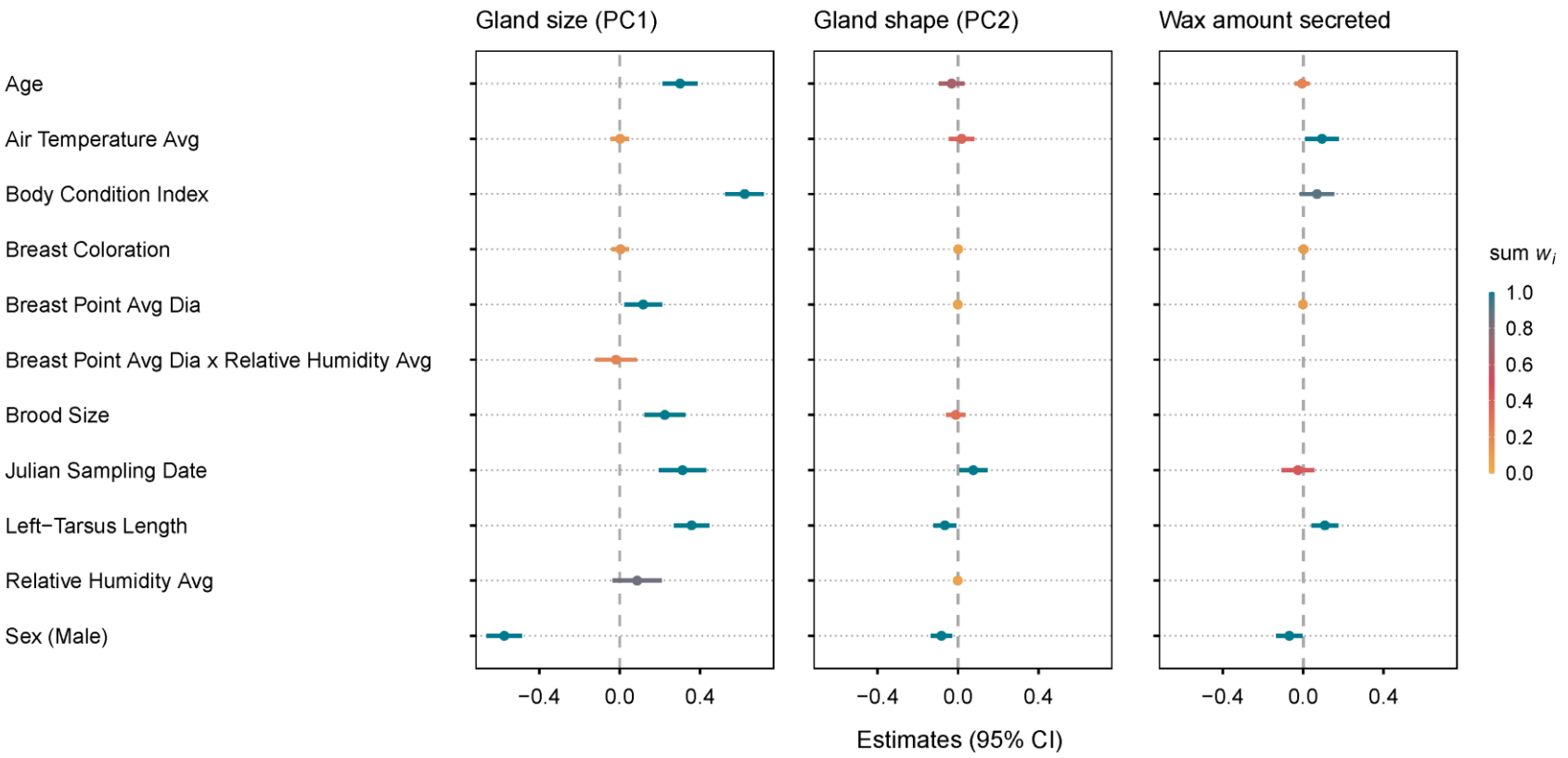

Figure 4. Standardized predictor estimates and 95 % confidence intervals from the full-model averaging method for preen gland size, shape and preen wax amount in fledgling barn owls. Predictors are colored according to their summed weights (sum of Akaike model weights in which a predictor appeared within the *confidence set* of best models [models with $\Delta AICc \leq 2$]). Air Temperature Avg = Air Temperature Average; Breast Point Avg Dia = Breast Point Average Diameter; Relative Humidity Avg = Relative Humidity Average.

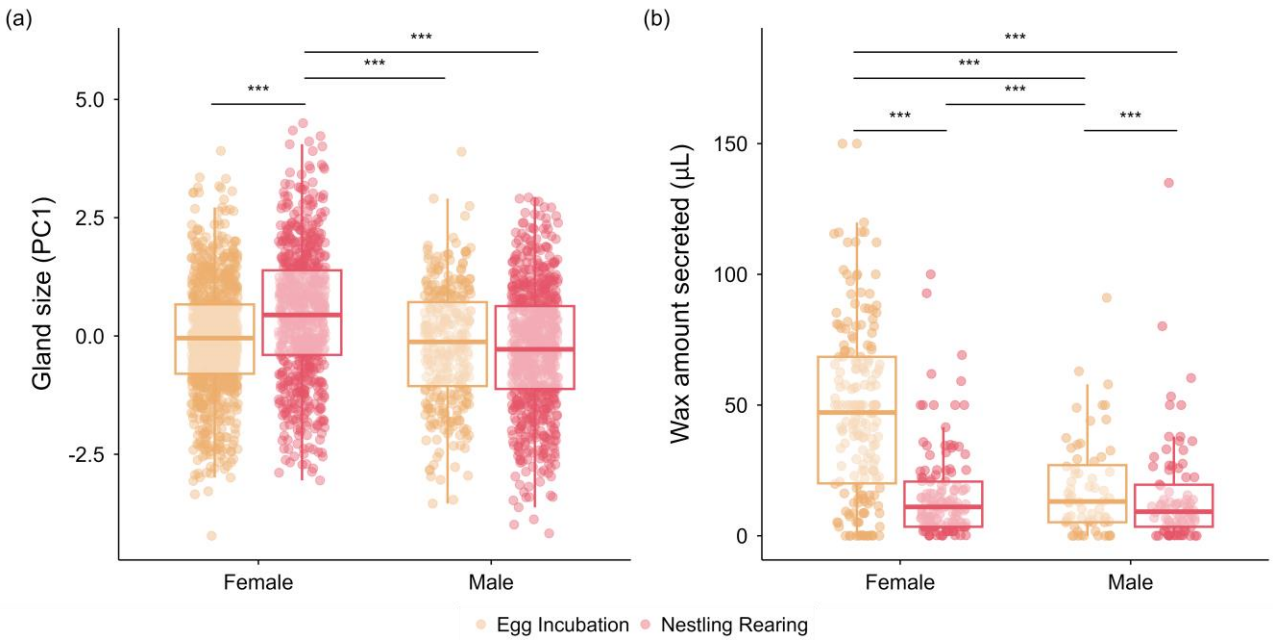

Figure 5. Boxplots showing variation in (a) preen gland size and (b) preen wax amount secreted between sexes depending on breeding stage in adult barn owls. Each dot represents a sample and colors denote breeding stage (egg incubation *vs* nestling rearing). Significance (*) was determined by statistical analyses.

Discussion

Prior research has proposed a wide range of biological functions for the preen gland in birds (*e.g.* feather maintenance and protection, waterproofing, visual and olfactory communication; Grieves *et al.*, 2022; Moreno-Rueda, 2017). Studying how different predictor types are associated with morphological and physiological aspects of the preen gland enabled us to highlight that both individual and environmental (climatic and social) factors influence preen gland traits. Assessing the relative importance of those associations sheds light on the complexity of preen gland's functioning in the barn owl.

Previous studies mainly focused on gland morphology (here, preen gland volume) owing to an existing positive correlation with gland physiology, *i.e.* the secretion of preen wax (Martín-Vivaldi *et al.*, 2009; Møller *et al.*, 2009; Pap *et al.*, 2010). Although the strength of such correlation is rarely reported, evidence to date suggests it might be of small magnitude (*e.g.* Pearson's correlation coefficient: $r < 0.1$; Møller *et al.*, 2009). Our study strongly highlighted that gland morphology and physiology deserve independent attention. On the one hand, we detected no (or a weak) correlation between preen gland volume and the amount of preen wax secreted in adult and fledgling barn owls despite a large sample size and thus a strong statistical power (see *Materials & Methods*). On the other hand, we observed gland morphology (here, preen gland size and shape) and physiology to be associated with different predictor types, and even when associated with the same predictors, the sign of associations can differ between preen gland size, shape and preen wax amount. For instance, body condition had an important effect on preen gland size but little or no effect on preen gland shape and wax amount (Fig. 3-4). In the same line, incubating females had smaller preen glands yet secreted more preen wax compared to rearing females (Fig. 5). Motivations for using gland morphology as a proxy for its secretory activity in previous studies especially relied on histological principles. Capsules within which preen wax is produced can indeed fill up to 68 % of the preen gland's inner volume in layer fowls (*Gallus gallus domesticus*; Sandilands *et al.*, 2004). Pending further histological studies on other bird species, studies like ours considering both morphological and physiological aspects of the preen gland and the different predictor types that are associated with them can provide interesting insights into preen gland's functioning.

While our study strongly highlighted the need to separate gland morphology and physiology, it also revealed that each of these aspects were associated with different predictor types in the barn owl. We observed that the preen gland was associated with individual (*e.g.* sex, age, body size and condition, plumage color traits) and environmental factors (*e.g.* weather conditions, brood size) as well as to the combination of both (*e.g.*

interactions of plumage color traits with weather conditions). Our study also hierarchized those factors regarding the strength of their association and importance in the retained statistical models. Sex was certainly among the most important predictors of preen gland size, shape and preen wax amount in both adults and fledglings (Fig. 3-4). Sex-specific differences might reflect behavioral, *e.g.* those tied to the distinct roles of males and females during reproduction (Roulin, 2020), or hormonal variations between sexes. Experimental evidence has indeed confirmed that sex steroid hormones (*e.g.* estradiol, testosterone) influence preen wax composition (Whelan *et al.*, 2010; Whittaker *et al.*, 2011), and that the preen gland expresses androgen and estrogen receptors (Daniel *et al.*, 1977).

Interestingly, differences between sexes in preen gland size and wax amount were strongly dependent on breeding stage (egg incubation *vs* nestling rearing) in adults. In short, incubating females had smaller preen glands than rearing females, but secreted more preen wax compared to rearing females and males at any stage (Fig. 5). Because females experience stronger selective pressures from the nest environment, these differences might reflect the extent to which they provide protection to the eggs, offspring and themselves throughout the breeding process (*antiparasitic defense hypothesis*; Moreno-Rueda, 2017). Nonetheless, why incubating and rearing females invest differently in preen gland traits and which of these aspects is most effective require further investigation.

Another important predictor of gland morphology (preen gland size in particular) was body condition (Fig. 3-4). Adults and fledglings in better condition had larger preen glands than those in poorer condition. Our finding is consistent with previous ones showing preen gland volume to be diminished in immune-challenged birds (Magallanes *et al.*, 2016; Moreno-Rueda, 2015; Piault *et al.*, 2008). One possible explanation is that preen gland's functioning may be costly to some extent, *e.g.* by demanding energy or nutrient resources that birds in poorer condition cannot afford. Preen gland's functioning may alternatively plastically adapt to birds' needs according to their condition. Regardless of the explanation, we found no evidence for an association between preen wax amount and body condition. We could expect body condition to affect the composition of preen wax instead, especially because such association would provide birds with the opportunity to signal their condition (Caro *et al.*, 2015).

Environmental factors were also relevant for preen gland's functioning, although they seemed generally less important than individual factors (Fig. 3-4). In adults, we observed that preen gland size was associated with environmental predictors, such as temperature and humidity, depending on plumage coloration (pheomelanin-based trait). Melanin pigments can strengthen biological structures and help protect plumage from microbial degradation (Bonser, 1995; Ruiz-De-Castañeda *et al.*, 2012), suggesting that the protective

roles of melanin pigmentation and preen wax against environmental microorganisms might function complementarily or that one might supersede the other to some extent (Roulin, 2007). We found some support for this latter idea. We showed that preen glands of paler adults increased in size when breeding in warmer and more humid environments. Paler adults may invest more in protecting their plumage as temperature and humidity increase (and potentially bacterial load as well; Burtt Jr. & Ichida, 2004) given the lack of melanin pigments and thus their higher plumage susceptibility to degradation. We also showed humidity – and expected temperature – to have little or no influence on preen glands of redder adults due to melanin pigments providing sufficient plumage protection regardless of humidity or temperature levels. Contrarily, we found a negative association between preen gland size and temperature, which we cannot conciliate with the explanations given above. Further work is needed to better understand how melanin pigmentation interacts with preen gland, and to what extent their combination is involved in explaining the yet-to-be understood Gloger's rule (*i.e.* why animals in warmer and more humid environments tend to have darker pigmentation; Delhey, 2017, 2019).

In fledglings, we found a positive association between preen gland size and brood size (though smaller in magnitude than the ones with sex or body condition; Fig. 4). Fledglings living in larger broods had larger preen glands. Larger broods can indeed entail higher bacterial load (Alt *et al.*, 2015; Lucas *et al.*, 2005) or require a higher allopreening effort to which fledglings may reply by enlarging their preen gland size or increasing their preen wax amount (S. Jacob *et al.*, 2014; Leclaire *et al.*, 2014). However, we never observed fledgling barn owls coating preen wax on each other, nor found an association between preen wax amount and brood size. Enlarging preen gland may then allow for adjusting preen wax composition to brood size and its associated bacterial load, although this requires further investigation.

In summary, our findings support the idea that the preen gland may function in diverse contexts – from antiparasitic defense to sensory communication – within the same species. While already known to influence gland morphology (Díez-Fernández *et al.*, 2021; Grieves *et al.*, 2019; Moreno-Rueda, 2010; Whittaker & Hagelin, 2021), we highlighted that individual factors (*e.g.* sex, age, body size and condition, plumage color traits) also differed in their relative importance. Future studies in other bird species would be useful to confirm if sex (particularly in relation to breeding stage) and body condition are major factors influencing gland morphology as observed here in the barn owl. Environmental factors also influenced preen gland's functioning to a minor extent, but such influence was likely to be dependent on other phenotypic traits (*e.g.* plumage color traits). Further studies in other color-polymorphic species would help understand how melanin pigmentation and preen gland are involved in the Gloger's rule. We finally strongly encourage

future studies to consider morphological and physiological aspects of the preen gland separately, while it might not appear biologically relevant to dissociate gland morphology into size and shape due to the low number of predictors associated with the latter.

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Supplementary Material

Table S1. Factor loadings, eigenvalues and percentage of variance explained for the first two principal components resulting from principal component analyses (PCAs) conducted on the three preen gland measurements in adult and fledgling barn owls.

	Adults		Fledglings	
	PC1	PC2	PC1	PC2
UG length	0.62	0.13	0.62	0.02
UG width	0.57	0.60	0.56	0.69
UG height	0.54	-0.79	0.55	-0.72
eigenvalues	1.7	0.7	1.8	0.7
% variance expl.	56.5	24.6	59.7	23.5

Table S2. Results of Akaike Information Criterion (AIC) model selection based on *ΔAICc* ≤ 2 for preen gland size and shape in adult barn owls.

Dependent variable	Predictors	*AICc*	*ΔAICc*	Weight	Acc. weight	ER
Gland size	Breeding Stage + Sex + Breeding Stage x Sex + Body Condition + Tarsus Length + Breast Col + Hum Avg + Temp Avg + Breast Col x Temp Avg + Sampling Date + Breast Col x Hum Avg + Age Class	8631.90	0.00	0.48	0.48	1.00
	Breeding Stage + Sex + Breeding Stage x Sex + Body Condition + Tarsus Length + Breast Col + Hum Avg + Temp Avg + Breast Col x Temp Avg + Sampling Date + Breast Col x Hum Avg + Age Class + Breast Point Dia	8633.84	1.95	0.18	0.67	2.65
Gland shape	Sex + Body Condition + Hum Avg + Breeding Stage	6213.62	0.00	0.02	0.02	1.00
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg	6213.63	0.01	0.02	0.05	1.01
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breast Point Dia	6214.24	0.62	0.02	0.06	1.37
	Sex + Body Condition + Hum Avg + Breeding Stage + Breast Point Dia	6214.26	0.64	0.02	0.08	1.38
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breeding Stage x Sex	6214.67	1.05	0.01	0.09	1.69
	Sex + Body Condition + Hum Avg + Breeding Stage + Breeding Stage x Sex	6214.86	1.25	0.01	0.11	1.87
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breast Col + Breast Col x Temp Avg	6214.89	1.28	0.01	0.12	1.89
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breast Point Dia + Breast Col + Breast Col x Temp Avg	6214.91	1.29	0.01	0.13	1.91
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Sampling Date	6215.26	1.65	0.01	0.14	2.28
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breast Col	6215.27	1.65	0.01	0.15	2.28
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breast Point Dia + Breeding Stage x Sex	6215.28	1.66	0.01	0.16	2.30
	Sex + Body Condition + Hum Avg + Breeding Stage + Breast Col	6215.35	1.73	0.01	0.17	2.38
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breast Point Dia + Breast Point Dia x Temp Avg	6215.35	1.73	0.01	0.18	2.38
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Breast Point Dia + Breast Col	6215.38	1.77	0.01	0.19	2.42
	Sex + Body Condition + Hum Avg + Breeding Stage + Tarsus Length	6215.44	1.83	0.01	0.20	2.49
	Sex + Body Condition + Hum Avg + Breeding Stage + Temp Avg + Tarsus Length	6215.49	1.88	0.01	0.21	2.56
	Sex + Body Condition + Hum Avg + Breeding Stage + Breast Point Dia + Breeding Stage x Sex	6215.51	1.89	0.01	0.22	2.57
	Sex + Body Condition + Hum Avg + Breeding Stage + Sampling Date	6215.55	1.93	0.01	0.23	2.63
	Sex + Body Condition + Hum Avg + Breeding Stage + Breast Point Dia + Breast Col	6215.56	1.94	0.01	0.24	2.64

Models with Akaike Information Criterion corrected for small sample size (*AICc*), AICc difference (*ΔAICc*), model weight (*weight*), accumulative model weight (*acc. weight*) and evidence ratio (*ER*). Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Table S3. Relative importance of preen gland size and shape predictors according to the summed weights (sum of Akaike model weights in which a predictor appeared within all candidate models and within the *confidence set* of best models [models with $\Delta AICc \leq 2$]) and the number of consecutive candidate models (ranked by their AICc) in which a predictor was included in adult barn owls. Predictors present within all models with $\Delta AICc \leq 2$ are highlighted in bold.

Gland size	Predictor importance			Gland shape	Predictor importance		
Predictors	*N* consecutive models	sum w_i (all models)	sum w_i (conf. set)	Predictors	*N* consecutive models	sum w_i (all models)	sum w_i (conf. set)
Breeding Stage	**1669**	**> 0.99**	**1.00**	**Sex**	**630**	**> 0.99**	**1.00**
Sex	**1291**	**> 0.99**	**1.00**	**Body Condition**	**447**	**0.99**	**1.00**
Breeding Stage x Sex	**610**	**> 0.99**	**1.00**	**Hum Avg**	**329**	**0.99**	**1.00**
Body Condition	**307**	**> 0.99**	**1.00**	**Breeding Stage**	**72**	**0.92**	**1.00**
Tarsus Length	**69**	**> 0.99**	**1.00**	Temp Avg	0	0.68	0.58
Breast Col	**25**	**> 0.99**	**1.00**	Breast Point Dia	0	0.59	0.40
Hum Avg	**20**	**> 0.99**	**1.00**	Breast Col	0	0.52	0.27
Temp Avg	**15**	**> 0.99**	**1.00**	Breeding Stage x Sex	0	0.33	0.19
Breast Col x Temp Avg	**15**	**0.99**	**1.00**	Sampling Date	0	0.29	0.08
Sampling Date	**13**	**0.99**	**1.00**	Tarsus Length	0	0.28	0.08
Breast Col x Hum Avg	**8**	**0.97**	**1.00**	Age Class	0	0.22	*NA*
Age Class	**4**	**0.90**	**1.00**	Breast Col x Temp Avg	0	0.21	0.10
Breast Point Dia	0	0.45	0.27	Breast Point Dia x Hum Avg	0	0.16	*NA*
Breast Point Dia x Hum Avg	0	0.15	*NA*	Breast Col x Hum Avg	0	0.14	*NA*
Breast Point Dia x Temp Avg	0	0.13	*NA*	Breast Point Dia x Temp Avg	0	0.14	0.04

Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Table S4. Results of Akaike Information Criterion (AIC) model selection based on *ΔAICc* ≤ 2 for preen wax amount in adult barn owls.

Dependent variable	Predictors	*AICc*	*ΔAICc*	Weight	Acc. weight	ER
Wax amount	Breeding Stage + Sex + Observation Year + Sampling Date + Breeding Stage x Sex + Hum Avg	841.62	0.00	0.10	0.10	1.00
	Breeding Stage + Sex + Observation Year + Sampling Date + Breeding Stage x Sex + Hum Avg + Temp Avg	842.58	0.96	0.06	0.16	1.62
	Breeding Stage + Sex + Observation Year + Sampling Date + Breeding Stage x Sex + Hum Avg + Body Condition	843.21	1.60	0.05	0.21	2.22
	Breeding Stage + Sex + Observation Year + Sampling Date + Breeding Stage x Sex + Hum Avg + Tarsus Length	843.33	1.71	0.04	0.25	2.36
	Breeding Stage + Sex + Observation Year + Sampling Date + Breeding Stage x Sex + Hum Avg + Breast Col	843.46	1.85	0.04	0.29	2.52

Models with Akaike Information Criterion corrected for small sample size (AICc), AICc difference (*ΔAICc*), model weight (weight), accumulative model weight (acc. weight) and evidence ratio (ER). Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Table S5. Relative importance of preen wax amount predictors according to the summed weights (sum of Akaike model weights in which a predictor appeared within all candidate models and within the *confidence set* of best models [models with $\Delta AICc \leq 2$]) and the number of consecutive candidate models (ranked by their AICc) in which a predictor was included in adult barn owls. Predictors present within all models with $\Delta AICc \leq 2$ are highlighted in bold.

Wax amount	Predictor importance		
Predictors	*N* consecutive models	sum w_i (all models)	sum w_i (conf. set)
Breeding Stage	**2795**	**> 0.99**	**1.00**
Sex	**871**	**> 0.99**	**1.00**
Observation Year	**691**	**> 0.99**	**1.00**
Sampling Date	**668**	**> 0.99**	**1.00**
Breeding Stage x Sex	**362**	**> 0.99**	**1.00**
Hum Avg	**134**	**> 0.99**	**1.00**
Temp Avg	0	0.44	0.21
Breast Col	0	0.39	0.14
Breast Point Dia	0	0.36	*NA*
Body Condition	0	0.30	0.16
Tarsus Length	0	0.30	0.15
Age Class	0	0.14	*NA*
Breast Col x Hum Avg	0	0.10	*NA*
Breast Point Dia x Hum Avg	0	0.10	*NA*
Breast Col x Temp Avg	0	0.06	*NA*
Breast Point Dia x Temp Avg	0	0.05	*NA*

Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Table S6. Results of Akaike Information Criterion (AIC) model selection based on *ΔAICc* ≤ 2 for preen gland size and shape in fledgling barn owls.

Dependent variable	Predictors	*AICc*	*ΔAICc*	Weight	Acc. weight	ER
Gland size	Body Condition + Sex + Tarsus Length + Age + Sampling Date + Brood Size + Breast Point Dia + Hum Avg	8597.80	0.00	0.17	0.17	1.00
	Body Condition + Sex + Tarsus Length + Age + Sampling Date + Brood Size + Breast Point Dia + Hum Avg + Breast Point Dia x Hum Avg	8598.78	0.98	0.10	0.28	1.63
	Body Condition + Sex + Tarsus Length + Age + Sampling Date + Brood Size + Breast Point Dia	8599.43	1.63	0.08	0.35	2.26
	Body Condition + Sex + Tarsus Length + Age + Sampling Date + Brood Size + Breast Point Dia + Hum Avg + Breast Col	8599.54	1.74	0.07	0.42	2.39
	Body Condition + Sex + Tarsus Length + Age + Sampling Date + Brood Size + Breast Point Dia + Hum Avg + Temp Avg	8599.76	1.96	0.06	0.49	2.67
Gland shape	Sex + Tarsus Length + Sampling Date + Age	6205.85	0.00	0.02	0.02	1.00
	Sex + Tarsus Length + Sampling Date + Age + Temp Avg	6206.24	0.39	0.02	0.04	1.22
	Sex + Tarsus Length + Sampling Date	6206.51	0.66	0.02	0.06	1.39
	Sex + Tarsus Length + Sampling Date + Age + Brood Size	6206.61	0.76	0.02	0.07	1.46
	Sex + Tarsus Length + Sampling Date + Temp Avg	6206.62	0.77	0.02	0.09	1.47
	Sex + Tarsus Length + Sampling Date + Age + Temp Avg + Brood Size	6206.76	0.90	0.01	0.10	1.57
	Sex + Tarsus Length + Sampling Date + Age + Hum Avg	6207.53	1.68	0.01	0.11	2.32
	Sex + Tarsus Length + Sampling Date + Age + Breast Point Dia	6207.57	1.72	0.01	0.12	2.36
	Sex + Tarsus Length + Sampling Date + Temp Avg + Brood Size	6207.66	1.81	0.01	0.13	2.47
	Sex + Tarsus Length + Sampling Date + Age + Breast Col	6207.75	1.90	0.01	0.14	2.59
	Sex + Tarsus Length + Sampling Date + Brood Size	6207.79	1.94	0.01	0.15	2.63

Models with Akaike Information Criterion corrected for small sample size (AICc), AICc difference (*ΔAICc*), model weight (weight), accumulative model weight (acc. weight) and evidence ratio (ER). Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Table S7. Relative importance of preen gland size and shape predictors according to the summed weights (sum of Akaike model weights in which a predictor appeared within all candidate models and within the *confidence set* of best models [models with $\Delta AICc \leq 2$]) and the number of consecutive candidate models (ranked by their AICc) in which a predictor was included in fledgling barn owls. Predictors present within all models with $\Delta AICc \leq 2$ are highlighted in bold.

Gland size	Predictor importance			Gland shape	Predictor importance		
Predictors	*N* consecutive models	sum w_i (all models)	sum w_i (conf. set)	Predictors	*N* consecutive models	sum w_i (all models)	sum w_i (conf. set)
Body Condition	**777**	**> 0.99**	**1.00**	**Sex**	**275**	**0.96**	**1.00**
Sex	**720**	**> 0.99**	**1.00**	**Tarsus Length**	**40**	**0.83**	**1.00**
Tarsus Length	**282**	**> 0.99**	**1.00**	**Sampling Date**	**18**	**0.82**	**1.00**
Age	**168**	**> 0.99**	**1.00**	Age	2	0.56	0.67
Sampling Date	**89**	**> 0.99**	**1.00**	Temp Avg	0	0.54	0.39
Brood Size	**47**	**> 0.99**	**1.00**	Brood Size	0	0.42	0.32
Breast Point Dia	**12**	**0.90**	**1.00**	Breast Point Dia	0	0.42	0.06
Hum Avg	2	0.79	0.84	Breast Col	0	0.40	0.06
Breast Col	0	0.42	0.15	Hum Avg	0	0.37	0.07
Temp Avg	0	0.38	0.13	Body Condition	0	0.27	*NA*
Breast Point Dia x Hum Avg	0	0.27	0.22	Breast Point Dia x Temp Avg	0	0.12	*NA*
Breast Col x Hum Avg	0	0.10	*NA*	Breast Col x Temp Avg	0	0.08	*NA*
Breast Point Dia x Temp Avg	0	0.10	*NA*	Breast Col x Hum Avg	0	0.07	*NA*
Breast Col x Temp Avg	0	0.06	*NA*	Breast Point Dia x Hum Avg	0	0.06	*NA*

Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Table S8. Results of Akaike Information Criterion (AIC) model selection based on *ΔAICc* ≤ 2 for preen wax amount in fledgling barn owls.

Dependent variable	Predictors	*AICc*	*ΔAICc*	Weight	Acc. weight	ER
Wax amount	Tarsus Length + Temp Avg + Sex + Body Condition	209.19	0.00	0.02	0.02	1.00
	Tarsus Length + Temp Avg + Sex + Body Condition + Sampling Date	209.45	0.26	0.02	0.04	1.14
	Tarsus Length + Temp Avg + Sex + Body Condition + Age	210.73	1.54	0.01	0.05	2.16
	Tarsus Length + Temp Avg + Sex + Body Condition + Sampling Date + Age	210.94	1.75	0.01	0.06	2.40
	Tarsus Length + Temp Avg + Sex + Body Condition + Breast Col	211.03	1.84	0.01	0.07	2.51
	Tarsus Length + Temp Avg + Sex + Sampling Date	211.07	1.87	0.01	0.08	2.55
	Tarsus Length + Temp Avg + Sex + Body Condition + Breast Point Dia	211.17	1.98	0.01	0.08	2.69

Models with Akaike Information Criterion corrected for small sample size (AICc), AICc difference (*ΔAICc*), model weight (weight), accumulative model weight (acc. weight) and evidence ratio (ER). Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Table S9. Relative importance of preen wax amount predictors according to the summed weights (sum of Akaike model weights in which a predictor appeared within all candidate models and within the *confidence set* of best models [models with $\Delta AICc \leq 2$]) and the number of consecutive candidate models (ranked by their AICc) in which a predictor was included in fledgling barn owls. Predictors present within all models with $\Delta AICc \leq 2$ are highlighted in bold.

Wax amount	Predictor importance		
Predictors	*N* consecutive models	sum w_i (all models)	sum w_i (conf. set)
Tarsus Length	**539**	**0.97**	**1.00**
Temp Avg	**18**	**0.77**	**1.00**
Sex	**11**	**0.76**	**1.00**
Body Condition	5	0.73	0.90
Sampling Date	0	0.49	0.43
Breast Col	0	0.44	0.10
Breast Point Dia	0	0.43	0.09
Hum Avg	0	0.42	*NA*
Age	0	0.36	0.22
Brood Size	0	0.26	*NA*
Breast Point Dia x Temp Avg	0	0.17	*NA*
Observation Year	0	0.14	*NA*
Breast Col x Temp Avg	0	0.10	*NA*
Breast Col x Hum Avg	0	0.08	*NA*
Breast Point Dia x Hum Avg	0	0.07	*NA*

Breast Col = Breast Coloration; Breast Point Dia = Breast Point Average Diameter; Hum Avg = Relative Humidity Average; Sampling Date = Julian Sampling Date; Temp Avg = Air Temperature Average.

Chapter 2

Exploring how individual and environmental factors shape plumage microbiota in the barn owl (*Tyto alba*)

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Author Contributions

L.A.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. A-L.D.: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing. C.D.: Conceptualization, Writing – review & editing. L.M.S-J.: Conceptualization, Methodology, Validation, Writing – review & editing. A.R.: Conceptualization, Methodology, Validation, Writing – review & editing, Funding acquisition.

Abstract

Strong selective pressures from microbial communities inhabiting avian plumage have prompted our investigation into the individual and environmental (climatic and social) factors influencing a bird's exposure to environmental microorganisms. Our study showed that a few factors shape the plumage bacterial diversity and composition in the barn owl (*Tyto alba*). Nestlings, fledglings and adults had different plumage microbiota. Individual factors, such as sex, breeding stage and their interaction, and a few environmental factors, such as ambient temperature and humidity, mainly influenced plumage microbiota in nestlings and adults. Whereas factors such as melanin-based plumage traits and preen wax amount did not influence plumage microbiota in fledglings and adults. Our study revealed that barn owl plumage microbiota is shaped by a few factors (if any in fledglings), highlighting other unexplored behavioral, morphological and physiological mechanisms used to maintain it stable and healthy.

Keywords: alpha diversity, beta diversity, breeding stage, brood size, environmental conditions, humidity, melanin-based plumage traits, plumage microbiota, preen wax, temperature

Introduction

Animal hosts and microorganisms have coexisted and interacted for millions of years, shaping each other's evolution over time (McFall-Ngai *et al.*, 2013). Host-microorganism interactions are dynamic evolutionary processes which have led to microbial colonization of the host's body surface, subsequently affecting its behavior, morphology and physiology. On the one hand, microorganisms are key players in mutualistic and commensal relationships. Mutualists and commensals can protect the host against invading parasites and pathogens, promote the development of its immune system (Belkaid & Hand, 2014; Hooper *et al.*, 2012; Sassone-Corsi & Raffatellu, 2015) and influence its behavior (*e.g.* personality, individual and kin recognition, mate choice; Archie & Theis, 2011; Ezenwa *et al.*, 2012). On the other hand, pathogenic microorganisms can cause diseases, illness and even death, exerting strong selective pressures on its life-history traits and fitness (Benskin *et al.*, 2009). Understanding the proximate mechanisms underlying such host-microorganism interactions and their broader effects on the host's ecological dynamics remains a scientific endeavor.

Host-microorganism interactions are essential for humans, other mammalian systems and various model organisms (Colston & Jackson, 2016), and are increasingly studied in wild birds (Evans *et al.*, 2017; Hird, 2017). For instance, the complex microbial communities inhabiting avian plumage (Bisson *et al.*, 2007; Shawkey *et al.*, 2005) can hinder the growth or establishment of pathogens through the production of antibiotics, alteration of the environment, competition for resources, or stimulation of the bird's immune system (Soler *et al.*, 2010). Alternatively, feather-degrading bacteria (FDB) can enzymatically break down feather β-keratin filaments (Burtt Jr. & Ichida, 1999; Gunderson, 2008), impeding flight efficiency when affecting flight feathers (Swaddle *et al.*, 1996) and disrupting thermoregulation and visual communication when affecting the entire avian plumage (Gunderson *et al.*, 2009; Shawkey *et al.*, 2007; but see S. Jacob, Colmas, *et al.*, 2014). Over time, such degradation can affect the bird's health and fitness (Merilä & Hemborg, 2000), underscoring the need to identify factors influencing its exposure to environmental microorganisms, as well as the physiological mechanisms it has evolved to shape them.

Numerous intrinsic and extrinsic factors are proposed to influence a bird's exposure to environmental microorganisms. Behavior or physiology, *e.g.* preening behavior, daily activities/reproductive duties and sex hormone levels, might lead to age or sex differences in microbial exposure throughout the breeding season (S. Jacob *et al.*, 2018; Leclaire *et al.*, 2019). For instance, prior studies have shown higher plumage bacterial load in female pied flycatchers (*Ficedula hypoleuca*) and great tits (*Parus major*) compared to males (Saag, Mänd, *et al.*, 2011; see Goodenough *et al.*, 2017; Saag, Tilgar, *et al.*, 2011 for bacterial richness) and in female

great tits during nest-building compared to brood stage (Kilgas *et al.*, 2012; Saag, Tilgar, *et al.*, 2011). Nest environment, *e.g.* nest material, microclimate (temperature and humidity) and brood size, might also have an influence as they can provide favorable conditions for microbial growth, proliferation and transmission (Burtt Jr. & Ichida, 2004). Accordingly, Goodenough & Stallwood (2012) found a significant association between the nest box orientation and the local microbial load in great tits, possibly resulting from nest temperature or humidity.

As a result, birds have evolved physiological mechanisms to shape plumage microbial communities and prevent any negative effects on its structural integrity (Clayton *et al.*, 2010; Gunderson, 2008). Self-preening is one time- and energy-consuming behavioral mechanism (Magallanes *et al.*, 2016; Moreno-Rueda, 2015; Piault *et al.*, 2008). During self-preening, birds not only smooth their feathers using their beak but also apply preen wax: a substance produced by the preen gland to maintain and protect the plumage (J. Jacob & Ziswiler, 1982; Moreno-Rueda, 2017). Preen wax can indeed form a protective physical barrier (S. Jacob *et al.*, 2018; Reneerkens *et al.*, 2008; Verea *et al.*, 2017), have a promicrobial action promoting mutualists and commensals able to compete with pathogens for trophic resources and ecological niches (as proposed by S. Jacob *et al.*, 2018; S. Jacob, Immer, *et al.*, 2014; Soler *et al.*, 2010), or a direct antimicrobial action preventing new pathogens from colonizing feather surfaces (Braun *et al.*, 2018; Carneiro *et al.*, 2020; Soini *et al.*, 2007). In this context, S. Jacob, Immer, *et al.* (2014) showed that great tits adjusted their preen gland size and preen wax composition in response to environmental microorganisms. Similarly, captive feral pigeons (*Columba livia*) decreased their investment in preen wax secretion and self-preening behavior when exposed to lower feather bacterial loads (Leclaire *et al.*, 2014). In addition to self-preening, feather structure and pigmentation are additional mechanisms shaping plumage microbial communities. Melanin pigments can indeed strengthen biological structures and help protect the plumage from damage caused by FDB (Ruiz-De-Castañeda *et al.*, 2012). In this regard, Justyn *et al.* (2017) reported higher susceptibility of white feather areas to colonization and attachment by the FDB *Bacillus licheniformis*, whereas Al Rubaiee *et al.* (2021) found higher microbial abundance and diversity on black feather areas. Despite recent advances in avian microbiota research, much remains to be explored regarding the factors shaping the complex microbial communities inhabiting avian plumage.

In the present study, we used the barn owl (*Tyto alba*) to better understand how individual and environmental (climatic and social) factors affect the microbial communities inhabiting its plumage. For this purpose, we analyzed feathers collected from the breast, belly and back of nestlings, fledglings and of adults at two breeding stages (egg incubation and nestling rearing) using high-throughput 16S rRNA gene

sequencing. We assigned an alpha and beta diversity metric for each body part separately and for the entire body of each individual. We combined this information with individual (age and sex), morphological (plumage coloration and spottiness) and physiological (wax amount secreted) data, along with data related to the nest environment (ambient temperature, humidity and brood size). Due to obvious behavioral, morphological and physiological differences, we first compared the plumage microbiota (*i.e.* bacterial diversity and composition) of the three age classes (nestlings, fledglings and adults). We then compared the plumage microbiota of the three body parts (breast, belly and back) within each age class. In our study population, barn owls display variation in melanin-based plumage coloration and spottiness among body parts. Breast and belly feathers typically range from immaculate white to spotted reddish-brown, whereas back feathers are predominantly dark (Roulin, 2020). Body parts also differ in contact with nest material or in accessibility during preening.

We then investigated how individual and environmental factors affect the plumage microbiota within each age class. We first expected sex, breeding stage and their interaction to influence plumage microbiota due to sex-specific exposure to environmental microorganisms throughout the breeding season. During reproduction, female barn owls care for the eggs, offspring and themselves in constant contact with nest microorganisms, while males provide them with food (Roulin, 2020). We also expected melanin-based plumage traits and preen wax amount to play a protective role in shaping plumage microbiota, and ambient temperature and humidity (Goodenough & Stallwood, 2012) and brood size (Alt *et al.*, 2015; Lucas *et al.*, 2005) to provide favorable conditions for microbial growth, proliferation and transmission (Burtt Jr. & Ichida, 2004). We finally compared the feather and nest microbiota – the nest serving as a major source of microorganisms for avian plumage (Goodenough *et al.*, 2017; S. Jacob, Immer, *et al.*, 2014; van Veelen *et al.*, 2017).

Materials & Methods

Study system, feather and nest material collection

We carried out the present study during the 2019 breeding season (3rd July – 4th November) on a wild population of barn owls nesting in wooden nest boxes installed across Western Switzerland. A detailed description of the study area can be found in Frey *et al.* (2011). We visited each nest box monthly to locate and date the clutches, monitored each breeding pair from egg incubation until they ended rearing their chicks, and each chick until it took its first flight at about 55-65 days of age. We collected multiple data and samples at each developmental stage on each of the monitored birds (*e.g.* breeding, individual and environmental variables).

We plucked three adjacent feathers from the mid-breast and belly, and the upper back of adult (*N* = 64 observations on 62 adults; 1.03 observations per individual on average) and chick (*N* = 79 observations on 41 chicks; 1.93 observations per individual on average) barn owls. Chicks were sampled at two developmental stages: between 15 and 30 days post-hatch when they were covered with down (*N* = 38 individuals, hereafter nestlings) and then between 45 and 70 days post-hatch once they have developed their feathers (*N* = 41 individuals, hereafter fledglings). A total of 34 broods was sampled between egg incubation and chick fledging, with 1.88 adults and 1.21 chicks sampled per brood on average. Each body part's feather samples were placed in individual sterile zip-lock bags, stored in ice in the field and then transferred within the next hours in a -80 °C freezer in the laboratory. Similarly, nest material (*e.g.* a mixture of pellets, droppings and dead prey; *N* = 26 samples, *mean* ± *standard error* [*SE*]: 0.18 ± 0.01 g) – a proxy for the microbiota to which individuals were daily exposed – was collected from the nest's central surface and stored in individual sterile 5-mL Eppendorf tubes under the same temperature conditions as feather samples. All handling and sampling of birds was done wearing 70 % ethanol-sterilized gloves to avoid exogenous contamination.

Individual data

Birds captured for the first time were ringed for identification. We estimated the age of chicks according to their left-wing length upon ringing (Roulin, 2004b), and that of adults based on their molt pattern if not previously ringed as chicks within our study area (Taylor, 1993). We determined the genetic sex of birds from a blood sample using sex-specific molecular markers (Py *et al.*, 2006). We assessed the melanin-based plumage traits by visually assigning a color score ranging from -8 for whitish to -1 for dark-reddish body parts, and by measuring the diameter of ten representative dark spots at the feather tips (in a 60 x 40 mm-

frame with a caliper to the nearest 0.1 mm) on the breast and belly of adults and fledglings only (Roulin, 2004a).

Physiological data

Each time we sampled feathers, we also sampled preen wax (except on nestlings whose preen gland was still too small for the manipulation, and one adult due to oversight). To do so, we gently pressed on the preen gland with the thumb fitted with a sterile glove until emptying it. We collected preen wax into 50-µL glass capillaries (Brand GmbH & Co. KG, Wertheim, DE), and immediately measured the amount of preen wax collected in the capillaries (with a caliper to the nearest 0.1 mm) before converting it into volume (*range*, *mean* ± *SE*: from 0.00 to 115.52 µL, 32.28 ± 4.14 µL in adults; from 0.17 to 24.83 µL, 7.57 ± 0.92 µL in fledglings).

Environmental data

We calculated air temperature (°C) and relative humidity (%) averages 24 hours prior to each feather sampling using climate data recorded at a ten-minute granularity by the MeteoSwiss weather station nearest to each brood (between 400 - 800 m alt., up to 15 km distance). The climate data was accessible from the climate database *IDAweb* provided by the Federal Office of Meteorology and Climatology MeteoSwiss (*Swiss Meteorological Institute IDAweb*). We kept 13 MeteoSwiss weather stations recording temperature data with which 1 to 9 broods were associated (*mean:* 3.54 broods, *median*: 3.0 broods per station; number of broods associated with more than one weather station within the year: 12 broods), and 12 MeteoSwiss weather stations recording humidity data with which 1 to 10 broods were associated (*mean*: 3.83 broods, *median*: 3.5 broods per station; number of broods associated with more than one weather station within the year: 12 broods). The average distance between a brood and a weather station was 6185 m for temperature data and 6204 m for humidity data. We calculated the brood size as the number of chicks present within each brood (*range*: from 0 to 7 chicks).

Ethics

Barn owl monitoring was performed according to a strict animal handling protocol approved by the 'Service de la consommation et des affaires vétérinaires', Switzerland (authorization numbers: VD 3213 and 3462).

DNA extraction, library preparation and sequencing

After incubation overnight and homogenization in a lysis solution using a Precellys Evolution homogenizer (Bertin Technologies, Montigny-le-Bretonneux, FR), we extracted total genomic DNA from feather samples

using the ZymoBIOMICS DNA Miniprep Kit (Cat. No. D4300; Zymo Research, Irvine, CA, USA) and from nest material using the DNeasy PowerSoil Pro Kit (Cat. No. 47014; QIAGEN, Hilden, DE) following the manufacturer's instructions. We quantified DNA concentration using a Qubit 2.0 Fluorometer (Invitrogen/Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

The V3-V4 and V5-V6 (*ca.* 295 bp long) hypervariable regions of the bacterial 16S rRNA gene were amplified using the universal primer pairs 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC), and BACTB-F (GGATTAGATACCCTGGTAGT) and BACTB-R (CACGACACGAGCTGACG; Fliegerova *et al.*, 2014; S. Jacob *et al.*, 2018), respectively. Each primer included an Illumina MiSeq adapter sequence, two to four randomized nucleotides and a unique barcode of eight nucleotides. PCR amplifications were performed in a total volume of 25 µL containing 7.5 µL of nuclease-free water, 0.75 µL of dimethyl sulfoxide (DMSO), 0.25 µL of bovine serum albumin (BSA) and 12.5 µL of Taq 2x Master Mix (Cat. No. M0270L; New England Biolabs, Ipswich, MA, USA) to which 1 µL (10 µM) of each primer and 2 µL of DNA were added. PCR conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 15 sec, annealing at 52 °C for 15 sec and extension at 72 °C for 30 sec, and a final extension step at 72 °C for 3 min. PCR products were run on 2 % agarose gels stained with GelRed Nucleic Acid Stain (Biotium, Fremont, CA, USA) and purified using AMPure XP beads (1:1 ratio; Beckman Coulter, Brea, CA, USA), and 3 ng of each (or all available material when PCR performance was lower) was pooled into one final library per amplicon. Amplicon libraries were prepared and sequenced in a single run with an Illumina MiSeq V3 platform using a 2 × 300-bp paired-end protocol for the V3-V4 amplicon and a 2 × 200-bp paired-end protocol for the V5-V6 amplicon by the Swiss company Fasteris (Fasteris, Plan-les-Ouates, CH).

We included 20 negative extraction controls (*i.e.* sample-free extractions) and five positive extraction controls (ZymoBIOMICS Microbial Community Standards, Cat. No. D6300; Zymo Research, Irvine, CA, USA) from DNA extraction to library sequencing to ensure DNA extraction efficiency, as well as to detect and withdraw potential contaminants. We included one negative PCR control per batch and four positive PCR controls (ZymoBIOMICS Microbial Community DNA Standards, Cat. No. D6305; Zymo Research, Irvine, CA, USA) to assess library preparation and sequencing reliability. We also considered eleven technical PCR replicates throughout the protocol for similar reasons.

Bioinformatics sequence processing

Raw paired-end sequence reads were bioinformatically processed using the *dada2* R package (version 1.20.0) following the DADA2 pipeline (version 1.16; Callahan *et al.*, 2016). Sequence reads were first

demultiplexed and had their barcode and adapter sequence trimmed by Fasteris. We then identified and trimmed the forward and reverse primers using the Cutadapt tool (version 3.4; Martin, 2011), and truncated the forward and reverse sequence reads to 215 and 235 bp for the V3-V4 amplicon and to 140 and 160 bp for the V5-V6 amplicon according to their per-base quality scores (*plotQualityProfile* function). We also truncated the sequence reads at the first instance of a quality score less than or equal to 2 and discarded those containing any ambiguous bases or more than 2 *expected errors* after trimming and truncation (truncQ = 2, maxN = 0, maxEE = 2; *filterAndTrim* function). We applied the DADA2 parametric error model to the forward and reverse sequence reads separately (nbases = 1e10; *learnErrors* function) and subsequently inferred the Amplicon Sequence Variants (ASVs) through the DADA2 core denoising algorithm (*dada* function) entitled to remove amplicon sequencing errors. We merged the forward and reverse sequence reads (minOverlap = 20, maxMismatch = 0; *mergePairs* function). We constructed the ASV tables (*makeSequenceTable* function), discarded the ASVs outside the expected amplicon size and filtered out the chimeric ASVs (method = consensus; *removeBimeraDenovo* function). We assigned a taxonomy status to the resulting ASVs using the DADA2 naïve Bayesian Ribosomal Database Project (RDP) classifier (minBoot = 80; *assignTaxonomy* function; Wang *et al.*, 2007) trained against the SILVA reference database (version 138.1; Quast *et al.*, 2013).

We combined the resulting ASVs and their taxonomic assignment with the sample metadata into phyloseq-class objects (*merge_phyloseq* function, *phyloseq* R package; McMurdie & Holmes, 2013) for downstream analysis. We filtered out the potential contaminant ASVs based on a higher prevalence in negative extraction controls than in true samples (method = prevalence, threshold = 0.5; *isContaminant* function, *decontam* R package; Davis *et al.*, 2018) and compared the expected bacterial composition of the two ZymoBIOMICS Microbial Community Standards to the ASVs inferred by the DADA2 algorithm. We considered both body part and individual plumage microbiota. We pooled samples – *i.e.* summed ASV abundances – from the three body parts of each individual (*merge_samples* function, *phyloseq* R package) to obtain individual plumage microbiota. From body part and individual samples, we discarded the singletons and non-bacterial ASVs (*i.e.* Eukaryota, Chloroplast or Mitochondria). We finally normalized and transformed the per-sample ASV counts into relative abundances (*transform_sample_counts* function, *phyloseq* R package) and retained only the ASVs with an average relative abundance above 0.001 %.

Statistical analysis

Statistical analyses were performed using R Statistical Software (R version 4.1.0; R Core Team, Vienna, Austria). We restricted our study to the V3-V4 hypervariable regions of the bacterial 16S rRNA gene but later

confirmed our main findings by analyzing the V5-V6 hypervariable regions (*data not shown*). We computed the Shannon Diversity Index as an alpha diversity metric (*estimate_richness* function, *phyloseq* R package) accounting for both ASV richness and evenness in each sample. We computed the Bray-Curtis Dissimilarity as a beta diversity metric (*vegdist* function, *vegan* R package; Oksanen *et al.*, 2022) considering the presence/absence of ASVs in each sample, as well as the abundance of ASVs shared between any two samples. We visualized the Bray-Curtis Dissimilarity using Non-Metric Multidimensional Scaling (NMDS). We calculated the inter-replicate repeatability (N = 11 replicates) which was significant for both alpha (ANOVA-based repeatability: R (SE) = 0.81 (0.11), $F_{10,11}$ = 9.24, P < 0.01; Nakagawa & Schielzeth, 2010) and beta diversity metrics (Procrustes analysis: *Procrustes SS* = 0.19, R = 0.90, P < 0.01) across replicates.

Age-class differences in plumage bacterial diversity and composition were analyzed using a linear mixed-effects model (LMM) on the Shannon Diversity Index (*lme4* function, *lmer* R package; Bates *et al.*, 2015) and pairwise permutational Multivariate Analyses of Variance (perMANOVA) on the Bray-Curtis Dissimilarity matrix (with 999 permutations; *pairwise.adonis2* function, *pairwiseAdonis* R package; Martinez Arbizu, 2020) based on individual-level data. We included the age class (nestling, fledgling or adult) as a single fixed factor in both model types, bird ID and clutch ID as random factors in LMM, and clutch ID as a strata within which to constrain permutations in pairwise perMANOVAs. Analyses on differences in plumage bacterial diversity and composition among body parts were conducted using LMMs on the Shannon Diversity Index and one-way perMANOVAs on the Bray-Curtis Dissimilarity matrix (with 999 permutations; by = margin, *adonis2* function, *vegan* R package) based on body part-level data within each age class. We included the body part (breast, belly or back) as a single fixed factor in both model types, bird ID and clutch ID as random factors in LMMs, and bird ID as a strata in perMANOVAs. With a view to studying how individual and environmental factors affect plumage microbiota, we performed LMMs on the Shannon Diversity Index and perMANOVAs on the Bray-Curtis Dissimilarity matrix based on individual-level data within each age class. In adult and fledgling models, we included sex (in interaction with breeding stage for adults only), plumage coloration and spottiness, wax amount secreted, ambient temperature and humidity and brood size, but ended up removing the brood size from adult models due to a too high collinearity with the breeding stage (variance inflation factor (VIF) ≥ 10). In nestling models, we only included sex, ambient temperature and humidity and brood size, as nestlings are not yet covered with feathers at this stage nor has preen wax been sampled. We included clutch ID as a random factor in LMMs and as a strata in perMANOVAs. Comparisons between feather and nest bacterial diversity and composition were carried out using a LMM on the Shannon Diversity Index and a one-way perMANOVA on the Bray-Curtis Dissimilarity matrix based on clutch-level

data. We included the sample type (feather or nest sample) as a single fixed factor in both model types, and clutch ID as a random factor in LMM and as a strata in perMANOVA.

We selected models by backward elimination of non-significant terms ($P > 0.1$). We applied post-hoc pairwise comparisons (adjusted using the Benjamini-Hochberg correction; Benjamini & Hochberg, 1995) among predictors levels (age class, body part, sex x breeding stage) to identify significantly different levels in plumage bacterial diversity and composition. We finally used the Linear discriminant analysis Effect Size (LEfSe) method (with default settings, *run_lefse* function, *microbiomeMarker* R package; Cao *et al.*, 2022) to detect microbial taxa with differential abundance among groups (age class, body parts, sex in adults, sample type) based on Kruskal-Wallis test and Wilcoxon Rank-Sum test, as well as to estimate the effect size of each differentially abundant taxon based on Linear Discriminant Analysis (Segata *et al.*, 2011).

We checked for the assumptions of collinearity of LMMs by computing the VIF among predictors, as well as of normality, linearity and homoscedasticity of residuals by plot diagnosis. We calculated the Cook's distance to detect any influential observations (Quinn & Keough, 2002). We also checked for the assumptions of multivariate homogeneity of group dispersions among age classes, body parts and sample types (*betadisper* function, *vegan* R package) using permutational Multivariate Analyses of Dispersion (PERMDISP; with 999 permutations, *permutest* function, *vegan* R package).

Results

Taxonomic composition of barn owl feathers and nests

In total, we detected 3104 ASVs across 439 body part-level observations distributed among 22 phyla, 47 classes, 112 orders, 213 families and 418 genera, with an ASV count ranging from 13 to 380 per sample and an average (± *standard deviation* [*SD*]) of 145 (± 56) ASVs. Of these 3104 ASVs, 2465 had a prevalence of less than 5 % (79.4 %), while 157 had a prevalence greater than 20 % (5.1 %). We also detected 2940 ASVs across 143 individual-level observations classified into 21 phyla, 42 classes, 100 orders, 189 families and 381 genera, with an ASV count ranging from 132 to 510 per sample and an average (± *SD*) of 274 (± 83) ASVs. Of these 2940 ASVs, 1706 had a prevalence of less than 5 % (58.0 %), while 347 had a prevalence greater than 20 % (11.8 %).

Microbiota inhabiting barn owl feathers was found to be predominantly composed of Firmicutes (76.2 and 76.4 %), Proteobacteria (12.3 and 12.4 %), Actinobacteriota (7.6 and 7.7 %) and Bacteroidota (2.5 and 2.7 % at body part- and individual-level, respectively), with the remaining phyla present at a frequency of less than 1 % (Fig. 1). Only 0.16 and 0.14 % of ASVs could not be classified at the phylum level. At the class level, Firmicutes were primarily represented by Bacilli (69.2 and 69.0 %), Clostridia (6.0 and 6.3 %) and Negativicutes (1.0 and 1.1 %), Proteobacteria by Gammaproteobacteria (6.8 and 6.6 %) and Alphaproteobacteria (5.5 and 5.8 %), Actinobacteriota by Actinobacteria (6.4 and 6.4 %) and Coriobacteriia (1.1 and 1.2 %), and Bacteroidota by Bacteroidia (2.5 and 2.7 %).

Finally, 635 ASVs were detected out of the 27 nest material samples (10 phyla, 17 classes, 44 orders, 71 families and 88 genera), with a minimum of 30 ASVs, a maximum of 160 ASVs and an average (± *SD*) of 96 (± 29) ASVs. Of these 635 ASVs, 281 ASVs had a prevalence of less than 5 % (44.3 %), while 131 had a prevalence greater than 20 % (20.6 %). Microbiota inhabiting nest material was also found to be dominated by the phyla Firmicutes (91.0 %), Proteobacteria (3.3 %), Actinobacteriota (3.2 %) and Bacteroidota (2.0 %; Fig. 1). Only 0.31 % of ASVs could not be classified at the phylum level. Similar to feather microbiota, Firmicutes were primarily represented by Bacilli (88.7 %) and Clostridia (2.3 %), Proteobacteria by Gammaproteobacteria (3.3 %), Actinobacteriota by Actinobacteria (3.0 %), and Bacteroidota by Bacteroidia (2.0 %) at class-level taxonomy. Most ASVs found in nest material were also identified on feathers (98.6 %), while only a few ASVs found on feathers were identified in nest material (20.2 %).

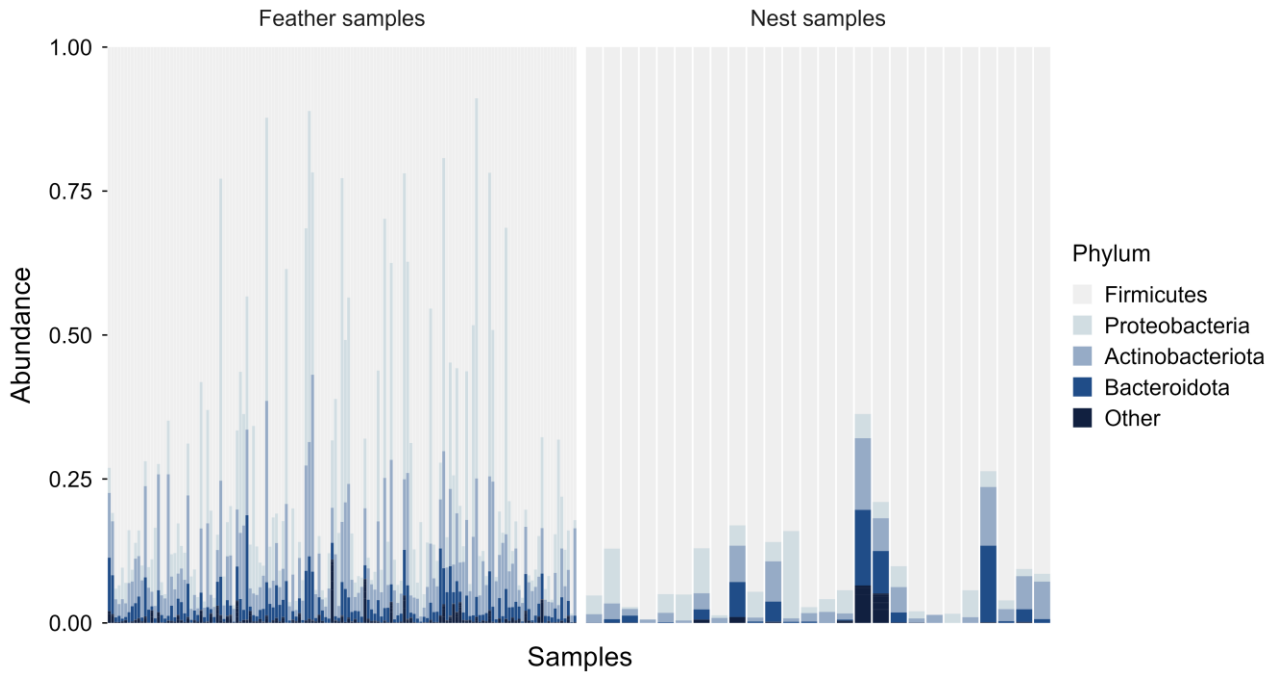

Figure 1. Taxonomic composition and relative abundance of the four most abundant phyla in barn owl feathers and nests. Both sample types were predominantly composed of Firmicutes, Proteobacteria, Actinobacteriota and Bacteroidota. Calculations were based on individual-level data. Each vertical bar represents a sample.

Differences among age classes

We found a significant difference in feather alpha diversity among the three age classes (LMM: $F_{2,80}$ = 25.08, P < 0.01). Adults had significantly greater alpha diversity compared to nestlings and fledglings (adults *vs* nestlings: estimate (*SE*) = 0.56 (0.12), t_{127} = 4.83, P < 0.01; adults *vs* fledglings: estimate (*SE*) = 0.77 (0.11), t_{127} = 6.82, P < 0.01), and nestlings had marginally greater alpha diversity compared to fledglings (nestlings *vs* fledglings: estimate (*SE*) = 0.21 (0.12), t_{111} = 1.72, P = 0.09; Fig. 2a). We also found a significant difference in feather beta diversity among the three age classes, with each pairwise comparison differing significantly from one another (adults *vs* nestlings: pseudo-F = 14.09, P < 0.01; adults *vs* fledglings: pseudo-F = 22.03, P < 0.01; nestlings *vs* fledglings: pseudo-F = 11.87, P < 0.01). Despite having observed multivariate heterogeneity of group dispersion (perMDISP: pseudo-F = 45.33, P < 0.01), NMDS plot still indicated variations in beta diversity centroid among age classes (Fig. 2b). LEfSe analysis identified 26 differentially abundant microbial classes among age classes, with 8 being enriched in nestlings, 2 in fledglings and 16 in adults (all P < 0.05).

Among them, Clostridia, Coriobacteriia, Negativicutes, Desulfovibrionia, Fusobacteriia, Spirochaetia, Campylobacteria and Vampirivibrionia were more abundant in nestlings, Bacilli and Limnochordia in fledglings, and Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidia, Cyanobacteriia, Thermoleophilia, Verrucomicrobiae, Desulfuromonadia, Phycisphaerae, Saccharimonadia, Acidimicrobiia, Myxococcia, Anaerolineae, Abditibacteria, Rubrobacteria and an unidentified class in adults. After reviewing these results, we decided to conduct the subsequent analyses separately by age class.

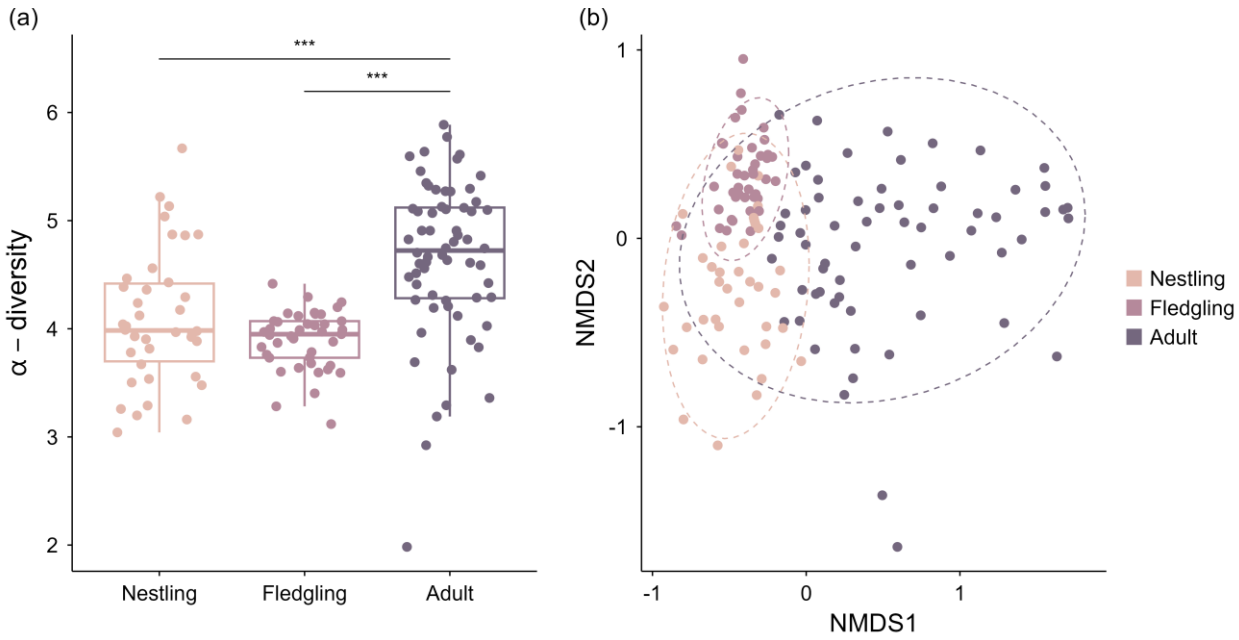

Figure 2. Plots showing variations in plumage bacterial diversity and composition among age classes. (a) Boxplots of Shannon Diversity Index and (b) NMDS plots based on Bray-Curtis Dissimilarity (stress value = 0.18) comparing plumage bacterial diversity and composition among nestling, fledgling and adult barn owls. Each dot represents a sample, each ellipse the 95 % confidence interval, and colors denote age class. Significance (*) was determined by statistical analyses.

Differences among body parts

In nestlings, we found a significant difference in feather alpha diversity among the three body parts (LMM: $F_{2,74}$ = 8.93, P < 0.01), with the back having significantly greater alpha diversity than the breast and belly (back *vs* breast: estimate (*SE*) = 0.42 (0.10), t_{91} = 4.22, P < 0.01; back *vs* belly: estimate (*SE*) = 0.22 (0.10), t_{91}

= 2.25, P = 0.04) and the belly greater alpha diversity than the breast (belly *vs* breast: estimate (*SE*) = 0.19 (0.10), t_{91} = 1.97, P = 0.05). We also found a significant difference in feather beta diversity (perMANOVA: pseudo-F = 1.02, R^2 = 0.02, P < 0.01) and observed multivariate homogeneity of group dispersion (perMDISP: pseudo-F = 1.57, P = 0.21), providing support for a difference in centroid rather than dispersion among the three body parts. However, post-hoc pairwise comparisons revealed no such significant differences (back *vs* breast: pseudo-F = 1.28, P = 0.22; back *vs* belly: pseudo-F = 1.30, P = 0.22; belly *vs* breast: pseudo-F = 0.50, P = 0.99). LEfSe analysis still identified 7 differentially abundant microbial classes among body parts (all P < 0.05), with Bacteroidia, Actinobacteria, Gammaproteobacteria, Fusobacteriia, Gracilibacteria, Desulfuromonadia and Blastocatellia being enriched in the back. No significant differences in feather alpha or beta diversity were found among body parts in fledglings (LMM: $F_{2,80}$ = 0.66, P = 0.52; perMANOVA: pseudo-F = 0.28, R^2 < 0.01, P = 0.12). In adults, we did not find a significant difference in feather alpha diversity among the three body parts (LMM: $F_{2,128}$ = 0.01, P = 0.99; Fig. 3a), but we did in feather beta diversity (perMANOVA: pseudo-F = 2.44, R^2 = 0.03, P < 0.01). However, we observed multivariate heterogeneity of group dispersion (perMDISP: pseudo-F = 5.86, P < 0.01), suggesting a difference in dispersion in addition to/instead of centroid among the three body parts (which could contribute to the significant result obtained in the perMANOVA). Breast differed significantly from the back (back *vs* breast: pseudo-F = 3.87, P = 0.01), whereas the other post-hoc pairwise comparisons revealed no such significant differences (back *vs* belly: pseudo-F = 3.06, P = 0.06; belly *vs* breast: pseudo-F = 0.53, P = 0.64; Fig. 3b). LEfSe analysis identified 6 differentially abundant microbial classes among body parts (all P < 0.05), with Alphaproteobacteria, Desulfovibrionia and Acidimicrobiia being enriched in the breast, Thermoleophilia in the belly, and Bacilli and Limnochordia in the back.

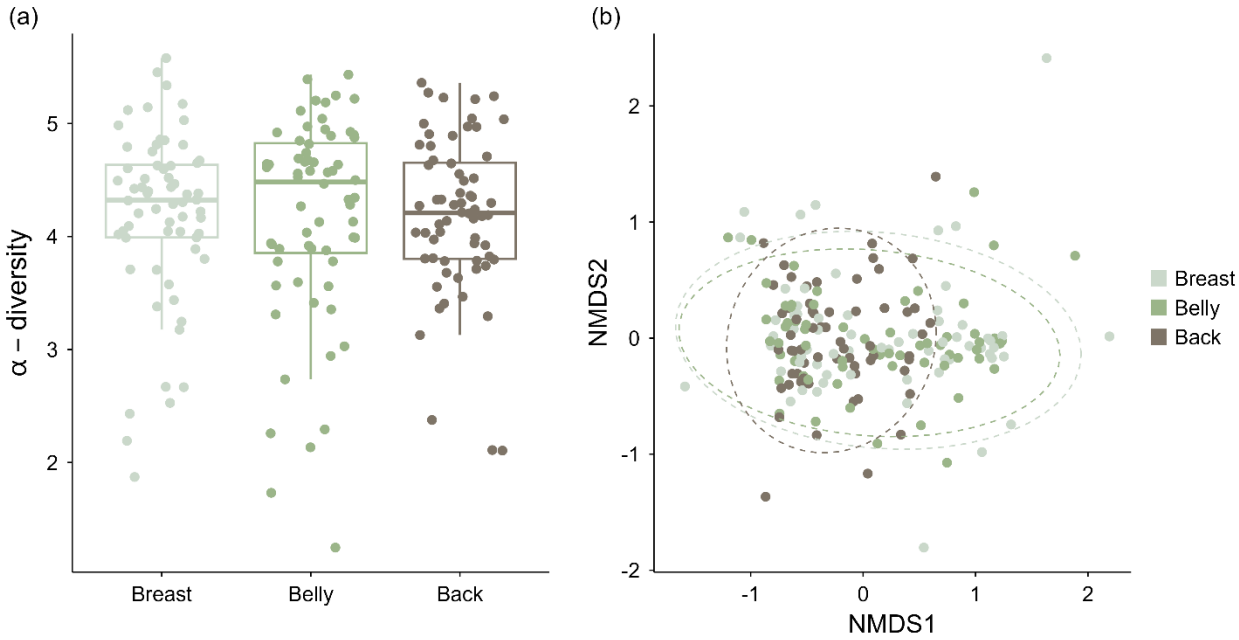

Figure 3. Plots showing variations in plumage bacterial diversity and composition among body parts in adults. (a) Boxplots of Shannon Diversity Index and (b) NMDS plots based on Bray-Curtis Dissimilarity (stress value = 0.18) comparing plumage bacterial diversity and composition among breast, belly and back in adult barn owls. Each dot represents a sample, each ellipse the 95 % confidence interval, and colors denote body part. Significance (*) was determined by statistical analyses.

Influence of individual and environmental factors

Models studying how individual and environmental factors affected bacterial diversity and composition in nestlings showed that feather alpha diversity was not significantly associated with any predictor (Table 1), whereas feather beta diversity was significantly associated with all predictors tested: namely sex, ambient temperature and humidity and brood size (Table 2). No predictors were significantly associated with feather alpha or beta diversity in fledglings (Tables 1-2). Models in adults showed that feather alpha diversity was significantly associated with sex, breeding stage and their interaction (Table 1). Females incubating eggs had significantly lower alpha diversity compared to males at the same breeding stage, and to both males and females rearing nestlings (incubating females *vs* incubating males: estimate (*SE*) = -1.27 (0.35), t_{52} = -3.61, P < 0.01; incubating females *vs* rearing males: estimate (*SE*) = -0.98 (0.16), t_{40} = -6.08, P < 0.01; incubating females *vs* rearing females: estimate (*SE*) = -0.87 (0.23), t_{56} = -3.85, P < 0.01; Fig. 4a). Feather beta diversity was significantly associated with sex and ambient temperature (Table 2; Fig. 4b). LEfSe analysis identified 11

differentially abundant microbial classes between sexes (all $P < 0.05$). Two microbial classes, namely Bacilli and Fusobacteriia, were more abundant in females, and 9 microbial classes, namely Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, Bacteroidia, Thermoleophilia, Saccharimonadia, Abditibacteria, Rubrobacteria and Bdellovibrionia, were more abundant in males.

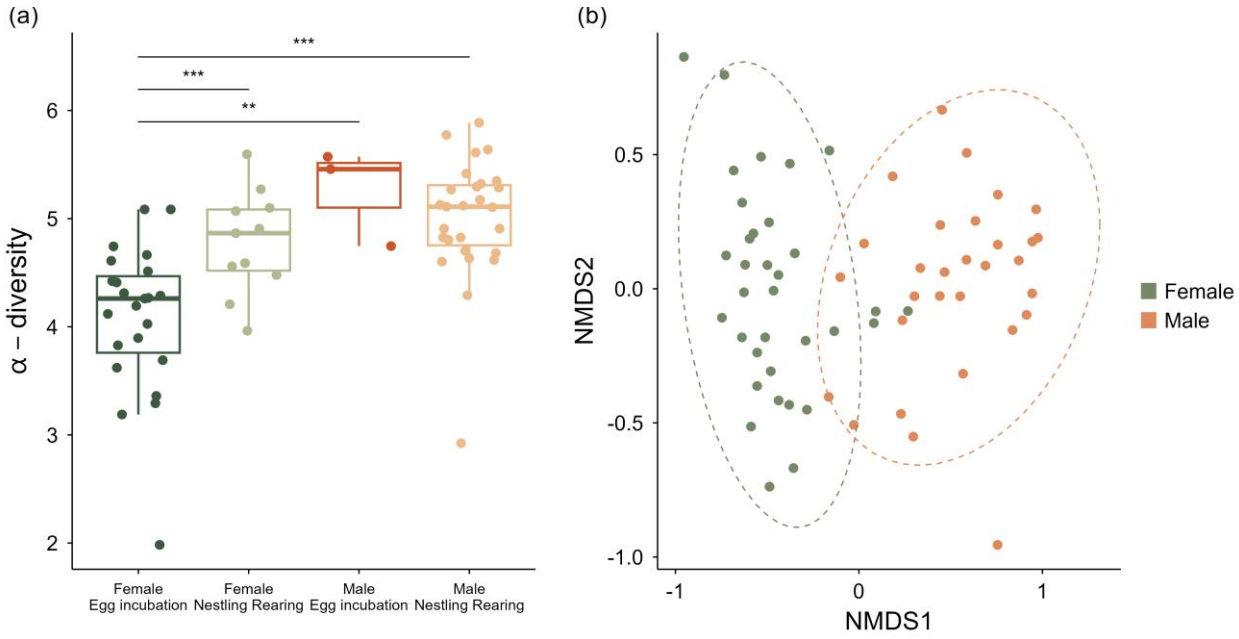

Figure 4. Plots showing variations in plumage bacterial diversity and composition between sexes depending on breeding stages in adults. (a) Boxplots of Shannon Diversity Index and (b) NMDS plots based on Bray-Curtis Dissimilarity (stress value = 0.16) comparing plumage bacterial diversity and composition between male and female barn owls. Each dot represents a sample, each ellipse the 95 % confidence interval, and colors denote sex. Significance (*) was determined by statistical analyses.

Table 1. Results from LMMs testing how individual and environmental predictors influenced Shannon alpha diversity in nestlings (*N* = 38), fledglings (*N* = 41) and adults (*N* = 63). Models after a stepwise backward selection are presented (*P* > 0.1). Significant results (*P* < 0.05) are highlighted in bold.

	Predictors	Estimate	Std Error	*F*-value	Df	Res. Df	*P*-value
Nestlings	Humidity Avg	< 0.01	0.01	0.01	1	18	0.93
	Sex (Male)	0.06	0.13	0.18	1	20	0.68
	Brood Size	0.11	0.12	0.88	1	18	0.36
	Temperature Avg	-0.03	0.04	0.70	1	19	0.41
Fledglings	Breast Coloration	-0.01	0.05	0.05	1	29	0.83
	Sex (Male)	0.02	0.08	0.07	1	29	0.79
	Wax Amount	< -0.01	0.01	0.09	1	34	0.77
	Breast Spottiness	0.01	0.01	0.52	1	31	0.48
	Temperature Avg	0.01	0.02	0.77	1	22	0.39
	Humidity Avg	< 0.01	< 0.01	1.10	1	29	0.30
	Brood Size	0.07	0.03	3.63	1	26	0.07
Adults	Breast Coloration	< 0.01	0.05	< 0.01	1	54	0.96
	Breast Spottiness	< 0.01	0.01	0.06	1	55	0.81
	Wax Amount	< -0.01	< 0.01	0.33	1	56	0.57
	Temperature Avg	0.02	0.03	0.49	1	42	0.49
	Humidity Avg	0.01	0.01	3.54	1	53	0.07
	Sex (Male) x Breeding Stage (Nestling Rearing)	**-1.16**	**0.41**	**7.42**	**1**	**49**	**0.01**
	Sex (Male)	**1.27**	**0.35**	**12.27**	**1**	**51**	**< 0.01**
	Breeding Stage (Nestling Rearing)	**0.87**	**0.23**	**13.92**	**1**	**55**	**< 0.01**

Std Error = standard error; Df = degrees of freedom; Res. Df = residual degrees of freedom

Table 2. Results from perMANOVAs testing how individual and environmental predictors influenced Bray-Curtis beta diversity in nestlings (*N* = 38), fledglings (*N* = 41) and adults (*N* = 63). Models after a stepwise backward selection are presented (*P* > 0.1). Significant results (*P* < 0.05) are highlighted in bold.

	Predictors	Df	Sum Of Sqs	R^2-value	*F*-value	*P*-value
Nestlings	**Humidity Avg**	**1**	**0.35**	**0.04**	**1.79**	**0.01**
	Temperature Avg	**1**	**0.51**	**0.06**	**2.56**	**0.01**
	Sex	**1**	**0.24**	**0.03**	**1.19**	**0.01**
	Brood Size	**1**	**0.32**	**0.04**	**1.63**	**0.03**
Fledglings	Wax Amount	1	0.13	0.02	0.96	0.96
	Brood Size	1	0.29	0.05	2.21	0.94
	Sex	1	0.10	0.02	0.77	0.74
	Breast Coloration	1	0.17	0.03	1.23	0.52
	Humidity Avg	1	0.31	0.05	2.24	0.48
	Temperature Avg	1	0.29	0.05	2.02	0.67
	Breast Spottiness	1	0.07	0.01	0.46	0.20
Adults	Sex x Breeding Stage	1	0.22	0.01	1.10	0.19
	Breast Coloration	1	0.14	0.01	0.66	0.71
	Breast Spottiness	1	0.24	0.01	1.19	0.42
	Wax Amount	1	0.21	0.01	1.01	0.40
	Humidity Avg	1	0.47	0.03	2.31	0.14
	BreedingStage	1	0.57	0.03	2.73	0.07
	Temperature Avg	**1**	**0.31**	**0.02**	**1.49**	**0.05**
	Sex	**1**	**1.57**	**0.10**	**7.55**	**< 0.01**

Df = degrees of freedom; Sum Of Sqs = sum of squares

Differences between feather and nest samples

We found a significant difference in feather alpha diversity between sample types, with feather samples having significantly greater alpha diversity compared to nest samples (LMM: estimate (*SE*) = 1.50 (0.10), $F_{1,28}$ = 228.53, *P* < 0.01; Fig. 5a). We also found a significant difference in feather beta diversity between sample types (perMANOVA: pseudo-*F* = 10.49, R^2 = 0.15, *P* < 0.01) and observed multivariate homogeneity of group dispersion (perMDISP: pseudo-*F* = 2.29, *P* = 0.14), providing support for a difference in centroid rather than dispersion between the two sample types (Fig. 5b). LEfSE analysis identified 22 differentially abundant microbial classes between sample types, with 18 being enriched in feather samples and 4 in nest samples (all *P* < 0.05). Among them, Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, Clostridia,

Bacteroidia, Negativicutes, Coriobacteriia, Cyanobacteriia, Thermoleophilia, Fusobacteriia, Desulfovibrionia, Spirochaetia, Myxococcia, Campylobacteria, Verrucomicrobiae, Phycisphaerae, Saccharimonadia and Acidimicrobiia were more abundant in feather samples, and Bacilli, Desulfuromonadia, Deinococci and an unidentified class in nest samples.

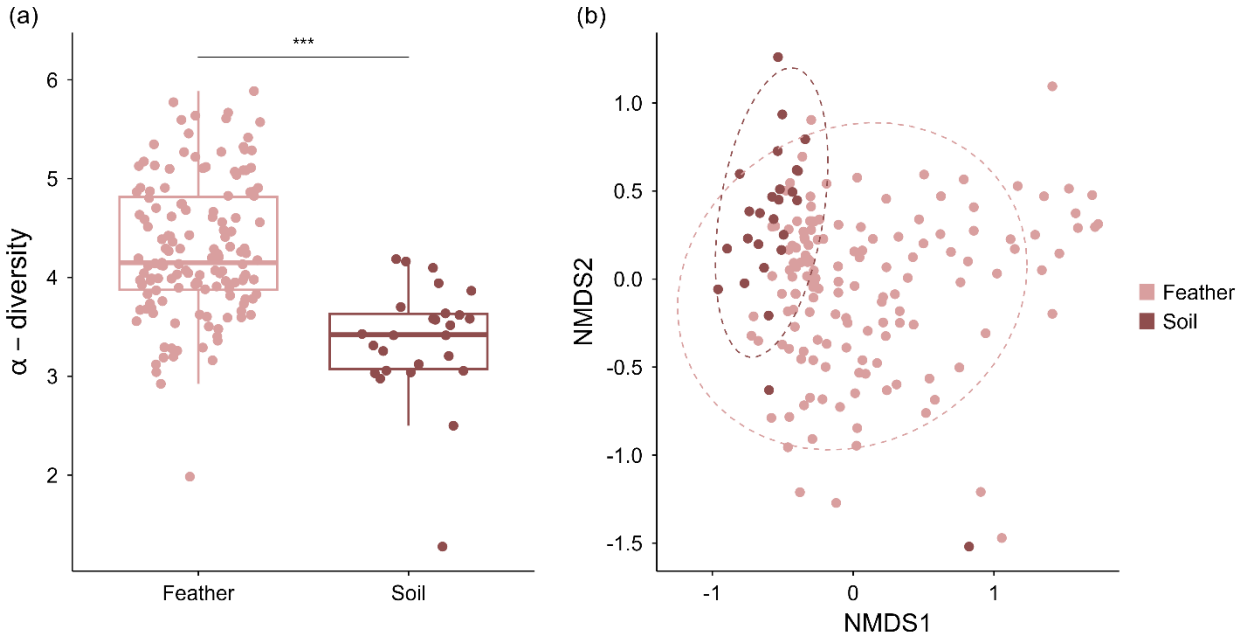

Figure 5. Plots showing variations in plumage bacterial diversity and composition between feather and nest samples. (a) Boxplots of Shannon Diversity Index and (b) NMDS plots based on Bray-Curtis Dissimilarity (stress value = 0.18) comparing plumage bacterial diversity and composition between feather and nest samples. Each dot represents a sample, each ellipse the 95 % confidence interval, and colors denote sample type. Significance (*) was determined by statistical analyses.

Discussion

The strong selective pressures from microbial communities inhabiting avian plumage have prompted our investigation into the individual and environmental (climatic and social) factors influencing a bird's exposure to environmental microorganisms, as well as the physiological mechanisms it has evolved to shape them. Investigating how these factors shape the plumage bacterial diversity and composition enabled us to highlight an age-specific plumage microbiota, and a body part-specific plumage microbiota in certain age classes. We also found that individual factors, such as sex, breeding stage and their interaction, and a few environmental factors, such as ambient temperature and humidity, mainly influenced plumage microbiota in nestlings and adults. Whereas melanin-based plumage traits and preen wax amount did not influence it in feathered individuals. We ultimately highlighted a different (but overlapping) microbiota between sample types, and we identified Firmicutes, Proteobacteria, Actinobacteriota and Bacteroidota as the four predominant phyla in both feather and nest samples (consistent with S. Jacob *et al.*, 2018; Leclaire *et al.*, 2019; van Veelen *et al.*, 2017).

Our study revealed an age-specific plumage microbiota, motivating us to consider age classes separately in our main analyses. Adults had greater bacterial diversity compared to nestlings and fledglings, and nestlings slightly greater bacterial diversity compared to fledglings. Also, bacterial composition varied among age classes. Plumage age and exposure to the nest environment might have resulted in an age-specific plumage microbiota. Fledglings recently replaced their down with new feathers. Fledgling feathers were then only briefly in contact with nest bacterial diversity when sampling unlike nestling down or adult feathers. Greater bacterial diversity in adults might alternatively have reflected exposure to the various habitats encountered during foraging trips (Alt *et al.*, 2015; Saag, Tilgar, *et al.*, 2011; see Corl *et al.*, 2020 for cloacal microbiota). Behavioral, morphological and physiological differences, *e.g.* in feather type, preening behavior or daily activities, might also have resulted in differences in bacterial diversity and composition among age classes.

Bird bodies harbor diverse microenvironments with unique pH, temperature, humidity and secretion types (as outlined by Leclaire *et al.*, 2019) which may favor distinct microbial niches. Previous studies have indeed highlighted differences among cloaca, skin and feather microbiota (Leclaire *et al.*, 2019; Pearce *et al.*, 2017; Silva *et al.*, 2022), while others have not (Engel *et al.*, 2018; van Veelen *et al.*, 2017). With a view to provide new insights into such contrasting results, our study investigated but did not reveal a body part-specific plumage microbiota within feathered individuals. In our study population, barn owls display variation in melanin-based plumage coloration and spottiness among body parts. Breast and belly feathers typically

range from immaculate white to spotted reddish-brown, whereas back feathers from gray to black (Roulin, 2020). Body parts also differ in contact with nest material, the belly being in constant contact with nest microorganisms, or in accessibility during preening. However, we do not know precisely which body parts are most often preened or covered with greater preen wax amount. Contrary to our predictions, our findings might support an alternative hypothesis, the *whole-body skin microbiome hypothesis* (Engel *et al.*, 2018), that a whole-body plumage microbiota can produce odors conveying individual-specific information (*e.g.* fitness relevant information; Maraci *et al.*, 2018). Future studies should investigate plumage microbiota similarity within an individual's body parts or between nestling and fledgling stages to support this hypothesis. While our study did not reveal a body part-specific plumage microbiota in fledglings and adults, it surprisingly found nestling back to be more diverse than the breast and belly, and the belly than the breast. Greater bacterial diversity in nestling back might have reflected a preference for allopreening in this area (Roulin *et al.*, 2016).

Our study not only considered age classes separately and body parts collectively, but it also revealed that individual factors, such as sex, breeding stage and their interaction, mainly influenced plumage microbiota in adults. In short, incubating females had lower bacterial diversity compared to males at the same stage, and to both rearing males and females. Females and males also harbored a distinct bacterial composition. During reproduction, male and female barn owls invest differently in parental care. Females care for the eggs, offspring and themselves in constant contact with nest microorganisms, while males provide them with food (Roulin, 2020). In contrast to females, males encounter a greater bacterial diversity when foraging in various habitats (Alt *et al.*, 2015; Saag, Tilgar, *et al.*, 2011; see Corl *et al.*, 2020 for cloacal microbiota). Greater bacterial diversity in rearing females compared to incubating females also supports our interpretation, as rearing females explore new nesting sites at that time. In addition to behavioral differences, males and females also exhibit physiological differences (*e.g.* sex or stress hormones, preen gland traits; Chapter 1), which might have resulted in sex differences in bacterial diversity and composition throughout the breeding season (S. Jacob *et al.*, 2018). Finally, barn owl pairs only partially share their nest (Roulin, 2020), which minimizes the microbial exchanges between them.

Interestingly, another of our studies revealed an inverse pattern in preen wax secretion. Incubating females secreted more preen wax (but had lower bacterial diversity) compared to males at the same stage, and to both rearing males and females (Chapter 1). Greater preen wax amount might have induced the lower plumage bacterial diversity we observed in incubating females. Preen wax can indeed form a protective physical barrier (S. Jacob *et al.*, 2018; Reneerkens *et al.*, 2008; Verea *et al.*, 2017), have a promicrobial action

promoting mutualists and commensals able to compete with pathogens for trophic resources and ecological niches (as proposed by S. Jacob *et al.*, 2018; S. Jacob, Immer, *et al.*, 2014; Soler *et al.*, 2010), or a direct antimicrobial action preventing new pathogens from colonizing feather surfaces (Braun *et al.*, 2018; Carneiro *et al.*, 2020; Soini *et al.*, 2007). However, we found no direct association between preen wax amount and plumage bacterial diversity or composition in feathered individuals.

Birds may have evolved a melanized plumage as an adaptation to environmental microorganisms. Melanin pigments can indeed strengthen biological structures and help protect the plumage from damage caused by FDB (Justyn *et al.*, 2017; Ruiz-De-Castañeda *et al.*, 2012; but see Al Rubaiee *et al.*, 2021). As females tend to be more melanized than males in our study population, sex differences in melanin-based plumage coloration and spottiness might have resulted in sex and breeding stage differences in plumage microbiota. However, we found no association between melanin-based plumage traits and bacterial diversity or composition in feathered individuals. Contrary to our predictions, lighter owls may have emphasized self-preening behavior to maintain comparable bacterial diversity and composition to darker owls (Roulin, 2007). So, neither preen wax amount nor melanin-based plumage traits appear to explain sex and breeding stage differences in plumage microbiota.

Unlike preen wax secretion and melanin-based plumage traits, environmental factors can provide favorable conditions for microbial growth, proliferation and transmission (Burtt Jr. & Ichida, 2004). Nestlings lacking melanin-based feathers and not yet preening (*i.e.* applying preen wax), nestling down may be more susceptible to warm and humid conditions – as well as to the environmental microorganisms they promote – than fledgling and adult feathers. Consistent with this, our study revealed that both climatic and social factors, namely ambient temperature and humidity and brood size, mainly influenced plumage bacterial composition (but not diversity) in nestlings. Ambient temperature (but not humidity) also influenced plumage bacterial composition in adults, emphasizing the need to further investigate how climatic factors affect plumage microbiota.

Due to our interest in the underlying mechanisms shaping plumage microbial communities, our study ultimately compared feather and nest microbiota, and revealed greater bacterial diversity in feather compared to nest samples as well as different (but overlapping) bacterial composition. About 98 % of ASVs found in nest material were also identified on feathers, while only about 20 % of ASVs found on feathers were identified in nest material. Nest material appeared to play a major role in shaping plumage microbiota (Goodenough *et al.*, 2017; S. Jacob, Immer, *et al.*, 2014; but see Bisson *et al.*, 2007, 2009). With this in mind, future studies should explore how barn owls acquire the plumage microbiota.

In summary, our findings revealed that barn owl plumage microbiota is shaped by a few factors (if any in fledglings). Sex, and sex depending on breeding stages in adults, are not only the most important factors influencing preen gland morphology and physiology (Chapter 1) but also plumage microbiota. Sex-specific behavior and physiology appear to consistently control host-microorganism interactions throughout the breeding season in our study species. Environmental (climatic and social) factors also influenced plumage microbiota to a minor extent. Future studies should consider melanin-based plumage traits and environmental factors together to understand whether they interact with each other and with plumage microbiota. Conducting a similar study on another color-polymorphic bird species would also help confirm our findings and shed light on host-microorganism dynamics.

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Chapter 3

Limited effect of preen wax lipids in regulating microbial communities on barn owl (*Tyto alba*) plumage

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Author Contributions

L.A.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. A-L.D.: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing. G.B.R.: Methodology, Investigation, Resources. D.S.: Methodology, Investigation, Resources. L.M.S-J.: Conceptualization, Methodology, Validation, Writing – review & editing. A.R.: Conceptualization, Methodology, Validation, Writing – review & editing, Funding acquisition.

Abstract

Preen wax is a widely-studied avian secretion coated on plumage to ensure a wide range of biological functions. Certain preen wax compounds may serve as antimicrobial defense and regulate microbial communities on plumage, while others may perform functions such as signaling and communication. However, the mechanisms of action and chemical compounds underlying the defensive and regulatory functions of preen wax are not fully understood to date. Hence, our study aimed to investigate whether and through which mechanism(s) of action preen wax' lipid composition regulates the microbial communities inhabiting barn owl (*Tyto alba*) plumage. Contrary to our expectation, we found only a very weak (if any) association between preen wax (lipid) and feather microbiota composition among fledglings and adults. Although preen wax may still play a role, our findings challenge the idea that its lipid composition mainly regulates the microbial communities on plumage. Instead, feather microbiota may be influenced by other factors, preen wax may still serve as antimicrobial defense through alternative mechanisms or perform functions such as signaling and communication in the barn owl. Therefore, our study helps to better understand the defensive and regulatory functions of preen wax in birds.

Keywords: antimicrobial defense, beta diversity, breeding stage, feather microbiota, gas chromatography-mass spectrometry, high-throughput sequencing, uropygial gland

Introduction

Interactions between host organisms and the microbial communities they harbor are complex and multifaceted. Maintaining microbial community stability is essential for host health and adaptation to environmental challenges, as these communities play a fundamental role in various biological processes (McFall-Ngai *et al.*, 2013). Beneficial microorganisms do indeed participate in host metabolism, behavior and protection against parasites and pathogens through bacterial interference (Archie & Theis, 2011; Ezenwa *et al.*, 2012; Fraune & Bosch, 2010). Bacterial interference refers to mutualists and commensals hindering the growth or establishment of pathogens through the production of antibiotics, alteration of the environment, competition for resources, or stimulation of host immune system (Soler *et al.*, 2010). Conversely, pathogenic microorganisms can cause dysbioses, diseases, and even death and thus exert strong selective pressures on host life-history traits and fitness. In response, hosts have evolved behavioral and physiological strategies (*e.g.* reproductive site selection, social distancing, grooming behavior, or release of chemical secretions; Curtis, 2014; Sarabian *et al.*, 2018) aimed at maintaining microbial community stability. For instance, certain endogenous and exogenous secretions produce antimicrobial substances or establish environments conducive to beneficial microorganisms while inhospitable to pathogenic ones (Akat *et al.*, 2022), making our understanding of how these secretions interact with microbial communities relevant to host ecology and evolutionary biology.

Preen wax, a widely-studied avian secretion, may be coated onto plumage to ensure maintenance, waterproofing, communication, antimicrobial defense, as well as regulation of microbial communities inhabiting it (reviewed in Grieves *et al.*, 2022; Moreno-Rueda, 2017). Several studies support the still-controversial hypothesis that preen wax may serve as an antimicrobial defense mechanism designed to regulate the plumage microbial communities. For instance, Møller *et al.* (2009) found a negative correlation between preen gland size and feather-degrading bacterial load in barn swallows (*Hirundo rustica*; but see Fülöp *et al.*, 2016). *In vitro* experiments also showed that preen wax inhibits the growth of naturally occurring bacterial isolates in several bird species (Alt *et al.*, 2020; Reneerkens *et al.*, 2008; Shawkey *et al.*, 2003), while *in vivo* experiments yielded contrasting results (Czirják *et al.*, 2013; Giraudeau *et al.*, 2013). S. Jacob *et al.* (2018) ultimately found a negative correlation between some preen wax chemicals and feather bacterial richness in great tits (*Parus major*), proposing preen wax as a non-specific/broad-spectrum antimicrobial defense mechanism. However, the precise mechanisms of action and chemical compounds underlying the defensive and regulatory functions of preen wax remain generally unresolved across bird species.

Preen wax is mainly composed of lipids (*e.g.* triglycerides, mono- and diester waxes of fatty acids and alcohols), fatty acids, alcohols, hydrocarbons and other organic compounds (Haribal *et al.*, 2005; J. Jacob & Ziswiler, 1982) thought to support its defensive and regulatory functions through (at least) four mechanisms of action (Gunderson, 2008). Firstly, the hydrophobic wax compounds may form a protective physical barrier on feathers preventing pathogens from colonizing and unbalancing the resident microbiota (S. Jacob *et al.*, 2018; Reneerkens *et al.*, 2008; Verea *et al.*, 2017). Secondly, the lipidic wax compounds may serve as energy stores promoting the growth of mutualists and commensals capable of competing with pathogens for trophic resources and ecological niches. Conversely, such a promicrobial action may also promote pathogenic colonization of the plumage (Soler *et al.*, 2010). Thirdly, the lipids, acids, alcohols, proteins and peptides may have a direct antimicrobial action against pathogens (Braun *et al.*, 2018; Carneiro *et al.*, 2020; Soini *et al.*, 2007). Recently, Carneiro *et al.* (2020) reported lysozymes and immunoglobulins Y as antimicrobial proteins in house sparrow (*Passer domesticus*) preen wax. Fourthly, the bacteriocins and other antimicrobial substances originating from symbiotic bacteria living in preen gland may also have a similar antimicrobial action (Martín-Vivaldi *et al.*, 2009, 2010; Soler *et al.*, 2008, 2010). However, such a mechanism has only been demonstrated in Eurasian hoopoes (*Upupa epops*) and green woodhoopoes (*Phoeniculus purpureus*) so far. Consequently, additional research is required to understand the ecological reasons behind selecting specific mechanisms of action and producing specific chemical compounds to regulate the plumage microbial communities.

After examining the individual and environmental (climatic and social) factors associated with preen gland and feather microbiota in Chapters 1 and 2, our current study focuses on understanding whether and through which mechanism(s) of action preen wax' lipid composition regulates the microbial communities inhabiting barn owl (*Tyto alba*) plumage. We selected 27 fledglings and 39 adults from which we sampled preen wax and analyzed the lipid composition using Gas Chromatography-Mass Spectrometry (GC-MS). We also collected body feathers and analyzed the microbial composition using high-throughput 16S rRNA gene sequencing. We considered the whole set of lipid and microbial compounds separately from the most common ones (*i.e.* present in over 90 % of samples) to balance capturing rare compounds with highlighting the biological importance of common ones. We conducted two types of statistical analyses per dataset (all compounds *vs* common compounds) to study the association between preen wax (lipid) and feather microbiota composition among fledglings and adults. We first expect preen wax' lipid composition to regulate the plumage microbial communities to some extent. More specifically, we predicted that greater differences in preen wax composition would result in greater differences in feather microbiota composition

between two samples (our first analysis). We also predicted co-occurrences or co-exclusions between certain lipid and microbial compounds (our second analysis; based on S. Jacob *et al.*, 2018 study) in both fledglings and adults. However, as we lack a detailed taxonomic classification of lipid or microbial compounds, we cannot yet provide more specific expectations. At the same time, preen wax is known to perform additional functions (*e.g.* signaling, communication) which may limit its importance in regulating the plumage microbial communities.

Materials & Methods

Study background

We selected fledgling and adult barn owls from which we collected both preen wax and body feathers of which we analyzed the lipid and microbial composition, respectively (see below). We focused on 27 fledglings and 39 adults sampled during egg incubation or nestling rearing between July 3rd and October 28th, 2019. Full details about our study area and its associated barn owl population can be found in Frey *et al.* (2011) and in Chapters 1 and 2. We ringed birds captured for the first time for identification. We sexed birds from a blood sample using sex-specific molecular markers in the laboratory (Py *et al.*, 2006).

Preen wax collection and analysis

We sampled preen wax by lightly pressing on the preen gland wearing clean gloves. We collected preen wax in 50-µL glass capillaries (Brand GmbH & Co. KG, Wertheim, DE), placed them in opaque 4-mL chromatographic vials sealed with a Teflon faced septum (La-Pha-Pack GmbH, Langerwehe, DE) and added 400 µL of *n*-hexane (for analysis, Sigma-Aldrich Chemie GmbH, Steinheim, DE) and dichloromethane (for analysis, Carl Roth GmbH and Co. KG, Karlsruhe, DE) in a 3:1 ratio to extract organic compounds. We also collected field blanks (*i.e.* empty capillaries placed in chromatographic vials to which solvent was typically added) at each sampling site to detect contamination. We stored preen wax and field blank samples on ice in the field for a few hours, then in a 4°C refrigerator in the laboratory until GC-MS analyses.

Prior to GC-MS analyses, we added 10 µL of *1*-docosanol (Sigma-Aldrich Chemie GmbH, Steinheim, DE) at 4.8 µg/µL to each sample (internal standard), vortexed and transferred 100 µL of the total volume to 1.5-mL chromatographic vials equipped with a glass insert and sealed with a Teflon faced septum (Macherey-Nagel GmbH & Co. KG, Düren, DE; see Burger *et al.*, 2004; Mardon *et al.*, 2010 for a more detailed protocol).

GC-MS analyses were conducted between January and June 2020 at Scitec Research SA (Lausanne, CH). Preen wax and field blank samples were analyzed using a GCMS-QP2010 Ultra instrument (Shimadzu Corporation, Kyoto, JP) coupled with an AOC-20i+s auto-sampler and equipped with an Optima 5-MS column (length = 30.0 m, inner diameter = 0.25 mm, film thickness = 0.25 µm; Macherey-Nagel GmbH & Co. KG, Düren, DE). Helium was used as carrier gas in a linear velocity flow control mode. The injection temperature was set at 320 °C. The oven temperature was set at 140 °C for 2 minutes, increased to 340 °C at 10 °C/min and then was held at 340 °C for 6 minutes, with a total analysis time of 28 minutes per sample. The MS ion

source temperature was set at 230 °C and the MS interface temperature was set at 280 °C. The MS was used in scan acquisition mode. No compounds were detected in the field blanks.

Bioinformatics analyses were conducted, and the identification of preen wax compounds is currently being carried out at the *Centre d'Ecologie Fonctionnelle et Evolutive* (CEFE, Montpellier, FR). Identification pending, we labeled preen wax compounds based on their elution order in GC-MS analyses (from C1 to C192, with C indicating compound).

Feather collection and analysis

We plucked three adjacent feathers from the mid-breast, mid-belly and upper back wearing 70 % ethanol-sterilized gloves. We placed each body part's feathers in sterile zip-lock bags. We stored feather samples on ice in the field for a few hours, then in a -80 °C freezer in the laboratory until DNA extraction.

We incubated feather samples overnight in a lysis solution, homogenized them and extracted total genomic DNA using the ZymoBIOMICS DNA Miniprep Kit (Cat. No. D4300; Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. Library preparation and sequencing were conducted at Fasteris (Fasteris, Plan-les-Ouates, CH). The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were PCR-amplified using the primer pair 341F/805R. Each primer contained an Illumina MiSeq adapter, two to four randomized nucleotides and a unique eight-nucleotide barcode. PCR products were visualized on agarose gels, purified, and 3 ng of each product was pooled into one final library. Libraries were sequenced in a single run on an Illumina MiSeq V3 platform using a 2 × 300-bp paired-end protocol. Full details about feather collection, DNA extraction, library preparation (including PCR protocols and conditions) and sequencing can be found in Chapter 2.

We processed paired-end sequence reads using the *dada2* R package (version 1.20.0) following the DADA2 pipeline (version 1.16; Callahan *et al.*, 2016). Sequence reads were demultiplexed and trimmed for barcodes and adapters at Fasteris. We trimmed primer pairs using the Cutadapt tool (version 3.4; Martin, 2011). We truncated sequence reads based on their per-base quality scores and at the first quality score less than or equal to 2. We discarded sequence reads with ambiguous bases or more than 2 *expected errors* after trimming and truncation. We applied the DADA2 parametric error model and inferred the Amplicon Sequence Variants (ASVs) through the DADA2 core denoising algorithm. We merged the forward and reverse sequence reads and constructed the ASV table. We filtered out chimeric and potential contaminant ASVs and discarded singletons and non-bacterial ASVs (*i.e.* Eukaryota, Chloroplast or Mitochondria). We pooled samples (*i.e.* summed ASV counts) from the three body parts per individual and retained only ASVs with a

relative abundance exceeding 0.001 % per sample. We finally taxonomically classified the ASVs using the DADA2 naïve Bayesian Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007) trained on the SILVA reference database (version 138.1; Quast *et al.*, 2013). Full details about bioinformatics sequence processing (including parameters used in R functions and packages) can be found in Chapter 2.

Ethics

Barn owl monitoring was performed according to a strict animal handling protocol approved by the 'Service de la consommation et des affaires vétérinaires', Switzerland (authorization numbers: VD 3213 and 3462).

Statistical analysis

Statistical analyses were done using R Statistical Software (R version 4.3.1; R Core Team, Vienna, Austria). In order to understand how preen wax' lipid composition regulates the microbial communities inhabiting barn owl plumage, we tested for the association between preen wax (lipid) and feather microbiota composition (hereafter lipid and microbial data, respectively) after removing the effects of sex and breeding stage (hereafter individual data). Microbial and lipid compounds present in none or a single sample were removed, and datasets were converted to relative abundance prior to statistical analyses. We ran the same statistical analyses on four different datasets: (a) all compounds in fledglings (776 ASVs × 164 wax compounds); (b) common compounds (*i.e.* compounds present in over 90 % of samples) in fledglings (573 ASVs × 156 wax compounds); (c) all compounds in adults (1693 ASVs × 153 wax compounds); and (d) common compounds in adults (992 ASVs × 129 wax compounds). Considering common compounds separately from all compounds allows us to balance capturing rare compounds with highlighting the biological importance of common ones. Less common compounds may indeed introduce noise, be inconsequential, and less functionally relevant than common compounds.

For each dataset, we first performed a Multiple Regression on distance Matrices (MRM) using the *MRM* function from the *ecodist* R package (Goslee & Urban, 2007). MRM, a combination of Mantel test and multiple regression, enables traditional regression analysis on two or more distance matrices using permutation tests to assess significance. We computed the Bray-Curtis Dissimilarity Indices on microbial and lipid data (*vegdist* function, *vegan* R package), considering the presence/absence of compounds in each sample, as well as the abundance of compounds shared between any two samples. We included the microbial distances as the response matrix and the lipid distances as the explanatory matrix (with 999 permutations, Pearson correlations and the linear method) in all four MRM.

Secondly, in addition to MRM, we conducted a partial Redundancy Analysis (pRDA) using the *rda* function from the *vegan* R package (Oksanen *et al.*, 2022). RDA, a multivariate multiple regression followed by a Principal Component Analysis (PCA) on the fitted values, finds linear combinations of explanatory variables that best explain the variance in response variables. pRDA also 'partials out' or 'controls for' the effects of some conditioning variables. Preen wax compounds being numerous and interdependent, we first reduced the lipid data using PCAs (*rda* function, *vegan* R package). We considered the first 16 and 20 Principal Components (PCs) as proxies for lipid data in downstream pRDAs in fledglings and adults, respectively. The first PCs accounted for approx. 80 % of the cumulative variance in lipid data (Suppl. Tables 1-2). We included the microbial data (scaled to unit variance) as the community data matrix and the PCs summarizing the lipid data which we previously selected in forward stepwise selection procedures (using permutation-based significance tests and the adjusted R^2 of global models as stopping criteria; *ordiR2step* function, *vegan* R package) as the constraining variables in all four pRDAs. We also added the sex as the conditioning variable in the pRDAs in fledglings, and the sex and breeding stage as the conditioning variables in the pRDAs in adults (*i.e*. variables whose effects are partialled out/controlled for). We assessed multicollinearity among variables using the variance inflation factor (all VIF < 2; *vif* function, *vegan* R package). We partitioned variance in the community data matrix with respect to the constraining and/or conditioning variables based on adjusted R^2 (*varpart* function, *vegan* R package) to estimate the distinct and shared contribution of lipid and individual data in explaining microbial data. Finally, we conducted ANOVA-like permutation tests to assess the significance of each pRDA, as well as each constrained axis and constraining variable (with 999 permutations; *anova* function, *vegan* R package).

Results

Relationships between microbial and lipid distances

MRM performed on all compounds and on common compounds (*i.e.* compounds present in over 90 % of samples) in fledglings explained 0.94 % and 0.96 % of variance in microbial distances, respectively. Microbial distances did not significantly associate with lipid distances in fledglings (*i.e.* fledglings with a similar preen wax composition did not have a similar feather microbiota composition; Table 1; Fig. 1a, 1b). MRM performed on all compounds and on common compounds in adults explained 1.12 % and 0.78 % of variance in microbial distances, respectively. Microbial distances did not significantly associate with lipid distances in adults either (Table 1; Fig. 1c, 1d).

Table 1. Results of Multiple Regressions on distance Matrices (MRM) showing the relationships between microbial and lipid distances (*i.e.* the Bray-Curtis Dissimilarity Indices computed on microbial and lipid data).

Models	**Lipid distances**	***P*-value**	***R²***	***F*-value**
MRM on all compounds in fledglings	-0.24	0.39	0.01	3.29
MRM on common compounds in fledglings	-0.25	0.38	0.01	3.40
MRM on all compounds in adults	0.23	0.19	0.01	8.35
MRM on common compounds in adults	0.20	0.25	0.01	5.83

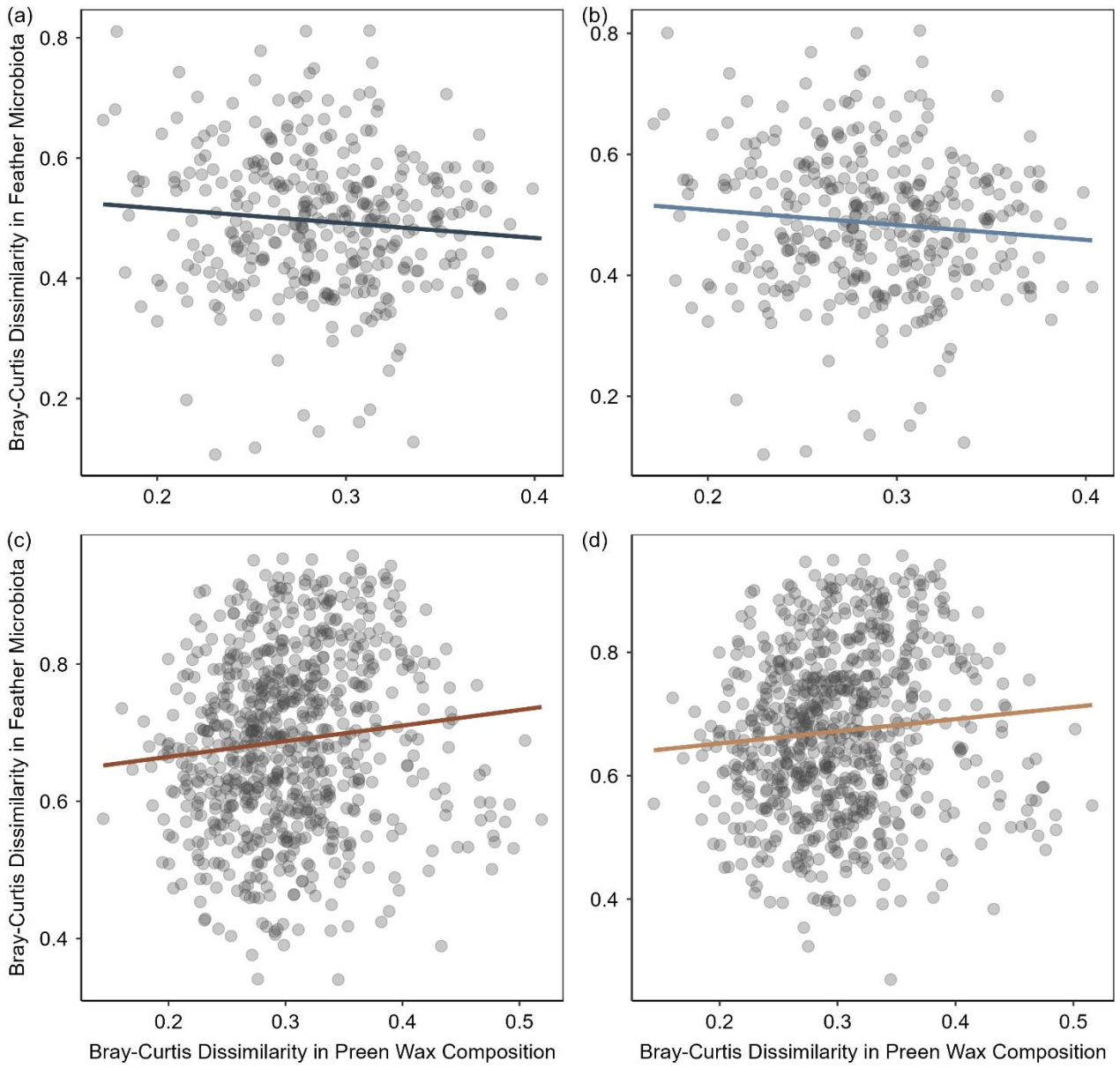

Figure 1. Linear relationships between microbial and lipid distances calculated on (a) all compounds in fledglings; (b) common compounds (*i.e.* compounds present in over 90 % of samples) in fledglings; (c) all compounds in adults; and (d) common compounds in adults.

Disentangling the influence of preen wax' lipid composition, sex and breeding stage on feather microbiota composition

A first RDA conducted on all compounds in fledglings suggested a significant association between microbial data and PC6 summarizing the lipid data (Table 2). PC6 was negatively correlated with C78 and positively correlated with C120 (Suppl. Table 1). However, this association was no longer significant after controlling for the effect of sex, and the variance explained by PC6, whether before or after controlling for sex, consistently remained very small (Table 3a; Fig. 2a, 3a). A second RDA conducted on common compounds (*i.e.* compounds present in over 90 % of samples) also suggested a significant association between microbial data and PC5 summarizing the lipid data (Table 2). PC5 was negatively correlated with C118 and positively correlated with C120 (Suppl. Table 1). Unlike the RDA conducted on all compounds, this association was still significant even after controlling for the effect of sex. However, the variance explained by PC5, whether before or after controlling for sex, consistently remained very small (Table 3b; Fig. 2b, 3b).

A first RDA conducted on all compounds in adults suggested a significant association between microbial data and PC1 and PC2 summarizing the lipid data (Table 2). PC1 was positively correlated with C139 and C144, and PC2 was negatively correlated with C34, C35 and C47 (Suppl. Table 2). This association was still significant even after controlling for the effects of sex and breeding stage. However, the variance explained by PC1 and PC2, whether before or after controlling for sex and breeding stage, consistently remained very small (Table 3c; Fig. 2c, 3c). A second RDA conducted on common compounds also suggested a significant association between microbial data and PC2 summarizing the lipid data (Table 2). PC2 was negatively correlated with C35 (Suppl. Table 2). Like the RDA conducted on all compounds, this association was still significant even after controlling for the effects of sex and breeding stage. However, the variance explained by PC2, whether before or after controlling for sex and breeding stage, consistently remained very small (Table 3d; Fig. 2d, 3d).

Table 2. Significant associations between microbial data and Principal Components (PCs) summarizing the lipid data ($P \leq 0.05$) through Redundancy Analyses (RDAs) with forward stepwise selection procedures.

Models	Predictors	Adj. R^2	Cum. R^2	Df	AIC	*F*-value	*P*-value
RDA on all compounds in fledglings	PC6	0.01	0.01	1	181.26	1.31	**0.04**
RDA on common compounds in fledglings	PC5	0.02	0.02	1	172.90	1.48	**0.02**
RDA on all compounds in adults	PC2	0.02	0.02	1	291.04	1.83	**< 0.01**
	PC1	0.03	0.05	1	291.59	1.36	**0.01**
RDA on common compounds in adults	PC2	0.03	0.03	1	269.75	2.28	**< 0.01**

Table 3. Results of variance partitioning showing the distinct and shared contribution of lipid and individual data in explaining microbial data through partial Redundancy Analyses (pRDAs) on (a) all compounds in fledglings; (b) common compounds (*i.e.* compounds present in over 90 % of samples) in fledglings; (c) all compounds in adults; and (d) common compounds in adults. Proportions of explainable variance and total variance are based on adjusted R^2.

(a) RDA on all compounds in fledglings	Inertia	R^2	Adj. R^2	Statistic (*P*-value)	Expl. var.	Total var.
Full model	62	0.08	0.01	pseudo-*F* = 1.05 (0.29)	1	0.01
Lipid data (PC6)	35	0.05	0.01	pseudo-*F* = 1.19 (0.11)	1	0.01
Individual data (sex)	24	0.03	0	pseudo-*F* = 0.80 (0.94)	0	0
Confounded	3				0	0
Total unexplained	714					0.99
Total inertia	776					1

(b) RDA on common compounds in fledglings	Inertia	R^2	Adj. R^2	Statistic (*P*-value)	Expl. var.	Total var.
Full model	49	0.09	0.01	pseudo-*F* = 1.11 (0.16)	1	0.01
Lipid data (PC5)	29	0.05	0.01	pseudo-*F* = 1.32 (**0.05**)	1	0.01
Individual data (sex)	17	0.03	0	pseudo-*F* = 0.76 (0.96)	0	0
Confounded	3				0	0
Total unexplained	524					0.99
Total inertia	573					1

(c) RDA on all compounds in adults	Inertia	R^2	Adj. R^2	Statistic (*P*-value)	Expl. var.	Total var.
Full model	274	0.16	0.06	pseudo-*F* = 1.64 (**< 0.01**)	1	0.06
Lipid data (PC1 & PC2)	105	0.06	0.01	pseudo-*F* = 1.26 (**< 0.01**)	0.22	0.01
Individual data (sex & breeding stage)	135	0.08	0.03	pseudo-*F* = 1.61 (**< 0.01**)	0.51	0.03
Confounded	34				0.28	0.02
Total unexplained	1419					0.94
Total inertia	1693					1

(d) RDA on common compounds in adults	Inertia	R^2	Adj. R^2	Statistic (*P*-value)	Expl. var.	Total var.
Full model	147	0.15	0.08	pseudo-*F* = 2.04 (**< 0.01**)	1	0.08
Lipid data (PC2)	30	0.03	0.01	pseudo-*F* = 1.26 (**0.03**)	0.09	0.01
Individual data (sex & breeding stage)	90	0.09	0.04	pseudo-*F* = 1.86 (**< 0.01**)	0.57	0.04
Confounded	27				0.34	0.03
Total unexplained	845					0.92
Total inertia	992					1

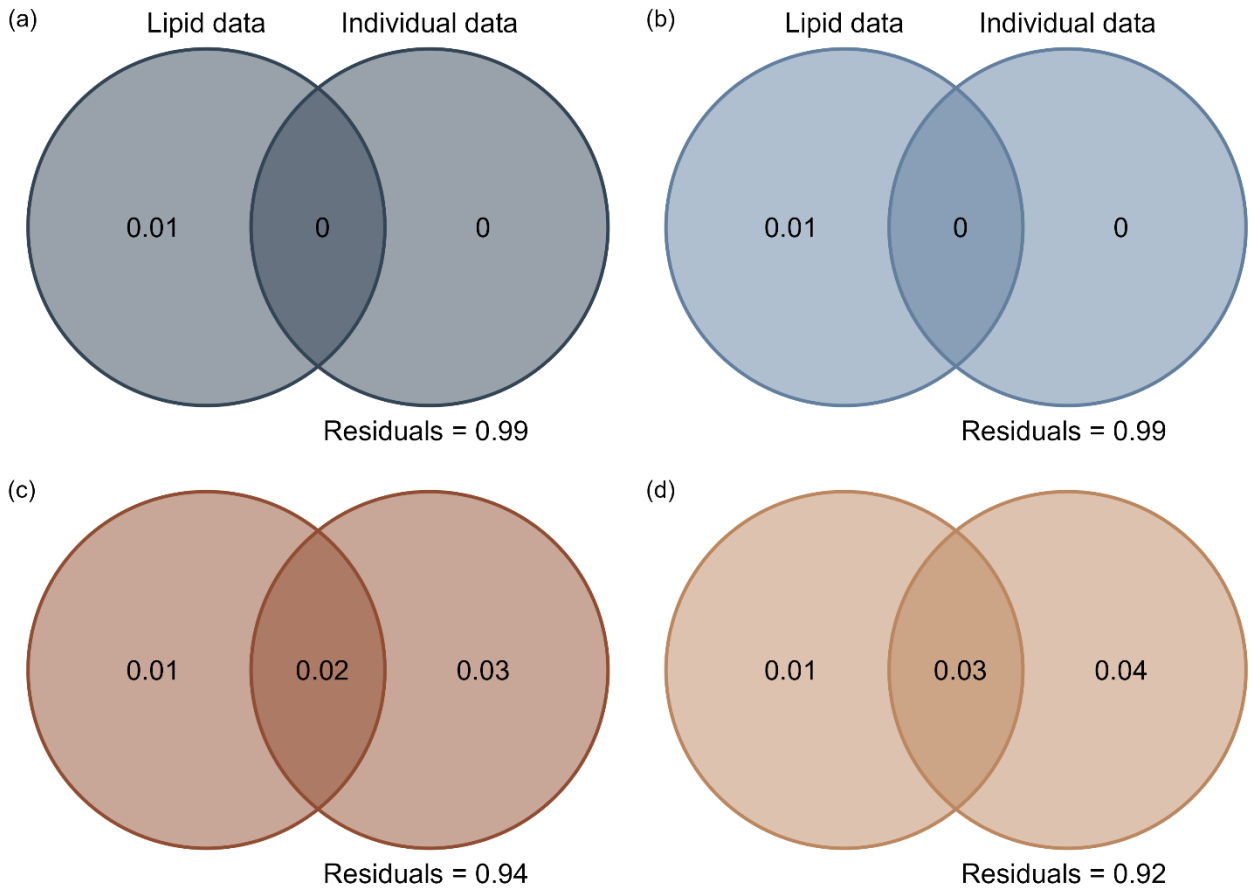

Figure 2. Representation of variance partitioning showing the distinct and shared contribution of lipid and individual data in explaining microbial data through partial Redundancy Analyses (pRDAs) on (a) all compounds in fledglings; (b) common compounds (*i.e.* compounds present in over 90 % of samples) in fledglings; (c) all compounds in adults; and (d) common compounds in adults.

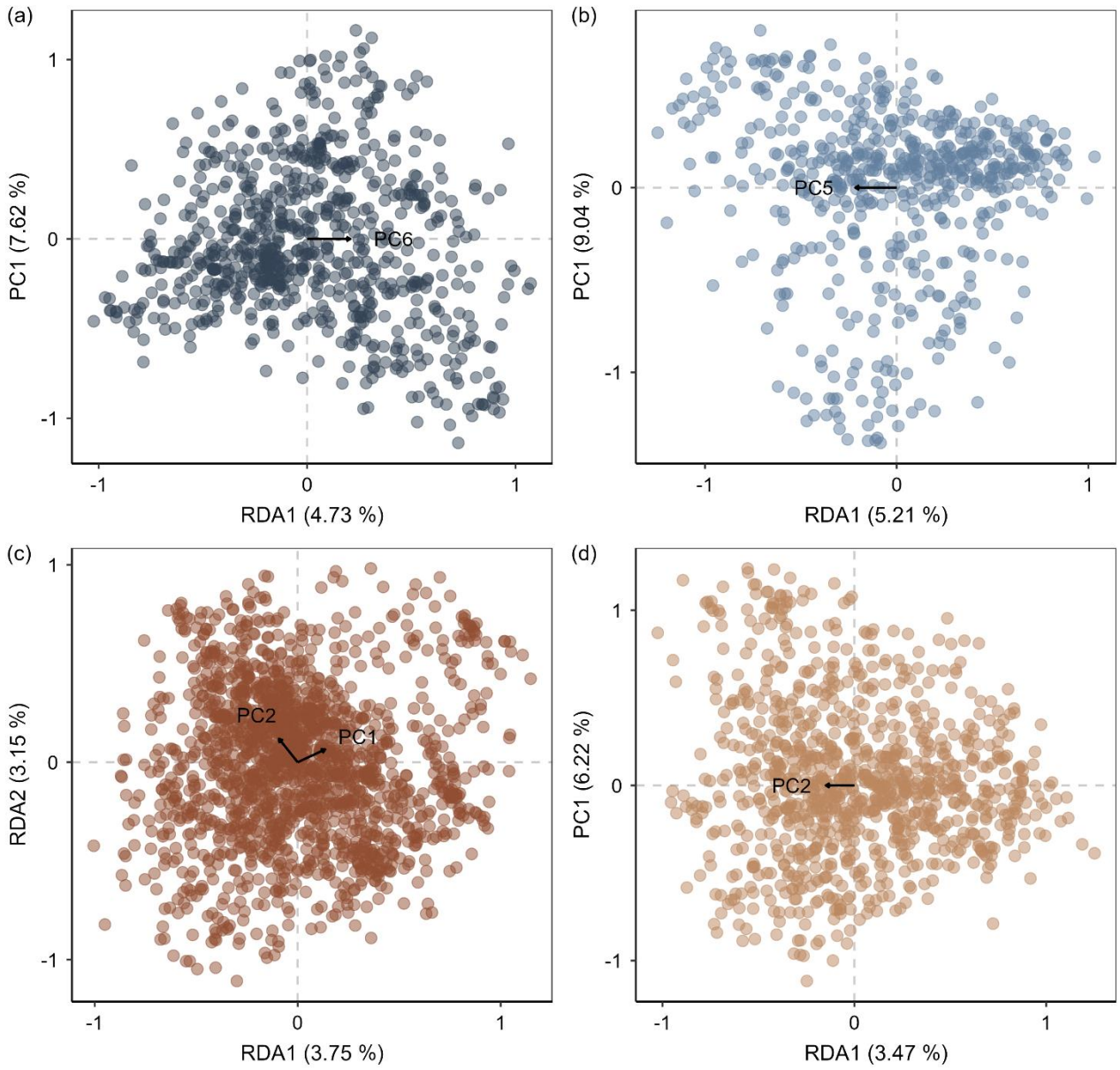

Figure 3. Representation of the first two constrained (RDA) or unconstrained (PC) axes of partial Redundancy Analyses (pRDAs) showing the relationships between microbial and lipid data on (a) all compounds in fledglings; (b) common compounds (*i.e.* compounds present in over 90 % of samples) in fledglings; (c) all compounds in adults; and (d) common compounds in adults (Suppl. Tables 3-4). Each dot represents an ASV and each arrow a Principal Component (PC) summarizing the lipid data.

Discussion

Preen wax is a widely-studied avian secretion for the numerous functions conferred on it (reviewed in Grieves *et al.*, 2022; Moreno-Rueda, 2017). Preen wax may serve as an antimicrobial defense mechanism designed to regulate the microbial communities inhabiting plumage. Various preen wax compounds may indeed support its defensive and regulatory functions through (at least) four mechanisms of action (see *Introduction*; Gunderson, 2008). At the same time, preen wax may perform additional functions such as signaling and communication which may limit its importance in regulating the plumage microbial communities. We therefore investigated in this study whether and through which mechanism(s) of action preen wax' lipid composition regulates the microbial communities inhabiting barn owl plumage. To do so, we analyzed preen wax composition using GC-MS and feather microbiota composition using high-throughput 16S rRNA gene sequencing. We considered the whole set of lipid and microbial compounds separately from the most common ones (*i.e.* present in over 90 % of samples) upon which we conducted two types of statistical analyses. Contrary to our expectation that preen wax' lipid composition would regulate the plumage microbial communities to a given extent, we found only a very weak (if any) association between preen wax (lipid) and feather microbiota composition among fledglings and adults. Although preen wax may still have a role, its lipid composition does not seem to play a significant one in regulating the microbial communities inhabiting barn owl plumage.

Our first analysis, the MRM performed on all compounds and on common compounds, showed no correlation between lipid and microbial distances, indicating that similar preen wax compositions do not necessarily entail similar feather microbiota compositions among fledglings or adults. This first result suggests that preen wax' lipid composition has little (if any) effect in regulating the plumage microbial communities. Our second analysis, the RDAs conducted on all compounds and on common compounds, yielded fairly similar results. Similarly to the MRM, the RDAs showed no clear association between microbial and lipid data. However, they revealed that some RDA axes summarizing the PCs summarizing the lipid data were positively or negatively correlated with certain ASVs among both age classes. This second result suggests that preen wax' lipid composition regulates a very few specific microbial compounds, but as suggested by the MRM, does not seem to serve as a key antimicrobial defense mechanism designed to regulate the microbial communities inhabiting barn owl plumage. Also, both MRM and RDAs, whether carried out on all compounds or on common compounds only, yielded no significant differences in results, supporting that less common compounds seem to be inconsequential and less functionally relevant than common ones (but see RDAs conducted in fledglings).

Unanimously, our findings challenge the idea that preen wax' lipid composition primarily regulates the plumage microbial communities (in agreement with Czirják *et al.*, 2013; Giraudeau *et al.*, 2013), highlighting that (1) feather microbiota may be influenced by other factors, (2) preen wax may still serve as antimicrobial defense through alternative mechanisms, or (3) preen wax may perform additional functions in the barn owl. Firstly, barn owls may not only regulate the microbial communities inhabiting their plumage through preening or preen wax application (S. Jacob *et al.*, 2018) but also rely on other strategies, explaining the weak or lack of association we found between preen wax (lipid) and feather microbiota composition. For instance, skin-derived lipids (*e.g.* free fatty acids and alcohols) may replace or support preen wax lipids in regulating the plumage microbial communities (as proposed by Braun *et al.*, 2018). Feather melanin may also contribute to influencing feather microbiota. Al Rubaiee *et al.* (2021) indeed found greater microbial abundance and diversity on melanized feather areas compared to unmelanized ones. Nevertheless, we found no association between melanin-based plumage traits and plumage bacterial diversity and composition in the barn owl (Chapter 2). Feather and preen gland microbiota may also contribute to it through bacterial interference, *i.e.* by hindering the growth or establishment of pathogens on feathers through the production of antibiotics, alteration of the environment, or competition for trophic resources and ecological niches (Soler *et al.*, 2010). Finally, annual molting was shown to reduce the pathogen load on plumage (Clayton *et al.*, 2010; Gunderson, 2008).

Secondly, preen wax may still serve as an antimicrobial defense mechanism designed to regulate the microbial communities inhabiting barn owl plumage but through other mechanisms of action than its lipidic nature. Certain preen wax compounds, such as acids, alcohols, proteins and peptides, may have a direct antimicrobial action against pathogens (Braun *et al.*, 2018; Carneiro *et al.*, 2020; J. Jacob *et al.*, 1997; Soini *et al.*, 2007). Not only did Carneiro *et al.* (2020) report lysozymes and immunoglobulins Y in house sparrow preen wax, we also found immune-related peptides and proteins in barn owl preen wax (Chapter 4). Bacteriocins and other antimicrobial substances originating from symbiotic bacteria living in preen gland may also have a similar antimicrobial action (Martín-Vivaldi *et al.*, 2009, 2010; Soler *et al.*, 2008, 2010). Yet, the preen gland microbiota has been little studied in the barn owl so far (Braun, Wang, Zimmermann, Boutin, *et al.*, 2019; Braun, Wang, Zimmermann, Wagner, *et al.*, 2019). However, gaining insight into the defensive and regulatory functions of preen wax would require taxonomically classifying the microbial and lipid compounds found on feathers and in preen gland.

Thirdly, barn owls may not only apply preen wax to regulate the microbial communities inhabiting their plumage in response to selective pressures (Moreno-Rueda, 2017) but also do so for signaling or

communication purposes. Preen wax may indeed function as (chemo)signals enabling visual signaling, olfactory camouflage, and visual/olfactory recognition and communication among conspecifics, mates or parents-offspring (*i.e. sex semiochemical hypothesis*; Grieves *et al.*, 2022; Whittaker & Hagelin, 2021). Yet, the importance of visual or olfactory communication remains little known in the barn owl.

In summary, our study does not support the idea that preen wax' lipid composition primarily regulates the microbial communities inhabiting barn owl plumage. Our analyses showed no clear association between lipid and microbial data among fledglings or adults, disregarding that preen wax' lipid composition may serve as an antimicrobial defense mechanism. Nevertheless, our findings suggest that feather microbiota may be influenced by other factors, that preen wax may still serve as antimicrobial defense through alternative mechanisms, or that preen wax may perform additional functions (signaling, communication) in the barn owl. Alternatively, the environmental and plumage microbial communities may in turn regulate/induce changes in preen gland traits (S. Jacob *et al.*, 2014; Leclaire *et al.*, 2014). Future experimental studies are however required before completely refuting the defensive and regulatory functions of barn owl's preen wax.

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Supplementary Material

Table S1. Correlations between the first 16 Principal Components (PCs) and the preen wax compounds based on a 2.5 standard deviation cut-off using all compound and common compound data (*i.e.* compounds present in over 90 % of samples) in fledglings.

PC axes	Wax compounds	Loadings using all compound data	Loadings using common compound data
PC2	C3		1.57
PC3	C100	1.87	1.83
PC3	C102	-1.45	
PC4	C79		-1.61
PC4	C146	-1.57	
PC5	C118		-1.66
PC5	C120		1.73
PC6	C78	-1.66	
PC6	C120	1.70	
PC6	C126		1.63
PC7	C71	1.60	
PC7	C132	-1.90	
PC7	C137	-1.72	
PC7	C143		-1.75
PC8	C33	-1.61	
PC8	C51	-1.62	
PC8	C132		1.74
PC8	C186		-1.67
PC9	C31	1.50	-1.74
PC9	C142	1.51	-1.59
PC10	C190	1.65	
PC11	C22		1.73
PC11	C62		1.69
PC11	C89	-1.52	-1.81
PC11	C95	-1.51	
PC11	C109	1.94	
PC11	C129		-1.84
PC12	C22	-2.41	-1.73
PC12	C62	-1.86	
PC13	C91	1.70	
PC13	C99	-1.78	
PC13	C102	1.67	
PC13	C123		1.61
PC14	C41	-1.67	-1.67

PC14	C97	1.67	1.78
PC15	C68	1.76	
PC15	C114	-1.58	
PC16	C114	-1.59	

Table S2. Correlations between the first 16 Principal Components (PCs) and the preen wax compounds based on a 2.5 standard deviation cut-off using all compound and common compound data (*i.e.* compounds present in over 90 % of samples) in adults.

PC axes	Wax compounds	Loadings using all compound data	Loadings using common compound data
PC1	C139	1.32	1.53
PC1	C144	1.29	
PC2	C34	-1.95	
PC2	C35	-2.02	-2.11
PC2	C47	-1.93	
PC3	C112		1.99
PC4	C25		-1.83
PC4	C79	1.84	
PC4	C88	1.92	
PC4	C107		-1.84
PC4	C124		-1.88
PC4	C159	1.99	
PC4	C188	2.09	
PC5	C80	-1.77	
PC6	C81		1.96
PC7	C107	-1.90	
PC8	C33		-1.97
PC8	C45		-2.08
PC8	C142	1.71	
PC9	C50	-1.99	
PC9	C89		1.95
PC9	C95	-1.96	
PC9	C102		1.97
PC10	C58		-1.89
PC11	C55		1.96
PC11	C84	1.99	
PC11	C173	1.86	
PC12	C135		-2.01

PC12	C146	2.02	
PC12	C162	-2.60	
PC12	C191	1.92	
PC13	C83		1.86
PC13	C85	1.94	
PC13	C135	2.03	
PC14	C57	-1.84	
PC14	C67		2.37
PC14	C71	1.90	
PC15	C13		-2.24
PC15	C129		-2.28
PC16	C102	1.76	
PC16	C105	1.86	
PC16	C126		-2.35
PC16	C170	-2.05	
PC17	C2	1.89	
PC17	C83	2.25	
PC17	C186		1.87
PC18	C152		-2.39
PC18	C190	-1.83	
PC19	C10	1.91	
PC19	C93		-2.32
PC19	C126	2.22	
PC20	C37	-2.28	
PC20	C118		-2
PC20	C128		2.08
PC20	C168	1.98	

Table S3. Correlations between the first Redundancy Axis and the ASVs based on a 2.5 standard deviation cut-off using all compound and common compound data (*i.e.* compounds present in over 90 % of samples) in fledglings.

RDA axes	ASVs	Loadings using all compound data	Loadings using common compound data
RDA1	ASV343		-1.25
RDA1	ASV655		-1.20

Table S4. Correlations between the first two Redundancy Axes and the ASVs based on a 2.5 standard deviation cut-off using all compound and common compound data (*i.e.* compounds present in over 90 % of samples) in adults.

RDA axes	ASVs	Loadings using all compound data	Loadings using common compound data
RDA1	ASV302	1.00	
RDA1	ASV471	1.05	
RDA1	ASV660	1.04	
RDA1	ASV1119		1.19
RDA1	ASV1187	-1.00	
RDA1	ASV1223	1.05	
RDA1	ASV2118	1.02	
RDA1	ASV2500	1.11	
RDA1	ASV2649		1.25
RDA1	ASV2783	1.04	
RDA1	ASV2899	1.15	
RDA1	ASV3149	1.02	
RDA1	ASV3989	1.05	
RDA2	ASV726	-1.04	
RDA2	ASV1249	-0.98	
RDA2	ASV1484	-1.05	
RDA2	ASV1768	0.98	
RDA2	ASV1859	-1.01	
RDA2	ASV2021	-0.99	
RDA2	ASV2152	-1.01	
RDA2	ASV2658	-1.11	
RDA2	ASV2806	0.97	
RDA2	ASV3264	-0.98	

Chapter 4

No evidence of preen wax proteome adapting to breeding duties in female barn owls (*Tyto alba*)

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Status

This chapter has not yet been submitted to a journal.

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Author Contributions

L.A.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. A-L.D.: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing. L.M.S-J.: Conceptualization, Methodology, Validation, Writing – review & editing. A.R.: Conceptualization, Methodology, Validation, Writing – review & editing, Funding acquisition.

Abstract

Avian reproduction encompasses challenges and trade-offs, such as egg incubation and nestling rearing in contact with nest and environmental microorganisms. Birds adapt to selective pressures they experience at that time through specific adjustments. For instance, preen wax reportedly protects eggshells, down feathers and adult plumage. However, the mechanisms of action underlying these adaptations still require investigation. Our study proposed the proteome as a possible mechanism of action for conferring and adjusting the protective function of preen wax in incubating and rearing female barn owls (*Tyto alba*). While it revealed immune-related peptides and proteins in preen wax, our study did not find any showing significant differential expression across breeding stages. Only one lipid transport protein, the apolipoprotein A-IV, was up-regulated during egg incubation. Also, peptide- and protein-related genes were found to be primarily associated with organic substance metabolic process, primary metabolic process and cellular metabolic process, involved in protein binding, organic cyclic compound binding and heterocyclic compound binding, and localized in intracellular anatomical structure, cytoplasm and organelle. Our study thus helps understand how female barn owls adjust preen wax proteome to reproduction timing and its associated breeding duties.

Keywords: antimicrobial function, egg incubation, immunity, microorganisms, nestling rearing, peptides, proteins, uropygial gland

Introduction

Birds consistently interact with the environment, exchanging diverse microorganisms ranging from beneficial to pathogenic ones. Beneficial microorganisms can hinder the growth or establishment of pathogens through the production of antibiotics, alteration of the environment, competition for resources, or stimulation of bird immune system (Soler *et al.*, 2010; Zepeda Mendoza *et al.*, 2018). Alternatively, pathogenic microorganisms can cause dysbioses, diseases, and even death, exerting strong selective pressures on bird health, life-history traits and fitness (Benskin *et al.*, 2009). To prevent any dysbioses, birds have developed a set of behavioral, morphological and physiological strategies – the mechanisms of which have not yet been fully elucidated to date – to protect their eggs, offspring and themselves (Clayton *et al.*, 2010).

Aside from strategically selecting or building nesting sites, birds actively protect their eggs, offspring and themselves by cleaning and coating them(selves) with antimicrobial materials or substances (Bush & Clayton, 2018; Clayton *et al.*, 2010; Gunderson, 2008). During preening, birds not only physically remove pathogens and parasites but also apply preen wax onto eggshells, down feathers and their own plumage to shape the resident microbiota (reviewed in Moreno-Rueda, 2017). Preen wax, an oily substance mainly composed of wax esters (*i.e.* fatty acids esterified to fatty alcohols; J. Jacob & Ziswiler, 1982), has however yielded controversial results in experimental studies regarding its purported protective function. *In vitro* studies tend to support the protective function of preen wax (Shawkey *et al.*, 2003) while *in vivo* studies have not yet provided sufficient evidence to support it (Czirják *et al.*, 2013; Giraudeau *et al.*, 2013, 2014).

Such divergence emphasizes the complexity in studying the protective function of preen wax. Unlike symbiotic microorganisms which are well-known for producing bacteriocins and antimicrobial volatile substances in hoopoe preen gland (Martín-Vivaldi *et al.*, 2009, 2010; Soler *et al.*, 2008, 2010), the specific mechanisms of action and chemical compounds underlying the protective function of preen wax need to be identified in other bird species (Braun *et al.*, 2018; Carneiro *et al.*, 2020; Soini *et al.*, 2007). Recently, Carneiro *et al.* (2020) successfully reported lysozymes and immunoglobulins Y – one innate and one adaptive immune protein – as potential antimicrobial agents in house sparrow (*Passer domesticus*) preen wax. In light of this study, both peptides and proteins – chains of amino acids varying in size – emerge as potential candidates for conferring the protective function purported to preen wax (Braun *et al.*, 2018; Carneiro *et al.*, 2020). Antimicrobial peptides and proteins are indeed able to disrupt the cellular membranes of microorganisms, bind to intracellular targets and interfere with essential cellular processes, or stimulate and modulate the

specific immune response in birds (Huan *et al.*, 2020; Q.-Y. Zhang *et al.*, 2021; see Cuperus *et al.*, 2013; Moreau *et al.*, 2022 for birds).

Our study precisely examined the proteome as a possible mechanism of action for conferring and adjusting the protective function of preen wax to reproduction timing. We analyzed preen wax samples collected from 20 female barn owls (*Tyto alba*) during egg incubation or nestling rearing using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). We identified and quantified the peptides and proteins detected, and extracted the Gene Ontology (GO; including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)) of these peptide- and protein-related genes. We tested for differential enrichment in (immune-related) peptides and proteins across breeding stages (egg incubation and nestling rearing), and of GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among peptide- and protein-related genes (see *Results* for further details).

Prior studies have shown variations in preen gland traits or feather microbial communities across breeding stages. For instance, Golüke & Caspers (2017) observed an increase in preen gland size as the breeding season progresses followed by a subsequent decrease in zebra finches (*Taeniopygia guttata*). Saag *et al.* (2011) noted a decrease in plumage bacterial load from nest-building to brood stage in female great tits (*Parus major*). We have also shown such variations between incubating and rearing female barn owls (Chapters 1 and 2). With this in mind, we expected female barn owls to adjust preen wax proteome to reproduction timing and the selective pressures they experience at that time (*e.g.* preening needs, exposure to nest/environmental microorganisms). However, we were unable to predict which (immune-related) peptides or proteins might have been up- or down-regulated in either breeding stage prior to conducting this study. Being the first to shed light on the untargeted preen wax proteome and its adjustment to reproduction timing (to the best of our knowledge), our study helps understand how female barn owls adjust the protective function of preen wax to breeding duties-associated selective pressures.

Materials & Methods

Sample collection

In order to study the protective function of preen wax, we selected 20 female barn owls captured between April and August 2021 for the study described in Chapter 1 (see Frey *et al.*, 2011 for a detailed description of the study area). Although preen gland activity also differs between breeding stages in males (Chapter 1), we only sampled females in this study due to the large amount of preen wax required for proteomic analysis. We sampled preen wax on incubating (*N* = 12) and rearing females (*N* = 8) by gently pressing on the preen gland with a gloved thumb until emptying it. We collected preen wax in 50-µL glass capillaries (Brand GmbH & Co. KG, Wertheim, DE) which we subsequently transferred into 1.5-mL Eppendorf tubes. Immediately after sampling, we measured the amount of preen wax collected in the capillary(ies) using a caliper to the nearest 0.1 mm (*range*, *mean* ± *standard error* [*SE*]: from 33.6 to 101.9 µL, 60.5 ± 4.3 µL). We stored preen wax samples in dry ice in the field for a few hours, then in a -80 °C freezer in the laboratory until proteomic analysis.

Each sampled female was fitted with a numbered metal ring for identification purposes. We determined birds' phenotypic sex by examining sex-specific reproductive characteristics (incubation behavior or brood patch) in the field, and then confirmed their genetic sex from a blood sample using sex-specific molecular markers in the laboratory (Py *et al.*, 2006).

Ethics

Barn owl monitoring was performed according to a strict animal handling protocol approved by the 'Service de la consommation et des affaires vétérinaires', Switzerland (authorization numbers: VD 3462 and 3571).

Proteomic analysis

Protocol development and proteomic analysis were outsourced to the Protein Analysis Facility (PAF, UNIL, Lausanne, CH).

Peptide and protein extraction. Preen wax samples were solubilized in 400 µL of a 3:1 mixture of *n*-hexane and dichloromethane. One volume (350 µL) of the organic wax phase was extracted with 2 volumes (700 µL) of 50 mM ammonium bicarbonate (AmBic) and 10 % methanol (MeOH). The resulting solutions were vigorously vortexed twice for 1 min and then sonicated for 30 sec. 600 µL of the aqueous phase (lower phase) were recovered after being centrifuged at 14,000 rpm for 2 min, and the recovered aqueous phase

was centrifuged again to remove any remaining upper phase. Approx. 500 µL of the recovered aqueous phase were transferred onto a Centricon 10 kDa cutoff filter previously washed with 500 µL of AmBic and 10 % MeOH, the volume was reduced to minimum and 20-30 µL of the peptide fraction were collected per sample. The supernatant (above in the cartridge) was recovered from the remaining aqueous phase, the Centricon 10 kDa cutoff filter was washed with 50 µL of AmBic, the outcome was pooled with the recovered supernatant and 70-80 µL of the protein fraction were collected per sample. For protein extraction only, 7 µL of 5X sample buffer were added to 15 µL of each protein fraction. The resulting solutions were loaded onto two 12 % gels along with quantitative standards. Both 12 % gels were run, stained with Candiano colloidal Coomassie for 3 h for batch 1 and overnight for batch 2 and scanned before quantification.

LC-MS/MS analysis. Peptide fractions were dried and re-dissolved in 25 µL of 50 mM AmBic. They were then reduced/alkylated with 15 µL of 20 mM TCEP (7 mM final concentration) and 2.5 µL of 0.5 M chloroacetamide (29.4 mM final concentration) and incubated for 1 h at 45°C on a shaker in the dark. They were finally split into two aliquots of 20 µL each, with one aliquot being digested with 0.1 µg of trypsin (1 µL of 0.1 µg/µL) for 3 h at 37°C. 2.5 µL of 20 % TFA were added to each aliquot, the pH was checked and 50 µL of 0.1 % TFA were added to each aliquot before being stored at 4 °C. Both aliquots were then desalted on a Sep-Pak C18 plate, eluted and dried. They were finally re-suspended in 25 µL of loading buffer A and 10 µL of final solutions were injected onto a Fusion MS system with a 65-minute gradient. Concurrently, protein fractions were dried and digested using the miST protocol. They were then re-suspended in 25-250 µL of loading buffer A and 2-10 µL of final solutions were injected onto a Fusion MS system with a 140-minute gradient.

Peptide and protein identification and quantification. LC-MS/MS raw files were searched using MaxQuant software (version 2.1.4.0; Cox & Mann, 2008) against the *NCBI_Tyto_alba* fasta file (version 22/10/2021, 42,066 sequences; Machado *et al.*, 2022). Protein fractions were searched with full tryptic cleavage, while peptide fractions with either semi-specific tryptic cleavage (digested aliquot) or unspecific cleavage (undigested aliquot). We identified 1,650 protein groups altogether in the peptide fractions, and 3,641 protein groups in the protein fractions (min. 2 peptides), showing strong heterogeneity between samples.

Statistical analysis

Data pre-processing and DEP analyses were conducted using the *DEP* R package (version 1.22.0; X. Zhang *et al.*, 2018) based on the *limma* algorithm (Ritchie *et al.*, 2015), and the GO and KEGG enrichment analyses using the *clusterProfiler* (version 4.8.2; Wu *et al.*, 2021; Yu *et al.*, 2012) and *org.Hs.eg.db* R packages (version

3.17.0; Carlson *et al.*, 2023) implemented in the R Statistical Software (R version 4.3.1; R Core Team, Vienna, Austria).

Data pre-processing. We first removed peptides and proteins marked as *only identified by site*, *reverse* and *potential contaminant* by MaxQuant, with less than two *razor + unique peptides* or without any quantitative value. We log2-transformed the quantitative values. We filtered peptides and proteins quantified in at least two-thirds of samples (type = fraction, min = 0.66; *filter_proteins* function). We normalized the log2-transformed quantitative values through variance-stabilizing normalization (*normalize_vsn* function). We imputed the missing values (*N* = 315 for peptides, *N* = 1377 for proteins) using random draws from a Gaussian distribution centered around a minimal value (fun = MinProb, q = 0.01; *impute* function) considering a non-random distribution (MNAR) for downstream analysis.

DEP analyses. We performed differential enrichment analyses based on protein-wise linear models and empirical Bayes statistics (*test_diff* function). Peptides and proteins with an FDR-adjusted *P*-value ≤ 0.05 and a |log2 fold change| ≥ 1.5 were considered significantly differentially expressed (DEPs; *add_rejections* function). We visualized the DEPs using Volcano plots (Fig. 1), and proteomic data using Principal Component Analysis (PCA) and heatmap plots (Fig. 2-3).

GO and KEGG enrichment analyses. We performed the GO and KEGG enrichment analyses to study the functional and pathway profiles of peptide- and protein-related genes with a |log2 fold change| ≥ 1.5 across breeding stages. We classified the genes based on their projection at a specific level of the GO terms (OrgDb = org.Hs.eg.db, level = 3; *groupGO* function), and then performed enrichment analyses for GO terms (OrgDb = org.Hs.eg.db; *enrichGO* function) and KEGG pathways (organism = tala; *enrichKEGG* function) based on hypergeometric distribution. GO terms and KEGG pathways with an FDR-adjusted *P*-value ≤ 0.05 were considered significantly enriched.

Results

We considered 141 out of the 1,650 peptides and 643 out of the 3,641 proteins identified in the 20 preen wax samples collected from incubating and rearing females after data pre-processing for DEP analyses.

DEP analyses.

DEP analyses did not reveal any differentially expressed peptides and only one significantly differentially expressed protein across breeding stages (Suppl. Tables 1 and 4). Specifically, the apolipoprotein A-IV (XP_042656345.1), a liver-produced protein involved in transporting lipids in the blood, was significantly up-regulated during egg incubation (DE analyses: *P*-value < 0.01, FDR-adjusted *P*-value < 0.01, average log2 fold change = 3.82; Fig. 1). PCA and heatmap plots also supported this result by showing no distinct clustering among replicates from the same breeding stage (Fig. 2-3).

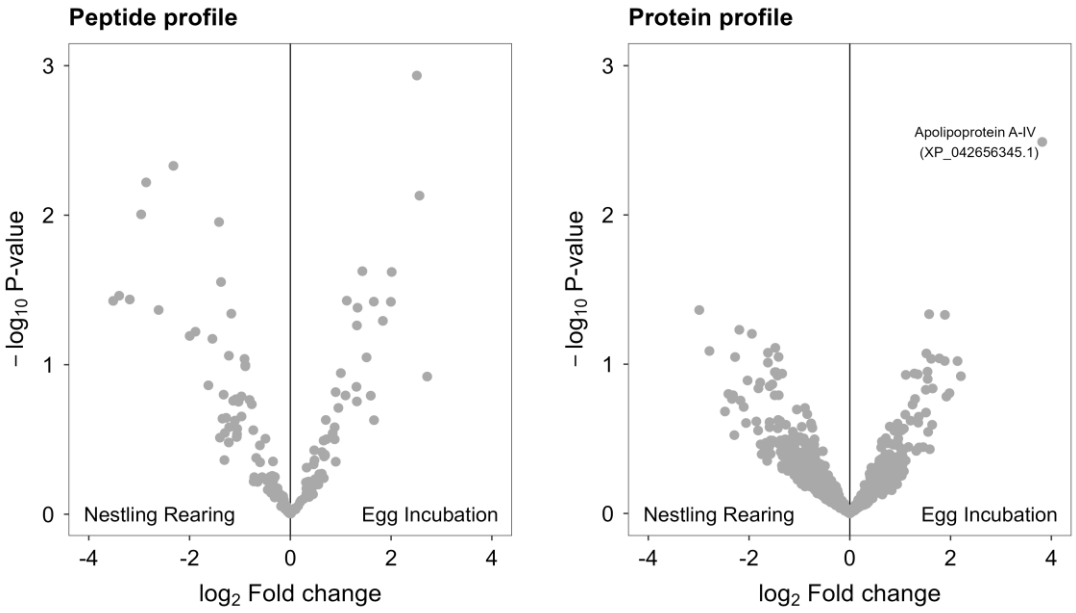

Figure 1. Volcano plots showing log2 fold change and -log10 (non-adjusted) *P*-value resulting from the comparison in peptides (*left*; *N* = 141) and proteins (*right*; *N* = 643) expressed in preen wax samples collected from incubating (*N* = 12) or rearing (*N* = 8) female barn owls. Each dot represents a peptide or a protein. Only the apolipoprotein A-IV (XP_042656345.1) was significantly up-regulated during egg incubation (DE analyses: *P*-value < 0.01, FDR-adjusted *P*-value < 0.01, average log2 fold change = 3.82).

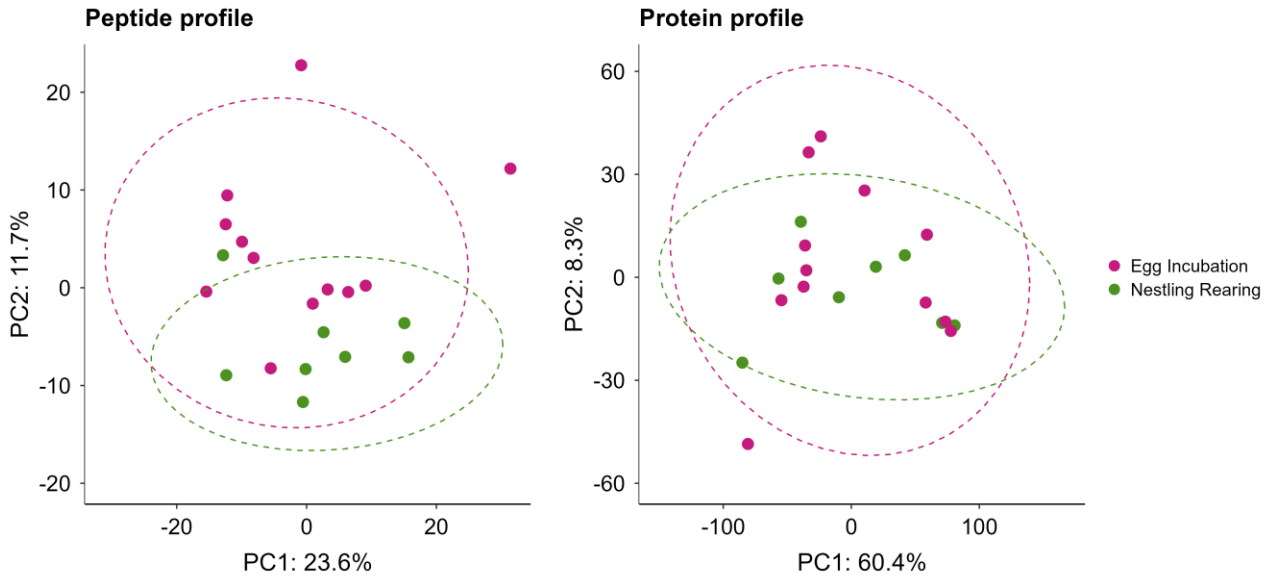

Figure 2. Principal Component Analysis (PCA) plots showing peptide (*left*; *N* = 141) and protein profiles (*right*; *N* = 643) in preen wax samples collected from incubating (*N* = 12) or rearing (*N* = 8) female barn owls. PC1 and PC2 together accounted for 35.3 % and 68.7 % of the total variance, respectively. PCA plots revealed no distinct clustering among replicates from the same breeding stage. Each dot represents a sample, each ellipse the 95 % confidence interval, and colors denote the breeding stage.

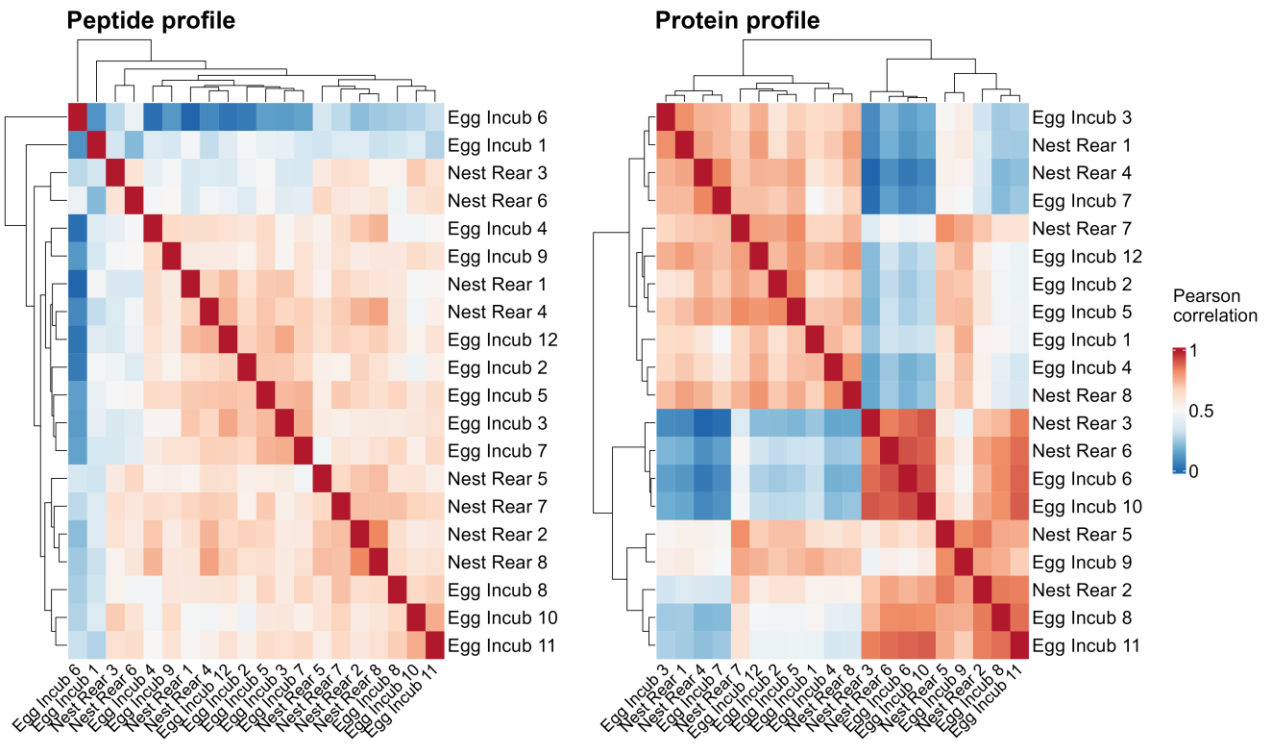

Figure 3. Heatmap plots showing Pearson correlation coefficients resulting from the comparison in peptides (*left*; *N* = 141) and proteins (*right*; *N* = 643) expressed in preen wax samples collected from incubating (*N* = 12) or rearing (*N* = 8) female barn owls. Sample-wise Pearson correlation coefficients revealed no distinct clustering among replicates from the same breeding stage. Rows and columns represent sample replicates, and colors denote Pearson correlation coefficients. Clustering is based on Euclidean distances.

GO and KEGG (enrichment) analyses.

GO analyses revealed that peptide- (*N* = 141) and protein-related genes (*N* = 643) were primarily associated with organic substance metabolic process, primary metabolic process and cellular metabolic process (BP), involved in protein binding, organic cyclic compound binding and heterocyclic compound binding (MF), and localized in intracellular anatomical structure, cytoplasm and organelle (CC) according to gene count and gene ratio (Fig. 4). Some of the peptide- and protein-related genes were also associated with immune system processes, such as immune response, (positive or negative) regulation of immune system process, immune effector process, activation of immune response, production of molecular mediator of immune response, immune system development, and somatic diversification of immune receptors (*data not shown*). Further details on GO description can be found in Tables 1-2.

With no peptide and only one protein showing significant differential expression, we decided to perform the GO and KEGG enrichment analyses on peptide- (*N* = 21) and protein-related genes (*N* = 55) with a |log2 fold change| ≥ 1.5 across breeding stages. GO enrichment analyses revealed significant enrichment in peptide-related genes in 54 BP, 27 MF and 9 CC, and in protein-related genes in 76 BP, 14 MF and 22 CC. Peptide-related genes were notably enriched in fatty acid metabolic process, cellular lipid catabolic process and fatty acid beta-oxidation within BP group, in acyltransferase activity transferring groups other than amino-acyl groups, thiolester hydrolase activity and acyltransferase activity within MF group, and in peroxisome, microbody and blood microparticle within CC group. While protein-related genes were enriched in BP such as fatty acid metabolic process, fatty acid beta-oxidation and cellular lipid catabolic process, in MF such as C-acyltransferase activity, acyltransferase activity and acyltransferase activity transferring groups other than amino-acyl groups, and in CC such as blood microparticle, axolemma and platelet alpha granule lumen, among others. KEGG enrichment analyses showed that sphingolipid metabolism, fatty acid degradation, lysosome, fatty acid metabolism, other glycan degradation, metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450 and PPAR signaling pathway were significantly enriched in peptide-related genes. Additionally, fatty acid degradation, fatty acid metabolism and valine, leucine and isoleucine degradation were significantly enriched in protein-related genes. Further details on enriched GO terms and KEGG pathways can be found in Suppl. Tables 2-3 (for peptide-related genes) and 5-6 (for protein-related genes).

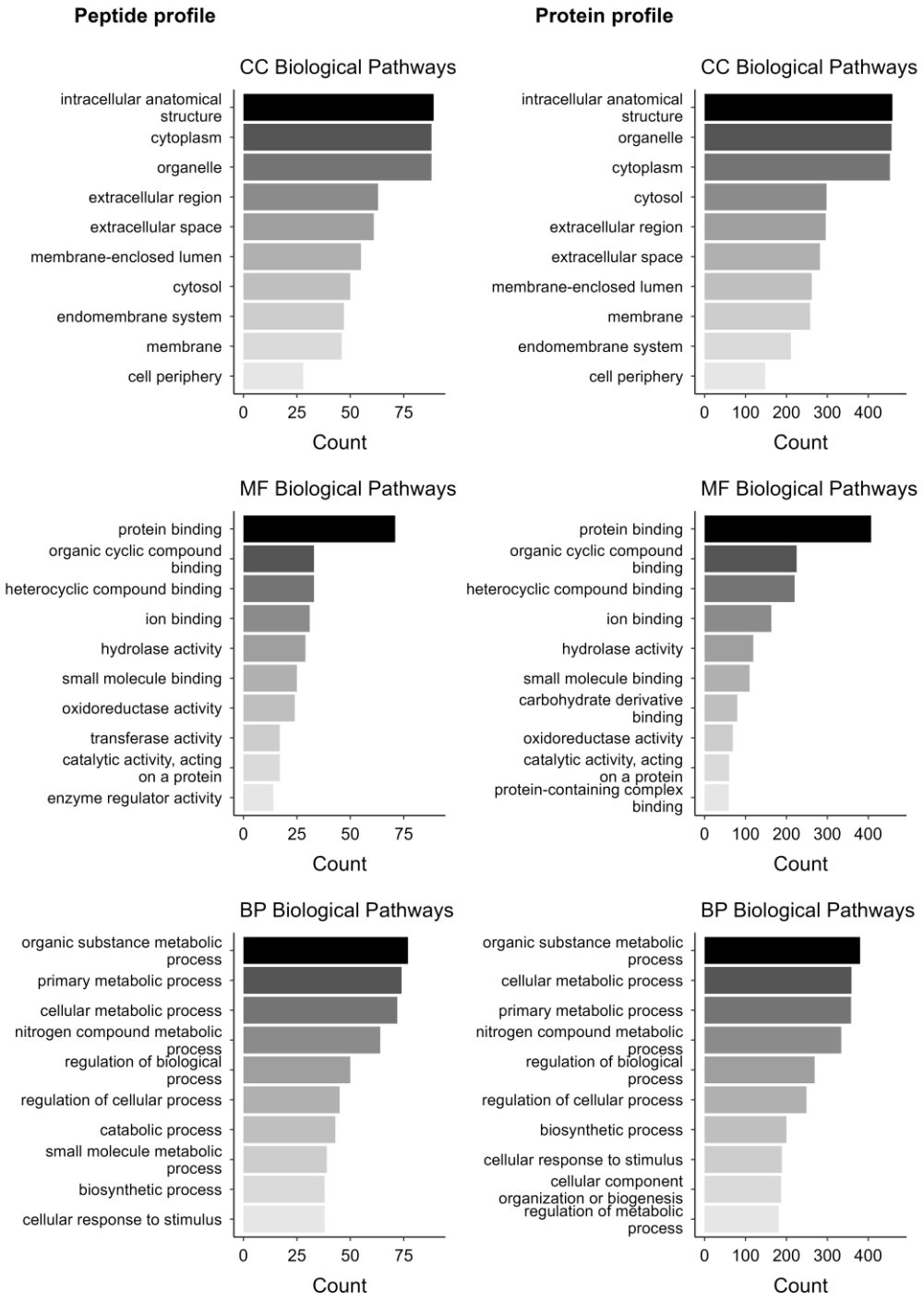

Figure 4. Barplots showing Gene Ontology (GO; including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)) of peptide- (*left*; *N* = 141) and protein-related genes (*right*; *N* = 643) expressed in preen wax samples collected from incubating (*N* = 12) or rearing (*N* = 8) female barn owls. Plots illustrate the top 10 GO terms for each GO group. A depth level 3 was considered.

Table 1. Results of Gene Ontology (GO; including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)) analysis of peptide-related genes (*N* = 141) expressed in preen wax samples collected from incubating (*N* = 12) or rearing (*N* = 8) female barn owls. Top 10 GO terms for each GO group are shown in the table. A depth level 3 was considered.

	GO ID	GO description	Count	Gene ratio	(a subset of 5) Gene ID
CC	GO:0005622	intracellular anatomical structure	89	89/141	VDAC2 / GOT1 / PRDX3 / LXN / PTMA
CC	GO:0005737	cytoplasm	88	88/141	VDAC2 / GOT1 / PRDX3 / LXN / PTMA
CC	GO:0043226	organelle	88	88/141	VDAC2 / GOT1 / PRDX3 / PTMA / SERPINE2
CC	GO:0005576	extracellular region	63	63/141	GOT1 / LXN / SERPINE2 / QSOX1 / PRDX6
CC	GO:0005615	extracellular space	61	61/141	GOT1 / LXN / SERPINE2 / QSOX1 / PRDX6
CC	GO:0031974	membrane-enclosed lumen	55	55/141	VDAC2 / GOT1 / PRDX3 / PTMA / QSOX1
CC	GO:0005829	cytosol	50	50/141	GOT1 / PRDX3 / PTMA / SERPINE2 / PRDX6
CC	GO:0012505	endomembrane system	47	47/141	VDAC2 / PRDX3 / SERPINE2 / HSD17B7 / QSOX1
CC	GO:0016020	membrane	46	46/141	VDAC2 / SERPINE2 / HSD17B7 / QSOX1 / PRDX6
CC	GO:0071944	cell periphery	28	28/141	SERPINE2 / HSPA5 / GRN / CTSL / GAPDH
MF	GO:0005515	protein binding	71	71/141	VDAC2 / PRDX3 / LXN / PTMA / SERPINE2
MF	GO:0097159	organic cyclic compound binding	33	33/141	VDAC2 / GOT1 / QSOX1 / DBT / FMO5
MF	GO:1901363	heterocyclic compound binding	33	33/141	VDAC2 / GOT1 / QSOX1 / DBT / FMO5
MF	GO:0043167	ion binding	31	31/141	VDAC2 / GOT1 / PTMA / QSOX1 / DBT
MF	GO:0016787	hydrolase activity	29	29/141	PRDX6 / DPP7 / HSPA5 / CTSL / GM2A
MF	GO:0036094	small molecule binding	25	25/141	VDAC2 / GOT1 / QSOX1 / DBT / FMO5
MF	GO:0016491	oxidoreductase activity	24	24/141	PRDX3 / HSD17B7 / QSOX1 / PRDX6 / FMO5
MF	GO:0016740	transferase activity	17	17/141	GOT1 / PRDX6 / DBT / SCP2 / CRAT
MF	GO:0140096	catalytic activity, acting on a protein	17	17/141	QSOX1 / DPP7 / TXN / CTSL / GAPDH
MF	GO:0030234	enzyme regulator activity	14	14/141	PRDX3 / LXN / SERPINE2 / CTSL / GAPDH
BP	GO:0071704	organic substance metabolic process	77	77/141	GOT1 / PRDX3 / LXN / PTMA / SERPINE2
BP	GO:0044238	primary metabolic process	74	74/141	GOT1 / PRDX3 / LXN / PTMA / SERPINE2
BP	GO:0044237	cellular metabolic process	72	72/141	GOT1 / PRDX3 / PTMA / QSOX1 / PRDX6
BP	GO:0006807	nitrogen compound metabolic process	64	64/141	GOT1 / PRDX3 / LXN / PTMA / SERPINE2
BP	GO:0050789	regulation of biological process	50	50/141	VDAC2 / GOT1 / PRDX3 / LXN / PTMA

BP	GO:0050794	regulation of cellular process	45	45/141	VDAC2 / GOT1 / PRDX3 / PTMA / SERPINE2
BP	GO:0009056	catabolic process	43	43/141	GOT1 / PRDX3 / SERPINE2 / QSOX1 / PRDX6
BP	GO:0044281	small molecule metabolic process	39	39/141	GOT1 / HSD17B7 / DBT / FMO5 / SCP2
BP	GO:0009058	biosynthetic process	38	38/141	GOT1 / PRDX3 / PTMA / HSD17B7 / SCP2
BP	GO:0051716	cellular response to stimulus	38	38/141	VDAC2 / GOT1 / PRDX3 / SERPINE2 / PRDX6

Table 2. Results of Gene Ontology (GO; including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)) analysis of protein-related genes (*N* = 643) expressed in preen wax samples collected from incubating (*N* = 12) or rearing (*N* = 8) female barn owls. Top 10 GO terms for each GO group are shown in the table. A depth level 3 was considered.

	GO ID	GO description	Count	Gene ratio	(a subset of 5) Gene ID
CC	GO:0005622	intracellular anatomical structure	459	459/639	APOA4 / VDAC2 / ANXA8L1 / PAOX / GSTO1
CC	GO:0043226	organelle	457	457/639	APOA4 / VDAC2 / PAOX / GSTO1 / SLK
CC	GO:0005737	cytoplasm	453	453/639	APOA4 / VDAC2 / ANXA8L1 / PAOX / GSTO1
CC	GO:0005829	cytosol	298	298/639	APOA4 / PAOX / GSTO1 / SLK / PPA1
CC	GO:0005576	extracellular region	296	296/639	APOA4 / GSTO1 / SLK / RBP4 / PPA1
CC	GO:0005615	extracellular space	282	282/639	APOA4 / GSTO1 / SLK / RBP4 / PPA1
CC	GO:0031974	membrane-enclosed lumen	262	262/639	APOA4 / VDAC2 / PAOX / NPM3 / ACADSB
CC	GO:0016020	membrane	258	258/639	VDAC2 / PGAM1 / CAMK2G / VCL / AADAC
CC	GO:0012505	endomembrane system	211	211/639	APOA4 / VDAC2 / PGAM1 / PRDX3 / CAMK2G
CC	GO:0071944	cell periphery	148	148/639	APOA4 / VCL / AHSG / NCL / SERPINE2
MF	GO:0005515	protein binding	407	407/639	APOA4 / VDAC2 / GSTO1 / SLK / RBP4
MF	GO:0097159	organic cyclic compound binding	225	225/639	VDAC2 / SLK / NPM3 / ACADSB / GOT1
MF	GO:1901363	heterocyclic compound binding	220	220/639	VDAC2 / SLK / NPM3 / ACADSB / GOT1
MF	GO:0043167	ion binding	163	163/639	APOA4 / VDAC2 / ANXA8L1 / SLK / ACADSB
MF	GO:0016787	hydrolase activity	119	119/639	PPA1 / PGAM1 / CAMK2G / AADAC / EIF4A2
MF	GO:0036094	small molecule binding	110	110/639	VDAC2 / SLK / RBP4 / ACADSB / GOT1
MF	GO:0097367	carbohydrate derivative binding	80	80/639	SLK / CAMK2G / LXN / EIF4G1 / EIF4A2
MF	GO:0016491	oxidoreductase activity	69	69/639	PAOX / GSTO1 / ACADSB / PRDX3 / IL4I1
MF	GO:0140096	catalytic activity, acting on a protein	60	60/639	SLK / CAMK2G / RNF13 / QSOX1 / PTPRF
MF	GO:0044877	protein-containing complex binding	59	59/639	EIF5A2 / RACK1 / PTPRF / GSN / HSPA5
BP	GO:0071704	organic substance metabolic process	380	380/639	APOA4 / ANXA8L1 / PAOX / GSTO1 / SLK
BP	GO:0044237	cellular metabolic process	359	359/639	APOA4 / PAOX / GSTO1 / SLK / RBP4
BP	GO:0044238	primary metabolic process	358	358/639	APOA4 / ANXA8L1 / PAOX / GSTO1 / SLK
BP	GO:0006807	nitrogen compound metabolic process	334	334/639	APOA4 / ANXA8L1 / PAOX / SLK / NPM3
BP	GO:0050789	regulation of biological process	269	269/639	APOA4 / VDAC2 / ANXA8L1 / PAOX / GSTO1

BP	GO:0050794	regulation of cellular process	249	249/639	APOA4 / VDAC2 / PAOX / GSTO1 / SLK
BP	GO:0009058	biosynthetic process	200	200/639	APOA4 / PAOX / RBP4 / NPM3 / GOT1
BP	GO:0051716	cellular response to stimulus	189	189/639	APOA4 / VDAC2 / GSTO1 / GOT1 / PRDX3
BP	GO:0071840	cellular component organization or biogenesis	187	187/639	APOA4 / VDAC2 / ANXA8L1 / SLK / NPM3
BP	GO:0019222	regulation of metabolic process	181	181/639	APOA4 / ANXA8L1 / PAOX / RBP4 / PGAM1

Discussion

Reproduction is a critical period for birds, encompassing challenges and trade-offs such as incubation, rearing, protection of eggs and offspring, as well as self-maintenance. Birds have not only developed specific adaptations such as preening and preen gland traits to fulfill these functions (Bush & Clayton, 2018; Clayton *et al.*, 2010; Gunderson, 2008) but also adjust their investment to reproduction timing and the selective pressures they experience at that time (*e.g.* preening needs, exposure to nest/environmental microorganisms). However, the mechanisms of action underlying potential adjustments in preen gland traits still require investigation (Moreno-Rueda, 2017). In the present study, we proposed the proteome as a possible mechanism of action for conferring and adjusting the protective function of preen wax (Braun *et al.*, 2018; Carneiro *et al.*, 2020). We analyzed preen wax samples collected from 20 female barn owls during egg incubation or nestling rearing using LC-MS/MS, identified and quantified the peptides and proteins detected, as well as extracted the GO (including BP, MF and CC) of these peptide- and protein-related genes. We found that peptide- and protein-related genes were primarily associated with organic substance metabolic process, primary metabolic process and cellular metabolic process (BP), involved in protein binding, organic cyclic compound binding and heterocyclic compound binding (MF), and localized in intracellular anatomical structure, cytoplasm and organelle (CC; see *Results* for top 10 GO terms). Some were also associated with immune system processes. We next tested for differential enrichment in (immune-related) peptides and proteins to understand how females adjust preen wax proteome to reproduction timing and its associated breeding duties. Contrary to our expectation, we did not find any immune-related peptides or proteins and only one lipid transport protein (the apolipoprotein A-IV) showing significant differential expression across breeding stages. The apolipoprotein A-IV was indeed up-regulated during egg incubation. We finally tested for differential GO and KEGG enrichment to gain insight into GO terms and KEGG pathways in which (differentially expressed) peptide- and protein-related genes were enriched.

Our study proposed the proteome as a possible mechanism of action not only for conferring but also for adjusting the protective function of preen wax to reproduction timing and the selective pressures female barn owls experience at that time (*e.g.* preening needs, exposure to nest/environmental microorganisms). As expected, we found some peptide- and protein-related genes to be associated with immune system processes, supporting the presence of immune-related peptides and proteins in barn owl's preen wax. However, we did not find any evidence supporting a differential enrichment in these immune-related peptides and proteins across breeding stages. In previous studies, we showed that incubating females

invested preferentially in preen wax amount while rearing females in preen gland size (Chapter 1). Both investment strategies (wax secretion *vs* storage) may then help adjust preen wax amount applied onto eggs, offspring and themselves to preening needs and exposure to nest/environmental microorganisms. Incubating females also harbored a less diverse feather microbiota compared to rearing females (Chapter 2) which may similarly arise from different microbial exposure or different investment strategies in preen gland traits if no proteome adjustment is effectively implemented across breeding stages. Additionally, we showed that males invested preferentially in preen wax amount during egg incubation, with no differences in preen gland size and feather microbiota diversity between egg incubation and nestling rearing. Unlike females, males do not engage in egg and offspring protection, restricting the use of preen wax to self-maintenance (Roulin, 2020). Comparing the proteomic profile of male and female preen wax would allow us to better understand what barn owls adjust the protective function of preen wax to (Whittaker & Hagelin, 2021).

In addition to/instead of immune-related peptides and proteins, barn owls may also rely on other mechanisms of action and chemical compounds for adjusting the protective function of preen wax. Preen wax may indeed form a protective physical barrier on eggshells, down feathers and adult plumage (S. Jacob *et al.*, 2018; Reneerkens *et al.*, 2008; Verea *et al.*, 2017). Certain preen wax compounds, such as acids, alcohols and other organic compounds, may also serve as energy stores promoting mutualists and commensals able to compete with pathogens (Soler *et al.*, 2010) or have a direct antimicrobial action against those pathogens (Braun *et al.*, 2018; Carneiro *et al.*, 2020; Soini *et al.*, 2007). Bacteriocins and other antimicrobial substances originating from symbiotic bacteria living in preen gland may have a similar antimicrobial action (Martín-Vivaldi *et al.*, 2009, 2010; Soler *et al.*, 2008, 2010). Yet, the preen gland microbiota has been little studied in the barn owl so far (Braun, Wang, Zimmermann, Boutin, *et al.*, 2019; Braun, Wang, Zimmermann, Wagner, *et al.*, 2019).

Interestingly, our study revealed one liver-produced protein involved in transporting lipids in the blood, the apolipoprotein A-IV, showing significant differential expression across breeding stages. The apolipoprotein A-IV was indeed up-regulated during egg incubation, suggesting a possible role in lipid transport at this stage. Nevertheless, further investigation is required to fully understand the functional significance and regulatory pathways associated with this up-regulation in incubating females. Additionally, our study revealed 21 peptides and 55 proteins with a $|\log2 \text{ fold change}| \geq 1.5$ across breeding stages. However, the extensive diversity of these peptides and proteins, along with the diversity and abundance of the GO terms and KEGG pathways they are enriched in, prevents us from drawing definitive conclusions.

We still need to acknowledge the methodological limitations and weaknesses of our study, as these issues may explain why we did not find any evidence supporting a differential enrichment in (immune-related) peptides and proteins across breeding stages. At first, we constrained our sample size to 20 individuals due to the high cost associated with developing protocols and conducting proteomic analysis. Increasing our sample size would certainly enhance the detection of low-abundance peptides and proteins, boost the statistical power of our analyses, and consequently improve the accuracy and reliability of our results. Additionally, despite following usual laboratory and bioinformatics procedures (De Livera *et al.*, 2013), our samples still clustered based on peptide or protein quantities post-normalization. Applying a more extensive normalization method, *e.g.* considering non-changing metabolites within preen wax as internal standards, would help reveal distinct clustering patterns among breeding stages (if present). Finally, conducting a targeted proteomic analysis, with a specific focus on immune-related peptides and proteins, would definitively provide valuable insights into key biological processes and pathways pertaining to preen wax proteomic profile.

Shedding light for the first time on the untargeted preen wax proteome and its adjustment to reproduction timing, our study supported the presence of immune-related peptides and proteins in barn owl's preen wax but did not find evidence supporting a differential enrichment in these immune-related peptides and proteins across breeding stages. In summary, female barn owls do not seem to consider the proteome as a possible mechanism of action for adjusting the protective function of preen wax to the selective pressures they experience during reproduction (*e.g.* preening needs, exposure to nest/environmental microorganisms). Further investigation is therefore required to explore the other mechanisms of action and chemical compounds likely to adjust the protective function of preen wax across bird species.

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Supplementary Material

Table S1. List of differentially expressed peptides (DEPs; *N* = 21) in preen wax samples with a |log2 fold change| ≥ 1.5 between egg incubation (*N* = 12) and nestling rearing (*N* = 8). DEPs, average log2 fold change, *P*-value and FDR-adjusted *P*-value resulting from the comparison are shown in the table. Significant DEPs are highlighted in bold.

Majority peptide ID	Majority peptide	Gene ID	Gene	Avg log2 fold change	*P*-value	Adj *P*-value
XP_032853370.2	keratin, type II cytoskeletal 6A	104363205	LOC104363205	2.72	0.12	0.96
XP_009970475.2	fatty acid-binding protein, adipocyte	104365394	LOC104365394	2.56	0.01	0.67
XP_042652391.1	annexin A5	104356827	ANXA5	2.51	< 0.01	0.44
XP_042641604.1	lysosomal acid glucosylceramidase-like	122152381	LOC122152381	2.01	0.02	0.85
XP_042640045.1	glycerol-3-phosphate dehydrogenase, mitochondrial-like	122152390	LOC122152390	2.00	0.04	0.89
XP_042660467.1	3-ketoacyl-CoA thiolase, mitochondrial isoform X2	104357364	ACAA2	1.84	0.05	0.92
XP_032848254.1	prosaposin isoform X3	104361126	LOC104361126	1.66	0.04	0.89
XP_042643167.1	junction plakoglobin	116963969	LOC116963969	1.66	0.23	0.97
XP_042641360.1	aldehyde dehydrogenase family 3 member B1-like isoform X1	116961870	LOC116961870	1.60	0.16	0.96
XP_042647097.1	galactocerebrosidase isoform X2	104356982	GALC	1.51	0.09	0.94
XP_042658783.1	complement C3	104361896	C3	-1.55	0.07	0.93
XP_032839718.2	histone H1.01	116959388	LOC116959388	-1.63	0.14	0.96
XP_009973220.2	protein S100-A7-like	104367745	LOC104367745	-1.88	0.06	0.93
XP_042640464.1	carnitine O-palmitoyltransferase 1, liver isoform isoform X1	104363336	CPT1A	-2.00	0.06	0.93
XP_042651478.1	microsomal glutathione S-transferase 1	104357091	MGST1	-2.32	< 0.01	0.64
XP_042641081.1	lysosomal acid glucosylceramidase isoform X2	104366019	LOC104366019	-2.61	0.04	0.90
XP_032854038.2	digestive cysteine proteinase 1-like	116962917	LOC116962917	-2.86	0.01	0.66
XP_042662788.1	enoyl-CoA delta isomerase 2 isoform X3	104367903	ECI2	-2.96	0.01	0.73
XP_032839426.1	hemoglobin subunit alpha-A	116959343	LOC116959343	-3.19	0.04	0.89
XP_032857738.2	fatty acid synthase	104367309	FASN	-3.40	0.03	0.89
XP_032862987.1	albumin	104361928	LOC104361928	-3.52	0.04	0.89

Table S2. Results of Gene Ontology (GO; including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)) enrichment analysis of peptide-related genes (*N* = 21) with a |log2 fold change| ≥ 1.5 between egg incubation (*N* = 12) and nestling rearing (*N* = 8). Significantly enriched GO terms, *P*-value, FDR-adjusted *P*-value and *Q*-value are shown in the table.

	GO ID	GO description	Gene ratio	Bg ratio	*P*-value	Adj *P*-value	*Q*-value	Gene ID	Count
CC	GO:0005777	peroxisome	2/8	143/19518	< 0.01	0.01	< 0.01	MGST1 / ECI2	2
CC	GO:0042579	microbody	2/8	143/19518	< 0.01	0.01	< 0.01	MGST1 / ECI2	2
CC	GO:0072562	blood microparticle	2/8	144/19518	< 0.01	0.01	< 0.01	ANXA5 / C3	2
CC	GO:0005766	primary lysosome	2/8	155/19518	< 0.01	0.01	< 0.01	MGST1 / C3	2
CC	GO:0042582	azurophil granule	2/8	155/19518	< 0.01	0.01	< 0.01	MGST1 / C3	2
CC	GO:0005775	vacuolar lumen	2/8	176/19518	< 0.01	0.01	< 0.01	GALC / C3	2
CC	GO:0005741	mitochondrial outer membrane	2/8	208/19518	< 0.01	0.01	0.01	MGST1 / CPT1A	2
CC	GO:0031968	organelle outer membrane	2/8	236/19518	< 0.01	0.01	0.01	MGST1 / CPT1A	2
CC	GO:0019867	outer membrane	2/8	238/19518	< 0.01	0.01	0.01	MGST1 / CPT1A	2
MF	GO:0016747	acyltransferase activity, transferring groups other than amino-acyl groups	3/8	226/18369	< 0.01	< 0.01	< 0.01	FASN / ACAA2 / CPT1A	3
MF	GO:0016790	thiolester hydrolase activity	2/8	40/18369	< 0.01	< 0.01	< 0.01	FASN / ACAA2	2
MF	GO:0016746	acyltransferase activity	3/8	253/18369	< 0.01	< 0.01	< 0.01	FASN / ACAA2 / CPT1A	3
MF	GO:0072341	modified amino acid binding	2/8	85/18369	< 0.01	0.01	< 0.01	ANXA5 / FASN	2
MF	GO:0016407	acetyltransferase activity	2/8	94/18369	< 0.01	0.01	< 0.01	FASN / ACAA2	2
MF	GO:0004859	phospholipase inhibitor activity	1/8	12/18369	0.01	0.03	0.01	ANXA5	1
MF	GO:0004312	fatty acid synthase activity	1/8	13/18369	0.01	0.03	0.01	FASN	1
MF	GO:0102991	myristoyl-CoA hydrolase activity	1/8	15/18369	0.01	0.03	0.01	ACAA2	1
MF	GO:0055102	lipase inhibitor activity	1/8	16/18369	0.01	0.03	0.01	ANXA5	1
MF	GO:0016290	palmitoyl-CoA hydrolase activity	1/8	17/18369	0.01	0.03	0.01	ACAA2	1
MF	GO:0016408	C-acyltransferase activity	1/8	20/18369	0.01	0.03	0.01	ACAA2	1
MF	GO:0004602	glutathione peroxidase activity	1/8	22/18369	0.01	0.03	0.01	MGST1	1
MF	GO:0047617	acyl-CoA hydrolase activity	1/8	22/18369	0.01	0.03	0.01	ACAA2	1
MF	GO:0004857	enzyme inhibitor activity	2/8	359/18369	0.01	0.03	0.01	ANXA5 / C3	2
MF	GO:0016289	CoA hydrolase activity	1/8	25/18369	0.01	0.03	0.01	ACAA2	1

MF	GO:0004364	glutathione transferase activity	1/8	26/18369	0.01	0.03	0.01	MGST1	1
MF	GO:0016628	oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	1/8	28/18369	0.01	0.03	0.01	FASN	1
MF	GO:0016417	S-acyltransferase activity	1/8	30/18369	0.01	0.03	0.01	FASN	1
MF	GO:0016409	palmitoyltransferase activity	1/8	37/18369	0.02	0.04	0.01	CPT1A	1
MF	GO:0005544	calcium-dependent phospholipid binding	1/8	52/18369	0.02	0.04	0.01	ANXA5	1
MF	GO:0008374	O-acyltransferase activity	1/8	54/18369	0.02	0.04	0.01	CPT1A	1
MF	GO:0004601	peroxidase activity	1/8	55/18369	0.02	0.04	0.01	MGST1	1
MF	GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	1/8	57/18369	0.02	0.04	0.01	MGST1	1
MF	GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors	1/8	60/18369	0.03	0.04	0.01	FASN	1
MF	GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	1/8	61/18369	0.03	0.04	0.01	MGST1	1
MF	GO:0001786	phosphatidylserine binding	1/8	62/18369	0.03	0.04	0.01	ANXA5	1
MF	GO:0016836	hydro-lyase activity	1/8	62/18369	0.03	0.04	0.01	FASN	1
BP	GO:0006631	fatty acid metabolic process	5/8	394/18614	< 0.01	< 0.01	< 0.01	FASN / C3 / ACAA2 / ECI2 / CPT1A	5
BP	GO:0044242	cellular lipid catabolic process	4/8	222/18614	< 0.01	< 0.01	< 0.01	GALC / ACAA2 / ECI2 / CPT1A	4
BP	GO:0006635	fatty acid beta-oxidation	3/8	76/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0016042	lipid catabolic process	4/8	330/18614	< 0.01	< 0.01	< 0.01	GALC / ACAA2 / ECI2 / CPT1A	4
BP	GO:0009062	fatty acid catabolic process	3/8	105/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0019395	fatty acid oxidation	3/8	109/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0034440	lipid oxidation	3/8	116/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0072329	monocarboxylic acid catabolic process	3/8	131/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0030258	lipid modification	3/8	197/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0016054	organic acid catabolic process	3/8	250/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0046395	carboxylic acid catabolic process	3/8	250/18614	< 0.01	< 0.01	< 0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0010883	regulation of lipid storage	2/8	53/18614	< 0.01	0.01	< 0.01	C3 / CPT1A	2
BP	GO:0044282	small molecule catabolic process	3/8	388/18614	< 0.01	0.01	0.01	ACAA2 / ECI2 / CPT1A	3
BP	GO:0019915	lipid storage	2/8	86/18614	< 0.01	0.01	0.01	C3 / CPT1A	2
BP	GO:0006641	triglyceride metabolic process	2/8	100/18614	< 0.01	0.02	0.01	C3 / CPT1A	2

BP	GO:0006639	acylglycerol metabolic process	2/8	128/18614	< 0.01	0.02	0.01	C3 / CPT1A	2
BP	GO:0006638	neutral lipid metabolic process	2/8	129/18614	< 0.01	0.02	0.01	C3 / CPT1A	2
BP	GO:0006839	mitochondrial transport	2/8	189/18614	< 0.01	0.05	0.02	ACAA2 / CPT1A	2
BP	GO:0030223	neutrophil differentiation	1/8	10/18614	< 0.01	0.05	0.02	FASN	1
BP	GO:2000425	regulation of apoptotic cell clearance	1/8	10/18614	< 0.01	0.05	0.02	C3	1
BP	GO:0008611	ether lipid biosynthetic process	1/8	11/18614	< 0.01	0.05	0.02	FASN	1
BP	GO:0032000	positive regulation of fatty acid beta-oxidation	1/8	11/18614	< 0.01	0.05	0.02	CPT1A	1
BP	GO:0046504	glycerol ether biosynthetic process	1/8	11/18614	< 0.01	0.05	0.02	FASN	1
BP	GO:0090557	establishment of endothelial intestinal barrier	1/8	11/18614	< 0.01	0.05	0.02	FASN	1
BP	GO:0097278	complement-dependent cytotoxicity	1/8	11/18614	< 0.01	0.05	0.02	C3	1
BP	GO:0097384	cellular lipid biosynthetic process	1/8	11/18614	< 0.01	0.05	0.02	FASN	1
BP	GO:1901503	ether biosynthetic process	1/8	12/18614	0.01	0.05	0.02	FASN	1
BP	GO:0002524	hypersensitivity	1/8	13/18614	0.01	0.05	0.02	C3	1
BP	GO:0002863	positive regulation of inflammatory response to antigenic stimulus	1/8	13/18614	0.01	0.05	0.02	C3	1
BP	GO:0009437	carnitine metabolic process	1/8	13/18614	0.01	0.05	0.02	CPT1A	1
BP	GO:0060100	positive regulation of phagocytosis, engulfment	1/8	13/18614	0.01	0.05	0.02	C3	1
BP	GO:1905155	positive regulation of membrane invagination	1/8	13/18614	0.01	0.05	0.02	C3	1
BP	GO:0016322	neuron remodeling	1/8	14/18614	0.01	0.05	0.02	C3	1
BP	GO:0046479	glycosphingolipid catabolic process	1/8	14/18614	0.01	0.05	0.02	GALC	1
BP	GO:1901029	negative regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway	1/8	14/18614	0.01	0.05	0.02	ACAA2	1
BP	GO:0002864	regulation of acute inflammatory response to antigenic stimulus	1/8	15/18614	0.01	0.05	0.02	C3	1
BP	GO:0060099	regulation of phagocytosis, engulfment	1/8	15/18614	0.01	0.05	0.02	C3	1
BP	GO:0098883	synapse pruning	1/8	15/18614	0.01	0.05	0.02	C3	1
BP	GO:1905153	regulation of membrane invagination	1/8	15/18614	0.01	0.05	0.02	C3	1

BP	GO:0046321	positive regulation of fatty acid oxidation	1/8	16/18614	0.01	0.05	0.02	CPT1A	1
BP	GO:0006577	amino-acid betaine metabolic process	1/8	17/18614	0.01	0.05	0.02	CPT1A	1
BP	GO:0006677	glycosylceramide metabolic process	1/8	17/18614	0.01	0.05	0.02	GALC	1
BP	GO:0019377	glycolipid catabolic process	1/8	17/18614	0.01	0.05	0.02	GALC	1
BP	GO:0006957	complement activation, alternative pathway	1/8	18/18614	0.01	0.05	0.02	C3	1
BP	GO:0033194	response to hydroperoxide	1/8	18/18614	0.01	0.05	0.02	MGST1	1
BP	GO:0006662	glycerol ether metabolic process	1/8	20/18614	0.01	0.05	0.02	FASN	1
BP	GO:0031998	regulation of fatty acid beta-oxidation	1/8	20/18614	0.01	0.05	0.02	CPT1A	1
BP	GO:0035795	negative regulation of mitochondrial membrane permeability	1/8	20/18614	0.01	0.05	0.02	ACAA2	1
BP	GO:0046485	ether lipid metabolic process	1/8	20/18614	0.01	0.05	0.02	FASN	1
BP	GO:0046514	ceramide catabolic process	1/8	20/18614	0.01	0.05	0.02	GALC	1
BP	GO:1902001	fatty acid transmembrane transport	1/8	20/18614	0.01	0.05	0.02	CPT1A	1
BP	GO:0019216	regulation of lipid metabolic process	2/8	344/18614	0.01	0.05	0.02	C3 / CPT1A	2
BP	GO:0002888	positive regulation of myeloid leukocyte mediated immunity	1/8	21/18614	0.01	0.05	0.02	C3	1
BP	GO:0051235	maintenance of location	2/8	347/18614	0.01	0.05	0.02	C3 / CPT1A	2

Table S3. Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of peptide-related genes (*N* = 21) with a |log2 fold change| ≥ 1.5 between egg incubation (*N* = 12) and nestling rearing (*N* = 8). Significantly enriched KEGG pathways, *P*-value, FDR-adjusted *P*-value and *Q*-value are shown in the table.

	KEGG ID	KEGG description	Gene ratio	Bg ratio	*P*-value	Adj *P*-value	*Q*-value	Gene ID	Count
	tala00600	sphingolipid metabolism	4/13	65/5413	< 0.01	< 0.01	< 0.01	104361126 / 104356982 / 104366019 / 122152381	4
	tala00071	fatty acid degradation	3/13	40/5413	< 0.01	< 0.01	< 0.01	104357364 / 104367903 / 104363336	3

tala04142	lysosome	4/13	136/5413	< 0.01	< 0.01	< 0.01	104361126 / 104356982 / 104366019 / 122152381	4
tala01212	fatty acid metabolism	3/13	55/5413	< 0.01	< 0.01	< 0.01	104367309 / 104357364 / 104363336	3
tala00511	other glycan degradation	2/13	19/5413	< 0.01	< 0.01	< 0.01	104366019 / 122152381	2
tala00980	metabolism of xenobiotics by cytochrome P450	2/13	30/5413	< 0.01	0.01	0.01	104357091 / 116961870	2
tala00982	drug metabolism - cytochrome P450	2/13	32/5413	< 0.01	0.01	0.01	104357091 / 116961870	2
tala03320	PPAR signaling pathway	2/13	68/5413	0.01	0.03	0.02	104363336 / 104365394	2

Table S4. List of differentially expressed proteins (DEPs; *N* = 55) in preen wax samples with a |log2 fold change| ≥ 1.5 between egg incubation (*N* = 12) and nestling rearing (*N* = 8). DEPs, average log2 fold change, *P*-value and FDR-adjusted *P*-value resulting from the comparison are shown in the table. Significant DEPs are highlighted in bold.

Majority protein ID	Majority protein	Gene ID	Gene	Avg log2 fold change	*P*-value	Adj *P*-value
XP_042656345.1	**apolipoprotein A-IV**	**116964985**	**APOA4**	**3.82**	**< 0.01**	**< 0.01**
XP_032861890.2	glutathione peroxidase 3	116964915	GPX3	2.20	0.12	0.38
XP_042653016.1	60S ribosomal protein L34	116958813	RPL34	2.14	0.10	0.36
XP_042644285.1	thymosin beta-4	116963585	TMSB4X	1.98	0.16	0.52
XP_042653848.1	prothymosin alpha isoform X4	116958989	PTMA	1.92	0.16	0.55
XP_009964701.2	alpha-2-HS-glycoprotein	104360452	AHSG	1.89	0.05	0.26
XP_042643167.1	junction plakoglobin	116963969	LOC116963969	1.88	0.10	0.36
XP_032858261.1	jupiter microtubule associated homolog 1 isoform X1	104356753	JPT1	1.78	0.09	0.36
XP_032841463.1	vitamin D-binding protein	104365691	GC	1.64	0.15	0.48
XP_042644158.1	60S ribosomal protein L8	104361816	RPL8	1.63	0.25	0.75
XP_032838066.1	annexin A8-like protein 1	104366785	ANXA8L1	1.62	0.09	0.36
XP_042652522.1	myosin light polypeptide 6 isoform X2	122153776	LOC122153776	1.59	0.37	0.87
XP_032840024.2	mesencephalic astrocyte-derived neurotrophic factor	104356656	MANF	1.57	0.05	0.26

XP_042641417.1	astrocytic phosphoprotein PEA-15	116959600	LOC116959600	1.55	0.28	0.79
XP_009967608.2	ly6/PLAUR domain-containing protein 2	104362924	LOC104362924	1.55	0.13	0.40
XP_042663219.1	SH3 domain-binding glutamic acid-rich-like protein	104357037	SH3BGRL	1.54	0.11	0.37
XP_009973690.2	annexin A4	104356766	ANXA4	1.52	0.08	0.35
XP_042643089.1	microtubule-associated protein tau isoform X1	104359825	MAPT	1.51	0.21	0.67
XP_032840275.1	extracellular superoxide dismutase [Cu-Zn	104361736	SOD3	1.51	0.15	0.49
XP_032846723.2	acetyl-CoA acetyltransferase, mitochondrial	104356949	ACAT1	-1.52	0.33	0.84
XP_042663455.1	sterol carrier protein 2 isoform X1	104358971	SCP2	-1.54	0.32	0.83
XP_009970614.3	prostatic acid phosphatase	104365504	LOC104365504	-1.54	0.14	0.44
XP_032857738.2	fatty acid synthase	104367309	FASN	-1.57	0.40	0.88
XP_032840864.1	cytosolic 5-nucleotidase 1A	104359556	LOC104359556	-1.57	0.32	0.83
XP_042649428.1	glutaryl-CoA dehydrogenase, mitochondrial	116963484	GCDH	-1.59	0.26	0.77
XP_042649754.1	adenylyl cyclase-associated protein 1	104360123	CAP1	-1.59	0.24	0.74
XP_042641804.1	2,4-dienoyl-CoA reductase [(3E)-enoyl-CoA-producing	104361179	DECR1	-1.59	0.14	0.46
XP_032858964.1	ras-related protein Rab-11B	104366926	RAB11B	-1.60	0.36	0.86
XP_042645199.1	spectrin beta chain, non-erythrocytic 1 isoform X1	104365519	SPTBN1	-1.62	0.08	0.35
XP_042658690.1	retinoid-inducible serine carboxypeptidase isoform X2	104362288	SCPEP1	-1.62	0.10	0.36
XP_042643157.1	ATP-citrate synthase isoform X2	104365621	ACLY	-1.64	0.44	0.90
XP_032857902.1	small glutamine-rich tetratricopeptide repeat-containing protein alpha	104355722	SGTA	-1.65	0.41	0.89
XP_032839426.1	hemoglobin subunit alpha-A	116959343	LOC116959343	-1.65	0.39	0.88
XP_032856568.1	dnaJ homolog subfamily B member 4	104364636	DNAJB4	-1.66	0.34	0.84
XP_042642201.1	regulator of microtubule dynamics protein 1	104363901	RMDN1	-1.69	0.38	0.87
XP_042654655.1	hemoglobin subunit alpha-2	116961035	LOC116961035	-1.75	0.40	0.88
XP_042642088.1	endoribonuclease LACTB2 isoform X2	104363297	LACTB2	-1.77	0.34	0.85
XP_032854038.2	digestive cysteine proteinase 1-like	116962917	LOC116962917	-1.78	0.13	0.43
XP_042652846.1	CD63 antigen	116964442	CD63	-1.81	0.15	0.48
XP_032843772.2	polyadenylate-binding protein 1 isoform X1	104356115	PABPC1	-1.82	0.28	0.79
XP_042662788.1	enoyl-CoA delta isomerase 2 isoform X3	104367903	ECI2	-1.86	0.24	0.73
NP_001289627.1	fatty acyl-CoA reductase 1	104367998	FAR1	-1.94	0.06	0.31
XP_032847126.2	1-acylglycerol-3-phosphate O-acyltransferase ABHD5	104369047	ABHD5	-2.03	0.13	0.41
XP_032860961.1	avidin-like	116964704	LOC116964704	-2.06	0.25	0.74

XP_009972798.2	fibrinogen gamma chain	104367389	FGG	-2.11	0.19	0.63
XP_042643044.1	migration and invasion enhancer 1	116962355	MIEN1	-2.17	0.18	0.58
XP_032837594.1	creatine kinase B-type isoform X2	104356674	CKB	-2.19	0.06	0.30
XP_042663062.1	cystathionine gamma-lyase	104364183	CTH	-2.28	0.09	0.35
XP_032864286.2	endoplasmic reticulum resident protein 29	104356478	ERP29	-2.29	0.30	0.81
XP_042657565.1	medium-chain specific acyl-CoA dehydrogenase, mitochondrial isoform X2	104366752	LOC104366752	-2.32	0.16	0.54
XP_042652508.1	retinol dehydrogenase 16 isoform X2	104361204	LOC104361204	-2.34	0.17	0.57
XP_032844719.2	trifunctional enzyme subunit beta, mitochondrial isoform X1	104357578	HADHB	-2.41	0.16	0.53
XP_032839970.2	peptidyl-prolyl cis-trans isomerase FKBP4	104360088	FKBP4	-2.48	0.21	0.67
XP_042663933.1	serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform isoform X2	104359234	PPP3CA	-2.79	0.08	0.35
XP_032865721.1	low molecular weight phosphotyrosine protein phosphatase isoform X1	104356332	ACP1	-2.99	0.04	0.25

Table S5. Results of Gene Ontology (GO; including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)) enrichment analysis of protein-related genes (*N* = 55) with a |log2 fold change| ≥ 1.5 between egg incubation (*N* = 12) and nestling rearing (*N* = 8). Significantly enriched GO terms, *P*-value, FDR-adjusted *P*-value and *Q*-value are shown in the table.

	GO ID	GO description	Gene ratio	Bg ratio	*P*-value	Adj *P*-value	*Q*-value	Gene ID	Count
CC	GO:0072562	blood microparticle	4/43	144/19518	< 0.01	0.01	0.01	APOA4 / AHSG / GC / FGG	4
CC	GO:0030673	axolemma	2/43	14/19518	< 0.01	0.01	0.01	MAPT / SPTBN1	2
CC	GO:0031093	platelet alpha granule lumen	3/43	67/19518	< 0.01	0.01	0.01	AHSG / TMSB4X / FGG	3
CC	GO:0005788	endoplasmic reticulum lumen	5/43	312/19518	< 0.01	0.01	0.01	APOA4 / AHSG / MANF / ERP29 / FGG	5
CC	GO:0034774	secretory granule lumen	5/43	322/19518	< 0.01	0.01	0.01	AHSG / ACLY / TMSB4X / CAP1 / FGG	5
CC	GO:0060205	cytoplasmic vesicle lumen	5/43	325/19518	< 0.01	0.01	0.01	AHSG / ACLY / TMSB4X / CAP1 / FGG	5
CC	GO:0031983	vesicle lumen	5/43	327/19518	< 0.01	0.01	0.01	AHSG / ACLY / TMSB4X / CAP1 / FGG	5
CC	GO:0031091	platelet alpha granule	3/43	91/19518	< 0.01	0.02	0.01	AHSG / TMSB4X / FGG	3

CC	GO:0042470	melanosome	3/43	112/19518	< 0.01	0.03	0.02	CD63 / FASN / ERP29	3
CC	GO:0048770	pigment granule	3/43	112/19518	< 0.01	0.03	0.02	CD63 / FASN / ERP29	3
CC	GO:0062023	collagen-containing extracellular matrix	5/43	415/19518	< 0.01	0.03	0.02	APOA4 / AHSG / ANXA4 / FGG / SOD3	5
CC	GO:0005777	peroxisome	3/43	143/19518	< 0.01	0.04	0.03	SCP2 / ECI2 / FAR1	3
CC	GO:0042579	microbody	3/43	143/19518	< 0.01	0.04	0.03	SCP2 / ECI2 / FAR1	3
CC	GO:0005759	mitochondrial matrix	5/43	484/19518	< 0.01	0.04	0.03	ACAT1 / GCDH / HADHB / DECR1 / LACTB2	5
CC	GO:0005766	primary lysosome	3/43	155/19518	< 0.01	0.04	0.03	ACLY / CAP1 / CD63	3
CC	GO:0042582	azurophil granule	3/43	155/19518	< 0.01	0.04	0.03	ACLY / CAP1 / CD63	3
CC	GO:0009898	cytoplasmic side of plasma membrane	3/43	159/19518	0.01	0.04	0.03	MIEN1 / ACP1 / PPP3CA	3
CC	GO:0005782	peroxisomal matrix	2/43	51/19518	0.01	0.04	0.03	SCP2 / ECI2	2
CC	GO:0031907	microbody lumen	2/43	51/19518	0.01	0.04	0.03	SCP2 / ECI2	2
CC	GO:0005775	vacuolar lumen	3/43	176/19518	0.01	0.05	0.04	ACLY / CAP1 / GC	3
CC	GO:0022625	cytosolic large ribosomal subunit	2/43	58/19518	0.01	0.05	0.04	RPL8 / RPL34	2
CC	GO:0032589	neuron projection membrane	2/43	60/19518	0.01	0.05	0.04	MAPT / SPTBN1	2
MF	GO:0016408	C-acyltransferase activity	3/43	20/18369	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / HADHB	3
MF	GO:0016746	acyltransferase activity	6/43	253/18369	< 0.01	< 0.01	< 0.01	SCP2 / ACLY / ACAT1 / FASN / HADHB / ABHD5	6
MF	GO:0016747	acyltransferase activity, transferring groups other than amino-acyl groups	5/43	226/18369	< 0.01	0.01	0.01	SCP2 / ACAT1 / FASN / HADHB / ABHD5	5
MF	GO:0019107	myristoyltransferase activity	2/43	11/18369	< 0.01	0.01	0.01	SCP2 / HADHB	2
MF	GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors	3/43	60/18369	< 0.01	0.01	0.01	GCDH / FASN / DECR1	3
MF	GO:0000062	fatty-acyl-CoA binding	2/43	18/18369	< 0.01	0.02	0.01	SCP2 / GCDH	2
MF	GO:1901567	fatty acid derivative binding	2/43	19/18369	< 0.01	0.02	0.01	SCP2 / GCDH	2
MF	GO:0016209	antioxidant activity	3/43	82/18369	< 0.01	0.02	0.01	APOA4 / GPX3 / SOD3	3
MF	GO:0120227	acyl-CoA binding	2/43	21/18369	< 0.01	0.02	0.01	SCP2 / GCDH	2
MF	GO:0016407	acetyltransferase activity	3/43	94/18369	< 0.01	0.02	0.02	ACAT1 / FASN / HADHB	3
MF	GO:0051087	chaperone binding	3/43	104/18369	< 0.01	0.03	0.02	DNAJB4 / MAPT / ERP29	3
MF	GO:0016628	oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	2/43	28/18369	< 0.01	0.03	0.02	FASN / DECR1	2

MF	GO:0033218	amide binding	5/43	400/18369	< 0.01	0.03	0.02	SCP2 / GCDH / FKBP4 / FASN / PPP3CA	5
MF	GO:0003779	actin binding	5/43	434/18369	< 0.01	0.04	0.03	MAPT / TMSB4X / SPTBN1 / CAP1 / GC	5
BP	GO:0006631	fatty acid metabolic process	10/42	394/18614	< 0.01	< 0.01	< 0.01	APOA4 / SCP2 / ACLY / ACAT1 / GCDH / FASN / HADHB / ABHD5 / ECI2 / DECR1	10
BP	GO:0006635	fatty acid beta-oxidation	6/42	76/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0044242	cellular lipid catabolic process	8/42	222/18614	< 0.01	< 0.01	< 0.01	APOA4 / SCP2 / ACAT1 / GCDH / HADHB / ABHD5 / ECI2 / DECR1	8
BP	GO:0009062	fatty acid catabolic process	6/42	105/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0019395	fatty acid oxidation	6/42	109/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0034440	lipid oxidation	6/42	116/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0072329	monocarboxylic acid catabolic process	6/42	131/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0016042	lipid catabolic process	8/42	330/18614	< 0.01	< 0.01	< 0.01	APOA4 / SCP2 / ACAT1 / GCDH / HADHB / ABHD5 / ECI2 / DECR1	8
BP	GO:0030258	lipid modification	6/42	197/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0016054	organic acid catabolic process	6/42	250/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0046395	carboxylic acid catabolic process	6/42	250/18614	< 0.01	< 0.01	< 0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0006637	acyl-CoA metabolic process	4/42	94/18614	< 0.01	0.01	< 0.01	ACLY / ACAT1 / GCDH / FAR1	4
BP	GO:0035383	thioester metabolic process	4/42	94/18614	< 0.01	0.01	< 0.01	ACLY / ACAT1 / GCDH / FAR1	4
BP	GO:0035337	fatty-acyl-CoA metabolic process	3/42	40/18614	< 0.01	0.01	0.01	ACAT1 / GCDH / FAR1	3
BP	GO:0035384	thioester biosynthetic process	3/42	46/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH	3
BP	GO:0071616	acyl-CoA biosynthetic process	3/42	46/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH	3
BP	GO:0033865	nucleoside bisphosphate metabolic process	4/42	122/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH / FAR1	4
BP	GO:0033875	ribonucleoside bisphosphate metabolic process	4/42	122/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH / FAR1	4

BP	GO:0034032	purine nucleoside bisphosphate metabolic process	4/42	122/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH / FAR1	4
BP	GO:1901570	fatty acid derivative biosynthetic process	3/42	52/18614	< 0.01	0.01	0.01	ACAT1 / GCDH / FAR1	3
BP	GO:0044282	small molecule catabolic process	6/42	388/18614	< 0.01	0.01	0.01	SCP2 / ACAT1 / GCDH / HADHB / ECI2 / DECR1	6
BP	GO:0061365	positive regulation of triglyceride lipase activity	2/42	10/18614	< 0.01	0.01	0.01	APOA4 / ABHD5	2
BP	GO:0008611	ether lipid biosynthetic process	2/42	11/18614	< 0.01	0.01	0.01	FASN / FAR1	2
BP	GO:0046504	glycerol ether biosynthetic process	2/42	11/18614	< 0.01	0.01	0.01	FASN / FAR1	2
BP	GO:0097384	cellular lipid biosynthetic process	2/42	11/18614	< 0.01	0.01	0.01	FASN / FAR1	2
BP	GO:0033866	nucleoside bisphosphate biosynthetic process	3/42	57/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH	3
BP	GO:0034030	ribonucleoside bisphosphate biosynthetic process	3/42	57/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH	3
BP	GO:0034033	purine nucleoside bisphosphate biosynthetic process	3/42	57/18614	< 0.01	0.01	0.01	ACLY / ACAT1 / GCDH	3
BP	GO:1901503	ether biosynthetic process	2/42	12/18614	< 0.01	0.01	0.01	FASN / FAR1	2
BP	GO:0051258	protein polymerization	5/42	276/18614	< 0.01	0.01	0.01	MAPT / TMSB4X / SPTBN1 / FKBP4 / FGG	5
BP	GO:0010896	regulation of triglyceride catabolic process	2/42	13/18614	< 0.01	0.01	0.01	APOA4 / ABHD5	2
BP	GO:0044272	sulfur compound biosynthetic process	4/42	155/18614	< 0.01	0.02	0.01	CTH / ACLY / ACAT1 / GCDH	4
BP	GO:0002181	cytoplasmic translation	4/42	159/18614	< 0.01	0.02	0.01	RPL8 / RPL34 / SH3BGRL / PABPC1	4
BP	GO:0006801	superoxide metabolic process	3/42	74/18614	< 0.01	0.02	0.02	APOA4 / MAPT / SOD3	3
BP	GO:0006085	acetyl-CoA biosynthetic process	2/42	17/18614	< 0.01	0.02	0.02	ACLY / ACAT1	2
BP	GO:1901568	fatty acid derivative metabolic process	3/42	77/18614	< 0.01	0.02	0.02	ACAT1 / GCDH / FAR1	3
BP	GO:0006790	sulfur compound metabolic process	5/42	319/18614	< 0.01	0.02	0.02	CTH / ACLY / ACAT1 / GCDH / FAR1	5
BP	GO:0015936	coenzyme A metabolic process	2/42	18/18614	< 0.01	0.02	0.02	ACLY / ACAT1	2
BP	GO:0033194	response to hydroperoxide	2/42	18/18614	< 0.01	0.02	0.02	APOA4 / GPX3	2
BP	GO:0090066	regulation of anatomical structure size	6/42	492/18614	< 0.01	0.02	0.02	MAPT / TMSB4X / SPTBN1 / SCPEP1 / FGG / SOD3	6
BP	GO:0032272	negative regulation of protein polymerization	3/42	80/18614	< 0.01	0.02	0.02	TMSB4X / SPTBN1 / FKBP4	3
BP	GO:0046394	carboxylic acid biosynthetic process	5/42	328/18614	< 0.01	0.02	0.02	APOA4 / CTH / SCP2 / ACLY / FASN	5

BP	GO:0051004	regulation of lipoprotein lipase activity	2/42	19/18614	< 0.01	0.02	0.02	APOA4 / ABHD5	2
BP	GO:1905897	regulation of response to endoplasmic reticulum stress	3/42	82/18614	< 0.01	0.02	0.02	SGTA / MANF / ERP29	3
BP	GO:0016053	organic acid biosynthetic process	5/42	331/18614	< 0.01	0.02	0.02	APOA4 / CTH / SCP2 / ACLY / FASN	5
BP	GO:0032271	regulation of protein polymerization	4/42	191/18614	< 0.01	0.02	0.02	MAPT / TMSB4X / SPTBN1 / FKBP4	4
BP	GO:0006662	glycerol ether metabolic process	2/42	20/18614	< 0.01	0.02	0.02	FASN / FAR1	2
BP	GO:0046485	ether lipid metabolic process	2/42	20/18614	< 0.01	0.02	0.02	FASN / FAR1	2
BP	GO:0060191	regulation of lipase activity	3/42	89/18614	< 0.01	0.03	0.02	APOA4 / ANXA8L1 / ABHD5	3
BP	GO:0019430	removal of superoxide radicals	2/42	23/18614	< 0.01	0.03	0.02	APOA4 / SOD3	2
BP	GO:0090208	positive regulation of triglyceride metabolic process	2/42	23/18614	< 0.01	0.03	0.02	APOA4 / ABHD5	2
BP	GO:0009152	purine ribonucleotide biosynthetic process	4/42	217/18614	< 0.01	0.03	0.02	ACLY / TMSB4X / ACAT1 / GCDH	4
BP	GO:0098869	cellular oxidant detoxification	3/42	99/18614	< 0.01	0.03	0.02	APOA4 / GPX3 / SOD3	3
BP	GO:0018904	ether metabolic process	2/42	25/18614	< 0.01	0.03	0.02	FASN / FAR1	2
BP	GO:0071450	cellular response to oxygen radical	2/42	25/18614	< 0.01	0.03	0.02	APOA4 / SOD3	2
BP	GO:0071451	cellular response to superoxide	2/42	25/18614	< 0.01	0.03	0.02	APOA4 / SOD3	2
BP	GO:0072330	monocarboxylic acid biosynthetic process	4/42	220/18614	< 0.01	0.03	0.02	APOA4 / SCP2 / ACLY / FASN	4
BP	GO:0019433	triglyceride catabolic process	2/42	27/18614	< 0.01	0.03	0.03	APOA4 / ABHD5	2
BP	GO:0046949	fatty-acyl-CoA biosynthetic process	2/42	27/18614	< 0.01	0.03	0.03	ACAT1 / GCDH	2
BP	GO:0050996	positive regulation of lipid catabolic process	2/42	27/18614	< 0.01	0.03	0.03	APOA4 / ABHD5	2
BP	GO:0009260	ribonucleotide biosynthetic process	4/42	231/18614	< 0.01	0.03	0.03	ACLY / TMSB4X / ACAT1 / GCDH	4
BP	GO:0000303	response to superoxide	2/42	28/18614	< 0.01	0.03	0.03	APOA4 / SOD3	2
BP	GO:0000305	response to oxygen radical	2/42	29/18614	< 0.01	0.04	0.03	APOA4 / SOD3	2
BP	GO:2000765	regulation of cytoplasmic translation	2/42	29/18614	< 0.01	0.04	0.03	SH3BGRL / PABPC1	2
BP	GO:0072593	reactive oxygen species metabolic process	4/42	237/18614	< 0.01	0.04	0.03	APOA4 / MAPT / GPX3 / SOD3	4
BP	GO:0046390	ribose phosphate biosynthetic process	4/42	238/18614	< 0.01	0.04	0.03	ACLY / TMSB4X / ACAT1 / GCDH	4
BP	GO:0042744	hydrogen peroxide catabolic process	2/42	30/18614	< 0.01	0.04	0.03	APOA4 / GPX3	2
BP	GO:1990748	cellular detoxification	3/42	115/18614	< 0.01	0.04	0.03	APOA4 / GPX3 / SOD3	3
BP	GO:0006084	acetyl-CoA metabolic process	2/42	32/18614	< 0.01	0.04	0.03	ACLY / ACAT1	2

BP	GO:0034976	response to endoplasmic reticulum stress	4/42	259/18614	< 0.01	0.05	0.04	CTH / SGTA / MANF / ERP29	4
BP	GO:0097237	cellular response to toxic substance	3/42	124/18614	< 0.01	0.05	0.04	APOA4 / GPX3 / SOD3	3
BP	GO:0006164	purine nucleotide biosynthetic process	4/42	262/18614	< 0.01	0.05	0.04	ACLY / TMSB4X / ACAT1 / GCDH	4
BP	GO:0009150	purine ribonucleotide metabolic process	5/42	445/18614	< 0.01	0.05	0.04	ACLY / TMSB4X / ACAT1 / GCDH / FAR1	5
BP	GO:0046461	neutral lipid catabolic process	2/42	37/18614	< 0.01	0.05	0.04	APOA4 / ABHD5	2
BP	GO:0046464	acylglycerol catabolic process	2/42	37/18614	< 0.01	0.05	0.04	APOA4 / ABHD5	2
BP	GO:0072522	purine-containing compound biosynthetic process	4/42	271/18614	< 0.01	0.05	0.04	ACLY / TMSB4X / ACAT1 / GCDH	4

Table S6. Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of protein-related genes (*N* = 55) with a |log2 fold change| ≥ 1.5 between egg incubation (*N* = 12) and nestling rearing (*N* = 8). Significantly enriched KEGG pathways, *P*-value, FDR-adjusted *P*-value and *Q*-value are shown in the table.

	KEGG ID	KEGG description	Gene ratio	Bg ratio	*P*-value	Adj *P*-value	*Q*-value	Gene ID	Count
	tala00071	fatty acid degradation	5/25	40/5413	< 0.01	< 0.01	< 0.01	104356949 / 116963484 / 104366752 / 104357578 / 104367903	5
	tala01212	fatty acid metabolism	5/25	55/5413	< 0.01	< 0.01	< 0.01	104358971 / 104356949 / 104366752 / 104367309 / 104357578	5
	tala00280	valine, leucine and isoleucine degradation	3/25	47/5413	< 0.01	0.02	0.02	104356949 / 104366752 / 104357578	3

General discussion

Organisms have developed diverse adaptive strategies, encompassing biological, physiological and behavioral mechanisms, to minimize exposure and protect themselves from environmental microorganisms (McFall-Ngai *et al.*, 2013). Birds employ common avoidance behaviors, such as nesting site selection, preening or the release of chemical defenses (Bush & Clayton, 2018; Clayton *et al.*, 2010), but also distinguish themselves through specific integumentary adaptations. Birds are covered with feathers, the maintenance and protection of which are necessary for flight, insulation, camouflage, protection, and communication (Stettenheim, 2000). Feather protection primarily relies on their keratin-based structure (Bonser, 1995, 1996) and melanin-based coloration (Galván & Solano, 2016). Feathers also receive protection from preen wax, an oily substance produced by the preen gland (J. Jacob & Ziswiler, 1982; Moreno-Rueda, 2017), and from beneficial yet potentially pathogenic microorganisms inhabiting them (*feather microbiota*) through bacterial interference (Soler *et al.*, 2010). Thus, inadequate feather maintenance and protection can negatively impact birds' health and fitness, emphasizing the importance of identifying key elements and adaptations influencing feather protection. With the aim of addressing the factors influencing feather protection, this thesis coupled lipidomic and proteomic analyses and high-throughput 16S rRNA gene sequencing from field-collected samples to investigate whether and how a wide range of individual and environmental factors – including preen gland traits – influences the barn owl's (*Tyto alba*) feather microbiota.

Barn owl displays a strong sexual dimorphism in breeding duties. Females lay and incubate the eggs for about 30 days and then care for the chicks for a few weeks in the nest, while males provide them with food. Chicks leave the nest at around 55-60 days old (Roulin, 2020). Exposure to nest/environmental microorganisms, along with plumage characteristics and sex hormone levels, may have led to the differences we found in preen gland and feather microbiota traits between chicks and adults, and males and females. Adults indeed had greater plumage bacterial diversity than nestlings and fledglings, and nestlings slightly greater plumage bacterial diversity than fledglings. Among adults, rearing females had larger preen glands than incubating females and males at any stage, and incubating females secreted more preen wax and had lower plumage bacterial diversity than rearing females and males at any stage. Nestlings, fledglings and adults, as well as males and females, also had different plumage bacterial composition (Chapters 1 and 2). Being in contact with nest environment, *i.e.* formed from prey cadavers, droppings and pellets, may have increased preen wax secretion in incubating females for protection purposes, and reduced plumage bacterial diversity in chicks and incubating females. Foraging and exploring new nesting sites may instead

have increased plumage bacterial diversity in males and rearing females (see also Goodenough *et al.*, 2017; Kilgas *et al.*, 2012; Saag, Mänd, *et al.*, 2011; Saag, Tilgar, *et al.*, 2011). Yet, why incubating and rearing females invest differently in preen gland traits, and whether greater or lower plumage bacterial diversity is beneficial, remain poorly understood (Reese & Dunn, 2018). Studying behavioral and physiological variations in female barn owls throughout reproduction would provide insights into the preening frequency, whether females indeed apply preen wax onto eggs and offspring (and for how long), and the selective pressures driving distinct investments in preening strategies and preen gland traits across breeding stages.

Barn owl also exhibits variation in two melanin-based plumage traits. Ventral plumage varies in coloration from white to dark reddish-brown (pheomelanic trait) and in spottiness from immaculate to strongly marked with dark spots (eumelanic trait; Roulin, 2020). Melanin not only contributes to pigmentation but also strengthens and protects feathers from wear, physical abrasion (Bonser, 1995, 1996) and feather-degrading microorganisms (Justyn *et al.*, 2017; Ruiz-De-Castañeda *et al.*, 2012) in a similar way to preen wax (see *General introduction* for mechanisms of action). For these reasons, melanin-based plumage traits could have (and did to some extent) influenced preen gland and feather microbiota traits in fledglings and adults. Darker adults on average had larger preen glands, although melanin-based color effects were found to depend on weather conditions. Lighter adults' preen gland indeed increased in size with temperature and humidity, while darker adults' preen gland decreased in size with temperature only (Chapter 1). However, no clear differences were found in feather microbiota traits according to melanin-based plumage traits in fledglings and adults (Chapter 2). Such a lack of differences may not be due to a lack of effect of melanin-based plumage traits (*e.g.* melanized individuals could have varying bacterial diversity and composition compared to unmelanized ones; see also Al Rubaiee *et al.*, 2021) but rather to an interactive effect between melanin-based plumage and preen gland traits. Increasing lighter adults' preen gland in response to temperature and humidity may have effectively adjusted plumage bacterial diversity and composition to levels comparable to darker adults. Both melanin-based plumage traits and preen gland may thus supersede each other in protecting feathers from environmental microorganisms (see also Roulin, 2007), supporting to some extent the Gloger's rule (Delhey, 2017, 2019). Further research is nevertheless required to understand how melanin pigmentation and preen gland interaction contributes to the Gloger's rule (*i.e.* animals in warmer and more humid environments tend to have darker pigmentation than those in colder and drier ones). Similar research to ours on barn owls living in more extreme temperature or humidity conditions, or on other bird species with unmelanized or completely melanized plumage, would provide further insights into the respective and

shared roles of melanin pigmentation and preen wax in protecting feathers from environmental microorganisms.

Barn owl, not appearing to rely on melanin-based plumage traits, may instead use the preen gland for feather protection. Preen wax may indeed form a protective oily barrier (S. Jacob *et al.*, 2018; Reneerkens *et al.*, 2008; Verea *et al.*, 2017), serve as energy stores promoting mutualists and commensals (Soler *et al.*, 2010) or exert an antimicrobial action against pathogens (Braun *et al.*, 2018; Carneiro *et al.*, 2020; J. Jacob *et al.*, 1997; Martín-Vivaldi *et al.*, 2009, 2010; Soini *et al.*, 2007; Soler *et al.*, 2008, 2010) to this end (see *General introduction* for mechanisms of action). For the same reasons as melanin-based plumage traits, preen gland traits could have (but did not clearly) influenced feather microbiota traits in fledglings and adults. As mentioned above, incubating females secreted more preen wax and had lower plumage bacterial diversity than rearing females and males at any stage (Chapters 1 and 2). Increasing preen wax secretion may have indirectly reduced plumage bacterial diversity in incubating females. However, no direct association was found between preen wax amount and plumage bacterial diversity or composition in fledglings and adults (Chapter 2), and only a very weak association between preen wax (lipid) and plumage bacterial composition in adults (Chapter 3; but see S. Jacob *et al.*, 2018). Modifying preen wax amount and composition applied onto feathers *in vitro* or bird plumage *in vivo*, while exposing them to various combinations of microorganisms (mutualists, commensals, pathogens), would shed light on the ecological dynamics of preen wax' protective function.

Barn owl does not appear to rely on preen gland traits (*e.g.* its lipid composition) for feather protection either, supporting that (1) feather microbiota may instead be influenced by other factors, (2) preen wax may still serve as antimicrobial defense through alternative mechanisms, or (3) preen wax may perform additional functions (signaling, communication) in the barn owl. Firstly, skin-derived lipids (*e.g.* free fatty acids and alcohols) along with symbiotic microorganisms residing on feathers and in preen gland (*resident microbiota*) may help preen wax protect feathers and thus influence feather microbiota through antimicrobial action or bacterial interference (Braun *et al.*, 2018; Soler *et al.*, 2010), respectively. Molting may also renew feathers and thus influence/reduce the overall feather microbial load (Clayton *et al.*, 2010; Gunderson, 2008). Analyzing feather chemical composition (*e.g.* skin-derived lipids), as well as the preen gland microbiota would help understand factors influencing barn owl feather microbiota. Secondly, if lipids do not protect feathers, other preen wax compounds, such as acids, alcohols, proteins and peptides, may instead exert a direct antimicrobial action against pathogens (Braun *et al.*, 2018; Carneiro *et al.*, 2020; J. Jacob *et al.*, 1997; Soini *et al.*, 2007). Lysozymes and immunoglobulins Y were not only reported in house

sparrow (*Passer domesticus*) preen wax (Carneiro *et al.*, 2020), several immune-related peptides and proteins were also found in barn owl preen wax (Chapter 4). Bacteriocins and other antimicrobial substances originating from symbiotic bacteria living in preen gland may also have a similar antimicrobial action (Martín-Vivaldi *et al.*, 2009, 2010; Soler *et al.*, 2008, 2010). Yet, the preen gland microbiota has been little but should be further studied in the barn owl (Braun, Wang, Zimmermann, Boutin, *et al.*, 2019; Braun, Wang, Zimmermann, Wagner, *et al.*, 2019). Thirdly, preen wax may not only protect feathers but also play a role in signaling and communication (Grieves *et al.*, 2022; Moreno-Rueda, 2017). Age and sex specificity (see above), as well as variations in preen gland traits according to body condition – *i.e.* adults and fledglings in better condition had larger preen glands, may support preen wax' involvement in such functions (Chapter 1). Preen wax may indeed function as (chemo)signals enabling visual signaling, olfactory camouflage, and visual/olfactory recognition and communication among conspecifics, mates or parents-offspring (Grieves *et al.*, 2022; Whittaker & Hagelin, 2021), perhaps limiting its importance in feather protection. Yet, the importance of visual and olfactory communication should also be further studied in the barn owl. Thoroughly investigating these three hypotheses would certainly contribute to a deeper insight into preen gland's complex functioning across bird species.

Prior research has proposed a wide range of biological functions for the preen gland in birds, ranging from feather maintenance, protection and waterproofing to visual and olfactory communication (Grieves *et al.*, 2022; Moreno-Rueda, 2017). Preen gland may serve well-defined specific functions in certain bird species, such as in sandpipers or hoopoes, while its primary function remains elusive or proves to be multifunctional in others (Moreno-Rueda, 2017). Consequently, exploring trade-offs among its functions and assessing its impact on fitness is essential to deepen our understanding of preen gland's functioning.

In summary, this thesis coupled a large sample size with different analytical techniques, such as lipidomic and proteomic analyses, as well as high-throughput 16S rRNA gene sequencing, to investigate whether and how a wide range of individual and environmental factors influences the barn owl's feather microbiota. Both individual and environmental factors were found to influence preen gland and feather microbiota traits to some extent. However, neither melanin-based plumage traits nor preen gland traits appeared to clearly protect barn owl's plumage despite their protective properties. Preen wax may provide some feather protection while also performing additional functions such as signaling and communication in this species (Grieves *et al.*, 2022; Moreno-Rueda, 2017). Future experimental studies, along with correlative research on avian species with diverse ecologies, are consequently required to decipher preen gland's complex functioning.

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