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1 **Chemical fingerprints suggest direct familiarisation rather than phenotype matching during olfactory**
2 **recognition in Australian sea lions (*Neophoca cinerea*)**

3 Kaja Wierucka*^{1,2}, Nicolas Barthes³, Robert Harcourt¹, Benoist Schaal⁴, Isabelle Charrier², Benjamin J.
4 Pitcher^{1,5}

5

6 ¹Department of Biological Sciences, Macquarie University, Sydney, Australia

7 ²Institut des Neurosciences Paris-Saclay, Université Paris-Saclay, CNRS (UMR 9197), Université Paris-Sud,
8 Orsay, France

9 ³Centre d'Ecologie Fonctionnelle et Evolutive, CNRS (UMR 5175), Université de Montpellier – Université
10 Paul-Valéry Montpellier 3 – EPHE, IRD, Montpellier, France

11 ⁴Developmental Ethology Group, Centre for taste, smell and feeding behavior science, CNRS (UMR
12 6265), Université de Bourgogne, Dijon, France

13 ⁵Taronga Conservation Society Australia, Mosman, NSW, Australia

14 *email: kaja.wierucka@hdr.mq.edu.au, phone: +61 2 9850 7975

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16

17 ABSTRACT

18 Olfaction is one of the most commonly used senses for communication among animals and is of
19 particular importance to mother-offspring recognition in mammals. The use of smell in offspring
20 recognition has been well studied, however, we often lack information about the underlying mechanistic
21 basis for olfactory recognition. Using gas chromatography-mass spectrometry (GC-MS), we examine
22 chemical profiles of two different colonies of Australian sea lions (*Neophoca cinerea*) and assess
23 similarity of chemical fingerprints in mother-pup pairs. This analysis allows us to examine whether a
24 chemical base for phenotype matching exists in this species. Our results showed no GC-detectible
25 mother-offspring similarity in the overall chemical fingerprints, suggesting that direct familiarisation is a
26 more likely mechanism used by Australian sea lion mothers for recognition of their young. Our analysis
27 also demonstrates that colony differences are encoded within chemical fingerprints and appear to be
28 highly influenced by environmental compounds. The study improves our understanding of how
29 Australian sea lion females use odours for selective offspring recognition and the potential importance
30 of environmentally acquired chemical compounds in the overall odour bouquet used in mother-pup
31 interactions.

32

33 Keywords: Chemical communication, mother-offspring recognition, olfaction, pinniped, *Neophoca*
34 *cinerea*.

35

36 1. INTRODUCTION

37 Chemical communication is the oldest communication channel used by animals and mediates many
38 social interactions among individuals (e.g., Brown and MacDonald, 1985; Bradbury and Vehrencamp,
39 2011). In mammals, olfactory cues play an important role in reproductive behaviours associated with
40 intra-sexual competition, mating, parental care, and filial responsiveness (Brennan and Kendrick, 2006).
41 Although the abilities of animals to recognise conspecifics, particularly kin, has been exhaustively
42 researched (e.g., Johnston, 2008), the cognitive/behavioural mechanisms through which olfactory
43 recognition occurs have rarely been examined in free-ranging mammals.

44 Animals use different mechanisms to recognise kin by smell (reviewed in Wyatt, 2014). However, the
45 source of the template the animal uses for recognition can differ. In *direct familiarisation* animals
46 associate with another individual and learn its distinct odour which they remember and use for
47 identification at the next encounter (e.g. Holmes and Sherman, 1982). *Phenotype matching* (indirect
48 familiarisation) does not require prior contact. Instead, animals match a known template, either from
49 other kin or from self (self-referent matching), to a newly encountered odour (e.g. Brennan and
50 Kendrick 2006; Mateo and Johnston, 2000). Although very uncommon, some animals can recognise each
51 other through *recognition alleles* – they have the capability to recognise others if they possess a specific
52 allele in common with themselves and this does not require learning (e.g. Keller and Ross, 1998). These
53 mechanisms are not mutually exclusive and animals may use them in combination or use different
54 templates in different contexts (Penn and Frommen, 2010).

55 Quantifying the chemical composition of natural odour mixtures emitted by animals may assist with
56 determining which mechanisms are available for the animal to rely on for recognition. The exact
57 mechanism used in recognition performances can then be revealed through behavioural assays (Wyatt,
58 2014). For olfactory recognition to occur, the chemical composition of a scent needs either to be stable

59 over time or at least change at a slower rate than consecutive interactions among animals, allowing an
60 update of a possibly changing template. We expect chemical fingerprints (or fractions of them) to be
61 similar among related individuals in cases where phenotype matching is possible. However, for direct
62 familiarisation, no similarity is required (Thom and Hurst, 2004).

63 Recognition is particularly important for mother-offspring interactions, and the pressures for successful
64 recognition are higher for colonial or gregarious species with exclusive maternal care of mobile young
65 and frequent separations (Corona and Lévy, 2015; Okabe et al., 2012), such as otariids (fur seals and sea
66 lions). Otariid females undertake regular foraging trips to sea throughout lactation, during which they
67 leave their pups on land. Following each return to the colony, they must find and identify their young to
68 feed them selectively (Renouf, 1991; Riedman, 1990). Otariid mother-offspring recognition has been
69 best explored in Australian sea lions (*Neophoca cinerea*) where a combination of olfactory, visual and
70 auditory cues are used (Charrier and Harcourt, 2006; Charrier et al., 2009; Pitcher et al., 2012, 2010a,
71 2010b, 2010c, 2009; Wierucka et al., 2018a, 2018b, 2017). Females can distinguish their pups using
72 olfactory cues (Pitcher et al., 2010c) but the underlying perceptual mechanism through which this
73 recognition occurs remains unknown. The aims of this study were to determine whether chemical
74 fingerprints of Australian sea lion mothers and pups are more similar to each other than to others, and
75 to explore the possible contribution of environmental and genotypic factors to the chemical compounds
76 encoding this similarity, if it exists.

77

78 2. ANIMALS, MATERIALS AND METHODS

79 Scent samples were collected in two wild populations of Australian sea lions inhabiting two
80 medium/large colonies – Olive Island (32°43'18.5" S, 133°58'6.3" E; 133 pups in 2014/15; Goldsworthy
81 et al., 2015) and Seal Bay (Kangaroo Island; 35°59'34.8" S, 137°19'4.8" E; 268 pups in 2014/15;

82 Goldsworthy et al., 2015) in April and October 2016, respectively. Olive Island consists mostly of non-
83 foliate granite and has limited, simple vegetation consisting mostly of marsh saltbush (*Atriplex paludosa*)
84 and nitre-bush (*Nitraria billardierei*; Robinson et al. 1996). Seal Bay has diverse habitats consisting of
85 sandy and pebble beaches, coves and sand dunes covered by low bushes (grey saltbush, *Atriplex*
86 *cinerea*) or succulent plants (New Zealand spinach, *Tetragonia implexicoma* and native pigface,
87 *Carpobrotus rossi*).

88 Pups (2-4 months of age) were caught in a hand net and restrained manually for a short period of time
89 (less than 15 minutes, during maternal absences) while sampled and individually marked (fur clipped).
90 Mothers were identified by association with marked pups (following Pitcher et al., 2009) and sampled
91 after their return to the colony from foraging trips and never on the same day as pups (mean number of
92 days between sampling pairs = 6.2). As capture of females was deemed to cause too much disturbance
93 both for the individuals and for the colony, samples were collected using a sampling device identical to
94 that used for pups but mounted on a 3-m pole. As the source of semiochemicals used for recognition is
95 unknown, and (excluding saliva) samples collected from different body regions are similar in chemical
96 composition (Wierucka et al. 2019), we collected scent samples from the lumbar area of animals, an
97 area easily accessible for sampling in both pups and females. This was done by firmly rubbing the
98 animals with a clean cotton swab (previously washed in chemical-grade methanol and hexane). Samples
99 were stored in dark chromatographic vials, with air expelled with Argon and kept frozen at -20° C until
100 chemical analysis. Control samples consisted of swabs exposed to air in the colony using the same
101 protocol as sample collection.

102 Following defrosting (30 min at 22° C), we exposed Twisters® (Gerstel Magnetic Stir Bar) to each sample
103 (2 h under a nitrogen flow of 50 ml.min⁻¹ at 30°C). The Twisters were desorbed in a Gerstel Thermal
104 Desorption Unit (by a Gerstel MPS autosampler, Gerstel GmbH & Co. KG, Mühlheim/Ruhr, Germany)
105 and injected via Gerstel Cooled Injection System (-80°C then 250°C) with a split ratio of 4:1 into a

106 coupled gas chromatography-mass spectrometry (GC-MS) system (Thermo Trace 1310-ISQ, Thermo
107 Fisher Scientific Inc., Bremen, Germany). The GC was equipped with a 30 m DB-5 MS column (methyl
108 siloxane, 0.25 μm film thickness, 250 μm ID, Macherey-Nagel, Düren, Germany) and helium was used as
109 the carrier gas (1 $\text{ml}\cdot\text{min}^{-1}$). Ionization was by electron impact (70 eV, source temperature 200°C). The
110 column temperature was kept at 40°C for 3 min with a following program of 5°C min^{-1} up to 200°C, then
111 10°C min^{-1} up to 250°C, kept for 2 min.

112 Chemical data obtained from the GC-MS were prepared for statistical analysis in an adapted version of
113 MZmine 2.18 (Pluskal et al., 2010; customised software available on request) allowing for fully
114 automated and reproducible compound integration and alignment among samples (MZmine batch file
115 available upon request). To account for contamination (from the environment and/or equipment) and
116 varying intensities of samples, we first removed compounds that were present in the controls and
117 discarded compounds present in single samples, then recalculated the data to relative proportions
118 within each sample. Furthermore, a Wisconsin and square root transformation was applied to the
119 dataset.

120 All statistical analyses were performed in R version 3.2.2 (R Core Team, 2015). We used an analysis of
121 similarities to assess mother-offspring chemical fingerprint similarity (nested within colony) and a two-
122 way nonparametric permutational multivariate analysis of variance with a Bray-Curtis dissimilarity index
123 to determine whether colony and age (adult female vs pup) differences exist (Anderson, 2001; Oksanen
124 et al., 2016) followed by a pairwise comparison (with a Holm's p-value adjustment for multiple
125 comparisons) to explore interaction among variables. We then ran a similarity percentage analysis
126 (SIMPER; Clarke, 1993) to evaluate which compounds contributed most to the observed differences
127 between various tested groups (if they occurred). Chemical similarity/dissimilarity was visualised using a
128 non-metric multidimensional scaling (NMDS) technique, which was based on the same index as the
129 statistical tests.

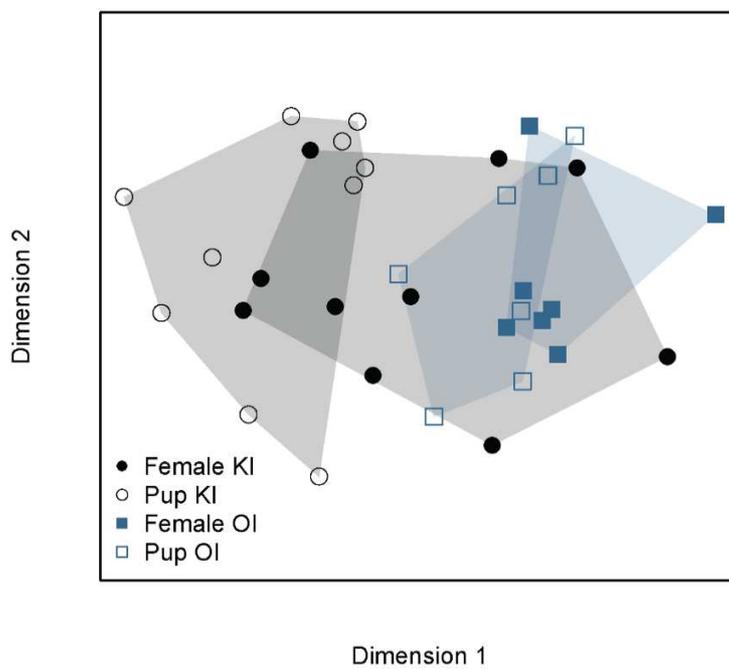
130

131 3. RESULTS

132 Seventeen mother-pup pairs were sampled for chemical compounds present on the lower back skin and
133 fur (Olive Island n=7, Kangaroo Island n=10) and used for analyses, four samples from the original
134 dataset were excluded based on too many zeros in the matrix for computation of similarity. Chemical
135 fingerprints of mothers and their pups were not significantly more similar to each other than to other
136 individuals ($R=0.035$, $p=0.778$). However, we found a difference in chemical compound composition of
137 the samples between colonies ($R^2=0.093$, $p=0.018$). No differentiation in mean chemical fingerprint from
138 back fur samples was found between pups and adult females ($R^2=0.038$, $p=0.088$), yet a significant
139 interaction between colony and age ($R^2=0.049$, $p=0.011$) indicated that chemical composition was
140 affected differently for different age classes depending on the colony (Figure 1, Table 1). Pairwise
141 comparisons showed that the chemical composition of samples from both adult females and pups
142 differed by colony (adjusted p-value: 0.044 and 0.006 for adult females and pups, respectively; Table 1).
143 Furthermore, differences among adult females and pups were much stronger (although not significant
144 after adjusting p values) for the Kangaroo Island colony, compared to the Olive Island colony (Table 1).

145

146



147

148 Figure 1. Visualisation of Australian sea lion mother-pup pairs' chemical fingerprints on Kangaroo Island

149 (KI) and Olive Island (OI). Points in close proximity indicate higher similarity.

150

151 Table 1. Pairwise comparisons of differences in chemical composition among colony and age-class in
 152 Australian sea lions. Significant results are marked in bold. Grey text indicates pairwise comparisons that
 153 were not relevant to the question of the study.

Pair compared				F	R ²	p	adjusted p
age	colony	vs	age colony				
Female	KI	vs	Pup KI	1.815	0.092	0.024	0.072
Female	KI	vs	Female OI	1.923	0.114	0.011	0.044
Pup	KI	vs	Pup OI	3.340	0.182	0.001	0.006
Female	OI	vs	Pup OI	1.392	0.104	0.132	0.132
Female	KI	vs	Pup OI	1.621	0.098	0.056	0.112
Pup	KI	vs	Female OI	3.099	0.171	0.001	0.006

154

155 The chemical analyses indicated that only 12 compounds contributed to over 50% of the differences in
 156 the chemical profiles seen between the colonies (Table 2), with two of the three top compounds (betulin
 157 and the cyclic monoterpene derivative) being plant-derived. Similarly, differences between pups and
 158 adult females on Kangaroo Island were related to plant compounds and long-chain compounds, with 10
 159 chemical compounds contributing to over 50% differences between these age classes (Table 3).

160

161 Table 2. Top 50% of chemical compounds making the largest contribution to the observed colony
 162 differences in chemical fingerprints of Australian sea lions.

No.	Compound name	Average RT	Cumulative contribution
1	Betulin (triterpene)	28.739	0.079
2	Alkane	26.056	0.155
3	Cyclic monoterpene derivative	20.764	0.225
4	Alkyl substituted phenol	30.518	0.266
5	Long chain aldehyde	29.869	0.308
6	Long chain alkane	32.944	0.340
7	Isopropylalkanoate	33.342	0.372
8	Silane	6.597	0.403
9	3-hydroxy-dodecanoic acid	22.429	0.430
10	Phenolic ester	8.614	0.455
11	Cholestan-3-ol derivative	28.750	0.481
12	Cholestan-3-ol derivative	26.134	0.504

163

164 Table 3. Top 50% of compounds making the largest contribution to observed differences in chemical
 165 fingerprints between pups and adult females of Australian sea lions from the Kangaroo Island colony.

No.	Compound name	Average RT	Cumulative contribution
1	Cyclic monoterpene derivative	20.764	0.127
2	Long chain aldehyde	29.869	0.204
3	Long chain alkane	32.944	0.256
4	<i>N</i> -substitued phtalate	14.886	0.297
5	Small alkylamide with an oxirane	15.943	0.337
6	Cholestan-3-ol derivative	28.750	0.374
7	<i>S</i> -substitued cysteamine	22.661	0.411
8	Small phenolic polar compound	16.494	0.447
9	Unknown small branched alkane	6.563	0.481
10	Silane	6.597	0.515

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169 4. DISCUSSION

170 Chemical composition of lumbar fur odours was not specific to mother-pup pairs, however we did find
171 pronounced colony differences indicating that broader population affiliation information can be
172 encoded in Australian sea lion body odours. Colony similarity seemed to be determined by compounds
173 obtained from their immediate environment rather than produced by the animals. Our results failed to
174 support the presence of a chemical basis for phenotype matching, suggesting that individual olfactory
175 recognition is more likely to occur through animals learning the scents of their conspecifics.

176 Chemical fingerprints of pups and mothers were not significantly similar to each other, indicating no
177 chemical basis for self-referent phenotype matching. Because there seems to be no genetically derived
178 template, it is likely that females learn the chemical signature mixture of their pup through direct
179 familiarisation. Australian sea lion females have been shown to learn individually distinct acoustic cues
180 (Pitcher et al., 2010b) and to recognise changing age-class characteristics visually (Wierucka et al., 2017).
181 It is therefore likely that the cognitive capacity to learn and update a sensory template could include
182 olfactory cues and would further support the notion that females learn the chemical signature mixture
183 of their pups through direct familiarisation. Our findings contrast with Stoffel et al. (2015), who showed
184 mother-offspring similarity and colony differences in chemical fingerprints of Antarctic fur seals
185 (*Arctocephalus gazella*). Antarctic fur seals live in much higher density colonies than Australian sea lions
186 (Doidge et al., 1984; Gales et al., 1994), and thus the selective pressure for individual recognition
187 between mothers and offspring is much higher in the former species, as the risk of confusion between
188 individuals is greater. The similarity in mother-pup chemical fingerprints could thus be a result of such
189 selective pressures. Although there is a chemical basis for Antarctic fur seal females to use phenotype
190 matching to recognise their pup, it does not exclude the possibility of learning also playing a role in the
191 process. It is possible that olfactory recognition is a two-step mechanism (phenotype matching

192 combined with learning of an individual profile, varying in importance among species) in order to secure
193 the conveyed information.

194 There always exists the possibility that similarities in mother-pup chemical fingerprints exist, yet we
195 simply did not detect them. However, the sampling and analysis methods we use have been tested
196 previously and shown to successfully detect chemical differences in Australian sea lions (Wierucka et al.
197 2019), making this unlikely. Importantly, GC-MS techniques only test machine detectable differences
198 and we do not have information about which of these compounds are biologically relevant, how they
199 are perceived by the animals and if they are used for recognition. Behavioural assays are required to
200 confirm the mechanism of template matching used by individuals for recognition and to identify
201 semiochemicals involved in the process.

202 We found pronounced differences in chemical compound composition among animals inhabiting
203 different populations/islands. These differences in chemical fingerprints between animals inhabiting the
204 two colonies seemed to be driven by plant-derived compounds present on the animals' bodies.
205 Kangaroo Island and Olive Island have markedly different vegetative habitat. The influence of the
206 ambient habitat in odour production is likely to be important over most spatial scales (Wäschke et al.,
207 2014) and may well play an important role in recognition systems in general and warrants further
208 investigation. Further, in addition to exogenous compounds inadvertently transferred to the animal
209 from the environment there is potential that some compounds may be deliberately acquired for specific
210 functions. Betulin is an interesting case in point, occurring in the bark of various *betulaceae* and a variety
211 of other plants (e.g., O'Connell et al., 1998). Betulic acid derivatives, among which is betulin, have been
212 shown to convey biological activities including antibacterial, anti-inflammatory, anthelmintic and
213 antioxidant properties (Yogeeswari and Sriram, 2005; Alakurtti et al., 2006). Intentional ingestion of
214 these compounds for the purpose of self-medication is often noted in other terrestrial carnivores (e.g.,
215 Rodriguez and Wrangham, 1993). So far, we have no evidence of occasional or intentional seeking of

216 body surface contact or ingestion of such plants in Australian sea lions or other pinnipeds. However,
217 future investigations of this and other pinniped species should be mindful of the potential functions of
218 environmentally contributed compounds, such as the presence of betulin in these samples, which raises
219 the possibility of previously undescribed behaviors.

220 We found some separation of pup and mother fingerprints in the Kangaroo Island colony. While
221 metabolic and behavioural differences (Thom and Hurst, 2004) between females and pups may in part
222 explain this, over 12% of the difference could explained by a cyclic monoterpene derivative (compound
223 3, Table 2), which was more abundant in pup samples. This is a plant-derived chemical, further
224 indicating that compounds found in the environment are an important contributor to chemical
225 fingerprint composition. In this case, pups (until moult) spent most of their time on land, while adult
226 females regularly foraged in the water allowing many external compounds to be washed off. Supporting
227 this is our earlier finding where we compared pre- and post-moult pups, with plant-derived compounds
228 being more frequent in pre-moult pups (Wierucka et al. 2019).

229 Our results provide a first insight into the olfactory recognition mechanism in Australian sea lions. The
230 data suggest the lack of a chemical basis for phenotype matching in the overall chemical fingerprint. It
231 still remains a question whether only a subset of this fingerprint is used by animals for individual
232 recognition and future studies should aim to identify whether our efforts in assessing similarity among
233 mother-pup pairs should focus only on a subset of chemicals. Furthermore, behavioural assays are
234 needed to confirm if compounds that contribute highly to observed differences in chemical fingerprints
235 are biologically relevant and used by animals. It is possible that other subsets of the chemical profile are
236 important and influence animal behavior. Complex behavioral experiments would also provide a good
237 test of our broad findings and would allow to confirm which olfactory recognition mechanism
238 (phenotype matching or direct familiarization, or a combination of the two) is used by females to
239 identify their young.

240

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245 6. COMPETING INTERESTS

246 Declarations of interest: none

247 7. AUTHOR CONTRIBUTIONS

248 *All authors designed the study. KW and IC collected data in the field, KW and NB processed chemical*
249 *data, KW conducted all statistical analyses and wrote the manuscript. All authors reviewed the*
250 *manuscript and gave final approval for publication.*

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261

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