Chem. Senses 35: 309–321, 2010
doi:10.1093/chemse/bjq021
Advance Access publication February 26, 2010

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

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Accepted February 1, 2010

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

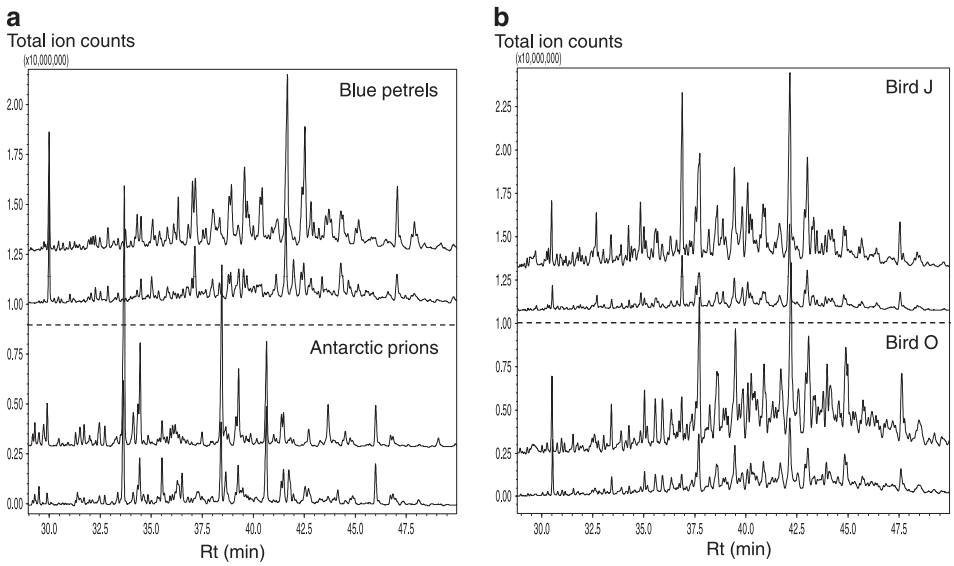

Figure 1 Selection of chromatograms illustrating the different analyses. For graphic clarity, only a 20-min section of the chromatograms (Rt = 30–50 min) is displayed. **(a)** interspecific comparison: the 2 top chromatograms are from BPs and the 2 bottom ones from APs. **(b)** intraspecific comparison: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

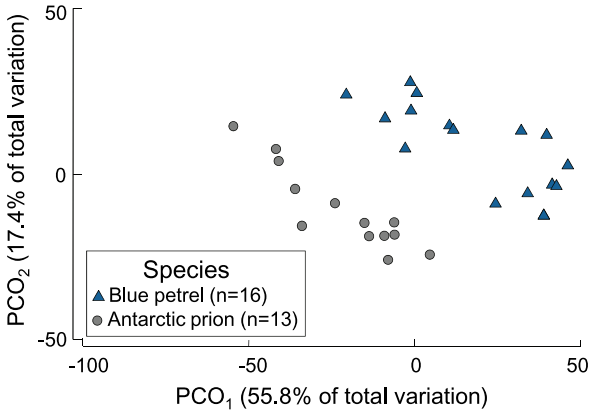

Figure 2 Bidimensional PCO ordination of the samples included in the interspecific analysis. Each data point corresponds to one sample, that is, one chemical profile. This figure appears in color in the online version of *Chemical Senses*.

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.

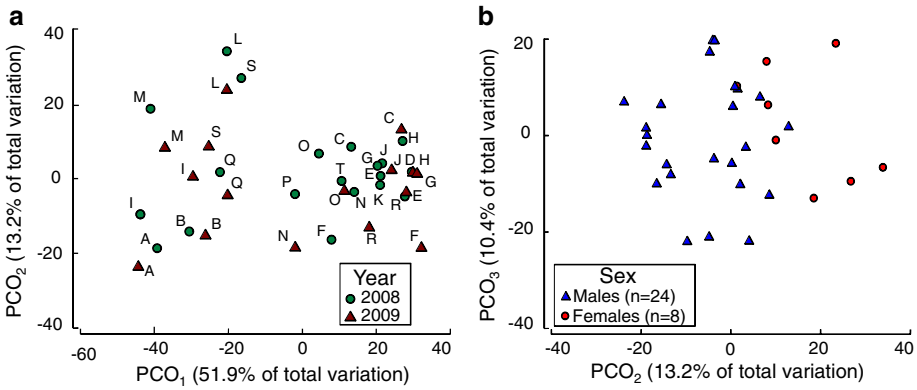

Figure 3 Bidimensional PCO ordinations of the BP samples included in the intraspecific analysis. **(a)** PCO_1 versus PCO_2 and **(b)** PCO_2 versus PCO_3. Each data point corresponds to one sample and each letter corresponds to a particular individual. This figure appears in color in the online version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

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 unsurprising (see also

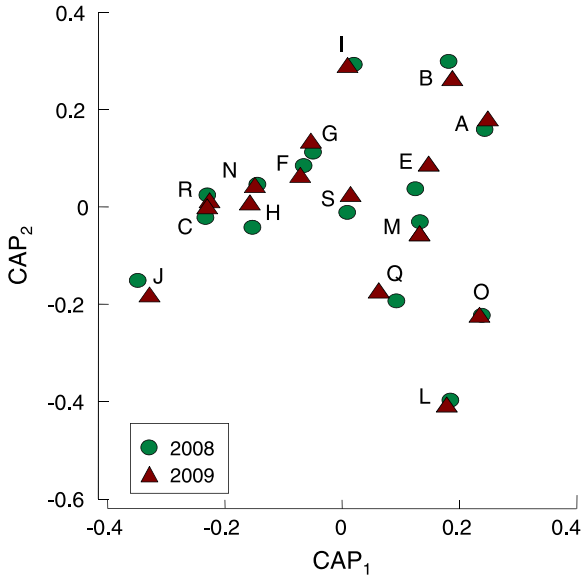

Figure 4 CAP analysis of the Individual factor (BP samples) showing 87.5% correct discrimination of chemical profiles between the different individuals. Each data point corresponds to one sample and each letter corresponds to a particular individual. Letters are not duplicated as the 2008 and 2009 samples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

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/

Funding

This work was supported by the Institut Polaire Français Paul-Emile Victor through the program [ETHOTAAF 354 to F.B.]; and the Agence Nationale de la Recherche Française [AMBO ANR-08-BLAN-0117-01 to F.B.].

Acknowledgements

All aspects of the study were performed according to guidelines established by the Institut Polaire Français Paul-Emile Victor (IPEV) and the Centre National de la Recherche Scientifique for the Ethical Treatment of Animals and complied with current French regulations. The authors wish to thank all field assistants on Ile Verte, the IPEV logistic team for amazing field support, and Tony Reader and Gavin Flemmati from the School of Biomedical, Biomolecular and Chemical Sciences of the University of Western Australia, for their expertise on GCMS techniques. We are also grateful to 2 anonymous reviewers and our many collaborators and friends for fruitful discussions and constructive feedback on earlier versions of the manuscript.

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