Chapter 4

Encoded information within urine influences behavioural responses among European badgers (*Meles meles*)

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Abstract

Metabolic by-products like sex steroid metabolites convey information regarding an individual's physiological fitness and reproductive state. Urine and faeces containing these excreted metabolites can function as cues for conspecifics within social contexts, particularly for mate selection. Our pilot study investigated in scent provisioning experiments, how donor- and responder-related characteristics influenced behavioural responses towards excreted sex steroid metabolites. We used 51 urine samples with known urinary hormone concentrations (determined by EIA corrected for specific gravity) collected from known individuals in a high-density population of European badgers (*Meles meles*) in Wytham Woods, Oxfordshire, UK during the spring mating season. Samples were provided in Eppendorff tubes pushed into the ground near badger setts to assess how response likelihood, sniff duration, and countermarking responses varied according to donor-related traits (age, sex, group familiarity, and hormone concentrations). From 143 videos containing 26 identifiable adult responders, we observed adult badgers modifying their behaviour towards urine samples based on the perceived relevance to their own traits with badgers investigating and countermarking urine from opposite-sex donors more often than urine from same-sex donors. This suggests that urine-marking can serve as an adaptive signal for individual advertisement. Additionally, all badgers were more likely to investigate male urine (containing testosterone) than female urine (containing oestradiol); however, no other clear effects of sex steroid metabolites were noticed. We consider that this could be a feature of the social evolution of badgers or arise because badgers signal reproductive status through other olfactory secretions, notably their unique subcaudal gland marking behaviour.

4.1 Introduction

Mammals generate metabolic by-products that individuals ultimately excrete via urination and defecation (Behringer & Deschner, 2017). Since these metabolic by-products result directly from biological pathways (e.g., digestion, muscle metabolism, endocrinological processes), their presence in urinary/faecal compositional profiles can convey information regarding an individual's physiological fitness to conspecifics (e.g. age [Linklater et al., 2013], health [Penn & Potts, 1998; Zala et al., 2004], relative body size [Sharpe, 2015]) and reproductive state [Converse et al., 1995]). Social information (e.g. social group membership) may also be conveyed (Henkel et al., 2015), particularly if individuals or populations reside in different habitats and/or exploit different dietary sources.

Sex steroid levels indicate sexual maturity (Lu et al., 1979), with lower levels present during adolescence (Courant et al., 2010) and senescence (Lu et al., 1979; badgers: Sugianto et al., in press a). In females, sex steroids also reveal oestrus cycle stage, where higher oestradiol levels promote ovulation, alter cervical mucus consistency for improved sperm transport, and prepare the uterine lining for implantation (Findlay et al., 2010; Wira et al., 2015; badgers: Sugianto et al., in press b). In males, higher testosterone levels promote spermatogenesis and secondary sex characteristics (Preston et al., 2012; badgers: Buesching et al., 2008, Sugianto et al., in press a, b). Once sex-steroids fulfil their biological function, species-specific filtration pathways through the kidneys and/or liver (Campbell et al., 2001; Snoj et al., 2014) transform these into metabolites (Amendolagine et al., 2018), in conjugated and/or unconjugated form (James et al., 1972; Thomas et al., 1990) excreted via urine or faeces. Consequently, excreted levels generally correlate with circulating plasma titres, and can provide reliable information concerning an individual's reproductive status (Brown et al., 1994; Snoj et al., 2014). Nevertheless, excretion filtration pathways are highly species-specific (Wasser et al., 1998) – for instance, linked to metabolic rate (Goymann, 2012) – and thus need to be validated carefully for each study species (Touma & Palme, 2005).

Importantly, these metabolites expose olfactory cues about the status of that individual to conspecifics (reviewed in Wyatt, 2014), and thus purposeful urinating/defecating behaviours have evolved to function as intentional signals for individual advertisement; for example, to elicit mating opportunities and enhance mate competition (Albone, 1984; Macdonald, 1979;

Thonhauser et al., 2013; Vogt et al., 2014). Responses stimulated by deposited marks may affect mate selection decisions, therefore ultimately influencing social and reproductive partners. Visual signals are less important for nocturnal mammals, making them particularly reliant on olfactory signals for conspecific assessment (Fobes & King, 1977; Wong et al., 2010).

Here we undertook a pilot study to begin to examine the behavioural responses of nocturnal European badgers (*Meles meles*; henceforth badgers) to the scent of conspecific urine in a high-density population (40 individuals/km²), where badgers live in groups, sharing dens (termed setts) in which more than 20 individuals can reside (see Macdonald et al., 2015).

Badgers are macrosmatic and rely heavily on olfactory communication using urine-marking, faecal deposits coated with anal gland secretions, inter-digital gland secretions and unique subcaudal gland secretions, originating from a specially evolved subcaudal pouch situated between the tail and anus (reviewed by Buesching & Stankowich, 2017; Stubbe, 1971). This subcaudal gland secretion plays an important role in badger olfactory communication (Buesching et al., 2002a, b, c), with badgers engaging in social allo-marking (Buesching et al., 2003) and object marking (Buesching & Macdonald, 2004), including over-marking of scents left by conspecifics (Buesching & Macdonald, 2001). Faeces and these specialised gland secretions encode individual- and group-specific information (reviewed in Buesching & Macdonald, 2001; Buesching & Stankowich, 2017; Tinnesand et al., 2015), yet thus far relatively little is known regarding the information encoded in urine (but see Service & Harris, 2001). Badger urine has been established to contain hormone metabolites indicative of reproductive status, albeit with a time lag (Sugianto et al., in press b).

Urination generally occurs on the ground during roaming and foraging (Buesching & Macdonald, 2001) with no alterations in position (e.g. handstands) to elevate the position of urine marks to signify dominance as observed in other species (Sharpe, 2015; White et al., 2002). Hutchings et al. (2002) observed that badgers urinated predominantly at latrines, which are also shared sites used for defecation (Buesching & Jordan, this volume; Stewart et al., 2002), although 38% of urinations occurred at non-latrine locations (e.g. associated with linear features such as fencing and thick undergrowth; see also White et al., 1993). Stewart et al. (2002) found that latrine visits last an average of 23 seconds, with nearly 54% of that duration involving sniffing. High sniffing activity along with preferences for latrines under tree cover (Stewart et

al., 2002) indicate that urine and other latrine scents are important enough to warrant protection from rainfall and other forms of environmental degradation.

While it has been established that badgers can distinguish sex and age from urine marks (Service & Harris, 2001), as it does in some other species (Converse et al., 1995; Hradecký, 1986; SankarGanesh et al., 2014), this was done without consideration of the responder's identity, sex, age, etc. The possibility that badger urine encodes reproductive status and conveys this to the responder has thus far not been fully explored. Here we use scent-playback experiments (following Tinnesand et al., 2015) to investigate how known, identified responders react to the provisioning of badger urine collected from known donors, a proportion of which was also analysed for sex steroid metabolite levels. We analyse, how donor- and responder-specific traits moderate responder behaviour.

4.2 Methods

4.2.1 Study population and sample collection

Our pilot study used 51 urine samples collected from 29 females and 22 males (42 adults and 9 cubs; Table 4.1) between 22 May and 3 June 2017 inhabiting a high-density population at Wytham Woods, Oxfordshire, UK (GPS reference: 51° 46'26"N; 1° 19'19"W; see Macdonald et al., 2009 for details of the badger population), an area of 424-ha of semi-natural mixed woodland (Savill et al., 2011). Cage traps were baited with peanuts and placed near sett entrances as part of a long term (1987-2018) capture-mark-recapture study, following the methodology described by Macdonald et al. (2015).

Badgers were sedated with an intramuscular injection of 0.2 ml ketamine hydrochloride/kg body weight (McLaren et al., 2005) prior to being identified by their unique tattoo number received at first capture (usually as cubs). Individuals were sexed and weighed (in kg to the nearest 100 g). Exact ages were known for individuals first captured as cubs (n = 41) while age of those individuals first captured as adults (n = 10) was estimated based on toothwear (categorised on a 1-5 scale) using the following formula (Macdonald et al., 2009; see da Silva & Macdonald, 1989 for limitations):

$$Age = -2.51 + 2.10 \times (toothwear)$$

To identify individuals during scent-playback experiments, all adults from the three focal setts were given unique clip marks by cutting a pattern into the black guard hair to expose the white under-fur (Stewart & Macdonald, 1997). Cubs were not clipped to minimise thermal compromise.

Badgers were assigned to social groups based on the location (social den, termed a ‘sett’) at which they were captured, and from their former capture history (following the rules in Annavi et al. 2014). This enabled us to experiment with scents provisioned from unfamiliar, i.e., donor badgers residing at other social groups from the responders, and familiar badgers, i.e., donor and responder residing in the same social group.

Urine was collected in glass vials with Teflon-coated screw lids (Sigma-Aldrich, Dorset, UK) by gentle manual palpation of the bladder (Vicente et al., 2007). Urine samples were then divided between 5-10 Eppendorf tubes (2 ml) filled to 0.5 ml each using plastic pipettes (one pipette per urine sample) for hormonal (enzyme immunoassay: EIA) analysis and scent-playback experiments. Subsamples were frozen at -20°C until further analysis.

Ethical review and approval were obtained for all trapping and handling procedures, and were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 currently under Home Office licence 30-3379 and Natural England licence 2017-27589-SCI-SCI.

4.2.2 Scent-playback experiments and behavioural observations

Scent-playback experiments using contemporaneous urine samples were conducted during the spring cub-rearing season from 24 May 2017 to 12 June 2017.

Urine samples were defrosted within 1-2 hours prior to scent playback and the lids of the Eppendorf tubes removed. A new pair of latex gloves was used for handling each sample to prevent cross-contamination. Around 1-2 hours before badger emergence, 1 urine sample and 1 control sample (an Eppendorf tube filled with 0.5 ml of water) were positioned 50 cm – about one head-body length of an average badger – apart on either side along badger paths near each of the 3 focal badger setts (between 2-8 m from the sett). The Eppendorf tubes were pushed into the ground to ensure that the rim was level with the soil. Eppendorf tubes were removed in the mornings and replaced in the afternoon with fresh urine and water samples. Urine and water placements were moved about 50 cm after each trial, and relative positions of urine and water (i.e., urine placement on left or right side of badger path) were alternated every day to prevent

familiarisation. Urine samples were presented randomly each night regarding donor sex, familiarity, and age to avoid scent habituation.

Badger reactions to urine and water were observed remotely using Infra-red Crenova 2.4” LCD Display 12MP 1080P HD camera traps activated by three motion sensors. Camera traps were set up on a nearby tree at a height of 1.5 m and about 1.5-3.0 m away from the sample, and the video length was set to 60 seconds with 15 frames per second. The previous night’s footage was downloaded each morning.

Badger proximity to the samples was estimated by sight using body size as an indicator. No response was recorded if a badger did not approach a sample within a 50 cm (i.e., 1 body length) radius. Responses were recorded as “potential” if a badger approached the samples within a 50 cm radius due to the likelihood that the tube content could be smelled (Tinnesand et al., 2015). Responses were recorded as “actual” if a badger investigated the tubes more closely (≤ 10 cm) by sniffing or licking/ direct contact.

For each potential and actual response, we recorded:

- Sniff duration (in seconds)
- Subcaudal over-marking (within 10 cm of tube) and proximity marking (between 10-50 cm of a vial)

4.2.3 Enzyme immunoassay (EIA) analyses of urinary oestradiol and testosterone metabolites

EIA analyses were performed at Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany. Sex-steroid levels were analysed by mixing 0.1 ml of urine with 0.5 ml of 0.05 M acetate buffer (pH = 4.8). β -glucuronidase/arylsulfatase from *Helix pomatia* was added to the mixture, and samples were incubated for 2 hours at 37°C to liberate the steroids from their conjugates. For extraction, 2.5 ml of a mixture of *tert*-butyl methyl ether and petroleum ether 30/70% v/v was added. After being shaken for 20 minutes, samples were frozen at -80°C. The organic phase was then decanted, dried at 50°C under a stream of nitrogen, and dissolved in 1 mL 40% methanol. For both oestradiol and testosterone analysis, 20 μ l aliquots were used.

4.2.3.1 Oestrogen EIA (ng/ml)

Total oestrogen analyses were carried out using a polyclonal antibody raised in rabbits immunised against 1,3,5(10)-oestratrien-3,17 β -diol-17-HS-BSA and 1,3,5(10)-oestratrien-3,17 β -

diol-17-HS-peroxidase. Cross-reactivities to oestrogens were as follows: 1,3,5(10)-oestratrien-3,17b-diol (17 β -oestradiol), 100%; 1,3,5(10)-oestratrien-3,17-one (oestrone), 100%; 1,3,5(10)-oestratrien-3,17 α -diol (17 α -oestradiol), 66%; 1,3,5(10)-oestratrien-3,16a,17b-triol (oestriol), 1.5%; and 0.1% for 19-nortestosterone, progesterone (P4), and testosterone.

Intra- and inter-assay coefficient of variations for biological samples with low and high concentrations were 2.4% and 6.9% (nZ12) and 1.3 and 4.7% (n = 18), respectively.

4.2.3.2 Testosterone EIA (ng/ml)

Antibodies (provided by Prof. Meyer, Weihestephan, Germany) were raised in rabbits immunised against 17a-OH-testosterone-HS-BSA. Testosterone-3-CMO-peroxidase was used as an enzyme conjugate. Cross-reactivity of the antibody with other steroids was as follows: 4-androsten-17 β -ol-3-one (testosterone), 100%; 5a-androstan-17 β -ol-3-one (dihydrotestosterone [DHT]), 13.6%; 5a-androst-2-en-17 β -ol (1.5%), and < 0.1% for 5a-androstan-3 β -ol-17-one (epi-androsterone), 5a-androstan-3a-ol-17-one (androsterone), and 5a-androst-2-en-17-one.

4.2.3.3 Normalisation of urinary hormone concentration with specific gravity

Specific gravity (SG) measurements for all urine samples were collected with a urine SG refractometer (Atago Uricon-PN; NSA Precision Cells, Inc.) after the first freeze-thaw cycle. The SG population mean was calculated to establish the SG_{target}. Following the methodology detailed in Miller et al. (2014), all sex-steroid levels were corrected using the equation:

$$\text{Raw hormone concentration} \times \frac{(SG_{\text{target}} - 1.0)}{(SG_{\text{sample}} - 1.0)}$$

4.2.4 Statistical analysis

4.2.4.1 Behavioural observations with scent-playback experiments

To analyse if urine encodes any biologically relevant information that influences responder behaviour, we conducted contingency chi-square tests comparing responses to urine versus water as a control by using response likelihood (proportion of actual responses to total [actual + potential] responses) and subcaudal countermarking responses (proportion of proximity marking and over-marking to total responses). No urine marking was observed in any of the recordings.

To improve the fit of the chi-square tests for countermarking analysis, categories were reclassified as “marking” (combining both over-marking and proximity marking) and “no marking.” Highly skewed sniff durations prompted the use of permutation tests (*lmPerm* package, Wheeler, 2010) to analyse any variation between urine and water responses. Variation in sniff duration was quantified as $\pm$ standard error (SE). To analyse if the duration samples were frozen for affected the sniff duration of urine, we also used permutation tests due to highly skewed data.

Additional contingency chi-square tests were conducted to relate donor and responder traits in the specific context of reproductive advertisement (i.e., age [adult or cub], sex, familiarity [resident or non-resident]) to response likelihood and countermarking responses. Sniff duration was analysed using permutation tests. A low number of observations of cubs as responders (n = 9) prevented any statistical analysis on responder age. Thus, all subsequent analyses focused solely on adult responders.

Analyses involving interactions between traits were conducted using general linear models (for response likelihood and countermarking responses) and a permuted two-way ANOVA using the *aovp* function (for sniff duration; *lmPerm* package, Wheeler, 2010). Significant interactions were analysed with a post-hoc pairwise test using the *lsmeans* package (Lenth, 2016).

4.2.3.2 Correlating behavioural observations with EIA analyses

To determine if sex-steroid metabolites affect behavioural responses, we ran three generalised linear models (GLMs) and linear models (only for sniff duration of opposite sex urine) with donor identity and responder identity as random effects:

- 1) *Response likelihood ~ Oestradiol/Testosterone levels (ng/ml)*
- 2) *Urine sniff duration ~ Oestradiol/Testosterone levels (ng/ml)*
- 3) *Marking response ~ Oestradiol/Testosterone levels (ng/ml)*

Male (n = 9) and female donors (n = 14) were analysed separately, as were male (n = 7) and female responders (n = 7). GLMs were modified to incorporate responder age in months or responder reproductive stage. Hormone levels and age in months were re-scaled using the *scale()*

function in base R when analysed together. Reproductive life stages of both donors and responders were based on age following Sugianto et al., submitted a, b (sexually immature: ≤ 2 years old [n = 9; 1 male donor, 6 female donors, 1 male responder, 1 female responder]; reproductively active: 2 – 8 years old [n = 24; 8 male donors, 6 female donors, 5 male responders, 5 female responders]; reproductively senescent: ≥ 9 years old [n = 2; 1 female donor, 1 male responder]).

All analyses were performed in R (version 3.5.1) and R Studio (version 1.1.453).

4.3 Results

4.3.1 Behavioural observations with scent-playback experiments

143 of the 197 videos analysed contained badger appearances; involving 26 different individually identifiable adults and two unmarked cubs. In 22 badger appearances, the adult individuals could not be identified unambiguously. However, in 33 appearances (23.0% of appearances), badgers did not come within 50 cm of either water or urine sample (i.e., no potential reaction). Potential, but not actual responses were recorded in a further 74 appearances (51.7%; 15 within urine only and 8 within water only), while 63 appearances (44.0%) included actual responses where badgers sniffed in 24 appearances (38.1%) only the urine, in 23 (36.5%) only the water sample, and in 16 appearances both (25.4%) urine and water. Because only two cubs responded to any of the samples, we analysed only adult responses in the following.

Badgers exhibited similar response likelihoods ($\chi^2 = 0.34$, $df = 1$, $p = 0.56$) for both urine and water. Regardless, badgers sniffed urine (n = 40, 5.02 ± 1.58 s) significantly longer than water (n = 23, 3.05 ± 0.49 s; estimate = 0.95, $F_{1,77} = 4.71$, $\text{Pr(Prob)} = 0.02$). Individuals that sniffed both urine and water (n = 16) were removed from countermarking analysis due to the uncertainty about which sample they countermarked against. Removing these individuals showed that badgers significantly countermarked urine more than water ($\chi^2 = 14.08$, $df = 1$, $p < 0.001$). Badgers demonstrated no significant variation in sniff duration regardless of sample freezing duration (estimate = -0.19, $F_{1,38} = 1.09$, $\text{Pr(Prob)} = 0.46$).

4.3.2 Effects of age-related information on behavioural responses

Cub urine elicited slightly more responses overall than did adult urine amongst adult responders, although this effect was not significant ($\chi^2 = 1$, $df = 1$, $p = 0.32$). Cub and adult urine were countermarked equally often, but only one single female and one male were observed countermarking cub urine (albeit at high frequency).

Both cub urine and adult urine elicited more responses from adult females than from adult males (Table 4.2, Fig. 4.1), with adult females generally sniffing urine longer than males (males: $n = 6$, 2.35 ± 0.86 s; females: $n = 4$, 5.37 ± 2.39 s; Fig. 4.2).

Because all cub donors were non-residents at the setts provisioned, we repeated this analysis including only unfamiliar donors of either age class. As before, adult badgers demonstrated similar response likelihoods ($\chi^2 = 1$, $df = 1$, $p = 0.32$) and countermarking responses (Table 4.2) towards adult and cub urine. Sniff duration, however, differed significantly between cub and adult urine samples (all donors: estimate = -1.78, $F_{1,38} = 4.32$, $\text{Pr(Prob)} = 0.03$; unfamiliar donors only: estimate: -1.78, $F_{1,38} = 4.42$, $\text{Pr(Prob)} = 0.02$) as responders sniffed cub urine (all cub donors: $n = 13$, 6.46 ± 1.86 s) longer than adult urine (all adult donors: $n = 27$, 2.90 ± 0.77 s; unfamiliar adult donors: $n = 17$, 3.05 ± 1.11 s; Fig. 4.3).

4.3.3 Effects of sex-related information on behavioural responses

Overall, males and females were equally likely to respond to conspecific urine (response likelihood: $\chi^2 = 1$, $df = 1$, $p = 0.32$) and did not differ in their propensity to countermark ($\chi^2 = 1.70$, $df = 1$, $p = 0.19$) or sniff duration (estimate = 0.86, $F_{1,13} = 0.96$, $\text{Pr(Prob)} = 0.64$). Similarly, overall, the sex of the donor urine sample donor did not elicit significantly different responses among responders (response likelihood: $\chi^2 = 1$, $df = 1$, $p = 0.32$; countermarking responses: $\chi^2 = 2.67$, $df = 1$, $p = 0.10$; sniff duration: estimate = 1.62, $F_{1,29} = 2.34$, $\text{Pr(Prob)} = 0.19$).

However, when responder sex was considered, adult females responded more to male donors (response likelihood = 0.80; Table 4.2; Fig. 4.4) than to female donors (response likelihood = 0.36), whereas adult males responded more to female donors (response likelihood = 0.36; Fig. 4.4) than to male donors (response likelihood = 0.20), although sniff duration was not affected by the interaction between donor-responder sex dyads (Table 4.2).

Both sexes countermarked male urine (female responders: countermarking proportion = 0.4; male responders: countermarking proportion = 0.2) significantly more than female urine (female responders: countermarking proportion = 0.18; male responders: countermarking

proportion = 0.14; Fig. 4.5), with all donor-responder combinations differing significantly from each other (Table 4.2).

Responder sex, donor age, and their interaction all significantly affected response likelihood (Table 4.2). Females responded more often than males to adult and to cub donors (Fig. 4.4). Responder sex and donor age, but not their interaction, also significantly affected sniff duration, with females ($n = 4$; 5.37 ± 2.39 s) investigating urine longer than males ($n = 6$; 2.35 ± 0.86 s; Fig. 4.2) and all responders investigating cub urine ($n = 2$; 7.54 ± 4.89 s) for longer durations than adult urine ($n = 8$; 2.56 ± 0.67 s; Fig. 4.6).

Countermarking responses were affected by responder sex, donor age, and their interaction (Tables 4.2).

4.3.4 Effects of familiarity on behavioural responses

Overall, donor familiarity did not affect behavioural responses (response likelihood: $\chi^2 = 1$, $df = 1$, $p = 0.32$; sniff duration: estimate = -0.55, $F_{1,13} = 0.33$, $Pr(Prob) = 1$; countermarking responses: $\chi^2 = 1.70$, $df = 1$, $p = 0.19$). All adults responded more to unfamiliar male donors than to familiar females, unfamiliar females, and familiar males (Table 4.2).

4.3.5 Correlating behavioural observations with sex-steroid levels

4.3.5.1 Effects of female donor oestradiol levels

Twenty-five of 54 videos (for which we had oestradiol levels for the provisioned donor sample) involved identifiable responders (adult females = 8, adult males = 17). For female donors, female response likelihood was not influenced significantly by donor oestradiol levels, responder age, and responder reproductive stage ($n = 6$). No female responders were observed countermarking urine, while only two female responders sniffed urine, thereby preventing further statistical analysis.

Male response likelihood to female donors was also not influenced significantly by donor oestradiol levels, responder age, and responder reproductive stage. However, male responders spent significantly more time sniffing urine containing lower oestradiol levels ($n = 5$, estimate = -2.05, standard error = 0.71, t -value = -2.88; Fig. 4.7). There were also no effects on countermarking .

4.3.5.2 *Effects of male donor testosterone levels*

Fifteen of 20 videos (for which we had testosterone levels for the provisioned donor sample) involved identifiable responders (adult females = 6, adult males = 9). There were no effects on response likelihood, and sniff duration by female responders to male urine was not influenced by testosterone levels, responder age, or responder reproductive stage. Limited responses ($n = 6$) and observed countermarking ($n = 1$) prevented further analysis. Response likelihoods and countermarking by male responders were not influenced by testosterone levels, responder age, or responder reproductive stage. Only two males sniffed urine, thereby preventing any further analysis.

4.4 Discussion

Adult badgers were demonstrably able to decipher relevant information concerning sex, age and group familiarity encoded in the urine of conspecifics. In response, they modified their behaviour according to the signals' pertinence to their own traits/condition, investigating and countermarking urine from opposite-sex donors more often than urine from same-sex donors. Heightened interest towards opposite-sex donors occurred regardless of donor familiarity. Additionally, cub urine stimulated more responses and countermarking as well as longer sniff durations than to adults (but limited by sample size).

Adult badgers responded more often to and countermarked urine from opposite-sex donors than they did same-sex donors. Badgers were likely responding to the potentially volatile sex-specific information encoded within urine, reflected by GC-MS profile variations between reproductively active males and oestrous females (Tinnesand et al., unpublished data). This aligns with previous sex-related differences in response behaviour amongst badgers (Service et al., 2001). Other species have been shown to exhibit considerable sex-related variation in urinary scent profiles (Barman et al., 2013), and these variations may augment olfactory sex discrimination abilities (Charlton, 2014; Gilfillan et al., 2017; Leonard et al., 2001; Linklater et al., 2013). Surprisingly, however, the excreted sex-steroid metabolite (oestradiol and testosterone) levels in urine did not elicit clear response patterns, aside from male (testosterone) samples receiving more attention than female (oestradiol) samples, conforming with Service & Harris (2001). Interestingly, in this context, our pilot study indicated that male responders sniffed

female urine with low oestradiol content significantly longer than samples with high oestradiol content.

Oestradiol fluctuations during oestrus, or elevated testosterone levels during the mating season, are likely reflected in altered urinary odours detectable by conspecifics. Surprisingly, however, we found that the excreted sex-steroid metabolite (oestradiol and testosterone) levels in urine did not elicit clear response patterns, except that male responders sniffed urine provisioned from females with low oestradiol for significantly longer durations. This is contrary to typical behaviour in other mammals where males typically show heightened interest in urine from oestrous females and engage in flehmen behaviour to transfer sex-steroid metabolites to their vomeronasal organ (Crump et al., 1984; Rajanarayanan & Archunan, 2011).

Our findings here, however, imply that either i) a donor's reproductive quality may take longer to discern when sex steroid metabolite levels are lowered or ii) any potential olfactory cue information that sex steroid metabolites might imbue to urine is not being used as any discernible quantitative signal for individual advertisement. This is consistent with the theory that urinary proteins mediate individual recognition (mice: Hurst et al., 2001), superseding former ideas that the Major Histocompatibility Complex (MHC) played a role (Hurst et al., 2005); thus, this mediator's signal quality might exceed the cue potential of sex metabolites, although we re-emphasise here that we analysed only small sample sizes in this pilot study.

Although we found no gross effect of familiarity, when re-examined by the sex of the donor/responder, unfamiliar male scent marks elicited the greatest responses and familiar females the least. Unfamiliar cubs also elicited a strong response, particularly from adult females since such signals would be very out of context, given that cubs would not be expected to visit other social groups (Macdonald et al., 2008). Within other mammalian species (e.g., Rostain et al. 2004), individuals – particularly males – often investigate unfamiliar scents more often than familiar scents. This may serve to either locate new females as part of their philandering search for mates or enable them to detect the risk of competing males intruding into their territory (Gosling & Roberts, 2001; Tinnesand et al., 2013). This has been formalised as the “Dear Enemy Phenomenon” (DEP) which theorizes that animals will respond less aggressively towards neighbours than strangers (Fisher, 1954). This DEP becomes evident in how this same badger population reacts to anal gland secretions from neighbouring and unfamiliar individuals (Tinnesand et al., 2013). Insofar as successive studies of the socio-spatial system of these same

badgers have provided little corroboration for the classical model of active territorial defence (Buesching & Macdonald, 2001; Kilshaw et al., 2009; Macdonald et al., 2015; Stewart et al., 1997), it is progressively less parsimonious to interpret urine (or other) marking in this context. Consequently, this adds weight to the possibility that individuals investigate unfamiliar scents longer to discern potential mates that have yet to be encountered.

These sex and age effects, but only weak effects of familiarity within this social system, and no strong evidence for any discrimination based on sex steroid metabolite signalling, illustrate the species- and context- specific nature of scent communication and the active use of urinary scents in advertisement. Moreover, the deficits in what badgers glean from conspecific scent marks are perhaps more informative to the olfactory communication paradigm than what we demonstrate they may be detecting, where there is a general trend for signalling individuality to increase with social group size and the extent of social behaviour within a clade, best established thus far in studies of acoustic communication (Pollard & Blumstein, 2011). Given that European badgers living at high-density in the UK display only rudimentary social behaviours, and given the high rate of trespassing and thus integration at the population level in our study population (Macdonald et al., 2015), our pilot study may be exposing a stage in the evolution of social scent-marking. The relatively recent adaptation of badgers to the higher carrying capacity supported by the agricultural mosaic of land use in parts of the UK (and Ireland; Macdonald et al., 2015) may have not yet led them to fully exploit urinary scent discrimination. Alternatively, given that we have established that badgers are discriminating a lot of information from the anal gland secretion coating faeces at latrines (Tinniesand et al., 2015), and given that badgers uniquely engage in quite subtle social signalling of individual qualities using their subcaudal gland secretion (Buesching et al., 2002b, c), then urinary marking may play a more subsidiary role in this macro-osmotic mammal species.

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Table 4.1: Age, sex, and familiarity distribution of urine sample donors ($n = 51$). Urine samples collected during spring 2017 and presented during scent-playback experiments were distributed across sex and social group. All cub urine was from non-residents.

Adults ($n = 42$)			Cubs ($n = 9$)	
	Females ($n = 24$)	Males ($n = 18$)	Females ($n = 5$)	Males ($n = 4$)
Residents ($n = 17$)	8	9	NA	NA
Non-residents ($n = 34$)	16	9	5	4

679 *Table 2a: Pairwise tests comparing behavioural responses based on donor sex / age and responder*
680 *sex/age. Asterisks denote statistical significance.*

Response Likelihood (Donor Sex*Responder Sex)				
Contrast	Estimate	SE	Z-ratio	P
Female,Female - Male,Female	-0.436363636	3.532708e-16	-1.235210e+15	<.0001*
Female,Female - Female,Male	0.006493506	3.532708e-16	1.838110e+13	<.0001*
Female,Female - Male,Male	0.163636364	3.532708e-16	4.632038e+14	<.0001*
Male,Female - Female,Male	0.442857143	3.532708e-16	1.253591e+15	<.0001*
Male,Female - Male,Male	0.600000000	3.532708e-16	1.698414e+15	<.0001*
Female,Male - Male,Male	0.157142857	3.532708e-16	4.448227e+14	<.0001*
Countermarking Responses (Donor Sex*Responder Sex)				
Contrast	Estimate	SE	Z-ratio	P
Female,Female - Male,Female	-0.21818182	6.798700e-17	-3.209170e+15	<0.0001*
Female,Female - Female,Male	0.03896104	7.447602e-17	5.231353e+14	<0.0001*
Female,Female - Male,Male	-0.01818182	7.447602e-17	-2.441298e+14	<0.0001*
Male,Female - Female,Male	0.25714286	6.798700e-17	3.782236e+15	<0.0001*
Male,Female - Male,Male	0.20000000	6.798700e-17	2.941739e+15	<0.0001*
Female,Male - Male,Male	-0.05714286	7.447602e-17	-7.672651e+14	<0.0001*
Countermarking Responses (Responder Sex*Donor Age)				
Contrast	Estimate	SE	Z-ratio	P
Female,Adult - Male,Adult	0.1190476	2.019047e-16	5.896228e+14	<0.0001*
Female,Adult - Female,Cub	-0.7142857	2.553915e-16	-2.796826e+15	<0.0001*
Female,Adult - Male,Cub	0.1190476	2.019047e-16	5.896228e+14	<0.0001*
Male,Adult - Female,Cub	-0.8333333	2.708836e-16	-3.076352e+15	<0.0001*
Male,Adult - Male,Cub	0.0000000	2.211755e-16	0.000000e+00	1.0000
Female,Cub - Male,Cub	0.8333333	2.708836e-16	3.076352e+15	<0.0001*
Response Likelihood (Donor Group * Donor Sex * Responder Sex)				

Contrast	Estimate	SE	Z-ratio	P
Familiar,Female,Female - Unfamiliar,Female,Female	-4.166667e-02	0.04014586	-1.038	0.9687
Familiar,Female,Female - Familiar,Male,Female	-3.347222e-01	0.05051576	-6.626	<0.0001*
Familiar,Female,Female - Unfamiliar,Male,Female	-6.263889e-01	0.05794555	-10.810	<0.0001*
Familiar,Female,Female - Familiar,Female,Male	4.166667e-02	0.04014586	1.038	0.9687
Familiar,Female,Female - Unfamiliar,Female,Male	1.526557e-16	0.05677482	0.000	1.0000
Familiar,Female,Female - Familiar,Male,Male	3.736111e-01	0.05794555	6.448	<0.0001*
Familiar,Female,Female - Unfamiliar,Male,Male	8.194444e-02	0.05051576	1.622	0.7370
Unfamiliar,Female,Female - Familiar,Male,Female	-2.930556e-01	0.05051576	-5.801	<0.0001*
Unfamiliar,Female,Female - Unfamiliar,Male,Female	-5.847222e-01	0.05794555	-10.091	<0.0001*
Unfamiliar,Female,Female - Familiar,Female,Male	8.333333e-02	0.05677482	1.468	0.8247
Unfamiliar,Female,Female - Unfamiliar,Female,Male	4.166667e-02	0.04014586	1.038	0.9687
Unfamiliar,Female,Female - Familiar,Male,Male	4.152778e-01	0.05794555	7.167	<0.0001*
Unfamiliar,Female,Female - Unfamiliar,Male,Male	1.236111e-01	0.05051576	2.447	0.2188
Familiar,Male,Female - Unfamiliar,Male,Female	-2.916667e-01	0.04916843	-5.932	<0.0001*
Familiar,Male,Female - Familiar,Female,Male	3.763889e-01	0.05051576	7.451	<0.0001*
Familiar,Male,Female - Unfamiliar,Female,Male	3.347222e-01	0.05051576	6.626	<0.0001*
Familiar,Male,Female - Familiar,Male,Male	7.083333e-01	0.04916843	14.406	<0.0001*

Familiar, Male, Female - Unfamiliar, Male, Male	4.166667e-01	0.05677482	7.339	<0.0001*
Unfamiliar, Male, Female - Familiar, Female, Male	6.680556e-01	0.05794555	11.529	<0.0001*
Unfamiliar, Male, Female - Unfamiliar, Female, Male	6.263889e-01	0.05794555	10.810	<0.0001*
Unfamiliar, Male, Female - Familiar, Male, Male	1.000000e+00	0.08029171	12.455	<0.0001*
Unfamiliar, Male, Female - Unfamiliar, Male, Male	7.083333e-01	0.04916843	14.406	<0.0001*
Familiar, Female, Male - Unfamiliar, Female, Male	-4.166667e-02	0.04014586	-1.038	0.9687
Familiar, Female, Male - Familiar, Male, Male	3.319444e-01	0.05794555	5.729	<0.0001*
Familiar, Female, Male - Unfamiliar, Male, Male	4.027778e-02	0.05051576	0.797	0.9933
Unfamiliar, Female, Male - Familiar, Male, Male	3.736111e-01	0.05794555	6.448	<0.0001*
Unfamiliar, Female, Male - Unfamiliar, Male, Male	8.194444e-02	0.05051576	1.622	0.7370
Familiar, Male, Male - Unfamiliar, Male, Male	-2.916667e-01	0.04916843	-5.932	<0.0001*

681

682

683 *Table 2b: Behavioural responses based on the interaction between donor-related traits and responder-*
684 *related sex / age. Asterisks denote statistical significance.*

Response Likelihood					
	Estimate	Std. Error	t-value	Pr(> t)	
Donor Sex	4.364e-01	3.533e-16	1.235e+15	<2e-16 *	
Responder Sex	-6.494e-03	3.533e-16	-1.838e+13	<2e-16 *	
Donor Sex*Responder Sex	-5.935e-01	4.996e-16	-1.188e+15	<2e-16 *	
Countermarking Responses					
	Estimate	Std. Error	t-value	Pr(> t)	
Donor Sex	2.182e-01	6.799e-17	3.209e+15	<2e-16 *	
Responder Sex	-3.896e-02	7.448e-17	-5.231e+14	<2e-16 *	
Donor Sex*Responder Sex	-1.610e-01	1.008e-16	-1.597e+15	<2e-16 *	
Sniff Duration					
	Df	R Sum Sq	R Mean Sq	Iterations	Pr(Prob)
Donor Sex	1	28.881	28.8806	807	0.1103
Responder Sex	1	12.890	12.8904	240	0.2958
Donor Sex*Responder Sex	1	11.060	11.0595	58	0.6379
Response Likelihood					
	Estimate	Std. Error	t-value	Pr(> t)	
Responder Sex	-1.190e-02	2.691e-16	-4.424e+13	<2e-16 *	
Donor Age	5.714e-01	3.296e-16	1.734e+15	<2e-16 *	
Responder Sex*Donor Age	-8.214e-01	4.255e-16	-1.931e+15	<2e-16*	
Countermarking Responses					
	Estimate	Std. Error	t-value	Pr(> t)	
Responder Sex	-1.190e-01	2.019e-16	-5.896e+14	<2e-16 *	
Donor Age	7.143e-01	2.554e-16	2.797e+15	<2e-16 *	

Responder Sex*Donor Age	-7.143e-01	3.379e-16	-2.114e+15	<2e-16 *
Sniff Duration				
	Estimate	Iterations	Pr(Prob)	
Responder Sex	2.593	2028	0.0473*	
Donor Age	-2.523	3444	0.0285*	
Responder Sex*Donor Age	-2.302	1369	0.0687	
Response Likelihood				
	Estimate	Std. Error	t-value	Pr(> t)
Donor Familiarity	0.04167	0.04015	1.038	0.329675
Donor Sex	0.33472	0.05052	6.626	0.000165 *
Responder Sex	-0.04167	0.04015	-1.038	0.329675
Donor Sex*Responder Sex	-0.66667	0.06348	-10.503	5.88e-06 *
Donor Familiarity* Donor Sex	0.25000	0.06348	3.938	0.004305 *
Countermarking Responses				
	Estimate	Std. Error	t-value	Pr(> t)
Donor Familiarity	0.04333	0.08495	0.510	0.6238
Donor Sex	0.12333	0.10967	1.125	0.2934
Responder Sex	-0.04000	0.08495	-0.471	0.6503
Donor Sex*Responder Sex	-0.24333	0.12014	-2.025	0.0774
Donor Familiarity* Donor Sex	0.15667	0.12014	1.304	0.2285

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Figure 4.1: Interactions between donor age and responder sex for response likelihood. When controlling for donor familiarity, female adults responded more often towards cub urine than adult urine (Table 2b).

Figure 4.2: Urine sniff duration in relation to donor age and responder sex. When analysed alongside donor age, adult females ($n = 4$; 5.37 ± 2.39 s) generally sniffed urine longer than adult males ($n = 6$; 2.35 ± 0.86 s).

Figure 4.3: Sniff duration of unfamiliar adult urine and cub urine by adult responders. When accounting for familiarity, adult badgers sniffed unfamiliar cub urine significantly longer ($n = 13$, 6.46 ± 1.86 s) than they did unfamiliar adult urine ($n = 17$, 3.05 ± 1.11 s; estimate: -1.78 , $F_{1,38} = 4.42$, $\text{Pr}(\text{Prob}) = 0.02$).

Figure 4.4: Interactions between donor sex and responder sex for response likelihood. Adult females showed more actual responses towards male urine than female urine, while adult males showed more actual responses towards female urine than male urine (Tables 2a and 2b).

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Figure 4.5: Interactions between donor sex and responder sex for countermarking responses. Urine from male donors elicited more countermarking responses than urine from female donors, particularly amongst adult female responders (Tables 2a and 2b).

Figure 4.6: Urine sniff duration in relation to donor age and responder sex. When analysed alongside responder sex, cub urine ($n = 2$; 7.54 ± 4.89 s) was sniffed longer than adult urine ($n = 8$; 2.56 ± 0.67 s). However, only two individuals sniffed cub urine.

Figure 4.7: Sniff duration of adult male responders towards urine with oestradiol. Male responders spent significantly longer time investigating urine with lower oestradiol levels (Table 3a).