

## Spontaneous discrimination of urine odours in wild African lions (*Panthera leo*)

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1 **Spontaneous discrimination of urine odours in wild African lions (*Panthera***  
2 ***leo*)**

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20 Olfactory communication is the primary mode of communication for many mammals, yet  
21 research on this form of signalling is still largely descriptive in most species. Thus, despite  
22 the apparent importance of scent-marking in the social lives of wild felids, experimental  
23 studies directly investigating the function of olfactory communication are lacking. We  
24 conducted scent presentation experiments to investigate whether wild African lions can  
25 discriminate another lion's social group and sex from a sample of its urine. Our results  
26 indicated that lion urine has the potential to signal depositor sex and social group, and that  
27 lions can use urine to discriminate males from females and residents from non-residents. The  
28 response of lions to urine was also dependent on both the sex and age of the subject receiving  
29 the presentation. Female lions responded less frequently to urine from resident females than  
30 to either non-resident females or resident males. Males responded more strongly to urine  
31 from resident males than resident females, but did not appear to differentiate urine from non-  
32 resident and resident females. Observations of flehmen and further scent-marking responses  
33 from lions provide additional evidence that lion urine functions in scent-marking. These  
34 results establish that urine scent-marks contain sufficient information for receivers to  
35 discriminate the sex and social affiliation of the signaller, and are the first to experimentally  
36 demonstrate the functional relevance of scent-marking in African lions.

37

38 Keywords: Felids; Group discrimination; Mammals; Olfactory communication; Scent-  
39 marking; Scent presentation; Sex discrimination.

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43 Olfactory communication is the primary means of communication among many mammals  
44 (Brown and MacDonald, 1985). Semiochemicals in urine, faeces and glandular secretions  
45 play a pivotal role in mediating social and sexual interactions (Bradbury and Vehrencamp,  
46 1998; Wyatt, 2003; Brennan and Kendrick, 2006; Apps, 2013). The ability to assess the sex  
47 and residence status of conspecifics through scent-marks may be particularly adaptive for  
48 wide-ranging territorial species that are under intense selection pressure to deter territorial  
49 intruders and locate mating partners (Bradbury and Vehrencamp, 1998). These selection  
50 pressures should apply in many felid species (Kleiman and Eisenberg, 1973), but little is  
51 known about the function of felid scent-marking in the wild (Soso, Koziel, Johnson, Lee and  
52 Fairbanks, 2014; Vogt, Zimmermann, Kölliker and Breitenmoser, 2014; Allen, Wallace and  
53 Wilmers, 2015; Vogt, Boos, Breitenmoser and Kölliker, 2016).

54 It is thought that felids make extensive use of olfactory communication (Kleiman and  
55 Eisenberg, 1973). Cats have numerous specialised scent glands and also use urine for scent-  
56 marking (Brown and Macdonald, 1985; Asa, 1993; Mellen, 1993; Pageat and Gaultier, 2003;  
57 Vogt et al., 2016). Both male and female cats either spray urine backwards onto vegetation,  
58 or rake their feet through urine on soil (Eaton, 1970; Kleiman and Eisenberg 1973; Verberne  
59 and De Boer, 1976). Although olfactory communication is apparently important in felid  
60 social systems, research results are scarce because cats are elusive and nocturnal (Vogt et al.,  
61 2014). Whilst detailed data on marking behaviour is accumulating for wild felids (e.g. Vogt  
62 et al., 2014; Allen, Whittmer and Wilmers, 2014; Allen et al., 2015; Allen, Yovovich and  
63 Wilmers, 2016), experimental investigations of the response of felids to potential scent-marks  
64 have been limited to the puma (*Puma concolor*: Allen et al., 2014). To understand the  
65 functions of scent-marking, we need to consider the information content of olfactory signals  
66 and investigate the responses of individuals to scent-marks. Previous behavioural (Schaller,  
67 1972; Asa, 1993; Brahmachary and Singh, 2000; Lehmann, Funston, Owen and Slotow,

68 2008; Barja and Miguel, 2010; Gilfillan, McNutt, Vitale, Iongh and Golabek, 2016) and  
69 chemical (Andersen and Vulpius, 1999; McLean, Hurst, Gaskell, Lewis and Beynon, 2007;  
70 Umapathy et al., 2007; Poddar-Sarkar, Chakroborty, Bhar and Brahmachary, 2008;  
71 Umapathy, Kumar, Kabra and Shivaji, 2013) work has suggested that scent-marking plays an  
72 important role in the social and sexual behaviour of lions (*Panthera leo*). For example,  
73 chemical analysis indicates the potential for lion urine to signal individual identity and sex  
74 (Andersen and Vulpius, 1999). We therefore conducted scent presentation experiments to  
75 investigate whether wild lions can discriminate another lion's sex and social group from a  
76 sample of its urine.

77 Lions live in a fission-fusion social system in which group members collectively defend  
78 territories or access to mates from other same-sex groups (Schaller, 1972; Packer, Scheel and  
79 Pusey, 1990; Grinnell, Packer and Pusey, 1995). Maintaining territories is essential for  
80 breeding, with fights between territory rivals often leading to injury and death (Schaller,  
81 1972; Packer et al., 1990; Heinsohn and Packer, 1995). Lions can detect potential mates and  
82 territorial threats from the long-distance calls of conspecifics based on caller sex, familiarity  
83 and group membership (McComb, Pusey, Packer and Grinnell, 1993; McComb, Packer and  
84 Pusey, 1994; Grinnell et al., 1995). We hypothesised that olfactory communication in lions  
85 will also extend to sex and social discrimination. We predicted that lions presented with urine  
86 from resident adult males and females, will be more likely to respond (e.g. sniff, lick or  
87 overmark the urine), and will spend longer responding to urine from opposite sex  
88 conspecifics. In this way, lions could assess the reproductive condition of mates (Charlton,  
89 2014; Tinnesand et al., 2015). We expected that olfactory sex discrimination will be most  
90 pronounced in adult lions than in sexually immature subadults and cubs. With the ability to  
91 discriminate between the scents of resident and non-resident individuals, females could detect  
92 the presence of potential rivals for their territory. We predicted that female lions will be more

93 likely to respond, and will spend longer responding to urine from non-resident females than  
94 from resident females. In contrast, male lions would not show a strong difference in response  
95 to resident and non-resident female urine as both signal the presence of a potential mate.  
96 Since lions of all ages are at risk during territory take-overs, we hypothesised that there  
97 should be no clear age differences in the ability to discriminate the urine of resident from  
98 non-resident females.

## 99 **METHODS:**

### 100 *Study Population and Site:*

101 The study area (ca. 1500 km<sup>2</sup> 19°31'S, 23°37'E; elevation ca. 950 m) was bordered by the  
102 Okavango Delta and included the Moremi Game Reserve and its surrounding Wildlife  
103 Management Areas (for further details see: McNutt, 1996). Life histories and demographic  
104 data were available on the study population since 2007, and all individuals were habituated to  
105 close approaches by vehicles. Lions were located using spoor- or radio-tracking, with up to  
106 three individuals in each social group fitted with VHF-GPS radio collars (Royal Veterinary  
107 College, U.K; < 1080 g) or VHF radio collars (African Wildlife Tracking, Pretoria, South  
108 Africa; < 780 g). Individual lions were identified from their unique whisker-spot patterns, and  
109 were divided into demographic categories based on their sex and age, with age categories  
110 defined as follows: cubs (below 18 months), subadults (18-48 months), adults (four years and  
111 above) (Packer et al., 1988). Where the birth date was not known, the individual was aged  
112 using body size, teeth wear, male mane development, and the observation of sexual activity  
113 (Whitman and Packer, 2007).

114 Sixty-eight scent presentation experiments were conducted between May 2014 and December  
115 2015 on 52 free-ranging lions from four female prides and three male coalitions. A pride was  
116 defined as a minimum of two sexually mature females that defend a shared territory, and

117 includes all offspring of pre-dispersal age. A coalition was defined as a group of post-  
118 dispersal males who attempt to associate with female prides (Schaller, 1972).

119 *Scent Sample Collection:*

120 Urine-soaked soil deposited with the following five body postures was collected for  
121 presentation: 1) Spray: backwards spray onto vegetation and surrounding soil whilst standing  
122 ( $N = 5$  samples), 2) Scrape: downwards spray onto soil whilst scraping the urine soaked soil  
123 with hind paws ( $N = 28$ ), 3) Squat: urination onto soil with genitals lowered to soil and no  
124 paw scraping ( $N = 22$ ), 4) Stand: urination onto soil whilst standing with no paw scraping ( $N$   
125  $= 5$ ), and 5) Lie: urination onto soil whilst lying down ( $N = 1$ ). In the statistical analyses (see  
126 below), we further grouped the body postures of the urinating lions into ‘urine-only’ postures  
127 (spray, squat, stand and lie postures) and scrape postures. In contrast to urine-only postures,  
128 scrape postures could involve deposits of both urine and secretions from pedal (feet) glands.  
129 Pedal glands are known to deposit important scent-marks for some mammals (white tailed  
130 deer (*Odocoileus virginianus*): Gasset et al., 1996; polar bear (*Ursus maritimus*): Owen et  
131 al., 2015; brown bear (*Ursus arctos*): Clapham, Nevin, Ramsey and Rosell, 2014), and felids  
132 also have inter-digital glands and sweat glands on their feet (Pageat and Gaultier, 2003). It is  
133 not known whether lions deposit semiochemicals from pedal glands.

134 Urine was collected from lions in different social (alone or in a group), sexual (oestrous or  
135 non-oestrous), and activity states (resting or mobile). Urine-moistened soil was collected  
136 using a clean metal spoon and placed into sterilised glass jars with aluminium foil-lined lids.  
137 Following collection, all samples were immediately stored in a 12 V cooler box (to keep  
138 contents 15 °C below the ambient temperature), and were later (within a few hours) frozen at  
139 -20 °C until thawed for presentation (average number of days frozen before presentation  $\pm$   
140 SD =  $129 \pm 112$  days; see discussion for the effect of freezing). Sixty-one urine samples from  
141 20 adult lions were used. Non-resident urine (see below) was collected from lions in social

142 groups with territory directly adjacent to the subjects receiving the presentation (determined  
143 through GPS radio-telemetry collars: Gilfillan, n.d).

144

145 *Experiment Procedure:*

146 Before an experiment, a clean metal spoon was used to place approximately 80 g of the  
147 frozen sample into a sterilised glass jar (with aluminium foil-lined lid). The sample was then  
148 allowed to thaw. A resting group of lions was approached with a vehicle. From the vehicle  
149 the urine sample was tipped out of the jar in a single pile on top of short grass or bare soil 7-  
150 25 m from the lions, and the vehicle was reversed away. Lions that moved to within 2 m of  
151 the sample were videoed with a Bell + Howell DNV16HDZ (North Carolina, U.S.A) digital  
152 camcorder. Observations stopped when the lions moved away from the area (Figure 1). Scent  
153 presentation experiments were conducted within two hours of sunset, when lions begin to  
154 become active. The sample was not re-collected. We put the samples upwind of the lion  
155 group as a whole, but for an observer sitting in an open-top vehicle judging whether any  
156 individual lion was downwind at any particular time was impossible.

157 To test whether lions can discriminate another lion's social group and sex from a sample of its  
158 urine, we presented three urine treatments from the following donors: 1) Absent adult female  
159 residents ("Female Resident"), 2) Absent adult male residents ("Male Resident"), and 3)  
160 Adult female non-residents ("Female Non-Resident"). Since the non-resident individual in  
161 treatment 3 was never present, urine samples from absent group members were used in  
162 Treatments 1 and 2 so that donors were absent in all three treatments. To further standardise  
163 the presentations, urine samples were presented to unisex (i.e. all adults were of the same sex)  
164 lion groups (two or more adult lions) that were not feeding. Two presentations (out of 68;  
165 2.9%) were made to a nomadic coalition of two males who were attempting to take over a



166 pride. Only one sample was presented during each trial, but 98.1% of subjects were presented  
167 with at least two treatment categories spread over the study period, and 55.8% were presented  
168 with all three. Whilst no control treatment was used, the experimental design controlled for  
169 the handling, storing and presentation methods by comparing the response of lions to  
170 different treatments, whilst also largely controlling for individual differences between lions  
171 by using a repeated measures design.

172

173 *Ethical Note:*

174 The collaring of lions and presentation experiments were performed under permits granted by  
175 the Botswana Ministry of Environment Wildlife and Tourism (8/36/4 XXV (8)), and the  
176 University of Sussex (Non-ASPAs 4 – November 2013).

177 *Behavioural Analysis of Response:*

178 Video recordings of lion responses (Table 1) were analysed frame-by-frame (frame =  
179 0.033/0.034 s) on a Fujitsu Siemens Amilo Pi2515 (Munich, Germany) laptop using  
180 Avidemux 2.6.9 (Mean Development Team, 2015) video analysis software. Distances  
181 between each lion and the urine were estimated by sight during the experiment.

182 *Statistical Analyses:*

183 All statistical analyses were conducted using the R statistical software (v. 3.3.1; R Core  
184 Development Team, 2016). Fisher's exact tests were used to compare the likelihood that lions  
185 of different age or sex would countermark or perform flehmen when responding to the urine.  
186 Where multiple comparisons were performed, the Bonferroni correction was applied.  
187 To assess what factors determine whether lions responded to urine samples, we ran a series of  
188 generalised linear mixed models (GLMMs) with a binomial error distribution (0 = sample

189 ignored, 1 = sample investigated/countermarked/overmarked) and a logit link function. The  
190 identity of the test subject ( $N = 52$ ) was included as a random term to account for multiple  
191 data from the same lions. Regarding the fixed effects, the global model was coded as follows:  
192 Respond or not  $\sim$  Treatment + Recipient Age + Recipient Sex + Number of Days Frozen +  
193 Time Between Deposition and Collection (mean  $\pm$  SD =  $18.8 \pm 18.1$  minutes, range = 5-72  
194 minutes) + Donor Posture (urine-only posture = 33 samples; scrape posture = 28 samples) +  
195 Closest Distance to Sample (m, see Table 1) + Treatment \* Recipient Sex + Treatment \*  
196 Recipient Age. To assist model convergence, the number of days each sample was frozen  
197 before presentation, and the time between deposition and collection were scaled and centered  
198 (using the 'scale' function in R) prior to running the models. The fixed effects were not  
199 strongly correlated (measured using the variance inflation factor and the Condition Number  
200 test). The GLMMs were run using the 'lme4' package (Bates, Maechler, Bolker and Walker,  
201 2015), and the dredge function in the 'MuMIn' package (Barton, 2016) was used to create a  
202 list of candidate models from the global model. We used Akaike's information criterion with  
203 a correction for small sample sizes (AICc) for model selection, where lower AICc values  
204 corresponded with better support for a given model. Akaike weights were calculated to show  
205 the relative importance of each candidate model (Akaike, 1974). Conditional model  
206 averaging (MuMIn package) was performed on the models within 2 AICc of the optimal  
207 model (lowest AICc) to extract averaged parameter estimates and their 95% confidence  
208 intervals (Symonds and Moussalli, 2011). Tukey's HSD post-hoc tests were run on the  
209 optimal model using the 'multcomp' package (Hothorn, Bretz and Westfall, 2008).

210 For those individuals that responded to the urine, we ran a series of generalised linear mixed  
211 models (GLMMs) with a Gaussian error distribution to assess what factors determine the  
212 duration of response (seconds). The cube-root transformation was applied to the response  
213 duration to correct for non-normality in the response variable. Regarding the fixed effects, the

214 global model was coded as follows: Treatment + Recipient Age + Recipient Sex + Number of  
215 Days Frozen + Time Between Deposition and Collection + Donor Posture + Treatment \*  
216 Recipient Age + Treatment \* Recipient Sex. The fixed effects were not strongly correlated.  
217 We incorporated the identity of the test subject ( $N = 36$ ), the sample number ( $N = 31$ ), and  
218 the recipient's social group ( $N = 7$ ) as random terms to account for multiple data from the  
219 same lions, when presented with the same sample of urine. As above, model averaging was  
220 performed on candidate models using AICc. Tukey's HSD post-hoc tests were run using the  
221 'multcomp' package on the optimal model.

## 222 **RESULTS:**

223 Thirty-six lions (out of 52 lions; 69.2%) responded to at least one urine sample. The average  
224 ( $\pm$  SD) distance from which lions first appeared to detect the urine (as evidenced by changing  
225 the position of the head in the direction of the sample location) was  $2\text{ m} \pm 4\text{ m}$  (range: 0-20  
226 m, mode: 1 m), but since this can be difficult to measure, lions were scored as investigating  
227 the sample only when within 0.5 m of the sample (consistent with Jordan, Golabek, Apps,  
228 Gilfillan and McNutt 2013; Allen et al., 2014; Allen et al., 2015). A typical response (93.1%  
229 of responses) involved sniffing the sample, with flehmen following sniffing in 59.7% of  
230 cases. The average ( $\pm$  SD) duration of response was  $35.3 \pm 34.1$  seconds. Overmarking was  
231 never observed, whilst countermarking was observed nine (12.5%) times (resident female  
232 urine = 4, non-resident female urine = 4, and resident male urine = 1). Countermarking  
233 involved defecation twice (22.2% of countermarks) and urination seven times (77.8% of  
234 countermarks), all within 2 m of the urine (Table 2). The average ( $\pm$  SD) time it took the  
235 urine to elicit a response from a lion was  $27 \pm 20$  minutes. The average ( $\pm$  SD) time that  
236 individual lions ignored the urine (irrespective of whether other group members responded)  
237 was  $66 \pm 18$  minutes.

238 When responding to urine, males and females were as likely to flehmen (Two-tailed Fisher's  
239 exact test:  $P = 0.106$ ), and to countermark the sample (Two-tailed Fisher's exact test:  $P =$   
240  $0.999$ ). Adults were significantly more likely to countermark the urine than either subadults  
241 (Two-tailed Fisher's exact test with Bonferroni correction of alpha:  $P < 0.001$ ), or cubs  
242 (Two-tailed Fisher's exact test with Bonferroni correction of alpha:  $P < 0.001$ ). However,  
243 there was no significant age-class difference in the likelihood of performing flehmen after  
244 investigating the urine (Two-tailed Fisher's exact test:  $P = 0.950$ ).

245 The optimal model of the likelihood of lions responding to the urine included the closest  
246 distance the lion moved to the sample, the donor posture, the age of the recipient, the sex of  
247 the recipient, the urine treatment, and the interaction between recipient sex and urine  
248 treatment. Model averaging of the best GLMMs revealed that the closest distance the lion  
249 moved to the sample, the donor posture, the age of the recipient, and the interaction between  
250 recipient sex and urine treatment are the best predictors of whether or not test subjects  
251 responded (Table 3). Post-hoc tests revealed that females were significantly less likely to  
252 respond to resident female urine than either resident male urine ( $P = 0.008$ ; Figure 2; Table  
253 4), or non-resident female urine ( $P = 0.039$ ; Table A2 in Appendix). The likelihood of males  
254 responding was independent of both the donor's sex ( $P = 0.869$ ) and social group ( $P =$   
255  $0.409$ ). Adults were as likely to respond as subadults ( $P = 0.150$ ) or cubs ( $P = 0.729$ ), but  
256 subadults were significantly more likely to respond than cubs ( $P = 0.027$ ; Table A3 in  
257 Appendix). Lions were more likely to respond to urine deposited with a scrape posture (45  
258 out of 152 trials; 29.6%) compared to a urine-only posture (27 out of 167 trials; 16.2%).  
259 Finally, the time between urine deposition and collection, and the number of days the urine  
260 was frozen before presentation were weak predictors of response likelihood (relative  
261 importance  $< 0.5$ ; as in Dala-Corte, Becker and Melo, 2016): lions were more likely to  
262 respond to urine that had been collected more quickly after deposition, and were less likely to

263 respond to urine that had been frozen for longer.

264 Regarding the duration of response of lions to the urine, the optimal model included the sex  
265 of the recipient, the urine treatment, and the interaction between recipient sex and urine  
266 treatment. Model averaging on the best GLMMs revealed that the urine treatment, the sex of  
267 the recipient, and the interaction between recipient sex and urine treatment are the best  
268 predictors of response duration (Table 5). Post-hoc tests revealed that males responded for  
269 longer to urine from resident males than from resident females ( $P = 0.017$ ; Figure 3; Table 4),  
270 but had similar response durations to urine from resident and non-resident females ( $P =$   
271  $0.995$ ; Table A5 in Appendix). The response duration of females was independent of both the  
272 donor's sex ( $P = 0.994$ ), and social group ( $P = 0.844$ ). Time between urine deposition and  
273 collection, and the number of days the urine was frozen before presentation were weak  
274 predictors of response duration: lions responded for longer to urine that was collected more  
275 quickly after deposition, and to urine that had been frozen for longer.

## 276 **DISCUSSION:**

277 Our scent presentation experiment indicated that lion urine has the potential to signal  
278 depositor sex and social group, and that lions can use urine to discriminate males from  
279 females and residents from non-residents. The response of lions to the urine also depended on  
280 both the sex and age of the subject receiving the presentation. These results provide evidence  
281 that lions perceive certain urine deposits as scent-marks with a communicatory significance.

282 Female lions were more likely to respond to resident male urine than to resident female urine.  
283 Females cooperate to defend cubs and territory and often hunt together (Packer et al., 1990).  
284 In contrast, male lions typically take food from females (Schaller, 1972) and consequently a  
285 high level of aggression can be seen between females and resident males (pers. obs.). It may  
286 be adaptive for females to use olfactory cues such as urine to monitor the movement and

287 presence of males, potentially allowing them to avoid moving through and hunting in areas  
288 with signs of male presence. Infanticidal non-resident males also pose a significant threat to  
289 females and their cubs (Pusey and Packer, 1994), and it may be adaptive for females to  
290 respond to male urine in order to establish the residency status (and thus risk of infanticide)  
291 of the urine donor. We were unable to test this hypothesis directly, but we predict that female  
292 lions will show elevated responses towards non-resident male urine compared to resident  
293 male urine.

294 We had hypothesised that male lions would show stronger responses towards resident female  
295 urine, but males responded more strongly to resident male urine. These results could reflect  
296 the fact that we had insufficient data to test for a three-way effect of recipient age, recipient  
297 sex and urine treatment. It seems likely that adult and sexually immature (subadults and cubs)  
298 males will respond differently to urine. Male lions disperse at sexual maturity (Schaller,  
299 1972). Adult males are aggressive towards related juvenile males (pers. obs.), and unrelated  
300 adult males pose a significant infanticidal threat to juveniles. Consequently, juvenile males  
301 could be expected to show strong responses to male urine.

302 We would still expect adult males to show stronger responses to female urine than resident  
303 male urine. Competition between adult males for reproductive opportunities is intense (West  
304 and Packer, 2002). Males are attracted to the urine of oestrous females in some mammals  
305 (e.g. Swaisgood, Lindburg, and Zhang, 2002; Charlton, 2014). We were unable to account for  
306 the oestrous state of female urine donors in our experiment, and urine from anoestrus females  
307 could be less interesting to males (Charlton, 2014). Keeping track of male companions  
308 (potentially through scent-marking) is also likely to be a key aspect of coalition formation  
309 and function, enabling males to cement and maintain social bonds with their coalition  
310 partners. Coalition cohesiveness would likely influence male tenure in prides and thus  
311 ultimately reproductive success.

312 As predicted, female lions, but not males, were more likely to respond to non-resident female  
313 urine than to resident female urine. For resident female lions, rival females (and their prides)  
314 pose a significant territorial threat (Schaller, 1972; Packer et al., 1990). Since mammalian  
315 scent-marking often functions for territory demarcation (Roberts and Gosling, 2001,  
316 Christensen, Kern, Bennitt and Radford, 2016), it would be adaptive for females to pay  
317 attention to the scent-marks of territory rivals (non-residents) to detect when their territory  
318 ownership is being challenged. While we provide evidence that female lions could use urine  
319 to determine the residency status of conspecifics, further research would be required to  
320 sufficiently test this in males. Non-resident adult male lions compete aggressively with  
321 resident males for access to reproductive females (Grinnell et al., 1995), and it may be  
322 adaptive for males to use scent-marking to distinguish coalition partners from non-resident  
323 males.

324 Flehmen was consistently exhibited by all age/sex classes of lion in response to urine. It is  
325 notable that flehmen is typically performed by males in response to sexual olfactory stimuli  
326 (Rasmussen, Schmidt, Henneous, Groves and Daves, 1982; Hart and Leedy, 1987; Stahlbaum  
327 and Houpt, 1989), but we regularly observed flehmen by females and sexually immature  
328 lions in response to urine. Flehmen transports non-volatile chemical signals (especially  
329 proteins and steroid conjugates) from the oral cavity to the vomeronasal organ for  
330 chemosensory analysis (Hart and Leedy, 1987; Igbokwe, 2009), and its occurrence gives  
331 further evidence that lion urine functions in scent-marking.

332 Lions of all ages had a similar pattern of response to the urine, but subadults were more likely  
333 to respond than cubs. Whilst little is known about the development of scent-marking in  
334 carnivores, scent-mark investigation and overmarking often increase with age, and adults  
335 scent-mark the most (Ferkin, 2010; Clapham et al., 2014, Vogt et al., 2014; Allen et al.,

336 2015). Subadult lions are approaching sexual maturity and male subadults disperse to locate  
337 mating partners (Schaller, 1972). Perhaps subadult lions pay particular attention to the  
338 olfactory signals of conspecifics to begin acquiring information regarding mating  
339 opportunities, and to assess the risk of intra-sexual competition (White, Swaisgood and  
340 Zhang, 2002; Clapham et al., 2014). However, only adult lions were observed to countermark  
341 the thawed urine ( $N = 9$ ). Countermarking establishes that both the urine and the countermark  
342 are scent-marks (Jordan et al., 2013).

343 Lions were more likely to respond to urine deposited with a scrape body posture compared to  
344 a urine-only posture. Urine deposited in combination with scraping could elicit a greater  
345 likelihood of response because it contains semiochemicals from pedal gland secretions as  
346 well as urine (Gasset et al., 1996; Clapham et al., 2014; Owen et al., 2015). The mixing of  
347 chemical signals from multiple sources may increase the information content of deposits, and  
348 increase the response likelihood of conspecifics (Greene et al., 2016). Further research would  
349 be required to test this hypothesis, but lions still investigate and overmark pedal gland  
350 secretions in the absence of urine (Gilfillan, Golabek, Vitale, McNutt and McComb, n.d).

351 Finally, our results provide valuable methodological insights that might aid future scent  
352 presentation experiments. Lions were less likely to respond, and responded for shorter  
353 durations to urine that was exposed to the environment for longer before collection. While the  
354 time between urine deposition and collection was a weak predictor of how lions responded,  
355 our results suggest that some semiochemicals important to lions were degraded or diffused in  
356 the time between deposition and collection.

357 There is also conflicting evidence about the effects of freezing scent, with some evidence that  
358 freezing has a significant effect on the subsequent response of animals (Hoffmann, Musolf  
359 and Penn, 2009), and some evidence that freezing has little or no effect (Smadja, Catalan and



360 Ganem, 2004; Bagley, Goodwin, Rasmussen and Schulte, 2006; Kwak et al., 2009;  
361 Lenochova, Roberts and Havlicek, 2009). In our experiments, the number of days that urine  
362 was stored at -20 °C had a weak effect on the likelihood of response, and the duration of  
363 response from lions. Lions were less likely to respond, but responded for longer to urine that  
364 had been frozen for longer. These results suggest that some semiochemicals important to  
365 lions were degraded over time in the frozen urine. Freezing may have had additional effects  
366 on the urine that were not directly evident in our results – such as influencing the distance  
367 over which lions were attracted to the scent, or leading to lower response rates than may have  
368 been possible with fresh urine.

369 In conclusion, we provide the first direct experimental evidence that urine functions in social  
370 and sexual communication in wild lions. Our results suggest that lions can use urine to  
371 discriminate males from females and residents from non-residents. The response of lions to  
372 urine was also dependent on the sex and age of the subject receiving the presentation. Further  
373 research is required to reveal the specific semiochemicals in urine and their functions for  
374 social and sexual scent communication in lions, as well as the longer-term behavioural  
375 changes of lions following the detection of scent-marks.

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596 **TABLES:**

597 **Table 1- Behavioural measurements of lions after the presentation of urine.**

Behavioural Measurement	Definition
Respond to urine	A lion responded to the urine when investigating it, countermarking it, or overmarking it.
Investigate urine	Nose pointed towards the ground within 0.5 m of the urine. Investigatory behaviours included sniffing (with or without flehmen) and licking the sample. Flehmen is a key response of mammals to semiochemicals (Hart and Leedy, 1987) and involves a curled upper lip facial expression, exposing the front teeth.
Countermark urine	Pawing the ground, urinating, and/or defecating within 2 m of the urine, without the two deposits touching (Rich and Hurst, 1999).
Overmark urine	Pawing the ground, urinating, and/or defecating on top of the urine, so that the two deposits are at least partially touching (Johnston, Chiang and Tung, 1994).
Duration of response	The combined total time each lion was scored as investigating, countermarking and/or overmarking the urine.
Closest distance to the urine	The closest distance the test subject moved to the urine sample (in metres), irrespective of whether they responded to the urine. If the subject moved away from the sample from their resting place, the closest distance to the sample was equal to the distance that the sample was originally placed from the lion. The closest distance that the subject moved to the sample was chosen rather than the distance the sample was first placed from the subject, since in only 2 cases (out of 72) the subject showed signs of detecting the sample (e.g. sniffing the air) from their resting spot.

598

599 **Table 2- Summary of the raw data on the responses to urine for lions of different sex and age.**

Individual class	Number of trials	Number of responses	Responses involving sniffing	Responses involving flehmen	Responses involving a countermark
Recipient Sex					
<i>Male</i>	99 (31.0% of total trials)	22 (22.2% of male trials)	21 (95.5% of male responses)	16 (76.2% of male sniffings)	3 (13.6% of male responses)
<i>Female</i>	220 (69.0%)	50 (22.7%)	46 (92.0%)	24 (52.2%)	6 (12.0%)
Recipient Age					
<i>Adult</i>	143 (44.8%)	23 (16.1%)	18 (78.3%)	10 (55.6%)	9 (39.1%)
<i>Subadult</i>	72 (22.6%)	25 (34.7%)	25 (100.0%)	15 (60.0%)	0 (0.0%)
<i>Cub</i>	104 (32.6%)	24 (23.1%)	24 (100.0%)	15 (62.5%)	0 (0.0%)
Total	319	72 (22.6% of total trials)	67 (93.1% of total responses)	40 (59.7% of total sniffings)	9 (12.5% of total responses)

600

601 **Table 3- Factors influencing whether lions responded to the urine (N = 319).**

Variable	Estimate	SE	CI (2.5:97.5%)	Relative importance	
Intercept	1.722	0.648	0.448:2.996*	-	
Closest Distance to Sample (m)	-3.398	0.582	-4.544:-2.253*	1.00	
Treatment					
	Female Non-Resident	-	-	1.00	
	Female Resident	-1.761	0.694	-3.125:-0.396*	-
	Male Resident	0.669	0.727	-0.761:2.099	-
Recipient Sex					
	Female	-	-	1.00	
	Male	-0.760	0.910	-2.551:1.030	-

Recipient Age	Adult	-	-	-	1.00
	Subadult	1.239	0.670	-0.080:2.558	-
	Cub	-0.395	0.553	-1.483:0.694	-
Recipient Sex * Treatment	Female * Female Non-Resident	-	-	-	1.00
	Male * Female Resident	3.378	1.319	0.782:5.973*	-
	Male * Male Resident	0.090	1.416	-2.695:2.875	-
Donor Posture	Scrape	-	-	-	0.80
	Urine-only	-1.028	0.550	-2.133:0.044	-
Time Between Deposition and Collection (scaled and centered)		-0.259	0.277	-0.804:0.286	0.21
Number of Days Frozen (scaled and centered)		-0.225	0.258	-0.733:0.283	0.20

602 Model parameters were generated using model averaging on the best GLMMs (with binomial error distribution) selected using  
603 AICc (see Table A1 in Appendix). SE = Standard Error. CI = Confidence Interval. \* indicates confidence intervals that do not cross  
604 zero.

605

606 Table 4- Summary of the raw data on the responses to the urine of each treatment for lions of different sex and age.

	Female Resident			Female Non-resident			Male Resident			Total		
Recipient Sex	Trials	Responses	Response duration (s ± SD)	Trials	Responses	Response duration (s ± SD)	Trials	Responses	Response duration (s ± SD)	Trials	Responses	Response duration (s ± SD)
Male	43	9 (20.9%)	48.67±48.54	29	6 (20.7%)	21.73±33.02	27	7 (25.9%)	58.69±24.03	99	22 (22.2%)	44.51±39.31
Female	79	9 (11.4%)	27.50±13.09	82	23 (28.0%)	37.15±40.64	59	18 (30.5%)	25.54±21.99	220	50 (22.7%)	31.24±31.12
Recipient Age												
Adult	58	7 (12.1%)	57.95±48.93	48	11 (22.9%)	44.34±43.62	37	5 (13.5%)	7.55±6.82	143	23 (16.1%)	40.48±31.12
Subadult	32	6 (18.8%)	36.09±17.38	28	13 (46.4%)	26.74±34.56	12	6 (50.0%)	45.72±35.84	72	25 (34.7%)	33.54±31.49
Cub	32	5 (15.6%)	12.67±9.37	35	5 (14.3%)	29.92±43.63	37	14 (37.8%)	39.89±21.17	104	24 (23.1%)	32.14±26.83
Total	122	18 (14.8%)	38.04±36.17	111	29 (26.1%)	33.96±39.15	86	25 (29.1%)	34.82±26.79	319	72 (22.6%)	35.29±34.11

607 ± SD = standard deviation.

608 Table 5- Factors influencing the duration of response ( $\sqrt[3]{s}$ ) of lions to urine (N = 72).

Variable	Estimate	SE	CI (2.5:97.5%)	Relative importance	
Intercept	3.198	0.375	2.451:3.944*	-	
Recipient Sex	Female	-	-	1.00	
	Male	-0.878	0.422	-1.722:-0.033*	-
Treatment	Female Non-resident	-	-	1.00	
	Female Resident	-0.333	0.407	-1.147:0.481	-
	Male Resident	-0.507	0.457	-1.420:0.406	-
Recipient Sex * Treatment	Female * Female Non-resident	-	-	1.00	

	Male * Female Resident	0.472	0.553	-0.633:1.577	-
	Male * Male Resident	2.409	0.560	1.289:3.528*	-
	Time Between Deposition and Collection	-0.017	0.011	-0.039:0.006	0.33
	Number of Days Frozen	0.001	0.001	-0.001:0.004	0.20

609 **Model parameters were generated using model averaging on the best GLMMs (with Gaussian error distribution) selected using**  
610 **AICc (see Table A4 in Appendix). SE = Standard Error. CI = Confidence Interval. \* indicates confidence intervals that do not cross**  
611 **zero.**

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628 **FIGURE CAPTIONS:**

629 **Figure 1. Experimental procedure: approximately 80 g of thawed urine-soaked soil was deposited on the ground 7-25 m away from**  
630 **resting lions. Responses were observed from a vehicle.**

631 **Figure 2. (a) Predicted probabilities of responding to the treatments of presented urine for male and female lions. Estimates were**  
632 **derived from the averaged model presented in Table 3. (b) Mean probabilities of responding to the treatments of urine for male and**  
633 **female lions calculated from the raw data in Table 4. Bars are binomial standard errors.**

634 **Figure 3. (a) Predicted mean duration ( $\sqrt[3]{s}$ ) of response to the treatments of urine for male and female lions. Estimates were derived**  
635 **from the averaged model presented in Table 5. (b) Mean duration ( $\sqrt[3]{s}$ ) of response to the treatments of urine for male and female**  
636 **lions calculated from the raw data in Table 4. Bars are standard errors.**

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