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

3
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19 The role and use of olfactory cues by penguins is largely under-investigated with only a few studies
20 suggesting that odours are involved in prey detection, orientation and for interspecific communication.
21 This also applies to King Penguins (*Aptenodytes patagonicus*) where little is known about their
22 abilities in chemoreception and, subsequently, importance of odours in their behavioural ecology.
23 Here, we investigated the chemical composition of volatile organic compounds (VOCs) from feathers
24 of King Penguins in the Kerguelen Archipelago and their potential to carry information on identity and
25 sex. We analysed VOCs using direct thermal desorption, a novel approach for extracting volatile
26 compounds directly from solid matrices. We were only able to test at desorption temperatures of 70 °C
27 and 100 °C to optimise conditions for VOC analysis. We found a profile of 26 VOCs, present in most
28 individuals, which varied significantly between individuals but not between sexes. Results suggested
29 that VOCs could be potentially used by King Penguins to locate the colony and recognize individuals
30 if similar VOCs are also present at ambient conditions. Further studies and behavioural experiments
31 are encouraged to explore olfactory-based communication in this species.

32

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

34

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

46 Sphenisciformes (penguins) and Procellariiformes (albatrosses and petrels) are
47 phylogenetically closely related (Ksepka *et al.* 2006, Hackett *et al.* 2008) and share many common
48 traits. For example, penguins and petrels exploit similar ocean habitats, tend to forage on similar types
49 of prey, such as krill, fish and squid (Warham 1990, Williams 1995). Also a number of species of both
50 orders nest in large colonies and are central place foragers during the breeding season (Stephens &
51 Krebs 1986, Williams 1995). Despite these similarities, it is assumed that these seabirds employ
52 different sensory modalities. Procellariiformes use chemical communication for orientation, homing,
53 reproduction and social interactions (Gagliardo *et al.* 2013, Bonadonna & Mardon 2013, Caro *et al.*
54 2015), whereas the role and function if any of chemical cues in sphenisciform species is unclear.
55 Studies on penguins have focused on calls in individual recognition (Aubin *et al.* 2000, Jouventin &
56 Aubin 2002) or visual cues in foraging (Kooyman *et al.* 1992). However, penguins may also utilize
57 chemical cues, at least food-related odour in foraging (Cunningham *et al.* 2008, Wright *et al.* 2011,
58 Cunningham *et al.* 2017). In a recent study, King Penguins (*Aptenodytes patagonicus*) responded
59 differentially to smell of sand, feathers or faeces, when exposed to samples while asleep (Cunningham
60 & Bonadonna 2015). In addition, Nesterova *et al.* (2009) reported that King Penguin chicks only
61 successfully oriented towards their colony at night when placed down-wind of the colony after having
62 been removed from the nest. Breeding King Penguins also walk towards their place in the colony in
63 complete darkness (Nesterova *et al.* 2010). Even though visual cues appear to be important in short-
64 distance orientation of King Penguins (Nesterova *et al.* 2009), it is sensible to assume that additional
65 information from olfactory, magnetic or acoustic cues could be involved in their navigating back to
66 their place in the colony in complete darkness (Nesterova *et al.* 2010). Therefore, King Penguins may
67 use scents to locate the colony or the island, similarly to many Procellariiformes which detect their
68 burrow by olfactory cues (Bonadonna *et al.* 2003). Olfaction might also be important in social
69 communication as already reported in Humbolt Penguins (*Spheniscus humboldti*), which show kin
70 recognition based on olfactory cues (Coffin *et al.* 2011). Odours from feathers and faeces may also

71 relay information on individual identity enabling recognition of their partner among a large number of
72 individuals and orientate animals back to the colony after foraging (Cunningham & Bonadonna 2015).

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

89

90

91 **METHODS**

92

93 **Species and field sampling**

94

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

104

105 **Sample preparation**

106

107 Three feathers from each sample were placed in 'Loose Fit' Teflon® inserts (Liner PTFE; Markes
108 International Limited, Llantrisant, UK) which were inserted into clean empty TD tubes (OD = 6 mm;
109 L = 88 mm; Perkin-Elmer France). A silanized glass wool plug (Perkin Elmer USA) was added at the
110 top to avoid any loss of feathers. All samples were spiked with 1 µL of 0.1 mg/mL biphenyl (MW =
111 154.21 g/mol, 99.5 % Sigma-Aldrich®, France) in a mixture of 1:3 dichloromethane/n-hexane
112 (Sigma-Aldrich®, France) as internal standard. Control tubes containing an empty insert (i.e. without
113 feather) were prepared in the same manner as feather samples and were run every 10 samples within
114 each sample batch. These empty tubes controlled for possible contamination during sample
115 preparation and from GC-MS instrument itself during extraction and analysis.

116

117 **Thermal desorption and chromatographic analysis**

118

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

124 The choice of desorption temperature for a sample is critical, ideally it should reflect natural
125 conditions, avoid pyrolysis and yield detectable amounts of VOCs. The combustion point of King
126 Penguin feathers is unknown and the value for feathers of blue petrels (*Halobaena caerulea*) of around
127 230 °C (J. Mardon personal data) was used as a reference. In order to reflect natural conditions, a
128 thermal desorption temperature close to their body surface temperature (30-35 °C, Schmidt *et al.*
129 (2006)) would have been ideal. Unfortunately, this temperature was not within the technical
130 specification of the instrument, which only allowed for a minimum desorption temperature of 70 °C.
131 We, therefore tested two desorption temperatures, 70 °C and 100 °C, which fell within the lower limit
132 of the instrument and avoided combustion or undue thermal stress and excessive desorption of waxes,
133 which was found at higher temperatures in preliminary trials. Desorption was repeated with a separate
134 feather sample for each individual at both desorption temperatures resulting in four chemical profiles
135 for each individual: two at 70 °C and two at 100 °C.

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

144 °C. Data were acquired in scan mode from 20 to 350 amu at scan speed = 1111, scan interval = 0.3 s
145 and electron ionization (EI) energy of 70 eV. A mixture of C₈-C₂₀ alkanes (Sigma Aldrich®,
146 Switzerland) was processed under the same conditions to calibrate for retention index calculation.

147

148 **Chromatographic Data Processing**

149

150 Raw data were processed and integrated using GC-MS Solution software v2.40 (Shimadzu Corp.). The
151 quality of all software-defined peak integrations was visually reviewed and manually corrected if
152 necessary. Data processing was 'blind' as uninformative codes were given to all samples and used in
153 all analytical steps until the final data set was obtained. Analytes were identified by comparison of
154 mass spectral data using the NIST (National Institute of Standards and Technology) Mass Spectral
155 Search Program v2.0© (Faircom Corp.; Columbia MO, USA) and Wiley Registry™ of Mass Spectral
156 Data and cross-checking spectral matches with the calculated Retention Index (RI) of the analytes. For
157 quantitative analyses, data were standardised to area of the internal standard (biphenyl, RT = 44.95
158 min, RI = 1439).

159

160 **Data pre-treatment, resemblance measure, and ordination**

161

162 GC-MS total ion current (TIC) profiles of samples with and without feathers were compared first (Fig.
163 1). The difference between the chromatographic profiles of controls and samples was clearly
164 noticeable by visual inspection. Consequently we removed all compounds of controls from further
165 analysis (e.g. molecules derived from GC-column). Only compounds eluting from C7 (n-heptane RT =
166 11.68 min, RI = 700) to C18 (n-octadecane, RT = 56.51, RI = 1800) were used for the analysis. Before
167 and after this range of chemical compounds (and retention time or retention index) we did not have a
168 good signal and resolution in chemical profile. This resulted in 26 compounds, which were (i)
169 putatively identified individually by matches of mass spectrum and RI, and (ii) classified to substance
170 class level (e.g. fatty acid ethyl ester) by matching mass spectrum. One profile, desorbed at 70 °C, was
171 excluded, as it showed no peaks, most probably due to problems during chromatography analysis. In
172 addition, there were not enough feathers of two individuals (one male and one female) to sample at
173 both temperatures and were only desorbed at 70 °C, which resulted in a total of 65 profiles (17 birds x
174 2 T x 2 replicates - 3). Standardized data were finally square-root transformed to reduce the influence
175 of the most abundant analytes on the analysis (Clarke & Warwick 2001). Euclidean distances between
176 every pair of samples were calculated to produce a resemblance matrix that formed the basis of
177 ensuing analyses. Principal coordinates (PCO) analysis based on the Euclidean resemblance matrix
178 (Gower 1966) was used as an ordination method in order to visualize the patterns of differences in the
179 multivariate chemical structure among samples (see Mardon *et al.* (2010)). All statistical analyses
180 were carried out using the computer program PRIMER V.7.0.5 Permanova+1 (Primer-E Ltd©).

181

182 **Effect of desorption temperature, sex and individual variability**

183

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

196

197

198 **RESULTS**

199

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

205 The comparison of profiles of all 17 individuals (males and females desorbed at 70 and
206 100 °C) indicated differences between the two desorption temperatures. In an unconstrained 3D PCO
207 the first three axes explained 80.46 % (Axis 1: 48.95 % - Axis 2: 20.79 % - Axis 3: 10.72 %) of the
208 total variation. The PCO plot using just the first and second axes was not very effective to see
209 differences between the chemical profiles associated with temperature of desorption. However when
210 we explored the first and third PCO axes (PCO1 x PCO3) and the second and third PCO axes (PCO2 x
211 PCO3, Fig. 2A), we saw a clear distinction between 70 °C and 100 °C. Using PERMANOVA,
212 significant differences between profiles of VOCs were found between temperatures of desorption and
213 between individuals (Table 2). There was no significant difference in profiles with respect to sex
214 (Table 2).

215 Chemical profiles distinguished desorption temperatures in CAP on a single axis obtained
216 from $m = 3$ PCO axes. The leave-one-out misclassification error was 4.8% for the samples used to
217 build the CAP model (Fig. 2B). The CAP model associated mostly dimethyl alkanes (3,8 dimethyl

218 decane, dimethyl undecane, Table 1) to 70 °C desorption and nonane, nonanal, dodecanoic acid ethyl
219 ester and 9-methylpentadecane to 100 °C (see Table 1 and Fig. 2B).

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

223

224

225 **DISCUSSION**

226

227 The volatile organic compounds present on feathers of King Penguins included ketones (2-pentanone,
228 2-hexanone, 3-heptanone), methyl alkanes (hexane, 2,4-dimethyl, decane, 3,8-dimethyl), aldehydes
229 (nonanal, undecanal), aromatic compound, furans and fatty acid ester (Table 1) showing a composition
230 that compared well with previous studies on semiochemicals in birds in general (review in Campagna
231 *et al.* (2012)). In particular, ketones and aldehydes have been encountered in feathers of other bird
232 species such as Domestic Ducks (*Anas platyrhynchos*, Bernier *et al.* 2008), Antarctic Prions
233 (*Pachyptila desolata*, Bonadonna *et al.* 2007), Crested Auklets (*Aethia cristatella*, Hagelin *et al.*
234 2003), Dark-eyed Juncos (*Junco hyemalis*, Soini *et al.* 2007) and Black-bellied Whistling Ducks
235 (*Dendrocygna autumnalis*, Robacker *et al.* 2000). The variance of profiles was found to be large
236 enough to discriminate between individuals but interestingly not between the sexes. This suggests the
237 distinct possibility that chemical communication is used for individual recognition in King Penguins.
238 Ultimately, behavioural studies can confirm or disprove occurrence of chemical communication.
239 However, such field tests are greatly aided by any knowledge of composition and nature of the
240 potential chemical signal.

241 Ideally, VOCs should be collected under natural conditions directly from the animal.

242 Nevertheless working on a remote island with King Penguins creates several problems. A direct
243 collection of headspace of animals would require capture and enclosure of individuals, which would
244 not be feasible and potentially affect results. Direct collection of odours or chemical mixtures in the
245 field implicates sorbent materials to capture molecules from animals or environments, which have to
246 be robust and easy to handle during field work. In addition, absorbed odours or chemical mixtures
247 should be stable on the sorbent until extraction and/or analysis with GC-MS. However, sorbent
248 materials such as in stir-bars and SPME fibres are not suited for longer term storage and extraction
249 from e.g. charcoal or Tenax with solvents results in unacceptable dilution of samples. As we
250 explained, the King Penguins colony studied here is situated in remote Island, at about one month
251 travelling to come back to laboratory. Therefore, collection of feathers provides a straightforward and
252 effective method to gather material that represents the actual scent of an individual. The collection of
253 VOCs from such material should optimally be carried out at body temperature and we tested several
254 options in the laboratory (active headspace, SPME) to do so. Although a scent was clearly discernible

255 by the human nose, we were not able to obtain a sufficiently strong signal in the GC-MS (MG & CTM
256 personal obs.). We, consequently, developed a novel approach, direct thermal desorption, to obtain
257 VOCs from feathers of King Penguins. This method essentially evaporates the chemical compounds
258 directly off feathers at elevated temperatures. Increasing the temperature is a common method to
259 increase vapour pressure and, hence, headspace concentration without much distortion of the VOC
260 profile as long as the temperature is low enough to avoid (i) breakdown of the sample or its
261 components e.g. alcohols, carboxylic acids (Baltussen *et al.* 2002) and (ii) accumulation of very high
262 boiling compounds e.g. high molecular weight waxes at a desorption temperature of 180 °C (MG and
263 CTM personal obs.). To explore the effect of desorption temperature on feathers, we compared the
264 VOCs obtained at temperatures of desorption of 70 and 100 °C. Such desorption temperatures are still
265 two to three times higher than body temperature and will result in increased abundance of observed
266 compounds by an estimated factor in the order of 4 to 8 respectively. This would mean that the less
267 volatile components reported here (No 17 – 26 in table 1) would still be emitted under field conditions
268 albeit at much lower concentrations. However, as olfaction can be orders of magnitude more sensitive
269 than detection with GC-MS they still may play a role and only behavioural assays will be able to
270 resolve this issue. Overall, higher desorption temperatures appear to be problematic and temperatures
271 of and lower than 100 °C are recommended for direct thermal desorption of feathers. Consequently
272 our method combines advantages from using a relative abundant material (feather lipids) with the
273 advantages of using thermal desorption (higher sensitivity due to the absence of dilution).

274 Only recently, discrimination between individuals based on olfaction and associated chemical
275 profiles from feathers have been reported in Blue Petrels (*Halobaena caerulea*) (by chemical analysis
276 and behavioural experiments) and in Antarctic prions (*Pachyptila desolata*) (by behavioural tests only)
277 (Mardon *et al.* 2010, Bonadonna & Mardon 2013). Individuality in odours has been also observed in
278 several mammals species including mice (*Mus musculus domesticus*) (by chemical analysis and
279 behavioural experiment in Singer *et al.* 1993), Bechstein's bats (*Myotis bechsteinii*) (by chemical
280 analysis in Safi & Kerth 2003), and humans (by chemical analysis in Penn *et al.* 2007). Chemical
281 recognition was also observed in Humboldt Penguins, which can discriminate between familiar and
282 unfamiliar non-kin odours (using prior association) and between unfamiliar kin and non-kin odours
283 (probably using phenotype matching) (Coffin *et al.* 2011). Variations between individual VOCs
284 profiles found in our study might give rise to the observed differential responses of adult King
285 Penguins to faeces and feathers of other adult King Penguins (Cunningham & Bonadonna 2015).
286 However, chemical variation could also be influenced by other factors such as age (Martín & López
287 2006) and reproductive state (Caro *et al.* 2015). More investigations such as behavioural experiments
288 are needed to explore the real potential of these VOCs in chemical recognition in this species.

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

292 (*Sturnus unicolor*) and Dark-eyed Juncos, in which females and males differed in uropygial scents
293 during the breeding season (Jacob *et al.* 1979, Zhang *et al.* 2010, Amo *et al.* 2012, Whittaker *et al.*
294 2013). However, sexual dimorphism in the composition of uropygial gland secretions is not
295 ubiquitous. For instance, male and female Magpies (*Pica pica*) and Cory's Shearwaters (*Calonectris*
296 *borealis*) exhibited no difference in the scent of the uropygial gland (Zhang unpubl. in Zhang *et al.*
297 2013, Gabriot *et al.* 2015). King Penguins might well use other traits such as calls to discriminate
298 between sexes (Jouventin 1982).

299 Recognition of individual identity can be used to discriminate a mate, offspring, sibling or
300 rival (Tibbetts & Dale 2007). Odours and chemical information could also be used by King Penguins
301 to find their colony and to locate the position of the chick and the partner within the colony
302 ("rendezvous zone") (Cunningham & Bonadonna 2015). King Penguins call during nest exchange to
303 find their reproductive partner (Lengagne *et al.* 1999b, Lengagne *et al.* 1999a, Robisson 1993).
304 Individuals returning from the ocean walk back to the colony and begins to call once within 8 m of the
305 rendezvous zone, the partner incubating the egg or rearing the chick then replies (Lengagne *et al.*
306 1999b). The returning bird utilized the response to identify the position of partner or chick in the
307 colony. However, beyond a distance of 14 m, penguins cannot discriminate calls from the background
308 noise (Aubin & Jouventin 1998). Mechanisms for detection or recognition of the rendezvous zone at
309 long distances are still unidentified. Chemical signals are known to work over long-distances from the
310 emitter even if there are barriers, wind or water currents (Wyatt 2003). In the case of penguins the
311 individual scents could well blend into a distinctive colony odour and returning birds could, therefore,
312 use this odour to locate the rendezvous zone before switching to acoustic cues to locate their partner or
313 chick.

314
315 To conclude our analysis showed variation of VOC profiles from feathers between individual
316 King Penguins. This species employ strong and efficient acoustic signatures to recognize mates
317 (Lengagne *et al.* 1999b) and discriminate between parents and chicks (Jouventin *et al.* 1999).
318 Similarly, the presence of these individual variations in chemical profiles from feathers might have
319 implications for ecological processes such as individual, kin recognition and mate choice. The
320 quantitative and qualitative findings from this present study suggested that the plumage odour of King
321 Penguins may play a role at least in social communication. Multiple traits (here acoustic and chemical
322 cues) may either signal different characteristics of individual or may be redundant as a way to
323 reinforce the reliability of signal (Rowe 1999). Animals can also take advantage of both sensory
324 channels according with the context (e.i. environment proprieties) and distances to the potential
325 receiving individual (mate or chick). Many animals (e.g. lizards, butterflies) use visual cues at short
326 range and they communicate by chemical signature over longer distances (Wyatt 2003). Nevertheless,
327 more studies and specifically behavioural tests are essential to establish the role of chemical

328 communication in King Penguins, and in penguins in general, to derive a more complete picture of the
329 mechanisms involved.

330

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337

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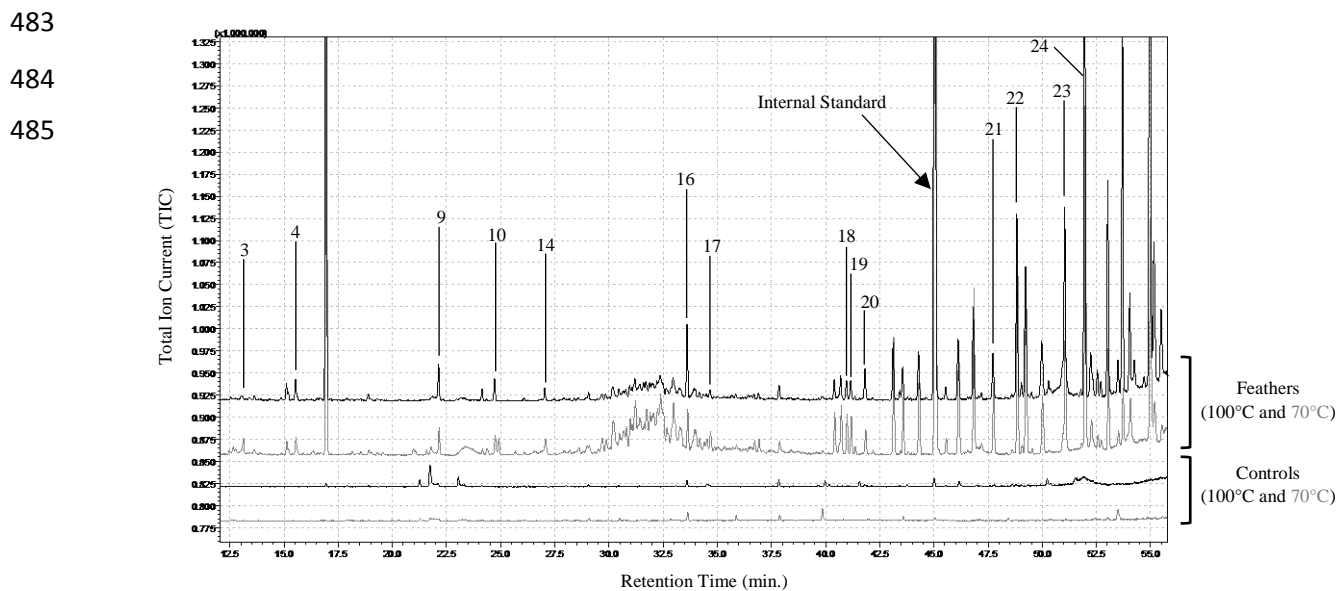
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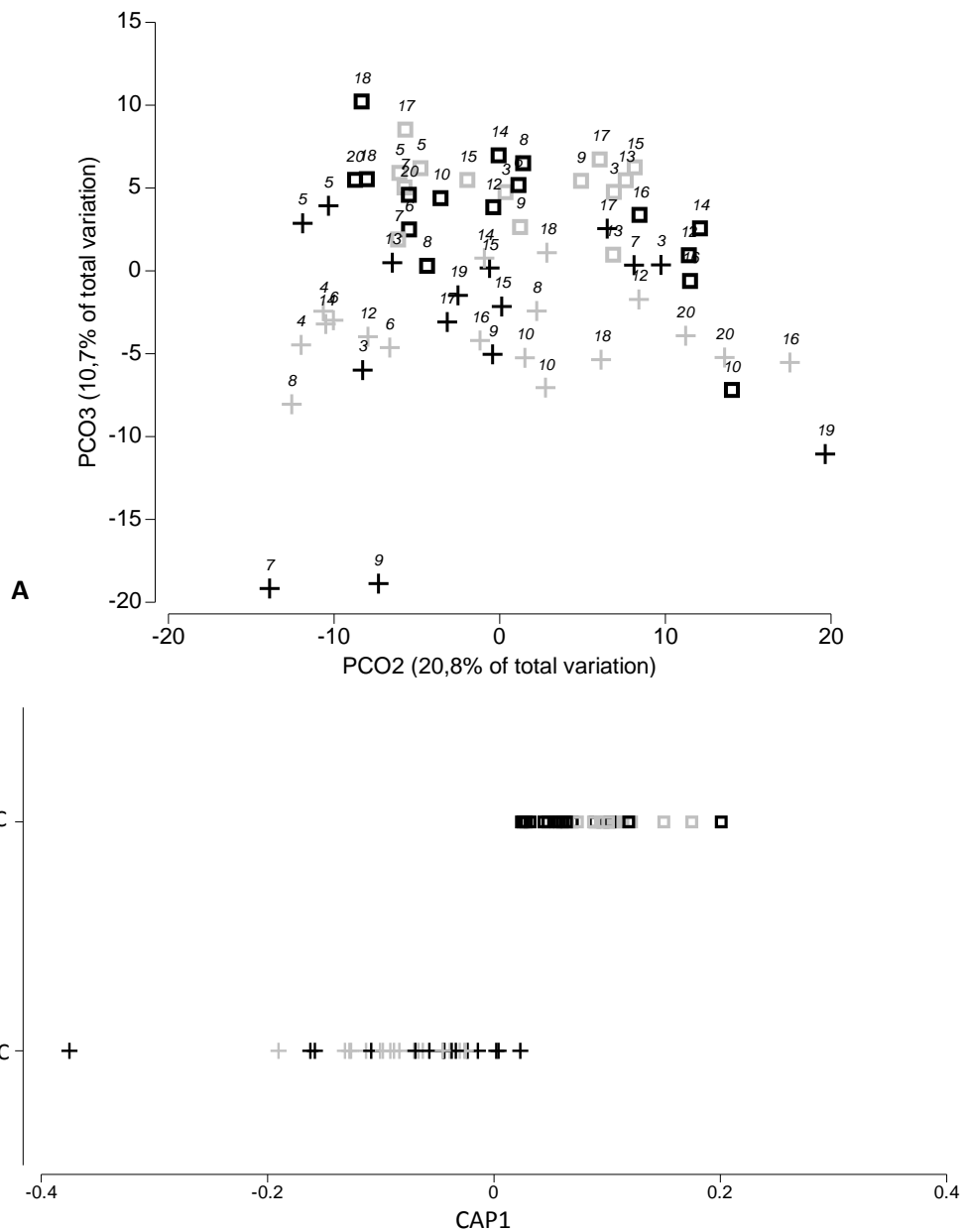
479 **FIGURES**

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



486 **Figure 2.** Comparison of VOCs profiles from feathers of King Penguins between two temperatures of
 487 desorption. A) two-dimensional PCO ordinations for all samples: PCO2 vs PCO3. B) CAP analysis of
 488 the temperature of desorption factor using a single CAP axis obtained from $m = 3$ PCO axes, showing
 489 95.23% correct discrimination of chemical profiles between the different temperatures. Each number
 490 corresponds to an individual: cross for 70 °C of desorption and rectangle for 100 °C of desorption;
 491 black symbols for males and grey for females.

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496 **TABLES**

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

509

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

514

515 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

517 Table 2.

518

519 A)

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

520

521 B)

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

522

523 C)

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

524

525

526

527