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

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ABSTRACT

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

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

1. INTRODUCTION

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

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

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

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

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

2. ANIMALS, MATERIALS AND METHODS

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

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

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

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

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

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

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

3. RESULTS

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

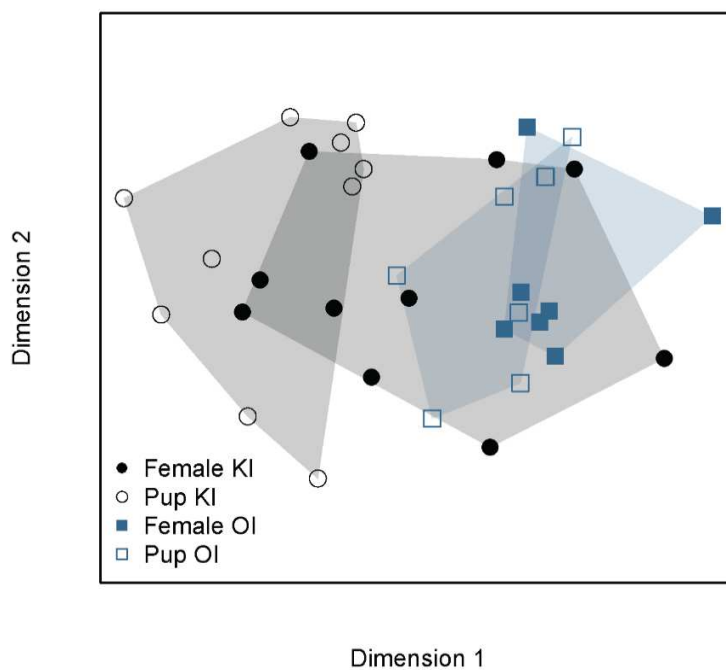


Figure 1. Visualisation of Australian sea lion mother-pup pairs' chemical fingerprints on Kangaroo Island (KI) and Olive Island (OI). Points in close proximity indicate higher similarity.

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		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

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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

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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 phthalate	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> -substituted 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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4. DISCUSSION

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

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

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

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

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

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

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

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