Citation for final published version:

Gabirot, Marianne, Buatois, Bruno, Muller, Carsten and Bonadonna, Francesco 2018. Odour of King Penguin feathers analysed using direct thermal desorption discriminates between individuals but not sexes. Ibis 160 (2) , pp. 379-389. 10.1111/ibi.12544 file

Publishers page: http://dx.doi.org/10.1111/ibi.12544 <http://dx.doi.org/10.1111/ibi.12544>

Odour of feathers of King Penguins analysed using direct thermal desorption discriminates individuals but not sex

MARIANNE GABIROT,[1,2,*] BRUNO BUATOIS,[2] CARSTEN T. MÜLLER[1] &
FRANCESCO BONADONNA[2]

[1] *Organisms and Environment research division, Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff CF10 3AX, Wales, UK*

[2] *Centre d'Ecologie Fonctionnelle et Evolutive CEFE, UMR 5175, CNRS, Université de Montpellier, Université Paul-Valéry Montpellier, EPHE, 1919 route de Mende, 34293 Montpellier Cedex 5, France*

*Corresponding author: Marianne Gabirot. Email: marianne.gabirot@gmail.com

N° words: 6673

The role and use of olfactory cues by penguins is largely under-investigated with only a few studies suggesting that odours are involved in prey detection, orientation and for interspecific communication. This also applies to King Penguins (*Aptenodytes patagonicus*) where little is known about their abilities in chemoreception and, subsequently, importance of odours in their behavioural ecology. Here, we investigated the chemical composition of volatile organic compounds (VOCs) from feathers of King Penguins in the Kerguelen Archipelago and their potential to carry information on identity and sex. We analysed VOCs using direct thermal desorption, a novel approach for extracting volatile compounds directly from solid matrices. We were only able to test at desorption temperatures of 70 °C and 100 °C to optimise conditions for VOC analysis. We found a profile of 26 VOCs, present in most individuals, which varied significantly between individuals but not between sexes. Results suggested that VOCs could be potentially used by King Penguins to locate the colony and recognize individuals if similar VOCs are also present at ambient conditions. Further studies and behavioural experiments are encouraged to explore olfactory-based communication in this species.

Keywords: *Aptenodytes patagonicus*, volatile organic compounds, plumage, individual variability.

Chemical signals play a meaningful role in behavioural ecology and intraspecific communication of many animals (Mason 1992, Penn & Potts 1998, Wyatt 2003). Despite ample behavioural evidence of importance of chemical signals in vertebrates, comparatively little is known about the actual chemical composition and nature of these signals (Roper 1999, Apps 2013). Signals, often consisting of mixtures of several chemical compounds with different properties (Muller-Schwarze 2006), can be crucial in social and reproductive behaviour, providing information on e.g. sex, age, social status (Wyatt 2003, Muller-Schwarze 2006, Bonadonna & Mardon 2013). Several recent studies have described chemical signals in birds (Mardon *et al.* 2010, Whittaker *et al.* 2010, Shaw *et al.* 2011), and reported their role and function in interactions with conspecifics (Bonadonna & Nevitt 2004, Hagelin & Jones 2007). In particular, procellariiform birds produce a characteristic scent that has been implicated in kin recognition (Strandh *et al.* 2012, Bonadonna & Mardon 2013).

Sphenisciformes (penguins) and Procellariiformes (albatrosses and petrels) are phylogenetically closely related (Ksepka *et al.* 2006, Hackett *et al.* 2008) and share many common traits. For example, penguins and petrels exploit similar ocean habitats, tend to forage on similar types of prey, such as krill, fish and squid (Warham 1990, Williams 1995). Also a number of species of both orders nest in large colonies and are central place foragers during the breeding season (Stephens & Krebs 1986, Williams 1995). Despite these similarities, it is assumed that these seabirds employ different sensory modalities. Procellariiformes use chemical communication for orientation, homing, reproduction and social interactions (Gagliardo *et al.* 2013, Bonadonna & Mardon 2013, Caro *et al.* 2015), whereas the role and function if any of chemical cues in sphenisciform species is unclear. Studies on penguins have focused on calls in individual recognition (Aubin *et al.* 2000, Jouventin & Aubin 2002) or visual cues in foraging (Kooyman *et al.* 1992). However, penguins may also utilize chemical cues, at least food-related odour in foraging (Cunningham *et al.* 2008, Wright *et al.* 2011, Cunningham *et al.* 2017). In a recent study, King Penguins (*Aptenodytes patagonicus*) responded differentially to smell of sand, feathers or faeces, when exposed to samples while asleep (Cunningham & Bonadonna 2015). In addition, Nesterova et al. (2009) reported that King Penguin chicks only successfully oriented towards their colony at night when placed down-wind of the colony after having been removed from the nest. Breeding King Penguins also walk towards their place in the colony in complete darkness (Nesterova *et al.* 2010). Even though visual cues appear to be important in short-distance orientation of King Penguins (Nesterova *et al.* 2009), it is sensible to assume that additional information from olfactory, magnetic or acoustic cues could be involved in their navigating back to their place in the colony in complete darkness (Nesterova *et al.* 2010). Therefore, King Penguins may use scents to locate the colony or the island, similarly to many Procellariiformes which detect their burrow by olfactory cues (Bonadonna *et al.* 2003). Olfaction might also be important in social communication as already reported in Humbolt Penguins (*Spheniscus humboldti*), which show kin recognition based on olfactory cues (Coffin *et al*. 2011). Odours from feathers and faeces may also relay information on individual identity enabling recognition of their partner among a large number of individuals and orientate animals back to the colony after foraging (Cunningham & Bonadonna 2015).

Although the importance of chemical signals in social interactions in some bird taxa is becoming more and more apparent (Caro *et al.* 2015), the exact nature of the chemical cues involved remains largely undocumented, and is completely unknown in the case of penguins. Identification of volatile organic compounds (VOCs) from avian secretory organs thus is a critical element and starting point for understanding the role of olfaction in the social life of birds. Most work on avian olfaction has focused on the analysis of either uropygial secretions or feather lipids, as the preen gland is often considered as the key source of avian chemical substances (Campagna *et al.* 2012). Yet to date no published study has focused on the actual airborne volatiles emitted by birds. There is only a small number of studies on chemical signals emitted from whole birds, with most studies focussing on insects and rodents (Moritz & Crewe 1988, Cardé & Millar 2004, Röck *et al.* 2006, Douglas III 2006). Sampling odours (and VOCs) from a relatively large vertebrates in a remote field location is a logistical challenge. Here we propose an innovative method, direct thermal desorption, to extract VOCs directly from feathers collected in King Penguins at the Kerguelen Archipelago. We tested this method at two temperatures of desorption aiming to maximise yield of VOCs and minimise formation of artefacts in the resulting VOCs profiles. Chemical profiles were subsequently assessed for information on their potential to discriminate sexes and individuals.

METHODS

Species and field sampling

Feather samples were obtained from 17 King Penguins (9 males and 8 females) at the Cape Ratmanoff colony (Courbet Peninsula, Kerguelen Islands (70°33’E, 49°42’S)) during the austral summer (December) 2011. Wearing clean nitrile gloves, 5-10 feathers were cut close to the uropygial gland with clean steel scissors rinsed with methanol (LR grade, Sigma-Aldrich). Feathers were wrapped in nalophan® (polyethylene terephthalate) first and aluminium foil second and stored at -20 °C until the extraction in the laboratory. All aspects of the study were performed according to guidelines established and approved by the French Polar Institute (IPEV), ‘Terre Australes et Antarctiques Françaises’ (TAAF) and French National Center for Scientific Research (CNRS) for the Ethical Treatment of Animals and complied with current French regulations.

Sample preparation

Three feathers from each sample were placed in 'Loose Fit' Teflon® inserts (Liner PTFE; Markes International Limited, Llantrisant, UK) which were inserted into clean empty TD tubes (OD = 6 mm; L = 88 mm; Perkin-Elmer France). A silanized glass wool plug (Perkin Elmer USA) was added at the top to avoid any loss of feathers. All samples were spiked with 1 µL of 0.1 mg/mL biphenyl (MW = 154.21 g/mol, 99.5 % Sigma-Aldrich®, France) in a mixture of 1:3 dichloromethane/n-hexane (Sigma-Aldrich®, France) as internal standard. Control tubes containing an empty insert (i.e. without feather) were prepared in the same manner as feather samples and were run every 10 samples within each sample batch. These empty tubes controlled for possible contamination during sample preparation and from GC-MS instrument itself during extraction and analysis.

Thermal desorption and chromatographic analysis

VOCs were desorbed directly from samples by heating in a flow of inert gas, re-trapped on a secondary adsorbent tube and desorbed directly into the gas chromatography – mass spectrometry (GC–MS). Although extraction efficiency of thermal desorption is lower than that of solvent extraction (Baltussen *et al.* 2002), the absence of a solvent dilution effect generally makes it more sensitive overall.

The choice of desorption temperature for a sample is critical, ideally it should reflect natural conditions, avoid pyrolysis and yield detectable amounts of VOCs. The combustion point of King Penguin feathers is unknown and the value for feathers of blue petrels (*Halobaena caerulea)* of around 230 °C (J. Mardon personal data) was used as a reference. In order to reflect natural conditions, a thermal desorption temperature close to their body surface temperature (30-35 °C, Schmidt *et al.* (2006)) would have been ideal. Unfortunately, this temperature was not within the technical specification of the instrument, which only allowed for a minimum desorption temperature of 70 °C. We, therefore tested two desorption temperatures, 70 °C and 100 °C, which fell within the lower limit of the instrument and avoided combustion or undue thermal stress and excessive desorption of waxes, which was found at higher temperatures in preliminary trials. Desorption was repeated with a separate feather sample for each individual at both desorption temperatures resulting in four chemical profiles for each individual: two at 70 °C and two at 100 °C.

Chromatographic analyses were carried out at the PACE-Labex CEFE-CNRS (Montpellier, France), on a Shimadzu QP2010 GC–MS (Shimadzu Corp.) equipped with a TD autosampler (Shimadzu AOC-20i+s; Shimadzu Corp.). VOCs were re-collected on a Tenax® TA trap at -10 °C, desorbed by rapidly heating it from -10°C to 250°C and injected into the GC with a split of 10:1. Samples were separated over a Rtx®624Sil-MS Low-Bleed GC-MS column (l = 30.0 m; ID = 0.25 mm; film thickness = 1.40 µm; Restek USA) using the following temperature program: initial temperature 30 °C for 4 min, then 4 °C/min to 270 °C and 3 min hold at end. The interface temperature to the mass-spectrometer (MS) was held at 250 °C and the ion source temperature at 200

°C. Data were acquired in scan mode from 20 to 350 amu at scan speed = 1111, scan interval = 0.3 s and electron ionization (EI) energy of 70 eV. A mixture of C_8-C_{20} alkanes (Sigma Aldrich®, Switzerland) was processed under the same conditions to calibrate for retention index calculation.

Chromatographic Data Processing

Raw data were processed and integrated using GC-MS Solution software v2.40 (Shimadzu Corp.). The quality of all software-defined peak integrations was visually reviewed and manually corrected if 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. Analytes were identified by comparison of mass spectral data using the NIST (National Institute of Standards and Technology) Mass Spectral Search Program v2.0© (Faircom Corp.; Columbia MO, USA) and Wiley Registry™ of Mass Spectral Data and cross-checking spectral matches with the calculated Retention Index (RI) of the analytes. For quantitative analyses, data were standardised to area of the internal standard (biphenyl, RT = 44.95 min, RI = 1439).

Data pre-treatment, resemblance measure, and ordination

GC-MS total ion current (TIC) profiles of samples with and without feathers were compared first (Fig. 1). The difference between the chromatographic profiles of controls and samples was clearly noticeable by visual inspection. Consequently we removed all compounds of controls from further analysis (e.g. molecules derived from GC-column). Only compounds eluting from C7 (n-heptane RT = 11.68 min, RI = 700) to C18 (n-octadecane, RT = 56.51, RI = 1800) were used for the analysis. Before and after this range of chemical compounds (and retention time or retention index) we did not have a good signal and resolution in chemical profile. This resulted in 26 compounds, which were (i) putatively identified individually by matches of mass spectrum and RI, and (ii) classified to substance class level (e.g. fatty acid ethyl ester) by matching mass spectrum. One profile, desorbed at 70 °C, was excluded, as it showed no peaks, most probably due to problems during chromatography analysis. In addition, there were not enough feathers of two individuals (one male and one female) to sample at both temperatures and were only desorbed at 70 °C, which resulted in a total of 65 profiles (17 birds x 2 T x 2 replicates - 3). Standardized data were finally square-root transformed to reduce the influence of the most abundant analytes on the analysis (Clarke & 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 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 (see Mardon *et al.* (2010)). All statistical analyses were carried out using the computer program PRIMER V.7.0.5 Permanova+1 (Primer-E Ltd©).

Effect of desorption temperature, sex and individual variability

All chemical data were initially analysed with an unconstrained PCO ordination. Chemical profiles were evaluated with a three-factor permutational multi-variate analysis of variance (PERMANOVA, (Anderson 2001, McArdle & Anderson 2001) using 9999 permutations (see Mardon *et al.* (2010)): temperature desorption, sex and individual (nested to sex). PERMANOVA allows distance-based tests of significance for comparing a priori groupings, as in a classical partitioning. *P* values were obtained using 9999 permutations of residuals under a reduced models (Freedman & Lane 1983) and Type I (sequential) sums of squares (SS). Pairwise comparisons were made using constrained permutation tests. Finally, profiles were compared using CAP (Canonical Analysis of Principal coordinates). CAP is a method based on a dissimilarity matrix to test differences in a priori groups of multivariate observations (Anderson & Robinson 2003, Anderson & Willis 2003). CAP calculates classification based on distances, estimation of error rates using cross-validation and Pearson rank correlation (r) between the individual analytes.

RESULTS

A good chromatographic signal was obtained from feather samples (Fig. 1). Resolution and peak symmetry overall was good with little co-elution, although most carboxylic acid ester peaks tailed significantly. Compounds detected and tentatively identified ranged from 2-pentanone to fatty acid dodecanoic acid ethyl ester. All compounds were shared by most males and females and recovered at both temperatures of desorption.

The comparison of profiles of all 17 individuals (males and females desorbed at 70 and 100 °C) indicated differences between the two desorption temperatures. In an unconstrained 3D PCO the first three axes explained 80.46 % (Axis 1: 48.95 % - Axis 2: 20.79 % - Axis 3: 10.72 %) of the total variation. The PCO plot using just the first and second axes was not very effective to see differences between the chemical profiles associated with temperature of desorption. However when we explored the first and third PCO axes (PCO1 x PCO3) and the second and third PCO axes (PCO2 x PCO3, Fig. 2A), we saw a clear distinction between 70 °C and 100 °C. Using PERMANOVA, significant differences between profiles of VOCs were found between temperatures of desorption and between individuals (Table 2). There was no significant difference in profiles with respect to sex (Table 2).

Chemical profiles distinguished desorption temperatures in CAP on a single axis obtained from m = 3 PCO axes. The leave-one-out misclassification error was 4.8% for the samples used to build the CAP model (Fig. 2B). The CAP model associated mostly dimethyl alkanes (3,8 dimethyl decane, dimethyl undecane, Table 1) to 70 °C desorption and nonane, nonanal, dodecanoic acid ethyl ester and 9-methylpentadecane to 100 °C (see Table 1 and Fig. 2B).

Because of the significant effect of temperature, profiles obtained at 70 °C and 100 °C were tested separately for discrimination between sex and individuals and again no difference between the VOC profiles of males and females was observed at either temperature (Table 2).

DISCUSSION

The volatile organic compounds present on feathers of King Penguins included ketones (2-pentanone, 2-hexanone, 3-heptanone), methyl alkanes (hexane, 2,4-dimethyl, decane, 3,8-dimethyl), aldehydes (nonanal, undecanal), aromatic compound, furans and fatty acid ester (Table 1) showing a composition that compared well with previous studies on semiochemicals in birds in general (review in Campagna *et al.* (2012)). In particular, ketones and aldehydes have been encountered in feathers of other bird species such as Domestic Ducks (*Anas platyrhynchos*, Bernier *et al.* 2008), Antarctic Prions (*Pachyptila desolata*, Bonadonna *et al.* 2007), Crested Auklets (*Aethia cristatella*, Hagelin *et al.* 2003), Dark-eyed Juncos (*Junco hyemalis*, Soini *et al.* 2007) and Black-bellied Whistling Ducks (*Dendrocygna autumnalis*, Robacker *et al*. 2000). The variance of profiles was found to be large enough to discriminate between individuals but interestingly not between the sexes. This suggests the distinct possibility that chemical communication is used for individual recognition in King Penguins. Ultimately, behavioural studies can confirm or disprove occurrence of chemical communication. However, such field tests are greatly aided by any knowledge of composition and nature of the potential chemical signal.

Ideally, VOCs should be collected under natural conditions directly from the animal. Nevertheless working on a remote island with King Penguins creates several problems. A direct collection of headspace of animals would require capture and enclosure of individuals, which would not be feasible and potentially affect results. Direct collection of odours or chemical mixtures in the field implicates sorbent materials to capture molecules from animals or environments, which have to be robust and easy to handle during field work. In addition, absorbed odours or chemical mixtures should be stable on the sorbent until extraction and/or analysis with GC-MS. However, sorbent materials such as in stir-bars and SPME fibres are not suited for longer term storage and extraction from e.g. charcoal or Tenax with solvents results in unacceptable dilution of samples. As we explained, the King Penguins colony studied here is situated in remote Island, at about one month travelling to come back to laboratory. Therefore, collection of feathers provides a straightforward and effective method to gather material that represents the actual scent of an individual. The collection of VOCs from such material should optimally be carried out at body temperature and we tested several options in the laboratory (active headspace, SPME) to do so. Although a scent was clearly discernible by the human nose, we were not able to obtain a sufficiently strong signal in the GC-MS (MG & CTM personal obs.). We, consequently, developed a novel approach, direct thermal desorption, to obtain VOCs from feathers of King Penguins. This method essentially evaporates the chemical compounds directly off feathers at elevated temperatures. Increasing the temperature is a common method to increase vapour pressure and, hence, headspace concentration without much distortion of the VOC profile as long as the temperature is low enough to avoid (i) breakdown of the sample or its components e.g. alcohols, carboxylic acids (Baltussen *et al.* 2002) and (ii) accumulation of very high boiling compounds e.g. high molecular weight waxes at a desorption temperature of 180 °C (MG and CTM personal obs.). To explore the effect of desorption temperature on feathers, we compared the VOCs obtained at temperatures of desorption of 70 and 100 °C. Such desorption temperatures are still two to three times higher than body temperature and will result in increased abundance of observed compounds by an estimated factor in the order of 4 to 8 respectively. This would mean that the less volatile components reported here (No 17 – 26 in table 1) would still be emitted under field conditions albeit at much lower concentrations. However, as olfaction can be orders of magnitude more sensitive than detection with GC-MS they still may play a role and only behavioural assays will be able to resolve this issue. Overall, higher desorption temperatures appear to be problematic and temperatures of and lower than 100 °C are recommended for direct thermal desorption of feathers. Consequently our method combines advantages from using a relative abundant material (feather lipids) with the advantages of using thermal desorption (higher sensitivity due to the absence of dilution).

Only recently, discrimination between individuals based on olfaction and associated chemical profiles from feathers have been reported in Blue Petrels (*Halobaena caerulea*) (by chemical analysis and behavioural experiments) and in Antarctic prions (*Pachyptila desolata*) (by behavioural tests only) (Mardon *et al.* 2010, Bonadonna & Mardon 2013). Individuality in odours has been also observed in several mammals species including mice (*Mus musculus domesticus*) (by chemical analysis and behavioural experiment in Singer *et al.* 1993), Bechstein's bats (*Myotis bechsteinii*) (by chemical analysis in Safi & Kerth 2003), and humans (by chemical analysis in Penn *et al.* 2007). Chemical recognition was also observed in Humboldt Penguins, which can discriminate between familiar and unfamiliar non-kin odours (using prior association) and between unfamiliar kin and non-kin odours (probably using phenotype matching) (Coffin *et al.* 2011). Variations between individual VOCs profiles found in our study might give rise to the observed differential responses of adult King Penguins to faeces and feathers of other adult King Penguins (Cunningham & Bonadonna 2015). However, chemical variation could also be influenced by other factors such as age (Martín & López 2006) and reproductive state (Caro et al. 2015). More investigations such as behavioural experiments are needed to explore the real potential of these VOCs in chemical recognition in this species.

Despite evidence for individual variation in VOC profiles of King Penguins, we did not find any evidence for sex discrimination. Sexual dimorphism in chemical signals has been reported in Domestic Ducks (*Anas platyrhynchos*), Budgerigars (*Melopsittacus undulatus*), Spotless Starlings

(*Sturnus unicolor*) and Dark-eyed Juncos, in which females and males differed in uropygial scents during the breeding season (Jacob *et al.* 1979, Zhang *et al.* 2010, Amo *et al.* 2012, Whittaker *et al.* 2013). However, sexual dimorphism in the composition of uropygial gland secretions is not ubiquitous. For instance, male and female Magpies (*Pica pica*) and Cory's Shearwaters (*Calonectris borealis*) exhibited no difference in the scent of the uropygial gland (Zhang unpubl. in Zhang *et al.* 2013, Gabirot *et al.* 2015). King Penguins might well use other traits such as calls to discriminate between sexes (Jouventin 1982).

Recognition of individual identity can be used to discriminate a mate, offspring, sibling or rival (Tibbetts & Dale 2007). Odours and chemical information could also be used by King Penguins to find their colony and to locate the position of the chick and the partner within the colony ("rendezvous zone") (Cunningham & Bonadonna 2015). King Penguins call during nest exchange to find their reproductive partner (Lengagne *et al.* 1999b, Lengagne *et al.* 1999a, Robisson 1993). Individuals returning from the ocean walk back to the colony and begins to call once within 8 m of the rendezvous zone, the partner incubating the egg or rearing the chick then replies (Lengagne *et al.* 1999b). The returning bird utilized the response to identify the position of partner or chick in the colony. However, beyond a distance of 14 m, penguins cannot discriminate calls from the background noise (Aubin & Jouventin 1998). Mechanisms for detection or recognition of the rendezvous zone at long distances are still unidentified. Chemical signals are known to work over long-distances from the emitter even if there are barriers, wind or water currents (Wyatt 2003). In the case of penguins the individual scents could well blend into a distinctive colony odour and returning birds could, therefore, use this odour to locate the rendezvous zone before switching to acoustic cues to locate their partner or chick.

To conclude our analysis showed variation of VOC profiles from feathers between individual King Penguins. This species employ strong and efficient acoustic signatures to recognize mates (Lengagne *et al.* 1999b) and discriminate between parents and chicks (Jouventin *et al.* 1999). Similarly, the presence of these individual variations in chemical profiles from feathers might have implications for ecological processes such as individual, kin recognition and mate choice. The quantitative and qualitative findings from this present study suggested that the plumage odour of King Penguins may play a role at least in social communication. Multiple traits (here acoustic and chemical cues) may either signal different characteristics of individual or may be redundant as a way to reinforce the reliability of signal (Rowe 1999). Animals can also take advantage of both sensory channels according with the context (e.i. environment proprieties) and distances to the potential receiving individual (mate or chick). Many animals (e.g. lizards, butterflies) use visual cues at short range and they communicate by chemical signature over longer distances (Wyatt 2003). Nevertheless, more studies and specifically behavioural tests are essential to establish the role of chemical communication in King Penguins, and in penguins in general, to derive a more complete picture of the mechanisms involved.

This work was supported by the Agence Nationale de la Recherche Française (AMBO ANR-08-BLAN-0117-01 to FB), by an IPEV grant (ETHOTAAF 354 to FB) and by Marie Curie Intra-European Fellowship (FP7-PEOPLE-2013-IEF – 625385 BIRDSCENTS by MG). We thank all the fieldworkers who collected samples in the field. We appreciate greatly Ismael Keddar and Yoanna Marescot for their work in the penguin's colony. We also thank the Institut Polaire Français Paul-Emile VICTOR (IPEV) for logistic support. All the stages of the study were performed following the IPEV and CNRS guidelines for the Ethical Treatment of Animals and complied with current French regulations.

REFERENCES

Amo, L., Avilés, J.M., Parejo, D., Peña, A., Rodríguez, J. & Tomás, G. 2012. Sex recognition by odour and variation in the uropygial gland secretion in starlings. *J. Anim. Ecol.* **81:** 605-613.

Anderson, M.J. 2001. Permutation tests for univariate or multivariate analysis of variance and regression. *Can. J. Fish. Aquat. Sci.* **58:** 626-639.

Apps, P.J. 2013. Are mammal olfactory signals hiding right under our noses? *Naturwissenschaften* **100:** 487-506.

Aubin, T. & Jouventin, P. 1998. Cocktail–party effect in king penguin colonies. *Proc. R. Soc. Lond. B Biol. Sci.* **265:** 1665-1673.

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.

Baltussen, E., Cramers, C. & Sandra, P. 2002. Sorptive sample preparation–a review. *Anal. Bioanal. Chem.* **373:** 3-22.

Bernier, U.R., Allan, S.A., Quinn, B.P., Kline, D.L., Barnard, D.R. & Clark, G.G. 2008. Volatile compounds from the integument of White Leghorn Chickens (*Gallus gallus domesticus L.*): Candidate attractants of ornithophilic mosquito species*. *J. Sep. Sci.* **31:** 1092-1099.

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. 2013. Besides colours and songs, odour is the new black of avian communication. In : Chemical Signals in Vertebrates 12. pp. 325-339. Springer New York.

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

Campagna, S., Mardon, J., Celerier, A. & Bonadonna, F. 2012. Potential semiochemical molecules from birds: a practical and comprehensive compilation of the last 20 years studies. *Chem. senses* **37:** 3-25.

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

Caro, S.P., Balthazart, J. & Bonadonna, F. 2015. The perfume of reproduction in birds: chemosignaling in avian social life. *Horm. Behav.* **68:** 25-42.

Clarke, K. & Warwick, R. 2001. A further biodiversity index applicable to species lists: variation in taxonomic distinctness. *Mar. Ecol. Prog. Ser.* **216:** 265-278.

Coffin, H.R., Watters, J.V. & Mateo, J.M. 2011. Odor-based recognition of familiar and related conspecifics: a first test conducted on captive Humboldt penguins (*Spheniscus humboldti*). *PloS one* **6:** e25002.

Cunningham, G.B. & Bonadonna, F. 2015. King penguins can detect two odours associated with conspecifics. *J. Exp. Biol.* **218:** 3374-3376.

Cunningham, G.B., Leclaire, S., Toscani, C. & Bonadonna, F. 2017. Responses of King penguin (*Aptenodytes patagonicus*) adults and chicks to two food-related odours. *J. Avian Biol.* **48***:* 235-242.

Cunningham, G.B., Strauss, V. & Ryan, P.G. 2008. African penguins (*Spheniscus demersus*) can detect ethyl sulphide, a prey-related odour. *J. Exp. Biol.* **211:** 3123-7.

Douglas III, H.D. 2006. Measurement of chemical emissions in crested auklets (*Aethia cristatella*). *J. Chem. Ecol.* **32:** 2559-2567.

Freedman, D.A. & Lane, D. 1983. Significance testing in a nonstochastic setting. A Festschrift for Erich L. Lehmann, edited by PJ Bickel, KA Docksum, and JL Hodges Jr. pp. 185-208. Wadsworth International Group, Wadworth, Belmont, California.

Gabirot, M., Raux, L., Dell'Ariccia, G., Bried, J., Ramos, R., González–Solís, J., Buatois, B., Crochet, P.A. & Bonadonna, F. 2015. Chemical labels differ between two closely related shearwater taxa. *J. Avian Biol.* **47***:* 540-551.

Gagliardo, A., Bried, J., Lambardi, P., Luschi, P., Wikelski, M. & Bonadonna, F. 2013. Oceanic navigation in Cory's shearwaters: evidence for a crucial role of olfactory cues for homing after displacement. *J. Exp. Biol.* **216:** 2798-2805.

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

Hackett, S.J., Kimball, R.T., Reddy, S., Bowie, R.C., Braun, E.L., Braun, M.J., Chojnowski, J.L., Cox, W.A., Han, K.-L. & Harshman, J. 2008. A phylogenomic study of birds reveals their evolutionary history. S*cience* **320:** 1763-1768.

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

Hagelin, J.C., Jones, I.L. & Rasmussen, L. 2003. A tangerine-scented social odour in a monogamous seabird. *Proc. R. Soc. Lond. B Biol. Sci.* **270:** 1323-1329.

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.

Jouventin, P. 1982. Visual and vocal signals in penguins, their evolution and adaptive characters. *Fortschritte der Verhaltensforschung*.

Jouventin, P. & Aubin, T. 2002. Acoustic systems are adapted to breeding ecologies: individual recognition in nesting penguins. *Anim. Behav.* **64:** 747-757.

Jouventin, P., Aubin, T. & Lengagne, T. 1999. Finding a parent in a king penguin colony: the acoustic system of individual recognition. *Anim. Behav*. **57:** 1175-1183.

Kooyman, G., Cherel, Y., Maho, Y.L., Croxall, J., Thorson, P., Ridoux, V. & Kooyman, C. 1992. Diving behavior and energetics during foraging cycles in king penguins. *Ecol. Monogr.* **62:** 143-163.

Ksepka, D.T., Bertelli, S. & Giannini, N.P. 2006. The phylogeny of the living and fossil Sphenisciformes (penguins). *Cladistics* **22:** 412-441.

Lengagne, T., Aubin, T., Lauga, J. & Jouventin, P. 1999a. How do king penguins (*Aptenodytes patagonicus*) apply the mathematical theory of information to communicate in windy conditions? *Proc. R. Soc. Lond. B Biol. Sci.* **266:** 1623-1628.

Lengagne, T., Jouventin, P. & Aubin, T. 1999b. Finding one's mate in a king penguin colony: efficiency of acoustic communication. *Behaviour* **136:** 833-846.

Mardon, J., Saunders, S.M., Anderson, M.J., Couchoux, C. & Bonadonna, F. 2010. Species, gender, and identity: cracking petrels' sociochemical code. *Chem. senses* **35**(4): 309-21. doi: 10.1093/chemse/bjq02

Mason, R.T. 1992. Reptilian pheromones. *Biology of the Reptilia* **18:** 114-228.

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

Moritz, R.F. & Crewe, R.M. 1988. Chemical signals of queens in kin recognition of honeybees, *Apis mellifera* L. *J. Comp. Physiol. A* **164:** 83-89.

Muller-Schwarze, D. (2006) *Chemical ecology of vertebrates*: Cambridge University Press.

Nesterova, A.P., Le Bohec, C., Beaune, D., Pettex, E., Le Maho, Y. & Bonadonna, F. 2010. Do penguins dare to walk at night? Visual cues influence king penguin colony arrivals and departures. *Behav. Ecol. Sociobiol.* **64:** 1145-1156.

Nesterova, A.P., Mardon, J. & Bonadonna, F. 2009. Orientation in a crowded environment: can King Penguin (*Aptenodytes patagonicus*) chicks find their creches after a displacement? *J. Exp. Biol.* **212:** 210-6.

Penn, D. & Potts, W.K. 1998. Chemical signals and parasite-mediated sexual selection. *Trends Ecol. Evol.* **13:** 391-396.

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. *J. R. Soc. Interface* **4:** 331-340.

Robacker, D.C., Garcia, J.A. & Bartelt, R.J. 2000. Volatiles from duck feces attractive to Mexican fruit fly. *J. Chem. Ecol.* **26:** 1849-1867.

Robisson, P. 1993. La reconnaissance individuelle chez deux espèces jumelles, le manchot empereur Aptenodytes forsteri et le manchot royal Aptenodytes patagonicus. PhD thesis, University of Rennes, France.

Röck, F., Mueller, S., Weimar, U., Rammensee, H.-G. & Overath, P. 2006. Comparative analysis of volatile constituents from mice and their urine. *J. Chem. Ecol.* **32:** 1333-1346.

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

Rowe, C. 1999. Receiver psychology and the evolution of multicomponent signals. Anim. behav. **58**(5): 921-931.

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.

Saraux, C., Le Bohec, C., Durant, J.M., Viblanc, V.A., Gauthier-Clerc, M., Beaune, D., Park, Y.-H., Yoccoz, N.G., Stenseth, N.C. & Le Maho, Y. 2011. Reliability of flipper-banded penguins as indicators of climate change. *Nature* **469:** 203-206.

Schmidt, A., Alard, F. & Handrich, Y. 2006. Changes in body temperatures in king penguins at sea: the result of fine adjustments in peripheral heat loss? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291:** R608-R618.

Shaw, C.L., Rutter, J.E., Austin, A.L., Garvin, M.C. & Whelan, R.J. 2011. Volatile and semivolatile compounds in gray catbird uropygial secretions vary with age and between breeding and wintering grounds. *J. Chem. Ecol.* **37:** 329-339.

Singer, A.G., Tsuchiya, H., Wellington, J.L., Beauchamp, G.K. & Yamazaki, K. 1993. Chemistry of odortypes in mice: fractionation and bioassay. *J. Chem. Ecol.* **19:** 569-579.

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*). *J. Chem. Ecol.* **33:** 183-198.

Stephens, D.W. & Krebs, J.R. (1986) *Foraging theory*: Princeton University Press.

Strandh, M., Westerdahl, H., Pontarp, M., Canbäck, B., Dubois, M.-P., Miquel, C., Taberlet, P. & Bonadonna, F. 2012. Major histocompatibility complex class II compatibility, but not class I, predicts mate choice in a bird with highly developed olfaction. *Proc. R. Soc. Lond. B Biol. Sci.* **279:** 4457-4463.

Tibbetts, E.A. & Dale, J. 2007. Individual recognition: it is good to be different. *Trends Ecol. Evol.* **22:** 529-537.

Warham, J. (1990) *The petrels: their ecology and breeding systems*: A&C Black.

Whittaker, D.J., Gerlach, N.M., Soini, H.A., Novotny, M.V. & Ketterson, E.D. 2013. Bird odour predicts reproductive success. *Anim. Behav.* **86:** 697-703.

Whittaker, D.J., Soini, H.A., Atwell, J.W., Hollars, C., Novotny, M.V. & Ketterson, E.D. 2010. Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. *Behav. Ecol.* **21:** 608-614.

Williams, C.R., Kokkinn, M.J. & Smith, B.P. 2003. Intraspecific variation in odor-mediated host preference of the mosquito *Culex annulirostris*. *J. Chem. Ecol.* **29:** 1889-1903.

Williams, T.D. (1995) *The penquins; Spheniscidae*: Oxford University Press.

Wright, K.L., Pichegru, L. & Ryan, P.G. 2011. Penguins are attracted to dimethyl sulphide at sea. *J. Exp. Biol.* **214:** 2509-11.

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

Zhang, J.-X., Wei, W., Zhang, J.-H. & Yang, W.-H. 2010. Uropygial gland-secreted alkanols contribute to olfactory sex signals in budgerigars. *Chem. Senses* **35:** 375-382.

Zhang, Y.-H., Du, Y.-F. & Zhang, J.-X. 2013. Uropygial gland volatiles facilitate species recognition between two sympatric sibling bird species. *Behav. Ecol.* **24:** 1271-1278.

FIGURES

Figure 1. Chromatograms of volatile organic compounds from feathers of one individual King Penguin and blanks at two temperatures of desorption: 70 °C in grey and 100 °C in black. Numbers represent chemical compounds used in further analysis and are listed in Table 1.

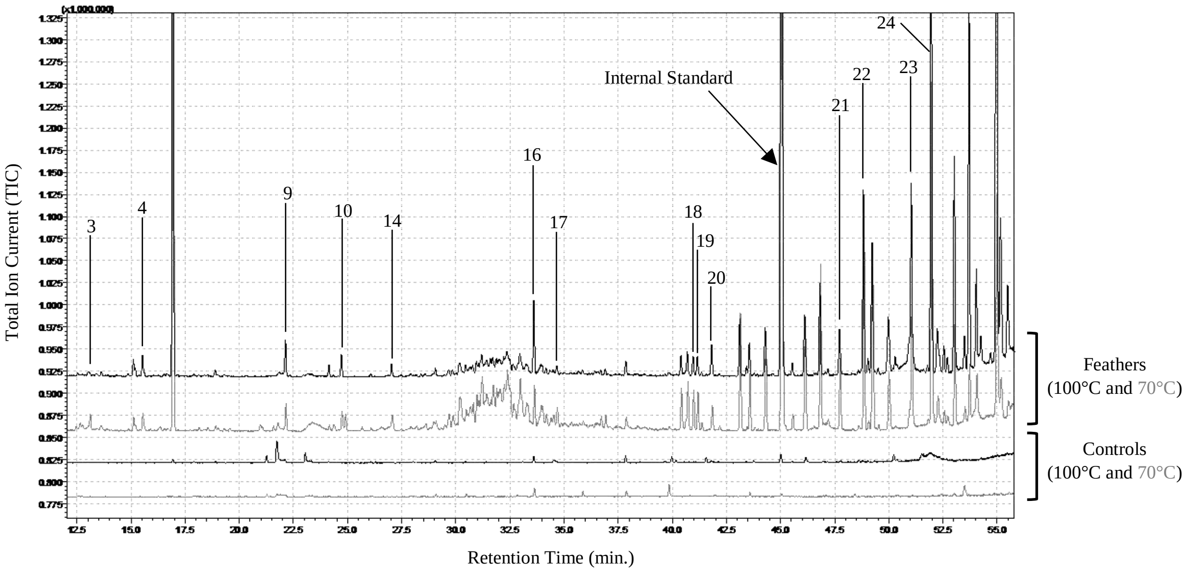
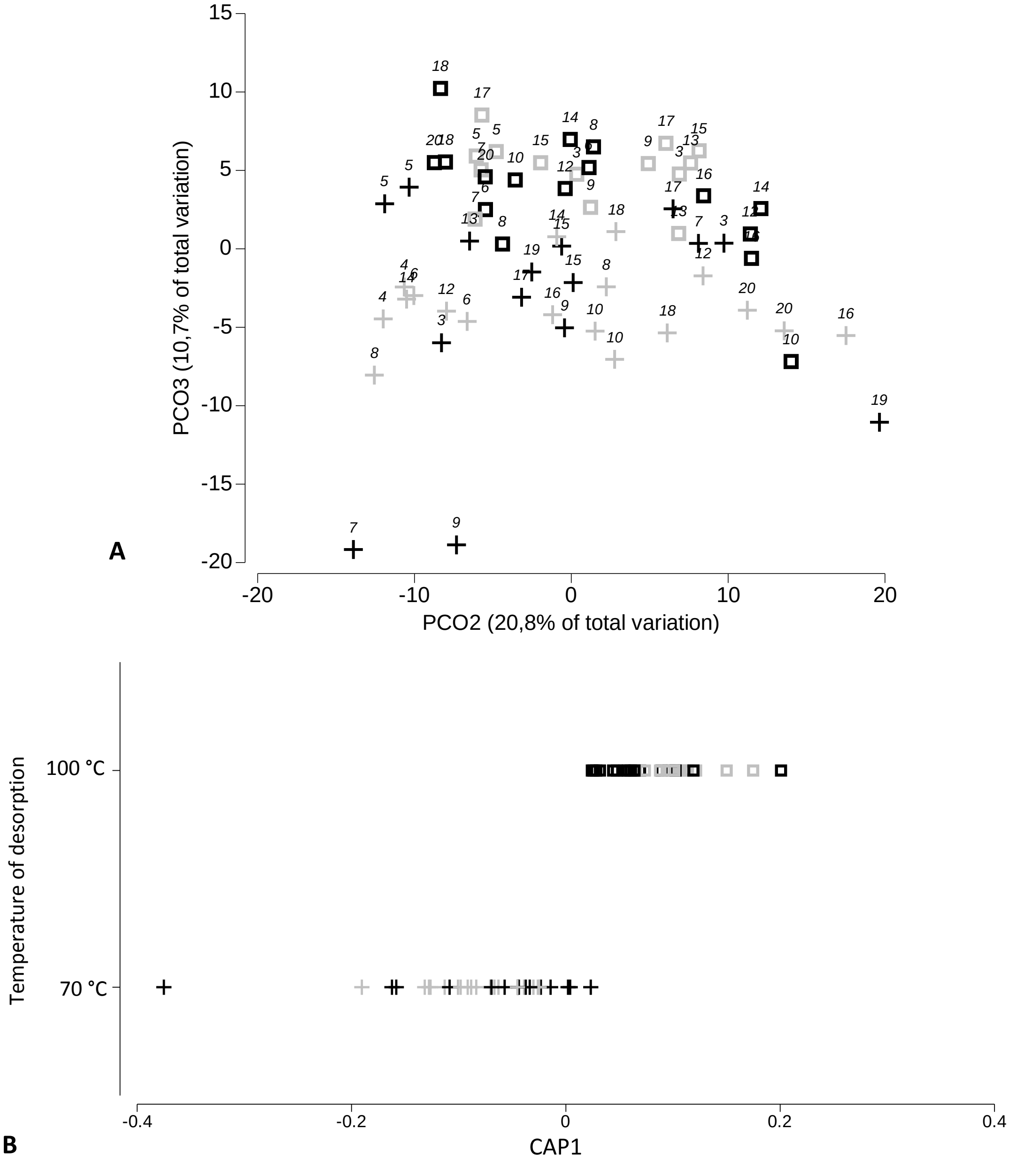


Figure 2. Comparison of VOCs profiles from feathers of King Penguins between two temperatures of desorption. A) two-dimensional PCO ordinations for all samples: PCO2 vs PCO3. B) CAP analysis of the temperature of desorption factor using a single CAP axis obtained from m = 3 PCO axes, showing 95.23% correct discrimination of chemical profiles between the different temperatures. Each number corresponds to an individual: cross for 70 °C of desorption and rectangle for 100 °C of desorption; black symbols for males and grey for females.



TABLES

Table 1. List of chemical volatile organic compounds (VOCs) extracted from feathers of King Penguins by thermal desorption. Avian occurrence reports the molecules from feathers, uropygial secretions, feet-skin and faeces in other species of birds: BWD (Black-bellied Whistling Duck, *Dendrocygna autumnalis*) in Robacker *et al.* (2000), DC (Domestic Chicken, *Anas platyrhynchos*) in Williams *et al.* (2003) and Bernier *et al.* (2008), AP (Antarctic Prion, *Pachyptila desolata*) in Bonadonna *et al.* (2007), DJ (Dark-eyed Junco, *Junco hyemalis*) in Soini *et al.* (2007), CA (Crested Auklet, *Aethia cristatella*) in Hagelin *et al.* (2003) (reviewed in Campagna *et al.* (2012)). ***r*** corresponds to the Pearson correlation coefficient of a particular compound with the CAP axis discriminating the two temperatures analysis (70 and 100 °C). For information, critical r values (at a level of α = 5%) would be 0.47. Strong contributions are in bold. Negative values of Pearson correlation (r) were related with samples desorbed at 70 °C and positive values of r with samples desorbed at 100 °C (see also Fig. 2B).

Table 2. Results from PERMANOVA tests examining factors effect on chemical profiles from feathers: A) the temperature, sex and individual effect; B) sex and individual effect at 70 °C; C) sex and individual effect at 100 °C. (df: degrees of freedom; SS: sum of squares; MS: mean square; significant effect at a level of α = 5 % are in bold)

Table 1.

N°	Retention Time (min.)	Retention Index Calculated	Molar Mass	Formula	Name proposed by the library (match percentage to the NIST library)	Family of compound	Avian occurrences	*r*
1	12.455	714.62	100	C6H12O	2,5-Dimethyl tetra-hydro-furan (87%)	furane		-0.01
2	12.635	718.02	98	C6H12O	2,3-Dihydro-2,5-dimethyl furan (89%)	furane		-0.099
3	13.105	726.89	86	C5H10O	2-Pentanone (94%)	ketone	BWD	-0.114
4	15.545	772.92	114	C8H18	2,4-Dimethyl hexane (91%)	methyl alkane		0.3
5	16.34	787.92	-	-	Compound unidentified 1	furane		-0.044
6	18.15	822.59	100	C6H12O	3-Hexanone (99%)	ketone	DC	0.07
7	18.545	830.21	100	C6H12O	2-Hexanone (95%)	ketone	DC	0.078
8	21.58	888.8	98	C6H10O	3-Hexen-2-one (95%)	ketone		-0.077
9	22.15	899.81	128	C9H20	Nonane (93%)	alkane		**0.58**
10	24.745	927.35	108	C7H8O	Methoxybenzene (Anisol) (77%)	benzene		**0.445**
11	24.91	929.1	114	C8H18	3,4-Dimethyl hexane (88%)	alkane		-0.078
12	25.68	937.25	114	-	Compound unidentified 2	alkanol		0.087
13	26.61	947.09	204	C10H20O4	2-(2-Butoxyethoxy)-ethanol acetate (87%)	ether ester		-0.04
14	27.075	952.01	114	C6H10O2	2,5-Hexanedione (98%)	ketone	DC	-0.1
15	28.495	967.04	114	C7H14O	3-Heptanone (85%)	ketone		0.0245
16	33.635	1147.65	142	C9H18O	Nonanal (96%)	aldehyde	DJ AP DC	**0.611**
17	34.69	1172.47	170	C12H26	3,8-Dimethyl decane (92%)	methyl alkane		**-0.631**
18	40.99	1330.65	-	C13H28	Undecane, dimethyl, Isomere I (92%)	methyl alkane		**-0.841**
19	41.19	1336.02	-	C13H28	Undecane, dimethyl, Isomere II (92%)	methyl alkane		**-0.849**
20	41.845	1353.63	170	C11H22O	Undecanal (95%)	aldehyde	DJ CA DC	0.111
21	47.755	1519.85	-	C12H24O2	Decanoic acid ethyl ester, Isomere I (78%)	fatty acid, ethyl ester		0.123
22	48.855	1553.18	-	C12H24O2	Decanoic acid ethyl ester, Isomere II (76%)	fatty acid, ethyl ester		0.034
23	51.075	1621.5	-	-	Compound unidentified 3	fatty acid, ethyl ester		0.353
24	51.985	1650.48	214	C13H26O2	Undecanoic acid ethyl ester (80%)	fatty acid, ethyl ester		0.401
25	53.73	1706.4	226	C16H34	9-Methylpentadecane (90%)	methyl alkane		**0.632**
26	54.995	1748.99	228	C14H28O2	Dodecanoic acid ethyl ester (80%)	fatty acid, ethyl ester		**0.54**

Table 2.

A)

Source	df	SS	MS	Pseudo-*F*	*P* (perm)
Temperature	1	2915.6	2915.6	13.43	**0.0001**
Sex	1	420.01	420.01	0.76	0.5118
Individual (Nested with Sex)	15	8126.6	541.78	2.93	**0.0001**
Residuals	31	5734.3	184.98		
Total	62	19840			

B)

Source	df	SS	MS	Pseudo-*F*	*P* (perm)
Sex	1	179.97	179.97	0.48	0.7547
Individual (Nested with Sex)	15	5661.3	377.42	1.91	**0.0107**
Residuals	16	3160.5	197.53		
Total	32	9001.7			

C)

Source	df	SS	MS	Pseudo-*F*	*P* (perm)
Sex	1	345.02	345.02	0.90	0.4333
Individual (Nested with Sex)	13	5003.9	384.92	2.24	**0.0114**
Residuals	15	2573.8	171.59		
Total	29	7922.8			