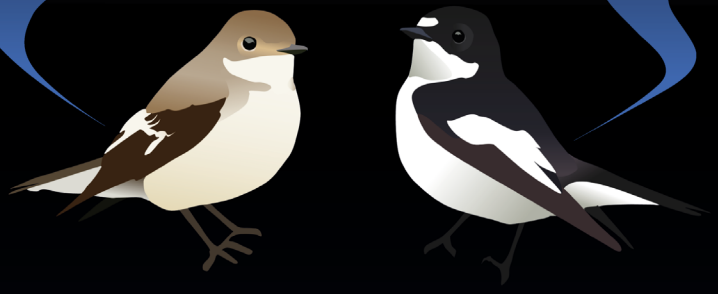

The role of preen oil and body odour in birds

A PhD thesis by

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The role of preen oil and body odour in birds

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CONTENTS

THESIS ABTSTRACT

While the roles of auditory and visual cues in avian communication have been studied extensively, the importance of olfactory cues has been largely overlooked. During preening, birds extract a waxy secretion called preen oil from their uropygial gland (or preen gland) and smear it over their plumage. Preen oil is an important source of avian body odour and may have perfuming (or odour-related) roles, in addition to other roles like plumage maintenance and waterproofing. In this thesis, I explored the odour-based roles of preen oil, notably olfactory crypsis against predators and olfactory intraspecific communication, including mate choice and parent-offspring recognition. To do so, I first investigated what information (e.g. season, sex) is encoded in avian olfactory cues by analysing the variation in the chemical composition of preen oil. In a systematic review (**Chapter 2**), I found that almost all bird species studied exhibit seasonal changes in preen oil composition, whereas only half of bird species studied show sex differences. Why would seasonal changes be common yet sex differences vary between species? To answer this question, I conducted a comparative analysis on 59 species and showed that both seasonal and sex differences can be predicted by the nesting ecology of the species (uni- *vs* biparental incubation, ground *vs* non-ground nesting) (**Chapter 2**). These results suggest that preen oil odours could be used to increase olfactory crypsis at the nest, especially in ground-nesting species, but also as a sex semiochemical for olfactory mate choice. Because the role of preen oil odours is probably species-specific, I sampled and analysed the preen oil of two specific species, the Kentish plover (*Anarhynchus alexandrinus*) and the European pied flycatcher (*Ficedula hypoleuca*). In Kentish plovers, a ground-nesting shorebird with biparental incubation, I found no sex difference during incubation (**Chapter 5**), corroborating results from the comparative analysis. In pied flycatchers, a hole-nesting songbird with uniparental incubation, I also found no sex differences during breeding (**Chapter 4**), which was contrary to the predictions from the comparative analysis. Furthermore, in pied flycatchers, preen oil changed rapidly across breeding stages (between incubation and nestling-rearing) in females and changed across ontogeny (from nestling to adulthood) (**Chapters 3 & 4**). Interestingly, I did not find individual chemical signatures, but breeding pair and family signatures, which suggest that preen oil is influenced by the nest environment (**Chapters 3 & 4**). To make sure that results from such studies are robust and reliable, I conducted an almost exact replication (**Chapter 4**) of the study on the preen oil of pied flycatchers (**Chapter 3**). Importantly, part of the results were not reproducible (e.g. the subtle sex differences detected in the first study were not found in the replication), highlighting the value of replication studies, which are still very scarce in chemical ecology. Based on the results from my descriptive studies, I speculate that preen oil may have a role in olfactory crypsis in Kentish plovers and in olfactory mate choice in pied flycatchers, although this remains to be experimentally tested. A few studies have shown that birds can use olfactory cues for parent-offspring recognition, but these studies did not use solely preen oil odour. I performed behavioural trials to test if chicks of white-fronted plovers (*Anarhynchus* marginatus), which are precocial and can thus lose contact with their

parents, can use preen oil odours to recognise their parents (**Chapter 6**). In a Y-maze, chicks showed no preference for the preen oil odour of parents or of unfamiliar adults. This result is inconclusive: it could be that chicks were not able to perceive the odours in the experimental setup, were not able to discriminate between these odours, or simply had no preference. In summary, I combined chemical analyses and behavioural experiments on multiple wild bird species to investigate the roles of preen oil and body odour in birds. Although I could not find any clear evidence for a specific role, my thesis provides valuable information and ideas to promote further study on the ecological significance of preen oil and body odour in birds. Notably, I recommend future studies to better assess the relative contribution of preen oil (and other sources) in whole-body odour, to measure nest odour rather than preen oil or body odour to study olfactory crypsis, and to test for olfactory parent-offspring recognition in colonially-nesting precocial species or in species with intraspecific brood parasitism. The importance of odours in the ecology of birds has long been neglected, but the field of avian chemical ecology is now growing rapidly and promises important discoveries.

ZUSAMMENFASSUNG

Während die Rolle akustischer und visueller Signale bei der Kommunikation von Vögeln eingehend untersucht wurde, ist die Bedeutung geruchlicher Kommunikation bisher weitgehend übersehen worden. Vögel nutzen ein wachsartiges Sekret aus ihrer Bürzeldrüse, das sogenannte Bürzeldrüsensekret, und verteilen es über ihr Gefieder. Dieses Bürzeldrüsensekret ist eine wichtige Quelle für den Körpergeruch von Vögeln und kann neben anderen Funktionen wie Gefiederpflege auch eine parfümierende (oder geruchsbezogene) Funktion haben. In dieser Arbeit untersuchte ich die geruchsbasierten Funktionen des Bürzeldrüsensekrets, insbesondere eine potentielle Bedeutung bei der Abwehr von Fressfeinden und die olfaktorische intraspezifische Kommunikation, einschließlich der Partnerwahl und der Erkennung von Eltern und Nachwuchs. Zu diesem Zweck untersuchte ich zunächst, welche Informationen (z. B. Jahreszeit, Geschlecht) in den Geruchssignalen von Vögeln kodiert sein könnten, indem ich die Variationen in der chemischen Zusammensetzung des Bürzeldrüsensekrets analysierte. In einer systematischen Übersichtsarbeit (**Kapitel 2**) stellte ich fest, dass fast alle untersuchten Vogelarten saisonale Veränderungen in der Zusammensetzung des Bürzeldrüsensekrets aufweisen, während nur die Hälfte der untersuchten Vogelarten geschlechtsspezifische Unterschiede zeigt. Damit stellte sich mir die Frage: Warum sind saisonale Veränderungen üblich, während die Geschlechtsunterschiede zwischen den Arten variieren? Zur Beantwortung dieser Frage habe ich eine vergleichende Analyse von 59 Arten durchgeführt und gezeigt, dass sowohl jahreszeitliche als auch geschlechtsspezifische Unterschiede durch die Nistökologie der Arten (ein- vs. birparentaler Bebrütung, Nester in Bodennähe bzw. nicht in Bodennähe) vorhergesagt werden können (**Kapitel 2**). Diese Ergebnisse deuten darauf hin, dass der Geruch des Bürzeldrüsensekrets dazu dienen könnte, die olfaktorische Krypsis am Nest zu verstärken, insbesondere bei bodenbrütenden Arten, aber auch als geschlechtsspezifische Gerüche für die olfaktorische Partnerwahl. Da der Geruch von Bürzeldrüsensekret wahrscheinlich artspezifisch ist, habe ich das Bürzeldrüsensekret zweier spezieller Arten, des Seeregenpfeifers (*Anarhynchus alexandrinus*) und des Trauerschnäppers (*Ficedula hypoleuca*), entnommen und analysiert. Bei Seeregenpfeifern, einem bodenbrütenden Küstenvogel mit biparentaler Brutzeit, fand ich keinen Geschlechtsunterschied während der Brutzeit (**Kapitel 5**), was die Ergebnisse der vergleichenden Analyse bestätigt. Bei Trauerschnäppern, einem in Höhlen brütenden Singvogel mit uniparentaler Bebrütung, fand ich ebenfalls keine Geschlechtsunterschiede während der Brutzeit (**Kapitel 4**), was den Vorhersagen der vergleichenden Analyse widersprach. Darüber hinaus veränderte sich bei Trauerschnäppern das Bürzeldrüsensekret bei den Weibchen in den verschiedenen Brutzeiträumen (zwischen Brutzeit und Nestlingsaufzucht) schnell und veränderte sich im Laufe der Ontogenese (vom Nestling bis zum Erwachsenenalter) (**Kapitel 3 & 4**). Interessanterweise fand ich keine individuellen chemischen Signaturen, sondern Signaturen von Brutpaaren und Familien, was darauf hindeutet, dass das Bürzeldrüsensekret von der Nestumgebung beeinflusst wird (**Kapitel 3 & 4**). Um sicherzustellen, dass die Ergebnisse solcher Studien robust und zuverlässig sind,

habe ich eine fast exakte Replikation (**Kapitel 4**) der Studie über das Bürzeldrüsensekret von Trauerschnäppern (**Kapitel 3**) durchgeführt. Wichtig ist, dass ein Teil der Ergebnisse nicht reproduzierbar war (z. B. wurden die subtilen Geschlechtsunterschiede, die in der ersten Studie festgestellt wurden, in der Wiederholung nicht gefunden), was den Wert von Wiederholungsstudien unterstreicht, die in der chemischen Ökologie immer noch sehr rar sind. Auf der Grundlage der Ergebnisse meiner deskriptiven Studien spekuliere ich, dass das Bürzeldrüsensekret eine Rolle bei der olfaktorischen Krypsis bei Seeregenpfeifern und bei der olfaktorischen Partnerwahl bei Trauerschnäppern spielen könnte, obwohl dies noch experimentell überprüft werden muss. Einige Studien haben gezeigt, dass Vögel den Geruchssinn für die Erkennung von Eltern und Nachkommen nutzen können, aber in diesen Studien wurde nicht ausschließlich der Geruch des Bürzeldrüsensekret verwendet. Ich habe Verhaltensversuche durchgeführt, um zu testen, ob Küken von Weißstirn-Regenpfeifern (*Anarhynchus marginatus*), die aufgrund ihrer Frühreife den Kontakt zu ihren Eltern verlieren können, den Duft des Bürzeldrüsensekrets nutzen können, um ihre Eltern zu erkennen (**Kapitel 6**). In einem Y-Labyrinth zeigten die Küken keine Vorliebe für den Geruch des Bürzeldrüsensekrets ihrer Eltern oder für den eines unbekannten Erwachsenen. Es könnte sein, dass sie die Gerüche in der Versuchsanordnung nicht wahrnehmen konnten, nicht in der Lage waren, zwischen diesen Gerüchen zu unterscheiden, oder einfach keine Präferenz hatten. Zusammenfassend kann ich sagen, dass ich chemische Analysen und Verhaltensversuche an mehreren Wildvogelarten kombiniert habe, um die Rolle von Bürzeldrüsensekret und Körpergeruch bei Vögeln zu untersuchen. Obwohl ich keine eindeutigen Beweise für eine bestimmte Rolle finden konnte, liefert meine Arbeit wertvolle Daten und Ideen zur Förderung weiterer Studien über die ökologische Bedeutung von Bürzeldrüsensekret und Körpergeruch bei Vögeln. Insbesondere empfehle ich künftige Studien, um den relativen Beitrag des Bürzeldrüsensekrets (und anderer Quellen) zum Ganzkörpergeruch besser einschätzen zu können, den Nestgeruch anstelle des Bürzeldrüsensekrets oder des Körpergeruchs zu messen, um die olfaktorische Krypsis zu untersuchen, und die olfaktorische Eltern-Nachwuchs-Erkennung bei in Kolonien brütenden, Nestflüchter Arten oder bei Arten mit intraspezifischem Brutparasitismus zu testen. Die Bedeutung von Gerüchen in der Vogelökologie wurde lange Zeit vernachlässigt, aber das Gebiet der chemischen Vogelökologie wächst jetzt schnell und verspricht wichtige Entdeckungen.

A great white pelican rubbing its bill on its uropygial gland to extract preen oil

Photo by Basile Morin

CHAPTER 1

General introduction

Marc Gilles

Animal chemical communication

Chemical senses are probably the most ancient and widespread senses. Indeed, all organisms, from bacteria to plants and animals, have evolved chemical senses (Wyatt 2014). Chemical senses include olfaction (olfactory system) which allows the detection of *odours* (see glossary in **Table 1**), taste (gustatory system) and chemesthesis (somatosensory system, including the trigeminal system in vertebrates) (Roper 1999). Using these senses, and particularly olfaction, animals have access to abundant chemical information in their environment, such as about food, predators or conspecifics. All animals produce odours, and some of these odours can transmit information about the emitter (referred to as *semiochemicals*, see Wyatt 2014). These semiochemicals – or informational odours – include *pheromones* and *signature mixtures* that allow the interaction between members of the same species (**Fig. 1**). For example, a dog can smell the sex or reproductive status of another dog based on pheromones, and may also recognise the individual's identity based on its signature mixture (Goodwin et al. 1979). Similarly, ants can communicate with conspecifics, producing pheromones to transmit information about their sex and caste, and signature mixtures to transmit information about their colony identity (Monnin et al. 1998, Monnin and Peeters 1999). Semiochemicals can also mediate the interaction between members of different species, and are then referred to as allelochemicals (including allomones, kairomones and synomones; further explained in **Fig. 1**). For example, moths are attracted and lured by allomones from bolas spider predators (Haynes et al. 2002), salamander larvae hide if they detect kairomones from newt predators (Hahn et al. 2023), and anemonefish are attracted by the synomones released by sea anemones (Murata et al. 1986). It is important to recall the distinction between *signals* and *cues*. The information emitted by the sender and affecting the receiver can be a signal if, and only if, it has evolved to produce this specific effect, but is otherwise termed a cue, if it has not (Maynard Smith and Harper 2003). For example, the pheromones from dogs and ants, as well as the

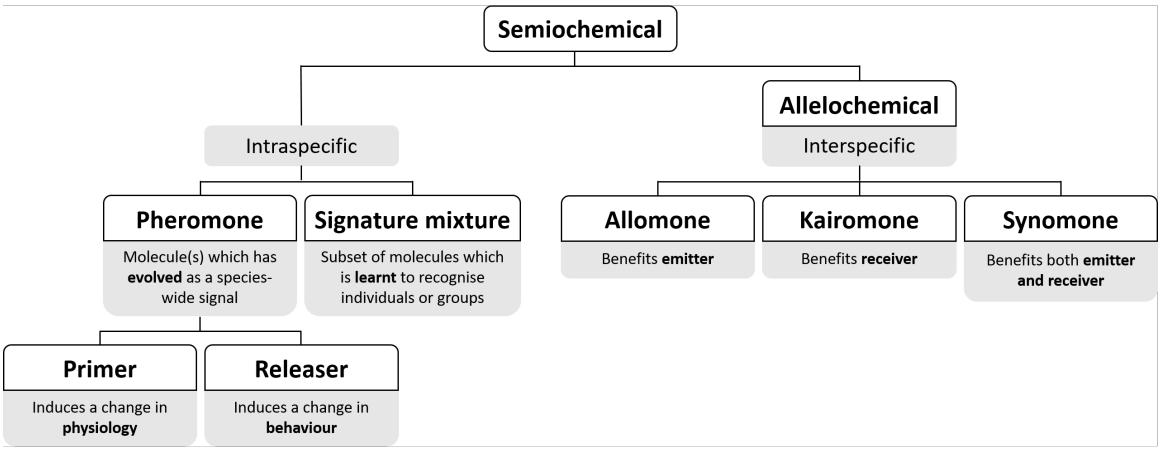

Fig. 1. Different categories of semiochemicals. Inspired by Wyatt (2014) and Schulte (2016).

synomones from sea anemones, are signals (in this case *chemosignals*), whereas the kairomones from newt predators are cues (Wyatt 2010).

Chemical communication has been extensively studied in mammals and insects, but also in amphibians, reptiles, fishes, echinoderms, non-insect arthropods, molluscs, flatworms and cnidarians (Buchinger and Li 2023). In contrast, the scarcity of studies on birds is striking (Johansson and Jones 2007). This is intriguing because birds are important models for the study of sensory ecology and animal communication. Indeed, the acoustic (e.g. songs and calls) and visual (e.g. plumage, ornaments and courtship displays) signals of birds have been catalogued for hundreds of species (Mason et al. 2014). In comparison, avian odours and olfaction were largely unexplored, at least until the 1960s (Clark et al. 2015, Alves Soares et al. 2024a). Why did it take so long for researchers to study this sensory modality in birds? Is it because birds do not produce, detect or use odours? Or is it because researchers neglected this possibility?

Smell: an overlooked aspect of avian biology

For a long time, birds were believed to be anosmic (i.e. with no sense of smell) or at best microsmatic (i.e. with a poor sense of smell) (Roper 1999). This misbelief probably originated in the 1820s, after John James Audubon conducted field experiments to test if vultures use smell to find food – a fact that was commonly accepted at that time (Audubon 1826, Stager 1967). The results indicated that vultures use vision rather than olfaction to locate carrion. Although the experiments were flawed (Stager 1967, Nevitt and Prada 2015), Audubon's aim to "explode" the consensual idea that birds use smell was achieved (Audubon 1826). Indeed, the (mis) belief that birds have no sense of smell (or too little to be important for their ecology) gained popularity and still persists today – two centuries later – in both public and academic circles (*personal observations*).

Several factors probably contribute to the persistence of this misconception. The first factor is theoretical: following the theory that nature is parsimonious, birds may have evolved developed auditory and visual senses only at the expense of the sense of smell (which was probably Audubon's reasoning; Souder 2005, Nevitt and Prada 2015). The second factor is anatomical: most birds have relatively small olfactory bulbs – the region of the brain involved in the sense of smell (especially passerines, Bang and Cobb 1968). The third factor is behavioural: in contrast to mammals which sniff with their rhinarium (or "wet nose") or insects which move their antennae towards odours, birds do not display obvious smelling behaviours with their rigid bill and nostrils (Roper 1999). Birds also do not engage in obvious scent-marking behaviours (Roper 1999). Despite this, in the 1960s, researchers started to question the anosmia of birds (Tucker 1965, Stager 1967, Wenzel 1967). Studies on avian olfaction started to accumulate (Caro et al. 2015), and it soon became evident that birds can smell and that odours play a significant role in their ecology.

Glossary

Camouflage Strategies involved in concealment, including prevention of detection (i.e. crypsis) and recognition (i.e. masquerade) (Stevens and Merilaita 2009)

Chemosignal Chemical signal

Cue Information produced by senders that affects receivers but that has not evolved because of this effect (Maynard Smith and Harper 2003)

Exocrine gland Type of gland that releases its secretion external to or at the surface of an organ by means of a canal or duct

Holocrine gland Type of exocrine glands in which the cell disintegrates to release its secretions (e.g. sebaceous gland)

Odour Airborne chemical

Pheromone Molecule (or combination of molecules in defined ratios) that has evolved as a species-wide signal that elicits a specific reaction in a conspecific (Wyatt 2010)

Sebaceous gland Type of holocrine exocrine gland that secretes an oily substance (sebum)

Semiochemical Informational molecule(s)

Signal Information produced by senders that affects receivers and that has evolved because of this effect (Maynard Smith and Harper 2003)

Signature mixture Variable subset of molecules from an animal's chemical profile learnt by conspecifics to recognise the animal as an individual or as a member of a particular group (e.g. family, clan, colony) (Wyatt 2010)

Table 1. Glossary.

Avian olfaction

Anatomical, electrophysiological and molecular evidence

Researchers first showed that birds are anatomically and neurologically equipped to detect odours (see Wenzel 2007, Balthazart and Taziaux 2009, Caro et al. 2015 for reviews). The olfactory system of birds is similar to that of other tetrapods (Caro et al. 2015). Air is inspired through a pair of external nares and transferred into internal nasal cavities, where it is filtered, warmed up, moistened and chemically sampled via olfactory receptors located on the olfactory epithelium. The chemical information is then transmitted via olfactory nerves to the olfactory bulb in the forebrain (Roper 1999). Electrophysiological studies

showed that odour stimuli stimulate olfactory nerves and neurons of the olfactory bulb in numerous bird species (Tucker 1965, Wenzel and Sieck 1972, Clark and Mason 1987, McKeegan et al. 2002). Molecular studies found that avian olfactory receptors are coded by large and diverse repertoires of functional genes, with only a limited proportion of pseudogenes, suggesting a well-developed sense of smell (Steiger et al. 2008, 2009). Birds, including passerines with small olfactory bulbs, have surprisingly high olfactory acuity (comparable to that of rats and rabbits, Clark et al. 1993, Avilés and Amo 2018). In conclusion, all the birds that have been studied possess a fully functional olfactory system and a relatively good sense of smell.

Behavioural and ecological evidence

Researchers then showed that birds actually use smell in many ecological contexts (see Balthazart and Taziaux 2009 and Abankwah et al. 2020 for reviews).

Birds use smell to find food. For example, using olfactory cues alone, turkey vultures (*Cathartes aura*) locate putrefying meat (Potier et al. 2019), North Island brown kiwis (*Apteryx mantelli*) locate mealworms (Cunningham et al. 2009), toucans (*Ramphastos spp.*) locate fruits (Hernández et al. 2023), Eurasian magpies (*Pica pica*) locate nuts (Molina-Morales et al. 2020), and great tits (*Parus major*) locate caterpillars (Amo and Saavedra 2021). On larger spatial scales, wandering albatrosses (*Diomedea exulans*) can smell food patches in the open sea (Nevitt et al. 2008) and white storks (*Ciconia ciconia*) can smell freshly mown pastures where their prey abound (Wikelski et al. 2021).

Birds use smell to navigate. The most studied and famous case is the feral pigeon (*Columba livia*), which uses odour cues from the environment for homing (Gagliardo 2013). Other species, such as gray catbirds (*Dumetella carolinensis)* and lesser black-backed gulls (*Larus fuscus*), use odours to navigate during migration (Holland et al. 2009, Wikelski et al. 2015, Bonadonna and Gagliardo 2021).

Birds use smell to detect predators. Red junglefowl (*Gallus gallus*) increase vigilance in the presence of faecal odours from dhole and tiger predators (Zidar and Løvlie 2012), and red-legged partridges (*Alectoris rufa*) avoid the odour of ferret predators (Mahr and Hoi 2018). However, it should be noted that a number of studies found that birds do not avoid predator scents during foraging or nesting (e.g. Amo et al. 2018, Avilés et al. 2019, Dotta et al. 2024).

Birds use smell to choose nest material. Several species of passerines, including blue tits (*Cyanistes caeruleus*), European starlings (*Sturnus vulgaris*) and spotless starlings (*Sturnus unicolor*), use odours to select aromatic plants to add to their nest, which may protect their offspring from pathogenic microbes (Petit et al. 2002, Gwinner and Berger 2008, Ruiz-Castellano et al. 2018). Interestingly, European starlings are only sensitive to the odours of these plants during the nest building period (De Groof et al. 2010).

Birds use smell to communicate with conspecifics. Several passerine birds, including dark-eyed juncos (*Junco hyemalis*), song sparrows (*Melospiza melodia*) and spotless starlings, can discriminate between sexes using olfactory cues alone (Whittaker et al. 2011a, Amo et al. 2012a, Grieves et al. 2019b, Krause et al. 2023). European storm-petrels (*Hydrobates pelagicus*) and Humboldt penguins (*Spheniscus humboldti*) can recognise kin by smell (Coffin et al. 2011, Bonadonna and Sanz-Aguilar 2012). Birds can even assess the similarity and diversity of the major histocompatibility complex (MHC) genotype of potential mates based on odours, as shown in blue petrels, song sparrows and black-legged kittiwakes (*Rissa tridactyla*) (Leclaire et al. 2017b, Grieves et al. 2019c, Pineaux et al. 2023). For a more complete overview of olfactory intraspecific communication in birds, excellent reviews are available (Hagelin 2007a, Hagelin and Jones 2007, Caro et al. 2015, Whittaker and Hagelin 2021), and I also provide more details in the section "**Hypothesized functions of avian body odour**" further below. If birds can use smell to assess sex, kinship or genotype, this information must be encoded in their body odour.

Avian body odour

Birds do not only smell (i.e. have a sense of smell) but also smell (i.e. have a body odour). Some species have a pungent odour (reviewed in Dumbacher and Pruett-Jones 1996 and Weldon and Rappole 1997). Among those are petrels and shearwaters (Procelariiforms) with their musky scent, and crested auklets (*Aethia cristatella*) with their citrusy scent (Hagelin 2007b). In contrast, some species are famous for their foul or unpleasant odour, like the hoatzin (*Opisthocomus hoazin*), the Eurasian hoopoe (*Upupa epops*), woodhoopoes (*Phoeniculus spp.*), anis (*Crotophaga spp.*) and several other species of cuckoos (Cuculidae) (Dumbacher and Pruett-Jones 1996, Weldon and Rappole 1997, Hagelin 2007a). Among passerines, caciques (*Cacicus spp.*) and grackles (*Quiscalus spp.*) appear to be particularly smelly, while Hawaiian honeycreepers (Carduelinae) are said to smell "like old canvas" (Weldon and Rappole 1997). Although a few bird species have a noticeable odour, many species are not particularly odorous to humans. But the fact that they do not smell strongly to humans does not mean that they do not smell to other birds. In fact, birds are well known to communicate using sensory channels that humans are unable to detect (e.g. infrasound, Freeman and Hare 2015; ultraviolet; Cuthill et al. 2000).

What chemical information is contained in the smell of birds? Odours are biogenic and are thus usually not used for rapid, versatile or mutually responsive communication, unlike visual or acoustic cues. Instead, odours allow the prolonged and stable broadcasting of genetic and physiological information, which is useful for social interactions (e.g. mate choice, individual or kin recognition; Caro et al. 2015). This is the case for bird odours. Avian chemical cues contain a wealth of information about individuals, including individual identity (Mardon et al. 2010, Jennings and Ebeler 2020), sex (Amo et al. 2012a, Caspers et al. 2022), age (Shaw et al. 2011, Díez-Fernández et al. 2021), genetic heterozygosity (Whittaker et al. 2019b), genetic relatedness (Potier et al. 2018), MHC

genotype (Leclaire et al. 2014, Slade et al. 2016), morph (Tuttle et al. 2014), group membership (Grieves et al. 2024) and population of origin (Whittaker et al. 2010, Grieves et al. 2019a). In dark-eyed juncos, body odour even predicts aggressiveness (Whittaker et al. 2018) and reproductive success (Whittaker et al. 2013). Avian odours also reflect the health and physiology of individuals, like blood parasite infection (Grieves et al. 2018, Talbott et al. 2022), exposure to metal pollution (Leclaire et al. 2019), food stress (Reneerkens et al. 2007b, Grieves et al. 2020), diet (Thomas et al. 2010, Kanakri et al. 2016), plumage microbiota (Jacob et al. 2014), uropygial gland microbiota (Martín-Vivaldi et al. 2010, Whittaker et al. 2019b) and hormone levels (Whelan et al. 2010, Whittaker et al. 2011b). In addition, in many species, body odour varies seasonally (e.g. Reneerkens et al. 2002, Soini et al. 2007). Most of these studies investigating the information available in avian chemical cues (> 70 %) used the chemical composition of preen oil as a proxy of bird body odour (Alves Soares et al. 2023, 2024a).

Preen oil: a major source of avian body odour

While mammals have many different scent-producing glands (Eisenberg and Kleiman 1972), birds have essentially one: the uropygial gland (or preen gland) (Jacob and Ziswiler 1982). The secretion from the uropygial gland is called preen oil and is considered as the main source of avian body odour (Soini et al. 2013, Caro et al. 2015). Other sources of avian odour have been suggested (e.g. skin, powder down feathers, faeces, stomach oils) but never tested (Hagelin and Jones 2007, Nevitt and Prada 2015). I expand on these hypothesized additional sources of odour in the **General discussion**.

Preen oil is the secretion from the uropygial gland – a large *sebaceous gland* located dorsally at the base of the tail (**Fig. 2**, reviewed in Jacob and Ziswiler 1982, Salibian and Montalti 2009). This gland is unique to birds and is present in all bird species during embryonic development. It is absent only in adults in ostriches (Struthioniformes), rheas (Rheiformes), cassowaries (Casuariiformes), bustards (Otididae), mesites (Mesitornithidae) and several species of pigeons (Columbidae), parrots (Psittacidae), woodpeckers (Picinae) and frogmouths (*Podargus spp.*) (Johnston 1988). During preening, birds extract preen oil from their uropygial gland by rubbing the papilla of the gland and smear it over their entire body (**Fig. 2**). Preen oil is odoriferous. Indeed, it is composed of volatile compounds, as well as nonvolatile compounds which can be degraded into volatile compounds once smeared over the plumage (via physical or microbial degradation; Mardon et al. 2011a, Maraci et al. 2018). These compounds mainly include wax esters, alkanes, alkenes, alcohols, aldehydes, ketones, carboxylic acids and aromatic compounds (reviewed in Campagna et al. 2012, Alves Soares et al. 2024). Many studies used preen oil chemical composition as a proxy for avian body odour, because **(1)** preen oil is likely the main source of avian body odour, and **(2)** it is easy to sample, store and extract (Alves Soares et al. 2024a). It should however be emphasized that preen oil composition may not be the best

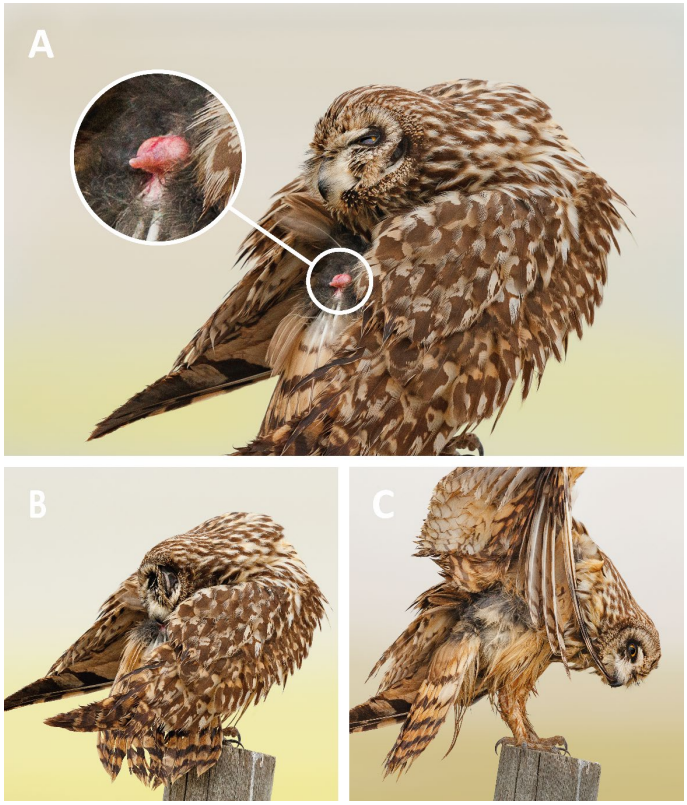

Fig. 2. Preening. Birds preen their feathers with preen oil, a waxy secretion from **(A)** the uropygial gland, located dorsally at the base of the tail. During preening, **(B)** birds collect preen oil by rubbing their bill or head on their uropygial gland, and **(C)** smear it onto their entire plumage. Photos of a preening short-eared owl (*Asio flammeus*) by Jacques van Wijlick.

proxy for body odour for all bird species (e.g. crested auklets, Hagelin 2007b; feral pigeons, Leclaire et al. 2019).

Preen oil is a multifunctional secretion (**Fig. 3**, reviewed in Moreno-Rueda 2017). Because of its odoriferous nature, preen oil may have a role in olfactory intraspecific communication (including mate choice and parent-offspring communication) and olfactory protection against predators or parasites via crypsis or repellence (detailed in the next section "**Hypothesized functions of avian body odour**"). However, the uropygial gland is not only a scent gland and also has non-odour-based functions. Those include plumage maintenance and waterproofing (Elder 1954, Giraudeau et al. 2010), visual intraspecific communication via cosmetic colouration (e.g. of plumage, Amat et al. 2011; skin, Soler et al. 2022; eggs, Díaz-Lora et al. 2021), protection against ectoparasites via antimicrobial activity (e.g. against eggshell bacteria, Martín-Vivaldi et al. 2014; feather bacteria, Alt et al. 2020) and pollutant excretion (Gutiérrez et al. 1998). While there is strong evidence of a role of preen oil in plumage maintenance and waterproofing, the other hypothesized functions received variable support from experimental studies (**Fig. 3**) (Moreno-Rueda 2017).

Hypothesized functions of avian body odour

I summarize here the hypothesized functions of avian body odour, mainly focussing on preen oil odours, and briefly review the evidence for each function. It is important to note that these functions are not mutually exclusive. For example, if body (or preen oil) odours

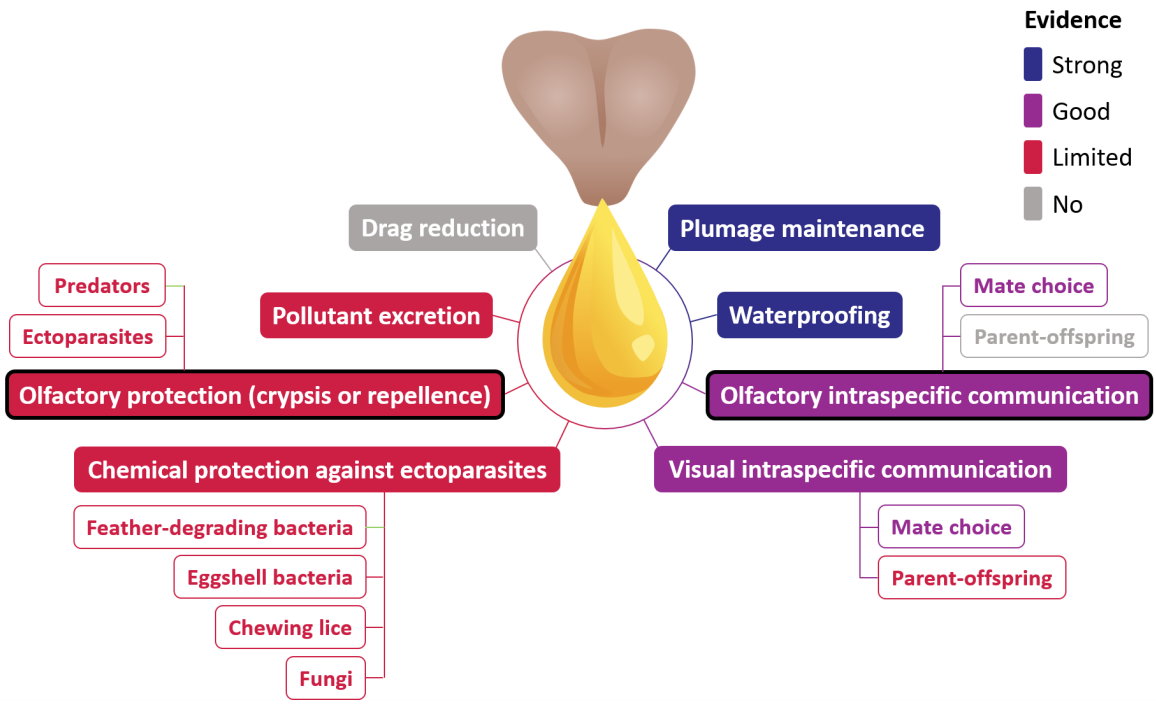

Fig. 3. Current evidence for the hypothesized functions of preen oil. The degree of evidence for each function was based on Moreno-Rueda (2017) and updated with the recent literature (2017-2024). Strong: experimental evidence of a direct effect of preen oil and fitness consequences in more than two species. Good: experimental evidence of a direct effect of preen oil and fitness consequences in one or two species. Limited: evidence of an effect of preen oil but not of fitness consequences. No: no evidence of an effect of preen oil. Odour-based functions are outlined in black.

offer a selective advantage (e.g. increased breeding success via enhanced olfactory crypsis against nest predators), they could become a sexually selected signal of quality – or "olfactory sexual ornament" – and be used in olfactory mate choice (Hagelin 2007b, Díaz-Lora et al. 2021).

Olfactory protection against predators and/or ectoparasites

Olfactory crypsis against predators. While many bird species are visually cryptic, some may also be olfactorily cryptic (Ruxton 2009). Olfactory *crypsis* is thought to be beneficial in birds especially during nesting, as the odour from eggs, chicks and brooding parents can attract olfactorily-searching (nest) predators (e.g. mammals, snakes, birds; Thompson III 2007, Shutler 2019). Most of the evidence for olfactory crypsis in birds comes from studies on ground-nesting shorebirds (Charadriiformes, 27 species; Reneerkens et al. 2002, 2006, 2007). In these species, preen oil composition switches from monoesters to diesters, which are presumably less odorous because of their higher molecular weight, during the time of incubation and chick-rearing, and may enhance olfactory crypsis by reducing odour cues at the nest. This was supported by the observation that the preen oil switch occurs essentially in the sex(es) that incubates (Reneerkens et al. 2007a), and by an experimental study where a trained dog took longer to detect the diester preen oil than the monoester preen oil of red knots (*Calidris canutus*) (**Fig. 4a**) (Reneerkens

et al. 2005). Inspired by these results, other studies have speculated on a cryptic role of preen oil after finding a reduced volatility of preen oil during breeding or in the incubating sex (Fluen 2008, Fischer et al. 2017, López-Perea and Mateo 2019). Olfactory crypsis may be achieved by the reduction of odour cues, but also by background matching. This was suggested for dark-eyed juncos, in which preen oil contains more linear alcohols during breeding, which may blend with the linear alcohols from the plants surrounding their nest, but this was never tested (Soini et al. 2007). While olfactory crypsis seems well supported in shorebirds, it is important to note that its support stems solely from one experimental study. To my knowledge, olfactory crypsis (via body odours) has never been documented in species other than shorebirds. Overall, evidence for olfactory crypsis against predators in birds is limited.

Olfactory repellence against predators. Avian body odour may be used as a predator deterrent. One documented case of olfactory deterrence of predators is the green woodhoopoe (*Phoeniculus purpureus*). When threatened by a predator at the nest, green woodhoopoes release a malodorous preen oil that they expose to the predator to repel it (Burger et al. 2004). Anecdotally, such a function may be evident only in species that produce malodorous preen oil, stomach oils, vomit or faeces. It has been suggested, but not tested, in a few other species (reviewed in Dumbacher and Pruett-Jones 1996).

Olfactory repellence (or crypsis) against ectoparasites. Avian body odour may be used to repel ectoparasites, notably blood-feeding arthropods. The malodorous preen oil of Eurasian hoopoes seem to repel haematophagous dipterans (mosquitoes, blackflies and biting midges) (Tomás et al. 2020). Chemicals associated with the citrusy scent of crested auklets and chemicals associated with the sour scent of pitohuis (*Pitohui spp.*) and ifritas (*Ifrita spp.*) may also deter ectoparasitic arthopods (lice, ticks and mosquitoes), but these chemicals do not seem to originate from preen oil, and evidence of their antiparasitic role is mixed (Hagelin and Jones 2007). When researchers find, in preen oil, chemicals that are known to be effective arthropod repellents, they often speculate on a possible repellent function (e.g. Whittaker et al. 2019a), but experimental studies are lacking (Moreno-Rueda 2017). In addition to repelling ectoparasites, preen oil odours may be used to avoid being detected by ectoparasites (i.e. crypsis). Many studies have tested whether bird (and preen oil) odours attract ectoparasites, especially blood-sucking dipterans that are vectors of malaria parasites (e.g. Díez-Fernández et al. 2020). Some studies have found that blood-sucking dipterans are attracted to bird (and preen oil) odours, but other studies did not find such an effect (reviewed in Martínez-de la Puente et al. 2020 and Marzal et al. 2022). Evidence for a function of bird (and preen oil) odour in repellence (or crypsis) against ectoparasites is still limited and, where available, only applies to a handful of species (see Marzal et al. 2022 and Weldon 2023 for reviews).

Olfactory intraspecific communication

Olfactory mate choice. The first step of mate choice is to identify potential partners (i.e. species and sex recognition). In all species tested, birds prefer the scent of a conspecific

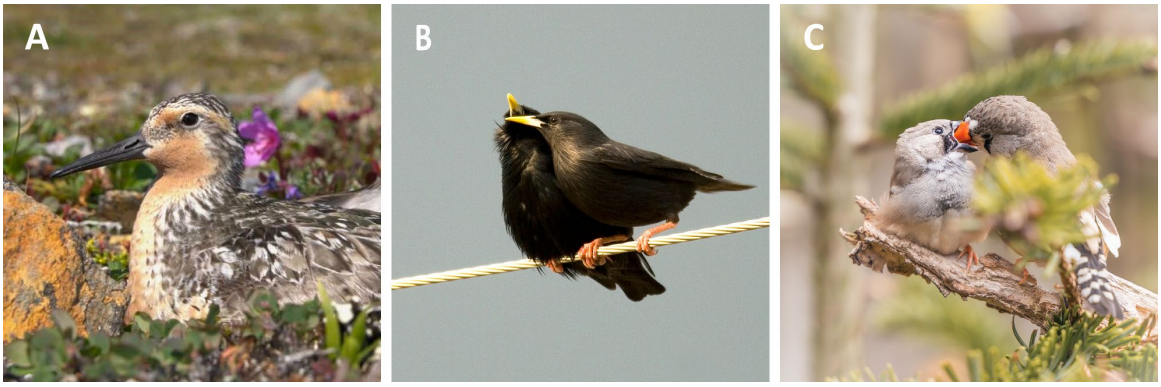

Fig. 4. Hypothesized functions of body odour in birds. (A) Olfactory crypsis against predators: body odours may be reduced during incubation and/or chick-rearing to limit detection by olfactorily-searching predators and thereby protect the eggs, chicks and/or brooding parents. Photo of a red knot by Jeroen Reneerkens. **(B) Olfactory mate choice:** body odours may be produced to transmit information (e.g. sex, kin, genetic quality and/or compatibility) to potential mates during breeding. Photo of spotless starlings by Coulanges. **(C) Olfactory parent-offspring communication:** body odours may be produced to facilitate parent-offspring recognition. Photo of zebra finches by Dafne Vos.

over that of a heterospecific, which may prevent hybridization (e.g. waxwings *Bombycilla spp.*, Zhang et al. 2013, chickadees *Poecile spp.*, Van Huynh and Rice 2019). Birds can also discriminate between female and male conspecifics, as found in spotless starlings (**Fig. 4b**) and a few other species (e.g. dark-eyed juncos, song sparrows, six species of Estrildid finches; Whittaker et al. 2011a, Amo et al. 2012, Grieves et al. 2019b, Krause et al. 2023). The second step is the assessment of the genetic and phenotypic quality of potential partners. Blue petrel and song sparrows prefer the scent of potential mates with MHC dissimilar genotypes (i.e. genetically more compatible), which may increase offspring survival through enhanced resistance to pathogens and reduced risk of inbreeding (Leclaire et al. 2017b, Grieves et al. 2019c). European storm-petrels and Humboldt penguins prefer the odour of nonkin individuals to that of kin individuals, which probably limits inbreeding (Coffin et al. 2011, Bonadonna and Sanz-Aguilar 2012). Preen oil odour correlates with breeding success in dark-eyed juncos, and may thus be used to assess the quality of potential mates, although this was not tested (Whittaker et al. 2013). After finding a partner, birds can recognise the scent of their partner, as found in Antarctic prions and blue petrels (Bonadonna and Nevitt 2004, Mardon and Bonadonna 2009). Many of these studies, but not all (e.g. studies on seabirds), were conducted using preen oil samples as odour stimuli. Overall, evidence that birds use body (and preen oil) odours for sexual signalling during mate choice is accumulating.

Olfactory parent-offspring communication. Recognising kin is not only useful for mate choice but also for parent-offspring recognition. Olfactory parent recognition was documented in two altricial passerines. Zebra finches (*Taeniopygia guttata*) and tree swallows (*Tachycineta bicolor*) nestlings beg longer to the odour of their parent (genetic and familiar) than to the odour of an unrelated adult (Caspers et al. 2017b, Griebel and Dawson 2020). Remarkably, zebra finch nestlings that were fostered in another nest before

hatching could recognise the odour of their genetic mother, suggesting learning of chemical cues in the egg (Caspers et al. 2017b) (**Fig. 4c**). Evidence of offspring recognition is mixed, as both zebra finch and spotless starling females did not discriminate between their own and foreign chicks by smell, but male zebra finches did (Amo et al. 2014, Golüke et al. 2021). Besides, parents can discriminate between the odour of their own and foreign eggs (zebra finches, Golüke et al. 2016; blue petrels, Leclaire et al. 2017a). Evidence of a role of body odour in parent-offspring communication is still limited to a handful of studies and species. Besides, none of these studies used preen oil as odour stimuli.

Dissertation aims and outline

In this dissertation, I combine chemical analyses of preen oil composition and behavioural trials to explore the role of preen oil in avian olfactory communication, both within species (mate choice, parent-offspring recognition) and between species (crypsis against nest predators).

The number of studies on preen oil is growing rapidly. Many studies describe the variation in preen oil composition in a given species, and speculate on its possible function. The two main hypotheses are olfactory crypsis and sexual signalling. How much evidence is there for either hypothesis? In **chapter 2**, my colleagues and I quantitatively answer this question by conducting a comparative study of sex and seasonal differences in preen oil composition. I also systematically review the current evidence for the two hypotheses, as the literature on avian olfactory camouflage has never been reviewed, and the last review on avian signalling is nine years old (Caro et al. 2015, but see Whittaker and Hagelin 2021 which was published shortly before my chapter). I also provide guidelines for future research in avian chemical ecology.

Following our own guidelines, in **chapter 3**, my colleagues and I describe the variation in preen oil chemical composition in pied flycatchers (*Ficedula hypoleuca*) during

Fig. 5. Study species. I studied the role of preen oil and body odour in a songbird (Passeriformes) species, **(A)** the European pied flycatcher (*Ficedula hypoleuca*), and two shorebird (Charadriiformes) species, **(B)** the Kentish plover (*Anarhynchus alexandrinus*) and **(C)** the white-fronted plover (*Anarhynchus marginatus*). Photos by Marc Gilles (A, C) and Hela Boughdiri (B).

breeding (**Fig. 5a**). This passerine is a model species for the study of mate choice and sexual selection (Alatalo et al. 1984, Sirkiä and Laaksonen 2009) but has never been studied with respect to olfactory communication. I investigate whether preen oil composition differs between sexes and breeding stages, and whether it is repeatable within breeding pairs and individuals. Doing so, I describe olfactory phenotypes in this species and speculate on their possible function(s).

Studies in chemical ecology are rarely replicated, notably due to the complexity of the methodology of chemical analyses and the complexity of ecological systems (Nakagawa and Parker 2015). However, replication studies are desperately needed in the current period of a "reproducibility crisis" (Baker 2016). In **chapter 4**, my colleagues and I conduct an almost exact replication of our previous study (**chapter 3**). I use the same protocol to collect and analyse preen oil samples, only with larger sample sizes. After pre-registration of the statistical analysis, I test whether the chemical patterns previously identified are reproducible. In addition, I explore the ontogeny of preen oil composition by comparing the preen oil of adults with that of nestlings.

As found in **chapter 2**, sex differences in preen oil composition are relatively rare in biparentally incubating species. In **chapter 4**, my colleagues and I test whether sex differences in preen oil composition occur in Kentish plovers (*Anarhynchus alexandrinus*), a shorebird with biparental incubation (**Fig. 5b**).

Few studies have investigated olfactory parent-offspring recognition in birds. Intriguingly, the only two studies that tested if chicks can recognise parental odours were conducted on altricial species (without cooperative breeding), where the risk of intermingling between chicks and unrelated adullts is minimal (Caspers et al. 2017b, Griebel and Dawson 2020). Precocial chicks, on the other hand, can lose contact with their parents and should especially benefit from parent recognition. Whether precocial chicks use odours for parental recognition is currently unknown. In **chapter 5**, I conduct behavioural trials in the field to test whether the precocial chicks of white-fronted plovers (*Anarhynchus marginatus*) discriminate between preen oil odour of parents and unfamiliar conspecifics (**Fig. 5c**).

These three study species (**Fig. 5**) were not chosen for their particular body (or preen oil) odour, nor for their known olfactory acuity. In fact, body odours and olfaction have never been investigated in these species. But now that it is established that probably all birds produce odours and have a good sense of smell, we should not focus our research on species with strong odours or renowned olfaction. Instead, it is now crucial to show how important odours are for birds in general.

A white-crowned sparrow collecting preen oil from its uropygial gland

Photo by Hayley Crews

CHAPTER 2

Olfactory camouflage and communication in birds

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ABSTRACT

Smell is a sensory modality that is rarely considered in birds, but evidence is mounting that olfaction is an important aspect of avian behaviour and ecology. The uropygial gland produces an odoriferous secretion (preen oil) that can differ seasonally and between the sexes. These differences are hypothesized to function in olfactory camouflage, i.e. minimizing detection by nest predators (olfactory crypsis hypothesis), and/or intraspecific olfactory communication, particularly during breeding (sex semiochemical hypothesis). However, evidence for seasonal and sex differences in preen oil is mixed, with some studies finding differences and others not, and direct evidence for the putative function(s) of seasonal variation and sex differences in preen oil remains limited. We conducted a systematic review of the evidence for such changes in preen oil chemical composition, finding seasonal differences in 95% of species (57/60 species in 35 studies) and sex differences in 47% of species (28/59 species in 46 studies). We then conducted phylogenetic comparative analyses using data from 59 bird species to evaluate evidence for both the olfactory crypsis and sex semiochemical hypotheses. Seasonal differences were more likely in the incubating than non-incubating sex in ground-nesting species, but were equally likely regardless of incubation strategy in non-ground-nesting species. This result supports the olfactory crypsis hypothesis, if ground nesters are more vulnerable to olfactorily searching predators than non-ground nesters. Sex differences were more likely in species with uniparental than biparental incubation and during breeding than non-breeding, consistent with both the olfactory crypsis and sex semiochemical hypotheses. At present, the data do not allow us to disentangle these two hypotheses, but we provide recommendations that will enable researchers to do so.

INTRODUCTION

All animals produce odours, either as metabolic by-products or as chemicals secreted by specialised glands. These odours can provide information about the producer that can be used during interspecific interactions (e.g. to detect the presence of potential predators or prey) or during intraspecific interactions (e.g. to assess the age, sex, relatedness, or genetic compatibility of a potential mate). In birds, body odours can derive from various sources, including faeces, blood, stomach oils, powder down, plumage, and from secretions of the anal gland, salt gland, salivary gland, ear glands, sebokeratocytes, or skin (Hagelin and Jones 2007). Recently, much attention has focused on the odour-producing role of the uropygial or preen gland (Moreno-Rueda 2017, Whittaker and Hagelin 2021). The preen gland, located near the base of the tail, is present in almost all bird species (Johnston 1988, Moreno-Rueda 2017). The gland secretes preen oil, a complex mixture of wax esters (monoesters, diesters, and triesters) and other compounds (e.g. alcohols, alkanes, aldehydes, carboxylic acids, ketones; reviewed in Campagna et al. 2012). Early work on preen oil was primarily descriptive, but there has been a remarkable growth in preen oil research, particularly with respect to its putative functions (reviewed in Moreno-Rueda 2017; summarized in **Fig. 1**).

Over the past 20 years, researchers have begun to explore preen oil from the perspectives of ecotoxicology [effects of environmental pollutants on preen oil composition, a role for preen oil in pollutant depuration (López-Perea and Mateo 2019, Grieves et al. 2020)]; chemical defence [antimicrobial/antiparasitic activity, predator repellence, olfactory crypsis (Burger et al. 2004, Reneerkens et al. 2007a, Martín-Vivaldi et al. 2010)]; vector attraction (preen oil as an attractant to parasite vectors such as mosquitoes; reviewed in Martínez-de la Puente et al. 2020); species recognition and speciation [testing for chemical signatures of preen oil useful for taxonomic classification (Zhang et al. 2013, Gabirot et al. 2016)]; and intraspecific communication [reproductive and social signalling (reviewed in Caro et al. 2015, Whittaker and Hagelin 2021)]. Researchers have also continued to study the mechanisms underlying preen oil production and chemistry [e.g. diet, endocrine regulation, symbiotic microbes (Thomas et al. 2010, Whelan et al. 2010, Whittaker et al. 2019b)]. Despite this growth in research, the mechanisms of preen oil production and variation – as well as the putative functions of preen oil – are still poorly understood across all research areas. Thus, there is ample opportunity for researchers to make novel and valuable contributions to our understanding of preen oil production and its function in birds.

Some of the functions of preen oil, including waterproofing, feather maintenance, and pollutant depuration, depend on its physical (i.e. oily, waxy) structure. In addition to these structural functions, preen oil is also odoriferous and considered to be a major source

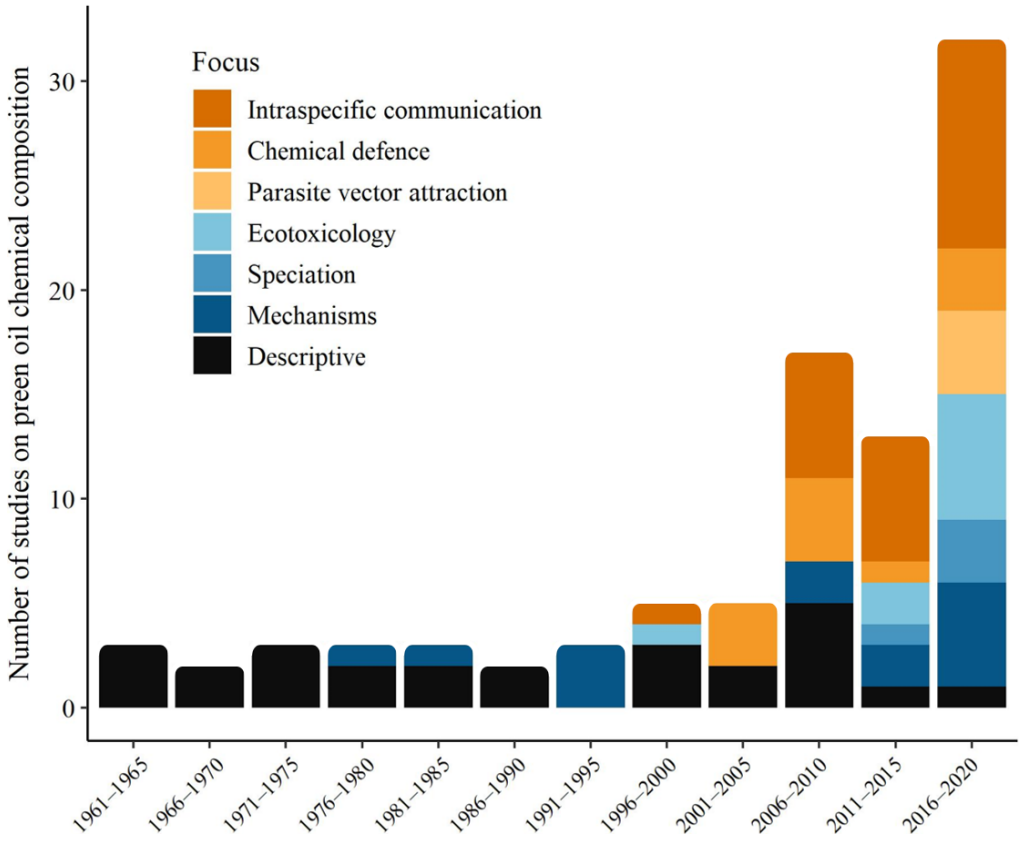


Fig. 1. Major study topics on preen oil chemical composition (97 studies). See Appendix S1 and Table S3 for further details.

of avian body odour (Hagelin and Jones 2007, Caro et al. 2015). Accordingly, preen oil has been hypothesized also to act as an infochemical (Müller et al. 2020) during intraspecific interactions (reviewed in Moreno-Rueda 2017), or as a deleterious cue that reduces detection by predators, such that downregulation of its production, or volatility, would be indicative of olfactory crypsis.

Crypsis is the avoidance of detection through camouflage (Stevens and Merilaita 2009). While most studies of crypsis involve vision, crypsis can also involve olfactory concealment (Ruxton 2009). Birds in a nest can emit odours at all life stages (as eggs, chicks, and adults) and may be vulnerable to olfactorily searching nest predators such as mammals as a result. Birds should therefore benefit from olfactory crypsis at the nest (Shutler 2019), especially since nest predation is a primary cause of reproductive failure (Martin 1993). As such, birds might alter their odours to become less detectable to predators, especially during the critical period of nesting. By contrast, the use of sex semiochemicals for intraspecific chemical communication during breeding suggests that individuals might alter their odours to convey information to and/or modulate their detectability by conspecifics.

The chemical composition of preen oil is dynamic and can be affected by diverse factors, including diet (Thomas et al. 2010), food stress (Reneerkens et al. 2007b, Grieves

et al. 2020), infection status (Grieves et al. 2018), plumage and preen gland microbiota (Jacob et al. 2014, Whittaker et al. 2019b), major histocompatibility complex (MHC) genotype (Leclaire et al. 2014, Slade et al. 2016), age (Shaw et al. 2011, Grieves et al. 2019a), hormone levels (Bohnet et al. 1991, Whittaker et al. 2018), season (Bhattacharyya and Chowdhury 1995, Soini et al. 2007), and sex (Jacob et al. 1979, Whittaker et al. 2010). Seasonal and sex differences in preen oil composition may translate into seasonal and sex differences in odour, which could be linked to specific functions for olfactory crypsis and/or intraspecific communication. Avian preen oil thus has the potential to act as an infochemical that conveys a diversity of information to conspecifics, or as a deleterious cue that masks information from heterospecifics.

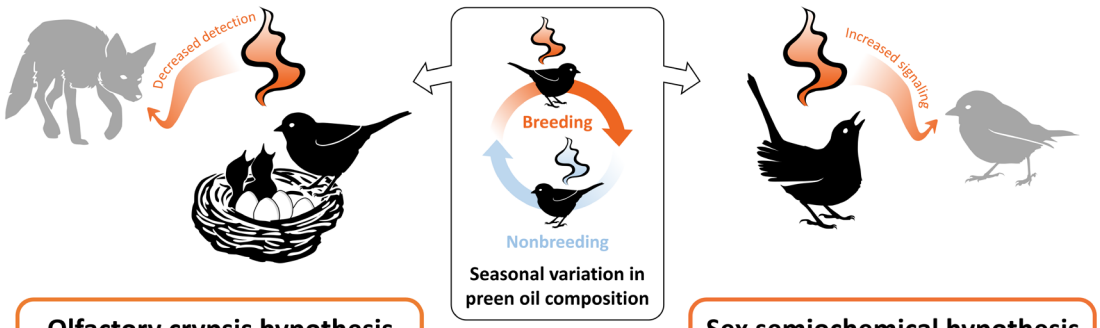
Avian chemical communication has been understudied because birds were historically believed to possess little to no sense of smell (Stager 1967, Bang and Cobb 1968). Fortunately, our understanding of avian chemical communication is growing rapidly. Indeed, birds use smell in intraspecific social contexts such as species discrimination (Zhang et al. 2013, Krause et al. 2014, Van Huynh and Rice 2019), mate recognition (Bonadonna and Nevitt 2004), kin recognition (Coffin et al. 2011, Bonadonna and Sanz-Aguilar 2012, Krause et al. 2012, Caspers et al. 2015a, Caspers et al. 2017), individual recognition (Bonadonna et al. 2007, Bonadonna et al. 2009, Fracasso et al. 2018), distinguishing sex (Hirao et al. 2009, Whittaker et al. 2011a, Amo et al. 2012a, Grieves et al. 2019b), and distinguishing the major histocompatibility complex (MHC) genotype of potential mates (Leclaire et al. 2017b, Grieves et al. 2019c).

We systematically reviewed the literature on seasonal and sex differences in preen oil composition to investigate two non-mutually exclusive hypotheses. First, the 'olfactory crypsis hypothesis' posits that incubating birds switch from more odorous to less odorous preen oil during incubation as a means of reducing odour cues at the nest, thereby protecting eggs and young from olfactorily searching predators (Reneerkens et al. 2002, Reneerkens et al. 2007a). Because less-odorous (higher molecular weight) preen oil is presumably more costly to produce, and perhaps also to apply (Reneerkens et al. 2007b), it is predicted to be secreted only during incubation, when the benefits of crypsis outweigh the costs of production (Reneerkens et al. 2006). This hypothesis predicts an effect of both breeding stage and incubation type on the chemical composition of preen oil. Preen oil changes should occur specifically during incubation and only in the incubating sex, leading to sex differences in uniparentally incubating, but not biparentally incubating, species. Changes in preen oil composition specifically associated with incubation should have evolved primarily in species under strong selective pressure from olfactorily searching nest predators (Reneerkens et al. 2006). Notably, this hypothesis assumes that nest predators should be better at detecting low molecular weight than high molecular weight preen oil (Reneerkens et al. 2005).

Next, we introduce the 'sex semiochemical hypothesis', which posits that sex differences in preen oil are associated with mate recognition (identifying the appropriate sex to mate with) and/or mate choice (identifying a suitable, e.g. genetically compatible,

mate). The sex semiochemical hypothesis predicts that sex differences in the chemical composition of preen oil should be found only during breeding (particularly during mate pairing and egg laying), and that birds should use preen oil odour cues to discriminate between the sexes and/or among individuals. We expand on these two hypotheses further in **Fig. 2**.

The olfactory crypsis and sex semiochemical hypotheses are based on the odoriferous nature of preen oil. However, preen oil may also serve as a chemical defence against a range of parasites, including eggshell bacteria, feather-degrading bacteria, chewing lice, and mosquitoes (reviewed in Moreno-Rueda 2017), and such antiparasitic defence does not require preen oil to be odoriferous (though chemical defences can indeed be odorous). Thus, the antiparasitic defence hypothesis is also non-mutually exclusive with the olfactory crypsis and sex semiochemical hypotheses. Due to a paucity of data, we were not able to conduct a comparative analysis to test for general support of this hypothesis, and therefore focused our analyses on the odour-based hypotheses.



Olfactory crypsis hypothesis

Preen oil of incubating birds becomes less odorous during incubation to reduce odour cues at the nest and protect eggs and young from olfactorily searching predators.

Function of preen oil changes: protection of eggs and young

Receiver of preen oil odour cues: nest predators (heterospecifics)

Sex and seasonal differences: associated with incubation

Predictions

1. Sex differences in preen oil are found during incubation and only in species with uniparental incubation.

2. Preen oil is less volatile during the breeding season.

3. Preen oil odour cues are used by predators to detect nests. Preen oil produced during breeding is less detected by nest predators than preen oil produced during non-breeding.

4. Seasonal changes in preen oil evolved primarily in species under selective pressure from olfactorily searching nest predators.

Sex semiochemical hypothesis

Preen oil odour is used for mate recognition (sex discrimination) and/or mate choice (identification of high-quality or genetically compatible mates).

Function of preen oil changes: mating signal

Receiver of preen oil odour cues: potential mates (conspecifics)

Sex and seasonal differences: associated with mate choice

Predictions

1. Sex differences in preen oil are found only during the breeding season.

2. Preen oil is more volatile during the breeding season.

3. Preen oil odour cues are used by conspecifics to discriminate between the sexes or among individuals of varying quality and/or genetic compatibility.

4. Seasonal changes in preen oil evolved primarily in species under selective pressure to engage in mate choice.

Fig. 2. Hypotheses and predictions to explain the function of seasonal and sex differences in the chemical composition of avian preen oil (a major source of avian body odour).

Under the olfactory crypsis hypothesis, we predicted that, in uniparentally incubating species, only the incubating sex would show a shift in preen oil composition while in biparentally incubating species, both sexes would show shifts; thus, we expected that seasonal differences in preen oil chemical composition would be more common in the incubating sex. We also expected to find seasonal differences more commonly in species with nests more vulnerable to olfactorily searching predators (i.e. nests that are located on or near the ground compared to nests placed at height or in remote, inaccessible locations such as on cliffs). Similarly, we also predicted that sex differences in preen oil would be more likely in species with uniparental than biparental incubation. Under the sex semiochemical hypothesis, we predicted that sex differences in the chemical composition of preen oil would be more likely during breeding than non-breeding. To test these predictions, we conducted a comparative analysis of the available literature that tested for seasonal and sex differences in the preen oil of all bird species for which data were available.

METHODS

Literature review

We systematically reviewed studies that tested for an effect of season and/or sex on the chemical composition of preen oil. We screened the abstracts of 187 publications and the full text of 66 publications, retaining 55 publications (35 on seasonal differences and 46 on sex differences, including 26 publications addressing both seasonal and sex differences) that corresponded to our inclusion criteria. Details of the systematic review and the data used for analysis are available as online Supporting Information (**Appendix S1**, **Fig. S1**, **Tables S1 and S2**).

Preen oil chemical differences

Various analytical and statistical methods have been used to evaluate chemical differences in preen oil composition (**Table S1**). Given the diversity of methodologies used, if a significant chemical difference was observed at $\alpha = 0.05$, we recorded it as such. Thus, we created binary response variables of 'sex difference' and 'seasonal difference' (yes/no).

Seasonal differences

We tested whether sex-specific seasonal changes are related to incubation and nest ecology. We obtained data on seasonal differences for 91 occurrences, defined as data on a given sex for a given species. For each occurrence, we recorded whether the sex exhibited a significant ($\alpha = 0.05$) seasonal change in preen oil composition (yes/no), whether the sex incubates (yes/no), whether the species nests on the ground (ground/non-ground; details below), and the timescale of the study (within breeding season/across breeding and non-breeding seasons; details below; **Table S4**). Thus, a species could be included multiple times in our analysis if it was included in multiple studies. Information about incubation and nest

ecology was obtained from the *Handbook of the Birds of the World* (del Hoyo et al. 2009). In some species, only one parent incubates, but the incubating parent can be of either sex (e.g. western sandpiper, *Calidris mauri*). Because a mix of both sexes would be incubating in any given study population for such species, we categorized these species as biparentally incubating. For studies on captive birds, we inspected the methods to confirm that seasonality was established using appropriate methods (e.g. by using artificial light cycles for birds kept indoors).

To estimate the vulnerability of different species to olfactorily searching nest predators, we described their nest ecology as 'ground nesting' (more vulnerable) or 'non-ground-nesting' (less vulnerable). Ground-nesting birds often suffer from higher nest predation rates than non-ground-nesting birds (Loiselle and Hoppes 1983, Wilcove 1985, but see Martin 1995), notably by mammals (Söderström et al. 1998, Zuria et al. 2007, Macdonald and Bolton 2008), which primarily rely on olfaction to detect nests (Reneerkens et al. 2005, Whelan et al. 2010). Species that nest in low shrubs (< 2 m) were considered 'ground nesting' because they are likely more exposed to mammalian nest predators (e.g. Schaefer 2004). Species that nest on cliffs were considered 'non-ground-nesting' because they are rarely exposed to such predators (Barros et al. 2016).

Seasonal changes can occur at different timescales (within the breeding season, within the non-breeding season, and across the breeding and non-breeding seasons). To interpret any biological functions of preen oil changes, it is necessary to consider the timescale of the changes. We categorized timescale as 'within breeding season' (spanning nest building, egg laying, incubation, and brood care), and 'across breeding and non-breeding seasons' (where non-breeding encompasses fledging through winter, up to the start of nest building the following year). Studies conducted within the breeding season either compared samples from different periods within the breeding season (e.g. across mating, incubation, and brood care) or measured the effect of date on preen oil composition. Studies conducted across the breeding and non-breeding seasons either compared samples from the breeding and non-breeding season, or compared samples collected regularly throughout the year (e.g. monthly).

In total, our data set on seasonal differences comprised 91 occurrences (where one occurrence corresponds to one sex) from 43 species and 25 studies (**Table S4**). Effect sizes (Cohen's *d*) could be calculated for only three studies [using an online calculator (Lenhard and Lenhard 2016), **Table S4**] and were therefore not used for analysis.

Sex differences

We tested whether sex differences in the chemical composition of preen oil are related to season and incubation type. For each species, we recorded whether a significant ($\alpha = 0.05$) sex difference was detected in the composition of preen oil (yes/no), the season in which preen oil was sampled (breeding/non-breeding; where breeding includes nest building, egg laying, incubation, and brood care, and non-breeding encompasses fledging through winter,

up to the start of nest building the following year), and the incubation type (uniparental/biparental; **Table S5**). Analysing sex differences during specific breeding periods (e.g. mate choice, incubation, chick rearing) would be more informative than distinguishing only breeding and non-breeding, but most studies sampled birds across multiple breeding stages, and we therefore could not conduct such an analysis. Also, in most cases, the nature and direction of sex differences were not explicitly recorded, so we could not include this information in our analyses. For studies on free-living birds, breeding stage dates and incubation type were verified using the *Handbook of the Birds of the World* (del Hoyo et al. 2009). For studies on captive birds, we inspected the methods to confirm that birds were brought into breeding condition using appropriate methods (e.g. using natural light cycles for birds in outdoor aviaries or by using artificial light to photostimulate birds kept indoors).

In total, our data set on sex differences comprised 75 occurrences (where one occurrence corresponds to one season) from 49 species and 39 studies (**Table S5**). As with seasonal differences, because effect sizes could be calculated for only a limited number of studies (21, **Table S5**), we did not use effect sizes in our analysis.

Statistical analyses

Our full data set included 59 species and 45 studies. We conducted comparative analyses for each model (seasonal differences, sex differences) using generalized linear mixed models (GLMMs) with Markov chain Monte Carlo techniques under a Bayesian statistical framework, using the package MCMCglmm (Hadfield, 2010) in R (R Development Core Team 2017) that allowed us to control for phylogenetic dependency. The first model (seasonal differences) was run for 13×10^6 iterations, with a burn-in phase of 10,000 and a thinning interval of 3500, resulting in a sample size of 3712. The second model (sex differences) was run for 10×10^6 iterations, with a burn-in phase of 5000 and a thinning interval of 2000, resulting in a sample size of 4998. These parameters were chosen to ensure model convergence (Hadfield 2010). Because we had no *a priori* predictions about the values of these parameters, both models were fit using a weakly informative inverse-gamma prior (Hadfield 2010). We verified the absence of autocorrelation, verified convergence with the Gelman–Rubin diagnostic (Gelman and Rubin 1992), and assessed the significance of fixed effects (at $\alpha = 0.05$) by checking whether their 95% credible interval spanned 0.

Our first model included *seasonal difference* as a binary response variable (yes/no) and the fixed effects *incubation* (sex incubates/sex does not incubate), *nest ecology* (ground/non-ground nesting), *timescale* (within breeding season/across breeding and non-breeding seasons), and the interaction term *incubation* × *nest ecology*. Our second model included *sex difference* as a binary response variable (yes/no) and the fixed effects *season* (breeding/non-breeding) and *incubation type* (uniparental/biparental).

For both models, we included *species* as a random effect because some species were used in multiple studies, and because some species were tested at two times of year (sex differences) or in both sexes (seasonal differences). We included *phylogeny* as a random effect to control for potential effects of phylogenetic relatedness. We calculated the phylogenetic relatedness between species using the consensus tree of 1000 phylogenetic trees (Stage2 MayrAll Hackett backbone) generated on birdtree.org (Jetz et al. 2012). Finally, we verified that the inclusion of random effects improved the fit of the models, indicated by a lower deviance information criterion (DIC) score. These analyses are detailed in the Supporting information (**Appendices S3** and **S4**). Detailed sample sizes used in each analysis are available in **Table S6**.

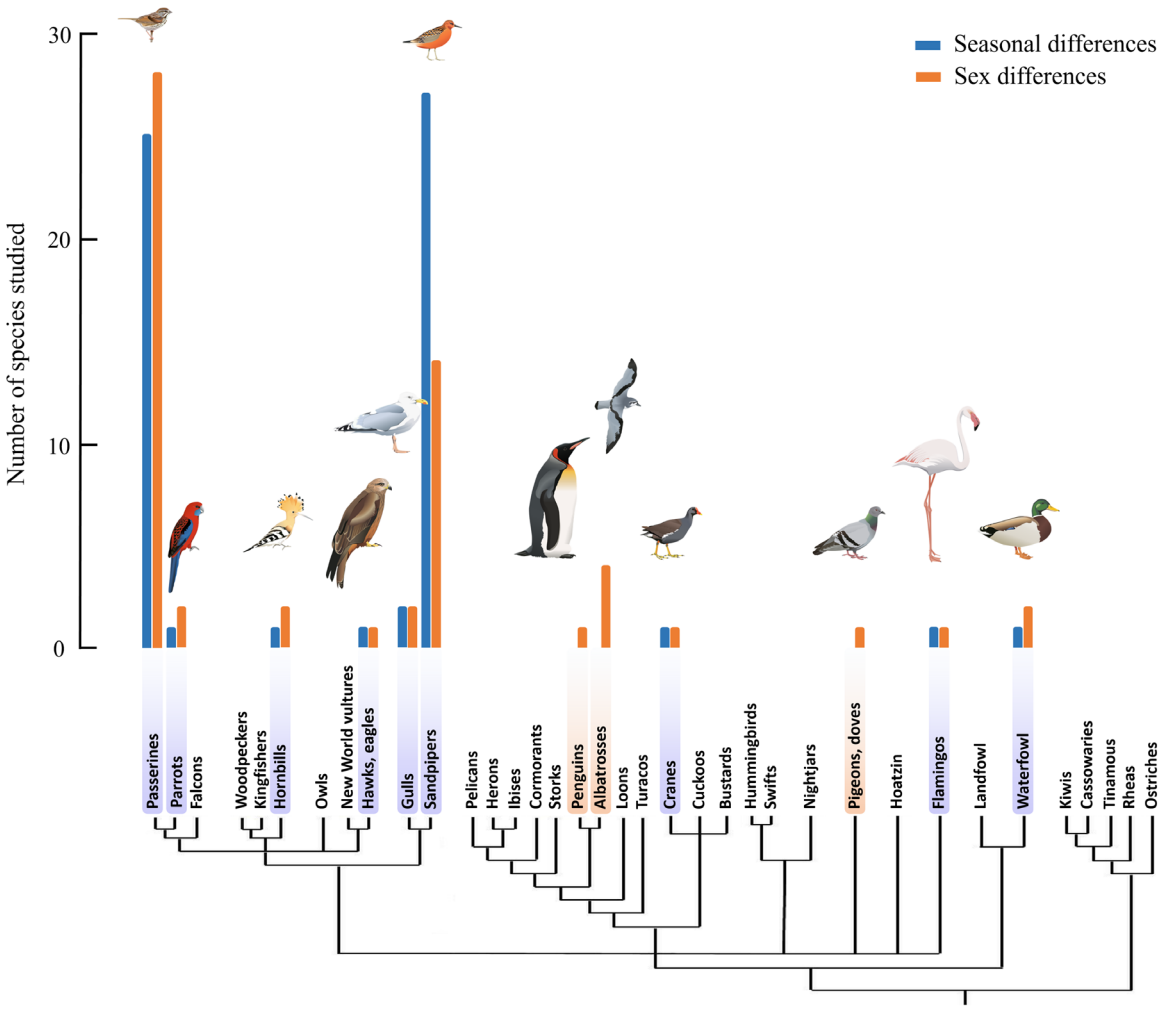


Fig. 3. Distribution of species studied with respect to seasonal (blue) and sex (orange) differences in preen oil chemical composition in birds. Orders highlighted in purple were studied with respect to both seasonal and sex differences. No order was studied with respect to seasonal differences only. Phylogeny is based on Hackett *et al.* (2008); gulls (family Laridae) and sandpipers (family Scolopacidae) belong to the order Charadriiformes. Illustrations by Marc Gilles.

RESULTS

Literature review

Of the 55 studies included in our systematic review, 35 investigated seasonal differences (60 species) and 46 investigated sex differences (59 species) in preen oil composition, with 26 of these papers investigating both seasonal and sex differences. While 76 species have been investigated, most studies (61) involved just two phylogenetic orders, Passeriformes (songbirds, 32 species) and Charadriiformes (gulls and shorebirds, 29 species; **Fig. 3**). Seasonal differences were found in 95% (57/60) of species studied and sex differences were detected in 47% (28/59) of species studied.

Seasonal differences

The probability of detecting a seasonal change in preen oil composition was related to the interaction between incubation and nest ecology (posterior mean = 287.64, 95% CI = [66.39, 543.24], P_{MCMC} = 0.01; **Table 1**, **Fig. 4**). To elucidate the direction of the interaction, we performed separate analyses for ground nesting species (45 occurrences) and non-ground nesting species (46 occurrences). For ground-nesting species, seasonal differences were more likely in the incubating than the non-incubating sex (posterior mean = 286.66; 95% CI = [98.27, 494.14], P_{MCMC} < 0.001), whereas for non-ground-nesting species, seasonal differences were apparent regardless of which sex incubated (posterior mean = 51.73, 95% CI = [-66.78, 182.74], P_{MCMC} = 0.29). Timescale had no effect on the probability of detecting seasonal changes (**Table 1**). Accounting for *phylogeny* and *species* increased the fit of the models slightly but had little effect overall (**Table S7**). *Phylogeny* and *species* explained 7% and 5% of the total variance, respectively (**Table S8**).

Sex differences

The probability of detecting sex differences in preen oil composition was related to both breeding stage and incubation type (**Table 1**, **Fig. 5**). Sex differences were more likely during breeding than non-breeding (posterior mean = 339.49, 95% CI = [108.42, 586.84], P_{MCMC} < 0.001), and in species with uniparental than biparental incubation (posterior mean = −221.20; 95% CI = [−388.98, −43.52], P_{MCMC} = 0.001; **Fig. 5**). Accounting for *phylogeny* nd *species* increased the fit of the models slightly but had little effect on the overall model results (**Table S7**). *Phylogeny* and *species* explained 9% and 5% of the total variance respectively (**Table S8**).

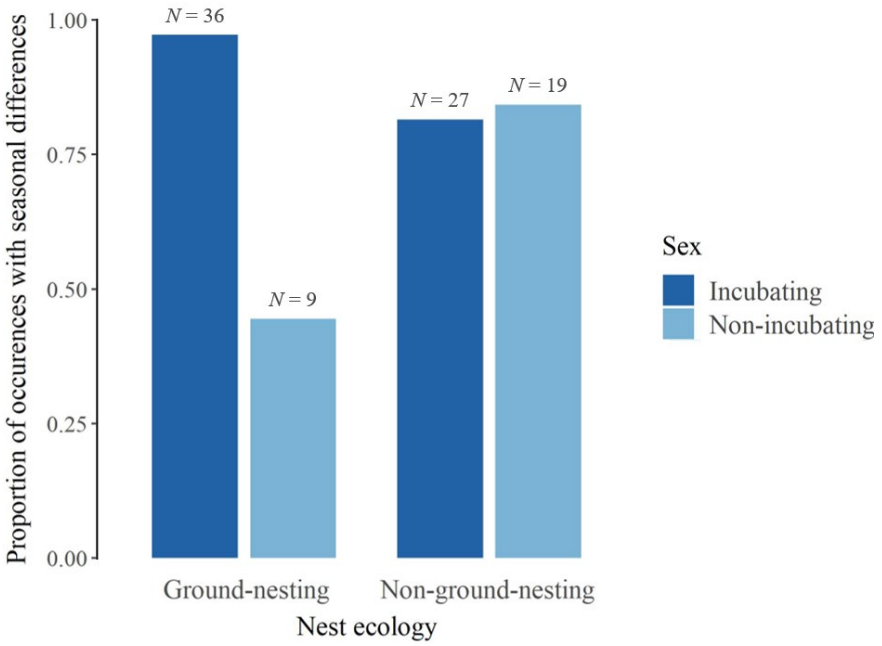


Fig. 4. Proportion of occurrences (i.e. sex within species) of seasonal differences in preen oil chemical composition in ground- *versus* non-ground-nesting species and if the sex incubates *versus* does not incubate. Sample size (91 occurrences) exceeds the number of species (43) because most studies sampled both sexes of a species, and some species were examined in multiple studies.

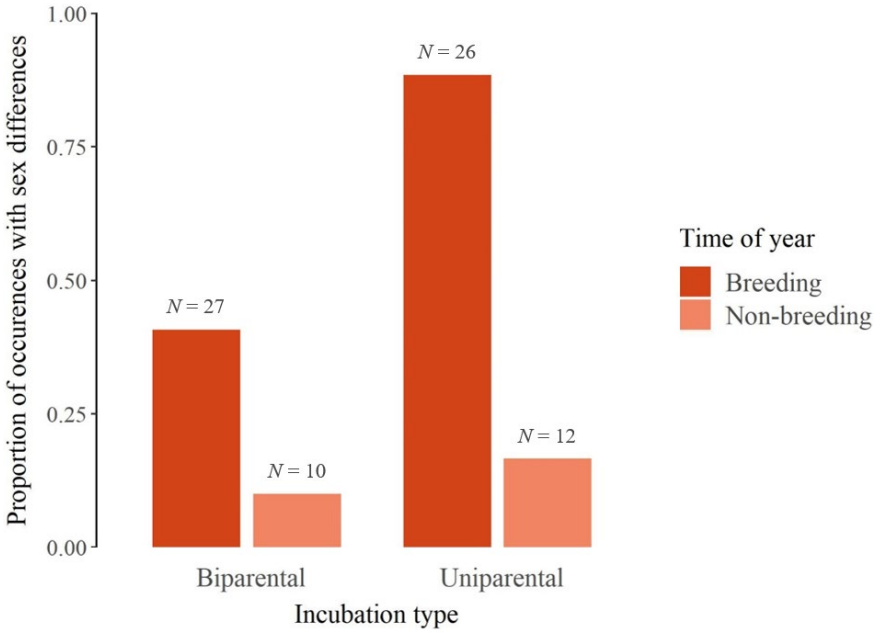


Fig. 5. Proportion of occurrences (i.e. season within species) of sex differences in preen oil chemical composition with biparental *versus* uniparental incubation and during breeding *versus* non-breeding. Sample size (75 occurrences) exceeds the number of species (49) because some species were tested during both breeding and non-breeding seasons, and some species were examined in multiple studies.

Table 1. Summary of phylogenetically controlled Markov chain Monte Carlo generalized linear mixed effects models to investigate factors affecting seasonal and sex differences in preen oil chemical composition. The model on seasonal differences (91 occurrences) tests whether the occurrence of seasonal differences (no = 0; yes = 1) depends on incubation (sex does not incubate = 0; sex incubates = 1), nest ecology (non-ground-nesting = 0; ground-nesting = 1), the timescale of the study (within breeding season = 0; across breeding and non-breeding season = 1), and the interaction between incubation and nest ecology. The model on sex differences (75 occurrences) tests whether the occurrence of sex differences (no = 0; yes = 1) depends on the species' incubation type (uniparental = 0; biparental = 1) and the season (non-breeding = 0; breeding = 1).

Dependent variable	Effect	Independent variable	Posterior mean	Lower 95% CI	Upper 95% CI	P_{MCMC}
Seasonal difference	Fixed	Intercept	71.68	–90.48	239.04	0.370
		Incubation	2.87	–142.84	146.06	0.961
		Nest ecology	–163.19	–365.86	34.83	0.084
		Timescale	100.72	–18.76	232.14	0.099
		Incubation × Nest ecology	287.64	66.39	543.24	**0.010**
	Random	Phylogeny	2865	3e-04	16492	–
		Species	1478	2e-04	9524	–
		Residual	23546	5337	44760	–
Sex difference	Fixed	Intercept	–142.77	–364.12	33.30	0.096
		Incubation type	–221.20	–388.98	–43.52	**0.001**
		Season	339.49	108.42	586.84	**<0.001**
	Random	Phylogeny	5855	2e-04	33352	–
		Species	2116	2e-04	14095	–
		Residual	39544	1056	84827	–

CI: credible interval; bold: $P_{MCMC} < 0.05$.

DISCUSSION

This study reviewed and analysed the literature on olfactory crypsis and sex semiochemicals and found support for both hypotheses. Seasonal changes in the chemical composition of preen oil were nearly ubiquitous. Consistent with predictions derived from the olfactory crypsis hypothesis, the likelihood of detecting a seasonal change in preen oil composition was related to the interaction between incubation and nest ecology such that seasonal differences were more likely in the incubating sex, but only in ground-nesting species. For non-ground-nesting species, seasonal changes were equally likely, regardless of which sex incubated. By contrast, sex differences were less ubiquitous than seasonal differences, occurring in less than half of the species studied. Consistent with predictions of both the sex semiochemical and olfactory crypsis hypotheses, the likelihood of detecting sex differences in preen oil composition was related to both breeding stage and incubation type. Specifically, sex differences were more likely during breeding than non-breeding, and in species with uniparental than biparental incubation. It should be noted that our results on the probabilities of seasonal and sex differences may be overestimates if there is publication bias in favour of significant results. On the other hand, the probabilities of

seasonal and sex differences may also be underestimated, since some studies were not designed specifically to test for such differences (e.g. in cases where studies sampled across breeding and/or non-breeding stages, and/or had small sample sizes), and as a result could not or did not detect any differences in preen oil composition. With a more appropriate design, such studies may have detected seasonal and/or sex differences in preen oil composition.

At present, there are insufficient data to disentangle these non-mutually exclusive hypotheses. Thus, our work is not the definitive test of these two hypotheses, but it is the best we can achieve to date. Below, we review current support for the olfactory crypsis and sex semiochemical hypotheses and offer recommendations for more direct hypothesis testing.

Olfactory crypsis

Evidence for a role of preen oil in olfactory crypsis is currently limited. Studies on the preen oil composition of 27 ground-nesting shorebird species (order Charadriiformes) revealed a seasonal shift from monoesters to diesters at the onset of breeding (Piersma et al. 1999, Reneerkens et al. 2002, 2006, 2007a), with diester secretion being maintained during incubation and chick-rearing (Reneerkens et al. 2002, 2006). Remarkably, diesters were secreted equally in both sexes in species where both sexes incubate, only in males in species where only males incubate, and mainly in females in species where only females incubate (Reneerkens et al. 2007a). Because diesters are less volatile than monoesters, these authors hypothesized that seasonal changes in the preen oil of incubating birds enhance olfactory crypsis by reducing olfactory cues at the nest, thereby limiting detection by olfactorily searching nest predators (Reneerkens et al. 2002, 2007a). In support of this hypothesis, a trained dog was better at detecting preen oil composed of monoesters than diesters (Reneerkens et al. 2005).

Other studies have also interpreted seasonal and sex differences in preen oil composition using the olfactory crypsis hypothesis. For example, the preen oil of Eurasian moorhens (*Gallinula chloropus*) is less volatile during breeding than non-breeding, and olfactory crypsis was proposed as an explanation (López-Perea and Mateo 2019). In mallards (*Anas platyrhynchos*), a shift from monoesters to diesters at the onset of breeding was observed in females but not in males (Jacob et al. 1979, Bohnet et al. 1991). The shift was first thought to be involved in mate choice, by providing an olfactory cue that males might use to identify females in breeding condition (see Section IV.2). But given that diesters are less volatile than monoesters, that only females incubate in this species, and that mallard nests are exposed to mammalian predators (Johnson et al. 1989), this shift may be more relevant for olfactory crypsis than intraspecific communication and mate choice.

Finally, a New Zealand study on 13 non-ground-nesting passerine species compared the preen oil of introduced species that co-evolved with mammalian predators to that of native species that have a long evolutionary history without mammalian predators.

Consistent with the olfactory crypsis hypothesis, preen oil was less volatile during breeding than non-breeding in introduced but not in native species (Fluen 2008). However, sample sizes were low and preen oil volatiles were not lower in females for species with female-only incubation (Fluen 2008), as would be predicted by the olfactory crypsis hypothesis. Overall, most studies speculate on the role of seasonal and sex variation in preen oil in maintaining olfactory crypsis without providing evidence. Additional studies on the ability of predators to detect preen oil secreted during various breeding and life-cycle stages are thus warranted.

Based on the olfactory crypsis hypothesis, we predicted that seasonal differences in preen oil composition would be more common in the incubating than non-incubating sex(es), especially in species with nests that are more vulnerable to olfactorily searching predators, such as ground nesters. We also predicted that sex differences in preen oil composition would be more common during breeding than non-breeding, and in uniparentally incubating than biparentally incubating species. We found support for all three predictions. Consistent with our first prediction, seasonal differences were more frequently detected in sexes that incubate than in sexes that do not incubate, but only in ground-nesting species (i.e. species more likely exposed to olfactorily searching predators). This suggests that preen oil changes are indeed associated with incubation in species that are under stronger selection pressure from olfactorily searching predators, supporting the olfactory crypsis hypothesis. However, our findings also highlight that olfactory crypsis cannot explain our findings for non-ground-nesting species. Birds exhibited seasonal changes in preen oil regardless of which sex(es) incubated, suggesting that there are also other explanations for seasonal changes – such as intraspecific chemical communication, as predicted under the sex semiochemical hypothesis.

Consistent with our second and third predictions, sex differences in preen oil composition were more common during breeding and in species with uniparental incubation. This is consistent with both the olfactory crypsis and sex semiochemical hypotheses. Additional information about the nature of sex differences could allow us to disentangle these two hypotheses. For example, less-volatile preen oil during breeding compared to non-breeding could corroborate a cryptic function, while more volatile preen oil during breeding compared to non-breeding could corroborate a signalling function.

We used a comparative analysis to re-evaluate Reneerkens' olfactory crypsis hypothesis, which predicts that seasonal changes in the preen oil of incubating birds is primarily due to mammalian predation (Reneerkens et al. 2002, 2007a). Based on our results, we propose expanding the definition of olfactory crypsis to consider other biologically relevant factors. First, nest predation can be as high during the nestling stage as during the incubation stage (Pietz and Granfors 2000; although we note this is not always the case), so olfactory crypsis could be important during both breeding stages. This is consistent with diesters being secreted until the end of the chick-rearing period in shorebirds (Reneerkens et al. 2006). Second, preen oil could reduce the detectability of nests in two main ways: preen oil could enhance the crypsis of brooding (i.e. incubating

and chick rearing) parents, thereby masking nest odours while adults are on the nest, but preen oil could also enhance crypsis of the eggs and chicks directly, if preen oil is transferred from parents to the offspring. Evidence that preen oil is transferred directly from parents to eggs and chicks is limited (but see Soler et al. 2014), so the mechanisms and efficacy of preen oil transfer at nests are worth exploring further. Third, olfactory crypsis may be applicable not only to non-volatile (e.g. diesters) but also to volatile compounds. For example, certain volatile compounds could enhance crypsis if they blend in with the olfactory background of the nest (i.e. background matching; Soini et al. 2007). Comparing preen oil compounds secreted during incubation and brooding with background odours of the nest and surrounding environment could help determine whether olfactory background matching is occurring. Finally, the olfactory crypsis hypothesis could apply not just to mammals, but to any olfactorily searching nest predators, regardless of taxon (e.g. insects, snakes, and even birds; Shutler, 2019).

In this study, we estimated the vulnerability to olfactorily searching nest predators by describing the nest ecology of the species and distinguishing ground-nesting and non-ground-nesting species. We assumed that ground nests are more vulnerable to olfactorily searching predators, because ground nests are more commonly depredated by mammals (Söderström et al. 1998, Zuria et al. 2007, but see Angelstam 1986, Mallord et al. 2012) and because mammalian nest predation usually occurs at night and is mostly olfactorily based (Whelan et al. 2010, Cox et al. 2013). However, this assumption is simplistic. Both ground nests and non-ground nests are susceptible to predation by three taxa in particular: mammals, snakes, and birds (and, to a lesser extent, insects; Thompson III 2007). The prevalence of nest predation by each taxon may depend more on habitat characteristics than nest ecology (Martin 1995, Thompson III 2007, Reidy and Thompson III 2012). Moreover, each predator taxon can use multiple cues to detect nests. Mammals and snakes depredate nests mostly at night (e.g. Cox et al. 2013, DeGregorio et al. 2014) using olfactory cues (Ford and Burghardt 1993, Whelan et al. 2010) but may also use other cues [e.g. visual cues (Mullin and Cooper 1998, Stake et al. 2005, Dawson et al. 2014)]. Birds commonly depredate nests during the day (Reidy and Thompson III 2012) and rely mainly on visual cues, but may also use olfactory cues (e.g. Buitron and Nuechterlein 1985, Molina-Morales et al. 2020). A more accurate proxy of vulnerability to olfactorily searching nest predators would be, for example, the incidence of such predators weighted by the likelihood of nest detection by olfaction, but such a measure was impossible to obtain for the species and populations included in our analyses. Although simplistic, we consider that nest ecology is a reasonable proxy of vulnerability to olfactorily searching predators in the absence of site-specific information on predation dynamics. Furthermore, if olfactory crypsis prevents some (although not all) predator detections, it could still be sufficiently beneficial to have evolved. Overall, although evidence from experimental studies with natural predators and from studies on taxa other than shorebirds are still lacking, our results and literature review provide compelling support for a role of preen oil in olfactory crypsis.

Sex semiochemicals

Preen oil chemical cues are increasingly thought to play a role in avian mate choice and reproduction (Balthazart and Taziaux 2009, Caro and Balthazart 2010, Caro et al. 2015, Whittaker and Hagelin 2021). Reproductive signals or cues should differ between the sexes and reflect aspects of quality or condition (Johansson and Jones 2007), and there is growing evidence that preen oil provides odour cues of sex that at least some bird species respond to. Thus, we proposed the sex semiochemical hypothesis, positing that sex differences in preen oil are associated with reproduction and preen oil odour cues are involved in mate recognition and/or mate choice. The sex semiochemical hypothesis predicts that there should be an effect of breeding stage (breeding *versus* non-breeding) on preen oil. Indeed, sex differences were more common in breeding birds, suggesting a role for preen oil in reproductive chemical signalling.

The preen oil of several passerine species becomes more volatile during the breeding season [e.g. white-throated sparrows, *Zonotrichia albicollis* (Tuttle et al. 2014), gray catbirds, *Dumetella carolinensis* (Shaw et al. 2011), dark-eyed juncos, *Junco hyemalis* (Soini et al. 2007)], and birds may use these preen oil odour cues to attract mates and compete with same-sex conspecifics (Whittaker and Hagelin 2021). Such findings argue against the chemical crypsis hypothesis, at least for some species. An increased volatility of preen oil chemical cues could serve to advertise for mates and/or to compete with same-sex conspecifics (e.g. *via* territorial scent marking), and such signals might reinforce or enhance other indicators of sex, breeding status, or dominance, such as song characteristics, plumage traits, and other sexually selected ornaments. However, sex differences in preen oil are often, but not always, associated with a greater abundance and/or diversity of chemical compounds in the preen oil of females (Whittaker and Hagelin 2021), who often display fewer sexually selected ornaments than males. This apparent female emphasis on chemical differences in preen oil may be driven by three main factors: intersexual advertisement (e.g. of female receptivity and/or quality) and physiological priming effects on males; intrasexual competition (e.g. territorial scent marking, dominance, and reproductive suppression); and maternal behaviours (e.g. maternal care, mother–offspring recognition, chemical protection of eggs and nestlings) (Whittaker and Hagelin 2021). Additional experiments testing for evidence of a role for preen oil in intersexual advertisement, intrasexual competition, and parental behaviours are warranted.

Shifts in the preen oil composition of breeding birds may also act as indicators of quality (Whittaker and Hagelin 2021). In dark-eyed juncos, females with more ‘female-like’ odour and males with more ‘male-like’ odour both produce more offspring (Whittaker et al. 2013a). Further, males with more ‘male-like’ odour have more surviving nestlings (regardless of nestling paternity) while males with more ‘female-like’ odour have more extrapair young in their home nest (Whittaker et al. 2013). In the lance-tailed manakin (*Chiroxiphia lanceolata*), the likelihood that offspring survive to fledging increases with male microsatellite heterozygosity (a proxy for genome-wide heterozygosity), and this almost certainly reflects genetic quality, because male manakins do not provide parental

care (Sardell et al. 2014). Furthermore, some preen oil components are correlated with increased heterozygosity in males, suggesting that females could use preen oil odour cues to evaluate male heterozygosity (Whittaker et al. 2019a).

In species where it has been investigated, the chemical composition of preen oil is associated with MHC genotype, part of the adaptive immune system, such that individuals with more similar preen oil composition are more similar at MHC [e.g. in black-legged kittiwake, *Rissa tridactyla* (Leclaire et al. 2014) and song sparrows, *Melospiza melodia* (Slade et al. 2016, Grieves et al. 2019c)]. This suggests that preen oil may provide cues of relatedness and/or genetic compatibility. Notably, such cues are detectable to at least some bird species [blue petrels, *Hydrobates caerulea* (Leclaire et al. 2017b) and song sparrows (Grieves et al. 2019c)]. To understand better the role of preen oil chemical cues in avian reproduction, more information is needed on which sexes exhibit changes in preen oil and in what directions, whether the volatility of preen oil compounds increases or decreases in each sex, and at what breeding stages such changes occur.

Most of the studies included in our comparative analysis did not test birds' ability to discriminate between the sexes, but evidence for sex discrimination was found in all six of the studies that did (Zhang et al. 2010, Whittaker et al. 2011a, Amo et al. 2012a, Mihailova 2014, Grieves et al. 2019b, Van Huynh and Rice 2019). In breeding-condition Passeriformes, both sexes spent more time with male odour in dark-eyed juncos (Whittaker et al. 2011a) and spotless starlings (*Sturnus unicolor*; Amo et al. 2012a). By contrast, both sexes spent more time with opposite sex odour in black-capped chickadees (*Poecile atricapillus*), Carolina chickadees (*Poecile carolinensis;* Van Huynh and Rice 2019), and song sparrows (Grieves et al. 2019b). In Psittaciformes, female budgerigars (*Melopsittacus undulatus*) spent more time with male odour (Zhang et al. 2010), and female crimson rosellas (*Platycercus elegans*) spent more time on nest boxes treated with male odour than female odour (Mihailova 2014), suggesting a preference for these odour types.

Evidence for sex discrimination was also found in studies that were not included in our analysis (because sex differences in preen oil composition were not measured). In Galliformes, male domestic chickens (*Gallus gallus domesticus*) more frequently mount and copulate with females that have an intact preen gland, but this preference is abolished in anosmic males (Hirao et al. 2009). In Charadriiformes, crested auklets (*Aethia cristatella*) of both sexes approached model birds treated with male odour more closely than they approached models treated with female odour; this study used a synthetic odour mimicking two major components of auklet odour (Jones et al. 2004). Importantly, these sex-discrimination tests were all performed on birds in breeding condition. Taken together, these results suggest that the ability to use odour cues to discriminate conspecific sex is widespread in birds.

Mechanisms of seasonal and sex differences

Seasonal and sex differences in preen oil composition may be related to changes in diet, preen gland microbes, and circulating hormone levels. Such shifts may provide both protection from predators (*via* olfactory crypsis) and indirect cues of reproductive status (i.e. readiness to breed) that play a role in both intersexual signalling and intrasexual competition (*via* sex semiochemicals; Whittaker and Hagelin 2021).

Diet

Many avian species change their diet at the onset of the breeding season (Bairlein and Gwinner 1994). As such, seasonal differences in preen oil composition may also be affected by changes in diet. To our knowledge, no studies have tested whether natural seasonal dietary changes affect preen oil composition, but laboratory studies have shown that diet affects preen oil composition in captive birds (Apandi and Edwards 1964, Thomas et al. 2010, Kanakri et al. 2016). However, captive birds fed a constant diet still exhibit seasonal changes in preen oil (Whelan et al. 2010, Tuttle et al. 2014, Potier et al. 2018, Grieves et al. 2020), demonstrating that differences in diet can only partly explain seasonal changes in preen oil composition. Sex differences in the chemical composition of preen oil may be driven partly by sex differences in diet, which is common in species with size dimorphism [e.g. seabirds (Phillips et al. 2011), raptors (Catry et al. 2016)] or with spatial segregation during foraging (e.g. shorebirds; Catry et al. 2012).

Symbiotic microbes

Preen gland microbes can also influence the chemical composition of preen oil (Martín-Vivaldi et al. 2009, 2010, Whittaker et al. 2019). Gland microbiota can differ seasonally, which may be associated with seasonal changes in bacterial loads (e.g. an increase during the breeding season; Rodríguez-Ruano et al. 2018) that can then affect preen oil composition. Preen gland microbiota can also differ between the sexes (Pearce et al. 2017; Rodríguez-Ruano et al. 2018, but see Whittaker et al. 2019b, Grieves et al. 2021b). Given that nests can harbour unique microbial communities (Jacob et al. 2014, van Veelen et al. 2017), sex differences in symbiotic microbes may be driven by sex differences in time spent at the nest (Saag et al. 2011, Goodenough et al. 2017). Seasonal changes in diet could also contribute to changes in preen gland microbes, but to our knowledge this has only been explored in avian gut microbiota (Grond et al. 2018).

Hormones

Seasonal and sex differences in the chemical composition of preen oil may be driven at least partly by endogenous changes in circulating levels of sex steroid hormones such as oestradiol and testosterone. Oestradiol injections trigger a shift from monoesters to diesters in mallard preen oil (Bohnet et al. 1991). Testosterone implants have variable effects on preen oil composition across species, triggering increases in some compounds (Abalain et

al. 1984, Whittaker et al. 2011b) and decreases in others (Whelan et al. 2010). Thus, seasonal and sex differences in preen oil are likely at least partly related to physiological changes associated with reproduction.

RECOMMENDATIONS FOR FUTURE RESEARCH

Sampling and study design

Based on our comparative analysis, we found support for both the olfactory crypsis and sex semiochemical hypotheses. In most cases, the studies we reviewed do not consider the nature of seasonal and sex differences; that is, information on which sex(es) exhibited changes, and details on which chemicals changed (and how they changed) are rarely reported. Seasonal changes in preen oil composition have been detected at fine timescales [e.g. less than a week in red knots, *Calidris canutus* (Reneerkens et al. 2007b), less than two weeks in dark-eyed juncos and song sparrows (Whittaker et al. 2011b; Grieves et al. 2018)], and preen oil appears to be more subject to these finer scale seasonal changes during the breeding than the non-breeding season (e.g. Reneerkens et al. 2002). Preen oil composition may thus be more stable (i.e. less variable) during the non-breeding season. That said, if there is selection on a specific mix of preen oil compounds during breeding, one might predict that preen oil should be less, not more, variable during the breeding season, or at least during specific stages of breeding; these contrasting possibilities are worth further study. Interestingly, the speed of seasonal changes in preen oil can be altered in captivity, as shown in red knots, where the shift to diesters was two times slower in captive (four weeks) than wild individuals (two weeks; Reneerkens et al. 2007b). Our comparative analysis revealed that seasonal changes in preen oil were detected independently of the timescale of the study (within breeding or across the breeding and non-breeding seasons). Seasonal changes may have occurred before incubation, supporting olfactory crypsis, but may also have occurred at other times. Sampling preen oil at regular intervals across breeding and non-breeding stages would clarify the timescale over which preen oil changes. In addition, knowing at which specific period(s) (e.g. mate choice, incubation, chick rearing) seasonal changes occur will allow more specific, testable predictions about the function of changes in preen oil composition to be made.

Based on our findings that sex differences depend on season, and seasonal differences were nearly ubiquitous, these factors should be carefully considered in sampling design and analysis. If sex differences are of interest, sampling should be conducted during the breeding season, and the breeding stages (e.g. pair formation, egg laying, incubation, brood care) during which sampling occurs should be recorded. If sex differences are not of interest, it may be ideal to sample during the non-breeding season when sex differences may be less likely to be observed, as this may reduce potential confounds. The conditions, date(s), and duration of sampling should always be taken into consideration.

Hypothesis testing

The olfactory crypsis and sex semiochemical hypotheses are not mutually exclusive. Studies that examine changes in preen oil over a finer timescale (e.g. comparing different periods within the breeding season, such as pre-breeding, incubation, and chick guarding; Reneerkens et al. 2002), quantify hatching and fledging success (Whittaker et al. 2013), and measure additional variables of interest (e.g. measures of quality; Whittaker et al. 2019a) should provide important insights into the functions of avian preen oil in crypsis and social signalling.

The existing literature concerns mainly two bird orders (shorebirds and passerines). Most studies supporting the olfactory crypsis hypothesis have been conducted on shorebirds (but see Fluen 2008), while studies supporting the sex semiochemical hypothesis have been conducted on predominantly passerines and shorebirds (**Table S4**). This could be due to a taxonomic bias, as evidence of olfactory crypsis was first collected in shorebirds (Reneerkens et al. 2002), and evidence for sex semiochemicals is rapidly accumulating in passerines (Whittaker and Hagelin 2021). Alternatively, this could be because seasonal and/or sexual variation in preen oil chemical composition has different functions in each taxon, or that such differences depend on the environment or ecology of a given species. These hypotheses could be tested by analysing variation in preen oil composition in ground-breeding passerines and shorebirds that co-occur in the same habitat, taking care to collect and analyse preen oil separately from the pre-breeding stage (mate choice, pairing, nest building) and during incubation and brood care.

Olfactory crypsis hypothesis

Here, we outline specific predictions that should be tested to evaluate support for the olfactory crypsis hypothesis.

(1) Predators should be less able to detect nests treated with the preen oil of incubating birds compared to preen oil of non-incubating birds. To test this, field experiments should be conducted, although we recognize that experiments on nest predation are difficult to implement in the field. For example, one could measure the predator detection rate of artificial nests where eggs are smeared with preen oil secreted during incubation *versus* outside of incubation, compared to nests with no preen oil treatment. Such an experiment should ideally be combined with chemical analyses to verify and quantify chemical differences among treatments.

(2) During incubation, preen oil should become less volatile in the incubating sex compared to the non-incubating sex. This can be tested by taking repeated measurements of preen oil collected from incubating and non-incubating birds and performing chemical analyses to measure the volatility of preen oil at different time points.

(3) Preen oil may be transferred from parents to eggs and/or chicks. To test for evidence of preen oil transfer, one could search for traces of preen oil on the eggs and/or chicks, or

determine (e.g. using video recording; Martín-Vivaldi et al. 2014) whether adults actively deposit preen oil onto the eggs and/or chicks. Currently, preen oil transfer has only been documented in a single species (Eurasian hoopoe, *Upupa epops*). In this species, preen oil becomes malodorous during breeding (Martín-Vivaldi et al. 2009), suggesting it does not provide olfactory crypsis but may instead repel predators and/or parasites.

(4) Preen oil could increase olfactory crypsis at the nest *via* background matching (Soini et al. 2007). To test this, one could analyse the chemical composition of preen oil secreted by the incubating parent(s) compared to the chemical composition of the nest and surrounding environment (e.g. using headspace sampling; Díez-Fernández et al. 2021) and assess whether preen oil is more chemically similar to the environment than would be expected by chance.

Sex semiochemical hypothesis

Here, we outline specific predictions that should be tested to evaluate support for the sex semiochemical hypothesis.

(1) Birds should use preen oil odour cues to discriminate between the sexes and among individuals of varying quality. There is growing evidence for avian olfactory sex discrimination in the literature (e.g. Zhang et al. 2010, Whittaker et al. 2011a, Amo et al. 2012a, Grieves et al. 2019a, Van Huynh and Rice 2019). Additional experiments should be designed to test for evidence of olfactory discrimination based on indicators of genetic quality, and compatibility such as genome-wide heterozygosity (Whittaker et al. 2019a) and MHC genotype (Grieves et al. 2019c).

(2) Preen oil should become more volatile and/or more abundant (e.g. to increase detectability) during breeding than non-breeding. This can be tested by taking repeated measurements of preen oil collected from breeding and non-breeding birds and performing chemical analyses to measure the volatility of preen oil and the abundance of compounds of interest (i.e. those that have been associated with sex or individual differences) at different time points and between the sexes.

(3) The preen oil preferences of birds tested in a laboratory should translate to mate choice in the wild. Such experiments are difficult to perform, not least because numerous factors affect mate choice in the field, but one could start by looking for evidence of non-random mating based on features birds have been shown to discriminate using odour cues, such as MHC genotype (Grieves et al. 2019c) or the relative 'maleness' of preen oil composition (Whittaker et al. 2011a).

(4) Mate choice based on preen oil odour cues should also be linked to measures of mate quality and fitness. For example, in lance-tailed manakins, male reproductive success is correlated with microsatellite heterozygosity – chicks of more heterozygous males are more likely to fledge, and heterozygosity is correlated with lower proportions of certain preen oil chemicals, but whether female manakins use preen oil odour during male mate choice

is still unknown (Whittaker et al. 2019a). Odour preferences can be tested using a two-choice maze, and preferences can subsequently be linked back to field data on metrics such as heterozygosity, quality, and fitness.

CONCLUSIONS

(1) Determining the functions of preen oil chemical differences in birds has the potential to shift our understanding of avian behaviour.

(2) We conducted an extensive literature review to evaluate the evidence for seasonal and sex differences in the chemical composition of avian preen oil (a proxy of avian body odour). Seasonal differences were nearly ubiquitous, while sex differences were found in almost half of the species studied.

(3) We conducted a comparative analysis to test two hypotheses that may explain seasonal and sex differences in preen oil: the olfactory crypsis and the sex semiochemical hypotheses.

(4) Our comparative analyses on both seasonal and sex differences supports the olfactory crypsis hypothesis. However, direct evidence of a role for preen oil differences in olfactory crypsis is still lacking, notably from experimental studies with natural predators and from studies on species other than shorebirds.

(5) Our comparative analysis supports the sex semiochemical hypothesis. Evidence for the sex semiochemical hypothesis is growing, but more research is needed to connect preen oil differences to odour preferences, measures of quality, and mating success in the wild.

(6) We suggest numerous predictions that can be tested to allow researchers to disentangle the olfactory crypsis and sex semiochemical hypotheses. Doing so will enable us to gain deeper insights into the role of chemical masking and chemical signalling in birds.

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Data availability

The data and code used for the literature review and the comparative analyses are available at the repository PUB – Publications at Bielefeld University (https://pub.uni-bielefeld.de/record/2956821, doi: https://doi.org/10.4119/unibi/2956821).

Supporting information

The complete supporting information (including **Appendices S2 and S3** and **Tables S1 and S2**) can be found online in the Supporting Information section at the end of the article (https://doi.org/10.1111/brv.12837).

Appendix S1. Supplementary methods.

Literature review

We systematically reviewed studies that tested for an effect of season and/or sex on the chemical composition of preen oil. We performed a literature search using the search string [TS=((preen* OR uropygial*) AND (gland* OR secret* OR oil* OR wax*) AND (season* OR sex*))] in *Web of Science*, including all document types, all years (1900–2021), and all languages. We also scrutinized the resultant publications for additional references. Our literature search end date was June 1st 2021. In total, we screened the abstracts of 187 publications (164 from *Web of Science* and 23 from additional sources). We then screened the full text of 66 of these publications, retaining 55 that corresponded to our inclusion criteria.

To be included, studies should have tested for seasonal and/or sex differences in the chemical composition of preen oil collected from adult birds. If multiple studies used the same data, we retained only the study with the largest sample size. We included studies that analysed feathers sampled close to the preen gland (i.e. circlet feathers located directly above the gland), as these probably contain fresh preen oil. We included one study that analysed the colour of preen oil, as colour is related to chemical composition in the study species (Martín-Vivaldi et al. 2010). We also included one study that measured the concentration of carotenoid pigments, as these are natural components of preen oil in this species (Amat et al. 2018). We excluded studies that did not investigate the effect of season or sex, studies that did not analyse chemical composition (e.g. those focused on preen gland size, preen gland microbes), studies that analysed chemicals not directly originating from the preen gland (e.g. from feathers or headspace analysis of volatiles emitted from live birds), and studies on immature birds. More details about the systematic review process and the data used for the analyses are available in **Fig. S1** and **Tables S1 and S2**.

Major study topics on preen oil chemical composition

Our aim was to report all studies ever conducted on the chemical composition of preen oil and categorise them according to their main focus. We performed a systematic literature search in *Web of Science*, with the search string TS = ((molecul* OR chemical* OR chemistry OR scent* OR olfact* OR odor* OR odour* OR volatile* OR compound* OR fatty acid* OR lipid) AND (composition* OR profile* OR signature*) AND (preen* OR uropygial*) AND (gland* OR wax* OR ester* OR oil* OR secretion*), in all languages, all document types and all years (1900–2020). The literature search was conducted in January 2021. We obtained 108 hits. We retained studies on preen oil and excluded studies on feathers or eggs. We included studies on chemical substances only, excluding studies on microbiota. In total, we retained 97 studies. Studies were categorized based on their main focus (**Table S3**).

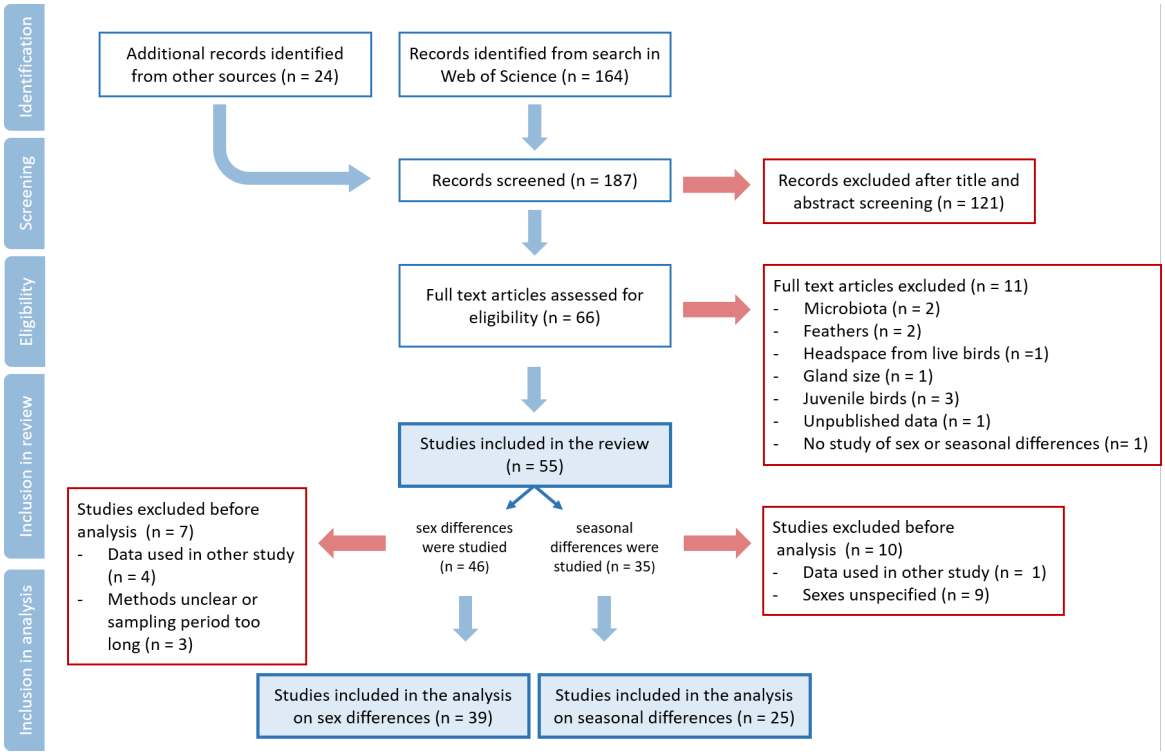

Fig. S1. PRISMA flowchart for the systematic review and comparative analysis on seasonal and sex differences in preen oil chemical composition.

Table S3. Definitions used to categorize studies on preen oil according to their main focus.

Main focus	Type of study
Chemical defence	Describe or test a role of preen oil for protection against bacteria, parasites and predators (crypsis or repellence)
Descriptive	Essentially descriptive (i.e. no *a priori* hypothesis on the function of preen oil) or compare species, possibly for taxonomic purposes (i.e. chemotaxonomy)
Ecotoxicology	Measure the concentration of specific pollutants in the preen gland, possibly for biomonitoring purposes
Intraspecific communication	Propose or test a role of preen oil for signalling (essentially *via* olfactory cues, but also *via* visual cues that may be produced *via* preen oil application; 3 studies)
Mechanisms	Investigate the proximal causes of preen oil chemical composition (e.g. biosynthesis, hormonal control, preen gland microbiota)
Parasite vector attraction	Test the role of preen oil in attracting parasite vectors (mostly mosquitoes) to study parasite transmission
Speciation	Investigate species-specific differences in preen oil composition and propose or test a role of preen oil for species recognition (essentially *via* olfaction) in the context of speciation

Table S4. Species and studies included in comparative analysis testing whether sex-specific seasonal changes in preen oil chemical composition are related to incubation and nest ecology. We recorded whether the sex of a given species exhibited a seasonal change in preen oil composition (Yes/No), whether the sex incubates (Inc = sex incubates; NInc = sex does not incubate), where the nest is typically located (Ground/Non-ground), and the timescale of the study (Br/NBr = across the breeding and non-breeding seasons; Br = within the breeding season). Effect sizes (Cohen's *d*) were calculated where possible using the online tool psychometrica.de/effect_size.html.

Order	Species	Sex	Seasonal diff.	Sex incub.	Nest ecology	Timescale	Effect size (Cohen's *d*)	Study
Accipitriformes	Black kite *Milvus migrans*	Female	Yes	Inc	Nonground	Br/NBr	–	Potier *et al.* (2018)
		Male	Yes	NInc		Br/NBr	–	Potier *et al.* (2018)
Anseriformes	Mallard *Anas platyrhynchos*	Female	Yes	Inc	Ground	Br/NBr	–	Jacob *et al.* (1979)
		Male	No	NInc		Br/NBr	–	Jacob *et al.* (1979)
		Male	Yes	NInc		Br/NBr	–	Kolattukudy *et al.* (1985)
		Female	Yes	Inc		Br/NBr	–	Kolattukudy *et al.* (1987)
Bucerotiformes	Eurasian hoopoe *Upupa epops*	Female	Yes	Inc	Nonground	Br	–	Martín-Vivaldi *et al.* (2009)
		Male	No	NInc		Br	–	Martín-Vivaldi *et al.* (2009)
Charadriiformes	Asian dowitcher *Limnodromus semipalmatus*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al.* (2002)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al.* (2002)
	Black-legged kittiwake *Rissa tridactyla*	Female	Yes	Inc	Nonground	Br	–	Leclaire *et al.* (2011)
		Male	No	Inc		Br	–	Leclaire *et al.* (2011)
	Black-tailed godwit *Limosa limosa*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al.* (2002)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al.* (2002)
	Buff-breasted sandpiper *Tryngites subruficollis*	Female	Yes	Inc	Ground	Br	–	Reneerkens *et al.* (2007*a*)
		Male	No	NInc		Br	–	Reneerkens *et al.* (2007*a*)
	Curlew sandpiper *Calidris ferruginea*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al.* (2002)
		Male	No	NInc		Br/NBr	–	Reneerkens *et al.* (2002)
	Dunlin *Calidris alpina*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al.* (2002)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al.* (2002)
	Herring gull *Larus argentatus*	Female	Yes	Inc	Ground	Br/NBr	–	Fischer *et al.* (2017)
		Male	Yes	Inc		Br/NBr	–	Fischer *et al.* (2017)
	Little stint *Calidris minuta*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al.* (2002)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al.* (2002)
	Red knot *Calidris canutus*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al.* (2007*b*)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al.* (2007*b*)

Table S4. *(continued)*

Order	Species	Sex	Seasonal diff.	Sex incub.	Nest ecology	Timescale	Effect size (Cohen's *d*)	Study
Charadriiformes	Red phalarope *Phalaropus fulicarius*	Female	No	NInc	Ground	Br	–	Reneerkens *et al*. (2007*a*)
		Male	Yes	Inc		Br	–	Reneerkens *et al*. (2007*a*)
	Redshank *Tringa totanus*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al*. (2002)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al*. (2002)
	Ruddy turnstone *Arenaria interpres*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al*. (2002)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al*. (2002)
	Ruff *Philomachus pugnax*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al*. (2002)
		Male	No	NInc		Br/NBr	–	Reneerkens *et al*. (2002)
	Short-billed dowitcher *Limnodromus griseus*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al*. (2002)
		Male	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al*. (2002)
	Temminck's stint *Calidris temminckii*	Female	Yes	Inc	Ground	Br	–	Reneerkens *et al*. (2007*a*)
		Male	Yes	Inc		Br	–	Reneerkens *et al*. (2007*a*)
	Western sandpiper *Calidris mauri*	Female	Yes	Inc	Ground	Br/NBr	–	Reneerkens *et al*. (2002)
		Male	Yes	Inc		Br/NBr	–	Reneerkens *et al*. (2002)
Gruiformes	Common moorhen *Gallinula chloropus*	Female	Yes	Inc	Ground	Br/NBr	–	López-Perea & Mateo (2019)
		Male	Yes	Inc		Br/NBr	–	López-Perea & Mateo (2019)
Passeriformes	Black-capped chickadee *Parus atricapillus*	Female	Yes	Inc	Nonground	Br/NBr	–	Van Huynh & Rice (2019)
		Male	Yes	NInc		Br/NBr	–	Van Huynh & Rice (2019)
	Blackbird *Turdus merula*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)
	Carolina chickadee *Parus carolinensis*	Female	Yes	Inc	Nonground	Br/NBr	–	Van Huynh & Rice (2019)
		Male	Yes	NInc		Br/NBr	–	Van Huynh & Rice (2019)
	Chaffinch *Fringilla coelebs*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)
	Dark-eyed junco *Junco hyemalis*	Female	Yes	Inc	Ground	Br/NBr	–	Soini *et al.* (2007)
		Male	No	Inc		Br/NBr	–	Soini *et al.* (2007)
		Female	Yes	Inc		Br	–	Whittaker *et al.* (2011*b*)
		Male	Yes	NInc		Br	–	Whittaker *et al.* (2011*b*)
	Dunnock *Prunella modularis*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)
	European goldfinch *Carduelis carduelis*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)

Table S4. *(continued)*

Order	Species	Sex	Seasonal diff.	Sex incub.	Nest ecology	Timescale	Effect size (Cohen's *d*)	Study
Passeriformes	European greenfinch *Chloris chloris*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)
	Gray catbird *Dumetella carolinensis*	Female	Yes	Inc	Nonground	Br/NBr	–	Shaw *et al.* (2011)
		Male	Yes	NInc		Br/NBr	–	Shaw *et al.* (2011)
		Male	Yes	NInc		Br/NBr	Acid acetic: 5.43, propanoic acid: 7.61, 2-methyl propanoic acid: 7.61, butanoic acid: 6.28, 3-methyl butanoic acid: 3.47	Whelan *et al.* (2010)
	House sparrow *Passer domesticus*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	Inc		Br/NBr	–	Fluen (2008)
	Lance-tailed manakin *Chiroxiphia lanceolata*	Female	Yes	Inc	Nonground	Br	PC3: – PC4: 0.35	Whittaker *et al.* (2019*a*)
		Male	Yes	NInc		Br	PC4: 1.19	Whittaker *et al.* (2019*a*)
	New Zealand Bellbird *Anthornis melanura*	Female	No	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	No	NInc		Br/NBr	–	Fluen (2008)
	New Zealand Fantail *Rhipidura fuliginosa*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	Inc		Br/NBr	–	Fluen (2008)
	Red-vented bulbul *Pycnonotus cafer*	Male	Yes	Inc	Ground	Br/NBr	–	Bhattacharyya & Chowdhury (1995)
	Common redpoll *Acanthis flammea*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)
	Silvereye *Zosterops lateralis*	Female	No	Inc	Nonground	Br	–	Azzani *et al.* (2016)
		Male	No	Inc		Br	–	Azzani *et al.* (2016)
		Female	Yes	Inc		Br/NBr	–	Fluen (2008)
		Male	Yes	Inc		Br/NBr	–	Fluen (2008)
	Song sparrow *Melospiza melodia*	Female	Yes	Inc	Ground	Br/NBr	–	Grieves *et al*. (2019*a*)
		Male	Yes	NInc		Br/NBr	–	Grieves *et al*. (2019*a*)
	South Island robin *Petroica australis*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)
	South Island saddleback *Philesturnus carunculatus*	Female	No	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	No	NInc		Br/NBr	–	Fluen (2008)

Table S4. *(continued)*

Order	Species	Sex	Seasonal diff.	Sex incub.	Nest ecology	Timescale	Effect size (Cohen's *d*)	Study
Passeriformes	Spotless starling *Sturnus unicolor*	Female	Yes	Inc	Nonground	Br	–	Amo *et al.* (2012)
		Male	Yes	NInc		Br	–	Amo *et al.* (2012)
	White-throated sparrow *Zonotrichia albicollis*	Male	Yes	NInc	Ground	Br/NBr	–	Tuttle *et al.* (2014)
		Female	Yes	Inc		Br	Alcohols: 8.16, carboxylic acids: 4.65	Forrette (2018)
	Yellowhammer *Emberiza citronella*	Female	Yes	Inc	Nonground	Br/NBr	–	Fluen (2008)
		Male	Yes	NInc		Br/NBr	–	Fluen (2008)
Psittaciformes	Crimson rosella *Platycercus elegans*	Male	Yes	NInc	Nonground	Br/NBr	–	Mihailova (2014)

Table S5. Species and studies included in a comparative analysis testing whether sex-specific changes in preen oil chemical composition are related to season (Breeding/Non-breeding) and incubation type (Uniparental/Biparental). Effect sizes (Cohen's d) were calculated where possible using the online tool psychometrica.de/effect_size.html.

Order	Species	Sex diff.	Season	Incubation type	Effect size (Cohen's *d*)	Study
Accipitriformes	Black kite *Milvus migrans*	No	Breeding	Uniparental	0.48	Potier *et al.* (2018)
		Yes	Non-breeding		0.29	Potier *et al.* (2018)
Anseriformes	Falkland steamer-duck *Tachyeres brachypterus*	Yes	Breeding	Uniparental	–	Livezey *et al.* (1986)
	Mallard *Anas platyrhynchos*	Yes	Breeding	Uniparental	–	Jacob *et al.* (1979)
Bucerotiformes	Eurasian hoopoe *Upupa epops*	Yes	Breeding	Uniparental	–	Martín-Vivaldi *et al.* (2009)
Charadriiformes	Asian dowitcher *Limnodromus semipalmatus*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)
		No	Non-breeding		–	Reneerkens *et al.* (2002)
	Black-legged kittiwake *Rissa tridactyla*	Yes	Breeding	Biparental	Volatiles: 0.56, non-volatiles: 0.63	Leclaire *et al.* (2011)
	Black-tailed godwit *Limosa limosa*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)

Table S5. *(continued)*

Order	Species	Sex diff.	Season	Incubation type	Effect size (Cohen's *d*)	Study
Charadriiformes	Buff-breasted sandpiper *Tryngites subruficollis*	Yes	Breeding	Uniparental	–	Reneerkens *et al.* (2007*a*)
		No	Non-breeding		–	Reneerkens *et al.* (2007*a*)
	Common Redshank *Tringa tetanus*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)
		No	Non-breeding		–	Reneerkens *et al.* (2002)
	Curlew sandpiper *Calidris ferruginea*	Yes	Breeding	Uniparental	1.29	Reneerkens *et al.* (2002)
		No	Non-breeding		–	Reneerkens *et al.* (2002)
	Dunlin *Calidris alpina*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)
		No	Non-breeding		–	Reneerkens *et al.* (2002)
	Herring gull *Larus argentatus*	Yes	Breeding	Biparental	–	Fischer *et al.* (2017)
		No	Non-breeding		–	Fischer *et al.* (2017)
		No	Non-breeding		–	Fischer *et al.* (2020)
	Little stint *Calidris minuta*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)
		No	Non-breeding		–	Reneerkens *et al.* (2002)
	Red knot *Calidris canutus*	No	Breeding	Biparental	–	Reneerkens *et al.* (2007*b*)
		No	Non-breeding		–	Reneerkens *et al.* (2007*b*)
	Red phalarope *Phalaropus fulicarius*	Yes	Breeding	Uniparental	–	Reneerkens *et al.* (2007*a*)
	Ruddy turnstone *Arenaria interpres*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)
		No	Non-breeding		–	Reneerkens *et al.* (2002)
	Ruff *Philomachus pugnax*	Yes	Breeding	Uniparental	–	Reneerkens *et al.* (2002)
		No	Non-breeding		–	Reneerkens *et al.* (2002)
	Short-billed dowitcher *Limnodromus griseus*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)
	Temminck's stint *Calidris temminckii*	No	Breeding	Biparental	–	Reneerkens *et al.* (2007*a*)
	Western sandpiper *Calidris mauri*	No	Breeding	Biparental	–	Reneerkens *et al.* (2002)
Columbiformes	Pigeon (feral) *Columba livia*	No	Non-breeding	Biparental	–	Montalti *et al.* (2005)
Passeriformes	Bengalese finch *Lonchura striata*	Yes	Breeding	Biparental	Hexadecanol: 2.5, unknown diester: 1.39, octadecanol: 1.11	Zhang *et al.* (2009)

Table S5. *(continued)*

Order	Species	Sex diff.	Season	Incubation type	Effect size (Cohen's *d*)	Study
Passeriformes	Black-capped chickadee *Parus atricapillus*	Yes	Breeding	Uniparental	–	Van Huynh & Rice (2019)
		Yes	Non-breeding		–	Van Huynh & Rice (2019)
	Carolina chickadee *Parus carolinensis*	Yes	Breeding	Uniparental	–	Van Huynh & Rice (2019)
		No	Non-breeding		–	Van Huynh & Rice (2019)
	Dark-eyed junco *Junco hyemalis*	Yes	Breeding	Uniparental	–	Soini *et al.* (2007)
		No	Non-breeding		–	Soini *et al.* (2007)
		Yes	Breeding		2.15	Whittaker *et al.* (2010)
		Yes	Breeding		1.25	Whittaker *et al.* (2011*b*)
		No	Breeding		R: –0.15, F: 0.42	Whittaker *et al.* (2016)
		No	Breeding			Whittaker *et al.* (2018)
	Eurasian blackbird *Turdus merula*	Yes	Breeding	Uniparental	All substances: 0.85, substances in > 70% of samples: 0.91	Díez-Fernández *et al.* (2021)
	Gray catbird *Dumetella carolinensis*	No	Non-breeding	Uniparental	Acid acetic: 3.41, propanoic acid: 2.86, 2-methyl propanoic acid: 2.75, butanoic acid: 2.98, 3-methyl butanoic acid: 2.75	Whelan *et al.* (2010)
	Great tit *Parus major*	Yes	Breeding	Uniparental	PC1: 1.28, PC2: 0.85, PC3: 1.29	Jacob *et al.* (2014)
	Grey gerigone *Gerygone igata*	Yes	Breeding	Uniparental	All substances: 0.90, 30 most abundant substances: 1.19	Rasmussen (2013)
	Japanese waxwing *Bombycilla japonica*	No	Non-breeding	Uniparental	–	Zhang *et al.* (2013)
	Lance-tailed manakin *Chiroxiphia lanceolate*	Yes	Breeding	Uniparental	0.25	Whittaker *et al.* (2019*a*)
	New-Zealand bellbird *Anthornis melanura*	Yes	Breeding	Uniparental	All substances: 0.10, 30 most abundant substances: 1.03	Rasmussen (2013)
	New-Zealand fantail *Rhipidura fuliginosa*	No	Breeding	Biparental	All substances: 0.71, 30 most abundant substances: 0.72	Rasmussen (2013)
	Pipipi *Mohoua novaeseelandiae*	Yes	Breeding	Uniparental	All substances: 1.60, 30 most abundant substances: 3.28	Rasmussen (2013)

Table S5. *(continued)*

Order	Species	Sex diff.	Season	Incubation type	Effect size (Cohen's *d*)	Study
Passeriformes	Rifleman *Acanthisitta chloris*	No	Breeding	Biparental	All substances: 1.05, 30 most abundant substances: 0.85	Rasmussen (2013)
	Silvereye *Zosterops lateralis*	Yes	Breeding	Biparental	All substances: 1.56, 30 most abundant substances: 2.42	Rasmussen (2013)
		No	Breeding		–	Azzani *et al.* (2016)
	Song sparrow *Melospiza melodia*	No	Non-breeding	Uniparental	0.47	Grieves *et al.* (2018)
		Yes	Breeding		Population 1: 0.66, population 2: 0.52	Grieves *et al.* (2019*a*)
		No	Non-breeding		Population 1: 0.41, population 2: 0.12	Grieves *et al.* (2019*a*)
		Yes	Breeding		0.54	Grieves *et al.* (2019b)
	South Island Robin *Petroica Australis*	Yes	Breeding	Uniparental	All substances: 1.07, 30 most abundant substances: 1.02	Rasmussen (2013)
	Spotless starling *Sturnus unicolor*	Yes	Breeding	Uniparental	–	Amo *et al.* (2012)
	White-throated sparrow *Zonotrichia albicollis*	Yes	Breeding	Uniparental	Average of 11 compounds: 1.94,	Tuttle *et al.* (2014)
		Yes	Breeding		PC1: 2.99	Forrette (2018)
Phoenicopteriformes	Greater flamingo *Phoenicopterus roseus*	Yes	Breeding	Biparental	–	Amat *et al.* (2018)
Procellariiformes	Antarctic prion *Pachyptila desolata*	Yes	Breeding	Biparental	–	Bonadonna *et al.* (2007)
		Yes	Breeding		–	Mardon *et al.* (2010)
	Blue petrel *Halobaena caerulea*	Yes	Breeding	Biparental	0.62	Mardon *et al.* (2010)
	Cory's shearwater *Calonectris diomedea*	No	Breeding	Biparental	–	Gabirot *et al.* (2016)
	Leach's storm petrel *Oceanodroma leucorhoa*	No	Breeding	Biparental	0.44	Jennings & Ebeler (2020)
Psittaciformes	Crimson rosella *Platycercus elegans*	Yes	Breeding	Uniparental	–	Mihailova (2014)
		Yes	Non-breeding		–	Mihailova (2014)
	Budgerigar *Melopsittacus undulates*	Yes	Breeding	Uniparental	Average of 6 compounds: 1.15	Zhang *et al.* (2010)
Sphenisciformes	King penguin *Aptenodytes patagonicus*	No	Breeding	Biparental	0.45	Gabirot *et al.* (2018)

Table S6. Sample sizes (number of occurrences) for the analyses testing for factors affecting the likelihood of detecting seasonal and sex differences in avian preen oil chemical composition. One occurrence corresponds to one sex in a given species for seasonal differences and to one season in a given species for sex differences.

Model	Variable	Sample size
Seasonal differences	**Response variable**	
	Seasonal differences	77
	No seasonal differences	14
	Fixed effects	
	Incubation	
	Sex incubates	63
	Sex does not incubate	28
	Nest ecology	
	Ground-nesting	45
	Non-ground-nesting	46
	Time scale	
	Breeding	19
	Breeding and non-breeding	72
Sex differences	**Response variable**	
	Sex differences	37
	No sex differences	38
	Fixed effects	
	Season	
	Breeding	53
	Non-breeding	22
	Incubation type	
	Uniparental	38
	Biparental	37

Table S7. Effect of the inclusion of the random effects (species, phylogeny) on the fit of the Markov chain Monte Carlo generalized linear mixed effects (MCMCglmm) models on seasonal differences and sex differences in preen oil composition. A lower deviance information criterion (DIC) score indicates a better model fit.

Random effects	DIC Model season	DIC Model sex
No random effect	1.60	1.66
Species	1.58	1.74
Phylogeny	1.58	1.64
Species and Phylogeny	1.57	1.60

Table S8. Mean proportion of the total variance explained by the random effects (phylogeny, species, and residual variance) in the two models fitted to explain seasonal and sex differences in preen oil chemical composition across species. The 95% posterior intervals are shown in square brackets. Large posterior intervals indicate poor precision in the estimates.

	Seasonal differences	Sex differences
Phylogeny	0.07 [7e-08, 0.58]	0.09 [5e-08, 0.62]
Species	0.05 [7e-08, 0.48]	0.05 [3e-08, 0.43]
Residuals	0.88 [0.35, 1.00]	0.86 [0.33, 1.00]

A breeding pair of pied flycatchers

Photo by Jörg Asmus

CHAPTER 3

Preen oil composition of pied flycatchers is similar between partners but differs between sexes and breeding stages

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ABSTRACT

Preen oil, the secretion of the uropygial gland, may be an important source of body odour in birds. By characterizing the chemical composition of preen oil, we can describe the olfactory phenotypes of birds and investigate whether odours could have a function in sexual signalling or other chemical communication. Here we analysed the preen oil of a wild passerine, the European pied flycatcher *Ficedula hypoleuca*, to find out whether it holds socially relevant information. We sampled both the female and male of breeding pairs during nestling rearing to test for sex differences and within-pair similarity. We additionally sampled the females during incubation to test for changes across breeding stages and for individual repeatability of chemical profiles. Pair mates had similar chemical profiles in comparison with other breeding adults. Furthermore, we found evidence for sex differences and for changes across breeding stages. Notably, the preen oil of females was more diverse and more volatile than that of males, and the preen oil secreted by females during incubation was more volatile than that secreted during nestling rearing. However, we found no evidence for individual repeatability of chemical profiles across breeding stages in females. Our results point towards a function of preen oil in sexual signalling, although other functions should not be excluded. Our study is a first step towards understanding the role of odours in the social life of an important avian model species used in the study of mate choice and sexual selection.

INTRODUCTION

Birds were long believed to have no or little sense of smell, notably because of their relatively small olfactory bulbs, their lack of a vomeronasal system, and the absence of sniffing behaviour (Roper 1999, Hagelin and Jones 2007, Caro et al. 2015). In addition, their often elaborate songs and colourful plumages mislead researchers into thinking that birds essentially rely on acoustic and visual cues for communication, overlooking the potential importance of olfaction (Bonadonna and Mardon 2013). However, in the last two decades, evidence has accumulated demonstrating that birds have a well-developed sense of smell (Clark and Smeraski 2022), which they use in a variety of contexts, like foraging (Nevitt 2008, Wikelski et al. 2021), navigation (Wallraff 2004, Gagliardo 2013), and nest building (Petit et al. 2002, Gwinner and Berger 2008). Evidence further suggests that birds also use olfaction to communicate with conspecifics, in particular during reproduction (reviewed in Hagelin and Jones 2007, Balthazart and Taziaux 2009, Caro et al. 2015, Whittaker and Hagelin 2021). However, how general and important chemical communication is across bird taxa remains to be established.

The occurrence of chemical communication is conceivable in birds because their olfactory phenotype often holds socially-relevant information. Preen oil is a waxy substance secreted by the preen (or uropygial) gland, which birds smear on their plumage during preening (Jacob and Ziswiler 1982). Preen oil, or chemical substances derived from preen oil (e.g. via physical or bacterial degradation), may be a major source of infochemicals in birds (Hagelin and Jones 2007, Caro et al. 2015), although not all avian infochemicals come from the preen oil (Hagelin and Jones 2007, Golüke et al. 2021). In fact, preen oil is often used as a proxy of avian body odour, for example in olfactory preference trials (e.g. Whittaker et al. 2011a, Grieves et al. 2019b). In addition to its potential role in chemical communication, preen oil serves diverse other functions like plumage maintenance, waterproofing, and protection against bacteria and ectoparasites (reviewed in Moreno-Rueda 2017).

Preen oil is usually composed of non-volatile compounds, including wax esters, and volatile compounds, including alcohols, aldehydes, carboxylic acids, methyl ketones, benzoates, terpenes, lactones, and phenols (Haahti et al. 1964, Jacob and Ziswiler 1982, Soini et al. 2013, reviewed in Campagna et al. 2012). The chemical composition of preen oil can be affected by diet (Thomas et al. 2010, Kanakri et al. 2016), season (Reneerkens et al. 2002, reviewed in Grieves et al. 2022), preen gland microbiota (Martín-Vivaldi et al. 2010, Whittaker et al. 2019b), and hormones (Bohnet et al. 1991, Whittaker et al. 2011b). Many studies have analysed the chemical composition of preen oil to decipher whether it contains socially-relevant information. Preen oil can hold information about species identity (Mardon et al. 2010, Van Huynh and Rice 2019), population identity (Whittaker et al. 2010, Grieves et al. 2019a), sex (Caspers et al. 2022, reviewed in Grieves et al. 2022), age (Sandilands et al. 2004, Díez-Fernández et al. 2021), breeding status (Reneerkens et al. 2007a, Martín-Vivaldi et al. 2009), individual identity (Mardon et al. 2010, Jennings and

Ebeler 2020), genetic heterozygosity (Whittaker et al. 2019a), major histocompatibility (MHC) genotype (Leclaire et al. 2014, Slade et al. 2016) and genetic relatedness (Potier et al. 2018). Importantly, experimental studies have shown that birds can perceive such socially-relevant variation in preen oil composition using their sense of smell (Whittaker et al. 2011a, Leclaire et al. 2017b, Grieves et al. 2019c).

Sex differences and seasonal changes in preen oil composition are major targets to study the role of odour in intraspecific communication in birds. For example, a recent review found that, in most species, the preen oil of females contains a higher number and diversity of volatile substances than that of males (Whittaker and Hagelin 2021). This suggests that preen oil could play a role in olfactory signalling between the sexes, although sex differences in preen oil composition could also point towards other sex-specific functions. For example, the sex and seasonal differences described in the preen oil of ground-breeding shorebirds (order Charadriiformes) may serve olfactory crypsis to avoid predation (Reneerkens et al. 2002). Indeed, the preen oil of individuals of the incubating sex becomes less volatile during incubation, which may hinder the olfactory detection of the nest by predators (Reneerkens 2005). A recent quantitative review analysed the occurrence of sex and seasonal differences in preen oil composition in 59 species and found chemical patterns to be consistent with a role of preen oil in sexual signalling during mate choice, as well as olfactory crypsis during incubation (Grieves et al. 2022). Investigating how the volatility of preen oil differs between the sexes and breeding stages would help assess the relative importance of these two hypotheses. A role in olfactory crypsis would be supported by a lower volatility in the incubating sex and during incubation, whereas a role in sexual signalling would be supported by any difference in volatility. Indeed, either high or low volatility compounds can transmit chemical information and act as honest, sexually selected signals—not just high-volatility compounds, as proposed in Grieves et al. (2022). Low-volatility compounds could act as sexually selected signals, for example, if they signal greater protection for the offspring (against predators via olfactory crypsis, or against pathogens via antimicrobial activity, Moreno-Rueda 2017). High concentrations of large, less-volatile compounds might also indicate mate quality, if the bearer must use more energy to produce them. In addition, it would be insightful to evaluate the (dis)similarity in preen oil composition between breeding partners. Birds may use odours to assess relatedness (Krause et al. 2012, Bonadonna and Sanz-Aguilar 2012, Caspers et al. 2015a) and evidence from several species suggests that similarity in preen oil composition covaries with genetic relatedness and MHC genotype (Leclaire et al. 2014, Slade et al. 2016, Potier et al. 2018, Grieves et al. 2021c). Individuals should generally pair up with unrelated mates to avoid inbreeding (e.g. Kruuk et al. 2002, but see de Boer et al. 2021), or with mates dissimilar at the MHC to maximize the disease resistance of their offspring (e.g. Consuegra & Garcia de Leaniz 2008), and therefore partners can be expected to have a rather dissimilar preen oil composition (Grieves et al. 2019c). Finally, individual chemical signatures (i.e. repeatable preen oil composition within individuals over time) are also of interest. Individual signatures are considered a fixed aspect of an individual, and their

presence may suggest that preen oil composition has a genetic component. Individual chemical signatures are essential for birds to individually recognize conspecifics and assess their relatedness and other characteristics via olfaction, notably during mate choice (Mardon et al. 2010).

In this study, we investigated the chemical composition of the preen oil of the European pied flycatcher *Ficedula hypoleuca* (hereafter pied flycatcher), a common passerine bird often used in studies on behaviour, ecology and evolution (e.g. Both et al. 2006, Ellegren et al. 2012, Nicolaus et al. 2022). While visual and acoustic traits and their role in sexual selection have received great attention in this species (e.g. Lampe and Espmark 2003, Sirkiä and Laaksonen 2009), the potential role of olfactory phenotypes has been completely unexplored. We sampled the preen oil of pied flycatchers to analyse its chemical composition using gas chromatography and investigated sex differences and partner (dis)similarity during the period of nestling rearing, as well as changes across breeding stages (incubation versus nestling rearing) and individual signatures in females.

MATERIALS AND METHODS

Study species and population

We studied pied flycatchers from an established nestbox population in a lowland mixed coniferous forest near Elbergen in NW Germany (52°27' N, 7°15' E; for details on the study site see Altenkirch & Winkel 1991). The pied flycatcher is a common medium-sized (13 cm; 9-22 g) passerine bird with a wide distribution in the Palaearctic, and is a trans-Saharan migrant that arrives between late March and early May on European breeding grounds. During the breeding season, pied flycatchers form social pair bonds (Glutz von Blotzheim and Bauer 1993). While social monogamy prevails in the study population, typically a small proportion of males is socially polygynous each year (Lubjuhn et al. 2000, Huk and Winkel 2006). Furthermore, some extra-pair paternity occurs (Brün et al. 1996, Lubjuhn et al. 2000). Pied flycatchers provide biparental care, but only females build the nest and incubate while both sexes contribute to nestling provisioning (Glutz von Blotzheim and Bauer 1993).

Field methods

During routine nestbox checks as part of a long-term monitoring program, pied flycatchers were caught by hand or with nest traps during the breeding season in May and June 2019, and were ringed with uniquely numbered metal rings (issued by "Vogelwarte Helgoland", Wilhemshaven, Germany). To collect preen oil, we gently swabbed the preen gland with a fresh cotton bud several times from both sides over a period of approximately five seconds, and immediately placed the cotton bud in a 20 mL screw neck glass vial (following Caspers *et al.* 2022). Upon return from the field site in the evenings of sampling days, vials were stored at –20 °C until further analysis. Females were sampled during incubation and both

pair partners were sampled during nestling rearing (often on the same day). In all, we collected 103 preen oil samples over a period of 22 days. In addition, we took field blank samples (four in total) for which an identical handling protocol was applied although without sampling a bird.

Laboratory methods

Vials were defrosted for at least 60 minutes prior to sample preparation. To extract preen oil, we injected 100 µl of dichloromethane directly into the cotton bud, and squeezed out the content of the bud (consisting of dichloromethane and preen oil) using a 100 µl blunt point glass syringe. We transferred the extracts into 2 ml glass vials equipped with a 100 µl glass inlet. The extracts were concentrated by evaporation—by leaving the glass vials open at ambient temperature under a fume hood for 10 to 30 min—to a volume of approximately 5 µl before analysis. Samples were analysed by gas chromatography (GC) with a flame ionisation detector (GC-FID, GC 2010 plus, Shimadzu, Duisburg, Germany) equipped with a VF-5ms capillary column (30 m x 0.25 mm ID, DF 0.25, 10 m guard column, Varian Inc., Lake Forest, USA). One microliter (1 µl) of each sample was injected into a deactivated glasswool-packed liner at an inlet temperature of 220 °C and processed in a split 10 mode with 20 ml/min split flow. Hydrogen was used as carrier gas and its flow rate was held at 1 ml/min. The GC temperature started at 60 °C initial time of 3 minutes, followed by a 10 °C/min increase rate to a final temperature of 280 °C, which was kept for 20 minutes. Three GC blank samples (containing dichloromethane only) were analysed among the preen oil and field blank samples.

Chromatographic data processing

For a given sample, GC-FID produces a chromatogram in which each substance is represented by a peak, the area of which is proportional to the abundance of that substance in the sample. Substances are distinguished by their specific retention times. Peak areas and retention times were extracted using GC Solutions v2.41 (see **Table S1** for details). The retention times of homologous substances may vary subtly among samples due to unavoidable stochastic variation in ambient temperature, flow rate of the carrier gas, or column ageing. However, homologous peaks should be considered as representing a single substance and therefore need to be aligned on a unique retention time. Chromatograms were aligned using the *GCalignR* package (Ottensmann et al. 2018) in R v3.6.1 (R Core Team 2022). In total, 110 samples were used for the alignment procedure, consisting of 103 preen oil samples, three GC blank samples and four field blank samples. Any substance detected in the GC blank or field blank samples were removed to control for possible contamination (e.g. from the cotton swabs, the observer, or the environment) during laboratory work or fieldwork, respectively. We excluded samples that contained no further substances after the removal of the substances detected in the blank samples from further analysis. Substances detected in only a single sample were ignored in further analysis. After

alignment and filtering, 98 preen oil samples were retained. Details about the alignment procedure are available in the Supporting Information (**Appendix S1**, **Table S1**). From the 98 retained samples, we discarded 21 samples with chromatograms similar to blank samples where no preen oil had apparently been collected, resulting in 77 successful samples. We calculated the relative abundance of each substance by dividing its peak area by the total chromatogram area, because the total amount of preen oil collected was not standardized and varied across samples. Relative abundances are pertinent to assess differences in potential information content, although we may miss differences in absolute abundances, which can also be important (Mardon et al. 2011b, Whittaker and Hagelin 2021). Relative abundances were log-transformed log(X+1)) to prevent high-abundance substances from having a disproportionate influence during the analysis (following Clarke *et al.* 2014). We verified that our chromatographic data were properly aligned and transformed by inspecting shade plots (**Fig. S1**) in PRIMER v7.0.20 (Clarke and Gorley 2015). We calculated the chemical richness (number of substances), diversity (Shannon index) and volatility (proportions of high-volatility and low-volatility substances) of each sample. The proportions of high-volatility and low-volatility substances were measured as the proportion of abundance (i.e. chromatogram area) *before peak C* and *after peak F*, respectively (**Fig. 1 & S2**). We chose these specific thresholds to exclude the central part of the chromatograms (which contains most of the abundance and shows little variation among samples) while conserving sufficient and equivalent abundances in the early part (high-volatility; mean ± SD proportion = 9.9 ± 2.1%; mean ± SD number of substances = 14.1 ± 8.6) and the late part (low-volatility; mean ± SD proportion = 9.0 ± 3.1%; *mean* ± SD number of substances = 13.6 ± 8.3) of the chromatograms. Alternatively, we could have chosen another relevant set of thresholds, that is *before peak B* and *after peak H*, to focus only on the most volatile and the least volatile substances, respectively. We repeated the

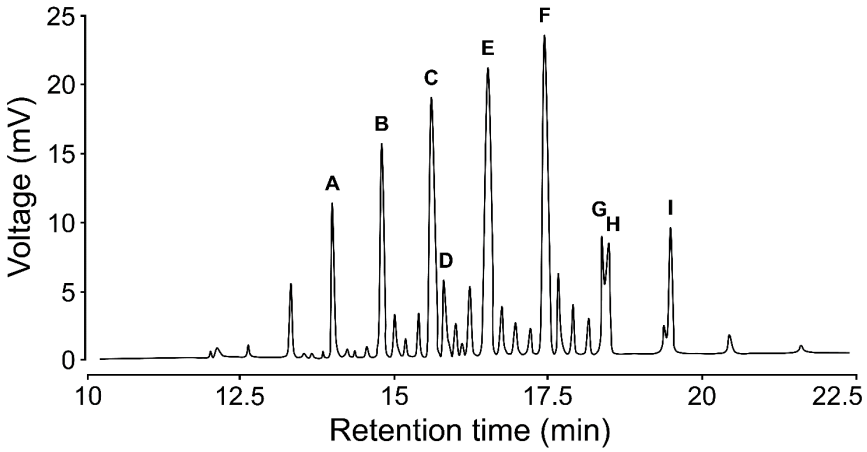

Fig. 1. Representative GC-FID chromatogram of the preen oil of a female pied flycatcher sampled during nestling rearing. Analyses were performed on the complete chromatograms (all substances) and on the nine most abundant substances (indicated with letters). The abundance of the most abundant substance across all samples (substance F) was used as a proxy for the concentration of preen oil in each sample.

analyses on volatility using this alternative set of thresholds and found similar results, suggesting that our method is robust (**Supporting Information**). These two measures of volatility inform us on two different mechanisms by which preen oil substances can affect the detectability of a bird (or its nest/clutch): high-volatility substances would directly increase its detectability, while low-volatility substances may reduce its detectability. The proportions of high-volatility and low-volatility substances were not correlated (Spearman test: *rho* = –0.11, *P* = 0.35), which confirms that these measures represent two distinct traits.

Concentration bias

Upon further examination of the chromatographic data, it appeared that some samples had a remarkably limited chemical richness and diversity. As we had no measurement of the generally minute quantities of preen gland secretion we collected, it is likely that only a very small amount of secretion was collected in these samples, and that this low concentration made low-abundance substances hard to detect, resulting in low richness and diversity (**Fig. S3**). We examined the relationship between diversity and concentration, using the abundance of the most abundant substance across samples (substance F in **Fig. 1**) as a proxy for the total concentration of a given sample. Diversity drops abruptly below a certain concentration threshold, revealing a likely concentration bias (**Fig. S4**). Scarcer substances are less well detected in low-concentration samples, resulting in an underestimated chemical richness and diversity. Because low-concentration samples may introduce noise in our data, we discarded samples below the concentration threshold (N = 16 samples) and conducted our analyses on the remaining 61 samples (**Fig. S4**). We ran additional analyses where we included the low-concentration samples (N = 77 samples) to verify the robustness of our results (see "complete dataset" in **Supporting Information**). .

Statistical analysis

To investigate differences between sexes and among breeding pairs, we used the samples from breeding pairs where both the female and the male were successfully sampled. Our data included 22 samples from 11 pairs. To investigate differences between breeding stages and among individuals, we used the samples from females sampled on two occasions, namely during incubation and during nestling rearing (mean ± SD number of days between the two samples = 16.8 ± 2.8). Our data included 14 samples from 7 females. We made sure that our designs were perfectly balanced (i.e. equal sample sizes across groups), in particular because permutational multivariate analyses of variance (PERMANOVA) can be sensitive to differences in dispersion under unbalanced designs (Anderson et al. 2008).

We tested for differences in the overall composition of preen oil using PERMANOVA with the PERMANOVA+ v1 add-on (Anderson et al. 2008) in PRIMER v7.0.20 (Clarke and Gorley 2015). We first constructed a resemblance matrix based on

pairwise Bray-Curtis dissimilarities between samples. Bray-Curtis dissimilarity, which is commonly used in chemical ecology studies (Brückner and Heethoff 2017), is a well suited measure for the analysis of abundance data because it ignores joint absences (Clarke et al. 2014). PERMANOVA models were run with 9 999 permutations and type III (partial) sums of squares. Although the type of sums of squares should not matter with our balanced designs, we chose type III because it is the most conservative (Anderson et al. 2008). To test for sex and pair differences, *sex* was included as a fixed effect and *pair ID* as a random effect. To test for breeding stage and individual differences, *breeding stage* was included as a fixed effect and *individual ID* as a random effect. We verified the homogeneity in dispersion with PERMDISP tests, even though PERMANOVA is robust to heterogeneity in dispersion under balanced designs (Anderson et al. 2008). We repeated all PERMANOVA models considering only the nine most abundant substances (i.e. only substances that were consistently the most abundant substances in all samples; **Fig. 1 & S1**) to assess whether differences observed in preen oil composition are solely driven by differences in the most abundant substances, or whether scarce substances also play a role. In combination with PERMANOVA, we visualized our data with metric multidimensional scaling (mMDS) plots in PRIMER.

In addition, we investigated differences among preen oil samples in chemical richness, chemical diversity and volatility (proportion of high-volatility substances and low-volatility substances). To do so, we ran generalized linear mixed models (GLMM) with Gaussian distribution and identity link using the *lmer* function of the *lme4* package (Bates et al. 2007) in R. In the models testing for sex and pair differences, *sex* was included as a fixed effect and *pair ID* as a random effect. In the models testing for breeding stage and individual differences, *breeding stage* was included as a fixed effect and *individual ID* as a random effect. The significance of fixed effects was assessed (at $\alpha = 0.05$) by checking whether their 95% confidence interval contained 0. We additionally indicated P-values for both fixed effects and random effects, which were obtained with the *lmerTest* (Kuznetsova et al. 2015) and the *rptR* (Stoffel et al. 2017) packages, respectively. We calculated the marginal R^2 explained by fixed effects using the *partR2* package (Stoffel et al. 2021) and the adjusted repeatability of traits based on random effects with the *rptR* package. Assumptions of normality and homoscedasticity of the residuals were verified by visual inspection of plots with the *performance* package (Lüdecke et al. 2021). Boxplots were produced with the *ggplot2* package (Wickham 2016). Details on the analyses in R are available in **Appendix S1**.

Discarding the low-concentration samples may not be sufficient to control for the concentration bias. Therefore, we repeated all models where significant effects were detected, adding *concentration* (area of the most abundant peak across samples) as a covariate, and checked whether the effects were robust. We also verified that there was (1) no difference in concentration between the groups (females *vs* males, incubation *vs* nestling rearing period) with Wilcoxon rank-sum tests, and (2) no correlation in concentration

between paired samples (both sexes of a pair, both breeding stages of an individual) with Spearman correlation tests.

RESULTS

The 61 preen oil samples that were retained for analysis contained a total of 119 different substances with on average 46 substances (SD = 16) per sample.

Sex differences during nestling rearing period

We found a sex difference in the overall composition of preen oil (PERMANOVA; $P = 0.035$, component of variation = 3.1%; **Table 1**, **Fig. 2**). However, no sex difference was detected when including the low-concentration samples (**Table S2**) or when considering only the nine most abundant substances (**Table S3**). Females had on average a slightly more diverse preen oil than males (GLMM; β [95% CI] = 0.08 [0.01; 0.15], marginal $R^2 = 0.07$; **Table S4**). The preen oil of females was also more volatile than that of males, as it contained more high-volatility substances (β = 1.35 [0.29; 2.46], marginal $R^2 = 0.21$; **Fig. 3**; **Table S4**). However, we found no evidence that sexes differed in chemical richness (males: β = –2.73 [–11.30; 5.80], marginal $R^2 = 0.01$) or in the proportion of low-volatility substances (males: β = 0.03 [–0.83; 0.95], marginal $R^2 = 0$; **Table S4**).

Partner similarity during nestling rearing period

We detected similarity between pair members (i.e. partners) in the overall composition of preen oil (PERMANOVA; $P = 0.006$, component of variation = 5.9%; **Table 1**; **Fig. 2**). This

Table 1. Results from PERMANOVA on the preen oil chemical composition of pied flycatchers. **(a)** Effect of sex (fixed effect) within breeding pairs (random effect) sampled during nestling rearing (N = 22 samples from 11 pairs). **(b)** Effect of breeding stage (fixed effect) within individual females (random effect) sampled during both incubation and nestling rearing (N = 16 samples from 8 females).

	df	SS	F (pseudo)	P (perm)	Component of variation
(a) Sex and pair					
Sex	1	156.7	3.23	**0.035**	3.14
Pair	10	1172.0	2.42	**0.006**	5.86
Residuals	10	484.6	—	—	6.96
(b) Breeding stage and individual					
Breeding stage	1	458.0	5.74	**0.014**	6.88
Individual	7	691.6	1.24	0.293	3.08
Residuals	7	558.5	—	—	8.93

Analysis based on Bray-Curtis dissimilarities of log-transformed values. P-values were obtained using 9 999 permutations under a reduced model with type III (partial) sums of square (SS), and are indicated in bold if the effect is significant at $\alpha = 5\%$. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray-Curtis units (i.e. % of Bray-Curtis dissimilarity).

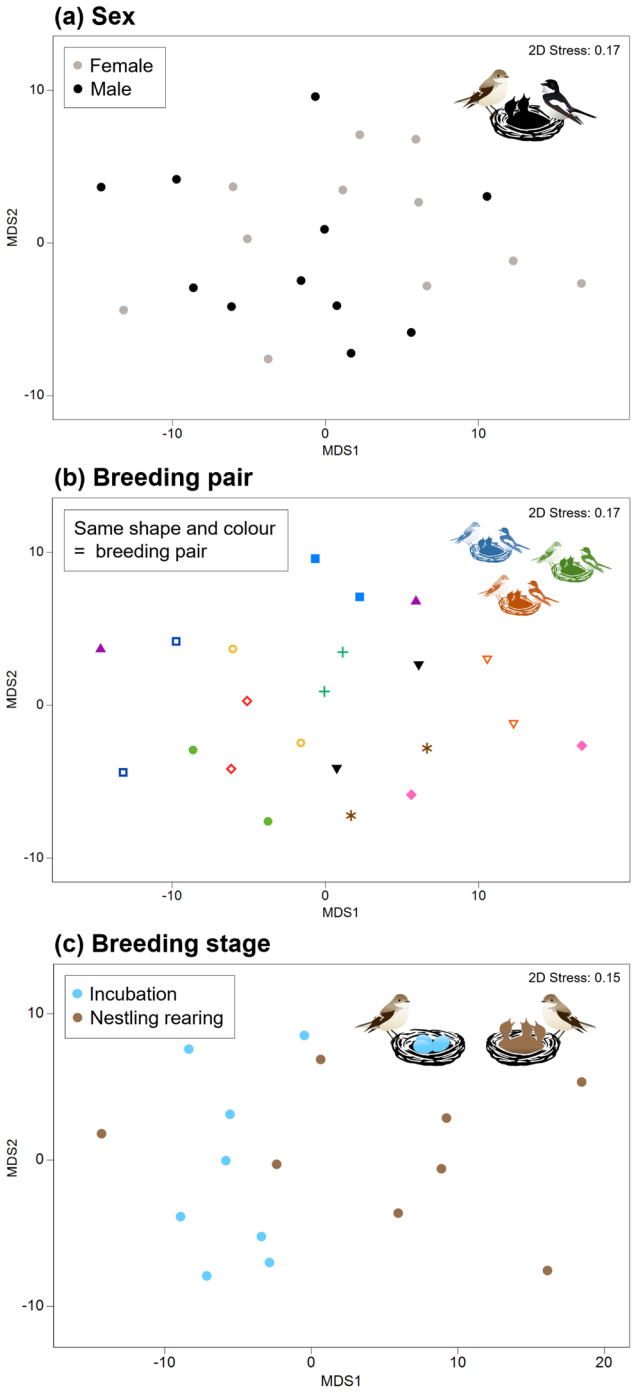

Fig. 2. Two-dimensional metric multidimensional scaling (mMDS) plots representing Bray-Curtis dissimilarity in the preen oil composition of pied flycatchers. (a) Sex differences within pairs during nestling rearing. **(b)** Differences among breeding pairs during nestling rearing. **(c)** Differences between breeding stages within individual females. 2D Stress is a measure (between 0 and 1) of the fit between the distance among samples in two-dimensional space and the actual distance among samples in multivariate space (values near 0 indicating a better fit).

pair effect was also evident when including the low-concentration samples (**Table S2**) and when considering only the nine most abundant substances (**Table S3**). The similarity between partners may be partly explained by the fact that they were sampled close in time, at a similar temperature and by the same observer. Possibly as a result of this, the concentration in preen oil in samples obtained from pair members was correlated (*rho* = 0.74, *P* = 0.01). However, when controlling for concentration, and for date and time of sampling, the pair effect remained (**Tables S5 & S6**). Partners also had similar preen oil in terms of chemical richness (GLMM; repeatability = 0.71), diversity (repeatability = 0.67), and proportion of low-volatility substances (repeatability = 0.58; **Table S4**). The proportion

of high-volatility substances, however, was not repeatable within pairs (repeatability = 0; **Table S4**).

Change across breeding stages within females

The overall composition of preen oil of females changed significantly from incubation to nestling rearing (PERMANOVA; $P = 0.014$, component of variation = 6.9%; **Fig. 2**; **Table 1**). The effect of breeding stage was also detected when including low-concentration samples (**Table S2**) and when considering only the nine most abundant substances (**Table S3**). The preen oil secreted during incubation contained a lower proportion of low-volatility substances (GLMM; $\beta = 1.84$ [0.89, 2.94], marginal $R^2 = 0.43$; **Fig. 3**; **Table S7**). However, no breeding stage differences were detected in chemical richness ($\beta = 1.12$ [–18.00, 21.40], marginal $R^2 = 0$), diversity ($\beta = 0.05$ [–0.13, 0.24], marginal $R^2 = 0.02$) or the proportion of high-volatility substances ($\beta = -1.12$ [–2.72, 0.34], marginal $R^2 = 0.06$; **Table S7**).

Individual repeatability within females

We found no evidence for individual-specific chemical signatures in the females sampled twice, neither for the overall composition (PERMANOVA; $P = 0.29$, component of variation = 3.1%; **Table 1**), nor for the nine most abundant substances (**Table S3**). In addition, neither richness, diversity nor volatility were repeatable within individual females (**Table S7**).

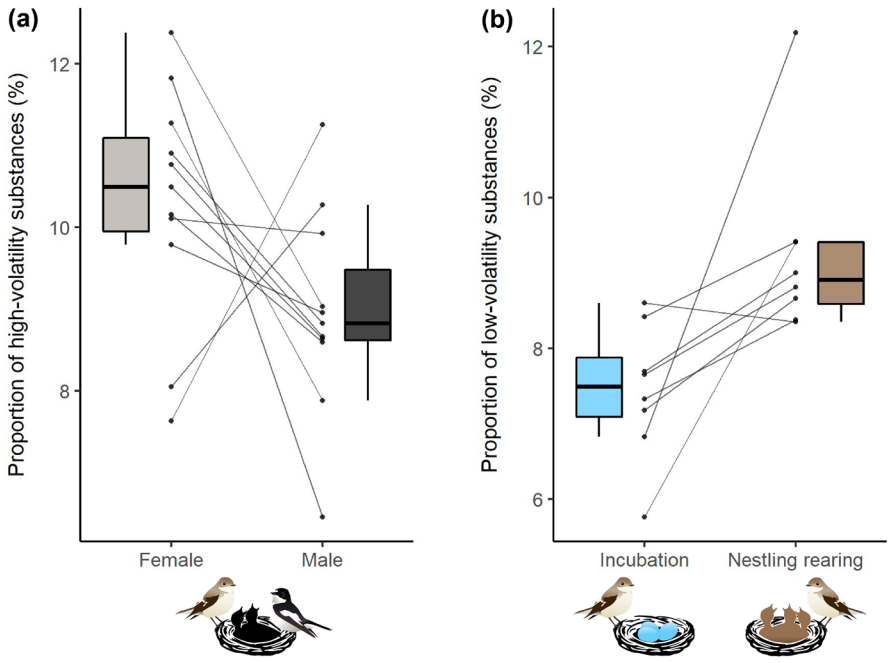

Fig. 3. Sex and breeding stage differences in the volatility of pied flycatcher preen oil. (a) Sex differences in the proportion of *high-volatility* substances within pairs during nestling rearing. Lines connect breeding pair mates. **(b)** Breeding stage differences in the proportion of *low-volatility* substances within individual females. Lines connect observations of individual females.

DISCUSSION

We investigated natural variation in preen oil chemical composition in a wild population of pied flycatchers. We found that preen oil composition is similar between pair mates and differs between the sexes during the period of nestling rearing, and differs between breeding stages in females. However, we found no evidence for any repeatable, individual chemical signature in females across breeding stages. Based on these findings, we discuss potential functions of preen oil and olfactory phenotypes in this species. We focus in particular on odour-related functions, due to the potential importance of preen oil for olfactory communication (Whittaker and Hagelin 2021, Grieves et al. 2022), but also touch upon other, non-olfactory functions of preen oil (reviewed in Moreno-Rueda 2017).

Sex differences

Our study revealed sex differences in preen oil composition during the period of nestling rearing. Sex differences were detected in the overall composition of preen oil only when excluding low-concentration samples, where scarce substances could not be reliably detected. This suggests that the sex difference is mainly driven by scarce substances. Hence, it is no surprise that, when only looking at the nine most abundant substances, we found no sex difference. In addition, sexes differed in chemical diversity and volatility, with a higher diversity and higher proportion of high-volatility substances in females. This is in line with findings of Whittaker and Hagelin (2021), who demonstrated that avian chemical signals are often more pronounced in females. Indeed, females often have larger preen glands (e.g. Golüke and Caspers 2017; therefore secreting greater amounts of preen oil, Martín-Vivaldi et al. 2009) and produce a preen oil with a higher diversity and a higher number of volatile substances than males (e.g. Jacob et al. 2014, Caspers et al. 2022, reviewed in Whittaker and Hagelin 2021).

Different functional hypotheses have been proposed to explain why females and males secrete a different preen oil during the breeding season. First, the sex semiochemical hypothesis posits that preen oil provides chemosignals that function in mate choice, which can be associated with a sex difference in the proportions of some substances (Whittaker et al. 2010, Grieves et al. 2022). Second, the olfactory crypsis hypothesis posits that the preen oil secreted by the incubating sex is used to minimise olfactory cues from eggs, nestlings or the incubating parent, thereby reducing nest predation from olfactorily-searching predators, and leading to sex differences in species with uniparental incubation (Reneerkens et al. 2002, Grieves et al. 2022). Third, the incubating and/or chick rearing sex may produce preen oil substances that limit infection of the eggs and/or chicks by parasitic bacteria, fungi or arthropods (Moreno-Rueda 2017), which could also lead to sex differences in preen oil composition in species with uniparental incubation and/or care (e.g. Martín-Vivaldi et al. 2009). Although this third hypothesis does not make any assumption on the odorous nature of preen oil, it is possible that preen oil odours are used as a signal

indicating an individual's ability to battle against pathogens, but this remains to be investigated.

The sex difference observed in pied flycatchers more likely reflects a function in sexual signalling than in olfactory crypsis, because the preen oil of females (incubating sex) was more volatile than that of males. It is nonetheless possible that the more volatile preen oil of females serves olfactory crypsis via chemical background matching, if the volatile substances blend with the odour of the environment surrounding the nest. This hypothesis has previously been proposed based on the observation that the preen oil of Dark-eyed Juncos *Junco hyemalis* contains volatile linear alcohols similar to that produced by plants surrounding their nest (Soini et al. 2007). Dark-eyed Juncos are however a ground-nesting species, and olfactory crypsis may be more important for such species with less protection from predators than for cavity-nesting species, like the pied flycatcher (Grieves et al. 2022).

Olfactory acuity may be highest during courtship (Groof et al. 2010) and the preen gland is often largest at the time of hatching (Martín-Vivaldi et al. 2009, Golüke and Caspers 2017), suggesting that the perception and production of chemical substances may be magnified during early breeding. It would be important to analyse sex differences in preen oil even before egg laying, when most of sexual selection occurs. In our study, sex differences were assessed during nestling rearing (*mean* ± *SD* number of days after hatching = 9.2 ± 2.2), that is after mate choice and incubation. For stronger inference on a role of preen oil in either sexual signalling or chemical protection, sex differences should ideally be evaluated during mate choice or incubation, respectively. We can speculate that the sex difference would be more pronounced if we had considered absolute abundances, instead of relative abundances, notably as females may have a larger preen gland size and produce more preen oil than males (Whittaker and Hagelin 2021). To allow such analyses, future studies should measure the mass of preen oil collected. We should not overlook the possibility that the observed sex differences may also be the result of non-adaptive mechanisms, such as sex differences in diet or preen gland microbiota (Grieves et al. 2022). For example, sex differences in diet have been documented in the pied flycatcher, with females foraging more often in trees searching for caterpillars and males foraging more often on aerial lepidopterans (Alatalo and Alatalo 1979).

Similarity between breeding partners

Despite the sex differences in preen oil composition, breeding partners secreted preen oil with relatively similar compositions (in terms of overall composition, chemical richness and diversity, proportion of low-volatility substances) in comparison to other synchronously breeding pairs, suggesting that partners may have similar odours. In another passerine species, the Dark-eyed Junco, it has also been found that breeding partners had similar preen oil composition (Whittaker et al. 2016). We cannot exclude that this similarity may partly be due to sampling bias, as both members of a pair were sampled on the same day and around the same time of day. Pair members were therefore sampled under similar

environmental conditions (e.g. temperature, humidity), which may have affected the viscosity and thereby the amount of the preen oil collected, and which could explain the correlation in preen oil concentration between the samples of pair mates. However, the pair effect was robust and remained similar in magnitude when statistically controlling for both concentration and date and time of sampling. We thus propose that the similarity in preen oil composition observed between mates may be due to **(1)** preferences for mates with similar odours, **(2)** a transfer of preen oil substances between mates, or **(3)** phenotype-environment correlations, as mates share the same environment (e.g. nest microbiota, available food).

The first possibility is that pied flycatchers mate preferentially with individuals that have an odour similar to their own, resulting in assortative mating. One of the possible functions of a preference for breeding partners with a similar odour is the avoidance of outbreeding, i.e. mating with individuals that are genetically very different (Luo et al. 2014). Indeed, like inbreeding, outbreeding can have deleterious consequences (outbreeding depression, Marshall and Spalton 2000, Szulkin et al. 2013). For this hypothesis to be relevant, it should be verified that similarity in preen oil composition covaries with genetic similarity in pied flycatchers, as has been shown in other bird species (Leclaire et al. 2014, Slade et al. 2016, Potier et al. 2018). However, we think it is unlikely that the similarity in preen oil observed within breeding pairs is the result of an increased relatedness between pair members, as highly dispersive passerines with large population sizes often have very low rates of inbreeding (e.g. less than 1% in the closely related Collared Flycatchers *Ficedula albicollis*; Kruuk *et al.* 2002).

The second possibility is that breeding partners transfer preen oil substances or preen gland microbes to one another. Such transfers could be direct during social interactions (copulation, allopreening) or indirect when sharing the same breeding environment (e.g. via nestbox surfaces oil, nest material, nestlings) (Hagelin 2007a, Hagelin and Jones 2007). Once smeared on the plumage, preen oil compounds could be exchanged directly between partners, just like feather microbes are transmitted during social interactions (Kulkarni and Heeb 2007). However, we are unsure how likely it is that such an exchange of preen oil would lead to a different composition of the freshly secreted preen oil that we collected. Mates may also secrete a similar preen because they have similar preen gland microbiota. Indeed, breeding partners often have similar microbiota (preen gland, Whittaker et al. 2016; skin around the preen gland, Engel *et al.* 2020; cloaca, Kreisinger *et al.* 2015, Whittaker et al. 2016), possibly due to their spatial proximity or similarity in diet. Preen gland microbiota may shape preen oil composition (Whittaker et al. 2019b, but see Whittaker et al. 2016, Grieves et al. 2021) by modifying chemical substances in the preen oil (Martín-Vivaldi et al. 2010, Whittaker et al. 2019b). However, it is currently not known whether pied flycatchers harbour microbes in their preen gland that produce specific substances in the preen oil.

A third possibility is that partners adjust their preen oil phenotypes to their shared environment in a similar way (i.e. phenotype-environment correlations; Snowberg &

Bolnick 2012, Fokkema et al. 2021, Trappes et al. 2022). Mated pairs may for instance exhibit a parallel chemical adjustment to the communities of bacteria and ectoparasites that are specific to their nests (i.e. nest microbiota). Indeed, it has been shown that in Great tits *Parus major* the preen oil of both females and males changes in response to experimental modifications of nest microbiota (Jacob et al. 2014). However, the induced changes were greater in females than in males, probably because females spend more time in the nest. In pied flycatchers, females are also considerably longer in contact with the nest environment than males, as they build the nest and incubate the eggs alone, whereas males only briefly enter the nest to feed the female during incubation and the chicks during brood care. The effect of nest microbiota is therefore expected to have a greater impact on females than on males. Accordingly, it was found that nest microbiota affected the plumage microbiota of female but not of male pied flycatchers (Goodenough et al. 2017). Furthermore, even though the sexes can differ in their overall diet (Alatalo and Alatalo 1979), breeding partners may still consume a relatively similar diet, which is known to affect preen oil composition (Thomas et al. 2010). Pied flycatcher pairs exploit the same territory, and therefore have the same food resources available (Grundel 1990, Moreno et al. 1995). Breeding partners may thus have a similar preen oil composition because they feed on similar food resources in their territory. This could be investigated by testing whether pairs with neighbouring or partly overlapping territories have similar preen oil, but such a test would require a larger sample size.

Change across breeding stages in females

Almost all species studied to date exhibit seasonal changes in preen oil composition (Whittaker and Hagelin 2021, Grieves et al. 2022). The preen oil of female pied flycatchers, in our study, changed from the incubation to the nestling rearing phase. Thereby, our study provides further evidence that systematic seasonal changes in preen oil can occur over relatively short periods of time, with a change detected over only 17 days (average time period between the two samples). Other rapid changes were documented in Red Knots *Calidris canutus* (Reneerkens et al. 2007d), Dark-eyed Juncos (Whittaker et al. 2011b) and Song sparrows *Melospiza melodia* (Grieves et al. 2018), where preen oil composition changed in less than two weeks.

We found that the preen oil secreted during incubation was on average more volatile, as it contained a lower proportion of low-volatility substances, than that secreted later in the breeding season. Despite the lack of change in chemical richness and diversity, this result is consistent with the sex semiochemical hypothesis (Grieves et al. 2022), as a change in volatility could be used for chemical communication. Similarly, a number of studies on other bird species found that the preen oil produced during breeding is more volatile than that produced during nonbreeding, and have hypothesized that the preen oil produced during breeding serves as a chemosignal for reproduction (e.g. White-throated Sparrows *Zonotrichia albicollis*, Tuttle et al. 2014; Gray Catbirds *Dumetella carolinensis*, Shaw et al. 2011; Dark-eyed Juncos, Soini et al. 2007). For example, in White-throated

Sparrows, individuals held in breeding conditions produced four volatile compounds that are not secreted under non-breeding conditions (Tuttle et al. 2014), and the preen oil of females contained higher abundances of volatiles before laying (i.e. during the mate choice period) than during incubation (Forrette 2018). However, our finding of a higher volatility of preen oil during incubation than nestling rearing seems inconsistent with a role of peen oil in olfactory crypsis (Grieves et al. 2022). In precocial species, like sandpipers, mobile chicks leave the nest shortly after hatching, and it is therefore important to avoid olfactory detection of the nest by predators during the egg phase (incubation), but not necessarily during the chick phase (Reneerkens et al. 2002). Sandpipers chemically camouflage their nest by secreting a less volatile preen oil specifically during the period of incubation (Reneerkens et al. 2002, 2005). In contrast, in altricial species like the pied flycatcher, chicks are raised in the nest, and olfactory detection of the nest by predators should be avoided both during the egg and chick phases. Presumably, the vulnerability of pied flycatchers to olfactorily-searching nest predators does not vary across breeding stages, and thus preen oil volatility should not change across breeding stages. It is also possible that the changes across breeding stages observed in the preen oil of females are related to a role in olfactory parent-offspring communication (Caspers et al. 2017b). An alternative explanation for our finding that female preen oil is more volatile during incubation is that it may contain specific volatile compounds that inhibit the growth of eggshell bacteria, as was shown in Eurasian Hoopoes *Upupa epops* (Martín-Vivaldi et al. 2010).

CONCLUSION AND FUTURE RESEARCH

This study provides the first characterization of the chemical composition of the preen oil of pied flycatchers. Our results warrant further investigation in the chemical ecology of an important model species for studying sexual selection and mate choice in birds. In future work, sampling females during mate choice (e.g. before egg laying) and sampling males during both mate choice and incubation would be highly valuable. More pronounced sex differences in preen oil during mate choice, possibly in combination with either increased or reduced volatility, can be an indication that it plays a role in sexual olfactory signalling (but note that a function in sexual signalling should not be ruled out in case there are no sex differences in preen oil composition). If the similarity within pairs is caused by the transmission of chemicals between mates, it is predicted to increase across the breeding stages, as mates spend more time closely together. The possible presence of individual signatures should be further investigated in females (with additional repeated samples during peak periods of sexual selection, and within and across breeding stages), as well as in males. Finally, behavioural trials should be conducted to test whether pied flycatchers can actually smell and use these differences in chemical profiles, particularly in the context of mate choice and reproduction.

Acknowledgements

We thank Meinolf Ottensmann and Martin A. Stoffel for advice on statistical analysis, and Julie Hagelin and an anonymous reviewer for comments that improved this manuscript.

Data availability

Data and code are available at the repository PUB – Publications at Bielefeld University (https://pub.uni-bielefeld.de/record/2965523,
DOI: https://doi.org/10.4119/unibi/2965523).

Supporting information

The complete supporting information (including **Appendix S1**) can be found online in the Supporting Information section at the end of the article (https://doi.org/10.1111/ibi.13246).

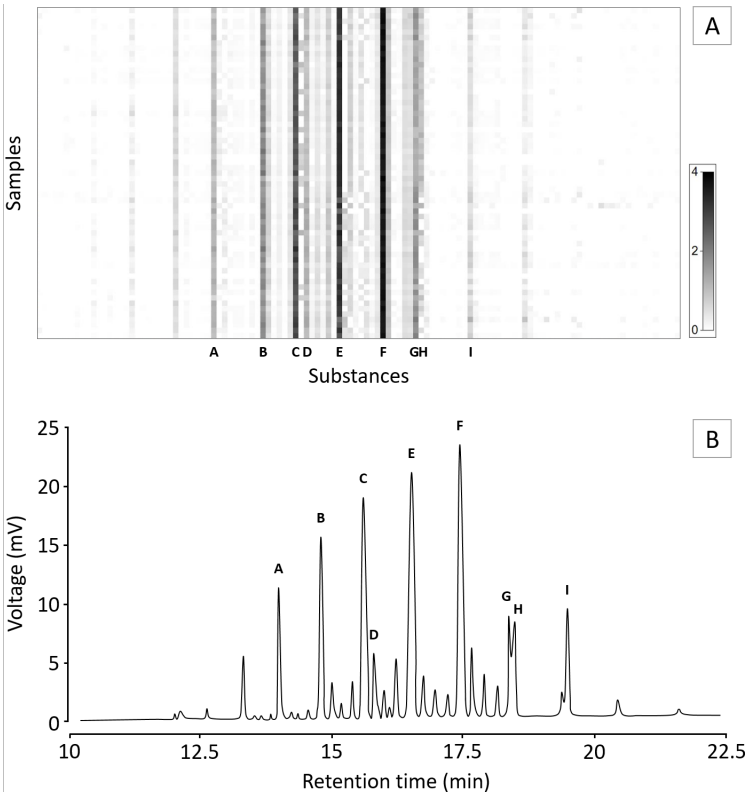

Fig. S1. Chromatographic data from the GC-FID analysis of 77 preen oil samples of pied flycatchers. (a) Shadeplot showing the relative log10-transformed abundance of each substance (columns) in the samples (rows) used for the statistical analysis (only high-concentration samples are shown). (b) Representative chromatogram of the preen oil of a female pied flycatcher sampled during nestling rearing. Letters indicate the nine most abundant substances across all samples. Substance F was the most abundant substance across all samples and we used its abundance as a proxy of overall preen oil concentration in the samples.

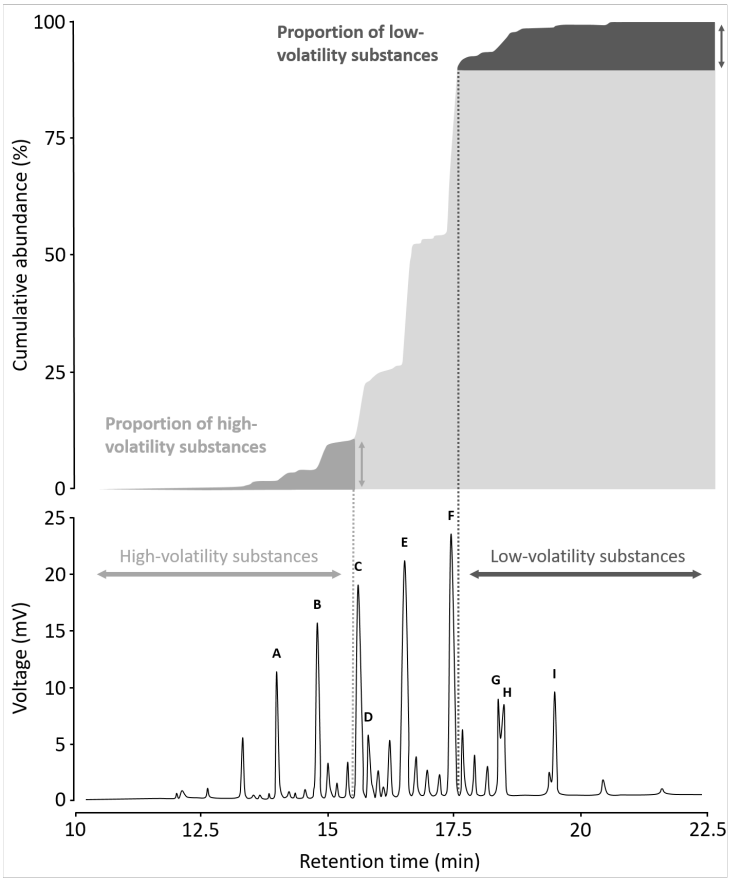

Fig. S2. Calculation of the proportion of high-volatility and low-volatility substances. The proportion of high-volatility substances was measured as the proportion of abundance (i.e. chromatogram area) before peak C (i.e. substances with short retention time). The proportion of low-volatility substances was measured as the proportion of abundance after peak F (i.e. substances with long retention time). These thresholds (before peak C and after peak F) were selected to exclude the abundant central peaks while conserving sufficient portions of the chromatograms at each end. The analysis was repeated with another set of thresholds (earlier threshold for high-volatility substances, before peak B; later threshold for low-volatility substances, after peak H) and yielded similar results (see Table S9).

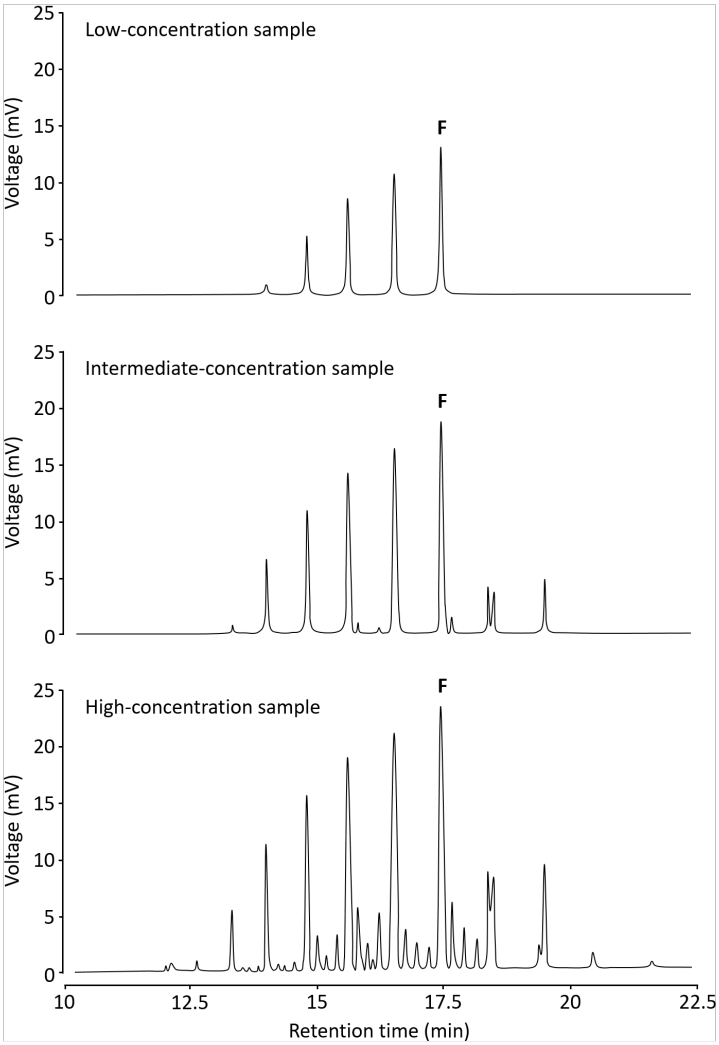

Fig. S3. Concentration bias, illustrated by representative GC–FID chromatograms of three samples with varying overall concentrations of preen oil. Less substances are detected in samples with lower concentration, resulting in reduced chemical richness and diversity. Substance F was the most abundant substance across all samples and we used its abundance as a proxy of overall preen oil concentration in the samples.

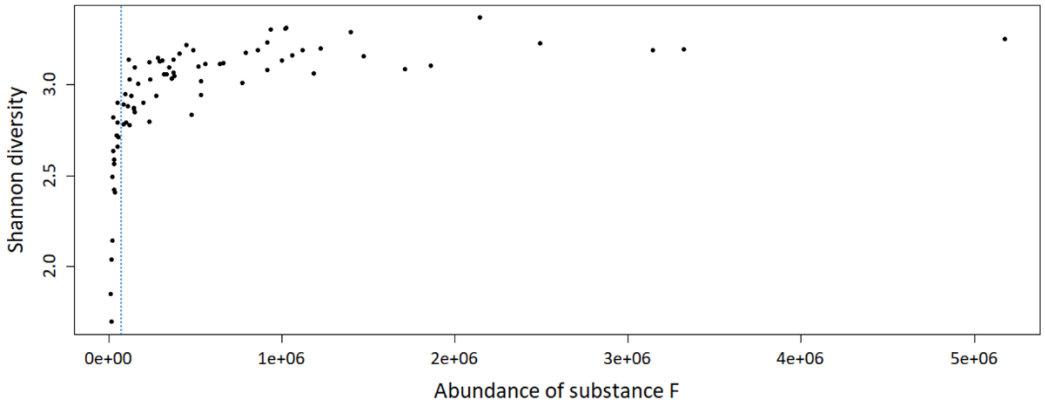

Fig. S4. Chemical diversity (Shannon diversity index) in relation to the overall concentration in preen oil (abundance of substance F), revealing a concentration bias. Each dot represents a sample. Shannon diversity drops sharply below a certain threshold of concentration (abundance of substance F = 70000; indicated by the dashed line). Less substances are detected in low-concentration samples (abundance of substance F < 70000; left of the dashed blue line), so that their chromatogram reflects poorly their real chemical composition and possibly underestimates chemical richness and diversity). Low-concentration samples may introduce noise in our analysis and we therefore discarded samples below the threshold before running an additional analysis (our reduced dataset).

Table S1. Settings used for the integration of chromatographic data using the software GC Solutions (version 2.41) and for the alignment of chromatographic data using the *align_chromatograms* function of the *GCalignR* package in R.

	Parameter	Value
Peak integration	Width	1 sec
	Slope	500 uV/min
	Drift	0 uV/min
	Doubling time (T.DBL)	500 min
	Min. Area/Height	500 counts
Peak alignment	max_linear_shift	0 min
	max_diff_peak2mean	0.025 min
	min_diff_peak2peak	0.05 min

Table S2. Results from permutational multivariate analysis of variance (PERMANOVA) on the preen oil chemical composition of pied flycatchers including the low-concentration samples (complete dataset). **(a)** Effect of sex (fixed effect) within breeding pairs (random effect) sampled during nestling rearing (N = 34 samples from 17 pairs). **(b)** Effect of breeding stage (fixed effect) within individual females (random effect) sampled during both incubation and nestling rearing (N = 24 samples from 12 females).

	df	SS	F (pseudo)	P (perm)	Component of variation
(a) Sex and pair					
Sex	1	156.1	1.45	0.210	1.69
Pair	16	3606.5	2.09	**0.007**	7.67
Residuals	16	1721.7	—	—	10.37
(b) Breeding stage and individual					
Breeding stage	1	748.1	8.25	**0.004**	7.40
Individual	11	1214.1	1.22	0.270	3.16
Residuals	11	997.8	—	—	9.52

Analysis based on Bray-Curtis dissimilarities of log-transformed values. P-values were obtained using 9 999 permutations under a reduced model with type III (partial) sums of square (SS), and are indicated in bold if the effect is significant at $\alpha = 5\%$. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray-Curtis units (i.e. % of Bray-Curtis dissimilarity).

Table S3. Results from permutational multivariate analysis of variance (PERMANOVA) on the preen oil chemical composition of pied flycatchers considering only the nine most abundant substances. **(a)** Effect of sex (fixed effect) within breeding pairs (random effect) sampled during nestling rearing (complete dataset: N = 34 samples from 17 pairs; reduced dataset: N = 22 samples from 11 pairs). **(b)** Effect of breeding stage (fixed effect) within individual females (random effect) sampled during both incubation and nestling rearing (complete dataset: N = 24 samples from 12 females; reduced dataset: N = 16 samples from 8 females). The complete dataset includes all samples, whereas the reduced dataset includes only high-concentration samples.

	Complete dataset					Reduced dataset				
	df	SS	F (pseudo)	P (perm)	Comp. of variation	df	SS	F (pseudo)	P (perm)	Comp. of variation
(a) Sex and pair										
Sex	1	20.5	1.11	0.358	0.34	1	14.5	1.35	0.277	0.34
Pair	16	639.5	2.17	**0.008**	3.28	10	265.7	2.48	**0.034**	2.82
Residuals	16	295.2	—	—	4.30	10	107.1	—	—	3.27
(b) Breeding stage and individual										
Breeding stage	1	189.43	9.61	**0.005**	3.76	1	115.3	5.42	**0.033**	3.43
Individual	11	285.7	1.31	0.271	1.77	7	163.0	1.09	0.439	1.00
Residuals	11	216.7	—	—	4.44	7	149.0	—	—	4.61

Analysis based on Bray-Curtis dissimilarities of log-transformed values. P-values were obtained using 9 999 permutations under a reduced model with type III (partial) sums of square, and are set in bold if the effect is significant at $\alpha = 5\%$. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray-Curtis units (i.e. % of Bray-Curtis dissimilarity).

Table S4. Results of generalized linear mixed models (GLMMs) investigating sex differences within breeding pairs in several chemical aspects of the preen oil of pied flycatchers: richness (number of substances), diversity (Shannon index), proportion of high-volatility substances and proportion of low-volatility substances. N = 24 samples from 12 pairs (12 females and 12 males).

Richness			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Sex (males)	–2.73 [–11.30, 5.80]	0.533	0.01 [0, 0.10]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Pair	241.36 (15.54)	0.013	**0.71 [0.30, 0.92]**
Diversity			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Sex (males)	**–0.08 [–0.15, –0.01]**	0.051	0.07 [0, 0.29]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Pair	0.01 (0.12)	0.011	**0.67 [0.21, 0.91]**
High-volatility			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Sex (males)	**–1.35 [–2.46, –0.20]**	0.029	0.21 [0.01, 0.53]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Pair	0 (0)	1	0 [0, 0.60]
Low-volatility			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Sex (males)	0.03 [–0.83, 0.95]	0.950	0 [0, 0.13]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Pair	1.61 (1.27)	0.037	**0.58 [0.06, 0.87]**

Fixed effects as well as repeatabilities were considered significant (α = 5%) if their 95% confidence interval does not include zero, and are indicated in bold. β [95% CI]: Beta estimate and 95% confidence interval. SD: standard deviation. Repeatability: adjusted repeatability. P-values are indicated but were not used to assess significance. P-values of random effects are based on permutations.

Table S5. Results from permutational multivariate analysis of variance (PERMANOVA) examining the effects of factors on the preen oil chemical composition of pied flycatchers while controlling for the concentration in preen oil (abundance of the most abundant substance). **(a)** Effect of concentration and sex (fixed effects) within breeding pairs (random effect) sampled during nestling rearing (N = 34 samples from 17 pairs using the complete dataset; N = 22 samples from 11 pairs using the reduced dataset). **(b)** Effect of concentration and breeding stage (fixed effects) within individual females (random effect) sampled both during incubation and during nestling rearing (N = 24 samples from 12 females using the complete dataset; N = 16 samples from 8 females using the reduced dataset). The complete dataset includes all samples, whereas the reduced dataset includes only high-concentration samples.

	Complete dataset					Reduced dataset				
	df	SS	F (pseudo)	P (perm)	Comp. of variation	df	SS	F (pseudo)	P (perm)	Comp. of variation
(a) Sex and pair										
Concentration	1	560.2	3.06	**0.033**	3.33	1	189.5	2.05	0.093	2.10
Sex	1	193.3	1.80	0.138	2.25	1	191.0	3.80	**0.027**	3.65
Pair	16	3127.6	1.83	**0.019**	6.83	10	995.6	2.05	**0.034**	5.27
Residuals	15	1603.2	—	—	10.34	9	437.2	—	—	6.97
(b) Breeding stage and individual										
Concentration	1	252.2	2.65	0.055	2.56	1	164.5	1.97	0.145	2.25
Breeding stage	1	727.8	8.32	**0.006**	7.41	1	459.3	6.11	**0.021**	7.05
Individual	11	1107.6	1.15	0.342	2.66	7	635.5	1.21	0.342	2.94
Residuals	10	872.4	—	—	9.34	6	448.9	—	—	8.65

Analysis based on Bray-Curtis dissimilarities of log-transformed values. P-values were obtained using 9 999 permutations under a reduced model with type I (sequential) sums of square, and are set in bold if the effect is significant at $\alpha = 5\%$. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray-Curtis units (i.e. % of Bray-Curtis dissimilarity).

Table S6. Results from permutational multivariate analysis of variance (PERMANOVA) examining sex differences (fixed effect) within breeding pairs (random effect) on the preen oil chemical composition of pied flycatchers sampled during nestling rearing, while controlling for the temporal effects of sampling date (fixed effect) and time of day (fixed effect). N = 34 samples from 17 pairs using the complete dataset; N = 22 samples from 11 pairs using the reduced dataset. The complete dataset includes all samples, whereas the reduced dataset includes only high-concentration samples.

	Complete dataset					Reduced dataset				
	df	**SS**	**F** (pseudo)	**P** (perm)	**Comp. of variation**	**df**	**SS**	**F** (pseudo)	**P** (perm)	**Comp. of variation**
Sampling date	1	439.5	2.10	0.081	2.63	1	187.9	1.93	0.114	2.06
Sampling time	1	351.8	1.70	0.149	2.10	1	193.3	2.00	0.105	2.14
Sex	1	157.8	1.52	0.196	1.78	1	156.7	3.01	**0.045**	3.08
Pair	15	2975.6	1.91	**0.021**	7.11	9	806.4	1.72	0.071	4.59
Residuals	15	1559.5	—	—	10.20	9	469.0	—	—	7.22

Analysis based on Bray-Curtis dissimilarities of log-transformed values. P-values were obtained using 9 999 permutations under a reduced model with type I (sequential) sums of square, and are set in bold if the effect is significant at α = 5%. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray-Curtis units (i.e. % of Bray-Curtis dissimilarity).

Table S7. Results of generalized linear mixed models (GLMMs) investigating the effect of breeding stage within individuals in several chemical aspects of the preen oil of pied flycatchers: richness (number of substances), diversity (Shannon index), proportion of high-volatility substances and proportion of low-volatility substances. N = 16 samples from 8 individual females (8 during incubation, 8 during nestling rearing).

Richness			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Breeding stage (rearing)	1.12 [–18.00, 21.40]	0.913	0 [0, 0.30]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Individual	0 (0)	1	0 [0, 0.68]
Diversity			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Breeding stage (rearing)	0.05 [–0.13, 0.24]	0.556	0.02 [0, 0.36]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Individual	0 (0)	1	0 [0, 0.73]
High-volatility			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Breeding stage (rearing)	–1.12 [–2.72, 0.34]	0.200	0.06 [0, 0.35]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Individual	2.42 (1.55)	0.134	0.49 [0, 0.89]
Low-volatility			
Fixed effect	**β [95% CI]**	**P**	**Marginal R^2 [95% CI]**
Breeding stage (rearing)	**1.84 [0.89, 2.94]**	0.004	0.43 [0.13, 0.74]
Random effect	**Variance (SD)**	**P**	**Repeatability [95% CI]**
Individual	0 (0)	1	0 [0, 0.69]

Fixed effects as well as repeatabilities were considered significant (α = 5%) if their 95% confidence interval does not include zero, and are indicated in bold. β [95% CI]: Beta estimate and 95% confidence interval. SD: standard deviation. Repeatability: adjusted repeatability. P-values are indicated but were not used to assess significance. P-values of random effects are based on permutations.

Table S8. Results of the tests for homogeneity of multivariate dispersions (PERMDISP) to test for differences between sexes and breeding stages in dispersion (i.e. in deviations from centroid) in preen oil chemical composition. Non-significant differences indicate that the groups (females and males, incubation and nestling rearing period) have homogeneous dispersions.

	Complete dataset					Reduced dataset				
	Size	**Mean**	**SE**	**F**	**P (perm)**	**Size**	**Mean**	**SE**	**F**	**P (perm)**
Sex	—	—	—	0.69	0.52	—	—	—	0.35	0.58
Female	17	12.10	1.23	—	—	11	8.59	0.95	—	—
Male	17	10.55	1.40	—	—	11	7.86	0.79	—	—
Breeding stage	—	—	—	3.90	0.08	—	—	—	3.50	0.10
Incubation	12	10.28	1.33	—	—	8	9.68	1.53	—	—
Nestling rearing	12	7.21	0.80	—	—	8	6.61	0.59	—	—

Analysis based on Bray-Curtis dissimilarities of log-transformed values. P-values were obtained using 9 999 permutations and significance was assessed at α = 5%. SE: standard error.

Table S9. Results of generalized linear mixed models (GLMMs) on volatility using the alternative thresholds to measure the proportion of high-volatility and low-volatility substances (see Fig. S2). Results (β estimates and P-values) are consistent with the results obtained using the first threshold (Tables S4 & S7).

(A) Sex and pair		
High-volatility (alternative threshold)		
Fixed effect	**β**	**P**
Sex (males)	**–1.06**	0.004
Random effect	**Variance (SD)**	
Pair	0 (0)	
Low-volatility (alternative threshold)		
Fixed effect	**β**	**P**
Sex (males)	0.20	0.618
Random effect	**Variance (SD)**	
Pair	1.15 (1.07)	
(B) Breeding stage and individual		
High-volatility (alternative threshold)		
Fixed effect	**β**	**P**
Breeding stage (rearing)	0.28	0.351
Random effect	**Variance (SD)**	
Individual	0.72 (0.84)	
Low-volatility (alternative threshold)		
Fixed effect	**β**	**P**
Breeding stage (rearing)	**1.24**	0.018
Random effect	**Variance (SD)**	
Individual	0 (0)	

Fixed effects were considered significant (α = 5%) if their 95% confidence interval does not include zero, and are indicated in bold.

A young pied flycatcher
Photo by V-M. Suhonen

CHAPTER 4

Preen oil composition in pied flycatchers: reproducibility and ontogeny

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ABSTRACT

Preen oil – the secretion from the uropygial gland of birds – may have a variety of functions in reproduction, such as protection against eggshell bacteria, olfactory crypsis against nest predators and olfactory mate choice. To investigate these functions, we should in a first step characterise natural variation in preen oil composition, but also verify that previously described patterns are robust. Replication studies are crucial to test the reproducibility of previous findings, but are rarely undertaken in chemical ecology. We conducted an almost exact replication of an own previous study on the chemical composition of the preen oil of a wild passerine bird, the pied flycatcher (*Ficedula hypoleuca*). We aimed to test the reproducibility of the previous results while following a pre-registered analysis plan and using larger sample sizes. In line with original findings, preen oil composition was similar between pair mates and not repeatable within individual females across breeding stages. The preen oil of females changed across breeding stages (higher richness, diversity and volatility during incubation than during nestling-rearing) more clearly than in the original study, and further refutes a role of preen oil in olfactory crypsis in this species. In contrast to the original study, we found no difference between sexes during nestling-rearing, casting doubt on the proposed role of preen oil as a sex signature in this species. Beyond our replication effort, we explored the ontogeny of preen oil composition. The preen oil of nestlings differed from adults, was more similar to adult males than adult females, but was not more similar to parents than non-parents. We also found breeding pair and family chemical signatures which suggest an influence of the nest environment on preen oil composition. Our study highlights the importance of replication and provides new insights into the function and development of preen oil.

INTRODUCTION

The uropygial gland (or preen gland) is the main secretory gland of the skin of birds, producing a waxy secretion (or preen oil) which birds spread onto their plumage during preening (Jacob and Ziswiler 1982). Preen oil consists of a complex cocktail of chemicals that varies in composition within and between individuals (Grieves et al. 2022). The adaptive function of preen oil has been extensively studied but remains debated (reviewed in Moreno-Rueda 2017). Preen oil appears to be a multifunctional secretion, serving notably plumage maintenance and waterproofing (Giraudeau et al. 2010), chemical protection against ectoparasites (Alt et al. 2020), olfactory crypsis against nest predators (Reneerkens et al. 2005) and olfactory intraspecific communication (Grieves et al. 2022). The function(s) of preen oil likely depend on the species, but also on the season (Grieves et al. 2022). Indeed, at the onset of the breeding period, the chemical composition of preen oil changes and the amount of preen oil secreted increases in many species (reviewed in Whittaker and Hagelin 2021 and Grieves et al. 2022). This suggests that, in addition to its year-round role in plumage maintenance, preen oil serves specific roles in reproduction. Three main hypotheses for the reproductive function of preen oil have been proposed. First, the preen oil may serve chemical protection against ectoparasites during breeding. For example, the preen oil from incubating female Eurasian hoopoes (*Upupa Epops*) has antimicrobial properties which may protect the eggs from eggshell bacteria (Martín-Vivaldi et al. 2009, 2010, 2014). Second, preen oil may serve olfactory crypsis during breeding. For instance, in several ground-nesting shorebirds, the preen oil of incubating birds becomes less odorous during breeding, which may reduce the detectability of the clutch or incubating parent(s) by olfactorily-searching nest predators (Reneerkens et al. 2005, 2007a). Third, preen oil has been hypothesised to play a role in intraspecific olfactory communication, such as sex signalling during mate choice (Whittaker and Hagelin 2021, Grieves et al. 2022a). For example, in two passerine species (dark-eyed juncos *Junco hyemalis* and song sparrows *Melospiza melodia*), the preen oil of females and males undergo different chemical changes before breeding. This leads to sex differences in preen oil composition during the breeding season, which allow birds to discriminate between sexes by smell (Whittaker et al. 2010, 2011a, Grieves et al. 2019a, b). The two latter hypotheses are based on the odoriferous nature of preen oil. Indeed, preen oil is a major source of avian body odour (Alves Soares et al. 2024a).

Before experimentally testing hypothetical functions of preen oil, it is important to describe the natural variation in its chemical composition (i.e. “chemical fingerprint” or “chemical profile”) within and among individuals. For example, the existence of sex differences in chemical fingerprints should be established before testing for olfactory sex discrimination (Grieves et al. 2019a, b). In addition, the detected chemical patterns should ideally be corroborated by replication studies to verify their robustness (i.e. their reproducibility). Dedicated replication studies are extremely valuable but still very rare in the fields of ecology and evolution (Nakagawa and Parker 2015, Kelly 2019), especially exact (or close) replication studies (i.e. with a high degree of fidelity to the original study

protocol). Studies on chemical fingerprints are particularly difficult to replicate, given the complexity of the methodology of chemical extraction and analysis, as well as processing and statistical analysis of the chromatographic data (Tebbe et al. 2020, Alves Soares et al. 2024a). Even when using the same methodology, studies conducted across several years commonly report strong among-year differences, which may be due to fluctuations in environmental conditions (e.g. weather, food availability) or differences in protocols like the preservation of the samples (Mardon et al. 2010). Besides, in the few replicated studies in this field, results are rarely reproducible (Wyatt 2015). Indeed, studies on chemical fingerprints often search for subtle effects based on relatively small sample sizes, increasing the risk of false positives or exaggerated effect sizes (Wyatt 2015). In recent years, studies on preen oil composition have accumulated and revealed differences between sexes, seasons, life stages and individuals (reviewed in Grieves et al. 2022 and Alves Soares et al. 2023), but also effects of diet (Thomas et al. 2010), food stress (Grieves et al. 2020), hormones (Whittaker et al. 2018), parasitic infection (Talbott et al. 2022), microbiota (Whittaker et al. 2019b) or major histocompatibility complex (MHC) genotype (Leclaire et al. 2014). Yet, only a handful of these studies have been replicated and successfully reproduced the original findings. For example, in the preen oil of song sparrows, sex differences were found repeatedly across populations and in both wild and captive individuals (Grieves et al. 2018, 2019a, b, 2020). Similarly, in this species, the covariation of preen oil composition with MHC genotype was reproducible (Slade et al. 2016, Grieves et al. 2019c, 2021a). Sex and seasonal differences in preen oil were also reproduced in six shorebird species (Reneerkens et al. 2002, 2007a, b), dark-eyed juncos (Soini et al. 2007, Whittaker et al. 2010, 2011b, 2013) and white-throated sparrows (*Zonotrichia albicollis*) (Tuttle et al. 2014, Forrette 2018). However, in the two latter species, only part of the chemical patterns were reproducible. For example, the sex differences in the preen oil of dark-eyed juncos reported in Whittaker et al. (2010, 2013) were not reproduced in (Whittaker et al. 2016). Similarly, the sex differences in the preen oil of white-throated sparrows in captivity (Tuttle et al. 2014) were mostly not reproducible in the wild (Forrette 2018).

In a previous study on the chemical composition of the preen oil of wild European pied flycatchers (*Ficedula hypoleuca*), we detected interesting patterns of natural variation (Gilles et al. 2024). Analysing samples from eleven breeding pairs during the period of nestling-rearing, we found a high similarity between pair mates and subtle differences between sexes, notably a higher volatility and a slightly higher diversity in females than in males (Gilles et al. 2024). In the preen oil from eight females that were sampled repeatedly during both incubation and nestling-rearing, we found no repeatability within individuals but changes across breeding stages, such as an increased volatility (lower proportion of low-volatility compounds) during incubation (Gilles et al. 2024). From these patterns, we speculated on the possible function of preen oil in this species. Observing sex differences during breeding, we hypothesised that pied flycatchers may use preen oil for olfactory sex signalling during mate choice ("sex semiochemical hypothesis", Grieves et al. 2022).

Further, the increased volatility observed during incubation in females suggested that preen oil does not play a role in olfactory crypsis at the nest in this species (Grieves et al. 2022). However, these results were based on relatively small sample sizes, and we should thus refrain from speculating further or from designing experiments on the possible function underlying these patterns. Instead, we need to verify that the patterns are reproducible.

In this study, we therefore conducted a close replication of Gilles et al. (2024). We returned to the study site one year later and collected preen oil samples from the same population of pied flycatchers. We used the same methodology for the sampling, storage and extraction of the preen oil, as well as for the processing and statistical analysis of the chromatographic data, which we pre-registered (Jeanjean et al. 2023, https://osf.io/tbcug). Pre-registered analyses increase the trustworthiness of results by limiting practices such as cherry-picking, p-hacking and HARKing (Fraser et al. 2018). The only methodological difference compared to the original study was the use of gas chromatography-mass spectrometry (GC-MS) instead of gas chromatography-mass spectrometry with flame ionisation detector (GC-FID) for the chemical analysis. For this reason, our study is not an exact but a close replication of Gilles et al. (2024) (Nakagawa and Parker 2015). Sample sizes were much larger than in the original study, with samples from 46 breeding pairs during nestling-rearing (compared to 11 in the original study) and from 29 females repeatedly sampled across breeding stages (compared to 8 in the original study). Like in the original study, we measured the alpha diversity (richness and Shannon diversity), volatility, and beta diversity (Bray-Curtis dissimilarity) in preen oil composition, and tested for sex differences and similarity between pair mates during nestling-rearing (N = 46 pairs), and for changes across breeding stages and individual signatures in females sampled both during incubation and nestling-rearing (N = 29 females). We expected to reproduce the results from the original study, i.e. sex differences (with higher volatility and diversity in females) and similarity between pair mates during nestling-rearing, and changes across breeding stages (with higher volatility during incubation) and no individual signature in females.

In addition to this replication, we explored proximate causes of variation in preen oil composition. First, we tested whether the similarity observed between pair mates is caused by their spatial proximity (same territory including potentially a similar diet) as suggested in the original study. Second, we investigated the ontogeny of preen oil composition in pied flycatchers, by sampling preen oil from nestlings (N = 69) and adults (N = 31) from 16 families during the nestling-rearing period. We predicted to find differences between life stages, as reported in other species (reviewed in Alves Soares et al. 2023), which can reflect differences in physiology (e.g. related to reproduction). We also predicted that nestlings would secrete a preen oil more similar to their mother than to their (social) father, as females spend more time brooding, potentially transferring more preen oil substances, or microbiota affecting preen oil substances, to the nestlings (Whittaker et al. 2016). Finally, we predicted to find family signatures (i.e. higher similarity between family members than with other individuals), as found in another passerine species

(dark-eyed juncos, Whittaker et al. 2016), which would indicate an effect of the rearing environment and/or genetics on the development of preen oil composition.

MATERIALS AND METHODS

Sampling

The sampling methods were the same, and were performed by the same person, as in Gilles et al. (2024), except that we sampled preen oil from nestlings in addition to adults. Fieldwork took place between May 1st and June 8th 2020, during the breeding season of pied flycatchers, in a nestbox population based in a lowland mixed coniferous forest near Elbergen in NW Germany (52°27' N, 7°15' E; for details on the study site see Altenkirch and Winkel 1991). The GPS position of the nestboxes was recorded as Northing and Easting coordinates. Adult females and males were sampled during both the incubation (6.1 days ± SD 3.6 before hatching) and the nestling-rearing period (10.6 days ± SD 2.6 after hatching). Nestlings from 16 broods (5.7 nestlings ± SD 1.0 per brood) were sampled at 12.2 days (± SD 1.4) after hatching on average. In total, 249 preen oil samples were taken (161 from adults during both incubation and nestling-rearing, and 88 from nestlings). Birds were caught directly in their nestbox, using custom-made wire swing traps. To capture males during incubation, we used mist-nets placed around the nestbox, as they do not enter the nestbox during this period. We sampled preen oil by gently rubbing a cotton swab on the preen gland of the bird during 5 s, and immediately placed the cotton swab in a 20 mL screw neck glass vial. In addition, 17 field blanks were taken by agitating a cotton swab in the air, to control for environment contamination of the preen oil samples. Samples were stored at -20 °C in the evenings of sampling days, and at -40 °C at the end of the field season pending chemical analysis. Bird capture and sampling were carried out with licence no. DEW-0772 issued to Tim Schmoll by 'Vogelwarte Helgoland' (Wilhelmshaven, Germany).

Chemical analysis

The preparation of the samples prior chemical analysis was the same, and were performed by the same person, as in Gilles et al. (2024). Samples were defrosted before sample preparation. We injected dichloromethane into the cotton swab, which was then squeezed out using a 100 µl blunt point glass syringe to extract the preen oil (and dichloromethane). Extracts were then concentrated by evaporation (for 10-30 min) to a volume of approximately 5 µl at ambient temperature, under hood, in 2 ml glass vials equipped with a 100 µl glass inlet. For the chemical analysis, we deviated from the methodology of the original study, as we used GC-MS instead of GC-FID, and helium instead of hydrogen as a carrier gas. This is because our laboratory transitioned from GC-FID to GC-MS analyses. The main difference is that the mass spectrometry (MS) of GC-MS allows for the identification of compounds in addition to the retention time, whereas GC-FID only provides retention times. However, in our case the identification of substances (using the

National Institute of Standards and Technology library) was not certain enough to be used in the study, but we used it to improve our confidence during manual adjustments of the data (see next section). We used a GC-MS model GC2030-QP2020NX (Shimadzu) with a VF-5ms capillary column (30 m x 0.25 mm ID, DF 0.25, 10 m guard column, Varian Inc., Lake Forest, USA) and helium (at a 1 ml/min flow rate) as a carrier gas. The GC temperature was first set at 60 °C for 3 minutes, and then increased at 10 °C/min to reach the final temperature of 280 °C, kept for 20 min. In addition to the preen oil samples and the field blanks, 33 GC blanks (containing dichloromethane only) were added to the analysis to control for instrument contamination of the preen oil samples.

Processing of chemical data

We processed the chemical data following Gilles et al. (2024). From the chromatograms, we extracted the retention time of each peak (i.e. substance) and its abundance (area under the peak) using GC Solutions v2.41 (see settings **Table S1**). When the chromatogram appeared to carry either too much noise or no preen oil substances, samples were excluded from the analysis (N = 23 samples). We also discarded two samples for which information on breeding stage was missing, and six samples from individuals that were sampled twice during nestling-rearing (in that case we kept the second sample, the first one being too close to the hatching date). Using the 218 remaining samples, we aligned the retention times with the *GCalignR* package (Ottensmann et al. 2018) in the R software v4.2.0 (R Core Team 2022) to associate each substance with a single retention time across all samples (**Table S1**). To make sure that major environmental and instrument substances were not analysed as preen oil substances, the substances present in field blanks and/or GC blanks were excluded during the alignment. We also performed manual adjustments to improve the *GCalignR* alignment, using shade plots in the PRIMER software v7.0.21 (Clarke and Gorley 2015) for visualisation (**Fig. S1**). Blank substances with retention times closely resembling those of preen oil substances were manually removed, after verification of their distinctiveness using the MS identification.

Because cotton swabs were used for sampling preen oil, the exact quantity of preen oil collected in each sample was unknown. We thus used relative abundances (area under the peak divided by the total area under the chromatogram) instead of absolute abundances of each substance in our analyses. This means that we were not able to study the variation in the absolute abundance of preen oil substances, although it could be important (Whittaker and Hagelin 2021). To attenuate the disproportionate influence of high-abundance substances compared to low-abundance substances, the chemical data were log-transformed (log(X+1)) prior to analysis (following Clarke et al. 2014). In samples containing little preen oil (i.e. with a relatively small total area under the chromatogram), low-abundance substances may not be detected ("concentration bias" identified in Gilles et al. 2024). To make sure that the concentration of the sample would not affect our analyses, we plotted the Shannon diversity in relation with the mean concentration of preen oil (the total area under the chromatogram divided by the number of substances recorded

in the sample) in each sample (**Fig. S2**). The plot shows that there is indeed a positive relationship between concentration and chemical diversity, but there was no obvious concentration threshold below which Shannon diversity dropped (i.e. no concentration threshold), in contrast to Gilles et al. (2024). Thus, we decided to keep all of the samples in the analysis.

To further analyse the variation in preen oil composition, we took measures of alpha diversity for each sample, namely chemical richness (number of substances) and diversity (Shannon index). We also measured the volatility of each sample i.e. the proportion of highly volatile compounds (total area under the chromatogram before peak C, retention time 10.12 min, **Fig. S1**) as in Gilles et al. (2024). Unlike the original study however, we decided not to use the proportion of low-volatility substances as a proxy of volatility, because we are uncertain whether the presence of low-volatility substances imply a lower volatility. Indeed, low-volatility substances may break into more volatile compounds and take part in body odour once applied on the plumage (Mardon et al. 2011a, Maraci et al. 2018).

Statistical analysis

The statistical analyses were conducted in accordance with the pre-registration (Jeanjean et al. 2023, https://osf.io/tbcug*)*. The data and code used in the analyses are available on Github (https://github.com/marc-gilles/preen-oil-replication-ontogeny).

Replication

Following Gilles et al. (2024), we tested the effect of sex and pair identity using samples from complete breeding pairs (i.e. where both the male and female were successfully sampled) during nestling-rearing (N = 92 samples from 46 pairs), and the effect of breeding stage and individual identity using samples from females with repeated samples (i.e. sampled successfully during both incubation and nestling-rearing) (N = 58 samples from 29 females). Because permutational analysis of variance (PERMANOVA) can be sensitive to differences in dispersion under unbalanced designs (Anderson et al. 2008), we made sure that our designs were perfectly balanced. Note that the sample sizes for the latter analysis are slightly lower than in the pre-registration (repeated samples for 29 females instead of 33), because we discarded four samples from incubating females that were identified as clear outliers.

We first investigated the effect of sex (fixed effect) and pair identity (random effect), and of breeding stage (fixed effect) and individual identity (random effect), on the beta diversity (overall composition) of preen oil composition using PERMANOVA (Anderson et al. 2008, Anderson 2014) on Bray-Curtis dissimilarities (Borcard et al. 2011) in PRIMER v7.0.21 (Clarke and Gorley 2015). In addition, we used non-metric multidimensional scaling (NMDS) on Bray-Curtis dissimilarities for visualisation (Borcard et al. 2011). P-values for the PERMANOVAs were obtained using 9 999 permutations

under a reduced model with type III (partial) sums of square (SS), and considered significant when below $\alpha = 0.05$. In addition to the statistical analyses outlined in the pre-registration, we performed dispersion tests (PERMDISP) using PRIMER. Given that a significant result in PERMANOVA may indicate differences in both location and dispersion between groups, conducting a PERMDISP enables us to account for variations in dispersion across sexes and breeding stages.

We then studied the same effects (one model with sex (fixed) and pair identity (random) and one model with breeding stage (fixed) and individual identity (random)) on the chemical richness, Shannon diversity and volatility of preen oil using linear mixed models (LMM) with Gaussian distributions, using the *lme4* package (Bates et al. 2014) in R v4.2.0 (R Core Team 2022). We assessed the significance of fixed effects by checking whether the 95% confidence interval of the beta estimates contained 0 using the *broom.mixed* package (Bolker et al. 2022), and also checked P-values using the *lmerTest* package (Kuznetsova et al. 2015). The significance of random effects was evaluated by checking whether the 95% confidence intervals of the repeatability estimates contained 0, and by checking the P-value based on permutations, using the *rptR* package (Stoffel et al. 2017). In addition, we measured the variance explained (marginal R^2) by each fixed effect using the *partR2* package (Stoffel et al. 2021). We verified the assumptions for LMMs using the *performance* package (Lüdecke et al. 2021).

To compare the results from the replication study and the original study, we calculated effect sizes for the LMM analyses (chemical richness, Shannon diversity and volatility). For fixed effects, we calculated a corrected version of the standardised effect size Cohen's d (Cohen 2013) for small sample sizes, the Hedges' g (Hedges and Olkin 2014) and its 95% confidence interval, using the *effsize* package (Torchiano and Torchiano 2020) in R. We used the repeatabilities with their confidence interval as effect sizes for random effects (Stoffel et al. 2017). To drive our inference on whether the results of the original study were reproduced (replication success), for each effect studied, we answered two questions (Valentine et al. 2011). **(1)** Is the effect significant or non significant in both studies, meaning is the p-value under or above the significance threshold of 0.05 in both studies? **(2)** Does the effect size estimate of the replication study fall into the confidence interval of the effect size estimate of the original study? For the PERMANOVAs, we could not calculate confidence intervals of effect sizes, and thus could not answer question **(2)**.

Exploratory analyses

In addition to the replication study, we conducted exploratory analyses, as mentioned in the pre-registration (Jeanjean et al. 2023).

Gilles et al. (2024) found a high similarity in preen oil composition between breeding partners and proposed that this may be due to their spatial proximity, as they share the same territory and the same food available. To test for the effect of spatial proximity on preen oil composition, we ran Mantel tests of the spatial versus the Bray-Curtis distance,

along with Mantel correlograms (Borcard et al. 2011) and scatterplots for visualisation, using the *vegan* package (Oksanen et al. 2019) in R. This method tests whether chemical similarity covaries with spatial proximity by comparing pairwise chemical distances with pairwise spatial distances. We used all the samples from adult males and females during nestling-rearing for which we had the GPS position of the nestbox (regardless of whether they were part of a complete breeding pair). We tested males (N = 42) and females (N = 44) separately to control for the effect of breeding partner proximity.

To explore the ontogeny of preen oil composition, we tested whether preen oil composition differs between nestlings and adults, and whether it contains (social) family signatures (i.e. high similarity between members of the same nest). We used samples from 16 broods (100 samples, 31 from adults and 69 from nestlings) collected during nestling-rearing, and employed the same analytical method as for the replication analysis. We tested the effect of life stage (fixed effect) and family identity (random effect) on beta diversity (Bray-Curtis dissimilarities) using PERMANOVA, PERMDISP and NMDS, and on chemical richness, Shannon diversity and volatility using LMMs. In addition, we tested whether the preen oil of nestlings is more similar to that of their mother or father (social father as we did not control for extra-pair paternity, but hereafter simply referred to as "father"), to that of adult females or males (other than their mother and father), and to that of their parents or other nestling-rearing adults. First, we extracted the pairwise Bray-Curtis similarity for each nestling-adult pair from the Bray-Curtis matrix, and separated them in pairs of nestling-mother, nestling-father, nestling-adult female and nestling-adult male (Raulo et al. 2021). We could then study the effect of adult and parent sex, as well as the effect of being the mother/father (fixed effect) on the similarity between samples, while controlling for the effect of family identity (random effect) and nestling identity (random effect nested within family identity). As Bray-Curtis similarity data ranges between 0 and 1, we used generalised linear mixed models (GLMM) with beta distribution using the *glmmTMB* package (Magnusson et al. 2017) in R.

As indicated in the pre-registration, we initially intended to test for the interaction between sex and breeding stage, and to test whether pair similarity changes across breeding stages. However, with only seven samples from males during incubation, we were not able to obtain meaningful results and did not run this analysis.

RESULTS

On the 218 samples retained for alignment, a total of 88 substances were detected, with an average of 24 substances per sample (SD = 7).

Sex differences

We found no sex difference in overall preen oil composition (Bray-Curtis dissimilarities) during nestling-rearing (PERMANOVA; $P = 0.17$, component of variation = 0.01, **Table 1**, **Fig. 1a**), despite the fact that this effect was significant in the original study. The dispersion

test however indicates that there is a slightly greater dissimilarity in preen oil composition among males than among females (PERMDISP; difference in mean distance to centroid = 2.99; $P = 0.013$). We also found no effect of sex on chemical richness (LMM; males: β = 1.09 [95% CI: –1.60, 3.38]), $P = 0.36$, marginal $R^2 = 0.01$), diversity (LMM; males: β = 0.04 [–0.06, 0.14]), $P = 0.45$, marginal $R^2 = 0.01$) and volatility (LMM; males: β = –0.36 [–0.77 ; –0.004], $P = 0.07$, marginal $R^2 = 0.04$) (**Table 2**, **Fig. S3**). In the original study, the effect of sex was either non-significant or marginally significant on richness and diversity, but significant on volatility (**Table 2**, **Fig. 2**). Note that the effect size estimates of the replication were all included in the 95% confidence intervals of the effect size estimates of the original study (**Fig. 2**).

Similarity between breeding partners

Pied flycatchers had a preen oil slightly more similar to that of their breeding partner than to that of other nestling-rearing individuals (PERMANOVA; $P = 0.05$, component of variation = 0.05; **Table 1**, **Fig. 1b**), corroborating the result from the original study. However, breeding partners did not show a similar preen oil in terms of chemical richness (LMM; repeatability = 0.06 (95%CI: [0, 0.33]), $P = 0.34$), diversity (LMM; repeatability = 0 [0, 0.29], $P = 1$) or volatility (LMM; repeatability = 0.01 [0, 0.32], $P = 0.51$) (**Table 2**). In the original study, the effect of pair identity was significant on chemical richness and

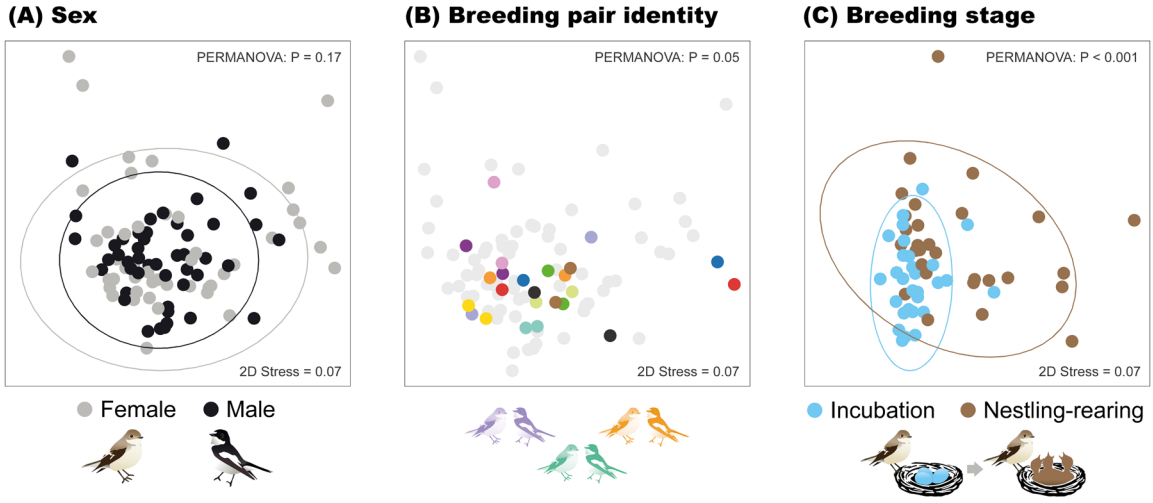

Fig. 1. Differences in overall preen oil composition between sexes, among breeding pairs and between breeding stages in pied flycatchers. Non-metric multidimensional scaling (NMDS) plots represent Bray–Curtis dissimilarities. Each circle represents a preen oil sample and ellipses the 95% confidence intervals for each group assuming a multivariate t-distribution. P-values were calculated from PERMANOVAs. 2D Stress is a measure of the fit between the distance among samples in the plot (i.e. in two-dimensional space) and the actual distance among samples in multivariate space, with values below 0.1 indicating a great fit. **(A)** Difference between sexes (N = 92 samples from 46 females and 46 males during nestling rearing). **(B)** Similarity between pair mates (N = 92 samples from 46 pairs during nestling rearing). Circles of the same colour represent samples from pair mates (only a random selection of 12 pairs is coloured for illustration purposes, while the remaining 34 pairs are greyed out). **(C)** Difference between breeding stages (N = 58 repeated samples from 29 individual females).

Table 1. Results from PERMANOVAs on the overall preen oil chemical composition (Bray-Curtis dissimilarities) of pied flycatchers from both the original and the replication study. 1) Effect of sex (fixed effect) within breeding pairs (random effect) sampled during nestling-rearing ($N_{original}$ = 22 samples from 11 pairs; $N_{replication}$ = 92 samples from 46 pairs). **2)** Effect of breeding stage (fixed effect) within individual females (random effect) sampled during both the incubation and nestling-rearing ($N_{original}$ = 16 samples from 8 females; $N_{replication}$ = 58 samples from 29 females).

	Original study				Replication			
	SS	$F_{(pseudo)}$	$P_{(perm)}$	Comp. variation	SS	$F_{(pseudo)}$	$P_{(perm)}$	Comp. variation
1) Effect of sex and breeding pair identity								
Sex	156.7	3.23	**0.035**	3.14	182.5	1.67	0.173	1.26
Breeding pair	1172.0	2.42	**0.006**	5.86	6831.6	1.39	**0.050**	4.63
Residuals	484.6	-	-	6.96	4904.1	-	-	10.44
2) Effect of breeding stage and individual identity								
Breeding stage	458.0	5.74	**0.014**	6.88	1124.5	9.44	**< 0.001**	5.89
Individual	691.6	1.24	0.293	3.08	3951.5	1.18	0.215	3.32
Residuals	558.5	-	-	8.93	3335.8	-	-	10.90

Analysis based on Bray-Curtis dissimilarities of log-transformed relative abundances. P-values were obtained using 9 999 permutations under a reduced model with type III (partial) sums of square (SS), and are indicated in bold if the effect is significant at α = 5%. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray-Curtis units (i.e. % of Bray-Curtis dissimilarity).

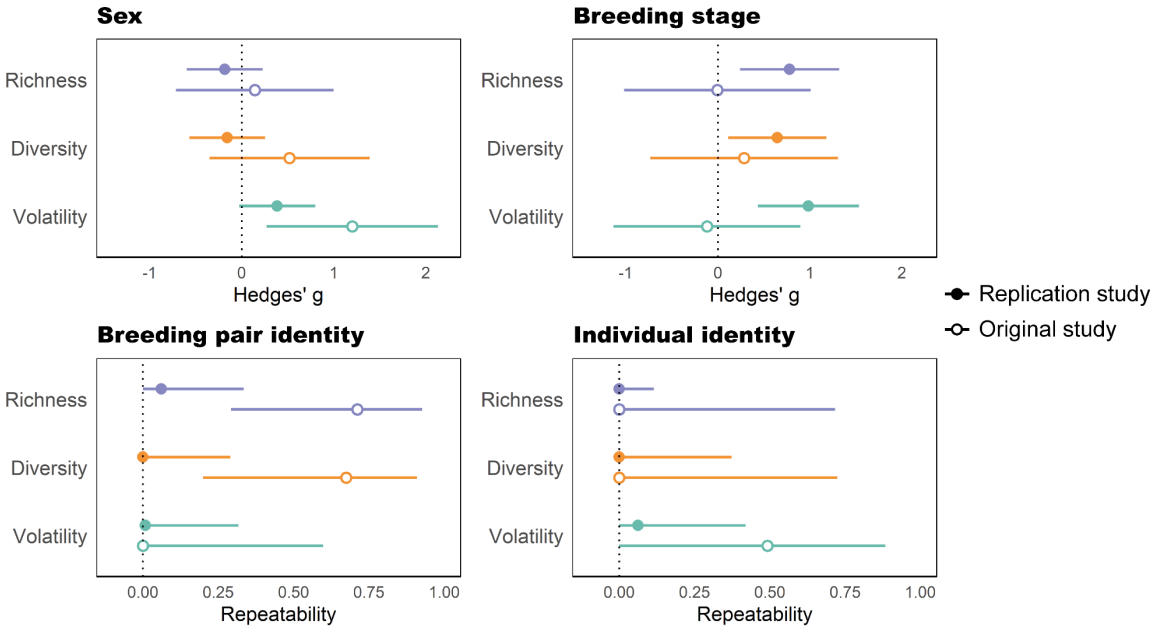

Fig. 2. Reproducibility of sex and breeding stage differences as well as pair and individual signatures in preen oil composition (chemical richness, chemical diversity and volatility). Circles represent effect sizes (Hedges' g for fixed effects of sex and breeding stage, repeatability for random effect variances of breeding pair and individual identity) obtained from linear mixed effects models in the replication (filled circles) and the original study (empty circles). Whiskers represent 95% confidence intervals of the effect sizes.

diversity, but not on volatility (**Table 2**, **Fig. 2**). The effect size from the replication study was only included in the confidence interval of the effect size estimate of the original study for the effect on volatility (**Fig. 2**). The Mantel tests and Mantel correlograms (**Fig. S4**) indicated no correlation between the chemical distance (Bray-Curtis dissimilarity) in preen oil composition and the spatial distance between nestboxes, either in females (r = –0.01, P = 0.5) or males (r = 0.03, P = 0.3).

Changes across breeding stages

Females differed in the overall preen oil composition during incubation and nestling-rearing (PERMANOVA; P < 0.001, component of variation = 0.059; **Table 1**, **Fig. 1c**). Furthermore females differed in dispersion (greater dissimilarity among nestling-rearing females than among incubating females: PERMDISP; difference in mean distance to centroid = 4.48; P = 0.002). The effect of breeding stage on the overall preen oil composition was significant in both studies (along with the difference in dispersion). We also detected a higher chemical richness (LMM; nestling-rearing: β = –4.90 [–8.25, –1.70], P = 0.004, marginal R^2 = 0.14), diversity (LMM; nestling-rearing: β = –0.15 [–0.27, –0.05], P = 0.016, marginal R^2 = 0.1) and volatility (LMM; nestling-rearing: β = –1.34 [–2.01, –0.68], P < 0.001, marginal R^2 = 0.20) during incubation than nestling-rearing (**Table 2**, **Fig. 3**). In contrast, the effects of breeding stage on chemical richness, diversity and volatility

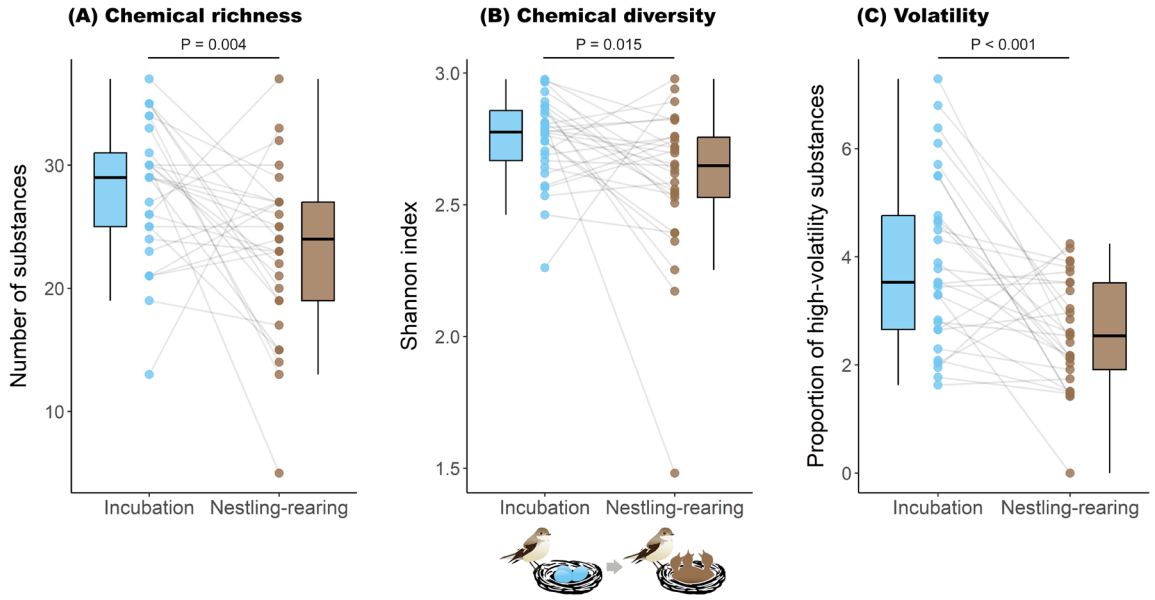

Fig. 3. Changes in preen oil composition between breeding stages within individual female pied flycatchers. (A) Chemical richness (number of substances). **(B)** Chemical diversity (Shannon index). **(C)** Volatility (proportion of high-volatility substances). N = 58 repeated samples from 29 individual females. P-values were calculated from linear mixed effects models. Each point represents a preen oil sample. Lines connect the repeated samples of an individual.

Table 2. Results of GLMMs on the chemcial richnees (nuber of substances), diversity (Shannon index) and volatility (proportion of high-volatility substances) from both the original and the replication study. 1) Sex differences within breeding pairs (N*original* = 22 samples from 11 pairs; N*replication* = 92 samples from 46 pairs). **2)** breeding stage differences within individual females (N*original* = 16 samples from 8 females; N*replication* = 58 samples from 29 females).

	Original study			Replication		
1) Effect of sex and breeding pair identity						
Richness						
Fixed effect	**β [95% CI]**	**P**	**R^2 [95% CI]**	**β [95% CI]**	**P**	**R^2 [95% CI]**
Sex	-2.73 [-11.30, 5.8]	0.53	0.01 [0,0.10]	1.09 [-1.60, 3.38]	0.36	0.01 [0,0.08]
Random effect	**Variance (SD)**	**P**	**Rep [95% CI]**	**Variance (SD)**	**P**	**Rep [95% CI]**
Breeding pair	**241.36 (15.54)**	**0.01**	0.71 [0.30,0.92]	2.12 (1.46)	0.34	0.06 [0,0.33]
Diversity						
Fixed effect	**β [95% CI]**	**P**	**R^2 [95% CI]**	**β [95% CI]**	**P**	**R^2 [95% CI]**
Sex	**-0.08 [-0.15, -0.01]**	**0.05**	0.07 [0, 0.29]	0.04 [-0.06, 0.14]	0.45	0.01 [0,0.08]
Random effect	**Variance (SD)**	**P**	**Rep [95% CI]**	**Variance (SD)**	**P**	**Rep [95% CI]**
Breeding pair	**0.01 (0.12)**	**0.01**	0.67 [0.21, 0.91]	0 (0)	1	0 [0,0.29]
Volatility						
Fixed effect	**β [95% CI]**	**P**	**R^2 [95% CI]**	**β [95% CI]**	**P**	**R^2 [95% CI]**
Sex	**-1.35 [-2.46, -2.20]**	**0.03**	0.21 [0.01, 0.53]	-0.36 [-0.77,-0.004]	0.07	0.04 [0, 0.14]
Random effect	**Variance (SD)**	**P**	**Rep [95% CI]**	**Variance (SD)**	**P**	**Rep [95% CI]**
Breeding pair	0 (0)	1	0 [0, 0.60]	0 [0, 0.60]	0.51	0.01 [0,0.32]
2) Effect of breeding stage and individual identity						
Richness						
Fixed effect	**β [95% CI]**	**P**	**R^2 [95% CI]**	**β [95% CI]**	**P**	**R^2 [95% CI]**
Breeding stage	1.12 [-18.00, 21.40]	0.91	0 [0, 0.30]	**-4.90 [-8.25, -1.70]**	**0.004**	0.14 [0.02, 0.31]
Random effect	**Variance (SD)**	**P**	**Rep [95% CI]**	**Variance (SD)**	**P**	**Rep [95% CI]**
Individual	0 (0)	1	0 [0,0.68]	0 (0)	0.38	0 [0, 0.38]
Diversity						
Fixed effect	**β [95% CI]**	**P**	**R^2 [95% CI]**	**β [95% CI]**	**P**	**R^2 [95% CI]**
Breeding stage	0.05 [-0.13,0.24]	0.56	0.02 [0,0.36]	**-0.15 [-0.27, -0.05]**	**0.016**	0.1 [0.01, 0.26]
Random effect	**Variance (SD)**	**P**	**Rep [95% CI]**	**Variance (SD)**	**P**	**Rep [95% CI]**
Individual	0 (0)	1	0 [0,0.73]	0 (0)	1	0 [0,0.37]
Volatility						
Fixed effect	**β [95% CI]**	**P**	**R^2 [95% CI]**	**β [95% CI]**	**P**	**R^2 [95% CI]**
Breeding stage	-1.12 [-2.72,0.34]	0.20	0.06 [0,0.35]	**-1.34 [-2.01, -0.68]**	**< 0.001**	0.20 [0.06, 0.39]
Random effect	**Variance (SD)**	**P**	**Rep [95% CI]**	**Variance (SD)**	**P**	**Rep [95% CI]**
Individual	2.42 (1.55)	0.13	0.49 [0,0.89]	0.11 (0.34)	0.41	0.06 [0,0.42]

Effect of sex (males) and breeding stage (nestling-rearing). R^2: marginal R^2. Rep.: repeatability. Effects that are significant at α = 5% are indicated in bold.

were never significant in the original study (**Table 2**, **Fig. 2**). The effect sizes from the study for the effect of breeding stage on both chemical richness and diversity (though marginally for chemical richness), but not for the effect on volatility (**Fig. 2**).

Individual signatures

We found no individual chemical signature in overall preen oil composition in females across breeding stages (PERMANOVA; $P = 0.22$, component of variation = 0.03; **Table 1**), as in the original study. We also found no repeatability in chemical richness (LMM; repeatability = 0 [0, 0.38], $P = 0.38$), diversity (LMM; repeatability = 0 [0, 0.37], $P = 1$) or volatility (LMM; repeatability = 0.06 [0, 0.42], $P = 0.41$) within individual females across breeding stages, as in the original study (**Table 2**, **Fig. 3**). All of the effect size estimates from the replication study were included in the confidence interval of the original study (**Fig. 3**).

Life stage differences and family signatures

Nestlings and adults had a different overall preen oil composition (PERMANOVA; $P < 0.001$, component of variation = 0.06; **Fig. 4a**, **Table S2**,), with no difference in dispersion (PERMDISP; difference in mean distance to centroid = 2.37; $P = 0.07$). There were no differences in terms of chemical richness (LMM; nestling : $\beta = -1.15$ [–3.33 ; 1.07], $P = 0.34$, marginal $R^2 = 0.01$) and diversity (LMM; nestling : $\beta = -0.05$ [–0.13, 0.04], $P = 0.27$, marginal $R^2 = 0.01$), but the preen oil from adults was more volatile (LMM; nestling: $\beta = -0.69$ [–1.04 ; –0.40], $P < 0.001$, marginal $R^2 = 0.12$) than that of nestlings (**Table S3**). We also found that family members (i.e. sharing the same nestbox) had a greater similar preen oil composition to each other than to members of other families (PERMANOVA; $P < 0.001$, component of variation = 0.05; **Fig. 4b**, **Table S2**). Preen oil composition was also repeatable within families in terms of chemical richness (LMM; repeatability = 0.17 [0, 0.36], $P = 0.014$) and volatility (LMM; repeatability = 0.25 [0.04, 0.44], $P = 0.001$) but not diversity (LMM; repeatability = 0.08 [0, 0.25], $P = 0.11$) (**Table S3**). In addition, the preen oil composition of nestlings was more similar to that of their father than to that of their mother (GLMM; mother: $\beta = -0.13$ [–0.24, –0.02], $P = 0.02$), and was more similar to that of other nestling-rearing males than that of other nestling-rearing females in the population (GLMM; nestling-rearing males: $\beta = 0.14$ [0.11, 0.17], $P < 0.001$). However, they did not exhibit greater similarity to their father than to other nestling-rearing males (GLMM; nestling-rearing males: $\beta = -0.01$ [–0.63, 0.62], $P = 0.98$), or to their mother than to other estling-rearing females (GLMM; nestling-rearing females: $\beta = -0.01$ [–0.59, 0.56], $P = 0.97$) (**Fig. 4c**, **Table S4**).

DISCUSSION

Replication studies are essential (Nakagawa and Parker 2015) but rarely ever done in the fields of ecology and evolution, especially close or exact replications (Parker 2013, Kelly

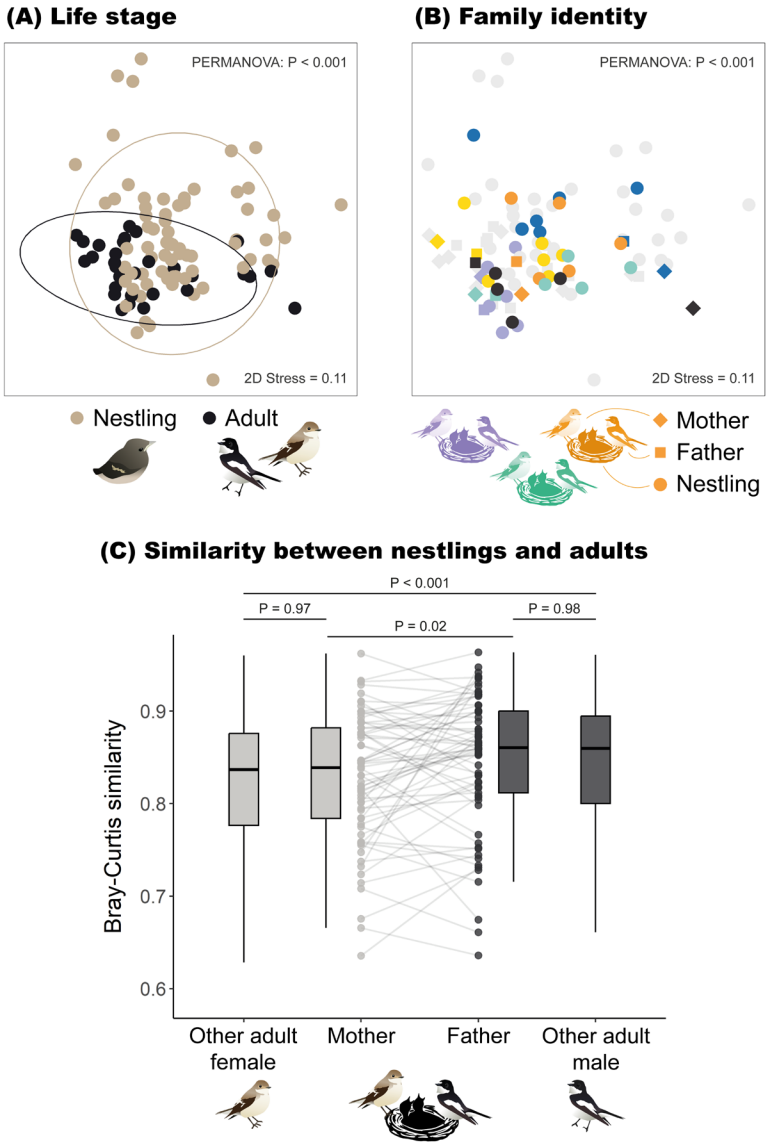

Figure 4. Life stage differences and family signatures in preen oil composition in pied flycatchers. **(A)** Differences between life stages (N = 100 samples, 31 from adults and 69 from nestlings). **(B)** Similarity between family members (N = 100 samples from 16 families). Points of the same colour represent samples from the same family (only a random selection of six families are coloured for illustration purposes, while the remaining ten families are greyed out). (A) and (B) are non-metric multidimensional scaling (NMDS) plots representing Bray–Curtis dissimilarities. Each circle represents a preen oil sample and ellipses the 95% confidence intervals for each group assuming a multivariate t-distribution. P-values were calculated from PERMANOVAs. 2D Stress is a measure of the fit between the distance among samples in the plot (i.e. in two-dimensional space) and the actual distance among samples in multivariate space, with values below 0.15 indicating a good fit. **(C)** Similarity in overall preen oil composition (Bray-Curtis similarity) between nestlings and adults (parents and other nestling-rearing adults). Each point represents the similarity between a nestling and a parent. Lines connect the similarity of a nestling with its mother and the similarity with its father. P-values were calculated from linear mixed effects models testing the effect of social relationship (mother, father, other adult nestling-rearing female and other adult nestling-rearing male) as a fixed effect while controlling for the random effects of nestling and family identities.

2019, Fraser et al. 2020). This is concerning, as many effects are assumed to be true in literature although they are actually not robust across studies, and likely heavily influenced by type I error (false positive) and publication bias (Seguin and Forstmeier 2012, Parker 2013, Sanchez-Tojar et al. 2018). We performed a close replication study of Gilles et al. (2024) investigating the variation in preen oil composition in pied flycatchers during the breeding season. As in the original study, we found that preen oil composition was similar between pair mates, differed between breeding stages in females, and contained no individual chemical signature in females. However, the evidence for the similarity between pair mates was more subtle than in the original study, while the evidence for a breeding stage difference in females was stronger. Importantly, we did not find any evidence that preen oil composition differs between sexes, although subtle sex differences were found in the first study. In addition to the replication, we explored proximate causes of variation in preen oil composition. First, we found no correlation between similarity in preen oil chemical composition and spatial proximity (of nestboxes). Second, we found that preen oil composition was similar between family members and differed between life stages (nestling and adult). Third, we found that the preen oil of nestlings was slightly more similar to that of their father than to that of their mother, and overall more "male-like" than "female-like".

Sex differences

Unlike the study from Gilles et al. (2024), we did not find any evidence for a sex difference in preen oil composition during nestling-rearing. In the original study, sex differences were marginally detected in overall preen oil composition, but this result was not reproduced in our study. Our replication study had greater sample sizes, and therefore more power to correctly reject a false null hypothesis (Asendorpf et al. 2013), making the replication findings more reliable. Our failure to reproduce the original result thus suggests that it was a false positive. Further, the original study found a more volatile preen oil composition in females than males, which could also not be reproduced here. But as the effect size from the replication is still close to the original and not far from significant (Valentine et al. 2011), we cannot completely dismiss the possibility of a sex-related effect on preen oil volatility in this species. Finally, the original study also found that females had a slightly greater chemical diversity than males, and this marginal effect was, yet again, not reproducible. Overall, these findings drastically reduce our confidence in the presence of a sex difference in preen oil.

This changes the inference made by Gilles et al. (2024) on the potential function of preen oil as an olfactory cue used to discriminate between sexes during mate choice (Grieves et al. 2022). However, as argued in the original study, sex differences should be investigated during mate choice, rather than nestling-rearing, before dismissing this hypothesis. In song sparrows for instance, sex differences were found during nest building and early egg laying (i.e. when mate choice still occurs) but not after fledging (i.e. after mate choice) (Grieves et al. 2019a). Similarly, in dark-eyed juncos, sex differences were

found repeatedly before egg laying (Whittaker et al. 2010, 2013) but not at the time of fledging (Whittaker et al. 2016). As we sampled preen oil in the middle of the nestling-rearing period (ca. 11 days after hatching), this may have been too long after mate choice for sex differences to be detected. But note that even if preen oil does not convey information about the sex of individuals, it may still be used as an olfactory signal during mate choice by encoding information on relatedness or individual quality (Whittaker et al. 2013, Potier et al. 2018). Our new results also diminish the credibility of preen oil playing a role in chemically protecting eggs and/or nestlings. Indeed, we would expect such a function to cause sex differences in preen oil, as females brood the eggs and nestlings, whereas males only feed nestlings.

Similarity between breeding partners

As in Gilles et al. (2024), our study brings evidence that breeding partners secrete a preen oil of similar composition. However, the evidence for a breeding pair chemical signature is much weaker than in the original study. Indeed, the effect of pair identity was only successfully reproduced on the overall preen oil composition. For this effect, the only method to assess replication success is to compare p-values, and with a p-value just under the significance threshold in the replication ($P = 0.0495$), whether the effect is considered significant or not strongly comes to chance (Piper et al. 2019). In addition, we could not reproduce the pair similarity in terms of chemical richness and diversity that were found in the original study. Overall, these results hint toward a very subtle (or even potentially absent) breeding pair similarity in preen oil composition.

As in the original study, pair similarity could be explained by a temporal autocorrelation. Indeed, as in Gilles et al. (2024), breeding pairs were often sampled on the same day and around the same time of day. But Gilles et al. (2024) proposed several other hypotheses. First, we suggested that pair similarity could be attributable to spatial autocorrelation, in case of phenotype-environment correlations outside of the nest (e.g. pairs share the same territory and thus possibly the same diet). For example, zebra finches (*Taeniopygia guttata*) that nest closer together have more similar skin microbiota (although this may also result from direct exchange of bacteria in addition to environmental effects) (Engel et al. 2020). But as we found no correlation between the spatial proximity between nests and the similarity in preen oil composition, this hypothesis now seems unlikely. Second, preen oil composition could be impacted by an exchange of substances through physical contact or allopreening. However, since we collected preen oil directly from the uropygial gland, such exchanges are unlikely. A third hypothesis is that preen oil composition is influenced by the social and/or microbial environment in the nestbox. This hypothesis seems particularly plausible as we also found an effect of family identity on preen oil composition. Nest and social environment seem to be key factors in shaping preen oil composition (Whittaker et al. 2016) and microbial communities (Kulkarni and Heeb 2007, Kreisinger et al. 2015, Leclaire et al. 2023), which was also found in pied flycatchers (plumage microbiota; Goodenough et al. 2017). Although the relationship between

microbiota and preen gland secretions remains unclear (Grieves et al. 2021a), there is some evidence that uropygial microbial communities influence preen oil composition (Martín-Vivaldi et al. 2010, Jacob et al. 2014, Whittaker and Theis 2016, Whittaker et al. 2019b). Lastly, the similarity in preen oil between pair mates may be the result of assortative mating based on preen oil odours. However, we believe that this hypothesis is unlikely, as such a mating strategy can hardly be advantageous (Gilles et al. 2024).

Changes across breeding stages

Both the original study and our replication study found that preen oil composition changes between the incubation and nestling-rearing periods in females. The breeding stage effects were clearer than in the original study. Indeed, we not only found changes in overall preen oil composition (as in the original study), but also in chemical richness, diversity and volatility. Note that an effect on volatility had also been detected in the original study, but on a different measure of volatility (i.e. proportion of low-volatility substances) which was not used in the replication (see **Methods**). The effect of breeding stage on chemical diversity was widely included in the confidence interval of the original study, suggesting that it may have been non-significant originally due to a lack of power (Valentine et al. 2011).

Overall, our new results reinforce the suggestions from Gilles et al. (2024) regarding the potential functions associated with the changes in preen oil across breeding stages in females. This shift may reflect a use of preen oil as an olfactory signal during mate choice (e.g. signalling individual quality or reproductive state; Grieves et al. 2022). However, we note that preen oil composition during incubation may not necessarily reflect preen oil composition during mate choice (e.g. abrupt drop in volatile compounds in female dark-eyed juncos after egg-laying; Whittaker et al. 2011b). Our results also align with previous evidence in favour of a role of preen oil in parental care, as in many species, seasonal changes in preen oil composition are found primarily in the incubating sex (Grieves et al. 2022) and often occur during (or shortly before) specific breeding stages, such as the incubation (Reneerkens et al. 2002) or nestling-rearing period (Pap et al. 2010, Amo et al. 2012a). Our results could therefore reflect a role of preen oil as an olfactory signal for parent-offspring communication (Caspers et al. 2017b) or as a chemical defence against eggshell bacteria (Martín-Vivaldi et al. 2010b). These hypotheses are not mutually exclusive and should be experimentally tested. Besides, our results could simply result from nonadaptive mechanisms, such as diet (Thomas et al. 2010) or hormonal changes during the course of the breeding season (Whittaker et al. 2011b). Finally, our results show preen oil composition can change rapidly (i.e. about two weeks), as found in other species (Reneerkens et al. 2007b, Whittaker et al. 2011b, Amo et al. 2012a, Grieves et al. 2018).

Replication outcome

While it is tempting to put more confidence in the (more powerful) replication study than in the original study, it is important to note that a replication study on its own can never

confirm or disconfirm the results of an original study (Earp and Trafimow 2015). This is because replication studies are never exact replications, especially when studying wild populations, where many factors cannot be controlled for (Fidler et al. 2017), and conducting chemical analyses, which are sensitive to slight alterations in methodology (Tebbe et al. 2020, Alves Soares et al. 2024a). For instance, although the chromatograms in the replication had a very similar appearance to the chromatograms from the original study, we found substantially fewer substances (88 instead of 119 in the original study), and the nine most abundant substances were not exactly the same as in the original study (see **Fig. S1** in comapison with Fig. S1 in Gilles et al. 2024). With only two studies, it is impossible to know whether these differences in chemical data are due to biological (i.e. among-year differences) or methodological differences (Tebbe et al. 2020). Instead, replication studies increase or decrease our confidence in a given hypothesis, while contributing to the general estimation of the effect studied (Heirene 2021). All the results from both studies should therefore be taken into account, especially when considering that our methods to assess replication outcome are not flawless. As already explained, using a dichotomous approach such as statistical significance to assess replication success is limited as it only tells us whether the effect was different from zero in both studies, not whether we found the same effect (Heirene 2021). This method is especially unreliable when comparing studies with different sample sizes as we did. Indeed, by changing the sample sizes, one could find a very different p-value associated with the same effect size (Piper et al. 2019, Heirene 2021). The comparison of effect sizes and their confidence intervals present an amelioration by giving information about the magnitude and direction of the effect on a continuous scale, making use of most of the statistical information available (Asendorpf et al. 2013, Heirene 2021). However, this method is still sensitive to low power analyses, like those from the original study, which provide uncertain estimates (i.e. with wide confidence intervals) (Verhagen and Wagenmakers 2014). Consequently, all the confidence intervals from the replication were overlapping with that of the original study, incorrectly implying that all of the effects were reproducible. This is why it may be interesting to look at multiple different proxies of replication success before drawing conclusions (Valentine et al. 2011, Asendorpf et al. 2013). Improving the quality of replication studies could be facilitated by the development of straightforward methods to compare results across studies, including Bayesian approaches like the Bayes Factor (Verhagen and Wagenmakers 2014).

Ontogeny of preen oil composition

As found in numerous bird species, we found a significant difference in overall preen oil composition between nestlings and adults (reviewed in Alves Soares et al. 2023). This difference could reflect non-adaptive processes such as differences in diet or physiology. It may also reflect a role of preen oil olfactory signalling for reproduction, with adults, and not nestlings, secreting sex semiochemicals. Preen oil odours may even be used to advertise sexual readiness or maturity, although this remains to be tested. In line with this, our study

showed that adults had a more volatile preen oil than nestlings, as in grey catbirds (*Dumetella carolinensis*) (Shaw et al. 2011). Juvenile birds, which are still developing adult preening behaviour, could benefit from a less volatile preen oil, as it may adhere longer to the plumage, therefore requiring less preening (Shaw et al. 2011), although this remains to be tested.

In addition, our study revealed that preen oil composition was similar within families, suggesting an influence of shared environments (i.e. the nest) and/or genetic relatedness. Although we cannot disentangle the relative influences of these two factors, a study on dark-eyed juncos showed that the social (nest) environment was a stronger predictor of preen oil composition than genes (Whittaker et al. 2016). A strong effect of the nest environment has also been suggested in cooperatively-breeding smooth-billed anis (*Crotophaga ani*), in which members of the same breeding group (i.e. sharing the same nest) have a similar preen oil composition (and preen gland microbiota) although they are not genetically related (Grieves et al. 2024). In line with this, we found that breeding partners, which are in theory rather unrelated (Kruuk et al. 2002), produce a similar preen oil, suggesting that the nest environment has an effect on preen oil composition in pied flycatchers (see "**Similarity between partners**" above). Several features of the nest environment could affect preen oil composition, notably the nest microbiome and the nest occupants themselves (i.e. social environment) (Jacob et al. 2014, Whittaker et al. 2016, Goodenough et al. 2017).

Our study also indicated that the preen oil composition of nestlings resembled marginally more that of their father than that of their mother. As mothers spend more time with nestlings than fathers, we expected to find the opposite. Interestingly, we also found that nestlings secrete a preen oil that resembled marginally more that of adult males (other than their father) than females (other than their mother), which was surprising since we found no difference between sexes in adults. Furthermore, the preen oil of nestlings was not more similar to the preen oil of their mother than to that of other adult females, nor was it more similar to the preen oil of their father than to that of other adult males. We expected the opposite, since we found pair and family signatures, which suggest an influence of the nest environment on preen oil composition. However, these unexpected results align almost exactly with the results of a study on dark-eyed juncos (Whittaker et al. 2016). Indeed, although dark-eyed junco chicks are more in physical contact with their mother, their preen oil is more similar to that of their father than to that of their mother. Besides, although no sex differences were found in adults at the time of fledgling, fledglings secrete a preen oil that is more similar to that of males than to that of females. Finally, even though the study found strong effects of the social environment in the nest on preen oil, the preen oil of chicks is not more similar to that of their father than to that of other adult males. In dark-eyed juncos, the male-like/father-like preen oil of nestlings has been suggested to be driven by the fact that nestlings have a lower abundance of compounds that are typically abundant in adult females (female-like) (Whittaker et al. 2016). The fact that the preen oil of adults often presents female-based patterns (reviewed in Whittaker and Hagelin 2021) may

explain why young birds secrete a more male-like preen oil, which may not contain (or in lower proportions) substances secreted by adult females. The male-like preen oil of nestlings could also be due to their reduced volatility, as we also found that volatility was marginally lower in males than females. But this remains speculative, especially since we found ne sex difference in adult pied flycatchers. Note also that since we did not determine the sex of the nestlings, it is possible that the male-like preen oil we observed in our results is due to a higher proportion of male nestlings in our data. To our knowledge, family signatures on preen oil composition had only been studied in dark-eyed juncos (Whittaker et al. 2016). But similarities within families regarding microbiota have been more extensively investigated. Many of these studies highlight the importance of nest and social environment on bird microbiota (Ruiz-Rodríguez et al. 2014, Engel et al. 2020, Maraci et al. 2022, Grieves et al. 2024). Taking inspiration from these studies, future research should conduct cross-fostering experiments to disentangle environmental and genetic effects on preen oil composition. Finally, future studies should measure the heritability of preen oil composition, for example by studying pedigreed populations (e.g. using "animal models", which is now possible with compositional data; Wilson et al. 2010, Sweeny et al. 2023).

CONCLUSION

In a dedicated replication study, we showed the robustness of the effect of breeding stage on preen oil composition in female pied flycatchers, while revealing the fragility of the effects of sex and pair identity. The non-reproducibility of a substantial portion of the original results emphasises the critical need for more replication studies in the field of avian chemical ecology and beyond. To more comprehensively describe chemical signatures in preen oil, future research should investigate sexual differences also during the mate choice period, and determine whether individual plasticity across breeding stages is exclusive to the incubating sex. Our study also provides novel insights into the ontogeny of preen oil composition in this species, but further investigations are needed to validate whether family signatures and male-like preen oil in nestlings are consistent patterns in this and in other species. Finally, future research should conduct experiments (e.g. bioassays on antimicrobial activity, behavioural trials on olfactory preferences) to examine the role of preen oil in reproduction in pied flycatchers.

Acknowledgements

We thank Barbara Fuchs for her help with the extraction and chemical analysis of preen oil samples.

Data availability

Data and code used in the analyses are available on Github (https://github.com/marc-gilles/preen-oil-replication-ontogeny).

Supporting information

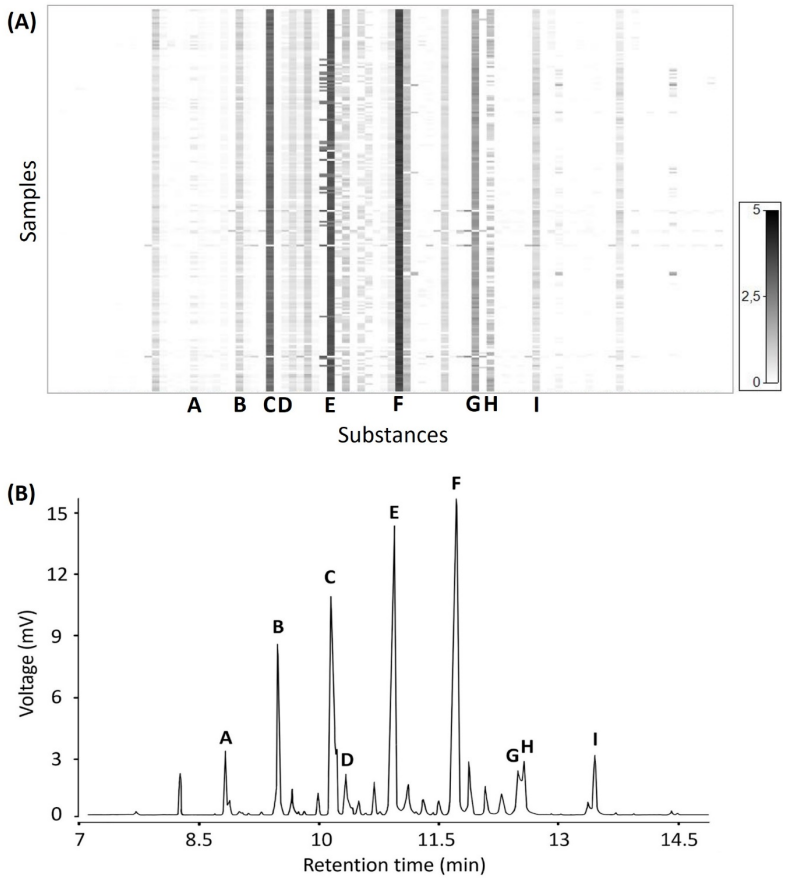

Fig. S1. Chromatographic data from the GC-MS analysis of the preen oil samples. **(A)** Shadeplot showing the relative log-transformed abundance of each substance (columns) in the samples (rows) used for the statistical analysis (N= 210). **(B)** Representative chromatogram of the preen oil of a female pied flycatcher sampled during incubation. Letters indicate the nine most abundant substances across all samples, as defined in Gilles et al. (2024).

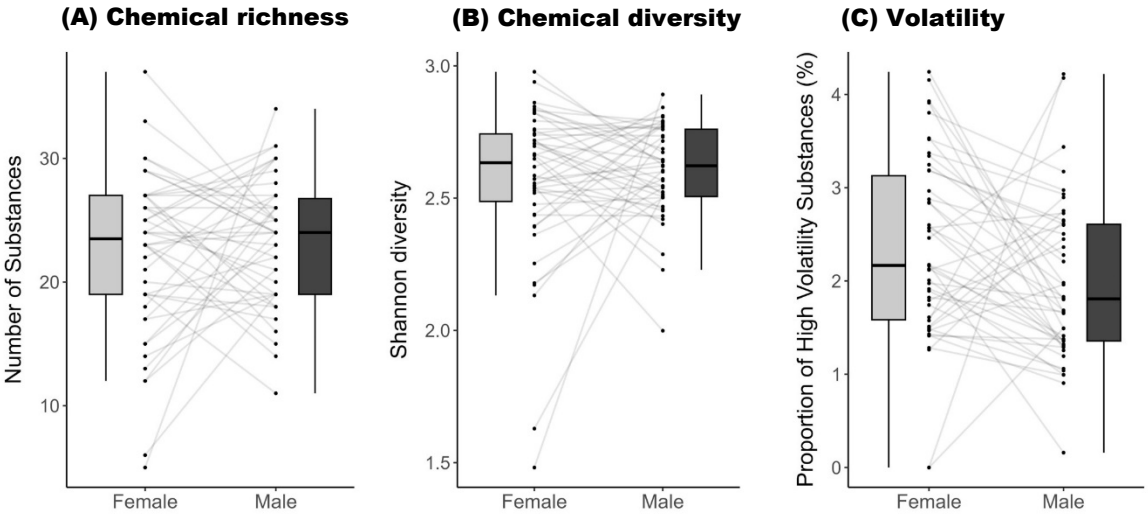

Fig. S2. Chemical diversity (Shannon diversity index) in relation to the overall concentration in preen oil (total area under the chromatogram divided by the number of substances recorded in the sample), revealing a concentration bias but no obvious concentration threshold below which Shannon diversity drops (N = 210 samples).

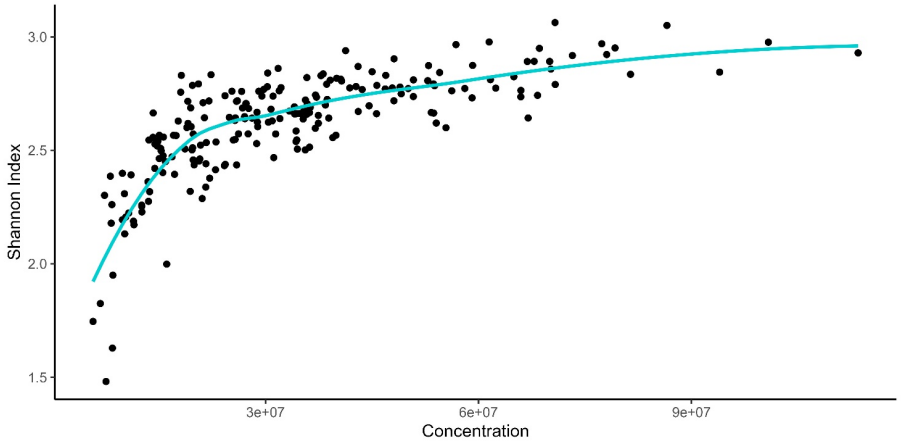

Fig. S3. Changes in preen oil composition between sexes in nestling-rearing pied flycatchers. Preen oil does not differ between females and males in **(A)** chemical richness, **(B)** chemical diversity (Shannon index) and **(C)** volatility (proportion of high-volatility substances). N = 92 samples from 46 breeding pairs. Each circle represents a preen oil sample. Grey lines connect the repeated samples of a single breeding pair.

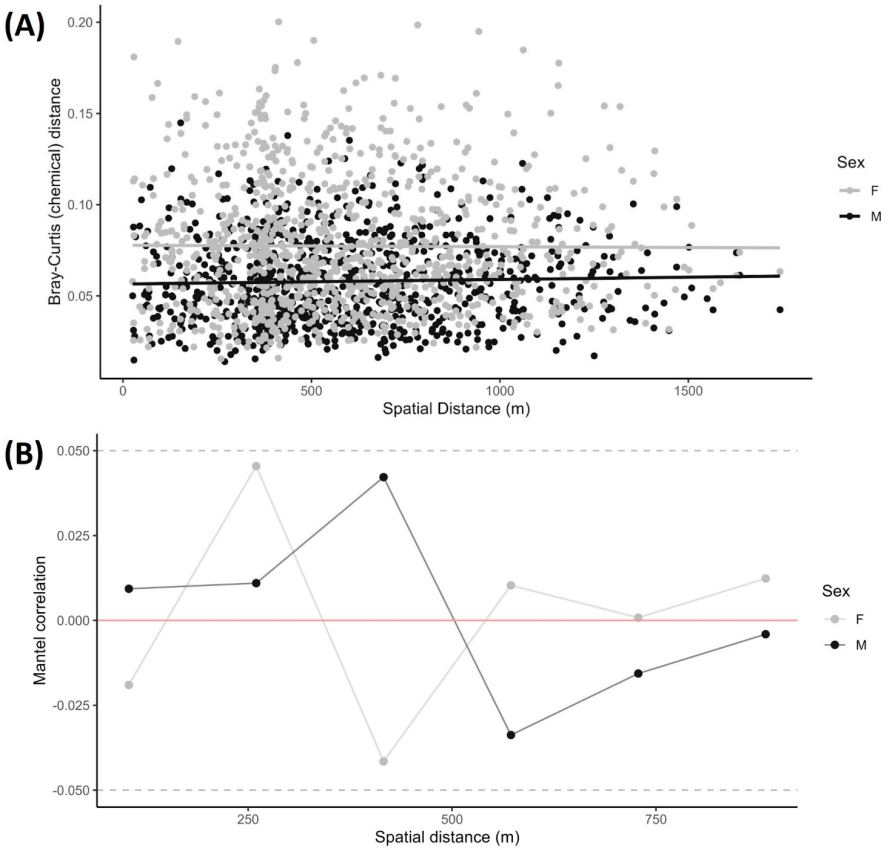

Fig. S4. Relationship between chemical distance (Bray–Curtis dissimilarity in preen oil composition) and spatial distance (between nestboxes) within females (grey) and males (black). **(A)** Scatterplot. Each point represents a female dyad (grey) or a male dyad (black), Lines are regression lines. **(B)** Mantel correlograms. Dashed lines represent the significance thresholds. The same data was used for the Mantel tests and Mantel correlograms (N = 44 females and 42 males).

Table S1. Settings used for the integration of chromatographic data using the software GC Solutions (version 2.41) and for the alignment of chromatographic data using the *align_chromatograms* function of the *GCalignR* package in R.

	Parameter	Value
Peak integration	Width	1 sec
	Slope	500 µV/min
	Drift	0 µV/min
	Doubling time (T.DBL)	500 min
	Min. Area/Height	500 counts
Peak alignment	max_linear_shift	0.03 min
	max_diff_peak2mean	0.015 min
	max_diff_peak2peak	0.035 min

Table S2. Results from permutational multivariate analysis of variance (PERMANOVA) on the preen oil chemical composition of adult and juvenile pied flycatchers. Effect of life stage (fixed effect) within families (random effect) sampled during nestling rearing. N = 100 samples from 16 families (31 adults and 69 nestlings.

	SS	F (pseudo)	P (perm)	Comp. of variation
Life stage	1547.5	13.2	0.0001	5.58
Family	4414.5	2.51	0.0001	5.34
Residuals	9729.4	-	-	10.83

Analysis based on Bray-Curtis dissimilarities of log-transformed values. P-values were obtained using 9999 permutations under a reduced model with type III (partial) sums of square (SS), and are indicated in bold if the effect is significant at α = 5%. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray-Curtis units (i.e. % of Bray-Curtis dissimilarity).

Table S3. Results of generalised linear mixed models (GLMMs) investigating life stage differences within families in several chemical aspects of the preen oil of pied flycatchers: richness (number of substances), diversity (Shannon index) and volatility (proportion of high-volatility substances). N = 100 samples from 16 families (31 adults and 69 nestlings).

	Fixed effect **Life stage**			Random effect **Family**		
	β [95% CI]	**P**	**R^2**	**Variance**	**P**	**Repeatability**
Richness	-1.15 [-3.66;1.19]	0.34	0.01 [0;0.07]	6.30 (2.5)	**0.01**	0.17 [0, 0.37]
Diversity	-0.05 [-0.12;0.04]	0.27	0.01 [0;0.08]	0.003 (0.05)	0.13	0.08 [0, 0.25]
Volatility	-0.69 [-1.02;-1.35]	**<0.001**	0.12	0.18 (0.43)	**0.001**	0.25 [0.03, 0.45]

P-values below *α* = 5% threshold are indicated in bold.

Male and female Kentish plovers taking turns during incubation

Photo by Melvin Grey

CHAPTER 5

No sex difference in preen oil chemical composition during incubation in Kentish plovers

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ABSTRACT

Preen oil – the secretion from the uropygial gland of birds – may have a specific function in incubation. Consistent with this, during incubation, the chemical composition of preen oil is more likely to differ between sexes in species where only one sex incubates than in species where both sexes incubate. In this study, we tested the generality of this apparent difference, by investigating sex differences in the preen oil composition of a shorebird species, the Kentish plover (*Anarhynchus*, formerly *Charadrius*, *alexandrinus*). As both sexes incubate in this species, we predicted the absence of sex differences in preen oil composition during incubation. In the field, we sampled preen oil from 9 females and 11 males during incubation, which we analysed with gas chromatography–mass spectrometry (GC–MS). Consistent with predictions, we found no sex difference in preen oil composition, neither in beta diversity (Bray-Curtis dissimilarities) nor in alpha diversity (Shannon index and number of substances). Based on these results, we cannot conclude whether preen oil has a function during incubation in Kentish plovers. Still, we discuss hypothetical roles, such as olfactory crypsis, protection against ectoparasites or olfactory intraspecific communication, which remain to be tested.

INTRODUCTION

Most birds possess a sebaceous gland at the base of the tail – the uropygial gland (or preen gland) – that produces an oily secretion (preen oil) (Jacob and Ziswiler 1982). Birds spread preen oil over their plumage during preening (Moreno-Rueda 2017). The chemical composition of preen oil typically consists of wax esters and other substances, such as alcohols, aldehydes, alkanes, carboxylic acids and ketones (reviewed in Campagna *et al.* 2012, Alves Soares *et al. accepted*). Preen oil is multifunctional, serving plumage maintenance, protection against ectoparasites (e.g. feather degrading bacteria, eggshell bacteria, chewing lice) and waterproofing (reviewed in Moreno-Rueda 2017). Preen oil is also an important source of body odour in birds (Hagelin and Jones 2007) and may have odour-related functions, namely olfactory crypsis and olfactory communication (reviewed in Grieves *et al.* 2022).

The first step to investigate the potential function(s) of preen oil is to describe the variation in its chemical composition, notably seasonal changes and sex differences (Grieves et al. 2022). In many species, preen oil composition changes during breeding, specifically at the time of incubation and specifically in the incubating sex (Reneerkens et al. 2007a), strongly suggesting that preen oil has a function associated to incubation. First, a function of preen oil during incubation could be protection against ectoparasites, in case incubating birds are exposed to high parasitic loads in the nest or to limit pathogenic infection of the eggs. This was shown in Eurasian hoopoes (*Upupa epops*) where only females (incubating sex) produce a dark preen oil that contains antibacterial substances during incubation, and that is smeared on the eggs to protect embryos from eggshell bacteria (Martín-Vivaldi et al. 2009, 2010). Second, a function of preen oil during incubation could be olfactory crypsis, in case the incubating birds (and their clutch or brood) are exposed to olfactorily searching nest predators (Reneerkens 2005b). This is the case in shorebirds (order Charadriiformes), where preen oil composition shifts from monoesters to diesters during incubation (seasonal change in preen oil; documented in 19 sandpiper, 6 plover and 1 oystercatcher species; Reneerkens et al. 2006), solely in the incubating sex (sex-specific seasonal change in preen oil; documented in 7 sandpiper species; Reneerkens et al. 2007a). The diester preen oil secreted during incubation is less volatile than the monoester preen oil, which makes the incubating birds (or their clutch or brood) less detectable to olfactorily searching nest predators (e.g. dog, Reneerkens 2005). Finally, a function of preen oil during incubation could be olfactory intraspecific signalling (e.g. for mate choice, "sex semiochemical hypothesis", Grieves *et al.* 2022). For example, in three passerine species (order Passeriformes) with uniparental incubation, preen oil composition differs between sexes during breeding (Whittaker et al. 2010, Amo et al. 2012a, Grieves et al. 2019a), which allows birds to discriminate the sex of conspecifics by smell (Whittaker et al. 2011a, Amo et al. 2012a, Grieves et al. 2019b).

Although several shorebird species (order Charadriiformes) have been studied with regard to sex differences in preen oil (14 species), they were all studied using a fairly

straightforward analytical method (Reneerkens et al. 2002, 2007a). This method consists in describing preen oil composition using a single categorical variable (i.e. ester composition) with three categories (i.e. monoesters only, mixture of monoesters and diesters, diesters only). Reducing the complexity of preen oil composition (usually hundreds of substances) to a single categorical variable is simple but effective. Indeed, this method revealed striking sex differences in preen oil during incubation in uniparentally incubating species (diesters in the incubating sex, monoesters in the non-incubating sex), but not in biparentally incubating species (diesters in both sexes). However, subtle sex differences in biparentally incubating species may have been missed using this categorisation, and may be uncovered using more advanced methods (e.g. multivariate analyses).

In this study, we sampled preen oil from female and male Kentish plovers (*Anarhynchus alexandrinus,* formerly *Charadrius alexandrinus*) during incubation, analysed their chemical composition using GC–MS, and tested for sex differences in alpha and beta diversity using multivariate statistical analyses. Given that both sexes incubate in this species (Kosztolányi and Székely 2002), and assuming that this species undergoes the same sex-specific seasonal changes in preen oil composition as the other shorebird species studied (Reneerkens et al. 2002, 2006, 2007a), we predicted an absence of sex differences in preen oil composition during incubation. Alternatively, sex differences in preen oil composition can be expected in case of sexual selection or other sex-dependent reason. It should be emphasized that, since we sampled preen oil only during the incubation period, our aim was not to investigate sex-specific seasonal changes and replicate studies from Reneerkens et al. (2002, 2007a). Rather, we used their findings to make predictions on whether we should find sex differences during incubation in Kentish plovers.

MATERIALS AND METHODS

Study site and species

Fieldwork was conducted on breeding Kentish Plovers at the Samouco saltpans complex (38°44′N, 8°59′W) on the south bank of the Tagus estuary, Portugal. In the study site, Kentish Plovers breed on dykes of abandoned saltpans, nesting in the ground sparsely covered by pebbles, wooden planks and salt marsh vegetation, isolated or in proximity to nests of black-winged stilts *Himantopus himantopus* and little terns *Sternula albifrons* (Rocha et al. 2016). The population (20-76 breeding pairs) is resident and presents an extended breeding season, from early March, when males start to defend nesting sites, to the end of July. During the breeding season, mates generally re-nest with a different mate (sequential polygamy), but monogamy is also observed. Both parents incubate the eggs for a period of 25-26 days (Kosztolányi and Székely 2002).

Field methods

As part of a colour-ringing marking program, from May to June 2019, Kentish Plovers were caught on their nests during incubation using walk-in funnel traps (Székely et al. 2008). The birds were sexed using plumage characteristics, measured and ringed (Székely et al. 2008). We collected preen oil from 20 birds (9 females and 11 males) by gently massaging the preen gland papilla with a 100 µl microcapillary and snapping the end of the microcapillary (containing the extracted preen oil) in a 2 ml glass vial with Teflon seal (Rotilabo ®) while wearing nitrile gloves. For some breeding pairs, both partners of a breeding pair could be sampled (N = 8 samples from 4 pairs), but for most pairs only a single bird was sampled (N = 12 samples). Samples were stored at –20 °C during seven months, before being transferred at –80 °C for seven months until analysis. The laying date of each nest was estimated by egg flotation (Székely et al. 2008). Bird capture and sampling were carried out in accordance with the Portuguese Institute of Nature Conservation and Forestry (ICNF) guidelines (license N°1/2019) and no additional institutional animal care approval was required. To ensure the well-being of the birds, we took all necessary measures to minimize any stress caused by capture and handling. After capture, birds were placed inside a dark cotton bag before being ringed, measured and sampled. This procedure took less than ten minutes per bird. Birds were released immediately after sampling, they showed no sign of discomfort or stress (e.g. increased respiratory rate, open mouth breathing, or closed eyes) and returned to incubate at their nest a few minutes after release.

Chemical analysis

All samples were first defrosted and then extracted by adding 500 µl of dichloromethane as a solvent to the vials containing the microcapillary and the preen oil. After briefly vortexing each sample, we transferred 100 µl of the solution (preen oil and dichloromethane) into a glass vial (2 ml, Rotilabo ®) containing a 100 µl glass inset, using a blunt point glass syringe (which was washed with dichloromethane between each sample). For chemical analyses, we performed GC-MS, using a gas-chromatograph (GC-2030, Shimadzu, Kyoto, Japan) equipped with a VF-5ms capillary column (30 m x 0.25 mm ID, DF 0.25, 10 m guard column, Varian Inc., Lake Forest, USA) and helium (at a 1 ml/min flow rate) as a carrier gas, coupled to a mass spectrometer (GCMS-QP2020NX, Shimadzu) in split (1/10) mode. The settings for the gas chromatography were as follows; injection temperature: 310 °C, starting temperature: 150 °C, followed by an increase in temperature of 20 °C per min until reaching 280 °C, followed an increase of 5 °C per minute until reaching the end temperature of 310 °C, which was kept for 20 min. For the mass spectrometry, ion source temperature was set at 230 °C and interface temperature at 310 °C. Seven GC blank samples (containing dichloromethane only) were analysed among the preen oil samples.

Chromatographic data processing

GC-MS produces chromatograms, where each peak is a substance (defined by its retention time) and the area of the peak represents the abundance of the substance (see **Fig. 1** for a typical chromatogram of Kentish Plover preen oil). We assumed that each peak represents a single substance, but we acknowledge that a single peak can represent multiple substances that coelute (i.e. with the exact same retention time). We extracted peak retention times and areas from chromatograms using LabSolutions GCMS solution v4.52 (Shimadzu). Because the retention time of a substance can vary subtly between samples, we aligned the chromatograms using the *GCalignR* package (Ottensmann et al. 2018). We used the 20 preen oil samples and the seven GC blank samples for the alignment. Substances detected in GC blank samples, as well as substances detected in single samples, were removed to control for potential contamination. We verified the quality of the alignment with a shadeplot (**Fig. S1**) in PRIMER v7.0.20 (Clarke and Gorley 2015). Because the amount of preen oil collected was not standardized, we used relative abundances (i.e. peak area divided by total chromatogram area) for the analysis. As we have no prior knowledge about the substances potentially involved in sex differences, we log-transformed (log(X+1)) the relative abundances, thereby increasing the weight of low-abundance substances in the analysis (Clarke et al. 2014). We calculated the chemical diversity (Shannon index) and richness (number of substances) of each sample, as measures of alpha diversity, using the *vegan* package (Oksanen et al. 2019). If the detectability of substances was positively correlated to their retention time, there could be a methodological issue (e.g. more volatile substances evaporating or breaking more than less volatile substances). We verified that this was not the case, as the effect of the retention time on the detectability of substances was not positive linear (polynomial beta regression: $\beta = 0.54$, $P = 0.48$), but negative quadratic (polynomial beta regression: $\beta = -5.57$, $P < 0.001$). This shows that both more

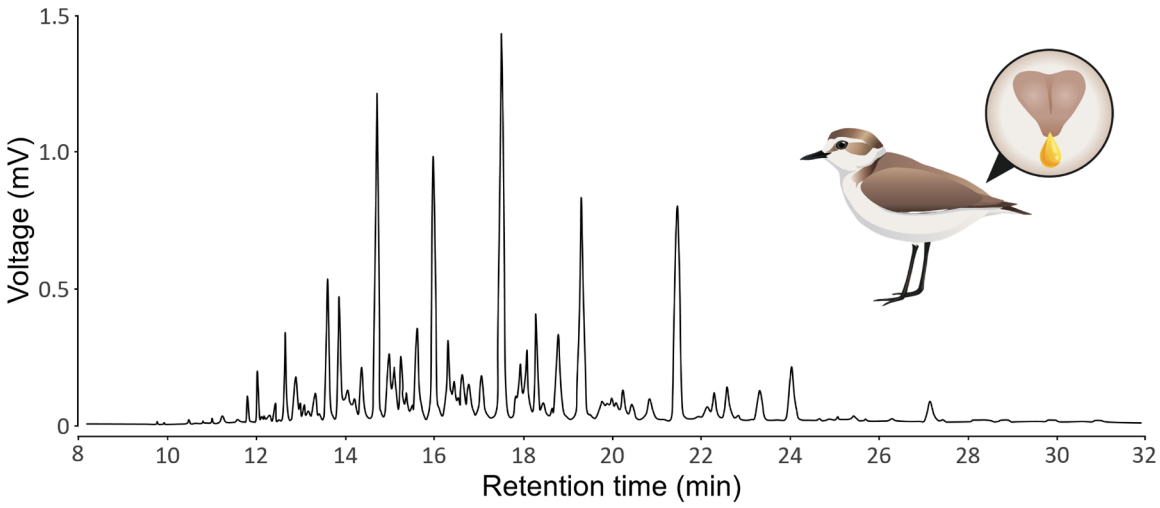

Fig. 1. Representative chromatogram of the preen oil of Kentish Plovers. The illustration depicts a female Kentish Plover with a zoom on its uropygial gland secreting preen oil.

volatile and less volatile substances were less detected than substances with intermediate retention time (**Fig. S2**). All the chromatographic data processing was conducted in R v4.2.2 (R Core Team 2022) and is detailed in an R Markdown document (Baumer and Udwin 2015) in the Supplemental Information.

An accurate identification of the substances would have required sophisticated analytical methods, including calculating retention indices, comparing substances with commercially available standards and using two columns of different polarity (e.g. Alves Soares *et al.* 2024). For structural identification of esters, other methods could be conducted, such as combined GC and GC-MS using synthesized standards (Sinninghe Damsté et al. 2000, Rijpstra et al. 2007). As we were interested in quantitative, rather than qualitative, chemical differences, we did not need to identify the substances and used retention times instead. We putatively identified the chemical substances by comparing their mass spectrometry (MS) to that of the NIST library (NIST/EPA/NIH Mass Spectral Library 2017) and recording the substance name with the highest match, but this method is not accurate enough to identify substances with certainty. For this reason, we do not provide the list of putative (and likely erroneous) substance names. However, we recorded the class of the substances, in case the class of the putatively identified substances was the same across all samples (see **Table S1** for the list of substances, including retention times, mean relative abundances and classes). The raw chromatographic data are available at the repository PUB – Publications at Bielefeld University (https://doi.org/10.4119/unibi/2965523).

Statistical analysis

We tested for sex differences in preen oil composition using 20 samples (9 females, 11 males). First, to test for sex differences in the overall chemical composition (i.e. beta diversity), we performed a permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities using the *adonis2* function from the *vegan* package (Oksanen et al. 2019). Bray-Curtis dissimilarity is pertinent for the analysis of abundance data, notably because it ignores joint absences (Clarke et al. 2014). PERMANOVA was run with 9,999 permutations and sequential effects (type I sums of squares). As fixed effects, we included *sex*, but also *number of days after laying* to test for a potential seasonal effect as preen oil composition can change over short periods (less than a week, Grieves *et al.* 2022), and the interaction between *sex* and *number of days after laying*, as seasonal changes may differ between sexes (Grieves et al. 2022). Some of our samples were collected from breeding partners (N = 8 "paired" samples from 4 breeding pairs), and preen oil can be more similar within than between breeding pairs (Gilles et al. 2024). However, using blocking permutations within breeding pairs is not appropriate to deal with the possible pseudoreplication in this dataset, because in 12 cases there is only one data point from a pair (i.e. only one possible choice within a pair); therefore we applied an alternative approach. First, we randomly excluded four of the "paired" samples so that we included only independent samples (N = 16 samples, only one sample from a breeding

pair). The four excluded samples always included two females and two males so that the ratio between the sexes was not distorted (seven females and nine males). Second, we ran iterated PERMANOVAs (1,000 iterations) on the 16 samples randomly selected before each run. We report the median (and interquartile range) of the SS, R^2 and F values from the iterated PERMANOVA runs. P values were calculated as

$$P = \frac{\sum_{i=1}^{N_{iterations}}(1+\sum_{j=1}^{N_{perm}} \mathrm{I}(F_j \geq F_{obs_i}))}{N_{iterations} \times (1+N_{perm})}$$

where $N_{iterations}$ is the number of iterations of PERMANOVAs, N_{perm} is the number of permutations per PERMANOVA, F_j is the F-statistic for the j^{th} permutation, F_{obs_i} is the observed F-statistic in the i^{th} iteration, and I(condition) is an indicator function that equals 1 if the condition is true and 0 otherwise. We also tested for a sex difference in dispersion (or variance) using the *betadisper* function from the *vegan* package (Oksanen et al. 2019). We used non-metric multidimensional scaling (NMDS) plots for visualization of differences in Bray-Curtis dissimilarity (beta diversity).

Second, to test for sex differences in chemical diversity (Shannon index) and richness (number of substances) (i.e. alpha diversity), we performed linear models (LMM) using the *lmer* function in the *lme4* package (Bates 2010). For both models, *sex* and *number of days after laying* were included as fixed effects, and *pair ID* as random effect. Using *pair ID* as a random effect, we controlled for the potential increased similarity within breeding pairs, and thus we could include all 20 samples (nine females and eleven males). We assessed the significance of fixed effects (α = 0.05) by checking whether their 95% confidence interval (95% CI) contained zero, using the *broom.mixed* package (Bolker et al. 2022). Assumptions of normality and homoscedasticity of the residuals were verified using the *performance* package (Lüdecke et al. 2021). All plots were created with *ggplot2* (Wickham 2016), all analyses were performed in R v4.2.2 (R Core Team 2022). Data and code are available in the Supplemental Information and at the repository PUB – Publications at Bielefeld University (https://doi.org/10.4119/unibi/2965523).

RESULTS

We detected a total of 95 chemical substances in the preen oil of Kentish plovers, with on average 63 substances (SD = 9) per sample (on average 62 substances in females and 63 substances in males). These numbers should be treated as minima, as they are based on the assumption that one peak represents one substance, but it is possible that one peak represents multiple substances (in case of coeluting substances). Most putative substances appeared to be monoesters, while no diester was detected (**Table S1**). About one third of the substances (32%, N = 35 substances) were detected in all 20 samples, and no substance was sex-specific (i.e. detected in females only or males only).

We found no sex difference in preen oil composition (beta diversity) based on Bray-Curtis dissimilarities (PERMANOVA: P = 0.35, R^2 = 0.11). The absence of a sex

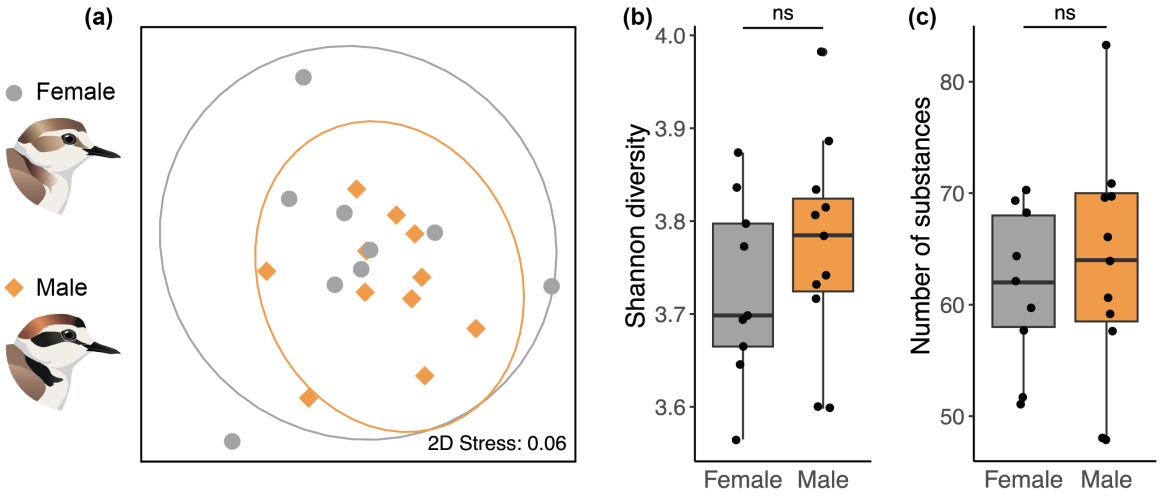

Fig. 2. No sex difference in the preen oil composition of Kentish Plovers. (a) Non-metric multidimensional scaling (NMDS) plot representing Bray-Curtis dissimilarity in chemical composition. 2D Stress measures the goodness of fit of the NMDS ordination, with a value < 0.1 indicating a good fit. The ellipses for each sex (95% confidence intervals assuming a multivariate t-distribution) overlap entirely, highlighting the absence of a sex difference in beta diversity. Besides, no sex difference was detected in alpha diversity, namely **(b)** chemical diversity (Shannon index) and **(c)** chemical richness (number of substances) of preen oil.

difference can be seen on the NMDS plot (**Fig. 2a**) where the 95% confidence intervals for each sex overlap entirely. The preen oil composition of females and males also did not differ in dispersion (P = 0.39). In addition, no sex difference was detected in alpha diversity, neither in chemical diversity (LMM: β [95% CI] = 0.09 [–0.15; 0.36], **Fig. 2b**) nor richness (LMM: β [95% CI] = 5.88 [–12.7; 26.4], **Fig. 2c**). Preen oil composition did not change over the course of incubation (from 1 day until 33 days after laying), neither in Bray-Curtis dissimilarities (PERMANOVA: P = 0.48, R^2 = 0.11), diversity (LMM: β [95% CI] = 0.00 [–0.01; 0.01]), nor richness (LMM: β [95% CI] = 0.02 [–0.81; 0.86]). Finally, we detected no effect of the interaction between sex and the number of days after laying, neither in Bray-Curtis dissimilarities (PERMANOVA: P = 0.34, R^2 = 0.06), diversity (LMM: β [95% CI] = 0.00 [–0.02; 0.01]), nor richness (LMM: β [95% CI] = –0.32 [–1.73; 0.93]). Detailed results are available in the supplemental information (**Tables S2 & S3**).

DISCUSSION

As predicted, we found no sex difference in the preen oil of Kentish plovers during incubation, neither in beta diversity nor in alpha diversity. This is consistent with previous findings that, in shorebirds with biparental incubation, both sexes secrete a similar preen oil during incubation (Reneerkens et al. 2007a, Grieves et al. 2022). Using more advanced statistics than the classical studies on the chemistry of the preen oil of shorebirds (Reneerkens et al. 2002, 2006, 2007a), we did not uncover subtle sex differences.

Our finding that both sexes secrete a similar preen oil during incubation may indicate a specific function of preen oil in incubation, but only if preen oil composition changes specifically during this period, as in other shorebird species (Reneerkens et al. 2002, 2006, 2007a). Because we sampled preen oil only during the incubation period, we could not test for such seasonal changes. We assumed that Kentish plover preen oil would follow the general pattern identified by Reneerkens et al. (2002) in other shorebirds, that is a switch from monoesters to diesters at the onset of incubation followed by a switch back to monoesters after incubation. It seems however that Kentish plovers do not follow this general pattern. Indeed, our putative identification of the class of substances revealed that the preen oil of incubating Kentish plovers contained predominantly monoesters, and no diesters (**Table S1**). Although surprising, this finding is consistent with a preliminary study (Reneerkens 2007), which found only monoesters in the preen oil of incubating Kentish plovers (as well as in incubating Northern Lapwings *Vanellus vanellus* and Eurasian dotterels *Anarhynchus morinellus*), and thus no seasonal change from monoesters to diesters. Together, these results suggest that, in some shorebird species including Kentish plover, preen oil composition may not switch to a diester mixture during incubation, and challenge the idea that preen oil has a role in incubation in these species. However, even if the preen oil of Kentish plovers does not contain any diester during incubation, it may still undergo seasonal changes, although not as dramatic as a complete shift to diesters. For example, preen oil may consist of a mixture of monoesters year-round, but the monoesters produced during incubation may be less volatile than those secreted the rest of the year. However, if the seasonal changes are only subtle, they may not affect volatility sufficiently to play a role in olfactory crypsis. In any case, we call for caution with these preliminary results, because the analytical methods used by Reneerkens (2007) (i.e. judging peak patterns from chromatograms) and our study (i.e. comparing mass spectrometry with the NIST library) are simplistic and prone to inaccuracies. This warrants a more accurate identification of the substances in the preen oil of Kentish plovers (e.g. Rijpstra *et al.* 2007, Alves Soares *et al.* 2024), as well as an estimation of volatility, using samples collected throughout the year.

From our descriptive results, we cannot conclude whether preen oil has a function in incubation in Kentish plovers. Still, we can speculate on possible incubation-related functions. Preen oil may have a role in olfactory crypsis at the nest, although there are hints that the preen oil of Kentish plovers does not follow the pattern observed in other shorebirds studied (Reneerkens 2007). Kentish plovers nest on the ground and are vulnerable to olfactorily searching nest predators, such as dogs, foxes, snakes and lizards (Fraga and Amat 1996, Kosztolányi et al. 2009). When producing a low-volatility preen oil, incubating birds (and/or their clutch or brood) may be less detectable to olfactorily searching nest predators, thereby increasing nest survival (Reneerkens 2005, Grieves et al. 2022). To further investigate this possibility, we should sample preen oil from Kentish plovers across several breeding stages (not only during incubation) and measure its volatility. Unfortunately, there is, to our knowledge, no consensual way to measure volatility,

although several methods have been proposed (e.g. Gilles et al. 2024). Future research should thus develop a standardised method to measure volatility (or detectability) from chromatograms or from biological samples. Alternatively, one can assess differences in volatility by conducting detection trials with predators or conspecifics (e.g. trained dog, Reneerkens 2005). Preen oil may also protect incubating birds from feather degrading bacteria (e.g. red knots *Calidris canutus*, Reneerkens et al. 2008; Eurasian hoopoes, Ruiz-Rodríguez et al. 2009) and their clutch from eggshell bacteria (e.g. Eurasian hoopoes, Martín-Vivaldi *et al.* 2010). To test this, we should assess the antimicrobial properties of the preen oil of Kentish plovers (e.g. Shawkey *et al.* 2003). Finally, preen oil may have a role in chemical signalling for mate choice. Sex recognition based on preen oil odours, like in dark-eyed juncos (*Junco hyemalis,* Whittaker et al. 2010, 2011a) and song sparrows (*Melospiza melodia*, Grieves et al. 2019a, b), is not likely in Kentish plovers because of the absence of sex differences during incubation. To confirm this, we should also test for sex differences in preen oil before incubation, when mate choice actually occurs. Also, preen oil may have signalling roles other than sex recognition. Birds may display their genetic compatibility (e.g. major histocompatibility complex) in their preen oil odours, like in black-legged kittiwakes (*Rissa tridactyla*, Leclaire et al. 2014) and song sparrows (Grieves et al. 2019c), and they may use preen oil to assess relatedness of a potential mate (Krause et al. 2012, Caspers et al. 2015a). It should be emphasized that preen oil could have a function for chemical protection and chemical signalling at the same time. Indeed, preen oil odours could signal greater protection of the offspring (e.g. against predators via olfactory crypsis, or against pathogens via antimicrobial activity) and thereby be sexually selected signals.

We acknowledge that our negative results (absence of sex differences) may be due to the limited sample size and thus limited statistical power (i.e. false negative, or type II error). To evaluate whether our negative results are more likely false negatives or true negatives, we can compare the effect sizes of positive results from other studies with the confidence intervals from our study (Nakagawa and Cuthill 2007). A study on the preen oil composition of blue tits found a significant sex difference in chemical richness, with females producing on average 38 substances more than males (Caspers et al. 2022). This effect size (38 substances) falls well outside our confidence interval ([–12.7; 26.4]), indicating that our study would have had the power to detect such an effect. Although this does not prove that our results are true negatives, it gives us confidence that they likely are. We note that we did not focus on the volatile fraction of preen oil, which would be the most relevant to study for its putative odour-related roles, like olfactory crypsis or chemical signalling. Instead, we analysed the whole preen oil composition, which includes both volatile and nonvolatile compounds. We did so because nonvolatile compounds may be precursors of volatile compounds involved in crypsis or signalling, and are thus also relevant for such studies (Mardon et al. 2011a). For example, the monoesters and diesters in the preen oil of red knots *Calidris canutus* are nonvolatile but still seem to have different odours or odour levels (different detection success of monoester and diester preen oil by a

dog, Reneerkens 2005). Another example is the preen oil of song sparrows, where the sex differences in nonvolatile esters seem to translate into sex differences in body odour, allowing the birds to discriminate sex by smell (Grieves et al. 2019b).

Our results are based on a single species and a single period, and thus cannot elucidate whether preen oil has a role (and which role) in incubation. However, our study provides valuable data on sex differences in preen oil. To investigate the function of preen oil in Kentish plovers, future studies should sample preen oil at different breeding stages (notably during mate choice and non-breeding) and measure its volatility. Importantly, future studies should conduct experiments, such as antimicrobial assays to test for antiparasitic protection (e.g. Reneerkens et al. 2008, Martín-Vivaldi et al. 2010), detectability trials or field experiments to test for olfactory crypsis (e.g. Reneerkens 2005, Selonen et al. 2022), and behavioural trials to test for olfactory communication (e.g. Caspers et al. 2015b, Grieves et al. 2019b).

CONCLUSION

Sex differences in preen oil composition could not be detected during incubation in Kentish plovers, a shorebird species in which both sexes incubate. This result is consistent with previous studies, where sex differences in preen oil occurred during incubation in uniparentally incubating species more than in biparentally incubating species. The similar preen oil secreted by females and males during incubation may have a function for olfactory crypsis, as proposed for other shorebird species, but also for protection against ectoparasites and/or olfactory communication, and may have no incubation-related function at all. To elucidate whether preen oil has a function in incubation, future studies should first test whether preen oil composition changes seasonally, specifically at the time of incubation.

Acknowledgements

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Data Availability

Data and code used for the analyses are available in the Supplemental Information and at the repository PUB – Publications at Bielefeld University (https://pub.uni-bielefeld.de/record/2965523, DOI: https://doi.org/10.4119/unibi/2965523).

Supporting information

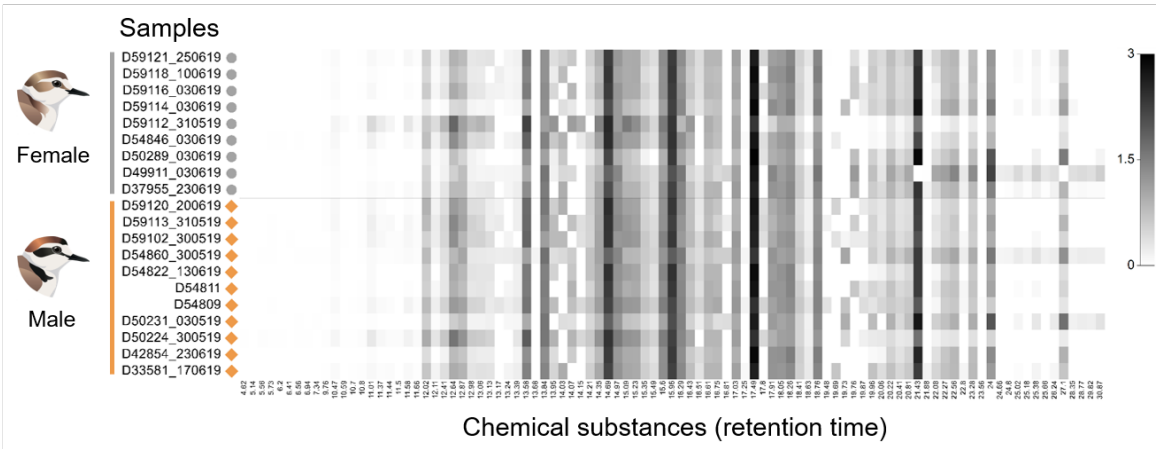

Fig. S1. Shadeplot of the relative (log-transformed) abundances of the chemical substances (N = 95) detected in the 20 preen oil samples from Kentish Plovers. Chemical substances are identified by their retention time (min).

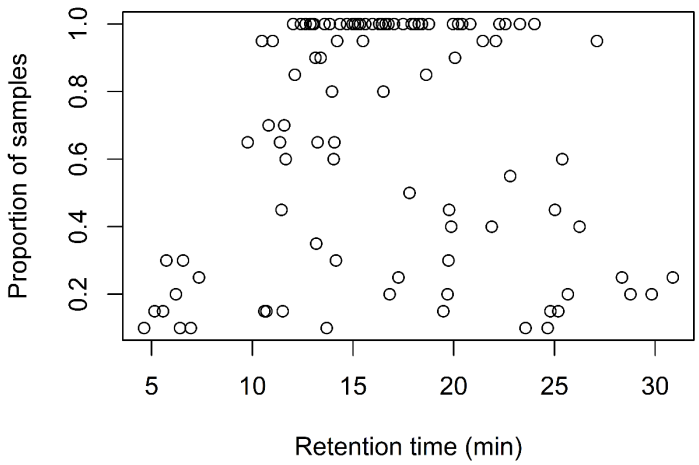

Fig. S2. Proportion of samples where a putative chemical substance occurs in relation to the retention time of the substance. Each circle represents a putative chemical substance.

Table S1. Chemical substances detected in the preen oil samples of female and male adult Kentish plovers during incubation (N = 20 samples). Chemical substances were defined by their retention time and are presented in descending order of mean relative abundance. Substances were first putatively identified at the substance level by comparing mass spectrometry (MS) with the NIST library, and then the class of the putatively identified substances was identified if it was matching across all samples. Our analytical method did not allow for an accurate identification of substance names (therefore not reported here).

Substance (retention time in min)	**Number of samples** containing the substance	**Relative abundance** (mean ± SD)	**Putative class**
17.49	20	13.70 ± 2.58	Monoester
21.43	19	8.86 ± 3.87	Monoester
15.96	20	8.53 ± 1.15	Monoester
14.69	20	8.50 ± 1.87	Monoester
13.58	20	3.69 ± 1.59	Monoester
13.84	20	3.16 ± 0.69	Monoester
18.76	20	2.93 ± 0.53	Unidentified
24.00	20	2.81 ± 2.00	Monoester
16.29	20	2.80 ± 0.59	Unidentified
18.26	20	2.72 ± 0.52	Unidentified
15.60	20	2.70 ± 0.57	Monoester
14.97	20	2.57 ± 0.47	Unidentified
18.05	20	2.23 ± 0.28	Unidentified
17.91	20	2.07 ± 0.58	Monoester
12.64	20	1.91 ± 1.14	Monoester
17.03	20	1.85 ± 0.33	Monoester
15.09	20	1.83 ± 0.66	Monoester
15.24	20	1.70 ± 0.52	Monoester
14.35	20	1.69 ± 0.56	Unidentified
12.87	20	1.40 ± 0.49	Alkene
16.61	20	1.26 ± 0.23	Monoester
27.11	19	1.22 ± 1.16	Monoester
22.56	20	1.20 ± 0.61	Unidentified
16.75	20	1.12 ± 0.34	Monoester
16.43	20	1.11 ± 0.40	Monoester
23.28	20	1.11 ± 0.53	Ether
20.22	20	0.99 ± 0.38	Unidentified
22.27	20	0.92 ± 0.43	Unidentified
12.02	20	0.89 ± 0.35	Unidentified
20.81	20	0.80 ± 0.37	Monoester
15.35	20	0.74 ± 0.24	Monoester
18.42	20	0.67 ± 0.15	Monoester
13.95	16	0.66 ± 0.62	Monoester
14.07	13	0.66 ± 0.63	Unidentified
14.21	19	0.66 ± 0.25	Unidentified
14.03	12	0.61 ± 0.62	Unidentified
19.96	20	0.60 ± 0.35	Monoester
20.41	20	0.52 ± 0.22	Unidentified
15.49	19	0.46 ± 0.16	Unidentified
12.41	20	0.45 ± 0.3	Monoester
19.76	9	0.45 ± 0.64	Unidentified
22.08	19	0.45 ± 0.40	Unidentified
12.98	20	0.43 ± 0.27	Monoester
13.06	20	0.43 ± 0.31	Monoester

Table S1. *(continued)*

Substance (retention time in min)	**Number of samples** containing the substance	**Relative abundance** (mean ± SD)	**Putative class**
16.51	16	0.36 ± 0.21	Monoester
20.06	18	0.34 ± 0.19	Monoester
19.73	6	0.32 ± 0.59	Unidentified
13.13	18	0.31 ± 0.22	Monoester
17.80	10	0.25 ± 0.28	Monoester
18.63	17	0.21 ± 0.11	Unidentified
14.15	6	0.19 ± 0.38	Unidentified
13.39	18	0.17 ± 0.10	Monoester
12.11	17	0.14 ± 0.13	Monoester
25.38	12	0.13 ± 0.18	Unidentified
11.01	19	0.12 ± 0.13	Monoester
16.81	4	0.11 ± 0.24	Unidentified
19.69	4	0.11 ± 0.27	Monoester
19.87	8	0.11 ± 0.15	Unidentified
13.24	13	0.09 ± 0.08	Monoester
22.80	11	0.09 ± 0.09	Monoester
26.24	8	0.08 ± 0.12	Unidentified
30.87	5	0.08 ± 0.19	Monoester
11.58	14	0.07 ± 0.08	Unidentified
13.17	7	0.07 ± 0.11	Monoester
25.02	9	0.07 ± 0.13	Monoester
10.47	19	0.05 ± 0.03	Unidentified
28.35	5	0.05 ± 0.11	Unidentified
28.77	4	0.05 ± 0.14	Unidentified
29.82	4	0.05 ± 0.12	Unidentified
11.37	13	0.04 ± 0.06	Monoester
17.25	5	0.03 ± 0.05	Unidentified
19.48	3	0.03 ± 0.09	Unidentified
21.88	8	0.03 ± 0.06	Unidentified
25.66	4	0.03 ± 0.09	Monoester
11.44	9	0.02 ± 0.03	Unidentified
11.66	12	0.02 ± 0.03	Unidentified
24.66	2	0.02 ± 0.06	Unidentified
24.80	3	0.02 ± 0.07	Monoester
25.18	3	0.02 ± 0.07	Monoester
9.76	13	0.01 ± 0.01	Unidentified
10.80	14	0.01 ± 0.02	Unidentified
11.50	3	0.01 ± 0.01	Unidentified
13.69	2	0.01 ± 0.03	Monoester
23.56	2	0.01 ± 0.02	Unidentified
4.62	2	< 0.01	Unidentified
5.14	3	< 0.01	Unidentified
5.56	3	< 0.01	Unidentified
5.73	6	< 0.01	Fatty acid
6.20	4	< 0.01	Unidentified
6.41	2	< 0.01	Unidentified
6.56	6	< 0.01	Unidentified
6.94	2	< 0.01	Fatty acid
7.34	5	< 0.01	Unidentified
10.59	3	< 0.01	Monoester
10.70	3	< 0.01	Unidentified

Table S2. Results from permutational multivariate analysis of variance (PERMANOVA) testing the effect of sex, the number of days after laying and the interaction between sex and the number of days after laying on the beta diversity (Bray–Curtis dissimilarities) of the preen oil of Kentish plovers. The PERMANOVA was run on 16 samples (N = 16; 7 females and 9 males) out of the 20 samples, excluding 4 samples (2 females and 2 males) among "paired" samples (i.e. from the same breeding pair), so that all samples were from different breeding pairs. The PERMANOVA was iterated 1,000 times with a randomized selection of four excluded samples at each iteration, and was run with 9,999 permutations and sequential (type I) sums of square. Sums of square (SS), R2 and F values are reported as median (interquartile range) across iterations.

Bray-Curtis dissimilarity	df	SS	R^2	F	P
Sex	1	0.02 (0.01–0.03)	0.11 (0.03–0.13)	1.77 (0.57–2.45)	0.35
Days after laying	1	0.02 (0.00–0.03)	0.11 (0.01–0.15)	1.79 (0.27–2.87)	0.48
Sex × Days after laying	1	0.01 (0.01–0.04)	0.06 (0.02–0.14)	1.06 (0.86–2.05)	0.34
Residuals	12	0.16 (0.14–0.20)	0.73 (0.64–0.80)	—	—

Table S3. Results from linear mixed models (LMMs) testing the effect of sex (fixed), the number of days after laying (fixed), the interaction between sex and number of days after laying (fixed) and pair ID (random) on the alpha diversity, namely chemical diversity (Shannon index) and richness (number of substances), of the preen oil of Kentish plovers (N = 20 samples; 9 females and 11 males).

Chemical diversity	β [95% CI]	Variance
Sex (male)	0.09 [–0.15, 0.36]	—
Days after laying	0.00 [–0.01, 0.01]	—
Sex × Days after laying	0.00 [–0.02, 0.01]	—
Pair ID	—	0

Chemical richness	β [95% CI]	Variance
Sex (male)	5.88 [–12.7, 26.4]	—
Days after laying	0.02 [–0.81,0.86]	—
Sex × Days after laying	–0.32 [–1.73, 0.93]	—
Pair ID	—	0

Testing olfactory preferences in a white-fronted plover chick using a Y-maze in the field in Madagascar

Photo by Marc Gilles

CHAPTER 6

Can chicks smell their parents? No evidence of olfactory parent recognition in a shorebird

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ABSTRACT

In many taxa, young can recognise their parents using olfactory cues. Yet this possibility has been overlooked in birds, because they were long assumed to have a poor sense of smell. While evidence is growing that birds use odours to communicate, olfactory parent recognition has only been documented in two altricial bird species. Whether chicks of precocial species use olfaction to recognise parents is currently unknown. Parent recognition is particularly important in precocial species, as chicks leave the nest shortly after hatching, and may lose contact with their parents and encounter other conspecific adults. We conducted Y-maze trials in the wild to test if chicks of a precocial shorebird, the white-fronted plover (*Anarhynchus*, formerly *Charadrius*, *marginatus*), can recognise parents via olfaction. We tested first if chicks show a preference for the odour (preen oil) of an unfamiliar adult over a control (no odour), and second if chicks show a preference for the odour of a parent over that of an unfamiliar adult. Plover chicks spent as much time with the odour of an unfamiliar adult as with the control, and as much time with the odour of a parent as with that of an unfamiliar adult. Therefore, we found no evidence that chicks react to the preen oil odour of a conspecific adult, nor that they can discriminate a parent using preen oil odours. It may be that chicks of this species recognise parents using other (e.g. auditory) cues, or the olfactory cues are perceptible, but not expressed in the context of the experiment.

INTRODUCTION

Kin recognition – the ability to recognise close relatives – has evolved in many animal taxa (Waldman 1988, Hepper 1991). It allows individuals to cooperate with and care for relatives, and avoid mating or competing with them, thereby increasing their inclusive fitness (Hamilton 1964, Waldman 1988). One important context of kin recognition is parental care, where it is advantageous for parents and offspring to recognise one another (Hepper 1986, Waldman 1988). Indeed, parents should recognise their offspring (offspring recognition) to direct their parental care towards their offspring rather than nonkin young (Waldman 1988). Offspring should also recognise their parents (parent recognition) to solicit parental care (e.g. begging) from their parents rather than nonkin adults (Jacot et al. 2010). Parent recognition is likely to evolve in species where young can confuse their parents with nonkin conspecific adults, such as colonially breeding species (Beecher et al. 1986, Aubin and Jouventin 1998) and precocial species (Mathevon et al. 2003, Scheiber et al. 2017). Precocial young are mature and mobile from the moment of hatching or birth (i.e. precocial) and can leave the nest shortly after (i.e. nidifugous). Thus, they are likely to lose contact with their parents and meet nonkin adults, from which soliciting care could have a cost. Indeed, chicks that solicit care from unrelated adults can be rejected (Beecher et al. 1981, Davis and McCaffrey 1989) or attacked (Proffitt and McLean 1990, Öst and Bäck 2003, Kalmbach et al. 2005). In addition, the unsuccessful begging by the chicks may attract predators (Lima 2009). However, we note that soliciting care from nonkin adults is not necessarily costly and may even be advantageous. For example, in case adults cannot discriminate between kin and nonkin young, young can take advantage of care provided by nonkin adults without risking eviction or aggression. Also, in case young have lost their parents, they may benefit from any care and should thus solicit care from other adults, even at the risk of eviction or aggression, or they may receive no care at all ("best of a bad job") (Kalmbach 2006). In such cases, parent recognition provides no (or only limited) selective advantage.

In birds, studies on parent-offspring recognition have focused on auditory and visual cues (Beecher 1988, Komdeur and Hatchwell 1999, Jacot et al. 2010), overlooking the potential role of olfactory cues. Yet, we now know that birds can use olfaction for intraspecific communication (reviewed in Hagelin and Jones 2007, Caro et al. 2015, Krause et al. 2018, Grieves et al. 2022), including parent-offspring recognition. Indeed, adult birds can recognise the odour of their own nest (four petrel species, Bonadonna et al. 2003a, Bonadonna et al. 2003b; two finch species although only females, Krause and Caspers 2012), eggs (zebra finch females, Golüke et al. 2016; blue petrel females *Halobaena caerulea*, Leclaire et al. 2017a) and chicks (zebra finch, although only males, Golüke et al. 2021). However, female spotless starlings (*Sturnus unicolor*) do not discriminate between their own and other chicks using smell (Amo et al. 2014). Several studies have shown that young birds recognise and prefer familiar nest odours (domestic chicken *Gallus gallus domesticus*, Burne and Rogers 1995, Jones and Gentle 1985; greylag geese *Anser anser*, Würdinger 1982; European storm-petrel *Hydrobates pelagicus*, Mínguez 1997; Leach's

storm-petrel *Oceanodroma leucorhoa*, O'Dwyer et al. 2008; zebra finch, Caspers et al. 2013, 2015, Caspers and Krause 2011). In contrast, only two studies have investigated whether young birds can recognise parental odours.

A first study found that zebra finch hatchlings prefer, i.e. beg longer in response to, the odour of a familiar parent over that of an unfamiliar adult (Caspers et al. 2017b). Moreover, zebra finch hatchlings prefer the odour of their genetic (unfamiliar) mother over that of their foster (nonkin familiar) mother (Caspers et al. 2017b). In line with these results, a second study found that tree swallow (*Tachycineta bicolor*) nestlings begged longer and more intensely at the odour of a familiar adult (parent) than at the odour of an unfamiliar adult (Griebel and Dawson 2020). Interestingly, these two species (zebra finches and tree swallows) are altricial, with chicks staying in the nest for an extended period after hatching (i.e. nidicolous), and may thus not need parent recognition, at least during the nestling phase (Scheiber et al. 2017). This begs the question whether chicks of precocial species, where selection pressure for parent recognition should be stronger, also show olfactory parent recognition.

In this study, we tested whether the precocial chicks of white-fronted plovers (*Anarhynchus marginatus*, formerly *Charadrius marginatus*) can recognise their parents via olfaction. White-fronted plover chicks leave the nest scrape shortly after hatching, and are attended and defended by their parents for an extended period of time (Safford and Hawkins 2020, Zefania and Székely 2022). Chicks can lose contact with their parents (e.g. after hiding from a threat, or during territorial fights between adults) and, in places with high nesting densities, encounter foreign conspecific adults, which increases the necessity for parent recognition. Using a Y-maze in the field, we conducted two behavioural trials to investigate the responses of chicks to olfactory cues (preen oil) from adult conspecifics (unfamiliar adults and parents). In a first trial, chicks were exposed to the odour of an unfamiliar (and nonkin) adult on one side and a control (no odour) on the other side. If chicks identify the odour of the unfamiliar adult as a non-parent, and if there is a cost of soliciting care from an unfamiliar adult, we predicted that chicks would avoid it and spend more time with the control. Alternatively, if there is no cost of soliciting care from an unfamiliar adult, chicks may be attracted to the odour of an unfamiliar adult and thus spend more time with it. In a second trial, chicks were exposed to the odour of an unfamiliar (and nonkin) adult on one side and the odour of a parent on the other side. If chicks can discriminate the odour from their parents, we predicted them to show a preference for the odour of a parent, especially if there is a cost of soliciting care from an unfamiliar adult.

MATERIALS AND METHODS

Study species and subjects

We studied white-fronted plovers at Andavadoaka (S°22.02, E°43.39) in southwest Madagascar in April–May 2022. At the study site, white-fronted plovers breed on salt marshes and sandy beaches (Jones et al. 2022). Nests are on average 250 m (SD = 170 m)

apart, but can be as close as 15 m apart (unpublished data). Breeding occurs year-round, but increases between February and June, after seasonal periods of heavy rainfall. White-fronted plovers are monogamous, with no extra-pair mating (Maher et al. 2017). They exhibit biparental care, with both partners defending nest territories, incubating the eggs (1–4 eggs, usually 2) and caring for the chicks (1–4 chicks, usually 2; Eberhart-Phillips 2019, Safford and Hawkins 2020). Chicks are precocial (**Fig. 1**) and leave the nest shortly after hatching, but still need care from their parents until fledging (28–38 days), for example for protection against predators or thermoregulation (Safford and Hawkins 2020). After losing contact with their parents (e.g. after hiding from a predator, or during territorial fights between adults from different families), chicks walk around in search of their parents and may encounter nonkin adults, especially in places with high nesting densities. As a result, broods can get mixed (chicks raised by unrelated adults), although rarely (Maher et al. 2017). It should be noted here that the occurrence of brood mixing does not imply the absence of recognition between parents and offspring. For example, Caspian tern (*Sterna caspia*) can adopt foreign young although they can discriminate between own and foreign young (Shugart 1978). Young white-fronted plovers remain with their parents for 2–3 months (Safford and Hawkins 2020).

When finding a family (i.e. at least one parent with at least one non-fledged chick), we first approached the chick(s) carefully and captured them by hand. We then placed the chick(s) under a sieve in a trap (spring or funnel trap) to attract and capture the parents (Székely et al. 2008). Chicks were captured to participate in odour preference trials (**Fig. 1**), whereas parents were captured to collect test odours for the trials. In total, we captured 44 chicks (from 33 families), all of which participated in odour preference trials. Chicks were between 1 and 23 days old, but most were less than 5 days old (average = 8 days; interquartile range = 12 days; age determined using tarsus length following Parra 2015). The sex of the chicks was unknown during the trials and was determined after molecular sexing (19 females, 23 males, 2 chicks of unknown sex). For sexing, blood (25–50 µl) was

Fig. 1. A white-fronted plover chick (left) released after participating in a behavioural trial in a Y-maze (right) in the wild in Madagascar. Photos by Marc Gilles.

sampled from the medial metatarsal (leg) vein (Székely et al. 2008) and stored in ethanol, DNA was extracted using a standard phenol-chloroform protocol, and sex was determined using established molecular methods developed for *Charadrius* species (described in Jones et al. 2022).

Test odours

As test odours, we used preen oil (secretion from the uropygial gland) swabs from adult white-fronted plovers collected during chick-rearing (between 1 and 23 days after hatching). Preen oil is a major source of bird odour (Hagelin and Jones 2007) and is commonly used as a proxy of body odour in behavioural trials (e.g. Grieves et al. 2019b, Whittaker et al. 2011a). We used preen oil swabs from chick-rearing birds only, because preen oil composition is known to change across breeding stages (Reneerkens et al. 2002, Gilles et al. 2024). In our odour preference trials, we used three types of test stimuli: parent odour (preen oil swab from a parent of the chick tested), unfamiliar adult odour (preen oil swab from an adult of another family than the chick tested), and no odour (swab with no preen oil). Because there is no extra-pair paternity and only a low probability of intraspecific brood parasitism or brood mixing (< 3.45%) in this species (Maher et al. 2017), familiarity and genetic relatedness were confounded. "Unfamiliar adults" were most likely both unfamiliar and unrelated (nonkin) to the tested chick, while "parents" were most likely both familiar and related (genetic parents) to the tested chick. For each family, we aimed to catch both parents, but in most cases we caught only one parent, and in some cases no parent at all. From our experience, it was more difficult to capture parents with older chicks (more than one week old), as they came less close to the captured chicks.

We collected preen oil from adult birds by swabbing their uropygial gland with a cotton swab, wearing nitrile gloves. To standardize the quantity of preen oil collected, we systematically swabbed the uropygial gland 20 times. Preen oil swabs were stored in Teflon-capped 20 ml glass vials (Labsolute, Th. Geyer, Renningen, Germany), which were put in a fridge upon return to the field station at the end of the day and kept refrigerated until use in odour preference trials. In total, 34 preen oil swabs (from 34 individuals) were used in trials, and they were used on average in 2.4 ± 1.4 different trials. We systematically used the most recent preen oil swabs (sampled maximum 8 days before the trials). Due to methodological constraints, preen oil swabs from unfamiliar adults were sampled on average 2.8 ± 2.3 days before use in trials, whereas preen oil swabs of parents were sampled shortly (less than one hour) before use in trials. We controlled for this confounding effect by verifying that the freshness of the preen oil swabs had no effect in the analysis. As white-fronted plovers are sexually monomorphic (Zefania et al. 2010), we were blind to the sex of the birds sampled, which was revealed only after molecular analyses (same method as for chicks except that blood was sampled from the brachial vein; Jones et al. 2022, Székely et al. 2008). We controlled for the potential effect of the sex of the test odours (see *Statistical analysis*), although we expected chicks to respond similarly to odours from

females or males, because both sexes care for the chicks in this species (Eberhart-Phillips 2019).

Y-maze apparatus

Odour preference trials were conducted directly in the field (**Fig. 1**) using a Y-maze consisting of one start arm and two test arms (**Fig. 2**, three symmetrical arms, 20 x 15 x 15 cm angled at 120°, PVC material, more details in **Fig. S1**). The floor of the maze was covered with a metal grid to allow chicks to walk without slipping. In the start arm, an acclimation chamber (15 x 15 x 15 cm) was covered with an opaque ceiling, and was separated by an opaque PVC door that could be slid open to allow the bird to explore the maze (**Fig. 2**). The bird was placed in the acclimation chamber using a sliding door made of perforated PVC which allowed airflow but kept the acclimation chamber dark. The acclimation chamber was dark so that chicks could calm down after the stress of capture. The test arms were covered with semi-transparent Plexiglas (**Fig. S1**), allowing video recording from above, while limiting the effect of the environment from above the maze (e.g. clouds, trees). Chicks were therefore in the dark in the acclimation chamber, but not during the trial. Test odours (i.e. cotton swabs) were placed at the end of the two test arms, separated by an opaque perforated PVC barrier, which allowed olfactory cues, but not visual cues, to be perceived from within the maze. Test odours were positioned vertically on a nail, with the cotton tip (containing preen oil, except for the "no odour" stimulus where it contained no preen oil) reaching a height of 5 cm. To control the air flow coming from each test odour, we placed fans (Pure Wings 2, BeQuiet, Glinde, Germany) at the end of the test arms, which circulated air from the test odours towards the start arm. Trials were video recorded

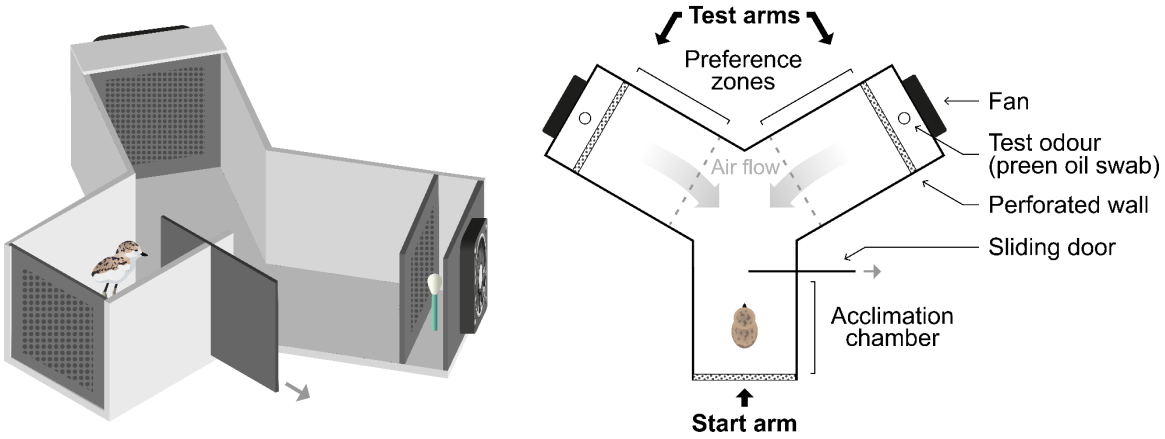

Fig. 2. Schematic representation of the Y-maze in 3D (left) and from above (right). The semi-transparent ceiling and one opaque wall of the right arm are not displayed on the 3D representation (left) for a better visualization of the inside of the Y-maze. At the start of a preference trial (i.e. when opening the door of the acclimation chamber), the plover chick was allowed to explore the two test arms, from which came the odours, circulated by an air flow. To measure olfactory preferences, we recorded videos of the trials from above and measured the amount of time the chick spent with each test odour (i.e. in the preference zones) and which test odour it visited first. Illustrations by Marc Gilles.

using a camera mounted on a tripod positioned above the maze. We set up the maze in the field at least 200 m from the territory of the family, to avoid any interference from non-captured parent(s) (e.g. auditory cues). We placed the maze in the shade to limit the effect of sunlight. Also, we chose a place protected from the wind and orientated the maze with the start arm facing the wind to limit the effect of the wind.

Odour preference trials

Chicks participated in two successive odour preference trials in the Y-maze:

(1) Conspecific odour preference trial: odour of an unfamiliar adult *versus* no odour.

(2) Parent odour preference trial: odour of an unfamiliar adult *versus* odour of a parent.

We conducted the trials in this order, because we wanted to test first whether chicks react to (prefer or avoid) the odour of a conspecific adult (even if not their parent). In the second trial, we tested whether chicks show a preference for (and thus discriminate) the odour of a parent over the odour of an unfamiliar adult. Using this fixed order, we reduced potential carryover effects (Bell 2013), as chicks might have behaved differently in the second trial depending on whether they had been exposed to the odour of their parent in the first trial or not. The location (left or right arm of the Y-maze) of the test odours (parent odour, unfamiliar adult odour, absence of odour) was alternated systematically for each trial.

After capture, the chick(s) was transported in a cotton bag to the Y-maze. The maze was washed with 96% ethanol and allowed to air dry before each trial to remove any odour residue. We placed the test odours at the end of the test arms and the chick in the acclimation chamber. When threatened, plover chicks hide and stay still for a while (from a few minutes to an hour) before going out to search for their parents. Thus, we decided on a 10 min acclimation period (in the dark acclimation chamber where chicks calmed down) to allow the chicks to feel confident enough to explore the Y-maze in search of their parents. After the acclimation period, we started video recording, turned on the fans, and opened the door of the acclimation chamber to start the trial. The chick was allowed to explore the maze during a trial period of 15 min. If the chick did not leave the acclimation chamber, the trial was considered unsuccessful and was discarded. During the trial period, the chick could go in the test arms but also return to the start arm. In total, we recorded 44 conspecific odour preference trials (29 successful, 15 unsuccessful) and 30 parent odour preference trials (24 successful, 6 unsuccessful). Most chicks participated in both trials (N = 30 chicks), but some chicks participated only in the first trial (conspecific odour preference trial, N = 14 chicks), in those cases where no parent odour had been sampled. Chicks did not participate more than once in each trial. In successful trials, chicks did not necessarily make a single choice but rather explored the entire maze and often switched between arms. We scored the amount of time spent by the chick in each arm of the maze (up to 15 cm from the end of the arm, see "preference zones" in **Fig. 2**) using the BORIS software (Friard and Gamba 2016). Trials were scored blind with respect to the identity of

the chick and the test odours. An example of a trial video is available in the Supplementary Material.

Ethical statement

This study was approved by the « Direction des Aires Protégées, des Ressources Naturellement Renouvelables et des Ecosystèmes » from Madagascar (permits no. 386/21 and 282/23/MEDD/SG/DGGE/DAPRNE/SCBE.Re). We attempted to minimize the stress of the birds at all stages of the study. Chicks were captured by hand after a careful observation and approach. The chick(s) were then placed under a shaded sieve in a trap (spring trap with remote trigger or funnel trap) to attract and capture the parents (Székely et al. 2008). If the parents were not trapped after 20 min, we stopped the capture attempt. After capture, birds were held in opaque cloth bags to minimize stress. The birds, when held in the cloth bags or during trials in the Y-maze, were always kept in a warm place (to avoid overcooling) in the shade (to avoid overheating). We started the behavioural trials immediately after the 20 min of capture attempt in case no parent could be captured, or immediately after sampling the preen oil of a parent in case a parent could be captured. Chicks were transported to the Y-maze in opaque cloth bags and were placed in a dark acclimation chamber in the Y-maze for 10 min to reduce stress before the trial. Parents were sampled, measured and ringed during the trials to minimize the handling time. After the trial(s), chicks were measured, sampled and ringed. We collected a small blood sample from all adults (25–50 µl blood) and chicks (10–25 µl blood) for sexing. Chicks were then released together with their sibling (if two chicks of a family were captured) and their parents (if their parents were captured) at the place where they were captured (Székely et al. 2008). Upon release, we made sure that the parents reunited with their chick(s). For families breeding on beaches, we tried to synchronise the release of the birds with the low tide so that they could feed directly after release. In total, birds were held between 45 min (in cases where only one chick of a family was tested for the first trial only) and 2 h 15 (in cases where two chicks of a family were tested for both trials).

Statistical analysis

Statistical analyses were conducted using all successful trials (N = 29 conspecific odour preference trials, N = 24 parent odour preference trials). We used as a preference measure the proportion of time spent in the arm with the unfamiliar adult odour relative to the time spent in both test arms, with values > 0.5 indicating a preference for the unfamiliar adult odour and values < 0.5 indicating a preference for the other odour (no odour in the first trial, parent odour in the second trial). For each trial, we ran a one sample t-test to test if the proportion of time spent with the unfamiliar adult odour differed from 0.5 (i.e. if chicks showed a preference for either odour). In addition, we recorded the odour which the chicks visited first, as their first choice may also indicate a preference. For each trial, we ran a two-tailed binomial test to test whether they consistently visited one type of odour first. Trials from sibling chicks (11 families where two siblings were tested) were considered

independent, as we used different test odours (although the same parent odour had to be used for both chicks in the parent odour preference trial, in case only one parent could be captured, N = 5 families) and in different locations (left or right arm of the Y-maze).

For each trial, we controlled for the potential effect of the sex and the freshness (number of days between sampling of the odour and use in the trial) of the test odours. For the proportion of time spent with the unfamiliar adult odour we ran a beta regression using the *glmmTMB* package (Brooks et al. 2017), and for the odour visited first we ran a generalized linear model (GLM) with binomial distribution, both with *sex* and *freshness* of test odour as fixed effects. For the parent odour discrimination trial, the *sex* of test odour included three levels (mother *versus* unfamiliar female, mother *versus* unfamiliar male, father *versus* unfamiliar male; coincidentally there was no trial with father *versus* unfamiliar female) to test if chicks showed stronger preferences when using the odour of their mother or that of their father. Because the sex of one test odour was unknown, these models included one less trial (N = 28 conspecific odour preference trials, N = 23 parent odour preference trials). We also investigated potential side biases (left or right) using one sample t-tests for the proportion of time spent in the test arms, and binomial tests for the arm visited first.

In an exploratory analysis, we investigated the behavioural response of the chicks to their first exposure to the Y-maze (i.e. novel environment). We measured the probability and latency to leave the acclimation chamber in the first trial, and tested whether these measures were affected by the sex and age of the chicks. We ran a generalized mixed model (GLMM) with binomial distribution to test for differences in the probability to leave the chamber (all first trials with sexed chicks, one trial per chick, N = 42 trials), and with gamma distribution to test for differences in the latency to leave the chamber (only successful first trials with sexed chicks, one trial per chick, N = 28 trials) using the *lme4* package (Bates *et al.* 2014). For both models, s*ex* and *age* of the chicks were included as fixed effects, while *brood* was included as a random effect to control for the non-independency of sibling chicks.

Model assumptions were verified using the *performance* package (Lüdecke et al. 2021) and plots were created using the *ggplot2* package (Wickham 2016). We assessed the significance of our tests at $\alpha = 0.05$ by checking *P*-values (significant if $P < 0.05$) for all tests, and 95% confidence intervals (significant if 95% CI does not contain 0) for the fixed effects of beta regressions, GLMs and GLMMs. The analysis was performed in R v4.2.2 (R Core Team 2022). Data and code (R Markdown document, Baumer and Udwin 2015) used in the analyses are available on Github (https://github.com/marc-gilles/parent-recognition-plovers).

RESULTS

Chicks showed no preference in the conspecific odour preference trial. They did not spend more time in the arm with the odour of an unfamiliar adult (average ± SD = 131 ± 102 s)

than in the arm with no odour (average ± SD = 123 ± 106 s) (one sample t-test: mean [95% CI] = 0.51 [0.42, 0.62], t = –0.88, P = 0.71, **Fig. 3**), nor did they show any preference in their first choice (binomial test: probability [95% CI] = 0.45 [0.26, 0.64], P = 0.71). Chicks also exhibited no preference in the parent odour preference trial. They spent as much time with the odour of an unfamiliar adult (average ± SD = 134 ± 123 s) as with that of their parent (average ± SD = 115 ± 81 s) (one sample t-test: mean [95% CI] = 0.46 [0.35, 0.57], t = –0.71, P = 0.48, **Fig. 3**), and they did not visit first the odour of their parent (binomial test: probability [95% CI] = 0.56 [0.33, 0.74], P = 0.83). The preference measures (time spent with odour and odour visited first) were neither affected by the sex, nor by the freshness of the test odours (**Tables S1 & S2**). The chicks showed no side bias, neither in duration (one sample t-test: mean time spent in the left arm [95% CI] = 0.46 [0.36, 0.56], t = –0.88, P = 0.38) nor in their first choice (binomial test: probability of choosing the left side first [95% CI] = 0.48 [0.30, 0.67], P = 1).

The exploratory analysis revealed that the behavioural responses of the chicks to their first exposure to the Y-maze (novel environment) was affected by sex and age (**Table**

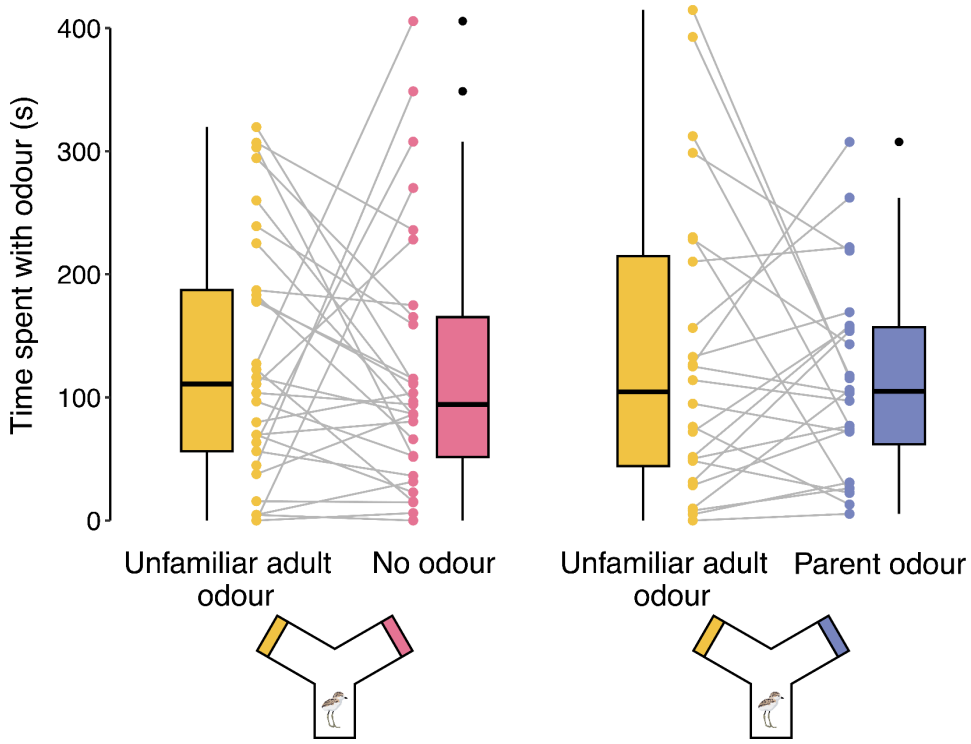

Fig. 3. Time spent by plover chicks with the odour of an unfamiliar adult *versus* no odour (left, conspecific odour discrimination trial, N = 29 trials) and with the odour of an unfamiliar adult *versus* the odour of a parent (right, parent odour discrimination trial, N = 24 trials). Each grey line represents a chick in a trial. Trials lasted 15 min (900 s). Note that chicks could be in either test arm but also in the start arm. To test for chicks' preferences, we tested whether the proportion of time spent in the arm with the unfamiliar adult odour relative to the time spent in both test arms differed from 0.5 using one sample t-tests (conspecific odour discrimination trial (left): mean [95% CI] = 0.51 [0.42, 0.62], P = 0.71; parent odour discrimination trial (right): mean [95% CI] = 0.46 [0.35, 0.57], P = 0.48).

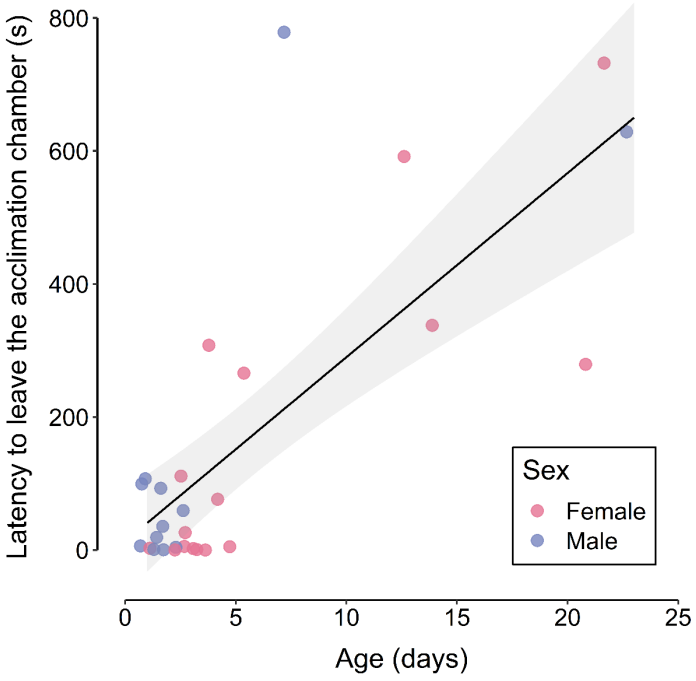

Fig. 4. Sex and age differences in the latency of plover chicks to leave the acclimation chamber during their first trial in the Y-maze. The black line represents the linear regression, and the grey area the 95% confidence interval, of latency on age.

S3). Males were less likely to leave the acclimation chamber than females (GLMM: odds ratio [95% CI] = 0.11 [0.02, 0.77], $P = 0.03$) and also waited longer than females before leaving the chamber (GLMM: estimate [95% CI] = 3.32 [1.05, 10.56], $P = 0.04$, **Fig. 4**). Older chicks were less likely to leave the chamber (GLMM: odds ratio [95% CI] = 0.83 [0.74, 0.93], $P = 0.002$) and waited longer before leaving the chamber (GLMM: estimate [95% CI] = 1.23 [1.08, 1.39], $P < 0.001$, **Fig. 4**). Because the model on the latency to leave the chamber included only chicks that left the chamber, and because older males were less likely to leave the chamber, the average age of males (3.6 days old) that left the chamber was lower than that of females (6.8 days old), although not significantly (t-test: $t = 1.30$, $P = 0.21$).

DISCUSSION

Only recently have researchers started to study if birds, as many other taxa, use olfactory cues for kin recognition. We tested for olfactory parent recognition in a precocial shorebird, where parent-offspring recognition should be important, due to the high mobility of chicks and hence the high risk of brood mixing. Contrary to our predictions, we found neither a preference for the odour of an unfamiliar adult over a control (no odour), nor a preference for the odour of a parent over that of an unfamiliar adult. These results fit four mutually non-exclusive hypotheses.

Ability to smell

First, white-fronted plover chicks may not be able to perceive odours. Although we did not investigate the ability of chicks to smell (e.g. using a neurological approach), we think that

this hypothesis is unlikely. Most of the bird species tested to date have been shown to have a functional sense of smell (Abankwah et al. 2020) and chicks of other bird species can discriminate odours shortly after hatching (Porter et al. 1999, Caspers et al. 2015b, 2017). In fact, olfaction is among the first sensory systems to develop and is functional even before hatching (Burne and Rogers 1999, Lickliter 2005, Bertin et al. 2010). In case olfactory parent recognition is acquired via associative learning (or imprinting), it may, however, be argued that chicks in our experiments were too young and did not have sufficient time to learn the odours from their parents. Indeed, associative learning seems to be the most common mechanism of parent recognition, and kin recognition in general (Komdeur and Hatchwell 1999). Nevertheless, odour learning can start early during development, even *in ovo*, and very young chicks are able to recognise familiar odours (1-day-old and 4-day-old domestic chickens, Bertin et al. 2010, Burne and Rogers 1999; 1-day-old and 2-day-old zebra finches, Caspers et al. 2015b, 2017; 2-day-old tree swallows, Griebel and Dawson 2020; 7-day-old blue tits *Cyanistes caeruleaus*, Rossi et al. 2017). On the contrary, we may even argue that chicks in our study were too old to respond to odours. Indeed, in some species, olfaction is mostly important during early life (i.e. shortly after birth or hatching) and becomes less important as visual and auditory senses develop (Lickliter 2005). We hypothesize that, during the first days after hatching, altricial chicks may rely on odours more than precocial chicks, because their visual and auditory senses are less developed (e.g. closed eyes). This could explain why altricial chicks of zebra finches and tree swallows could discriminate parental odours (Caspers et al. 2017, Griebel and Dawson 2020), while precocial plover chicks do not seem to. More studies on precocial species are needed to confirm this. In any case, we would rule out the possibility that white-fronted plover chicks are anosmic.

Need for parent recognition

Second, white-fronted plover chicks may not need parent recognition. Because white-fronted plovers are precocial, chicks are likely to encounter non-parent adults, from which they should not solicit parental care, in order to avoid unnecessary energy expenditure, brood mixing, aggression or increased exposure to predators (Beecher et al. 1981, Davis and McCaffrey 1989, Kalmbach et al. 2005, Lima 2009). However, even at high nesting densities, white-fronted plover chicks will not encounter hundreds of conspecific adults, as is the case in colonially-breeding species (Aubin and Jouventin 1998). In addition, the costs of misidentification of parents may not be high. In fact, soliciting care from unrelated adults may bear no cost at all for the chicks, and may even be beneficial (Kalmbach 2006). For example, adults may respond favourably to any begging chick (e.g. if they are unable to discriminate between kin and nonkin offspring, or if there is a low risk of misidentification combined with a low cost of caring for unrelated chick), and chicks may therefore safely and successfully beg towards unrelated adults (Beecher 1988). This is for example the case in pied avocets (*Recurvirostra avosetta*), where chicks seem to benefit from joining other families (Lengyel 2002). In conclusion, the selection pressure in this species may not be

strong enough for parent-offspring recognition to evolve. Alternatively, if the selection pressure for parent-offspring recognition is only moderate, unidirectional recognition might be sufficient (Knörnschild and von Helversen 2008): chicks may not recognise their parents but their parents may recognise them. This way, chicks could simply respond to any adult providing care to them. If this is the case (i.e. offspring recognition only), we can question whether isolated chicks actually search for their parents actively (i.e. in a goal-oriented way, responding to parental cues), which was an assumption of this study, or whether they randomly move around to maximise the chance that their parents will find them. Interestingly, in ungulate species in which young hide between nursing periods ("hider" species), parent-offspring recognition is often unidirectional (either offspring recognition only or parent recognition only), and this has been assumed to be an anti-predatory adaptation (limiting the emission of cues on which predators could eavesdrop, Torriani et al. 2006). In plovers, chicks have a similar "hider" anti-predatory strategy, and we can speculate whether this shaped a similar unidirectional parent-offspring recognition (offspring recognition only). To investigate this further, we should first test whether parent plovers can discriminate between their own chicks and foreign chicks (e.g. by auditory cues, olfactory cues, or contextual cues like spatial cues or the size of the chicks). In such a scenario, where chicks should hide from olfactorily-searching predators, we can also hypothesize that chicks would have a reduced odour to be olfactorily cryptic (Grieves et al. 2022).

It should be noted that olfactory parent recognition has been reported in two altricial species (zebra finches and tree swallows, Caspers et al. 2017, Griebel and Dawson 2020), where parent-offspring recognition is presumably not highly beneficial (Scheiber et al. 2017). Indeed, in altricial species, parents can locate their offspring simply by locating their nests, as chicks do not switch between nests, and discriminating between own and foreign nest would be sufficient (Becciu et al. 2021). Finally, parent-offspring recognition can be beneficial not only during parental care, but also during subsequent breeding when offspring have reached sexual maturity. Indeed, parents and offspring should recognise each other during mate choice to avoid inbreeding (Bonadonna and Sanz-Aguilar 2012), notably in philopatric species (regardless of altricial or precocial development). For example, in the philopatric European storm-petrels, parents and their past offspring seem to discriminate and avoid each other (also based on olfactory cues) during mate choice (Bonadonna and Sanz-Aguilar 2012). To summarize, **(1)** parent recognition may not be highly beneficial in this species, and it would be interesting to investigate olfactory parent recognition in a species with higher risk of intermingling between families (e.g. colonially-nesting species with precocial chicks); **(2)** parent-offspring recognition may be unidirectional in this species, so we should test for olfactory offspring recognition in adults; **(3)** however, extrapolating from the zebra finch and tree swallow studies mentioned above, even if not highly beneficial during chick-rearing, we might expect that parent recognition might still be present in this species.

Need for olfactory cues

Third, chicks may use other cues than odours to recognise their parents. Chicks from other *Charadriiformes* species (i.e. shorebirds, gulls, auks and allies) seem to rely on auditory cues for parent recognition (e.g. laughing gull *Larus atricilla*, Beer, 1969; thick-billed murre *Uria lomvia*, Lefevre et al. 1998, although more evidence is needed for shorebirds, Johnson et al. 2008). A hint for acoustic parent-offspring recognition in white-fronted plovers, is that both adults and chicks were calling when they were reuniting after the behavioural trials (*personal observations*). It would be interesting to investigate if white-fronted plover chicks recognise parents based on their calls. Nonetheless, even if *Charadriiformes* species (including white-fronted plovers) use calls for recognition, they may use odours in addition (multimodal communication, Higham and Hebets 2013). Indeed, in several species, parent recognition is not based on calls only, but on a combination of sensory cues. For example, in domestic sheep (*Ovis aries*), young use auditory and visual cues to recognise their mother over long distances, and olfactory cues at a closer range (Lindsay and Fletcher 1968). Following this hypothesis, it could be that odours alone (not in combination with calls) are not sufficient for chicks to recognise their parents. To our knowledge, our study is the first to investigate olfactory parent recognition in a *Charadriiformes* species (in fact in any other avian order than *Passeriformes*). The absence of evidence reported in this study should not discourage researchers from studying olfactory communication in these species. Quite the contrary, olfactory communication was already reported in some *Charadriiformes* species (crested auklet *Aethia cristatella*, Hagelin 2007b; black-legged kittiwake *Rissa tridactyla*, Pineaux et al. 2023) and more research is needed to establish if there is olfactory parent-offspring recognition in this order.

Methodological limitations

Fourth, the absence of preferences in our experiments might be due to methodological limitations. First and foremost, we acknowledge that this study lacks as a positive control, i.e. evidence that the experimental setup can elicit preferences in plover chicks. To validate the methodology, we could have run preference trials using stimuli that chicks are expected to prefer (e.g. warmth *vs* cold, food *vs* no food). However, our methodology should be valid, because we followed methods from other studies (Y-maze setup, odour stimuli, duration of habituation and test periods, scoring of preferences) that successfully detected odour preferences in various bird species, both in captivity (13 songbird species and one parrot species; Grieves et al. 2019b, c, Krause et al. 2023, Van Huynh and Rice 2019, Whittaker et al. 2011a, Zhang et al. 2010, Zhang et al. 2013) and in the wild (three seabird species; Bonadonna and Mardon 2010, Bonadonna and Sanz-Aguilar 2012, Leclaire et al. 2017a, b, Mardon and Bonadonna 2009). We note that the studies performed in the wild were all conducted on burrowing seabirds, for which the Y-maze may be less stressful. It is possible that the chicks were too agitated (e.g. because of the isolation or the apparatus) during the trials to exhibit any preference. Yet we believe that our trials are ecologically

relevant, because we investigated parent recognition in the context of chicks losing contact with their parents (e.g. after hiding from a predator), which is also stressful. To overcome the possible hindering effect of the agitation of the chicks, we would need to capture complete families and keep them in captivity for a few days to habituate the chicks, before sampling parental odours and conducting trials with habituated chicks. Second, we used preen oil swabs from adult birds as odour stimuli. Although preen oil is an important source of avian body odour (Mardon et al. 2011a), it is not the only one (e.g. plumage, skin, faeces, Hagelin and Jones 2007). Furthermore, preen oil substances may be altered once spread onto the plumage (e.g. by feather microbiota, Jacob et al. 2014), and as a result plumage and overall body odour may differ from preen oil odour (e.g. Alves Soares et al. 2024b, Leclaire et al. 2019). In contrast to our study, the two previous studies on parent recognition in zebra finches (Caspers et al. 2017b) and tree swallows (Griebel and Dawson 2020) used overall body odours as odour stimuli. Thus, we cannot rule out the possibility that chicks in our study can recognise their parents by smell, but not based on preen oil odours only. However, it should be noted that dark-eyed juncos (*Junco hyemalis*) and song sparrows (*Melospiza melodia*) responded to preen oil odours in similar Y-maze trials (Whittaker et al. 2011a, Grieves et al. 2019b). Third, it might be possible that the test odours have faded after sampling and were not strong enough to be perceived by the chicks. This is however unlikely, as chicks did not respond to parent odours, which were sampled right before the trial, and we found no effect of the freshness of the test odours (number of days between sampling of the odour and use in the trial) on the behaviour of the chicks. Fourth, it is possible is that the chicks could discriminate between the odours but, as they could not find any adult bird in the Y-maze, they ceased searching after a while. This was however probably not the case, since we also found no preference at the beginning of trials (i.e. first odour visited). Finally, it could be that there was no preference in the second trial (unfamiliar adult odour *versus* parent odour) because of the simultaneous presentation of odours, which can hamper preferences (Krause and Caspers 2012). However, this is unlikely as an explanation here since the chicks also showed no preference in the first trial (unfamiliar adult odour *versus* no odour), where only one odour was presented at a time.

Sex and age differences in behaviour

Although this was not part of our initial study questions, we found sex and age differences in the behavioural response of chicks to a novel environment. During their first trial in the Y-maze, females were more likely (and took less time) to leave the acclimation chamber than males, and younger chicks were more likely (and took less time) to leave the acclimation chamber than older chicks. These differences in movement in a novel environment may reflect differences in exploration, risk-taking, boldness or proactivity (Verbeek et al. 1994, Réale et al. 2007). The age effect was expected from our field observations, as young chicks did not stay still as long as older chicks during capture attempts, which facilitated their capture. This may be explained by the fact that younger chicks need more brooding from their parents (Colwell et al. 2007, *personal observations*)

and may thus be more motivated (or more quickly motivated) to search for them. The sex effect, however, is an exciting finding. Indeed, early sex differences in behaviour were unexpected and may have long term consequences (e.g. behaviour at adulthood, survival, dispersal). Indeed, in the same white-fronted plover population, it was found that females have a higher apparent survival during their first year than males (Eberhart-Phillips et al. 2018). This female-biased survival may be explained by early sex differences in behaviour, for example if more explorative female chicks are more efficient at foraging (Verbeek et al. 1994, but see Bijleveld et al. 2014), but may also be explained by demographic causes (e.g. male-biased dispersal, Eberhart-Phillips et al. 2018). In any case, these preliminary results should be considered with caution, as the study was not designed to test for personality (a single trait measured only once per individual, Beckmann and Biro 2013, Réale et al. 2007). Nevertheless, this interesting result warrants further research on inter-individual differences in behaviour in this species.

CONCLUSION

Although parent-offspring recognition can be mediated by odour cues in birds, and although parent-offspring recognition should be important in this precocial species, we found no evidence of olfactory parent recognition in white-fronted plover chicks. It may be that plover chicks do not need to discriminate between parents and foreign adults, or that they rely on other (e.g. acoustic) cues. It is also possible that chicks can discriminate parental and conspecific odours but that our experiment failed to detect it. More research is needed to understand how common and important olfaction is for parent recognition in birds. Precocial species are well suited to address this question, as 1) they probably need parent-offspring recognition, and 2) Y-maze trials can easily be conducted on mobile chicks. Finally, our study revealed that female and male chicks may differ in their behaviour, which calls for more research on individual differences in behaviour in this species.

Acknowledgements

We thank Thomas Schmitz for building the Y-maze, Ignace Delula “Vavaly” for his assistance in searching for plover families, and two anonymous reviewers for their comments that improved this manuscript.

Data Availability

Data and R code used in the analyses are available on Github (https://github.com/marc-gilles/parent-recognition-plovers).

Supplementary information

Fig. S1. Y-maze apparatus used to conduct olfactory preference trials on plover chicks in the field. The Y-maze consisted of one start arm (with opaque cover) and two test arms (with semi-transparent cover). At the end of each of the two test arms, we placed **(a)** a test odour (cotton swab) and **(b)** an electric fan powered by a battery, which circulated air from the test odour towards the inside of the maze through a perforated wall. We recorded the preference trials **(c)** from above, **(d)** using a camera mounted on a tripod, directly in the field.

Table S1. Effect of the sex and freshness of the test odours on two preference measures, i.e. the proportion of time spent by the plover chick with the unfamiliar adult odour relative to the time spent in both test arms (beta regression) and the type of odour visited first (binomial linear regression), in conspecific odour trials (unfamiliar adult odour *vs* no odour). Odour freshness is the number of days between the sampling of the unfamiliar adult odour and the trial. N = 28 trials. P < 0.05 indicated in bold.

Conspecific odour trial (unfamiliar adult odour *vs* no odour) N = 28 trials			
Proportion of time spent with unfamiliar adult odour			
Fixed effects	**β estimate**	**95% CI**	**P**
Intercept	–0.23	[–1.28, 0.81]	0.66
Odour sex (male)	0.25	[–0.67, 1.17]	0.59
Odour freshness	0.02	[–0.18, 0.22]	0.84
Odour visited first			
Fixed effects	**β estimate**	**95% CI**	**P**
Intercept	–2.52	[–6.47, 0.02]	0.11
Odour sex (male)	2.06	[–0.09, 5.16]	0.10
Odour freshness	0.32	[–0.15, 0.94]	0.23

Table S2. Effect of the sex and freshness of the test odours on two preference measures, i.e. the proportion of time spent by the plover chick with the unfamiliar adult odour relative to the time spent in both test arms (beta regression) and the type of odour visited first (binomial linear regression), in parent odour trials (unfamiliar adult odour *vs* parent odour). The reference level of odours sex is unfamiliar female *vs* mother. There was no trial with odours from unfamiliar female *vs* father. Odour freshness is the number of days between the sampling of the unfamiliar adult odour and the trial (the parent odour was always sampled on the day of the trial). N = 23 trials. P < 0.05 indicated in bold.

Parent odour trial (unfamiliar adult odour *vs* parent odour) N = 23 trials			
Proportion of time spent with unfamiliar adult odour			
Fixed effects	**β estimate**	**95% CI**	**P**
Intercept	–0.31	[–1.00, 0.37]	0.37
Odours sex (unfamiliar male *vs* mother)	0.81	[–0.24, 1.88]	0.13
Odours sex (unfamiliar male *vs* father)	0.54	[–0.28, 1.35]	0.20
Odour freshness	0.09	[–0.25, 0.08]	0.30
Odour visited first			
Fixed effects	**β estimate**	**95% CI**	**P**
Intercept	–0.69	[–2.50, 0.88]	0.41
Odours sex (mother *vs* unfamiliar male)	1.62	[–0.83, 4.86]	0.23
Odours sex (father *vs* unfamiliar male)	1.16	[–0.69, 3.22]	0.23
Odour freshness	0.11	[–0.28, 0.57]	0.57

Table S3. Results from Generalised Mixed Models (GLMMs) testing the effect of the sex and age of plover chicks on their probability and latency to leave the acclimation chamber during their first trial (conspecific odour preference trial). Probability to leave the chamber was recorded for all chicks that participated in the trial (N = 42 chicks), while latency to leave the chamber was measured only in chicks that left the chamber (N = 28 chicks). Estimates of of probability to leave the chamber are log-odds and should be exponentiated to obtain odds ratios. P < 0.05 indicated in bold.

Probability to leave the chamber N = 42 chicks from 32 broods

Fixed effects	**β estimate**	**95% CI**	**P**
Intercept	3.76	[1.57, 5.96]	**<0.001**
Sex (male)	–2.22	[–4.18, –0.26]	**0.027**
Age	–0.18	[–0.30, –0.07]	**0.002**
Random effect	**Variance**	**SD**	
Brood ID	0	0	

Latency to leave the chamber (s) N = 28 chicks from 21 broods

Fixed effects	**β estimate**	**95% CI**	**P**
Intercept	1.68	[0.31, 3.06]	**0.016**
Sex (male)	1.20	[0.05, 2.36]	**0.041**
Age	0.21	[0.08, 0.33]	**<0.001**
Random effect	**Variance**	**SD**	
Brood ID	3.26	1.81	

A preening white-fronted plover in Madagascar

Photo by Marc Gilles

CHAPTER 7

General discussion

Marc Gilles

In this thesis, I have explored putative roles of preen oil and body odour in birds. I have shown that preen oil composition undergoes seasonal changes in nearly all bird species studied, and differs between sexes in about half of the species studied (**Chapter 2**). Why would the smell of birds change across seasons and differ between females and males? I and my colleagues proposed two hypotheses: olfactory crypsis and sex semiochemical. I then conducted a phylogenetic comparative analysis on 59 bird species, which supported both hypotheses (**Chapter 2**). Following this, I analysed the chemical composition of preen oil in two species, the Kentish plover and the pied flycatcher. In Kentish plovers, a shorebird with biparental incubation, I found no sex difference in preen oil during breeding, which fits the results from the comparative analysis (**Fig. 1**, **Chapter 5**). In pied flycatchers, a songbird with uniparental incubation, I also found no sex difference in preen oil during breeding, which was contrary to predictions (**Fig. 1**, **Chapter 4**). I also found that the preen oil of pied flycatchers changes across breeding stages and life stages, and does not seem to contain individual signatures (**Chapter 3 & 4**). However, I found breeding pair and family signatures, which suggests that preen oil composition is affected by the social or microbial environment in the nest (**Chapter 4**). Most, but not all, chemical patterns I detected in the preen oil of pied flycatchers were reproducible, highlighting the importance of replication studies (**Chapter 4**). For example, sex differences were detected in the first study, but not in the replication study. Preen oil odours may be used as semiochemicals not only for mate

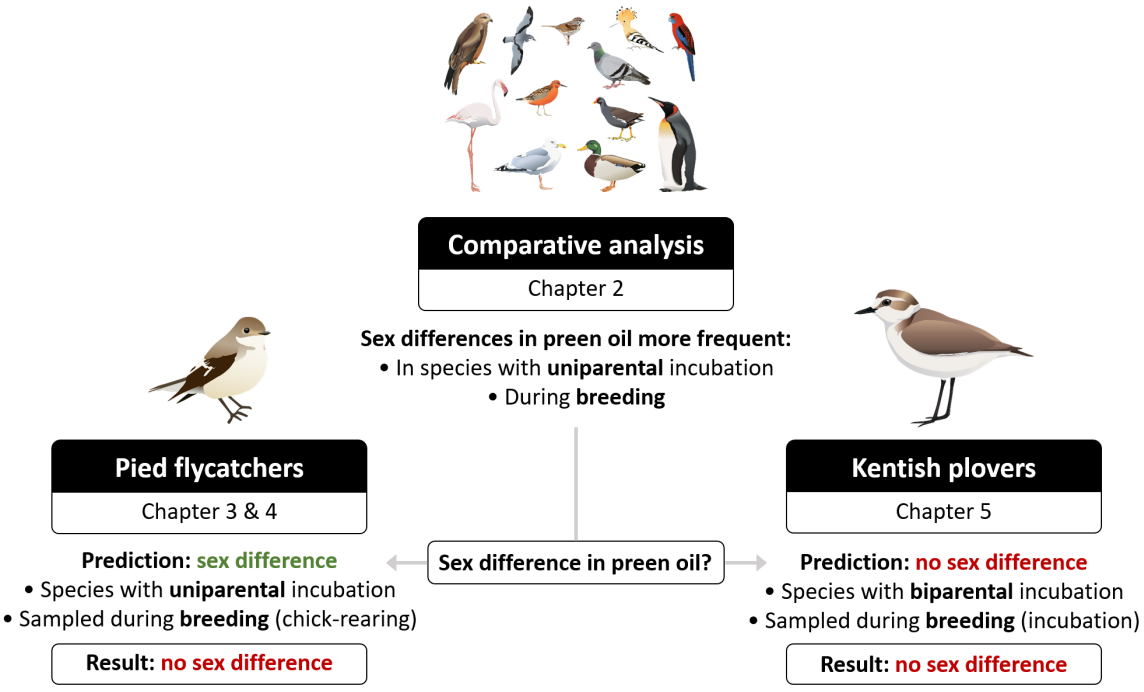

Fig. 1. Predicted *vs* actual sex differences in preen oil composition in pied flycatchers and Kentish plovers, based on the results from the comparative analysis.

choice, but also for parent-offspring communication. I performed behavioural trials to test if the precocial chicks of white-fronted plovers can discriminate between preen oil odours of parents and that of conspecific adults (**Chapter 5**), but I found no evidence for olfactory parent recognition in this species.

Role of preen oil in olfactory crypsis

What do we know now?

In its original version, the olfactory crypsis hypothesis posited that the preen oil of incubating birds becomes less odorous during incubation to reduce the detection of nests by mammalian predators (Reneerkens et al. 2007a). In **Chapter 2**, I and my colleagues broadened this hypothesis for several reasons. **(1)** Olfactory crypsis may be important until fledging, and not only during incubation (nest predation can be as high during chick-rearing as during incubation; Pietz and Granfors 2000). **(2)** Crypsis may be achieved not only by reducing the production of odours, but also by matching odours to the olfactory background (background matching; Soini et al. 2007). **(3)** It could apply not only to mammalian predators, but to any olfactorily-searching predators regardless of taxon (e.g. birds, reptiles, insects; Shutler 2019). **(4)** Crypsis may protect the eggs and young from predators, but also the incubating or chick-rearing parents themselves. In its updated version, the olfactory crypsis hypothesis thus posits that preen oil becomes less odorous or more similar to the olfactory background during incubation and chick-rearing, to reduce nest detection by olfactorily-searching predators, thereby protecting eggs, young and/or parents. In a comparative analysis (**Chapter 2**), I found that sex differences in preen oil are more common during breeding than non-breeding, and in species with uniparental incubation than in species with biparental incubation. I also found that seasonal changes in preen oil composition are more common in the incubating sex(es), but only in ground-nesting species, which may be more vulnerable to olfactorily-searching predators. Together my results suggest that the sex-specific seasonal changes in preen oil could have a role in olfactory crypsis in ground-nesting birds.

In pied flycatchers, which nest in cavities above ground, preen oil likely does not provide olfactory crypsis. Indeed, I and my colleagues found that **(1)** preen oil is more volatile during incubation than during nestling-rearing in females, and that **(2)** females and males produce a similar preen oil during chick-rearing, although only females incubate and females spend much more time in the nest with chicks than males (**Chapter 3 & 4**). In Kentish plovers, which nest on the ground and are vulnerable to olfactorily-searching nest predators (e.g. dogs, foxes, snakes and lizards; Fraga & Amat 1996, Kosztolányi *et al.* 2009), preen oil was similar between sexes during incubation (**Chapter 5**). This is in line with the olfactory crypsis hypothesis. However, I did not study seasonal changes in this species, and if preen oil does not vary seasonally, this result does not provide any evidence of a specific function (e.g. crypsis) in incubation. Furthermore, surprisingly, the preen oil of incubating Kentish plovers consisted essentially of monoesters, and no diesters. In contrast, most other

shorebirds studied secrete diesters during incubation (Reneerkens et al. 2006), and these diesters are assumed to provide olfactory crypsis (Reneerkens et al. 2005). Therefore, it is uncertain whether the monoesters from the preen oil of incubating Kentish plovers can have a role in chemical camouflage. To date, most of the evidence on olfactory crypsis comes from the studies of Reneerkens and colleagues on 27 shorebird species (Reneerkens et al. 2002, 2005, 2006, 2007a), including a single experimental study on the preen oil of red knots, which was not performed in natural conditions (Reneerkens et al. 2005). Our understanding of how birds may use preen oil and body odour for olfactory crypsis therefore remains limited.

What should we study next?

(1) We need more experimental evidence. Importantly, we need evidence for an effect of preen oil on nest detectability in the wild, which would suggest an effect on fitness. To test this, we could prepare artificial nests with eggs smeared with either preen oil from incubating individuals, preen oil from non-incubating individuals, or a control, and compare predator detection rates between treatments. This should be conducted during the night only, to make sure that nests are detected primarily by smell. I acknowledge that such field experiments are difficult to conduct. In fact, I tried to perform one myself, and failed because the rate of nest predation in the study site was too low to collect enough data. However, studies with a similar setup have been successful (e.g. Selonen et al. 2022). Alternatively, and more practically, we could conduct these detection experiments using predators (e.g. rats) in captivity.

(2) We should investigate whether olfactory crypsis is achieved by masking the odour of the eggs, of the chicks or of the incubating/chick-rearing parent. In fact, it is still unclear whether preen oil substances are transferred to eggs and/or chicks. To test for such a transfer of preen oil, one could search for traces of preen oil on the eggs and/or chicks, or observe if parents actively apply preen oil onto the eggs and/or chicks (e.g. using video recording; Martín-Vivaldi et al. 2014). To my knowledge, an active transfer of preen oil on eggs has been documented only in Eurasian hoopoes (Martín-Vivaldi et al. 2014, Soler et al. 2014).

(3) To test if preen oil can increase olfactory crypsis via background matching, as proposed by Soini et al. (2007), we could test whether the preen oil odours are similar to odours from the nest environment (e.g. sampled using headspace sampling; Díez-Fernández et al. 2020), and whether a higher similarity results in a reduced nest predation rate. However, because it would be difficult to disentangle the many possible causes of predation in the wild, we should conduct experiments in controlled conditions. We could prepare artificial nests with different degrees of chemical background matching, and test whether it affects the detection rate by captive predators (e.g. rats) in a dark chamber.

Role of preen oil in olfactory mate choice

What do we know now?

In **Chapter 2**, my colleagues and I formalized a new hypothesis: the sex semiochemical hypothesis. It posits that sex differences in preen oil arise during breeding (especially during mate choice) because preen oil odour cues are used for mate choice, including sex discrimination and assessment of mate quality and compatibility. Our comparative analysis showed that sex differences in preen oil are more common during breeding than nonbreeding, and in species with uniparental incubation than in species with biparental incubation. These results support the sex semiochemical hypothesis.

Interestingly, the preen oil of pied flycatchers, in which only females incubate, did not differ between sexes during breeding, although our comparative analysis predicted a sex difference (**Fig. 1, Chapter 4**). As a result, pied flycatchers probably cannot use preen oil odours for sex discrimination, in contrast to other species (e.g. Whittaker et al. 2011a, Grieves et al. 2019b). However, it should be noted that sex differences were tested only during chick-rearing (i.e. several weeks after mate choice), and it may be that sex differences occurred at the time of mate choice. In fact, in dark-eyed juncos and song sparrows, sex differences were not found during chick-rearing but were found before and during egg laying (i.e. when mate choice occurs; Whittaker et al. 2016, Grieves et al. 2019a). Furthermore, even if the preen oil of pied flycatchers does not contain information about sex, it may still contain information about mate quality or compatibility, which could be used for mate choice, although this remains to be tested. In pied flycatchers, I also found that breeding partners produce a similar preen oil and might therefore smell alike (**Chapters 3 & 4**). I then hypothesized that they might mate assortatively with respect to body odour, although this is not likely, as this would promote inbreeding (Mardon and Bonadonna 2009). This similarity between partners is more likely the result of them sharing the same social and microbial environment in the nest (**Chapter 4**), as found in dark-eyed juncos (Whittaker et al. 2016). In Kentish plovers, both sexes participate in incubation and, as predicted, females and males secrete a similar preen oil during incubation (**Chapter 5**). Like in pied flycatchers, with no sex difference in preen oil, it is unlikely that Kentish plovers can use preen oil odours to discriminate between sexes. It is unknown whether they otherwise use preen oil odours for sexual signalling (e.g. to assess mate quality or compatibility).

My studies did not provide clear evidence for a use of preen oil for olfactory mate choice. Evidence for a direct role of preen oil in olfactory sexual signalling is limited to one parrot and two songbird species. Indeed, budgerigars, song sparrows and dark-eyed juncos can discriminate between sexes based on preen oil odours (Zhang et al. 2010, Whittaker et al. 2011a, Grieves et al. 2019b). Song sparrows can even assess MHC similarity and diversity via preen oil odours (Grieves et al. 2019c). There is more evidence of a role of whole-body odour, rather than solely preen oil odour, for mate choice (e.g. discrimination of sexes, kinship or MHC genotype; Coffin et al. 2011, Amo et al. 2012a,

Bonadonna and Sanz-Aguilar 2012, Leclaire et al. 2017b, Van Huynh and Rice 2019, Krause et al. 2023). Because preen oil is a major constituent of whole-body odour, these studies may provide indirect evidence for a role of preen oil in olfactory sexual signalling.

What should we study next?

(1) While olfactory discrimination of sexes has been documented in several bird species already, we need more evidence for olfactory assessment of mate quality or compatibility. Olfactory discrimination of MHC diversity and similarity has been found in blue petrels (Leclaire et al. 2017b), black-legged kittiwakes (Pineaux et al. 2023) and song sparrows (Grieves et al. 2019c), but not in house sparrows (Amo et al. 2022). Olfactory assessment of body condition has been shown in zebra finches and house finches *Haemorhous mexicanus* (Amo et al. 2012b, Amo and López-Rull 2024). Additional experiments in other species, especially other than songbirds or seabirds, should be conducted. For example, we could study whether birds can evaluate genome-wide heterozygosity by smell (Rivers 2023).

(2) We should show that odour preferences in laboratory conditions translate to mate choice in natural conditions. Such studies are difficult because mate choice is affected by numerous factors in the wild. A first step could be to investigate whether birds mate non-randomly with regard to traits that birds can discriminate by smell, like MHC genotype (Leclaire et al. 2017b), heterozyogisity (Whittaker et al. 2019a), body condition (Amo and López-Rull 2024) or relative "maleness" or "femaleness" of preen oil composition (Whittaker et al. 2013).

(3) I propose that chemical sexual dimorphism (e.g. sex differences in preen oil) may have evolved as an alternative way to discriminate between sexes in species that are visually sexually monomorphic. To test this, we could conduct a comparative study to test whether sex differences in preen oil composition are more common (or stronger) in visually monomorphic species than in visually dimorphic species. Such an analysis should be possible using the data collected in **Chapter 2**.

Role of preen oil in olfactory parent–offspring communication

What do we know now?

In the first chapters of this thesis, I presented the possible odour-related roles of preen oil in a rather dichotomous way – crypsis and/or mate choice. However, preen oil may have an additional odour-based role that is much less studied: olfactory parent-offspring recognition. For offspring recognition, evidence is very limited. Zebra finch males discriminate their own offspring by smell, but females do not (Golüke et al. 2021). This recognition is probably not mediated by preen oil odours, because the chicks tested were at an age where their uropygial gland was not yet functional. Similarly, female spotless starlings do not discriminate between own and foreign chicks by smell, regardless of whether the uropygial gland of the chicks was functional or not (Amo et al. 2014). In

contrast, evidence for parent recognition is clearer, although limited to only two species. Indeed, zebra finch and tree swallow nestlings can recognise the odour of their parents (Caspers et al. 2017b, Griebel and Dawson 2020). The test stimuli used in these studies were whole-body odours and not preen oil odours. But because preen oil is an important source of body odour (e.g. in zebra finches, Alves Soares et al. 2024), these studies may provide indirect evidence of a role of preen oil for olfactory parent recognition.

To my knowledge, no study had evaluated the direct effect of preen oil on parent-offspring recognition. In **Chapter 5**, I found that white-fronted plover chicks did not show any preference for preen oil odours of parents over unfamiliar adults. Chicks also did not seem to prefer nor avoid the preen oil odour of unfamiliar adults compared to a control. It could be that chicks **(1)** did not detect the odours in the experimental setup, **(2)** detected but did not discriminate between these odours, or **(3)** detected and discriminated between these odours but showed no preference. Other than recognition, parental odours may have calming effects on chicks. I found a single study that tested for such an effect. It showed that a synthetic analogue of mother preen oil reduces corticosterone (a proxy of stress) in chicks of domestic chickens (Madec et al. 2006). The study was conducted in farm conditions, and it is unclear whether this could apply to non-domestic species in the wild.

What should we study next?

(1) Both parent and offspring olfactory recognition should be further investigated. We should start by studying species in which there is a high risk of intermingling between families, for which parent-offspring recognition should be important (e.g. colonially-nesting species, precocial species and species with intraspecific brood parasitism).

(2) A role of preen oil in parent-offspring communication could drive sex and seasonal differences in the preen oil composition of the parents. Indeed, in species where only one sex cares for the chicks, the preen oil of the caring sex may change during chick-rearing, for example to increase recognition or to reduce the stress of the chicks (e.g. if specific compounds are secreted for these purposes). This is highly speculative at this stage, but represents an interesting avenue for research, especially for species where parent recognition has already been shown (e.g. zebra finches).

Keeping alternative hypotheses on the table

In my thesis, I have focussed on three odour-based roles of preen oil: crypsis, mate choice and parent-offspring communication. There is an additional putative function of preen oil that is also based on odours: repellence against predators and ectoparasites. Preen oil odours may be used to repel predators, like in green woodhoopoes, but this probably only applies to species with especially odorous (or rather malodorous) preen oil (Burger et al. 2004). Preen oil odours may also be used to repel ectoparasites, like blood-feeding dipterans. This seems to be the case in Eurasian hoopoes (Tomás et al. 2020), but otherwise evidence is scarce (Marzal et al. 2022). Preen oil has also other functions that are not based

on odours, such as waterproofing, visual mate choice via cosmetic colouration, and protection against ectoparasites via antimicrobial activity (see **Fig. 3** in **General introduction**). Variation in preen oil could thus reflect a role of preen oil in any of these functions, and even possibly several functions at the same time. Indeed, these functions are mutually non-exclusive. An exciting possibility is that, if preen oil serves a specific function (e.g. protection via crypsis or antimicrobial activity), it may subsequently evolve as a sexually selected signal for mate choice. This has been suggested in Eurasian hoopoes, in which breeding females deposit a dark (odorous) antimicrobial preen oil on their eggs that limits infection by eggshell bacteria. The altered colour of the eggs is then used by males as a post-mating sexual signal of female quality (Díaz-Lora et al. 2021). It would be interesting to test if females signal the antimicrobial activity of their preen oil, not only via visual cues on the eggs, but also via olfactory cues (possibly before egg laying). With descriptive studies, such as my studies on the preen oil of pied flycatchers (**Chapters 3 & 4**) and Kentish plovers (**Chapter 5**), it is difficult to disentangle the different functions of preen oil, and one can only speculate. Experiments are therefore necessary to unravel if preen oil differences play a role in chemical camouflage, communication, or both, or if they have no function and are only by-products (e.g. of physiology).

Beyond preen oil

Is preen oil a good proxy of avian body odour?

Preen oil composition may not be the best proxy of body odour for all bird species. Obvious examples are species that have been described as odorous but that lack the uropygial gland, such as macaws (*Anodorhynchus spp.*) and amazon parrots (*Amazona spp.*) (Johnston 1988, Weldon and Rappole 1997). Other examples include feral pigeons, wood pigeons (*Columba palumbus*) and smooth-billed anis, where most of the chemicals found on feathers could not be found in preen oil (Jacob and Grimmer 1975, Leclaire et al. 2019, Grieves et al. 2024). Preen oil is partly composed of nonvolatile (i.e. nonodorous) substances, which can contribute indirectly to body odour. Indeed, once smeared onto the plumage, the nonodorous substances can be broken down, notably by plumage microbes, into smaller, more odorous substances (Mardon et al. 2011a, Maraci et al. 2018). How much the variation in the nonvolatile fraction of preen oil reflects the variation in odour is still poorly understood, and likely varies greatly among species (Leclaire et al. 2011, Alves Soares et al. 2024a). At least in some species, variation in nonvolatile preen ol composition seems to translate well to variation in odour. For example, song sparrows can discriminate between sexes based on preen oil odours, and sex differences have been found in whole preen oil (essentially composed of nonvolatile wax esters) (Grieves et al. 2019a, b). In such cases, preen oil composition can be a good proxy of body odour. Given the multifunctional nature of oil, it is likely that only some subset of compounds (probably from the more volatile fraction), and not the whole preen oil (all compounds), is used for communication.

To understand the degree to which preen oil contributes to body odour, studies have compared the chemical composition of preen oil and plumage (e.g. Mardon et al. 2011a, Leclaire et al. 2011, Grieves et al. 2024, Alves Soares et al. 2024). To analyse plumage odour, feathers are generally cut from the bird, but volatiles can also be sampled directly by rolling a stir bar on the wing surface (Soini et al. 2005, Whittaker et al. 2010). An important next step would be to compare preen oil composition, not to plumage odour, but to whole-body odour. Whole-body odour can be collected using headspace sampling (e.g. Douglas 2006, Díez-Fernández et al. 2020). Headspace sampling extracts the volatile substances from the air surrounding the bird (or biological sample), thus sampling the actual odour of the bird (or biological sample). The main problem with headspace sampling is that it is rather unpractical in the field, as it takes time (e.g. 15 min in Díez-Fernández et al. 2020, 50 min in Douglas 2006, 2 h 30 min in Spanoudis et al. 2020) and can be unwieldy (especially for large species). Another way to collect whole-body odour is to place birds in cotton bags or nylon socks to impregnate the fabric with their body odour (e.g. Bonadonna and Nevitt 2004, Krause et al. 2014). This method also takes time (e.g. 1 h in Bonadonna and Nevitt 2004 and Krause et al. 2014). Headspace sampling is usually used for chemical analyses, whereas impregnation of fabric is rather used to prepare test odours for behavioural trials. The most evident disadvantage of sampling whole-body odour (via headspace sampling or impregnation of fabric), in comparison to sampling preen oil, which takes less than 20 sec, is that it is more time-consuming and might thus impose a greater stress or disturbance to the birds. Another possible limitation, which to my knowledge has never been mentioned, is that whole-body odour may be biased by faeces odour, in case the birds defecate during sampling. In conclusion, preen oil odour may not be the best proxy of body odour, but it is a good proxy for at least some species, and is especially practical for field studies. Future research should develop methods, like headspace sampling or thermal desorption (Kücklich et al. 2017), to allow the sampling of whole-body odour also in the field.

Whether we study preen oil odour or whole-body odour, a persistent problem is: how to measure volatility? In our review in **Chapter 2**, I encouraged future studies to measure volatility, as it can help to investigate the possible function of bird odours. Indeed, a higher volatility (i.e. potentially increased odour) would rather suggest a signalling function, whereas as a lower volatility (i.e. potentially reduced odour) would rather suggest a masking function. In our first study on the preen oil of pied flycatchers (**Chapter 3**), I used two measures of volatility: the proportion of high-volatility substances and the proportion of low-volatility substances. For this, I chose arbitrary thresholds of retention time to exclude the central part of the chromatograms (which contains most of the abundance and shows little variation among samples) while conserving sufficient and equivalent abundances (ca. 10 %) in the early and late parts of the chromatograms. I argued that a higher proportion of high-volatility substances would represent an increased volatility, whereas a higher proportion of low-volatility substances would represent a decreased volatility. Because I was not certain about the latter assumption, I refrained from using the

proportion of low-volatility substances in the replication study (**Chapter 4**). Similarly, other studies have assessed the volatility of substances based on retention times (e.g. Reneerkens et al. 2002, Leclaire et al. 2011, Fischer et al. 2017). Yet, retention times are probably not the best measure of volatility. The volatility of substances is better measured by their vapour pressure (or boiling point), which represents their readiness to evaporate from a solid or liquid matrix and enter into the air, thereby becoming odorous. Studies should thus use vapour pressures (or boiling points) rather than retention times to measure the volatility of single substances (Nevitt and Prada 2015). Even though one can measure the volatility of single substances, there is, to my knowledge, no standardized method to measure the overall volatility, or the odour level, of an entire biological sample. I propose to measure the total abundance of volatile substances extracted by headspace sampling in a given period of time as a possible proxy of overall volatility (or odour level).

Other sources of avian body odour

Other sources of bird odour have been suggested (reviewed in Nevitt and Prada 2015) and should be explored, as they may also play a role in inter- or intraspecific communication.

Skin and feathers. In birds, feather follicles (i.e. invaginations of the skin) do not contain glands like the hair follicles of mammals. Instead, the avian skin contains sebokeratinocytes, which are epidermal cells that secrete lipids (Menon and Menon 2000). Therefore, the entire skin can be considered as a lipid-producing *holocrine* gland, and may contribute to body odour (Nevitt and Prada 2015). Interestingly, lipid production may be higher in featherless regions of the skin, such as bare parts (e.g. caruncles, combs, wattles, ceres) and brood patches (Nevitt and Prada 2015). Nevitt and Prada (2015) proposed that birds could use bare parts, which are already used for sexual signalling via size and colour (e.g. in Phasianidae, Zhao et al. 2024), to additionally transmit odours for sexual signalling. In addition, birds could use brood patches to transmit odours to eggs and/or chicks. Nevitt and Prada (2015) also hypothesized that birds could sequester chemical compounds from their diet in their skin, like the neurotoxins found in the skin of pitohuis and ifritas (Dumbacher et al. 2009), thereby producing dietary derived odours through the skin. These ideas remain to be tested. Skin and feather odours (independently of preen oil odour) are involved in olfactory mate choice in crested auklets (Hagelin 2007b), and also possibly in crimson rosellas (*Platycercus elegans*) (Mihailova et al. 2014). Skin and feather odours may also have a role in deterrence of ectoparasites in crested auklets, pitohuis and ifritas, but evidence is mixed (reviewed in Weldon 2023).

Powder down feathers. Powder downs produce a talcum-like powder, which is composed of keratinized cells with lipid-type characteristics (Menon and Menon 2000). Powder downs have a role in plumage maintenance and waterproofing, and have been hypothesized to complement preen oil for this role. Consistent with this, species that do not possess a uropygial gland tend to produce more powder downs (Johnston 1988). Powder downs may contribute to body odour (Nevitt and Prada 2015), especially for species that lack a uropygial gland, although this remains to be studied.

Faeces. Despite being a rather obvious source of odour in birds, its role for olfactory communication has been largely overlooked. Like in mammals, avian faecal odours could transmit information about individuals, such as sex, reproductive status and relatedness (e.g. Beynon and Hurst 2004). In domestic chickens, the odour of faeces of stressed individuals differs from that of unstressed individuals, and may thus function as a silent alarm signal, but this has not been tested (Jones and Roper 1997). Some studies have shown that faeces can be used as an olfactory deterrent against nest predators. For example, when alarmed during the incubation period, common eiders (*Somateria mollissima*) and Northern shovelers (*Anas acuta* spray faeces on their eggs, which repels mammalian predators (Swennen 1968). Similarly, chicks of great spotted cuckoos (*Clamator glandarius*) defend themselves with a malodorous cloacal secretion that deters predators (Canestrari et al. 2014). In most altricial bird species, parents remove nestling faeces (faecal sacs) from the nest, which may be considered as a form of olfactory crypsis, as it may reduce odour cues at the nest (Ibáñez-Álamo et al. 2017). Although a cryptic function of this behaviour has long been assumed (Weatherhead 1984), it has not been confirmed (Ibáñez-Álamo et al. 2014, Rubio et al. 2018). Another way for parents to reduce odour cues at the nest is to produce less faeces, or less odorous faeces, during the nesting period. This was suggested in a study on red grouse (*Lagopus lagopus scotica*) which found that females (incubating sex) stop producing caecal faeces during the incubation period (Hudson et al. 1992). This change in faeces production (and possibly faeces odour) is believed to reduce the detectability of incubating females. Indeed, according to the study, females can be detected by trained dogs from a distance of 50 m during most of the year, but cannot be detected from more than 0.5 m during incubation. This possible adaptation, which is reminiscent of the preen oil changes in incubating sandpipers (Reneerkens et al. 2005, 2007a), is worth exploring further.

Stomach oils. Some bird species, notably seabirds, can regurgitate stomach oils or vomit as a defence mechanism against predators. For instance, when threatened by predators, Northern fulmars (*Fulmarus glacialis*) propel stomach oils (Swennen 1974), turkey vultures vomit carrion (Dumbacher and Pruett-Jones 1996) and chicks of Eurasian rollers (*Coracias garrulus*) vomit an odorous orange liquid (Parejo et al. 2012). Substances from stomach oils can be found on the birds' plumage (e.g. in Leach's storm petrels *Oceanodroma leucorhoa*), showing that they can contribute to body odours (Nevitt and Prada 2015). However, these substances are derived from the diet and are thus poor candidates to transmit information about individuals (Nevitt and Prada 2015), although diet could be an indicator of current quality. Interestingly, the vomit from roller chicks may be used by their parents as an olfactory cue of danger, suggesting that scents produced during interspecific interactions can have a role in intraspecific communication (Parejo et al. 2012).

Eggs. Even before hatching, birds (or rather bird embryos) can emit odours that are transferred through the eggshell outside of the egg, and could play a role in parent-offspring communication. Remarkably, volatiles emanating from eggs of domestic chickens and

Japanese quails (*Coturnix japonica*) contain information about sex, fertility and development (Webster et al. 2015, Borras et al. 2023). Sex differences were also found in the volatiles from eggs of barn swallows (*Hirundo rustica*) (Costanzo et al. 2016). However, whether this information can be smelt and used by parents remains to be elucidated.

Other suggested sources of body odour in birds include anal glands, salt glands, saliva and conspecific blood (Hagelin 2007a, Nevitt and Prada 2015), but to my knowledge, their possible olfactory role has never been investigated.

Measuring nest odour to study olfactory crypsis

Olfactory crypsis of nests can be achieved by reducing the odour of incubating or chick-rearing parents (Reneerkens et al. 2005). But nest odour is not only made of the body odour of parents, but also of odours of eggs, nestlings, substances produced by nest occupants (mainly faeces) and nest material. To make nests olfactorily cryptic, birds may use three strategies (reviewed in Shutler 2019). **(1)** They can remove odour cues from the nests, such as faecal sacs from nestlings (Weatherhead 1984, but see Ibáñez-Álamo et al. 2017). **(2)** Birds can add specific nest material, such as dung (Levey et al. 2004, but see Smith and Conway 2007) or mammalian hair (Coppedge 2010), that masks nest odour. **(3)** Birds may select a nest location which provides olfactory concealment or which makes odours dissipate rapidly, for example with greater wind turbulences (Fogarty et al. 2018). Future research on olfactory crypsis should probably study nest odour rather than solely the body odour of the incubating or chick-rearing parents.

Conclusion

In my research, I have shown that seasonal changes in preen oil composition are the rule rather than the exception across species. Furthermore, I have demonstrated that these seasonal changes, as well as sex differences, can be explained by the breeding biology of bird species, and may reflect a role of preen oil in both olfactory crypsis and signalling (including mate choice and parent-offspring communication). I have shown that, in Kentish plovers, a ground-nesting shorebird with biparental incubation, females and males secrete a similar preen oil during breeding, as predicted by the comparative analysis in my first chapter. Surprisingly, in pied flycatchers, a hole-nesting songbird with uniparental incubation, I also found no sex difference in preen oil, although the contrary was expected. It is important to note that, in a first study on pied flycatchers, I had found subtle sex differences and speculated that they could reflect a role of preen oil as a sex semiochemical. However, after conducting a replication study, I realised that this result was not reproducible. This highlights the importance of replications, especially in a field where they are still very rare. I further found that the preen oil of pied flycatchers changes rapidly across breeding stages, as well as across ontogeny. I detected no chemical signature of individuals, but chemical signatures of breeding pairs and families, which suggest that preen oil composition is influenced by the nest environment. Based on these descriptive

results alone, it is impossible to tell whether preen oil has a role in olfactory crypsis or signalling in these two species (although a role in crypsis is unlikely in pied flycatchers). For this, experiments are required. In my last chapter, I performed an experiment and found that white-fronted plover chicks do not discriminate between preen oil odours of parents and other conspecific adults. Overall, my studies did not provide clear evidence for a role of preen oil and body odour in olfactory crypsis, mate choice or parent-offspring communication. Nevertheless, I added replication and experiments where needed, and tested new species in what is still a very limited field. My studies provide important information and the basis from which to further explore the still poorly understood roles of odours in birds.

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DECLARATION OF ACADEMIC INTEGRITY

I herewith formally declare that this work, submitted in fulfilment of the requirements for the doctoral degree, has been developed and written by myself and I have not used any sources without declaration in the text. Any citations and references are marked as such. I am aware of the doctoral regulations of the biological faculty and I certify that the work is original and that I have not submitted this thesis, in whole or parts, or any other essay in support of a doctoral degree or other qualification from this or any other university. I confirm that no third party has earned monetary benefits, direct or indirect, for any work related to this thesis.

ERKLÄRUNG DER UHREBERSCHAFT

Hiermit erkläre ich, dass ich diese Arbeit, eingereicht zur Erlangung des Doktortitels (Dr. rer. nat.), eigenständig entwickelt und verfasst und ich keine Quellen ohne entsprechende Angabe im Text genutzt habe. Jegliche Hilfsmittel und Quellen sind als solche kenntlich gemacht. Die Promotionsordnung der biologischen Fakultät ist mir bekannt und ich versichere, dass diese Arbeit ein Original ist und weder in Teilen, noch als Ganzes und ebenso keine andere Abhandlung zur Erlangung eines Doktortitels oder einer anderen Qualifikation bei dieser oder einer anderen Universität eingereicht wurde. Ich versichere, dass Dritte weder mittelbar noch unmittelbar geldwerte Leistungen für Arbeiten erhalten haben, die mit dieser Thesis in Verbindung stehen.

Marc Gilles

Bielefeld, April 2024

STATEMENT OF CONTRIBUTION

This thesis is the result of a collaborative work with numerous researchers worldwide. My personal contribution to the conception, data collection, analysis, and writing of each of the following chapters is detailed below. All co-authors have read this statement and have given their consensual agreement that my contributions are correct and that I may use these manuscripts in this thesis.

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Chapter 2

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Chapter 3

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Chapter 4

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Chapter 5

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Chapter 6

Gilles, M.*, Zefania, S., Mijoro, T. J., Cuthill, I. C., Székely, T., & Caspers, B. A. Can chicks smell their parents? No evidence of olfactory parent recognition in a shorebird. (*in revision*).

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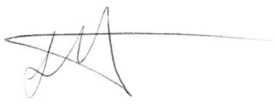

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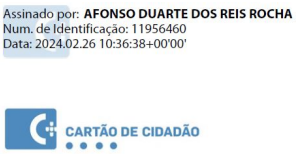

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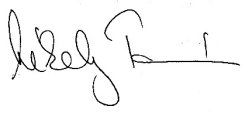

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