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Chemical communication in petrel seabirds

Submitted March 2010 by

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In loving memory of Dr. Philippe Barbazan (cousin, godfather and scientific mentor)
independent soul back to the cycle of life,

...to Claire
lighthouse in all weathers,

...and to life
whatever the reason, the secret and the fate.

THESIS SYNOPSIS (ENGLISH)

TITLE - **Chemical communication in petrel seabirds**

ABSTRACT - Chemical communication, the transfer of information from an emitter to a receiver via molecular signals, occurs in all animal phyla. Although such processes have been largely overlooked in birds, recent results suggest that chemical signals may play a more significant role than previously assumed in the social lives of birds. Procellariiform seabirds, and burrow-nesting petrels in particular, are appropriate models to investigate these questions. They indeed possess a well-developed olfactory anatomy, a noticeable musky scent and a life-history which favours the evolution of olfactory-mediated social behaviours (Chapter1).

We have explored the role of chemical signals in the ecology of the blue petrel (*Halobaena caerulea*), a burrow-nesting seabird from the Subantarctic Ocean, using existing and innovative methods from field ornithology, analytical chemistry and multivariate statistics (Chapter 2). We first demonstrate that the uropygial secretions of these birds, their main source of endogenous chemical substances, contain social information including species, sex and individual identity (i.e. a chemical signature). We also show evidence that these signals are still present, in a virtually identical form, on the plumage of the birds and are thus a likely contributor to the animals' scent (Chapter 3). Furthermore, we show that blue petrels, as receivers of the sociochemical information, are able to discriminate between the odours from different species, and between the odours of different conspecifics. There is however no evidence of olfactory capabilities of sex discrimination in this species (Chapter 4).

The study of avian olfactory behaviours, historically limited to foraging and orientation (Chapter 5), is rapidly expanding to incorporate social functions. In this regard, our results provide the first multidisciplinary case-study of avian chemical communication. The elucidation of the origin, nature and function of chemical communication in birds has major eco-evolutionary implications for our understanding of avian ecology (Chapter 6).

KEY-WORDS - Chemical communication; social behaviour; olfaction; bird; petrel; procellariiform seabird; Antarctic prion; *Pachyptila desolata*; blue petrel; *Halobaena caerulea*; GC/MS; behavioural experiment; non-parametric multivariate analysis; chemical signature; chemosignal; recognition; mate choice; chemical ecology; behavioural ecology.

DISCIPLINE - Evolutionary Biology and Ecology

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THESIS SYNOPSIS (FRENCH)

TITRE - **Communication chimique chez les pétrels**

RESUME - La communication chimique, c'est-à-dire le transfert d'information d'un émetteur à un receveur via signaux moléculaires, est présente dans tous les embranchements animaux. Longtemps négligée, l'étude récente de ces processus chez les oiseaux suggère que les signaux chimiques (ou 'chémosignaux') ont une fonction beaucoup plus importante que longtemps anticipée. Les pétrels hypogés (ordre: procellariiformes) fournissent un model approprié pour examiner ces questions. Ces oiseaux marins possèdent en effet une neuro-anatomie olfactive développée, une odeur musquée caractéristique et des traits d'histoire de vie favorisant l'évolution d'une composante olfactive aux comportements sociaux (Chapitre 1).

En utilisant une combinaison de méthodes, existantes et spécifiquement développées, d'écologie comportementale, de chimie analytique et de statistique multivariées, nous avons examiné le rôle des chémosignaux dans l'écologie du pétrel bleu (*Halobaena caerulea*), un pétrel hypogée de l'océan Subantarctique (Chapitre 2). Nous avons ainsi démontré que la sécrétion uropygiale des pétrels bleus, leur source principale de substances chimiques exogènes, contient des informations sociales telles que l'espèce, le sexe et l'identité (i.e. une signature chimique). De plus, cette information est encore présente, de manière quasi-identique, sur le plumage des oiseaux et participe donc vraisemblablement à l'odeur des individus (Chapitre 3). En termes de perception des signaux chimiques, nous avons établi que les pétrels bleus sont capables de percevoir et distinguer entre les odeurs de différentes espèces de pétrels, ainsi qu'entre les odeurs de différents conspécifiques. Cependant, aucune capacité de discrimination olfactive intersexuelle n'a été observée (Chapitre 4).

Longtemps restreinte aux comportements de recherche alimentaire et d'orientation (Chapitre 5), l'étude de l'olfaction aviaire est en pleine expansion pour désormais intégrer des aspects sociaux. Nos résultats fournissent en ce sens une première étude multidisciplinaire du sujet. La clarification de l'origine, de la nature et de la fonction de la communication chimique chez les oiseaux devrait avoir des implications éco-évolutives majeures pour la compréhension de leur biologie (Chapitre 6).

MOTS-CLES - Communication chimique; comportement social; olfaction; oiseau; pétrel; procellariiformes; prion de la désolation; *Pachyptila desolata*; pétrel bleus; *Halobaena caerulea*; GC/MS; expérience comportementale; analyse multivariée non-paramétrique; signature chimique; chemosignal; reconnaissance; choix du partenaire; écologie chimique; écologie comportementale.

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(UNDERSTAND "*MERCI BEAUCOUP*")

"By way of personal instinct, I have an inherent distaste for grandiose rhetorical statements, which don't have any substantive dimension to them"

Kevin Rudd, Australian prime minister

One of the many good things about Australians (or most of them) is that they do not '*beat around the bush*', i.e. they usually go straight to the point. As a tribute to these magnificent people, I have thus tried not to turn my acknowledgements into an endless compilation of names and wordy private jokes.

A controversial French adage says that "*L'argent est le nerf de la guerre*" (meaning "*Money lies behind all wars*"). I came to realise during my PhD that money also is the '*nerve*' of Science, and controls the fate of the later. I am therefore grateful to the Institut Polaire Paul-Emile Victor (grant to the Program ETHOTAAF n°354), the 'Agence National pour la Recherche' (funding AMBO ANR-08-BLAN-0117-01 to Francesco Bonadonna), Dr Sandra Saunders (UWA research funds), the endangered French ministry of research (for my 'Allocation de Recherche') and the UWA Graduate School (for their scholarship for cotutelle students), for the nervous impulse they gave to my project.

Many scientific collaborators, helpers, assistants, and mentors have enhanced my scientific experience throughout the last three years. For the sake of sustainable PhD-thesis printing, however, I will restrict the following acknowledgements to those persons whose insistence on being repeatedly helpful makes it impossible to ignore, no matter how much French arrogance I may muster. Among those, I am critically indebted to Dr A.P. Nesterova, Dr A. Charmantier, Dr A. Grégoire, Dr M. De Dinechin and Dr M.P. Dubois from the CEFE CNRS of Montpellier, Pr J.M. Bessière and Dr N. Charbonnel from the broader Montpellier scientific community, M. Grocott, H. Woods and N. West from the ChemCentre of Perth and Dr G. Flemmati from The University of Western Australia.

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Finally, to all the people whose love, care and/or respect have influenced not only this work but also the person I am and the path I follow: I relish real-life gratitude more than digitally-printed appreciation, and intend to convey most of my gratefulness to you through availability, handshakes and earnest smiles. I am now setting on a new project whose focus is you.

DECLARATION FOR THESES CONTAINING PUBLISHED WORK (AND/OR PREPARED FOR PUBLICATION)

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UNIVERSITÉ MONTPELLIER 2
SCIENCES ET TECHNIQUES

This thesis contains published material, and material prepared for publication, some of which has been co-authored. This material results from work carried out by the author within the Centre d'Ecologie Fonctionnelle et Evolutive (CNRS) de Montpellier at The Université de Montpellier 2 and within the School of Biomedical, Biomolecular and Chemical Sciences at The University of Western Australia between October 2006 and February 2010.

Bibliographical details of these works, and where they appear in the thesis, are outlined in the following pages. For each, the relative contribution of the PhD-candidate to the work is also indicated (as a percentage). A more detailed description of the nature of the candidate's contribution to each work is further provided within the thesis, on the introductory page of each publication.

To the best of the author's knowledge and belief, the work presented contains no material previously published or written by another person, except where due reference is made in the text.

Student: Jérôme Mardon

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Coordinating Supervisor: Dr. Sandra M. Saunders

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PUBLICATIONS & CONFERENCE ABSTRACTS RELATED TO THIS THESIS

Peer-reviewed journal articles

1. **Mardon J.**, Bonadonna F., N. West & Saunders S.M. (unpublished manuscript). Existing and innovative experimental protocols for the study of avian chemosignals. Comparative case study with a procellariiform seabird. *Journal of Chemical Ecology*: submitted.

Location in the thesis	Chapter 2 Section 2
Overall candidate contribution	80%

2. **Mardon J.**, Saunders S.M. & Bonadonna F. (unpublished manuscript). From preen secretions to plumage: the ontogeny of petrels' social scents. *Journal of Avian Biology*: submitted.

Location in the thesis	Chapter 3 Section 3
Overall candidate contribution	80%

3. **Mardon J.**, Saunders S.M., Anderson M.J., Couchoux C. & Bonadonna F. (2010). Species, gender and identity: cracking petrels' socio-chemical code. *Chemical Senses* **Advance.access**: *doi: 10.1093/chemse/bjq021*.

Location in the thesis	Chapter 3 Section 2
Overall candidate contribution	75%

4. **Mardon J.**, Nesterova A.P., Traugott J., Saunders S.M. & Bonadonna F. (2010). Insight of scent: experimental evidence of olfactory capabilities in the wandering albatross (*Diomedea exulans*). *Journal of Experimental Biology* **213**: 558-563.

Location in the thesis	Chapter 5 Section 2
Overall candidate contribution	70%

5. Bonadonna F. & **Mardon J.** (2010). One house two families: petrel squatters get a sniff of low-cost breeding opportunities. *Ethology* **116**: 176-182.

Location in the thesis	Chapter 4 Section 2
Overall candidate contribution	50%

6. Nesterova A.P., **Mardon J.** & Bonadonna F. (2009). Orientation in a crowded environment: can King Penguin (*Aptenodytes patagonicus*) chicks find their crèches after a displacement? *Journal of Experimental Biology* **212**: 210-216.

Location in the thesis	Chapter 5 Section 3
Overall candidate contribution	33%

7. **Mardon J.** & Bonadonna F. (2009). Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own. *Behavioral Ecology and Sociobiology* **63**: 537-542.

Location in the thesis	Chapter 4 Section 3
Overall candidate contribution	60%

Peer-reviewed conference proceedings

1. Francesco Bonadonna, **Jérôme Mardon** (2009). Do blue petrels get a sniff of low-cost breeding opportunities? International Ethological Conference 2009 – University of Rennes1, France.

2. Anna N. Nesterova, **Jérôme Mardon**, Charline Couchoux, Emeline Pettex, Francesco Bonadonna (2009). King penguin chicks orientation and the importance of visual landmarks. 46th Animal Behavior Meeting. Pirenópolis, Brazil.

3. Anna P. Nesterova, **Jérôme Mardon**, Francesco Bonadonna (2009). Orientation in a crowded environment: Can king penguin chicks find their crèches after a displacement? International Ethological Conference 2009 – University of Rennes1, France.

4. Anna P. Nesterova, **Jérôme Mardon,** Francesco Bonadonna (2008). Orientation in the crowded environment: Can king penguin chicks (*Aptenodytes patagonicus*) find their crèches? Royal Institute of Navigation 2008 - The 6th International Conference on Animal Navigation, University of Reading, UK.

5. **Jérôme Mardon**, Sam Saunders, Francesco Bonadonna (2007). Chemical communication in birds: petrel seabirds' olfactory make up. 6th International Zoo and Wildlife Research Conference on Behaviour, Physiology and Genetics, Berlin 2007. Advances in Ethology 39 (2007), Supplements to Ethology.

COAUTHORS PERMISSION FOR THE USE OF PUBLICATIONS

By signing the present statement, I wish to confirm that:

- I have read all the information contained within and the bibliographical details of our co-authored publications, as presented above are exact, to the best of my knowledge;
- The student contribution to our co-authored work(s), as indicated above, is accurate;
- I authorise the author (PhD candidate Jerome Mardon) to include our co-authored publication(s) in his PhD thesis.

Dr. S.M. Saunders (Coordinating supervisor)	03/02/2010 Date	 Signature
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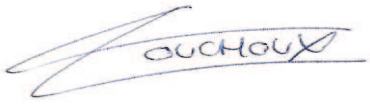
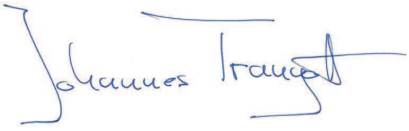
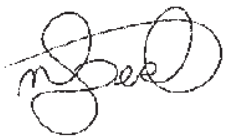
Dr. M. Anderson	16/02/2010 Date	 Signature
C. Couchoux	10/02/2010 Date	 Signature
J. Traugott	12/02/2010 Date	 Signature
Dr. A.P. Nesterova	08/02/2010 Date	 Signature
N. West	26/02/2010 Date	 Signature

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Chapter 1

INTRODUCTION

AIM & CONTENT

The nature of this PhD thesis, essentially a compilation of published and submitted articles, means that related and relevant aspects of the literature are reviewed in the introduction of the different manuscripts. Consequently, this introductory chapter positions the research topic and its overarching questions within the current field of evolutionary and behavioural ecology. It contains an introduction to the topic of chemical communication, with a particular focus on the avian case (Section 1.1), and a rationale for the structure of the project and the organisation of this thesis (Section 1.2).

From Birdbrains (March 2009), by T.Bluemel

Section 1.1

TOPIC PRESENTATION

ANIMAL COMMUNICATION

Communication underlies all sociality, regardless of its level of complexity, and is thus a keystone of animal behaviour. In its simplest form, communication is a prerequisite for any sexual reproduction (Bradbury & Vehrencamp, 1998); in an elaborate form, it is the essence of eusociality in which it controls virtually all behaviours (Wilson, 1975). As for most concepts fundamental to ecology and evolution, however, its definition has been historically rather contentious (Maynard Smith & Harper, 2003). The difficulty in restricting animal communication to a categorical definition notably arises from the fact that communication is not a behaviour *per se*. It is instead a concept incorporating the hugely diverse range of signalling solutions that animals have evolved to facilitate their interactions with one another.

In most situations, *communication* can be defined as a transfer of information (e.g. biological characteristics, emotions, intentions), carried by a *signal*, from one organism, the *emitter*, to another organism, the *receiver* (Bradbury & Vehrencamp, 1998). Biological processes involved in animal communication can therefore be sorted into three main categories: i) the *expression* component, which relates to the production of signal(s) by the emitter; ii) the *perception* component, which relates to how these signals are perceived and processed by the receiver; and finally iii) the *action* component, which relates to the responses, often behavioural, from the receiver (Tsutsui, 2004). The concept of *true communication,* however, further requires this transfer of information to be beneficial to all parties, i.e. to both the producer and the receiver(s) of the signal (Lewis & Gower, 1980). The deception of receivers or exploitation of senders, as observed for example in many predatory interactions, cannot really be considered communication in this sense. Therefore, biologists often consider that in true animal communication systems, selection should favour both the production and the reception of the signal(s).

Animal communication is involved in a huge range of behaviours so that its constitutive components are under strong evolutionary pressure (Hauser, 1996; Johnstone, 1997). Signals, for example, are extended phenotypes that contribute to the adaptation of an animal to its environment (Dawkins, 1982). Together with perception mechanisms, they will be selected in the context of the local physical and biological conditions (e.g. background noise, propagation properties, predators, competitors) (Endler, 1992). The study of signal optimisation within different ecological contexts is therefore particularly appropriate to examine the nature and intensity of natural selection. Signals will also be selected for their efficacy at eliciting beneficial responses from receivers (Johnstone, 1997). In this regard, signalling systems are shaped by the evolution of both emitters and receivers, and can therefore be used to reconstruct evolutionary scenarios and phylogenies (Macedonia & Stanger, 1994; Shaw, 1995; Stoka, 1999). Finally, signals involved in sexual selection can play a crucial role in processes such as reproductive isolation and speciation (Stratton & Uetz, 1981; Gleason & Ritchie, 1998). The study of animal communication is therefore a prime contribution to our understanding of evolution.

CHEMICAL COMMUNICATION

Among the numerous forms of communication that animals have evolved, chemical signalling, i.e. the transfer of information from sender to receiver(s) by molecular signals, is one of the least apparent to human observation. Indeed, the general (and incorrect) belief that our chemical senses (smell and taste) have nothing to do with our social lives has long hindered our appreciation of the ubiquity of chemical communication in nature (Wyatt, 2003). Chemical signals (or *chemosignals*) can be transmitted by direct contact and perceived through sensory organs such as antennae and taste receptors; or they can be medium-borne (e.g. air or water) and be perceived through olfactory channels. This chapter focuses exclusively on airborne chemosignals and olfactory perception, as they are the primary channels for sociochemical communication in terrestrial air-breathing vertebrates, and particularly birds (Bradbury & Vehrencamp, 1998; Wyatt, 2003).

Definitions & function

Many terms and definitions are currently used in the field of chemical ecology, sometimes with little agreement between authors. *Semiochemicals*, the broadest term, encompasses any chemical involved in animal communication. It includes signals used for either intraspecific or interspecific communication. *Pheromones*, on the other hand, are a subclass of semiochemicals used exclusively for intraspecific chemocommunication (Wyatt, 2003). Originally, pheromones were defined as "*substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction, for instance a definite behaviour or developmental process*" (Karlson & Lüscher, 1959). Arguably, some authors have subsequently broadened the original definition of pheromones to include signals of social recognition (kin, clan, individuality) even though such signals do not necessarily elicit a specific response from the receiver (Wyatt, 2003). This thesis adopts the conservative approach of not applying the term pheromone to social signals of recognition. Instead, these signals are referred to simply as *social chemosignals* throughout.

Chemosignals involved in animal communication (e.g. pheromones, scent marks, personal odours) typically contribute to relatively short-range interactions such as territoriality, recognition and mate-choice. The biomolecular nature of these chemosignals implies that, in contrast to the visual or acoustic channels, they are often not suited for immediate, modulated and mutually responsive communication. Yet this is not always true, as some species such as many hymenopterans have developed a chemical language of amazing complexity and responsiveness (Wyatt, 2003; Cardé & Millar, 2004). In most vertebrates, however, chemosignals are used for the prolonged and maintained broadcasting of personal characteristics. This is why some authors consider them to be *state* signals, i.e. signals that remain 'on' for a prolonged time; in contrast to *event* signals, which are typically very short-term manifestations (Hauser, 1996). Importantly, the biogenic origin of social chemosignals makes them particularly appropriate for the transport of subtle physiological and genetic information about the emitter. Accordingly, social chemosignals in vertebrates are often used as signals of recognition and/or quality assessments.

Recognition & assessment

Recognition and assessment processes are some of the most ubiquitous aspects of animal communication. When two animals encounter, much of the information first exchanged serves these two behavioural functions. Recognition is a cognitive process based on a wide range of information that can provide simple class dichotomies (e.g. conspecific versus heterospecific, male versus female, familiar versus unfamiliar), or much finer variations such as relatedness, or individual identity (Thom & Hurst, 2004). Upon perception and discrimination of this information, animals can sort encounters and produce appropriate behavioural responses. In mammals for example, chemosignals can carry social information such as species (Bowers & Alexander, 1967), group membership (Safi & Kerth, 2003; Burgener *et al.*, 2008), relatedness (Ables *et al.*, 2007), hierarchical status (Zhang *et al.*, 2001) or individuality (Smith *et al.*, 2001; Hagey & MacDonald, 2003; Burgener *et al.*, 2009).

Assessments, on the other hand, consist of the evaluation by the receiver of some of the emitter's qualities, using qualitative or quantitative properties of the signal emitted. Secondary sexual traits (such as plumage coloration, call intensity, ornament size) provide many examples of signals used by prospective partners for quality assessments of potential mates (Maynard Smith & Harper, 2003). Yet, assessments based on communication signals are not restricted to the context of sexual selection. They can also occur in various situations such as between conspecific rivals judging each other's competitiveness, or between members of a social group gauging each other's physiological condition. The use of chemical signals for such assessments is common in mammals (Rich & Hurst, 1998; Burgener *et al.*, 2009).

Of particular interest for this thesis are situations in which a signal can be used for both recognition and quality assessment. This is the case, for example, for genetically–based individuality signals. The strong genetic determinism of these signals indeed provides an opportunity for the receiver, not only to uniquely identify the emitter, but also to assess its genetic make-up (Brown & Eklund, 1994; Penn, 2002). By then comparing this information to a reference (itself for instance), an individual may obtain a proxy of the genetic distance between itself and the emitter which is crucial in mate choice systems based on genetic compatibility. In such systems, individuals typically try to avoid partners with genomes either overly dissimilar (e.g. new migrant into a locally adapted population), or overly similar (e.g. kin) to their own (Tregenza & Wedell,

2000). Importantly, these genetically-based recognition/assessment systems require the ability to contrast one's own genetic makeup with that of a conspecific. This complex task appears, based on current evidence, to be achieved primarily through chemical signalling (Penn & Potts, 1999; Tregenza & Wedell, 2000; Schaefer *et al.*, 2002).

Chemical communication in birds

Chemosignals participate in a whole range of social behaviours in mammals including territorial marking, maternal bonding, young-born feeding chemotaxis, mate choice and social structuring (see Burger, 2005; Brennan & Kendrick, 2006 for reviews). Known examples of social chemosignalling in other vertebrate species are much scarcer but include fish (Reusch *et al.*, 2001), amphibians (Waldman & Bishop, 2004), reptiles (Martín & López, 2000) and, of interest for this thesis, birds (Hagelin & Jones, 2007).

Avian chemical communication has long been overlooked because birds were historically considered microsmatic or anosmic (i.e. having little or no smell). Several findings over the last 50 years have, however, progressively led biologists to reconsider the question. First, anatomical evidence emerged supporting claims of functional olfaction in most birds (Bang, 1960). Second, several avian groups (in particular pigeons, vultures, kiwis and procellariiform seabirds) were shown to possess acute olfactory capabilities, used for foraging or orientation (see Roper, 1999 for a review). Finally, a few experiments have drawn the attention of ornithologists to the role of olfaction in avian social interactions. For example, sexual differences in the chemical composition of the uropygial secretions of domestic ducks have been detected before the nesting period (Jacob *et al.*, 1979), and may explain the alteration of sexual behaviours observed in males that were made anosmic (Balthazart & Schoffeniels, 1979). Recently, a similar study on domestic chickens reported that while intact males preferred intact females over uropygial glandectomised females, the preference disappeared in anosmic males (Hirao *et al.*, 2009). Neurophysiological findings on Japanese quails also indicate that brain activation induced by sexual interactions with a female is significantly affected by olfactory deprivation (Balthazart & Taziaux, 2009).

In the light of these and other results discussed below, chemosignals are now suspected to play a more significant role than previously assumed in the social lives of birds (Hagelin & Jones, 2007), highlighting the need to examine these questions.

THE CASE STUDY OF PROCELLARIIFORM SEABIRDS

Why some particular avian groups rely more heavily than others on social chemosignals is a fascinating topic of its own. Yet, regardless of the evolutionary scenarios involved, good avian candidates for the study of chemical communication should all share three key features: i) a reasonably developed olfactory anatomy, ii) a favourable ecological context for the evolution of olfactory mechanisms, and iii) a relatively apparent form of chemosignalling.

Olfactory anatomy of tube-noses

Procellariiform seabirds are an avian order including albatrosses, shearwaters, fulmars and petrels. These birds, sometimes referred to as tubinares or 'tube-noses', all share the anatomical feature of the presence of a tubular nasal passage, on top of the beak, which is used for olfaction (Bang, 1966). Importantly, procellariiforms also possess the most developed olfactory neuroanatomy of all birds. Their average olfactory bulb ratio, i.e. the ratio between the length of the olfactory bulb and the total length of a brain hemisphere, ranges from 18% to 37% (Bang & Cobb 1968). By comparison, the maximum olfactory bulb ratio values in other groups are 24% for anseriforms, 18% for passeriforms and 15% for the galliforms.

The ecological niche for olfactory mechanisms

The olfactory neuroanatomical development of procellariiforms has been hypothetically related to several aspects of the lifestyle of these animals.

First, they are pelagic and thus spend the majority of their life in the open ocean. The absence of obvious visual and/or acoustic landmarks in such an environment may have contributed to the development of other sensory modalities such as olfaction (Wallraff & Andreae, 2000). For example, most procellariiform species respond to food-related olfactory cues, suggesting they can use smell for foraging (Nevitt & Bonadonna, 2005a). Results of experiments at sea further suggest that tube-nosed seabirds follow species-specific foraging strategies that are interdependent, and are more complex than simply tracking prey by scent (Nevitt *et al.*, 2004). Olfactory navigation has also long

been posited for the order (Grubb, 1979; Bonadonna *et al.*, 2003a; Wallraff, 2004; Nevitt & Bonadonna, 2005b). The hypothesis is that seabirds may be able to use a combination of several atmospheric/biogenic gradients as an olfactory map to navigate the open ocean. Although theoretically appealing, to date there is no clear experimental evidence of such a mechanism in procellariiforms.

During the summer months, procellariiforms return to land to breed colonially on remote islands. While larger species such as albatrosses nest at the surface, smaller species nest underground, in burrows that are dug by the males (Warham, 1990). These burrow-nesting species, including most petrels, then become nocturnal around the colony to reduce the still heavy predation from other avian species present such as skuas and gulls (Healy & Guilford, 1990; Bonadonna & Bretagnolle, 2002). It is thus possible that the combination of burrowing, nocturnal, and colonial behaviours during the breeding season has contributed to the evolution of refined olfactory mechanisms in hypogean petrels (Healy & Guilford, 1990). Accordingly, olfaction is required for nest location and homing in these species (Grubb, 1974; Benvenuti *et al.*, 1993; Minguez, 1997; Bonadonna *et al.*, 2001; Bonadonna & Bretagnolle, 2002). What is more, olfactory cues are sufficient to allow identification of the nest (Bonadonna *et al.*, 2003b; Bonadonna *et al.*, 2003c) and even predominate over visual cues for this task (Bonadonna *et al.*, 2004).

The scent of procellariiforms

Because the burrows of hypogean petrels essentially consist of bare earth, roots and a few feathers, the above results on olfactory homing have shed new light on another characteristic of the order: its particular scent. Indeed, procellariiforms are well-known to ornithologists for the distinct musky scent emanating from their plumage (Jacob & Ziswiler, 1982; Weldon & Rappole, 1997). This scent, easily perceptible to the human nose, is still noticeable from empty burrows, months after a breeding season (personal observation). This prompted some pioneering investigations of social chemosignals in hypogean petrels which found that European storm-petrels chicks are able to recognise and orient to their own odour when presented against the odour of a conspecific (De Leon *et al.*, 2003), and that Antarctic prions (small hypogean petrels) are able to recognise their own odour and that of their mate (Bonadonna & Nevitt, 2004).

This introduction presented and discussed the scientific work that led to a realisation of the existence and importance of avian social chemosignalling. While such a realisation has major implications for the fields of behavioural ecology, and avian biology in general, comprehensive study cases examining both the chemical, sensory, and behavioural aspects of the question are yet to be conducted. Petrel seabirds provide an ideal model for such investigation as their ecology presents optimal conditions for the development of olfactory-mediated social behaviours. The project presented in this thesis is built on these scientific realisations.

Section 1.2

PhD structure & Thesis organisation

This thesis explores the topic of chemical communication in procellariiform seabirds, as introduced in the previous section. I have worked in particular with two closely-related species of hypogean petrels, the Antarctic prion (*Pachyptila desolata*) and the blue petrel (*Halobaena caerulea*), two ideal candidates for such investigation. The research project was designed specifically to consider both the *expression* and the *action* components of communication in relation to each other, in order to provide a comprehensive and multidisciplinary case study of avian social chemosignalling.

Chapter 2 is dedicated to the methodology. A particular emphasis is placed on methods developed during the course of the project, whether experimental, chemical, or statistical. As avian chemosignalling is a relatively new field of research, there is no established methodological framework for studies in the field. This chapter thus provides some innovative directions and solutions for future studies.

Chapter 3 considers the expression component of the communication, i.e. the production of social chemosignals. The results from chemical analyses examining the nature of the signals produced by petrels, their ontogeny, and the social information contained within, are presented.

Chapter 4 considers the action component, i.e. the behavioural responses to social chemosignals. The results from field experiments investigating olfactory capabilities of petrels, in relation to the chemical signals unveiled in chapter 3, are presented.

Chapter 5 presents two adjunctive examples of behavioural studies that I designed, or contributed to, during the course of my PhD fieldwork. These experiments, which investigate sensory mechanisms (including olfaction) in other species of seabirds and in different behavioural contexts from the context of my primary research, broaden the scope of this thesis.

Finally, Chapter 6 reviews these findings, and provides an integrated discussion of their ecological and evolutionary implications.

Section 1.3

REFERENCES

Ables E.M., Kay L.M. & Mateo J.M. (2007). Rats assess degree of relatedness from human odors. *Physiology & Behavior* **90**: 726-732.

Balthazart J. & Schoffeniels E. (1979). Pheromones are involved in the control of sexual behavior in birds. *Naturwissenschaften* **66**: 55-56.

Balthazart J. & Taziaux M. (2009). The underestimated role of olfaction in avian reproduction? *Behavioural Brain Research* **200**: 248-259.

Bang B.G. (1960). Anatomical evidence for olfactory function in some species of birds. *Nature* **188**: 547-549.

Bang B.G. (1966). The olfactory apparatus of tubenosed birds (Procellariiformes). *Acta Anatomica* **65**: 391-415.

Bang B.G. & Cobb S. (1968). The size of the olfactory bulb in 108 species of birds. *The Auk* **85**: 55-61.

Benvenuti S., Ioale P. & Massa B. (1993). Olfactory experiments on Cory's shearwater (*Calonectris diomedea*): the effect of intranasal zinc sulphate treatment on short-range homing behaviour. *Italian Journal of Zoology* **60**: 207-210.

Bonadonna F., Benhamou S. & Jouventin P. (2003a). Orientation in "featureless" environments: the extreme case of pelagic birds. In: Berthold P., Gwinner E., Sonnenschein E. (eds). *Avian migration*. Springer, Berlin, Heidelberg: p.367-377.

Bonadonna F. & Bretagnolle V. (2002). Smelling home: a good solution for burrow-finding in nocturnal petrels? *Journal of Experimental Biology* **205**: 2519-2523.

Bonadonna F., Cunningham G.B., Jouventin P., Hesters F. & Nevitt G.A. (2003b). Evidence for nest-odour recognition in two species of diving petrel. *Journal of Experimental Biology* **206**: 3719-3722.

Bonadonna F., Hesters F. & Jouventin P. (2003c). Scent of a nest: discrimination of own-nest odours in Antarctic prions, *Pachyptila desolata*. *Behavioral Ecology and Sociobiology* **54**: 174-178.

Bonadonna F. & Nevitt G.A. (2004). Partner-specific odor recognition in an Antarctic seabird. *Science* **306**: 835.

Bonadonna F., Spaggiari J. & Weimerskirch H. (2001). Could osmotaxis explain the ability of blue petrels to return to their burrows at night? *Journal of Experimental Biology* **204**: 1485-1489.

Bonadonna F., Villafane M., Bajzak C. & Jouventin P. (2004). Recognition of burrow's 'olfactory signature' in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. *Animal Behaviour* **67**: 893-898.

Bowers J.M. & Alexander B.K. (1967). Mice: individual recognition by olfactory cues. *Science* **158**: 1208-1210.

Bradbury J.W. & Vehrencamp S.L. (1998). Principles of animal communication. Sinauer Associates, Inc., Sunderland, Massachusetts.

Brennan P.A. & Kendrick K.M. (2006). Mammalian social odours: attraction and individual recognition. *Philosophical Transactions of the Royal Society of London B Biological Sciences* **361**: 2061-2078.

Brown J.L. & Eklund A. (1994). Kin recognition and the major histocompatibility complex: an integrative review. *American Naturalist* **143**: 435-461.

Burgener N., Dehnhard M., Hofer H. & East M.L. (2009). Does anal gland scent signal identity in the spotted hyaena? *Animal Behaviour* **77**: 707-715.

Burgener N., East M.L., Hofer H. & Dehnhard M. (2008). Do spotted hyena scent marks code for clan membership? In: Hurst J.L., Beynon R.J., Roberts S.C., Wyatt T.D. (eds). *Chemical Signals in Vertebrates*, vol XI. Springer, New York: p.169-177.

Burger B.V. (2005). Mammalian semiochemicals. *Topics in Current Chemistry* **240**: 231-278.

Cardé R.T. & Millar J.G. (2004). Advances in insect chemical ecology. Cambridge University Press, Cambridge.

Dawkins R. (1982). The Extended Phenotype. W.H. Freeman, San Francisco, CA,.

De Leon A., Minguez E. & Belliure B. (2003). Self-odour recognition in European storm-petrel chicks. *Behaviour* **140**: 925-933.

Endler J.A. (1992). Signals, signal conditions, and the direction of evolution *American Naturalist* **139**: S125-S153.

Gleason J.M. & Ritchie M.G. (1998). Evolution of courtship song and reproductive isolation in the drosophila willistoni species complex: do sexual signals diverge the most quickly? *Evolution* **52**: 1493-1500.

Grubb T.C. (1974). Olfactory navigation to the nesting burrow in Leach's petrel (*Oceanodroma leucorrhoa*). *Animal Behaviour* **22**: 192-202.

Grubb T.C. (1979). Olfactory guidance of Leach's storm petrel to the breeding island. *Wilson Bulletin* **91**: 141-143.

Hagelin J.C. & Jones I.L. (2007). Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? *The Auk* **124**: 741-761.

Hagey L. & MacDonald E. (2003). Chemical cues identify gender and individuality in giant pandas (*Ailuropoda melanoleuca*) *Journal of Chemical Ecology* **29**: 1479-1488.

Hauser M.D. (1996). The evolution of communication. MIT Press, Cambridge, Massachusetts.

Healy S. & Guilford T. (1990). Olfactory-bulb size and nocturnality in birds. *Evolution* **44**: 339-346.

Hirao A., Aoyama M. & Sugita S. (2009). The role of uropygial gland on sexual behavior in domestic chicken *Gallus gallus domesticus*. *Behavioural Processes* **80**: 115-120.

Jacob J., Balthazart J. & Schoffeniels E. (1979). Sex differences in the chemical composition of uropygial gland waxes in domestic ducks. *Biochemical Systematics and Ecology* **7**: 149-153.

Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Johansson B.G. & Jones T.M. (2007). The role of chemical communication in mate choice. *Biological Reviews* **82**: 265-289.

Johnstone R.A. (1997). The evolution of animal signals. In: Krebs J.R., Davies N.B. (eds). *Behavioral ecology: an evolutionary approach*. Blackwell Publishing, Oxford: p.155-178.

Karlson P. & Lüscher M. (1959). 'Pheromones': a new term for a class of biologically active substances. *Nature* **183**: 155-156.

Lévy F., Locatelli A., Piketty V., Tillet Y. & Poindron P. (1995). Involvement of the main but not the accessory olfactory system in maternal behaviour of primiparous and multiparous ewes. *Physiology & Behavior* **57**: 97-104.

Lewis D.B. & Gower D.M. (1980). Biology of communication. Blackie, Glasgow and London.

Macedonia J.M. & Stanger K.F. (1994). Phylogeny of the Lemuridae revisited: evidence from communication signals. *Folia Primatologica* **63**: 1-43.

Martín J. & López P. (2000). Chemoreception, symmetry and mate choice in lizards. *Proceedings of the Royal Society of London Series B-Biological Sciences* **267**: 1265-1269.

Maynard Smith J. & Harper D. (2003). Animal Signals. Oxford University Press, Oxford.

Minguez E. (1997). Olfactory recognition by British storm-petrel chicks. *Animal Behaviour* **53**: 701-707.

Nevitt G.A. & Bonadonna F. (2005a). Seeing the world trough the nose of a bird: New developments in the sensory ecology of procellariiform seabirds. *Marine Ecology Progress Series* **287**: 292-295.

Nevitt G.A. & Bonadonna F. (2005b). Sensitivity to dimethyl sulphide suggests a mechanism for olfactory navigation by seabirds. *Biology Letters* **1**: 303 - 305.

Nevitt G.A., Reid K. & Trathan P.N. (2004). Testing olfactory foraging strategies in an Antarctic seabird assemblage. *Journal of Experimental Biology* **207**: 3537-3544.

Penn D. (2002). The scent of genetic compatibility: sexual selection and the Major Histocompatibility Complex. *Ethology* **108**: 1-21.

Penn D.J. & Potts W.K. (1999). The evolution of mating preferences and major histocompatibility complex genes. *American Naturalist* **153**: 145-164.

Ralls K. (1971). Mammalian Scent Marking. *Science* **171**: 443-449.

Reusch T.B.H., Häberli M.A., Aeschlimann P.B. & Milinski M. (2001). Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. *Nature* **414**: 300-302.

Rich T.J. & Hurst J.L. (1998). Scent marks as reliable signals of the competitive ability of mates. *Animal Behaviour* **56**: 727-735.

Roper T.J. (1999). Olfaction in birds. *Advances in the Study of Behavior* **28**: 247-332.

Safi K. & Kerth G. (2003). Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. *Animal Behaviour* **65**: 363-369.

Schaal B., Coureaud G., Langlois D., Ginies C., Semon E. & Perrier G. (2003). Chemical and behavioural characterization of the rabbit mammary pheromone. *Nature* **424**: 68-72.

Schaefer M.L., Yamazaki K., Osada K., Restrepo D. & Beauchamp G.K. (2002). Olfactory fingerprints for major histocompatibility complex-determined body odors II: Relationship among odor maps, genetics, odor composition, and behavior. *Journal of Neuroscience* **22**: 9513-9521.

Shaw K. (1995). Phylogenetic tests of the sensory exploitation model of sexual selection. *Trends in Ecology & Evolution* **10**: 117-120.

Smith T.E., Tomlinson A.J., Mlotkiewicz J.A. & Abbott D.H. (2001). Female marmoset monkeys (*Callithrix jacchus*) can be identified from the chemical composition of their scent marks. *Chemical Senses* **26**: 449-458.

Stoka A.M. (1999). Phylogeny and evolution of chemical communication: an endocrine approach. *Journal of Molecular Endocrinology* **22**: 207-225.

Stratton G.E. & Uetz G.W. (1981). Acoustic communication and reproductive isolation in two species of wolf spiders. *Science* **214**: 575-577.

Thom M.D. & Hurst J.L. (2004). Individual recognition by scent. *Annales Zoologici Fennici* **41**: 765-787.

Tregenza T. & Wedell N. (2000). Genetic compatibility, mate choice and patterns of parentage: Invited review. *Molecular Ecology* **9**: 1013-1027.

Tsutsui N.D. (2004). Scents of self: The expression component of self/non-self recognition systems. *Annales Zoologici Fennici* **41**: 713-727.

Waldman B. & Bishop P.J. (2004). Chemical communication in an archaic anuran amphibian. *Behavioral Ecology* **15**: 88-93.

Wallraff H.G. (2004). Avian olfactory navigation: its empirical foundation and conceptual state. *Animal Behaviour* **67**: 189-204.

Wallraff H.G. & Andreae M.O. (2000). Spatial gradients in ratios of atmospheric trace gases: a study stimulated by experiments on bird navigation. *Tellus* **52B**: 1138-1157.

Warham J. (1990). The petrels: their ecology and breeding systems. Academic Press, London, UK.

Weldon P.J. & Rappole J.H. (1997). A survey of birds odorous or unpalatable to humans: Possible indications of chemical defense. *Journal of Chemical Ecology* **23**: 2609-2633.

Wilson E.O. (1975). Sociobiology: the new synthesis. Harvard University Press, Cambridge (Massachussetts).

Wyatt T.D. (2003). Pheromones and animal behaviour: communication by smell and taste. Cambridge University Press, Cambridge.

Zhang J.-X., Zhang Z.-B. & Wang Z.-W. (2001). Scent, social status, and reproductive condition in rat-like hamsters (*Cricetulus triton*). *Physiology & Behavior* **74**: 415-420.

Chapter 2

METHOD DEVELOPMENTS FOR THE STUDY OF AVIAN SCENTS

AIM & CONTENT

This chapter presents the methodological developments for the chemical characterisation of avian chemosignals made during the project, and provides the background and rationale for these developments. It contains: (i) background considerations on methodological aspects (Section 2.1); (ii) a manuscript, submitted to the *Journal of Chemical Ecology* in March 2010, presenting a comparison of existing and innovative methods for the study of avian chemosignals (Section 2.2); (iii) a presentation of previously available and recently developed approaches for the processing of large chromatographic datasets and their statistical analysis (Section 2.3).

Adapted from Abeona forum (2010)

Section 2.1

GENERAL CONSIDERATIONS ON METHODOLOGY

Note from the author
Subsequent sections of this chapter and Chapter 3 require a basic knowledge in analytical chemistry, in particular Gas-Chromatography and Mass-Spectrometry (GC/MS) techniques. An exhaustive description of such instrumentation is beyond the scope of this thesis, but a brief presentation is provided in Appendix A1. A more comprehensive description can be found in Perry (adapted from 1981).

As described in Section 1.2, questions arising from our research were approached from two different yet complementary biological perspectives: animal behaviour and chemical ecology. Materials and methods used for most field behavioural experiments have already been tested, optimised and used in previous studies. This methodology is described in detail within the publications presented in Chapter 4 of this thesis and elsewhere (Bonadonna & Nevitt, 2004). On the other hand, many field, laboratory, and data processing techniques associated with the chemical aspects of our work are novel. These are the focus of this second chapter.

Being a relatively recent field of investigation, the study of avian chemical communication does not benefit from a wealth of repeatedly tested and optimised protocols. For example, only three studies were found reporting appropriate methods for the sampling of airborne chemosignals (such as body scents) from live animals or secreted material (Moritz & Crewe, 1988; Perrin *et al.*, 1996; Röck *et al.*, 2006). These protocols would require, however, further refinements to be usable with wild birds in the field.

There are nevertheless published studies that investigate the chemical substances secreted by birds, through either uropygial secretions or feather lipids. In this regard, the work of Dr. Jürgen Jacob on the uropygial gland of birds, between 1960 and 1980, provided an invaluable methodological basis for our work (Jacob & Ziswiler, 1982). Several studies have also recently considered the chemicals present on the plumage of

birds for widely different reasons: phylogenetic investigation (Sweeney *et al.*, 2004), sexual selection (Piersma *et al.*, 1999), ectoparasite repellence (Hagelin, 2008) or even organic pollutants biomonitoring (Jaspers *et al.*, 2007). Only one such study, whose methods proved sub-optimal, was however directly motivated by the question of avian chemical communication (Bonadonna *et al.*, 2007). As a consequence, there is not yet a commonly tested and accepted chemical procedure in the field and authors often use their own combination of existing and innovative experimental protocols (e.g. Soini *et al.*, 2007).

Finally, another methodological limitation that has hindered the power of many eco-chemical studies is the availability of procedures for processing and analysing large chromatographic datasets. Indeed, animal chemical profiles usually consist of tens or hundreds of peaks, each corresponding to a specific compound, which may have to be quantified and/or identified. The manual processing of such a dataset is a daunting task which also leaves room for human error and bias. This has led most previous studies on animal chemosignals to either aggregate and/or summarise the chemical information into a few clusters (Jacob & Ziswiler, 1982; Sweeney *et al.*, 2004), to focus on only a few particular compounds within the chromatograms (Douglas *et al.*, 2001) or to restrict the analysis to qualitative descriptions (Burger *et al.*, 2004). What is more, every sample analysed typically results in rich multivariate information as every compound quantified can behave as an independent variable. The inadequacy of traditional statistics to treat such datasets is another factor that had led previous studies to reduce the span of their examination.

Instrumentation used in analytical chemistry, and statistical tools used for processing resulting data, have progressed considerably in the last few decades allowing the emergence of new analytical techniques and procedures. The present chapter introduces some of these new analytical opportunities developed or applied in our research.

Section 2.2

SAMPLING & EXTRACTION TECHNIQUES

CONTEXT

Avian chemosignalling being a relatively novel field, many aspects such as the ontogeny of avian scents, for example, remains unclear. The commonly accepted hypothesis that bird odours originate from the preening of uropygial waxes on to the feathers (Bonadonna *et al.*, 2007; Hagelin & Jones, 2007) is not really supported by the existing evidence (see Section 3.3). This is why different types of samples including uropygial secretions, feathers and airborne volatiles were considered in our research.

This versatility of samples has led us to develop and apply both existing and innovative protocols in our research. The following article therefore presents a comparison of the various combinations of sampling and extracting techniques that were tested.

PRESENTATION OF THE ARTICLE

Title	Existing and innovative experimental protocols for the study of avian chemosignals. Comparative case study with a procellariiform seabird.
Authors	Mardon J., Bonadonna F., West N. & Saunders S.M.
Journal	*Journal of Chemical Ecology*
Date of publication	Submitted
Contribution of PhD-candidate	I have contributed, at a level of about 80%, to all stages of this particular work, including the design of experimental and analytical methods, data sampling, data analysis and redaction/submission of manuscript.

Existing and innovative experimental protocols for the study of avian chemosignals: Comparative case study with a procellariiform seabird

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Abstract

Recent research on bird chemical communication has highlighted the need for new appropriate protocols for studying avian chemosignals. Indeed, although many studies have actually examined chemical substances secreted by birds, only a few have done so from the perspective of chemical communication and none have focused on the actual airborne compounds which compose the olfactory signals. As well as the relative infancy of the field, this gap originates from the absence of an appropriate methodological framework.

In this study, we provide a methodological comparison of various combinations of sampling and analytical techniques that have been used in the field, or that were recently developed and tested in our research. These include: (i) analysis of uropygial secretion samples by solvent extraction, (ii) analysis of feather lipids by solvent extraction; (iii) analysis of airborne signals by solvent extraction, (iv) analysis of feather lipids by direct solid-phase thermal desorption, (v) analysis of airborne signals by thermal desorption. To do so, we use the particular case study of blue petrels (*Halobaena caerulea*), a Subantarctic procellariiform seabird known for its musky smell and good olfactory capabilities. The different methods are compared in terms of chromatographic quality, the number and properties of identified analytes and the biological results they led to. Advantages and limitations of each method are discussed together with challenges that remain to make the new protocols presented more robust.

Keywords: Adsorbent, avian chemosignals, chemical communication, methods, olfaction, petrel seabirds, solvent extraction, thermal desorption.

Running title: Mardon *et al.*, methods for the study of avian chemosignals

ABBREVIATIONS

TD	Thermal desorption
SE	Solvent extraction
GC/MS	Gas chromatograph coupled to a mass spectrometer
RI	Retention index
MW	Molecular weight
SBSE	Stir bar sorptive extraction

INTRODUCTION

For many animals, chemical signals or 'chemosignals' (i.e. pheromones, scent marks, body odours) are an important feature of social behaviours. Bird chemosignalling is, however, a relatively unexplored field of research because avian olfactory capabilities and avian chemosignals (essentially limited to plumage scent) have long been overlooked. A handful of case studies have only recently drawn the attention of biologists to these questions (see Hagelin and Jones, 2007). For example, sex-related chemosignals produced by the preen gland of domestic ducks (Jacob et al., 1979) and chickens have a significant influence on the sexual behaviours and preferences of these species (Balthazart and Schoffeniels, 1979; Hirao et al., 2009). Another example are petrel seabirds (Order: Procellariiformes), an avian group known for their developed olfactory anatomy and good associated capabilities, whose uropygial waxes and plumage lipids contain social information (Bonadonna et al., 2007; Mardon et al., 2010) which are related to olfactory capabilities of both interspecific and intraspecific discrimination/recognition (Bonadonna and Nevitt, 2004; Mardon and Bonadonna, 2009; Bonadonna and Mardon, 2010). These results (among others) have not only invigorated the field, but they have also stressed the need for ornithologists to possess appropriate, accessible and robust protocols for the study of avian chemosignals.

Many studies have actually examined the chemical substances produced by birds for either taxonomic (Jacob, 1978), phylogenetic (Sweeney et al., 2004), physiological (Sandilands et al., 2004), functional (Bolliger and Varga, 1961; Douglas et al., 2001; Reneerkens et al., 2002; Burger et al., 2004) or descriptive (Montalti et al., 2005) reasons. Very few, however, have done so from the perspective of chemical

communication (Jacob et al., 1979; Bonadonna et al., 2007; Mardon et al., 2010). In addition, all of these works have focused the analysis on either uropygial secretions or feather lipids. The uropygial gland, located at the dorsal base of the tail, is indeed the only sebaceous gland of birds and as such, is often considered as the key source of avian chemical substances. It produces large amounts of waxy fluids that are spread on feathers as part of plumage maintenance (Jacob and Ziswiler, 1982). Importantly, the chemical communication framework requires olfactory signals to be perceptible by surrounding individuals, which means that the compounds involved should be of reasonable volatility. Yet to date no published study has focused on the actual airborne signals emitted by birds, as has been done for some insects and rodents (Moritz and Crewe, 1988; Cardé and Millar, 2004; Röck et al., 2006). As well as the relative infancy of the field, a major reason for this gap is undoubtedly the absence of an appropriate methodological framework.

In the present study, we provide a methodological comparison of various combinations of sampling and analytical techniques that have been used in the field, or recently developed and tested in our research. To do so, we use the particular case study of blue petrels (*Halobaena caerulea*), a Subantarctic procellariiform seabird known for its musky smell and the behavioural functions associated with it (Mardon and Bonadonna, 2009; Bonadonna and Mardon, 2010). The sampling approaches considered involve either uropygial secretions, feather lipids or airborne volatiles emitted by the birds. Two different extraction approaches are also compared: solvent extraction (SE) and thermal desorption (TD). In all cases, extracted materials were chemically analysed with a Gas Chromatograph coupled to a Mass Spectrometer (GC/MS). Explicitly, the experimental approaches considered in our study are: (i) analysis of uropygial secretion samples by SE, (ii) analysis of feather lipids by SE, (iii) analysis of airborne signals by SE, (iv) analysis of feather lipids by direct solid-phase TD, and (v) analysis of airborne signals by TD.

The different methods are compared in terms of chromatographic quality, number and properties of identified analytes, and biological results obtained. Although designed to target avian chemosignals, the protocols developed should be readily adaptable to other animal models.

STUDY CONTEXT

Samples used for the present study were collected during three successive austral summers (November to January 2006-07, 2007-08 and 2008-09), on a small Subantarctic island (Ile Verte, 49°51'S, 70°05'E) from the Kerguelen archipelago, a French territory located in the southern Indian Ocean. Chemical analyses were carried out from April 2008 to March 2009 at the University of Western Australia and at the ChemCentre (Perth, Australia).

Blue petrels (*Halobaena caerulea,* Gmelin 1789) live in the Southern Ocean and breed on small oceanic islands around Antarctica where they form dense colonies. These procellariiform seabirds nest in underground burrows and are commonly found around the Kerguelen archipelago. Like most other burrow-nesting petrels, they have a well developed olfactory system (Bang, 1966) and good associated capabilities which are used for foraging (Nevitt, 2000), homing (Bonadonna et al., 2004) and nesting (Bonadonna and Mardon, 2010). Importantly, there is also evidence that chemical communication, via the musky odour of the birds' plumage, is occurring in the species (Mardon and Bonadonna, 2009). Blue petrels therefore provide an appropriate model for the investigation of avian social chemosignals.

METHODS & RESULTS

METHOD 1: ANALYSIS OF UROPYGIAL SECRETIONS BY SE

Field sampling

Secretion samples were obtained from the same 16 blue petrels, during two successive field campaigns (2007-08 and 2008-09), using a protocol adapted from Burger and colleagues (2004). Briefly, uropygial gland contents were collected by gently squeezing the area around the gland, whilst wearing clean nitrile gloves, until a small amount of waxy secretion was discharged. The secretion was then collected with a 100µl glass capillary. The end of the capillary containing the secretion was inserted into a small chromatographic vial. The back end of the capillary, which served as a handle during the collection process, was then broken off and discarded. Finally the vial was sealed with a Teflon® PTFE-faced septum and stored at -4ºC until extraction.

Sample preparation & extraction

When extracting complex organic mixtures, combining polar and non-polar solvents is important to obtain high recovery yields across a wide range of molecular classes. Using solvents of relatively high volatilities also reduces the overlapping between solvent peaks and light analytes in GC/MS analyses (Burger, 2005). Accordingly, uropygial samples were solvent extracted in 400µl of a 1:3 mix of dichloromethane (distilled HPLC grade, ≥99.9%, Sigma-Aldrich®) and n-hexane (distilled AR grade, ≥99.0%, Fluka®), poured directly into the field vial containing the capillary tube. The vial was then resealed and left to stand 7min in a beaker of ice to keep the extracting temperature as low as possible. The extraction mixture in the vial was subsequently transferred into a second clean chromatographic vial, passing through a Pasteur pipette filled with a glass wool plug to filter out impurities (dust, feather debris). Finally, all samples were spiked with 10µl of a standard solution of 2-bromophenol (purum, ≥99.0%, Fluka®) in methanol (AR grade, ≥99.6%, Sigma-Aldrich®) at 504ng/µl for indicative quantification purposes. At this stage, samples were ready for chromatographic analyses as the extracts were sufficiently concentrated to be used without any preconcentrating step.

Chromatographic analysis

Chromatographic analyses were carried out at the University of Western Australia, using a GC/MS (Shimadzu QP2010, Shimadzu Corp.) equipped an autosampler (Shimadzu AOC-20i+s, Shimadzu Corp.) and a generalist Rtx®-5MS capillary column (L=30.0m; Diameter=0.25mm; Thickness=0.10µm). The injection port temperature was set at 250°C and helium was used as carrier gas at a constant linear velocity of 35cm/sec. A volume of 1µl of secretion extracts was injected, in splitless mode, and cold-trapped at 40°C on the column tip for 3min. Samples were subsequently separated using a temperature program of 8°C/min from 40 to 150°C, then 6°C/min from 150 to 200°C and then 2°C/min from 200 to 280°C (hold 15min). The interface temperature was held at 280°C and the ion source temperature at 200°C. The MS was used in scan mode (scan speed=625; scan interval=0.5sec) with an electron source voltage of 70eV and over the mass range of 45 to 350amu.

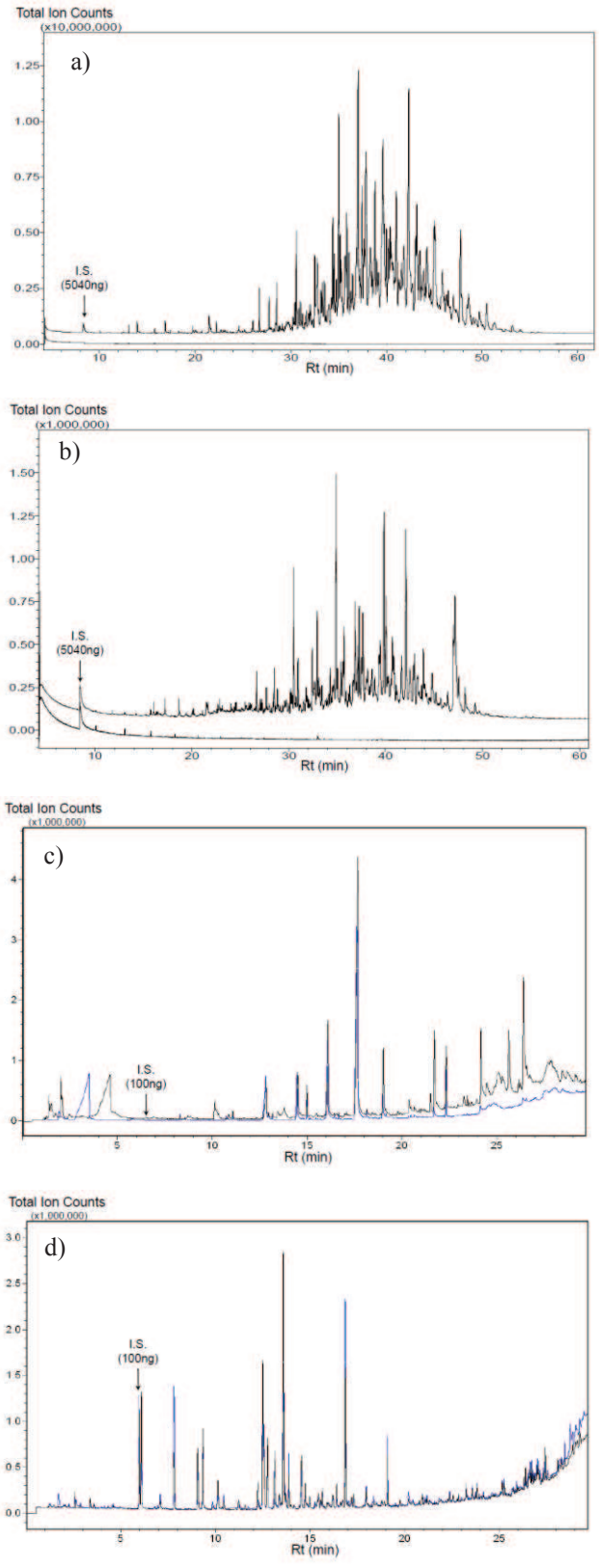

Figure 1: Examples of blank (bottom) and sample (top) chromatograms obtained with the various methods. a: Method 1; b: Method 2; c: Method 4; d: Method 5 (the two chromatograms are hardly distinguishable). The peak of the internal standard (I.S.) and the quantity injected in the sample are indicated.

Chromatographic data processing

Chemical data processing of these samples was carried out with the GCMS Solution software v2.40© (Shimadzu Corp.). In all analyses, background noise was first removed from the data by subtracting the signals obtained from blank samples run regularly within our sample batches (see Supplementary Appendix 2.2-1 for details on analytical blanks). Blanks were designed to account for potential noise from the sampling procedure, the extraction protocol and the instrument. In addition, the quality of all software-defined peak integrations was visually reviewed and manually corrected when necessary.

Qualitative identification of all analytes of interest was determined by cross-checking the best matches obtained from the NIST Mass Spectral Search Program v2.0© (Faircom Corp.) with the calculated Retention Index (RI) of the analytes. Calculated RIs were obtained by calibrating the GCMS Solution software with the retention times

of various linear alkanes between C_{10} and C_{40} (n=15), run under identical chromatographic conditions.

For quantitative analyses, standardisation across the peak areas of the internal standard (2-bromophenol) was used to account for variations in the GC/MS instrument response. In addition, calibration curves were built for various compounds whose size and class were chosen on the basis of preliminary analyses of our samples (see Supplementary Appendix 2.2-2 for details on calibration procedures). Each analyte identified in our samples could thus be quantified by referring to the calibration curve of the most chemically similar calibrated standard.

Results

Method 1 resulted in chromatograms of more than 60min (Fig.1a). Chemical profiles showed in particular a 30min section (25-55min) containing many abundant analytes, essentially fatty esters and alcohols. The sample signal was strong compared to background noise (instrument, sampling), and peak chromatography was good overall. A slower ramp than the one used could provide even better separation of fatty contents although considerably extending the total analysis time.

Compounds detected and tentatively identified ranged from lower semi-volatiles (octanoic acid, nonanal, tetradecane) to large fatty esters (iso-nonadecanoic acid, eicosyl ester). The lower edge of detection sensitivity, essentially for the smaller compounds, was around 100ng per sample (Table 1).

Data analysis led to the identification of clear sociochemical information within the uropygial secretions of blue petrels including species-specific, sex-specific and individually-specific signals (Mardon et al., 2010). Importantly, chemical compounds associated with these signals were large fatty esters and alcohols of relatively low volatility, which are unlikely to be the final carriers of the olfactory information. Method 1 therefore did not lead to the identification of the final olfactory signals but provided instead chemical correlates or precursors of these signals.

METHOD 2: ANALYSIS OF FEATHER LIPIDS BY SE

Field sampling

Feather samples were obtained from the same 16 blue petrels during two successive field campaigns (2007-08 and 2008-09). Wearing clean nitrile gloves, we cut between 100 to 200mg of feathers from the ventral duvet of the birds with steel scissors, rinsed with methanol (LR grade, Sigma-Aldrich®) between samples. Feathers were then packed in aluminium foil, placed in a sealed plastic bag, and stored at -4ºC until chemical analysis.

Sample preparation & extraction

Feather samples were solvent extracted with the same solvent mix as in Method 1. To do so, 60mg of feather were placed in a 50ml conical flask, together with 4ml of the solvent mix and a magnetic stirrer. The flask was then sealed with a hermetic stopper, placed in a beaker of ice to minimise volatilisation of lighter compounds, and the content left to macerate on a magnetic stirring apparatus for 2.5hr. After the maceration, the extract was transferred into a 4ml vial through a Pasteur pipette filled with a glass wool plug, again to filter out impurities from the extract. The extract volume was then reduced to 400µl by attaching the vial to a low pressure liquid nitrogen cold finger manifold (the vacuum was provided by a rotary vacuum pump Genevac® type GRS2), thus concentrating the extract samples approximately 10 times. This drying approach, which relies on the lowering of the pressure to promote evaporation of the solvent, was found to be more effective in minimising losses of light volatile compounds than the usual method of exposing samples to a slow stream of purified nitrogen (Burger et al. 2004). Finally, the 400µl concentrated extract was transferred into a second clean chromatography vial and spiked with 10µl of the same standard solution of 2-bromophenol in methanol as in Method 1.

Chromatographic analysis & data processing

GC/MS conditions for the analysis of the feather lipid extracts were identical to the ones detailed in Method 1.

Results

As for the previous method, Method 2 resulted in chromatograms of more than 60min (Fig.1b). The sample signal again largely dominated the background noise and peak chromatography was satisfactory. The lower edge of detection sensitivity was also similar to Method 1 (Table 1).

Although, the quantity of chemical material retrieved was slightly less, more analytes were detected in this analysis than in the previous one. This is explained by the appearance of new compounds, essentially semi-volatile compounds and cyclic hydrocarbons, on the bird feathers due to different processes (J. Mardon, S. Saunders and F. Bonadonna, unpublished data). The feather chemical profiles were nevertheless analogous to the uropygial signal with a similar 30min section (25-55min) containing abundant analytes. What is more, 95% (253 out of 266) of the secretion analytes were present on the feathers while 79% (253 out of 321) of the feather signal originated from the preen secretions; thus confirming, in petrels at least, the uropygial origin of most feather lipids.

Data analysis of the blue petrel feather lipids logically led to the identification of sociochemical signals similar to the ones found within the uropygial secretions (J. Mardon, S. Saunders and F. Bonadonna, unpublished data). The chemical nature of these feather-borne sociosignals was consistent with the ones previously elucidated within uropygial contents.

METHOD 3: ANALYSIS OF AIRBORNE VOLATILES BY SE

Paradoxically, few studies on vertebrates' chemosignalling have so far focused directly on the airborne volatiles emitted by an animal. This is possibly due to experimental difficulties associated with collecting such samples, particularly in the field. Below, we present a method inspired by laboratory studies of plant, insect or rodent semiochemicals (Moritz and Crewe, 1988; Dicke et al., 1990; Soini et al., 2005; Röck et al., 2006), allowing the quantitative sampling of a known volume of air passing through a chamber containing the animal.

Field sampling

The sampling apparatus (Fig.2) consisted of an activated charcoal trap (400cc in-line gas purifier, filled with charcoal refill kit, Grace/Alltech®) connected with Teflon®

PTFE tubing (OD=6mm, Clean Air Engineering, Inc.) to a chamber formed by an hermetic stainless steel autoclave (modified SEB® pressure cooker model Clipso Ovale, size 44 x 24 x 24cm). A constant laminar air flow was created through the apparatus using a portable air sampling pump (Escort® ELF Pump, MSA) located at the end of the circuit. The air, filtered through the activated charcoal filter, was drawn through the chamber where a bird sat, and then out of the chamber through a glass tube containing 150mg of adsorbent polymer (Fig.3). All junctions to and from the chamber were made with Teflon® PTFE connectors.

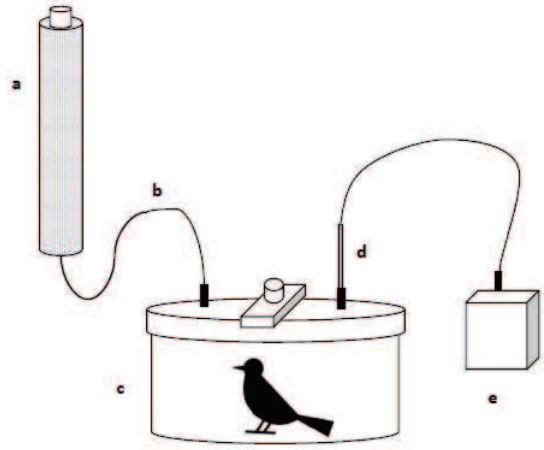

Figure 2: Experimental apparatus for the collection of airborne compounds emitted by birds. Legend: **a**: activated charcoal air filter; **b**: Teflon® tubing connectors; **c**: air chamber; **d**: adsorbent tube; **e**: air sampling pump.

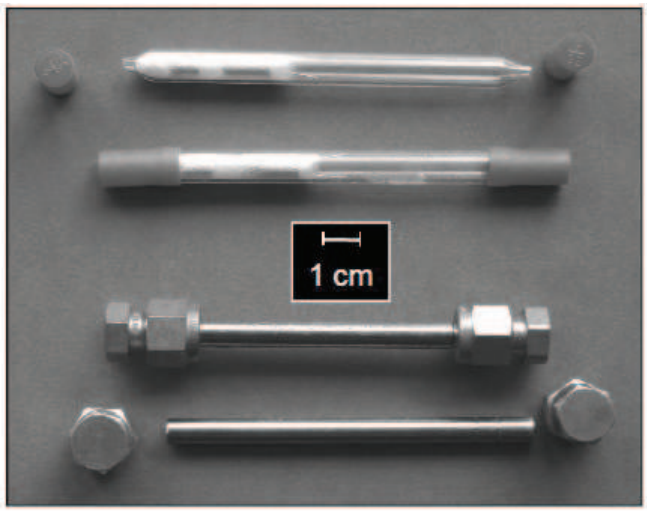

Figure 3: Two different types of adsorbent tubes packed with Tenax® TA (mesh size 35/60) used in our protocols. Top: Glass tubes (Supelco ORBO^{tm} 402) for SE (Method 3); Bottom: Stainless steel tubes for TD (Method 4 & 5).

Among the various adsorbents commercially available for the trapping of volatile compounds, Tenax® TA (mesh size: 35/60) was selected in this study. Tenax® TA, a commonly used organic adsorbent, indeed possesses the most wide-ranging affinities in terms of chemical class and size and is hydrophobic (Scientific Instrument Services Inc., 1995), making it appropriate for the study of complex mixtures of volatiles such as scents, particularly in areas where there may be significant amount of moisture. Air flow was set at 500ml.min^{-1}. Adsorbance efficiency of Tenax® is maximised at low air velocities, and remains fairly consistent for flow rates up to 500ml.min^{-1} (Guillot et al., 2000). A high flow rate, within the range of optimal adsorption efficiency, was chosen considering the volume of air required (dependant on the concentration of the chemosignal) and the level of disturbance for the animal (dependant on sampling time). Air sampling was carried out for 30min, corresponding to 15L of air sampled. These sampling conditions further ensured a virtually zero level of breakthrough for most biogenic organic volatiles at the temperatures where the samples were collected, generally 0-10°C (Scientific Instrument Services Inc., 1995).

Birds were captured from their burrow, transported to the apparatus in a cotton bag and placed in the chamber. After the air sampling, the adsorbent tube was sealed at both ends with Teflon® PTFE caps and stored at -20°C until analysis. Birds were transported back to their burrow, using a cotton bag again. Removing petrels from their burrow does not appear to affect incubation behaviour in the long term or the hatchability of the eggs (Bonadonna and Nevitt, 2004; Bonadonna et al., 2004; Mardon and Bonadonna, 2009), and no bird deserted the nest following experiment in the present study. Interestingly, birds seemed more stressed during handling phases (capture or ringing) than while in the apparatus chamber, during which they often sat quietly in the dark. Between two consecutive samples, the chamber was cleaned with water then methanol (LR grade, Sigma-Aldrich®), and allowed to dry. Activated charcoal from the air filter was renewed every 10 to 15 samples.

The above protocol was used to collect the ‘odours’ from 15 blue petrels during the austral summer 2006-07. Several field and apparatus blank samples were also collected to provide information regarding background noise in subsequent analyses (see Supplementary Appendix 2.2-1).

Sample preparation & extraction

Glass adsorbent tubes (Orbo^{tm} 402) packed with 150mg (2 beds: 100mg+50mg) of Tenax® TA (mesh size 35/60) were used during the 2006-07 field season for several reasons (Fig.3). First, these tubes are reportedly designed for an easy removal of the adsorbent polymer, for subsequent SE. Second, the equipment complexity (and associated costs) required for solvent-based chromatographic techniques is much lower than for thermal desorption (as presented in Method 4 and 5).

The protocol we developed for the SE of Tenax® is adapted from the few studies reporting similar approaches (Barro et al., 2005; Muir et al., 2005). First, glass tubes were scored to recover the 150mg of adsorbent into a clean vial. A volume of 2ml of a 3/2 mix of acetone (distilled AR grade, ≥99.5%, Sigma-Aldrich®) and n-hexane (distilled AR grade, ≥99.0%, Fluka®) was then added to the vial. This solvent mix was chosen following recovery tests of various combinations involving, for the polar fraction, either methanol, dichloromethane, ethyl acetate, diethyl ether, or acetone, and for the non-polar fraction, either hexane or isooctane (see Supplementary Appendix 2.2-3 for details). Vials containing the recovered Tenax® and the extraction mix were capped and sonicated in an ultra-sound bath for 30min. The liquid extract was then quantitavely transferred into chromatographic vials.

Importantly, preliminary work (Supplementary Appendix 2.2-3) showed that Tenax® TA is incompatible with dichloromethane (CH_2Cl_2) in which it reacts and degrades, even after neutralisation of potential HCl in the solvent. This incompatibility is not indicated by suppliers and is only allusively mentioned in another study (Teske et al., 1998).

Results

While the SE technique provided satisfactory results in the cases of uropygial contents and feather lipids, it proved inadequate when working at the low levels of volatiles trapped on the adsorbent. Indeed, no matter the combination of solvent quantity, sonication time, evaporating technique (cold finger manifold or stream of purified nitrogen), level of pre-concentration or GC/MS parameters tried on the samples, we were unable to reach a satisfactory level of sensitivity for this analysis. Although minor peaks were detected, indicating the verge of sensitivity, most chemical profiles were not usable (Table 1). Therefore, optimised chromatographic conditions are

not provided but methodologically-related information can be found in Barro et al. and 2005 and Muir et al., 2005.

To overcome this problem, an alternative extraction/desorption approach, thermal desorption (TD), was investigated. Traditional TD consists of exposing samples to a flow of hot dry inert gas so that chemical compounds present within (or on) are progressively volatilised. Released analytes eventually pass, via the gas flow, to an analytical instrument (a GC/MS in most cases) without any solvent dilution or pre-concentration. Although the retrieving efficiency of TD is lower than through SE (Baltussen et al., 2002), the absence of a dilution effect generally makes it more sensitive than the latter.

METHOD 4: ANALYSIS OF FEATHER LIPIDS BY DIRECT TD

The innovative approach described below was partly inspired by the work of another team investigating mosquitoe-chicken chemical interactions (Santos et al., 2005). However, no outcomes of their work have been published to date (Bernier, personal communication).

Field sampling

Feather samples used in this analysis were the same as for Method 2.

Sample preparation

Using clean nitrile gloves, 15mg of feather from each sample were packed in TD stainless steel tubes (Perkin Elmer; OD=6mm; L=88mm). Feathers were first placed in a 'Loose Fit' Teflon® insert (Liner PTFE, Markes International) which was then dropped in a clean empty TD tube. For quantitation purposes, all samples were then spiked with 10ul of a 10 ng/ul solution of toluene-D8 (99.6% atom D, Aldrich®) in methanol (AR grade, ≥99.6%, Sigma-Aldrich®) (100 ng injected in each TD tube). Once capped, the tubes were ready for TD-GC/MS analyses.

Chromatographic analysis

Chromatographic analyses of these samples were carried out at the ChemCentre, using a Varian Saturn 2000 GC/MS (ion-trap), equipped with a TD autosampler (Perkin Elmer ATD 400 Automatic Thermal Desorption System).

Following the definition of the combustion point of feathers at around 230°C, samples were thermally desorbed for 10min at 210°C to avoid combustion products collecting during analysis. Volatilised analytes were sent to a secondary Tenax® TA trap held at -10°C which was then desorbed in turn, by rapidly heating it from -10°C to 270°C within a few seconds. From there, samples were injected with a split of 10:1 into the GC capillary column (DB-5MS; L=30.0m; Diameter=0.25mm; Thickness=0.25µm). The GC oven temperature was programmed as 30°C (hold 1min), then 5°C/min to 100°C, 10°C/min to 200°C, 15°C/min to 290°C (hold 4min). The transfer line was set at 170°C, the ion trap at 150°C. Standard positive electron ionization (70eV) was used with the scanning rate of 2 scans/sec over the mass range of 45 to 270amu.

Chromatographic data processing

Chemical data processing of these samples was carried out with the Varian MS Workstation v6.5 SP1© (Varian Inc.). In all analyses, background noise was first removed from the data by subtracting the signals obtained from blank samples run regularly within our sample batches (Supplementary Appendix 2.2-1). In addition, the quality of all software-defined peak integrations was visually reviewed and manually corrected when necessary.

Qualitative identification was carried out, for each analyte, by cross-checking the matches obtained from the NIST05 Mass Spectral Database with the calculated RI of the analyte. For quantitative analyses, standardisation across the peak areas of the internal standard (toluene-D8) was used to account for variations in the GC/MS instrument response. In addition, calibration curves were constructed from a suite of standards of various masses and chemical classes (Supplementary Appendix 2.2-2), using air-monitoring NATA (National Association of Testing Authorities, Australia) accredited methods. All analytes detected and identified in our samples could thus be tentatively quantified by referring to one of these curves.

Results

Method 4 resulted in chromatograms (Fig.1c) of 30min. Although a good signal from the feather lipids was obtained, chromatograms also showed background noise from the instrument. Peak chromatography was good overall with little co-elution, although most carboxylic acid peaks significantly tailed. The lower edge of detection sensitivity for this method was found to be about 5 times lower than for the SE technique (Table 1).

The TD approach permitted the detection of several analytes that were more volatile and of a much smaller size than in Method 1 and 2. Interestingly, some of these early eluting compounds were of a different chemical nature than those previously encountered. These included pyrrole, sulphides (dimethyl disulphide) and furans (furan-methanol). The remainder of the analytes detected were from already encountered chemical classes (acids, esters, alkanols and alkanes).

Data analysis of these samples showed that the volatiles desorbed directly from the feather still carried a significant signal of individuality, i.e. a chemical signature, but no clear-cut sex-specific signal (Mardon J., unpublished data). Unfortunately, the background noise present in the data, as well as the use an ion-trap MS, affected the identification of the analytes involved in the signatures.

METHOD 5: ANALYSIS OF AIRBORNE VOLATILES BY TD

Field sampling

The sampling procedure used in this particular protocol was similar to the one detailed for Method 3 (Fig.2) except for the type of adsorbent tubes used. Stainless steel TD tubes (OD=6mm, L=88mm; Perkin-Elmer®; Fig.3) packed with a single bed of 150mg of Tenax® TA (mesh size 35/60) sealed with glass wool, were used (instead of glass tubes) so that the samples could later be thermally desorbed. Samples from the same 20 blue petrels were collected with this protocol in both 2007-08 and 2008-09.

Sample preparation

For quantitative analyses, all sample tubes were spiked with the same solution of toluene-D8 in methanol described in Method 4.

Chromatographic analysis & data processing

All chromatographic aspects of this analysis were similar to the ones detailed in Method 4 except for the desorbing temperature of the tubes. A 270°C temperature (below the 350°C upper limit of Tenax® TA) was used to maximise analyte recovery and minimise the background contribution from the thermal degradation of Tenax® TA.

Results

Method 5 produced chromatograms of 30min that were dominated by background noise (fig.1d). This noise made the discrimination of the bird-associated signal significantly harder, resulting in fewer analytes detected in this analysis (table 1). Again, peak chromatography was satisfactory, with the exception of carboxylic acid peaks. This method achieved the best sensitivity of all with a lower threshold of 10ng of material per sample.

Interestingly, the smaller early eluting analytes identified in Method 4, in particular furanes and sulphides, were also found with this method. Most of the analytes detected were however from the more usual acid, alkanes, alkanols and ester classes.

Data analysis did not provide clear-cut evidence of social-chemosignals, although a significant sex-effect, potentially due to one particular compound, was obtained. The high background, however, prevents any definitive conclusion at this stage of our research.

Table 1: Chromatographic performances of the different methods

Method #	Sample type	Extraction method	N analytes detected	Ranges for bird-derived detected analytes				Class of analytes detected	Signal to noise ratio [a]	Origin of noise
				Carbon chain size	MW ($g.mol^{-1}$)	RI	Amount in samples (in ng)			
1	Uropygial secretions	SE	266	C_8 - C_{39}	142 - 578	1105 - 4020	100 – 29095	Aldehydes, Alkanes, Alkanols, Alkenes, Amides, Carboxylic acids, Esters	High	Instrument
2	Feathers lipids	SE	321	C_8 - C_{39}	142 - 638	1105 – 4335	100 – 8360	Aldehydes, Alkanes, Alkanols, Alkenes, Amides, Aromatic hydrocarbons, Cholestanol, Carboxylic acids, Esters	High	Instrument
3	Airborne volatiles	SE	5	C_{12} - C_{17}	168 - 268	1030 – 1955	NA	Alkanes, Alkanols	Very low	Instrument Adsorbent Ambient
4	Feathers lipids	TD	71	C_2[b] – C_{18}	94 - 270	750 - 2075	14 – 169380[c]	Aldehydes, Alkanes, Alkanols, Aromatic hydrocarbons, Carboxylic acids, Esters, Furanes, Pyrroles, Sulphides	Fair	Instrument
5	Airborne volatiles	TD	22	C_2[b] – C_{13}	86 – 214	660 - 1550	10 – 1095[c]	Aldehydes, Alkanes, Alkanols, Carboxylic acids, Esters, Furanes, Sulphides	Low	Instrument Adsorbent Ambient

TD = Thermal desorption, MW = Molecular Weight, RI = Retention Index, NA = Non-applicable

[a] Relative contribution of the sample signal compared to the instrument/experimental background

[b] C_2 is Dimethyl disulphide (DMDS) measured at around 20ng on average per sample

[c] Value linearly extrapolated because outside the calibration range

DISCUSSION

The merits of the three different sampling approaches (uropygial secretions, feather lipids or airborne volatiles) and two different extraction approaches considered in our research (SE and TD) are compared below, highlighting the advantages and limitations of each method.

Sampling techniques

Collection of uropygial secretions and/or feathers from birds is relatively common and straightforward (Bolliger and Varga, 1961; Reneerkens et al., 2002; Burger et al., 2004; Sweeney et al., 2004; Bonadonna et al., 2007; Soini et al., 2007). These collection techniques present significant advantages such as their rapidity, their logistical simplicity and the relatively high quantity of chemical material they can provide. In addition, they involve only minor environmental interferences, such as atmospheric pollutants deposited on the feathers (J. Mardon, S. Saunders and F. Bonadonna, unpublished data), which do not affect the clarity of the biogenic signals. There are however a few important issues to consider. First, these approaches, and the collection of uropygial contents in particular, require a significant amount of handling which can be stressful for birds (Le Maho et al., 1992). In addition, the species biology, and particularly the timing of moulting, has to be considered when deciding between cutting feathers or pulling them whole so that the effect on thermal insulation is minimised.

Sampling of airborne volatiles with the novel apparatus described in Method 3 is logistically more complex than the above techniques. It also requires a longer time so that sufficient levels of airborne signals can be collected on the adsorbent traps. The stress induced appears however reduced as animals were globally much calmer during such sampling than while being handled. Most importantly, this technique successfully collects bird-emitted airborne compounds which are the targets of chemical communication research. The apparatus described here led to significant environmental noise from unfiltered anthropogenic ambient volatile organic compounds (VOCs). Methodological refinements are currently being tested to address this issue.

Extracting techniques

Solvent extracting approaches are based on the chemical affinity between sample materials and solvents. As such, they require reasonable chemical skills in order to tune

protocols to samples (Burger et al., 2004). They can lead to good chromatographic data provided there is sufficient chemical material extracted. Some downsides of these techniques are, however, critical to consider when studying animal chemosignals. Because there is no universal solvent (or solvent mix) able to achieve a comprehensive recovery of polar and non-polar mixtures, SE inevitably results in a partial qualitative loss of chemical signals. Solvents can also directly impact upon the most reactive/fragile fraction of organic mixtures (e.g. amines). What is more, the addition of solvent itself induces an important dilution effect which is undesired when examining subtle olfactory chemosignals. Finally, although generally chosen for their low boiling points, solvents result in large chromatographic peaks that affect the exploration of the most volatile fraction of chromatograms.

All these limitations are particularly apparent in the poor outcome from Method 3 which also confirms that solvent desorption is not a particularly sensitive technique (Ligocki and Pankow, 1985; Baltussen et al., 2002). Although solvents can theoretically break stronger adsorbent–analyte interactions than TD, leading to better recoveries (Baltussen et al., 2002), sample dilution reduces the sensitivity of this approach. The combination of adsorbent trapping and SE thus appears not suited for the profiling of whole animal scents. The approach may nevertheless prove useful for the analysis of a-priori identified targets, such as pheromones or environmental pollutants (Barro et al., 2005). Indeed, the use of restricted search options (e.g. the specific ion mode of mass spectrometers) may provide sufficient sensitivity in these cases.

As illustrated by our results, TD provides a higher sensitivity than SE due to the absence of dilution and the quantitative transfer of desorbed material to the analytical instrument. What is more, the absence of solvent facilitates the examination of early eluting compounds, a key aspect for chemosignal research. For example, the detection of dimethyl disulphide (DMDS) in both the desorbed feather lipids and airborne volatiles is intriguing as it has been shown that blue petrel chicks and adults respond to the odour of related compound (dimethyl sulphide or DMS) (Nevitt et al., 1995; Bonadonna et al., 2006). Although more work is required to ascertain the origin of the DMDS detected in our samples, a biogenic origin and behavioural function is not improbable (Singer et al., 1976).

Several issues however need to be addressed before the combination of adsorbent trapping and TD (Method 5) leads to the types of unequivocal results obtained with SE of feathers and secretions. First, the high temperatures needed to achieve sufficient

recoveries from the adsorbent may result in the breakdown of the trapped analytes, particularly for polar analytes such as alcohols, carboxylic acids, and so on (Baltussen et al., 2002). Second, there is a clear need for better generalist adsorbents that could reduce noise while increasing the sample signal. Indeed, not only does Tenax® (and most other adsorbents) release significant amounts of breakdown products in the GC/MS, but its strong affinity for polar analytes leads to poor recovery rates of these (Baltussen et al., 2002). This is particularly problematic for chemical ecologists as polar compounds are very often involved in biogenic chemosignals (Soini et al., 2005). Until the development of such adsorbents, this technique will remain essentially suited for environmental studies monitoring non-polar analytes (e.g. hydrocarbons such as alkanes, alkenes and aromatics).

Direct solid-phase TD however, such as presented in Method 4, is not exposed to most of these restrictions. It combines advantages from using an abundant material (i.e. feather lipids) with the advantages of using TD (sensitivity and focus on volatile compounds), while avoiding most environmental and adsorbent noise. The lower number of analytes obtained from this method (compared to SE) should also facilitate the targeting of biologically active compounds. This method therefore presents particularly interesting experimental prospects and results.

Final considerations

The choice of a particular sampling or extraction technique is generally influenced by the theoretical framework of a study. For instance, the scarcity of studies examining avian chemosignals from the perspective of chemical communication has led to a methodological bias towards SE of uropygial secretions and feathers. This is critical to consider when examining the resolution and relevance of data obtained so far. Avian secretions and feather lipids typically contain a complex mix of both large waxy non-volatile and smaller potentially volatile compounds (Jacob, 1978). To screen these whole mixtures in search of potential olfactory chemosignals present some analytical shortcomings. First, the relative abundances of volatile and waxy contents may be very dissimilar. As a result, the levels of analytical sensitivity required to explore the full range of chemical fractions has proven to be extremely different. On a similar note, highly abundant analytes may be 'masking' the presence of other less abundant compounds (for instance because of coelution, detector saturation or scale distortion). Third, the often very large number of compounds detected in avian chemical substances

can compel biologists to select a-priori the compounds, or class of compounds, which could be relevant (Soini et al., 2007). Such a decision, apart from requiring a good chemical expertise, is not entirely satisfactory as the nature of the biologically active signals remain in most cases completely unknown. Finally, screening secretions or feather lipids for volatile signals might overlook the various degradation processes (oxidation, enzymatic breakdown and photolysis) which may exogenously convert secreted lipid precursors into their biologically active forms (Wisthaler and Weschler, 2009; J. Mardon, S. Saunders and F. Bonadonna, unpublished data). This is, in essence, why we have recently designed and started optimising alternative techniques as presented here.

There are, in addition, other recent and newly emerging alternative methods for the study of animal chemosignals which are not considered in our comparison, due of limitations of available resources and equipment in our study. The technique of stir bar sorptive extraction (SBSE) (Baltussen et al., 1999), has recently been successfully applied to biological media and avian endogenous chemicals (Soini et al., 2005; Soini et al., 2007). SBSE uses a polymer-coated magnetic stir bar which allows sorption of the sample and subsequent thermal desorption. Applicability, advantages and limits of this technique have been described elsewhere (Soini et al., 2005). In comparison to the adsorptive techniques presented here, the extraction efficiency of SBSE is better for semi-volatiles but slightly lower for more volatile compounds. SBSE nevertheless provides good quantitative reproducibility and a wider range of compounds on thermal desorption than Tenax®. Another potential avenue of future methodological development is the use of Proton-Transfer-Reaction Mass-Spectrometry (PTR-MS) (Lindinger et al., 1998), a technique recently developed and classically used in atmospheric chemistry. PTR-MS does not require any extraction or separation step such as traditional GC techniques, so that there is less chance of chromatographic losses or external interferences with the analysis. It is well suited to the measurement of very low concentrations of volatiles (Schwarz et al., 2009) and could potentially be adapted to the measurement of volatile emissions from feathers, secretions or whole animals (E. Zardin, personal communication).

In conclusion, ecologists working in the field of avian chemical communication (and animal chemosignalling in general) still await the development of satisfactory, sensitive and logistically reasonable techniques. We have presented here several techniques

specifically used for the study of avian chemical signals, and have compared their relative properties. The limits of traditional techniques, such as SE, are illustrated and new alternative techniques, such as adsorbent trapping or solid-phase thermal desorption, are introduced. Advantages of these approaches and the challenges to make them entirely robust are discussed. This study contributes to the general methodological effort dedicated to these questions, and there is little doubt that exciting discoveries are around the corner.

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REFERENCES

Balthazart J. & Schoffeniels E. (1979). Pheromones are involved in the control of sexual behavior in birds. *Naturwissenschaften* **66**: 55-56.

Baltussen E., Cramers C.A. & Sandra P.J.F. (2002). Sorptive sample preparation – a review. *Analytical and Bioanalytical Chemistry* **373**: 3-22.

Baltussen E., Sandra P., David F. & Cramers C. (1999). Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: Theory and principles. *Journal of Microcolumn Separations* **11**: 737-747.

Bang B.G. (1966). The olfactory apparatus of tubenosed birds (Procellariiformes). *Acta Anatomica* **65**: 391-415.

Barro R., Ares S., Garcia-Jares C., Llompart M. & Cela R. (2005). A simple and fast micromethod for the analysis of polychlorinated biphenyls in air by sorbent enrichment and ultrasound-assisted solvent extraction. *Analytical and Bioanalytical Chemistry* **381**: 255–260.

Bolliger A. & Varga D. (1961). Feather Lipids. *Nature* **190**: 1125.

Bonadonna F., Caro S., Jouventin P. & Nevitt G.A. (2006). Evidence that blue petrel, Halobaena Caerulea, fledglings can detect and orient to dimethyl sulfide. *Journal of Experimental Biology* **209**: 2165-2169.

Bonadonna F. & Mardon J. (2010). One house two families: petrel squatters get a sniff of low-cost breeding opportunities. *Ethology* **116**: 176-182.

Bonadonna F., Miguel E., Grosbois V., Jouventin P. & Bessiere J.-M. (2007). Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? *Journal of Chemical Ecology* **33**: 1819-1829.

Bonadonna F. & Nevitt G.A. (2004). Partner-specific odor recognition in an Antarctic seabird. *Science* **306**: 835.

Bonadonna F., Villafane M., Bajzak C. & Jouventin P. (2004). Recognition of burrow's 'olfactory signature' in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. *Animal Behaviour* **67**: 893-898.

Burger B.V. (2005). Mammalian semiochemicals. *Topics in Current Chemistry* **240**: 231-278.

Burger B.V., Reiter B., Borzyk O. & du Plessis M.A. (2004). Avian exocrine secretions I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. *Journal of Chemical Ecology* **30**: 1603-1611.

Cardé R.T. & Millar J.G. (2004). Advances in insect chemical ecology. Cambridge University Press, Cambridge.

Dicke M., Beek T.A.V., Posthumus M.A., Dom N.B., Bokhoven H.V. & Groot A.D. (1990). Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *Journal of Chemical Ecology* **16**: 381-396.

Douglas H.D., III, Co J.E., Jones T.H. & Conner W.E. (2001). Chemistry, production and potential functions of aldehyde odorants in the crested auklet (*Aethia cristatella*). *American Zoologist* **41**: 1641-1641.

Guillot J.-M., Fernandez B. & Cloirec P.L. (2000). Advantages and limits of adsorption sampling for physico-chemical measurements of odorous compounds. *Analusis* **28**: 180-187.

Hagelin J.C. & Jones I.L. (2007). Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? *The Auk* **124**: 741-761.

Hirao A., Aoyama M. & Sugita S. (2009). The role of uropygial gland on sexual behavior in domestic chicken *Gallus gallus domesticus*. *Behavioural Processes* **80**: 115-120.

Jacob J. (1978). Uropygial gland secretions and feather waxes. In: Florkin M., Scheer B.T. (eds). *Chemical zoology*, vol 10 Aves. Academic press, New York: p.165-211.

Jacob J., Balthazart J. & Schoffeniels E. (1979). Sex differences in the chemical composition of uropygial gland waxes in domestic ducks. *Biochemical Systematics and Ecology* **7**: 149-153.

Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Le Maho Y., Karmann H., Briot D., Handrich Y., Robin J.-P., Mioskowski E., Cherel Y. & Farni J. (1992). Stress in birds due to routine handling and a technique to avoid it. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **263**: 775-781.

Ligocki M.P. & Pankow J.F. (1985). Assessment of Adsorption/Solvent Extraction with Polyurethane Foam and Adsorption/Thermal Desorption with Tenax-GC for the Collection and Analysis of Ambient Organic Vapors. *Analytical chemistry* **57**: 1138-1344.

Lindinger W., Hansel A. & Jordan A. (1998). On-line monitoring of volatile organic compound at pptv levels by means of Proton-Transfer-Reaction Mass Spectrometry (PTR-MS): Medical applications, food control and environmental research. Review paper. *International Journal of Mass Spectrometry and Ion Processes* **173**: 191-241.

Mardon J. & Bonadonna F. (2009). Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own. *Behavioral Ecology and Sociobiology* **63**: 537-542.

Mardon J., Saunders S.M., Anderson M.J., Couchoux C. & Bonadonna F. (2010). Species, gender and identity: cracking petrels' socio-chemical code. *Chemical Senses* **Advance access**: doi:10.1093/chemse/bjq021.

Montalti D., Gutiérrez A.M., Reboredo G. & Salibián A. (2005). The chemical composition of the uropygial gland secretion of rock dove *Columba livia*. *Comparative Biochemistry and Physiology A Comparative Physiology* **140**: 275-279.

Moritz R.F.A. & Crewe R.M. (1988). Chemical signals of queens in kin recognition of honeybees, *Apis mellifera L. Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **164**: 83-89.

Muir B., McDonald G., Cooper D.B. & Moranb M.C. (2005). Optimisation of solvent desorption conditions for chemical warfare agent and simulant compounds from Porapak QTM using experimental design. Part 2: Extraction of sulphur mustard from steel and glass PorapakTM tubes. *Journal of Chromatography A* **1076**: 1-6.

Nevitt G.A. (2000). Olfactory foraging by Antarctic procellariiform seabirds: life at high Reynolds numbers. *Biological Bulletin (Woods Hole)* **198**: 245-253.

Nevitt G.A., Veit R.R. & Kareiva P. (1995). Dimethyl sulphide as a foraging cue for Antarctic procellariiform seabirds. *Nature* **376**: 680-682.

Reneerkens J., Piersma T. & Sinninghe-Damsté J.S. (2002). Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proceedings of the Royal Society of London B Biological Sciences* **269**: 2135-2139.

Röck F., Mueller S., Weimar U., Rammensee H.-G. & Overath P. (2006). Comparative analysis of volatile constituents from mice and their urine. *Journal of Chemical Ecology* **32**: 1333-1346.

Sandilands V., Savory J. & Powell K. (2004). Preen gland function in layer fowls: factors affecting morphology and feather lipid levels. *Comparative Biochemistry and Physiology A Comparative Physiology* **137**: 217-255.

Santos S.M., Bernier U.R., Allan S.A., Quinn B.P., Booth M.M., Kline D.L., Barnard D.R. & Yost R.A. (2005). Identification of candidate attractant compounds from

chicken feathers for the mosquito vector of the west nile virus by gc/ms. Proceedings of the 53rd ASMS Conference on Mass Spectrometry and Allied Topics. In: 53rd ASMS Conference on Mass Spectrometry and Allied Topics. , San Antonio, Texas

Schwarz K., Filipiak W. & Amann A. (2009). Determining concentration patterns of volatile compounds in exhaled breath by PTR-MS *Journal of breath research* **3**: 027002.

Scientific Instrument Services Inc. (1995). Adsorbent resins guide (updated: 2009). In, vol 2010. http://www.sisweb.com/index/referenc/resins.htm

Singer A.G., Agosta W.C., O'Connell R.J., Pfaffmann C., Bowen D.V. & Field F.H. (1976). Dimethyl disulfide: an attractant pheromone in hamster vaginal secretion. *Science* **191**: 948-950.

Soini H.A., Bruce K.E., Wiesler D., David F., Sandra P. & Novotny M.V. (2005). Stir bar sorptive extraction: a new quantitative and comprehensive sampling technique for determination of chemical signal profiles from biological media. *Journal of Chemical Ecology* **31**: 377-392.

Soini H.A., Schrock S.E., Bruce K.E., Wiesler D., Ketterson E.D. & Novotny M.V. (2007). Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*). *Journal of Chemical Ecology* **33**: 183-198.

Sweeney R.J., Lovette I.J. & Harvey E.L. (2004). Evolutionary variation in feather waxes of passerine birds. *The Auk* **121**: 435-445.

Teske J., Efer J. & Engewald W. (1998). Large-Volume PTV Injection: Comparison of Direct Water Injection and In-Vial Extraction for GC Analysis of Triazines. *Chromatographia* **47**: 35-41.

Wisthaler A. & Weschler C.J. (2009). Reactions of ozone with human skin lipids: sources of carbonyls, dicarbonyls, and hydroxycarbonyls in indoor air. *Proceedings of the National Academy of Sciences of the United States of America* **Advance access**: doi10.1073/pnas.0904498106.

SUPPLEMENTARY MATERIAL

APPENDIX 2.2-1: ANALYTICAL BLANKS USED IN THE VARIOUS METHODS

Table 2.2-1 : Description of the experimental blanks used in each method

Method #	Blank type	Origin of noise targeted	Description	Minimum frequency
1 & 2	A)	Sampling procedures	Clean capillary tube in vial transported with the other samples	2 / sample batch
	B)	Extraction procedures (solvent, filtering, drying)	All extraction steps applied to a solvent aliquot.	1 / 10 samples
	C)	GC/MS instrument	Pure solvent ran in GC/MS	2 / sample batch (start and end)
3	A)	Adsorbent tubes	Unused sealed tube (transported with other samples)	2 / sample batch
	B)	Ambient contaminants	Tubes used in the field to sample ambient air directly	2 / sample batch
	C)	Sampling procedures	Tubes used in the field with an empty apparatus	1 / 7 samples
	D)	Bird's faeces	Tubes used with the apparatus to collect volatiles from birds' faeces	2 / sample batch
4	A)	PTFE-inserts	TD tube with an empty insert only	1 / 10 samples
	B)	TD-GC/MS instrument	Empty TD tube ran in GC/MS	2 / sample batch (start and end)
5	A)	Adsorbent tubes	Unused sealed tube (transported with other samples)	2 / sample batch
	B)	Ambient contaminants	Tubes used in the field to sample ambient air directly	2 / sample batch
	C)	Sampling procedures	Tubes used in the field with an empty apparatus	1 / 7 samples
	D)	Bird's faeces	Tubes used with the apparatus to collect volatiles from birds' faeces	2 / sample batch
	E)	TD-GC/MS instrument	Clean Tenax® tube ran in GC/MS	2 / sample batch (start and end)

APPENDIX 2.2-2: DETAILS OF CALIBRATION PROCEDURES

Obtaining absolute quantitative information (i.e. the abundance) about an analyte present in a chromatographic sample requires building a calibration curve for this particular, or a similar compound. This calibration provides a relationship between the amount of compound injected in the GC/MS and the peak area observed on the chromatogram. Because the nature of this relationship varies from compound to compound, in particular as a function of molecular classes (e.g. alkanes, aldehydes and so on), several relevant calibration curves usually need to be constructed.

Calibration for Methods 1 & 2

For analyses using Method 1 or Method 2, calibration curves were built for 17 different compounds, representing 12 different chemical classes (Table 2.2-2a). Standard solutions were prepared in the same solvent mix as used for extraction. To reduce analytical time, the 17 compounds were organised into 4 standard mixtures which were designed to be chemically relatively homogenous to minimise reactivity. For each standard mixture, two completely independent sets of five dilutions were prepared representing a 1000 fold range of concentrations. The duplicate sets associated with the four mixtures (i.e. 5 dilutions/set x 2 sets/mixture x 4 mixtures = 40 solutions) were run on the GC/MS before each sample batch.

Calibration for Methods 4 & 5

For analyses using Methods 4 and 5, calibration curves were built for 14 different compounds, representing 12 different chemical classes (Table 2.2-2b). Standard solutions were prepared in methanol (AR grade, ≥99.6%, Sigma-Aldrich®). As described above, the 14 compounds were organised into 3 mixtures to reduce analytical time while minimising reactivity. The 3 standard mixtures were prepared, in terms of relative abundance of each standard, so that the injection of 1µl of a given mixture in a 6.0L canister, passivated as per US EPA TO14A and brought to a pressure of 3atm, resulted in a 1ppm (v/v) concentration for each of its standard constituents in the canister. The three canisters prepared were then sub-sampled and a known volume (corresponding to a known mass load) was taken from the canister and spiked on to Tenax® TA-packed TD tubes according to the accredited method US EPA TO17A. For each calibration and each mixture (i.e. each canister), a new set of 6 TD tubes was freshly prepared corresponding to a 23 fold range of mass abundance. A complete

calibration (i.e. 6 tubes/set x 1 set/mixture x 3 mixtures = 18 tubes) was run on the TD-GC/MS before each sample batch.

Table 2.2-2a: Calibration standards for Methods 1 & 2

Chemical class (& Mixture #)	Standard(s)	MW ($g.mol^{-1}$)	Calibration range (ng/sample)	Average r^2 [a]
Alkane (1)	n-Decane	142	57 – 11504	0.9967
	n-Pentadecane	212	95 – 19040	
Alkene (1)	Tridecene	182	112 – 22400	0.9951
Alkyne (1)	1,8-Nonadiyne	120	77 – 15520	0.9978
Homocyclic hydrocarbon (1)	Cyclooctene, (Z)-	110	40 – 8000	0.9962
Heterocyclic hydrocarbon (1)	1-Butyl Imidazole	124	90 – 18080	0.9991
Aromatic (1)	4-tert-Butyl-Phenol	150	67 – 13440	0.9994
Alkanol (2)	n-Octanol	130	97 – 19520	0.9930
	n-Tetradecanol	214	85 – 17120	0.9914
Aldehyde (2)	Decanal	156	103 – 20640	0.9938
Acid (3)	Nonanoic acid	158	84 – 16960	0.9990
	Tetradecanoic acid	228	112 – 22400	0.9891
Ester (3)	Nonanoic acid, methyl ester	172	80 – 16160	0.9998
	Dodecanodioic acid, 1,12-dimethyl ester	258	147 – 29440	0.9985
Amide (4)	Trimethylacetamide	101	118 – 23680	0.9999
	Undecenylamide	186	70 – 14080	0.9964
Amine (4)	Dibutylamine	129	132 – 26560	0.9892

[a] A r^2 value was obtained for each standard and for each calibration by a least-square linear regression including data points from both duplicates. r^2 shown in the table are the averages of values obtained across all calibrations run during our analyses.

Table 2.2-2b: Calibration standards for Methods 4 & 5

Chemical class (& Mixture #)	Standard(s)	MW ($g.mol^{-1}$)	Calibration range (ng/tube)	Average $r^{2\,a}$
Alkane (1)	n-Decane	142	20 - 479	0.9984
	n-Pentadecane	212	31 - 720	0.9898
Alkene (1)	1-Octene	182	17 – 396	0.9994
Alkyne (1)	1,8-Nonadiyne	120	16 – 388	0.9990
Homocyclic hydrocarbon (1)	Cyclooctene, (Z)-	110	16 – 376	0.9956
Heterocyclic hydrocarbon (1)	1-Butyl Imidazole	124	19 – 439	0.6808
Aromatic (1)	p-xylene	106	16 – 385	0.9947
Amide (1)	Undecenylamide	186	27 – 621	0.7411
Alkanol (2)	n-Octanol	130	18 – 426	0.9645
	n-Tetradecanol	214	31 - 732	0.9873
Aldehyde (2)	Decanal	156	23 – 533	0.9561
Acid (3)	Nonanoic acid	158	23 – 547	0.9816
Ester (3)	Nonanoic acid, methyl ester	172	25 – 591	0.9422
Sulphide (3)	Disulphide, dimethyl	94	13 – 311	0.9940

[a] A r^2 value was obtained for each standard and for each calibration by a least-square linear regression. r^2 shown in the table are the averages of values obtained across all calibrations run during our analyses.

APPENDIX 2.2-3: CHOICE OF A SOLVENT FOR THE EXTRACTION OF TENAX®

While developing Method 3, we set up a preliminary test to identify an optimal extracting solvent for Tenax® TA. As described in Method 1, combining polar and non-polar solvents is important, when extracting complex organic mixtures, to obtain high recovery yields across a wide range of molecular classes. We therefore tried all possible combinations of 2 solvents using either methanol, dichloromethane, ethyl acetate, diethyl ether, or acetone (for the polar fraction), and hexane or isooctane (for the non-polar fraction). For each combination, a recovery test was carried out in duplicate. Recovery tests were done using a 10ppm mixture of 8 standards (n-decane, n-heptadecane, p-xylene, 1-nonanol, 1-tetradecanol, nonanoic acid methyl ester, n-hexanal, n-decanal) loaded on 150 mg of Tenax® TA (mesh size: 35/60). A qualitative summary of the results from these tests is presented in Table 2.2-3.

Table 2.2-3: Solvents tested for extraction of complex mixtures from Tenax® TA

Solvent	Polarity	Boiling point	Efficiency[a] on Tenax® TA	Additional comments
Methanol	Polar	65 ºC	Poor	Does not mix well with non-polar solvents or Tenax
Dichloromethane	Polar	40 ºC	NA	Incompatible with Tenax® TA
Ethyl acetate	Polar	77 ºC	Good	High boiling point so not suitable for the study of volatiles
Diethyl ether	Polar	35 ºC	Good	Very high volatility complicating sample extraction and conservation. Also a safety hazard
Acetone	Polar	56 ºC	Good	Good extraction efficiency and volatility
Hexane	Non-polar	69 ºC	Good	Good extraction efficiency and volatility
Isooctane	Non-polar	98 ºC	Acceptable	Lower extraction efficiency than hexane and higher boiling point.

[a]*Recovery efficiency ratings: Poor = 0-50 %, Acceptable = 50-70%, Good = 70-90%, Excellent > 90%*
NA : Not applicable

Section 2.3

DATA PROCESSING & STATISTICS

POST-PROCESSING OF CHROMATOGRAPHIC DATA

Processing GC/MS data involves various tasks which, depending on the type of information sought, may include: (i) alignment of chromatograms, (ii) detection/definition of peaks, (iii) characterisation and removal of noise, (iv) integration of peak areas (or heights), (v) qualitative identification of analytes of interest. When multiple samples are involved (which is usually the case), peak matching/correspondence across samples is also necessary. All these steps, if conducted manually, require a huge amount of time. For example, Dixon *et al.* (2006) estimated that manually processing a chromatographic dataset of 50 chromatograms consisting of 30 peaks would take 125 hours, i.e. around three weeks of work.

Fortunately, most current GC/MS software include post-processing tools that facilitate such analysis. These automated programs generally all use a similar approach. First, the user must build a *target list* of peaks/analytes of interest encountered in any of the chromatograms. Then an *automated method* has to be set up that will allow the software to search, within a specified time-window, for a particular target peak that would match a specified mass spectrum (at a specified minimum level of similarity). If the target peak is found and identified, the software may then integrate the peak according to pre-specified parameters. Provided that the quality of peak chromatography is good, and that the dataset is of reasonable size, these software tools save considerable processing time for users. When the resolution of peak chromatography is not optimal however, for example in the case of co-eluted analytes, experience shows that the analytical power of these programs often collapses. In such cases, it is crucial to individually review all integrations performed, and manually correct erroneous ones. With an average reviewing/correcting time of 1.5min per peak, plus the time necessary to previously build a target list and set-up a method, processing

the dataset mentioned above (50 chromatograms consisting of 30 peaks) with software assistance would still require a minimum of 50 hours.

GC/MS techniques are becoming increasingly used for the study of complex mixtures. In areas such as metabolomics (Dixon *et al.*, 2007) or the study of animal chemosignals (see Chapter 3), chromatograms can typically contain several hundreds of identifiable peaks. Achieving optimal chromatographic quality across the whole range of compounds present in such samples is often impossible. What is more, the complexity of these mixtures requires researchers to collect a large number of samples to obtain sufficient analytical power. Finally, building target lists for this type of data can be problematic as there is often considerable variation from sample to sample which leads to unrealistic numbers of targets (e.g. 70000 in Dixon *et al.*, 2006). The processing of such chromatographic data, whether manually or with software assistance, is time-consuming at best.

Consequently, a more efficient automated method has been recently developed that can detect, integrate, cluster and match peaks across enormous datasets as well as compare their relative amounts (Dixon *et al.*, 2006). This new method relies on a 3-step procedure. First, data are pre-processed by (i) restricting the analysis to informative m/z channels, (ii) removing the noise, and (iii) smoothing the data. The second step deals with peak definition by (i) detecting peaks in each informative m/z channel, (ii) validating these peaks and (iii) grouping m/z peaks from the same compound. Finally, the last and hardest step is to sort all detected peaks into appropriate clusters by matching and merging similar peaks/clusters into one single target that can be compared across all samples (see Dixon *et al.*, 2006 for details).

Although appealing, several aspects of the above method have thwarted its propagation among ecological chemists. First, it requires an advanced level of computational and programming skills and is thus not directly usable by a non-specifically trained scientist. Second, Dixon and co-authors found that for datasets up to 200 chromatograms (such as in the present thesis), simpler traditional approaches were similarly efficient. Finally and most importantly, the automated routine and associated tools are not openly available to users and require an expensive software package.

These restrictions had two important consequences for my research project. The first one is that a 'semi-automated' approach, relying on the post-processing tools offered by classical GC/MS software, was used. The second is that I became interested in the development of simpler and more easily accessible automated methods for the processing of chromatographic data, as this is becoming critical to research in chemical ecology. Accordingly, I have been collaborating with a team from the University Jean Monnet (St Etienne, France) developing an easy, robust and free automated method for processing complex chromatographic datasets (Nicolè, 2009). The method, now optimised, is analogous to the one described above and involves: (i) smoothing the data (ii) detecting peaks, (iii) correcting the noise and other artefacts, (iv) compiling mass spectral information, (v) clustering mass spectra from the same compound/peak, (vi) checking for cluster redundancy, and (vii) qualitatively identifying the compound corresponding to each cluster. Importantly, this automated procedure is planned to be uploaded and available from the Open Source statistical software R with the corresponding scripts and packages (targeted for September 2010). I am currently contributing to the preparation of a manuscript on this method (Nicolè *et al.*, in prep).

STATISTICAL ANALYSIS OF COMPLEX CHROMATOGRAPHIC DATASETS

The statistical challenge posed by chromatographic data & previous approaches

Once processed, GC/MS data are often presented in the form of a final matrix containing, for each sample (columns), the peak areas of all target analytes (rows). Analytical difficulties however do not necessarily end with obtaining such a matrix. Chromatographic data, particularly in ecological studies, are often characterised by a large number of variables (i.e. peak areas of all analytes) compared to the number of sample units. In addition, the relative abundances of chemical analytes are rarely normally distributed, typically displaying a high right-skewness (concentrations being either quite high or low). Unfortunately, usual parametric multivariate statistics, such as MANOVA, are not particularly robust to departures from the assumption of multivariate normality (Olson, 1974; Johnson & Field, 1993) and simply cannot be computed when there are more variables than sampling units (Anderson, 2001). This generally precludes the use of traditional parametric multivariate statistics on chromatographic data.

Faced with this issue, authors have used various approaches. For example, some have limited their analysis to qualitative observations (Jacob *et al.*, 1979; Reneerkens *et al.*, 2002; Burger *et al.*, 2004). Others have focused their analysis on a subset of analytes chosen *a-posteriori* within the chromatograms (Douglas *et al.*, 2001; Hagey & MacDonald, 2003). Arguably, some studies have used repeated parametric univariate tests (Montalti *et al.*, 2005; Dixon *et al.*, 2007) or repeated non-parametric univariate tests (Soini *et al.*, 2007), depending on the level of normality reached by the data. Other authors have also used finer approaches such as first reducing the dimensionality of the data with canonical analyses, before applying multivariate parametric analyses on the principal components obtained (Safi & Kerth, 2003; Bonadonna *et al.*, 2007). Finally, some studies report rather obscure statistical procedures whose robustness is hard to assess (Smith *et al.*, 2001). None of the above, however, constitutes an entirely satisfactory approach because none allows a systematic, unbiased, unrestricted and statistically robust quantitative investigation of the undiminished dataset.

In a recent study on the chemical fingerprints present within human sweat (Xu *et al.*, 2007), an elegant solution is suggested to circumvent the problematic aspects of large ecological chromatographic datasets: the use of *similarity* indices. This work provided the first illustration of how qualitative measures of similarity (or dissimilarity) between chromatographic profiles could facilitate the exploration of such data. By allowing the construction of *similarity matrices* representing the whole chromatographic dataset, the use of such indices opens a vast field of statistical opportunities that even the authors did not fully realise at the time.

Distance-based statistical methods: principles & applicability

The concept of *similarity* (*S*) between samples has been introduced in biology from the field of community ecology, where comparing qualitative and quantitative patterns of species assemblages across various environments also requires condensing hugely multivariate information into summarised variables (Clarke *et al.*, 2006). Interestingly, community ecology data have very similar properties to chemical ecology data. They both consist of the abundance of a large number of variables (species in the first case, chemical analytes in the second) measured in a large number of samples (sampling stations in the first case, chromatograms in the second). In addition, data obtained are in both cases rarely normally distributed and typically contain many zeros. This is why

analytical methods developed in community ecology are readily usable for large chromatographic datasets.

The level of similarity between a pair of samples is an indication of how much the variables within each sample vary in a similar way. Because the information for each sample is multivariate (many analytes in one chromatogram), there are inevitably many ways of defining similarity and many corresponding indices that can be computed (Clarke & Warwick, 2001). Each index will give a different weight to different aspects of the data. For instance, some may put the emphasis on the few common analytes shared across all samples while other definitions may concentrate on the rarer analytes. A comprehensive review of the numerous similarity (or *dissimilarity* or *distance*) indices and their properties can be found in Legendre & Legendre (1998). A simplistic illustration of similarity calculation between two multivariate samples, using the Euclidean distance (as in our research), is also provided in Text box 1.

Text box 1: Similarity calculation example

Consider an imaginary multivariate dataset containing two samples: sample 1 (50, 60, 40, 0, 10, 20, 50) and sample 2 (0, 0, 10, 0, 0, 20, 40). As the Euclidean distance between two samples (a and b) is defined by:

$$D_{ab} = \sqrt{\sum_i (y_{ia} - y_{ib})^2}$$

Then, the distance between samples 1 and 2 is:

$$D_{12} = \sqrt{\left((50-0)^2 + (60-0)^2 + (40-10)^2 + (0-0)^2 + (10-0)^2 + (20-20)^2 + (50-40)^2\right)}$$

$$D_{12} = \sqrt{(7200)} = 84.85$$

Importantly, the value calculated in this case is a distance, i.e. a dissimilarity instead of a similarity.

For most of the similarity indices (not Euclidean distance though), calculation for any pair of samples will result in a value between 0 (if totally dissimilar) and 1 or 100% (if identical). Repeating this calculation for every single pair of samples in a dataset results in a triangular matrix called a *similarity matrix* (Text box 2) which thus contains all the information about inter-sample comparisons. Importantly, similarity matrices can be used as a basis for many multivariate methods including clustering analyses, ordination methods, and some statistical tests (Clarke, 1993). The use of similarity indices allows these distance-based statistical techniques to circumvent the problem of the relative number of variables and sample units; every pair of samples being

associated with a distance, regardless of the number of variables used to compute this distance.

Text box 2: From a multivariate dataset to the similarity matrix

Original Dataset

Samples Variables	1	2	...	n
1	x_{11}	x_{21}	...	x_{n1}
2	x_{12}	x_{22}	...	x_{n2}
...	...	...	...	
m	x_{1m}	x_{2m}	...	x_{nm}

Similarity calculation between every pair of samples

►

(e.g Euclidean distance)

$$D_{ab} = \sqrt{\sum_i (y_{ia} - y_{ib})^2}$$

Similarity matrix

Samples Samples	1	2	3	...	n
1					
2	S_{12}				
3	S_{13}	S_{23}			
...	...	...	...		
n	S_{1n}	S_{2n}	S_{3n}	...	

Many distance–based statistical techniques are completely non-parametric in that they use the ranks of the calculated similarities. As such, they are also very robust to the non-normality of data (Clarke, 1988). As for all rank-based analyses, however, these non-parametric techniques inevitably lead to a partial loss of the quantitative aspect of the data, by converting all the inter-sample variation (the distance values) into ranks. To circumvent this issue, some recently developed distance-based techniques, such as PERMANOVA (Permutational ANOVA - Anderson, 2001), use a 'semi-parametric' approach, being able to retain the quantitative information within the similarity matrices while being completely oblivious to the non-normality of the data. PERMANOVA, for example, uses the general property that the sum of squared distances between points and their centroid/average, is equal to (and can be calculated directly from) the sum of squared inter-point distances divided by the number of points (Fig.2-1).

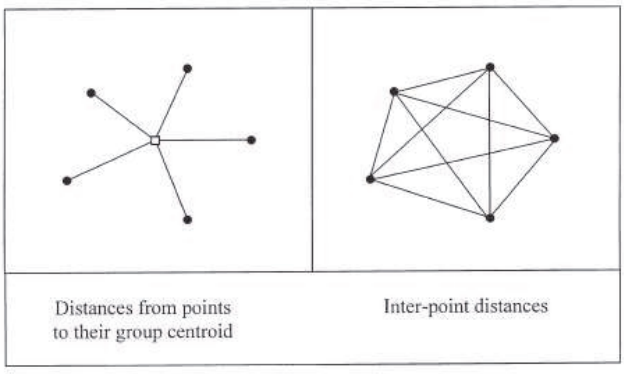

Figure 2-1 (adapted from Anderson, 2001): The sum of squared distances from individual points to their centroids is equal to the sum of squared interpoint-distances divided by the number of points.

This means that a quantitative variation (or 'variance') can be obtained from the inter-sample distances without having to calculate group means, i.e. regardless of the normality of the data. Consequently, any additive partitioning of variance, similar to the ones from traditional ANOVA techniques, can be achieved from distance matrices. This includes partitioning of variation for complex designs such as factorial, hierarchical (nested), and interacting factors. As for traditional ANOVA, a test statistic called *pseudo-F* (conceptually analogous to the traditional Fisher *F*) can be constructed using the ratios of within-group and between group variations. The distribution of this *pseudo-F* and the associated *p*-values (different from the traditional Fisher's *F*-ratio) are simply obtained by permutation of the observations. PERMANOVA thus combines the flexibility of traditional MANOVA approaches with the robustness of distance-based and permutational approaches (Anderson, 2001).

Reviewing the numerous distance-based statistical techniques that could be useful to chemical ecologists for the analysis of chromatographic data is beyond the scope of this section. Table 1, however, provides an overview including the names of some common statistical techniques, their distance-based equivalent, and relevant literature references.

Table 1: Some statistical techniques traditionally used in ecology and distance-based equivalents

Common statistical method	Recently developed distance-based equivalent	Reference
t-test & Mann-Whitney	ANOSIM (i.e. Analysis of Similarity)	Clarke & Warwick, 2001
MANOVA (i.e. Multivariate ANOVA)	PERMANOVA (i.e. Permutational MANOVA)	Anderson, 2001
PCA (i.e. Principal Component Analysis)	Non-parametric: NMDS (i.e. Non-Metric Multi-Dimensional Scaling)	Kruskal, 1964
	Semi-parametric: PCO (i.e. Principal Coordinates analysis)	Gower, 1966
General Linear Models	DISTLM (i.e. Distance-based Linear Models)	Legendre & Anderson, 1999 McArdle & Anderson, 2001
Test of homogeneity of variances	PERMDISP (i.e. Test of homegenity of dispersions)	Anderson, 2006
Canonical analysis	CAP (i.e Canonical Analysis of Principal coordinates)	Anderson & Robinson, 2003 Anderson & Willis, 2003

Researching appropriate statistical solutions for my data analysis, I became particularly interested in applying multivariate distance-based statistical techniques, (which were essentially confined to the field of community ecology) to complex chromatographic data. In June 2009, I thus participated in a statistical workshop organised in Auckland (New-Zealand) by Pr. Bob Clarke and Pr. Marti Anderson, two primary developers of these techniques. This intensive course proved to be a crucial experience for my work as it provided me with (i) a better understanding of these techniques (ii) a computer program specially designed for my needs [PRIMER V6.1.12 (Clarke & Gorley, 2006) with the PERMANOVA+ V1.0.2 add-on package (Anderson *et al.*, 2008)], and (iii) a statistical mentor and collaborator for my thesis in the person of Pr. Marti Anderson (Massey University, Auckland). The novel and valuable application of recently developed distance-based techniques for questions of chemical ecology was intriguing to Pr. Anderson who agreed to contribute to some of my published work (see Mardon *et al.*, 2010 in Section 3.2).

The potential benefits of using distance-based multivariate techniques for the study of animal chemosignalling are illustrated by the work presented in Chapter 3.

Section 2.4

REFERENCES

Anderson M.J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology* **26**: 32-46.

Anderson M.J. (2006). Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* **62**: 245-253.

Anderson M.J., Gorley R.N. & Clarke K.R. (2008). *PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods.* PRIMER-E Ltd, Plymouth, UK.

Anderson M.J. & Robinson J. (2003). Generalized discriminant analysis based on distances. *Australian & New Zealand Journal of Statistics* **43**: 75-88.

Anderson M.J. & Willis T.J. (2003). Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecology* **84**: 511-525.

Bonadonna F., Miguel E., Grosbois V., Jouventin P. & Bessiere J.-M. (2007). Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? *Journal of Chemical Ecology* **33**: 1819-1829.

Bonadonna F. & Nevitt G.A. (2004). Partner-specific odor recognition in an Antarctic seabird. *Science* **306**: 835.

Burger B.V., Reiter B., Borzyk O. & du Plessis M.A. (2004). Avian exocrine secretions I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. *Journal of Chemical Ecology* **30**: 1603-1611.

Clarke K.R. (1988). Statistical design and analysis for a 'biological effects' study. *Marine Ecology Progress Series* **46**: 213-226.

Clarke K.R. (1993). Non-parametric multivariate analyses of changes in community structure. *Austral Ecology* **18**: 117-143.

Clarke K.R. & Gorley R.N. (2006). *PRIMER v6: user manual/tutorial*. PRIMER-E Ltd, Plymouth, UK.

Clarke K.R., Somerfield P.J. & Chapman M.G. (2006). On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. *Journal of Experimental Marine Biology and Ecology* **330**: 55-80.

Clarke K.R. & Warwick R.M. (2001). Changes in marine communities: An approach to statistical analysis and interpretation, 2nd edition. edn. Primer-E., Plymouth.

Dixon S.J., Brereton R.G., Soini H.A., Novotny M.V. & Penn D.J. (2006). An automated method for peak detection and matching in large gas chromatography-mass spectrometry data sets. *Journal of chemometrics* **20**: 325-340.

Dixon S.J., Xu Y., Brereton R.G., Soini H.A., Novotny M.V., Oberzaucher E., Grammer K. & Penn D.J. (2007). Pattern recognition of gas chromatography mass spectrometry of human volatiles in sweat to distinguish the sex of subjects and determine potential discriminatory marker peaks. *Chemometrics and Intelligent Laboratory Systems* **87**: 161-172.

Douglas H.D., III, Co J.E., Jones T.H. & Conner W.E. (2001). Heteropteran chemical repellents identified in the citrus odor of a seabird (crested auklet: *Aethia cristatella*): evolutionary convergence in chemical ecology. *Naturwissenschaften* **88**: 330-332.

Gower J.C. (1966). Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* **53**: 325-338.

Hagelin J.C. (2008). New data and new questions for Crested Auklet research. *The Auk* **125**: 497-198.

Hagelin J.C. & Jones I.L. (2007). Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? *The Auk* **124**: 741-761.

Hagey L. & MacDonald E. (2003). Chemical cues identify gender and individuality in giant pandas (*Ailuropoda melanoleuca*) *Journal of Chemical Ecology* **29**: 1479-1488.

Jacob J., Balthazart J. & Schoffeniels E. (1979). Sex differences in the chemical composition of uropygial gland waxes in domestic ducks. *Biochemical Systematics and Ecology* **7**: 149-153.

Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Jaspers V.L.B., Voorspoels S., Covaci A., Lepoint G. & Eens M. (2007). Evaluation of the usefulness of bird feathers as a nondestructive biomonitoring tool for organic pollutants: A comparative and meta-analytical approach. *Environment International* **33**: 328-337.

Johnson C. & Field C. (1993). Using fixed-effects model multivariate analysis of variance in marine biology and ecology. *Oceanography and Marine Biology: an annual review* **31**: 177-221.

Kruskal J.B. (1964). Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika* **29**: 1-27.

Legendre P. & Anderson M.J. (1999). Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. *Ecological Monographs* **69**: 1-24.

Legendre P. & Legendre L. (1998). Numerical ecology, 2nd English edition edn. Elsevier, Amsterdam.

Mardon J., Saunders S.M., Anderson M.J., Couchoux C. & Bonadonna F. (2010). Species, gender and identity: cracking petrels' socio-chemical code. *Chemical Senses* **Advance access**: doi:10.1093/chemse/bjq021.

McArdle B.H. & Anderson M.J. (2001). Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82**: 290-297.

Montalti D., Gutiérrez A.M., Reboredo G. & Salibián A. (2005). The chemical composition of the uropygial gland secretion of rock dove *Columba livia*. *Comparative Biochemistry and Physiology A Comparative Physiology* **140**: 275-279.

Moritz R.F.A. & Crewe R.M. (1988). Chemical signals of queens in kin recognition of honeybees, *Apis mellifera L. Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **164**: 83-89.

Nicolè F. (2009). Un outil statistique simple et non supervise pour automatiser et faciliter l'identification des molecules dans de grands jeux de donnees complexes. Wokshop on Biogenic Volatile Organic Compounds (BVOCs). Montpellier, France (February 2009).

Nicolè F., Courtois E., Guitton Y., Mardon J., Legendre L. & Hossaert-McKey M.. R package for an exhaustive pre-processing of GC/LC-MS data. In preparation for *Bioinformatics (Oxford)*.

Olson C.L. (1974). Comparative robustness of six tests in multivariate analysis of variance *Journal of the American Statistical Association* **69**: 894-908.

Perrin T.E., Rasmussen L.E.L., Gunawardena R. & Rasmussen R.A. (1996). A method for collection, long-term storage, and bioassay of labile volatile chemosignals *Journal of Chemical Ecology* **22**: 207-221.

Perry J.A. (1981). Introduction to analytical gas chromatography: history, principles, and practice. Marcel Dekker Inc., New York, USA.

Piersma T., Dekker M. & Sinninghe-Damsté J.S. (1999). An avian equivalent of make-up? *Ecology Letters* **2**: 201-203.

Reneerkens J., Piersma T. & Sinninghe-Damsté J.S. (2002). Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proceedings of the Royal Society of London B Biological Sciences* **269**: 2135-2139.

Röck F., Mueller S., Weimar U., Rammensee H.-G. & Overath P. (2006). Comparative analysis of volatile constituents from mice and their urine. *Journal of Chemical Ecology* **32**: 1333-1346.

Safi K. & Kerth G. (2003). Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. *Animal Behaviour* **65**: 363-369.

Smith T.E., Tomlinson A.J., Mlotkiewicz J.A. & Abbott D.H. (2001). Female marmoset monkeys (*Callithrix jacchus*) can be identified from the chemical composition of their scent marks. *Chemical Senses* **26**: 449-458.

Soini H.A., Schrock S.E., Bruce K.E., Wiesler D., Ketterson E.D. & Novotny M.V. (2007). Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*). *Journal of Chemical Ecology* **33**: 183-198.

Sweeney R.J., Lovette I.J. & Harvey E.L. (2004). Evolutionary variation in feather waxes of passerine birds. *The Auk* **121**: 435-445.

Xu Y., Gong F., Dixon S.J., Brereton R.G., Soini H.A., Novotny M.V., Oberzaucher E., Grammer K. & Penn D.J. (2007). Application of dissimilarity indices, principal coordinates analysis, and rank tests to peak tables in metabolomics of the gas chromatography/mass spectrometry of human sweat. *Analytical Chemistry* **79**: 5633-41.

Chapter 3

THE CHEMISTRY OF PETRELS SOCIAL SCENTS

AIM & CONTENT

This chapter presents a chemical investigation of petrels' social scents. Chemosignals are considered at different 'life-stages', from the uropygial precursors to the feather lipids and their final airborne form, in order to inform on the ontogeny of avian scent. For each type of signal, the chemical nature and social information contained within are examined. The following sections contain: (i) background considerations on the investigation of avian chemical substances (Section 3.1); (ii) an article, published in 2010 in the journal *Chemical Senses*, focusing on the social signals within the uropygial secretions of petrels (Section 3.2); (ii) a manuscript, submitted to the *Journal of Avian Biology* in December 2009, focusing on the chemical transition from uropygial secretions to plumage lipids (Section 3.3); (iii) final considerations on our chemical analyses including preliminary results on airborne volatile signals (Section 3.4).

Adapted from M.Parisi (2009)

Section 3.1

BACKGROUND

Note from the author
As in the previous chapter, Chapter 3 requires a basic knowledge in analytical chemistry, in particular Gas-Chromatography and Mass-Spectrometry (GC-MS) techniques (see Appendix A1). A more comprehensive description of this technique can be found in Perry (1981).

Several studies have examined the chemical substances produced by birds for either taxonomic (Jacob, 1978), phylogenetic (Sweeney *et al.*, 2004), physiological (Sandilands *et al.*, 2004), functional (Bolliger & Varga, 1961; Douglas *et al.*, 2001; Reneerkens *et al.*, 2002; Burger *et al.*, 2004), or purely descriptive (Montalti *et al.*, 2005) reasons. Few, however, have done so from the perspective of chemical communication and only one has focused on possible cues of social recognition (Bonadonna *et al.*, 2007).

Importantly, all these works have focused their analysis on either uropygial secretions or feathers lipids. The uropygial gland, located at the dorsal base of the tail, is indeed the only sebaceous gland of birds and as such, is often considered as the key source of avian chemical substances (Jacob, 1978). It produces large amounts of waxy fluids that are spread on feathers as part of the plumage maintenance (Jacob & Ziswiler, 1982). The suitability of such sample substrates for studying avian social chemosignals is, however, not entirely guaranteed because our understanding of the ontogeny of avian scents remains speculatory. A commonly accepted hypothesis proposes that birds' odour originates from the uropygial waxes after being preened on the feathers (Bonadonna *et al.*, 2007; Hagelin & Jones, 2007). The scarce existing evidence, however, does not always support this assumption (see Section 3.3).

In addition, the chemical communication framework requires the olfactory signals to be perceptible by surrounding individuals, which means that the compounds involved should be of reasonable volatility. Yet, to date no study has examined the actual airborne chemosignals emitted by birds, as has been done for some insects and

mammals (Moritz & Crewe, 1988; Cardé & Millar, 2004; Röck *et al.*, 2006). As discussed in Chapter 2, reasons for this gap are the relative infancy of the field and the absence of an appropriate methodological framework.

Much remains to be done regarding avian social chemosignals, in particular regarding their nature and ontogeny. In order to address these questions, this project considers various types of samples including uropygial secretions (Section 3.2), feathers (Section 3.3) and airborne volatiles (Section 3.4).

Section 3.2

SOCIAL CHEMOSIGNALS PRESENT IN THE UROPYGIAL SECRETIONS

CONTEXT

The first step in our investigation of petrels' chemosignals was to elucidate whether social information is already present in the uropygial secretions of the birds. This was critical to confirm an endogenous, and thus potentially genetically-determined, origin of olfactory signals.

Accordingly, I present in this section a chemical examination of the uropygial contents of both Antarctic prions (*Pachyptila desolata*) and blue petrels (*Halobaena caerulea*). The two species were considered together because field observations suggested that chemical communication may be involved in their interspecific interactions (see Section 4.2). The chemical profiles obtained were screened for various social information including species, gender and individual identity.

PRESENTATION OF THE ARTICLE

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Species, Gender, and Identity: Cracking Petrels' Sociochemical Code

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Abstract

Avian chemosignaling remains relatively unexplored, but its potential importance in birds' social behaviors is becoming recognized. Procellariiform seabirds provide particularly appropriate models for investigating these topics as they possess a well-developed olfactory system and unequalled associated capabilities. We present here results from a detailed chemical examination of the uropygial secretions (the main source of avian exogenous chemicals) from 2 petrel species, Antarctic prions and blue petrels. Using gas chromatography–mass spectrometry techniques and recently developed multivariate tools, we demonstrate that the secretions contain critical socioecological information such as species, gender, and individual identity. Importantly, these chemosignals correlate with some of the birds' olfactory behaviors demonstrated in the field. The molecules found to be associated with social information were essentially large unsaturated compounds, suggesting that these may be precursors of, or correlates to the actual airborne signals. Although the species-specific chemosignal may be involved in interspecific competition at the breeding grounds, the role of the sexually specific chemosignal remains unclear. The existence of individually specific signals (i.e., chemical signatures) in these birds has important implications for processes such as individual recognition and genetically based mate choice already suspected for this group. Our results open promising avenues of research for the study of avian chemical communication.

Key words: chemical communication, compatibility-based mate choice, distance-based multivariate statistics, GCMS, individual signature, olfaction

Introduction

Chemical signals or ''chemosignals,'' and their associated olfactory processes, play an important role in animal social behaviors. In vertebrates, chemosignals have been examined extensively in mammals (Burger 2005; Brennan and Kendrick 2006) where they can carry different sorts of social information including group membership (Safi and Kerth 2003; Burgener et al. 2008), relatedness (Ables et al. 2007), or individuality (Penn et al. 2007; Burgener et al. 2009). In contrast, examples of social chemosignaling in other phyla are much scarcer (but see Martín and López 2000 for reptiles; Reusch et al. 2001 for fish; Waldman and Bishop 2004 for amphibians). Avian chemosignals, in particular, remain a relatively unexplored field of study (Hagelin and Jones 2007). Indeed, since birds' olfactory capabilities were first unveiled, most physiological research has investigated if and how chemical signals are perceived and processed by birds (Roper 1999), whereas field studies have typically focused on birds' reactions to environmental scents for behaviors such as foraging (Smith and Paselk 1986; Nevitt 2000), predator avoidance (Amo et al. 2008; Roth et al. 2008), or navigation (Wallraff 2004). Research over the last 30 years, however, has slowly drawn attention to the potential significance of chemosignals for the social lives of birds (see Hagelin and Jones 2007 for a review).

Petrel seabirds from the Procellariiform order possess a particularly developed olfactory neuroanatomy, with an average olfactory bulb ratio (i.e., the ratio between the length of the olfactory bulb and the total length of the brain hemisphere) ranging from 18% to 37% (Bang and Cobb 1968). This anatomical development is thought to be related to the nocturnal and colonial ecology of these seabirds during their breeding season, which involves selective pressures favoring the evolution of refined olfactory mechanisms (Healy and Guilford 1990; Bonadonna and Bretagnolle 2002). Accordingly, many petrel species posses good olfactory capabilities that are used in different behavioral contexts such as foraging (Nevitt 2000) and homing (Bonadonna et al. 2004). Hypogean (i.e., burrow nesting) petrels, for instance, predominantly use olfactory cues to locate their burrow (Bonadonna et al. 2003) and can recognize the odor of their own burrow when presented against the odor of a conspecific (Bonadonna et al. 2004). Importantly, olfaction could also be involved in social aspects of these birds' ecology, including individual recognition and mate choice. Indeed, hypogean petrels (Antarctic prions [APs], Wilson's storm petrels, and blue petrels [BPs] in particular) are, to date, the only bird species known to possess olfactory discrimination capabilities beyond self/non-self recognition (Bonadonna and Nevitt 2004; Jouventin et al. 2007; Mardon and Bonadonna 2009). Chemosignals may thus play a wider role in the social lives of petrels than in any other avian group.

The uropygial gland (or "preen" gland), located at the dorsal base of the tail, is the principal cutaneous gland of birds (Pycraft 1910; Jacob and Ziswiler 1982). It produces large amounts of volatile and nonvolatile compounds in the form of waxy fluids that are spread on feathers while preening. Consequently, it is often considered as the main source of avian exogenous chemical substances (Jacob and Ziswiler 1982; Sweeney et al. 2004; Hagelin and Jones 2007). The potential implication of uropygial secretions in avian social behaviors remains unclear although experimental evidence is slowly emerging. For example, the presence at the nest of heterospecific odors derived from uropygial contents can influence the parental behavior of dark-eyed juncos that are commonly exposed to brood parasitism by cowbirds (Whittaker et al. 2009). At the intraspecific level, sex differences in the chemical composition of the uropygial secretions of domestic ducks have been detected prior to the nesting period (Jacob et al. 1979) and hypothetically related to the alteration of sexual behaviors observed in anosmic males (Balthazart and Schoffeniels 1979). Recent behavioral and neurophysiological results on domestic chickens and Japanese quails (Balthazart and Taziaux 2009; Hirao et al. 2009) similarly suggest that the uropygial gland could play a role in birds' sexual behavior.

Here, we present results from a detailed chemical examination of uropygial secretions from 2 closely related burrowing petrel species, the AP (*Pachyptila desolata*, Gmelin 1789) and the BP (*Halobaena caerulea*, Gmelin 1789) using chromatographic techniques (gas chromatography [GC] and mass spectrometry [MS]). Exploiting recent statistical tools, we investigated, in particular, whether these secretions contain specific chemical signals that could contribute to some of the olfactory behaviors mentioned above. Therefore, we explicitly tested our multivariate chemical data for the presence of:

(i) a "Species" signal, whereby the chemical profiles from 2 different species can be reliably distinguished,
(ii) a "Sex" signal, whereby the chemical profiles from males and females of the same species can be reliably distinguished,
(iii) an "Individual" signal, whereby the chemical profiles from different individuals of the same species can be reliably distinguished and consistently identified over time.

Materials and methods

Study period, location, and species

Fieldwork was carried out during 2 successive campaigns, in December–January 2007–2008 and 2008–2009, on the Kerguelen Archipelago, a French Subantarctic territory located in the southern Indian Ocean. We worked on "Ile Verte," a small island of the archipelago (lat 49°51′S, long 70°05′E), which is a breeding site for BPs (*H. caerulea*) and APs (*P. desolata*).

BPs and APs are hypogean seabirds from the Procellariiform order. Phylogenetically, the genus *Halobaena* (the BP only) is the closest sister clade to the genus *Pachyptila* (all prion species) (Rheindt and Austin 2005), which partly explains the ecological similarity of these birds. Both species live in the Southern Ocean and breed on small oceanic islands around Antarctica where they form dense colonies. Each pair occupies a burrow dug by the male and typically made of a curved gallery leading to an incubating chamber around 30 cm below the surface. Once established, pairs remain stable for life and return to the same burrow year after year. During incubation, partners alternate foraging shifts, relieving each other from the nest every 8–12 days (Warham 1990). They return from their foraging trip to the colony only during the dark of night to avoid predation by skuas (*Catharacta skua lönnbergi*; Stercorariidae) (Warham 1996; Mougeot et al. 1998; Mougeot and Bretagnolle 2000). Deprived of night vision adaptations (Warham 1996), they primarily rely on olfaction to relocate their burrow in the dark (Bonadonna et al. 2004).

Both species are common around the Kerguelen archipelago and 2 colonies, consisting of about 50 burrows each, have been studied since 2001 on Ile Verte. Most birds from these nests are ringed, and burrows have been fitted with a closable aperture above the incubating chamber to facilitate capture. Removing birds from the burrow for a brief

time does not appear to affect incubation behavior or the hatchability of the eggs (Bonadonna et al. 2003, 2004; Bonadonna and Nevitt 2004) and no petrel deserted the nest following the experiments in the present study. Hatching success was 73% for the study burrows (11 nests of 15) and around 70% for control burrows in the same colony (11 nests of 16).

Sampling procedure

Uropygial secretions were sampled using a protocol adapted from Burger et al. (2004). Briefly, uropygial gland contents were collected by gently squeezing the area around the gland, wearing clean nitrile gloves, until a small amount of waxy secretion was discharged. The secretion was collected with a 100-μL glass capillary, which was then placed into an opaque chromatographic vial sealed with a Teflon faced septum. Interindividual differences in the volumes of secretions obtained were not controlled during sampling, but standardized analytically instead (see the section on data pretreatment). We attempted to keep all samples in the dark and at –4 °C from the day of collection until their extraction in the laboratory. However, the cold chain between the field and the chemical laboratory was broken in 2008, when our 2007–2008 secretion samples were retained (partially at ambient temperature) by Australian quarantine (AQIS) for 2 weeks. In contrast, the 2009 samples were consistently kept refrigerated until analysis.

Samples were obtained from 20 breeding BP in 2007–2008 (4 females and 16 males) and from 16 of these 20 initial BP (4 females and 12 males) in 2008–2009. A second secretion sample (a replicate) was also taken for 2 of the 16 BP in 2008–2009. In addition, we collected samples from 16 breeding AP in 2008–2009 (6 females and 10 males). Overall, a total of 54 secretion samples were collected from 36 different birds.

Sample preparation and extraction

Chemical analyses were carried out shortly after returning from the field, in March–April 2008 and 2009, at the University of Western Australia (Perth, Australia). Uropygial secretion samples were solvent extracted in 400 μL of a mix of dichloromethane and n-hexane (ratio 1:3) poured directly in the field vial containing the capillary tube. The vial was resealed and left to stand 7 min in a beaker of ice, to minimize volatilization of lighter compounds. The extraction mixture in the vial was then transferred into a second chromatographic vial, passing through a clean Pasteur pipette filled with a glass wool plug, to filter out impurities. Finally, all samples were spiked with 10 μL of a standard solution of 2-bromophenol in methanol at 504 ng/μL (equivalent to a 12.6 ng load in the GCMS instrument) for quantification purposes. At this stage, samples were ready for chromatographic analyses as extracts were sufficiently concentrated to be used without any preconcentrating step.

Chromatographic analysis

Chromatographic analyses were carried out on a GC coupled with a MS (GC-MS Shimadzu QP2010), equipped with an autosampler (Shimadzu AOC-20i+s) and a generalist Rtx-5MS (Restek) capillary column (L = 30.0 m; Thickness = 0.10 μm; Ø = 0.25 mm). The injection port temperature was set at 250 °C, and helium was used as carrier gas at a constant linear velocity of 35 cm/s. A volume of 1 μL of secretion extracts was injected, in splitless mode, and cold-trapped at 40 °C on the column tip for 3 min. Samples were subsequently separated using a temperature program of 8 °C/min from 40 to 150 °C, then 6 °C/min from 150 to 200 °C, and then 2 °C/min from 200 to 280 °C (hold 15 min). The interface temperature was held at 280 °C and the ion source temperature at 200 °C. The MS was used in scan mode (scan speed = 625; scan interval = 0.5 s).

Chromatographic data processing

Chemical data processing was carried out with the GCMS Solution software v2.40 (Shimadzu Corp.). In all analyses, background noise was first removed from the data by subtracting the signals obtained from blank samples run regularly within our sample batches. Blanks were designed to account for potential noise from the sampling procedure, the extraction protocol, or the instrument. In addition, the quality of all software-defined peak integrations was visually reviewed and manually corrected when necessary. Data processing was "blind" as uninformative codes were given to all samples and used in all analytical steps until the final data set was obtained.

All nonbackground analytes encountered during the processing of our data were included in the analysis, without any a priori criterion of size or class. Qualitative identification of all analytes of interest was determined by cross-checking the best suggested matches obtained from the NIST Mass Spectral Search Program v2.0 (Faircom Corp.) with the calculated retention index (RI) of the analytes. Calculated RIs were obtained by calibrating the GCMS solution software with retention times (Rts) of various unbranched alkanes between C_{10} and C_{40} (n = 15), run under identical chromatographic conditions. We thus obtained accurate estimates of all our analytes RIs, despite the nonlinear nature of the temperature program. In addition, we also used the ion relative abundances at m/z 74, 87, 88, and 101 to estimate the type of methyl-substitution of esterified acids as described by Sweeney et al. (2004). Four types of methylations, nonbranched (NB), 2-methyl branched (2MB), 3-methyl branched (3MB), and 4-methyl branched (4MB), were thus discriminated. These methylation types are not mutually exclusive as compounds can have several methyl branching, such as "2-4MB." Exact identification of each compound (through injection of commercial or synthetised standards), in particular regarding isomers, was considered unnecessary

and unimportant for the present study. Indeed, our focus was instead on the presence of the different signals, the type of chemical coding involved (whether qualitative or quantitative), and the general class of compounds involved.

Interspecific analysis: the Species signal

Only samples from 2009, that is, 16 AP and 16 (+2) BP, were considered here to avoid interannual noise in the data. The difference between the chromatographic profiles of the 2 species being visibly noticeable (Figure 1a), we restricted the analysis to a subset of chromatographic peaks. For each of the 2 species, we first selected the 50 analytes displaying the largest peak areas (on average) in the chromatographic profiles. These 100 initials candidates were then checked for any redundancy and/or poor chromatographic quality, yielding a final target list of 70 analytes whose qualitative identification was sought using the procedure described above. This target list was then searched, and quantified, in each sample chromatogram, resulting in an output table containing the peak areas of the 70 analytes for each sample involved in this analysis (n = 34).

Intraspecific analysis: the Sex and Individual signals

To investigate the possibility of a Sex or an Individual signal, we considered BP samples from 2008 (n = 20) and 2009 (n = 16). The chromatograms from the 4 individuals for which we had only a 2008 sample were also processed and used for validation of statistical models (see next section). An exhaustive target list, containing all analytes encountered in the samples (n = 266), was first constructed. After chemical identification of all analytes, the resulting target list was again searched and quantified, in terms of peak areas, for each one of the 36 sample chromatograms.

Statistical analyses

Chromatographic data were characterized by a large number of variables (i.e., peak areas for all analytes) compared with the number of sample units ($n \leq 36$) and a high right skewness of variables, precluding the use of classical multivariate analysis of variance. Thus a number of more robust distance-based multivariate approaches were used instead, as described below. All statistical analyses were carried out using the computer program PRIMER V6.1.12 (Clarke and Gorley 2006) with the PERMANOVA+ V1.0.2 add-on package (Anderson et al. 2008).

Data pretreatment, resemblance measure, and ordination

Peak areas for each analyte were successively standardized twice across all samples. The first standardization used the peak area of the internal standard (2-bromophenol), to account for variation in the instrument response among samples (particularly across years). The second standardization used the peak area of a particular target analyte (no. 211: dodecanoic acid, hexadecyl ester, RI = 3045), which was one of the highest (if not the highest) peak in all samples. This relativized the values for different analytes within a sample in order to account for the total quantity of secretion, which varied among samples. Standardized data were then square-root transformed to reduce the influence of the most abundant analytes on the analysis (Clarke and Warwick 2001). Euclidean distances between every pair of samples were calculated to produce a resemblance matrix that formed the basis of ensuing analyses. Principal coordinates (PCO) analysis based on the

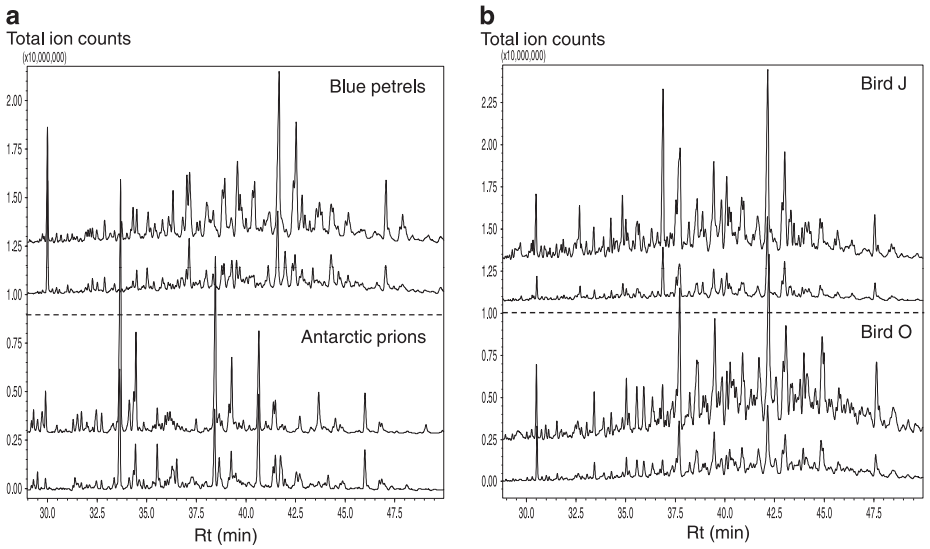

Figure 1 Selection of chromatograms illustrating the different analyses. For graphic clarity, only a 20-min section of the chromatograms (Rt = 30–50 min) is displayed. **(a)** interspecific comparison: the 2 top chromatograms are from BPs and the 2 bottom ones from APs. **(b)** intraspecific comparison: 2009 (above) and 2008 (below) chromatograms from 2 different BPs.

Euclidean resemblance matrix (Gower 1966) was used as an ordination method in order to visualize the patterns of differences in the multivariate chemical structure among samples.

Interspecific analysis: the Species signal

Uropygial secretion profiles from the 2 species were compared with a single factor PERMANOVA (Anderson 2001; McArdle and Anderson 2001) using 9999 permutations. Significant interspecific differences were examined further using canonical analysis of principal coordinates (CAP, Anderson and Willis 2003). Indeed, although PERMANOVA allows distance-based tests of significance for comparing a priori groupings, as in a classical partitioning, CAP is useful for obtaining predictive models that search the multivariate data for the best discrimination between a priori groups. The number of PCO axes to use in the CAP model, and the predictive capability of the model to discriminate the 2 species, was assessed by a leave-one-out cross-validation method (Anderson and Robinson 2003). Validation of the model was also carried out using 3 AP samples (run in a different batch from the other samples) and 2 BP samples (the 2 repeats) that had been excluded from our initial analyses. These 5 "validation samples," treated as new unknown samples, were classified as one of the 2 species according to the CAP model derived from the original set of samples (Anderson et al. 2008).

Intraspecific analysis: the Sex and Individual signals

Secretion profiles from BPs were first analyzed using PERMANOVA with 3 factors: "Year" (fixed), Sex (fixed), and Individual (random, nested within Sex). *P* values were obtained using 9999 permutations of residuals under a reduced model (Freedman and Lane 1983) and Type I (sequential) sums of squares (SS). Interaction terms were removed from the model because neither were significant nor corresponded to any particular biological hypothesis. Predictive discriminatory models for the main effects were obtained using CAP, as described above. Only the individuals sampled in both 2008 and 2009 ($n = 16$) were used to build these CAP models; the 4 birds for which we only had a 2008 sample (4 males) were later used as "unknown" samples for model validation. Note that the 2 alternative and complementary statistical perspectives offered by PERMANOVA and CAP analyses are well illustrated in the present study by the different outcomes obtained with regard to the Sex effect (see Results).

Identification of analytes associated with the different signals

CAP models that had a good discriminating capability between biological groups were examined to identify the analytes associated with the different chemical signals. For each model, we calculated the Pearson correlation (r) between the individual analytes and the model CAP axes. As analytes having high correlations are likely to contribute to group differences in chemical profiles, we considered, for each model, up to 20 analytes having $r > 0.62$ in absolute value as this corresponded to a level of correlation which would be deemed statistically significant in a classical linear correlation analysis (for the number of samples and variables involved). The purpose here was not to attribute significance (no tests performed), nor infer direct biological causation, but only to characterize the nature of group differences in chemical profiles.

Results

Interspecific analysis: the Species signal

A sample of the chromatographic profiles involved in the interspecific comparison is displayed in Figure 1a (the figure only shows the most relevant section of the chromatograms but examples of full chromatographic profiles are provided in Supplementary Appendix 1). An unconstrained 2D PCO ordination explained 73.2% of the total variation in these data and showed a clear separation between the 2 species in terms of their uropygial secretion profiles (Figure 2). The interspecific segregation does not completely dominate the data set though as interindividual variation is also apparent. This indicates the existence, despite a species-specific signal, of a certain amount of chemical similarity between the 2 species.

The visually apparent interspecific difference in the ordination was statistically significant by PERMANOVA (pseudo-$F_{1,27} = 14.8$, $P = 0.0001$). Furthermore, a single canonical axis using just the first 2 PCO axes ($m = 2$) was very effective at discriminating between the chemical profiles associated with the 2 different species. The leave-one-out misclassification error was 0% for the samples used to build the CAP model (Table 1), and all 5 validation samples (3 AP and 2 BP) were correctly classified using this model (Supplementary Appendix 2).

Individual compounds associated with the CAP model discriminating the 2 species' chemical profiles were primarily fatty esterified acids and alcohols between C_{17} and C_{30} (Table 2). This Species signal included both compounds that were associated with BPs and others that were associated

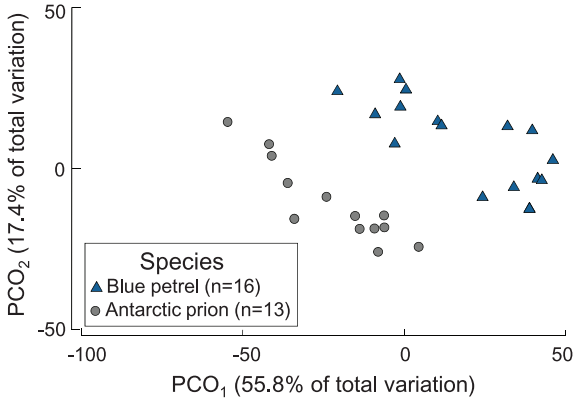

Figure 2 Bidimensional PCO ordination of the samples included in the interspecific analysis. Each data point corresponds to one sample, that is, one chemical profile.

Table 1 Results from CAP analyses examining the effect of species, sex, and individual identity

	Original groups	Classified group		% correct classification	*m*	Trace statistic	*P* value
Species		BPs	APs				
	BPs	16	0	100	2	0.8967	**0.0001**
	APs	0	13	100			
Sex		Females	Males				
	Females	6	2	75.0	3	0.54882	**0.0001**
	Males	2	22	91.7			
Individual		Same individual	Different individual				
	16 individuals (16 ≠ groups)	28	4	87.5	9	6.86931	**0.0001**

The left part of the table presents cross-validation results (leave-one-out allocation of observations). The last two columns show permutation test outputs (n = 9999 permutations in each case); significant outcomes (at a level α = 5%) are bolded.

Table 2 Main analytes associated with the chemical Species signal

Peak number	RI	Reference m/z ions (main ID ion in bold)	Best identification (and methyl substitution)	Formula	Dir	r^a
30	2680	**187**, 210, 167, 182, 255	Iso-Undecanoic acid, tetradecyl ester (4MB)	$C_{25}H_{50}O_2$	BP	−0.98
9	2342	**97**, 83, 69, 252, 280	Iso-Heneicosanol	$C_{21}H_{44}O$	BP	−0.96
41	2900	**210**, 201, 183, 297, 87	Iso-Dodecanoic acid, hexadecyl ester (4MB)	$C_{28}H_{56}O_2$	BP	−0.93
58	3181	**215**, 224, 311, 87	Iso-Tridecanoic acid, heptadecyl ester (4MB)	$C_{30}H_{60}O_2$	BP	−0.91
16	2445	**97**, 83, 69, 266, 294	Heneicosyl formate	$C_{22}H_{44}O_2$	BP	−0.88
37	2816	**187**, 110, 74, 87, 311	Iso-Undecanoic acid, pentadecyl ester (3MB)	$C_{26}H_{52}O_2$	BP	−0.88
32	2706	**173**, 74, 87, 224, 269	Iso-Decanoic acid, hexadecyl ester (3MB)	$C_{26}H_{52}O_2$	BP	−0.87
59	3185	**201**, 183, 238, 325, 97	Iso-Dodecanoic acid, octadecyl ester (4MB)	$C_{30}H_{60}O_2$	BP	−0.87
25	2595	**187**, 182, 167, 74, 87	Iso-Undecanoic acid, tridecyl ester (NB)	$C_{24}H_{48}O_2$	BP	−0.85
42	2905	**187**, 224, 311, 169, 87	Iso-Undecanoic acid, heptadecyl ester (NB)	$C_{28}H_{56}O_2$	BP	−0.84
45	2945	**201**, 196, 181, 159, 97	Iso-Dodecanoic acid, hexadecyl ester (2MB)	$C_{28}H_{56}O_2$	BP	−0.84
48	2978	**201**, 87, 224, 311, 87	Iso-Dodecanoic acid, hexadecyl ester (4MB)	$C_{28}H_{56}O_2$	BP	−0.84
4	2242	**97**, 83, 69, 55, 266	Iso-Eicosanol	$C_{20}H_{42}O$	BP	−0.72
2	1890	**83**, 69, 97, 111, 139	Iso-Heptadecanol	$C_{17}H_{36}O$	BP	−0.66
11	2365	**159**, 167, 196, 141, 57	Iso-Nonanoic acid, tridecyl ester (3MB)	$C_{22}H_{44}O_2$	AP	0.76
13	2410	**131**, 224, 269, 74, 87	Iso-Heptanoic acid, hexadecyl ester (NB)	$C_{23}H_{46}O_2$	AP	0.72
29	2670	**159**, 224, 325, 74, 101	Iso-Nonanoic acid, hexadecyl ester (3MB)	$C_{25}H_{50}O_2$	AP	0.72
66	3480	**243**, 224, 185, 101	Unidentified peak	*NA*	AP	0.71
46	2960	**187**, 169, 238, 283, 74	Iso-Undecanoic acid, heptadecyl ester (3MB)	$C_{28}H_{56}O_2$	AP	0.69
33	2715	**159**, 195, 210, 238, 101	Iso-Nonanoic acid, hexadecyl ester (3MB)	$C_{25}H_{50}O_2$	AP	0.67

Dir, direction of contribution.
[a]r is the Pearson correlation of a particular compound with the CAP axis discriminating the 2 species in the corresponding model. Correlations presented would all be deemed significant at a level of α = 5% (r_{crit} = 0.6).

more with APs, although the contributions of the former appeared to be stronger. Besides, the chemical dichotomy between the 2 species was dominated by a high level of 4-methyl substituted esters in BP's secretions, whereas AP's secretions had more 3-methyl substituted esters.

Intraspecific analysis: the Sex and Individual signals

A sample of the chromatographic profiles involved in the intraspecific comparison (16 individuals in each of the 2 years) is displayed in Figure 1b. For this analysis, the first 2 PCO axes explained 65.1% of the total variation in the multivariate data (Figure 3a), with the third axis explaining a further 10.4% (Figure 3b). Individual birds measured in the 2 years have similar chemical signatures, but variation from year to year is also apparent in this ordination along with a partial chemical dichotomy between the profiles of males and females.

Accordingly, the 3-factor PERMANOVA analysis demonstrated a significant interannual effect, a trend toward chemical dimorphism between males and females ($0.05 < P < 0.1$) and highly significant interindividual variability in uropygial secretion profiles (Table 3). None of the interactions among factors was statistically significant ($P > 0.1$), and results were not altered substantially by changing the order of fit of individual factors in the unbalanced PERMANOVA model using Type I SS. Note that the PERMANOVA design used, which tested the Sex factor before the Individual factor nested within it, rules out the possibility that the weaker intensity of the former is a consequence of some chemical redundancy in the 2 types of signals.

Regarding the significant interannual effect, we identified 49 compounds that were present in only one of the 2 sampling years: 48 analytes present only in 2008 and 1 analyte present only in 2009. All of these annually specific compounds were contained in the early portion of the chromatograms, within the first 26 min (corresponding to RI < 2200). Chemical identification of the compounds specific to 2008 indicated that most were small free acids between C_8 and C_{18} ($n = 19$) and alcohols between C_7 and C_{17} ($n = 11$).

Regarding a possible Sex signal, chemical profiles of males and females were successfully distinguished using a single CAP axis obtained from $m = 3$ PCO axes. The leave-one-out allocation success was 87.5% for the samples used to build the CAP model (Table 1), and all 4 validation samples (4 males) were correctly classified using this CAP model (Supplementary Appendix 3). Interestingly, the coexistence of a trend from the PERMANOVA results and of a significant discrimination from the CAP analysis suggests that the Sex signal identified involves a different direction of chemical variability from the 2 other factors tested and whose overall contribution is lessened by the interannual and interindividual chemical effects. Compounds strongly correlated to the sex-discriminating CAP axis were all esterified acids between C_{23} and C_{28} (Table 4). Importantly, all these analytes had a higher occurrence in females' uropygial secretions than in males, suggesting the Sex signal is essentially female-derived. In addition, the types of methyl-substitution of the esterified acids involved in the Sex signal also appeared to differ between sexes, with 4MB making up all "female-associated" compounds, whereas 2MB dominated the "male associated" ones.

Finally, examination of the Individual signal, through a CAP analysis (Figure 4), showed that chemical signatures were successfully attributed to the correct individual in 87.5% of cases (Table 1), using a subset of $m = 9$ PCO axes. The higher number of PCO axes required to obtain a correct

Table 3 PERMANOVA table of results for the intraspecific analysis

Source	df	SS	MS	Pseudo-*F*	*P* (perm)
Year	1	5437	5437	14.69	**0.0001**
Sex	1	4796	4796	2.26	0.0954
Individual identity (Nested within Sex)	18	34728	1929	7.24	**0.0001**
Residuals	15	3994	266		

df, degrees of freedom; SS, sum of squares; MS, mean square; significant effects (at a level $\alpha = 5\%$) are bolded.

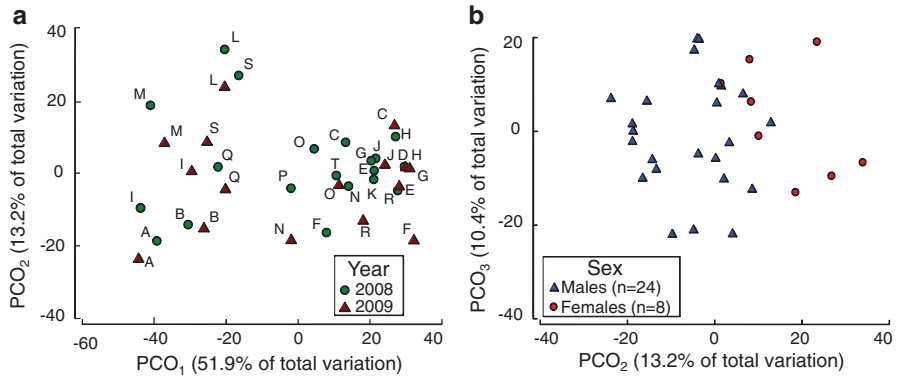

Figure 3 Bidimensional PCO ordinations of the BP samples included in the intraspecific analysis. **(a)** PCO_1 versus PCO_2 and **(b)** PCO_2 versus PCO_3. Each data point corresponds to one sample and each letter corresponds to a particular individual.

Table 4 Main analytes associated with the chemical Sex signal

Peak number	RI	Reference m/z ions (main ID ion in bold)	Best identification (and methyl substitution)	Formula	Dir	r^a
195	2920	**173**, 155, 238, 61, 87	Iso-Decanoic acid, octadecyl ester (4MB)	$C_{28}H_{56}O_2$	Females	−0.92
180	2820	**173**, 84, 210, 195, 238	Iso-Decanoic acid, heptadecyl ester (4MB)	$C_{27}H_{54}O_2$	Females	−0.88
154	2650	**159**, 224, 61, 311, 87	Iso-Nonanoic acid, hexadecyl ester (2-4MB)	$C_{25}H_{50}O_2$	Females	−0.88
194	2910	**187**, 224, 169, 311, 87	Iso-Undecanoic acid, heptadecyl ester (2-4MB)	$C_{28}H_{56}O_2$	Females	−0.83
161	2690	**173**, 196, 181, 311, 87	Iso-Decanoic acid, pentadecyl ester (4MB)	$C_{25}H_{50}O_2$	Females	−0.83
189	2870	**187**, 224, 311, 157, 87	Iso-Undecanoic acid, hexadecyl ester (2-4MB)	$C_{27}H_{54}O_2$	Females	−0.82
173	2780	**173**, 224, 155, 311, 87	Iso-Decanoic acid, hexadecyl ester (2-4MB)	$C_{26}H_{52}O_2$	Females	−0.82
146	2600	**173**, 155, 297, 87	Iso-Decanoic acid, tetradecyl ester (4MB)	$C_{24}H_{48}O_2$	Females	−0.81
152	2645	**173**, 155, 210, 297, 87	Iso-Decanoic acid, pentadecyl ester (2-4MB)	$C_{25}H_{50}O_2$	Females	−0.77
133	2525	**159**, 210, 141, 297, 85	Iso-Nonanoic acid, pentadecyl ester (2-4MB)	$C_{24}H_{48}O_2$	Females	−0.77
141	2555	**173**, 182, 167, 155,	Iso-Decanoic acid, tetradecyl ester (4MB)	$C_{24}H_{48}O_2$	Females	−0.73
134	2535	**145**, 224, 87	Iso-Octanoic acid, hexadecyl ester (4MB)	$C_{24}H_{48}O_2$	Females	−0.72
120	2440	**173**, 167, 155, 196	Iso-Decanoic acid, tridecyl ester (4MB)	$C_{23}H_{46}O_2$	Females	−0.70
174	2785	**159**, 141, 238, 325, 87	Iso-Nonanoic acid, heptadecyl ester (4MB)	$C_{26}H_{52}O_2$	Females	−0.67
181	2825	**201**, 182, 167, 241, 101	Iso-Dodecanoic acid, pentadecyl ester (2MB)	$C_{27}H_{54}O_2$	Males	0.59
221	3135	**215**, 143, 225, 297,	Iso-Tridecanoic acid, heptadecyl ester (2MB)	$C_{30}H_{60}O_2$	Males	0.56
164	2710	**201**, 182, 167, 124,	Iso-Dodecanoic acid, tetradecyl ester (2MB)	$C_{26}H_{52}O_2$	Males	0.56
196	2915	**187**, 215, 167, 74, 87	Iso-Undecanoic acid, heptadecyl ester (NB)	$C_{28}H_{56}O_2$	Males	0.55
198	2940	**201**, 124, 224, 74, 87	Iso-Dodecanoic acid, hexadecyl ester (NB)	$C_{28}H_{56}O_2$	Males	0.53

Dir, direction of contribution.
[a] r is the Pearson correlation of a particular compound with the CAP axis discriminating the 2 sexes in the corresponding model. Correlations presented would be deemed significant at a level of $\alpha = 5\%$ if above $r_{crit} = 0.62$. The 5 analytes most strongly associated with males' chemical signal are shown for information only as their relationship with the CAP axis is below this threshold.

classification for this signal reflects the higher number of groups to be discriminated (16 different individuals). It also suggests that the Individual signal is chemically more elaborate and multidimensional than the 2 previous signals considered. Accordingly, examination of the analytes associated with the 9 CAP axes showed that at least 63 compounds had high correlations (above 0.62). The exhaustive list of these, and their comparison from one individual to the next, is of little pertinence for the hypotheses tested in the present study. These 63 compounds were present, however, in all samples, thus indicating that birds' chemical signatures are not made up of individually specific compounds but rather are identifiable by differences in the relative proportions of a large number of omnipresent compounds.

Discussion

In this study, we used GCMS techniques to investigate the chemical composition of the uropygial (preening) secretion of hypogean petrels, a group of seabirds known for their developed olfactory capabilities. The chemical data were tested for the presence of 3 particular signals that potentially play key roles in the social ecology of these species: species, gender, and individual identity.

The Species signal: a competition-driven chemical divergence?

BPs and APs are closely related and have relatively similar ecologies. Phylogenetically, the genus *Halobaena* (BPs only) is the closest sister clade to the genus *Pachyptila* (all prion species), with a nucleotide distance between the 2 genera of less than 3% (Penhallurick and Wink 2004; Rheindt and Austin 2005). Accordingly, morphological and behavioral similarities between these birds are numerous and include aspects of flight, call, mating system, and foraging behavior (Bretagnolle 1990; Warham 1996; Cherel, Bocher, De Broyer, and Hobson 2002; Cherel, Bocher, Trouve, and Weimerskirch 2002). Both species also use their good olfactory capabilities in similar behavioral functions such as foraging (Nevitt 2000), homing (Bonadonna et al. 2003, 2004), or social recognition (Bonadonna and Nevitt 2004; Mardon and Bonadonna 2009). Our finding of a certain amount of chemical similarity in their secretion contents is therefore

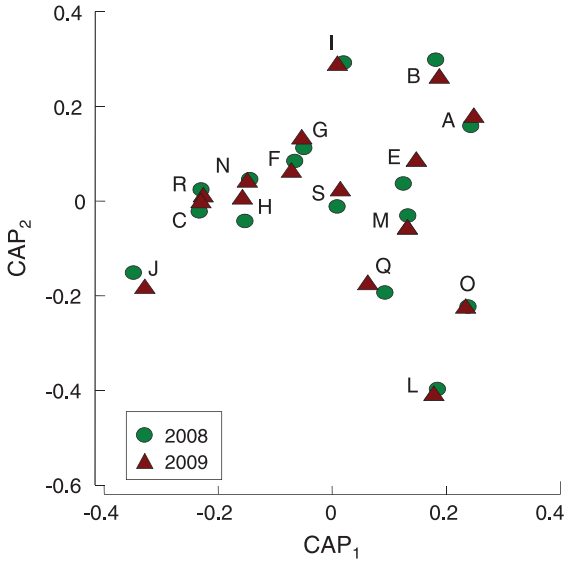

Figure 4 CAP analysis of the Individual factor (BP samples) showing 87.5% correct discrimination of chemical profiles between the different individuals. Each data point corresponds to one sample and each letter corresponds to a particular individual. Letters are not duplicated as the 2008 and 2009 samples from each individual are clearly paired. Note that the figure only displays 2 CAP axes out of the 9 generated in this model.

unsurprising (see also Jacob and Ziswiler 1982). Incidentally, this could also explain why odors from the 2 species appear somehow similar to the human nose (Mardon J, personal observation).

Nevertheless, our results demonstrate the existence of a strongly significant species-specific chemical signal within the secretions of the 2 species. The chemical nature of this signal, in particular the type of ester methyl-substitution found in the compounds involved in each species, is consistent with previous taxonomic investigations of these substances (Jacob and Ziswiler 1982). Given the level of biological affinity, one may wonder whether this Species signal is a simple by-product of genetic differentiation or the consequence of divergent selection. Divergence of chemical signals is expected indeed between ecologically similar species when interspecific competition favors species recognition capabilities (Johansson and Jones 2007).

During the breeding season, BPs and prions nest in dense colonies, made of hundreds of burrows, which can largely overlap (Warham 1990). Faced with a high predation risk from avian predators (Mougeot et al. 1998), many birds try to avoid the cost of digging their own nest by squatting in empty burrows. Due to the sympatric but asynchronous nesting behavior of the 2 species, there is important interspecific competition for burrows and thus potentially a strong selective pressure which should favor species discrimination olfactory capabilities, at least in BPs (Bonadonna and Mardon 2010). Accordingly, BPs have been showed to discriminate and prefer their conspecific odor over the AP odor (Bonadonna and Mardon 2010). The Species signal characterized here in 2 closely related petrel species may therefore be an example of chemosignal divergence led by a strong interspecific competition at the breeding ground. The view is also supported by a taxonomic comparison of uropygial contents, within the Procellariiform order, completed by Jacob and Ziswiler (1982, p. 268) which suggests that closely related species within several burrowing petrel families (e.g., *Pachyptila*, *Procellaria*, and *Puffinus*) show a greater chemical divergence from one another (in terms of ester methyl-substitutions) than they do from some species in other families (*Macronectes* and *Diomedea*).

The Year signal: a potential insight into the scent emission process

The year of sampling had a significant effect on the chemical profiles of BPs. Possible explanations for these annual chemical variations include: 1) environmental fluctuations, such as climatic conditions or food availability, which could have affected the birds' metabolism or diet (Cherel, Bocher, Trouve, and Weimerskirch 2002) and 2) age, which is known to influence concentrations of uropygial lipids in fowls and chickens (Kolattukudy and Sawaya 1974; Sandilands et al. 2004). However, a more likely explanation is that preliminary breakdown occurred for the 2008 samples which were kept at ambient temperatures for several days before extraction (see Materials and methods). Indeed, the 48 compounds specific to the 2008 samples were comparatively smaller than all the other analytes. This episode provides, however, an interesting insight into the degradation process that these secretions may undertake once spread on the bird's feathers; a question that is critical for the understanding of avian olfactory signals' emission (Mardon J, Saunders SM, Bonadonna F, unpublished data). Indeed, the nature of the 2008-specific compounds, essentially free acids and alcohols, has already been proposed to underlie the strong plumage scent of the Procellariiforms (Jacob and Ziswiler 1982, p. 306).

The Sex signal: which role for sexual behaviors?

Our results demonstrate the existence, during the breeding season, of a sexually specific chemosignal in the uropygial secretions of petrels. This clarifies results from a previous study of APs' feather lipids (Bonadonna et al. 2007) which could not positively resolve this question. Previous reports of a chemical sexual dimorphism in birds are so far limited to the domestic duck, in which females shift from monoester to diester waxes during the breeding season (Jacob et al. 1979). Importantly, current behavioral evidence supports the idea that such dimorphism can contribute to avian behaviors. For example, altered sexual behaviors were observed in male ducks whose olfactory nerves had been sectioned (Balthazart and Schoffeniels 1979). More recently, a similar study on domestic chickens reported that while normal males preferred control females over uropygial glandectomised females, the preference was not expressed by anosmic males (Hirao et al. 2009).

There is no evidence, however, at this stage of our research, that the Sex signal identified affects the sexual behaviors of hypogean petrels. Indeed, field experiments did not find any supportive evidence of olfactory sexual discrimination capabilities, whether in APs (Bonadonna et al. 2009) or BPs (Mardon J, unpublished data). These results may be explained by the relatively ''uneventful'' sexual life of hypogean petrels, when compared with lekking or extrapair mating species. Indeed, the lifelong and faithful monogamy of petrels may emphasize capabilities of individual, rather than sexual, recognition. In this context, the olfactory task of sexual discrimination may only apply to the first encounter, when sexually dimorphic acoustic signals can also be used (Bretagnolle 1990). Once formed, each pair only needs to ascertain each other's identity when they annually meet underground. Again, this most likely involves personal scents rather than a generic chemical sexual signal. Note that although the intense Individual signal may preside over mating decisions, the Sex signal may still have a role in the activation of actual sexual behaviors (copulations and mounts), in conjunction with sexual displays or postures (Balthazart and Taziaux 2009).

The female-specific nature of the chemical sexual dimorphism identified contrasts with the norm for other vertebrates, for which males often bear secondary sexual traits. In petrels, however, there is no clear disequilibrium in the direction of sexual competition, which may explain the morphological similarity of the 2 sexes. The female-caused Sex signal we report may thus originate from the genetic mechanism of sex determinism in birds. Indeed, avian gonosomes work in an opposite pattern to mammals, with males being homogametic (ZZ), whereas females are heterogametic (ZW) (Fridolfsson and Ellegren 1999). This view is also supported by the observation that in domestic ducks, female secretions express qualitative and quantitative variations, whereas male secretions remain consistent (Jacob et al. 1979).

The Individual signal: chemical signatures and implications

Although identified in several mammals including mice (Singer et al. 1997), bats (Safi and Kerth 2003), and humans (Penn et al. 2007), the first avian chemical signatures were only recently discovered on the feathers of APs (Bonadonna et al. 2007). The analytical protocol used in that study, however, did not prove sensitive enough to identify the chemical complexity of this signature (Bonadonna et al. 2007). The elucidation here of repeatable individual signatures in the uropygial secretions of another petrel has therefore important implications regarding individual recognition and mate choice in this group.

Petrel seabirds are long-lived, monogamous, completely faithful (Mauck et al. 1995; Bried et al. 2003) and philopatric to their native island (Warham 1996). This particular life history should have favored the evolution of mating preferences promoting genetic compatibility between partners as a suboptimal mate choice would dramatically reduce a bird's fitness over a lifetime (Zelano and Edwards 2002). The major histocompatibility complex (MHC) is often suspected to participate in these processes as it provides both a genetic determinism, thus reducing environmental influences on signals (Brennan and Kendrick 2006), and a high level of polymorphism, thus allowing sufficient phenotypic variation between individuals (Tregenza and Wedell 2000; Penn 2002).

Choosing a mate on genetic grounds requires, however, the ability to contrast one's own genetic makeup to that of a potential mate; a task for which the olfactory system, in the light of the current evidence, seems the most apt to achieve (Penn 2002). Mating preferences for particular MHC-profiles based on chemical assessment have indeed been observed in fish and mammals (Wedekind and Furi 1997; Reusch et al. 2001; Penn 2002). These processes remain, however, undocumented in birds probably because of the limited amount of behavioral and chemical data available to date on avian chemosignals (Hagelin and Jones 2007). In this regard, the coupling of our chemical results with behavioral data reported elsewhere (Bonadonna and Nevitt 2004; Mardon and Bonadonna 2009) provides the most comprehensive case study of avian chemosignals to date. APs and BPs, for example, express a self-odor avoidance behavior that is directly consistent with a possible olfactory mechanism of inbreeding avoidance. The results documented here thus provide a chemical basis for these behaviors and support the hypothesis of an MHC-based mate choice mediated by olfaction in these birds. Research involving the MHC screening of large populations is currently investigating possible genetic evidence of such mating systems, as well as the relationship between the genetic and chemical signals.

Chemical nature of avian social chemosignals

The analytes associated with the different ecological signals identified in our study, that is, esterified fatty acids and alcohols, are consistent with previous investigations of uropygial secretion contents in Procellariiforms and other avian groups (Jacob and Ziswiler 1982). Large wax esters, for instance, are present in the preen oils of most species (Jacob et al. 1979; Piersma et al. 1999; Burger et al. 2004) and have received particular attention due to the seasonal shift typically observed in their production (Dekker et al. 2000). Potential functions of these esters in other birds include feather waterproofing (Burger et al. 2004), sexual attractiveness of the plumage (Jacob et al. 1979; Piersma et al. 1999), or olfactory crypticism of the nest against predators (Reneerkens et al. 2002). Fatty alcohols (C_{10}–C_{18}) have also been found in dark-eyed juncos' uropygial secretions where their expression increases during the breeding season, potentially serving an antimicrobial/fungal function (Soini et al. 2007).

Nevertheless, the compounds found in the present study to be associated with the different signals should not be interpreted as the direct carriers of the odorous biological information. First, the fatty molecules identified have low vapor pressures so that their volatilities at ambient temperature

would be minimal. In addition, our targeting procedure, which highlights correlations between signals and analytes, makes no causative assumption. It is thus possible that the compounds presented are actually proxies for or precursors of the actual odorous signals. For example, uropygial secretions may contain some smaller, volatile and biologically active compounds that follow, for genetic reasons, the same patterns as the large ones identified. These smaller compounds could have been present in concentrations too low to be detected. In such a case, the large fatty molecules secreted together with the smaller active compounds could act as controlled-release materials, allowing a durable emission of scents (Burger 2005). Alternatively, some of the compounds identified in this study could form the chemical precursors of the olfactory signals. Various chemical processes such as oxidation, enzymatic breakdown, hydrolysis, and photolysis, could then exogenously convert large secreted precursors into smaller volatiles. The presence of small free fatty acids and alcohols in our 2008 samples supports this idea. Regardless of the actual chemical trajectory from the secreted uropygial waxes to the airborne odorants, our findings demonstrate the existence of a substrate for various social chemosignals for the first time in a bird species. Further research investigating avian chemosignals at different life-stages, including uropygial secretions, feathers and airborne volatiles, should contribute to further elucidate the ontogeny of social scents in birds.

The present study has demonstrated that the uropygial secretion of hypogean petrels, a group of seabirds known for their developed olfactory capabilities, encapsulates some critical eco-chemical information including species, gender, and individual identity. This is the most biologically informative chemical signal yet described in a bird species. The presence of these chemosignals, which relate to olfactory behaviors demonstrated in the field, have many implications for ecological processes such as interspecific competition, individual recognition, and mate choice.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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References

Ables EM, Kay LM, Mateo JM. 2007. Rats assess degree of relatedness from human odors. Physiol Behav. 90:726–732.

Amo L, Galván I, Tomás G, Sanz JJ. 2008. Predator odour recognition and avoidance in a songbird. Funct Ecol. 22:289–293.

Anderson MJ. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26:32–46.

Anderson MJ, Gorley RN, Clarke KR. 2008. PERMANOVA+ for PRIMER: guide to software and statistical methods. Plymouth (UK): PRIMER-E Ltd.

Anderson MJ, Robinson J. 2003. Generalized discriminant analysis based on distances. Aus N Z J Stat. 43:75–88.

Anderson MJ, Willis TJ. 2003. Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology. 84: 511–525.

Balthazart J, Schoffeniels E. 1979. Pheromones are involved in the control of sexual behavior in birds. Naturwissenschaften. 66:55–56.

Balthazart J, Taziaux M. 2009. The underestimated role of olfaction in avian reproduction? Behav Brain Res. 200:248–259.

Bang BG, Cobb S. 1968. The size of the olfactory bulb in 108 species of birds. The Auk. 85:55–61.

Bonadonna F, Bretagnolle V. 2002. Smelling home: a good solution for burrow-finding in nocturnal petrels? J Exp Biol. 205:2519–2523.

Bonadonna F, Caro SP, Brooke MdL. 2009. Olfactory sex recognition investigated in Antarctic Prions. PLoS one. 4:e4148.

Bonadonna F, Hesters F, Jouventin P. 2003. Scent of a nest: discrimination of own-nest odours in Antarctic prions, *Pachyptila desolata*. Behav Ecol Sociobiol. 54:174–178.

Bonadonna F, Mardon J. 2010. One house two families: petrel squatters get a sniff of low-cost breeding opportunities. Ethology. 116:176–182.

Bonadonna F, Miguel E, Grosbois V, Jouventin P, Bessiere J-M. 2007. Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? J Chem Ecol. 33:1819–1829.

Bonadonna F, Nevitt GA. 2004. Partner-specific odor recognition in an Antarctic seabird. Science. 306:835.

Bonadonna F, Villafane M, Bajzak C, Jouventin P. 2004. Recognition of burrow's 'olfactory signature' in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. Anim Behav. 67: 893–898.

Brennan PA, Kendrick KM. 2006. Mammalian social odours: attraction and individual recognition. Philos Trans R Soc Lond Ser B Biol Sci. 361: 2061–2078.

Bretagnolle V. 1990. Behavioural affinities of the blue petrel *Halobaena caerulea*. Ibis. 132:102–123.

Bried J, Pontier D, Jouventin P. 2003. Mate fidelity in monogamous birds: a re-examination of the Procellariiformes. Anim Behav. 65:235–246.

Burgener N, Dehnhard M, Hofer H, East ML. 2009. Does anal gland scent signal identity in the spotted hyaena? Anim Behav. 77:707–715.

Burgener N, East ML, Hofer H, Dehnhard M. 2008. Do spotted hyena scent marks code for clan membership? In: Hurst JL, Beynon RJ, Roberts SC, Wyatt TD, editors. Chemical signals in vertebrates. New York: Springer. p. 169–177.

Burger BV. 2005. Mammalian semiochemicals. Top Curr Chem. 240:231–278.

Burger BV, Reiter B, Borzyk O, Plessis MAD. 2004. Avian exocrine secretions I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. J Chem Ecol. 30:1603–1611.

Cherel Y, Bocher P, De Broyer C, Hobson KA. 2002. Food and feeding ecology of the sympatric thin-billed *Pachyptila belcheri* and Antarctic *P. desolata* prions at Iles Kerguelen, southern Indian Ocean. Mar Ecol Prog Ser. 228:263–281.

Cherel Y, Bocher P, Trouve C, Weimerskirch H. 2002. Diet and feeding ecology of blue petrels *Halobaena caerulea* at Iles Kerguelen, Southern Indian Ocean. Mar Ecol Prog Ser. 228:283–299.

Clarke KR, Gorley RN. 2006. PRIMER v6: user manual/tutorial. Plymouth (UK): PRIMER-E Ltd.

Clarke KR, Warwick RM. 2001. Changes in marine communities: an approach to statistical analysis and interpretation. Plymouth (UK): PRIMER-E Ltd.

Dekker MHA, Piersma T, Sinninghe-Damsté JS. 2000. Molecular analysis of intact preen waxes of *Calidris canutus* (Aves: Scolopacidae) by gas chromatography/mass spectrometry. Lipids. 35:533–541.

Freedman D, Lane D. 1983. A nonstochastic interpretation of reported significance levels. J Bus Econ Stat. 1:292–298.

Fridolfsson A-K, Ellegren H. 1999. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol. 30:116–121.

Gower JC. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika. 53:325–338.

Hagelin JC, Jones IL. 2007. Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? The Auk. 124:741–761.

Healy S, Guilford T. 1990. Olfactory-bulb size and nocturnality in birds. Evolution. 44:339–346.

Hirao A, Aoyama M, Sugita S. 2009. The role of uropygial gland on sexual behavior in domestic chicken *Gallus gallus domesticus*. Behav Process. 80:115–120.

Jacob J, Balthazart J, Schoffeniels E. 1979. Sex differences in the chemical composition of uropygial gland waxes in domestic ducks. Biochem Syst Ecol. 7:149–153.

Jacob J, Ziswiler V. 1982. The uropygial gland. In: Farner DS, King JR, Parkes KC, editors. Avian biology. New York: Academic Press. p. 199–324.

Johansson BG, Jones TM. 2007. The role of chemical communication in mate choice. Biol Rev. 82:265–289.

Jouventin P, Mouret V, Bonadonna F. 2007. Wilson's storm petrels *Oceanites oceanicus* recognise the olfactory signature of their mate. Ethology. 113:1228–1232.

Kolattukudy PE, Sawaya WN. 1974. Age dependent structural changes in the diol esters of uropygial glands of chicken. Lipids. 9:290–292.

Mardon J, Bonadonna F. 2009. Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own. Behav Ecol Sociobiol. 63:537–542.

Martín J, López P. 2000. Chemoreception, symmetry and mate choice in lizards. Proc R Soc Lond Ser B Biol Sci. 267:1265–1269.

Mauck R, Waite T, Parker P. 1995. Monogamy in Leach's storm petrel: DNA-fingerprinting evidence. The Auk. 112:473–482.

McArdle BH, Anderson MJ. 2001. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecology. 82: 290–297.

Mougeot F, Bretagnolle V. 2000. Predation risk and moonlight avoidance in nocturnal seabirds. J Avian Biol. 31:376–387.

Mougeot F, Genevois F, Bretagnolle V. 1998. Predation on burrowing petrels by the brown skua at Mayes islands, Kerguelen. J Zool (Lond). 244:429–438.

Nevitt GA. 2000. Olfactory foraging by Antarctic procellariiform seabirds: life at high Reynolds numbers. Biol Bull. 198:245–253.

Penhallurick J, Wink M. 2004. Analysis of the taxonomy and nomenclature of the Procellariiformes based on complete nucleotide sequences of the mitochondrial cytochrome b gene. Emu. 104:125–147.

Penn D. 2002. The scent of genetic compatibility: sexual selection and the major histocompatibility complex. Ethology. 108:1–21.

Penn DJ, Oberzaucher E, Grammer K, Fischer G, Soini HA, Wiesler D, Novotny MV, Dixon SJ, Xu Y, Brereton RG. 2007. Individual and gender fingerprints in human body odour. J R Soc Interface. 4: 331–340.

Piersma T, Dekker M, Sinninghe-Damsté JS. 1999. An avian equivalent of make-up? Ecol Lett. 2:201–203.

Pycraft WP. 1910. A history of birds. London: Methuen and Co.

Reneerkens J, Piersma T, Sinninghe-Damsté JS. 2002. Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? Proc R Soc Lond B Biol Sci. 269:2135–2139.

Reusch TBH, Häberli MA, Aeschlimann PB, Milinski M. 2001. Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. Nature. 414:300–302.

Rheindt FE, Austin JJ. 2005. Major analytical and conceptual shortcomings in a recent taxonomic revision of the Procellariiformes—a reply to Penhallurick and Wink (2004). Emu. 105:181–186.

Roper TJ. 1999. Olfaction in birds. Adv Study Behav. 28:247–332.

Roth TCII, Cox JG, Lima SL. 2008. Can foraging birds assess predation risk by scent? Anim Behav. 76:2021–2027.

Safi K, Kerth G. 2003. Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. Anim Behav. 65:363–369.

Sandilands V, Savory J, Powell K. 2004. Preen gland function in layer fowls: factors affecting morphology and feather lipid levels. Comp Biochem Physiol A. 137:217–255.

Singer AG, Beauchamp GK, Yamazaki K. 1997. Volatile signals of the major histocompatibility complex in male mouse urine. Proc Natl Acad Sci U S A. 94:2210–2214.

Smith SA, Paselk RA. 1986. Olfactory sensitivity of the turkey vulture (*Cathartes aura*) to three carrion-associated odorants. The Auk. 103: 586–592.

Soini HA, Schrock SE, Bruce KE, Wiesler D, Ketterson ED, Novotny MV. 2007. Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*). J Chem Ecol. 33:183–198.

Sweeney RJ, Lovette IJ, Harvey EL. 2004. Evolutionary variation in feather waxes of passerine birds. The Auk. 121:435–445.

Tregenza T, Wedell N. 2000. Genetic compatibility, mate choice and patterns of parentage: invited review. Mol Ecol. 9:1013–1027.

Waldman B, Bishop PJ. 2004. Chemical communication in an archaic anuran amphibian. Behav Ecol. 15:88–93.

Wallraff HG. 2004. Avian olfactory navigation: its empirical foundation and conceptual state. Anim Behav. 67:189–204.

Warham J. 1990. The petrels: their ecology and breeding systems. London: Academic Press.

Warham J. 1996. The behaviour, population biology and physiology of the petrels. London: Academic Press.

Wedekind C, Furi S. 1997. Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? Proc R Soc Lond Ser B Biol Sci. 264:1471–1479.

Whittaker DJ, Reichard DG, Dapper AL, Ketterson ED. 2009. Behavioral responses of nesting female dark-eyed juncos *Junco hyemalis* to hetero- and conspecific passerine preen oils. J Avian Biol. 40:579–583.

Zelano B, Edwards VE. 2002. An Mhc component to kin recognition and mate choice in birds: predictions, progress, and prospects. Am Nat. 160:225–238.

Section 3.3

FROM SECRETIONS TO FEATHERS

CONTEXT

Once the presence of social information within the preen secretions of petrels was established, the next step of our investigation was to examine whether (and if so in which form) this chemical information appeared once spread on the plumage. The characteristic musky scent of most procellariiform birds (Weldon & Rappole, 1997) emanates indeed from the whole plumage and remains clearly perceptible on the feathers of old carcasses (personal observation).

In this section, I present a chemical examination of the feather lipids of blue petrels (*Halobaena caerulea*). Only blue petrels were considered in this work to avoid a redundant interspecific comparison. Chemical profiles were screened for the same social information found within the secretions (i.e. gender and identity). Significant efforts were also directed at elucidating the chemical trajectory of the social signals from secretions to feathers. To do so, compositional similarities and differences were examined.

PRESENTATION OF THE ARTICLE

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From preen secretions to plumage: the ontogeny of petrels' social scents

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Abstract

Petrel seabirds use chemosignals more than any other birds and are thus appropriate models for the study of avian chemical communication. The uropygial secretions of blue petrels (*Halobaena caerulea*), for instance, have recently been shown to encapsulate elaborate sociochemical information including species, gender and identity. Yet, it is the plumage, and not preening secretions *per se*, which acts as the final substrate of avian scents. Although it is often considered as the primary source of avian exogenous substances, the participation of the uropygial gland, located at the dorsal base of the tail, to avian scents is not established. Furthermore, the chemical relationship between uropygial secretions and plumage lipids has been considered in only a handful of studies which reported large qualitative differences.

To further examine the ontogeny of blue petrels' social chemosignals, we compared secretion and feather samples using Gas-Chromatography-Mass-Spectrometry (GCMS) and recently developed multivariate statistics. Our results indicate that (i) 85.2% of the feather chemical signal comes from uropygial secretions; (ii) chemical differentiation between secretions and feather lipids includes qualitative and quantitative variations, which both have interesting implications for scent production; (iii) social chemosignals contained within the secretions (i.e. a sex-specific signal and chemical signatures) are present in very conserved forms on the plumage. In the light of these results, it is now apparent that the uropygial gland plays a critical role for chemical communication in petrels and possibly other avian groups.

Keywords: avian scent; chemical communication; feather waxes; olfaction; procellariiform seabirds; uropygial secretions.

Running title: Mardon et al.: Ontogeny of petrels' social scent

INTRODUCTION

Birds' chemical communication is an emergent topic of research, although the origin and nature of the signals potentially involved in this communication remain unresolved. The uropygial gland (or 'preen' gland), located at the dorsal base of the tail, is the only cutaneous gland of birds (Pycraft, 1910; Jacob & Ziswiler, 1982) besides the sebaceous glands of the external ear and the anal glands. It is often considered as the main source of avian exogenous chemical substances (Borchelt & Duncan, 1974; Sweeney *et al.*, 2004; Hagelin & Jones, 2007) because it produces large amounts of volatile and non-volatile compounds, in the form of waxy fluids that are spread on feathers while preening. The chemistry and biochemistry of uropygial secretions has been considered in many studies (see Jacob, 1978; Jacob & Ziswiler, 1982; Salibián & Montalti, 2009 for reviews) whose primary focus was on the interspecific diversity and taxonomic specificity of uropygial contents. Importantly, the biological function of these secretions has been, and still is, controversial. Among the most commonly suggested and reasonably supported roles for these preening materials are: (i) waterproofing, especially for waterbirds which possess large preen glands; (ii) plumage maintenance and feather flexibility; (iii) feather hygiene in a large sense (microflora regulation, antifungal, antibacterial, antimycotic, antiparasitic, cleaning) (Jacob & Ziswiler, 1982; Shawkey *et al.*, 2003); (iv) vitamin D production (Elder, 1954); (v) sexual signalling through feather sheen (Piersma *et al.*, 1999); (vi) olfactory crypticism of the nest (Reneerkens *et al.*, 2002); and finally, of interest for the present study, (vii) scent production (Jacob, 1978).

As early as 1910, the uropygial gland was suggested to be the avian equivalent of mammalian scent glands, thus participating in the production of bird odours, as some of the wax constituents were found to be intensely odorous (Pycraft, 1910). Other sources of avian odours however also exist, as several strongly scented bird groups lack the gland altogether (Weldon & Rappole, 1997; Hagelin & Jones, 2007). These alternative odour-sources could include a feather-derived powder (in parrots, pigeons, woodpeckers), epidermal lipogenesis (Jacob & Ziswiler, 1982) or contributions from the feather microbial fauna (Hagelin & Jones, 2007). Thus, volatile compounds participating to avian scents may be derived from different sources and mechanisms, varying from one species to the next. Nevertheless, it is considered, in most cases, that the scent emanating from birds' plumage originates from some strong-smelling constituents of the preen waxes (Jacob & Ziswiler, 1982; Bonadonna *et al.*, 2007;

Hagelin & Jones, 2007). This commonly accepted hypothesis has seldom been tested (Jacob, 1978), and in the few instances where it was, results indicated instead important qualitative differences between uropygial secretions and feather lipids (Bolliger & Varga, 1961; Jacob & Pomeroy, 1979; Jacob and Grimmer, 1975 in Jacob & Ziswiler, 1982).

Several observations suggest that chemosignals may play a wider role in the lives of procellariiform seabirds, and petrels in particular, than in any other avian group, making them an ideal model for the investigation of avian chemical communication. Firstly, their plumage bears a strong musky scent which is easily perceptible to the human nose (Weldon & Rappole, 1997), even on feathers several years old (personal observation). Secondly, with large olfactory bulbs whose size can represent from 18% up to 30% of the total brain length, petrels possess the most developed olfactory neuroanatomy of all birds (Bang & Cobb, 1968). Accordingly, many of these species posses good olfactory capabilities that are used in different behavioural contexts such as foraging (Nevitt, 2000), homing (Bonadonna *et al.*, 2004), nesting (Bonadonna & Mardon, 2010) and, importantly, social recognition. Indeed, petrels are the only birds shown to date to possess olfactory capabilities of individual discrimination beyond simple self/non-self recognition (Bonadonna & Nevitt, 2004; Jouventin *et al.*, 2007; Mardon & Bonadonna, 2009). Finally, we have recently reported the existence of rich sociochemical information encapsulated within the uropygial secretions of blue petrels (*Halobaena caerulea,* Gmelin 1789), namely a species-specific, a sex-specific and an individual-specific signal (Mardon *et al.*, 2010)

The final substrate of avian odours is not the uropygial secretion however but the plumage, from which emanates the characteristic musky scent of petrels. Consequently, a further step in our investigation of petrels' chemical communication was to consider the nature and origin of the chemical signals present on the feathers of the birds. To address this question also required an examination of the chemical differentiation from uropygial secretions to feather lipids, which remains a rather obscure and scarcely documented aspect of avian biology. Accordingly, we present here results from a detailed chemical comparison of blue petrels' uropygial secretion and feather chemosignals. To do so, both types of samples were collected from breeding birds in the field, and analysed using Gas-Chromatography Mass-Spectrometry (GCMS). Data analysis was organised into three sections, each corresponding to a particular aspect of

our investigation: i) the origin of the feather chemical signal, ii) the nature of the chemical differentiation between uropygial secretions and feathers, and iii) the presence of social chemosignals on blue petrels' feathers.

MATERIALS AND METHODS

Study period, location and species

Fieldwork was carried out during two successive campaigns, in December-January 2007-08 and 2008-09, on the Kerguelen Archipelago, a French subantarctic territory located in the southern Indian Ocean. We worked on 'Ile Verte', a small island of the archipelago (49°51'S, 70°05'E), which is a breeding site for blue petrels (*Halobaena caerulea*).

Blue petrels are hypogean (i.e. burrow-nesting) seabirds from the Procellariiform order. They live in the Southern Ocean and breed on small oceanic islands around Antarctica where they form dense colonies. Each pair occupies a burrow typically made of a curved gallery leading to an incubating chamber around 30cm below the surface. Once established, pairs remain stable for a lifetime and return to the same burrow year after year (Warham, 1996). During the breeding season, birds only approach the colony at night to avoid avian predators (Mougeot *et al.*, 1998; Mougeot & Bretagnolle, 2000). Deprived of night vision adaptations, they primarily use olfaction to locate their burrow in the dark (Bonadonna *et al.*, 2004).

A blue petrel colony, consisting of about 70 burrows, has been studied since 2001 on Ile Verte. Burrows from the colony have been fitted with a closable aperture above the incubating chamber to facilitate capture and most birds from these nests are ringed. Removing birds from the burrow for a brief time does not appear to affect incubation behaviour nor the hatchability of the eggs (Bonadonna & Nevitt, 2004; Bonadonna *et al.*, 2004), and no petrel deserted the nest following our sampling procedure. Hatching success in our study burrows was 73% (11 out of 15) and 69% for control burrows in the same colony (11 out of 16).

Field sampling

Two different types of samples were considered in the present study: uropygial secretions and ventral feathers. In the field, birds were removed from their burrow and

transported to the field lab in an opaque cotton bag to reduce handling time and stress on the animal. Clean nitrile gloves were used for all subsequent sampling procedures.

Feather samples were collected first to avoid any possible contamination from fresh secretions. To do so, 100 to 200 mg of feathers were cut from the ventral duvet of the birds with steel scissors, rinsed with methanol between samples. Feathers were then packed in aluminium foil, placed in a sealed plastic bag, and stored at -4ºC. Uropygial secretions were collected from the same individuals, immediately after the feather samples, using a protocol adapted from Burger et al. (2004). Briefly, waxy secretions from the uropygial gland were collected with a clean 100µl glass capillary. The end of the capillary containing the secretion was then placed into a small chromatographic vial, sealed with a Teflon faced septum, and stored at -4°C.

Samples were obtained from 20 breeding blue petrels (4 females and 16 males) in 2007-08 (hereafter referred to as '2008 samples') and from 16 of these 20 initial birds (4 females and 12 males) in 2008-09 (hereafter referred to as '2009 samples'). Therefore, a total of 36 secretion samples and 36 feather samples were collected. Note that we attempted to keep all samples in the dark and at -4°C from the day of collection until their extraction in the lab. However, the cold chain between the field and the chemical lab was broken in 2008, when our samples were retained (partially at ambient temperature) by the Australian quarantine (AQIS) for 2 weeks. In contrast, the 2009 samples were consistently kept refrigerated until analysis.

Sample preparation and extraction

Chemical analyses were carried out shortly after returning from the field, in March-April 2008 and 2009, at the University of Western Australia (Perth, Australia). Uropygial samples were solvent extracted in 400µl of a mix of dichloromethane and n-hexane (ratio 1:3), placed directly in the field vial containing the capillary tube with secretions. The vial was then resealed and left to stand 7min in a beaker of ice, to minimise volatilisation of the lighter compounds. The liquid phase in the vial was then transferred into a second chromatographic vial, passing through a Pasteur pipette filled with a glass wool plug, to filter out impurities (i.e. dust, feather bits). At this stage, samples were ready for chromatographic analyses as extracts were sufficiently concentrated to be used without any preconcentrating step.

Feather samples were solvent extracted in a similar mix of dichloromethane and n-hexane (ratio 1:3). To do so, 60mg of feather were placed in a 50ml conical flask,

together with 4ml of the solvent mix and a magnetic stirrer. The flask was then sealed with a hermetic stopper, placed in a beaker of ice, and the content left to macerate on a magnetic stirring apparatus for 2.5h. After the maceration, the extract was transferred into a 4ml vial through a Pasteur pipette filled with a glass wool plug, again to filter out impurities from the extract. The extract volume was then reduced to 400 µl by attaching the vial to a low pressure liquid nitrogen cold finger manifold (vacuum provided by a rotary vacuum pump), thus concentrating the extract samples approximately 10 times. This drying approach, which relies on the lowering of the pressure to promote evaporation of the solvent, was found in preliminary tests to be more effective in minimising losses of light volatile compounds than the usual method of exposing samples to a slow stream of purified nitrogen (Burger *et al.*, 2004).

Finally, all samples were spiked with 10µl of a standard solution of 2-bromophenol in methanol at 504ng/µl (equivalent to a 12.6ng load in the GCMS instrument) for quantification purposes.

Chromatographic analysis

Chromatographic analyses were carried out on a Gas Chromatograph coupled with a Mass Spectrometer (GC-MS Shimadzu QP2010), equipped with an autosampler (Shimadzu AOC-20i+s) and a generalist Rtx®-5MS (Restek) capillary column (L=30.0m; Thickness=0.10µm; Ø=0.25mm). The injection port temperature was set at 250°C and helium was used as carrier gas at a constant linear velocity of 35cm/sec. A volume of 1µl of secretion extract was injected, in splitless mode, and cold-trapped at 40°C on the column tip for 3min. Samples were subsequently separated using a temperature program of 8°C/min from 40 to 150°C, then 6°C/min from 150 to 200°C and then 2°C/min from 200 to 280°C (hold 15min). The interface temperature was held at 280°C and the ion source temperature at 200°C. The MS was used in scan mode (scan speed=625; scan interval=0.5sec).

Chromatographic data processing

Chemical data processing was carried out with the GCMS Solution software v2.40© (Shimadzu Corp.). In all analyses, background noise was first removed from the data by subtracting the signals obtained from blank samples run regularly within our sample batches. Blanks were designed to account for potential noise from the sampling procedure, the extraction protocols or the instrument. Data processing was 'blind' as

uninformative codes were attributed to all samples before chromatographic analyses, and used until the final dataset was obtained.

Initially, an exhaustive target list of all analytes (or 'peaks' or 'compounds) encountered in the samples, secretions and feathers, was built. The resulting target list of 330 compounds was then searched and quantified, in terms of peak areas, for each one of the 72 sample chromatograms. The chromatograms from the four individuals for which we only had 2008 samples were also processed as they were used for validation of statistical models (see below).

Chemical identification of all analytes of interest was sought by cross-checking the best suggested matches obtained from the NIST Mass Spectral Search Program v2.0© (Faircom Corp.) with the calculated Retention Index (RI) of the analytes. Calculated RIs were obtained by calibrating the GCMS Solution software with retention times (Rt) of various unbranched alkanes between C_{10} and C_{40} (n=15), run under identical chromatographic conditions. We thus obtained accurate estimates of all our analytes RIs, despite the non-linear nature of the temperature program. In addition, we also used the ion relative abundances at m/z 74, 87, 88 and 101 to estimate the type of methyl-substitution of esterified acids as described by Sweeney et al. (2004). Four types of methylations, non-branched (NB), 2-methyl branched (2MB), 3-methyl branched (3MB) and 4-methyl branched (4MB), were thus discriminated. These methylation types are not mutually exclusive though as compounds can have several methyl-branching such as '2-4MB'. The exact chemical identification of each compound (through injection of commercial or synthetised standards), in particular regarding isomers, was considered unnecessary for the present study. Indeed, our focus was instead on the general class and size of the compounds involved, as well as the presence of biological signals in the samples.

Data pre-treatment, resemblance measure and ordination

Peak areas were successively standardised twice across all samples. The first standardisation used the peak area of the spike (2-bromophenol), to account for variation in the instrument response among samples (particularly across years). The second standardisation used the peak area of a particular target analyte (#265: dodecanoic acid, hexadecyl ester, RI=3045), which was one of the highest (if not the highest) peak in all samples, to account for the variation in the total quantity of wax, which could not be controlled during sampling. After standardisation, data were square-

root transformed to reduce skewness and so that the resemblance measure calculations, while retaining the relative abundances of analytes, would not be overly dominated by the most abundant analytes (Clarke & Warwick, 2001). Euclidean distances between every pair of samples were then calculated to produce a resemblance matrix that formed the basis of ensuing analyses. Principal coordinates analysis based on the Euclidean resemblance matrix (PCO; Gower, 1966) was used as an ordination method in order to visualise the patterns of differences in the multivariate chemical structure among samples.

Statistical analyses

Variation among chemical profiles was explored using recent non-parametric multivariate techniques which are briefly explained below. Readers will also find more details about the principles and applications of these analyses in Appendix 3.3-1.

Two different types of distance-based multivariate approaches were used in our study: PERMANOVA, which is a permutational distance-based equivalent of traditional MANOVA analyses (Anderson, 2001; Mc Ardle & Anderson, 2001) and CAP (Canonical Analysis of Principal coordinates; Anderson & Willis, 2003). Importantly, PERMANOVA indicates how the various factors included in the model contribute to the overall variation in the data while CAP models search the multivariate space for a separation between *a-priori* groups which can then be used for predictive modelling. Therefore, these two types of analysis offer alternative and complementary statistical perspectives on the multivariate data, which prove particularly useful when the multivariate direction of segregation between the groups of interest is fundamentally different from the main direction(s) of variation in the whole dataset (Anderson & Willis, 2003).

CAP models that had a good discriminating capability between biological groups were examined to identify the analytes associated with the different chemical signals. For each model, we calculated the Pearson correlation (r) between the individual analytes and the model CAP axes. As analytes having high correlations are likely to contribute to group differences in chemical profiles, we considered, for each model, up to 20 analytes whose Pearson correlation r to the CAP axes was higher than the level of that would be deemed statistically significant in a classical linear correlation analysis. The purpose here was not to attribute significance (no tests performed), nor infer direct biological causation, but only to characterise the nature of group differences in chemical

profiles. All statistical analyses were carried out using the computer program PRIMER V6.1.12 (Clarke & Gorley, 2006) with the PERMANOVA+ V1.0.2 add-on package (Anderson *et al.*, 2008). We organised our analysis into three sections, each corresponding to one of the arching questions of the study.

Origin of the feather chemical signal

In a first analysis, the origin of the chemical signal present at the surface of blue petrels' feathers was explored by simply surveying the compounds (i.e. peak or analytes) identified in different subsets of samples corresponding to the different combination of year and sample type. The level of chemical similarity between the secretion and feather signals was estimated here by the amount of compound co-occurrences among the two sample types.

Chemical trajectory from uropygial secretions to feathers

The second part of our analysis focused on the differentiation of the chemical signal from the uropygial secretions to the feathers. First, feather-specific compounds were examined in the light of the published literature (especially Jacob, 1978; Jacob & Ziswiler, 1982) to estimate their most likely origin.

We then compared the profiles from secretion and feather samples using single factor ('sample type') PERMANOVA analyses applied to two different datasets: (i) the first one included all the variables (n=330 analytes), (ii) the second one included only those compounds that were found in both sample types and in both years (n=253 analytes). The PERMANOVA analysis of this second subset of data enabled testing as to whether the 'sample type' effect was purely qualitative, being solely caused by the additional compounds found on feathers only.

Finally, the chemical differentiation between sample types was investigated further using CAP analyses applied to the two different datasets mentioned above. Validation of these CAP models was carried out using the 4 birds for which we only had 2008 samples (i.e. 4 secretion and 4 feather samples). These 'validation samples' were treated as unknowns, and were classified as one of the a-priori group according to the CAP model derived from the original samples (Anderson *et al.*, 2008). Analytes associated with the secretion-feather dichotomy were identified following the procedure already described.

Presence of social chemosignals on feathers

In a last analysis, we investigated whether the social chemosignals recently elucidated in blue petrels uropygial secretions (Mardon *et al.*, 2010) were also present, and of a similar nature, on the feathers of the birds. To do so, we used a PERMANOVA model including the two factors 'Sex' (fixed) and 'Individual identity' (random, nested within Sex). To examine the continuity of these signals from secretions to feathers, this analysis was applied to three different subsets of data: (i) secretions samples only, with variables limited to the analytes present in secretions in both years (n=259), (ii) feather samples only, with variables limited to the analytes present in feathers in both years (n=297) and (iii) all samples from birds obtained in both 2008 and 2009 (n=16), with variables limited to the analytes present in both secretions and feathers in both years (n=253).

The 'Sex' and 'Individual identity' effects were then further examined using various CAP analyses on the different subsets of data mentioned above. Validation of the CAP models retained was done again using the 4 birds for which we only had 2008 samples and the analytes associated to these models were identified.

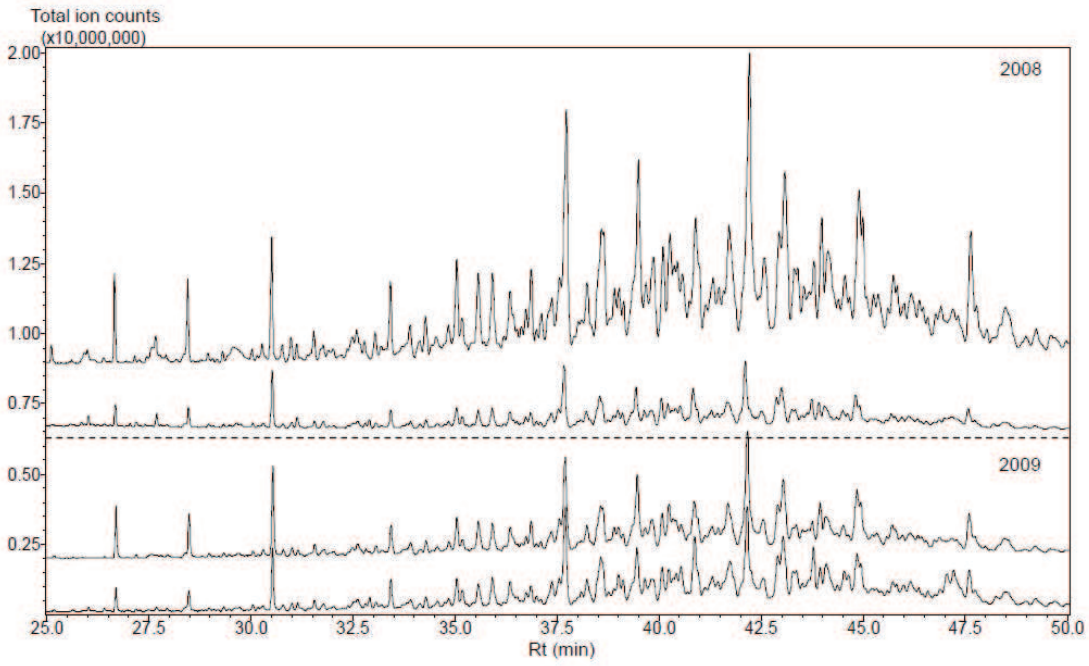

Figure 1: Examples of chromatograms obtained with the 4 samples from the same bird (from top to bottom: 2008 secretion, 2008 feather, 2009 secretion, 2009 feather). For graphic clarity, only a 25min section of the chromatograms (Rt=25-50min) is displayed.

RESULTS

A sample of the chromatographic profiles obtained in our study is displayed in Figure 1 (note that for graphic purposes, the figure only shows a 25min section of the chromatograms; examples of full chromatograms are provided in Appendix 3.3-2).

Origin of the feather chemical signal

The numbers of compounds identified in the different subsets of samples, corresponding to the different combinations of year and sample type, are presented in Table 1.

Table 1: Number of compounds present in the different sample subsets

Sample type \ Year	2008	2009	Both*
Uropygial secretions	308	261	259
Feathers	321	301	297
Both*	298	257	253

* Figures provided here are the number of compounds present in each of the two categories considered (e.g. 2008 AND 2009, or secretions AND feathers).

More compounds were found in the 2008 samples, for both sample types. Interestingly, most of the compounds specific to the 2008 samples eluted early in the chromatograms, before Rt=25min (i.e. RI< 2130). This was the case for 43 of 49 analytes from secretions, and 21 of 24 analytes from feathers. This suggests that the 2008 samples underwent more chemical breakdown before extraction than the 2009 samples, maybe due to the quarantine episode (see methods).

More compounds were also recovered from feathers than from uropygial secretions with a total of 44 compounds being specific to feathers (both years considered). This particular aspect, namely the chemical differentiation from secretions to feathers, is further explored in the next section of our analysis.

Finally, a large proportion of the secretion and feather signals were redundant. Indeed, respectively 97% (298 out of 308), and 98% (257 out of 261), of the secretion analytes were also present in the feather signal in 2008, and 2009. Inversely, respectively 93% (298 out of 321), and 85% (257 out of 301), of the feather analytes

originated from the preen secretions in 2008 and 2009. Both years considered, 98% (253 out of 259) of the secretion analytes were present on the feathers while 85% (253 out of 297) of the feather signal originated from the preen secretions.

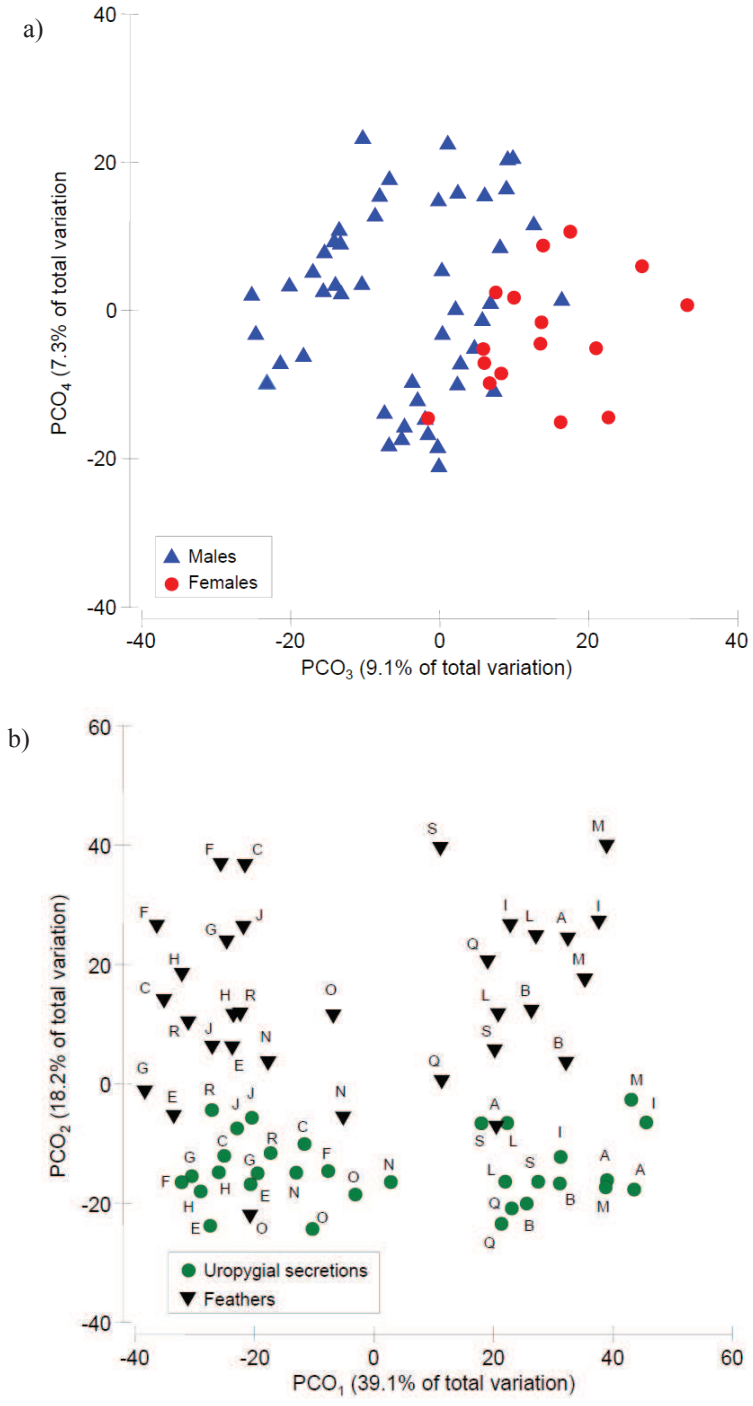

Figure 2: Bi-dimensional PCO ordinations of all samples included in the analysis. a) PCO_1 Vs PCO_2: each symbol corresponds to a sample type, each letter to a particular individual; b) PCO_2 Vs PCO_3: symbols represent the two sexes.

Chemical trajectory from uropygial secretions to feathers

Despite the fact that about 85% of the feather signal originates from the preening secretions, 44 compounds specific to feathers were also identified (Table 2). The chemical nature of those compounds, essentially free fatty acids, aldehydes and alkanes, indicates that preening secretions may be the main contributor to 'feather-specific' analytes, through pathways that are discussed further. A small fraction of these feather-specific analytes however seemed to originate from environmental pollutants or from sources which could not be resolved.

Table 2: List of all feather-specific analytes, sorted by likely origin

Peak ID#	RI	Best Identification	Peak ID#	RI	Best Identification
	Secretion-related compounds		107	2190	Octadecanamide
10	1315	Nonanoic acid	109	2205	Iso-Docosane
11	1360	Iso-Decanoic acid	114	2235	Iso-Tricosane
13	1400	n-Tetradecane	119	2260	Docosane, 2,21-dimethyl
14	1405	n-Decanoic acid	121	2270	Iso-Heneicosanol
19	1500	n-Pentadecane	148	2375	Nonadecanamide
23	1505	Undecanoic acid, 2-methyl	155	2400	Tetracosane
26	1585	n-Dodecanoic acid			
27	1595	1-Tridecanol			**Environmental pollutants**
37	1705	Hexadecane, 2,6,10-trimethyl	33	1635	Benzophenone
39	1720	Pentadecanal	38	1715	Benzoic acid, 2-ethylhexyl ester
40	1730	Iso-Tetradecanoic acid, dimethyl ester	54	1850	Benzene, (1-propyldecyl)
43	1775	Tetradecanoic acid	58	1875	Benzene, (1-ethylundecyl)
49	1820	Hexadecanal	76	2020	Ambreinolide(cis-A/B)
57	1865	Octadecane, 2-methyl	122	2270	Padimate O
70	1970	n-Hexadecanoic acid			
77	2020	9-Octadecen-1-ol			**Unresolved origin**
80	2055	Iso-Hexadecen-1-ol acetate	56	1860	Unidentified peak
82	2060	Iso-Heneicosane	123	2275	Tributyl acetylcitrate
84	2065	Eicosane, 2-methyl	124	2285	Unidentified peak
85	2070	Iso-Nonadecanol	138	2335	15-Isobutyl-(13αH)-isocopalane
88	2085	2-Nonadecanol	252	2945	Unidentified peak
99	2160	Heneicosane, 5-methyl	297	3290	Cholestane-3,5-diol, 5-acetate
103	2175	Iso-Docosane	333	4360	Iso-Dodecanoic acid, propanetriyl ester
104	2175	Octadecanoic acid			

A PCO ordination of all samples, whose first two axes explained 57.3% of the total variation among samples, showed that the 'Sample type' factor had an important influence on chemical profiles (Figure 2a). Also apparent on the ordination is the consistent direction of the secretion-feather trajectory, suggesting that the differentiation process has a certain level of homogeneity across all individuals. Accordingly, the two single-factor PERMANOVA analyses, testing the 'Sample-type' effect on either the whole range of compounds or on the subset of compounds common to both sample types, indicated the existence, in both cases, of a highly significant chemical differentiation between the two signals (whole dataset: df=1, Pseudo-F=15.351, p=0.0001; subset of compounds: df=1, Pseudo-F=9.3401, p=0.0001). Because only analytes common to both sample types were considered in the second PERMANOVA analysis, its outcome implies that the chemical differentiation examined goes beyond the qualitative appearance of extra compounds on feathers, and also includes a quantitative variation of common compounds.

Table 3: Results from CAP analyses examining the effect of a) Sample type b) Sex and c) Individual identity

	Original groups	Classified group		% correct allocation	*m**	Trace statistic *	*P (perm)*
		Secretions	Feathers				
a)	Secretions	32	0	100	5	0.8303	**0.0001**
	Feathers	2	30	93.8	7	0.8275	
		Females	Males				
b)	Females	12	4	75.0	18	0.7346	**0.0001**
	Males	2	46	95.8			
		Correct individual	Different individual				
c)	16 individuals (16 ≠ groups)	59	5	92.2	11	6.7251	**0.0001**

Note: The left part of the table presents cross-validation results (leave-one-out allocation of observations). The right part shows permutation test outputs (n=9999 permutations in each case).
*: For the 'Sample type' CAP analysis, top value corresponds to model 1 (all compounds considered), bottom value to model 2 (compounds present in both sample type only).

Finally, a further examination of this chemical process led to two CAP models, the first one including all identified compounds (n=330) and the second one limited to compounds common to both sample types, which were equally as successful at discriminating between sample types (Table 3a). Using respectively 5 and 7 PCO axes,

both models reached a 96.9% allocation success and both perfectly allocated all eight validation samples (4 secretion and 4 feather samples) to the correct sample type.

Table 4: Main analytes associated with the chemical differentiation between uropygial secretion and feather signals

Key target analytes				Average peak areas (transformed & standardised)					
				Secretions		Feathers			
Peak ID #	RI	Best Identification	Formula	Mean	± SE	Mean	± SE	r_1	r_2
36	1700	n-Heptadecane	$C_{17}H_{36}$	0.8	0.1	114.1	14.1	**0.95**	**0.94**
297	3290	Cholestane-3,5-diol, 5-acetate	$C_{29}H_{50}O_3$	0.0	0.0	107.2	14.9	**0.91**	*NA*
300	3295	Iso-Cholestanol	$C_{27}H_{48}O$	0.3	0.2	247.8	30.5	**0.91**	**0.93**
121	2270	Iso-Heneicosanol	$C_{21}H_{46}O$	0.0	0.0	14.5	2.2	**0.88**	*NA*
305	3350	Unidentified peak	*NA*	14.5	1.3	138.0	15.6	**0.87**	**0.91**
252	2945	Unidentified peak	*NA*	0.0	0.0	29.6	4.1	**0.87**	*NA*
155	2400	Iso-Tetracosane	$C_{24}H_{50}$	0.0	0.0	28.9	3.7	**0.87**	*NA*
19	1500	n-Pentadecane	$C_{15}H_{32}$	0.0	0.0	25.9	3.8	**0.85**	*NA*
37	1710	Iso-Octadecane	$C_{18}H_{38}$	0.0	0.0	4.5	0.7	**0.84**	*NA*
62	1900	n-Nonadecane	$C_{19}H_{40}$	3.0	0.6	62.3	10.5	**0.84**	**0.83**
27	1590	1-Tridecanol	$C_{13}H_{28}O$	0.0	0.0	6.9	1.2	**0.82**	*NA*
85	2070	Iso-Nonadecanol	$C_{19}H_{38}O$	0.0	0.0	13.8	2.4	**0.81**	*NA*
46	1800	n-Octadecane	$C_{18}H_{38}$	1.3	0.2	56.1	9.9	**0.80**	**0.78**
82	2060	Iso-Heneicosane	$C_{21}H_{44}$	0.0	0.0	8.2	1.3	**0.80**	*NA*
72	1990	Iso-Octadecanol	$C_{18}H_{38}O$	0.7	0.1	8.3	1.3	**0.79**	**0.82**
99	2155	Heneicosane, 5-methyl	$C_{22}H_{46}$	0.0	0.0	12.5	2.3	**0.79**	*NA*
13	1400	n-Tetradecane	$C_{14}H_{30}$	0.0	0.0	8.2	1.2	**0.78**	*NA*
44	1795	Iso-Hexadecanol	$C_{16}H_{34}O$	6.4	1.0	32.8	3.6	**0.78**	**0.82**
48	1805	Benzene, 1-methylundecyl	$C_{18}H_{30}$	0.0	0.0	18.5	3.7	**0.77**	*NA*
29	1600	n-Hexadecane	$C_{16}H_{34}$	1.0	0.1	27.2	4.8	**0.76**	**0.74**
59	1875	Phthalic acid, diisobutyl ester	$C_{16}H_{22}O_4$	3.5	0.5	68.3	13.7	**0.75**	**0.73**
35	1680	Iso-Hexadecanol	$C_{16}H_{34}O$	2.5	0.5	17.9	2.6	**0.75**	**0.76**
52	1830	Benzene, (1-pentylheptyl)	$C_{18}H_{30}$	0.0	0.0	13.3	3.1	**0.75**	*NA*
101	2175	n-Pentadecylcyclohexane	$C_{21}H_{42}$	0.4	0.1	24.8	5.4	**0.71**	**0.70**
42	1755	Iso-Hexadecanol	$C_{16}H_{34}O$	1.1	0.3	16.4	3.5	**0.66**	**0.71**

Note: ***r*** corresponds to the Pearson correlation of a particular compound with the CAP axis discriminating the two sample types (r_1 is from the first CAP model including all analytes, r_2 is from the second model limited to analytes common to both sample types). All contributions presented are significant (critical ***r*** value, at a level of α=5%, was 0.45).

Individual compounds associated with these CAP models were essentially alkanes and alcohols between C_{13} and C_{22} (Table 4), and all had a higher occurrence on feathers than in secretions. Yet, two large feather-specific cholestanol-based analytes (#297 and #300 – Table 4) whose origin remain uncertain, and a few pollutants (#48, #59 and #52 – Table 4), also played an important role in the secretion/feather chemical dichotomy.

Presence of social chemosignals on feathers

All chemical profiles involved in this analysis were initially input in an unconstrained PCO ordination whose four first axes explained respectively 39.1%, 18.2%, 9.1 % and 7.3% of the total variation of the multivariate data (Figures 2a & 2b). Individual identities of the birds appeared to be represented essentially along the first PCO axis while the second axis discriminated well between secretion and feather samples as seen before. The third and fourth PCO axes, on the other hand, were efficient at discriminating between sexes.

Accordingly, the different PERMANOVA analyses carried out on sample subsets and on the overall dataset, using the two factors 'Sex' and 'Individual identity', consistently indicated a weak evidence towards chemical dimorphism between males and females and a highly significant inter-individual variability in chemical profiles (Table 5). Note that the PERMANOVA design used, which tested the 'Sex' factor before the 'Individual' factor nested within, rules out the possibility that the weaker intensity of the former is a consequence of some chemical redundancy in the two types of signals.

Table 5: PERMANOVA table of results for the analysis of social chemosignals

Source	Sample subset	df	SS	MS	Pseudo *F*	*p* (perm)
Sex	Secretions	1	4406	4406	1.8382	0.1307
	Feathers	1	5402	5402	1.8477	0.1219
	All samples	1	9029	9029	1.93	0.1182
Individual identity (nested within Sex)	Secretions	14	33554	2397	5.5692	**0.0001**
	Feathers	14	40928	2923	2.394	**0.0001**
	All samples	14	65383	4670	5.4082	**0.0001**
Residuals	Secretions	16	6886	430		
	Feathers	16	19538	1221		
	All samples	48	41450	864		

Regarding the 'Sex' factor, PERMANOVA results indicate that the sex-associated chemical variability does not constitute a significant portion of the overall variation of the multivariate data. Yet, a CAP analysis using all samples could successfully distinguish the chemical profiles of males and females, using a single axis obtained from *m*=18 PCO axes. The overall leave-one-out allocation success was 90.6% (Table 3b) for the samples used to build the CAP model and all 8 validation samples (from 4 different males) were correctly classified using this model (Figure 3). Note that the outcome of this analysis was affected by the set of chemical profiles from a particular female individual. For information, excluding this individual (H - see Figure 3) from the dataset resulted in a much simpler CAP discriminating model, using only m=4 PCO axes, and presenting an overall allocation success of 96.7% (100% for males and 95.8% for females, p=0.0001). When considered together, outcomes from the PCO (i.e. a partial sexual dichotomy along the third and fourth dimensional axes), the PERMANOVA and the CAP (i.e. existence of a highly successful sex-discriminating model) analyses suggest that the nature of the chemical variability induced by the 'Sex' factor is different from the main directions of variability in the multivariate data. The contribution of this signal to the overall chemical variability seems to be lessened in particular by the high inter-individual variability.

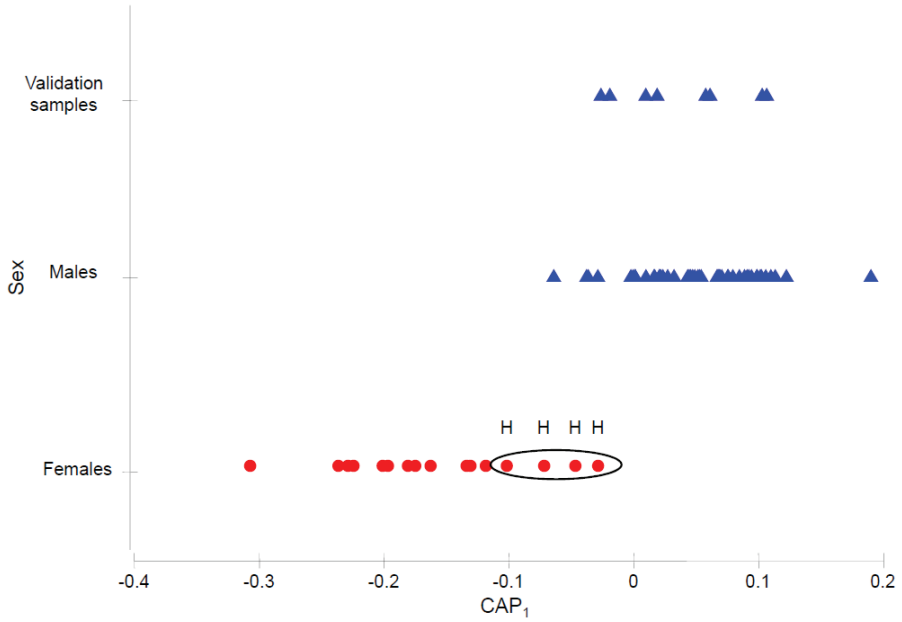

Figure 3: CAP analysis of the 'Sex' factor showing 90.6% correct discrimination of chemical profiles between the two sexes and the correct classification of all validation samples. Data points corresponding to the H female are circled on the graph.

Table 6: Main analytes associated with the chemical 'Sex' signal in secretions, feathers and all samples together

Key analytes					Pearson *r* (with CAP axis)			
Peak ID #	RI	Best Identification	Methyl subt.	Formula	Secret.	Feathers	All samples	Signal direction
247	2920	Iso-Decanoic acid, octadecyl ester	4	$C_{28}H_{56}0_2$	**0.93**	**0.73**	**0.79**	Females
201	2650	Iso-Nonanoic acid, hexadecyl ester	2-4	$C_{25}H_{50}O_2$	**0.89**	**0.68**	**0.78**	Females
246	2910	Iso-Undecanoic acid, heptadecyl ester	2-4	$C_{28}H_{56}O_2$	**0.89**	**0.64**	**0.72**	Females
239	2870	Iso-Undecanoic acid, hexadecyl ester	2-4	$C_{27}H_{54}O_2$	**0.87**	**0.65**	**0.70**	Females
208	2685	Iso-Decanoic acid, pentadecyl ester	4	$C_{25}H_{50}O_2$	**0.87**	0.53	**0.70**	Females
230	2820	Iso-Decanoic acid, heptadecyl ester	4	$C_{27}H_{54}O_2$	**0.88**	0.57	**0.67**	Females
199	2645	Iso-Decanoic acid, pentadecyl ester	2-4	$C_{25}H_{50}O_2$	**0.76**	0.58	**0.66**	Females
261	3005	Iso-Undecanoic acid, octadecyl ester	4	$C_{29}H_{58}0_2$	**0.73**	**0.64**	**0.65**	Females
192	2600	Iso-Decanoic acid, tetradecyl ester	4	$C_{24}H_{48}O_2$	**0.83**	**0.64**	**0.64**	Females
223	2780	Iso-Decanoic acid, hexadecyl ester	2-4	$C_{26}H_{52}O_2$	**0.80**	0.54	**0.63**	Females
177	2525	Iso-Nonanoic acid, pentadecyl ester	2-4	$C_{24}H_{48}O_2$	**0.76**	0.52	**0.63**	Females
222	2770	Iso-Undecanoic, pentadecyl ester	2-4	$C_{26}H_{52}O_2$	**0.67**	0.56	**0.62**	Females
253	2955	Iso-Undecanoic, heptadecyl ester	4	$C_{28}H_{56}O_2$	**0.72**	0.60	**0.62**	Females
234	2840	Iso-Hexacosanol		$C_{26}H_{54}O$	0.60	0.49	**0.54**	Females
236	2855	Iso-Nonanoic acid, octadecyl ester	3	$C_{27}H_{54}O_2$	0.55	0.51	**0.53**	Females
160	2440	Iso-Decanoic acid, tridecyl ester	4	$C_{23}H_{46}O_2$	**0.68**	0.49	**0.53**	Females
186	2555	Iso-Decanoic acid, tetradecyl ester	4	$C_{24}H_{48}O_2$	**0.69**	0.47	**0.52**	Females
179	2535	Iso-Octanoic acid, hexadecyl ester	4	$C_{24}H_{48}O_2$	**0.75**	0.41	**0.50**	Females
213	2710	Iso-Dodecanoic acid, tetradecyl ester	2	$C_{26}H_{52}O_2$	-0.55	-0.61	**-0.60**	Males
204	2660	Iso-Dodecanoic acid, tridecyl ester	NB	$C_{25}H_{50}O_2$	-0.49	-0.56	**-0.51**	Males
250	2940	Iso-Dodecanoic acid, hexadecyl ester	NB	$C_{28}H_{56}O_2$	-0.47	-0.53	**-0.50**	Males

Note: ***r*** corresponds to the Pearson correlation of a particular compound with the CAP axis discriminating the two sexes in the corresponding model. Strong contributions are bold. For information, critical ***r*** values (at a level of α=5%) would be respectively 0.62 (secretions), 0.62 (feathers) and 0.45 (all samples).

Individual compounds associated with the CAP models discriminating the two sexes' chemical profiles, were consistent across sample types, consisting primarily of esterified acids between C_{23} and C_{29} (Table 6). Most of these analytes had a higher occurrence in females than in males suggesting the 'Sex' signal is essentially a female signal. In

addition, the number of compounds significantly contributing to the 'Sex' signal in feather samples was limited to a handful of esters, all showing at least a 4 methyl-substitution. In contrast, the three marginally contributing compounds which had higher presence in males showed either a 2-methyl substitution or none.

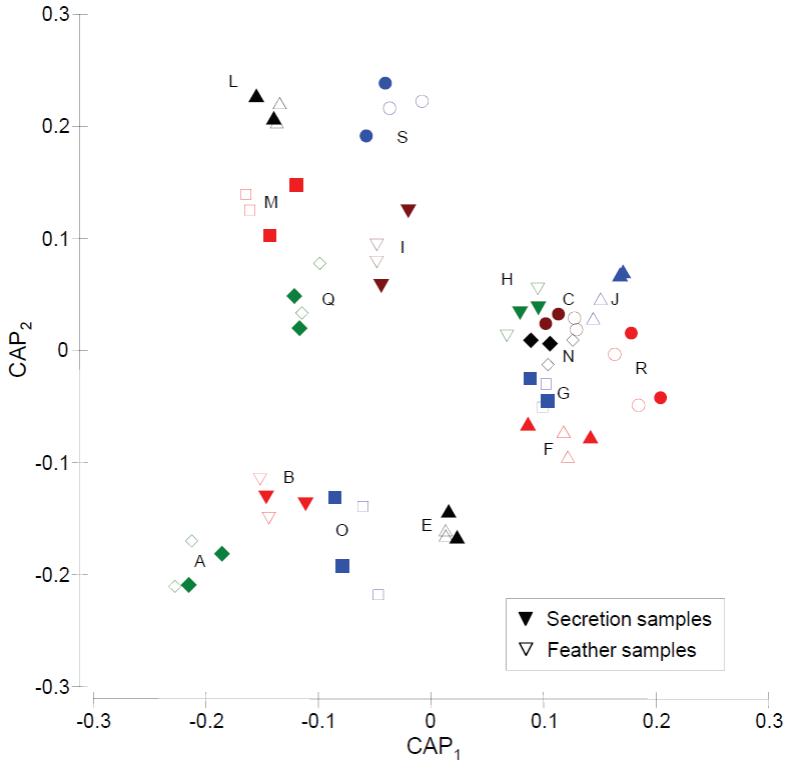

Figure 4: CAP analysis of the 'Individual' factor showing 92.2% correct discrimination of chemical profiles between the different individuals. Note that the figure only displays CAP_1 and CAP_2 axes out of the 11 generated in this model. Each data point corresponds to one sample, each letter to a particular individual, and fillings (open or full) represent the two sample types.

Examination of the 'Individual' signal, through a CAP analysis (Figure 4), yielded a model that successfully attributed the chemical profiles to the correct individuals in 92.2% of cases (Table 3c), using a subset of only *m*=11 PCO axes. Highly apparent on Figure 4, displaying CAP_1 and CAP_2 axes only, is the continuity of the 'Individual' signal from secretions to feathers and across years, as the four samples from each individual (2 secretions and 2 feathers) remain closely clustered. This demonstrates that not only is the 'Individual' signal (i.e. chemical signatures) a predominant component of the overall chemical variability within all samples as indicated by the PERMANOVA results, but it is also remarkably consistent across years and substrates.

Analytes associated with this strong 'Individual' signal were targeted once more by looking at their correlation (Pearson ***r***) to the CAP axes (m=11). When all samples were

considered, 182 compounds out of the 253 selected for this analysis (72%) had a significant contribution to the CAP model discriminating individuals. Moreover, these compounds were present in all samples. This suggests that birds' chemical signature, both within secretions and on feathers, may not be made up of individually specific compounds, but would rather lie in the relative proportions of a large number of omnipresent compounds.

DISCUSSION

This detailed investigation of blue petrels' chemosignals indicates a high level of chemical similarity between uropygial secretions and feather lipids. Uropygial contents form the principal source of feather lipids in these birds although a few feather-specific compounds have been identified. Importantly, the social information (sex and individual identity) contained in the secretions is still present on the plumage in a very consistent form.

Origin of the feather chemical signal

Results from our first analysis indicate that uropygial secretions are by far the main contributor to plumage lipids in blue petrels. Across the two years of the study, we found that 98% of the secretion contents were present on the feathers, representing 85% of the whole feather signal. The finding is striking in the light of the few previous accounts of such comparisons, which typically reported important qualitative differences between the two signals. For instance, Bolliger and Varga (1961)'s examination of feather lipids across 14 bird species (unfortunately not explicitly provided) led them to the conclusion that "*feather lipids are of dissimilar qualitative composition to the preen or oil gland secretion...*". As a result, the authors hypothesise that feather lipids could be by-products of the keratinisation process associated with feather development. Similarly, a chemical investigation of marabou feather lipids showed a significant difference with those of the uropygial secretions by the presence of sterols, sterols esters, di and mono-glycerides, and free fatty acids (Jacob & Pomeroy, 1979). Finally, the most significant result on the question comes from wood pigeons where only 6.7% of the whole-plumage lipids were considered to originate from the uropygial contents (Jacob and Grimmer, 1975 in Jacob & Ziswiler, 1982). Our

contrasting results from blue petrels are supported however by the anecdotal mention by Jacob that "*extraction of the plumage and comparison of the lipids extracted with those of the preen gland did not show any significant differences in case of Puffinus griseus",* i.e. another procellariiform seabird ("J. Jacob, unpublished results, 1975" in Jacob, 1978).

Importantly, the above considerations indicate that there is no general answer to the question of the origin of plumage chemicals. Relative contributions of the different potential sources (uropygial gland, epidermis, feather by-products and others) likely varies from one avian group to the next, depending on their ecology. It is reasonable indeed to expect different chemical processes to control the plumage fate in waterbirds and landbirds, or in groups with large a uropygial gland compared to groups without it. With a mainly-pelagic lifestyle and a large uropygial gland, the strong chemical redundancy between secretions and feather lipids reported here for a procellariiform seabird is thus ecologically sound.

Finally, the relationship between secretions and feather lipids may also vary within one species; it can involve inter-individual variation in the presence and size of the uropygial gland (Johansson, 1927), as well as intra-individual variation depending on the type and location of feathers considered for a particular bird (Bolliger & Varga, 1961).

Chemical trajectory from uropygial secretions to feathers

Despite the high redundancy of the secretion and feather signals discussed above, the chemical differentiation from one to the next significantly contributed to the chemical variation within our samples. Our results also indicate that this differentiation includes both qualitative and quantitative variations. The qualitative component of the differentiation lies in the appearance of new relatively short-chained analytes on the feather surface, principally free fatty acids (C_9-C_{18}), aldehydes (C_{15}-C_{18}) and alkanes (C_{15}-C_{24}). Likewise, the quantitative component essentially consists in the increased presence of several short–chain alkanes (C_{15}-C_{21}) and alcohols (C_{16}-C_{18}) on the plumage, compared to the secretions. Regarding the origin of the 'feather-specific' compounds, we cannot rule out the possibility that these were already present in secretion samples, but remained undetected because of the absence of pre-concentrating step. However, the co-occurrence of significant quantitative changes between secretion and plumage rather suggests that these chemical variations result from the partial

breakdown of secretion contents once on the plumage. This breakdown could involve various bio-chemical processes such enzymatic actions from integument lipases (Jacob & Pomeroy, 1979; Jacob & Ziswiler, 1982 - p306), microbial activity (Hagelin & Jones, 2007), photolysis, oxidation, etc.

In this regard, the combined appearance and/or increase of semi-volatile fatty acids, aldehydes and alcohols have a high potential significance regarding the process of odour-production. Indeed, these classes of compounds often constitute key components of animal scents and have been found in the preen secretions (and/or on the plumage) of many birds (Jacob & Ziswiler, 1982; Bonadonna *et al.*, 2007; Soini *et al.*, 2007). Fatty acids, for instance, and their relative proportion on the skin, have long been proposed to be the chemical basis for human individual olfactory signatures (Nicolaides, 1974; Penn *et al.*, 2007). Fatty linear alcohols present on the plumage of dark-eyed juncos have, on the other hand, been hypothetically related to ectoparasite repellence and/or nest olfactory crypticism (Soini *et al.*, 2007). Importantly, the relative amounts of the two former class of compounds could be interdependent as fatty linear alcohols can be produced by the reduction of fatty acids (Soini *et al.*, 2007). Furthermore, both have been anecdotically suggested to participate to the strong scent of procellariiform seabirds (Jacob & Ziswiler, 1982 - p306). Finally aldehydes, found on the plumage of many birds including petrels (Bonadonna *et al.*, 2007), chicken (Allan *et al.*, 2006) and auklets (Hagelin *et al.*, 2003), have been shown to be effective repellents to some avian parasites (Douglas *et al.*, 2005). To further determine which particular compounds, or class of compounds, play a key role in the conduction of petrels' olfactory signals, current research is examining the airborne volatiles surrounding birds.

Our findings also indicate that the seemingly odourless nature of uropygial secretions, in some species, should not be considered as an indication that these secretions do not participate in the scent-production process (Hagelin & Jones, 2007). Indeed, our results support the view that, in procellariiform seabirds at least, plumage scents may be associated with particular compounds which are produced and/or released once the secretions are spread on the feathers (Jacob & Ziswiler, 1982; Burger *et al.*, 2004).

Finally, it is interesting to note the appearance of several benzene-based compounds on the feathers as well, which likely originated from environmental pollution of some sort (e.g. ship fumes, oil slicks). Importantly, no trace of such contaminants was present within our secretion samples, which suggest that this environmental pollution

essentially originated from external deposition. In contrast, recent bioenvironmental studies indicated an endogenous origin of feather pollutants by reporting significant correlations between the levels of organic pollutants found in birds' feathers, preen oils and internal tissues (Yamashita *et al.*, 2007; Jaspers *et al.*, 2008). These studies, which advocate the use of bird feathers as a useful monitoring tool for organic pollutants (Jaspers *et al.*, 2007), however essentially considered bird specimens from urbanised areas.

Presence of social chemosignals on feathers

Our distance-based multivariate analyses indicate that the social chemosignals present on blue petrels' feathers are very similar, in both nature and intensity, to those found within their uropygial secretions. In both cases, we found a sex-specific chemical signal, whose direction of chemical variation was different from the main multivariate chemical variability. Individual chemical signatures, revealed in blue petrels secretions (Mardon *et al.*, 2010), are also present in a remarkably consistent form on the feathers of these birds. The ecological and evolutionary implications of these chemosignals are extensively discussed elsewhere (Mardon *et al.*, 2010) and the following discussion is essentially restricted to the significance of their presence on the birds' feathers.

The notable continuity of the 'Sex' and 'Individual' chemosignals, from uropygial secretions to the feathers of blue petrels, is an important result for the study of petrels' chemical communication as it indicates that all endogenous social information is still present, in a much conserved form, on the final odour substrate which is the plumage. The chemical processes undergone by these signals when they are converted to personal scents are not fully resolved but the second section of our analysis has pointed towards some interesting directions.

In petrels, strong chemical personal signatures may preside over mating decisions. Indeed, a genetic-based mate choice ensuring compatibility between lifelong partners, and mediated by olfactory cues is suspected in this group (Zelano & Edwards, 2002). The remarkable chemical continuity of the 'Individual' signal from the secretions of blue petrels to their feathers indicates that it could constitute a very reliable basis for individual recognition and/or assessment in this species (Bonadonna & Nevitt, 2004; Mardon & Bonadonna, 2009). In addition, the presence of chemical signatures on the feathers of Antarctic prions, another closely-related petrel species (Bonadonna *et al.*, 2007), supports the extension of our finding to the whole group.

The relatively weak, though significantly dichotomous, 'Sex' chemosignal also remains consistent from secretions to feathers. Its contribution to the chemical profile is greatly reduced by the strong personal signature, possibly reflecting the fact that olfactory individual discrimination is much more important in petrels' reproductive ecology than olfactory sexual discrimination (Bonadonna *et al.*, 2009; Mardon *et al.*, 2010). Nevertheless, the presence of this signal on the birds' feathers indicates that it could have a role in close range behaviours, such as in the activation of actual sexual behaviours (copulations, mounts, etc.), as suggested by other studies (Hagelin & Jones, 2007; Balthazart & Taziaux, 2009).

The function of the uropygial gland and its secretions has been debated for a long time. Much of the debate however overlooked the fact that these secretions may serve widely different purposes across groups of different ecology. In the light of previous findings on petrel seabirds, and of the results presented here, it is now scientifically reasonable to consider that the uropygial gland plays a crucial role in scent production in petrel seabirds. Indeed, secretion contents represent more than 85% of the chemical signal from the birds' musky plumage and may provide the precursors for most of the remaining 15%. The potential importance of chemical communication in birds' social lives has only been recently appreciated and our study confirms how valuable this investigation is promising to be.

SUPPLEMENTARY MATERIAL

- ➔ Appendix 3.3-1: Details of statistical methods
- ➔ Appendix 3.3-2: Examples of full chromatograms

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REFERENCES

Allan S.A., Bernier U.R. & Kline D.L. (2006). Laboratory evaluation of avian odors for mosquito (Diptera : culicidae) attraction *Journal of medical entomology* **43**: 225-231.

Anderson M.J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology* **26**: 32-46.

Anderson M.J., Gorley R.N. & Clarke K.R. (2008). *PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods.* PRIMER-E Ltd, Plymouth, UK.

Anderson M.J. & Willis T.J. (2003). Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecology* **84**: 511-525.

Balthazart J. & Taziaux M. (2009). The underestimated role of olfaction in avian reproduction? *Behavioural Brain Research* **200**: 248-259.

Bang B.G. & Cobb S. (1968). The size of the olfactory bulb in 108 species of birds. *The Auk* **85**: 55-61.

Bolliger A. & Varga D. (1961). Feather Lipids. *Nature* **190**: 1125.

Bonadonna F., Caro S.P. & Brooke M.d.L. (2009). Olfactory Sex Recognition Investigated in Antarctic Prions. *PLoS ONE* **4**: e4148.

Bonadonna F. & Mardon J. (2010). One house two families: petrel squatters get a sniff of low-cost breeding opportunities. *Ethology* **116**: 176-182.

Bonadonna F., Miguel E., Grosbois V., Jouventin P. & Bessiere J.-M. (2007). Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? *Journal of Chemical Ecology* **33**: 1819-1829.

Bonadonna F. & Nevitt G.A. (2004). Partner-specific odor recognition in an Antarctic seabird. *Science* **306**: 835.

Bonadonna F., Villafane M., Bajzak C. & Jouventin P. (2004). Recognition of burrow's 'olfactory signature' in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. *Animal Behaviour* **67**: 893-898.

Borchelt P.L. & Duncan L. (1974). Dustbathing and feather lipid in bobwhite (*Colinus virginianus*). *The Condor* **76**: 471-472.

Burger B.V., Reiter B., Borzyk O. & du Plessis M.A. (2004). Avian exocrine secretions I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. *Journal of Chemical Ecology* **30**: 1603-1611.

Clarke K.R. & Gorley R.N. (2006). *PRIMER v6: user manual/tutorial*. PRIMER-E Ltd, Plymouth, UK.

Clarke K.R. & Warwick R.M. (2001). Changes in marine communities: An approach to statistical analysis and interpretation, 2nd edition. edn. Primer-E., Plymouth.

Douglas H.D., III , Malenke J.R. & Clayton D.H. (2005). Is the citrus-like plumage odorant of crested auklets (*Aethia cristatella*) a defense against lice? *Journal of Ornithology* **146**: 111-115.

Elder W.H. (1954). The oil gland of birds. *Wilson Bulletin* **66**: 6-31.

Gower J.C. (1966). Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* **53**: 325-338.

Hagelin J.C., Jones I.J. & Rasmussen L.E.L. (2003). A tangerine-scented social odour in a monogamous seabird. *Proceedings of the Royal Society of London B Biological Sciences* **270**: 1323-1329.

Hagelin J.C. & Jones I.L. (2007). Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? *The Auk* **124**: 741-761.

Jacob J. (1978). Uropygial gland secretions and feather waxes. In: Florkin M., Scheer B.T. (eds). *Chemical zoology*, vol 10 Aves. Academic press, New York: p.165-211.

Jacob J. & Pomeroy D.E. (1979). The feather lipids of the marabou stork (*Leptoptiles crumeniferus*). *Comparative biochemistry and physiology* **64B**: 301-303.

Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Jaspers V.L.B., Covaci A., Deleu P., Neels H. & Eens M. (2008). Preen oil as the main source of external contamination with organic pollutants onto feathers of the common magpie (*Pica pica*). *Environment International* **34**: 741-748.

Jaspers V.L.B., Voorspoels S., Covaci A., Lepoint G. & Eens M. (2007). Evaluation of the usefulness of bird feathers as a nondestructive biomonitoring tool for organic pollutants: A comparative and meta-analytical approach. *Environment International* **33**: 328-337.

Johansson I. (1927). Studies on inheritance in pigeons. VI. Number of tail feathers and uropygial gland. *Genetics* **12**: 93-107.

Jouventin P., Mouret V. & Bonadonna F. (2007). Wilson's storm petrels *Oceanites oceanicus* recognise the olfactory signature of their mate. *Ethology* **113**: 1228-1232.

Mardon J. & Bonadonna F. (2009). Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own. *Behavioral Ecology and Sociobiology* **63**: 537-542.

Mardon J., Saunders S.M., Anderson M.J., Couchoux C. & Bonadonna F. (2010). Species, gender and identity: cracking petrels' socio-chemical code. *Chemical Senses* **Advance access**: doi:10.1093/chemse/bjq021.

Mc Ardle B.H. & Anderson M.J. (2001). Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82**: 290-297.

Mougeot F. & Bretagnolle V. (2000). Predation risk and moonlight avoidance in nocturnal seabirds. *Journal of Avian Biology* **31**: 376-387.

Mougeot F., Genevois F. & Bretagnolle V. (1998). Predation on burrowing petrels by the brown skua at Mayes islands, Kerguelen. *Journal of Zoology, London* **244**: 429-438.

Nevitt G.A. (2000). Olfactory foraging by Antarctic procellariiform seabirds: life at high Reynolds numbers. *Biological Bulletin (Woods Hole)* **198**: 245-253.

Nicolaides N. (1974). Skin lipids: their biochemical uniqueness. *Science* **186**: 19-26.

Penn D.J., Oberzaucher E., Grammer K., Fischer G., Soini H.A., Wiesler D., Novotny M.V., Dixon S.J., Xu Y. & Brereton R.G. (2007). Individual and gender fingerprints in human body odour. *Journal of the Royal Society Interface* **4**: 331-340.

Piersma T., Dekker M. & Sinninghe-Damsté J.S. (1999). An avian equivalent of make-up? *Ecology Letters* **2**: 201-203.

Pycraft W.P. (1910). A history of birds. Methuen and Co., London.

Reneerkens J., Piersma T. & Sinninghe-Damsté J.S. (2002). Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proceedings of the Royal Society of London B Biological Sciences* **269**: 2135-2139.

Salibián A. & Montalti D. (2009). Physiological and biochemical aspects of the avian uropygial gland. *Brazilian Journal of Biology* **69**: 437-446.

Shawkey M.D., Pillai S.R. & Hill G.E. (2003). Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. *Journal of Avian Biology* **34**: 345-349.

Soini H.A., Schrock S.E., Bruce K.E., Wiesler D., Ketterson E.D. & Novotny M.V. (2007). Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*). *Journal of Chemical Ecology* **33**: 183-198.

Sweeney R.J., Lovette I.J. & Harvey E.L. (2004). Evolutionary variation in feather waxes of passerine birds. *The Auk* **121**: 435-445.

Warham J. (1996). The behaviour, population biology and physiology of the petrels. Academic Press, London, UK.

Weldon P.J. & Rappole J.H. (1997). A survey of birds odorous or unpalatable to humans: Possible indications of chemical defense. *Journal of Chemical Ecology* **23**: 2609-2633.

Yamashita R., Takada H., Murakami M., Fukuwaka M.-A. & Watanuki Y. (2007). Evaluation of Noninvasive Approach for Monitoring PCB Pollution of Seabirds Using Preen Gland Oil. *Environmental Science & Technology* **41**: 4901-4906.

Zelano B. & Edwards V.E. (2002). An Mhc component to kin recognition and mate choice in birds: predictions, progress, and prospects. *American Naturalist* **160**: 225-238.

APPENDIX 3.3-1: STATISTICAL METHODS

Chromatographic data from our study were characterised by a large number of variables (i.e. peak areas for all analytes) compared to the number of sample units ($n \leq 72$). In addition, the relative abundances of the chemical analytes were rarely normally distributed, typically displaying a high right-skewness. Unfortunately, MANOVA test statistics are not particularly robust to departures from the assumption of multivariate normality (Olson, 1974) and simply cannot be computed when there are more variables than sampling units (Anderson, 2001). Thus a number of more robust distance-based multivariate approaches which are described below were used instead.

DATA PRE-TREATMENT, RESEMBLANCE MEASURE AND ORDINATION

Peak areas were successively standardised twice across all samples. The first standardisation used the peak area of the spike (2-bromophenol), to account for variation in the instrument response among samples (particularly across years). The second standardisation used the peak area of a particular target analyte (#265: dodecanoic acid, hexadecyl ester, RI=3045), which was one of the highest (if not the highest) peak in all samples. This relativised the values for different analytes within a sample in order to account for the total quantity of secretion, which varied among samples. After standardisation, data were square-root transformed to reduce skewness and so that the resemblance measure calculations, while retaining the relative abundances of analytes, would not be overly dominated by the most abundant analytes (Clarke & Warwick, 2001).

Euclidean distances between every pair of samples were then calculated to produce a resemblance matrix that formed the basis of ensuing analyses. Note that Euclidean distance was considered an appropriate choice here, because analytes were measured in similar units and were on similar scales after transformation. In addition, the joint absence of any given analyte was considered to indicate similarity between two samples, and Euclidean distances do not exclude joint absence information. As an illustration, a chemical sexual dimorphism may lie in the systematic absence of certain analytes in one sex compared to the other.

Principal coordinates analysis based on the Euclidean resemblance matrix (PCO; Gower, 1966) was used as an ordination method in order to visualise the patterns of differences in the multivariate chemical structure among samples. Note that although PCO on a Euclidean distance matrix is equivalent to a PCA on the original data, we used PCO here because of the intrinsic over-parameterisation of the problem (many more variables than sampling units).

STATISTICAL METHODS

We used two different types of distance-based multivariate approaches in our study, PERMANOVA (PERmutational Multivariate ANalysis Of VAriance; Anderson, 2001; Mc Ardle & Anderson, 2001) and CAP (Canonical Analysis of Principal coordinates; Anderson & Willis, 2003). These two types of analysis (PERMANOVA and CAP) offer two alternative statistical perspectives on the data. PERMANOVA indicates how the various factors included in the model contribute to the overall variation in the data. As such, the importance of a given factor is influenced by the quantity of overall variation in the data. CAP models, on the other hand, search the multivariate space for a separation between *a-priori* groups, which can then be used for predictive modelling. This kind of analysis is particularly useful when the direction of segregation between the groups of interest in the multivariate space is fundamentally different from the main direction(s) of overall variation in the dataset (Anderson & Willis, 2003) which is the case for the 'Sex' factor in the present study.

CAP models that had a good discriminating capability between groups were used to identify the key analytes associated with the various chemical signals. This was done by examining the linear relationships between each of the individual variables (analytes) and the discriminating axes of the corresponding CAP analysis. In each case, we retained the first 20 analytes which had a Pearson correlation ***r*** to the CAP axis higher than a specific threshold value. This specific value was calculated to correspond to the minimum level of correlation that would be deemed statistically significant (after correction for the number of variables tested) in a classical linear correlation analysis (for instance $n_{analytes}$=330, $n_{samples}$=64, r_{min}=0.45). This procedure provides correlation-based chemical associations between compounds and the different signals which should not be interpreted in a causative way.

All statistical analyses were carried out using the computer program PRIMER V6.1.12 (Clarke & Gorley, 2006) with the PERMANOVA+ V1.0.2 add-on package

(Anderson *et al.*, 2008). We organised our analysis into three sections, each corresponding to one of the arching questions of the study.

Origin of the feather chemical signal

In a first analysis, the origin of the chemical signal present at the surface of blue petrels' feathers was explored by simply surveying the compounds (i.e. peak or analytes) identified in different subsets of samples corresponding to the different combination of year and sample type. The level of chemical similarity between the secretion and feather signals was estimated here by the amount of compound co-occurrences among the two sample types.

Chemical trajectory from uropygial secretions to feathers

In a second analysis, we focused on the differentiation of the chemical signal from the uropygial secretions to the feathers. To do so, we first examined feather-specific compounds in the light of the published literature (especially Jacob, 1978; Jacob & Ziswiler, 1982) to estimate their most likely origin.

We also compared the profiles from secretion and feather samples using a single factor PERMANOVA analysis. P-values were obtained using 9999 permutations of the raw data and Type I (sequential) sum of squares. The analysis was applied to two different datasets: (i) the first one included all the variables (n=330 analytes), (ii) the second one included only those compounds that were found in both sample types and in both years (n=253 analytes). The PERMANOVA analysis of this second subset of data enabled testing as to whether the 'sample type' effect was purely qualitative, being solely caused by the additional compounds found on feathers only.

Finally, the chemical differentiation between sample types was investigated further using a CAP analysis which is a distance-based discriminant analysis, in this case yielding a model to discriminate between sample types on the basis of their chemical profile. Again, this analysis was applied to the two different datasets mentioned above. A leave-one-out cross-validation method was used to determine the number of PCO axes to use for the CAP models (Anderson & Robinson, 2003) and to assess their predictive capability. Validation of the models was carried out using the 4 birds for which we only had 2008 samples (i.e. 4 secretion and 4 feather samples). These 'validation samples' were treated as unknowns, and were classified as one of the a-priori group according to the CAP model derived from the original samples (Anderson

et al., 2008), allowing an independent further assessment of the model's quality. Analytes associated with the secretion-feather dichotomy were then targeted by looking at their Pearson correlation ***r*** to the discriminating CAP models following the procedure described in the previous section.

Presence of social chemosignals on feathers

In a last analysis, we investigated whether the social chemosignals recently elucidated in blue petrels uropygial secretions (Mardon *et al.*, submitted) were also present, and of a similar nature, on the feathers of the birds. To do so, we used a PERMANOVA model which included the two factors 'Sex' (fixed) and 'Individual identity' (random, nested within Sex). P-values were obtained using 9999 permutations of residuals under a reduced model (Freedman & Lane, 1983). The design was unbalanced and Type I (sequential) sum of squares were used. In order to examine the continuity of these signals from secretions to feathers, this analysis was applied to three different subsets of data: (i) secretions samples only, with variables limited to the analytes present in secretions in both years (n=259), (ii) feather samples only, with variables limited to the analytes present in feathers in both years (n=297) and (iii) all samples from birds obtained in both 2008 and 2009 (n=16), with variables limited to the analytes present in both secretions and feathers in both years (n=253).

The 'Sex' and 'Individual identity' effects were then further examined using various CAP analyses on the different subsets of data mentioned above. Validation of the CAP models retained was carried out again using the 4 birds for which we only had 2008 samples. Analytes associated with the different discriminating CAP models were targeted following the procedure described above.

REFERENCES

Anderson M.J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology* **26**: 32-46.

Anderson M.J., Gorley R.N. & Clarke K.R. (2008). *PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods.* PRIMER-E Ltd, Plymouth, UK.

Anderson M.J. & Robinson J. (2003). Generalized discriminant analysis based on distances. *Australian & New Zealand Journal of Statistics* **43**: 75-88.

Anderson M.J. & Willis T.J. (2003). Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecology* **84**: 511-525.

Clarke K.R. & Gorley R.N. (2006). *PRIMER v6: user manual/tutorial*. PRIMER-E Ltd, Plymouth, UK.

Clarke K.R. & Warwick R.M. (2001). Changes in marine communities: An approach to statistical analysis and interpretation, 2nd edition. edn. Primer-E., Plymouth.

Freedman D. & Lane D. (1983). A nonstochastic interpretation of reported significance levels. *Journal of Business and Economic Statistics* **1**: 292-298.

Gower J.C. (1966). Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* **53**: 325-338.

Jacob J. (1978). Uropygial gland secretions and feather waxes. In: Florkin M., Scheer B.T. (eds). *Chemical zoology*, vol 10 Aves. Academic press, New York: p.165-211.

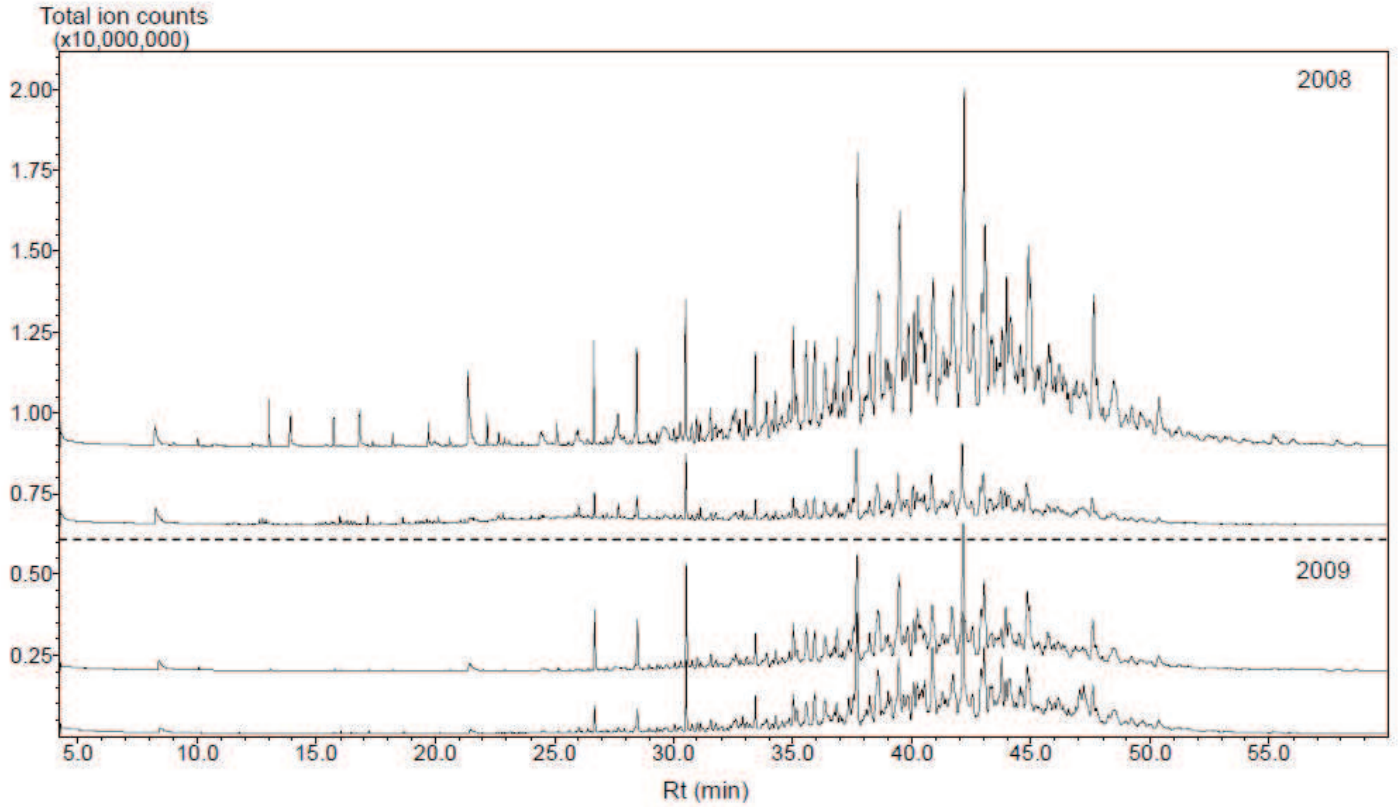
Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Mardon J., Saunders S.M., Anderson M.J., Couchoux C. & Bonadonna F. (submitted). Species, gender and identity: cracking petrels' socio-chemical code. *Chemical Senses*:

Mc Ardle B.H. & Anderson M.J. (2001). Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* **82**: 290-297.

Olson C.L. (1974). Comparative robustness of six tests in multivariate analysis of variance *Journal of the American Statistical Association* **69**: 894-908.

APPENDIX 3.3-2: FULL CHROMATOGRAMS



Appendix 3.3-2: Full chromatograms obtained with the 4 samples from the same bird (from top to bottom: 2008 secretion, 2008 feather, 2009 secretion, 2009 feather).

Section 3.4

FINAL CONSIDERATIONS ON CHEMICAL INVESTIGATIONS

The results presented so far in this chapter show that the uropygial secretions of hypogean petrels contain sociochemical information including species, sex and individual identity; and that this information is still present, in a very similar form, on the plumage of the birds. What is more, the chemical trajectory from secretions to feathers involves the appearance of highly volatile compounds whose chemical nature suggests they could be either products of secondary reactions, or be contained within the uropygial secretions. The presence on the plumage of these smaller compounds, in particular carboxylic acid and alcohols, is particularly interesting from the perspective of chemical communication as these two classes of compounds have been suggested as contributors to animal odorous signals (Nicolaides, 1974; Soini *et al.*, 2007).

An important goal of our research is to elucidate the final form of these signals, i.e. the airborne chemicals released from the plumage. These represent indeed the real signal that is perceived by other individuals. Hence the need for the development of the several new sampling and analytical methods we presented in Chapter 2. In theory, focusing the analysis exclusively on airborne signals has many advantages: it reduces the amount of chemical information to process, and directly provides the most relevant candidates for causative action. In practice, however, the sampling of airborne chemosignals is a complex task that can result in a significant level of data noise (see Chapter 2).

Besides the issue of noise, for which methodological refinements are currently being tested, our preliminary results on airborne signals have led to the identification of a significant sex-specific signal (PERMANOVA analysis: Pseuso-$F_{1/56}$=2.6454, p=0.0115). This chemical dimorphism is coded essentially through an oxygenated C_{10} compound, possibly an aldehyde (with RI=1251), whose abundance overall is higher in

females (Fig.3-1). Although somewhat consistent with results from secretions and feathers, further work (both chemical and behavioural) is required to support any claim regarding this 'sex pheromone' candidate.

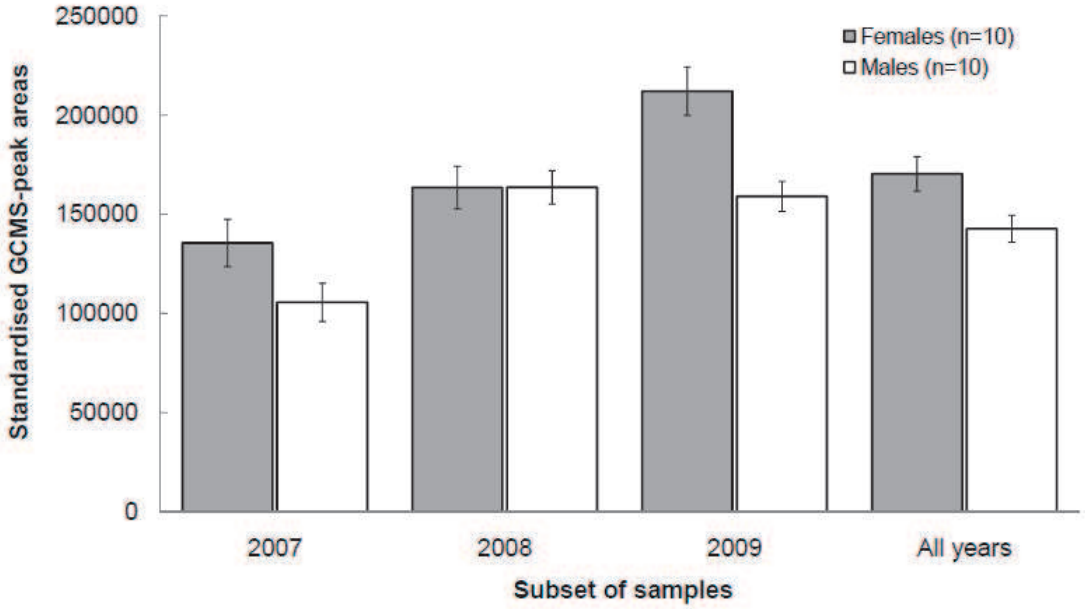


Figure 3-1: Intersexual comparison of the mean standardised peak areas of the potential sex pheromone candidate

An alternative approach to circumvent the issue of data noise was also tested in the form of direct thermal-desorption of feather lipids. This technique, which bypasses the problems related to adsorbent or ambient noise, is an intermediate between solvent extracting feather lipids and analysing only airborne signals. By thermally desorbing the chemicals present on feathers, only the reasonably volatile fraction of the lipid mixture is considered. Preliminary analysis of these data indicates the existence of chemical signatures within the volatiles recovered (PERMANOVA analysis: Pseuso-$F_{14/31}$=1.3627, p=0.0164; Fig.3-2). No sex-signal, however, could be resolved (PERMANOVA analysis: Pseuso-$F_{1/31}$=0.9488, p=0.4813).

These last two findings, even though discordant, are somewhat consistent with results presented in the previous sections. In this sense, the detection of a sex or an individual signal with our new techniques is an encouraging result. Current work including the refinement of our methods and additional sampling should provide more definitive answers in the near future.

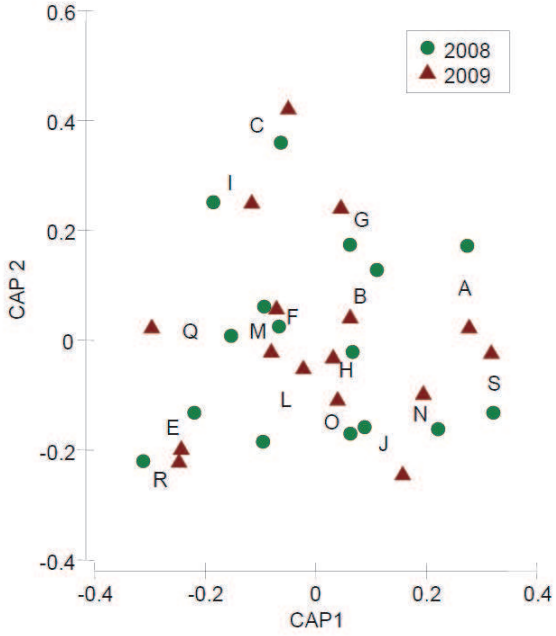


Figure 3-2: CAP analysis of the 'Individual' factor from the profiles obtained by direct thermal desorption of blue petrel feathers. Each data point corresponds to one sample. Each letter corresponds to a particular individual and is placed between the two samples from this individual (2008 and 2009).

In conclusion, the chemical results presented in this chapter provide a robust support for claims that (i) chemosignals endogenously produced and exogenously secreted by petrels contain rich social information, and (ii) the characteristic scent of procellariiforms originates from their uropygial secretions. In addition, preliminary results from our new methods focusing on airborne volatiles suggest that the social information exogenously secreted is eventually broadcast in the scent of the bird. This last point is further corroborated by the olfactory discrimination capabilities of these birds as presented in Chapter 4.

Note from the author
As this thesis was going to print, personal communication with adjunct professor Jian-Xu Zhang has brought to my attention some of his recent work on avian chemosignals which is remarkably closely related to the work presented in this chapter. A discussion of the results from these peer-reviewed publications has therefore been added as Appendix A2.

Section 3.5

REFERENCES

Bolliger A. & Varga D. (1961). Feather Lipids. *Nature* **190**: 1125.

Bonadonna F., Miguel E., Grosbois V., Jouventin P. & Bessiere J.-M. (2007). Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? *Journal of Chemical Ecology* **33**: 1819-1829.

Burger B.V., Reiter B., Borzyk O. & du Plessis M.A. (2004). Avian exocrine secretions I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. *Journal of Chemical Ecology* **30**: 1603-1611.

Cardé R.T. & Millar J.G. (2004). Advances in insect chemical ecology. Cambridge University Press, Cambridge.

Douglas H.D., III, Co J.E., Jones T.H. & Conner W.E. (2001). Chemistry, production and potential functions of aldehyde odorants in the crested auklet (*Aethia cristatella*). *American Zoologist* **41**: 1641-1641.

Hagelin J.C. & Jones I.L. (2007). Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? *The Auk* **124**: 741-761.

Jacob J. (1978). Uropygial gland secretions and feather waxes. In: Florkin M., Scheer B.T. (eds). *Chemical zoology*, vol 10 Aves. Academic press, New York: p.165-211.

Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Montalti D., Gutiérrez A.M., Reboredo G. & Salibián A. (2005). The chemical composition of the uropygial gland secretion of rock dove *Columba livia*. *Comparative Biochemistry and Physiology A Comparative Physiology* **140**: 275-279.

Moritz R.F.A. & Crewe R.M. (1988). Chemical signals of queens in kin recognition of honeybees, *Apis mellifera L. Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **164**: 83-89.

Nicolaides N. (1974). Skin lipids: their biochemical uniqueness. *Science* **186**: 19-26.

Perry J.A. (1981). Introduction to analytical gas chromatography: history, principles, and practice. Marcel Dekker Inc., New York, USA.

Reneerkens J., Piersma T. & Sinninghe-Damsté J.S. (2002). Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proceedings of the Royal Society of London B Biological Sciences* **269**: 2135-2139.

Röck F., Mueller S., Weimar U., Rammensee H.-G. & Overath P. (2006). Comparative analysis of volatile constituents from mice and their urine. *Journal of Chemical Ecology* **32**: 1333-1346.

Sandilands V., Savory J. & Powell K. (2004). Preen gland function in layer fowls: factors affecting morphology and feather lipid levels. *Comparative Biochemistry and Physiology A Comparative Physiology* **137**: 217-255.

Soini H.A., Schrock S.E., Bruce K.E., Wiesler D., Ketterson E.D. & Novotny M.V. (2007). Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*). *Journal of Chemical Ecology* **33**: 183-198.

Sweeney R.J., Lovette I.J. & Harvey E.L. (2004). Evolutionary variation in feather waxes of passerine birds. *The Auk* **121**: 435-445.

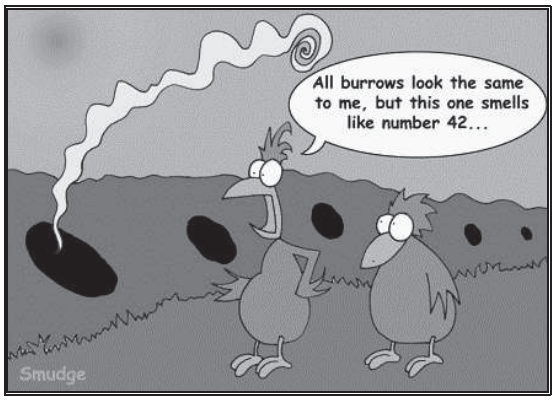
Weldon P.J. & Rappole J.H. (1997). A survey of birds odorous or unpalatable to humans: Possible indications of chemical defense. *Journal of Chemical Ecology* **23**: 2609-2633.

Chapter 4

OLFACTORY CAPABILITIES & SOCIAL BEHAVIOURS IN PETRELS

AIM & CONTENT

This chapter presents results from behavioural experiments conducted between 2006 and 2009, while working in the field on the Kerguelen archipelago during the austral summer. These experiments were designed to explore petrels' olfactory discrimination capabilities in relation to the different social chemosignals described in Chapter 3. Explicitly, the following sections contain: (i) a general presentation of the literature and questions associated with the experiments (Section 4.1); (ii) an article published in 2010 in the journal *Ethology*, examining olfactory capabilities of interspecific discrimination in the blue petrel (Section 4.2); (iii) an article published in 2009 in the journal *Behavioral Ecology and Sociobiology,* examining olfactory capabilities of intraspecific discrimination in the blue petrel (Section 4.3); and concluding comments on the findings of these experiments (Section 4.4).



Adapted from Bonadonna & Bretagnolle (2002). JEB **205** il604

Section 4.1

BACKGROUND

Field investigations on the olfactory behaviours of procellariiform seabirds essentially started with Grubb's pioneering work between 1970 and 1975. In a few years, Grubb had demonstrated with supportive evidence that (i) several species could positively respond to food-related odorous stimuli (Grubb, 1972), and that (ii) Leach's storm petrels (*Oceanodroma leucorhoa*) used olfaction to locate their island, colony and burrow in the dark (Grubb, 1973; 1974; 1979). Despite the wide-ranging implications of Grubb's work, most of the subsequent behavioural research focused exclusively on the response of these birds to food-related scents (e.g. Hutchison & Wenzel, 1980; Verheyden & Jouventin, 1994; Nevitt, 2000). These experiments provided however, a wealth of data highlighting the critical role of smell in the ecology of tubenose birds. Olfactory foraging by procellariiforms thus became an illustrative case for those advocating the reconsideration of the long-neglected avian olfaction.

Recent studies have examined the role of procellariiforms' developed sense of smell in non-foraging contexts, such as pelagic navigation (Wallraff, 2004; Nevitt & Bonadonna, 2005) and orientation around the colony (Benvenuti *et al.*, 1993; Bonadonna & Bretagnolle, 2002). Importantly, rapid progress of the research on olfactory homing by petrels (Minguez, 1997; Bonadonna *et al.*, 2001; Bonadonna *et al.*, 2003; Bonadonna *et al.*, 2004) suggested that the range of behaviours affected by olfactory mechanisms in these species is broader than previously anticipated, and may involve social aspects.

Subsequent studies on hypogean petrels soon revealed olfactory capabilities of self/non-self discrimination (De Leon *et al.*, 2003) and partner recognition (Bonadonna & Nevitt, 2004). These fascinating behavioural results, constituting the first case of olfactory individual discrimination in birds, provided the starting point for my PhD research with regards to the two experiments presented in this chapter.

Section 4.2

INTERSPECIFIC OLFACTORY DISCRIMINATION

CONTEXT

I have reported, in Chapter 3, the existence of an unambiguous species-specific chemical signal contained within the uropygial secretions of Antarctic prions and blue petrels (Mardon *et al.*, 2010). Interspecific variations in uropygial contents have long been reported (Jacob, 1978), but the ramification of these findings have so far been essentially limited to taxonomic and phylogenetic considerations (Jacob & Ziswiler, 1982; Sweeney *et al.*, 2004). The possible behavioural implications of such chemical divergence, for example in relation to interspecific interactions, had never been considered to date.

In this section, I present a study completed during the second field campaign on the Kerguelen archipelago. The rationale for this experiment emerged from several complementary field observations. First, Antarctic prions (*Pachyptila desolata*) and blue petrels (*Halobaena caerulea*), sympatrically breed in colonies on the archipelago. What is more, individuals from both species are regularly observed to squat and recycle nests from their own species but also, sometimes, from the other species. The risk and costs associated with the squatting strategy in blue petrels, however, varies greatly depending on the owner species (whether an Antarctic prion or a blue petrel).

Consequently, we tested whether blue petrels could use olfactory cues to discriminate between the scent of their own species and the one of a closely-related species; and whether this capability could be used when deciding between alternative nesting strategies.

PRESENTATION OF THE ARTICLE

Title	One house two families: petrel squatters get a sniff of low-cost breeding opportunities.
Authors	Bonadonna F. & Mardon J.
Journal	*Ethology* **116** (2): 176-182
Date of publication	6th of January 2010 (online); printed in 2010
Contribution of PhD-candidate	I have contributed, at a level of about 50%, to all stages of this particular study, including experimental design, data sampling, data analysis and redaction/submission of manuscript.

ethology international journal of behavioural biology

Ethology

One House Two Families: Petrel Squatters Get a Sniff of Low-Cost Breeding Opportunities

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Summary

Burrowing is a widespread nesting behaviour, found in vertebrates and invertebrates. It is particularly common in small procellariiform seabirds such as blue petrels (*Halobaena caerulea*) and Antarctic prions (*Pachyptila desolata*), two closely related petrel species. However, digging a burrow is costly and alternative strategies may evolve. Accordingly, blue petrel males can adopt two alternative nesting strategies: digging a new burrow or squatting in an empty one. Importantly, a blue petrel squatter arriving at the colony to breed is more likely to find empty Antarctic prion burrows than empty blue petrel burrows, since the former species only start breeding a month later. However, squatting in a prion's burrow is risky for blue petrels as the legitimate owner very often returns and claims the burrow back, thus ruining the squatter's breeding attempt. We present here results of a survey of two sympatric colonies of blue petrels and Antarctic prions on Kerguelen Island. Our data show that blue petrel squatters preferentially occupy blue petrel empty burrows. To investigate potential underlying mechanisms behind this preference, we used a simple Y-maze design to show that blue petrels can discriminate and prefer their specific odour over the prion odour. Our results confirm the existence of alternative burrowing strategies in blue petrels and suggest that squatters could use olfaction to avoid the less suitable Antarctic prion burrows.

Introduction

Digging a nest underground, that is, a burrow, is a widespread nesting behaviour that can be found in vertebrates and invertebrates. A burrow protects eggs or offspring against predators and buffers environmental changes such as wind and temperature (Kinlaw 1999). Digging a burrow, however, involves various costs, such as the energetic expenditure and a higher predation risk because of the extended exposition in the open and reduced vigilance levels (Jarvis & Bennett 1990; Ebensperger & Bozinovic 2000). The burrowing strategy may be further complicated in situations where there is a limited availability of suitable sites. It is thus not surprising that alternative strategies to the digging behaviour might evolve (Sullivan & Wilson 2001; Brousseau et al. 2003). A classic study on the digger wasp *Sphex ichneumoneus*, showed, for instance, that two alternative strategies can coexist in this situation: digging a new nest or 'squatting' in an existing empty nest (Brockmann & Dawkins 1979).

Burrowing behaviour is found in bird species such as puffins, kingfishers, bee-eaters and many procellariiform seabirds (albatrosses, shearwaters and petrels including prions). Among the latter, hypogean (i.e., burrow nesting) petrels exhibit long-term pair bonding and a high site fidelity, coming back to the

same nest year after year (Warham 1996; Brooke 2004). The nest is classically dug by the male before pair formation and then used as a protected call position to attract females (Warham 1990, 1996). Importantly, these burrowing species are threatened by avian predators such as gulls or skuas, and the burrow constitutes the only protection while at the colony. Predation pressure is so high that most of the burrowing petrels approach the nesting colony at night and without singing to escape these predators (Mougeot & Bretagnolle 2000a,b). Indeed, a large proportion of the birds caught by skuas at night are individuals wandering in the colony above ground (personal observation). As digging likely exposes petrels to predators, alternative strategies may evolve in this case also.

On Kerguelen Islands (southern Indian Ocean), blue petrels, *Halobaena caerulea*, sympatrically breed in crowded colonies with other similar petrel species, such as thin-billed prions, *Pachyptila belcheri*, Antarctic prions, *P. desolata*, white headed petrels, *Pterodroma lessonii*, or diving petrels, *Pelecanoides* sp. Although species-specific preferences in nesting sites have been observed on some islands (Schramm 1986), burrows of the different species are intermixed in many colonies of the Kerguelen archipelago, including our study site where blue petrels and Antarctic prions' nesting areas largely overlap. Importantly, burrows of the two latter species are similar in shape, diameter, length, depth, and entrance orientation (Jouventin et al. 1985; Warham 1990, 1996; personal observation for Verte Island, Kerguelen) which probably explains why pairs from both species have been repeatedly observed to 'recycle' or squat unoccupied burrows originally dug by others (personal observation; Genevois & Buffard 1994). In addition, blue petrels and Antarctic prions' nests differ greatly from those of other sympatrically nesting species such as diving petrels (whose nests are much smaller), or white-headed petrels (whose nests are much bigger) (Warham 1990, 1996; Brooke 2004).

Squatting however also involves certain risks. When a petrel (a blue petrel or an Antarctic prion in our study) returns to its nest to find squatters, anecdotal observation suggests that the legitimate owner usually evicts the squatting pair. The cost of eviction for the squatters then depends on the extent of the investment wasted in this breeding attempt, and thus of the timing of eviction. At the onset of the blue petrels' breeding period, most of the blue petrel nests and all Antarctic prion nests are empty. Indeed, blue petrels' incubation period starts in October (incubation 45–50 d) whereas Antarctic prions arrive later in the season, starting incubating only in late December. If a blue petrel nest has been squatted by a blue petrel pair and the original owners finally return, then the squatters are evicted only a few days after starting their breeding season, before the egg is even laid, and they can still try to complete their breeding attempt somewhere else. On the contrary, if a blue petrel squatting pair is evicted by a returning Antarctic prion in December, after an investment of approx. 45 d of incubation, then their whole breeding season is ruined as no replacement laying has been ever observed in petrels (Warham 1990). Hence there should be a strong selective pressure on squatters for evolving mechanisms to distinguish between burrows used by different species.

Olfaction plays a critical role in the burrow recognition processes of hypogean petrel species (see Bonadonna 2009). Importantly, the scent of petrels is very persistent and can still be perceived by a human nose, in burrows or on lone feathers, a year after the end of a breeding period (personal observation). We therefore considered olfaction as a primary candidate for the mechanism allowing blue petrels to discriminate between burrows previously occupied by different species. The aim of this study was to investigate the selective pressure on the burrowing strategies of blue petrels by (1) estimating the proportion of burrows squatted by blue petrels, (2) monitoring the destiny of the squatting pairs' breeding attempts and (3) testing whether blue petrels may discriminate between blue petrels and Antarctic prions' odour, using a Y-maze experiment.

Methods

Demographic Survey

The study was conducted on a small sub-Antarctic island (Ile Verte, 49°51′S, 70°05′E) of the Kerguelen Archipelago where a study colony of blue petrels and Antarctic prions has been followed since 2001. A total of 141 nests, surveyed between 2001 and 2006, were considered for the present study and sorted in two groups: the 'prion-nests' occupied from 2001 to 2006 principally by Antarctic prions (n = 62), and the 'blue-nests' occupied from 2001 to 2006 principally by blue petrels (n = 79). In cases where a particular nest had been used over the years by pairs of the two species (20 nests), we assigned that nest to the species that occupied it the most over the years. If the residential switch only applied to the last year of occupation, then the nest was still considered to be in a transitional state as petrels can

skip a particular breeding season. The survey started in early November 2007 and ended in late January 2008.

Y-Maze Experiment

A total of 28 blue-nests were randomly selected within non-squatted nests only, and at least one of their incubating occupants was tested in this experiment. Using a Y-maze apparatus, blue petrels were presented with a choice between their own species' odour against Antarctic prions' odour. Only one trial per bird was performed.

The Y-maze (Fig. 1) has three symmetrical arms (arm length: 60 cm; width: 12 cm; height: 11 cm; angle between each arm approx. 120°), made from standard opaque PVC wire housing pipe. One arm, used as the starting point, is fitted with two trap doors (30 cm apart) to provide a temporary holding compartment for the bird being tested. The end of the two remaining arms is equipped with a separate compartment for the odour source (scented cotton bags), also accessible via a trap door to the outside. A second partition, positioned at 20 cm from each end, is equipped with a CPU cooling fan (Globe Fan Technology Co. Ltd., product number S05010, Chung Ho City, Taiwan) to provide low-noise and controlled airflow (9 CFM) through each choice arm. The fans are set so as to draw the air from the odour source compartments and blow it down the choice arms towards the entrance arm. Thus, birds do not have direct access to scented bags, but are in contact with scented air flowing over the bags at a constant rate. The maze is carefully washed after each trial with methanol (70%) to remove odour residues.

Fig. 1: Y-maze apparatus. Arms of the maze are pictured closed, as during experiments. Fans are mounted in the innermost partition of each top arm. The entrance arm is showed in an opened position, with the inner trap door laid on top of the maze. A cotton bag similar to those used as odour sources is shown on top of the entrance.

Odours of non-breeding individuals of both species (five blue petrels and five Antarctic prions), found in burrows of the colony, were collected to be used as odour sources. To do so, birds were held individually in a cotton bag (21 × 20 cm; 10 g) for 1 h before being returned to the burrow where they were found. The use of non-breeding birds as odour donors was necessary to remain consistent across species since there are no breeding Antarctic prions in November. The odour collection was completed in 4 d (i.e. the last bag being 4 d older than the first) to avoid any effect of differential odours' freshness on birds' choice. Odour bags were stored separately in Ziplock® (S. C. Johnson & Son Inc., Racine, WI, USA) plastic storage bags and kept in the dark at ambient temperatures (5–10 °C) for the time of experiments (up to 10 d).

During trials, birds to be tested were presented with a choice between two different odour sources (blue petrels and Antarctic prions), each made of two bags (i.e. odour duos) per species. The reason behind the pairing of odour bags was that nests are typically used by a pair of breeding birds. For each species, the 10 possible combinations of odour duos, available from our sampling, were used randomly, paying attention to use each duo equally. Odour stimuli were alternated between arms for each trial to eliminate possible bias between either the choice arms themselves or their spatial positions. We did not consider the sex of the odour donors in the pairing of scented bags. Indeed, previous work on the same species, using the same experimental setup, has shown that birds do not express sex-related olfactory preferences in this particular context (Bonadonna et al. 2009; Mardon et al. submitted for publication). Consequently, a potential influence of the donors' sex on a crude interspecific olfactory discrimination task, when such an influence was never observed at the intraspecific level, was considered unlikely.

In the field, blue petrels were removed from burrows, transported to the maze in a cotton bag (different from the scented bags), placed in the temporary holding compartment of the maze's entrance, and allowed to settle for 5 min. At the end of this period, a trap door was lifted and the bird was allowed to make a choice in the maze. A choice, easily assessable by the noise of the bird walking in the maze, consisting of the bird walking down one of the

maze's arms to the end and staying there for more than 30 s. No-choice birds (removed after 15 min.) either never settled down, passing continuously from one arm to the other, or sat calmly in the holding compartment.

This Y-maze experiment, initially motivated by our demographic observations of nest squatting, was designed to test whether blue petrels can discriminate between conspecific and heterospecific odours and whether they tend to choose conspecific ones. Because of the rather high occurrence of no-choice outcomes (23%), we tested whether bird choices were random using a Pearson's chi-square test on the absolute frequencies of the three different possible outcomes (blue petrel odour, Antarctic prion odour or no-choice).

As already shown by previous studies on petrels (Bonadonna et al. 2001, 2003a,b, 2004, 2009; Bonadonna & Bretagnolle 2002; Bonadonna & Nevitt 2004) removing birds does not affect incubation behaviour nor the hatchability of the eggs. In the present study, no petrel deserted breeding following the experiment, and hatching success was 80% for the experimental burrows and approx. 70% for control burrows in the same colony. We left Ile Verte before fledging and therefore could not assess fledging success.

Results

Demographic Survey

Out of 79 blue-nests, 11 were empty and 68 actually occupied by blue petrel pairs in early November 2007. However, 12 of the latter 68 pairs were not the birds found in these nests the previous years, but non-ringed (i.e. new) individuals. In petrels, the burrow represents the yearly 'rendez-vous' site (Warham 1990, 1996) where birds easily meet their lifelong partner. Thus, the motivation to hang on to the same nest should be very strong and eviction of a previous 'official' owner by a new squatter highly unlikely. Consequently, we considered that the 12 blue-nests occupied by new blue petrel pairs were empty at the beginning of the breeding season, meaning that a total of 23 empty blue-nests (n = 11 + 12) were available to squatters at this time. Out of these available nests, 52% (12 out of 23) were eventually squatted. Note that the real proportion is probably higher considering that we did not include those squatted blue-nest that were rapidly won back by their legitimate owners. This behaviour is in fact barely noticeable because of the synchronous arrival of blue petrel breeders (Warham 1990, 1996).

From early November to late December, 13 of the 62 prion-nests were occupied by non-ringed incubating blue petrels (20.9%) whereas the others were empty. Out of these 13 nests, nine had been regularly occupied by Antarctic prions from 2001 to 2006 (see below for details on the others). Frequency comparison revealed a difference between the proportion of squatted blue-nests and prion-nests, when considering the ratio of squatted nests on available empty nests (Fisher exact test, p = 0.008).

When surveyed at the end of January 2008, well after breeding Antarctic prions arrived at the colony in late December (Antarctic prions were then incubating while blue petrels were rearing their chick), nine out of the 12 squatted blue-nests sheltered a healthy blue petrel chick (75%), one was occupied by non-ringed Antarctic prions, and two were empty. The survey also revealed that out of the 13 squatted prion-nests, only four (31%) sheltered a healthy blue petrel chick (hereafter referred to as *Resistant*), five were now occupied by the legitimate Antarctic prions, that is, the previous year occupant (hereafter referred to as *Reconquest*) and four were empty (hereafter referred to as *Deserted*). Frequency comparison revealed a difference in the hatching success of blue petrels squatting either blue-nests or prion-nests (Fisher's exact test p = 0.047).

Interestingly, the history of the 13 squatted prion-nests (Table 1) indicates that out of the four *Resistant* nests, two were already occupied by blue petrels and two were empty during the previous breeding season in 2006. Although occupied by Antarctic prions from 2001 to 2005, the history of these four nests suggests that they were probably in a usage transition from Antarctic prions to blue petrels and thus may have presented a lesser eviction risk from Antarctic prions in 2007–2008. Nevertheless, these four *Resistant* nests were included as prion-nests to remain consistent with our *a priori* design. This is also conservative as their classification as blue-nests would have resulted

Table 1: The 2006–2007 history of the 13 prion-nests squatted by blue petrel pairs during the 2007–2008 breeding season

Breeding season 2007–2008	Breeding season 2006–2007		
Final situation	AP inside	BP inside	Empty
4 Resistant (kept by BP pairs)	0	2	2
5 Reconquest (replaced by AP pairs)	4	0	1
4 Deserted	1	1	2

AP, Antarctic prion; BP, blue petrel.

in even higher differences in proportion and hatching success between the two squatting options.

Y-Maze Experiment

A total of 31 blue petrels (6 females and 25 males) performed the experiment (choice time mean ± SD: 3.5 ± 3.3 min). Seventeen birds preferred their conspecific odour (5 females and 12 males), seven birds preferred the heterospecific odour (all males) and seven birds (1 female and 6 males) did not choose but stayed in the entryway of the maze (Fig. 2). Results show that blue petrels were able to discriminate the two odours presented and significantly preferred the odour of their own species over the odour of Antarctic prions ($\chi^2 = 6.452$, df = 2, p = 0.0397; pooling females and males).

Discussion

At the start of the breeding season, a new burrowless blue petrel male has two options: to dig a new burrow or squat in an existing burrow. The yearly appearance of new nests in and around the studied colony (personal observations) indicates that many birds still dig their own burrow. However, our results confirm that a non-negligible proportion of blue petrels also squat in existing nests. If this squatting strategy was caused by a lack of nesting sites, then one would expect all empty burrows to be occupied. In contrast, 11 blue-nests out of 79 remained empty in our survey, thus suggesting that the squatting strategy is a true alternative strategy emerging from the trade-off between predation and eviction risks. Although the nature of our survey does not allow testing this hypothesis at this stage, we can hypothesise that the digging and squatting strategies are likely to be frequency-dependent as a decrease in new burrows would increase the risk of eviction and thus reduce the benefit of squatting.

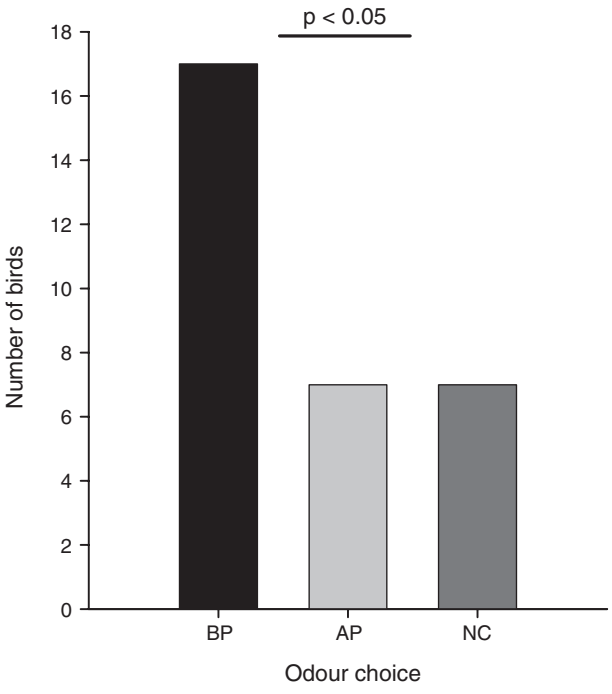

Fig. 2: Absolute frequencies of the different outcomes in the Y-maze experiment performed on blue petrels. AP, Antarctic prion; BP, blue petrel; NC, No choice.

Interestingly, the observed percentage of empty blue-nests squatted (52%) is higher than that observed in the best studied case of the digger wasp *Sphex ichneumoneus* (Brockmann & Dawkins 1979), where only 37% of empty burrows were occupied by squatting wasps. This probably reflects a higher predation risk experienced by digging petrels while at the colony (Mougeot et al. 1998). In fact digging exposes the petrels to predation for several nights, until the gallery is deep enough to escape skuas, the main predator of these birds (Mougeot et al. 1998).

Our results also show that blue petrels squatters preferentially occupy blue-nests rather than prion-nests. This is reasonable as in the case of blue petrels, the squatting strategy has two possible outcomes, with different consequences on the breeding success which could act as strong selective pressures.

If the usurped burrow is a prion-nest, the low hatching success found in this study indicates that the blue petrel has a high chance of losing its incubation investment. Out of 13 prion-nests occupied by blue petrel pairs only four *Resistants* were able to breed until hatching and these were probably the less exposed to any eviction risk. This confirms previous anecdotal observation that prions, in spite of their small body size (50 g smaller than blue petrels), are able to evict usurping blue petrels. Besides, although petrels exhibit long-term pair bonds, these are not strictly defined at the first breeding attempts and divorces may occur before a lifetime stable pair is established (Bradley et al. 1990; Jouventin & Bried 2001). Consequently, a high rate of desertion may be found in inexperienced birds whose pair bonds are not yet strengthened by successful breeding attempts (Warham 1990, 1996). Given the high desertion rate of prion-nest squatters observed (30.7%; Table 1), it is possible that blue petrels adopting the squatting strategy are essentially individuals at the beginning of their reproductive life (Chastel et al. 1995).

If the usurped nest is a blue-nest, then the higher hatching success found in this study (75%) suggests that the choice is safer. Considering the high synchrony in laying (Brooke 2004), a squatter freshly settled in an empty blue-nest has a good chance that the usurped burrow will remain empty or, at worst, that the legitimate pair will arrive within a few days before laying. In this case, the evicted squatters will still have the possibility of looking for another empty burrow without having wasted a large reproductive investment. Therefore, the blue petrel squatting strategy is much more profitable in empty blue-nests.

Unlike wasps (Brockmann & Dawkins 1979), it seems that blue petrels do not occupy whatever empty nest they find. They are able, at least, to distinguish between the nests of the two species, thus optimising their squatting strategy. The existence of a species-specific chemical signal, contained in the uropygial secretions of the two species (Mardon et al. submitted for publication), suggests that olfaction could play a major role in interspecific discrimination behaviours. Accordingly, results from our Y-maze experiment indicate that blue petrels are able to discriminate and prefer blue petrels' odour over Antarctic prions' odour (or they may avoid the Antarctic prions' odour). Independent of the nature of the behaviour, preference or avoidance, the outcome for a squatter in its process of burrow selection will be the same, namely choosing a blue-nest. Note that stress, escape attempts, or other factors, could well have been the main motivations leading the birds' choice in the maze experiment. However, what is of primary significance in Y-maze experiments is not the motivation behind a given choice but rather the capability to discriminate between two stimuli.

It is possible that 'errors' in the squatting behaviour of blue petrels are due to the degradation over time of nest odours, as burrows remained empty for the previous 10–12 mo. Nevertheless, our data show that legitimate pairs are able to find and recognise their own nest after 1 yr of absence, when coming back for a new breeding season. Besides, olfaction is the primary mechanism of nest recognition in blue petrels and does not require any other positional information (Bonadonna et al. 2001, 2004). It is also worth mentioning that the percentage of birds choosing prions' odour in the Y-maze (29%) is similar to the percentage of 'errors' in the actual squatting behaviour (20%). Whether this correspondence has any ecological basis or is simply an effect of random sampling cannot be resolved and leaves too much room for speculation.

It may be argued that other burrows' characteristics allow to distinguish between the two species' nests, and then that olfaction may be not the only mean birds may use. At our study site, blue petrels and Antarctic prions' nesting areas largely overlap, and nests of the two species are often found side by side. Therefore, birds walking around the colony have virtually similar chances to encounter conspecific or heterospecific nests. Moreover, burrows of the two species are similar in shape, diameter, length, depth and entrance orientation (Jouventin et al. 1985; Warham 1990, 1996; personal observation for Verte Island, Kerguelen), so that physical characteristics are likely to be the same. Consequently, physical or landscape characteristics are not likely to be an alternative hypothesis to explain the capability observed.

Blue petrels possess good olfactory capacities, from burrow (Bonadonna et al. 2004) to mate recognition (Mardon & Bonadonna 2009), even as chicks (Cunningham et al. 2003; Bonadonna et al. 2006). Our results broaden the range of behaviours based on olfaction in this species, suggesting once more that burrowing petrels live in a 'world of odours' presiding over various aspects of their lives, from foraging to breeding. Our study shows that a mixed burrowing strategy exists in blue petrels. The strategy of squatting empty nests is constrained by the necessity to avoid the more risky Antarctic prion nests, which could be achieved using olfactory cues.

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Literature Cited

Bonadonna, F. 2009: Olfaction in petrels: from homing to self-odor avoidance. Ann. N Y Acad. Sci. **1170**, 428—433.

Bonadonna, F. & Bretagnolle, V. 2002: Smelling home: a good solution for burrow-finding in nocturnal petrels? J. Exp. Biol. **205**, 2519—2523.

Bonadonna, F. & Nevitt, G. A. 2004: Partner-specific odor recognition in an Antarctic seabird. Science **306**, 835.

Bonadonna, F., Spaggiari, J. & Weimerskirch, H. 2001: Could osmotaxis explain the ability of blue petrels to

return to their burrows at night? J. Exp. Biol. **204**, 1485—1489.
Bonadonna, F., Cunningham, G. B., Jouventin, P., Hesters, F. & Nevitt, G. A. 2003a: Evidence for nest-odour recognition in two species of diving petrel. J. Exp. Biol. **206**, 3719—3722.
Bonadonna, F., Hesters, F. & Jouventin, P. 2003b: Scent of a nest: discrimination of own-nest odours in Antarctic prions, *Pachyptila desolata*. Behav. Ecol. Sociobiol. **54**, 174—178.
Bonadonna, F., Villafane, M., Bajzak, C. & Jouventin, P. 2004: Recognition of burrow's ''olfactory signature'' in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. Anim. Behav. **67**, 893—898.
Bonadonna, F., Caro, S., Jouventin, P. & Nevitt, G. A. 2006: Evidence that blue petrel, *Halobaena Caerulea*, fledglings can detect and orient to dimethyl sulfide. J. Exp. Biol. **209**, 2165—2169.
Bonadonna, F., Caro, S. & Brooke, M. 2009: Olfactory sex recognition investigated in Antarctic prions. PLoS ONE **4**, e4148. doi:4110.1371/journal.pone.0004148
Bradley, J. S., Wooller, R. D. & Serventy, D. L. 1990: The influence of mate retention and divorce upon reproductive success in short-tailed shearwaters *Puffinus-Tenuirostris*. J. Anim. Ecol. **59**, 487—496.
Brockmann, H. J. & Dawkins, R. 1979: Joint nesting in a digger wasp as an evolutionarily stable preadaptation to social-life. Behaviour **71**, 203—245.
Brooke, M. 2004: Albatrosses and Petrels Across the World. Oxford Univ. Press, New York.
Brousseau, D. J., Kriksciun, K. & Baglivo, J. A. 2003: Fiddler crab burrow usage by the Asian crab, *Hemigrapsus sanguineus*, in a Long Island sound salt marsh. Northeast. Nat. **10**, 415—420.
Chastel, O., Weimerskirch, H. & Jouventin, P. 1995: Influence of body condition on reproductive decision and reproductive success in the Blue Petrel. Auk **112**, 964—972.
Cunningham, G. B., Van Buskirk, R. W., Bonadonna, F., Weimerskirch, H. & Nevitt, G. A. 2003: A comparison of the olfactory abilities of three species of procellariiform chicks. J. Exp. Biol. **206**, 1615—1620.
Ebensperger, L. A. & Bozinovic, F. 2000: Communal burrowing in the hystricognath rodent, Octodon degus: a benefit of sociality? Behav. Ecol. Sociobiol. **47**, 365—369.
Genevois, F. & Buffard, E. 1994: Sites de nidification et caracteristiques des terriers chez deux especes de petrels sympatriques aux Iles Kerguelen: Le Petrel Bleu *Halobaena caerulea* et le Prion de Belcher *Pachyptila belcheri*. Alauda **62**, 123—134.
Jarvis, J. U. M. & Bennett, N. C. 1990: The evolutionary history, population biology and social structure of African mole-rats: family Bathyergidae. In: Evolution of Subterranean Mammals at the Organismal and Molecular Levels (Nevo, E. & Reig, O. A., eds). Liss, New York, pp. 97—128.
Jouventin, P., Mougin, J. L., Stahl, J. C. & Weimerskirch, H. 1985: Comparative biology of the burrowing petrels of the Crozet Islands. Notornis **32**, 157—220.
Jouventin, P. & Bried, J. 2001: The effect of mate choice on speciation in snow petrels. Anim. Behav. **62**, 123—132.
Kinlaw, A. 1999: A review of burrowing by semi-fossorial vertebrates in arid environments. J. Arid Environ. **41**, 127—145.
Mardon, J. & Bonadonna, F. 2009: Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own Behav. Ecol. Sociobiol. **63**, 537—542.
Mougeot, F. & Bretagnolle, V. 2000a: Predation as a cost of sexual communication in nocturnal seabirds: an experimental approach using acoustic signals. Anim. Behav. **60**, 647—656.
Mougeot, F. & Bretagnolle, V. 2000b: Predation risk and moonlight avoidance in nocturnal seabirds. J. Avian Biol. **31**, 376—387.
Mougeot, F., Genevois, F. & Bretagnolle, V. 1998: Predation on burrowing petrels by the brown skua at Mayes islands, Kerguelen. J. Zool. London **244**, 429—438.
Schramm, M. 1986: Burrow densities and nest site preferences of Petrels (Procellariidae) at the Prince-Edward-Islands. Polar Biol. **6**, 63—70.
Sullivan, W. & Wilson, K. J. 2001: Differences in habitat selection between Chatham petrels (*Pterodroma axillaris*) and broad-billed prions (*Pachyptila vittata*): implications for management of burrow competition. N. Z. J. Ecol. **25**, 65—69.
Warham, J. 1990: The Petrels: Their Ecology and Breeding Systems. Academic Press, London.
Warham, J. 1996: The Behaviour, Population Biology and Physiology of the Petrels. Academic Press, London.

Section 4.3

INTRASPECIFIC OLFACTORY DISCRIMINATION

CONTEXT

The investigation of social chemosignals in the blue petrel confirms the existence of highly conserved individual chemical signatures in the uropygial secretions and on the plumage of these birds (sees Chapter 3). Previous behavioural results obtained with different species suggest that such chemical signatures could support olfactory mechanisms of individual discrimination beyond self/non-self (Bonadonna & Nevitt, 2004; Jouventin *et al.*, 2007).

In the following section, I present a behavioural study completed during the first field campaign on the Kerguelen archipelago. Results confirm that blue petrels can use chemical signatures for olfactory inter-individual recognition, identifying both their own odour and that of their mate. The ecological and evolutionary implications of these findings are discussed below.

PRESENTATION OF THE ARTICLE

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ORIGINAL PAPER

Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own

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Abstract Among procellariiform seabirds, many burrowing petrels show good olfactory abilities especially in recognising their nest. In particular, it has been reported that Antarctic prions (*Pachyptila desolata*) discriminate their own and their mate's odours and, in Y-maze experiments, prefer the odour of a conspecific bird to their own. While traditionally examined from the perspective of homing mechanisms, these recent results have drawn attention to the possible use of chemical signals in birds' social behaviours. Indeed, the life history of petrels suggests that a mate choice mediated by olfactory mechanisms may have evolved in this group to ensure genetic compatibility. This study was undertaken to validate and extend results obtained on petrels' olfactory discrimination capabilities. Following the Y-maze experiment protocol, blue petrels (*Halobaena caerulea)* were offered three different choices: (1) mate versus conspecific's odour, (2) own versus mate's odour and (3) own versus conspecific's odour. We discovered that birds prefer the odour of their mate not only when presented against conspecific's odour but also against their own. We further verified that blue petrels also avoid their own odour when presented against conspecific's odour. Our results confirm that olfactory discrimination in burrowing petrels goes beyond self-recognition and that self-odour avoidance may be widespread. We use two mutually non-exclusive behavioural frameworks for the interpretation of our results, homing and mate choice, and explain why homing mechanisms cannot account for all of our observations. This study opens the door to further research on olfactory mechanisms that, in petrels, might mediate individual recognition and mate choice.

Keywords Petrel · Olfaction · Individual recognition · Behaviour · Seabirds

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Signals broadcasting quality of individuals govern optimal mate choice processes, just as signals broadcasting identity are used for individual recognition. In the literature, visual- or acoustic-based communication systems are frequently noted as preferential channels to acquire information on the identity and quality of a signalling individual (Maynard Smith and Harper 2003). This is particularly true in birds, where vision and hearing are considered the principal communication means for a wide range of behavioural processes. Calls may broadcast information on sex, species, body condition and identity (Bretagnolle 1989; Genevois and Bretagnolle 1994; Galeotti et al. 1997; Aubin et al. 2000), and colours may indicate parasitic loads, age, hierarchical status and sex (Fenoglio et al. 2004; Nolan et al. 2004; Pryke and Griffith 2006; Nicolaus et al. 2007). However, an increasing number of studies indicates that chemical signals are also broadly used in vertebrates' recognition systems (Brown and Eklund 1994; Yamazaki and Beauchamp 2005). Indeed, such signals may constitute reliable cues of quality and compatibility and could thus be used for social interactions (Wyatt 2003).

Procellariiform seabirds have become notorious for their well-developed olfactory neuroanatomy (Bang 1966) as well as for the attraction exhibited by some species for food-related scents (reviewed in Nevitt and Bonadonna 2005). Among procellariiforms, hypogean petrels nest in burrows they dig and to which they come back year after year. Returning from a foraging trip at sea, most of the burrowing species approach the nesting colony at night and without singing, probably to escape avian predators such as gulls and skuas (Mougeot and Bretagnolle 2000a, b). Olfaction thus became critical in the homing processes of these petrels, which are able to find and recognise their nest through an olfactory signature (Grubb 1974; Minguez 1997; Bonadonna et al. 2003a, b, 2004; Jouventin et al. 2007). This is, for instance, the case of European storm petrel chicks, *Hydrobates pelagicus*, which return to their burrow after night-exploring walks (Minguez 1997). Using a T-maze experiment, De Leon et al. (2003) showed that these chicks are able to recognise their own odour and that this odour leads them back to the nest. This study therefore demonstrated the first required step for individual recognition to proceed: self/non-self discrimination. Further work on the question was carried out on a subantarctic petrel species, the Antarctic prion *Pachyptila desolata* (Bonadonna and Nevitt 2004). Using a similar kind of maze experiment, the authors first showed that birds preferred their own odour when presented against an odourless blank, thus checking for the birds' capacity to perceive self-odour. The study also showed that whilst adult prions prefer their mate's odour to the odour of an unknown conspecific, they also prefer the odour of an unknown conspecific to their own odour. This work brought the debate beyond a simple self-discrimination process by highlighting the ability of burrowing petrels to recognise not only their own but also their mate's odours (see also Jouventin et al. 2007). Moreover, the presence of a 'random conspecific' preference over self-odour in Antarctic prions was unexpected, as it is not consistent with homing motivations that appear to explain results on European storm petrel chicks. Interpretation of such finding is therefore difficult and should be approached cautiously.

In a recent review, Zelano and Edwards (2002) suggested that a genetically based mate-choice system, particularly ones involving major histocompatibility complex (MHC) preferences, might be expected in long-lived species engaged in lifetime monogamy, such as many procellariiform seabirds. MHC-dependent mating preferences can indeed potentially increase the genetic compatibility between mates (Penn 2002) and have been documented in mammals, fish and reptiles (Tregenza and Wedell 2000; Olsson et al. 2003). Several studies on birds also reported that female mate choice may be driven by genetic compatibility in some species (Johnsen et al. 2000; Blomqvist et al. 2002; Bonneaud et al. 2006), although it remains unclear in most cases how females may identify the genetic makeup of potential partners. As olfactory signals from urine or body odours have been associated with MHC genotypes in several species of vertebrates (Singh 2001), a role for chemical signalling in mediating mate choice and inbreeding avoidance is not out of the question for birds, although it has received relatively little attention. Indeed, evidence of a functional olfaction has been found in every bird species tested so far (Roper 1999). Moreover, with their very acute olfactory capabilities, procellariiform seabirds are certainly the best candidates for the exploration of such processes in birds (Zelano and Edwards 2002; Bonadonna 2008).

If a genetically based mate choice relying on olfactory cues has evolved in burrow-nesting petrels, it is reasonable to expect two major behavioural processes in these species: olfactory individual discrimination and kin odour avoidance during mate choice. Therefore, before starting any further research on mate choice in petrel seabirds, we considered that it is important to confirm and validate results obtained with Antarctic prions regarding olfactory discrimination capabilities and preferences. To do so, we repeated and extended our maze experiments to blue petrels, *Halobaena caerulea*, phylogenetically close to Antarctic prions.

Materials and methods

This study was conducted on a small sub-Antarctic island (Ile Verte, 49°51′ S, 70°05′ E) in the Kerguelen Archipelago between November 2006 and January 2007. Blue petrels are a common burrow-nesting species in this region, and a study colony made of 70 burrows has been followed since 2001 on this island. Burrows are fitted with a closable aperture over the incubating chamber to facilitate capture. During incubation, partners alternate incubation shifts, relieving each other from the nest every 8 to 12 days (Warham 1996). Incubating birds were presented with odour choices in a Y-maze. To trap individual odours, incubating birds were collected from their burrows and held individually in cotton bags (23×23 cm) for half an hour. Bags were then stored separately in plastic storage bags (ziplock®) and kept in the dark in a cardboard box. Bags were stored between 2 and 20 days at ambient temperatures (5–10°C) before being used in experiments.

Choice experiments between two scented bags were carried out using a standard Y-maze. The maze was made from opaque polyvinyl chloride wire housing and had three symmetrical arms (arm length, 60 cm; width, 12 cm; height, 11 cm). The angle between each arm was ~120°. One arm was used as starting point and was fitted with two trap doors (30 cm apart) to provide a temporary holding

compartment for the bird to be tested. The end of each odour choice arm was equipped with a separate compartment for the odour source (a scented cotton bag), also accessible via a trap door to the outside. A second partition was positioned at 20 cm from each end and was equipped with a CPU cooling fan (Globe Fan Technology, product number S05010, Taiwan) to provide low-noise and controlled airflow (9 CFM; 243 l min^{-1}) through each choice arm. Thus, the bird did not have direct access to scented bags but was in contact with scented air flowing over the bags at a constant rate. The maze was washed after each trial with methanol (70%) to remove any odour residue. Odour stimuli were alternated between arms for each trial to eliminate possible bias between either the choice arms themselves or their spatial positions. Birds were removed from burrows, transported to the maze in a cotton bag (different from scented bags), placed in the entryway of the maze, and allowed to settle down for a 3-min period. At the end of this period, the inner trap door was lifted and the bird was allowed to make a choice. The choice was easily assessed by the noise of the bird walking in the maze. Birds that either never settled down or sat calmly in the holding compartment facing away from the maze arms were removed after 15 min and reported as no-choice birds (n=4). Three different odour choice experiments were performed on subject birds in a random order: (1) mate versus conspecific's odour, (2) own versus mate's odour and (3) own versus conspecific's odour.

Ten occupied burrows were selected, and the incubating birds found inside were held in cotton bags to collect their odour. Two days minimum after odour collection, burrows were checked again, and birds inside were picked to perform one of the choice experiments. When partners were found in the burrow, they were held in turn in clean cotton bags to collect their odour before performing one of the experiments. From then on, burrows were visited every second day (except on very windy and/or wet days), and the bird inside was tested in one of the three experiments according to the availability of odour bags. Our sampling design was to test each bird once for each type of experiment. However experiments were stopped with a given pair of birds as soon as the chick started to hatch. Consequently, some individuals did not perform all three experiments.

As already shown by previous studies on petrels (Bonadonna et al. 2003a,b, 2004; Bonadonna and Nevitt 2004), removing birds does not appear to affect incubation behaviour or the hatchability of the eggs. In the present study, no petrel deserted the nest following an experiment, and hatching success has been 80% for the study burrows (eight nest out of ten), about 70% for control burrows in the same colony (11 nests out of 15) and between 30% and 40% in a control study colony in another island, 6 km apart (Mayes island, more than 100 burrows).

Results

Six males and ten females performed experiment 1 (mean choice time±SD, 3.5±2.2 min), seven males and nine females performed experiment 2 (mean choice time±SD, 4.2±2.9 min), and ten males and seven females performed experiment 3 (mean choice time±SD, 2.9±2.1 min). Both sex-confounded, birds significantly preferred the odour of their mate over the odour of a conspecific in experiment 1 (binomial test, $p<0.01$, Fig. 1a), the odour of their mate over their own in experiment 2 (binomial test, $p<0.001$, Fig. 1b) and the odour of a conspecific bird over their own odour in experiment 3 (binomial test, $p<0.01$, Fig. 1c). Figure 1 also displays sex-specific results for the three experiments.

Discussion

Our results show that blue petrels are able to recognise and discriminate individual odour cues, in particular their mate's and their own odours. This behaviour is consistent with behaviours observed in Antarctic prions (Bonadonna and Nevitt 2004) for which the existence of an individual olfactory signature has recently been suggested (Bonadonna et al. 2007). However, if these clear cut results provide an unambiguous demonstration of blue petrels' olfactory discrimination capabilities, the birds' motivation underlying these choices are more challenging to explain. Traditionally, burrowing petrels' olfactory abilities have been investigated within the framework of homing behaviours (Grubb 1974; Minguez 1997; Bonadonna et al. 2003a,b, 2004). As the birds used in our experiment were displaced from their nest and placed in the novel environment of the Y-maze, the prime hypothesis is, therefore, that birds' decisions were driven by desires to escape and/or return to the nest.

In blue petrels, breeding partners alternatively leave the burrow to forage at sea for up to 12 days (Warham 1996). It follows that the last bird to occupy the nest before an individual comes back from its foraging trip is its partner. Therefore, the partner's scent should be the strongest odorous signal from the nest at that time, representing a major part of the burrow olfactory signature. When given a two-way choice, blue petrels preferred their mate's odour to the one of an unknown conspecific (experiment 1). A similar olfactory preference for the mate's odour has recently been reported for Wilson's storm petrels (*Oceanites oceanicus*; Jouventin et al. 2007). Such preference is consistent with the 'homing' hypothesis in that it would drive a bird back to its burrow under natural conditions. The novel finding of the birds' preference for their mate's odour over their own (experiment 2) could similarly be explained by this mechanism. Yet, we did not expect such an unequivocal

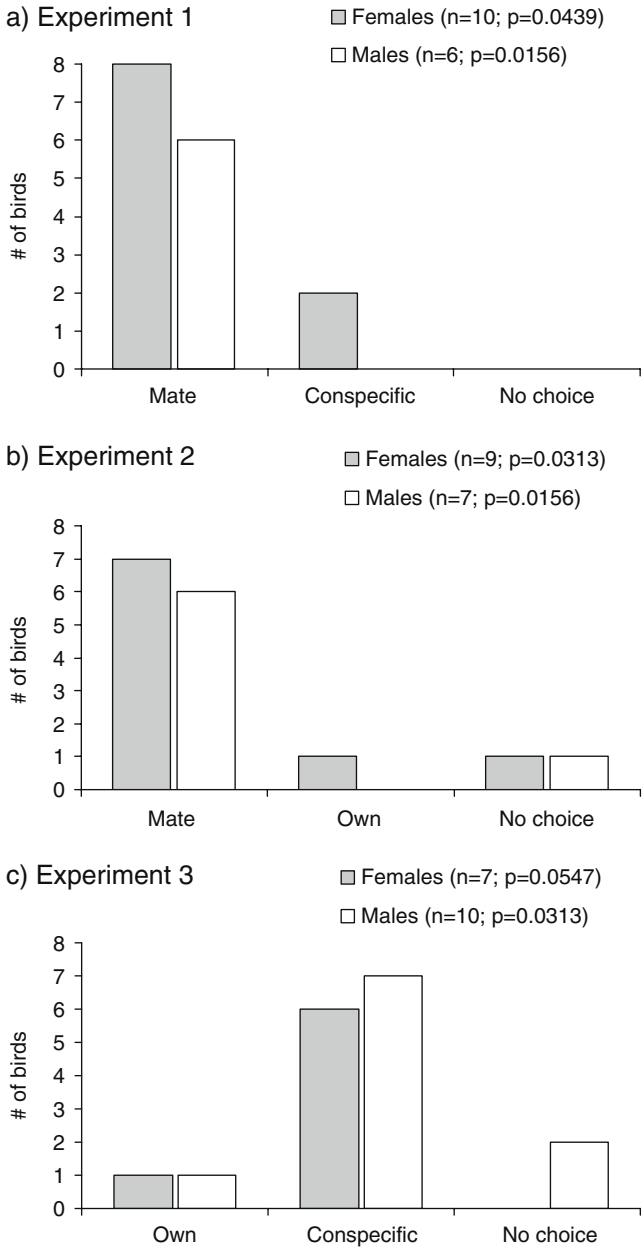

Fig. 1 Absolute frequencies of the different choice outcomes in the three experiments. Sex-specific results are shown (*dashed areas* females, *open areas* males). Sex specific *p* values are indicated above the graphs and refer to binomial tests between the two choice options

preference for the partner's scent as observed in this second experiment. Indeed, orienting to self-odour has been shown to be an efficient homing mechanism leading European storm petrel chicks back to their nests (Minguez 1997; De Leon et al. 2003). In adult petrels, it would also be useful in specific cases when the partner skipped an incubating shift or gave up breeding. In this regard, results of experiment 3 are striking. The preference for an unknown conspecific odour over self-odour observed in both blue petrels and Antarctic prions (Bonadonna and Nevitt 2004) challenges the results of De Leon et al. (2003) on European storm petrel chicks and more generally the 'homing' hypothesis. Indeed, such an olfactory mechanism would drive a homing bird away from its nest. Different behavioural processes that could explain this apparent self-odour avoidance need to be discussed. First, birds may simply not be able to perceive their own odour and are therefore just attracted to the only odour perceived (perception hypothesis). It is also possible that self-odour detection is perceived by the bird as an indication that it has already explored this arm of the maze (confusion hypothesis). However, several arguments challenge these two hypotheses. First, it was shown in both Antarctic prions (Bonadonna and Nevitt 2004) and European storm petrels (De Leon et al. 2003) that birds do perceive their own odour as they preferred it to an odourless blank. The latter result, together with the short length and simplicity of the Y-maze approach we used, also advocates against the 'confusion hypothesis'. Finally, these two hypotheses would not constitute a satisfying explanation of our data, as they are both inconsistent with results on self-odour perception obtained from European storm petrel chicks (De Leon et al. 2003).

The preference patterns described in this study, when compared with preferences observed in European storm petrel chicks (De Leon et al. 2003), suggest an alternative hypothesis. Petrels' olfactory preferences may vary with age and/or social contexts so that self-odour avoidance may be developed only at sexual maturity. In such case, olfactory preferences should be examined from the perspective of sexual behaviours and mate choice. Blue petrels are monogamous and faithful year after year to their mate and burrow (Bried and Jouventin 2002). After breeding, they disperse at sea until the next breeding season (Warham 1996). In September, males and females have to meet and recognise each other in the darkness of their burrow. Singing in the burrow is costly because of predation (Mougeot and Bretagnolle 2000a) so that olfactory partner recognition may be adaptive to burrow-nesting petrels. Moreover, if petrels' odour constitutes an individually specific (Bonadonna et al. 2007) and honest signal reflecting the genetic make-up of a bird, it could be used as a secondary sexual trait. In such a case, one would expect blue petrels to prefer their mate's odour to the odour of a random conspecific (experiment 1) as would have been the case during pair formation. Consistent with this hypothesis is the preference of birds for their mate's odour over their own (experiment 2).

What is more, most petrel species breed on remote islands and are philopatric with regard to the colony (Warham 1996). Their life-history traits (philopatry, genetic lifelong monogamy) suggest that kin recognition may be important for

discriminating between potential mates and to avoid inbreeding. Therefore, a kin-odour avoidance mechanism, based on a self-odour template, could account for the observed preference for an unknown conspecific odour over self-odour (experiment 3, Bonadonna and Nevitt 2004). Self-referent phenotype matching, the so-called armpit effect (Dawkins 1982), has been implicated as a mechanism for assessing relatedness in other systems including birds and rodents (Heth et al. 1998; Hauber et al. 2000; Mateo and Johnston 2000). To explore whether such a mechanism is used by burrowing petrels for olfactory discrimination among individuals is a fascinating prospect of future research.

Our results clearly demonstrate that blue petrels can discriminate between their own, their mate's and unknown conspecifics' odours. To our knowledge, this is only the third bird species (after Antarctic prions and Wilson's storm petrels) proven to possess olfactory mechanisms of individual recognition beyond simple self-discrimination. We have developed two mutually non-exclusive behavioural frameworks for the interpretation of our results: homing and mate choice. Although simpler, the homing hypothesis cannot account for all of our results. The mate choice hypothesis, or a combined effect of the two, therefore appears more robust. Zelano and Edwards's (2002) suggestion of a MHC-based mate choice, mediated by olfactory mechanisms, in procellariiform seabirds is still the object of current research, but all these results taken together support this intriguing hypothesis.

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References

Aubin T, Jouventin P, Hildebrand C (2000) Penguins use the two-voice system to recognize each other. Proc R Soc Lond B Biol Sci 267:1081–1087

Bang BG (1966) The olfactory apparatus of tubenosed birds (Procellariiformes). Acta Anat 65:391–415

Blomqvist D, Andersson M, Kupper C, Cuthill IC, Kis J, Lanctot RB, Sandercock BK, Szekely T, Wallander J, Kempenaers B (2002) Genetic similarity between mates and extra-pair parentage in three species of shorebirds. Nature 419:613–615

Bonadonna F (2008) Olfaction in petrels: from homing to self-odor avoidance. Ann NY Acad Sci (in press)

Bonadonna F, Nevitt GA (2004) Partner-specific odor recognition in an Antarctic seabird. Science 306:835

Bonadonna F, Cunningham GB, Jouventin P, Hesters F, Nevitt GA (2003a) Evidence for nest-odour recognition in two species of diving petrel. J Exp Biol 206:3719–3722

Bonadonna F, Hesters F, Jouventin P (2003b) Scent of a nest: discrimination of own-nest odours in Antarctic prions, *Pachyptila desolata*. Behav Ecol Sociobiol 54:174–178

Bonadonna F, Villafane M, Bajzak C, Jouventin P (2004) Recognition of burrow's "olfactory signature" in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. Anim Behav 67:893–898

Bonadonna F, Miguel E, Grosbois V, Jouventin P, Bessiere J-M (2007) Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? J Chem Ecol 33:1819–1829

Bonneaud C, Chastel O, Federici P, Westerdahl H, Sorci G (2006) Complex MHC-based mate choice in a wild passerine. Proc R Soc B Biol Sci 273:1111–1116

Bretagnolle V (1989) Calls of Wilson's storm petrel: functions, individual and sexual recognitions, and geographic variation. Behaviour 111:98–112

Bried J, Jouventin P (2002) Site and mate-choice in seabirds: an evolutionary approach. In: Schreiber EA, Burger J (eds) Biology of marine birds. CRC, Boca, pp 263–305

Brown JL, Eklund A (1994) Kin recognition and the major histocompatibility complex—an integrative review. Am Nat 143:435–461

Dawkins R (1982) The extended phenotype. Freeman, San Francisco, CA

De Leon A, Minguez E, Belliure B (2003) Self-odour recognition in European storm-petrel chicks. Behaviour 140:925–933

Fenoglio S, Cucco M, Fracchia L, Martinotti MG, Malacarne G (2004) Shield colours of the Moorhen are differently related to bacterial presence and health parameters. Ethol Ecol Evol 16:171–180

Galeotti P, Saino N, Sacchia R, MØller AP (1997) Song correlates with social context, testosterone and body condition in male barn swallows. Anim Behav 53:687–700

Genevois F, Bretagnolle V (1994) Male blue petrels reveal their body weight when calling. Ethol Ecol Evol 6:377–383

Grubb TC (1974) Olfactory navigation to the nesting burrow in Leach's petrel (*Oceanodroma leucorrhoa*). Anim Behav 22:192–202

Hauber ME, Sherman PW, Paprika D (2000) Self-referent phenotype matching in a brood parasite: the armpit effect in brown-headed cowbirds (*Molothrus ater*). Anim Cogn 3:133–117

Heth G, Todrank J, Johnston RE (1998) Kin recognition in golden hamsters: evidence for phenotype matching. Anim Behav 56:409–417

Johnsen A, Andersen V, Sunding C, Lifjeld JT (2000) Female bluethroats enhance offspring immunocompetence through extra-pair copulations. Nature 406:296–299

Jouventin P, Mouret V, Bonadonna F (2007) Wilson's storm petrels *Oceanites oceanicus* recognise the olfactory signature of their mate. Ethology 113:1228–1232

Mateo JM, Johnston RE (2000) Kin recognition and the 'armpit effect': evidence of self-referent phenotype matching. Proc R Soc Lond B 267:695–700

Maynard Smith J, Harper D (2003) Animal signals. Oxford University Press, Oxford

Minguez E (1997) Olfactory recognition by British storm-petrel chicks. Anim Behav 53:701–707

Mougeot F, Bretagnolle V (2000a) Predation as a cost of sexual communication in nocturnal seabirds: an experimental approach using acoustic signals. Anim Behav 60:647–656

Mougeot F, Bretagnolle V (2000b) Predation risk and moonlight avoidance in nocturnal seabirds. J Avian Biol 31:376–387

Nevitt GA, Bonadonna F (2005) Seeing the world trough the nose of a bird: new developments in the sensory ecology of procellariiform seabirds. Mar Ecol Prog Ser 287:292–295

Nicolaus M, Bohec CL, Nolan PM, Gauthier-Clerc M, Maho YL, Komdeur J, Jouventin P (2007) Ornamental colors reveal age in the king penguin. Polar Biol 31:53–61

Nolan PM, Jouventin P, Dobson FS (2004) Plumage color predicts immune response in king penguins. Integr Comp Biol 44:612–612

Olsson M, Madsen T, Nordby J, Wapstra E, Ujvari B, Wittsell H (2003) Major histocompatibility complex and mate choice in sand lizards. Proc R Soc B Biol Sci 270(Suppl):S254–S256

Penn D (2002) The scent of genetic compatibility: sexual selection and the major histocompatibility complex. Ethology 108:1–21

Pryke SR, Griffith SC (2006) Red dominates black: agonistic signalling among head morphs in the colour polymorphic Gouldian finch. Proc R Soc B Biol Sci 273:949–957

Roper TJ (1999) Olfaction in birds. Adv Study Behav 28:247–332

Singh PB (2001) Chemosensation and genetic individuality. Reproduction 121:529–539

Tregenza T, Wedell N (2000) Genetic compatibility, mate choice and patterns of parentage: invited review. Mol Ecol 9:1013–1027

Warham J (1996) The behaviour, population biology and physiology of the petrels. Academic, London

Wyatt TD (2003) Pheromones and animal behaviour. Cambridge University Press, Cambridge

Yamazaki K, Beauchamp GK (2005) Chemosensory recognition of olfactory individuality. Chem Senses 30:142–143

Zelano B, Edwards VE (2002) An MHC component to kin recognition and mate choice in birds: predictions, progress, and prospects. Am Nat 160:225–238

Section 4.4

ADDITIONAL REFLECTIONS

Results from the experiments presented in this chapter suggest that blue petrels possess sufficient olfactory capabilities to perceive some of the social chemosignals elucidated in Chapter 3. The species-specific chemosignal for example supports mechanisms of interspecific discrimination which are likely used in nesting decisions (Bonadonna & Mardon, 2010). Individual chemical signatures, on the other hand, support mechanisms of intraspecific discrimination (of at least partner and self), and could contribute to various behaviours such as homing, mate choice, and individual recognition (Mardon & Bonadonna, 2009).

A natural question arising from the results presented so far is thus the possible behavioural function of the third chemosignal mentioned in Chapter 3: the sex-specific signal. Although not yet published, I carried out a field experiment with blue petrels to explore this particular question, i.e. their capabilities of olfactory sex discrimination. To do this, I followed a protocol similar to the one used and described in our other Y-maze experiments (Mardon & Bonadonna, 2009; Bonadonna & Mardon, 2010). Methodological specificities to the 'Sex' Y-maze experiment were that (i) I used non-breeding birds, based on the hypothesis that unpaired individuals may be more receptive to a Sex signal; (ii) the odour choice offered was between a 'female odour', obtained from the combination of the odours of 3 females, and a 'male odour', similarly obtained from the combination of the odours from 3 males. Sexes of odour donors were known from previous lab biomolecular analyses (Fridolfsson & Ellegren, 1999); (iii) after each trial, I collected a blood sample from the bird so as to genetically determine the sex of all birds tested once back in the lab.

Overall, I tested 23 non-breeding blue petrels consisting of 12 females and 11 males. In terms of results, neither males nor females showed any significant sex-based pattern of odour choice. Indeed, 6 males chose the 'male' odour, 2 chose the 'female' odour and 3 failed to choose. Similarly, 4 females chose the 'male' odour, 5 chose the 'female'

odour and 3 failed to choose. Overall, 6 birds chose the odour of the opposite sex while 11 preferred their own (two-tailed binomial tests, p=0.0944).

The results therefore do not provide any evidence of sexual discrimination olfactory capabilities in blue petrels; a finding that is consistent with a previous similar investigation in Antarctic prions (Bonadonna *et al*., 2009). Importantly, the Y-maze protocol can only test for a discriminative/recognition function of odours. Such results would thus be expected if, as suggested by recent evidence, avian Sex chemosignals serve principally a physiological function in activating and/or stimulating sexual behaviours (see Chapter 6).

It is also worth mentioning that I have repeatedly tried to extend the behavioural findings on individual odours presented in Section 4.3. My intention was in particular to elucidate the question of the heritability and genetic determinism of personal odours in petrels. To do so, I designed and set up some cross-fostering experiments, whereby chicks of different pairs are exchanged, to dissociate the influence of environmental (nest, adoptive parents) and genetic (true parents) factors on chemical signals and olfactory preferences.

Unfortunately, these attempts were thwarted, for two consecutive field seasons (2008 and 2009), because of logistical problems in the field. Critically, the boat allowing me to access my field site (Ile Verte) within the Kerguelen archipelago broke down in both years, at the time of chick rearing and fledging. As a result, neither I nor my collaborators were able to access the study colony at the appropriate time to carry out the chemical sampling or the behavioural experiments planned.

Section 4.5

REFERENCES

Benvenuti S., Ioale P. & Massa B. (1993). Olfactory experiments on Cory's shearwater (*Calonectris diomedea*): the effect of intranasal zinc sulphate treatment on short-range homing behaviour. *Italian Journal of Zoology* **60**: 207-210.

Bonadonna F. & Bretagnolle V. (2002). Smelling home: a good solution for burrow-finding in nocturnal petrels? *Journal of Experimental Biology* **205**: 2519-2523.

Bonadonna F., Caro S.P. & Brooke M.d.L. (2009). Olfactory Sex Recognition Investigated in Antarctic Prions. *PLoS ONE* **4**: e4148.

Bonadonna F., Cunningham G.B., Jouventin P., Hesters F. & Nevitt G.A. (2003). Evidence for nest-odour recognition in two species of diving petrel. *Journal of Experimental Biology* **206**: 3719-3722.

Bonadonna F. & Mardon J. (2010). One house two families: petrel squatters get a sniff of low-cost breeding opportunities. *Ethology* **116**: 176-182.

Bonadonna F. & Nevitt G.A. (2004). Partner-specific odor recognition in an Antarctic seabird. *Science* **306**: 835.

Bonadonna F., Spaggiari J. & Weimerskirch H. (2001). Could osmotaxis explain the ability of blue petrels to return to their burrows at night? *Journal of Experimental Biology* **204**: 1485-1489.

Bonadonna F., Villafane M., Bajzak C. & Jouventin P. (2004). Recognition of burrow's 'olfactory signature' in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. *Animal Behaviour* **67**: 893-898.

De Leon A., Minguez E. & Belliure B. (2003). Self-odour recognition in European storm-petrel chicks. *Behaviour* **140**: 925-933.

Fridolfsson A.-K. & Ellegren H. (1999). A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology* **30**: 116-121.

Grubb T.C. (1972). Smell and foraging in shearwaters and petrels. *Nature* **237**: 404-405.

Grubb T.C. (1973). Colony location by Leach's petrel. *The Auk* **90**: 78-82.

Grubb T.C. (1974). Olfactory navigation to the nesting burrow in Leach's petrel (*Oceanodroma leucorrhoa*). *Animal Behaviour* **22**: 192-202.

Grubb T.C. (1979). Olfactory guidance of Leach's storm petrel to the breeding island. *Wilson Bulletin* **91**: 141-143.

Hutchison L.V. & Wenzel B.M. (1980). Olfactory guidance in foraging by procellariiforms. *Condor* **82**: 314-319.

Jacob J. (1978). Uropygial gland secretions and feather waxes. In: Florkin M., Scheer B.T. (eds). *Chemical zoology*, vol 10 Aves. Academic press, New York: p.165-211.

Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Jouventin P., Mouret V. & Bonadonna F. (2007). Wilson's storm petrels *Oceanites oceanicus* recognise the olfactory signature of their mate. *Ethology* **113**: 1228-1232.

Mardon J. & Bonadonna F. (2009). Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own. *Behavioral Ecology and Sociobiology* **63**: 537-542.

Mardon J., Saunders S.M., Anderson M.J., Couchoux C. & Bonadonna F. (2010). Species, gender and identity: cracking petrels' socio-chemical code. *Chemical Senses* **Advance access**: doi:10.1093/chemse/bjq021.

Minguez E. (1997). Olfactory recognition by British storm-petrel chicks. *Animal Behaviour* **53**: 701-707.

Nevitt G.A. (2000). Olfactory foraging by Antarctic procellariiform seabirds: life at high Reynolds numbers. *Biological Bulletin (Woods Hole)* **198**: 245-253.

Nevitt G.A. & Bonadonna F. (2005). Sensitivity to dimethyl sulphide suggests a mechanism for olfactory navigation by seabirds. *Biology Letters* **1**: 303 - 305.

Sweeney R.J., Lovette I.J. & Harvey E.L. (2004). Evolutionary variation in feather waxes of passerine birds. *The Auk* **121**: 435-445.

Verheyden C. & Jouventin P. (1994). Olfactory behavior of foraging procellariiformes. *The Auk* **111**: 285-291.

Wallraff H.G. (2004). Avian olfactory navigation: its empirical foundation and conceptual state. *Animal Behaviour* **67**: 189-204.

Chapter 5

OLFACTORY/SENSORY BEHAVIOURS IN OTHER SEABIRDS

AIM & CONTENT

Chapter 5 presents the results from two adjunct field experiments, which broaden the scope of my research. These experiments explore olfaction-related behaviours in seabird species different from those considered in my main research; and within theoretical frameworks different from that of chemical communication. The following sections contain: (i) preliminary comments on the human and scientific context of these complementary works (Section 5.1); (ii) an article published in 2010 in the *Journal of Experimental Biology*, exploring the existence of olfactory capabilities in breeding wandering albatrosses (Section 5.2); (iii) an article published in 2009 in the *Journal of Experimental Biology*, exploring mechanisms of orientation used at the colony by king penguin chicks (Section 5.3).

From The Rut (2007-09), by Phil Selby

Section 5.1

PRELIMINARY COMMENTS

Carrying out my fieldwork in the French subantarctic territories gave me the invaluable opportunity to experience a very collegial approach to science. Indeed, logistic, geographic and human conditions on these islands favour mutual assistance and, importantly, scientific exchanges and collaborations.

Unfortunate circumstances during my 2nd field campaign (namely the breakdown, halfway through the field season, of the boat necessary to access my field site, Ile Verte) led me to rethink and redesign my scientific objectives. As a result, I spent about 45 days in one of the biological hotspots of Kerguelen, Cape Ratmanoff (49°14' S, 70°34' E). The place is home to, among many other extraordinary features, one of the largest colonies of king penguins in the world and a colony of wandering albatrosses. The realisation that my unfortunate situation may provide new opportunities to extend the scope of my research led me to explore the role of olfaction in different seabird species and behavioural contexts.

In close collaboration with Dr. A.P. Nesterova, a post-doctoral colleague from Montpellier (supervised by F.Bonadonna), I thus designed and completed various behavioural experiments on the seabirds present at Ratmanoff. The following sections present two of these experiments which respectively examine the existence of olfactory sensitivity in wandering albatrosses, and mechanisms of orientation at the colony in king penguin chicks.

Section 5.2

OLFACTION IN THE WANDERING ALBATROSS

CONTEXT

There has been abundant research on olfactory foraging by procellariiform seabirds using at-sea observations and/or experiments (see Nevitt, 2000). As a result, olfactory guidance to foraging cues is clearly established for many species including most petrels. However, results remain inconclusive and sometimes inconsistent for some larger species such as albatrosses, and wandering albatrosses in particular. Recent telemetry studies suggest that some spatial behaviours of the latter species are consistent with the *multimodal foraging strategy* hypothesis which proposes that birds use a combination of olfactory and visual cues while foraging at sea (Nevitt *et al.*, 2008). The *multimodal foraging strategy* hypothesis however suffers from a lack of experimental evidence, particularly regarding the olfactory capabilities of wandering albatrosses.

In this section, I present a simple behavioural study completed collaboratively during my second and third field campaigns on the Kerguelen archipelago, confirming that wandering albatrosses possess a reasonable level of olfactory sensitivity.

PRESENTATION OF THE ARTICLE

Title	Insight of scent: experimental evidence of olfactory capabilities in the wandering albatross (*Diomedea exulans*).
Authors	Mardon J., Nesterova A.P., Traugott J., Saunders S.M. & Bonadonna F.
Journal	*Journal of Experimental Biology* **213** (4): 558-563
Date of publication	29th of January 2010 (online); printed in 2010
Contribution of PhD-candidate	For this particular study, most of the experimental design, data analysis, and redaction/submission of the manuscript are the results of my personal work (about 90%). Several colleagues assisted, to a level of 50%, with data collection in the field. My supervisors provided helpful improvements on the manuscript.

The Journal of Experimental Biology 213, 558-563

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Insight of scent: experimental evidence of olfactory capabilities in the wandering albatross (*Diomedea exulans*)

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SUMMARY

Wandering albatrosses routinely forage over thousands of kilometres of open ocean, but the sensory mechanisms used in the food search itself have not been completely elucidated. Recent telemetry studies show that some spatial behaviours of the species are consistent with the 'multimodal foraging strategy' hypothesis which proposes that birds use a combination of olfactory and visual cues while foraging at sea. The 'multimodal foraging strategy' hypothesis, however, still suffers from a lack of experimental evidence, particularly regarding the olfactory capabilities of wandering albatrosses. As an initial step to test the hypothesis, we carried out behavioural experiments exploring the sensory capabilities of adult wandering albatrosses at a breeding colony. Three two-choice tests were designed to investigate the birds' response to olfactory and visual stimuli, individually or in combination. Perception of the different stimuli was assessed by comparing the amount of exploration directed towards an 'experimental' display or a 'control' display. Our results indicate that birds were able to perceive the three types of stimulus presented: olfactory, visual and combined. Moreover, olfactory and visual cues were found to have additional effects on the exploratory behaviours of males. This simple experimental demonstration of reasonable olfactory capabilities in the wandering albatross supports the 'multimodal foraging strategy' and is consistent with recent hypotheses of the evolutionary history of procellariiforms.

Key words: *Diomedea exulans*, behaviour, multimodal, olfaction, vision, signal-detection theory.

INTRODUCTION

Procellariiform seabirds (albatrosses, petrels, shearwaters) are unusual among other avian orders in that most of these so-called 'tube-nose' birds have a well-developed olfactory neuroanatomy (Bang, 1966) and good associated capabilities. Procellariiforms use, or have been suggested to use, their sense of smell in various behaviours including foraging (Hutchison and Wenzel, 1980), homing (Bonadonna et al., 2001), ocean navigation (Nevitt and Bonadonna, 2005) and even some social aspects such as individual recognition and mate choice (Bonadonna and Nevitt, 2004; Hagelin and Jones, 2007; Mardon and Bonadonna, 2009). Following Grubb's pioneering experiments (Grubb, 1972), most of the early work investigated sensitivity to food-related scents by exposing wild seabirds to odorous stimuli such as cod liver oil-soaked sponges (Jouventin and Robin, 1983; Lequette et al., 1989), scented oil slicks (Hutchison and Wenzel, 1980; Nevitt et al., 1995; Nevitt, 1999; Nevitt et al., 2004) or aerosol plumes (Nevitt et al., 1995). These experiments provided an extensive list of procellariiform species for which olfactory foraging was supported, including storm petrels (*Oceanites oceanicus*, *Oceanodroma leucorhoa*), petrels (*Pagodroma nivea*, *Macronectes giganteus*, *Daption capense*, *Procellaria aequinoctialis*), shearwaters (*Puffinus gravis*, *P. creatopus*, *P. griseus*, *P. puffinus*, *P. tenuirostris*), fulmars (*Fulmarus glacialis*, *F. glacialoides*), albatrosses (*Diomedea nigripes*, *D. chrysostoma*, *D. melanophris*, *Phoebetria palpebrata*) and prions (*Pachyptila* sp.).

The wandering albatross (*Diomedea exulans*, Diomedeidae, Linnaeus 1758) is the largest of the procellariiform seabirds and has the largest wingspan of any living bird. Its foraging activity usually takes it over thousands of kilometres of open ocean, where it feeds on a variety of squids that are captured or found dead at the surface (Cherel and Weimerskirch, 1999). Yet, the sensory mechanisms used in this foraging search are still not completely understood. Early experiments on the response of albatrosses to olfactory foraging cues did not provide conclusive results. For instance, black-footed (Hutchison and Wenzel, 1980) and light-mantled sooty albatrosses (Lequette et al., 1989) are regularly attracted to food-related odours. In contrast, wandering, grey-headed and black-browed albatrosses do not appear to be attracted to either cod liver oil or dimethyl sulphide (DMS)-scented oil (Lequette et al., 1989; Nevitt et al., 1995), though black-browed albatrosses significantly respond to pyrazine- and herring-scented stimuli (Nevitt et al., 2004). Such intricacy probably explains why albatrosses are commonly thought to hunt visually (Prince and Morgan, 1990; Warham, 1990; Nevitt et al., 1995).

New elements from telemetric studies (Weimerskirch et al., 2005; Phalan et al., 2007) have recently improved our understanding of wandering albatrosses' behaviours. For instance, foraging activity is greater during daylight, when they feed mainly on large, isolated squids using active flight search (Phalan et al., 2007). At night, however, they feed on small, aggregated and bioluminescent squid by switching to a 'sit-and-wait' strategy at the water surface, probably because of the limited visual cues available for an active search (Phalan et al., 2007). Using the same GPS data, Nevitt and colleagues (Nevitt et al., 2008) showed that some spatial behaviours of foraging wandering albatrosses are consistent with the

'multimodal (vision and olfaction) foraging strategy' hypothesis (VanBuskirk and Nevitt, 2008). This hypothesis proposes that birds use more than a single mechanism of sensory detection when foraging, taking advantage of olfactory cues for initial detection and localisation of potential prey, whereas vision would be predominant during prey capture. The 'multimodal foraging strategy' hypothesis, however, still suffers from a lack of experimental evidence, particularly regarding the olfactory capabilities of wandering albatrosses, probably due to the difficulty of carrying out controlled experiments on large marine predators.

The purpose of our study was to provide an initial test of the 'multimodal foraging strategy' hypothesis by exploring the sensitivity of wandering albatrosses to relevant types of stimuli. Therefore, we investigated the birds' behavioural response to olfactory and visual cues. To do so, three similar experiments were carried out on incubating adults (at the colony), in which birds were offered the choice between an experimental/stimulus display and a control/empty display. Displays were designed so that they could provide (i) olfactory stimuli alone in the first experiment (olfaction test), (ii) visual stimuli alone in the second experiment (vision test), and (iii) both olfactory and visual stimuli in the third experiment (combined test). We assumed that a bird perceived and responded to a stimulus (olfactory, visual or both) if the experimental display elicited more exploration behaviours than the control display.

MATERIALS AND METHODS

Study site and animals

We studied wandering albatrosses in January 2009 at Cape Ratmanoff (49°14′S, 70°34′E) on the west coast of Kerguelen Island, a French sub-Antarctic territory in the Southern Indian Ocean. A total of 32 breeding pairs (N=64 birds), forming a loose colony around the area, were monitored daily for foraging and incubating shifts. Birds were sexed using reliable morphological secondary sexual traits such as plumage patterns, beak morphology and size (Weimerskirch et al., 1989).

Experimental procedure

Three behavioural experiments were designed to test the sensory capabilities of adult wandering albatrosses, focusing on vision and olfaction. In each experiment, birds were offered a choice between two sample displays: an experimental display containing chunks of tuna and a control display left empty. A roughly similar quantity (about 25 g) of freshly opened, unflavoured, canned tuna ('Thon au naturel' Albacore®) was used as the experimental stimulus for all tests. Canned tuna was not chosen in order to simulate an artificial foraging situation but simply to provide a shapeless, unfamiliar yet naturally intense stimulus. All displays were similarly made of a 50 ml transparent centrifuge tube (with a conical bottom) taped to a metal peg, used to secure them to the ground during tests. We used three different sets of experimental displays, each one being adapted to one of the three experiments performed (Fig. 1). In experiment 1, the 'olfaction test', we tested the effect of olfactory stimuli alone by covering the whole surface of the centrifuge tubes with black opaque masking tape while leaving the top of the tubes open. Care was taken that the depth of the tubes made it impossible for the bird to see the tuna at the bottom. In experiment 2, the 'vision test', we tested the effect of visual stimuli alone by sealing the top of the tubes with Parafilm® while leaving the surface of the tubes uncovered and therefore transparent. In experiment 3, the 'combined test', we tested the combined effects of olfactory and visual stimuli by leaving the surface and top of the tubes uncovered. Note that the design of the displays ensured that the intensity of a given stimulus (olfactory or visual) was similar in the isolated and combined tests. Indeed, the visual stimulus was as readily accessible in the vision test as it was in the combined test (transparent tubes). Similarly, the olfactory stimulus was equivalent in the olfaction and the combined tests as diffusion of volatile chemicals was unconstrained in both cases.

Wandering albatrosses have historically been relatively unexposed to human or predatory disturbances on their colony sites and do not show a strong response to slow ground-level approaches. For each trial, an incubating bird was therefore approached by slowly crawling to the nest. The two sample displays (experimental and control) were placed on the turf surrounding a nest, within 30 cm of each side of the bird's head. The experimenter then crawled back and the bird's response to the displays was recorded from 15 m away by a focal animal sampling observation of 10 min. For each trial, we recorded three complementary variables: the direction of the initial peck, the number of pecks on each display and the total time spent exploring each display. In our experiments, we defined a 'peck' event as a head movement from the normal incubating position towards one of the displays resulting in at least one contact between the bird's bill and the display. We considered a peck event to be finished when the bird returned to its normal incubating position. The durations of all peck events were recorded using a stopwatch and summed to obtain the total exploring time over a trial. Sample containers were removed immediately after the end of each trial.

To reduce disturbance of the animals and obtain independent data between treatments, each individual participated in only one of the three experiments. Overall, 21 birds (9 females and 12 males) were tested in the olfaction test, 18 birds (9 females and 9 males) in the vision test, and 21 birds (8 females and 13 males) in the combined test. The position of the two different displays (control and experimental) was randomised between trials with respect to the bird's side (left or right) to avoid lateralisation effects. The order of the trials, with regard to the type of experiment, was also randomised to reduced possible environmental effects. Finally, trials were carried out only under low-wind conditions (Beaufort wind force scale <3) to reduce possible wind effects on odour dispersion. The variable nature of the direction faced by incubating birds, together with the randomisation of the position of the two displays, should have further reduced possible wind-induced bias.

Animal ethics

All aspects of the study were performed according to guidelines established by the IPEV (Institut Polaire Français Paul-Emile Victor) and the CNRS for the Ethical Treatment of Animals and complied with current French regulations. Several factors indicate that the

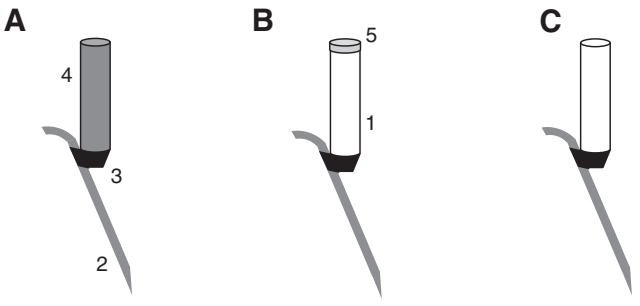

Fig. 1. Schematic layout of the different types of sample display used in the experiments: (A) Olfaction test, (B) vision test, (C) combined test. Key: 1, transparent plastic vial; 2, metal peg; 3, attaching tape; 4, opaque masking tape; 5, Parafilm® seal.

experiment is unlikely to have caused any major disturbance to the birds. No bird was handled and each was approached only once as mentioned above. Moreover, none of the tested individuals deserted the nest or moved away from the experimenter, and all resumed resting activity on the nest during or soon after the trial. Disturbance was therefore much less than in most current monitoring and telemetry studies, which have been reported to have no effect on albatross survival and breeding success (Weimerskirch et al., 2007).

Analysis

For clarity, our data analysis is organised into two sections. The first considers each experiment individually (olfaction, vision and combined tests), exploring whether birds did or did not perceive the stimulus associated with this particular experiment. In the second, the 'signal detection theory' framework is used to compare albatrosses' relative sensitivities to the different types of stimuli.

(A) Intra-experiment analysis

For each experiment, we compared the extent of exploration expressed by the incubating birds towards the two displays using several variables: (i) the direction of the initial peck, (ii) the number of pecks (no. pecks) and (iii) the total exploring time (T_{expl}). We also created a fourth summary variable, the total score, combining the first three variables for each trial. To compute this summary variable, a score out of 3 was calculated for each display; each of the above three variables contributing one point to the total score of the experimental or control display, depending on the direction of disparity. As an illustration, a trial in which the initial peck was directed to the control display, the experimental display received 12 pecks while the control received 5, and the total exploring time was 25 s on the experimental display and 11 s on the control, received a total score of 2 for the experimental display and 1 for the control. No point was attributed, for a given variable, when the two displays received an equal amount of investigation for this variable, a situation hereafter referred to as a 'draw'.

In a first analytical approach, we calculated for each of the four variables the proportion of trials in which the experimental display received more exploration than the control. For instance, out of the 21 olfaction trials, the experimental display received a longer exploring time in 12 instances, the control display received a longer exploring time in 4 instances and the two displays received equal exploring time in 5 instances (draws). The significance of all calculated proportions was then assessed using one-tailed exact binomial tests; that is, we tested the specific hypothesis that the presence of the canned tuna stimulus in the experimental display would increase exploratory behaviours compared with the control display. Note that draw outcomes were not considered in our statistical analyses. Indeed, excluding these while providing their frequency appeared to be the most biologically appropriate and statistically relevant option for several reasons. First, there was no correlation between the occurrence of draws and other variables such as the time of the day, the bird sex or the stimulus involved. Moreover, a large proportion of these draws (43%) were 'zero-draws', i.e. trials in which the bird did not respond at all to the displays. In most of these cases, the bird returned to rest (beak under the wing) straight after deployment of the displays, or did not wake up at all during deployment. Therefore the inclusion of draw outcomes would provide virtually no additional biological information while increasing data noise and the number of statistical tests involved.

In a second approach, we compared, for each experiment, the absolute values of no. pecks and T_{expl} between experimental and control displays. As an acceptable level of normality could not be obtained with these variables, regardless of the transformation applied, we used Wilcoxon rank-sum non-parametric tests. Again, one-tailed tests were chosen to test for the specific hypothesis that the extra stimulus in the experimental display would elicit more exploratory behaviours from the birds.

(B) Inter-experiment analysis

Results from two-choice experiments are generally not well suited for comparison across various treatments as they do not consist of a single variable. Therefore, to allow comparison of the results from our olfaction, vision and combined tests, we used the framework of 'signal detection theory' (Green and Swets, 1966). Signal detection theory was specifically developed by neuropsychologists as a way to analyse sensitivity experiments, in which sensory signals must be distinguished from a noisy background. It provides a method for assessing sensory performance and a framework for analysing this performance. Individuals are considered as decision makers, with four possible outcomes: hit (if signal present and detected), miss (if signal present but undetected), false alarm (if signal absent but detected), and correct rejection (if signal absent and undetected). The probability of the various outcomes can then be calculated from the total number of trials and converted into a z-score using z-tables (for normalised standardised data). In the 'signal detection theory', the sensitivity d' to a signal is defined as 'z (hits)–z (false alarm)'. This framework therefore provided us with a way to quantitatively estimate the birds' sensitivity to the different stimuli. Indeed, in our experiments, exploration of the experimental display could be considered as a 'hit' (signal present and detected) and exploration of the control display as a 'false alarm' (signal absent but detected). This analytical approach has the advantage of accounting for the simple effect of curiosity to new objects around the nest, and for biases associated with the personalities of the tested animals, such as high curiosity or shyness. In the present study, a positive value for sensitivity means that the extra stimulus in this experiment increased detection and/or exploration by the birds compared with the control display.

For each experiment, we first calculated the sensitivities d'_1 and d'_2, associated respectively with each of the no. pecks and T_{expl} variables. To do so, we converted the number of pecks on each display (no. pecks) into a probability, simply by dividing by the maximum number of pecks performed on a display, all trials confounded. Similarly, we converted the exploring time on each display (T_{expl}) into a probability, simply by dividing by the total time of the trial (600 s). Finally, in order to create a summary sensitivity variable, the two initial sensitivities d'_1 and d'_2 were scaled and averaged to obtain an overall sensitivity D', comparable across experiments. Note that data from the three experiments were independent as we used different birds in each.

The combined effects of sex and stimulus type ('Experiment') on the sensitivity D' were examined with a global fixed-effects ANOVA model. Based on the outcome of this initial model, we further investigated the experiment effect within each sex separately also using fixed-effects ANOVA models. *Post-hoc* pair-wise comparisons were carried out using standard t-tests.

RESULTS

(A) Intra-experiment analysis

In the olfaction test (21 trials), albatrosses could only discriminate between the two displays based on the odour cues emanating from the vials. Significantly more initial pecks were directed at the experimental display (initial peck: 14 out of 17; 4 trials with no

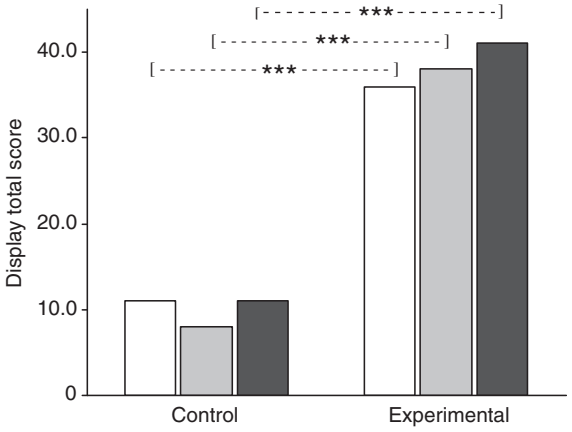

Fig. 2. Total score for each display, control and experimental, for the three experiments. Open bars, olfaction test; grey bars, vision test; black bars, combined test. Asterisks indicate a significant difference between the two displays (***P<0.001; one-tailed exact binomial test).

peck; P=0.0064). Moreover, there was a higher proportion of trials in which the experimental display elicited more exploratory behaviours than the control (no. pecks: 10 out of 14, 7 draws, P=0.0898; T_{expl}: 12 out of 16, 5 draws, P=0.0384; total score: 36 out of 47, 16 draws, P=0.0002) (Fig. 2). Comparison of the absolute values of no. pecks and T_{expl} between the two displays (Fig. 3) shows that the olfactory experimental display elicited a higher number of pecks and a longer exploring time than the control display, although these contrasts do not reach significance (no. pecks: Wilcoxon rank-sum test, Z=0.9831, P=0.1628; T_{expl}: Wilcoxon rank-sum test, Z=1.5723, P=0.0579).

In the vision test (18 trials), albatrosses could only discriminate between the two displays using the visual cues observed through the transparent vials. As in the previous experiment, significantly more initial pecks were directed at the experimental display (initial peck: 14 out of 18; P=0.0154). Moreover, there was a higher proportion of trials in which the experimental display elicited more exploratory behaviours than the control (no. pecks: 11 out of 14, 4 draws, P=0.0287; T_{expl}: 13 out of 14, 4 draws, P=0.0009; total score: 38 out of 46, 8 draws, P=0.0009) (Fig. 2). Comparison of the absolute values of no. pecks and T_{expl} between the two displays (Fig. 3) shows that the visual experimental display elicited a significantly higher number of pecks and exploring time than the control display (no. pecks: Wilcoxon rank-sum test, Z=2.1953, P=0.0141; T_{expl}: Wilcoxon rank-sum test, Z=1.7484, P=0.0402).

In the last experiment, the combined test (21 trials), albatrosses could use both odour and visual cues from the vials to discriminate between the two displays. In this case again, more initial pecks were directed at the experimental display although this proportion did not reach significance (initial peck: 14 out of 20; 1 trial with no peck; P=0.0577). Moreover, there was a higher proportion of trials in which the experimental display elicited more exploratory behaviours than the control (no. pecks: 14 out of 17, 4 draws, P=0.0064; T_{expl}: 13 out of 15, 6 draws, P=0.0037; total score: 41 out of 52, 11 draws, P<0.0001) (Fig. 2). Comparison of the absolute values of no. pecks and T_{expl} between the two displays (Fig. 3) shows that the combined experimental display elicited a significantly higher number of pecks and exploring time than the control display (no. pecks: Wilcoxon rank-sum test, Z=2.9619, P=0.0015; T_{expl}: Wilcoxon rank-sum test, Z=2.5025, P=0.0062).

The outcome of the three experiments thus indicates that albatrosses are able to perceive each of the three different types of stimulus presented.

(B) Inter-experiment analysis

The different sensitivities d'_1, d'_2 and D' calculated are presented in Fig. 4. Note that, consistent with the results reported above, sensitivity values are all positive, suggesting that the extra stimuli in the experimental displays all increased detection and/or exploration by the birds compared with the control display.

The fixed-effects ANOVA model investigating the influence of sex and stimulus type on the sensitivity D' (Table 1) shows a significant interaction term. This suggests that the sensitivity varies differently with the type of stimulus, according to the sex of the birds. Thus, we subsequently explored the influence of the stimulus type on the sensitivity D' of wandering albatrosses within each sex separately. The type of stimulus presented did not significantly affect females' sensitivity D' (d.f.=2, sum of squares (SSq) [Type1]=1.3028, F-value=1.1378, P=0.3387). In contrast, it significantly affected males' sensitivity D' (d.f.=2, SSq

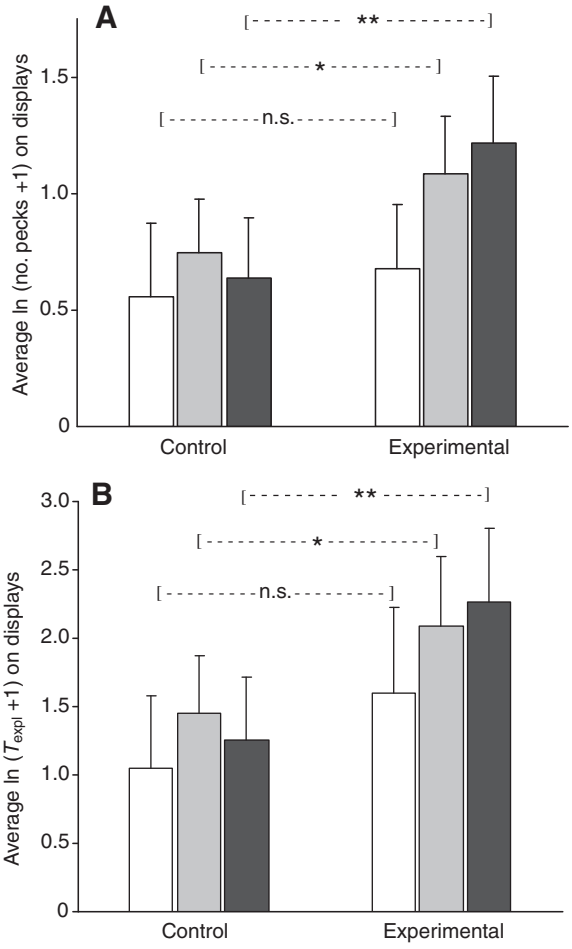

Fig. 3. (A) Average number of pecks (no. pecks) and (B) average exploring time (T_{expl}, in s) on each display, control and experimental, for the three experiments. Open bars, olfaction test; grey bars, vision test; black bars, combined test. Error bars correspond to 95% confidence intervals (calculated as $t_{0.95}$×s.e.m.). Asterisks indicate a significant difference between the two displays (*P<0.05; **P<0.01; one-tailed Wilcoxon rank-sum non-parametric tests). Note that data were ln(x+1) transformed for graphic purposes.

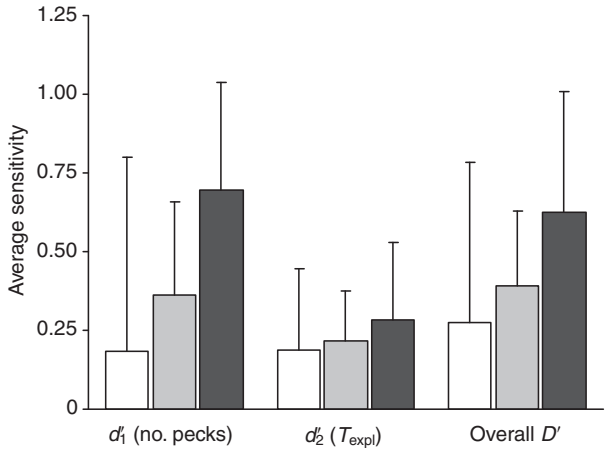

Fig. 4. Average sensitivities d'_1, d'_2 and D' of wandering albatrosses in the three experiments. Open bars, olfaction test; grey bars, vision test; black bars, combined test. Error bars correspond to 95% confidence intervals (calculated as $t_{0.95}\times$s.e.m.). Note that sensitivity is a dimensionless score.

[Type1]=5.6228, *F*-value=5.6246, *P*=0.0091). More specifically, males' sensitivity to the combined stimulus was significantly higher than that to either olfactory (*t*=–2.9848, d.f.=19, *P*=0.0076) or visual (*t*=–2.2226, d.f.=19, *P*=0.0386) stimuli, while the latter two were not significantly different (*t*=–1.3036, d.f.=16, *P*=0.2108).

DISCUSSION

In order to explore the sensitivity of wandering albatrosses to olfactory and visual stimuli, alone and combined, three similar experiments were carried out on incubating adults of both sexes, at the breeding colony. Birds showed a significant response to the experimental displays for the three types of cue presented: olfactory, visual or a combination of the two, indicating that they perceive all types of stimuli. While the finding of visual capabilities comes as no surprise, our simple experimental demonstration of olfactory capabilities in the wandering albatross finds special significance in the number of descriptive investigations and hypotheses that have resulted from the study of albatross behaviour so far (Jouventin and Weimerskirch, 1990; Akesson et al., 2001; Bonadonna et al., 2005; Weimerskirch et al., 2005; Phalan et al., 2007; Nevitt et al., 2008).

Among the procellariiforms, most nocturnal species are burrow nesters and possess an acute sense of smell that they use to locate their nest (Bonadonna and Bretagnolle, 2002). In contrast, diurnal species tend to be ground nesters and probably rely on visual cues to home. Interestingly, the ancestral condition for the whole procellariiform clade was probably nocturnal/burrow nesting with independent adaptations to surface nesting in different subgroups (VanBuskirk and Nevitt, 2008). In addition, the foraging style of these birds has probably evolved in conjunction with nesting behaviours, as attraction to DMS has been found to be associated with burrow-nesting behaviour (VanBuskirk and Nevitt, 2008). The shift that occurred in several independent procellariiform species from nocturnal to diurnal habits, associated with a shift from burrow nesting to ground nesting (Bonadonna and Bretagnolle, 2002), may thus have also decreased the reliance on olfactory cues to locate prey (VanBuskirk and Nevitt, 2008). In these species, visual cues would have become increasingly important while maintaining some olfactory capabilities, thus promoting the emergence of multimodal mechanisms. Such a scenario, supported by our finding of olfactory capabilities in the wandering albatross, could explain why, although all species still exhibit a well-developed olfactory neuroanatomy, the observed responses to food-related scents have remained inconclusive for many species.

Unexpectedly, the comparison of the relative sensitivities to the different stimuli showed some sex-specific patterns. While females' sensitivity was not affected by the type of cues presented, males' sensitivity to the combined stimuli (visual and olfactory) was significantly greater than sensitivity to each stimulus alone. Interestingly, a sexual dimorphism has been observed in the foraging strategies of many seabirds (González-Solís et al., 2000; Lewis et al., 2002; Phillips et al., 2004), including the wandering albatross (Weimerskirch et al., 1993; Shaffer et al., 2001). It consists typically of the sexual segregation of foraging areas, which has been attributed to size-related differences in flight performance between sexes (Shaffer et al., 2001). However, variations in the foraging behaviours themselves, independent of any size dimorphism, have also been reported but not explained (Lewis et al., 2002). Whether the sex-specific nature of our sensitivity results is somehow related to these considerations is intriguing but further discussion at this stage of the research would be highly speculative. Besides, the difference observed between the two sexes may have simply resulted from a combination of limited sample sizes and some sex-specific personality traits, the males tested being generally less shy and more explorative of the displays than females.

Nevertheless, the higher sensitivity observed in males exposed to the combined cues, when compared with exposure to each cue alone, suggests that olfactory and visual cues may have additional effects on exploratory behaviours, at least in males. If the amount of exploration is directly related to the quantity of stimuli perceived, the combination of visual and olfactory cues would be expected to elicit up to twice as many exploratory behaviours as the single-mode tests, depending on how each modality is integrated. This is consistent with the increase in the combined test response reported here.

Finally, to correctly understand our findings, it is important to identify the limitations of our approach, testing the sensitivity of wandering albatrosses during incubating shifts on land. As discussed in earlier similar studies (Nevitt and Haberman, 2003), we caution that showing a bird's response to a given stimulus at the breeding colony does not prove that such a stimulus is used while foraging at sea. Birds in our study were not placed in a foraging context and,

Table 1. Results of the fixed-effects ANOVA model investigating the influences of sex and stimulus type on the overall sensitivity D'

Source	d.f.	SSq	Mean Sq	*F*-value	*P*
Sex	1	0.5267	0.5267	0.9891	0.3249
Stimulus type	2	1.0753	0.5376	1.0097	0.3718
Sex and stimulus type	2	5.8504	2.9252	5.4935	0.0070*
Residuals	49	26.0916	0.5325		

Sum of squares (SSq) reported in the table are Type I sum of squares. Significant effects are indicated by an asterisk.

consistently, none of them exhibited a frantic response to the tuna-filled display nor manifested any intent to feed. This seems to confirm that birds did not consider these experiments as foraging tasks. However, our study does not focus on a bird's attraction to a particular foraging stimulus but rather explores the sensory pathways leading to detection and/or exploration. In this respect, our simple experimental demonstration of olfactory capabilities in the wandering albatross has a general relevance to the ecology of these birds, potentially including foraging at sea (Nevitt et al., 2008), navigation (Nevitt and Bonadonna, 2005) and even social behaviours (Bonadonna and Nevitt, 2004). It may thus be used as a basis for future studies on this species. Despite a bias towards single-modality studies, it is now recognised that detection and attraction behaviours often involve multimodal sensory mechanisms (for details, see Dusenbury, 1992) and our study constitutes one more suggestion that seabirds are no exception.

LIST OF SYMBOLS AND ABBREVIATIONS

d'_1	sensitivity associated with the no. pecks variable
d'_2	sensitivity associated with the T_{expl} variable
D'	overall sensitivity
DMS	dimethyl sulphide
no. pecks	number of pecks on the display
T_{expl}	total exploring time

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REFERENCES

Åkesson, S., Luschi, P., Papi, F., Broderick, A. C., Glen, F., Godley, B. J. and Hays, G. C. (2001). Oceanic long-distance navigation: do experienced migrants use the Earth's magnetic field? *J. Navigation* **54**, 419-427.

Bang, B. G. (1966). The olfactory apparatus of tubenosed birds (Procellariiformes). *Acta Anat.* **65**, 391-415.

Bonadonna, F. and Bretagnolle, V. (2002). Smelling home: a good solution for burrow-finding in nocturnal petrels? *J. Exp. Biol.* **205**, 2519-2523.

Bonadonna, F. and Nevitt, G. A. (2004). Partner-specific odor recognition in an Antarctic seabird. *Science* **306**, 835.

Bonadonna, F., Spaggiari, J. and Weimerskirch, H. (2001). Could osmotaxis explain the ability of blue petrels to return to their burrows at night? *J. Exp. Biol.* **204**, 1485-1489.

Bonadonna, F., Bajzak, C., Benhamou, S., Igloi, K., Jouventin, P., Lipp, H. P. and Dell'Omo, G. (2005). Orientation in the wandering albatross: interfering with magnetic perception does not affect orientation performance. *Proc. R. Soc. Lond. B Biol. Sci.* **272**, 489-495.

Cherel, Y. and Weimerskirch, H. (1999). Spawning cycle of onychoteuthid squids in the southern Indian Ocean: new information from seabird predators. *Marin. Ecol. Prog. Ser.* **188**, 93-104.

Dusenbury, D. B. (1992). Sensory ecology: how organisms acquire and respond to information. New York: W. H. Freeman and Company.

González-Solís, J., Croxall, J. P. and Wood, A. G. (2000). Sexual dimorphism and sexual segregation in foraging strategies of northern giant petrels, *Macronectes halli*, during incubation. *Oikos* **90**, 390-398.

Green, D. M. and Swets, J. A. (1966). Signal detection theory and psychophysics. New York: Wiley and Sons, Inc.

Grubb, T. C. (1972). Smell and foraging in shearwaters and petrels. *Nature* **237**, 404-405.

Hagelin, J. C. and Jones, I. L. (2007). Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? *Auk* **124**, 741-761.

Hutchison, L. V. and Wenzel, B. M. (1980). Olfactory guidance in foraging by procellariiforms. *Condor* **82**, 314-319.

Jouventin, P. and Robin, J. P. (1983). Olfactory experiments on some antarctic birds. *Emu* **84**, 46-48.

Jouventin, P. and Weimerskirch, H. (1990). Satellite tracking of wandering albatrosses. *Nature* **343**, 746-748.

Lequette, B., Verheyden, C. and Jouventin, P. (1989). Olfaction in Subantarctic seabirds: its phylogenetic and ecological significance. *Condor* **91**, 732-735.

Lewis, S., Benvenuti, S., Dall'Antonia, L., Griffiths, R., Money, L., Sherratt, T. N., Wanless, S. and Hamer, K. C. (2002). Sex-specific foraging behaviour in a monomorphic seabird. *Proc. R. Soc. Lond. B Biol. Sci.* **269**, 1687-1693.

Mardon, J. and Bonadonna, F. (2009). Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own. *Behav. Ecol. Socbiol.* **63**, 537-542.

Nevitt, G. A. (1999). Olfactory foraging in Antarctic seabirds: a species-specific attraction to krill odors. *Marin. Ecol. Prog. Ser.* **177**, 235-241.

Nevitt, G. A. and Bonadonna, F. (2005). Seeing the world trough the nose of a bird: new developments in the sensory ecology of procellariiform seabirds. *Marin. Ecol. Prog. Ser.* **287**, 292-295.

Nevitt, G. A. and Haberman, K. (2003). Behavioral attraction of Leach's storm-petrels (*Oceanodroma leucorhoa*) to dimethyl sulfide. *J. Exp. Biol.* **206**, 1497-1501.

Nevitt, G. A., Veit, R. R. and Kareiva, P. (1995). Dimethyl sulphide as a foraging cue for Antarctic procellariiform seabirds. *Nature* **376**, 680-682.

Nevitt, G. A., Reid, K. and Trathan, P. N. (2004). Testing olfactory foraging strategies in an Antarctic seabird assemblage. *J. Exp. Biol.* **207**, 3537-3544.

Nevitt, G. A., Losekoot, M. and Weimerskirch, H. (2008). Evidence for olfactory search in wandering albatross, *Diomedea exulans*. *Proc. Natl. Acad. Sci. USA* **105**, 4576-4581.

Phalan, B., Phillips, R. A., Silk, J. R. D., Afanasyev, V., Fukuda, A., Fox, J., Catry, P., Higuchi, H. and Croxall, J. P. (2007). Foraging behaviour of four albatross species by night and day. *Marin. Ecol. Prog. Ser.* **340**, 271-286.

Phillips, R. A., Silk, J. R. D., Phalan, B., Catry, P. and Croxall, J. P. (2004). Seasonal sexual segregation in two Thalassarche albatross species: competitive exclusion, reproductive role specialization or foraging niche divergence? *Proc. R. Soc. Lond. B Biol. Sci.* **271**, 1283-1291.

Prince, P. A. and Morgan, R. A. (1990). Diet and feeding ecology of Procellariiformes. In *Seabirds: Feeding Biology And Role In Marine Ecosystems* (ed. J. P. Croxall), pp. 135-171. Cambridge: Cambridge University Press.

Shaffer, S. A., Weimerskirch, H. and Costa, D. P. (2001). Functional significance of sexual dimorphism in wandering albatrosses, *Diomedea exulans*. *Funct. Ecol.* **15**, 203-210.

VanBuskirk, R. W. and Nevitt, G. A. (2008). The influence of developmental environment on the evolution of olfactory foraging behaviour in procellariiform seabirds. *J. Evol. Biol.* **21**, 67-76.

Warham, J. (1990). The petrels: their ecology and breeding systems. London: Academic Press.

Weimerskirch, H., Zotier, H. and Jouventin, P. (1989). The avifauna of the Kerguelen Islands. *Emu* **89**, 15-29.

Weimerskirch, H., Salamolard, M., Sarrazin, F. and Jouventin, P. (1993). Foraging strategy of wandering albatrosses through the breeding season: a study using satellite telemetry. *Auk* **110**, 325-342.

Weimerskirch, H., Gault, A. and Cherel, Y. (2005). Prey distribution and patchiness: factors in foraging success and efficiency of wandering albatrosses. *Ecology* **86**, 2611-2622.

Weimerskirch, H., Pinaud, D., Pawlowski, F. and Bost, C.-A. (2007). Does prey capture induce Area-Restricted Search? A fine-scale study using GPS in a marine predator, the wandering albatross. *Am. Nat.* **170**, 734-743.

Section 5.3

ORIENTATION AT THE COLONY BY KING PENGUIN CHICKS

CONTEXT

Short range-navigation, especially in colonial species remains very poorly understood. This is particularly true for king penguins (*Aptenodytes patagonicus*) which live in densely populated colonies that can stretch for over several kilometres and count several hundred of thousands of breeding pairs. In these dense colonies, individuals obstruct most of the local visual cues. Although sound is a good guiding cue for approaching king penguins, it has been shown to be ineffective at distances of more than nine meters for individual recognition (Lengagne *et al.*, 1999). Therefore, we still do not understand the orientation mechanisms used by colonial seabirds to reach their usual place in the colony. Visual, acoustic, olfactory or even magnetic cues may all participate, alone or in combination, with orientation behaviours.

In the following section, I present a behavioural study completed in collaboration during my second field campaign on the Kerguelen archipelago, investigating the short-range orientation abilities in king penguin chicks and the mechanisms involved.

PRESENTATION OF THE ARTICLE

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Orientation in a crowded environment: can King Penguin (*Aptenodytes patagonicus*) chicks find their crèches after a displacement?

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SUMMARY

For seabird species, the presence of conspecifics in a crowded breeding colony can obstruct locally available orientation cues. Thus, navigation to specific locations can present a challenging problem. We investigated short-range orientation in King Penguin (*Aptenodytes patagonicus*) chicks that live in a large and densely populated colony. The two main objectives were to determine whether chicks displaced to a novel location away from the colony (i) can orient towards the colony and return to their crèche and (ii) rely on visual or non-visual cues for orientation. To address these questions, a circular arena was constructed 100 m away from the colony. Chicks were released in the arena during the day and at night. After the orientation experiment in the arena, chicks were allowed to return to their home crèche, if they could. Our results showed that, during day trials, chicks preferred the half of the arena closer to the colony, but not at night. However, at night, birds spent more time on 'the colony half' of the arena if the wind blew from the colony direction. When animals were allowed to leave the arena, 98% of chicks homed during the day but only 62% of chicks homed at night. Chicks that homed at night also took longer to find their crèche. The experiments suggest that King Penguin chicks can find their crèche from a novel location. Visual cues are important for homing but, when visual cues are not present, animals are able to make use of other information carried by the wind.

Key words: short-range orientation, King Penguin, *Aptenodytes patagonicus*, chick, visual landmark.

INTRODUCTION

Navigation in many colonial species poses a riddle, given the current understanding of short-range navigation. Once an animal arrives closer to its goal, it must rely on local methods of orientation that allow fine-scale positioning (reviewed in Shettleworth, 1998). For example, pigeons (*Columba livia*), when arriving in the familiar area of the loft, pay attention to the visual features of the landscape around it; and birds that have been prevented from learning these features have problems in locating the loft (Gagliardo et al., 2007). Several species of nocturnal petrels (*Procellariiformes*) are known to use olfactory cues at the last stages of homing when they look for their burrows at night (Bonadonna and Bretagnolle, 2002). However, the presence of many conspecifics in dense colonies can obstruct any locally available cues, whether they be visual, olfactory or auditory, making short-range orientation especially challenging. The sun, stars and the Earth's magnetic field seem to be unaffected by the proximity of many conspecifics but such types of cues usually provide useful information for orientation over larger distances (at least several kilometres) (Lohmann et al., 2007; Wiltschko and Wiltschko, 1999; Wiltschko and Wiltschko, 2003; Wiltschko and Wiltschko, 2005) (but see Phillips et al., 2002). In spite of these challenges, colonial animals are successful at homing within a colony.

This conundrum of orientation is especially apparent in the case of King Penguins (*Aptenodytes patagonicus*). King penguins live in densely populated colonies that can stretch for over several kilometres along the shore and sometimes consist of as many as 300,000 breeding pairs (Aubin and Jouventin, 1998; Weimerskirch et al., 1992). The flightless nature of penguins adds an additional layer of complexity for orientation in the terrestrial breeding colony. Unlike other colonial birds, King Penguins cannot hover above the colony to obtain an aerial view of its structure and its potential landmarks. King penguins do not build nests; instead, they incubate a single egg and brood a chick on the top of their feet. Mates alternate parental duties, with one foraging at sea while the other attends to the egg or young chick. Once chicks become capable of self-thermoregulation, they are left alone in the colony while both parents forage and periodically come back for feedings. While waiting for their parents to return, chicks form groups called 'crèches' (Barrat, 1976; Stonehouse, 1960). As a result, each parent returning from a foraging trip at sea is faced with the complicated task of finding its partner on the egg or on a young chick, or an older chick in a crèche in the colony. Chicks, as well as adults, are faced with navigational challenges. In order to be found by the parents, they must remain in the crèche. These places are known as 'rendezvous' zones, and are the locations where the chicks were last fed (Dobson and Jouventin, 2003; Stonehouse, 1960). Within these zones, parents and chicks are extremely efficient at identifying each other based on their individual vocalizations (Aubin and Jouventin, 1998; Jouventin, 1982; Jouventin et al., 1999). However, it is not a trivial task for a chick to maintain fidelity to a rendezvous zone. Extensive rainfall can cause flooding, which forces chicks to temporarily abandon their places. Giant petrels (*Macronectes* spp.) that prey on chicks can split and drive crèches away from their original location or separate individuals from a crèche (Descamps et al., 2005; Le Bohec et al., 2003; Stonehouse, 1960). In addition, colony growth, disturbances created by elephant seals (*Mirounga leonina*) passing through and inclement weather conditions can all force chicks to move away from a rendezvous zone (A.P.N., J.M. and F.B., unpublished observations).

Possession of an ability to find a specific place in the colony is crucial for the survival of King Penguins but little is known about their orientation on land. On the flat relatively featureless beaches, densely distributed individuals obstruct most of the local visual cues. Sound from the colony can be a good guiding cue as King Penguins approach the colony. However, the sound has been shown to be ineffective at distances of more than ~8.8 m for individual recognition (Dobson and Jouventin, 2003; Lengagne et al., 1999). Therefore, we still do not understand the orientation mechanisms used by colonial sea birds to reach their place in the colony.

The goal of the present study was to investigate the short-range orientation abilities in King Penguin chicks and to analyse the mechanisms involved. We hypothesised that visual cues are important for orientation and designed two experiments to test this. In the arena experiment, we manipulated visual cues and observed the directional preference of chicks (towards *vs* away from the colony). Then, in the homing experiment, we tested the ability of chicks to home during the day (all visual cues present) and during the night (limited visual cues present).

METHODS

Animals and study area

We studied King Penguins (*Aptenodytes patagonicus* Miller 1778) from December 2007 to January 2008 at a colony situated at Cape Ratmanoff, Courbet Peninsula, Kerguelen Island (70 deg. 33′E, 49 deg. 42′S). This large colony stretches for over a kilometre from north to south on a flat sandy beach. Experiments were performed on chicks that were approximately 10 months old and had not yet moulted into adult plumage. We used the size and moulting condition to estimate the age of the chicks (Stonehouse, 1960; Weimerskirch et al., 1992). We based our experiments on the fact that chicks are motivated to remain in the crèches in order to be fed by the parents and to reduce predation risks (Le Bohec et al., 2005). On multiple occasions, we observed chicks that participated in the experiments being fed by a parent. It is unlikely that removal of the chicks from a crèche had an affect on their feeding. Chicks were removed from a crèche only for relatively short periods of time, never more than 90 min. During this time of the year, parents usually come to feed chicks at intervals of several days, and adults can also remain on shore for several days (Weimerskirch et al., 1992). If a parent cannot find its chick right away, it repeats its efforts (Dobson and Jouventin, 2003).

Experimental arena

A circular arena (radius 3.2 m) was located 100 m south of the three experimental crèches (94 m from crèche 1, 101 m from crèche 2 and 107 m from crèche 3, Fig. 1). It was located on a small plateau that was elevated ~2 m above the colony level, and as a result the colony was not visible from inside or just outside the arena. The arena barrier was made from fabric, with a 1 mm mesh size, that was supported by metal poles. Raising or lowering the barrier during experiments allowed manipulation of the availability of visual cues. The ground in the arena was divided into four quadrants with painted lines running through the middle of the arena in north–south and east–west directions. A release box ($0.8\times0.55\times0.4$ m) was located at the west end of the arena. This box had two opposite doors to allow the introduction of the animal inside the box through the outside door and its release inside the arena through the other door. Two observation posts were established 5 m west and 5 m east of the arena. Such placement of the experimenters minimized the potential effect on the behaviour of the chicks as no bias was introduced in a chick's choice between the north and the south sides of the arena. The observer at the west post carried out the video recording and weather measurements. The observer at the east post timed animal movements in the arena. No chicks were seen in the arena location before the experiments.

Experimental procedure

Chicks were captured with a net at their crèches and fitted with a cotton hood that prevented them from seeing. We marked and recorded the coordinates of the capture location. Animals were hand-carried towards the arena along one of two L-shaped routes (Fig. 1). At the arena, chicks were rotated three times in order to prevent chicks from using path integration on their return. Indeed, other birds such as domestic geese (*Anser* spp.) are able to home after passive displacement if they have been able to see during their outward journey but not if the view has been shielded, presumably by using path integration (Von Saint Paul, 1982).

Chicks were then marked with colored Tesa tape on their chest and back and fitted with a global positioning system (GPS) collar on the neck. The GPS collar consisted of a Velcro band to which a 13 g GPS (TechnoSmArt) was attached, which constitutes approximately 0.1% of the mass of a bird. A 1.5 m string was also attached to allow removal of the collar without recapturing birds.

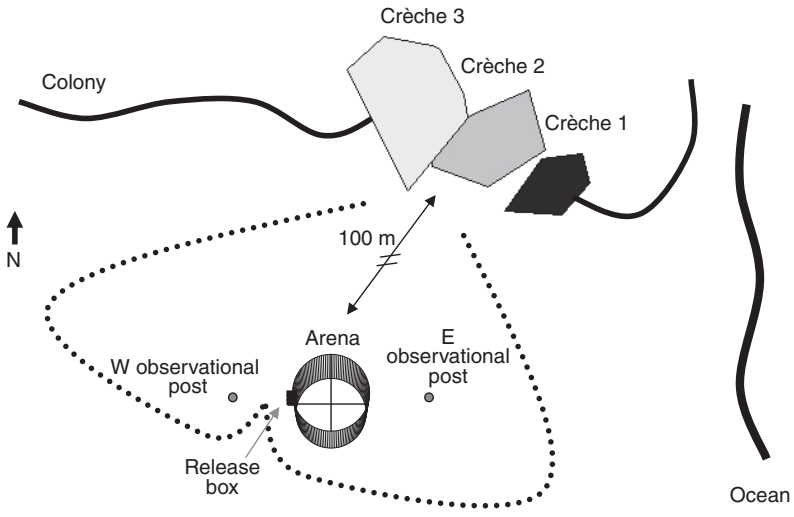

Fig. 1. Experimental arena. A circular arena was constructed 100 m away from the three experimental crèches. Chicks were carried from the crèches to the arena along one of the two routes (dotted lines) and were later released through the release box (black rectangle at the west end of the arena). The observations were conducted from two observational posts – east and west (gray circles). The thick gray line represents the edge of the colony, and the thick black line indicates the ocean. Drawing is not to scale.

Marked chicks were placed into a release box. The hood was removed once chicks were in the box, and the first door was closed. After 1 min, the second door – allowing access to the arena – was opened from a distance using a system of strings. The timing of the trial started once the chick stepped out of the box.

Before each trial, we measured the extent of any cloud cover and the direction and speed of wind at the height of the chick. Sky conditions were visually assessed using a 0–8 scale, where 0 represented a completely clear sky and 8 a completely overcast sky. To determine the direction of the wind, we used a weather vane and compass, while the wind speed was measured with a digital anemometer to the nearest decimetre per second.

Trials were organised in two parts. The first part tested the orientation of the chick in the arena, and the second part tested the ability of the chick to home to its crèche. We manipulated the availability of visual cues in two ways: (i) by conducting trials during day and night and (ii) by using different heights of the arena walls (high *vs* low barrier) during day trials. With the 'high-barrier night' configuration, the view of any landmarks was extremely limited. The 'high-barrier day' configuration (0.9 m) prevented animals from seeing the detailed landscape during the day but potentially allowed them to see the very general features of the environment, such as the land–sky outline, through the fabric mesh. The 'low-barrier day' configuration (0.7 m) was lower than the height of a chick and allowed the animal to see all the features of the landscape. Trials were partially videotaped.

Day trials

Each day trial started with the high-barrier configuration. During a 15 min period, we noted movements of the chick between quadrants and the amount of time it spent in each quadrant. After the first 15 min in the arena, the high barrier was lowered to 0.7 m (low-barrier treatment). When lowering the barrier, two researchers simultaneously approached the arena at the east and west ends and pushed the fabric down the metal poles while moving clockwise. Chicks were observed for another 15 min with the low barrier. Then the barrier was lowered completely and chicks were allowed to leave the arena. The homing chick was focally observed at a distance of approximately 50 m until it got to what was considered a homing distance from the capture location in its crèche (20 m). We also ensured that chicks remained in their home crèche for 5 min before ending the trial. At the end of the trial, one observer crawled towards the chick and pulled on the string to retrieve the GPS collar.

A total of 42 chicks were tested in day trials. All chicks expressed searching behaviour in the arena. On a few occasions, chicks escaped the arena before the end of the low-barrier test. These tended to be bold individuals that repeatedly pushed on the barrier, and eventually they were able to fall over it. As a result, 37 chicks completed high-barrier trials, and 26 completed both high- and low-barrier trials.

Night trials

Night trials were conducted between 23:30 h and 02:30 h when no sunlight was present. These trials were similar to the day trials, with a few exceptions. Animals were not subjected to the lower barrier treatment and were released after the first 15 min in the arena. Reflective tape was used to mark chicks and important locations in the colony such as crèches and capture locations.

At night, birds are more wary and removal of the GPS collar could have disturbed them. Therefore, chicks were not fitted with the GPS collar. Instead, an observer carrying the GPS device followed each chick at a distance of approximately 15 m, retracing its path. Chicks did not show any reaction to observers at such a distance. Particular care was also taken not to disturb other birds in a crèche, and the observers always walked very slowly. In the arena, chick movements were monitored by a camcorder with infrared lamps (Sony DCR-HC38 night shot and IRlamp6 from Bat Conservation and Management, Carlisle, PA, USA). Night-vision binoculars were used to follow animals outside of the arena. A total of 22 chicks were tested at night.

Any animals that failed to come back to their crèche within 1 h were recaptured and returned to their crèche.

Data analysis

To determine whether chicks can orient towards the colony, we analysed the amount of time they spent on the north and south halves of the arena as the colony was located north of it (Fig. 1). The chick was said to prefer the north half of the arena if it spent more than half of the total testing time (>450 s) there. A chi-square test was used in this analysis.

To compare the performance of the chicks between different treatments such as 'high-barrier day' *versus* 'high-barrier night', we analysed the number of seconds animals spent in the north half of the arena using Mann–Whitney tests. When the same animal performed in several treatments, such as in 'high-barrier day' and 'low-barrier day' treatments, a Wilcoxon signed ranks test was used. We also analysed whether northerly winds (coming from north, north-west or west directions), blowing approximately from the direction of the colony, and southerly winds (coming from south, south-east or south-west) had an influence on the behaviour of chicks (no north-east winds were recorded during experiments; west winds were grouped together with north and north-west winds because they also carried the noise from the colony, based on the perception of a human observer). As in the previous comparisons, we analysed the number of seconds chicks spent in the north half of the arena by means of Mann–Whitney tests.

The level of activity of a chick in the arena was assessed based on the number of transitions between each quadrant it made. A chick was considered to have moved from one quadrant to another if it had completely crossed the line separating the quadrants. The activity level was compared between different treatments by means of Mann–Whitney or Wilcoxon signed ranks tests.

After chicks left the arena, we noted whether they homed and how long it took them to reach their crèche. We compared the ability to home (Fisher exact test) and homing duration (Mann–Whitney test) between the day and night trials. To investigate the influence of sky conditions on homing, we compared homing time under a partially cloudy sky (1–7) with the homing time under a completely overcast sky (8) (Mann–Whitney test).

GPS trajectory data were collected for 33 chicks during the day and 18 chicks at night. From GPS homing data, we extracted the total length of the path (D_t) and the beeline distance (D_s) between the starting point (arena) and the end-point of the path. As a measure of the 'straightness' (optimality) of the path of a chick, we used the 'linearity index', LI, defined as $LI=D_s/D_t \leq 1$. Consequently, LI values approaching 1 would indicate animals following a path close to the shortest one. We also analysed the distribution of chicks at 10 m and 30 m from the arena, distances at which the colony was still not visible (Rayleigh and Watson U^2 tests).

Non-parametric tests were chosen for the analysis because not all data satisfied normality assumptions. All reported tests are two tailed.

RESULTS

Orientation in the arena

All chicks walked freely the around arena upon release. During day trials, more chicks preferred the north half of the arena: in the high-barrier treatment, 27 out of 36 animals and in the low-barrier treatment 21 out of 26 animals (χ^2 test, day trials, high barrier: N=36, χ^2_1=9, P=0.003; day trials, low barrier: N=26, χ^2_1=9.846, P=0.002, Fig. 2). This preference for the north side was less pronounced during night trials, where 14 out of 20 animals preferred the north half (χ^2 test, night trials, high barrier: N=20, χ^2_1=3.2, P=0.074, Fig. 2). The obstruction of visual cues with the high barrier during the day did not affect the behaviour of chicks in the arena. The amount of time chicks spent on the north side of the arena during high- or low-barrier treatment was not significantly different (Wilcoxon signed ranks test, N=26, Z=–1.486, P=0.137, Fig. 2). The activity levels of chicks were similar during day and night trials (Mann–Whitney test, N_{day}=33, N_{night}=22, U=294.5, P=0.238) and between low- and high-barrier treatments during the day (Wilcoxon signed ranks test, N=25, Z=–1.458, P=0.145).

Indeed, the direction of the wind affected the position of chicks in the arena during night trials, but not during day trials (Fig. 3). At night, birds spent more time in the north half of the arena if they experienced northerly winds (Mann–Whitney test, night trials, high barrier: N_N=16, N_S=6, U=18.500, P=0.030). During day trials, the wind direction did not significantly influence the orientation of the chicks (Mann–Whitney test, day trials, high barrier: N_N=26, N_S=11, U=100.0, P=0.153; day trials, low barrier: N_N=16, N_S=10, U=76.0, P=0.856).

Homing

The ability to home was drastically different between daytime and night-time. During the day, 98% of animals reached their crèche, whereas only 62% of animals homed at night (Fisher exact test, N=63, P<0.0001). Chicks that were able to return to their crèches at night took longer compared with the chicks that homed during the day ($median_{night}$=27 min, IR=41.50; $median_{day}$=14 min, IR=15.75; Mann–Whitney test, N_{day}=42, N_{night}=13, U=153.0, P=0.017). Examples of some paths of chicks during the night and day are shown in Fig. 4. Northerly winds coming from the colony did not improve homing time during the day (Mann–Whitney test, N_N=27, N_S=14, U=187.5, P=0.968). The effect of the wind during the night could not be determined owing to a low sample size.

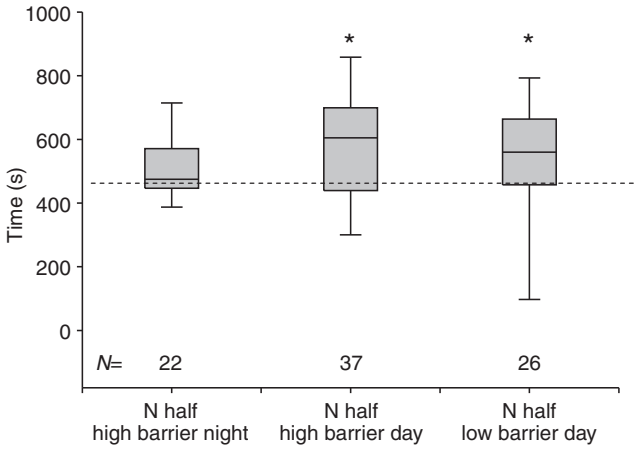

Fig. 2. Time spent in the north half of the arena. Box plots show medians and interquartile ranges for the time (s) chicks spent in the north half of the arena for the high-barrier night, high-barrier day and low-barrier day treatments. The dashed line indicates 450 s, one half of the testing time. Numbers above the *x*-axis indicate the number of animals that completed the test. The medians for each condition are the following: high-barrier night northern half=475, high-barrier day northern half=605, low-barrier day northern half=560. Significant effects are indicated with an asterisk (*).

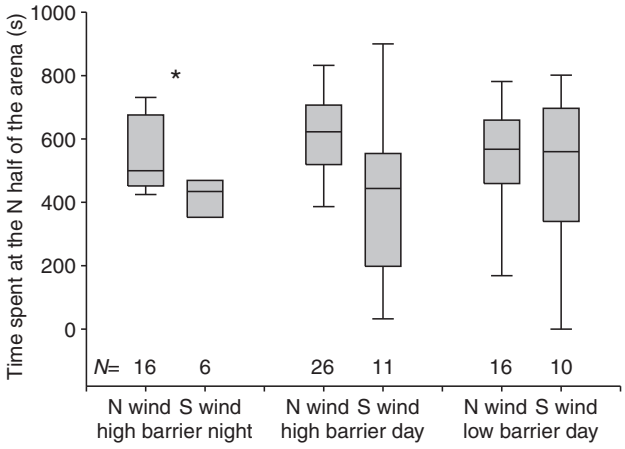

Fig. 3. The effect of wind on the orientation of chicks in the arena. Box plots show medians and interquartile ranges for the time (s) chicks spent in the north half of the arena when winds blew from the north or from the south directions for the high-barrier night, high-barrier day and low-barrier day treatments. Numbers above the *x*-axis indicate the number of animals that completed the test. The medians for each condition are the following: high-barrier night northerly wind=500, southerly wind=434; high-barrier day northerly wind=622.5, southerly winds=444, low-barrier day northerly wind=567, southerly wind=560. The significant effect is indicated with an asterisk (*).

We also investigated the influence of sky conditions on the orientation of chicks. At night, we compared the homing abilities of chicks under completely overcast (8) and partially overcast skies (1–7). Sky conditions did not seem to affect their performance (Fisher exact test, N=21, P=0.377). Some birds were able to home even under completely overcast conditions (four chicks out of six homed), whereas others did not find their crèches with star or moonlight present (four chicks out of 15 did not home). In addition, homing time was not affected by the level of cloud cover during the day (Mann–Whitney test, day trials: $N_{1–7}$=33, N_8=9, U=126.5, P=0.507). The effect of the sky condition during the night could not be determined owing to the low sample size.

Neither during day nor during night did chicks go to their crèche along straight paths (day trials: N=33, LI median=0.32, IR=0.20; night trials: N=18, LI median=0.27, IR=0.30). Linearity index comparison for chicks that reached their crèches gave no indication that day and night paths differ in their straightness (Mann–Whitney test, N_{day}=33, N_{night}=11, U=135.0, P=0.216). Already at 10 m and 30 m away from the arena chicks were oriented towards their crèches (Rayleigh test, 10 m: N_{day}=34, Z=11.16, P<0.001; N_{night}=18, Z=3.644, P=0.024; 30 m: N_{day}=34, Z=20.624, P<0.001; N_{night}=18, Z=5.608, P=0.003; Fig. 5). There was no significant difference in the distributions of homing animals at 10 m (or 30 m) during the day and during the night (Watson U^2 test, 10 m: N_{day}=34, N_{night}=18, U^2=0.074, 0.5>P>0.2; 30 m: N_{day}=34, N_{night}=18, U^2=0.115, 0.5>P>0.2).

Once in their crèches, chicks often stopped near the capture site ($median_{day}$=9.0 m, IR=10.0; $median_{night}$=5.0 m, IR=5.5). Chicks approached their capture site more closely at night than during the day (Mann–Whitney test, N_{day}=36, N_{night}=13, U=148.0, P=0.050).

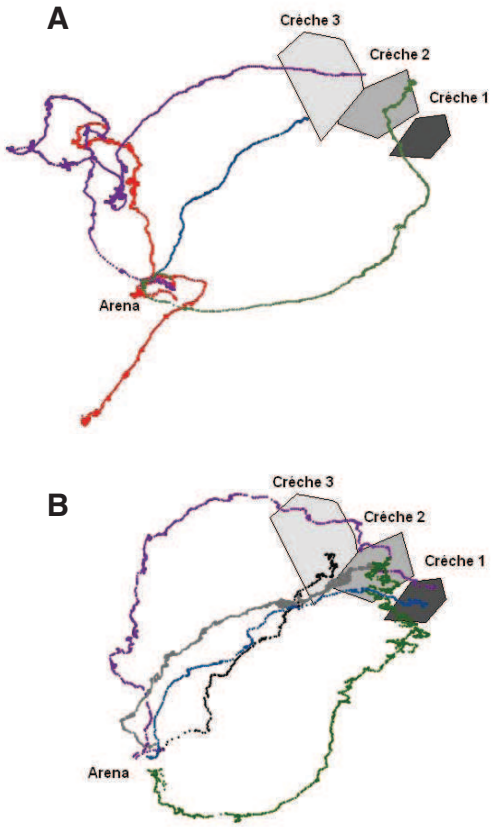

Fig. 4. The homing paths of chicks. (A) Five paths undertaken during the night and (B) five paths undertaken during day trials. Three crèches are represented by the gray polygons. In red are two paths of chicks that did not home at night. All chicks homed during the day.

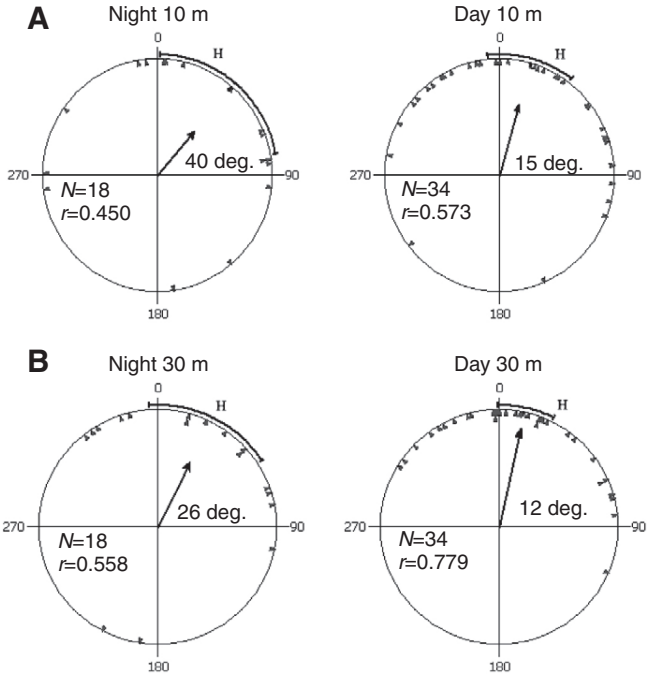

Fig. 5. Homing directions at distances of 10 m and 30 m away from the arena. Circular diagrams show the heading of chicks (blue triangles at the periphery) at (A) 10 m and (B) 30 m away from the arena during night and day trials. The arrow from the centre of the diagram indicates the mean heading direction vector. H, homing direction; *N*, number of birds; *r*, length of mean vector.

Visual examination of homing trajectories revealed no dependence of the shape of the homing path on the particular crèche from which chicks were taken or on the route along which chicks were transported to the arena.

DISCUSSION

Our experiments showed that, during day trials (high- or low-barrier configurations), more chicks preferred the half of the arena that was closer to the colony. This preference was less pronounced during night trials. However, at night, birds spent more time on the north half of the arena if the wind blew from the direction of the colony. During the day, homing success was higher, and chicks homed faster than at night. Chicks homing at night stopped closer to their original capture location than chicks homing during the day.

Our results suggest that chicks at the age of ten months can orient towards the colony and find their specific place after a passive displacement. They remain close to the colony and do not usually wander away so far inland. Accidental displacement, however, due to bad weather or predation can occur, but over smaller distances [10–40 m (A.P.N., J.M. and F.B., unpublished observations)] than our experimental displacement (100 m). In this situation, the ability to home is adaptive for survival.

At night, crèches are more condensed for thermoregulatory purposes (Le Bohec et al., 2005) and, probably, owing to nocturnal predation by giant petrels (A.P.N., J.M. and F.B., unpublished observations). This might account for the observed differences in homing accuracy between the day and night. Interestingly, the distance between the point where a chick stopped its homing trip and the original capture location is close to the average range of vocal recognition of 8.8 m reported for King Penguins (Lengagne et al., 1999). This suggests that chicks tended to return to their rendezvous zone where they could hear their parents. Chicks did not approach their crèches in a straight path, either during the day or during the night. When possible, they tried to join small groups of resting or moulting adults that could be found all around the colony. Moving from one group to another while homing might reduce predation risk through a dilution effect (Hamilton, 1971).

The drastic difference in homing rate between the day and night trials suggests that some cues that are available only during day time are especially important for orientation. The potential candidates are the sun and visual cues provided by the landscape. Our results suggest that the visibility of the sun did not affect the homing abilities of chicks. Even under completely overcast conditions during the day, chicks homed as fast as when the sky was visible. These findings are different from what was observed with Adélie Penguins (*Pygoscelis adeliae*) (Penney and Emlen, 1967; Penney and Riker, 1969). When released 340–1500 km away from the colony, Adélie Penguins were disoriented and lost under overcast skies but headed in the direction of their colony if the sun was visible. However, in the case of Adélie Penguins, the homing took place over a much larger scale – several hundreds of kilometres, whereas King Penguin chicks had to cover only 100 m. Many species of birds are known to use the sun for orientation but it usually functions as a compass to get the general bearings and not for fine-scale positioning (reviewed in Wiltschko and Wiltschko, 2003). At

the fine scale, information provided by the sun is presumably not useful or is not precise enough.

Most likely, King Penguin chicks relied on the visual cues of the landscape for orientation. The colony itself can serve as a visual landmark for the general direction of travel. However, in our experiments, chicks could not see the colony from the arena. The colony was also not visible at 10 m and 30 m away from the arena; nevertheless, the majority of the chicks headed in the correct direction.

Visual landscape landmarks other than the colony itself appear to be important for both initial orientation and homing. Even a limited view of the landscape was sufficient for initial orientation. During day trials with a high barrier, animals could potentially see through the fabric only the most prominent features of the landscape. A human observer could distinguish the outline of the horizon when looking through the fabric. The full view of the landscape, as with the low-barrier configuration, did not increase the amount of time chicks spent in the half of the arena that was closer to the colony. However, the presumably drastic reduction in visual cues due to darkness affected the behaviour of the chicks. The preference for the north half of the arena was more pronounced during day than night. Also, not all chicks homed at night, and homing time at night was longer.

The reduced ability to home and slow homing speed at night are unlikely to be explained by the difference in the activity levels as chicks were as active in the arena during day and night. Non-homing behaviour might also be attributed to the difference in the motivation to come back to a crèche. For example, chicks that were recently fed by the parents might not be as eager to return. We do not think that this is the case for two reasons. First, being in a crèche reduces predation risks (Le Bohec et al., 2005) and this should provide a strong motivation to home under any circumstances. Second, this potential bias and our random choice of birds for the experiment during the day and night should have affected the homing motivation of some chicks during the day as well but this was not observed.

Some chicks were able to home at night, and this ability seemed to be unaffected by the sky conditions. A few chicks homed even under completely overcast conditions, whereas other failed even with a moon and star light. This suggests that visual cues are not the only cues that penguins attend to. When visual cues were limited by the darkness, animals probably paid more attention to cues that were carried by the wind. At night, chicks spent more time in the north half of the arena if the wind blew from the colony direction. On the day trials with few visual cues available (high barrier), there was also a tendency for chicks to spend more time in the north half of the arena when the northerly (colony) winds blew, suggesting integration of visual and other cues whenever possible. This trend completely disappeared when full view of visual cues became available (low barrier), and the behaviour of animals was no longer influenced by the direction of the wind (Fig. 3). Surprisingly, wind direction affected the initial orientation but had no effect on the speed of homing. It is possible that cues carried by the wind are useful for choosing the general direction but are not useful for fine-scale homing.

The cues that can be carried by the wind are auditory and olfactory. Large colonies, as at Ratmanoff, produce a lot of noise. Humans can hear this colony as far as a kilometre away under favourable meteorological conditions. Knowing the superior auditory abilities of King Penguins, it seems likely that they use the colony noise as a compass, at least for initial orientation. Observations of Emperor Penguins suggest the same as later arrivals in the season have little trouble finding the colony, probably because they are guided by its sound (Jouventin, 1971). Similarly, nocturnally migrating passerines such as Eurasian Reed and Sedge warblers (*Acrocephalus* spp.) pay attention to acoustic stimuli, such as songs of conspecifics, when they look for stopover sites at night (Mukhin et al., 2008).

It is harder to assess the importance of olfactory cues as little is known about the ability of King Penguins to perceive odours. In Adélie penguins (*Pygoscelis adeliae*), the olfactory bulb constitutes 17% of the cerebral hemisphere. Other species with an olfactory bulb of similar size such as diving petrels (*Pelecanoides georgicus*) and pigeons (*Columba livia*) – both 18% – have been known to use olfaction for orientation (Bonadonna et al., 2003; Wallraff, 2004). If the olfactory bulbs of King Penguins are alike, they potentially can use odours for orientation as well. Furthermore, preliminary Y maze experiments with African Penguins (*Spheniscus demersus*) suggest that they can orient towards dimethyl sulfide (Cunningham et al., 2006).

At the scale of movements we described, information of high resolution is necessary for the animals to home. We have demonstrated that visual cues are of primary importance as their absence at night cannot be completely compensated by the other cues present. Also, the present experiments proved that chicks can use different cues for orientation, probably by integrating information from all the sources. Future experiments will test the homing abilities of chicks during the day and night when the ears of the birds are covered or when chicks are made anosmic. The systematic removal of each set of cues should reveal its relative importance for orientation.

Another interesting aspect of chick homing that remains to be investigated is the development of orientation abilities. The need to find rendezvous zones in the crèches seems to be ever present because chicks have to be fed by their parents. There must be a strong selection pressure on the development of these abilities from a very early age. However, in order to home, birds might need to develop a cognitive apparatus or simply require time to learn the surroundings of the colony. Also, it would be interesting to know whether knowledge of the colony landscape (visual, auditory or olfactory) acquired during the crèching period is used later when the adults come back to breed at the colony.

Our experiments were the first of short-range orientation in a colonial seabird. King Penguin chicks demonstrated a strong ability to home to their crèches, and, at a finer scale, to find their rendezvous zone within the crèches. Even at the age of ten months, before chicks moult and first go to sea, they already have a well-developed orientation system.

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REFERENCES

Aubin, T. and Jouventin, P. (1998). Cocktail-party effect in king penguin colonies. *Proc. R. Soc. Lond. B, Biol. Sci.* **265**, 1665-1673.

Barrat, A. (1976). Quelques aspects de la biologie et de l'ecologie du Manchot royal (*Aptenodytes patagonicus*) des Iles Crozet. *Comité National Français des Recherches Antarctiques (CNFRA)* **40**, 9-52.

Bonadonna, F. and Bretagnolle, V. (2002). Smelling home: a good solution for burrow-finding in nocturnal petrels? *J. Exp. Biol.* **205**, 2519-2523.

Bonadonna, F., Cunningham, G. B., Jouventin, P., Hesters, F. and Nevitt, G. A. (2003). Evidence for nest-odour recognition in two species of diving petrel. *J. Exp. Biol.* **206**, 3719-3722.
Cunningham, G. B., Strauss, V. and Ryan, P. G. (2006). First experimental evidence demonstrating that African penguins (Spheniscus demersus) have a functioning sense of smell. *Integr. Comp. Biol.* **46**, E29-E29.
Descamps, S., Gauthier-Clerc, M., Le Bohec, C., Gendner, J. P. and Le Maho, Y. (2005). Impact of predation on king penguin Aptenodytes patagonicus in Crozet Archipelago. *Polar Biol.* **28**, 303-310.
Dobson, F. S. and Jouventin, P. (2003). Use of the Nest Site as a rendezvous in penguins. *Waterbirds* **26**, 409-415.
Gagliardo, A., Ioale, P., Savini, M., Lipp, H. P. and Dell'Omo, G. (2007). Finding home: the final step of the pigeons' homing process studied with a GPS data logger. *J. Exp. Biol.* **210**, 1132-1138.
Hamilton, W. D. (1971). Geometry for the selfish herd. *J. Theor. Biol.* **31**, 295-311.
Jouventin, P. (1971). Comportement et structure sociale chez le manchot empereur. *La Terre et la Vie* **25**, 510-586.
Jouventin, P. (1982). *Visual and Vocal Signals In Penguins, Their Evolution And Adaptive Characters*. Berlin und Hamburg: Verlag Paul Parey.
Jouventin, P., Aubin, T. and Lengagne, T. (1999). Finding a parent in a king penguin colony: the acoustic system of individual recognition. *Ani. Behav.* **57**, 1175-1183.
Le Bohec, C., Gauthier-Clerc, M., Gendner, J. P., Chatelain, N. and Le Maho, Y. (2003). Nocturnal predation of king penguins by giant petrels on the Crozet Islands. *Polar Biol.* **26**, 587-590.
Le Bohec, C., Gauthier-Clerc, M. and Le Maho, Y. (2005). The adaptive significance of creches in the king penguin. *Ani. Behav.* **70**, 527-538.
Lengagne, T., Jouventin, P. and Aubin, T. (1999). Finding one's mate in a king penguin colony: efficiency of acoustic communication. *Behaviour* **136**, 833-846.
Lohmann, K. J., Lohmann, C. M. F. and Putman, N. F. (2007). Magnetic maps in animals: nature's GPS. *J. Exp. Biol.* **210**, 3697-3705.
Mukhin, A., Chernetsov, N. and Kishkinev, D. (2008). Acoustic information as a distant cue for habitat recognition by nocturnally migrating passerines during landfall. *Behav. Ecol.* **19**, 716-723.
Penney, R. L. and Emlen, J. T. (1967). Further experiments on distance navigation in Adelie Penguin *Pygoscelis adeliae*. *Ibis* **109**, 99-109.
Penney, R. L. and Riker, D. K. (1969). Adelie Penguin orientation under Northern Sun. *Antarct. J. US* **4**, 116-117.
Phillips, J. B., Freake, M. J., Fischer, J. H. and Borland, S. C. (2002). Behavioral titration of a magnetic map coordinate. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **188**, 157-160.
Shettleworth, S. J. (1998). *Cognition, Evolution, and Behavior*. New York: Oxford University Press.
Stonehouse, B. (1960). The king penguin *Aptenodytes patagonica* of South Georgia. I. Breeding behaviour and development. In *Falkland Islands Dependencies Survey.* Scientific Reports, 23.
Von Saint Paul, U. (1982). Do geese use path integration for walking home? In *Avian Navigation* (eds. F. Papi and H. G. Wallraff), pp. 298-307. Berlin: Springer-Verlag.
Wallraff, H. G. (2004). Avian olfactory navigation: its empirical foundation and conceptual state. *Ani. Behav.* **67**, 189-204.
Weimerskirch, H., Stahl, J. C. and Jouventin, P. (1992). The breeding biology and population-dynamics of King Penguins Aptenodytes-Patagonica on the Crozet-Islands. *Ibis* **134**, 107-117.
Wiltschko, R. and Wiltschko, W. (1999). The orientation system of birds–I. Compass mechanisms. *J. Ornithol.* **140**, 1-40.
Wiltschko, R. and Wiltschko, W. (2003). Avian navigation: from historical to modern concepts. *Ani. Behav.* **65**, 257-272.
Wiltschko, W. and Wiltschko, R. (2005). Magnetic orientation and magnetoreception in birds and other animals. *J. Comp. Physiol. A Neuroetho. Sens. Neural Behav. Physiol.* **191**, 675-693.

Section 5.4

REFERENCES

Lengagne T., Jouventin P. & Aubin T. (1999). Finding one's mate in a king penguin colony: efficiency of acoustic communication. *Behaviour* **136**: 833-846.

Nevitt G.A. (2000). Olfactory foraging by Antarctic procellariiform seabirds: life at high Reynolds numbers. *Biological Bulletin* **198**: 245-253.

Nevitt G.A., Losekoot M. & Weimerskirch H. (2008). Evidence for olfactory search in wandering albatross, *Diomedea exulans*. *Proceedings of Natural Academy of Science USA* **105**: 4576-4581.

Chapter 6

CONCLUSION

AIM & CONTENT

As described in Chapter 1, the presentation of this thesis as a series of papers means that the various results reported have been discussed specifically in the different journal articles. This final chapter integrates these findings and their general eco-evolutionary implications into the current understanding of avian olfaction and avian chemical communication, and emphasises promising avenues for future research.

From Z-Man (2009)

Section 6.1

CONCLUSIONS & PERSPECTIVES

Note from the author
As this thesis was going to print, personal communication with adjunct professor Jian-Xu Zhang has brought to my attention some of his recent work on avian chemosignals which is remarkably closely related to the topic of this thesis. The results from these peer-reviewed publications are not considered in this conclusion but are discussed in Appendix A2.

A century after the first evidence (and/or speculations) regarding avian olfaction (Benham, 1906; Pycraft, 1910), the existence of olfactory capabilities in virtually all birds is generally accepted. The old claim that birds are anosmic or microsmatic (Soudek, 1927; Walter, 1943) has indeed been convincingly refuted by both anatomical, physiological and behavioural evidence (see Roper, 1999 for a review). Rather than being denied however, the contribution of olfaction to avian behaviours has been largely ignored by ornithologists. This is probably because the general lifestyle of birds seems to emphasise essentially their vocal and visual functions (Wenzel, 1973). Another explanation is that research on avian olfaction has long been restricted to the responses of a few species (vultures, kiwis, pigeons, and procellariiform seabirds) to environmental cues only (Benham, 1906; Stager, 1967; Wenzel, 1968; Grubb, 1972; Papi *et al.*, 1974; Shallenberger, 1975; Benvenuti *et al.*, 1977; Smith & Paselk, 1986). This traditional perspective, essentially limited to olfactory foraging (see Nevitt, 2000) and olfactory navigation (see Wallraff, 2004), is illustrated by the work presented in Chapter 5. The role and importance of biogenic chemosignals in avian ecology, on the other hand, has been essentially overlooked.

This conclusion reviews and discusses how recent advances in the field, and particularly the results from this thesis, should invigorate the field of avian ecology.

INTERSPECIFIC INTERACTIONS: PREDATION, PARASITISM & COMPETITION

Many avian taxa are strongly scented (Weldon & Rappole, 1997); a characteristic that ornithologists have essentially interpreted as a mechanism of chemical defense against predators or ectoparasites (Hagelin & Jones, 2007). There are, however, only a few robust examples of predator-deterring avian scents. Some birds from the *Pitohui* or *Ifrita* genera, for instance, emit a sour odour (Dumbacher *et al.*, 1992; Dumbacher *et al.*, 2000) that may deter potential predators such as snakes, raptors and some arboreal marsupials. Importantly, tissues of these birds (in particular feather and skin) contain some batrachotoxins, a potent toxic alkaloid making them poisonous. The acrid odour emitted by these birds, whose nature and origin remains unclear, could thus serve a chemical aposematic function (Diamond, 1992). Similarly, the foul-scented uropygial secretion of green woodhoopoes that is released upon disturbance (see Hagelin & Jones, 2007 for references), is efficient at deterring feline and lizard predators (du Plessis et al., unpublished results in Burger *et al.*, 2004). Another aspect of predation, that can significantly affect the fitness of ground-nesting birds in particular, is nest-depredation by olfactory-searching mammals (Whelan *et al.*, 1994). In that regard, the switch from monoester to diester preen waxes observed in many species of sandpipers during the breeding season has been hypothesised to favour olfactory crypticism at the nest (Reneerkens *et al.*, 2002). Consistent with this hypothesis, mixtures of monoesters are more easily detected by a dog than mixtures of the less volatile diesters (Reneerkens *et al.*, 2005).

Examples of avian chemical defense against ectoparasites are more abundant. A review of this topic, which is beyond the scope of this conclusion, can be found in Hagelin & Jones (2007). Interestingly, some of the predator-deterring chemicals mentioned above are also efficient at repelling ectoparasites. This is the case, for example, of *Pitohui* feathers (Dumbacher, 1999) and of the secretions of the red-billed woodhoopoe (Law-Brown, 2001). A particularly comprehensive case of avian chemical defense against ectoparasites is the study of the tangerine-scented crested auklets (Hagelin *et al.*, 2003). Evidence shows that the chemicals present on the feathers of these birds can efficiently deter ectoparasites (Douglas, 2008; Hagelin, 2008). What is more, the increased intensity of the citrusy scent emitted during the breeding season suggests that it may serve as an olfactory ornament during courtship and mate-choice (Hagelin, 2007; see also next section).

As receivers, birds can use their olfactory capabilities in interspecific interactions such as predation and parasitism. For example, blue tits are able to detect predator-related chemical cues and show antipredatory behaviours when exposed to such cues (Amo *et al.*, 2008). Similarly, house finches respond to the odour of mammalian faeces while feeding by reducing the length of feeding bouts (Roth *et al.*, 2008). Heterospecific olfactory cues can also affect parental behaviours as illustrated by a study on dark-eyed juncos (Whittaker *et al.*, 2009). The authors observed that females, from a population commonly subjected to brood parasitism by brown-headed cowbirds, reduced incubation bout length (i.e. parental investment) following the application of heterospecific preen oils onto their nest.

None of the above examples, however, can be considered as *true* chemical communication because none involves a transfer of information that is mutually beneficial for both the emitter and the receiver of the chemosignal (Bradbury & Vehrencamp, 1998). The results presented in this thesis on the existence and importance of a Species chemosignal in hypogean petrels (Bonadonna & Mardon, 2010; Mardon *et al.*, 2010) are particularly valuable in this sense.

Interspecific variations in the chemical content of procellariiforms' uropygial secretion have been previously studied (Jacob, 1976; Jacob & Hoerschelmann, 1982; Jacob & Ziswiler, 1982). Jacob's investigation, however, was primarily directed at elucidating and defining chemotaxonomical relationships among birds. He therefore elegantly restricted his interspecific comparison of the complex preen secretions to the analysis of the relative ratio of the different methyl-substituted acids (Fig.6-1). Consistent with Jacob's work, our comprehensive analysis of the preen waxes from blue petrels and Antarctic prions confirm the existence of a clear Species chemosignal in these secretions (Mardon *et al.*, 2010). What is more, our behavioural results show that blue petrels can discriminate between conspecific and heterospecific odours, and our demographic data indicate that this capability may contribute to nesting decisions (Bonadonna & Mardon, 2010). Indeed, several hypogean petrels breed sympatrically on Subantarctic islands (Cherel *et al.*, 2002a; Cherel *et al.*, 2002b; Cherel *et al.*, 2006) which expose them to a certain degree of interspecific competition for nesting sites (i.e. burrows). For various reasons (see Section 4.2), the life-history and breeding ecology of these species should favour a clear broadcasting of burrow ownership so that

prospective breeders avoid using heterospecific nests which are more risky. Importantly, this broadcasting of a Species signal is beneficial to both the sender (i.e. the original owner of the burrow that needs to find its nest available when it returns to breed), and the receiver (i.e. a prospective breeder that is looking for an empty and safe burrow to complete its breeding attempt). These results provide therefore an original illustration of interspecific avian chemical communication.

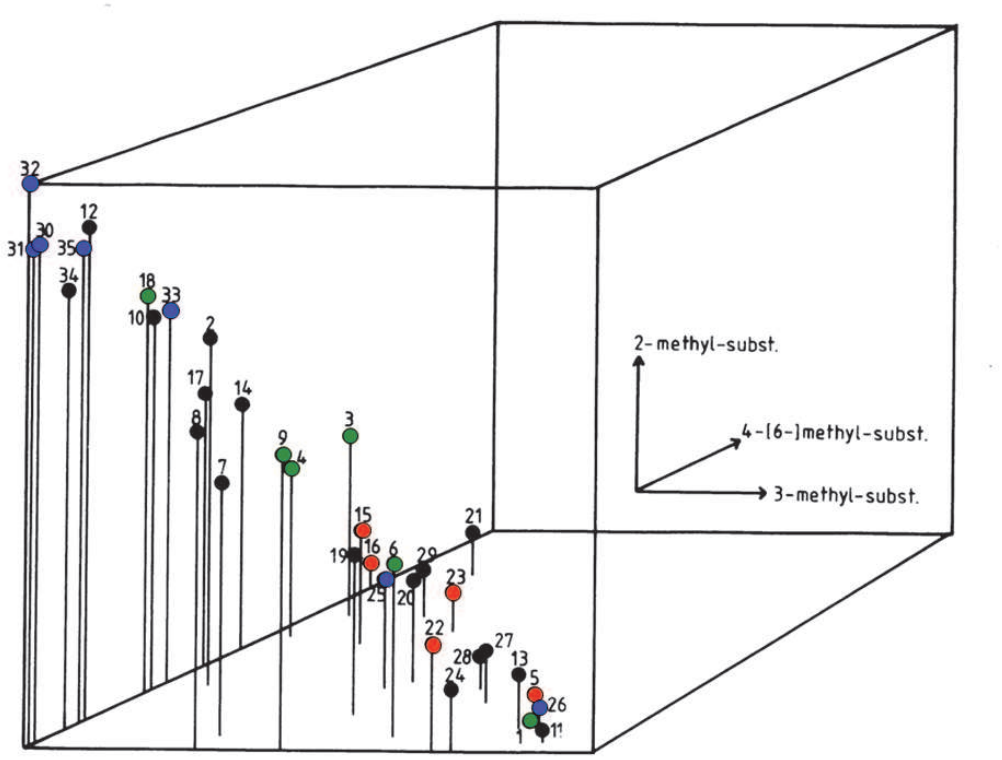


Figure 6-1 (adapted from Jacob & Ziswiler, 1982): Chemical composition of the wax acids from some procellariiform species. Each species is symbolised by a circle: black=not nesting on Kerguelen; blue=surface-nesting on Kerguelen; green=burrow-nesting on Kerguelen; red=sympatric burrow-nesting on Ile Verte (Kerguelen). *(1) Pelecanoides urinatrix; (2) Fregatta grallaria; (3) Fregatta tropica; (4) Garrodia nereis; (5) Oceanites oceanicus; (7) Procellaria aequinoctialis; (8) Procellaria westlandica; (9) Procellaria parkinsoni; (10) Procellaria cinerea; (11) Puffinus huttoni; (12) Puffinus griseus; (13) Puffinus assimilis; (14) Puffinus tenuirostris; (15) Puffinus gavial; (16) Halobaena caerulea; (17) Pterodroma lessonii; (18) Pterodroma inexpectata; (19) Pterodroma brevirostris; (20) Pterodroma cooki; (21) Pachyptila turtur; (22) Pachyptila crassirostris; (23) Pachyptila desolata; (24) Pachyptila belcheri; (25) Pachyptila vittata; (26) Macronectes giganteus; (27) Macronectes halli; (28) Thalassoica Antarctica; (29) Fulmarus glacialis; (30) Fulmarus glacialoides; (31) Diomedea chrysostoma; (32) Diomedea melanophris; (33) Diomedea epomophora; (34) Diomedea exulans; (35) Diomedea cauta; (36) Phoebetria palpebrata.*

The contribution of a Species chemosignal to petrels' interspecific interactions suggests that this signal is more than a physiological side-effect of genetic divergence between species. From an evolutionary perspective, processes driving the evolution of chemosignals involved in species recognition are considered to be selection for pre-mating reproductive isolation, for closely-related species, and interspecific competition for communication channels (Johansson & Jones, 2007). The possible involvement of chemosignals in the reproductive isolation and speciation events of petrels is entirely speculative at this stage but offers a fascinating avenue of future phylogenic/chemical research. In contrast, the competitive interactions of closely-related, and sympatrically breeding, burrow-nesting petrels offers a realistic context for the evolution of chemical divergence between these species. What is more, this hypothesis could be tested by comparing the chemical distance between several species of procellariiform while accounting for phylogenetic effects. Indeed, a prediction of competition-driven chemical divergence is that chemosignals of sympatric and/or sister-taxa should display particularly dramatic differences (Johansson & Jones, 2007). Jacob's chemotaxonomical work on the methyl-substitution of acids contained within procellariiform's uropygial secretions provides, in that regard, a preliminary illustration. Figure 6-1 (adapted from Jacob & Ziswiler, 1982) indeed shows that burrowing, and sympatrically breeding, species (red dots) are chemically more divergent than families of surface nesters such as giant petrels (25-26) or albatrosses (30-35). Further exploration of the relationship between interspecific interactions and the divergence of chemical signals in petrel seabirds may therefore prove particularly informative.

INTRASPECIFIC INTERACTIONS: SEXUAL BEHAVIOURS & RECOGNITION

Several recent studies have drawn attention to the contribution of chemosignals to avian intraspecific interactions, with a main emphasis on avian sexual behaviours.

Chemical communication & avian sexual behaviours

In 1979, a study reported the existence of a chemical sexual dimorphism in the uropygial waxes of domestic ducks (Jacob *et al.*, 1979). During the breeding season,

female ducks (but not males) shift their secretions from monoester to diester waxes, under the hormonal control of estradiol (Bohnet *et al.*, 1991). This chemical dimorphism was later hypothetically related to the inhibition of sexual behaviours observed in male ducks whose olfactory nerves had been sectioned (Balthazart & Schoffeniels, 1979). A similar contribution of uropygial chemicals to sexual behaviours has also been reported in domestic chickens (Hirao *et al.*, 2009). In this study, intact males courted and copulated significantly more with control females than with uropygial glandectomised females. This preference was, however, not expressed by anosmic males. An olfactory-mediated control of avian sexual behaviours is further supported by the observation that the brain activation of male Japanese quails, normally induced by sexual interaction with a female, is significantly affected by olfactory deprivation (Balthazart & Taziaux, 2009). Together, these results strongly suggest that the uropygial secretions of some avian taxa contain olfactory cues that favour, possibly through activation of key brain areas, the expression of sexual behaviours such as courtship displays, mounts and copulations.

Interestingly, the latter hypothesis could account for some ambiguous results on the role of olfactory cues in the sexual behaviours of seabirds. The plumage of the highly-social crested auklet, for example, emits a strong citrusy odour during the breeding season (Hagelin *et al.*, 2003), when the species exhibit some characteristic courtship behaviours involving essentially an intertwining of necks and the burying of bills in the nape and neck feathers (Jones & Hunter, 1993). This has led authors to suggest that the plumage odour of these birds may serve as an olfactory ornament (Hagelin *et al.*, 2003; Jones *et al.*, 2004; Hagelin, 2007) and that the so-called 'ruff-sniff' display could constitute a case of alloanointing of ectoparasite-repelling substances between prospective mates (Douglas, 2008). According to the authors, chemicals present on the feathers could act as a sexual trait (on top of an anti-parasite function – see previous section) whereby the most scented individuals are also the most attractive. Yet, field experiments using scented models provided ambiguous results as only male scented models (but not female) were approached more than controls. In contrast with artificial visual ornaments (Jones & Hunter, 1993), artificial scents also failed to elicit more stereotyped sexual displays (Jones *et al.*, 2004). However, such results may be expected if, as hypothesised above, sexual olfactory cues in birds act essentially as brain

activators and/or in conjunction with other displays (Hagelin & Jones, 2007) to facilitate the expression of sexual behaviours.

Similarly, there is no evidence, at this stage of the research, that the Sex chemosignal identified in petrels (Mardon *et al.*, 2010; Mardon *et al.*, unpublished manuscript) affects their sexual behaviours. Indeed, field experiments did not find any supportive evidence of olfactory sexual discrimination capabilities, whether in Antarctic prions (Bonadonna *et al.*, 2009) or in blue petrels (see Section 4.4). Again, such results may be expected if sexual olfactory cues act through a targeted brain activation to elicit specific and context-dependent sexual behaviours. Note that the very presence of this Sex chemosignal in petrels' secretions supports claims of a behavioural function, as such a signal is absent from many other birds' secretions (e.g. Piersma *et al.*, 1999; Burger *et al.*, 2004; Montalti *et al.*, 2005).

Finally, it is worth mentioning that most current evidence on the role of olfactory cues on avian sexual behaviours (with the exception of crested auklets - Jones *et al.*, 2004) indicates that the associated signalling is female-biased. In the above examples, male ducks, chickens and japanese quails all responded to the deprivation of access to a female signal (Balthazart & Schoffeniels, 1979; Balthazart & Taziaux, 2009; Hirao *et al.*, 2009). In addition, the two instances of avian chemical sexual dimorphisms identified to date (ducks and blue petrels) consist of female-specific variations of the chemosignal (Jacob *et al.*, 1979; Mardon *et al.*, 2010). This female bias in sexual chemosignals may originate from the genetic mechanism of sex determinism in birds. Indeed, avian gonosomes work in an opposite pattern to mammals, with males being homogametic (ZZ) while females are heterogametic (ZW) (Fridolfsson and Ellegren 1999). Further work on this particular question should prove particularly valuable.

Chemical communication & individual recognition

Recent research on the homing mechanisms of petrels has proved a fruitful gateway for the study of avian chemical communication as it has revealed the existence of social odours in these birds (see Section 4.1). Indeed, petrels are to date the only birds shown to possess olfactory capabilities of individual recognition beyond self/non-self discrimination. Following the initial demonstration of self-odour recognition by European storm-petrel chicks (De Leon *et al.*, 2003), three species of burrow-nesting

petrels (Antarctic prions, Wilson's storm petrels, and blue petrels) were later shown to recognise not only their own odour, but also the odour of their mate (Bonadonna & Nevitt, 2004; Jouventin *et al.*, 2007; Mardon & Bonadonna, 2009). The chemical results obtained in blue petrels (see Chapter 3), as well as a preliminary study on the feather lipids of Antarctic prions (Bonadonna *et al.*, 2007), show that petrels' individual chemical signatures are secreted through the uropygial waxes, and are still present on the plumage of the birds. This work not only provides a robust chemical basis to petrels' recognition behaviours, but it also contributes to elucidating the chemical nature of these chemosignals.

Chemical signatures in the uropygial secretions of blue petrels are not made of individually-specific bouquets of compounds. Instead they appear to involve the relative proportions of as many as 63 compounds present in most individuals (Mardon *et al.*, 2010). Similar quantitatively-coded chemical signatures have been reported in many mammals including mice (Singer *et al.*, 1997), monkeys (Smith *et al.*, 2001), pandas (Hagey & MacDonald, 2003), bats (Safi & Kerth, 2003) and hyenas (Burgener *et al.*, 2009); in humans, however, individuality appears to be best reflected in the qualitative variations of compounds within the axillary sweat (Penn *et al.*, 2007).

Importantly, birds (and petrels in particular) are well equipped to deal with the level of olfactory complexity suggested in this thesis. The structure of the avian neuro-olfactory system is indeed similar to that of most vertebrates (although birds do not have vomeronasal organs - Bertmar, 1981). Odour perception and discrimination relies on olfactory sensory neurons distributed over the epithelium of the third nasal chamber (Roper, 1999). These olfactory neurons possess a particular receptor protein, different from neuron to neuron, which determines their range of responsiveness. These ranges are not excessively specific, so a given odorant molecule can activate different types of neuroreceptor and one neuroreceptor may respond to several different odorants (Brennan & Kendrick, 2006). The neurons then project their axons in the olfactory bulbs, where each type of neuron (i.e. all neurons sharing the same receptor protein) connects to a particular olfactory glomerulus. A complex mixture of odorants, such as a personal scent, will quantitatively activate a particular subset of olfactory neuroreceptors, which in turn will cause a particular pattern of glomeruli activation across the olfactory bulb (Brennan & Kendrick, 2006). These patterns of glomerular activation allow the discrimination of complex chemical variations and were found, in

mice, to be predictive of both the differences in urine compositions, and the genetic differences among urine donors (Schaefer *et al.*, 2002). Therefore, this olfactory neuro-organisation offers sufficient sensitivity for the type of chemical coding reported in this thesis.

ORIGIN OF AVIAN CHEMOSIGNALS & IMPLICATIONS

The complex and polymorphic nature of petrels' chemical signatures raises questions about their determinism, a topic which has major implications for avian chemical communication.

Origin of chemosignals

Volatile signals emitted by birds are essentially limited to the odour of the plumage (but see Hagelin & Jones, 2007 for other sources) which is a particular form of olfactory signal. Indeed, this complex volatile emission is not released in a particular behavioural context, or aimed at a particular individual. Instead, it is a durable and passive feature which permanently accompanies its bearer. Furthermore, personal odours, unlike pheromones, rarely trigger a specific response from the receiver (Karlson & Lüscher, 1959), nor are they suited for immediate and mutually responsive communication. This is why some authors refer to them as *state* signals, i.e. signals that remain 'on' for a prolonged time; as opposed to *event* signals, which are typically short-term manifestations (Hauser, 1996).

Existing evidence in chickens (Hirao *et al.*, 2009) and blue petrels (Chapter 3) now clearly indicates that the social signals contained in birds' odour have an endogenous origin. This is a particularly important finding because it makes these odours appropriate for the transport of subtle physiological and genetic information, such as the one typically used by animals for social recognition and quality assessments. Endogenous chemosignals have indeed been found to carry information such as species, group, sex or individual identities (Safi & Kerth, 2003; Penn *et al.*, 2007; Burgener *et al.*, 2008; Whittaker *et al.*, 2009), social status (Moore *et al.*, 1997), physiological or health status (Zala *et al.*, 2004), relatedness (Ables *et al.*, 2007) or even particular genotypes (Reusch *et al.*, 2001).

Animal chemosignals used for recognition are generally considered to be based essentially on genotypic variation. Other pathways may indeed be subjected to environmental and physiological influences, such as aging, changes in diet or microbial flora (Brennan & Kendrick, 2006). The existence of complex chemical signature in petrels (Chapter 3), and their contribution to recognition behaviours, therefore advocates for a genetic determinism (at least partial) of these signals in birds. This hypothesis cannot, however, be definitively resolved at this stage and future experiments, designed to disentangle the influences of environment and heredity (e.g. cross-fostering experiments – see Section 4.4), should address this question.

Implications for mate choice & other behaviours

Any genetic determinism of identity signals should be polymorphic enough to allow a considerable amount of phenotypic variation among individuals. This is why biologists have considered and explored a possible role of the Major Histocompatibility Complex (MHC) in these processes (Penn, 2002; Johansson & Jones, 2007). The relationship between chemical signals, the MHC, and behaviours such as individual recognition and mate choice is rich and complex (Tregenza & Wedell, 2000; Penn, 2002; Johansson & Jones, 2007). MHC-dependent mating preferences, for example, may provide two different benefits: a better immunocompetence to the offspring and/or a way to avoid mating with genetically similar partners (Penn, 2002). These processes, although essentially studied in mammals (Brennan & Kendrick, 2006), are directly relevant to birds (Zelano & Edwards, 2002). Indeed, MHC-dependent mating preferences have been reported for many avian species (Johnsen *et al.*, 2000; Freeman-Gallant *et al.*, 2003; Richardson *et al.*, 2005; Bonneaud *et al.*, 2006; Gillingham *et al.*, 2009) (but see also Ekblom *et al.*, 2004; Westerdahl, 2004) although the proximate mechanisms leading to these preferences are still unknown.

Choosing a mate on genetic grounds requires the ability to somehow contrast one's own genetic makeup to that of a potential mate. Current evidence suggests that this task is most achievable through olfactory cues. MHC-dependent preferences based on chemical assessment have indeed been observed in fish and mammals (Wedekind & Furi, 1997; Reusch *et al.*, 2001; Penn, 2002). What is more, among the many pathways in which MHC can affect sexual traits (Fig.6-2), its influence on individual odours

provides the most direct (and hence reliable) route. To date the processes remain undocumented in birds, essentially because of the limited amount of behavioural and chemical data available on avian chemosignals (Hagelin & Jones 2007). The chemical elucidation of complex chemical signatures in petrel seabirds (Bonadonna *et al.*, 2007; Mardon *et al.*, 2010; Mardon *et al.*, unpublished manuscript) is an important contribution to the field in this regard. Research investigating the existence of MHC-based mating preferences in petrels is currently being undertaken and a fascinating prospect of avian chemical communication is therefore to manage the integration of genetic and chemical data to unveil the origin of social scents.

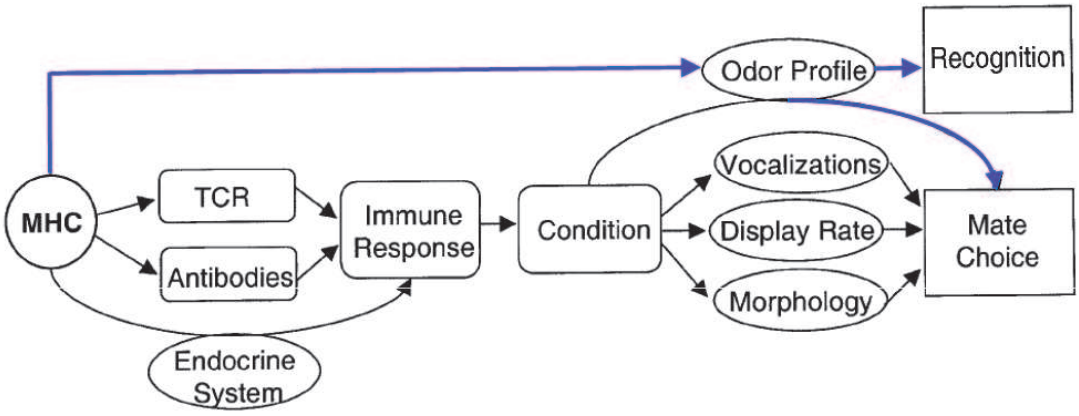


Figure 6-2 (adapted from Zelano & Edwards, 2002): Flow chart outlining the major relationships between Major Histocompatibility Complex variation, individual condition, mate choice, and recognition. MHC role in modulating the immune response (T-cell repertoire and antibodies) is indicated on the left. MHC could influence mate choice through condition-dependent traits or through odour profiles as for recognition. The linear nature of the 'MHC-odours-mate choice' pathway is highlighted in blue.

CONCLUSION

Biogenic chemical substances are a significant aspect of avian biology, and have been shown to contribute to various interspecific (e.g. predation, parasitism, competition) and intraspecific (e.g. sexual behaviours, recognition) interactions. Among these, social signals used for communication are essentially limited to the plumage odour, a *state* signal that permanently accompanies the emitter. Avian chemical communication (as it is known today) is therefore principally concerned with the

transfer of information supporting neurophysiological activation (as in Japanese quails), social recognition (as in petrels) and/or possibly genetically-based quality assessments.

The results presented in this thesis have contributed to a better understanding of these questions by providing a multidisciplinary and comprehensive investigation of chemical communication in petrel seabirds.

Section 6.2

REFERENCES

Ables E.M., Kay L.M. & Mateo J.M. (2007). Rats assess degree of relatedness from human odors. *Physiology & Behavior* **90**: 726-732.

Amo L., Galván I., Tomás G. & Sanz J.J. (2008). Predator odour recognition and avoidance in a songbird. *Functional Ecology* **22**: 289-293.

Balthazart J. & Schoffeniels E. (1979). Pheromones are involved in the control of sexual behavior in birds. *Naturwissenschaften* **66**: 55-56.

Balthazart J. & Taziaux M. (2009). The underestimated role of olfaction in avian reproduction? *Behavioural Brain Research* **200**: 248-259.

Benham W.B. (1906). The olfactory sense in Apteryx. *Nature* **74**: 222-223.

Benvenuti S., Fiaschi V. & Foà A. (1977). Homing behaviour of pigeons disturbed by application of an olfactory stimulus. *Journal of Comparative Physiology A* **120**: 175-179.

Bertmar G. (1981). Evolution of vomeronasal organs in vertebrates. *Evolution* **35**: 359-366.

Bohnet S., Rogers L., Sasaki G. & Kolattukudy P.E. (1991). Estradiol induces proliferation of peroxisome-like microbodies and the production of 3-Hydroxy fatty acid diesters, the female pheromones, in the uropygial glands of male and female mallards. *The Journal of Biological Chemistry* **266**: 9795-9804.

Bonadonna F., Caro S.P. & Brooke M.d.L. (2009). Olfactory Sex Recognition Investigated in Antarctic Prions. *PLoS ONE* **4**: e4148.

Bonadonna F. & Mardon J. (2010). One house two families: petrel squatters get a sniff of low-cost breeding opportunities. *Ethology* **116**: 176-182.

Bonadonna F., Miguel E., Grosbois V., Jouventin P. & Bessiere J.-M. (2007). Individual odor recognition in birds: an endogenous olfactory signature on petrels' feathers? *Journal of Chemical Ecology* **33**: 1819-1829.

Bonadonna F. & Nevitt G.A. (2004). Partner-specific odor recognition in an Antarctic seabird. *Science* **306**: 835.

Bonneaud C., Chastel O., Federici P., Westerdahl H. & Sorci G. (2006). Complex Mhc-based mate choice in a wild passerine. *Proceedings of the Royal Society of London Series B-Biological Sciences* **273**: 1111-1116.

Bradbury J.W. & Vehrencamp S.L. (1998). Principles of animal communication. Sinauer Associates, Inc., Sunderland, Massachusetts.

Brennan P.A. & Kendrick K.M. (2006). Mammalian social odours: attraction and individual recognition. *Philosophical Transactions of the Royal Society of London B Biological Sciences* **361**: 2061-2078.

Burgener N., Dehnhard M., Hofer H. & East M.L. (2009). Does anal gland scent signal identity in the spotted hyaena? *Animal Behaviour* **77**: 707-715.

Burgener N., East M.L., Hofer H. & Dehnhard M. (2008). Do spotted hyena scent marks code for clan membership? In: Hurst J.L., Beynon R.J., Roberts S.C., Wyatt T.D. (eds). *Chemical Signals in Vertebrates*, vol XI. Springer, New York: p.169-177.

Burger B.V., Reiter B., Borzyk O. & du Plessis M.A. (2004). Avian exocrine secretions I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. *Journal of Chemical Ecology* **30**: 1603-1611.

Cherel Y., Bocher P., De Broyer C. & Hobson K.A. (2002a). Food and feeding ecology of the sympatric thin-billed *Pachyptila belcheri* and Antarctic *P.desolata* prions at Iles Kerguelen, Southern Indian Ocean. *Marine Ecology-Progress Series* **228**: 263-281.

Cherel Y., Bocher P., Trouve C. & Weimerskirch H. (2002b). Diet and feeding ecology of blue petrels *Halobaena caerulea* at Iles Kerguelen, Southern Indian Ocean. *Marine Ecology-Progress Series* **228**: 283-299.

Cherel Y., Phillips R.A., Hobson K.A. & McGill R. (2006). Stable isotope evidence of diverse species-specific and individual wintering strategies in seabirds. *Biology Letters* **2**: 301-303.

De Leon A., Minguez E. & Belliure B. (2003). Self-odour recognition in European storm-petrel chicks. *Behaviour* **140**: 925-933.

Diamond J.M. (1992). Rubbish birds are poisonous. *Nature* **360**: 19-20.

Douglas H.D., III (2008). Prenuptial perfume: Alloanointing in the social rituals of the crested auklet (*Aethia cristatella*) and the transfer of arthropod deterrents. *Naturwissenschaften* **95**: 45–53.

Dumbacher J., Spande T.F. & Daly J.W. (2000). Batrachotoxin alkaloids from passerine birds: A second toxic bird genus (*Ifrita kowaldi*) from New Guinea. *Proceedings of Natural Academy of Science USA* **97**: 12970-12975.

Dumbacher J.P. (1999). Evolution of Toxicity in Pitohuis: I. Effects of Homobatrachotoxin on Chewing Lice (Order Phthiraptera). *The Auk* **116**: 957-963.

Dumbacher J.P., Beehler B.M., Spande T.F., Garraffo H.M. & Daly J.W. (1992). Homobatrachotoxin in the genus Pitohui: chemical defense in birds? *Science* **258**: 799-800.

Ekblom R., Saether S.A., Grahn M., Fiske P., Kalas J.A. & Hoglund J. (2004). Major histocompatibility complex variation and mate choice in a lekking bird, the great snipe (Gallinago media). *Molecular Ecology* **13**: 3821-3828.

Freeman-Gallant C.R., Meguerdichian M., Wheelwright N.T. & Sollecito S.V. (2003). Social pairing and female mating fidelity predicted by restriction fragment length polymorphism similarity at the major histocompatibility complex in a songbird. *Molecular Ecology* **12**: 3077-3083.

Gillingham M.A.F., Richardson D.S., Løvlie H., Moynihan A., Worley K. & Pizzari T. (2009). Cryptic preference for MHC-dissimilar females in male red junglefowl, *Gallus gallus*. *Proceedings of the Royal Society of London Series B-Biological Sciences* **276**: 1083-1092.

Grubb T.C. (1972). Smell and foraging in shearwaters and petrels. *Nature* **237**: 404-405.

Hagelin J.C. (2007). The citrus-like scent of crested auklets: reviewing the evidence for an avian olfactory ornament. *Journal of Ornithology* **148**: S195-S201.

Hagelin J.C. (2008). New data and new questions for Crested Auklet research. *The Auk* **125**: 497-198.

Hagelin J.C., Jones I.J. & Rasmussen L.E.L. (2003). A tangerine-scented social odour in a monogamous seabird. *Proceedings of the Royal Society of London B Biological Sciences* **270**: 1323-1329.

Hagelin J.C. & Jones I.L. (2007). Birds odors and other chemical substances: defense mechanism or overlooked mode of intraspecific communication? *The Auk* **124**: 741-761.

Hagey L. & MacDonald E. (2003). Chemical cues identify gender and individuality in giant pandas (*Ailuropoda melanoleuca*) *Journal of Chemical Ecology* **29**: 1479-1488.

Hauser M.D. (1996). The evolution of communication. MIT Press, Cambridge, Massachusetts.

Hirao A., Aoyama M. & Sugita S. (2009). The role of uropygial gland on sexual behavior in domestic chicken *Gallus gallus domesticus*. *Behavioural Processes* **80**: 115-120.

Jacob J. (1976). Chemotaxonomical Relationships between Penguins and Tubenoses. *Biochemical Systematics and Ecology* **4**: 215-221.

Jacob J., Balthazart J. & Schoffeniels E. (1979). Sex differences in the chemical composition of uropygial gland waxes in domestic ducks. *Biochemical Systematics and Ecology* **7**: 149-153.

Jacob J. & Hoerschelmann H. (1982). Chemotaxonomische Untersuchungen Zur Systematik Der Rohrennasen (Procellariiformes). *Journal of Ornithology* **123**: 63-84.

Jacob J. & Ziswiler V. (1982). The uropygial gland. In: Farner D.S., King J.R., Parkes K.C. (eds). *Avian biology*, vol 6. Academic Press, New York: p.199–324.

Johansson B.G. & Jones T.M. (2007). The role of chemical communication in mate choice. *Biological Reviews* **82**: 265-289.

Johnsen A., Andersen V., Sunding C. & Lifjeld J.T. (2000). Female bluethroats enhance offspring immunocompetence through extra-pair copulations. *Nature* **406**: 296-299.

Jones I.L., Hagelin J.C., Major H.L. & Rasmussen L.E.L. (2004). An experimental field study of the function of Crested Auklet feather odor. *Condor* **106**: 71-78.

Jones I.L. & Hunter F.M. (1993). Mutual sexual selection in a monogamous seabird. *Nature* **362**: 238–239.

Jouventin P., Mouret V. & Bonadonna F. (2007). Wilson's storm petrels *Oceanites oceanicus* recognise the olfactory signature of their mate. *Ethology* **113**: 1228-1232.

Karlson P. & Lüscher M. (1959). 'Pheromones': a new term for a class of biologically active substances. *Nature* **183**: 155-156.

Law-Brown J. (2001). Chemical defence in the Red-billed Woodhoopoe *Phoneiculus purpureus*. M.Sc. Thesis. University of Cape Town, Rondebosch, South Africa.

Mardon J. & Bonadonna F. (2009). Atypical homing or self-odour avoidance? Blue petrels (*Halobaena caerulea*) are attracted to their mate's odour but avoid their own. *Behavioral Ecology and Sociobiology* **63**: 537-542.

Mardon J., Saunders S.M., Anderson M.J., Couchoux C. & Bonadonna F. (2010). Species, gender and identity: cracking petrels' socio-chemical code. *Chemical Senses* **Advance access**: doi:10.1093/chemse/bjq021.

Mardon J., Saunders S.M. & Bonadonna F. (unpublished manuscript). From preen secretions to plumage: the ontogeny of petrels' social scents. *Journal of Avian Biology*: submitted.

Montalti D., Gutiérrez A.M., Reboredo G. & Salibián A. (2005). The chemical composition of the uropygial gland secretion of rock dove *Columba livia*. *Comparative Biochemistry and Physiology A Comparative Physiology* **140**: 275-279.

Moore P.J., Reagan-Wallin N.L., Haynes K.F. & Moore A.J. (1997). Odour conveys status on cockroaches. *Nature* **389**: 25-26.

Nevitt G.A. (2000). Olfactory foraging by Antarctic procellariiform seabirds: life at high Reynolds numbers. *Biological Bulletin (Woods Hole)* **198**: 245-253.

Papi F., Ioalè P., Fiaschi V., Benvenuti S. & Baldaccini N.E. (1974). Olfactory navigation of pigeons: the effect of treatment with odorous air currents. *Journal of Comparative Physiology A* **94**: 187-193.

Penn D. (2002). The scent of genetic compatibility: sexual selection and the Major Histocompatibility Complex. *Ethology* **108**: 1-21.

Penn D.J., Oberzaucher E., Grammer K., Fischer G., Soini H.A., Wiesler D., Novotny M.V., Dixon S.J., Xu Y. & Brereton R.G. (2007). Individual and gender fingerprints in human body odour. *Journal of the Royal Society Interface* **4**: 331-340.

Piersma T., Dekker M. & Sinninghe-Damsté J.S. (1999). An avian equivalent of make-up? *Ecology Letters* **2**: 201-203.

Pycraft W.P. (1910). A history of birds. Methuen and Co., London.

Reneerkens J., Piersma T. & Sinninghe-Damsté J.S. (2002). Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proceedings of the Royal Society of London B Biological Sciences* **269**: 2135-2139.

Reneerkens J., Piersma T. & Sinninghe-Damsté J.S. (2005). Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues *Journal of Experimental Biology* **208**: 4199-4202.

Reusch T.B.H., Häberli M.A., Aeschlimann P.B. & Milinski M. (2001). Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. *Nature* **414**: 300-302.

Richardson D.S., Komdeur J., Burke T. & von Schantz T. (2005). MHC-based patterns of social and extra-pair mate choice in the Seychelles warbler. *Proceedings of the Royal Society of London Series B-Biological Sciences* **272**: 759-767.

Roper T.J. (1999). Olfaction in birds. *Advances in the Study of Behavior* **28**: 247-332.

Roth T.C., II, Cox J.G. & Lima S.L. (2008). Can foraging birds assess predation risk by scent? *Animal Behaviour* **76**: 2021-2027.

Safi K. & Kerth G. (2003). Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. *Animal Behaviour* **65**: 363-369.

Schaefer M.L., Yamazaki K., Osada K., Restrepo D. & Beauchamp G.K. (2002). Olfactory fingerprints for major histocompatibility complex-determined body odors II: Relationship among odor maps, genetics, odor composition, and behavior. *Journal of Neuroscience* **22**: 9513-9521.

Shallenberger R.J. (1975). Olfactory use in the wedge-tailed shearwater (*Puffinus pacificus*) on Manana is. (Hawaii). In: Denton A., Coughlan J.P. (eds). *Olfaction and Taste* vol V. Academic Press, London: p.355-359.

Singer A.G., Beauchamp G.K. & Yamazaki K. (1997). Volatile signals of the major histocompatibility complex in male mouse urine. *Proceedings of Natural Academy of Science USA* **94**: 2210-2214.

Smith S.A. & Paselk R.A. (1986). Olfactory sensitivity of the Turkey Vulture (*Cathartes avra*) to three carrion-associated odorants. *The Auk* **103**: 586-592.

Smith T.E., Tomlinson A.J., Mlotkiewicz J.A. & Abbott D.H. (2001). Female marmoset monkeys (*Callithrix jacchus*) can be identified from the chemical composition of their scent marks. *Chemical Senses* **26**: 449-458.

Soudek S. (1927). The sense of smell in birds. X^{e} Congres International de Zoology. Budapest.

Stager K.E. (1967). Avian Olfaction. *Am. Zool.* **7**: 415-419.

Tregenza T. & Wedell N. (2000). Genetic compatibility, mate choice and patterns of parentage: Invited review. *Molecular Ecology* **9**: 1013-1027.

Wallraff H.G. (2004). Avian olfactory navigation: its empirical foundation and conceptual state. *Animal Behaviour* **67**: 189-204.

Walter W.E. (1943). Some experiments on the sense of smell in birds. *Archives Néerlandaises de Physiologie. L'homme et Animaux* **27**: 1-72.

Wedekind C. & Furi S. (1997). Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? *Proceedings of the Royal Society of London Series B-Biological Sciences* **264**: 1471-1479.

Weldon P.J. & Rappole J.H. (1997). A survey of birds odorous or unpalatable to humans: Possible indications of chemical defense. *Journal of Chemical Ecology* **23**: 2609-2633.

Wenzel B. (1973). Chemoreception. In: Farner D.S., King J.R. (eds). *Avian Biology*, vol 3. Academic Press, New York & London: p.389-415.

Wenzel B.M. (1968). The olfactory prowess of the kiwi. *Nature* **220**: 1133-1134.

Westerdahl H. (2004). No evidence of an MHC-based female mating preference in great reed warblers. *Molecular Ecology* **13**: 2465-2470.

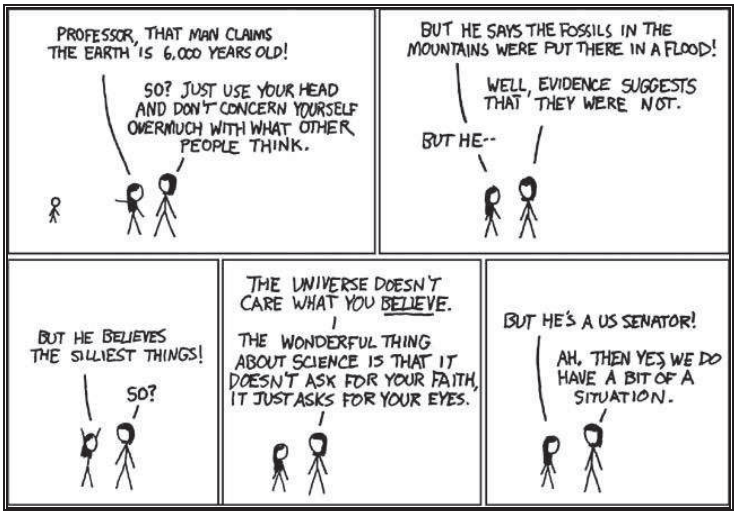
Whelan C.J., Dilger M.L., Robson D., Hallyn N. & Dilger S. (1994). Effects of olfactory cues on artificial-nest experiments *The Auk* **111**: 945-952.

Whittaker D.J., Reichard D.G., Dapper A.L. & Ketterson E.D. (2009). Behavioral responses of nesting female dark-eyed juncos *Junco hyemalis* to hetero- and conspecific passerine preen oils. *Journal of Avian Biology* **40**: 579-583.

Zala S.M., Potts W.K. & Penn D.J. (2004). Scent-marking displays provide honest signals of health and infection. *Behavioral Ecology* **15**: 338-344

Zelano B. & Edwards V.E. (2002). An Mhc component to kin recognition and mate choice in birds: predictions, progress, and prospects. *American Naturalist* **160**: 225-238.

APPENDICES



Anonymous

Appendix A1

BASIC PRINCIPLES OF GAS CHROMATOGRAPHY & MASS SPECTROMETRY

Animal secretions are often made of relatively complex chemical mixtures. For biologists studying either the composition, mode of action or function of these secretions, important analytical requirements are: (i) to individually separate all the constituents of such mixtures, (ii) to obtain qualitative and quantitative information about each isolated analyte (i.e. compound). Currently, the most common technique achieving these objectives is using a coupled *Gas-Chromatograph Mass-Spectrometer* (GC/MS) instrument (Fig.A1-1).

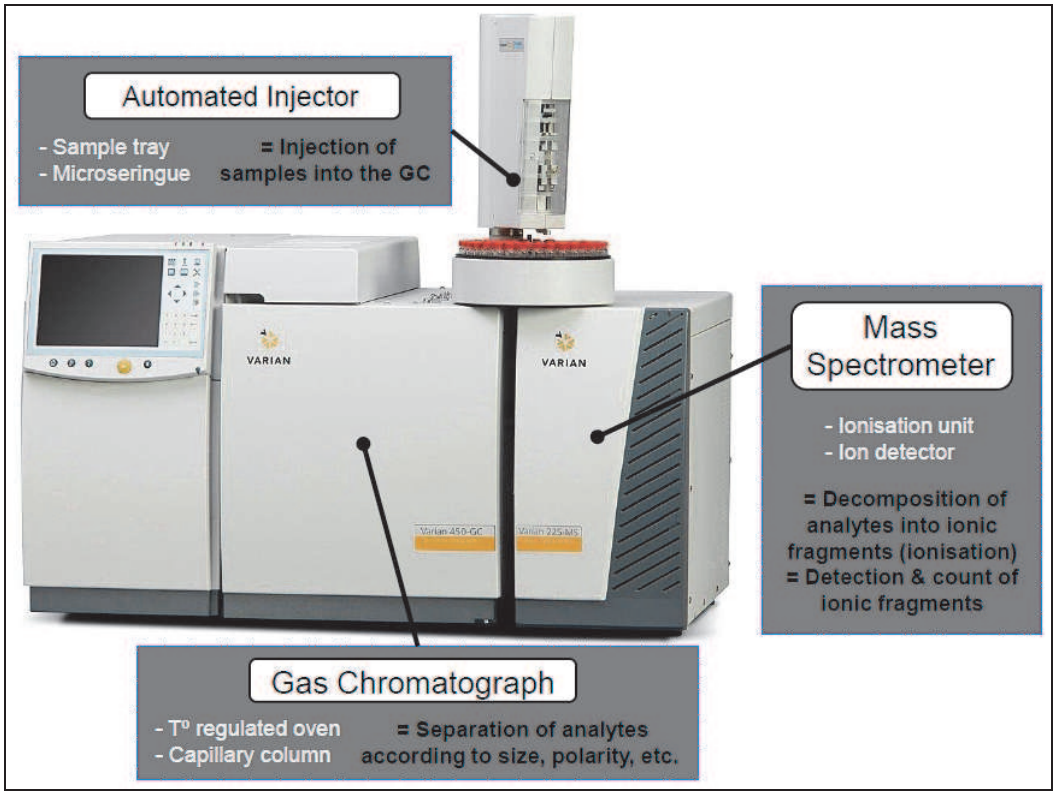


Figure A1-1: Essential components, equipment and functions of a GC/MS instrument (picture from www.directindustry.com)

In traditional GC/MS procedures, liquid phase samples are injected into the GC. Hence, non-liquid types of samples must be liquid extracted, although more recent techniques (presented in Chapter 2) now also allow the injection of gas-transferred samples into the GC. Once injected, regardless of the medium, the sample is volatilised (due to the high temperature of the *injection port*) and transported by a mobile gas phase, the *carrier gas*, through a *chromatography column*. The most commonly used columns are *capillary columns*. They consist of long (e.g. 30m or 60m) narrow (e.g. 0.25mm) tubes internally coated with a specific substance that varies with the type of column. The role of this coating is to retain and slow down the sample analytes as they are being transported through the column by the carrier gas. Components of a sample mixture will therefore move through the GC at different rates based on their affinity with the column material as well as their size. Accordingly, each compound will exit the column (i.e. *elute*) at a particular time called the *Retention time* (Rt), at which it will form a *peak* on the resulting *chromatogram* (Fig.A1-2). The relative times at which the different analytes of a mixture elute can also be optimised using temperature variations within the GC oven which will slow down or accelerate the transport of analytes.

In the GC/MS technique, sample analytes eluting out of the GC are then transported through a *transfer line*, to a MS. In the MS, they are first *ionised*, in most cases by a bombardment of electrons (e^-) which breaks them into several smaller charged fragments called *ions*. The different fragments formed have various sizes and charges which give them a characteristic *mass-to-charge ratio* (m/z). The detector part of the mass spectrometer then scans all the m/z values and provides a pattern of distribution and abundance of ions called a *mass spectrum* (Fig.A1-2). Because a particular compound ionised under similar conditions will always result in a similar mass spectrum, one can therefore tentatively identify analytes by comparing a mass spectrum observed within a sample to available databases. Quantification is also possible by calibrating the instrument so that the *Total Ion Count* (TIC) obtained for a particular analyte can be converted back to an actual quantity.

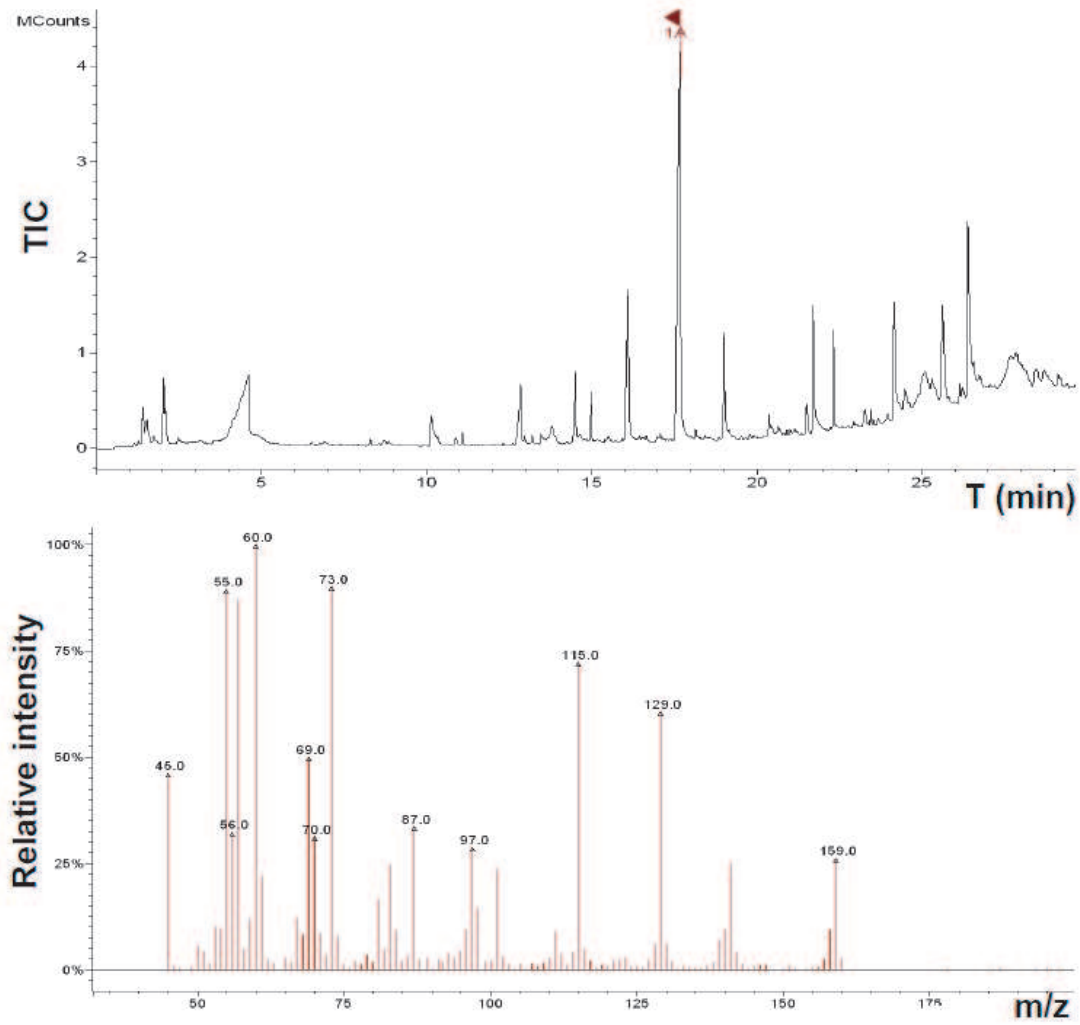


Figure A1-2: Example of a GC/MS output showing a chromatogram (top) and the mass spectrum (bottom) corresponding to the peak selected.

In summary, the usual output from a GC/MS analysis is a chromatogram, which is a graphic representation of the TIC recorded by the detector as a function of time. As analytes eluting out of the GC are being processed by the MS, the chromatogram line displays peaks. Ideally (if there is no co-elution of compounds), each chromatographic peak corresponds to a particular analyte that eluted at a particular Rt, and to which is associated a specific mass spectrum.

Appendix A2

COMMENTS ON WORKS BY ZHANG & CO-WORKERS

As this thesis was going to print, personal communication with adjunct professor Jian-Xu Zhang (Institute of Zoology, Chinese Academy of Sciences, Beijing) brought to my attention some of his recent peer-reviewed publications that are so remarkably related to my work that it seems necessary and appropriate to discuss them in this thesis.

COMMENTS ON ZHANG ET AL. 2009

In a first study published in 2009, Zhang & co-workers explored the uropygial secretions of domesticated Bengalese finches for social information about sex, individuality and species (Zhang *et al.*, 2009). This chemical investigation approaches key aspects of avian chemical communication although the robustness of the findings reported is somewhat limited by several methodological shortcomings.

First, the use of Bengalese finches (*Lonchura striata domestica*), erroneously confused with their wild siblings white-rumped munias (*Lonchura striata*), for such an investigation is questionable. Bengalese finches have indeed been domesticated as an avian pet at least 250 years ago in Japan. They are not a naturally-occurring species but a fertile hybrid whose ancestry is uncertain. Importantly, it has been shown the species has undergone many morphological and behavioural changes under domestication (Honda & Okanoya, 1999). The sample sizes used in the study are also relatively small (9 males and 8 females); probably for ethical reasons as all birds were sacrificed.

The analytical protocol of the study is relatively similar to the one described in Section 3.2 of this thesis, including solvent extraction and GC/MS analyses. The authors, however, used dichloromethane alone as the extracting solvent, probably to focus on the polar compounds within samples. While this is biologically justifiable, this would have inevitably resulted in a significant loss of the apolar fraction of the signals which then calls into question their choice of an apolar column (DB-WAX) for

chromatography. In addition, the temperature program set for the GC/MS, and particularly the starting temperature of 100ºC, is not optimal for studying the volatile fraction of their samples.

Several of the authors' decisions regarding the processing of their data are not justified. For example, their selection of 16 compounds retained for quantitative analyses (while the chemical profiles presented clearly indicate there are more) is unexplained. What is more, the authors assume, based on the evidence from mammalian social odours, that avian social information is coded through the relative abundance of compounds. This sound assumption however does not require the conversion of absolute abundances to percentages as applied in the study. This approach is particularly flawed by their restriction of the analysis to a subset of *a-priori* chosen compounds. Instead, the use of the whole chromatogram area to calculate percentages, or the standardisation of quantitative data using an internal standard, would be more satisfactory.

The authors nevertheless report some interesting results such as their qualitative comparison of the chemical signals from uropygial secretions and feather lipids. The chromatographic profiles presented indeed support the idea that feather lipids, and therefore possible social olfactory signals, originate from uropygial secretions (see Section 3.3 of this thesis).

The qualitative interspecific comparison of uropygial contents between Bengalese finches, Zebra finches (*Taeniopygia guttata*), yellow-browed buntings (*Emberiza chrysophrys*), and rooks (*Corvus frugilegus*) is also unambiguous. It confirms the existence of inter-specific variation of uropygial contents (Jacob, 1978). The ecological implications of this observation are, however, limited because Bengalese finches are a domesticated hybrid species with virtually no interspecific interaction.

Rightly pointing out that sexual dimorphism is a better indicator than seasonality when looking for sexual pheromones, the authors identify two interesting sex-pheromones candidates: hexadecanol (possibly a male pheromone) and octadecanol (possibly a female pheromone). Although a pheromonal function role for these compounds is biologically plausible, the quantitative basis of this hypothesis is incorrect. Indeed, not only can the use of percentage artificially inflate the sexual dimorphism shown by these compounds (see also comments on Zhang *et al.*, 2010), but

the statistical robustness of the claim is flawed as the number of tests applied (n=16) is not accounted for.

Finally, the possible existence of individual signals in Bengalese finches is an exciting finding. The authors suggest indeed that the inter-individual variation of chemical profiles is significantly greater than the intra-individual variations. Unfortunately, the validity of this finding cannot be assessed as nowhere in the study is the origin of the values used for intra-individual variation explained.

COMMENTS ON ZHANG ET AL. 2010

In a study conducted in 2007 (see Zhang *et al.*, 2008 for a non peer-reviewed preliminary report) and published very recently (Zhang *et al.*, 2010), Zhang & co-workers explored the uropygial secretions of budgerigars (*Melopsittacus undulatus*) with a particular focus on their role as precursors of olfactory sex signals. This study also contains attempts to bioassay components from these secretions. Again, the chemical and behavioural investigation presented by the authors contains exciting results whose robustness is again thwarted by major analytical shortcomings.

Many methodological limitations of this study are similar to the ones already discussed (e.g. use of dichloromethane alone as the extracting solvent, starting temperature of the chromatography). The unjustified selection of 23 compounds among the complex chemical profiles of budgerigars is again particularly questionable. Similarly, the ad-hoc statistical comparison, between the two sexes, of the abundances of compounds *a-priori* selected for being sexually dimorphic is problematic.

According to the authors, the results of this study robustly demonstrate that a blend of three long-chain alkanols (octadecanol-18OH, nonadecanol-19OH, and eicosanol-20OH) synergistically acts as a male pheromone in budgerigars. The authors' analysis of their chromatographic data is, however, highly arguable. Indeed, the amounts of the three alkanols (18OH, 19OH and 20OH) in 1mg of uropygial secretion are found to be respectively 3.58 ± 3.06 µg, 2.78 ± 2.67 µg, and 5.32 ± 3.10 µg in males (note the huge inter individual variation) but are not indicated for females. This is unfortunate because this information would clearly show that females' secretions have a higher content of these alkanols (as indicated by the GC area values provided in a table). Nevertheless,

once converted into relative abundances (in percent, using the total area of the 23 subjectively selected compounds), the contribution of the alkanols becomes around 4 times more important in males than females; a result that the authors used as a basis for all subsequent behavioural bioassays. The authors, however, failed to notice two important aspects of their data: (i) the wide and overlapping spread of the alkanols' absolute abundances in males and females, (ii) the fact that the higher relative contribution of alkanols in males exclusively results from the presence, in the secretions of females, of additional highly abundant compounds (pentanoates). Therefore, the chromatographic data of Zhang & co-workers indicate, if anything, the presence of a clear female signal in the uropygial secretions of budgerigars.

The authors have unfortunately propagated their misinterpretation even further by converting the 4 fold ratio in the relative abundance of alkanols into a 4 fold ratio of absolute abundances for their bioassays. Namely, they mimicked a 'male' odour by preparing a blend of the three alkanols 4 times more concentrated than the one supposed to mimic a 'female' odour, despite the fact that the absolute quantities of these compounds in the female secretions is higher than in males. Consequently, the outcomes from the behavioural bioassays presented by the authors are ambiguous. In a majority of cases, they can indeed be interpreted simply as a preference of the birds for the strongest stimulus (the most concentrated blend over the least concentrated one) which, as argued above, does not appropriately reflect the reality of the chemical sexual dimorphism. An attraction to familiar (or conspecific) odours could, for example, explain the preferences reported.

Nevertheless, the authors also observed that female budgerigars preferred both the uropygial odour and the body odour of males over their female counterparts. Although the protocol used for odour presentation is sometimes hard to follow, this interesting result suggests that budgerigars have olfactory capabilities of sexual discrimination. The evidence presented by Zhang and co-workers does not, however, resolve whether such olfactory capability originates from the attraction of females to some 'male' sex-pheromones, or from the avoidance of female-associated odours. The latter hypothesis, in particular, is completely overlooked by the authors. More generally, the design of this study is weakened by the bias of the author towards a 'male' sexual signal (probably originating from their mammalian research background). For example, only females

were tested in behavioural assays despite the ambiguity of chemical results. This is not consistent with current evidence from the field, as discussed in Chapter 6.

Overall, the work of Zhang and co-workers discussed above supports the current realisation of avian chemical communication, and extends the range of species investigated. The comments developed in this appendix (which may be the subject of a letter to the journal *Chemical senses*) do not aim at refuting the evidence reported by these authors. They advocate, instead, for higher methodological and analytical standards in the investigation of avian chemical communication.

REFERENCES

Honda E. & Okanoya K. (1999). Acoustical and syntactical comparisons between songs of the white-backed munia (*Lonchura striata*) and its domesticated strain, the bengalese finch (*Lonchura striata var. domestica*). *Zoological Science (Tokyo)* **16**: 319-326.

Jacob J. (1978). Uropygial gland secretions and feather waxes. In: Florkin M., Scheer B.T. (eds). *Chemical zoology*, vol 10 Aves. Academic press, New York: p.165-211.

Zhang J.-X., Sun L. & Zuo M.-X. (2009). Uropygial gland volatiles may code for olfactory information about sex, individual, and species in Bengalese finches *Lonchura striata*. *Current Zoology* **55**: 357-365.

Zhang J.-X., Wei W. & Zhang J.-H. (2008). Preen gland-secreted alkanols enhance male attractiveness in parrots. *Nature Precedings*: *hdl:10101/npre.2008.2305.1*.

Zhang J.-X., Wei W., Zhang J.-H. & Yang W.-H. (2010). Uropygial gland-secreted alkanols contribute to olfactory sex signals in budgerigars. *Chemical Senses* **Advanced access**: doi:10.1093/chemse/bjq025.

Appendix A3

FAREWELL

The Journal of Godly Creative & Intelligent Design

Erratum

IDENTIFIED SHORTCOMINGS ON THE INTELLIGENT DESIGN OF *SCIENCE*

M.Y. Lord†

In a recent model examining the *Evolution-Assisted Rational Trajectory of Humanity* (***EARTH***), I described how the probability of *Humanity Acting Propitiously towards Positive Illumination* (***p*****$_{happi}$**) positively correlates with the development of her *Understanding of Nature* (***UN***). Using Past's theorem[1], I then went on to demonstrate that ***UN*** is a sole function of humanity's objective appreciation of her surroundings, a quality that I have called ***SCIENCE*** (*Sufficiently Convincing Information Establishing New Controversies for Everyone*).

The contribution of ***SCIENCE*** to ***p*****$_{happi}$** is so important that I intelligently designed a simple scientific system to ensure a thorough, collective and growing knowledge of ***UN***. Explicitly, I modelled ***SCIENCE*** so that its *Impact Factor* (***IF***) would be a function of the quality of the research done (***a***), the scientists' contribution to humans general knowledge (***b***), and the total number of scientists (***c***).

Working on the ***SCIENCE*** model on the sixth day (and being therefore understandably exhausted), I committed three important errors while reporting the design of the above parameters.

(i) Regarding the first parameter ***a***, I defined the quality of the research done by a scientist by:

$$a = K \times \left(CT_{self} - CT_{other}\right)$$

Where: ***K*** = *a constant based on the importance of the topic;* ***CT*****$_{self}$** = *Critical thinking applied to their own work;* ***CT*****$_{other}$** = *Critical thinking applied to the work of others*

The actually correct definition of ***a*** is however:

$$a = K \times \left(CT_{self} + CT_{other}\right)$$

Indeed, the latter equation clearly indicates how ***a*** (and thus ***IF***) will increase with the capacity of scientists to apply a positive critical thinking to both their own work and the work of others. The incorrect equation reported previously, however, results in ***a*** (and thus ***IF***) being positively correlated to the capacity of scientists to apply positive critical thinking to their own work but a negative one to the works of others. Ironically, this erroneous design has led, in some instances, scientists to harshly criticise work of others even though of higher standards than their own.

(ii) Regarding the second parameter ***b***, I defined a scientists' contribution to the general knowledge of humanity by:

$$b = \sum \left(a + 1\right) \times n_{pub}$$

Where: ***a*** = *quality of work of the research done;* ***n*****$_{pub}$** = *Number of publications resulting from the research*

The actually correct definition of ***b*** however should have been:

$$b = \sum a \times \left(n_{pub} + 1\right)$$

Indeed, the latter equation implies that ***b*** (and thus ***IF***) will increase with the quality of the work done and the effort made to disseminate its outcomes. It also emphasises the quality of the work so that high quality research, even without publications, will have a positive

[1] "*The relative contributions from all personal/spiritual/religious subjectivities to* ***UN*** *cancel each other out so that their total sum is inevitably null.*"

† *You wish...*

contribution nonetheless. In contrast, the erroneous equation reported in my previous model causes ***b*** (and thus ***IF***) to emphasise the number of publications from a research even if the quality of a research is null. This erroneous design has led, for example, to an increase in the relative number of publications from literature reviews compared to original research. What is more, it has also favoured a prolific yet boring standard of scientific literature (Sand-Jensen, 2007).

(iii) Regarding the last parameter ***c***, I ensured that the number of scientists would increase by naively designing humans' life-history so that their population would increase. Furthermore, I included several functions of demographic self-regulation (reported in the ***HISTORY*** model) so as to avoid a population explosion. Unfortunately, the self-regulating functions reported failed to take into account the very development of ***SCIENCE*** itself so that they rapidly became inefficient.

These three fortuitous errors have led to a much different ***SCIENCE*** trajectory than originally designed. The unregulated demographic increase has, for example, brought down the relative average ***IF*** of each scientist. The resulting increase in the level of intradisciplinary competition has, in turn, aggravated the erroneous importance given to $\boldsymbol{CT_{other}}$ and $\boldsymbol{n_{pub}}$. As a consequence, ***IF*** has become an absurd and uninformative variable to the fate of ***SCIENCE*** (Petsko, 2008).

More importantly, these shortcomings have deviated ***SCIENCE*** from its originally designed purpose so that its contribution to ***UN*** and $\boldsymbol{p_{happi}}$ can be questioned. I have provided here the necessary corrections that scientists need to address, on an individual basis, to rectify the ***EARTH***. Hopefully, more relevant contributions to ***UN*** and $\boldsymbol{p_{happi}}$ may emerge in the meantime for enthused scientists to have a real impact.

REFERENCES

Petsko G.A. (2008). Having an impact (factor). *Genome Biology* **9**: 107.

Sand-Jensen K. (2007). How to write consistently boring scientific literature. *Oikos* **116**: 723-727.

“It's dead easy to die; it's the keeping on living that's hard.“

Douglas Mawson - Scientist and polar survivor