MULTIMODAL MOTHER-OFFSPRING RECOGNITION IN THE AUSTRALIAN SEA LION (*NEOPHOCA CINEREA*)

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STATEMENT OF ORIGINALITY

I certify that the work in this thesis, entitled "Multimodal mother-offspring recognition in the Australian sea lion (*Neophoca cinerea*)" is an original piece of research and has been written by me. Any help and/or assistance that I have received have been appropriately acknowledged.

This thesis will be submitted to Macquarie University and Université of Paris-Saclay in accordance with the Cotutelle agreement dated 25th of September 2015.

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ABSTRACT

Recognition plays an important role in animal communication systems and individuals often employ different sensory modalities to enact this activity. Although recognition has been widely investigated, especially for mother-offspring interactions, there is a dearth of information about multimodal recognition and the relative importance and interactions of various sensory cues. In this thesis, I explored multimodal communication in a colonial mammal – the Australian sea lion (*Neophoca cinerea*). Communication during mother-pup reunions is known to be multimodal in this species, yet the underlying processes of olfactory and visual recognition, as well as the interactions between acoustic, visual and olfactory cues remain unclear. Through chemical analyses, I determined whether chemical profiles differ among sex and age classes, colonies, and body regions of animals. Chemical similarities between mothers and pups indicate that phenotype matching may be used by Australian sea lions for olfactory recognition. I examined the role of visual cues in mother-pup recognition and found that age-specific visual cues assist mothers to refine their search for their offspring in the colony. Pups are capable of distinguishing various visual cues that can be used in the assessment of conspecifics. Having provided baseline information about the role of sensory cues in isolation, I determined how acoustic, olfactory, and visual cues are used in a synergistic way to ensure accurate mutual recognition and then interpreted the results using a cost-benefit perspective to disentangle the evolutionary pressures on each component of this communication system. I showed that although cues have the ability to convey given information in isolation, their role may be different when other sensory cues are present. Furthermore, there is a mutual dependency in the communication system, where the limitations imposed on one participant of the dyad affect cue use by the other. These findings contribute to a better understanding of mammal mother-offspring recognition and communication mechanisms in vertebrates.

STATEMENT OF PUBLICATION AND CO-AUTHORSHIP

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CONTRIBUTIONS OF CO-AUTHORS TO CHAPTERS

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CHAPTER 1

General introduction

Australian sea lion mother and pup reunited after the female's foraging trip (photo credit: Kaja Wierucka)

COMMUNICATION

Communication is an essential component of social interactions among individuals (Enquist et al. 2010). It plays an important role in a wide variety of behaviours involved in mating, parental care, predator-prey interactions, and foraging (Higham and Hebets 2013). By doing so it is instrumental in shaping the two major elements of life history of any animal species – reproduction and survival (Bolhuis and Giraldeau 2005). Because of the complexity of communication systems and the close relationship with other aspects of species' biology, to understand the underlying principles of signalling we are often compelled to explore different aspects of model systems and investigate and discuss their development, mechanism and function. While this complexity presents obvious challenges, communication studies are integral to a complete understanding of animal behaviour (McGregor 2005).

Communication occurs whenever individuals exchange information (Bolhuis and Giraldeau 2005). The process requires a sender – the counterpart producing the stimulus, usually modifying it in order to convey the desired information and coupling it to the medium (Shannon and Weaver 1949). The signal then propagates through the environment, and during this process it may be distorted, obstructed or modified (Shannon and Weaver 1949). Finally, it reaches the receiver of the information, who using specialised sensory organs, gathers the signal (Shannon and Weaver 1949). The receiver perceives it and then decodes and classifies the information conveyed within and responds accordingly (Shannon and Weaver 1949). This layout of communication involving the production, reception, perception, understanding and reaction is a simple representation of the process. In a natural environment there may be multiple animals involved in the exchange of information, and each individual can play multiple roles within the same system (i.e., being both receiver and sender) either simultaneously or sequentially often making it a network of interactions rather than a simple two-way exchange. Furthermore, communication is often associated with active communication, where animals intentionally convey information to specific recipients. However, it is important to remember that although the same general layout of transfer occurs, some communication may be passive and may be propagated involuntarily (e.g., body odour), or perceived by undesired receivers.

Regardless of intentions, the information acquired by animals through signals or cues influences the receiver's response (Seyfarth et al. 2010). Shannon (1948) defined information as a statistical measure of uncertainty. Although developed for technical purposes, this definition has been successfully applied to animal behaviour (Seyfarth et al. 2010). When a particular signal predicts a social situation, we assume that the cue has the ability to provide the receiver with information and thus reduces their uncertainty about a particular event (Seyfarth et al. 2010). By considering information as a measure

of uncertainty, and taking into account the costs and benefits of acquiring it, we are able to analyse the mechanisms through which animals employ different communication cues (Seyfarth et al. 2010).

SENSORY CUES

Prior to discussing different sensory cues, their functions, applications and limitations, it is important to note the distinction between cues and signals. Historically, there have been inconsistencies in the definitions and different authors have assigned a slightly different meaning to these terms (e.g., Hauser 1996; Bradbury and Vehrencamp 2011). However, in multimodal communication literature, the commonly accepted definition of 'cue' and 'signal' is related to their origin. Cues provide information to other animals, however, they may have not evolved for this purpose (Maynard Smith and Harper 2003; Ruxton and Schaefer 2011). They can provide information that is a 'by-product' with a different function from that they have been selected for. By contrast, signals have evolved for the purpose of communication (Maynard Smith and Harper 2003; Ruxton and Schaefer 2011). By this definition, signals are always cues, however not all cues are signals. Seeing that a majority of my work is discussed in a multimodal communication framework, I will adhere to the definitions provided above throughout this thesis. As we are lacking information about the origins of many cues, I use the term 'cues' instead of 'signals' throughout this body of work.

Animals have the ability to use a broad range of sensory cues to obtain information about other individuals or their environment. Each sensory modality is subject to different limitations, resulting from the physical properties of cues as well as anatomical limitations of the animals, which will determine which sensory channels are used in a given context (Higham and Hebets 2013; Yorzinski 2017). The active space of cues dictates their functional range and is affected by the speed, persistence and directionality as well as the environment through which they travel (including conditions as well as obstacles; McGregor 2005). Furthermore, different cues will be prone to different types of obstruction that the animals may have to adjust for. The production and perception of cues and well as their plasticity will also depend on their physical properties and the physiological abilities of animals. Due to taxa diversity, many different types of cues can be used by animals, however here I focus on the three senses most commonly used by mammals: hearing, sight and olfaction (Bradbury and Vehrencamp 2011).

Hearing is the perception of sounds, which are perturbations in local density and pressure of the medium through which they travel (Bradbury and Vehrencamp 2011). The methods by which animals produce sounds are diverse and are dependent on their anatomy and the medium they are

surrounded by. Mammals will typically force air through a valve (the larynx) to produce vibrations, however non-verbal sounds are also common (Bradbury and Vehrencamp 2011). Similarly, while sound reception organs vary among taxa, all function similarly – through perception of changes in motion between specialised mechanoreceptors and the rest of their body (Bradbury and Vehrencamp 2011). The environment greatly affects the propagation of acoustic cues, with various factors such as temperature and pressure influencing the cue before it reaches the receiver (Bradbury and Vehrencamp 2011). However, despite being susceptible to environmental degradation and being prone to noise obstruction, sound usually overcomes obstacles better than visual cues, propagates better than olfactory cues (Yorzinski 2017) and is relatively fast. Thus, acoustic cues have the ability to function over large ranges (Wiley and Richards 1983) and this is often exploited by animals. In contrast with the other two cues described below, where information can be conveyed passively with animal presence, the production of acoustic cues requires the existence of specialised organs and animals need to actively produce an acoustic cue (Rosenthal and Ryan 2000). Accordingly, physiological as well as physical constraints are higher for acoustic cues than for visual and olfactory cues (Thery and Heeb 2008).

Sight is based on the detection of electromagnetic radiation. Visible light, which can be used by animals for communication, consists of only a narrow frequency range of this radiation. Most animals use reflected light and thus all animals are capable of producing simple visual cues by passively reflecting light off their bodies (Rosenthal and Ryan 2000). However, while most visual cues are permanent (reflected light) they can also include postures and transitory cues (only visible at particular times, e.g. the pattern on butterfly wings that is visible only when the wings are spread; McGregor 2005). Animals collect and concentrate light with specialised organs – eyes, but also require a complex nerve system for spatial pattern analysis and resolution. Visual perception abilities are severely influenced by physiological eye performance abilities, neuromuscular predispositions and cognitive capabilities of a given species, with body size and phylogenetic history playing an important role (Thery and Heeb 2008). The transmission of visual cues occurs at speed of light and is therefore almost immediate (Rosenthal and Ryan 2000). However, this sensory channel is particularly easily obstructed by the presence of vegetation, topographic elements and/or the presence of conspecifics, making it not effective over large ranges in most natural habitats except in open environments (Rosenthal and Ryan 2000) and is thus considered a mid-short range modality.

Communication through the olfactory modality involves information transmitted through volatile compounds. Chemical compounds used in olfactory communication are produced either by specialised glands (e.g., territorial scent-marking, sexual attraction) or are a subset of the general body

odour – the overall chemical profile of an animal (Wyatt 2014). The sources of volatiles are diverse, with endogenous compounds, those resulting from microbial activity or acquired externally (from the environment or diet) all possibly containing important information for conspecifics (Thom and Hurst 2004; Brennan and Kendrick 2006). Terrestrial vertebrates perceive volatile compounds through specialised sensory cells located on the olfactory epithelium, within the respiratory system, where the medium passes and can be 'scanned' for odoriferous compounds (Bradbury and Vehrencamp 2011). Diffusion occurs along the medium gradient, which will determine the speed and directionality of compound transmission (Bradbury and Vehrencamp 2011). It is significantly slower than vision and hearing, but also allows the cue to persist in the environment following the signallers' departure. The receiver may approach the source and in that way obtain information faster, however, olfactory cues are usually only effective at relatively close range (Bradbury and Vehrencamp 2011). Compounds can be deposited by the animals intentionally or may involuntarily propagate through diffusion in the medium (McGregor 2005), however in either case, the control over transmission of the cue by the sender is very limited.

MULTIMODAL COMMUNICATION

As illustrated above, cues vary in their physical properties and their use also depends on the animals' physiological abilities determined by their evolutionary history. However, many other factors such as the economics of communication (the balance of relative benefits of signalling for both the receiver and sender), potential for signals being exploited by undesired receivers, relative costs and benefits of social interactions, and probability of cheating (Bradbury and Vehrencamp 2011), will also play a role in which sensory channels are applied by individuals in a given context. Because of this complexity and the differences in limitations that the use of a specific modality imposes on the transfer of information, animals will often employ multiple channels simultaneously to convey a message (Rosenthal and Ryan 2000; Higham and Hebets 2013; Uy and Safran 2013). Communication is multimodal when more than one sensory modality is used (Partan and Marler 1999; Hebets and Papaj 2005; Partan and Marler 2005). Using multiple cues ensures a more robust transmission of the information (redundant cues), or allows for the transfer of more information at once (non-redundant cues; Hebets and Papaj 2005; Partan and Marler 2005; Ay et al. 2007; Bro-Jørgensen 2010; Higham and Hebets 2013; Ratcliffe et al. 2016). The effect of the presence of multiple cues at once is not always simply additive, with many types of possible interactions (Partan and Marler 1999). Redundant cues can cause the intensity of the response to multimodal cues to remain the same (equivalence) or increase (enhancement) when compared to each unimodal cue alone. Non-redundant cues are

independent when the combination of cues prompts all of the responses elicited by each of the cues in isolation. Dominance occurs when one cue overrides the other and only the response of the dominant cue is evoked in the receiver. Some cues have the ability to interact with each other and modulate the receiver's response by enhancing or suppressing the response to one of the cues in the presence of others. Non-redundant cues may also result in the emergence of a new response that is different than the one elicited by each of the component cues.

In many cases, the use of multimodal cues is advantageous, however there are also certain costs associated with this type of communication (Hebets and Papaj 2005; Partan and Marler 2005; Ay et al. 2007; Bro-Jørgensen 2010; Higham and Hebets 2013; Ratcliffe et al. 2016). Apart from increased energetic expenditure potentially associated with the production, perception and cognitive processing of a higher number of cues, one of the main risks is being overheard by undesired parties, which for example may result in increased chances of exposure to predation (Partan and Marler 2005). Furthermore, when animals are engaged in communication, less attention is spent on other activities that might be important to fitness (Partan and Marler 2005). Consequently, where there are costs of multimodal communication, animals should choose to not employ more modalities than necessary (Partan and Marler 2005; Munoz and Blumstein 2012). Munoz and Blumstein (2012) provide a theoretical framework explaining the reasons for and against the use of bimodal cues over unimodal cues, evaluating the mechanisms of communication systems from the perspective of the receiver's costs in communication. They define three scenarios: enhancement, antagonism and equivalence/dominance. Enhancement is an elevated response to multimodal cues and occurs when the costs of missed opportunities are high. For example, when the uncertainty of information may lead to animals not finding a mate, not detecting a predator or not identifying filial offspring. When more cues predict higher likelihood of a situation, utilising additional cues is highly beneficial. By contrast, when the probability of an event is lower when multimodal cues are present, animals will decrease their response to allocate time to other activities (antagonism). Finally, when the costs of obtaining additional information encoded in multiple modalities is too high, equivalence and dominance will occur.

This diversity of possible interactions among cues and their influence on behavioural responses make it incredibly difficult to determine the mechanisms underlying communication and recognition. The effects of the environment and the differences in costs and benefits of interactions often vary for different participants within the exchange (Schaefer and Ruxton 2015) and further complicate the interpretation of results. Studying cues in isolation is useful for understanding the physiological and cognitive abilities of animals, the range of cues and the information contained in each of them.

However, in a natural environment, cues co-occur and so interactions among them will be present. Thus, it is important to explore the whole system and investigate all parties involved in the exchange as well as the relative costs and benefits of interactions. This will allow us to obtain a better understanding of the rules that these systems are governed by and gain knowledge about the intricacies of communication systems and the evolutionary pressures for their development (Candolin 2003; Hebets and Papaj 2005; Ratcliffe et al. 2016; Hebets et al. 2016).

RECOGNITION

For many social interactions to be successful, animals must have the ability to not only perceive cues, but also identify the sender of the information. Therefore, recognition must occur (Tibbetts and Dale 2007). Recognition can vary in specificity, from animals distinguishing only species, to having the ability to recognise sex, kin, mates, rivals or even specific individuals (Yorzinski 2017). The evolutionary pressures for recognition are particularly strong for social or group-living animals, in which repeated interactions occur between individuals and remembering prior encounters with conspecifics, their context and outcome can be beneficial (Talbot 2016).

Individual recognition is one of the more complex forms of communication and takes place when individually distinctive characteristics are used for identification (Tibbetts and Dale 2007). In order to be useful, information encoded in an individually distinctive cue has to be unique to a specific individual and different from that of others. It must also be stable - the rate of change in a cue must be less than the frequency of interactions between individuals. However, individual recognition is always associated with another, broader type of recognition (e.g., mate, rival, offspring, kin) and it is often difficult to ascertain whether animals have the ability to identify a particular individual or just distinguish a specific individual from others based on a broader category. Therefore, some researchers acknowledge a distinction between individual discrimination, where animals have the ability to differentiate one individual from others, and individual recognition (i.e. 'true individual recognition' *sensu* Tibbetts and Dale (2007)) – the most precise form of recognition, where each individual can be discerned from all others and individually distinct cues are associated with corresponding information about the individual (Beecher 1989; Talbot 2016). The specificity of the information learnt about the sender of the information is the key difference between the two definitions.

For individual recognition to evolve, obtaining identity information must be advantageous for the receiver, but also, the sender must benefit from providing information about their identity (Bradbury and Vehrencamp 2011). However, each party has different and often conflicting interests, and thus

the relative pressures for recognition may be different for each of them, making the interpretation of discrimination systems and underlying mechanisms exceptionally challenging. However, we can expect distinct individual identity cues and high pressures for accurate recognition when the cost of providing information/resources to a random individual are high and/or when the risk of confusion of individuals (many potential receivers) is increased (Bradbury and Vehrencamp 2011). The presence of individual discrimination has been shown for a wide variety taxa (Yorzinski 2017) and in different contexts, primarily competition, territoriality and parental care (Tibbetts and Dale 2007).

There are three main mechanisms used for individual distinction (Hepper 1991; Bradbury and Vehrencamp 2011). Animals may have the ability to recognise conspecifics based on familiarity – by learning individually distinctive characteristics of another individuals, remembering them and being able to associate the cue to the signaller of the information. Phenotype matching involves matching a cue to an existing template and often presents itself as self-referent matching. Finally, some recognition is determined genetically and in those cases organisms have the ability to distinguish the presence of genetically determined cues. Individual recognition however, is always based on familiarity as animals have to distinguish all individuals from each other

MOTHER-OFFSPRING RECOGNITION

Parental care is essential for any species in which offspring cannot survive on their own (Royle et al. 2012). In mammals, the majority of parental care of the young is provided by the mother (Enquist et al. 2010). Mother-offspring recognition is thus an excellent system to investigate the intricacies of recognition. We have a good understanding of the pressures and drivers for interactions by both the mother and offspring (Trivers 1972), therefore despite the diversity of maternal care strategies it is possible to interpret the reasons for specific communication systems to evolve from a cost-benefit perspective. Recognition is incredibly important for maternal care (Royle et al. 2012) as in order to direct costly resources (e.g., milk) only to filial young, females need to be able to distinguish their offspring from others (Hepper 1991; Broad et al. 2006; Royle et al. 2012). It is also often beneficial for the offspring to be able to identify their mothers, as it limits energy wasted on unsuccessful begging attempts (towards nonrelated individuals) and limits the risk of injury associated with approaching nonrelated individuals (Bradbury and Vehrencamp 2011). However, mother-offspring recognition does not take place in all species. Therefore, in some cases the information may not be needed or the costs of obtaining such information might be too high relative to the benefits it brings (Bradbury and Vehrencamp 2011). Although in theory, there is mutual benefit of recognition for both the mother and offspring, the costs and benefits of these interactions are different for each (Clutton-Brock 1991).

Recognition between a mother and young can be mutual, where both parties have the ability to recognise each other, however it also happens that only the mother can distinguish the young (Bradbury and Vehrencamp 2011). Mother-offspring recognition is especially important for species exhibiting extended maternal care, dense aggregations, exclusive care and frequent separations (Tibbetts and Dale 2007). In these systems, mutual recognition will also be favoured (Bradbury and Vehrencamp 2011).

Similarly to other types of communication, mother-offspring recognition is often multimodal (Hepper 1991; Bradbury and Vehrencamp 2011). Yet while there has been a significant amount of research on unimodal recognition (Deecke 2006; Corona and Lévy 2015; Mora-Medina et al. 2016), there is considerably less information about how cues interact with each other in a range of contexts. Olfactory cues have been shown to enhance the response to acoustic cues in mice (*Mus musculus;* Cohen et al. 2011) and rats (*Rattus norvegicus;* Farrell and Alberts 2002). Visual cues have been suggested to be of higher significance than acoustic cues in recognition of goat (*Capra hircus*) kids (Ruiz-Miranda 1993). However that study was designed to test visual cue (pelage colour) use by goat kids and conclusions were made based on no significant difference in successful recognition when females vocalised compared to when they did not. No attempt was made to test the relative importance of cues or present the acoustic cues in a controlled way. The relative importance of cues has been also tested in sheep. Alexander (1977) showed that while both acoustic and visual cues convey some information to Merino (*Ovis aries*) ewes about lambs, incongruent visual cues had a greater effect on mothers than removing acoustic cues, indicating higher importance of visual cues for recognition. However, in these experiments, the visual cue was never fully removed (lambs blackened, but present). Shilito-Walser (1978) showed that ewes are able to locate lambs when acoustic cues were absent, yet this was not the case when visual cues were not present. Although this study supports Alexander's findings, it tested the ability of ewes to locate, not recognise lambs. Another study (Alexander and Shillito 1977) experimentally showed lower identification rates of lambs by ewes when visual cues were removed, compared to treatments with acoustic cues removed, further confirming that sheep rely more on visual cues. Only one study experimentally tested the relative importance of visual, acoustic and olfactory cues and found an enhancement in recognition rates when all three cues were present, compared to treatments with two (acoustic and olfactory; visual and olfactory) or one (olfactory) cues. Similarly to previous findings, the importance of visual cues was higher than that of acoustic cues. Only one study has investigated the relative importance of cues by offspring for maternal recognition. While in the first week of life, lambs are insensitive to altered visual and/or acoustic maternal cues, the importance of visual cues relative to acoustic cues increases in lambs as they age with older lambs relying more on visual cues (Alexander 1977).

While this information is useful, until now the topic has only been explored for domestic species. As by definition domestication alters many behavioural processes, it is important to explore the topic on a free-ranging, non-domesticated species in its natural environment. Only then is it possible to clearly understand the processes underlying natural mother-offspring recognition and the intricacies of cue use by individuals involved in the exchange of information. Furthermore, where mutual recognition occurs, emphasis must be put on investigating the use of each modality in both the mother and offspring as an interdependent dyad where cue use by one participant of the exchange may be dependent on constraints acting on the other individual.

MOTHER-OFFSPRING RECOGNITION IN OTARIIDS

Pinnipeds (*Pinnipedia*) belong to the order *Carnivora* and consist of three families – otariids (fur seals and sea lions; *Otariidae*), phocids (true seals; *Phocidae*) and odobenids (the walrus, 2 subspecies; *Odobenidae*). All pinnipeds are amphibious, with a majority of pupping occurring either on land or ice, however there are two main maternal strategies within the clade (Riedman 1990; Reynolds III and Rommel 1999). Phocid females are typically capital breeders – they rely on body reserves during the relatively short lactation period (4-80 days; Riedman 1990; Renouf 1991), and stay on land/ice with their pups until they are weaned (Boyd 1998). Otariids and odobenids are income breeders – following a short perinatal period, mothers undertake regular foraging trips during lactation. The length of foraging trips varies among species, ranging from a few days to several weeks. Otariid mothers leave the pup on land during foraging trips, whereas walrus calves follow mothers on trips (Boyd 1998) and lactation times for both these families are longer than for phocids, ranging from 4-24 months for otariids, and up to 36 months in the walrus (Riedman 1990; Renouf 1991). Furthermore, pronounced differences in animal density exist during the breeding period. Otariids and odobenids are colonial animals, often breeding in high density colonies, while phocids (with the exception of grey (*Halichoerus grypus*) and elephant seals (*Mirounga sp.*)) tend to be either dispersed or clustered at low density (Riedman 1990; Renouf 1991). The high risk of misidentification of individuals associated with colonial breeding, mobile young and regular separations, as well as high female aggression towards nonfilial pups (Harcourt 1992) increase selective pressures for mother-pup recognition and make otariids an ideal system for investigating the mechanisms of mother-offspring recognition (Tibbetts and Dale 2007).

Mother-offspring reunion follows a similar pattern in all otariids and various sensory cues are likely to be involved in this process. Females come back from foraging trips and exchange vocalisations with pups, following that, animals actively look for each other and when in contact, naso-nasal

investigations occur which seem to be used as a final confirmation of identity (Insley et al. 2003). Although researchers have known for a long time that otariids use various cues during mother-pup reunions, most information about the use of sensory cues in recognition has been anecdotal or observational (e.g., Bartholomew 1959; Peterson and Bartholomew 1969; Stirling 1970; Stirling 1971; Marlow 1975; McNab and Crawley 1975; Gisiner and Schusterman 1991; Dobson and Jouventin 2003; Phillips 2003; Trimble and Insley 2010), with experimental studies focusing on acoustic recognition (Trillmich 1981; Hanggi 1992; Insley 2000; Charrier et al. 2001; Insley 2001; Charrier et al. 2002; Charrier et al. 2003; Charrier et al. 2009; Pitcher et al. 2009; Pitcher et al. 2010a; Aubin et al. 2015; but see Pitcher et al. (2010c) for olfactory studies). Multimodal cues are known to be used by otariids (at least by adult females), yet we lack information about the role of sensory cues other than acoustic in mother-offspring recognition (but see Pitcher et al. 2010c; Stoffel et al. 2015), and no studies have tested the relative importance of sensory modalities between mothers and pups.

THE AUSTRALIAN SEA LION

The Australian sea lion (*Neophoca cinerea*; Peron, 1816) is an endangered otariid species endemic to Australia (Goldsworthy 2015). They inhabit the south and southwestern coast of the Australian continent. Australian sea lions exhibit pronounced sexual dimorphism (Jefferson et al. 2015). Adult, mature males weigh 180-250 kg and can reach 2-2.5 m in length. They have dark brown fur, with a blond patch that originates on their head and extends towards the neck as the individuals get older (Jefferson et al. 2015; Figure 1.1). Females are smaller, reaching 1.7-1.8 m and 61-105 kg, and have dual colour pelage – silver on the dorsal side and cream on the ventral (Jefferson et al. 2015; Figure 1.2). From birth until about two months of age, pups are black (Jefferson et al. 2015; Figure 1.3a). As they grow the pelage lightens, becoming a chocolate brown colour when the pups are 2-4 months old (Jefferson et al. 2015; Figure 1.3b). Following their first moult (at around 4 months of age) pups acquire the dual colour pelage characteristic for females (Jefferson et al. 2015; Figure 1.3c). Sub-adult males slowly start becoming darker and gain more mass as they reach sexual maturity at around 8-9 years of age (Jefferson et al. 2015). Females do not drastically change their appearance and grow in size, reaching reproductive age when they are 4-6 years old (Jefferson et al. 2015).

Figure 1.1. Australian sea lion (*Neophoca cinerea*) male (photo credit: Kaja Wierucka).

Figure 1.2. Australian sea lion (*Neophoca cinerea*) female (photo credit: Kaja Wierucka).

Figure 1.3. Australian sea lion (*Neophoca cinerea*) pups a) under 2 months of age, b) 2-4 months old, c) post-moult pup, over 4 months old (photo credit: Kaja Wierucka).

Australian sea lions exhibit a unique breeding cycle (Figure 1.4). The species does not breed annually, but every 17-18 months (Ling and Walker 1978; Higgins 1993; Gales et al. 1994) and breeding is asynchronous among colonies (Gales et al. 1992). Males mate-guard one or two females at a time, not allowing rival males to approach them when they are close to oestrus (Higgins and Tedman 1990), rather than defending typical territories with harems (Boness 1991). The pupping period is quite long and lasts approximately 5 months on average, which is the longest among pinnipeds (Mcintosh et al. 2012). This results in pups of different ages and thus of different physical appearance being present in the colony simultaneously. After giving birth to a single pup, the female remains with it on land for up to two weeks (Marlow 1975; Higgins and Gass 1993). By the end of the perinatal attendance period, the female mates (oestrus occurs 4-10 days post-partum) following which she begins regular foraging trips that last about 1-2 days (Higgins and Gass 1993). These trips occur throughout the whole lactation period that lasts 15-18 months (Higgins and Gass 1993). During this time, following a delayed implantation of 3.5-5 months, most females become pregnant again (Gales et al. 1997) and after 14 months of placental gestation give birth to a new pup (Gales et al. 1997). Pups from the previous seasons are usually weaned just before the new ones are born, thus, Australian sea lion females have a dependent pup during most of their adult life.
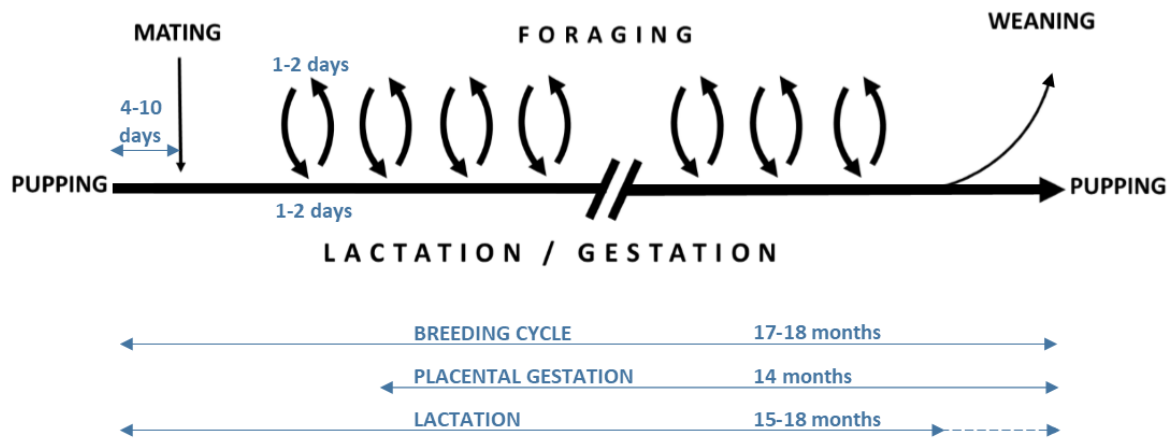
Figure 1.4. Breeding cycle of Australian sea lions.

Similarly to other otariids, the pressures for successful mother-offspring recognition are high. Females and pups have to find each other in the colony every 1-2 days (Higgins and Gass 1993), the suckling locations change and pups are very mobile during maternal absence (Marlow 1975). Furthermore allosuckling is rare (Marlow 1972; Pitcher et al. 2011) and females are aggressive towards nonfilial pups (Marlow 1972; Higgins and Tedman 1990; Gales et al. 1994) increasing the necessity for accurate recognition by pups.

Recognition is extremely important for the species, and has been shown to be of significance for different age and sex classes of animals. Males produce individually distinctive calls and are capable of distinguishing calls of different sexes, as well as differentiating their own species calls from that of others (Gwilliam et al. 2008). They also have the ability to discriminate among local males (inhabiting the same colony) and unfamiliar individuals (Attard et al. 2010; Ahonen et al. 2018). However, what sets Australian sea lions apart from other species of otariids is the amount of knowledge accumulated about mother-offspring recognition. It has been shown that both mothers and pups produce individually stereotyped calls (Charrier and Harcourt 2006). Mothers are capable of recognising filial pups within 48 hours post-partum (Pitcher et al. 2010b), while pup ability to recognise mothers based on acoustic cues develops between the perinatal attendance period and the time they are two months of age (Pitcher et al. 2009). Pups retain the ability to distinguish their mother's calls even two years post-weaning (Pitcher et al. 2010a). Both amplitude and frequency modulations as well as exact frequency values are used by pups to recognise their mother's voice (Charrier et al. 2009) whereas females only use amplitude modulation and frequency values to identify their pups (Pitcher et al. 2012). Propagation tests showed that the individual vocal signatures used by both mothers and pups is quite resistant to degradations during propagation, but degradation is highly dependent on the type

of habitat in the colony (Charrier et al. 2009; Pitcher et al. 2012). Furthermore, Australian sea lion mothers are capable of distinguishing their pup from others based solely on olfactory cues (Pitcher et al. 2010c). This abundance of information about cues used in mother-offspring recognition in the Australian sea lion provides a basis for further exploration of the process and allows for the exploration of underlying mechanisms and integration of cues.

RESEARCH OBJECTIVES

The main objective of this PhD is to experimentally explore how the response of a receiver to different sensory cues may change in a multimodal context and for multiple animals within a reciprocal communication system. I do this by investigating multimodal mother-offspring recognition in the Australian sea lion. Although evidence exists for recognition between a mother and her offspring being multimodal, we have very limited information about the interactions between modalities and how that impacts animal behaviour. In a natural setting, multiple cues are often available for inspection simultaneously and have the ability to influence information conveyed in one another. Furthermore, although the ability for cues to convey useful information and for animals to perceive it may be present, the relative costs and benefits of social interactions may influence what cues are used by animals in a given context. In order to fully understand animal behaviour, cue integration and the rules they are governed by, we must use a holistic approach and take into account all cues and participants involved in the exchange of information.

Considering the knowledge already acquired on mother-pup recognition, Australian sea lions are an ideal study system and provide a unique opportunity to further our understanding about the intricacies of cue use for recognition in mammals. My thesis aims to answer the following questions:

What are the characteristics of Australian sea lion chemical profiles?

In Chapter 3 I explore the chemical profiles of Australian sea lions. Olfaction has long been suggested to be of importance to otariids (e.g., Renouf 1991b; Insley et al. 2003), however few experimental studies have been done and we still lack knowledge about the processes underlying social olfaction. A way to explore this complexity is to ascertain whether instrumentally detectable differences in chemical composition exist among animals. I describe the differences in chemical compounds found in scent samples collected from pups. I examine whether differences exist among body regions, if differences between sexes are present, and how this varies for pups of different age. Due to differences in secretions and physical location on the body, I expect to find significant differences

among various body regions. Sex differences in adults have been anecdotally reported (Stirling 1972; Marlow 1975), however no one has tested this experimentally. Here, I examine whether sex differences in chemical composition are detectable at a young age, i.e. before reaching sexual maturity. Acquiring information about the characteristics of chemical profiles allows us to determine whether there is a chemical basis for recognition that can potentially be used by animals in a social context.

Is there a similarity between chemical profiles of mothers and pups?

Building on information obtained in the previous chapter, in Chapter 4 I further investigate the underlying mechanisms for olfactory recognition of Australian sea lion pups by mothers. I measure whether chemical profiles of mother-pup pairs are similar to each other and distinctly different from other pairs. I test this for two different colonies. While I expect to find significant differences in chemical profiles among animals inhabiting different regions, I predict a similar trend of differences in the two tested colonies. This chapter allows for determining the probable recognition mechanism used by Australian sea lions during mother-pup reunions. I hypothesise that chemical profiles of mothers and pups will be similar, pointing to phenotype matching being a likely mechanism used for recognition in the species.

Can visual cues facilitate mother-offspring reunion?

In Chapter 5 I examine the role of visual cues in mother-pup reunions of Australian sea lions. Spatial cues have been proposed to facilitate reunions. However, Australian sea lion pups are quite mobile, and they tend to cluster together during maternal absences. I hypothesise that in addition to acoustic and olfactory cues, mothers also use visual cues to refine their search for their pups. As pups of different age-classes exhibit different morphological characteristics and co-occur within one colony, with controlled behavioural experiments in the field, I determine whether these visual traits can be used by mothers to discriminate among pups of different ages, and potentially allow for an additional tier of recognition through another modality.

Do bimodal cues enhance pup response to maternal cues?

While some previous research has examined multimodal cue use in mammalian mothers, there is a dearth of information about the interactions of cues in the recognition process of mothers by offspring, with only one study published in the topic (Ruiz-Miranda 1993). It is known that Australian sea lion pups can recognise their mother's calls (Pitcher et al. 2009; Charrier et al. 2009). In Chapter 6,

I assess whether visual cues have the ability to enhance the pups' response to maternal cues when combined with acoustic cues. I first verify whether pups can distinguish broad categories of conspecifics based solely on visual cues. Having obtained information about the visual assessment abilities of mothers by pups, I proceed to experimentally evaluate whether visual and acoustic cues induce a synergetic effect on the behavioural response of pups and apply a cost and benefit framework assessment to the results, as I expect the use of cues by pups to be influenced by the nature of social interactions between pups and adult females.

How are acoustic, visual and olfactory cues used in a synergetic way in pup recognition by the mother?

The evolution of complex communication systems involving multiple sensory modalities is an important topic in vertebrate communication. Yet, a major limitation in previous studies of has been that while they may have attempted to address multimodal communication, most studies have focused on individual modalities rather than on how cues interact and influence one another's function. Having baseline knowledge from prior research as well as previous chapters of this thesis about the information conveyed in each cue in isolation, in Chapter 7 I explore how acoustic, olfactory, and visual cues are used in a synergistic way to permit accurate recognition in a mammalian species. I first analyse whether the presence of multiple sensory cues enhances Australian sea lion females' response to pup cues, and then assess the relative importance of acoustic and olfactory cues in the recognition process of pups by females. This will determine whether the importance, function or role of a sensory cue can change in a multimodal context, compared to its ability to convey specific information in isolation. I expect cues to be used differently by the mothers when presented simultaneously and for this to be determined by differences in cue properties combined with the characteristics of social interactions between mothers and pups. Thus, the results are interpreted using a cost-benefit perspective to disentangle the evolutionary pressures on each component of this communication system, and the social limitations for such an effect to take place are discussed.

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CHAPTER 2

Extended methods

Recording vocalisations of an Australian sea lion pup (photo credit: Kaja Wierucka)

STUDY LOCATIONS

Fieldwork was carried out in November 2015, April 2016, September-October 2016 and September 2017. Data were collected in wild populations of Australian sea lions, inhabiting one of two colonies in South Australia: Olive Island and Seal Bay, Kangaroo Island (Figure 2.1). Olive Island is a small (700x400 m), remote, uninhabited island located 8.5 km west of Cape Bauer (32°43'18.5" S, 133°58'6.3" E). The island consists mostly of non-foliate granite and has limited, simple vegetation consisting mostly of Marsh Saltbush (*Atriplex paludosa*) and Nitre-bush (*Nitraria billardierei*; Robinson et al. 1996). The local population of Australian sea lions was estimated to have 135 (95% CI 118-151) pups born in the 2015/2016 breeding season (most recent published data available; Goldsworthy et al. 2016).
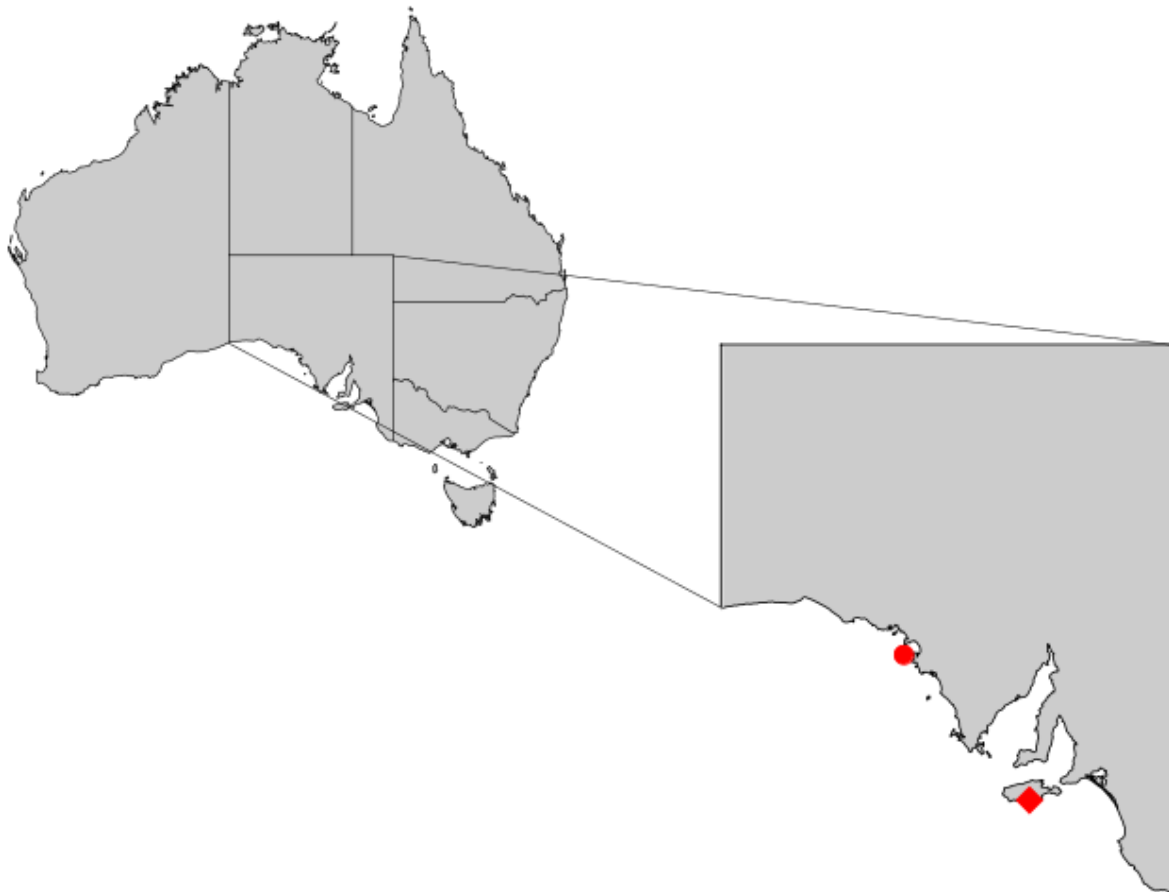
Figure 2.1. Map of study site locations: Olive Island (circle), Seal Bay (diamond).

Kangaroo Island, at approximately 145 km in length and 55 km wide, is the third largest Island in Australia. Seal Bay Conservation Park is located on the south side of the island (35°59'34.8" S, 137°19'4.8" E) and hosts Australia's third largest Australian sea lion population, with 268 (range: 259-277) pups estimated to be born in 2013 (last published estimate, Goldsworthy et al. 2015). The

colony is quite spread out, stretching for 5 km along the coast, with 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*). Seal Bay Conservation Park is a popular tourist attraction, and although visitor access is limited to sealed pathways and guided walks on the beach, due to the frequent presence of people, the animals are habituated to people and far easier to approach than on Olive Island.

In Chapter 5 I also use behavioural data collected on Beagle Island, Western Australia (29° 48' 24"S, 114° 52' 39"E) in March 2010.

Access to the colonies and permission for all data collection was granted by the South Australian Wildlife Ethics Committee (approval 30/2015) and the Department of Environment, Water and Natural Resources (permit E26447). For research conducted in 2010, permission was granted by the Department of Environment and Heritage South Australia (SA DEH; Scientific permit E24934) and research was approved by the SA DEH Wildlife Ethics Committee (approval 61/2005). All experimental procedures followed the Australian code of practice for the care and use of animals for scientific purposes.

IDENTIFICATION OF ANIMALS

Marking

Australian sea lion pups were captured and individually marked. Animals were caught using either a large hoop net, or by hand – by grabbing the hind flippers. When restrained, animals were sexed, and olfactory samples were collected (see further). Unique symbols were made on the lumbar region of each pup by clipping distinct shapes representing numbers (Figure 2.2, 2.3) in their fur and dying the remaining fur with blond hair dye (Clairol Nice'n Easy©; following Pitcher et al. 2009). This allowed for easy identification of pups at a distance, avoiding unnecessary approaches.
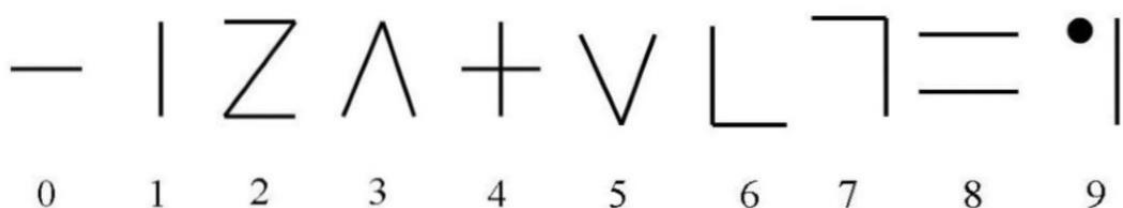
Figure 2.2. Symbols with corresponding assigned digits used for marking pups.

Figure 2.3. Pup marked by fur clipping and applying hair dye (here, pup with identification number 4).

Every pup used in the study was captured only once. Therefore, if pups also needed to be caught for monitoring programs or other research studies, we ensured that all procedures were coordinated and conducted simultaneously to limit animal disturbance.

Association

Mothers of marked pups were identified based on association. Allosuckling is rare in Australian sea lions (Marlow 1972; Pitcher et al. 2011), with females very aggressive towards nonfilial pups (Marlow 1972; Higgins and Tedman 1990; Gales et al. 1994) making it highly unlikely that females suckled by pups were not their mothers. This method of identification has been previously used in research studies and monitoring programs and is considered a reliable way of identifying females in the species (e.g., Pitcher et al. 2010; Goldsworthy et al. 2015).

Pelage colour

For estimating age classes of pups (Chapter 6) I used age-class specific pelage characteristics. As described in Chapter 1, pups change pelage colour and size as they age. Pups under two months old are black (A1), when 2-4 months old they turn a chocolate brown colour (A2), and finally, at

approximately four months old they moult and acquire the characteristic dual silver and beige pelage (A3) also exhibited by adult females (Figure 1.2, 1.3).

<div align="center">SAMPLE COLLECTION AND STIMULI PREPARATION</div>

Acoustic stimuli

For experiments that included playback procedures, I used both pup-attraction calls of mothers and mother-attraction calls of pups. To ensure that the correct type of call was recorded, samples were taken when mothers and pups were interacting in the colony during the naturally occurring reunion process. I used a BeyerDynamic M69 TG microphone (frequency response: 50 Hz-16 kHz ± 2.5 dB; BeyerDynamic, Heilbronn, Germany) mounted on a 3 m boom that was connected to a Marantz PMD 671 digital recorder (Marantz Europe, Eindhoven, Netherlands). Calls were recorded as close to the animal as possible (usually within 1-2 meters) and recorded at a 44.1 kHz sampling frequency. Good quality calls, with no or low background noise and no overlap with vocalisations from non-targeted individuals, were selected for building playback series used in the experiments. Calls were first high-pass filtered at 200 Hz using Avisoft SAS Lab Pro (Avisoft Bioacoustics, R. Specht) to remove any residual background noise generated by wind or waves. They were then assembled into playback series consisting of six calls, each separated by three seconds of silence, to imitate a natural calling pattern.

Visual stimuli

Visual models (dummies) imitating animals have been used in previous research and shown to be successful in communication studies in Australian sea lions (Pitcher et al. 2010) as well as other taxa (Klein et al. 2012). For my experiments, dummies imitating an adult female, three visually distinct age classes of pups, as well as naturally and unnaturally coloured pups were constructed. The size of the visually distinct age class dummies were based on the average size of pups of a given age class (Figure 2.4). The outer layer was made out of black (A1 natural), white (A1 unnatural), brown (A2), grey (dorsal side) and beige (ventral side; A3 and adult females) synthetic fur, and an inner wire skeleton kept the models in an upright position. The dummies were also filled with polyester stuffing to fill out their shape. Images or videos of dummies that were used are available in Chapters 6 and 7, as well as the supplementary materials for Chapter 5.

Dummy	Dimension [cm]			
	1	2	3	4
A1 natural/unnatural	63	20	19	16
A1	76	20	23	17
A2	87	23	26	19
A3	120	32	37	27
Adult female	156	40	47	35
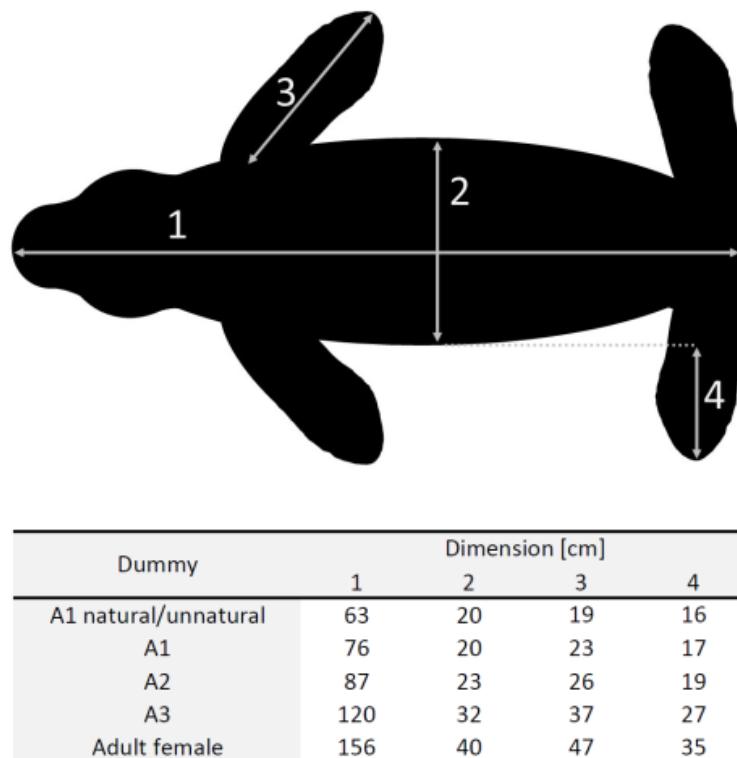
Figure 2.4. Dimensions of visual models (dummies) made for visual cue presentations.

Olfactory stimuli

Olfactory stimuli (used and referred to as 'scent samples' in Chapter 8) collection swabs were custom made for the experiments. Black cotton flannel (following Pitcher et al. 2010) was used to sew half-oval-shaped pouches that fit on the head of the visual pup model presented to females. These pouches were washed three times (1 h each time) in 90°C water and dried, following which they were packed individually in airtight polyethylene bags. Pup sampling occurred while pups were captured for marking, prior to applying hair dye, to not contaminate samples. Furthermore, olfactory stimuli were not collected by researchers who had handled hair dye that day and nitrile gloves were worn at all times to prevent human odours from entering the sample. Pouches were taken out of the bag immediately prior to sample collection and animals were sampled by firmly rubbing both sides of the pouch on their facial area, collecting various secretions from the nose, mouth, eyes, and skin. Stimuli were then places in sterile bags (Nasco Whirl-Pak®) and frozen at -20°C until behavioural presentation.

Scent samples (used in Chapters 3 and 4) consisted of pieces of pharmaceutical cotton wool. To remove any contaminants, they were prepared by washing three times in methanol (Sigma-Aldrich; ACS reagent ≥99.8%) and hexane (Sigma-Aldrich, ACS reagent 99%) with a magnetic bar. They were dried under a laboratory fume hood for 4 h before being placed in 4 ml dark chromatographic vials (45 x 14.7 mm, screw cap with PTFE septum, Chromoptic, Courtaboeuf, France). All samples (including

controls) were transported together, to be able to account for any contamination that might have occurred during travel. Similarly to olfactory stimuli, the collection of scent samples occurred during pup capture following the same procedures, to minimise contamination. Inox tweezers were used to take the cotton wool out of the vial and hold it while sampling the animal. The cotton was firmly but carefully rubbed on the nose (including mucus and skin secretions from the peri-narinal area), eyes (including Harderian and lachrymal fluids, caruncular mucus, and local secretions from skin glands on the eye-lids), mouth (including saliva, labial skin gland secretions, and externalised intraoral deposits due to oral microbiota), and/or the back fur (lumbar area, providing local skin glands' secretions; a video of the sampling procedure is available in SM 2.1). Adults and sub-adults were sampled with the same cotton wool mounted in clasped tweezers, attached to a 3 m pole. Animals were approached while maintaining a low profile (crawling or bending over and using topography and vegetation to minimise visibility to the animal) and were rubbed with cotton wool once within reach. Following collection, samples were placed back in the vial, Argon (Vinotech Pty Ltd) was added to expel air and limit the degradation of compounds, following which the sample was frozen at -20°C until defrosted for chemical analyses. Hands were gloved (nitrile gloves) at all times and tweezers were cleaned with 100% ethanol before being reused (Chem-Supply Pty Ltd).

To be able to account for and potentially eliminate contaminants (of environmental and instrumental origin), I also collected control samples. Pieces of cotton wool were exposed to the ambient air in the colony for approximately 30 s before being put back into the vial and treated according to the same procedures as the samples.

Gas chromatography-mass spectrometry (GC-MS) techniques allow for the separation and identification of volatile and semi-volatile compounds present in odour samples (Sneddon et al. 2007). The mixture of compounds present in a sample travels through the column of the gas chromatograph and molecules are separated based on differences in their chemical properties. As a result, the time it takes for molecules to travel through the column will vary (this is called the retention time (RT) of a compound). The mass spectrometer then ionises the molecules and separates them based on their mass-to-charge (m/z) ratios. Both RT and m/z ratios are used to identify the compounds present in samples. To analyse my results, I used a newly developed way of performing GC-MS analyses that involves Twisters® (Gerstel Magnetic Stir Bar, 20 mm length, 0.5 mm polydimethylsiloxane film thickness) – phase coated rods with multi-position stir plates that allow for sorptive-free extraction. After defrosting samples in a chemical incubator (30 min at 22°C), the Twisters were exposed to the compounds present in the sample. This was done by flushing out the compounds from the cotton wool for 2 h under a nitrogen flow of 50 ml min^{-1} at 30°C in specially designed containers. The Twisters were desorbed in a Gerstel Thermal Desorption Unit (by a Gerstel MPS autosampler, Gerstel GmbH & Co.

KG, Mühlheim an der Ruhr, Germany) and injected via a Gerstel Cooled Injection System (-80°C then 250°C) with a split ratio of 4:1 into a coupled gas chromatography-mass spectrometry system (Thermo Trace 1310-ISQ, Thermo Fisher Scientific Inc., Bremen, Germany). A GC containing a 30 m DB-5 MS column (methyl siloxane, 0.25 μm film thickness, 250 μm ID, Macherey-Nagel, Düren, Germany) was used, with Helium used as the carrier gas (1 ml min^{-1}). Electron impact ionization (70 eV, source temperature 200°C) was used. The column temperature program was as follows: 40°C for 3 min, then 5°C min^{-1} up to 200°C, 10°C min^{-1} up to 250°C, and kept for 2 min. This program was chosen as it allows for the incorporation of a broad range of compounds from the samples. As scent samples have never been analysed for Australian sea lions, I wanted to include all collected compounds. To control for contamination and monitor appropriate functioning of the machine, a reference Twister (a clean Twister not exposed to a scent sample) was processed every 5 samples, under the same settings and procedures as the scent samples.

In order to compare the chemical composition among samples, prior to statistical analyses the raw chemical data obtained from the GC-MS must be processed and prepared. The main and most important step of this involves determining which peaks (compounds) are the same across samples. Peak alignment methods vary across literature and many studies in the past have aligned peaks in a semi-automatic way (e.g., Penn et al. 2007; Poirotte et al. 2017; Wieß et al. 2018). This introduces the problem of a lack of full reproducibility as well as likely human errors and is not always a reliable method of alignment, especially when dealing with a high number of compounds present in each sample and retention times that are likely to overlap. In this thesis I use recently developed methods and software (MZmine 2.18; Pluskal et al. 2010) that use both RT and m/z ratios for compound alignment and allow for fully automatic and easily reproducible peak integration across samples. The method allows for consistency in data processing and makes it possible to compare different studies.

All GC-MS data were processed with the same protocol. MZmine batch processing files are provided in the supplementary materials of Chapter 3, and information about the algorithms used by MZmine are available in the software documentation, therefore they will not be described here in detail. I implemented a round resampling filter and retention times were kept at 5-35 min. I used a rolling ball baseline correction to account for shifts in the chromatographic baseline. Mass detection was set to 'centroid', which assumes that data above noise level is a detected ion (noise level set to 200). When building the final chromatogram, the minimum time span over which an ion must be observed to be recognised as a chromatogram was set to 0.08 min and minimum height of incorporated peaks was 100 with an m/z tolerance of 0.5 m/z or 0.001 ppm. I then smoothed the data with a filter width of 7. Deconvolution (peak separation) was done using the local minima search method. Here, the chromatographic threshold (noise removal) was set to 5%, search minimum in RT range (the point that

separates two adjacent peaks) was set to 0.04 min, the minimum relative height set at 5%, minimum absolute height of 20, minimum ratio of a peak's top/edge intensity was set to 1 and the range of acceptable peak durations was set to 0.04-2 min. The peaks were aligned using mass and retention times of each peak. The range of m/z (m/z tolerance) was 600-6000 ppm, the retention time tolerance was 0.3 min and 0.15 min post-recalibration. The assigned weights for m/z and RT for match score calculation were set to 0.2 and 0.8, relatively.

EXPERIMENTAL DESIGN

The experimental design varies for each chapter, therefore the details about each experiment as well as the presentation of cues and statistical analyses are included in the relevant sections of each chapter and are not included in the extended methods section.

VIDEO PROCESSING

Behavioural experiments used in Chapters 6 and 7 ('visual experiment' only) and 8, were video-recorded in the field, and videos were analysed later by observers blind to the treatments. All analyses were conducted according to the same protocols and conducted by trained observers.

The number of behaviours occurring from the beginning of the experiment (defined as the moment of cue presentation) were counted. Behaviours were defined as follows:

- Olfactory investigation ('investigatory behaviour' in Chapter 6): animal sniffs the dummy. This can be exhibited as either touching the dummy with the nose or coming into close proximity to it (approximately 50 cm) with erect whiskers and presenting a "head nod" in the direction of the dummy.

- Call: attraction call (pup: mother-attraction calls, females: pup-attraction calls) produced by an animal. These are very characteristic, loud calls that are easy to identify. Pups produce calls approximately 800 ms long, while females call for 1 s (Charrier and Harcourt 2006) and a distinct silent break occurs between each call if the animal is producing a series of calls.

- Aggressive behaviours: animal opens the mouth, exposes teeth (open mouth display), rapidly exhales air through the nose (puff sounds), bites the dummy (bite), or grabs the dummy with teeth and tosses it aside (sometimes associated with shaking; toss).

- Return to rest: following the presentation of a treatment, the animal looks at the dummy and then returns to its initial (resting) position.

- Vigilance: following the presentation of a treatment and looking at it, the animal does not return to its initial position and either remains alert or moves away from the object.

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SUPPLEMENTARY MATERIALS

SM 2.1. Video of scent sample collection (overall cephalic scent) from pup. Available at: https://mqoutlook-my.sharepoint.com/:u:/g/personal/kaja_wierucka_hdr_mq_edu_au/EQpzyO3H S91EmHGMDKDIDVsBa_zf2GQq8rE4M8NjZDW5Ag?e=wbZHne.

CHAPTER 3

Chemical profiles of integumentary and glandular substrates in Australian sea lion pups (*Neophoca cinerea*)

Australian sea lion pup (2-4 months old), Kangaroo Island 2016 (photo credit: Kaja Wierucka)

ABSTRACT

Recognition of individuals or classes of individuals plays an important role in the communication systems of many mammals. The ability of otariid (i.e. fur seal and sea lion) females to locate and identify their offspring in colonies after returning from regular foraging trips is essential to successful pup rearing. It has been shown that olfaction is used to confirm the identity of the pup by the mother when they reunite, yet the processes by which this chemical recognition occurs remain unclear. Using gas chromatography-mass spectrometry, we examined chemical profiles of integumentary and glandular secretions/excretions from pre- and post-moult Australian sea lion pups (*Neophoca cinerea*) and compared fur and swab samples to assess data collection methods. Multivariate statistics were applied to assess differences in chemical composition between body regions and sexes. We found differences among secretions from various body regions, driven by the distinctiveness of the oral odor mixture. The fine-scale trends in pre- and post-moult pups seem to differ due to changes in the behaviour of pups and consequent decrease in the transfer of compounds among adjacent body regions in older pups. Volatile compounds from exocrine substrates were not distinct for different sexes. We also show that swab samples provide better data for exploring social olfaction than fur samples for this species. Obtaining fundamental chemical information, in this case chemical profiles of animals, and discerning differences in chemical composition is an important step towards fully exploring the intricacies of mother-offspring olfactory recognition and its underlying processes.

Keywords: Chemical communication, gas chromatography-mass spectrometry (GC-MS), marine mammals, mother-offspring recognition, olfactory recognition, pinniped.

INTRODUCTION

Olfaction is a prominent sensory modality, regulating a large range of social interactions among mammals (e.g., Eisenberg and Kleiman 1972; Albone 1984; Brown and Macdonald 1985; Wyatt 2003; Müller-Schwarze 2006; Campbell-Palmer and Rosell 2011; Wyatt 2014). It is a critical component of many social behaviours associated with reproduction, such as mating, parental care and competition, which are contingent upon recognition of specific categories of animals and/or distinguishing specific individuals. While olfactory cues and signals are claimed to be essential to social recognition for many mammals (e.g., Colgan 1983; Brennan and Kendrick 2006), the chemical and olfacto-semiotic processes underlying olfactory recognition have to date received relatively little attention in wild mammals (e.g., Sorensen and Hoye 2010; Wyatt 2014).

For olfactory cues to be useful for animals, consistent, detectable olfactory differences must exist among categories of interest. Differences in chemical profiles, i.e. the presence/absence of certain compounds and/or relative abundance of given compounds, should indicate whether there is potential to convey given information by cues. Although chemical profiles will contain compounds of various origins, including those produced by the individual, those produced by other organisms (e.g., symbiotic micro-organisms), and those acquired from their diet and environment (Thom and Hurst 2004; Brennan and Kendrick 2006), they all contribute to the overall bouquet of compounds that is present on, or surrounds the animal, and so represent what is available for investigation by other individuals.

Otariids (fur seals and sea lions; clade *Pinnipedia*) are in many ways an excellent group for investigating social olfaction in wild mammals. Odours have long been imputed to play a significant role in social interactions (Renouf 1991a; Insley et al. 2003) such as mother-pup recognition. Otariid females are income breeders as they are incapable of storing large amounts of energy reserves that are required during lactation. This means that following a short perinatal period, females leave their pups on land for several days at a time to forage at sea (Bowen 1991). This pattern of departures and returns lasts throughout the whole lactation period (Bowen 1991) and, consequently, pups are suckled only during the short period when their mother is on land between foraging trips. The ability to locate and identify offspring by these females after returning from each trip is crucial (Renouf 1991a) and affects not only the pups' survival, but also the females' reproductive success. Mother-pup reunion is known to be a multimodal process, with vocalisations allowing the returning female to locate and identify the pup, visual cues assisting with the search and olfaction acting as a final check of identity by the mother before she allows the pup to suckle (Bowen 1991; Insley et al. 2003). However, to our knowledge, the role of olfaction between mothers and pups is based almost exclusively on observations of naso-nasal investigations (Bartholomew 1959; Peterson and Bartholomew 1969; Trillmich 1981; Renouf 1991b;

Dobson and Jouventin 2003; Insley et al. 2003; Philips 2003; Trimble and Insley 2010) and only one study has experimentally demonstrated that olfaction can be discriminative in otariid mother-pup recognition (Pitcher et al. 2010b).

Australian sea lions (*Neophoca cinerea*) have been well studied with regards to mother-offspring communication. Detailed information about the importance of various cues in mother-offspring interactions exists for this species (Charrier and Harcourt 2006; Charrier et al. 2009; Pitcher et al. 2009; Pitcher et al. 2010a; Pitcher et al. 2010b; Pitcher et al. 2012; Wierucka et al. 2017 – Chapter 5; Wierucka et al. 2018 – Chapter 6) giving us a good basis for further exploration of the processes underlying this social behaviour. However, most of the research has focused on acoustic and multimodal communication. Apart from showing that mother Australian sea lions are capable of recognising filial pups based solely on scent (Pitcher et al. 2010b), and observational data claiming that olfaction is likely used very shortly after birth (Stirling 1972; Marlow 1975), the chemical composition, and source and nature of active odour cues remain unknown.

Chemical compounds responsible for social interactions in mammals can have various origins and differ among species (e.g., Eisenberg and Kleiman 1972; Brown 1979; Burger 2005). Many mammals have specialised glands that produce compounds used to convey specific information (e.g., anal glands for scent marking), yet chemical cues can often be a by-product of secretions that serve other functions (Quay 1986; Thom and Hurst 2004). Pinnipeds do not seem to possess distinct, specialised glands that could be used exclusively for chemical communication and little is known about the exact role of olfaction in their social behaviour (Renouf 1991b). However, studies have shown that facial glands (either sweat or sebaceous or both) most likely play a role in sexual signalling in grey seals (Ling 1965), ringed seals (Hardy et al. 1991; Ryg et al. 1992), and Cape fur seals (Rotherham et al. 2005). Furthermore, sweat glands around the mouth of the walrus have been suggested to be important in mother-calf interactions (Berta et al. 2005). We currently have no information about other types of glands, secretions, or substances that could play a role in olfactory communication in pinnipeds.

Although research has shown that Australian sea lion females are capable of distinguishing between filial and nonfilial pup odours (Pitcher et al. 2010b), and despite calls for further exploration of their chemosensory bouquet (Pitcher et al. 2015) there have been no investigations on how these cues are produced and released and what type of secretions may be of significance. The single published study of chemical profiles in an otariid reported that Antarctic fur seal mothers and pups show similar chemical profiles (Stoffel et al. 2015). To fully understand the intricate process of social olfaction, it is essential to explore its underlying mechanisms, from the production and release of odour-borne information, to its detection, integration and response induction. In this study, we develop and assess

scent sample collection methods, and describe chemical profiles of secretions/excretions from various body regions of pups to determine whether intra-individual and inter-individual differences occur in the volatiles composing these biological substrates.

MATERIALS AND METHODS

Setting and season

The study was conducted in a single colony of Australian sea lions (*Neophoca cinerea*) inhabiting Olive Island, South Australia (32°43'18.5" S, 133°58'6.3" E) in November 2015 and April 2016. Olive Island is composed of non-foliated granite and supports simple and limited vegetation consisting mostly of Marsh Saltbush (*Atriplex paludosa*) and Nitre-bush (*Nitraria billardierei*; Robinson et al. 1996).

Sample Collection

Samples of integumentary secretions or excretions as well as fur samples of pre- and post-moult Australian sea lion pups were collected. Pre-moult pups were divided into two categories, pre-moult 1 – pups aged under 2 months, and pre-moult 2 – pups aged 2-4 months. The two different pre-moult age-classes of pups were distinguished as the pups' fur changes around 2 months. Pups under 2 months of age have black fur that changes to a chocolate brown/cinnamon colour around 2 months of age. At around 4 months, pups moult and acquire a dual silver and beige pelage colour characteristic for adult females (Jefferson et al. 2015). Post-moult pups were approximately 1-1.5 years of age at the time of the study and were still suckling, however would be weaned within a few months of the end of the study.

Pups were sampled during maternal absences to avoid unnecessary disturbance. They were caught in a net and restrained for a short period of time (less than 15 minutes) while samples from different exocrine secretions/excretions and body substrates were collected. Swab samples were collected by firmly rubbing a cotton swab onto different body regions: the back fur (lumbar area, providing local skin glands' secretions), the nose (including mucus and skin secretions from peri-narinal area), the eyes (including Harderian and lachrymal fluids, caruncular mucus, and local secretions from skin glands on eye-lids), and the mouth (including saliva, labial skin glands' secretion, and externalised intraoral deposits due to oral microbiota; Table 3.1). Fur samples (B_F; Table 3.1) were collected by clipping fur around the lumbar area with stainless steel scissors.

Table 3.1. Number and type of samples used for analysis. Notations: Pre-moult 1 – pups aged 2 months, Pre-moult 2 – pups 2-4 months, Post-moult – pups over 4 months, B – back, E – eye, M – mouth, N – nose, B_F – fur from the back.

Pup	No. individuals	female	male	unknown sex	body regions				
					B	E	M	N	B_F
Pre-moult 1	10	6	4	0	8	10	9	10	5
Pre-moult 2	10	5	5	0	9	8	9	10	10
Post-moult	10	5	3	2	10	10	10	10	9

Cotton swabs were prepared prior to fieldwork by vigorously stirring pharmaceutical cotton wool with a magnetic stir bar in methanol (Sigma-Aldrich – for analysis – ACS reagent ≥99.8%) and hexane (Sigma-Aldrich – for analysis – ACS reagent 99%) to remove most of the contaminants, especially volatiles. Swabs were washed three times. After the final wash with hexane, the cotton pieces were left to dry for 4 h under a clean chemical fume hood. They were then stored in 4 ml dark chromatographic vials (45 x 14.7 mm, screw cap with PTFE septum – Chromoptic, Courtaboeuf, France). In the field, cotton swabs were briefly taken out of the vials with tweezers (using gloved hands) for sampling and re-introduced to the vials immediately after sampling was completed. When collecting fur samples, the vial was briefly opened while fur was inserted with tweezers. A clean pair of tweezers was used during each sampling occasion (separate instruments for swab and fur samples) and both tweezers and scissors were cleaned with 100% ethanol (Chem-Supply Pty Ltd) prior to being reused. Argon (Vinotech Pty Ltd) was added directly after sample collection to expel air from the vial and limit oxidation, optimising the preservation of samples until analyses. Samples were kept frozen at -20°C until analysed. Several control samples were collected following the same protocols and tools, with the cotton merely exposed to air within the seal colony.

Chemical analysis

Samples were defrosted for 30 min at 22°C in a thermoregulated incubator. We then exposed Twisters® (Gerstel Magnetic Stir Bar – 20 mm length, 0.5 mm polydimethylsiloxane film thickness) to the headspace of the sample for 2 h under a nitrogen flow of 50 ml min^{-1} at 30°C. The Twisters were desorbed in a Gerstel Thermal Desorption Unit (6 min, by a Gerstel MPS autosampler, Gerstel GmbH & Co. KG, Mühlheim an der 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 as the carrier gas (1 ml min^{-1}). Ionization was by

electron impact (70eV, source temperature 200°C). The full GC-MS cycle lasted 42 min, with the column temperature 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. To monitor proper functioning of the machine and control for potential residual contamination, a reference Twister (clean Twisters not exposed to a swab sample) was processed every 5 samples, under identical settings and procedures as described above.

Data processing and statistical analysis

All GC-MS data were processed in a forked version of MZmine 2.18 (Pluskal et al. 2010) adapted to gas chromatography data processing (customised software available on request) using the same automated protocol, ensuring the consistency of peak integration. Following baseline correction and normalisation of the dataset, peaks were detected by local minima chromatogram deconvolution and were aligned across samples based on mass spectra and retention times (see SM 3.1 for full details on MZmine data processing). To eliminate possible contamination resulting from transporting samples, swab structure or chemical analyses (compounds originating from both the GC-MS and the Twister), we removed any compounds that were present in the control samples (2015 season n=6 controls; 2016 season n=3 controls) or reference Twisters (2015 season n=34, 2016 season n=110) from swab samples and removed molecules found in reference Twisters from fur samples. Furthermore, we also eliminated compounds that were present in only one sample. To account for varying intensities of the collected samples (as a result of the nature of sampling wild animals in a remote location), instead of using absolute values, peak areas of compounds were recalculated to represent their relative proportion within each sample. Due to the lack of prior information about Australian sea lion chemical profiles and the possible presence of important but less abundant compounds, we incorporated a broad range of compounds for analysis and, as a result, further restrictions to the dataset were not applied.

All statistical analyses were performed in R version 3.2.2 (R Core Team 2015). To compare the chemical profiles in *a priori* defined groups we applied a nonparametric permutational multivariate analysis of variance using distance matrices (npPERMANOVA; package vegan; Anderson 2001; Oksanen et al. 2016) to a Wisconsin and square root transformed dataset. This method compares the presence as well as relative abundance of chemical components within samples. We used the Bray-Curtis dissimilarity index and examined differences among body regions and between sexes, as well as the interactions between these variables (for samples in which sex information was obtained). Furthermore, we incorporated age differences for pre-moult 1 and pre-moult 2 samples. We did not test for age differences among all three age classes, as post-moult samples were collected during a

different field season and we did not want sampling season to influence our results. Where appropriate, we applied pairwise analyses to compare sampled groups, implementing Holm's adjustment of p-values for multiple comparisons (Holm 1979). To compare sampling techniques, we tested differences among compounds present on fur and back swab samples. A non-metric multidimensional scaling (NMDS) technique based on a Bray-Curtis dissimilarity index was used to visualise differences between age and sex classes. This method maps ranks non-linearly onto an ordination space, producing a clear representation of the level of chemical similarity on a two dimensional plot, with points in close proximity representing a higher similarity.

As no prior research has been done on chemical profiles of body odour in this species and only one otariid species has been studied before (Stoffel et al. 2015), information about the importance of given skin secretions or glandular excretions is limited. In order to designate whether several compounds were responsible for a majority of the differences observed between given groups or whether the overall chemical profile of the animal played a role, we also performed a similarity percentage analysis (SIMPER; Clarke 1993). This technique allows for pairwise comparisons of groups and the assessment of average contributions of each compound to the overall Bray-Curtis dissimilarity index. We then, where possible, identified or annotated the top 15 discriminating variables (compounds) from this analysis. This was done by 1) comparing the mass spectra to libraries (Wiley registry of Mass Spectral Data – 9th ed., NIST MS Database 2011, and Adams (2001)), and 2) manual confirmation of the identification by comparing Kovats indices in literature (Adams 2001).

RESULTS

A total of 137 scent samples from 30 Australian sea lion pups (Table 3.1) were collected and analysed to assess whether pup samples contained different chemical compounds detected by the GC. We focused on assessing whether the chemical composition of samples was characteristic for body regions and sexes. Following data restriction (cf. Methods section), 450 different compounds were detected across all samples and used for statistical analysis.

Pre-moult pups

The multivariate analysis of pup chromatographic profiles showed no significant differences among pre-moult 1 and pre-moult 2 pups (R^2=0.015, p=0.285, n individuals=20, n samples=73; Table 3.2), therefore we combined them into a single pre-moult group. Significant differences among body regions were present (R^2=0.073, p=0.001, Table 3.2), with cephalic samples (E, M, N) all being similar

to each other and different from the back (B) samples (Table 3.2; Figure 3.1a). No sex differences among pups were observed (R^2=0.010, p=0.786, n=20; Table 3.2).
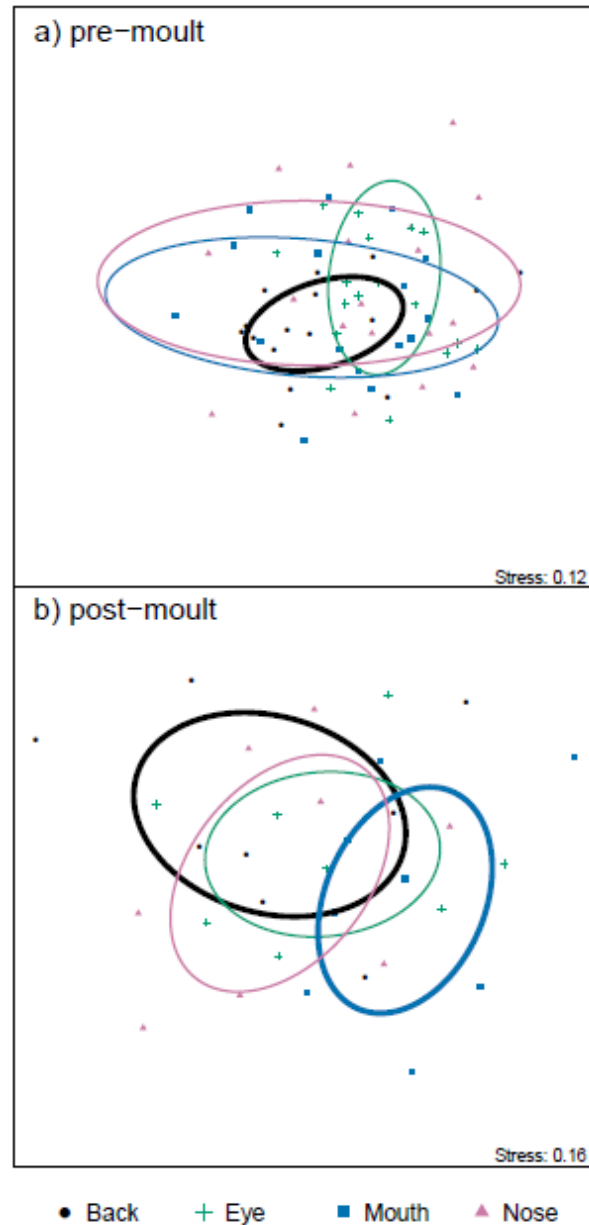
Figure 3.1. NMDS visualisation of differences in chemical profiles among pups' (a – pre-moult; b – post-moult) body regions. Points in close proximity represent a higher similarity. Dispersion ellipses were drawn using the standard deviation of point scores, with the weighted correlation defining the direction of the principal axis of the ellipse. Thicker ellipses indicate body regions significantly differing in chemical profiles. For ease of viewing, two outliers (1 mouth and 1 nose sample) are beyond the limits of the x axis however have been taken into account in the dispersion ellipses.

Table 3.2. Differences in chemical composition among body regions, sexes and individuals of post-moult Australian sea lion pups. Statistically significant results (p≤0.05; Holm's correction for multiple comparisons applied) are marked in bold.

Model	Factor	pre-moult				post-moult			
		Df	F	R^2	p	Df	F	R^2	p
body region*sex (post-moult) body region*sex*age (pre-moult)	body region	3	1.785	0.073	0.001	3	1.225	0.119	**0.050**
	sex	1	0.768	0.010	0.786	1	0.795	0.026	0.851
	age	1	1.128	0.015	0.285	-	-	-	-
	body:sex	3	0.849	0.035	0.792	3	0.847	0.082	0.929
	body:age	3	1.227	0.050	0.099	-	-	-	-
	sex:age	1	0.792	0.011	0.753	-	-	-	-
	body:sex:age	3	0.652	0.027	0.991	-	-	-	-
		pairwise comparisons (body regions)				pairwise comparisons (body regions)			
	back vs eye	-	3.026	0.084	**0.006**	-	1.143	0.075	0.630
	back vs mouth	-	2.372	0.067	**0.016**	-	1.750	0.111	**0.006**
	back vs nose	-	2.652	0.070	**0.010**	-	0.940	0.063	1.000
	eye vs mouth	-	1.453	0.041	0.186	-	1.264	0.083	0.552
	eye vs nose	-	0.881	0.024	1.000	-	0.905	0.061	1.000
	mouth vs nose	-	0.808	0.022	1.000	-	1.569	0.101	0.080
Sampling method*age	sampling method	1	9.372	0.234	**0.001**	1	3.691	0.780	**0.001**
	age	1	0.950	0.023	0.479	-	-	-	-
	sampling method:age	1	0.831	0.021	0.593	-	-	-	-

We performed a SIMPER routine prior to compound identification to assess which substances were most responsible for the observed statistical differences among body regions. As can be seen by the relatively gentle slope in Figure 3.2a there was a relatively high degree of equity or evenness in contribution from multiple compounds to the observed chemical profile differences among pre-moult Australian sea lion scent samples, rather than a small number of compounds predominating. This trend was consistent among all the statistically significant pairwise comparisons of body regions (Figure 3.2, Table 3.2). To explore this further, we annotated the top 15 compounds of the SIMPER analysis (Table 3.3). Compounds contributing to differences among the back and other body regions consisted mainly of polar volatile compounds: aldehydes and esters. Over 70% of the 45 compounds occur in more than one SIMPER pairwise comparison.
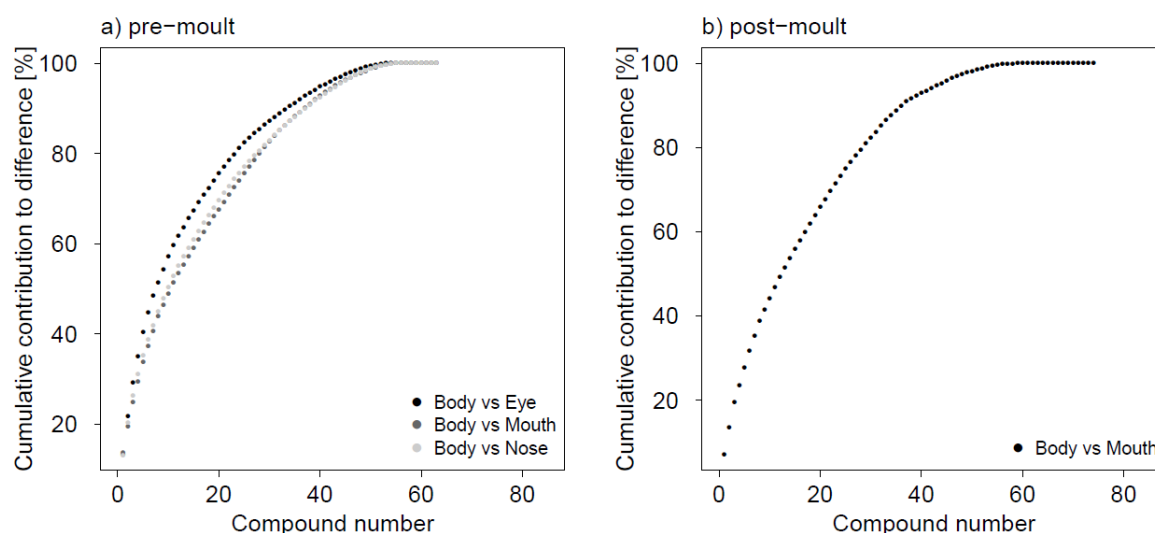
Figure 3.2. Cumulative contribution of compounds to the observed chemical profile differences between Australian sea lion pup scent samples (a – pre-moult; b – post-moult) taken from different body regions that showed significant differences in chemical composition. Compounds were ordered from the highest to the lowest individual contributions to the observed differences.

Table 3.3. Top 15 chemical compounds most contributing to the observed differences in chemical profiles among body region samples of pre-moult Australian sea lion pups. Where identification to a specific compound was not possible, an annotation of the compound is provided. Notations: ARA – Average relative abundance in a given sample type, ND – impossible to identify.

Contrast [1 vs 2]	No.	Compound name	Average RT	Contribution to difference	Cumulative contribution	ARA 1	ARA 2
B vs E	1	alkane	26.0561	0.1230	0.1313	0.2464	0.0086
	2	alkyl substituted phenol or quinone	30.5490	0.0797	0.2164	0.0790	0.1137
	3	long chain ester	23.5951	0.0699	0.2910	0.0752	0.1089
	4	long chain aldehyde	29.8686	0.0559	0.3506	0.0074	0.1082
	5	benzaldehyde	14.6224	0.0501	0.4041	0.0000	0.1001
	6	isopropylalkanoate	33.3417	0.0404	0.4472	0.0808	0.0000
	7	alkyl substituted phenol	30.5180	0.0351	0.4847	0.0479	0.0282
	8	ND - coelution	26.0277	0.0278	0.5143	0.0000	0.0556
	9	terpene	18.2214	0.0272	0.5433	0.0257	0.0324
	10	long chain alkane	32.9436	0.0257	0.5708	0.0288	0.0278
	11	dimethylsilandiol	6.2328	0.0248	0.5972	0.0000	0.0495
	12	aldehyde	32.4128	0.0185	0.6170	0.0119	0.0295
	13	trace - ND	32.2213	0.0183	0.6365	0.0320	0.0061
	14	trace - ND	7.1163	0.0178	0.6556	0.0303	0.0070
	15	lactone	31.6078	0.0171	0.6738	0.0067	0.0317
B vs M	16	alkane	26.0561	0.1269	0.1353	0.2464	0.0553
	17	long chain ester	23.5951	0.0555	0.1945	0.0752	0.0593

	18	alkyl substituted phenol or quinone	30.5490	0.0498	0.2476	0.0790	0.0319
	19	methyl-3-benzylpropanoate	24.3878	0.0438	0.2942	0.0000	0.0875
	20	isopropylalkanoate	33.3417	0.0404	0.3373	0.0808	0.0000
	21	ramified long chain ketone	31.1907	0.0332	0.3727	0.0000	0.0663
	22	long chain aldehyde	29.8686	0.0310	0.4057	0.0074	0.0586
	23	N-substituted phtalate	14.8863	0.0303	0.4381	0.0284	0.0447
	24	long chain alcohol acetate	21.5515	0.0241	0.4638	0.0000	0.0482
	25	alkyl substituted phenol	30.5180	0.0239	0.4893	0.0479	0.0000
	26	silane	6.5972	0.0235	0.5143	0.0092	0.0401
	27	ND	31.2812	0.0192	0.5348	0.0120	0.0278
	28	long chain alkane	32.9436	0.0178	0.5538	0.0288	0.0090
	29	trace - ND	32.2213	0.0176	0.5725	0.0320	0.0042
	30	benzaldehyde	14.6224	0.0176	0.5913	0.0000	0.0351
B vs N	31	alkane	26.0561	0.1229	0.1293	0.2464	0.0280
	32	long chain ester	23.5951	0.0687	0.2016	0.0752	0.0866
	33	alkyl substituted phenol or quinone	30.5490	0.0578	0.2624	0.0790	0.0538
	34	long chain aldehyde	29.8686	0.0448	0.3095	0.0074	0.0872
	35	isopropylalkanoate	33.3417	0.0404	0.3520	0.0808	0.0000
	36	ramified long chain ketone	31.1907	0.0342	0.3879	0.0000	0.0684
	37	benzaldehyde	14.6224	0.0296	0.4191	0.0000	0.0593
	38	long chain alcohol acetate	21.5515	0.0282	0.4488	0.0000	0.0565
	39	Nitrogen heterocycle	14.9179	0.0276	0.4778	0.0040	0.0521
	40	trace - ND	6.5760	0.0242	0.5033	0.0097	0.0407
	41	alkyl substituted phenol	30.5180	0.0239	0.5285	0.0479	0.0000
	42	trace - ND	25.8105	0.0217	0.5513	0.0000	0.0434
	43	long chain alkane	32.9436	0.0184	0.5707	0.0288	0.0101
	44	aldehyde	32.4128	0.0181	0.5898	0.0119	0.0280
	45	long chain alkane	27.8216	0.0181	0.6088	0.0138	0.0252

Post-moult pups

We found significant differences in chemical composition among the various body regions of post-moult pups (R^2=0.123, p=0.050; n individuals=10, n samples=40; Table 3.2, Figure 3.1b), however dissimilarities between the samples collected from the back and mouth regions seemed to drive this

result (adjusted p=0.006, Table 3.2). We observed no sex differences among samples overall (R^2=0.026, p=0.851, n=8; Table 3.2) or for any of the sampled regions (body region:sex R^2=0.0819, p=0.929, Table 3.2).

Similarly to pre-moult pup data, no individual compounds stood out as being particularly important in differences occurring between pup back and mouth swab samples of post-moult pups (Figure 3.2b). We annotated compounds that most contributed to differences among back and mouth samples of pups (Table 3.4). We found mostly small polar compounds and some molecules (compound 4 and 15; Table 3.4) that are not produced by mammals and have an environmental origin (found in terpene or phenylpropanoid metabolic pathways).

Table 3.4. Top 15 chemical compounds most contributing to the observed differences in chemical profiles between back and mouth samples of post-moult Australian sea lion pups. Where identification to a specific compound was not possible, an annotation of the compound is provided. Notations: B – back, E – eye, M – mouth, N – nose, ARA – Average relative abundance in a given sample type, ND – impossible to identify.

No.	Compound name	Average RT	Contribution to difference	Cumulative contribution	ARA Back	ARA Mouth
1	3-hydroxybutan-2-one	6.6286	0.0682	0.0695	0.0000	0.1364
2	N-substituted phtalate	14.8863	0.0641	0.1347	0.1259	0.0033
3	furane squeleton	9.4000	0.0585	0.1943	0.1171	0.0057
4	sesquiterpene	15.0132	0.0406	0.2357	0.0000	0.0813
5	4-methylphenol	18.1667	0.0406	0.2770	0.0811	0.0000
6	ND - coelution	23.2741	0.0383	0.3160	0.0000	0.0765
7	alkyl substituted phenol or quinone	30.5490	0.0355	0.3521	0.0651	0.0118
8	long chain ester	34.9754	0.0347	0.3875	0.0000	0.0695
9	butanoic acid	8.4325	0.0276	0.4155	0.0225	0.0382
10	ND - trace	5.9896	0.0257	0.4417	0.0000	0.0514
11	alkyl substituted phenol	30.5180	0.0251	0.4673	0.0503	0.0000
12	ND - coelution	16.9823	0.0241	0.4918	0.0481	0.0000
13	ND - trace	12.3137	0.0229	0.5151	0.0459	0.0000
14	benzaldehyde	14.6224	0.0215	0.5370	0.0000	0.0430
15	2-phenylethan-1-ol	19.6259	0.0206	0.5580	0.0181	0.0276

Fur and swab sample comparison

To evaluate and compare different methods of sample collection, we compared the chemical composition of fur and swab samples collected from the same body region. We found significant differences between these samples for both pre-moult (R^2=0.234, p=0.001; Table 3.2) and post-moult

(R^2 =0.780, p=0.001; Table 3.2) pups, with fur samples being much more dispersed than swab samples for post-moult pups, and the opposite trend visible for pre-moult pups (Figure 3.3). No age differences were present between pre-moult 1 and 2 pups (R^2=0.023, p=0.479, Table 3.2). The top 20 compounds for each pup type were annotated to determine which compounds contributed to differences among fur and swab samples (Table 3.5). Fur samples contained a higher number of heavier, polar compounds – heterocycles, long chain aldehydes and esters, than swab samples.
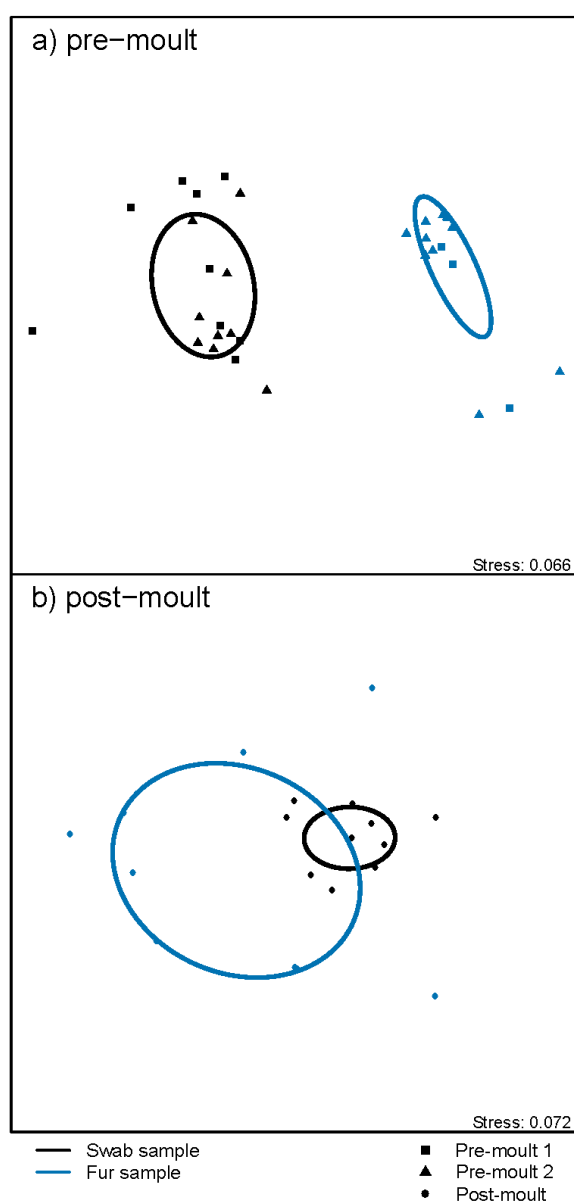

Figure 3.3. NMDS visualisation of differences in chemical profiles of fur and swab samples of Australian sea lion pups (a – pre-moult; b – post-moult). Points in close proximity represent a higher similarity. Dispersion ellipses were drawn using the standard deviation of point scores, with the weighted correlation defining the direction of the principal axis of the ellipse.

Table 3.5. Top 15 chemical compounds most contributing to the observed differences in chemical profiles between fur and swab samples of pre- and post-moult Australian sea lion pups. Where identification to a specific compound was not possible, an annotation of the compound is provided. Notations: ARA – Average relative abundance in a given sample type, ND – impossible to identify.

Pup	No.	Compound name	Average RT	Contribution to difference	Cumulative contribution	ARA Fur	ARA Swab
pre-moult	1	alkyl substituted phenol or quinone	30.5490	0.1766	0.1767	0.3532	0.0000
	2	ramified long chain ketone	31.1907	0.0751	0.2519	0.1502	0.0000
	3	coelution - ND	5.9109	0.0258	0.2777	0.0516	0.0000
	4	coelution - ND	6.5988	0.0180	0.2957	0.0350	0.0013
	5	2-(dialkylamino)-ethanol	16.8937	0.0169	0.3125	0.0333	0.0005
	6	Nitrogen heterocycle	25.8388	0.0168	0.3294	0.0336	0.0001
	7	long chain ester	25.8551	0.0166	0.3461	0.0333	0.0000
	8	sugar derivative	19.8455	0.0154	0.3615	0.0307	0.0002
	9	menthol or isomer	21.5496	0.0145	0.3760	0.0289	0.0000
	10	ND	18.3079	0.0130	0.3890	0.0253	0.0008
	11	phenol or quinone squeleton	33.6991	0.0130	0.4020	0.0259	0.0000
	12	amide compound	18.1406	0.0121	0.4140	0.0234	0.0010
	13	ND	8.0984	0.0116	0.4256	0.0224	0.0009
	14	hydroxylated long chain carboxylic acid (C18)	29.9219	0.0099	0.4355	0.0198	0.0000
	15	trace - ND	7.5907	0.0086	0.4441	0.0172	0.0000
	16	propanol derivative	5.9009	0.0085	0.4527	0.0171	0.0000
	17	Nitrogen heterocycle	14.9179	0.0083	0.4609	0.0153	0.0014
	18	sterol derivative	27.1431	0.0081	0.4690	0.0152	0.0012
	19	trace - ND	6.5760	0.0079	0.4769	0.0153	0.0005
	20	terpene	18.2214	0.0071	0.4840	0.0140	0.0003
post-moult	21	alkyl substituted phenol or quinone	30.5490	0.1088	0.1092	0.2150	0.0048
	22	ramified long chain ketone	31.1907	0.1063	0.2159	0.2126	0.0000
	23	ND	6.6823	0.0875	0.3037	0.1749	0.0002
	24	alkyl substituted phenol	30.5180	0.0486	0.3525	0.0885	0.0113
	25	Nitrogen heterocycle	16.9170	0.0314	0.3841	0.0628	0.0002
	26	sugar derivative	19.8455	0.0289	0.4131	0.0570	0.0010
	27	quinoline derivative	34.7186	0.0285	0.4417	0.0556	0.0019
	28	Nitrogen heterocycle	11.2621	0.0282	0.4700	0.0556	0.0010
	29	N-substituted phtalate	14.8863	0.0279	0.4980	0.0556	0.0004
	30	long chain alkane	32.9436	0.0137	0.5118	0.0227	0.0062

31	highly functionalized aromatic compound - might be phosphorylated	12.6011	0.0125	0.5243	0.0000	0.0250
32	hexan-3-ol or 2-methylbutan-2-ol	8.9134	0.0115	0.5359	0.0000	0.0230
33	polyphenol	10.0746	0.0108	0.5467	0.0000	0.0216
34	sugar derivative	8.5002	0.0103	0.5570	0.0000	0.0206
35	1-(2-aminophenyl)ethanone oxime	8.3817	0.0103	0.5673	0.0000	0.0205
36	small acetate compound	7.4684	0.0100	0.5774	0.0000	0.0201
37	branched aldehyde C9	19.2230	0.0099	0.5874	0.0000	0.0199
38	branched alkane C10	17.4224	0.0095	0.5969	0.0000	0.0190
39	ND	7.6816	0.0082	0.6051	0.0000	0.0163
40	aldehyde	24.3979	0.0081	0.6133	0.0000	0.0163

DISCUSSION

Olfaction has long been suggested to be of importance to otariids (e.g., Renouf 1991b; Insley et al. 2003), however few experimental studies have been conducted and we still lack knowledge about the processes underlying social olfaction. A way to explore this complexity is to ascertain whether instrumentally detectable differences in chemical profiles exist between body regions or sexes, and what determines them chemically. We found that differences in chemical profiles were present across body regions of Australian sea lion pups, with different trends for pre- and post-moult pups. No significant differences among sexes were detected. The overall chemical mixture, not particular chemical compounds, seemed to be important for the observed differences.

Significant differences in chemical composition were present between the cephalic (eye, nose and mouth) and noncephalic (back) body regions for pre-moult pups. For older, post-moult pups, only the mouth and back samples varied significantly. Combining these results and considering the behaviour of the different age classes of pups we suggest that it is the back, nose, and eye samples that are all relatively similar in terms of GC profile, with mouth chemical profiles being detectably different. Saliva, labial skin glands' secretions, breath compounds, derivatives from microbiota and other compounds present in the mouth spread to the surrounding body locations and a transfer of compounds may occur, which we suggest explains the observed similarity of all of the cephalic regions, particularly in younger pups. The secretions most likely spread throughout the whole cephalic region by natural diffusion or by the animal rubbing its face against natural substrates in its immediate surrounds. A very high chemical compound similarity was observed for mouth and nose samples, which can be explained

by the connectivity and proximity of the two body regions. Eye samples seemed less heterogeneous (Figure 3.1) and overlapped with nose and mouth samples, confirming the likely spread of secretions around the whole cephalic area. The large overlap in compounds contributing to differences among the different pairwise comparisons of body regions (Table 3.3) further confirmed this. We suggest that the greater distance between the mouth and back region, combined with the observation that seals do not lick their fur when grooming (Marlow 1975), contributed to our observation of pronounced differences in chemical composition among the two regions for post-moult pups. The difference in behaviour of pre- and post-moult pups further supports this hypothesis. Pre-moult pups stay on land and do not undergo foraging trips or long swimming sessions. Consequently, they do not have many opportunities to remove copious secretions. By contrast, post-moult pups spend more time in the water (Fowler et al. 2006) where secretions will wash off. The observed distinctiveness of mouth samples could have been due to the source of the secretions being different because of the presence of microbial activity or compounds of environmental origin. 2-phenylethan-1-ol (compound 15, Table 3.4) most likely derives from plant or microbial sources and sesquiterpene (e.g., compound 4, Table 3.4) is of environmental origin. Both of these compounds were more abundant in the mouth than in the back samples. Detecting less environmental compounds on the pups' back than in the mouth is consistent with our hypothesis that many external compounds get washed off during foraging trips.

Although mucus, saliva, and ocular secretions (Harderian secretion, tears) have been shown to carry semiochemical information in other mammals (e.g., Eisenberg and Kleiman 1972; Brown 1979; Albone 1984; Burger 2005; Drea et al. 2013), to our knowledge, there have been no studies looking at the role of compounds present in saliva, mucus, or ocular secretions in pinniped olfactory communication. Previous studies have proposed that sebaceous and sweat glands influence chemical communication in pinnipeds (Weiner and Hellmann 1960; Ling 1965; Hardy et al. 1991; Ryg et al. 1992; Berta et al. 2005; Rotherham et al. 2005). Considering the abundance and relatively equal distribution of skin glands on the body of otariids (Ling 1965), and the apparent absence of specialised scent glands, the lack of chemical differences between a majority of body regions found in our study may be due to the secretions of skin glands comprising a large contribution to overall chemical profiles of Australian sea lion pups. We expect that compounds important for social recognition are produced by the pup, rather than the mother (i.e., through labelling, as is the case for e.g., goats; Gubernick 1980), as no licking occurs (Marlow 1975). However, considering the abundance and instrumental distinctiveness of chemical profiles of the oral odour mixture, we cannot rule out the possibility that these compounds play a semiochemical role in mother-pup recognition, especially considering that the muzzle is the primary region investigated by mothers during reunions (Renouf 1991a; Insley et al. 2003).

Olfactory communication, particularly in mammals, tends to be reliant on animals distinguishing the relative abundance of common compounds in more or less complex mixtures, rather than the presence of specific chemicals (Brennan and Kendrick 2006; Johnston 2008). The differences in chemical composition that we found were determined by the overall GC profile of the samples, with no specific compounds identified as significantly contributing to the dissimilarity. As many body regions were chemically similar, and multiple compounds determined the differences for distinct regions, we suggest that it is the overall odour mosaic (or its subpart) which an animal has to learn or match that encodes information in Australian sea lions.

We found no detectable sex differences in the chemical profiles of pups. Observational studies have reported a distinct odour of mature Australian sea lion males during the breeding season that is more pronounced at the time they start exhibiting aggressive behaviours towards other males (Marlow 1975). Based on our sampling method and analytic results, pre- or post-moult pups do not show any sex differences in their chemical profiles. Thus it seems that these distinct odours develop later, either as, or coincident with, secondary sexual characteristics as the animals achieve sexual maturity, as is the case for many mammals (Brown 1979).

Finally, we found that swab samples proved to be a better sampling method than fur samples for investigating compounds related to social olfaction. Pronounced differences were noted between fur and swab samples collected from the same body region of the pups. We found heavier and more polar compounds in fur samples, which is consistent with water activity and skin secretions that impregnate the fur. Swab samples consisted of lighter and more volatile compounds, making them better for examining social chemical communication.

Out of 65 unique compounds that were identified, we only found 2 contaminants (compound 11 and 26; Table 3.3) that came from the GC-MS column. The equipment releases contaminants at random and thus some may have not been captured in the control or reference samples. However, the low number of contaminants should not have influenced the results.

Obtaining chemical profiles of animals is an important step to exploring the intricacies of chemical communication and the processes underlying it. Olfaction appears to play a role in otariid mother-offspring recognition and here we provide information about both inter-and intra- individual variation of the chemical profile of pup odours. Furthermore, based on chemo-analytic data and statistical computations, we show that a wide variety of compounds present in the pup's chemical profile that are available for inspection by females seem to be important, rather than only a few specific compounds. Future research could focus on unravelling the complexity of otariid olfactory communication through extracting fragments of chemical profiles that contain salient olfactory cues,

assessing whether stable individual olfactory signatures are present, and determining the type of recognition mechanisms through which mothers identify their pups through olfaction.

ACKNOWLEDGEMENTS

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CHAPTER 4

Mother-offspring similarity, age and colony affiliation are reflected in chemical profiles of Australian sea lions

Australian sea lion mother and pup performing olfactory investigations (photo credit: Kaja Wierucka)

Wierucka K, Barthes N, Harcourt R, Schaal B, Charrier I, Pitcher BJ (2018) Mother-offspring similarity, age and colony affiliation are reflected in chemical profiles of Australian sea lions. In review: J Exp Mar Biol Ecol.

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. However, most studies on free-ranging animals are based on behavioural and/or observational data and we lack information about the underlying basis for olfactory recognition. Using gas chromatography-mass spectrometry, we show chemical profile similarities for Australian sea lion (*Neophoca cinerea*) mother-pup pairs. These are not a result of compounds acquired from the immediate environment, but are either genotypic or mother-transferred. Our findings support a chemical basis for phenotype matching, a plausible recognition mechanism for mother-offspring recognition in this species. We also demonstrate that both colony and age differences are encoded within these chemical profiles. The study contributes to understanding how females use odours for selective offspring recognition and provides insight into the contributions of environmental and genotypic factors to this process in a mammalian species.

Keywords: Chemical communication, gas chromatography-mass spectrometry (GC-MS), marine mammals, mother-offspring recognition, olfactory recognition, pinniped.

INTRODUCTION

Chemical communication is the oldest communication channel used by animals and mediates many social interactions among individuals (Bradbury and Vehrencamp 2011). In mammals, olfactory cues play an important role in reproductive behaviours associated with intra-sexual competition, mating and parental care (Brennan and Kendrick 2006). Although the abilities of animals to recognise conspecifics, particularly kin, has been exhaustively researched (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). They involve learning a template, however its source can differ. In direct familiarisation animals associate with another individual and learn its odour that they remember, and use for identification at the next encounter. Phenotype matching (indirect familiarisation) does not require prior contact. Instead, animals match a known template, either from other kin or self (self-referent matching), to a newly encountered odour. Few species have the capability to recognise others possessing an allele in common with themselves without requiring learning. These mechanisms are not mutually exclusive and animals can use them in combination or use different templates in different contexts (Penn and Frommen 2010).

Determining which recognition mechanism is used by animals requires an in-depth analysis of behaviour as well as the chemical composition of odours, especially when dealing with complex scents that contain many compounds (Wyatt 2014). Quantifying the chemical composition of odours assists with determining which mechanisms are available for the animal to use for recognition. For olfactory recognition to occur, the chemical composition of a scent needs to be stable over time, or change at a slower rate than consecutive interactions among animals, to allow for relearning of the template. We expect chemical profiles (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 (Okabe et al. 2012; Corona and Lévy 2015), such as otariids (fur seals and sea lions). Otariid females undergo 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 (Riedman 1990; Renouf 1991). 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. 2009; Pitcher et al. 2010a; Pitcher et al. 2010b; Pitcher et al. 2010c; Pitcher et al. 2012; Wierucka et al. 2017 – Chapter 5; Wierucka et al. 2018 – Chapter 6). 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 profiles 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.

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) and Seal Bay (Kangaroo Island; 35°59'34.8" S, 137°19'4.8" E; 268 pups in 2014/15) in April and October 2016, respectively (Goldsworthy et al. 2015). Pups (2-4 months of age) were sampled and individually marked (fur clipped) during maternal absences, while mothers were identified by association with marked pups (following Pitcher et al. 2009). Mothers were 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 the source of semiochemicals is unknown, and animals seem to use general body odour for recognition, we collected scent samples from the lumbar area of animals. 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 ml 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 (SM 4.1). 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 profile similarity and a 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). Chemical similarity/dissimilarity was visualised using a non-metric multidimensional scaling (NMDS) technique, which was based on the same index as the statistical tests.

RESULTS

Twenty-one mother-pup pairs were sampled for lower back skin secretions (Olive Island n=8, Kangaroo Island n=13). Chemical profiles of mothers and their pups were significantly more similar to each other than other individuals (R=0.226, p=0.031; Figure 4.1). However, we also found a clear difference in composition among adult females and pups (R^2=0.047, p=0.001) and between colonies (R^2=0.108, p=0.001; age:colony R^2=0.063, p=0.001; Figure 4.1).
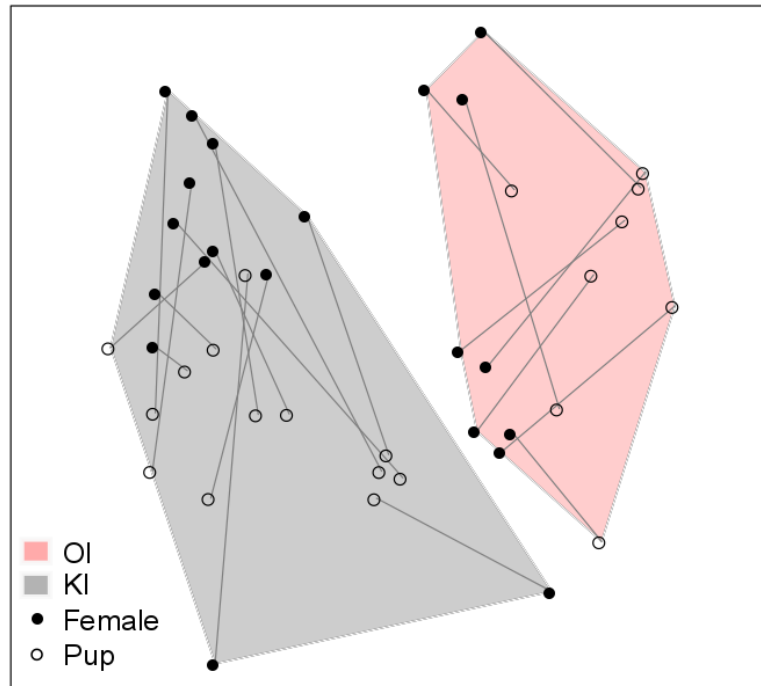
Figure 4.1. Visualisation of Australian sea lion mother-pup pairs' chemical profiles. Points in close proximity represent a higher similarity. Chemical profiles significantly differed by age and colony, while mother-pup pairs were significantly similar to each other. Mother-pup pairs are indicated with connecting lines. KI: Kangaroo Island, OI: Olive Island.

DISCUSSION

Chemical composition of dorsal fur odour was similar for mother-pup pairs, however we also found pronounced age and colony differences, indicating that mother-offspring similarity, as well as broader age/sex class and population affiliation information are all encoded in Australian sea lion body odours. Mother-offspring and within colony similarity seem to be determined by compounds produced on/by the animals rather than those obtained from their immediate environment. Our results demonstrate the presence of a chemical basis for phenotype matching, indicating that this recognition mechanism could be used for olfactory recognition of pups by mothers in this species.

Chemical profiles of pups and mothers were highly similar to each other, indicating that a chemical basis for self-referent phenotype matching exists. Although it does not rule out the possibility of females learning the chemical signature mixture of their pup through direct familiarisation, we show that despite females and pups having different metabolism, diet and behaviour, and occupying a different range of habitats (all of which have can influence the chemical make-up of individuals' body surface; Thom and Hurst 2004), the similarity was retained. Our findings are consistent with Stoffel et al. (2015), who showed mother-offspring similarity and colony differences in chemical profiles of Antarctic fur seals (*Arctocephalus gazella*). *A. gazella* are closely related to the Australian sea lion, with

a similar female foraging/suckling cycle (Doidge and Croxall 1989; Higgins and Gass 1993) and allosuckling being rare in both species (Lunn 1992; Pitcher et al. 2011). Considering they face similar pressures for successful recognition, the same mechanisms may be used in all otariids. However, GC-MS techniques only allow for testing machine detectable differences and we do not have information about which of these compounds are biologically relevant, perceived by the animals and 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.

Compounds used by animals as a template can have various origins (Wyatt 2014). The underlying basis for mother-offspring similarity of odours may be genotypic (specific genes, genotypic similarity correlating with phenotypic similarity) or environmental (e.g., maternal labelling, microbiota transfer, chemical by-products; Halpin 1986; Lévy et al. 2004). Due to chemical compound cross-individual contamination, mother-pup pairs must be sampled on different occasions and following natural separations. In this study mother and pup samples were always collected on different days, yet we found strong similarities in the chemical bouquet within mother-pup pairs. This provides strong grounds for our findings being inherent to the mother-pup pairs rather than local environmental contamination. First, we collected environmental controls within each colony and subtracted those compounds from the samples. Second, female sea lions undergo regular foraging trips (Marlow 1975), and so compounds on the skin or fur are washed off. Third, while mothers are away, the pups remain in the colony (Marlow 1975) playing with other pups and exploring the colony, increasing the likelihood of exposure to multiple environmental chemical compounds from different habitats and climatic conditions. Fourth, when mothers return to the colony they suckle their pups in different locations (Marlow 1975), reducing the chance of repeated exposure to compounds characteristic for a given locality. This also means that mothers were always sampled at a different location within the colony than their pups. Fifth, Australian sea lions do not lick their young (Marlow 1975) and labelling of other sorts has not been reported. Therefore, we suggest the observed similarities are not a result of environmental compounds acquired by animals from their immediate surrounding or labelling, but must reflect intrinsic similarities resulting either from direct genetic relatedness, or from compounds/processes that the offspring acquire from the mother when in contact or through suckling, e.g., microbiota (Archie and Theis 2011).

By contrast, mother-offspring similarity in Antarctic fur seals was shown to be influenced by both genetic and environmental factors, with compounds from the local environment being an important contributor to similarity (Stoffel et al. 2015). In contrast to Australian sea lions, Antarctic fur seals

exhibit high parturition and suckling site-fidelity (Lunn and Boyd 1991), therefore females and their pups would be exposed to similar habitat-derived compounds at the time of sampling.

We found pronounced differences in chemical compound composition among animals inhabiting different populations. We would expect differences among different geographical regions to be influenced by the environment – resulting from varying conditions in the different locations or compounds acquired from the habitat, as was the case for Antarctic fur seals (Stoffel et al. 2015). However, the studied fur seal colonies were genetically indistinguishable. Australian sea lions are philopatric and highly genetically differentiated among colonies (Ahonen et al. 2016). Although this means that both genetic and environmental factors could influence sea lion colony odours, the experimental design was constructed in a way that allowed disentanglement of the two. Control samples that were exposed to the overall 'colony odour' and included a majority of general environmental compounds, were removed from the samples prior to analysis. This, combined with the chemical similarity of mother-offspring pairs, suggests the chemical profiles may have an intrinsic origin (whether genetic and/or microbial community composition) which then plays a role in shaping the distinctive colony odour.

Complementing the within mother-pup and within colony similarity we found distinct separation between pup and female profiles. This implies that some chemical compounds are characteristic of an animals' age and reproductive status. Various differences (metabolic and behavioural) among females and pups might explain these differences, over and above the observed mother-offspring similarities (Thom and Hurst 2004). Animal age and colony may be encoded by different subsets of the chemical profile and future studies should aim to identify the compounds conveying each piece of information.

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CHAPTER 5

The role of visual cues in mother-pup reunions in a colonially breeding mammal

Australian sea lion female searching for her pup (photo credit: Kaja Wierucka)

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ABSTRACT

Parental care is an important factor influencing offspring survival and adult reproductive success in many vertebrates. Parent–offspring recognition ensures care is only directed to filial young, avoiding the costs of misallocated resource transfer. It is essential in colonial mammal species, such as otariids (fur seals and sea lions), in which repeated mother–offspring separations increase the risk of misdirecting maternal effort. Identification of otariid pups by mothers is known to be multi-modal, yet the role of visual cues in this process remains uncertain. We used three-dimensional visual models to investigate the importance of visual cues in maternal recognition of pups in Australian sea lions (*Neophoca cinerea*). We showed that the colour pattern of pup pelage in the absence of any other sensory cues served to attract the attention of females and prompt investigation. Furthermore, females were capable of accurately distinguishing between models imitating the age-class of their own pup and those resembling older or younger age-classes. Our results suggest that visual cues facilitate age-class discrimination of pups by females and so are likely to play an important role in mother–pup reunions and recognition in otariid species.

Keywords: Age recognition, mother–pup reunion, parent–offspring recognition, pinniped, visual cues.

INTRODUCTION

Parental care is an important component of the life history of many animals, influencing offspring survival and consequently adult reproductive success (Royle et al. 2012). To reduce costs, parents should direct care only towards filial young. Consequently, recognition of filial offspring is an essential element of parental care for many species (Royle et al. 2012). Information about offspring identity may be conveyed through various modalities (Hepper 1991; Bradbury and Vehrencamp 2011). While the roles of acoustic and olfactory cues have been well investigated for many mammals, there is considerably less information about the role and accuracy of visual cues in the parent–offspring recognition process. Visual cues appear important in mother–offspring reunions of ungulates and primates (Ruiz-Miranda 1993; Parr and de Waal 1999; Mora-Medina et al. 2016), and are likely to be particularly important for gregarious, colonial species, in which mother–offspring separations are frequent (Royle et al. 2012).

Otariid females and pups face extremely high selection pressures for successful recognition. Throughout lactation, females leave their pups on land while they forage at sea (Renouf 1991). Following each return, they must find and identify their pup in a colony. Furthermore, females are aggressive towards non-filial pups (Harcourt 1992) and allosuckling is rare (Pitcher et al. 2011), making successful reunion necessary for pup survival. Otariid mothers and pups use vocal, and olfactory cues to both localize and recognize individuals (Insley et al. 2003; Pitcher et al. 2010b), but while the ability to use visual cues to discriminate appears to be present (Insley et al. 2003), its role and precision in reunions remains uncertain.

Australian sea lions (*Neophoca cinerea*; ASL) have a 17–18-month long breeding cycle (Ling and Walker 1978) with a prolonged pupping season lasting up to eight months (Ling and Walker 1978). Consequently, pups of different ages co-occur in the colony. Over their first four months, ASL pups undergo significant morphological changes in pelage colour pattern and size. There are three visually distinct age-classes: A1—pups under two months of age, small and black; A2—pups two to four months old, larger with brown to cinnamon colour; A3—post-moult pups, over four months old, very large with silver-beige pelage (SM 5.1; Jefferson et al. 2015), enabling investigation of their role as visual cues in mother–pup recognition and in reunions.

MATERIALS AND METHODS

We investigate the role of visual cues in mother–pup reunions with two experiments. The first experiment, natural versus unnatural (N/UN), tested the response of females to pup models with a

natural or an unnatural pelage colour pattern. The second, age-classes (AC), tested the ability of females to distinguish pup models using age-class-specific pelage patterns. Data were collected on Beagle Island (N/UN) and Olive Island (AC), Australia in 2010 and 2016. For N/UN, we tested 15 adult ASL females with A1 pups. For AC, we used 28 ASL females, 15 with A1 filial pups and 13 with A2 pups.

We constructed three-dimensional visual models of ASL pups. Models used in N/UN were the size and shape of A1 pups and were either black (natural) or white (unnatural brightness). For AC, model pups were constructed to approximate the size, shape and colour brightness of the three visually distinct age-classes (A1, A2 and A3; SM 5.1). Sea lion pup models have been previously used in similar studies and shown to successfully imitate pups (Pitcher et al. 2010b). During presentations, we simultaneously placed two models on the ground, approximately 1 m apart, and 2–4 m in front of a targeted female (SM 5.2, SM 5.3). This distance range is well known to elicit a response from females (Pitcher et al. 2010b). For N/UN, a black (congruent) and a white (incongruent) model were presented to each female. For AC, each female was presented with one model imitating her pup's age-class (congruent model) and one imitating a different age-class pup (incongruent model), thereby providing four possible combinations of models: A1 (congruent) versus A2 (incongruent), n=8; A1 (congruent) versus A3 (incongruent), n=7; A2 (congruent) versus A1 (incongruent), n=7; A2 (congruent) versus A3 (incongruent), n=6. All models were randomly allocated to left or right.

We expected the mothers to act aggressively towards incongruent models, and not aggressively (displaying investigatory behaviour) towards congruent models. To ensure that the principle focus was the females' response to visual cues, we recorded which model was approached first and whether the female was aggressive upon approach. Based on this, we classified each response as appropriate or inappropriate. Appropriate was defined as the female (i) approaching the congruent model first in a non-aggressive manner or (ii) approaching the incongruent model first and exhibiting aggressive behaviour. Inappropriate was defined when the female (i) approached the congruent model first and exhibited aggressive behaviour or (ii) approached the incongruent model first and was not aggressive. An approach was considered as the female moving directly towards a model in a linear manner, stopping before it and exhibiting either an appropriate or an inappropriate response. We defined aggression as the female presenting an open mouth display, producing 'puffs' (i.e. air expulsion through nostrils) and/or biting the model. We tested for significant differences between appropriate and inappropriate responses with an exact binomial test. We later implemented a Fisher's exact test to assess whether differences in success ratios occur between the different combinations of presented models for AC.

Furthermore, for AC following data centring and scaling, we performed a principal component analysis (PCA) that included the number of sniffs, puffs and bites exhibited by the female within 90 seconds of approach and used a Wilcoxon signed-rank test to determine the differences in principal components (PC) between female response to congruent and incongruent models. For N/UN, we compared only the number of sniffs (as no aggression occurred) with a Wilcoxon signed-rank test. All statistical analyses were performed in R v. 3.2.2 (R Core Team 2015).

RESULTS

Natural versus unnatural

Females showed a significant preference for black over white pup models (p<0.001; Table 5.1) demonstrating that visual cues influence female reunion behaviour. Fourteen out of 15 females investigated the black model first and none displayed aggression towards the model they approached first. Females sniffed the congruent model significantly more times than the incongruent (p= 0.001; Figure 5.1a).
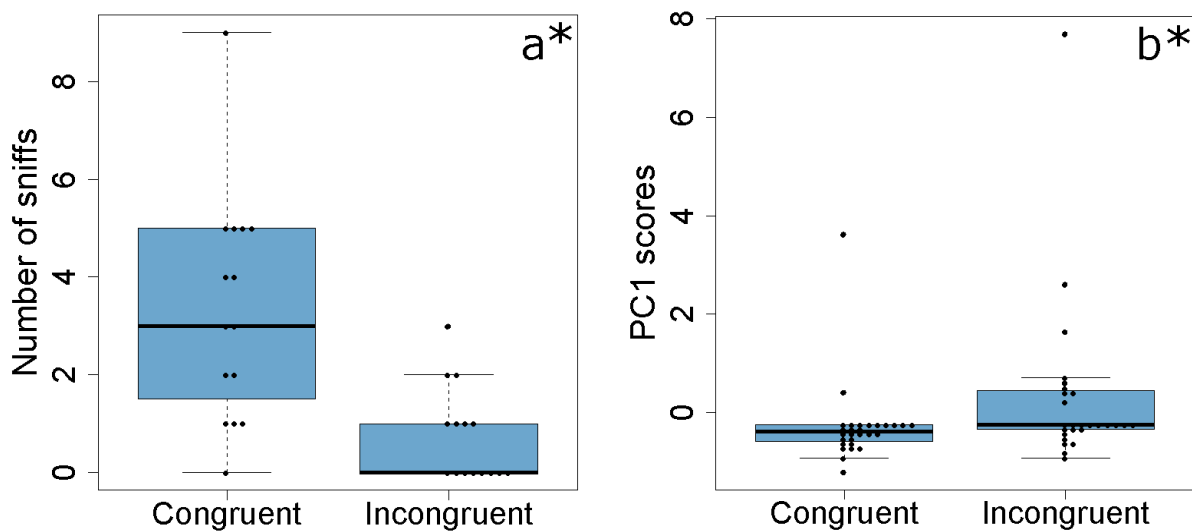

Figure 5.1. Differences in Australian sea lion female response towards congruent and incongruent models. Notations: (a) experiment N/UN, (b) experiment AC. *Statistical significance (p<0.05) determined by a Wilcoxon signed-rank test.

TABLE 5.1. Number of appropriate and inappropriate responses of Australian sea lion females towards congruent and incongruent pup models based on visual cues. Notations: A1/2/3, age-class of pup model; AC, age-classes experiment; N/UN, natural versus unnatural experiment. The p-value is the statistic of an exact binomial test for experiments N/UN and AC, and Fisher's exact test for comparison of different treatments for AC experiment.

Experiment	N success	N failure	N total	F model	NF model	p
N/UN	14	1	15	natural	unnatural	0.001
AC	21	7	28	filial age class	non-filial age class	0.013
AC	5	3	8	A1	A2	
AC	6	1	7	A1	A3	0.705
AC	6	1	7	A2	A1	
AC	4	2	6	A2	A3	

Age-classes

Significantly more ASL females behaved appropriately towards models representing different pup age-classes (i.e. 'appropriate' defined in methods; p=0.01; Table 5.1), confirming that they accurately distinguished the current age-class of their pup based solely on visual cues. Twelve females approached the congruent model first and behaved non-aggressively, while nine females approached the incongruent model first, but showed aggression. All inappropriate responses (n=7) were when females approached the incongruent model first but did not exhibit aggressive behaviours. We did not find any significant differences across the various combinations of presented models (p=0.7; Table 5.1; Figure 5.2) indicating equivalent accuracy of female visual discrimination of pup age-classes, irrespective of filial pup age or level of difference between the age-classes of presented models.
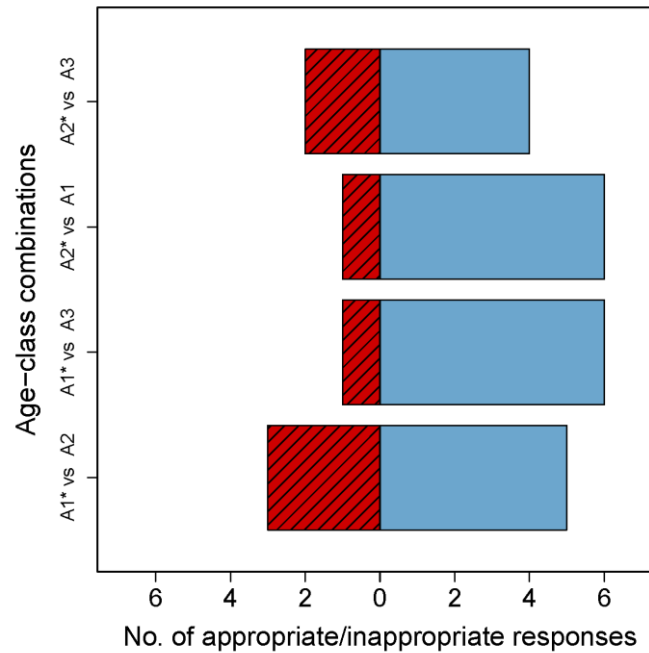
Figure 5.2. Number of appropriate (blue) and inappropriate (red) responses of Australian sea lion females towards pup models representing visually distinctive age-classes. Notations: A1/2/3, age-class represented by pup model. *Congruent model.

The PCA compiled three PCs, with only PC1 having an eigenvalue>1, explaining 57% of the variance in female response (Table 5.2). Aggressive behaviours were correlated with PC1 (puffs=0.68; bites=0.70). There was a higher number of aggressive behaviours exhibited by the females towards incongruent models than congruent models (p=0.006; Figure 5.1b).

Table 5.2. Results of a PCA including the number of sniffs, puffs and bites exhibited by Australian sea lion females towards pup models representing different age-classes. The PCA compiled three PCs, with only PC1 scoring an eigenvalue>1. PC1 was positively correlated with aggressive behaviours and explained 57% of the variance in female response.

	PC1	PC2	PC3
Rotation			
sniff	-0.22	-0.96	0.18
puff	0.68	-0.29	-0.68
bite	0.70	-0.03	0.71
Eigenvalue	1.71	0.99	0.30
Proportion of variance	0.57	0.33	0.10
Cumulative proportion	0.57	0.90	1.00

DISCUSSION

Australian sea lion mothers were more attracted to natural than to unnatural pup pelage, and could accurately differentiate pup age-class visual characteristics in favour of those resembling their own pup, suggesting that visual cues may play an important role in mother–pup reunions and recognition in wild otariids.

Females showed a clear preference towards congruent pup models and most ignored pup models with unnatural pelage colour patterns. While model size and shape were held constant, a change in the pelage completely changed maternal behaviour indicating that it is used in distinguishing pups and is important in attracting the attention of females during mother–pup reunion. Females also clearly recognized their own pup's age-class regardless of which other age-class it was compared against. This suggests that females are aware of the current morphological characteristics of their pup (size and pelage). Given that this will change through the lactation period, females must also have to adjust their recognition of their pup's appearance. The revision of the pup visual template by the female seems to be extremely accurate. Six of seven tested females with A2 pups successfully recognized the correct model when presented simultaneously with A1 models, yet their pup would have presented with A1 visual characteristics only a few weeks prior to the experiment. The ability to distinguish pup age-classes based on visual cues is likely to facilitate mother–pup reunions to the benefit of both. When many pups are present within the same area and aggression by non-mother females is common, the ability to quickly distinguish pups of the appropriate age and appearance not only increases the accuracy and speed of reunion, but also reduces potential harm to pups by non-mother females.

This study builds on earlier research which showed that both acoustic and olfactory cues reliably convey individual identity information of ASL pups (Pitcher et al. 2010a; Pitcher et al. 2010b). Here, we have demonstrated that visual cues also play a role in the recognition process with age-class discrimination. Visual cues thus create an additional tier of recognition, complementing the longer range vocal and shorter range olfactory individual recognition. While the exact contribution and relative importance of each sensory modality is still to be determined, it is likely that visual cues form part of a multimodal communication system in conjunction with vocal and olfactory cues and interact with them (Partan and Marler 1999).

Our study demonstrates behaviourally that ASL mothers can use visual cues derived from the pelage of their pups to discriminate between age-classes. Examination of pinniped visual systems indicate that despite being monochromatic, they might be capable of obtaining some colour information in mesopic light conditions or potentially use differences in brightness or contrast (Hanke et al. 2009).

While it is not possible from our study to determine which particular aspects of visual information ASL mothers are using, we have shown that differences in pelage facilitate differentiation. Further research is needed to identify the parameters used by ASL in these contexts.

Our findings illustrate that visual cues function to attract the attention of females during mother–pup reunions and refine the ability of a female to search for her pup. Their role in individual recognition and their interactions with olfactory and acoustic cues in the recognition process await further investigation.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

SM 5.1. Morphological differences among age-classes of Australian sea lions (*Neophoca cinerea*) pups. Images show three visually distinct age classes of Australian sea lion pups: A1 – pup under 2 months of age, small and black; A2 – pup 2-4 months old, larger with brown to cinnamon colour; A3 – post-moult pup, over 4 months old, very large with silver-beige pelage.

SM 5.2. Presentation of a natural (black) and unnatural (white) model pup to an Australian sea lion female. A female is presented with two pup models, one black (natural visual cues, congruent) and one white (unnatural cues, incongruent). She approaches the models and begins to sniff the black model, sniffs the white model once and then returns to the black model to sniff it again.

Video available at: https://figshare.com/articles/Video_S2_Presentation_of_pup_models_imitating _different_age_classes_to_an_Australian_sea_lion_female_from_The_role_of_visual_cues_in_moth er_pup_reunions_in_a_colonially_breeding_mammal/5573554.

SM 5.3. Presentation of pup models imitating different age-classes to an Australian sea lion female.

Example 1: Non-aggressive response to congruent model

A female is presented with two pup models, one imitating an A2 pup (on the right; congruent), one representing visual characteristics of an A1 pup (left; incongruent). The female approaches the congruent model first, in a non-aggressive manner and investigates (sniffs) it more times than the incongruent model.

Example 2: Aggressive response to incongruent model

A female is presented with two pup models, one imitating an A1 pup (on the right; congruent), the other imitating an A3 pup (left; incongruent). The female approaches the incongruent model and starts biting it and finally grabs and throws the model several meters away.

Pups of the tested females were present at the time of the experiments (visible at the end of the video), however they were at least one body length away from their mother and models were presented on the opposite side of the female than the pup, such that the pup was not in the same field of view as the pup models.

Video available at: https://figshare.com/articles/Video_S1_Presentation_of_a_natural_black_and_ unnatural_white_model_pup_to_an_Australian_sea_lion_female_from_The_role_of_visual_cues_in _mother_pup_reunions_in_a_colonially_breeding_mammal/5573548.

SM 5.4. Raw data used for analysis.
Available from the Dryad Digital Repository at: http://dx.doi.org/10.5061/dryad. qq435.

CHAPTER 6

Visual cues do not enhance sea lion pups' response to multimodal maternal cues

Australian sea lion pup calling to its mother (photo credit: Kaja Wierucka)

ABSTRACT

Mammals use multiple sensory cues for mother-offspring recognition. While the role of single sensory cues has been well studied, we lack information about how multiple cues produced by mothers are integrated by their offspring. Knowing that Australian sea lion (*Neophoca cinerea*) pups recognise their mother's calls, we first tested whether visual cues are used by pups to discriminate between conspecifics of different age classes (adult female vs pup). We then examined if adding a visual stimulus to an acoustic cue enhances vocal responsiveness of Australian sea lion pups, by presenting wild individuals with either a visual cue (female 3D-model), an acoustic cue (mother's call), or both simultaneously, and observing their reaction. We showed that visual cues can be used by pups to distinguish adult females from other individuals, however we found no enhancement effect of these cues on the response in a multimodal scenario. Audio-only cues prompted a similar reaction to audio-visual cues that was significantly stronger than pup response to visual-only cues. Our results suggest that visual cues are dominated by acoustic cues and that pups rely on the latter in mother recognition.

Keywords: Acoustic cues, enhancement, mammal, mother-offspring recognition, multimodal communication, visual cues.

INTRODUCTION

Animal communication can be extremely complex and may use multiple sensory modalities (Otovic and Partan 2010). Due to differences in environmental conditions, cue structure and limitations of sensory systems themselves, the costs and benefits of conveying information through each modality vary (Higham and Hebets 2013). Accordingly, animals often invoke multiple sensory modalities simultaneously, presumably to increase the robustness or diversity of transmitted information (Hebets and Papaj 2005; Partan and Marler 2005; Ay et al. 2007; Bro-Jørgensen 2010; Higham and Hebets 2013; Ratcliffe et al. 2016). Multimodal communication occurs when composite signals or cues are received through more than one sensory channel (Partan and Marler 1999; Hebets and Papaj 2005; Partan and Marler 2005). When multiple cues are present, they may be redundant, i.e. conveying multiple copies of the same information, or non-redundant, i.e. conveying multiple, different messages thereby enabling the transmission of more information (Partan and Marler 1999). Furthermore, when combined, cues may induce different responses from the receiver (Partan and Marler 1999). Multiple redundant cues may elicit either an equivalent or an enhanced response compared to a single cue, and non-redundant cues may be independent, cause dominance or modulation, or lead to the emergence of a new response (Partan and Marler 1999). Because of these interactions, investigating how animals respond to multiple cues simultaneously is necessary, as it provides greater understanding about complex behaviour than simply looking at cues in isolation. However, cues may be used and integrated differently by animals, depending on the interactions between the costs and benefits of obtaining them (Munoz and Blumstein 2012). Given there are limitations for specific cues to convey information (e.g., transmission distance) and costs associated with multisensory signal production and perception, using multiple cues is not always favoured (Munoz and Blumstein 2012). Determining why and when animals use specific combinations of cues, gives insight into the intricacies of multimodal communication. To understand how communication systems evolved and what rules they are governed by, the interactions and relevant importance of sensory cues in a given context need to be explored.

Mother-offspring recognition is known to involve different and usually multiple modalities simultaneously, with acoustic, olfactory and visual cues playing varying roles for different mammalian species (Hepper 1991; Bradbury and Vehrencamp 2011). The ability to identify young by mothers and its reciprocal is usually beneficial to both parties. It allows mothers to direct their care only towards filial offspring thereby enhancing potential reproductive output (Royle et al. 2012). For offspring, identifying their mother may limit the risk of injury caused by approaching non-kin and limit energy wasted in unsuccessful begging attempts (Royle et al. 2012). While extensive literature has

investigated mother-young recognition abilities of many mammalian species (Bachevalier et al. 1985; Insley et al. 2003; Okabe et al. 2012; Corona and Lévy 2015; Guo et al. 2015; Mora-Medina et al. 2016), most studies have investigated only the role of single sensory modalities. Under natural conditions, sensory cues co-occur and will rarely be available for inspection in isolation, yet there is not much information about the combined effect of different cues on the response of the receiver. No wild studies exist, although there is some evidence from domestic and laboratory mammals on the relative importance of individual sensory cues used in concert by mother and offspring sheep (*Ovis aries*; Alexander 1977; Alexander and Shillito 1977; Shillito Walser 1978; Shillito Walser 1980; Shillito Walser and Alexander 1980; Shillito Walser et al. 1981; Nowak 1991; Terrazas et al. 2002), goats (*Capra hircus;* Ruiz-Miranda 1993) mice (*Mus musculus;* Cohen et al. 2011) and rats (*Ratus norvegicus*; Farrell and Alberts 2002). While a vast majority of these studies focus on recognition of the offspring by mothers, it is likely that mothers and offspring utilise cues differently, as apart from discrimination abilities, their motivation and therefore costs and benefits of obtaining cues are very different.

Mother-offspring recognition is especially important for colonial mammals with mobile young and frequent mother-offspring separations occurring due to the mother needing to leave periodically to forage, such as fur seals and sea lions (Renouf 1991). Acoustic, olfactory and visual cues are all used in the mother-pup reunion process (Insley et al. 2003). For different otariid species, a similar pattern of the reunion has been observed – the female and pup call to each other, they look for each other, and when at close range nasal investigations are performed (Renouf 1991). Although observational studies exist for multiple species (Marlow 1975; Gisiner and Schusterman 1991; Dobson and Jouventin 2003; Philips 2003; Trimble and Insley 2010) extensive experimental work about recognition through different sensory modalities has been done only for the Australian sea lion (*Neophoca cinerea*). Previous research demonstrated that both pups and adults produce individually stereotyped calls (Charrier and Harcourt 2006), and females use acoustic (Charrier et al. 2009; Pitcher et al. 2010a; Pitcher et al. 2012), olfactory (Pitcher et al. 2010b) and visual (Wierucka et al. 2017 – Chapter 5) cues to recognise filial pups. Vocal recognition is mutual as pups can accurately distinguish their mother's calls from that of other females (Charrier et al. 2009), yet the onset of this ability is delayed compared to mothers (Pitcher et al. 2009). Although information is available for pup recognition by females, what role non-vocal cues play, and the interaction between cues, in the pups' recognition abilities of mothers remains to be evaluated. Australian sea lions provide a unique opportunity to look at the role of the receivers' costs in shaping recognition systems. The main constraints for the use of multimodal cues are perception and production costs of cues, as well as the risk of increased eavesdropping and therefore higher detection rates by predators (Partan and Marler 2005). This species does not have terrestrial predators and the cost of producing cues is negligible. Therefore, in a situation where cues

are capable of conveying useful information, the only limitations for using multimodal cues are the costs of obtaining, receiving, processing and integrating cues, and their survivorship consequences (Partan and Marler 2005).

In this study we first test whether visual cues can be used by pups to discriminate among conspecifics (adult females vs pups) and then examine whether visual and acoustic cues induce a synergistic effect on the behavioural response of Australian sea lion pups during mother-pup reunion.

MATERIALS AND METHODS

Study site and animals

The use of visual cues by pups (visual experiment) was studied in a wild population of Australian sea lions inhabiting Olive Island (32°43'18.5" S, 133°58'6.3" E) and Kangaroo Island (35°59'34.8" S, 137°19'4.8" E) in April and October 2016, respectively. Experiments examining multimodal cue use by pups (bimodal experiment) were conducted in September-October 2017 on Olive Island. Pups used in both experiments were less than 4 months old and were approached for procedures when mothers were away on foraging trips, to avoid mother-pup separation and thus limit disturbance. Pups used in the bimodal experiment (only 2–4 month olds) were captured and restrained for a short period of time where they were individually marked by clipping a unique symbol into their fur and applying hair dye (Clairol Nice'n Easy©). This allowed us to identify pups at a distance without the need to approach them and to identify their mother in order to record their pup attraction calls.

Sample collection

Pup attraction calls were recorded from mothers of marked pups during interactions with their pups in the colony using a BeyerDynamic M69 TG microphone (frequency response: 50Hz– 16 kHz ± 2.5 dB; BeyerDynamic, Heilbronn, Germany) mounted on a 3 m boom connected to a Marantz PMD 671 digital recorder (Marantz Europe, Eindhoven, Netherlands). Calls were recorded at a 44.1 kHz sampling frequency. Good quality calls (i.e., no background noise and no overlap with other vocalizing animals) were selected and high-pass filtered at 200 Hz using Avisoft SAS Lab Pro (Avisoft Bioacoustics, R. Specht) to remove low frequency noise caused by wind and/or waves. Experimental playback series were composed of six calls separated by 2–3 seconds of silence, similar to a natural calling sequence of a female searching for her pup. The playback series were broadcast using a portable amplified speaker (JBL Flip 3, 2 × 8 W, frequency response: 85 Hz-20 kHz) connected by Bluetooth to an audio

player. Calls were played at an approximately natural amplitude of 83 ± 3 dB SPL measured 1 meter from the source (Charrier et al. 2009; Pitcher et al. 2009).

3D-models imitating an adult female as well as a 1–2 and a 2–4 month old pup were constructed using synthetic fur with polyester filling, and fitted with a wire skeleton to maintain an upright posture (Figure 6.1). To examine the role of class-level visual cues in recognition, the size and fur colour pattern were chosen based on the average body size and colouration of adult females as well as 1–2 and 2–4 month old pups (body length: 156, 76 and 87 cm respectively). As all animals within a given age/sex class appear similar and no information currently exists about whether individual visual recognition is possible in pinnipeds, models approximated the size, shape and colour brightness of the respective age/sex groups. Our pup models have been previously shown successful in imitating animals for research purposes (Wierucka et al. 2017 – Chapter 5).
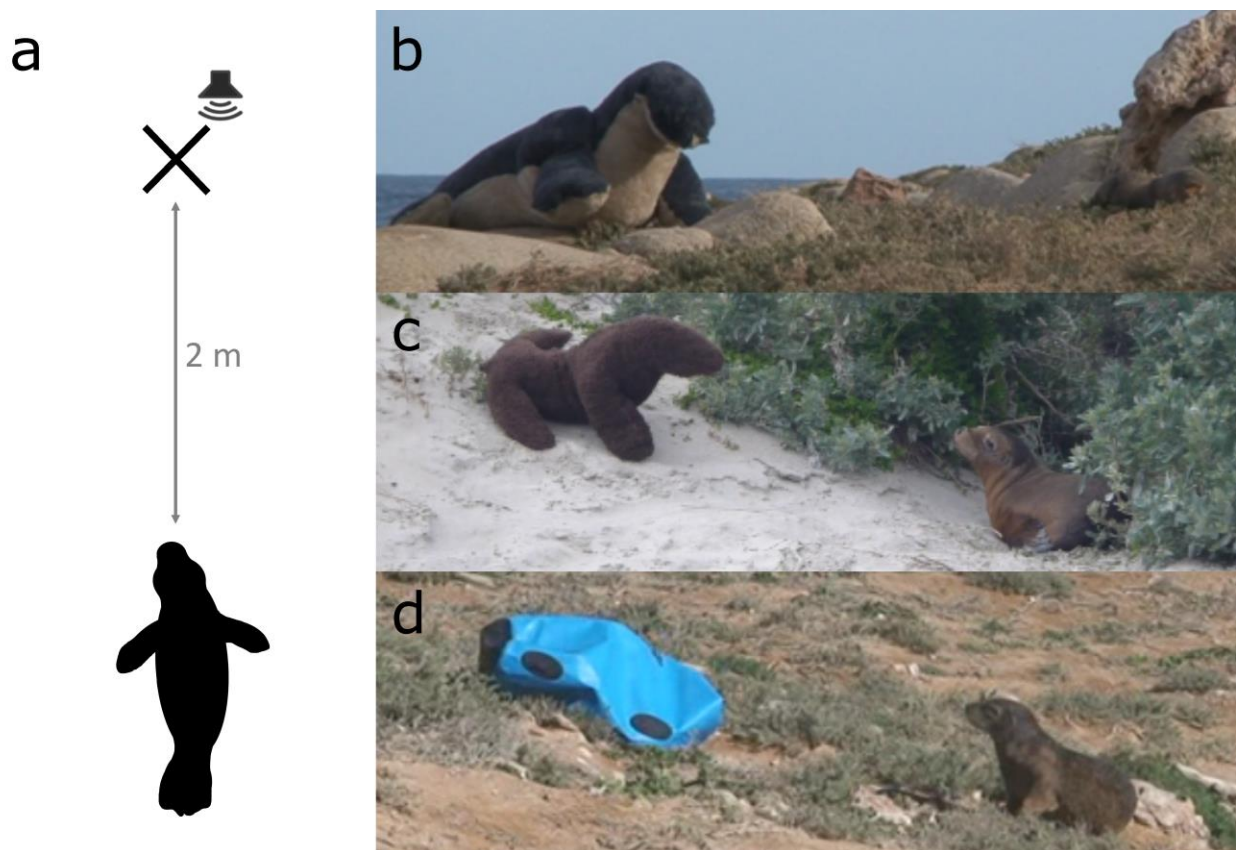

Figure 6.1. Behavioural experiment setup (a). Pups were presented with a stimulus (marked with 'x') – either a female model (b), a pup model (c), or a foreign object (d), placed 2 meters away from the animal, directly in line of sight, facing toward the individual. The speaker (where applicable) was placed adjacent to the model. When the visual model was absent (audio-only cues), the speaker was present in the same location.

Experimental design

In both the visual and bimodal experiments, the stimulus was presented 2 meters away from the pup, facing the pup's head or at least within 45° to ensure a clear visibility of the model presented (Figure 6.1). The models did not contain any sea lion olfactory cues, and were placed beyond the range that olfactory assessment appears to occur (Pitcher et al. 2010b). Objects were placed while the pup was sleeping as to not disrupt, startle or otherwise confound the response of the pup with human presence. The pup was woken up with a natural sound (i.e., a hand clap) immediately prior to presentations.

For the visual experiment, 25 pups were presented with one of three treatments: (1) female model – a life-size model of an adult female sea lion (n=8); (2) pup model – a life-size model of pup of the same age as the tested pup (n=9); (3) control – a foreign object (i.e., a 65 L blue dry bag filled with air; n=8). We expected pups to be vigilant if they identified the presented object as a female as nonrelated females are aggressive towards non-filial pups (Marlow 1972; Marlow 1975; Higgins and Tedman 1990). We also predicted that pups would not change their location and return to their initial behaviour if they identified the presented object as another pup, as pups associate with each other in the colony during maternal foraging trips on a regular basis and pose no threat to each other (Marlow 1975). Therefore, a significant difference in pup response to different models would indicate the use of visual cues for conspecific assessment, whereas no differences would point to the adult female models not being identified as non-mothers, and the possibility that visual cues are not used by pups. Based on this, an ethological scale was created to assess whether the pups could distinguish different categories of conspecifics/items based solely on visual cues. The behaviour of the pup following it looking at the object was scored and two patterns were defined: "return to rest" – when no change in location occurred and the pup returned to its initial resting position following the presentation, and "vigilance" – when the pup moved away from the object, or stayed in the general area without returning to a resting position.

During the bimodal experiment, one of three treatments was presented to 30 pups: (1) audio – pup attraction calls of their mother (n=10); (2) visual – the life-size model of an adult female sea lion (n=10); (3) audio-visual – pup attraction calls of their mother paired with the life-size adult female model (n=10). As we were measuring whether there is an enhancement effect following the addition of the visual cue to the acoustic cue, we noted the number of calls produced by the pup as well as the latency to call (if a call occurred) during 60 seconds after the beginning of each presentation.

Statistical analysis

A Fisher's exact test, with Holm's correction for multiple comparisons was used to assess whether differences in response among treatments occurred in the visual experiments. The number of calls produced by pups among treatments in the bimodal experiment were compared using a Kruskal-Wallis test with a Dunn's post-hoc test (Pohlert 2014). An exact Wilcoxon rank sum test was used to examine differences in latency to call between audio and audio-visual treatments (Hothorn and Hornik 2017). All statistical analyses were performed in R version 3.2.2 (R Core Team 2015).

The research was carried out under the permission of the South Australian Wildlife Ethics Committee (approval 30/2015) and the Department of Environment, Water and Natural Resources (permit E26447). All experimental procedures followed the Australian code of practice for the care and use of animals for scientific purposes. All data analysed during this study are included in this published article (and its Supplementary Information files; SM 6.1).

RESULTS

Pup response varied depending on the presented visual cue (p=0.031). Pairwise comparisons showed that this was due to a significant difference between pup responses to the female and pup models (female model vs pup model: p=0.046, control vs pup model: p=0.262; control vs female model: p=0.608, Figure 6.2). Eight out of nine pups presented with a pup model returned to a resting state after looking at the object. In contrast, six out of eight pups that were shown the female model responded to the treatment with vigilance. Pup response to the control varied, with half of the tested pups returning to a resting position and half staying alert or moving away from the object.
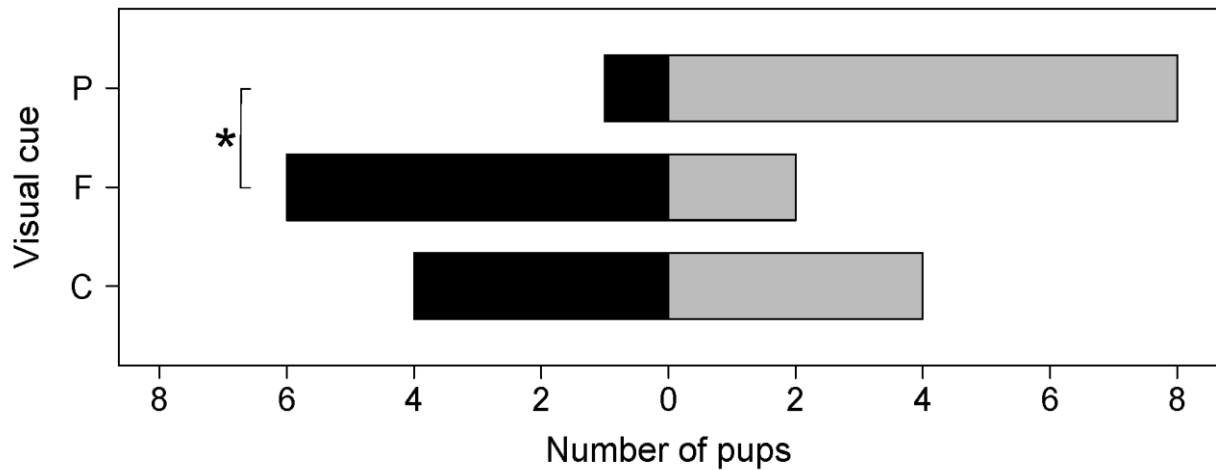
Figure 6.2. Number of pups returning to a resting state (grey) and displaying vigilance (black) in response to different presented visual stimuli. Notations: P – pup model, F – female model, C – control. The asterisk indicates statistically significant differences (p=0.046) among treatments.

When exploring bimodal cue use, we found significant differences in the number of calls produced among treatments (χ2=14.72, df=2, p=0.0006; Figure 6.3). The audio and audio-visual presentations elicited a statistically similar response that was significantly different from that exhibited during visual-only treatments (Dunn's test: visual vs audio: p=0.0007; visual vs audio-visual: p=0.01; audio vs audio-visual: p=0.35). Seven out of ten pups produced calls following audio-visual presentations, nine out of ten pups exposed to acoustic-only presentations responded vocally to the playback, and none of the animals presented with just the visual treatment produced calls. Furthermore, we found no significant differences in the latency to call between audio-only and audio-visual presentations (W=30, p=0.95, n=16; Figure 6.4).
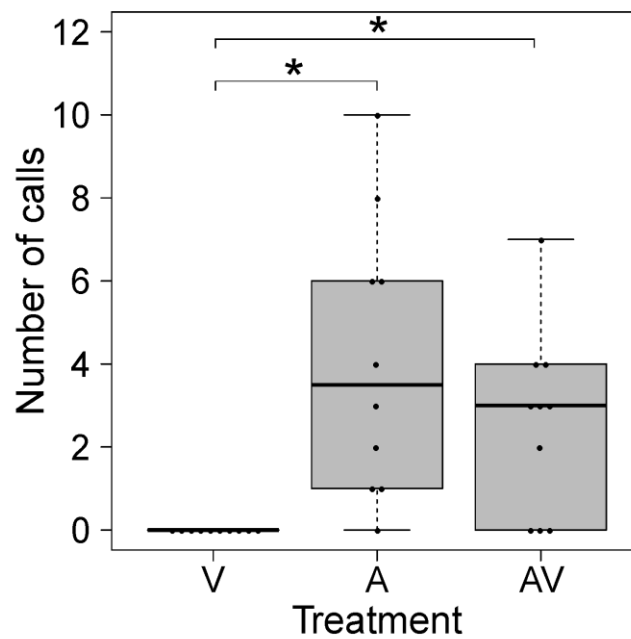
Figure 6.3. Number of calls produced by pups during visual-only (V), audio-only (A) and audio-visual (AV) treatments. Boxplots show the median, quartiles and minimum and maximum values within the inter-quartile range. Asterisks indicate statistically significant differences (V vs A p=0.0007, V vs AV p=0.01) among treatments.
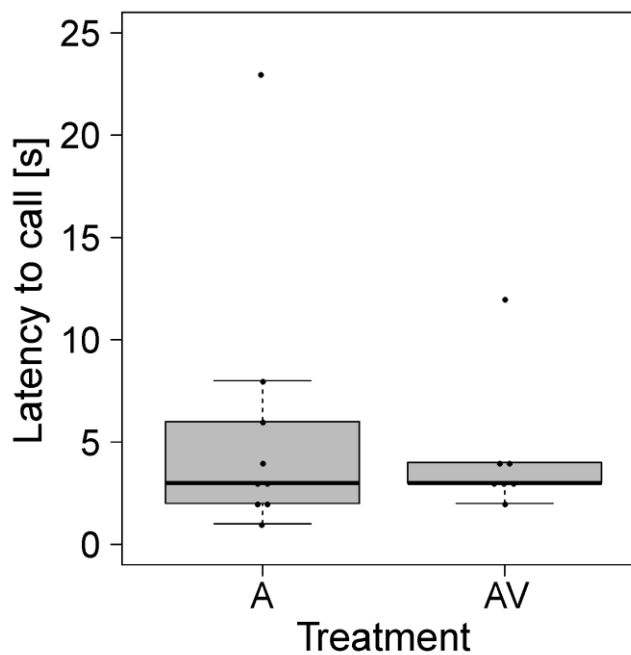

Figure 6.4. Pup latency to call during audio-only (A) and audio-visual (AV) treatments. Boxplots show the median, quartiles, and minimum and maximum values within the inter-quartile range. No significant difference was found between treatments.

DISCUSSION

We showed that visual cues can be used by sea lion pups to distinguish between pups and adult females. However, we found no enhancement of the pups' response in the presence of multiple cues, with combined audio-visual cues having the same effect as audio-only presentations, and both prompting a stronger response than the visual-only treatment. Our findings demonstrate that class-level visual cues (i.e., indicating an adult female) are dominated by acoustic cues, and are not used by pups to facilitate reunion, with offspring relying mainly on information conveyed in acoustic cues for mother recognition.

Australian sea lion pups showed pronounced differences in response when presented with a range of objects that either simulated conspecifics or were not biologically relevant and were able to distinguish adult female morphs from pup morphs based solely on visual cues. Pups displayed vigilance when presented with female models, but returned to a resting state following pup model presentations. When presented with the control which had no biologically relevant cues, but which was novel, there was no distinct response, with pups either responding with vigilance or without in equal measure. Overall this clearly demonstrated that pups can visually distinguish broad age classes of conspecifics. Our visual models were indicative of an adult female and did not provide any cues that might be used for individual recognition. Yet overwhelmingly (75%) pups responded as they would to an unfamiliar female, with vigilance. Our experiment is the first step towards understanding information perceived by pups through visual cues. Although we were unable to test individual visual recognition, we demonstrate that visual cues provide a broad assessment of animals at least to a given sex/age class and to the presence/absence of an animal in close proximity to the receiver.

Being able to identify the correct age/sex class of an individual using broad-brush cues may be beneficial when attempting to find a specific individual within a colony, as it refines the search to a subset of animals. Australian sea lion pups produce more calls in response to calls of their mothers compared to that of other females (Pitcher et al. 2009). We thus expected them to further increase call rates once a potential mother is within sight, as it would allow them limit energetic expenditure by increasing call rates only when chances of reunion are higher or decrease call rates if the model was visually identified as being non-mother. However, pups tested in our study showed no enhancement in behavioural response when presented with multimodal cues compared to unimodal ones. Pups produced a similar number of calls to the audio/visual stimulus as to the audio-only stimulus, with no vocal reaction to the visual-only cues. The absence of enhancement points to a lack of interaction between acoustic and visual cues and the pups' lack of use of class-level visual cues

when identifying their mothers. Based on the response of pups to female models when testing the role of visual cues, we ruled out the possibility that the absence of enhancement was simply a result of the pups identifying the female models as non-mothers. In our experiment, pups showed vigilance when presented with female models. If the model was identified as a non-mother in the bimodal experiments, we would have expected a decrease in call production, which was not the case. Ruiz-Miranda (1993) suggested that for goat kids, visual cues are more important than acoustic and olfactory cues. Only broad cues were tested (pelage colour) while acoustic cues were individually distinctive and olfactory cues were masked. Although the tested visual cues contained only broad information, they were of higher importance than individually distinctive acoustic cues, thus showing that even when broad, visual cues have the ability to induce increased response. In our study, adding the visual cue did not change the pups' response, therefore the most parsimonious explanation is that while pups are capable of differentiating classes of individuals based on visual cues, they do not use class-level visual cues in a multimodal context, suggesting the presence of other factors that limit the use of both cues simultaneously.

The active space of cues varies as a function of the characteristics of a given cue, its production and perception, as well as the environment through which it travels (Rosenthal and Ryan 2000; Higham and Hebets 2013; Uy and Safran 2013). Acoustic cues are generally considered to function at long range and visual cues are classified as mid to short range cues (Uy and Safran 2013). Differences in cue active space are regarded to be one of the main factors favouring multimodal communication (Uy and Safran 2013). However, for otariid pups, the differences in active space of sensory cues are important in context of risk of injury, as females can be extremely aggressive towards non-filial pups that approach them (Marlow 1972; Higgins and Tedman 1990; Harcourt 1992). In this case, differences in active space could limit the use of multiple modalities, as cues with a smaller range may require pups to come out of hiding and become exposed to getting attacked or trampled by other individuals, or if they approach an individual to obtain useful information it may put them at risk of injury. Consequently, it seems that pups rely on hearing – the one modality that allows them to acquire accurate and reliable information at long range (Charrier et al. 2009; Pitcher et al. 2012) for the assessment of female identity prior to approach.

Munoz and Blumstein (Munoz and Blumstein 2012) proposed a framework within which there is a plausible explanation for the evolution of bimodal responses, from the cost-benefit perspective of the receiver. The authors define three predictions for multisensory integration: enhancement – when the costs of missing information are high and outweigh the costs of obtaining cues; antagonism – when combined cues point to a lower likelihood of an event; and equivalence/dominance – when obtaining

more information is too costly and therefore multimodal cues are not used (Munoz and Blumstein 2012). This framework may help explain why pups do not combine acoustic and visual cues, and the evolutionary significance of this choice. We found the pup response to the bimodal and acoustic presentations to be the same or higher than the response to visual-only cues, and from our visual experiment we know that visual cues can be used in age-class conspecific assessment. Accordingly, our results fit the equivalence/dominance scenario, suggesting that acquiring information conveyed in visual cues does not outweigh the cost of obtaining them. This might be due to the risks of obtaining useful information being high or to the information encoded within them not providing any more useful information than the acoustic cues. The evolutionary pressures and mechanisms for this scenario to evolve could be investigated in more detail. However, regardless of which explanation plays a larger role, our findings indicate that the costs associated with obtaining information limit the use of multimodal cues in mother recognition by pups, with the characteristics of female-pup interactions as well as the consequences of differences in cue active space discussed above, also supporting this argument.

We have demonstrated that although Australian sea lion pups have the ability to use visual cues for conspecific assessment, they are not used in a multimodal context and are dominated by acoustic cues. By allowing the offspring to obtain detailed information at a distance, the use of acoustic cues does not entail a risk of injury from non-mother females and provides a stable and reliable way of mother identification on their own. Although reliance on a single modality may be disadvantageous (Partan and Marler 1999), we show that using cues in a multimodal context is not always beneficial, even when the risk of increased predation caused by eavesdropping and cue production costs are low or absent. The cost-benefit ratio of obtaining information seem to play a significant role in limiting the use of multimodal cues and this role in the evolution of communication systems should be examined in more detail.

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SUPPLEMENTARY MATERIALS

SM 6.1. Raw data used for analyses.
Available at: https://www.nature.com/articles/s41598-018-28171-w#Sec9.

CHAPTER 7

Multimodal mother-offspring recognition – the relative importance of acoustic, visual and olfactory cues in a colonial mammal

Australian sea lion mother and pup reunited after the female's foraging trip (photo credit: Kaja Wierucka)

ABSTRACT

Animals often employ multiple sensory modalities for communication and recognition, however, the combination of sensory cues used by individuals in given contexts will vary. Although mother-offspring recognition has been widely investigated and is known to be a multimodal process in gregarious mammal species, there is a dearth of information about the interactions among various sensory cues. Here we show how acoustic, olfactory and visual cues are used in a synergistic way in Australian sea lion (*Neophoca cinerea*) mother-offspring recognition. We interpret the results using a cost-benefit perspective to disentangle the evolutionary pressures on each component of this communication system. Although olfactory cues have the ability to convey individual identity information, it was their presence, not their congruency that prompted female sniffs. We found that pup calls needed to be filial for the identification process to be successful, with the information encoded in acoustic cues overriding that of olfactory cues. Despite each sensory cue accurately conveying identity information when presented in isolation, in a multimodal setting their importance, function or role may change and seems to be driven by the costs and benefits of obtaining information resulting from the constraints imposed by the active space of cues.

Keywords: Individual recognition, maternal behaviour, mother-offspring cues, multimodal communication, pinniped, sensory channels.

INTRODUCTION

Communication is essential for almost all social interactions between animals, and individuals will use different sensory cues to convey information to others (Higham and Hebets 2013). Which sensory modalities are used in the transmission of information in a given context will vary depending on the environment, the physical characteristics of sensory cues and the production and perception limitations of the animals (Higham and Hebets 2013). All of these elements determine the active space of cues – the range within which a cue can provide functionally relevant information to the animal. Visual cues are almost instantaneous, yet as they can be easily obstructed by the surrounding habitat and require the receiver of the information to be facing the sender, they are considered mid-range cues (Rosenthal and Ryan 2000; Bradbury and Vehrencamp 2011). Olfactory cues propagate through diffusion of chemicals in the environment and thus are considerably slower and have a very short range (Bradbury and Vehrencamp 2011). However, they have the ability to persist in the environment longer (Bradbury and Vehrencamp 2011). Although being prone to masking and degradation while traveling through the environment, acoustic cues propagate beyond visual barriers and better than olfactory cues and thus are typically used by animals to communicate over large distances (Wiley and Richards 1983; Bradbury and Vehrencamp 2011; Yorzinski 2017).

Differences among cues and the restrictions they impose on the transfer of information are suggested to favour the simultaneous use of multiple sensory channels (Rosenthal and Ryan 2000; Higham and Hebets 2013; Uy and Safran 2013). Multimodal communication is achieved when composite cues are received through more than one sensory modality (Partan and Marler 1999; Hebets and Papaj 2005; Partan and Marler 2005), and may be employed to increase the robustness of a message (i.e., redundant cues) or to allow the provision of more detailed information to the receiver (i.e., non-redundant cues; Hebets and Papaj 2005; Partan and Marler 2005; Ay et al. 2007; Bro-Jørgensen 2010; Higham and Hebets 2013; Ratcliffe et al. 2016). The simultaneous presence of multiple cues may result in interactions among them which may then affect the receivers' response (Partan and Marler 1999). Redundant cues can be equivalent, prompting the same response in the receiver as each unimodal cue, or result in the enhancement of the response to levels greater than each unimodal cue alone. Non-redundant cues may be independent – individually, each cue elicits a unique response, and when presented together all responses are observed. One cue may override the other, in which case we will see only the response to the dominant cue. Non-redundant cues are capable of interacting, resulting in the modulation of the receiver's behaviour, causing an enhancement or suppression of one response when another cue is present. Finally, some cues have the ability to elicit the emergence of a completely new response, different than the one induced by

each cue separately (Partan and Marler 1999). Exploring the intricacies of these relationships and the relative importance of cues in a multimodal context provides an understanding of the evolutionary pressures that promote their occurrence (Candolin 2003; Hebets and Papaj 2005; Ratcliffe et al. 2016; Hebets et al. 2016).

For an individual, using multisensory cues may have benefits, however encoding information within multiple cues is also associated with potential costs such as higher energetic expenditure, increased exposure to predation/injury as a result of eavesdropping or decreased vigilance to surroundings due to directing attention towards communication (Hebets and Papaj 2005; Partan and Marler 2005; Ay et al. 2007; Bro-Jørgensen 2010; Higham and Hebets 2013; Ratcliffe et al. 2016). Therefore, it may not always be beneficial to employ multiple modalities simultaneously (Partan and Marler 2005; Munoz and Blumstein 2012). Furthermore, although each sensory cue has constraints on its use, the consequences or costs of the same limitations may vary for different parties involved in the exchange of information (Schaefer and Ruxton 2015). Thus, to understand communication systems and the evolutionary pressures for their development, it is important to examine the multiple sensory stimuli involved in what appears a putative multimodal signal, while taking into account the costs and benefits for both the sender and the receiver (Hebets and Papaj 2005).

While studies on multimodal communication and cue integration on invertebrates have produced some great results, vertebrate research has focussed on anurans, birds and lizards, with mammalian experiments severely underrepresented (Hebets 2011; Higham and Hebets 2013; Starnberger et al. 2014; Hebets et al. 2016). With ethical considerations and the complexity of behaviours and factors influencing them, mammals are challenging to study. However, mother-offspring recognition provides an excellent model system for investigating multimodal communication and interactions between the sender and receiver. Mother-offspring recognition is known to involve different sensory modalities, with acoustic, olfactory and visual cues playing various roles in mammals (Hepper 1991; Bradbury and Vehrencamp 2011). Furthermore, the selective pressures for offspring recognition are high. In most mammal species, the ability to recognise offspring is beneficial to mothers as it allows the provision of costly resources only to filial young (Hepper 1991; Royle et al. 2012). Utilising multimodal cues has the potential to increase the amount of information received about identity and decrease the likelihood of misidentification (Hebets and Papaj 2005; Partan and Marler 2005; Ay et al. 2007; Bro-Jørgensen 2010; Higham and Hebets 2013; Ratcliffe et al. 2016). The ability of different sensory cues to provide mothers with specific identity information about their young has been relatively well explored in the past (for reviews see: Deecke 2006; Corona and Lévy 2015;

Mora-Medina et al. 2016). However, most studies have investigated cues in isolation and ignored possible interactions that may arise when multiple cues are simultaneously present.

In attempting to discern the function and information conveyed by each modality in systems where cues naturally occur in tandem and have the potential to interact, the first step is to determine the relative importance of each of the sensory cues. Despite mother-offspring recognition providing an excellent opportunity to investigate this, and calls for more empirical research on multimodal cue use and integration (Hebets 2011; Smith and Evans 2013; Starnberger et al. 2014; Hebets et al. 2016), few studies have attempted this in the past on domestic/lab species, and no one recently has tried to parse out the different roles and interactions of cues. In rodents, olfactory cues have been shown to enhance female response to acoustic cues in both rats (*Rattus norvegicus;* Farrell and Alberts 2002) and mice (*Mus musculus*; Cohen et al. 2011). By contrast Ruiz-Miranda (1993) suggested that in goats (*Capra hircus*), visual cues are more important than acoustic and olfactory cues for the mother's recognition by kids. Similarly, in sheep (*Ovis aries*), the presence of acoustic, visual and olfactory cues have been shown to all contribute to lamb recognition by the ewe, yet visual cues play a more important role than acoustic cues (Alexander 1977; Alexander and Shillito 1977; Shillito Walser 1978; Shillito Walser and Alexander 1980; Alexander and Stevens 1981). For these ungulates, olfactory cues are effective only at close range, and their importance declines as the lambs get older (Alexander and Shillito 1978; Shillito Walser and Alexander 1980). All of these multimodal studies on mammals provide useful insights, yet they have focussed on domestic species. Domestication may alter many aspects of behaviour, often in ways not readily apparent (Price 1999) and so while the mother-recognition process may be unaltered, this remains in question unless wild animals are also investigated. We suggest that in order to fully understand the processes underlying mother-offspring recognition and the reason for specific cue integration, we need to explore the topic in free-ranging wild animals performing behaviours in natural conditions, and only then consider different factors influencing cue use by individuals.

Breeding colonies of otariids (fur seals and sea lions) provide excellent opportunities to investigate the relative importance of sensory cues in mother-offspring recognition. Being colonial breeders with mobile young and frequent mother-offspring separations, the selective pressures for mother-pup recognition are high (Renouf 1991). Furthermore, acoustic, olfactory and visual cues are all involved in the process (Insley et al. 2003). Previous research on Australian sea lions (*Neophoca cinerea*) has shown that females and pups produce individually distinct calls (Charrier and Harcourt 2006). Females are capable of recognising filial pups based solely on acoustic (Pitcher et al. 2010a; Pitcher et al. 2012), as well as olfactory cues (Pitcher et al. 2010c). Pups have been shown to distinguish their mothers calls

(Charrier et al. 2009; Pitcher et al. 2009; Pitcher et al. 2010b) and rely solely on this acoustic cue during mother-pup reunions, with visual cues not influencing their response (Wierucka et al. 2018 – Chapter 6). Visual cues prompt investigations and allow adult females to distinguish pup age-classes, facilitating reunion (Wierucka et al. 2017 – Chapter 5). Understanding that information is conveyed by cues in isolation allows for further exploration of the intricacies of their interactions during multimodal communication.

Examining Australian sea lion recognition provides an opportunity to examine the receiver's role in shaping communication systems. Two main limitations for the use of multimodal cues have been identified: high costs of perception or production of cues, as well as increased risk of eavesdropping resulting in higher probability of predation (Partan and Marler 2005). However, Australian sea lions do not have terrestrial predators. Furthermore, the costs of producing cues are relatively low and consist mainly of production costs of vocalisations (as olfactory and visual cues require simply the presence of an animal). Therefore, the main factor limiting or altering the use of cues would likely be the direct and indirect costs of obtaining, receiving, processing and integrating cues (Partan and Marler 2005). Here, we examine if acoustic, olfactory and visual cues are used in a synergistic way to permit accurate mother-offspring recognition, by first determining whether the presence of multiple sensory cues enhances the females' response, and then testing the relative importance of sensory cues in the pup recognition process.

MATERIALS AND METHODS

Study site and animals

The data were collected in a wild population of Australian sea lions inhabiting Kangaroo Island, South Australia (35°59'34.8" S, 137°19'4.8" E) during the 2016 breeding season. Thirty mother-pup pairs (pups aged 2-4 months; pup age was determined based on size and age-specific pelage characteristics) were used for experiments. Pups were captured and restrained for a short period of time while they were individually marked by clipping a unique symbol into their fur and applying hair dye (Clairol Nice'n Easy©; following Pitcher et al. 2009). This allowed us to identify pups in order to record their calls and to identify and test their mothers.

Sample collection

Acoustic, visual and olfactory cues were collected prior to the experiments. A visual model designed to imitate the size, shape and colour of a 2-4 month old pup was created out of synthetic fur and was fitted with a polyester filling and wire skeleton to maintain an upright position so as to better resemble a live pup (Figure 7.1). Responses by female sea lions to these models in previous investigations suggest that they do represent an accurate visual representation of pups to adult females (Wierucka et al. 2017 – Chapter 5).

Figure 7.1. Behavioural experiment setup. Females were presented with a visual model representing their filial pup that was fitted with a swab containing olfactory cues (where applicable). The speaker (where applicable) was placed adjacent to the model. Here, the female is seen sniffing the model. Filial pups were present during the experiment, but away from their mother and not in the same field of view as the pup model when it was presented. The figure also illustrates the typical mix of vegetation and open space that limits the active space of visual cues during mother-offspring reunion.

Female attraction calls produced by pups were collected using a Marantz PMD 661 digital recorder (Marantz Europe, Eindhoven, Netherlands) connected to a BeyerDynamic M69 TG microphone (frequency response: 50 Hz-16 kHz ± 2.5 dB; BeyerDynamic, Heilbronn, Germany) and recorded at a 44.1 kHz sampling frequency. Good quality calls (i.e., low background noise and no overlap with other vocalising animals) were selected and high-pass filtered at 200 Hz using Avisoft SAS Lab Pro (Avisoft Bioacoustics, R. Specht) to remove low-frequency noise caused by wind and/or waves. One playback series consisted of three calls separated by three seconds of silence. The playback series were

broadcast using a portable amplified speaker (Western Rivers Nite Stalker, Midway USA, MO, USA) and calls were played at a natural amplitude level (83 ± 3 dB SPL measured 1 m from the source).

Olfactory samples were obtained during routine chipping of pups in the colony. Using methods outlined by Pitcher et al. (2010c) we prepared cotton swabs (i.e., two pieces of black cotton flannelette stitched to form a pocket shaped to fit over the head of the model) that were firmly rubbed on the head of pups collecting saliva, nasal mucus, ocular fluids and skin secretions of the pups. The samples were then placed in sterile bags (Nasco Whirl-Pak®) and frozen at -20°C until used in the experiments. Samples were unfrozen in ambient temperature for 20 minutes prior to the beginning of the presentations. For the experiments, swabs were put on the head of the visual pup model (Figure 7.1), separated from it by a poly-ethylene bag to avoid odour transfer between the swab and the pup model.

Method validation: freezing samples

Freezing samples containing odorants prior to behavioural presentations has been a method used in the past (Nilsson et al. 2014; Owen et al. 2015; Gil et al. 2017). However, as compound composition varies among species, it is difficult to determine whether compounds conveying identity information degrade with time and/or in low temperatures. To ensure that olfactory samples were still viable after freezing, we replicated the experiment conducted by Pitcher et al. (2010c) that investigated whether females are capable of distinguishing between the odour of their own pup and those from a nonfilial pup within the same colony. Females were presented with a choice test – two identical pup visual models were placed in front of the female, one was scented with the female's filial pup odour, while the other was marked with the odour of a different pup from the same colony (for details see Pitcher et al. 2010c). The number of sniffs performed by tested females towards the two pup models presented was recorded. Experiments were conducted in the same colony as in Pitcher et al. (2010c) and the only variation to the original experiment was that scent samples were collected 2-3 days prior to presentation (while the pup was alone during the mother's foraging trip) and frozen at -20°C until used.

Enhancement effect of cues

In the first experiment (Ex1), we tested whether the simultaneous presence of an increased number of cues originating from different sensory modalities would enhance the behavioural response of the females. The order of cue addition for simultaneous presentation was based on the natural course of mother-offspring reunion. When mothers return from their foraging trips, they begin calling and

actively look for pups, but the final olfactory check only occurs at a close range through naso-nasal contacts (Bowen 1991; Insley et al. 2003; Pitcher et al. 2010c). Ten females were each presented with three treatments, differing in the number of filial cues presented: 1) the acoustic cue alone (A); 2) the acoustic and the visual cue (A+V); or 3) the acoustic, visual and olfactory cues (A+V+O).

Relative importance of cues

In the second experiment (Ex2), we tested the relative importance of acoustic and olfactory cues in the individual identification process. As visual cues have not been experimentally tested to determine whether they alone allow for individual recognition, we kept this cue constant in all of our presentations. We used a pup model whose visual characteristics were congruent with the filial pups' age-class in all treatments and only altered the identity of acoustic and olfactory cues (both of which convey individual identity information (Charrier and Harcourt 2006; Charrier et al. 2009; Pitcher et al. 2009; Pitcher et al. 2010a; Pitcher et al. 2010c; Pitcher et al. 2012)) in all possible combinations. We presented four treatments to each of the 20 target females: 1) both filial acoustic and olfactory cues (A_fO_f); 2) filial acoustic cue and nonfilial olfactory cue (A_fO_{nf}); 3) nonfilial acoustic cue and filial olfactory cue ($A_{nf}O_f$); 4) both cues nonfilial ($A_{nf}O_{nf}$).

The order of treatments presented to females was balanced. Cues were presented simultaneously, 1-2 meters away from resting females (Figure 7.1). We incorporated breaks between treatment presentation of a minimum of 20 minutes and waited until the females returned to a resting state. To minimise disturbance and avoid separating mother-pup pairs, pups of the target females were present at the time of the experiments. However, we tested the females while pups were at least one body length away from their mother and pup models were presented on the opposite side of the female to the pup, so that the pup was not in the same field of view as the model (following Wierucka et al. 2017 – Chapter 5; Figure 7.1), limiting visual as well as olfactory cues that might be originating from the female's real pup. If the filial pup called during the experiment, or the female sniffed her pup, all behaviours after these interactions were not included in the analysis. All presentations were video recorded for later behavioural scoring and statistical analysis.

Behavioural responses

The females' behavioural response was recorded from the video files. For both experiments, we analysed the response of females to each treatment for 60 seconds following the beginning of the experiment. The number of sniffs and calls produced by tested females were scored by an observer blind to the treatments. We considered female sniffs and calls as indicators of recognition based on

the natural reunion process (Marlow 1975; Riedman 1990; Renouf 1991), following previous studies on communication and recognition in offspring recognition by the mother in the species (Pitcher et al. 2010a; Pitcher et al. 2010c). Half of the videos of Ex2 and all of Ex1 were scored by a second observer (blind to the treatments) to determine whether inter-observer scores were consistent.

Statistical analyses

Due to small sample sizes, and wanting to confirm the results were consistent regardless of the statistical approach, we analysed the results of Ex 1 in two different ways: 1) the Friedman's test with a Conover post-hoc test (Pohlert 2014), and 2) following a rank transformation of data, a repeated measures analysis of variance (R package nlme; Pinheiro et al. 2018) with a multiple comparison of means (R package multcomp; Hothorn et al. 2008) was used to obtain pairwise comparisons of treatments. In both cases, the individual identity was considered the random effect, to account for individual variability in the response. The differences in female response among treatments in Ex2 were analysed by implementing a mixed effects generalised linear model (R package lme4 and glmmTMB; Bates et al. 2015; Brooks et al. 2017; Magnusson et al. 2017). To look at differences in female response to treatments, we set the treatment as a fixed effect and considered individual identity as a random effect. As our data was overdispersed, we used negative binomial distributions (family='nbinom1') in our models. Furthermore, sniffs were zero-inflated, and therefore required the use of a zero-inflated model (ziformula=~1) for this response variable. A multiple comparison of means (R package multcomp; Hothorn et al. 2008) was used to obtain pairwise comparisons of treatments. To confirm whether there is an interaction between the olfactory and acoustic cues, we ran another set of models with the treatment encoded as two separate fixed variables: olfactory cue and acoustic cue, each with two levels: filial and nonfilial, whilst keeping individual identity as a random effect. All statistical analyses were performed in R version 3.2.2 (R Core Team 2015).

RESULTS

Method validation: freezing samples

Out of ten attempted trials with frozen olfactory samples, only six females were presented with cues, as four females moved away making it impossible to conduct the experiment. However, for these six successful trials we found that 5/6 females sniffed the filial model more frequently than the nonfilial one on approach, which was a similar response to the 9/10 reported by Pitcher et al. (2010c) where non-frozen olfactory samples were used. These positive responses, in combination with evidence from

a chemical analysis study with a closely related species showing that volatile compounds were indeed still present following freezing (Stoffel et al. 2015), and other behavioural studies conducted using frozen olfactory cues (Nilsson et al. 2014; Owen et al. 2015; Gil et al. 2017) provides us with confidence that females in our study were detecting volatile compounds from our swabs.

Enhancement effect of cues

When measuring the difference in responsiveness towards presentations including one, two or three sensory cues simultaneously, we found a clear enhancement effect of the presence of multiple cues on the number of sniffs presented by the female (Friedman: χ^2=7.05, p=0.029; ANOVA: F=4.719, p=0.023; Figure 7.2 I), with A+V+O prompting a stronger response from the females than other treatments (Conover post-hoc test: A+V+O vs A+V: p=0.004; A+V+O vs A: p=0.0002; A vs A+V: p=0.120; multiple comparison ANOVA: A+V+O vs A+V: p=0.118; A+V+O vs A: p=0.007; A vs A+V: p=0.547). There was no enhancement effect in the vocal response. Although the number of calls increased when visual cues were added to acoustic cues, the difference was not significant (ANOVA: F=2.896, p=0.0812; Friedman: χ^2=4.778, p=0.092; Figure 7.2 II).
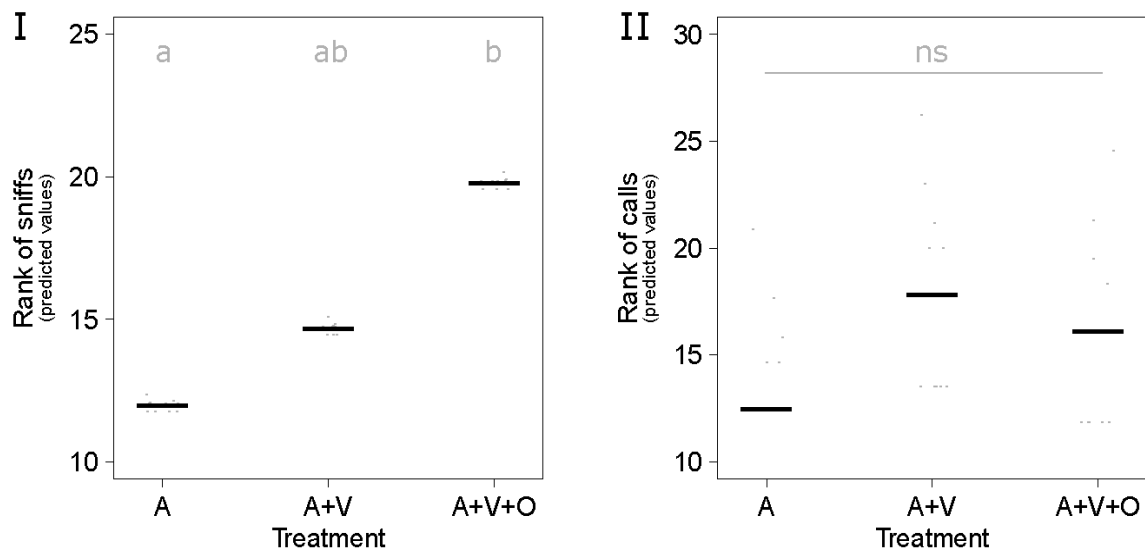

Figure 7.2. Rank of number of sniffs (I) and calls (II) of Australian sea lion females in response to treatments differing in the number of presented cues (Ex1). Notations: A – acoustic cue, O – olfactory cue, f – filial cue, nf – nonfilial cue. Predicted values and means of treatment levels from the fitted linear mixed models are shown on a response scale. Different letters (i.e. a, b) indicate differences among treatments.

Relative importance of cues

When comparing treatments with an altered identity of olfactory and acoustic cues, we observed no significant difference among treatments for sniffs (Table 7.1, Figure 7.3 I). By contrast, the vocal response differed significantly among treatments (Table 7.1, Figure 7.3 II). Both combinations containing filial acoustic cues (A_fO_f and A_fO_{nf}) elicited similar vocal responses (Table 7.1) and resulted in more calls produced by females than presentations including nonfilial acoustic cues ($A_{nf}O_{nf}$ and $A_{nf}O_f$; Figure 7.3 II, Table 7.1). Treatments that had the same identity of acoustic cues, but different identity of olfactory cues ($A_{nf}O_f$ and $A_{nf}O_{nf}$ as well as A_fO_f and A_fO_{nf}) were similar to each other, indicating no influence of olfactory cues on the vocal response. This was further confirmed by the second model with treatment encoded as two separate variables (olfactory cue and acoustic cue). We found a non-significant interaction between the acoustic and olfactory cues (p=0.943), indicating that the effects of each of these variables does not depend on the value of the other. The model also confirmed the importance of acoustic cues for the call response. Compared to the intercept condition of filial acoustic and filial olfactory cues, we found a significant negative effect of the acoustic cue (nonfilial compared to filial: estimate=-1.100, se=0.389, z-value=-2.830, p=0.004), and a non-significant effect of 'olfactory cue' (p=0.892). There were no differences for different levels of the variables (acoustic cue: p=0.786, olfactory cue p=0.134) and no interaction among them (p=0.649) for the females' sniff response.
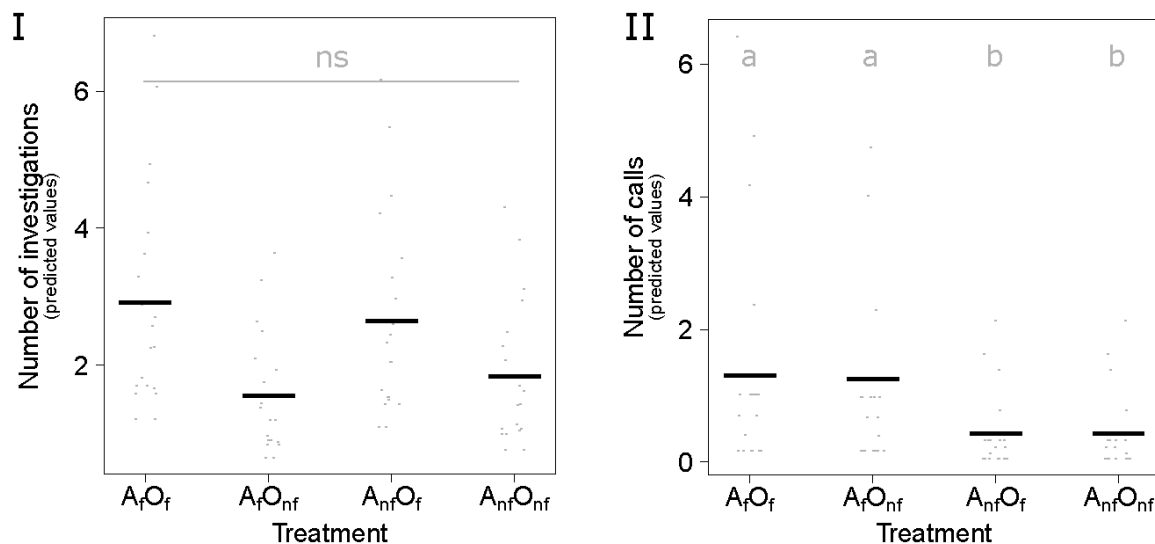

Figure 7.3. Number of sniffs (I) and calls (II) of Australian sea lion females in response to treatments differing in the identity of presented cues (Ex2). Notations: A – acoustic cue, O – olfactory cue, f – filial cue, nf – nonfilial cue. Predicted values and means of treatment levels from the fitted generalised linear mixed models are shown on a response scale. Different letters (i.e., a, b) indicate significant differences among treatments.

Table 7.1. Generalised linear mixed model estimates and pairwise comparisons shown for models fitted to Australian sea lion acoustic and olfactory response data to treatments differing in the identity of presented cues (Ex2). Statistically significant (p≤0.05) results are marked in bold.

Response	Treatment	Estimate	Standard error	Z value	P
Number of sniffs	A_fO_f (intercept)	0.8773	0.33	2.665	0.008
	A_fO_{nf}	-0.625	0.416	-1.5	0.189
	$A_{nf}O_f$	-0.1	0.369	-0.272	0.455
	$A_{nf}O_{nf}$	-0.459	0.401	-1.143	0.218
Number of calls	A_fO_f (intercept)	-0.652	0.477	-1.137	0.171
	A_fO_{nf}	-0.038	0.28	-0.136	0.892
	$A_{nf}O_f$	-1.1	0.389	-2.83	**0.005**
	$A_{nf}O_{nf}$	-1.099	0.389	-2.827	**0.005**
	Pairwise comparisons				
	A_fO_{nf} - A_fO_f	-0.038	0.28	-0.136	0.999
	$A_{nf}O_f$ - A_fO_f	-1.1	0.389	-2.83	**0.023**
	$A_{nf}O_{nf}$ - A_fO_f	-1.099	0.389	-2.827	**0.023**
	$A_{nf}O_f$ - A_fO_{nf}	-1.062	0.391	-2.719	**0.032**
	$A_{nf}O_{nf}$ - A_fO_{nf}	-0.061	0.391	-2.716	**0.032**
	$A_{nf}O_{nf}$ - $A_{nf}O_f$	0.001	0.475	0.002	1

Inter-observer reliability

The number of recorded behaviours was comparable between observers (Spearman's correlation: Ex1 sniffs: R=0.86; Ex 1 calls: R=0.90; Ex2 sniffs: R=0.81; Ex2 calls: R=0.94), therefore scores were considered reliable and not observer-biased.

DISCUSSION

A majority of mammals use multiple sensory cues for recognising conspecifics (Higham and Hebets 2013). While the function and role of each cue separately has often been investigated, until now we lacked knowledge about the interactions among different sensory cues and their relative or combined effect on the receiver of the information. We examined the synergistic effect of various sensory cues in mother-offspring recognition in a colonially breeding mammal, the Australian sea lion, and found that although sensory cues have the ability to convey the same information in isolation, in a multimodal context their relative importance and function differ. Olfactory cues are capable of encoding individual identity information in the Australian sea lion (Pitcher et al. 2010c), however in a multimodal setting it is only their presence, not their filial identity, that is important to prompting the

females' response. By contrast, recognising the specific identity of acoustic cues remains critical for the recognition of pups by mothers and the information conveyed in acoustic cues overrides olfactory cues. These patterns are surprising and seem to be driven by the costs and benefits of obtaining information resulting from the constraints imposed by the active space of each of the cues.

Females identified presentations as either filial or nonfilial based solely on the information encoded in the acoustic cue, with their vocal response not influenced by the identity or presence of other cues. Females increased their vocal production only when pup calls were filial and the congruency of olfactory cues did not affect their vocal response. Neither experiment showed an enhancement interaction between cues for the vocal response. For enhancement to occur, we would expect either the presence of multiple sensory cues to induce an increase in call production in Ex1, or the response to A_fO_f to be higher than both A_fO_{nf} and $A_{nf}O_f$ in Ex2, or a significant interaction among olfactory and acoustic cues when treatments were split into two variables, none of which occurred. The lack of differences in vocal response in Ex1 was likely due to the presence of a filial pup call in all three treatments, which further supports our finding that acoustic cues are more important in eliciting a vocal response than other cues.

We found that contrary to expectation, olfactory cues seemed to serve simply as a prompt for females to sniff the models rather than being a cue influencing decisions. The presence of an olfactory cue influenced the number of times a female sniffed a model, however the congruency of the odour did not. The difference in female response was significantly greater when all three sensory cues were present. However, we obtained varying results from both tests in regards to the difference in response between when two and three cues were present, with A+V significantly different from A+V+O in the Friedman test, yet overlapping with both A+V+O and A in the ANOVA. These inconsistencies are due to small sample sizes and differences in algorithms used by both tests. Due to this we will not attempt to determine whether the presence of the odour was solely responsible for this effect, or if the simultaneous presence of all three sensory cues was needed. However, in either case the enhancement effect in the presence of more cues is clear. The congruency of acoustic cues did not influence the number of sniffs. It appears that females will sniff any pup that is calling, presenting an odour, and is close enough for inspection, regardless of its identity.

During mother-pup reunions, nose to nose contact has long been considered the final step of identity confirmation of pup by females, with mothers gathering information encoded in olfactory cues prior to rejecting or accepting a pup (Bowen 1991; Insley et al. 2003). Previous studies (Pitcher et al. 2010c), as well as our results (i.e., our experience with frozen samples), demonstrate that females can indeed differentiate filial from non-filial pups based solely on olfactory cues. However, despite having the

ability to convey information about identity, our results show, surprisingly, that olfactory cues may not always be crucial in mother-offspring recognition in a multimodal setting. When multiple cues are present, there is a hierarchy in the use of information encoded within cues and in our study, acoustic cues overrode olfactory cues. It is important to note that the full importance of olfactory cues and their influence on the females' responses may not have been evident due to limitations in the presentation design. Our pup model was static and thus effectively unresponsive, for example they could not attempt to suckle the female as would occur with a live pup. This meant that we were only able to measure investigatory behaviour towards models, not whether a female would finally accept it to suckle.

While both olfactory and acoustic cues have the ability to provide individual identity information (Charrier and Harcourt 2006; Charrier et al. 2009; Pitcher et al. 2009; Pitcher et al. 2010a; Pitcher et al. 2010c; Pitcher et al. 2012) in a multimodal context, these cues are not equally important and are used differently. Therefore, there must be different underlying drivers such as other costs and/or constraints resulting from differences in active space, and/or the nature of female-pup interactions, that determine which cues are used and when.

The hierarchy in the role of different cues that we identified in the recognition process of pups by mothers could be caused by the order of cue perception, which is in turn driven by the active space of cues. Although multiple cues were available for simultaneous inspection, and potentially perceived concurrently, they may be processed in a specific order (Uy and Safran 2013). In a sequential perception setting, the cue that is processed first elicits the strongest response from the receiver (Partan and Marler 2005; Partan 2013). Many species assess multimodal cues sequentially, with large range cues attracting individuals for further assessment at close proximity (Uy and Safran 2013). In otariid colonies acoustic cues are used at both short and long ranges. However, acoustic cues have a larger active space than other cues (Insley et al. 2003; Pitcher et al. 2012) and as reunions occur on islands with complex topography covered in thick vegetation (Figure 7.1), they are often acquired first. Even when multiple cues are present, acoustic cues may be processed or even perceived earlier than other cues and therefore elicit an increase in response, hence the overriding by acoustic cues in a multimodal setting. This suggests that even when cues are presented simultaneously, sequential processing determines the use of cues.

The costs and benefits of obtaining various cues differ between Australian sea lion mothers and pups, and this may result in different use of cues by both parties in the information exchange. Australian sea lion mothers are highly aggressive towards nonfilial pups (Marlow 1972; Higgins and Tedman 1990) so it is necessary for the young to identify females at a distance in order to avoid being injured or even

killed by nonrelated females. Long distance acoustic cues have been shown to be essential for eliciting a call response in Australian sea lion pups with visual cues not increasing the occurrence of this behaviour (Wierucka et al. 2018 – Chapter 6). As a pup needs to hear and identify its mother's call in order to safely approach her, it is essential for the female to call back when her offspring vocalises. This may explain the increased vocalisations by females following the broadcast of filial pup calls in the current study. Although acoustic cues are clearly important during mother-pup reunions, they are not the only cues used by the mothers. As there is no direct risk of injury for females when approached by pups, the costs of obtaining information conveyed in multiple cues are low. Our results indicate that females use olfactory cues and investigate any pup that is available for inspection, regardless of whether the odour or other cues are congruent. With low costs and many potential benefits by decreasing the uncertainty of identity information, the action of investigating pups will be highly beneficial to females by adding an additional tier of recognition. However, mothers did not vocalise more when both olfactory and acoustic cues were filial, compared to treatments where only the calls were filial, despite olfactory cues having the ability to convey individual identity information in isolation (Pitcher et al. 2010c). This indicates that the female's call serves mainly as a prompt inducing the pup to approach. Once the pup is within detection range for olfactory cues, the female proceeds to sniff it. As the pup is already in close proximity, even if the cues are recognised as filial there will no longer be the need for the female to call.

Mutual mother-offspring recognition is an example of a reciprocal communication system in which individuals are both a receiver and sender. In cases like these, despite the costs and benefits of using various cues vary for different parties, the hierarchy of cues used may be determined by this interdependency. The importance of acoustic cues is evident for pups – it is dictated by the need to use long-distance cues because of the nature of interactions with females. However, this constraint on pups influences which cues are available for females to use in identification. This leads to a mutual interdependency, where the restrictions caused by active space acting on one of the animals in the dyad shapes the hierarchy of cues used. Consequently, while the active space of cues directly affects cue use by one participant, it also indirectly impacts the whole communication system. We suggest that cue active space is an evolutionary driver for the importance of given modalities within a recognition system, not only by promoting the use of multiple cues (Rosenthal and Ryan 2000; Higham and Hebets 2013; Uy and Safran 2013), but also by limiting them.

To our knowledge, this is the first experimental study on wild mammals showing that even though a given cue may have the ability to convey specific information, in a multimodal context, its importance, function or role may be different. We also show that even when individual identity information is

conveyed in the cue, its congruency is not always valuable and its mere presence can be sufficient to elicit a response from the receiver. We suggest that the active space of cues is not only a factor favouring the use of multimodal cues (Rosenthal and Ryan 2000; Higham and Hebets 2013; Uy and Safran 2013), but plays a significant role in shaping communication and recognition systems. It does this by causing limitations or increased costs of obtaining information that may arise during social interactions between animals.

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CHAPTER 8

General discussion

Slide from 3MT (three minute thesis) competition: 'Look, smell and listen: Multisensory recognition between sea lion mothers and pups (credit: Ver)

The overarching aim of this thesis was to use an experimental approach to explore how cues are used in a multimodal context by different receivers within a reciprocal communication system – in this case, mother-pup recognition. A further aim was to explore the reasons for any differences found in cue use within a multimodal as compared to a unimodal setting. Due to the diversity of taxa, both in respect to physiological capabilities as well as social and selective pressures imposed on them, different species will use cues differently. This will also vary in different contexts. Therefore, my thesis focused on investigating cue use during otariid mother-offspring reunion as it is a system under high selection pressure for successful recognition and the process is known to involve multiple cues. Prior to my thesis, it was acoustic cue use that had been most thoroughly explored, albeit previous research by Pitcher et al. (2010) had pioneered experimental investigation of olfactory mechanisms. Therefore, in this thesis I aimed to further knowledge about the use of olfactory and visual cues for recognition in the Australian sea lion, and then assess synergies among acoustic, visual and olfactory cues and their relative importance in the mother-offspring recognition process.

By analysing the chemical composition of Australian sea lion odours, I showed that general body scent shows distinct inter- and intra-individual trends and thus has the ability to provide a variety of information to animals (Chapters 3 and 4). Pup chemical profiles vary among body regions, with varying trends for pre- and post-moult pups (Chapter 3). Furthermore, colony differences are also encoded within the chemical profiles of animals (Chapter 4) and mother-pup pairs show chemical profile similarity, indicating a chemical basis allowing for phenotype matching to be a recognition mechanism used during reunion (Chapter 4). I found that visual cues provide useful information which sea lion mothers can use to refine their search for filial pups in the colony (Chapter 5). Mothers actively distinguish pup age-class specific visual cues and will match these templates to the resemblance of their own pup, while pups are capable of using visual cues to distinguish between females and pups (Chapter 6). For both pups and mothers, I found that the role and/or relative importance of sensory modalities changes in a multimodal setting compared to when cues are present in isolation. Acoustic cues dominated over visual cues for mother recognition by pups (Chapter 6), indicating that hearing is the primary modality that pups rely on during reunion in a natural setting. I also show that for mothers, although olfactory cues have the ability to convey individual identity information in isolation, they serve more as a prompt initiating investigations rather than a factor influencing decisions when other cues are present (Chapter 7). The congruency of acoustic cues however, is very important in eliciting female vocal response and initiating initial contact between the mother and pup (Chapter 7). These differences in the use of cues are not entirely a result of the animals' physiological abilities, as previous research has shown that females can use both olfactory and acoustic cues for individual identification (Chapter 7). Results from multimodal experiments conducted on both pups and mothers

indicate that the active space heavily influences which cues are used by animals in a natural setting. This is due to very specific characteristics of social interactions between mothers and pups, and the limitations that the active space imposes on the perception of cues. This body of work represents the first experimental study on multi-sensory mother-offspring recognition in a free-ranging gregarious mammal. It gives new insights into the role and function of various cues in a multimodal setting and provides a holistic view of recognition and discrimination processes in a mammalian species, contributing to understanding how different cues are used and how social constraints drive communication systems.

OLFACTORY MOTHER-OFFSPRING RECOGNITION

Animals often use many modalities for recognition, yet olfaction is considered to be fundamental for mother-offspring recognition (Schaal and Al Aïn 2014; Corona and Lévy 2015). Despite this, surprisingly little is known about the underlying chemical basis of olfactory communication in free-ranging animals. Australian sea lions are capable of distinguishing the smell of their own young from that of other pups in the colony (Pitcher et al. 2010) and nose to nose investigations between mother-pup pairs are frequently observed (Marlow 1975), yet we had no knowledge about the characteristics of chemical profiles, which secretions may be important or what mechanism is used by the female for offspring recognition.

In Chapter 3 I provided the first account of chemical profiles of Australian sea lions. When examining multiple types of secretions collected from pups, I found that body regions were distinct and these dissimilarities were likely driven by the distinctiveness of chemical compounds found in the mouth. It is possible that oral odour mixtures are important for recognition, especially considering that nose-to-nose investigations are prominent during mother-pup reunions (Marlow 1975), indicating that the cephalic region may be of importance. However, most body regions were similar to each other, showing that many compounds were shared across these regions and a subset of compounds important for recognition may be contained in the general body odour and be consistently present in a subset of chemicals that is included in every body region.

In Chapter 4 I showed that a variety of information can be reflected in the compound composition of Australia sea lion odours. Age/reproductive status, colony affiliation as well as mother-offspring similarity were all encoded within the chemical profiles. The chemical composition of odours from individual mothers differed from pups within and between colonies yet maternal mother and pup odours were more similar to each other than to others within the colony. This indicated a chemical

basis for phenotype matching that may be used for mother-offspring recognition. I suggest that the base for this similarity is determined primarily by similarities resulting from direct genetic components or long-lasting odour characteristics that offspring obtain from the mother (e.g., microbiota), rather than environmental compounds acquired from the immediate surrounds of the animals.

The two chapters referred to above provide information about chemical compounds found in Australian sea lion odours and their origin, confirm that there is a chemical basis for olfactory recognition in sea lions and point to the probable mechanism by which this happens. This advances our ability to understand olfactory recognition in Australian sea lions and provides useful insights into the mechanisms underlying olfactory communication in pinnipeds that will ultimately contribute to a better understanding of communication processes in mammals.

I have shown that collecting general body odour samples, in a relatively non-invasive and low-disturbance way (e.g., without capture for females) can provide high quality samples that allow for thorough chemical analysis. Many studies examining olfactory communication have focused on scent gland secretions, urine or faeces. These sources provide concentrated odours that have a known role in olfactory communication. Unfortunately, glands used for communication have not been identified in Australian sea lions. Furthermore, excrements and urine are difficult to obtain and have not been observed to play a big role in communication in the studied species. Males sniff the hind regions of females during mating behaviours (Marlow 1975) however nose to nose investigations are considered the primary behaviour associated with olfactory recognition among conspecifics, especially

mother-pup pairs (Marlow 1975). Therefore, I used overall body odours for chemical analysis. My results indicate that even general body odours collected on a wild population and sampled at a distance can be successfully chemically analysed (Chapters 3 and 4). This method of collecting general scent on swabs provides a better indication of potential chemicals used for olfaction than analysing fur samples (Chapter 3). Although swab samples are likely to have low concentrations of compounds and contain external chemicals, the overall trends and differences among chemical profiles are clearly visible, even with the relatively small sample sizes, showing the efficacy of the method. In a natural setting, all compounds contained in my samples are available to the females for inspection. Although animals may be selectively sensitive to a certain subset of these compounds, as we possess no information about which compounds might be important for females, the first step to understanding olfactory communication is to investigate whole chemical profiles.

OLFACTORY MOTHER-OFFSPRING RECOGNITION – LIMITATIONS AND FUTURE DIRECTIONS

Research included in this thesis (Chapters 3 and 4) implemented a newly-developed fully-automated protocol for post processing of chemical data allowing for full reproducibility. While GC-MS techniques are certainly useful and provide valuable information as to the chemical composition of odours, the lack of a unified methodology, and more importantly the shortage of reproducible research, is a serious problem. While excellent reviews have encompassed sample collection, GC-MS processing and data analysis methods (e.g., Sneddon et al. 2007; Charpentier et al. 2012; Drea et al. 2013), no such information is provided for processing of data (post GC-MS analyses and prior to statistical analysis). Deconvolution, peak integration, compound alignment and data restrictions are either not reported or reported very vaguely in many publications. The reasons for this are varied, but often stem from peaks being separated, aligned and identified manually or semi-automatically (e.g., Penn et al. 2007; Poirotte et al. 2017; Wieß et al. 2018). However, as I have shown, there now exists methods that provide entirely automated protocols that support fully reproducible results. The implications for such an approach are tremendous – they minimise human error and bias, but more importantly allow for better comparisons among studies and species. When dealing with a small number of compounds in samples, the inconsistencies resulting from manual analysis and their consequences on the interpretation of data may not affect the results. However, an increasing amount of studies now deal with complex mixtures of compounds in which hundreds of compounds are found in each sample. Even minute adjustments to the methods or parameters may drastically change the results and thus the interpretation of the communication system. There would be a clear benefit from a methodological study analysing the same dataset with different chemical pre-processing and statistical techniques. This would provide important information about the biases of each approach and determine the most robust and least biased methods for approaching specific data or questions concerning chemical ecology.

I have provided basic information about the intra- and inter-individual differences in chemical profiles of Australian sea lions. However, due to limitations of working with wild populations of an endangered species, I was unable to repeatedly collect samples from the same animals over multiple sampling occasions. Thus it was not possible to look at whether chemical differences among individuals are stable i.e., if they have an individually distinct olfactory signature. Individual signatures would indicate a chemical basis allowing for true individual recognition (*sensu* Tibbetts and Dale 2007). It would provide information about whether direct familiarisation can be used by Australian sea lions as a mechanism for olfactory recognition. I have shown that due to mother-pup chemical profile similarity, phenotype matching could be used in pup recognition. However, this does not rule out the possibility

that mothers learn the chemical "fingerprint" (in whole or its subset) of their pup as well and use the two mechanisms in different contexts or in combination (Wyatt 2014). Determining whether an olfactory signature exists for pups would allow us to gain additional information about the ability of mothers to accurately distinguish filial pup scent evidenced by Pitcher et al. (2010) and should be investigated in future studies.

For olfactory recognition to be successful, detectable differences in chemical composition among individuals must be present. Thus, if information is encoded within a chemical profile, differences in compound composition should inform us whether there is a chemical basis by which recognition may occur. Gas chromatography-mass spectrometry techniques have been increasingly used in recent years for the analysis of scent samples and interpretation of olfactory communication in different organisms (Sneddon et al. 2007; Wyatt 2014). However, a limitation to this method is that these are producing machine-detectable differences without necessarily taking into account animal organ sensitivity or the selective importance of particular compounds. The next and very important step would be to identify semiochemicals – compounds that convey information and are used for communication. Odours are complex and contain many compounds of various origin. Generally, not all of these compounds will be used for transmitting information. Chemical analysis of body odour scent samples only allows for broad-brush conclusions for several reasons. First, in this study as there was no prior information about chemical composition in this species, I examined entire chemical profiles and could not apply restrictions to the type of compounds that were included in the analysis. Secondly, although the method used allows the detection of differences in compound composition, what is analysed are machine-detectable differences. Animal sensory systems almost certainly have different sensitivity and are likely to be more receptive towards certain compounds or combinations of compounds. Therefore, although my analyses resulted in basic information about compounds that could be important in communication, to confirm these findings and determine which compounds or combinations of chemicals allow for the transfer of information, complex bioassays and real-life experiments are needed.

VISUAL MOTHER-OFFSPRING RECOGNITION

Although visual cues have long been suspected to play a role in otariid mother-offspring reunion, this knowledge came from anecdotal sources or observational studies, mostly focusing on animals using geographical cues, rather than cues distinct for a given individual or subset of animals (e.g., Bartholomew 1959; Stirling 1970; Marlow 1975; McNab and Crawley 1975; Dobson and Jouventin 2003; Phillips 2003; Trimble and Insley 2010). However, as otariid pups are quite mobile and

frequently change location, the presence of another level of recognition allowing females to narrow down their search to only a subset of individuals would not be surprising. In Chapter 5 I found that visual cues allow for age-class discrimination of pups by mothers. This is the first experimental study examining pinniped visual social recognition abilities and these unique findings show that animal specific (as opposed to spatial) visual cues can be used by females in the reunion process. Using more cues provides females with more information that can optimise the reunion process. The ability of Australian sea lions to use age-class specific recognition (Chapter 5) points to the use of visual cues in a more sophisticated way. Mothers can remember the broad visual characteristics of their pups and are able to use this information to classify offspring into congruent and incongruent categories thereby facilitating reunion.

I also explored visual cue use by offspring (as a subpart of Chapter 6) and found that sea lion pups are capable of distinguishing adult females from pups solely using visual cues. No knowledge or even speculation was previously available about visual cue use by pups. This is surprising considering the importance of accurate conspecific assessment by pups. Within a colony, pups are concurrently surrounded by animals of all age and sex categories, yet the interactions and consequences of approaching each of them greatly vary. Females are very aggressive towards non-filial pups and will attack them if they attempt to suckle (Marlow 1972; Higgins and Tedman 1990; Gales et al. 1994). Males pose a threat as they can attack pups or trample them during fights with rivals (Marlow 1975; Higgins and Tedman 1990). By contrast, pups often associate with each other in an affiliative manner while their mothers are away on foraging trips (Marlow 1975). Each of these groups of conspecifics vary significantly in their size, shape and colour, thus even broad-scale recognition would be beneficial to pups as it would allow a rapid classification to a given age-sex class and assessment of whether approach puts them at risk of injury. Pups have been shown to recognise females through calls, however, relying on one sensory channel for obtaining crucial information might be risky. Furthermore, during pup-male or pup-pup interactions animals do not vocalise. Therefore, other cues must be used by pups to evaluate the danger of approaching another animal. I have shown that visual cues allow for this and may be used by pups for rapid and broad conspecific assessment. This study is the first account of such information. It provides important baseline knowledge that animal-specific visual cues are recognised and used by individuals and paves the way to further exploration of the topic, discovering how detailed the conveyed information can be.

VISUAL MOTHER-OFFSPRING RECOGNITION – LIMITATIONS AND FUTURE DIRECTIONS

I showed that age-class specific cues can be used by mothers for refining their search for filial pups in the colony (Chapter 5) and that pups have the ability to distinguish females from pups based solely on visual cues (Chapter 6). However, the dummies I presented were of both different colour and size. I focused on overall characteristics of a given age/sex-classes of animals rather than specific components of the cue and thus was unable to determine which of these influenced the sea lions' response. In the future, it would be beneficial to examine which components of the visual cue are important for recognition, whether both size and colour need to be congruent for recognition to occur or if there is a hierarchy among these visual components.

I examined age/sex-class visual recognition, yet the question of whether individual visual recognition is possible remains unanswered. Visual capabilities of otariids above water, although worse than other related terrestrial mammals, do not rule this out (Schusterman 1972; Hanke et al. 2009). Australian sea lions do not possess obviously individualised pelage patterns, however facial recognition at short range may be possible. Visual recognition based on facial characteristics has been shown for other taxa, such as primates (e.g., Parr et al. 2000; Talbot et al. 2015; Talbot et al. 2016; Parr and de Waal 1999; Parr et al. 2010), sheep (Kendrick et al. 1995; Kendrick et al. 1996), and cattle (Coulon et al. 2009; Coulon et al. 2011) and thus it is likely that other species possess the ability as well. The preparation, logistics and interpretation of such experiments are extremely challenging, however are definitely worth undertaking and would improve our knowledge of the physiological and cognitive capabilities of otariids.

MULTIMODAL MOTHER-OFFSPRING RECOGNITION

I investigated multimodal cue use for both pup and mother Australian sea lions. I considered multiple sensory channels for produced cues as well as receiver response, and explored cue use by different participants of the interaction. This allowed me to examine the system as a whole, rather than the individual components that make up a communication network. Although pups can distinguish age/sex classes of conspecifics based on visual cues, when both visual and acoustic cues are present, pups rely on the latter and the simultaneous presence of cues do not enhance their response (Chapter 6). Females increase call rates if they hear their filial pup's call, while olfactory cues serve only as a prompt for investigations, with mothers sniffing any pup that is present, calling and producing an odour, regardless of its identity (encoded either through acoustic or olfactory cues; Chapter 7).

The hierarchy of cues used by both mothers and pups can be explained when taking into account the social interactions between them. Females are aggressive towards nonfilial offspring (Marlow 1972; Higgins and Tedman 1990; Gales et al. 1994), therefore pups have to rely on acoustic cues for mother recognition (Chapter 6). It is the only modality that provides offspring with reliable information at a distance, thus allowing pups to avoid injury resulting from approaching unrelated females. Yet in order for this to be possible, mothers have to respond to their pups' calls, which is clear in my results – female response was stronger when filial pup calls were played (Chapter 7). However, the costs and benefits of social interactions vary for females and pups. As there is little risk of injury upon approach and many potential benefits in the form of decreased uncertainty of identity information, mothers investigate any pup that approaches her and is available for investigation, regardless of whether the cues perceived by the female are congruent with her pups. Taking all information into account, it seems that successful reunion, or at least the first stages of this process, is reliant on the pup's ability to recognise its mother's call and initiate approach. Therefore, although the female plays a central role in the reunion, the pup's active engagement in the process is essential.

The nature of social interactions among pups and females and its effect on the costs of obtaining information through specific cues influences cue use in the studied system. These costs are mostly associated with direct contact with other individuals and the risk of injury or lack thereof. Thus, it is the functional range of a cue that determines whether it will be used by an individual, indicating that the active space of sensory cues plays a significant role in shaping this communication and recognition system. I demonstrate that using multiple cues is not always beneficial (Chapter 6). Although some information is encoded in multiple sensory channels, accessing some of these cues in a natural setting might entail additional costs that outweigh the benefits of obtaining information. Thus, although using a single modality is considered risky as it is more prone to noise obstruction, from a broader perspective it might be more advantageous to use a single modality. After an exhaustive literature search, to my knowledge only one other study suggested that the active space of cues plays a role in the hierarchy of cue use (that were presented simultaneously in a multimodal context) for recognition. The relative importance of olfactory and visual cues were tested in mating behaviours of spiders (*Pardosa milvina*; Rypstra et al. 2009). Here, cues also varied in specificity – chemical cues indicated the sex and reproductive state of the animal, and the visual cues represented the exact location and receptivity to mate. While both cues were deemed largely redundant, the olfactory cue elicited a stronger response in males. This was apparently due to females often remaining hidden in a natural environment, hindering visual cues. Therefore, the active space of cues influenced the hierarchy of cues used for mate discrimination (i.e., olfactory cues were more reliable). Although these are single examples, a similar mechanism in both invertebrates and vertebrates shows how universal the

principles of communication could be. Despite many differences in the physiology and behaviour of these taxa, similar rules apply to the systems. Extracting underlying mechanisms for communication, discovering the similarities and differences in trends across species, and applying the findings to broader frameworks are beneficial to explaining the behaviours of the studied species. Once enough knowledge is accumulated, comparative studies will be possible, which will be extremely insightful in understanding how communication systems evolved.

THE IMPORTANCE OF A MULTIMODAL NETWORK APPROACH

The results presented in this thesis (Chapters 6 and 7) demonstrate the importance of undertaking studies that investigate multiple cues whilst also taking into account various participants in the exchange of information when interpreting behaviour. Due to the complexity of communication and recognition, and the different modalities being intertwined in both, most studies in the past have focused on only the primary modality within a given context and looked at the one-sided interaction of the sender and receiver (Hebets 2011). Although this research provides useful information about the capabilities of each cue to convey given information and the ability of animals to produce and perceive them, the interpretation of the use of cues and the reasons for doing so in a natural setting based on such research may be incomplete. Animals are exposed to many cues simultaneously and the communication network is usually more complex than a one-sided sender-receiver scenario (McGregor and Peake 2000).

I have confirmed that in a multimodal context the role or function of cues is different than in isolation (Chapter 7). In some instances, despite a cue containing higher-order information such as identity information, the presence of a cue (regardless of the message it has the ability to convey) is sufficient to elicit a response (Chapter 7). This is an example of communication that differs with system architecture – the composition of cues that are present determine the function of the cues (Hebets et al. 2016). This term is broad and is often used in reference to changes in cue use occurring due to differences in external factors such as environmental conditions (e.g., Rundus et al. 2011; Wilgers and Hebets 2011). However, my study presents a unique example where cue use (not the intensity of the response, but in the way the cue is used) varies in response to the presence of other cues (Chapter 7). A cue has a function in isolation, but that function and the information used by the receiver changes in a multimodal setting. Several studies in the past have shown a similar cue interaction for other species. E.g., in wolf spiders (*Schizocosa crassipes*), the function of the visual cue in courtship displays varies depending on the presence of the vibratory cue (Stafstrom and Hebets 2013). Similar dependencies occur in frogs (*Epipedobates femoralis*), where only the simultaneous presence of

acoustic and visual cues elicits an aggressive response, and neither unimodal treatment evokes aggressive behaviour (Narins et al. 2003).

While multiple sensory cues have been measured in many studies, in a majority of them only a unidirectional transfer of information is assessed and explored. In Australian sea lions, the range at which cues can be useful not only determined cue use by one participant of the dyad, but indirectly affected other individuals within the network. I showed an interdependency between costs imposed on one participant of the system and the cues used by the other (Chapter 7). This illustrates that in order to better understand the use of cues by one participant in a communication network, as many relevant factors as possible should be taken into account as they might profoundly influence the conclusions we draw about the behaviour of animals (McGregor and Peake 2000; Patricelli and Hebets 2016).

When interpreting multimodal cue use, it is valuable to also consider limitations other than the ones typically acknowledged, such as the physical properties of cues and the production and perception capabilities of animals. Although there will always be additional factors that can be included in analyses, the two discussed here – the presence of multiple cues and multiple participants – have now been demonstrated to influence the studied communication system in mammals and should be further examined in other taxa. Overall, findings from Chapters 6 and 7 point to the importance of multimodal studies and looking beyond just single interactions and cues in isolation. A multimodal network approach shows how cues work together and influence the animals' responses in a natural environment. This allows us to identify the reasons for the use of different modalities by individuals and explain more about animal behaviour and the evolutionary drivers behind them, than just investigating cues in isolation.

MULTIMODAL MOTHER-OFFSPRING RECOGNITION – LIMITATIONS AND FUTURE DIRECTIONS

As a result of many communication studies undertaken in the past, as well as research encompassed within this thesis, the Australian sea lion is now an excellent species for more detailed investigation of the underpinnings of multimodal recognition. Having thorough information about the abilities of cues to convey information in isolation and knowledge about their interactions and role in a multimodal context gives the possibility for further exploration of interactions between sensory modalities. The simplest and most obvious extension to the conducted research would be to undertake a series of experiments extending the work done in this thesis by presenting animals with more varied combinations of cues. Displaying all cues in isolation, in pairs (all combinations) and then all together,

would give us a better understanding of any enhancement in response caused by the presence of multiple cues. Furthermore, in experiments conducted for the purpose of this thesis, all cues were presented simultaneously. However, as we determined that active space clearly plays an important role in cue use by animals, and the range at which different modalities can be used vary, it would be beneficial to present cues sequentially, and with varied delay times. By doing so, it would be possible to measure whether cues that are processed first have a greater impact on the animal's response and also investigate the maximum length of time shift in presentation of cues for an animal to still perceive them as multimodal.

It could be argued that due to the difference in specificity of cues presented in my study (Chapters 6 and 7; age-class specific for visual cues and individually distinct for olfactory and acoustic cues), the results did not fully reflect the interactions that occur among them. Considering previous literature (Ruiz-Miranda 1993) demonstrated that broad cues can affect cue use in mammals, and well as additional experiments conducted to confirm behavioural responses to single cues (Chapter 7) it is unlikely. However, adding a congruent and incongruent visual cue variable to the relative importance experiments would provide further insight into the interactions of cues. Once more knowledge is obtained about the detail of information conveyed through visual cues, studies presenting individually distinct visual cues (if such recognition occurs) would also be greatly beneficial.

All pups tested in the multimodal studies were 2-4 months old. This age was chosen as acoustic recognition of mothers is already developed by that age (Pitcher et al. 2009). Showing that acoustic cues play an essential role for pups in the reunion process raises new questions as to how the reunion process functions before pups learn and remember their mothers' calls. During the perinatal period pups remain with their mothers on land and at that time acoustic recognition of mothers by pups is not functional (Pitcher et al. 2009). This ability develops between the perinatal period and about two months of age, however, the exact time, or its variation among pups is not known. Furthermore, no studies have investigated pup home ranges and how they vary with age. It is reasonable to assume that when pups are very young, they do not have the ability to recognise their mother through acoustic cues and thus do not move far from their last suckling site or birth site during maternal absences. At that time the mother would play an active and primary role in reunion and perhaps olfaction may be of greater significance. I hypothesise that as pups age and gain new recognition abilities, their home ranges will increase, and they play a more active and important role, approaching calling mothers to initiate reunion. Future studies should aim to investigate the relationship among the movements of pups, their ability to recognise their mothers' calls and the relative importance of cues used by both mothers and pups during the reunion process.

When simplified, animal communication can be broken down to a pattern of cue production by the sender, cue transmission, perception, a decision made by the receiver based on the information provided by the cue and their response. What my study simulated was the production of cues. I then measured the response of the individuals. While this method of investigating various questions regarding communication is commonly used (Hebets 2011), it possesses certain limitations when interpreting the data. To observe a response, all previous elements of the process must be successful. Therefore, if a response occurred we can be certain that the cue was perceived and that it conveyed information. However, even in this case, which behavioural response is measured may have affected the results we obtained and their interpretation. If we observed no change in behaviour, the reasons for this could be fourfold: 1) the cue was perceived by the receiver, however did not convey information and thus did not evoke a response, 2) the cue was perceived and conveyed information but did not result in a response of the receiver, 3) the cue was not perceived due to presentation malfunction such as cue degradation or poor presentation methods, 4) the cue was not perceived due to limitations of the sensory capabilities of animals. Realising these limitations is important for the interpretation of data. While many measures were undertaken (in the form of literature searched or additional experiments confirming the efficacy of a given methodology) to account for these issues, future work should aim to quantify the error rates caused by these factors and their potential influence on the accuracy of results.

Studying communication in a wild population has allowed me to investigate the effect that different cues have on the receiver's response, and then apply cost-benefit frameworks to these empirical results. The research showed that although cues have the ability to convey certain information in isolation, when combined their function and use is different. We know that environmental constraints exist and the active space of cues plays a major role in sea lions' choice of when to use specific cues. However, learning how to distinguish cues, and the development of their production and perception, is tightly connected to cognitive abilities of animals. Studies on captive, trained animals could determine their fine-scale cognitive abilities (along with their neurological underpinnings) in respect to communication. This would provide an explanation of how detailed the information conveyed through each sensory modality can be and how information from multiple sensory channels is integrated in varying contexts on a behavioural and neurological level. Once this information is acquired it would be possible to determine whether sensory cues are used to their full potential in a natural environment. This in turn would allow to further investigate the reasons and limitations that are imposed by the natural environment on cue use, how that varies in different contexts, and in the long term determine the relative importance of cognitive/neurological and environmental constraints on this communication system.

Having obtained a good understanding of the communication system between Australian sea lion mother-pup pairs, expanding the research to other interaction contexts for this species would provide more information about the pressures and limitations that influence cue use. When testing the same species, physiological and cognitive abilities of animals remain the same. Any difference in cue use, integration, or hierarchy will be influenced by other factors such as social pressures or the cost-benefit ratios of a particular interaction. Conducting comparative multimodal experiments in other social contexts (e.g., male-female interactions, male-male aggressive behaviours) and examining the differences and similarities of the ways cues are combined would allow us to examine the underlying pressures for cue use that are not related to the abilities of animals or the physical properties of cues. Maintaining the same premise, and looking further into the future, investigations should continue in the direction of conducting similar research on other study systems, either species facing similar pressures, or similar species facing different pressures. This, as well as the other ideas listed above, would give an additional breadth of knowledge that would eventually allow for the creation of a more detailed framework about communication mechanisms bringing us closer to disentangling the intricacies of multimodal communication and determining what rules the integration and use of sensory cues are governed by.

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Visual cues do not enhance sea lion pups' response to multimodal maternal cues

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Mammals use multiple sensory cues for mother-offspring recognition. While the role of single sensory cues has been well studied, we lack information about how multiple cues produced by mothers are integrated by their offspring. Knowing that Australian sea lion (*Neophoca cinerea*) pups recognise their mother's calls, we first tested whether visual cues are used by pups to discriminate between conspecifics of different age classes (adult female vs pup). We then examined if adding a visual stimulus to an acoustic cue enhances vocal responsiveness of Australian sea lion pups, by presenting wild individuals with either a visual cue (female 3D-model), an acoustic cue (mother's call), or both simultaneously, and observing their reaction. We showed that visual cues can be used by pups to distinguish adult females from other individuals, however we found no enhancement effect of these cues on the response in a multimodal scenario. Audio-only cues prompted a similar reaction to audio-visual cues that was significantly stronger than pup response to visual-only cues. Our results suggest that visual cues are dominated by acoustic cues and that pups rely on the latter in mother recognition.

Animal communication can be extremely complex and may use multiple sensory modalities[1]. Due to differences in environmental conditions, cue structure and limitations of sensory systems themselves, the costs and benefits of conveying information through each modality vary[2]. Accordingly, animals often invoke multiple sensory modalities simultaneously, presumably to increase the robustness or diversity of transmitted information[2–7]. Multimodal communication occurs when composite signals or cues are received through more than one sensory channel[4,5,8]. When multiple cues are present, they may be redundant, i.e. conveying multiple copies of the same information, or non-redundant, i.e. conveying multiple, different messages thereby enabling the transmission of more information[8]. Furthermore, when combined, cues may induce different responses from the receiver[8]. Multiple redundant cues may elicit either an equivalent or an enhanced response compared to a single cue, and non-redundant cues may be independent, cause dominance or modulation, or lead to the emergence of a new response[8]. Because of these interactions, investigating how animals respond to multiple cues simultaneously is necessary, as it provides greater understanding about complex behaviour than simply looking at cues in isolation. However, cues may be used and integrated differently by animals, depending on the interactions between the costs and benefits of obtaining them[9]. Given there are limitations for specific cues to convey information (e.g. transmission distance) and costs associated with multisensory signal production and perception, using multiple cues is not always favoured[9]. Determining why and when animals use specific combinations of cues, gives insight into the intricacies of multimodal communication. To understand how communication systems evolved and what rules they are governed by, the interactions and relevant importance of sensory cues in a given context need to be explored.

Mother-offspring recognition is known to involve different and usually multiple modalities simultaneously, with acoustic, olfactory and visual cues playing varying roles for different mammalian species[10,11]. The ability to identify young by mothers and its reciprocal is usually beneficial to both parties. It allows mothers to direct their care only towards filial offspring thereby enhancing potential reproductive output[12]. For offspring, identifying their mother may limit the risk of injury caused by approaching non-kin and limit energy wasted in unsuccessful begging attempts[12]. While extensive literature has investigated mother-young recognition abilities of many mammalian species[13–18], most studies have investigated only the role of single sensory modalities. Under natural

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conditions, sensory cues co-occur and will rarely be available for inspection in isolation, yet there is not much information about the combined effect of different cues on the response of the receiver. No wild studies exist, although there is some evidence from domestic and laboratory mammals on the relative importance of individual sensory cues used in concert by mother and offspring sheep (*Ovis aries*)[19–27], goats (*Capra hircus*)[28] mice (*Mus musculus*)[29] and rats (*Ratus norvegicus*)[30]. While a vast majority of these studies focus on recognition of the offspring by mothers, it is likely that mothers and offspring utilise cues differently, as apart from discrimination abilities, their motivation and therefore costs and benefits of obtaining cues are very different.

Mother-offspring recognition is especially important for colonial mammals with mobile young and frequent mother-offspring separations occurring due to the mother needing to leave periodically to forage, such as fur seals and sea lions[31]. Acoustic, olfactory and visual cues are all used in the mother-pup reunion process[18]. For different otariid species, a similar pattern of the reunion has been observed – the female and pup call to each other, they look for each other, and when at close range nasal investigations are performed[31]. Although observational studies exist for multiple species[32–36] extensive experimental work about recognition through different sensory modalities has been done only for the Australian sea lion (*Neophoca cinerea*). Previous research demonstrated that both pups and adults produce individually stereotyped calls[37], and females use acoustic[38–40], olfactory[41] and visual[42] cues to recognise filial pups. Vocal recognition is mutual as pups can accurately distinguish filial mother's calls from that of other females[40], yet the onset of this ability is delayed compared to mothers[43]. Although information is available for pup recognition by females, what role non-vocal cues play, and the interaction between cues, in the pups' recognition abilities of mothers remains to be evaluated. Australian sea lions provide a unique opportunity to look at the role of the receivers' costs in shaping recognition systems. The main constraints for the use of multimodal cues are perception and production costs of cues, as well as the risk of increased eavesdropping and therefore higher detection rates by predators[4]. This species does not have terrestrial predators and the cost of producing cues is negligible. Therefore, in a situation where cues are capable of conveying useful information, the only limitations for using multimodal cues are the costs of obtaining, receiving, processing and integrating cues, and their survivorship consequences[4].

In this study we first test whether visual cues can be used by pups to discriminate among conspecifics (adult females vs pups) and then examine whether visual and acoustic cues induce a synergistic effect on the behavioural response of Australian sea lion pups during mother-pup reunion.

Methods

Study site and animals.
The use of visual cues by pups (visual experiment) was studied in a wild population of Australian sea lions inhabiting Olive Island (32°43′S, 133°58′E) and Kangaroo Island (35°59′S, 137°19′E) in April and October 2016, respectively. Experiments examining multimodal cue use by pups (bimodal experiment) were conducted in September-October 2017 on Olive Island. Pups used in both experiments were less than 4 months old and were approached for procedures when mothers were away on foraging trips, to avoid mother-pup separation and thus limit disturbance. Pups used in the bimodal experiment (only 2–4 month olds) were captured and restrained for a short period of time where they were individually marked by clipping a unique symbol into their fur and applying hair dye (Clairol Nice'n Easy©). This allowed us to identify pups at a distance without the need to approach them and to identify their mother in order to record their pup attraction calls.

Sample collection.
Pup attraction calls were recorded from mothers of marked pups during interactions with their pups in the colony using a BeyerDynamic M69 TG microphone (frequency response: 50Hz–16kHz $\pm$ 2.5 dB; BeyerDynamic, Heilbronn, Germany) mounted on a 3 m boom connected to a Marantz PMD 671 digital recorder (Marantz Europe, Eindhoven, Netherlands). Calls were recorded at a 44.1 kHz sampling frequency. Good quality calls (i.e., no background noise and no overlap with other vocalizing animals) were selected and high-pass filtered at 200 Hz using Avisoft SAS Lab Pro (Avisoft Bioacoustics, R. Specht) to remove low frequency noise caused by wind and/or waves. Experimental playback series were composed of six calls separated by 2–3 seconds of silence, similar to a natural calling sequence of a female searching for her pup. The playback series were broadcast using a portable amplified speaker (JBL Flip 3, 2×8 W, frequency response: 85Hz-20 kHz) connected by Bluetooth to an audio player. Calls were played at an approximately natural amplitude of 83 ± 3 dB SPL measured 1 meter from the source[40,43].

3D-models imitating an adult female as well as a 1–2 and a 2–4 month old pup were constructed using synthetic fur with polyester filling, and fitted with a wire skeleton to maintain an upright posture (Fig. 1). To examine the role of class-level visual cues in recognition, the size and fur colour pattern were chosen based on the average body size and colouration of adult females as well as 1–2 and 2–4 month old pups (body length: 156, 76 and 87 cm respectively). As all animals within a given age/sex class appear similar and no information currently exists about whether individual visual recognition is possible in pinnipeds, models approximated the size, shape and colour brightness of the respective age/sex groups. Our pup models have been previously shown successful in imitating animals for research purposes[42].

Experimental design.
In both the visual and bimodal experiments, the stimulus was presented 2 meters away from the pup, facing the pup's head or at least within 45° to ensure a clear visibility of the model presented (Fig. 1). The models did not contain any sea lion olfactory cues, and were placed beyond the range that olfactory assessment appears to occur[41]. Objects were placed while the pup was sleeping as to not disrupt, startle or otherwise confound the response of the pup with human presence. The pup was woken up with a natural sound (i.e., a hand clap) immediately prior to presentations.

For the visual experiment, 25 pups were presented with one of three treatments: (1) female model – a life-size model of an adult female sea lion (n = 8;); (2) pup model – a life-size model of pup of the same age as the tested pup (n = 9); (3) control – a foreign object (i.e a 65 L blue dry bag filled with air; n = 8). We expected pups to be
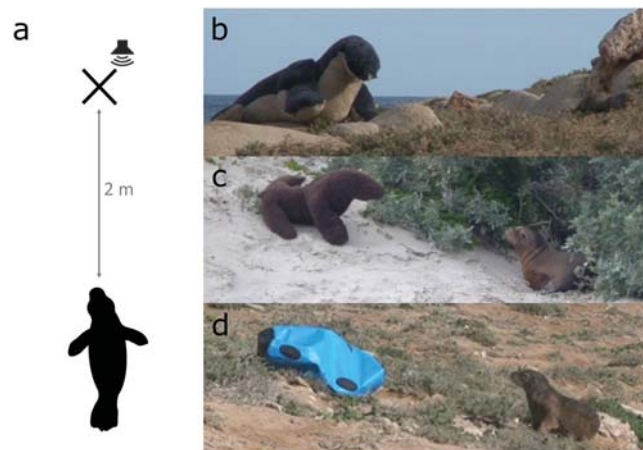
Figure 1. Behavioural experiment setup (**a**). Pups were presented with a stimulus (marked with 'x') - either a female model (**b**), or pup a model (**c**), or foreign object (**d**), placed 2 meters away from the animal, directly in line of sight, facing toward the individual. The speaker (where applicable) was placed adjacent to the model. When the visual model was absent (audio-only cues), the speaker was present in the same location.

vigilant if they identified the presented object as a female as non-related females are aggressive towards non-filial pups[32,44,45]. We also predicted that pups would not change their location and return to their initial behaviour if they identified the presented object as another pup, as pups associate with each other in the colony during maternal foraging trips on a regular basis and pose no threat to each other[32]. Therefore, a significant difference in pup response to different models would indicate the use of visual cues for conspecific assessment, whereas no differences would point to the adult female models not being identified as non-mothers, and the possibility that visual cues are not used by pups. Based on this, an ethological scale was created to assess whether the pups could distinguish different categories of conspecifics/items based solely on visual cues. The behaviour of the pup following it looking at the object was scored and two patterns were defined: "return to rest" – when no change in location occurred and the pup returned to its initial resting position following the presentation, and "vigilance" – when the pup moved away from the object, or stayed in the general area without returning to a resting position.

During the bimodal experiment, one of three treatments was presented to 30 pups: (1) audio – pup attraction calls of their mother (n = 10); (2) visual – the life-size model of an adult female sea lion (n = 10); (3) audio-visual – pup attraction calls of their mother paired with the life-size adult female model (n = 10). As we were measuring whether there is an enhancement effect following the addition of the visual cue to the acoustic cue, we noted the number of calls produced by the pup as well as the latency to call (if a call occurred) during 60 seconds after the beginning of each presentation.

Statistical analysis. A Fisher's exact test, with Holm's correction for multiple comparisons was used to assess whether differences in response among treatments occurred in the visual experiments. The number of calls produced by pups among treatments in the bimodal experiment were compared using a Kruskal-Wallis test with a Dunn's post-hoc test[46]. An exact Wilcoxon rank sum test was used to examine differences in latency to call between audio and audio-visual treatments[47]. All statistical analyses were performed in R version 3.2.2[48].

The research was carried out under the permission of the South Australian Wildlife Ethics Committee (approval 30/2015) and the Department of Environment, Water and Natural Resources (permit E26447). All experimental procedures followed the Australian code of practice for the care and use of animals for scientific purposes. All data analysed during this study are included in this published article (and its Supplementary Information files).

Results

Pup response varied depending on the presented visual cue (p = 0.031). Pairwise comparisons showed that this was due to a significant difference between pup responses to the female and pup models (female model vs pup model: p = 0.046, control vs pup model: p = 0.262; control vs female model: p = 0.608, Fig. 2). Eight out of nine pups presented with a pup model returned to a resting state after looking at the object. In contrast, six out of eight pups that were shown the female model responded to the treatment with vigilance. Pup response to the control varied, with half of the tested pups returning to a resting position and half staying alert or moving away from the object.

When exploring bimodal cue use, we found significant differences in the number of calls produced among treatments ($\chi^2 = 14.72$, df = 2, p = 0.0006; Fig. 3). The audio and audio-visual presentations elicited a statistically similar response that was significantly different from that exhibited during visual-only treatments (Dunn's test: visual vs audio: p = 0.0007; visual vs audio-visual: p = 0.01; audio vs audio-visual: p = 0.35). Seven out of ten pups produced calls following audio-visual presentations, nine out of ten pups exposed to acoustic-only presentations responded vocally to the playback, and none of the animals presented with just the visual treatment produced calls. Furthermore, we found no significant differences in the latency to call between audio-only and audio-visual presentations (W = 30, p = 0.95, n = 16; Fig. 4).
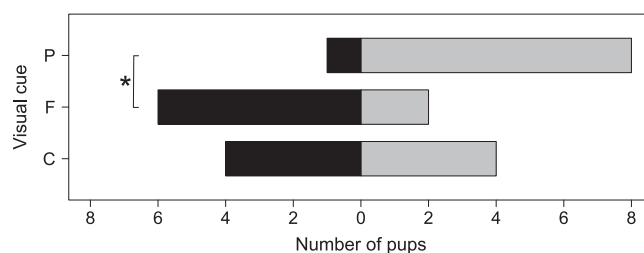
Figure 2. Number of pups returning to a resting state (grey) and displaying vigilance (black) in response to different presented visual stimuli. Notations: P – pup model, F – female model, C – control. The asterisk indicates statistically significant differences (p = 0.046) among treatments.
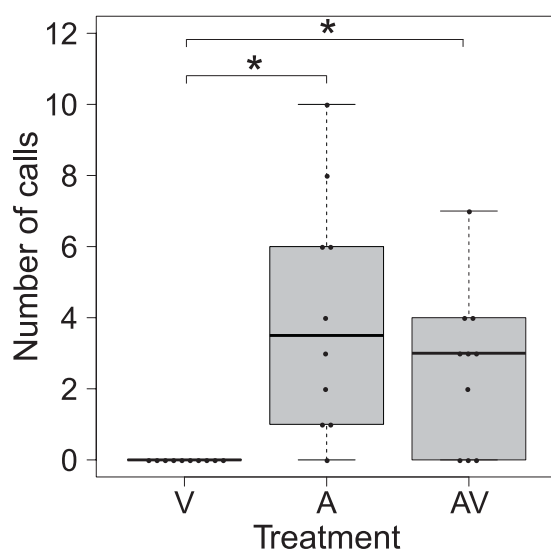

Figure 3. Number of calls produced by pups during visual-only (V), audio-only (A) and audio-visual (AV) treatments. Boxplots show the median, quartiles and minimum and maximum values within the inter-quartile range. Asterisks indicate statistically significant differences (V vs A p = 0.0007, V vs AV p = 0.01) among treatments.
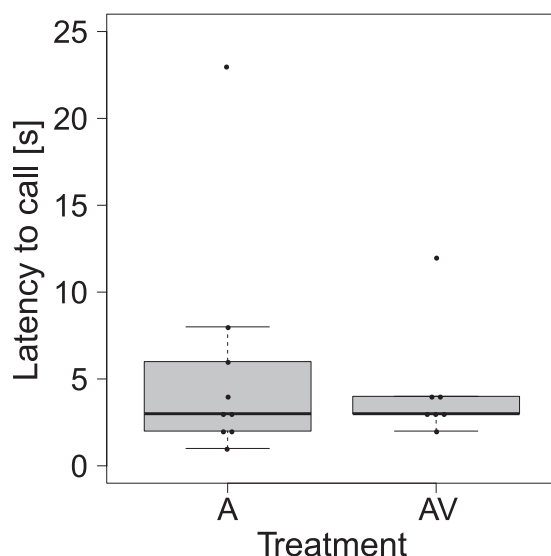
Figure 4. Pup latency to call during audio-only (A) and audio-visual (AV) treatments. Boxplots show the median, quartiles, and minimum and maximum values within the inter-quartile range. No significant difference was found between treatments.

Discussion

We showed that visual cues can be used by sea lion pups to distinguish between pups and adult females. However, we found no enhancement of the pups' response in the presence of multiple cues, with combined audio-visual cues having the same effect as audio-only presentations, and both prompting a stronger response than the visual-only treatment. Our findings demonstrate that class-level visual cues (i.e., indicating an adult female) are dominated by acoustic cues, and are not used by pups to facilitate reunion, with offspring relying mainly on information conveyed in acoustic cues for mother recognition.

Australian sea lion pups showed pronounced differences in response when presented with a range of objects that either simulated conspecifics or were not biologically relevant and were able to distinguish adult female morphs from pup morphs based solely on visual cues. Pups displayed vigilance when presented with female models, but returned to a resting state following pup model presentations. When presented with the control which had no biologically relevant cues, but which was novel, there was no distinct response, with pups either responding with vigilance or without in equal measure. Overall this clearly demonstrated that pups can visually distinguish broad age classes of conspecifics. Our visual models were indicative of an adult female and did not provide any cues that might be used for individual recognition. Yet overwhelmingly (75%) pups responded as they would to an unfamiliar female, with vigilance. Our experiment is the first step towards understanding information perceived by pups through visual cues. Although we were unable to test individual visual recognition, we demonstrate that visual cues provide a broad assessment of animals at least to a given sex/age class and to the presence/absence of an animal in close proximity to the receiver.

Being able to identify the correct age/sex class of an individual using broad-brush cues may be beneficial when attempting to find a specific individual within a colony, as it refines the search to a subset of animals. Australian sea lion pups produce more calls in response to calls of their mothers compared to that of other females[43]. We thus expected them to further increase call rates once a potential mother is within sight, as it would allow them limit energetic expenditure by increasing call rates only when chances of reunion are higher or decrease call rates if the model was visually identified as being non-mother. However, pups tested in our study showed no enhancement in behavioural response when presented with multimodal cues compared to unimodal ones. Pups produced a similar number of calls to the audio/visual stimulus as to the audio-only stimulus, with no vocal reaction to the visual-only cues. The absence of enhancement points to a lack of interaction between acoustic and visual cues and the pups' lack of use of class-level visual cues when identifying their mothers. Based on the response of pups to female models when testing the role of visual cues, we ruled out the possibility that the absence of enhancement was simply a result of the pups identifying the female models as non-mothers. In our experiment, pups showed vigilance when presented with female models. If the model was identified as a non-mother in the bimodal experiments, we would have expected a decrease in call production, which was not the case. Ruiz-Miranda[28] suggested that for goat kids, visual cues are more important than acoustic and olfactory cues. Only broad cues were tested (pelage colour) while acoustic cues were individually distinctive and olfactory cues were masked. Although the tested visual cues contained only broad information, they were of higher importance than individually distinctive acoustic cues, thus showing that even when broad, visual cues have the ability to induce increased response. In our study, adding the visual cue did not change the pups' response, therefore the most parsimonious explanation is that while pups are capable of differentiating classes of individuals based on visual cues, they do not use class-level visual cues in a multimodal context, suggesting the presence of other factors that limit the use of both cues simultaneously.

The active space of cues varies as a function of the characteristics of a given cue, its production and perception, as well as the environment through which it travels[2,49,50]. Acoustic cues are generally considered to function at long range and visual cues are classified as mid to short range cues[50]. Differences in cue active space are regarded to be one of the main factors favouring multimodal communication[50]. However, for otariid pups, the differences in active space of sensory cues are important in context of risk of injury, as females can be extremely aggressive towards non-filial pups that approach them[44,45,51]. In this case, differences in active space could limit the use of multiple modalities, as cues with a smaller range may require pups to come out of hiding and become exposed to getting attacked or trampled by other individuals, or if they approach an individual to obtain useful information it may put them at risk of injury. Consequently, it seems that pups rely on hearing – the one modality that allows them to acquire accurate and reliable information at long range[39,40] for the assessment of female identity prior to approach.

Munoz and Blumstein[9] proposed a framework within which there is a plausible explanation for the evolution of bimodal responses, from the cost-benefit perspective of the receiver. The authors define three predictions for multisensory integration: enhancement – when the costs of missing information are high and outweigh the costs of obtaining cues; antagonism – when combined cues point to a lower likelihood of an event; and equivalence/dominance – when obtaining more information is too costly and therefore multimodal cues are not used[9]. This framework may help explain why pups do not combine acoustic and visual cues, and the evolutionary significance of this choice. We found the pup response to the bimodal and acoustic presentations to be the same or higher than the response to visual-only cues, and from our visual experiment we know that visual cues can be used in age-class conspecific assessment. Accordingly, our results fit the equivalence/dominance scenario, suggesting that acquiring information conveyed in visual cues does not outweigh the cost of obtaining them. This might be due to the risks of obtaining useful information being high or to the information encoded within them not providing any more useful information than the acoustic cues. The evolutionary pressures and mechanisms for this scenario to evolve could be investigated in more detail. However, regardless of which explanation plays a larger role, our findings indicate that the costs associated with obtaining information limit the use of multimodal cues in mother recognition by pups, with the characteristics of female-pup interactions as well as the consequences of differences in cue active space discussed above, also supporting this argument.

We have demonstrated that although Australian sea lion pups have the ability to use visual cues for conspecific assessment, they are not used in a multimodal context and are dominated by acoustic cues. By allowing the offspring to obtain detailed information at a distance, the use of acoustic cues does not entail a risk of injury from non-mother females and provides a stable and reliable way of mother identification on their own. Although reliance on a single modality may be disadvantageous[8], we show that using cues in a multimodal context is not always beneficial, even when the risk of increased predation caused by eavesdropping and cue production costs are low or absent. The cost-benefit ratio of obtaining information seem to play a significant role in limiting the use of multimodal cues and this role in the evolution of communication systems should be examined in more detail.

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Author Contributions

All authors designed the study. K.W. wrote the main manuscript, conducted all statistical analyses and prepared figures. K.W. and I.C. conducted experiments in the field. All authors reviewed the manuscript.

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Appendix (Wildlife Ethics approval) of this thesis has been removed as it may contain sensitive/confidential content