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Discrimination of sex and reproductive state in koalas, *Phascolarctos cinereus*, using chemical cues in urine

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Although marsupials have well-developed olfactory systems and complex scent-marking behaviours, relatively little is known about the actual function of chemical signals in this group of mammals compared to eutherian species. In this study I investigated whether koalas are able to assess the sex and reproductive state of signallers using chemical cues present in urine. Male urine induced more chemosensory investigation by males and oestrous females than female urine, and nonoestrous female koalas displayed an aversion to male urine. When presented with oestrous versus nonoestrous female urine, males but not females displayed a significant investigatory preference for oestrous urine. Taken together these results indicate that koala urine contains chemical cues permitting the discrimination of sex and female oestrous stage. These findings also add to a growing body of literature showing that chemical cues serve to advertise female reproductive state in mammals, and the first clear evidence of this in a marsupial.

Chemical communication plays a key role governing many aspects of mammal social behaviour (Beauchamp & Yamazaki, 2003; Eisenberg & Kleiman, 1972; Petrulis, 2013). In reproductive contexts, chemical signals may be important for mate assessment, activating sexual motivation/physiology, and advertising female reproductive condition (Doty & Dunbar, 1974; Dunbar, 1977; Fadem, 1987; Ferkin, Gorman, & Zucker, 1991; Fisher, Swaisgood, & Fitch-Snyder, 2003; Karthikeyan et al., 2013; Rasmussen, Lee, Zhang, Roelofs, & Daves, 1997; Swaisgood, Lindburg, Zhou, & Owen, 2000). Furthermore, the ability to assess the sex and reproductive state of signallers via chemical cues could be especially important for solitary species that are required to locate and assess dispersed mating partners during the breeding season. For example, male giant pandas, *Ailuropoda melanoleuca*, and Indian rhinoceros, *Rhinoceros unicornis*, follow females for several days leading up to their fertile period (Laurie, 1982; Zhu, Lindburg, Pan, Forney, & Wang, 2001). In both these solitary species male reproductive success may hinge on the ability to avoid unnecessary confrontations with rival males and detect female reproductive stage via chemical cues, in order to concentrate reproductive efforts on oestrous females that are most likely to conceive. Numerous studies also show that female mammals are attracted to male scent (dogs, *Canis familiaris*, Dunbar, 1977; hamsters, *Mesocricetus auratus*, Johnston, 1979; meadow voles, *Microtus pennsylvanicus*, Ferkin & Seamon, 1987; rats, *Rattus norvegicus*, Gao, 1991; giant pandas, Swaisgood et al., 2000; black rhinoceros, *Diceros bicornis*, Linklater, Mayer, & Swaisgood, 2013), demonstrating that male chemical signals can also provide important information to female mammals about the location of potential mating partners.

Descriptive accounts of scent-marking behaviour in marsupials indicate that olfactory communication is also important in this group of mammals (Biggins, 1984; Croft, 1981; Eisenberg, Collins, & Wemmer, 1975; Ewer, 1968; Fadem & Cole, 1985; Kaufmann, 1974; Oakwood, 2002; Schultze-Westrum, 1969). Experimental studies that have systematically presented animals with scent stimuli show that marsupials can discriminate between male and female scent (Descovich, Lisle, Johnston, Nicolson, & Phillips, 2012; Zuri, Dombrowski, & Halpern, 2005), distinguish between the scent of different individuals (Walker & Croft, 1990) and also assess the relatedness of male signallers in mate choice contexts using chemical cues (Parrott, Ward, & Temple-Smith, 2007). In addition, female short-tailed opossums, *Monodelphis domestica*, are brought into oestrus by exposure to male scent (Fadem, 1987). Male short-tailed opossums, however, are not attracted to oestrous female urine (Zuri, Su, & Halpern, 2003), suggesting that urinary excretions do not advertise female reproductive condition in this Didelphidae marsupial species. Indeed, while it is often postulated that male marsupials use chemical cues to assess female reproductive state (Croft, 1981, 1982; Kaufmann, 1974; Oakwood, 2002; Walker & Croft, 1990), no evidence that males can actually

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discriminate between the scent of oestrous and nonoestrous females exists.

The koala is an arboreal marsupial that inhabits the *Eucalyptus* forest environments of eastern Australia (Lee & Carrick, 1989). As a relatively sedentary and solitary-living species, koalas are likely to depend on effective communication to coordinate their social lives. A suite of recent studies has confirmed the importance of vocal communication in this species' sexual communication, in particular, the information content and function of male bellowing (Charlton, Ellis, Brumm, Nilsson, & Fitch, 2012; Charlton, Ellis, Larkin, & Fitch, 2012; Charlton, Ellis, McKinnon, Brumm, 2011; Charlton, Ellis, McKinnon, Cowin, 2011; Charlton, Frey, et al. 2013; Charlton, Whisson, & Reby, 2013; Ellis et al., 2011). However, several observations also indicate that olfactory communication plays an important role in the koala's social organization.

Male koalas have prominent glands in the middle of their chest that they use to mark trees and substrates in their environment, usually when they are in unfamiliar surroundings or entering new trees (Mitchell, 1990; Smith, 1980a). Although no clear conclusions have been drawn on the function of this male scent-marking behaviour, it may play a role in regulating interindividual spacing or facilitating mate location (Salamon, Davies, & Stoddart, 1999). Males also urinate at the base of trees and dribble urine on the branches and trunks (Mitchell, 1990). This observation suggests that male koalas may be using urine to make their presence known to neighbouring individuals, allowing temporal and spatial relationships to be established without direct physical or visual contact. It is also possible that female urinary cues could serve to advertise sexual receptivity in this species. Chemical cues in urine are believed to be important for signalling female reproductive condition in other mammals (Johansson & Jones, 2007; Lydell & Doty, 1972; Randall, 1986; Rasmussen et al., 1997; Swaisgood, Lindburg, & Zhang, 2002), and captive oestrous female koalas are often observed to deposit small amounts of urine on branches after mounting females in their enclosures (during 'pseudosexual' behaviour; B. Charlton, personal observation), suggesting that they are using urinary cues to advertise their oestrous status and readiness to mate.

In this study I ran two separate experiments to investigate whether koalas can assess the sex and reproductive state of signallers via chemical cues in urine. In the first experiment I tested whether koalas could discriminate signaller sex solely on the basis of chemical cues in urine, and also whether olfactory investigation varies according to the sex and reproductive state of the signal receiver. I predicted that male koalas would show more interest in urine derived from male conspecifics because these individuals represent a potential threat. I also predicted that oestrous female koalas would display a heightened interest towards urine from males because they represent potential mating partners. In contrast, nonoestrous females are expected to show an aversion to male urine (by changing tree forks) because they are completely unreceptive to male advances during this stage of their reproductive cycle (Mitchell, 1990; Smith, 1980b). In the second experiment I presented koalas with urine from oestrous versus nonoestrous females to determine whether they could use cues present in urine to determine female reproductive stage. I predicted that male koalas would show greater interest in urine from oestrous females that are likely to be receptive to their copulation attempts. If males can extract information about female reproductive state using chemical cues in urine, they could use these olfactory abilities to target their reproductive attempts on oestrous females, and in doing so, maximize the likelihood of conception. Conversely, I did not expect female koalas to show a differential response when presented with urine from oestrous versus nonoestrous females because female reproductive stage is less likely to be relevant to their social interactions.

METHODS

Experimental Site and Animals

This study was conducted at Lone Pine Koala Sanctuary, Brisbane, Australia, during the 2013 breeding season (September–November). The study group for the first experiment consisted of 17 adult female koalas aged 4–10 years (mean = 6.9) and 12 adult male koalas aged 4–10 years (mean = 5.8). The subjects for the second experiment were 12 male koalas aged 4–10 years (mean = 6.3) and 12 female koalas aged 4–11 years (mean = 6.8). Subjects were used only once in the experiments. The koalas were fed a diet of *Eucalyptus* leaves once a day and housed with three to six other individuals in enclosures measuring approximately 5×3 m. All experimental tests were conducted while subjects resided in these enclosures.

Although several koalas were present in the enclosures during the experiments, the scent presentation tests were only conducted when all the animals were resting in separate tree forks. In addition, test subjects always had an unoccupied tree fork well within jumping range (<2 m) so that their movement (aversion) responses were not restricted during the experiments. Care was also taken to avoid conducting experiments during periods of increased activity when other individuals would be more likely to move around the enclosure and interfere with the test subject (for example, just after the daily feed had been introduced).

This study followed the ASAB/ABS guidelines for the use of animals in research, and was approved by the Animal Ethics Committee at the University of Sussex (ERC/34/E-CIRC/CHA).

Assessment of Female Reproductive Stage

The objective of experiment 1 was to systematically expose males, oestrous females and nonoestrous females to male and female urine. This required the accurate assessment of female reproductive stage. Behavioural indicators of oestrus in female koalas include bellowing, pseudomale behaviour (mounting other females and being mounted), jerking or convulsive behaviour and increased activity levels (Smith, 1980b). In the current study, females were only considered to be in oestrus when they showed all of the above signs on the day of the experiment. Females that displayed none of the above signs were considered to be nonoestrus. The above criteria were also used to identify oestrous and nonoestrous females to be used as urine donors.

Urine Collection

Observations of male and nonoestrous female koalas indicated that they often urinate on the ground, whereas oestrous females often urinate directly after mounting other females (B. Charlton, personal observation). Consequently, fresh urine was collected opportunistically in both of these behavioural contexts by placing a clean kidney dish under animals while they urinated. The urine was then transferred to a sterile plastic container and frozen at $-20\,^{\circ}C$ for up to 10 days prior to testing. To avoid any contamination during the urine collection the experimenter wore disposable gloves. A total of 40 adult koalas served as urine donors for the experiments: 15 males, 15 nonoestrous females and 10 oestrous females. The use of multiple urine donors allowed me to minimize the problem of pseudoreplication of experimental stimuli (Hurlbert, 1984).

Scent Presentation

The experiments were initiated when subjects were stationary and awake. Urine was thawed and allowed to reach ambient