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- 1 Testing for links between female urine odor and male sexual behaviors in Japanese macaques
- 2 (Macaca fuscata)
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12 Abstract

13 Urine is involved in sexual communication in New World monkeys and lemurs, but most studies of 14 the role of olfaction in sexual communication in Old World monkeys have focused on vaginal 15 secretions rather than urine. We investigated whether female urine promotes male sexual behaviors 16 (approaches and inspections of genital area) in Japanese macaques (Macaca fuscata). We used a 17 sequential presentation paradigm in behavioral experiments, presenting unfamiliar female urine and 18 control cotton swabs to males living in a social group with cycling females. We tested whether males 19 (N=3) showed more processing behaviors (licking, sniffing, tasting) towards female urine (22 stimuli 20 per male from pre-fertile, fertile, and post-fertile phases, based on urinary estrone and progesterone 21 conjugate profiles) than control odor (8 stimuli per male). We then compared male sexual behaviors 22 towards resident females pre- and post-exposure to stimuli and in relation to the females' 23 reproductive status (peri-fertile, non-fertile, and pregnancy periods, based on fecal estrone and 24 progesterone conjugate profiles). We found that males showed significantly more processing 25 behaviors towards urine stimuli than to controls but that male behavior did not vary across urine 26 samples from pre-fertile, fertile, and post-fertile phases. Exposure to unfamiliar female urine stimuli 27 did not modulate male approaches to and inspections of resident females at any stage of the female 28 reproductive cycle. Although our study is limited by its small sample size, the results suggest that 29 female urine contains compounds that males detect, but we found no evidence that female urine is 30 related to male sexual behaviors.

- 31
- 32 Keywords: sexual signaling, olfactory communication, female urine, *Macaca fuscata*
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- 34

35 Introduction

36 Female primates display multiple sexual signals that contain information about reproductive status 37 and influence male sexual behaviors. Studies of fertility signaling have mostly focused on visual 38 (e.g., Dubuc et al. 2009; Higham et al. 2008; Nunn 1999), behavioral (e.g., Brauch et al. 2007; Engelhardt et al. 2005; O'Neill et al. 2004), and auditory channels (e.g., Maestripieri et al. 2005; 39 40 Pfefferle et al. 2008; Pradhan et al. 2006). However, there is also evidence that odors are involved in 41 sexual communication in primates, with males investigating the female genital area and/or urine 42 (chacma baboons, Papio hamadryas ursinus: Clarke et al. 2009; pygmy marmoset, Cebuella 43 pygmaea: Converse et al. 1995; spider monkeys, Ateles belzebuth and Ateles geoffroyi: Klein 1971; 44 olive baboons, Papio anubis: Rigaill et al. 2013; gray mouse lemurs, Microcebus murinus: Schilling 45 1979) or showing behavioral responses such as flehmen after exposure to odors from cycling females 46 (mandrills, Mandrillus sphinx: Charpentier et al. 2013). Males thus might have access to some 47 odorant information about female reproductive status that could influence their sexual behaviors and that they could use in mating decisions to concentrate their reproductive effort on the most 48 49 conceptive days of the cycle. Studies show evidence for a signaling function in female urine and 50 labial/vaginal secretions in human and non-human primates (ring-tailed lemurs, Lemur catta: 51 Crawford and Drea 2015; rhesus macaques, Macaca mulatta: Curtis et al. 1971; Michael et al. 1971; 52 chimpanzees, Pan troglodytes: Fox 1982; Matsumoto-Oda et al. 2003; humans, Homo sapiens: 53 Michael et al. 1974). Odors may therefore be critical elements in primate sexual communication; 54 female odors may play a larger role in the regulation of primate sexual behavior than is currently 55 recognized.

Most studies of the role of olfaction in sexual communication in Old World monkeys have 56 57 focused on vaginal secretions. Early studies identified some compounds named "copulins" in these 58 secretions, i.e. volatile fatty acids such as acetic, propanoic, methylpropanoic, butanoic, and 59 methylbutanoic acids (Curtis et al. 1971; Michael et al. 1971; Michael and Keverne 1970). The 60 production of these compounds is enhanced by estrogen stimulation and suppressed by progesterone, 61 suggesting that they are linked to the menstrual cycle and may have a role in fertility signaling. 62 Studies of humans and stump-tailed macaques (Macaca arctoides) found that male testosterone and 63 cortisol concentrations increased after exposure to female vaginal odors from the peri-ovulatory period (stump-tailed macaques: Cerda-Molina et al. 2006; humans: Cerda-Molina et al. 2013). 64 65 Moreover, exposure to vaginal secretions from the most conceptive period of the cycle promotes 66 male inspections of female genital area in stump-tailed (Cerda-Molina et al. 2006) and rhesus 67 macaques (Michael and Keverne 1970; Michael and Zumpe 1982), although another study of rhesus 68 macaques could not replicate those results (Goldfoot et al. 1976). The studies of rhesus macaques 69 involved ovariectomized, androgen-treated females and used pairs of individuals housed alone. The 70 use of reproductively inactive but hormonally stimulated females (to promote female proceptive 71 behaviors) might produce results that are not representative of natural fertility signaling and mating 72 choices. Furthermore, although vaginal secretions seem to play a role in the multimodal framework 73 of reproductive signaling in Old World monkeys, it is likely that males have access to other 74 information through inspections of genital area, such as urine odor.

75 Female urine has been hypothesized to play a role in sexual communication in several New 76 World monkey species, with males directing more inspections of female genital area and of female 77 urine scent-marks during the mating season (squirrel monkeys, Saimiri sciureus: Hennessy et al. 78 1978; woolly spider monkeys, Brachyteles arachnoides: Milton 1985; Muriqui sp.: Nishimura et al. 79 1988; tufted capuchins, Cebus apella: Phillips et al. 2011; Ueno 1994). Captive male marmosets 80 responded more to urine scent-marks from ovulatory females compared to ovariectomized ones 81 (marmosets, *Callithrix jacchus*: Ferris et al. 2001). Similarly, studies reported urine-related behaviors 82 in sexual context have been also reported in several strepsirrhine, such as greater galagos (Galago 83 crassicaudatus crassicaudatus: Eaton et al. 1973), ring-tailed lemurs (Evans and Goy 1968; 84 Reynolds and Van Horn 1977) and ruffed lemurs (Varecia variegata: Foerg 1982; Lemur variegatus: 85 Shideler et al. 1983). To our knowledge, no studies have investigated the potential role of female 86 urine on male sexual behaviors in Old World monkeys.

87 Japanese macaques (Macaca fuscata) live in multi-male multi-female groups. In this seasonal 88 species, mating and conception occur during the coldest months of winter (Fooden and Aimi 2003), 89 which are often associated with the lowest food availability and variety (Go 2009; Hill 1997; 90 Iwamoto 1982; Nakagawa 1989). The high energetic costs (thermoregulation and reproductive costs, 91 see Garcia et al. 2011) associated with seasonality might have modulated the expression of fertility 92 signaling to avoid wasting energy on non-reproductive mating (i.e., mating outside the fertile phase). 93 Sexual signals (proceptive behaviors, estrous calls, and face color) in female Japanese macaques 94 convey broad information about female reproductive status (cycling vs. pregnancy, Rigaill et al. 95 2015), but they do not pinpoint the timing of ovulation as their expression does not vary between the 96 pre-fertile, fertile and post-fertile phases (behaviors and vocalizations, Garcia et al. 2009). Males are 97 nevertheless able to focus their reproductive effort on the most conceptive days of the cycle (in 98 captivity see Garcia et al. 2009; O'Neill et al. 2004) and refrain from mating with pregnant females 99 (Rigaill et al. 2015) which suggests that they might use other female signals. Captive and free-100 ranging females produce a strong odor (perceptible to humans) during periods of high sexual activity 101 and males frequently inspect the female genital area during the mating season (Garcia et al. 2009). It 102 is therefore possible that female odors (urine and/or vaginal secretions) modulate male sexual arousal or contain information about the probability of ovulation that males could detect; and olfactory
 signaling might be a crucial element of the Japanese macaque sexual communication.

105 We tested the hypothesis that female urine odor stimulates male sexual arousal or contains 106 information about the probability of ovulation, which ultimately modulates the expression of male 107 sexual behaviors (i.e. approaches and inspections of genital area) towards females in Japanese 108 macaques. We predicted that (a) males should show more processing behaviors (sniffing, licking, 109 and tasting the odor stimuli) toward urine than control stimuli, and (b) male processing behaviors 110 should vary with the reproductive state of the urine donor with an increase after exposure to urine 111 from the most fertile days of the menstrual cycle relative to the pre- and post-fertile phases. Then, (c) 112 if urine odor modulates male sexual arousal or is related to female reproductive state, male sexual 113 behaviors (i.e. approaches and inspections of genital area) should be greater after exposure to urine 114 compared with control stimuli, and finally (d) male sexual behaviors should vary with the 115 reproductive state of the donor with males displaying more sexual behaviors after exposure to odors 116 from the fertile phase than to other phases of the menstrual cycle.

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118 Methods

119 Ethical Note

We carried out our study in captivity using non-invasive methods. The Center for Human Evolution Modeling Research of the Primate Research Institute reviewed and approved our research protocol in agreement with the Guidelines for the Care and Use of Nonhuman Primates of the Kyoto University Primate Research Institute.

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125 Subjects and housing

We conducted our study during the 2015-2016 mating season, from early October to late January, 126 using a captive population of Japanese macaques living in a 1210 m² outdoor enclosure at the 127 128 Primate Research Institute of Kyoto University (Inuyama, Japan). Individuals in this group (Wakasa 129 group) are habituated to humans and easily identifiable through facial tattoos. Animals were fed 130 twice daily between 09:30h and 10:30h and between 15:00h and 15:30h. Water was supplied ad 131 *libitum.* The group was composed of 37 individuals consisting of 6 adult males $(10.0 \pm \text{SD } 4.4 \text{ years})$ 132 old, range = 6-18) and 7 sexually immature males ($2.5 \pm SD$ 1.4 years old, range = 1-5), 12 adult 133 females (9.3 \pm SD 2.9 years old, range = 6-16) and 9 sexually immature females (2.0 \pm SD 1.3 years 134 old, range = 1-4), and 3 infants less than 1 year old. Three adult females did not resume cycling at 135 the beginning of the study and were therefore not included for behavioral analyses. We initially 136 selected all adult and sexually active males for the study, but three males were later removed from the experimental protocol (see below) limiting our sample size to 3 adult males (male A: 12 years
old, rank number 2; male B: 7 years old, rank number 4; male C: 6 years old, rank number 5).

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140 **Collection of urine and fecal samples**

141 Due to logistical limitations (e.g., concrete surface, possibility of contamination), we were unable to 142 collect urine from females living in the social group with the focal males so we collected urine from 143 unfamiliar donor females. This reduces potential confounding factors related to familiarity which 144 could affect male response to odor stimuli such as past experience and mate preferences. We 145 collected urine samples during the previous mating season between November 2014 and late January 146 2015 from five adult and cycling donor females $(12.2 \pm SD 3.0 \text{ years old, range} = 8-16 \text{ years})$ housed 147 in individual cages at the Primate Research Institute. We placed a plastic container under the 148 females' individual cages, and covered it with mesh to prevent contamination from feces. We 149 collected urine samples every two days (except during menstruation) between 08:30h and 11:00h, 150 directly after micturition. We stored the samples in glass vials at -80°C to prevent chemical 151 degradation of the volatile compounds (Drea et al. 2013). In parallel, we kept a fraction of urine 152 samples at -20°C for hormonal analyses to determine female reproductive status. We followed the 153 females during two consecutive cycles and collected 169 samples (mean per female = $33.8 \pm SD 6.7$ 154 samples).

To determine the reproductive status of the resident females housed with the focal males, we 155 156 collected 275 fecal samples from the nine cycling females (mean per female = $30.56 \pm SD 6.06$ fecal 157 samples, range = 18-38). We collected fecal samples mainly between 8:00 AM and 12:00 PM, as 158 well as opportunistically when defecation was observed. We collected the fecal samples in their 159 entirety immediately after voiding; we bagged, labeled, and placed them on ice packs inside a 160 container until transfer to a freezer 0-2 hours later. We maximized our effort to collect samples 161 every two to three days to assess the ovarian cycle (Hodges and Heistermann 2011). All samples 162 were kept at -20°C until processing.

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164 **Determination of ovulation dates and cycle phases for urine and fecal samples**

We analyzed urine and fecal samples for estrone conjugates (E₁C) and pregnanediol-3-glucuronide (PdG) using previously described and validated enzyme immunoassays (EIA) (Fujita et al. 2001; Shimizu et al. 2003).

We thawed and centrifuged (3000 rpm, 5 min, Hitachi koki, himac CT13R) frozen urine samples (from donor females). We diluted the supernatant between 1:20 and 1:50 for E_1C and between 1:1 and 1:10 for PdG assays. We shook 0.25g of dry feces (from resident females) with 2.5 171 ml of modified phosphate buffer (0.1 M, pH 7.0, 0.1% BSA with 0.05% tween-20) at room 172 temperature for 24 hr, and then centrifuged (3000 rpm, 10 min, Hitachi koki, himac CF7F2). We 173 decanted the supernatant into clean tubes and discarded the residual pellet. We used fifty microliters 174 of urine and solubilized fecal samples for PdG and E₁C EIA, using pregnanediol-3-glucuronide-BSA 175 antibody and estrone-3-glucuronide-BSA antibody (Cosmo, FKA334E and FKA224E, Japan). We 176 calculated the PdG and E₁C concentrations for each sample by fitting the absorbance of the standard 177 curve using a curve-fitting program (LS-PLATE manager 2004, Wako Pure Chemical Industries, 178 Japan). We conducted dose response and parallelism tests to assess the validity of the assays. 179 Recovery of known amounts of E_1C standards to urine and fecal samples was 95.0 ± 13.0 % and 108.0 ± 7.5 % (mean \pm SD) respectively. Recovery of known amounts of PdG standards to urine and 180 181 fecal samples was 113.0 ± 18 % and 119.1 ± 26.6 % (mean \pm SD) respectively. Serial dilutions of 182 fecal and urine extracts showed that the slopes generated from the serially diluted samples were not 183 statistically different from the slope of the standard curve of E₁C and PdG (ANCOVA, range of P 184 values > 0.1). The E₁C assay had a sensitivity of 0.05-0.70 ng/ml with an intra-assay coefficient of 185 variation (CV) of 5.09 % and inter-assay CV of 4.70% for urine samples. The PdG assay had a 186 sensitivity of 0.03-0.61 µg/ml with an intra-assay CV of 5.38% and inter-assay CV of 2.61% for 187 fecal samples. We indexed all urine samples by creatinine to compensate for variations in individual 188 urine sample concentration (Taussky 1954). We used fifty microliters of urine, sodium hydroxide 189 and picric acid for creatinine assays. We determined the dilution factors of the urine samples 190 beforehand so that creatinine concentrations were in the range of creatinine standards. We calculated 191 the creatinine concentrations for each sample by fitting the absorbance of the standard curve using a 192 curve-fitting program (LS-PLATE manager 2004, Wako Pure Chemical Industries, Japan).

193 To determine the presumed days of ovulation of the donor females, we used urinary E₁C 194 profiles following a previous study of hormonal profiles in Japanese macaques which reported that 195 ovulation occurs one day after the urinary E_1C peak (Fujita et al. 2001). With the exception of one 196 abnormal cycle, we used data from 2 consecutive cycles per donor female. We defined the fertile 197 phase as the 5-day period covering the three preceding days before the urinary E₁C peak, and the 2 198 following days to account for the life span of sperm in the female tract (macaques: Behboodi et al. 199 1991). We defined the pre-fertile and the post-fertile phases as the 5 days preceding and following 200 the fertile phase (Heistermann et al. 2007). To determine the presumed days of ovulation of the 201 resident females, as well as possible pregnancy, we used the fecal PdG profiles (details in Rigaill et 202 al. 2015). Since fecal collection was not conducted daily, PdG profiles provided a better assessment 203 of the reproductive cycle and ovulation date than E1C profiles (e.g. higher possibility of not 204 detecting E1C peak). We determined hormonal profiles for eight of the resident females, and seven females got pregnant during the study period. During the study period, resident females went through all different stages of their reproductive cycle (i.e. pregnancy, pre-fertile, fertile, post-fertile, and non-fertile periods). To compare reproductive periods of similar lengths, we categorized the resident females as in their peri-fertile period (covering the pre-fertile, fertile, and post-fertile phases), nonfertile period (outside the peri-fertile period), and pregnancy.

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211 Experimental protocol and behavioral observations

LR conducted all behavioral experiments. Following the ethical guidelines for the Care and Use of Nonhuman Primates of the Kyoto University Primate Research Institute, the experimenter conducted the experiments and observations inside the enclosure (from Monday to Friday between 08:30h and 16:00h) wearing authorized field clothes and hat, facial mask, and plastic gloves. There were no physical interactions between the animals and the experimenter.

We used all urine samples corresponding to the pre-fertile, fertile and post-fertile phases as urine stimuli for the experiments in the enclosure, leading to a total of 66 samples (pre-fertile phase: N = 24, mean per female = $4.8 \pm SD 1.6$ samples; fertile phase N = 18, mean per female = $3.6 \pm SD$ 0.6 samples; post-fertile phase: N = 24, mean per female = $4.8 \pm SD 1.8$ samples). We restricted the data set to a small part of the follicular and luteal phases and we thereby conducted a more stringent test than if we had included the whole cycle or the whole luteal phase.

223 Prior to the beginning of the experiments in the enclosure, we habituated all males (N = 6) to 224 a control stimulus (neutral odor) over three weeks. Five days per week, the same familiar 225 experimenter entered the enclosure and presented one sterile cotton-swab (with wooden sticks) 226 soaked in 1 ml of distilled water to each male, resulting to the exposure to 15 swabs per male over 227 three weeks. At the end of this habituation phase, we excluded three males who did not interact with 228 the swab. We tested the three remaining males each for 22 urine stimuli, i.e. cotton-swabs soaked in 229 1 ml of urine (pre-fertile phase N = 8, fertile phase N = 6, post-fertile phase N = 8), and 6 control 230 swabs (to allow comparable analyses with the different cycle phases) soaked in 1 ml of distilled 231 water.

To answer our research question, i.e. whether female urine influences male processing and sexual behaviors (approaches and inspections of female genital area), we used a sequential presentation, i.e. presenting one odor stimulus per male for each experimental session. We tested males between 2 and 4 times per week depending on weather conditions (i.e. experiments were not carried out on rainy days to prevent from confounding factors linked to weather conditions that might affect odor detection) and on male accessibility; because samples could only be used during one session, when males were not accessible (i.e. engaged in social interactions or physically not 239 accessible) or did not grab the swab after several trials, we ended the experiment. We conducted 240 behavioral experiments from Monday to Friday, outside feeding time, and we balanced between 241 morning (08:30h to 11:30h) and afternoon sessions (13:00h to 15:30h). Before beginning the 242 experiments, we randomized the order of stimuli presentation so that each male had access to swabs 243 from different phases of different female donors, and we randomized between control and urine 244 stimuli throughout the experimental period. We presented stimuli only once (i.e. different males 245 never had access to the same sample) to limit pseudo-replication in exposure to odors. We placed 246 urine samples at -20 °C on the night preceding the experiment, and brought the samples in a cooler 247 box inside the enclosure just before the beginning of the experiment. We prepared swabs inside the 248 enclosure (i.e. soaked in 1 ml of urine or distilled water) before exposure to males.

249 The behavioral experiments consisted of three phases: 1) a first 30-min focal sample of the 250 focal male, 2) the exposure of the focal male to a stimulus, and 3) a second 30-min focal sample of 251 the focal male. During the focal sample (Altmann 1974), we recorded all socio-sexual behaviors (e.g. 252 mounts, holding behaviors, and grooming bouts) with a particular focus on approaches and 253 inspections of female genital area, i.e. deliberate placing of the nostrils within 5 cm of a female's 254 genital area directed by the male toward any resident female in the social group. We recorded 255 interactions between the focal male and females per dyad to later control for the reproductive status 256 of the resident females. We used male sexual behaviors directed toward resident females as a proxy 257 to estimate male sexual arousal. We used the first 30-min focal sample to assess the baseline level of 258 male sexual behaviors displayed naturally toward resident cycling females on that day. Within the 15 259 minutes following the end of the first 30-min focal sample, we gave to the focal male either a control 260 or a urine stimulus. We recorded any processing behavior (sniffing, licking, and tasting i.e. putting 261 the swab in the mouth without eating it) directed by the focal male toward the swab within the 1-min 262 exposure as a proxy for male interest. Male inspections of the female genital area generally last for 263 less than 30 s in Japanese macaques; making the 1-min exposure to the odor stimulus (female urine) 264 a good proxy to test the possible effect of female odor on male perception and behavior. We allowed 265 males to keep the swab after they grabbed it and to continue processing it. If the focal male rejected 266 the swab without interacting with it, we considered that the focal male was not exposed to the 267 stimulus (urine or control) and tried to give him another swab. If the focal male was engaged in 268 social or sexual interactions with other individuals, we waited until the end of the interaction to give 269 him the swab. We conducted the second 30-min behavioral observation at the end of the 1-minute 270 exposure. In total, we tested 53 urine and 18 control stimuli (see Table 1).

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Table 1. Number of successful trials exposing three male Japanese macaques to odor and control

stimuli at the Primate Research Institute, Inuyama, Japan (data from October 2015 to January 2016).

274 (males A to C: from highest to lowest dominance rank)

Stimuli	Male A	Male B	Male C
Control stimuli	6	6	6
Pre-fertile phase	5	8	7
Fertile phase	3	5	5
Post-fertile phase	5	7	8
Total	19	26	26
Rate of success ^a	70.37 %	92.86 %	92.86 %

^a (Number of trials with males taking the swab / total number of presentations)*100

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277 Statistical analyses

We could not analyze variation in male holding behaviors and mounts due to infrequency of occurrence during focal observations. Because of the small sample size, we could not assess the effect of male dominance rank or age. We used the glmmadmb and lmerTest packages (Bates et al. 2015; Fournier et al. 2012; Skaug et al. 2014) in R to fit the models in a series of generalized linear mixed-effect models (GLMMs) with negative binomial distributions for "processing behaviors", "approaches", and "inspections" variables. We accounted for temporal variation and pseudoreplication by using the date on which the experiment was conducted in all models.

285 To determine whether male processing behaviors vary (a) between urine and control stimuli 286 and (b) with respect to female donor reproductive status, we compared the number of processing 287 behaviors (sum of 3 behaviors: sniffing, licking, and tasting the swab) displayed by the focal male 288 (within the 1-min exposure to the stimuli) after exposure to urine (pre-fertile, fertile, post-fertile 289 phases) or control stimuli. We included the type of stimuli as a predictor variable (4 levels: pre-290 fertile, fertile, post-fertile phases, and controls). We added the identity of the focal male and the 291 identity of the female donor as crossed random effects. We compared the fitted model via a 292 likelihood ratio test (LRT) with a null model in which all predictor variables were removed but the 293 random effects structure was maintained.

To determine whether male approaches and inspections toward resident females vary (c) after exposure to urine vs. control stimuli and (d) with respect to female donor reproductive status, we compared the occurrence of approaches and inspections displayed by the focal male toward each resident female (i.e. per dyad) between the pre- and post-exposure phases. We included in the models 298 the exposure (2 levels: before and after exposure) and the reproductive status (3 levels: peri-fertile, 299 non-fertile, and pregnancy periods) of the resident female for each dyad as predictor variables. We 300 added the identity of the dyad (i.e. focal male and resident female) as crossed random effects. We 301 compared the fitted models via a LRT with their respective null models. If the models revealed that 302 male approaches or inspections differed between pre- and post-exposure to stimuli, we then 303 investigated the post-exposure period separately. For these models, along with the reproductive 304 status of the resident females for each dyad, we included the type of stimuli (4 levels: pre-fertile, 305 fertile, post-fertile phases, and controls) as predictor variables and tested for interactions between 306 donor and resident female reproductive status (i.e., do males exposed to urine from fertile phase 307 respond more strongly toward resident females in their peri-fertile period?).

We ensured that all relevant model assumptions were met by visually inspecting histograms of the residuals and plots of the residuals against fitted values. Finally, because this statistical approach forces comparisons of all categorical predictor levels against a baseline intercept term (i.e. controls for urine, peri-fertile period for resident female reproductive status), we re-categorised the baseline intercept term to compare all levels. This approach had no effect on other model parameters. We report all parameter estimates with 95% confidence intervals.

314

315 **Results**

Variation in male processing behaviors depended on the stimulus type (LRT: $\chi 2 = 8.44$, $\Delta df = 3$, $\Delta loglik = 4.22$, P = 0.038). Males displayed significantly more processing behaviors (i.e. sniffing, licking, and tasting) toward urine stimuli than to control stimuli (Figure 1 and Table 2). In contrast, we found no significant differences in the occurrence of processing behaviors between cycle phases, with no increase in those behaviors after exposure to urine stimuli from the fertile phase vs. pre- and post-fertile phases (Table 2).

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Table 2. Results of a GLMM testing the influence of stimulus type on the number of processing behaviors (i.e. sniffing, licking, and tasting) of three male Japanese macaques towards a swab during a 1-min exposure at the Primate Research Institute, Inuyama, Japan (data from October 2015 to January 2016).

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Duadiatana	Estimate	Lower confidence interval;	Std.	7	Р
rredictors		Upper confidence interval	Error	L	(> z)
Control vs. pre-fertile	1.077	0.28; 1.88	0.407	2.64	0.008
Control vs. fertile	1.141	0.30; 1.99	0.430	2.65	0.008
Control vs. post-fertile	0.869	0.05; 1.69	0.420	2.07	0.038
Fertile vs. pre-fertile	-0.065	-0.68; 0.55	0.315	-0.20	0.838
Fertile vs. post-fertile	-0.273	-0.92; 0.38	0.331	-0.82	0.410
Post-fertile vs. pre-fertile	0.208	-0.38; 0.80	0.301	0.69	0.489

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Figure 1. Number of processing behaviors during a 1-min exposure to odor stimuli in three male Japanese macaques at the Primate Research Institute, Inuyama, Japan (data from October 2015 to January 2016). Plots show the median (black horizontal line), the first and third quartiles (top and bottom of the box), and the maximum and minimum (top and lower whiskers) number of behaviors displayed. P values were generated by GLMMs. NS indicates non significant results at the 0.05 probability level



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343 We used a total of 376 approaches (183 before exposure and 193 after exposure) and 71 inspections of female genital area (32 before exposure and 39 after exposure) to analyze variation in 344 345 male sexual behaviors. The fitted model explaining the number of male approaches outperformed its respective null model (LRT: $\chi 2 = 17.50$, $\Delta df = 3$, $\Delta loglik = 8.75$, P < 0.001). Males approached 346 347 females significantly fewer times during pregnancy compared to the peri-fertile and non-fertile 348 periods, but there were no statistical differences in the occurrence of male approaches between the 349 peri-fertile and non-fertile periods (Tables 3 and 4). Exposure to odor stimuli did not modulate male 350 approaches toward females (Table 3). The fitted model of male inspections did not outperform its 351 respective null model (LRT: $\chi 2 = 1.33$, $\Delta df = 3$, $\Delta loglik = 0.66$, P = 0.721). Since the statistical 352 analyses suggested that male approaches and inspections did not vary after exposure to stimuli, we 353 did not investigate the effect of urine (pre-fertile, fertile, and post-fertile phases) vs. control stimuli 354 on these behaviors further.

355

Table 3. Results of a GLMM testing the influence of exposure to donor female urine and resident female reproductive status in the number of approaches toward resident females of three male Japanese macaques at the Primate Research Institute, Inuyama, Japan (data from October 2015 to January 2016).

Predictors	Estimate	Lower confidence interval;	Std. Error	Z	P (> z)
		Upper confidence interval			
Exposure	0.133	-0.23; 0.49	0.185	0.72	0.471
Peri-fertile vs. non-fertile	-0.586	-1.28; 0.11	0.353	-1.66	0.097
Peri-fertile vs. pregnancy	-1.394	-2.02; -0.77	0.321	-4.34	< 0.001
Non-fertile vs. pregnancy	-0.808	-1.58; -0.04	0.393	-2.06	0.040
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Table 4. Male approaches toward female genital area in relation to the reproductive status of resident
female Japanese macaques at the Primate Research Institute, Inuyama, Japan (data from October
2015 to January 2016).

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Mean ± SD number of approaches during 30-min focal sample

	e	1
Female reproductive status	Pre-exposure	Post-exposure
Non-fertile period	0.38 ± 1.67	0.58 ± 2.10
Peri-fertile period	0.80 ± 2.15	0.66 ± 1.88
Pregnancy period	0.22 ± 0.74	0.24 ± 0.79

369

370 Discussion

371 Our results did not fully support our hypothesis that female urine odor stimulates male sexual 372 arousal. We found that males showed more processing behaviors after exposure to female urine 373 stimuli than to control stimuli, which suggests that female urine contains olfactory and/or gustative 374 compounds that might have triggered their interest. However, male sexual behaviors (approaches, 375 inspections of female genital area) did not increase in response to exposure to female odors, 376 suggesting that among the olfactory and/or gustatory compounds that males could detect in female 377 urine, none stimulated their sexual arousal, or that urine alone is not sufficient to promote male 378 sexual behaviors. We did not find evidence that males could have information about the probability 379 of ovulation through female urine odor as males did not display more processing behaviors towards 380 odor from the most fertile days of the menstrual cycle than to pre- and post-fertile phases. We cannot 381 rule out the possibility that males did not respond to urine odor because the source (i.e., the donor 382 female) of the stimuli was unknown which could have affect the signal relevance. Indeed, in some 383 mammal species, urine contains compounds involved in individual recognition mechanisms 384 (Johnston et al. 1993; Hurst et al. 2001) and studies found evidence for volatile markers (in scent-385 marks and gland secretions) of identity in primates (Setchell et al. 2010; Smith 2006; Vaglio et al. 386 2016). To fully interpret our results, future studies should focus on the chemical analyses of urine 387 composition (volatile compounds, reviewed in Drea 2015) in relation to female chemosignature and 388 reproductive state to better understand the possible role of urine in individual recognition and in the 389 modulation of male sexual behaviors in Japanese macaques.

390 Besides female urine odor, males may access other information through close inspection of 391 female genital area, i.e. vaginal secretions and sperm remains. Vaginal secretions of human and some

392 non-human primate species contain volatile fatty acids (rhesus macaques: Curtis et al. 1971; Keverne 393 and Michael 1971; chimpanzees: Fox 1982; Matsumoto-Oda et al. 2003; humans: Michael et al. 394 1974) that elicit male behavioral (stump-tailed macaques: Cerda-Molina et al. 2006; rhesus 395 macaques: Michael and Keverne 1970; Michael and Zumpe 1982) and endocrinological responses 396 (stump-tailed macaques: Cerda-Molina et al. 2006; humans: Cerda-Molina et al. 2013). In Japanese 397 macaques, both female cervical mucus and vaginal scent increase during the follicular phase of the 398 menstrual cycle (Enomoto 1974; Nigi 1975), this suggests that males may be inspecting for 399 information contained in vaginal odors (but not urine). Males might also inspect females for 400 information about previous copulations. In several primate species, including Japanese macaques, 401 sperm plugs or sperm remaining in the female vagina or in the genital area after ejaculation are 402 visible from a distance (Dixson and Anderson 2002). There are several hypotheses for the physical 403 functions of sperm plugs, such as forming a physical barrier to future inseminations, preventing 404 sperm leakage and facilitating sperm release into the female reproductive tract (Voss 1979). Sperm 405 remains and/or plugs are more likely to occur in species with multi-male multi-female mating 406 systems (Dixson 1998; Parga 2003) and therefore they have been hypothesized to play a crucial role 407 in male-male competition. If males can assess the occurrence of previous copulations (sperm 408 remains) and simultaneously discriminate female reproductive status (vaginal secretions) through 409 visual, tactile, and/or olfactory cues, they might efficiently time their mating effort and maximize 410 their chance of siring, while females might gain benefits by promoting sperm and male-male 411 competition. Further studies compiling behavioral data (e.g. relationship between male copulations 412 and inspections of females), experiments to test signal perception (e.g. testing whether males can 413 discriminate individual identity in urine samples), and chemical analyses for signal content (e.g. 414 volatile fatty acids, identity markers in urine and vaginal secretions) are needed to better understand 415 the role of female odors in male and female mating strategies in Japanese macaques.

416

417 **Data availability**

The datasets analyzed in this study are available from the corresponding author on reasonable request.

420

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- 429
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