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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 temperature (circa 30 °C on the days the experiments were conducted) before 1 ml was transferred to a sterile cotton swab measuring 5×5 cm using a clean syringe immediately prior to presentation. The swab was then attached to the end of a microphone stand (Proel, Italy). This allowed the scent stimuli to be presented in a controlled manner to koalas in their tree forks. To limit the possibility of cross-contamination the experimenter wore disposable rubber gloves that were replaced after each experimental trial. In addition, the end of the microphone stand was covered with a new piece of thin plastic film (Cling Film, U.K.) before the swab was attached at the onset of every trial.

Two separate experiments were conducted: In the first experiment I tested the ability of koalas to discriminate between male and nonoestrous female urine (hereafter referred to as 'female urine'); in the second experiment I presented koalas with urine from oestrous versus nonoestrous females to test the effect of the donor's oestrous state on chemosensory investigation. For experiment 1 the subjects were split into three groups: males, nonoestrous females and oestrous females. This allowed me to determine whether the response of receivers to male versus female urine differed across these categories. My primary aim in the second experiment was to test whether male koalas can discriminate between oestrous and nonoestrous female urine, and, hence, whether they could use urine to assess female reproductive state. However, I also tested the response of females to oestrous versus nonoestrous urine in order to confirm whether any differential responses displayed by male koalas were related to reproduction, and not just the result of an arbitrary preference.

During the discrimination trials subjects were presented with stimuli in a repeated measures design. For the sex discrimination task (experiment 1) subjects were first of all presented with either male or female urine. Five minutes later, subjects that had been presented with male urine were presented with female urine, and vice versa. This ensured that test stimuli from both conditions were presented at the same ambient temperature. The same approach was used to test for behavioural discrimination between urine from oestrous and nonoestrous females (experiment 2). At the beginning of each trial the microphone stand was used to place the scent stimuli approximately 15 cm from the subject's head. Each of the experimental trials then commenced upon the onset of investigation of the stimuli and continued for 2 min. The class of scent stimuli presented first was alternated across trials, and each subject received a unique combination of urine donors. To minimize familiarity with scent stimuli, individuals were not used as donors for trials with test subjects if they had been housed in the same enclosure. All behaviour during the 2 min experimental period was captured using a Sony hard drive digital camera (model DCR-SX65) mounted on a tripod.

Behavioural Analysis

The videotapes were analysed using Gamebreaker version 7.0.121 (SportsTec, Sydney, Australia). To assess the chemosensory response of subjects to the different classes of stimuli I measured the duration of each sniff given towards the swab, and also noted whether subjects changed tree forks during the 2 min experimental period. Sniffing was defined as starting when the subject placed its nose within 5 cm of the swab before maintaining a fixed head position, and ended when the head moved away from this first fixed position. All subjects had finished investigating the stimuli before the end of the 2 min observation period.

Statistical Analysis

Log_{10} transformations were used to normalize the data distribution for the total duration of sniffing (Kolmogorov–Smirnov test: $P > 0.05$) before linear and binary logistic generalized linear models (GLMs) fitted with maximum likelihood estimation were used to examine the data. For each experiment, scent type (male versus female urine, or oestrous versus nonoestrous female urine) was entered as a within-subjects factor and subject group (experiment 1: males, nonoestrous females and oestrous females; experiment 2: males and nonoestrous females) was entered as a between-subjects factor. The interaction term subject group*scent type was entered into the GLMs to determine whether different subject groups showed different patterns of discrimination. Separate GLMs were also run for each of the subject groups with scent type entered as a within-subjects factor. The statistical tests were conducted using IBM SPSS statistics version 20 for Mac OS X, and significance levels were set at 0.05.

RESULTS

Experiment 1: Discrimination of Signaller Sex

The total duration of sniffing (Wald $\chi^2_1 = 6.038$, $P = 0.014$) was significantly greater to male versus female urine. Although subject group did not affect the total time spent sniffing the test stimuli (Wald $\chi^2_2 = 0.342$, $P = 0.843$), a significant interaction between subject group and scent type for total sniffing duration (Wald $\chi^2_2 = 9.500$, $P = 0.009$) indicated that males, nonoestrous females and oestrous females showed different patterns of discrimination. When considered separately, males (Wald $\chi^2_1 = 4.212$, $P = 0.040$) and oestrous females (Wald $\chi^2_1 = 7.246$, $P = 0.007$) both spent more time sniffing male urine than female urine (Fig. 1a), whereas no significant difference in the amount of time spent sniffing male versus female urine was detected for nonoestrous females (Wald $\chi^2_1 = 1.498$, $P = 0.221$; Fig. 1a).

Koalas changed tree forks significantly more often when presented with male urine than female urine (Wald $\chi^2_1 = 13.142$, $P < 0.001$). Subject group did not affect whether subjects changed tree forks (Wald $\chi^2_2 = 0.573$, $P = 0.751$) and no interaction between subject group and scent type was detected (Wald $\chi^2_2 = 2.917$, $P = 0.233$). When the subject groups were considered separately, however, only nonoestrous female koalas were significantly more likely to change tree forks when presented with male than female urine (Wald $\chi^2_1 = 8.690$, $P = 0.003$; Fig. 1b). No significant difference in the tendency to change tree forks when presented with male versus female urine was detected for male (Wald $\chi^2_1 = 2.616$, $P = 0.106$) and oestrous female koalas (Wald $\chi^2_1 = 2.471$, $P = 0.116$; Fig. 1b).

Experiment 2: Discrimination of Female Reproductive Stage

The amount of time koalas spent sniffing the test stimuli did not differ significantly according to scent type (Wald $\chi^2_1 = 1.909$, $P = 0.167$); however, males spent more time investigating the test stimuli than females (Wald $\chi^2_1 = 6.058$, $P = 0.014$), and a significant interaction between subject group and scent type for the total amount of time spent sniffing was detected (Wald $\chi^2_1 = 7.531$, $P = 0.006$). When males and females were considered separately, male koalas spent more time sniffing urine donated by females in oestrus than nonoestrus (Wald $\chi^2_1 = 8.482$, $P = 0.004$; Fig. 2a). In contrast, female koalas did not discriminate behaviourally between oestrous and nonoestrous female urine (Wald $\chi^2_1 = 0.932$, $P = 0.334$; Fig. 2a).

When both sexes were included in the analysis, the tendency of koalas to change tree forks was not affected by scent type (Wald $\chi^2_1 = 0.970$, $P = 0.325$) or subject group (Wald $\chi^2_1 = 2.670$, $P = 0.102$). In addition, no interaction between subject group and scent type was observed (Wald $\chi^2_1 = 0.000$, $P = 0.987$). When the sexes were

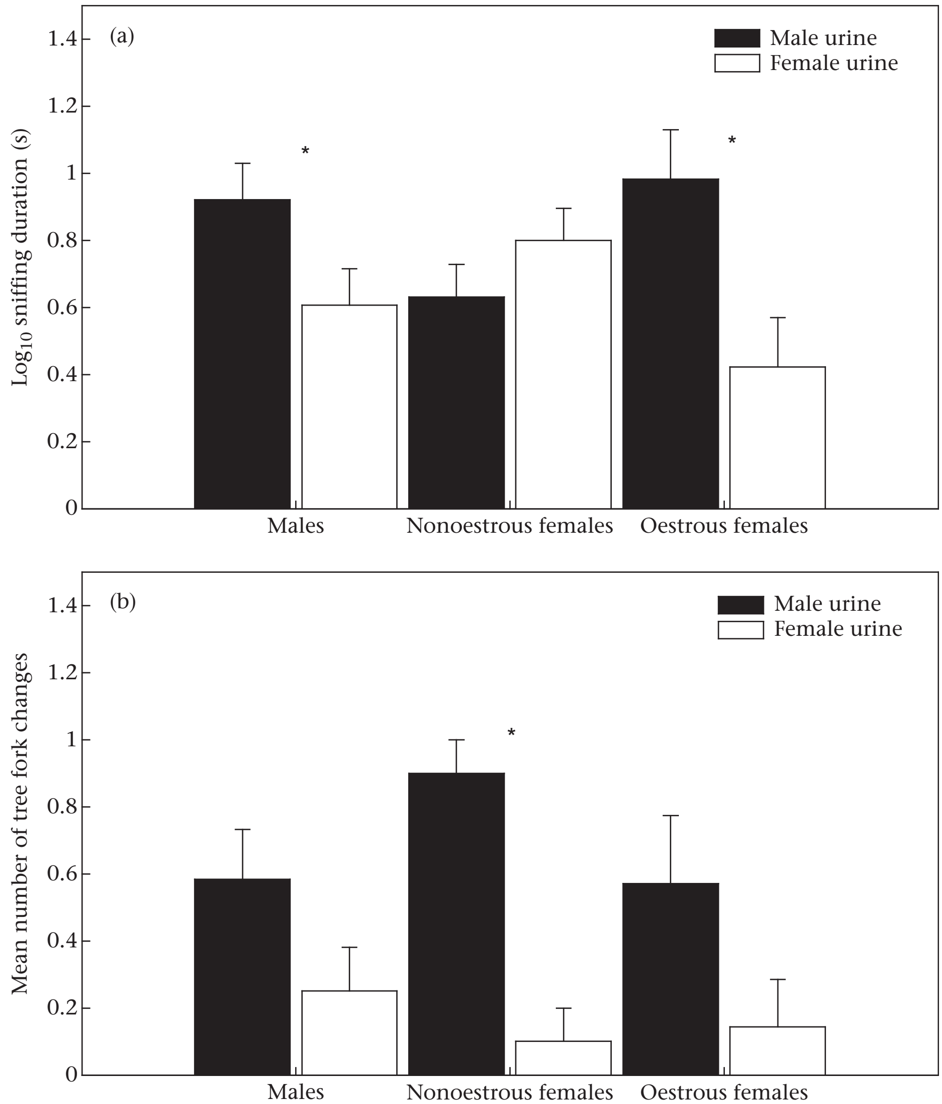

Figure 1. Discrimination of sex via urine. Error bar charts show means + SE of behavioural responses to male versus female urine ($N = 29$): (a) sniffing duration of males ($N = 12$), nonoestrous females ($N = 10$) and oestrous females ($N = 7$); (b) number of times males ($N = 12$), nonoestrous females ($N = 10$) and oestrous females ($N = 7$) changed tree forks. $^{*}P < 0.05$.

considered separately, the test stimuli presented had no effect on the tendency of male (Wald $\chi^2_1 = 0.368$, $P = 0.544$) or female (Wald $\chi^2_1 = 0.738$, $P = 0.390$) koalas to change tree forks (Fig. 2b).

DISCUSSION

This study shows that koalas can assess the sex and oestrous stage of signallers using chemical cues present in urine. There are three main results generated by the olfactory discrimination tests. First, male and oestrous female koalas showed more interest in male urine than nonoestrous female urine. Second, nonoestrous female koalas displayed a strong aversion to male urine. The final result is that male (but not female) koalas showed more investigatory behaviour towards oestrous female urine than nonoestrous female urine, suggesting that they use urinary cues to assess female reproductive state during the breeding season.

Discrimination of Signaller Sex

In line with the predictions, both male and oestrous female koalas showed more interest in male urine than nonoestrous female urine. The greater interest displayed by male koalas to male urine may reflect the importance of male–male competition in this species. Male koalas could assess the sex of conspecifics using urine that has been deposited at the base or on the branches of trees before risking a direct encounter with an unfamiliar rival male that would represent a potential threat to them. The preferential investigation of male urine by oestrous female koalas that was absent in nonoestrous females may indicate that they use urine to identify potential mates. Indeed, female koalas are only receptive to male copulation attempts when they are in oestrus (Smith, 1980b), whereas nonoestrous females reject male advances, with aggression and loud snarl, squark or wail vocalizations (Mitchell, 1990).

Furthermore, male koalas can be aggressive towards nonoestrous females that reject their advances (Smith, 1980c) and, hence, are likely to present a threat to them. Female koalas would therefore be expected to minimize the costs of associating with males when they are not in oestrus. Indeed, nonoestrous females showed a clear aversion to male urine by consistently moving tree forks when presented with this class of stimuli. These findings in nonoestrous females lend further support to the notion that

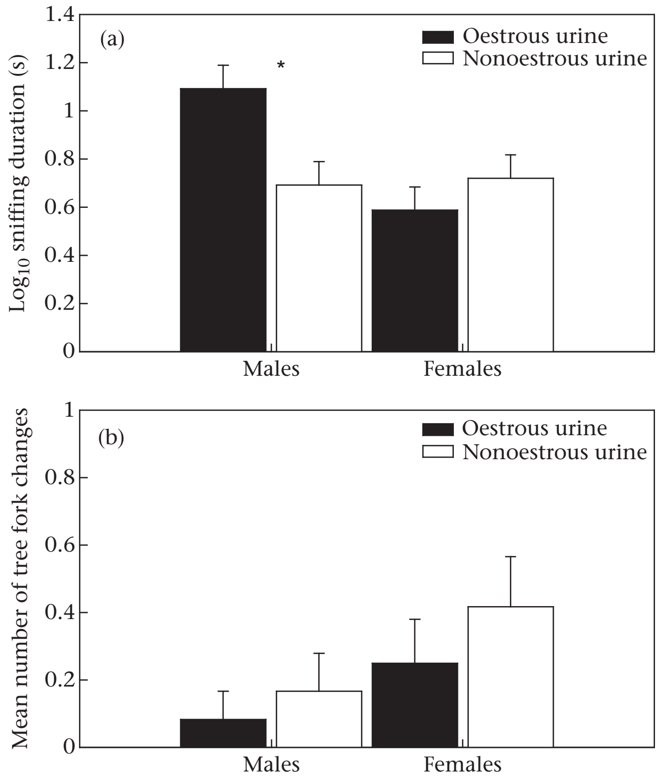

Figure 2. Discrimination of female reproductive state via urine. Error bar charts show means + SE of behavioural responses to oestrous versus nonoestrous female urine ($N = 24$): (a) sniffing duration of males ($N = 12$) and females ($N = 12$); (b) number of times males ($N = 12$) and females ($N = 12$) changed tree forks. $^*P < 0.05$.

females use urinary cues to identify potential mates when they are in oestrus, and facilitate the earlier detection and avoidance of males outside of this time. In addition, they emphasize that female responses appear to be sensitive to functionally relevant factors and are unlikely to be explained by an arbitrary preference or aversion for male urine.

Koalas may also use cues present in urine to assess male hormonal quality. For instance, because testosterone is an important component of male competitive ability and sperm quality in mammals (Minter & DeLiberto, 2008; Zielinski & Vandenbergh 1993), urinary cues to male testosterone levels may be important in this species' sexual communication for intrasexual assessment and/or mate choice, respectively. Male and female meadow voles both spend more time investigating scent from males with higher testosterone levels (Ferkin, Sorokin, Renfroe, & Johnston, 1994). Female preferences for the urinary odours of males treated with high dosages of testosterone versus males treated with low dosages of testosterone are also documented in other rodents (Taylor, Haller, & Regan, 1982; Taylor, Regan, & Haller, 1983; White, Fischer, & Meunier, 1984). Accordingly, future studies on koalas should attempt to isolate cues to male testosterone levels in urine, and also quantify the response of male and oestrous female koalas to urine from males with high versus low testosterone.

Discrimination of Female Reproductive Stage

The results from this study demonstrate that male koalas can discriminate female reproductive stage via urinary chemosignals. Oestrous female urine induced more chemosensory investigation by males than nonoestrous female urine. In contrast, female koalas did not show a differential response when presented with oestrous versus nonoestrous urine. It is possible that female koalas can still distinguish between oestrous and nonoestrous urine but the reproductive state of other females is not relevant to their social interactions and, therefore, does not warrant differential investigation. Accordingly, these results provide convincing evidence that the investigatory preference of male koalas for oestrous urine is related to reproduction, and are consistent with the hypothesis that female urinary cues serve to advertise sexual receptivity to male koalas.

Observations of other marsupial species suggest that males may be using chemical cues to assess female reproductive state (Croft, 1981, 1982; Kaufmann, 1974; Walker & Croft, 1990). For example, male northern quolls, *Dasyurus hallucatus*, are thought to monitor female scent marks and scats for the onset of oestrus because they regularly visit several females during the breeding season (Oakwood, 2002). This assumption, however, is almost entirely based on the fact that females increase their levels of scat production during the breeding season, and not on any observed differential behaviour when males are exposed to oestrous versus nonoestrous scent. To my knowledge, the findings of the current study provide the first unequivocal evidence that a male marsupial can actually discriminate between oestrous and nonoestrous females using chemical cues.

The ability to assess female reproductive condition using urine and the use of urine to advertise oestrous stage may play an important role governing the reproductive success of male and female koalas, respectively. Males could use chemical cues in urine to target their reproductive attempts on oestrous females to maximize the chances of fertilization. In addition, by signalling their oestrous status female koalas would encourage male–male competition, and in doing so, ensure insemination by a high-quality mate that can outcompete other rivals. Male koalas will also attempt to copulate with females that are not in peak oestrus (Mitchell, 1990; Smith, 1980b), and this has led to the conclusion that they cannot discern female reproductive state. It is possible, however, that males are sensitive to increased levels of oestrogen metabolites in urine before females are in peak oestrus and receptive to copulation attempts. This could prove to be adaptive for female koalas by serving to recruit males and activate sexual motivation before they enter peak oestrus (Matochik, White, & Barfield, 1992; Swaisgood et al., 2000), leading to intense male–male competition and increasing the likelihood of mating with a high-quality male (Cox & Leboeuf, 1977).

Future studies should determine the chemical constituents in urine that vary according to female oestrous stage. Blood oestradiol levels increase markedly in female koalas as they start to show behavioural signs of oestrus (Johnston, McGowan, O'Callaghan, Cox, & Nicolson, 2000), and this seems likely to cause a concomitant rise in urinary oestrogen metabolites (Hindle, Mostl, & Hodges, 1992; Murata, Masayuki, & Noboru, 1986). Once the chemical cues of oestrus have been isolated, synthetic analogues could be used in further experiments (sensu Jemiolo, Alberts, Sochinskiwiggins, Harvey, & Novotny, 1985; Rasmussen, Lee, Roelofs, Zhang, & Daves, 1996; Rasmussen et al., 1997) to investigate how this information mediates male sexual behaviour, the distances over which this information is perceived and the time periods over which the information remains biologically active.

In conclusion, the findings of the current study indicate that koalas use chemical signals present in urine to identify potential rivals and mates. They also add to a growing body of literature showing that female mammals advertise reproductive state via chemical cues (Doty & Dunbar, 1974; Dunbar, 1977; Ferkin et al., 1991; Gildersleeve, Haselton, Larson, & Pillsworth, 2012; Jemiolo et al., 1995; Karthikeyan et al., 2013; Lydell & Doty, 1972; Rasmussen et al., 1997; Swaisgood et al., 2002) and, to my knowledge, provide the first clear evidence that a marsupial species can discriminate female oestrous stage using chemical signals. Future studies should examine how the chemical profile of koala urine varies according to the sex and reproductive state of signallers, and investigate whether male and female koalas use male urine to assess the hormonal quality of potential rivals and mating partners, respectively. Odours play a critical role in the behaviour and physiology of many species (Beauchamp & Yamazaki, 2003; Eisenberg & Kleiman, 1972; Petrulis, 2013). The findings of the current study not only further our understanding of koala chemical communication, but also emphasize how olfactory discrimination tests can help to untangle some of the proximate mechanisms underlying observed social behaviour in solitary species that are often difficult to study in the wild.

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