Honest fitness advertisement in European badgers (*Meles meles*)



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Abstract

By using European badgers (*Meles meles*) as a model species, my thesis examines how wild mammalian populations utilise olfactory communication via specialised glandular secretions and metabolic by-products when advertising fitness-related information to conspecifics. This research utilised bomb calorimetry to quantify energetic content within subcaudal gland secretion (a uniquely evolved scent gland), enzyme immunoassays to measure sex-steroid levels within urine, and scent-provisioning experiments which assessed behavioural responses to particular scent marks.

Compared to other communication modalities, olfactory communication allows conspecifics to exchange individual- and group-related information without both parties (signaller and receiver) being present simultaneously. Section I addresses the factors that olfactory signals/cues must possess in order to be effective and common methodologies used within semiochemical research. Section II addresses olfactory advertisement with specialised glandular secretions with a focus on the specially evolved subcaudal gland and the secretion production costs incurred by individuals. By using bomb calorimetry and scent-provisioning experiments, I found that i) the energetic content of subcaudal secretion varies significantly based on individual fitness and season, and ii) conspecific responses vary their behavioural responses based on the perceived energetic costs along with donor-related traits (i.e., age and sex).

Metabolic by-products (e.g., urine) incur minimal production costs compared to specialised glandular secretions. Chemical profiles within urine vary depending on individual parameters, and Section III addresses if/how information encoded within urine influences conspecific responses. My findings indicated that, throughout the year, adult females responded

more than adult males to urine marks while urine marks from non-resident badgers – particularly badgers from neighbouring setts – elicited more responses. Additionally, adults and cubs adjusted their behaviour accordingly when presented with urine from different species, suggesting that badgers utilise olfactory cues in risk determination.

Section IV concludes my thesis by comparing the utilisation of subcaudal gland secretions and urine within olfactory fitness advertisement. Overall, subcaudal gland secretions appear to function as a primary source for individual fitness information while urine serves a subsidiary role in fitness advertisement. Avenues for future research – particularly longitudinal studies involving the correlation of behavioural responses to reproductive output – are discussed.

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Section I: The rules of effective advertisement



"Secret Scents" by Sophie Grainger and Erin Wilkinson

Chapter 1:
General introduction and methodology:
The rules of effective advertisement

The rules of effective advertisement

'Communication' – from the Latin root *communicare* meaning 'to share' – refers to the transmission of information between individuals (Cobley, 2008). To maintain their somatic fitness and maximise their reproductive output, animals must gather information about intra- and interspecific individuals (e.g., potential mating partners, competitors, and predators) within their environment (Wyatt, 2014a). Animals must also advertise information about their own fitness when seeking mating opportunities (Johansson & Jones, 2007) or defending resources against competitors (Gosling, 1982). Transmitting and receiving information via signals and cues therefore forms the underlying basis of fitness advertisement. Though signals and cues both encode information, it is important to note that signals evolved – typically from exaggerated cues – specifically to elicit certain reactions from the signal receiver. In this context, a cue is defined as a feature that can aid the receiver in decision-making and may alter the receiver's behaviour, but the cues did not evolve in the emitter specifically for that purpose (Maynard Smith & Harper, 1995; Wyatt, 2014a). For example, mosquitoes use the carbon dioxide and heat emitted by mammals as cues to find them as potential blood meals but these are not signals evolved by mammals to attract mosquitoes.

Conspecific communication amongst the class Mammalia occurs via numerous sensory modalities. Conveying visual (e.g., tail positioning in Eastern gray squirrels (*Sciurus carolinensis*); Pardo *et al.*, 2014), tactile (e.g., physical contact between ewes (*Ovis aries*) and lambs after parturition; Vince, 1987), auditory (e.g., vocalisations in primates; Fischer & Price,

2017), and vibrational (e.g., seismic vibrations in African elephants (*Loxodonta africana*); Mortimer, 2017) messages requires the signaller and the receiver to be within a certain range of each other. Yet, if either party lies out of range, or if any 'noise' – unreliable or irrelevant signals/cues (Bytheway *et al.*, 2013) – or physical barriers interfere with the signal, then messages conveyed with these modalities can remain undetected. Meanwhile, olfactory communication (a subset of 'chemical communication', 'chemical signalling', and 'chemosignalling'; Wyatt, 2014a) conveys information via molecules within metabolic by-products (i.e., urine and faeces) and specialised scent secretions (Albone, 1984). Unlike the visual, tactile, auditory and vibrational sensory modalities, olfactory cues/signals remain within the environment from anywhere between hours to weeks (Buesching *et al.*, 2002c; Buesching & Macdonald, 2004). Thus, information transmission can occur without the simultaneous presence of both parties. Furthermore, 'pheromones' – species-specific molecules or mixtures of molecules that trigger certain responses (Wyatt, 2014a) – can reduce any ambiguity within messages to benefit both the signaller and receiver.

Regardless of modality, signals/cues must have certain traits to maximise their effectiveness. To pinpoint these traits, one could draw upon Daniel Starch's (1923) marketing research and his proposed rules for effective advertisement. According to Starch, an effective advertisement has:

- 'To be seen'
- 'To be read'
- 'To be believed'
- 'To be remembered'
- 'To be acted upon'

This general introduction will discuss olfactory communication within the framework of Starch's five rules of effective advertisement, identify gaps within the literature, introduce European badgers (*Meles meles*) as a suitable study species to address these gaps, and finally, outline the remainder of my thesis.

'To be seen': Spatial and temporal patterns of scent deposition

Individuals deposit scent marks where conspecifics are likely to encounter them. Oftentimes, this involves depositing marks along commonly used paths (Gosling, 1982) as well as on visually conspicuous objects (e.g., rocks, trees; Vogt *et al.*, 2014) along such paths. Trees, in particular, are marked by animals making handstands to place the mark high on the trunk (dwarf mongooses (*Helogale parvula*): Sharpe, 2015; giant pandas (*Ailuropoda melanoleuca*): White *et al.*, 2002) and also via urination (domestic dogs (*Canis familiaris*): Lisberg & Snowdon, 2011; bush dogs (*Speothos venaticus*): Macdonald, 1996). In certain species, when depositing scent marks via handstands, both the height at which scent marks are deposited and the scent mark's size provide an 'index' – a direct correlation between a physical characteristic and a signal's quality (Maynard Smith & Harper, 1995) – to conspecifics regarding the signaller's body size.

Regularly frequented feeding/breeding grounds (Remonti *et al.*, 2011), territorial borders (Stewart *et al.*, 2002), and latrines (Green *et al.*, 2015) are also commonly scent-marked areas, particularly during periods of peak sexual receptivity (Buesching & Macdonald, 2004; Coombes *et al.*, 2018). In most species, both sexes may scent mark when advertising themselves for mating opportunities while also investigating marks deposited by sexually receptive, opposite-sex conspecifics (Allen *et al.*, 2015; Converse *et al.*, 1995). When individuals investigate marks

deposited by same-sex conspecifics, they often do this to assess potential competitors for access to mates (particularly among the limited sex, usually males; Gosling, 1986) and resources required for gestating/rearing offspring (especially among females; Coombes *et al.,* 2018). Such competition may result in over-marking (when individuals mark on top of a recently deposited scent mark; Ferkin & Pierce, 2007) and/or allo-marking (when individuals mark conspecifics; Buesching *et al.,* 2003). However, one must note that over-marking and allo-marking can also occur within non-competitive contexts (e.g., maintaining a common group odour, aiding in navigation, increasing foraging efficiency; reviewed in Ferkin & Pierce, 2007).

These aforementioned scent-marking patterns can vary considerably depending on both age and sex. Generally, sexually mature individuals mark more frequently than sexually immature individuals (Carnivora: Macdonald, 1985). Within most species, males mark more than females, although there are some species where females mark more than males or where sex differences are minimal (Carnivora: reviewed in Macdonald, 1985).

‘To be read’: Assessing the chemical composition of substances used for scent-marking

Assessing scent mark composition can shed insight on what information these marks encode (e.g., age, sex, reproductive status) along with how this information interacts with the receiver’s sensory receptors. Scent marks generally contain two types of compounds: volatile organic compounds (VOCs) and non-volatile organic compounds (non-VOCs; including proteins/peptides; Wyatt, 2014b). Once vaporisation occurs, VOCs become airborne and are detected by the main olfactory system (Brennan & Zufall, 2006). In contrast, the high molecular weight of non-VOCs prevents these compounds from becoming easily airborne, thus requiring

individuals to detect them via the vomeronasal organ (Jacobsen's organ) upon close contact with the scent mark (Brennan & Zufall, 2006).

The range of different molecules in scent marks, with different chemical properties, means that a variety of different analytical methods are required (Wyatt, 2014a). First, samples generally undergo sample pre-processing techniques to extract the compounds of interest (Drea *et al.,* 2013). Common extraction methods include headspace solid-phase microextraction (used for extracting VOCs in the air above a sample; Ouyang *et al.,* 2011), thermal desorption (used for extracting VOCs; Butrym, 1999), and alcohol extraction (also known as liquid-liquid extraction; used for extracting both VOCs and non-VOCs; Webster *et al.,* 2008). Extracted compounds then undergo analysis to identify specific molecules and their relative/absolute abundance within a sample, often via gas chromatography-mass spectrometry (GC-MS; Drea *et al.,* 2013). Within the gas chromatograph, the injected sample goes into the gas phase and is then carried by non-reactive gas (typically helium) through a heated capillary column. Compounds within the sample are separated based on their volatility and the amount of time spent in the moving gas phase and the stationary liquid phase as they travel through the heated column. The molecules become ionized once they reach the mass spectrometer. Afterwards, the mass spectrometer filters the ions based on mass and quantifies the number of ions, ultimately producing a graph where compound identity (along with compound abundance and travel time through the column) are represented by individual peaks (Drea *et al.,* 2013). Conducting a principal component analysis (PCA) on multiple GC-MS outputs can help when comparing chemical profiles between age groups, sexes, reproductive statuses, and social groups. These analyses require sophisticated statistical treatment and vigilance for biases (e.g. Noonan *et al.,* 2019).

Other semiochemical studies focus on protein/peptide composition, especially because of their role in facilitating the binding, transport, and release of VOCs within scent marks (Wyatt, 2014b). Identifying proteins within scent-marking substances often uses RNA sequencing (Zhang *et al.*, 2017), while protein assays and enzyme immunoassays (EIAs) detect the presence/quantity of proteins by binding the protein of interest to specific reagents (protein assays; Mistry *et al.*, 2013) or antibodies (EIAs; Wild, 2013). EIAs also provide a useful tool when measuring sex steroid (e.g., oestrogens, testosterone) levels within scent-marking substances (e.g., urine; Rajanarayanan & Archunan, 2011), particularly when analysing scent-marking within the context of reproductive advertisement.

‘To be believed’: Assessing the biological relevance of the chemical composition

Identifying the potential information encoded within scent marks does not address whether or not individuals find these particular compounds (or their relative combinations) biologically relevant. Addressing the biological relevance of scent mark composition requires behavioural studies via scent-provisioning experiments.

Scent-provisioning experiments can take several forms. Habituation/dishabituation experiments observe how individuals react towards novel versus familiar odours (Wyatt, 2014a). These experiments present individuals with an odour sample several times, measuring how an individual’s investigation behaviour (i.e., duration of time spent sniffing; Baum & Keverne, 2002) varies upon each presentation. Habituation is evidenced when an individual spends less time sniffing an odour during subsequent presentations. Meanwhile, discrimination experiments observe if/how individuals respond towards different odours (Tinnesand *et al.*, 2013; Tinnesand *et al.*, 2015). These experiments expose individuals to an odour along with a control treatment

(Descovich *et al.,* 2012), or to two odours simultaneously (Rostain *et al.,* 2004). Common behavioural responses measured during discrimination experiments are response frequency/likelihood, sniffing duration, countermarking frequency/likelihood, and avoidance behaviours (when assessing how prey species react to odours from predator species; Müller-Schwarze, 2006). Regardless of the experimental set-up, these behavioural experiments provide useful insight into if/how the chemical compositions that we as humans detect analytically via the methods mentioned in the previous section are perceived directly by at least some other mammals.

'To be remembered': Factors influencing olfactory signal memorability and discernment

As social interactions within a species become more complex, signals also increase in complexity to enable individual discrimination (Freeberg *et al.,* 2012; Pollard & Blumstein, 2011). Discrimination of individuals (and clans, of in-group and out-group) relies on learning (Wyatt, 2014a). In the context of recognition by smell, animals learn the individual signature mixtures (a combination of compounds that varies between individuals and/or social groups) of other conspecifics (Wyatt, 2010). Some species can use their own individual odour as a template (the so-called armpit effect, self-matching; Wyatt, 2010) when encountering unknown conspecifics. In other species, the odours of other clan members are learnt, allowing them to be recognized. Whatever the mechanism, such discrimination can provide a useful mechanism for inbreeding avoidance and enhancing their offspring's immunity by diversifying their major histocompatibility (MHC) profile (Milinski, 2006). Animals living in territories with stable boundaries may learn the neighbour's signature mixture which can be matched to the scent marks they leave (Gosling & Roberts, 2001).

‘To be acted upon’: Behavioural responses towards conspecific scent marks

Behavioural responses towards deposited scent marks vary depending on the donor’s and responder’s characteristics (e.g., age, reproductive status, sex, familiarity, dominance; Wyatt, 2014a). Sexually mature adults often respond more often and spend more time investigating conspecific marks compared to sexually immature juveniles (African lions (*Panthera leo*): Gilfillan *et al.*, 2017; Rodentia: Roberts, 2007). Likewise, scent marks from sexually receptive adults generally elicit more responses and longer investigation times – especially from opposite-sex conspecifics – compared to scent marks from non-receptive adults (e.g., pre-oestrous or dioestrus females; Rodentia: Roberts, 2007; koalas (*Phascolarctos cinereus*): Charlton, 2014) or sexually immature juveniles. Within agonistic settings, scent marks from unfamiliar conspecifics often trigger more responses and more countermarking than scent marks from familiar (group members and neighbours) individuals (e.g., banded mongoose (*Mungos mungo*): Müller & Manser, 2007). Meanwhile, dominant individuals mark more frequently than subordinate individuals (e.g., laboratory mice (*Mus musculus*): Gosling *et al.*, 2000), and their scent marks tend to elicit aversive behaviours (e.g., avoiding marked areas) from their subordinate conspecifics (e.g., Rodentia: Roberts, 2007).

Gaps within the literature

Individual advertisement via olfactory communication has been studied within numerous contexts. Nevertheless, certain gaps still remain in the literature. For instance, signal quality has long been considered to reflect an individual’s fitness in that only high-quality individuals possess the ability to produce high-quality, ‘costly’ signals (Zahavi, 1975; but see Penn & Számadó, 2020). However, while there are studies of song in crickets, frogs, and birds (see

Wyatt, 2014a) there is a notable lack of research of olfactory signals which directly quantifies the energetic costs associated with signal production or if conspecifics detect variations in production costs.

Additionally, regarding scent-marking with metabolic by-products (which are relatively 'free' to produce compared to specialised scent-marking substances), many field-based studies on wild populations have focused on conspecific responses to faecal deposits. This likely results from the comparative ease with which faeces can be collected along with faecal material lasting longer in the environment than urine. Just like faeces, urine also encodes fitness-related information regarding an individual's sex, age, and reproductive status (e.g., Indian blackbuck (*Antelope cervicapra L.*): Archunan & Rajagopal, 2013). Studies on domesticated (e.g., large white pig (*Sus scrofa domesticus*): Silambarasan *et al.,* 2019) and captive animals (e.g., koalas (*Phascolarctos cinereus*): Charlton, 2014) demonstrate that conspecifics vary their responses according to encoded information within urine, yet similar studies investigating this in wild populations – especially nocturnal mammals which rely predominantly on olfactory signals/cues – are comparatively scarce. My thesis aims to address these gaps by using a wild population of European badgers (*Meles meles*) as a study species.

European badgers (*Meles meles*) as a study species

Communication modalities used by European badgers

European badgers are nocturnal, group-living mammals which live in social dens, termed 'setts' (Tsunoda *et al.,* 2018) throughout Europe and Asia (Roper, 2010). Their social interactions, often between group members or individuals from neighbouring setts, can involve about 16 different types of vocalisations which vary depending on age, sex, and social context

(e.g., agonistic encounters, mating; Wong *et al.,* 1999). These vocalisations can sometimes accompany visual communication via its facial markings (Newman *et al.,* 2005) or tactile communication in the forms of allo-grooming between group members (Macdonald & Newman, 2017) or nudging individuals during foraging (Macdonald *et al.*, 2002).

However, due to their nocturnal activity and their poor eyesight from their fossorial lifestyle (Kitchener *et al.,* 2017; Noonan *et al.,* 2015), visual signals have minimal effectiveness. Furthermore, because badgers forage solitarily (Macdonald & Newman, 2017) and live in relatively low-density populations within most of their geographic range (Roper, 2010), tactile communication also has minimal effectiveness since messages are only conveyed when individuals are in close contact with each other. These lifestyle factors ultimately result in badgers depending largely on olfactory communication via metabolic by-products (i.e., urine and faeces; Buesching & Stankowich, 2017) and specialised glandular secretions (i.e., interdigital glands, anal glands, and the subcaudal gland that is unique to the genus *Meles* and the related genus *Arctonyx* (Figs. 1 and 2); Buesching & Stankowich, 2017).



Figure 1: *The uniquely evolved subcaudal pouch of the European badger circled here.*



Figure 2: *The subcaudal gland secretion which has been gently scooped out from the subcaudal pouch. This secretion has resulted in a yellow stain on the surrounding fur.*

The role of scent-marking in social interactions

The specially evolved subcaudal gland remains one of the more fascinating features of badgers. Composed predominantly of water, proteins, and short- and long-chained fatty acids (Buesching *et al.*, 2002a; Buesching *et al.*, 2002b), the composition of subcaudal gland secretion significantly varies based on age, sex, social group, and season (Buesching *et al.*, 2002a; Buesching *et al.*, 2002b). Part of this variation arises from microbial diversity within the subcaudal pouch and the resulting bacterial fermentation of the primary gland products (Buesching *et al.*, 2016b; Sin *et al.*, 2012). Badgers begin producing subcaudal secretion around 4-6 months old (Fell *et al.*, 2006), and the volume of secretion production varies with sex, age, and body condition (Buesching *et al.*, 2002a). Badgers of the genera *Meles* and *Arctonyx* are the only species with this particular gland, and little is known regarding the potential costs associated with producing this margarine-like secretion and if/how conspecifics respond to secretions with varying production costs.

Scent-marking with subcaudal gland secretion facilitates most social interactions amongst badgers. Like most mammals, badgers mark along commonly used paths and on various substrates (Buesching & Stankowich, 2017). Allo-marking also occurs among badgers, enabling the maintenance of a common group odour via the transfer of microbiota (Buesching *et al.*, 2016b; Sin *et al.*, 2012) while also using conspecifics as ‘mobile billboards’ when advertising their presence and/or sexual receptivity (Buesching *et al.*, 2003). Two forms of allo-marking have been observed amongst badgers – sequential allo-marking where one individual marks on top of another (Buesching *et al.*, 2003), and mutual allo-marking where two individuals press their subcaudal pouches against each other (usually practiced between group members; Buesching *et al.*, 2003).

Most badgers reside in their natal sett (Macdonald *et al.,* 2008), and many badger setts remain active for generations (Roper, 2010). These stable territories result in relatively stable borders marked by boundary latrines (Buesching *et al.,* 2016a). At these boundary latrines, badgers from neighbouring territories regularly deposit faeces coated with anal gland secretions along with urine and subcaudal gland secretions while also investigating recent deposits (Stewart *et al.,* 2002). Adult males typically use boundary latrines more than adult females and juveniles, especially during the peak mating season in spring (January-May; Sugianto *et al.,* 2018). Meanwhile, adult females and juveniles predominantly use hinterland latrines within their territory with peak usage also occurring during the peak mating season (Buesching & Macdonald, 2004). Although defecation occurs predominantly at latrines, urination can occur both at latrines and at non-latrine sites (Hutchings *et al.,* 2002). However, despite the knowledge that the VOCs within badger urine varies with season and sex (Service *et al.,* 2001), little is known regarding the function of urine-marking amongst badgers.

Thesis structure and aims

By using a wild population of European badgers living in Wytham Woods, Oxfordshire, U.K. (GPS reference: 51° 46’26”N; 1° 19’19”W; Macdonald *et al.,* 2009), my thesis aims to address the comparative adaptive significance of olfactory advertisement via specialised glandular secretions (subcaudal gland secretions) and metabolic by-products (urine) with the following research questions:

1. Scent-marking via specialised glandular secretions incurs production costs, but can these costs be quantified, does the level of investment differ between individuals and can this cost be detected by conspecifics?

2. Since the chemical composition within urine varies with season and sex, do these compositional variations encode biologically relevant information for conspecifics?
3. Since many badger populations live alongside humans and domesticated animals, do badgers rely on innate or learned knowledge when discriminating between scent marks from different species?

The first two data chapters focus on the subcaudal gland secretion by i) assessing the use of bomb calorimetry as a method for quantifying the secretion's energetic content, and ii) analysing conspecific responses to low- versus high-energy secretion in relation to donor/responder characteristics. The remaining three data chapters focus on the adaptive significance of urine-marking by i) analysing how excreted sex-steroid metabolites within urine influence conspecific behavioural responses, ii) observing seasonal variations in conspecific responses to urine marks, particularly outside the peak mating season, and iii) assessing olfactory landscapes of fear and evaluating whether badgers possess an innate or learned response towards species-specific urine marks. Finally, the general discussion compares the significance of scent-marking with subcaudal gland secretion versus urine as well as possibilities for future research.

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Section II: Olfactory advertisement via specialised glandular secretions



"The Autumn Badger" by Emily Palmer

Chapter 2:
Measuring the minimum cost of animal secretions: Using bomb calorimetry of badger secretions as a case-study[1]

Abstract

Evolutionary theory posits that fitness advertisement incurs costs to ensure signal honesty. To date, however, production costs of olfactory advertisement secretions have never been quantified because traditional chemical analyses are specific to component classes (necessitating additive analyses) and measure relative rather than absolute amounts (requiring post-analytical data-processing). Here we used a novel time- and cost-effective approach – bomb calorimetry – which bypasses these limitations by oxidising all chemical bonds within a chemically complex sample simultaneously to quantify the overall energetic content ('Calorific Value': CV_{dry}) of subcaudal gland secretions used by European badgers (*Meles meles*) for fitness advertisement. From this, we calculated three biologically meaningful metrics: the CV of natural secretion ($CV_{natural}$), the minimum daily energy allocation to scent production (CV_{pouch}), and the CV of individual scent marks ($CV_{scentmark}$). We then used linear mixed models to correlate these values with biological factors known to influence subcaudal gland secretion composition (season, sex, body condition, and reproductive life stage). Our proof-of-concept study found that all energetic metrics showed significant seasonal variations with females having higher CV_{dry}, $CV_{natural}$, and $CV_{scentmark}$ values during autumn, while males had higher CV_{pouch} values during spring. Only one metric (CV_{pouch}) showed sex-specific differences as males had higher CV_{pouch} values than females. Meanwhile, body condition and reproductive life stage only affected

[1] This chapter is the basis of a paper that was submitted to ***BioEssays,*** co-authored by Tanesha M. Allen, Paul Johnson, Chris Newman, David W. Macdonald & Christina D. Buesching

$CV_{natural}$ in females. We discuss our findings within the context of energetic investments in scent-marking and the usage of bomb calorimetry as a versatile tool for semio-chemical research.

Introduction

Many animals use scent from specialised glandular secretions alongside faeces/urine for intra- and inter-specific communication, where olfactory signals facilitate territorial defence, individual recognition, mate choice, and reproductive advertisement (e.g., Musteloidea: Buesching & Stankowich, 2017). Faeces/urine are free by-products from heterotrophic digestion, but the production and distribution of secretions (often involving elaborate scent-marking behaviours: e.g., Sharpe, 2015) incur energetic costs which may be further exacerbated if scent-marking increases risk of detection by predators/competitors (Roberts *et al.*, 2002). Therefore, theory predicts that only "fit" individuals (i.e., healthy animals in good condition) possess surplus energy to invest in secretion and undertake and/or intensify marking activity while maintaining their somatic requirements (Gosling & Roberts, 2001). Glandular scent-mark "quality" should thus correlate with marker "quality" – a concept known as an "index", allowing conspecifics to infer fitness-related information about the signaller (Számadó, 2011; Zala *et al.*, 2004). Indeed, inter-individual differences in scent-marking behaviour, glandular activity, and secretion composition correlate with physiological fitness indicators (Buesching & Stankowich, 2017; Petrulis, 2013), causing variation in conspecific responses (Petrulis, 2013).

Consequently, scent-marking is widely assumed to function as honest fitness advertisement (Coombes *et al.*, 2018; Gosling *et al.*, 2000), and modified responses based on scent-mark quality likely act as selective forces on signal design and evolution. Signalling individuals must afford production costs (particularly 'efficacy costs' for accurately conveying

information; Maynard Smith & Harper, 1995) alongside design practicalities to ensure that their signals are detectable, discriminable, and memorable (Guilford & Dawkins, 1991); receivers must accurately decipher the encoded information to prevent negative fitness consequences due to misjudging competitive ability or mate quality (Gosling & Roberts, 2001). To date, several studies have investigated investment in scent-marking behaviour (Coombes *et al.*, 2018; Gosling, 1982), but energetic costs associated with glandular secretion production have never been quantified. A further question is how the costs associated with glandular secretions compare with daily energy intake.

The cost of production in biochemical terms of specialised secretions may be beyond straightforward analysis (see e.g. milk, Bauman *et al.*, 2006), but an estimate of approximate minimum energetic investment can be sought from chemical analysis of the secretions themselves. Here we investigate inter-individual differences in the direct energy content of secretions using a different technique. While long used in ecological food web research (Phillipson, 1964) and animal dietary studies (Finley *et al.*, 2011), bomb calorimetry has not before been used to study the energetics of animal chemical communication. We use it here to provide a comprehensive measurement of the calorific value (CV) of scent secretions as an estimate of the approximate minimum energetic investment. Bomb calorimetry can quantify the energetic content of a complex chemical mixture in a single step: a standardised amount of sample wet weight is freeze-dried (to determine the dry mass and ensure proper ignition/combustion of the sample being analysed) and placed into a “bomb” – a thick walled, sealed, stainless steel container. The bomb is filled/charged with oxygen, creating a high-pressure environment within a larger, insulated container filled with water. An electrical charge then initiates combustion. Oxidation breaks all chemical bonds within the sample, releasing

energy that raises the surrounding water temperature and produces a quantifiable value of the energetic content within the sample ("heat of combustion" or "calorific value (CV)"; Parr Instrument Company, 2007).

To test the efficiency and biological validity of bomb-calorimetry analysis of scent-secretions, we use the subcaudal gland secretion (SGS; Stübbe, 1971; Buesching *et al.,* 2002a) of European badgers (*Meles meles*) as a model. Badgers have a seasonal lifecycle switching between catabolic metabolism during spring/summer and anabolic metabolism in during the winter months. SGS is secreted by sebaceous and apocrine cells and stored in the subcaudal pouch that opens in a 30-50 mm crescent-shaped slit between tail and anus (Buesching *et al.,* 2002a; Stübbe, 1971). SGS is deposited by brief contact of pouch opening and substrate when squatting down (Buesching & Macdonald, 2004). Distribution costs are minimal (Buesching & Macdonald, 2004). Because marking activity correlates with stored SGS volume (Buesching & Macdonald, 2004) and SGS pouch volumes get replenished to similar levels within 24 hours (Buesching *et al.,* 2002a), we posit that SGS pouch volume indicates glandular activity.

Badger SGS is a suitable model for our purposes for three reasons: i) SGS has been suggested to function as honest fitness advertisement with considerable inter-individual differences in marking activity (Buesching *et al.,* 2003; Buesching & Macdonald, 2004); ii) SGS composition and production depend on fitness-related parameters (Buesching *et al.,* 2002b); iii) SGS contains a complex mixture of short-, medium- and long-chained carboxylic acids, esters and proteins (Buesching *et al.,* 2002b). Thus, by linking secretion CV to donor sex, age/reproductive life stage, body condition and season (as a proxy for reproductive status in this seasonally breeding species), we can explore if SGS energy content accurately reflects body condition.

We analyse four metrics directly related to the energetic content of glandular scent marks: i) CV of freeze-dried secretion as determined by the bomb calorimeter (Joules/gram$_{secretion}$), ii) CV of natural secretion taking into account moisture content eliminated during freeze-drying (J/ml$_{secretion}$), iii) CV of the secretion amount stored in the pouch as a proxy for investment in scent-marking potential, and iv) CV of a subcaudal scent-mark taking the individual-specific size of the scent gland opening into account. We relate these to physiological and seasonal factors known to impact SGS chemical composition, SGS volume, and scent-marking.

Methods

Study animals and sample collection

SGS samples were collected between January 2010 and November 2017 from a high-density badger population in Wytham Woods, Oxfordshire, UK (GPS reference: 51° 46’26”N; 1° 19’19”W; Macdonald *et al.,* 2009) – a 424-ha area of semi-natural mixed woodland (Savill *et al.*, 2011). Using methodology described by Macdonald *et al.* (2015), badgers were live-trapped 3-4 times annually in cage traps baited with peanuts placed near sett entrances.

Following sedation by intramuscular injection of 0.2 ml ketamine hydrochloride/kg body weight (McLaren *et al.,* 2005), individuals were identified by unique tattoo numbers applied at first capture (typically as cubs: Sun *et al.,* 2015), sexed, and their body condition categorised as 1 = emaciated, 2 = intermediate, or 3 = very good condition (Buesching *et al.,* 2002a). Exact age (from birth year) was known for all sampled individuals. Reproductive life stage was based on age as sexually immature: ≤ 2 years old and sexually mature: 2+ years old (Buesching *et al.,* 2009; Sugianto *et al.,* 2020). No cubs (≤ 1 year old) were included in this study.

Subcaudal pouch opening was measured (in mm) using string to conform with the pouch curvature and referenced against a ruler. We emptied SGS from the subcaudal pouch with a rounded, stainless steel spatula, and the secretion volume was estimated (to the nearest 0.1 ml; Buesching *et al.,* 2002a). All samples were placed in glass vials with Teflon-coated screw lids (Sigma-Aldrich, Dorset, UK) and frozen immediately at -20°C to prevent bacterial decay (Buesching *et al.,* 2016; Sin *et al.,* 2012) from altering the calorific value and/or scent profile.

Ethical review and approval was obtained for all trapping and handling procedures, and performed under UK Animals (Scientific Procedures) Act, 1986, licence (currently PPL 30/3379) and Natural England licence (currently 2019-38863-SCI-SCI).

Bomb calorimetry analysis

Samples (n = 84; 51 males [spring = 21, summer = 8, autumn = 17, winter = 5] and 33 females [spring = 13, summer = 5, autumn = 15; no female winter samples were substantial enough for analysis]) were frozen overnight before being freeze-dried for 24h using a ScanVac CoolSafe (LaboGene ApS, Allerød, Denmark) freeze dryer. This eliminated any water content that could interfere with calorimetric analysis. Each sample was weighed (in g) before ('wet weight') and after ('dry weight') freeze-drying. Dry matter (in %) from each sample was determined with the following formula:

$$\frac{Dried\ sample\ weight\ [g]}{Wet\ sample\ weight\ [g]} * 100\%$$

Moisture content (in %) was calculated by subtracting dry matter from the total percentage. Moisture content was then converted into ml by multiplying the percentage by the

total secretion volume. The collected secretion volume was converted into collected dry matter by multiplying the percentage of dry matter by the total secretion volume.

CV analyses (in MJ/kg) were performed at Sciantec Analytical Services, Selby, UK, using a 6100 Parr bomb calorimeter (Parr Instrument Company, Illinois, USA). Freeze-dried samples were placed inside a crucible within a sealed, stainless steel bomb containing enough oxygen to ensure complete sample combustion. The resulting 'bomb' was placed inside a larger, insulated container with 2424 ml of water, and the combustion heat transferred to the surrounding water bath was measured in °C. Benzoic acid was used to calibrate the bombs. Sample combustion took about 10 minutes.

Assuming that i) the moisture content from the complete sample is comparable to the moisture content within the analysed subsample, and ii) wet mass provides a reasonable measure for secretion volume (because 1 ml of water weighs 1g), we then calculated four biological metrics:

i) CV of dried SGS (CV_{dry}) (in J/g).

ii) CV of natural (i.e., not dried) secretion ($CV_{natural}$; in J/ml) accounting for individual variation in water content: calculated as CV_{dry} multiplied by moisture content (in ml).

iii) Energetic investment into secretion (CV_{pouch}): total CV (in J/ml) of the secretion in the subcaudal pouch calculated as $CV_{natural}$ * pouch secretion volume.

iv) Energetic investment into each scent mark ($CV_{scentmark}$): total CV deposited with each scent mark (J/ml) calculated as $CV_{natural}$ * scent mark dimensions, where scent mark dimensions were calculated in mm^3 as pouch opening length (mm) *

pouch width (3 mm) * scent mark thickness (0.1 mm; pers. obs.) before being converted to ml.

Statistical analysis

Statistical analyses were performed in R (version 3.4.3) and R Studio (version 1.1.383). Linear mixed models (LMMs) were fitted with the *lme4* package (Bates *et al.*, 2015). Model convergence was checked with the *performance* package (Ludecke *et al.*, 2020), and statistically significant parameters were visualised with the *effects* package (Fox, 2003).

Inter-sexual differences in SGS calorific content were analysed using LMMs with body condition, season, and reproductive life stage as covariates. Sex-specific linear models analysing intra-sexual variation from physiological and seasonal factors were performed to account for differing reproductive strategies. SGS calorific content was log transformed in all analyses to improve model fit. Confidence intervals and standard deviations were recorded for point estimates.

Results

CV of freeze-dried secretion (CV_{dry})

CV_{dry} ranged from 4190 to 35020 $J/g_{dry\ weight}$ (median = 9175 ± 6056.26 $J/g_{dry;\ 95\%\ CI}$ [9311.21 $J/g_{dry,}$ 12825.93 J/g_{dry}]) in males (n = 50) and 6640 to 36660 J/g_{dry} (median = 9520 ± 8468.39 J/g_{dry}; 95% CI [9633.47 J/g_{dry}, 15542.99 J/g_{dry}) in females (n = 34). However, there was no evidence of sex-specific differences (LMM: estimate = -0.11, t-value = -1.15, p = 0.25). In females, CV_{dry} was significantly lower in spring (linear mixed model: estimate = -0.51, t-value = -2.71, p = 0.01) and somewhat lower in summer (LMM: estimate = -0.37, t = value = -1.56, p = 0.13; Fig.

1) than in autumn. Meanwhile, there was no evidence that season affected male CV_{dry}. Neither body condition nor reproductive life stage affected CV_{dry} in either sex.

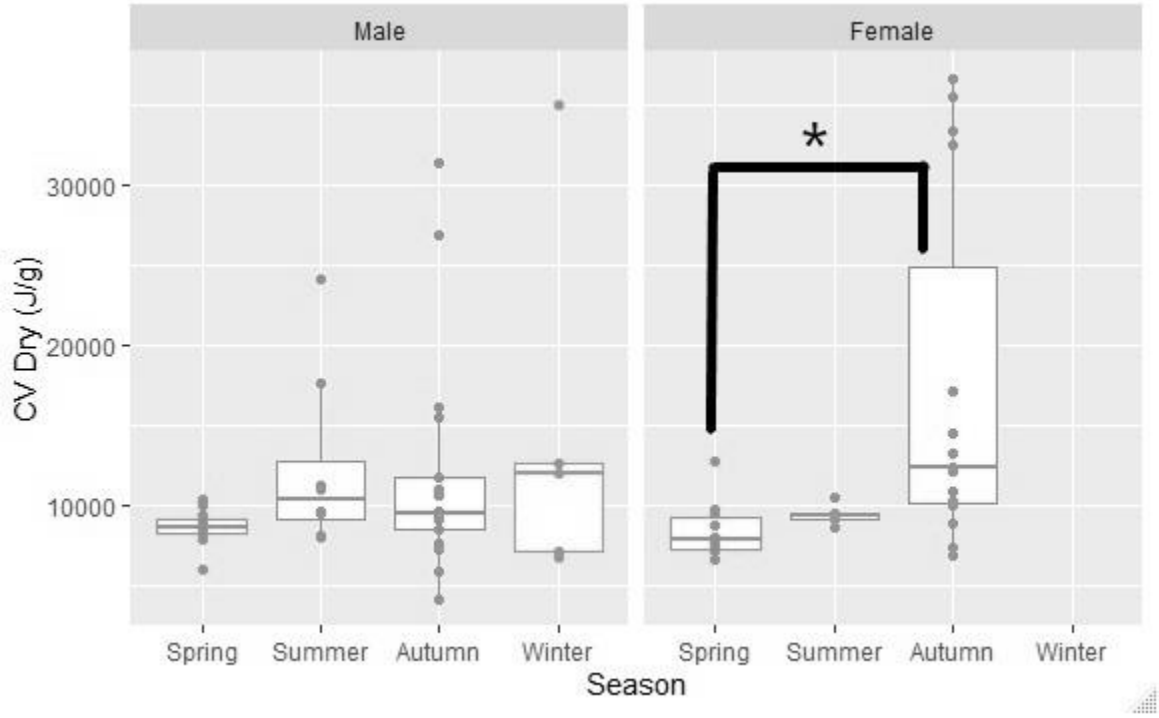


Figure 1: *Seasonal variation in male and female CV_{dry}. Asterisks denote significance.*

CV of natural secretion ($CV_{natural}$)

$CV_{natural}$ ranged from 3695.58 to 25914.80 $J/ml_{natural}$ (median = 6524.93 ± 2765.05 $J/ml_{natural}$; 95% CI [6396.37 $J/ml_{natural}$, 8610.44 $J/ml_{natural}$]) in males (n = 50) and 5000.44 to 27366.69 $J/ml_{natural}$ (median = 6774.41 ± 5676.45 $J/ml_{natural}$; 95% CI [6690.76 $J/ml_{natural}$, 10651.97$J/ml_{natural}$]) in females (n = 34). There was no evidence for sex-specific differences (LMM: estimate = -0.11, t-value = -1.33, p = 0.19). Season affected female (but not male) $CV_{natural}$ where females had lower $CV_{natural}$ values during spring (LMM: estimate = -0.57, t-value

= -3.30, p = 0.002) and summer than in autumn (LMM: estimate = -0.46, t-value = -2.06, p = 0.048; Fig. 2).

In females, but not males, body condition approached significance (LMM: estimate = -0.08, t-value = -2.92, p = 0.08; Fig. 3) with females in better condition having higher $CV_{natural}$ values than thinner females. Sexually mature females (but not males) had higher $CV_{natural}$ values than immature females (LMM: estimate = 0.09, t-value = 3.57, p = 0.06).

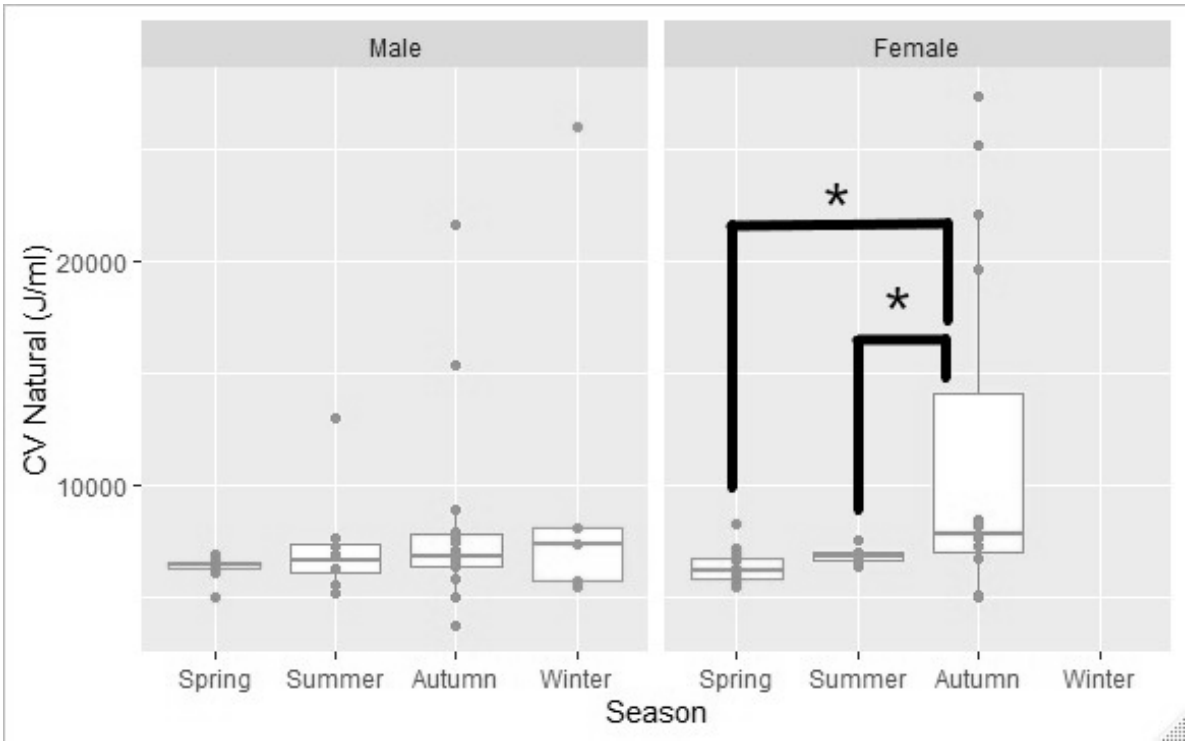


Figure 2: *Seasonal variation in male and female* $CV_{natural}$*. Asterisks denote significance.*

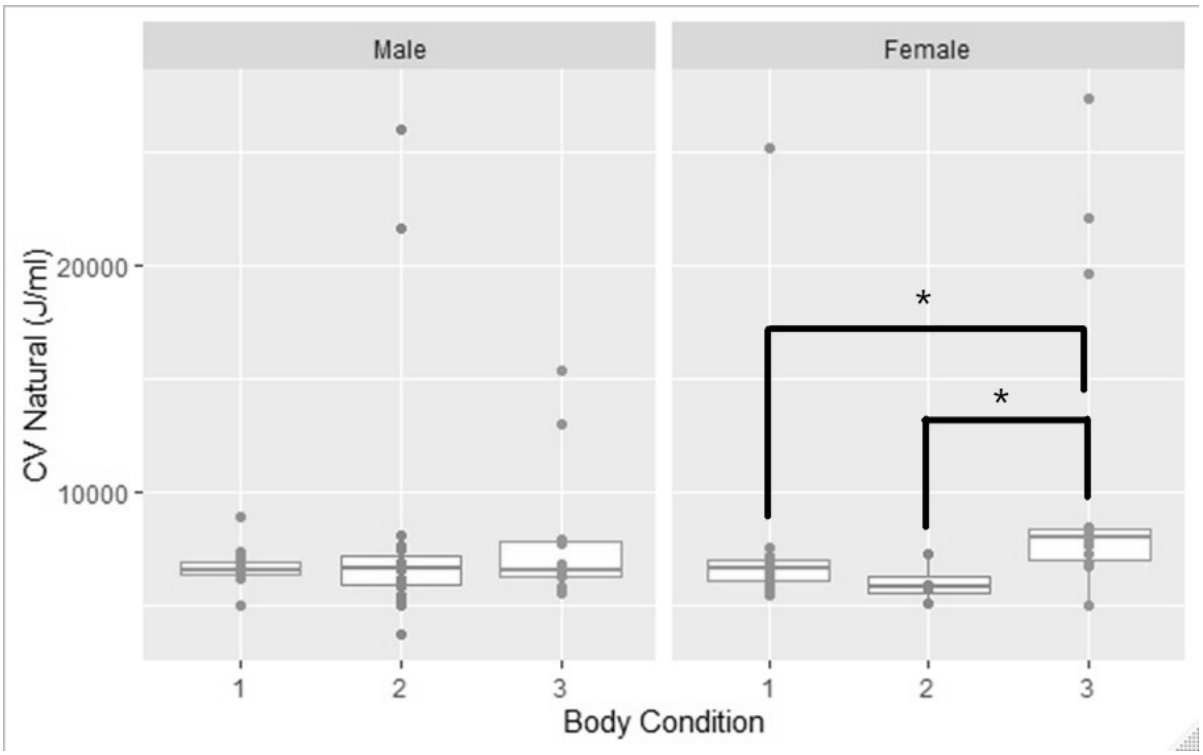


Figure 3: *Effects of body condition on male and female* $CV_{natural}$*. Asterisks denote significance.*

CV of the pouch content (CV_{pouch})

CV_{pouch} ranged from 2196.03 to 32116.74 J (median = 7790.36 ± 7011.78 J; 95% CI [7782.84 J, 11879.11 J]) in males (n = 49) and 752.08 to 24630.02 J (median = 4095.98 ± 5830.32 J; 95% CI [4751.23 J, 8885.91 J]) in females (n = 33). There was evidence that sex affected CV_{pouch} as CV_{pouch} was significantly higher in males than in females (LMM: estimate = 0.46, t-value = 2.89, p = 0.005). There was evidence that season affected CV_{pouch} in males (but not females) with male CV_{pouch} being significantly higher during spring (LMM: estimate = 0.77, t-value = 3.45, p = 0.001; Fig. 4). There was no evidence that either body condition or reproductive life stage affected CV_{pouch} in either sex.

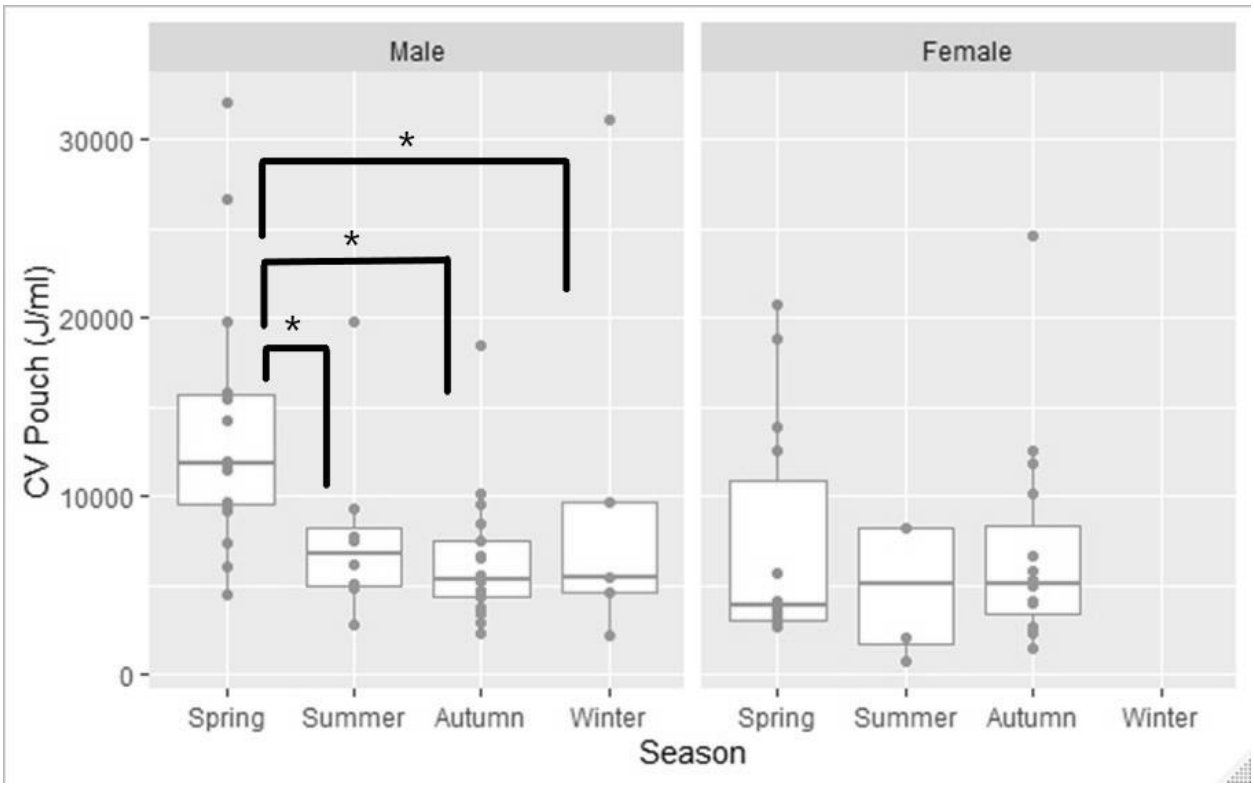


Figure 4: *Seasonal variation in male and female* CV_{pouch}*. Asterisks denote significance.*

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CV of scent marks ($CV_{scentmark}$)

$CV_{scentmark}$ ranged from 58.71 to 388.65 J (median = 112.11 ± 62.67 J; 95% CI [104.87 J; 141.19 J]) in males (n = 50) and 64.47 to 451.55 J (median = 112.47 ± 92.97 J; 95% CI [112.16 J; 177.04 J]) in females (n = 34). There was no evidence of sex-specific differences (LMM:

235 estimate = 0.10, t-value = 1.16, p = 0.25).

Female $CV_{scentmark}$ was significantly higher in autumn than in other seasons (LMM: estimate = -0.51, t-value = -2.31, p = 0.03; Fig. 5). Meanwhile, there was no evidence that either body condition or reproductive life stage affected $CV_{scentmark}$ in either sex.

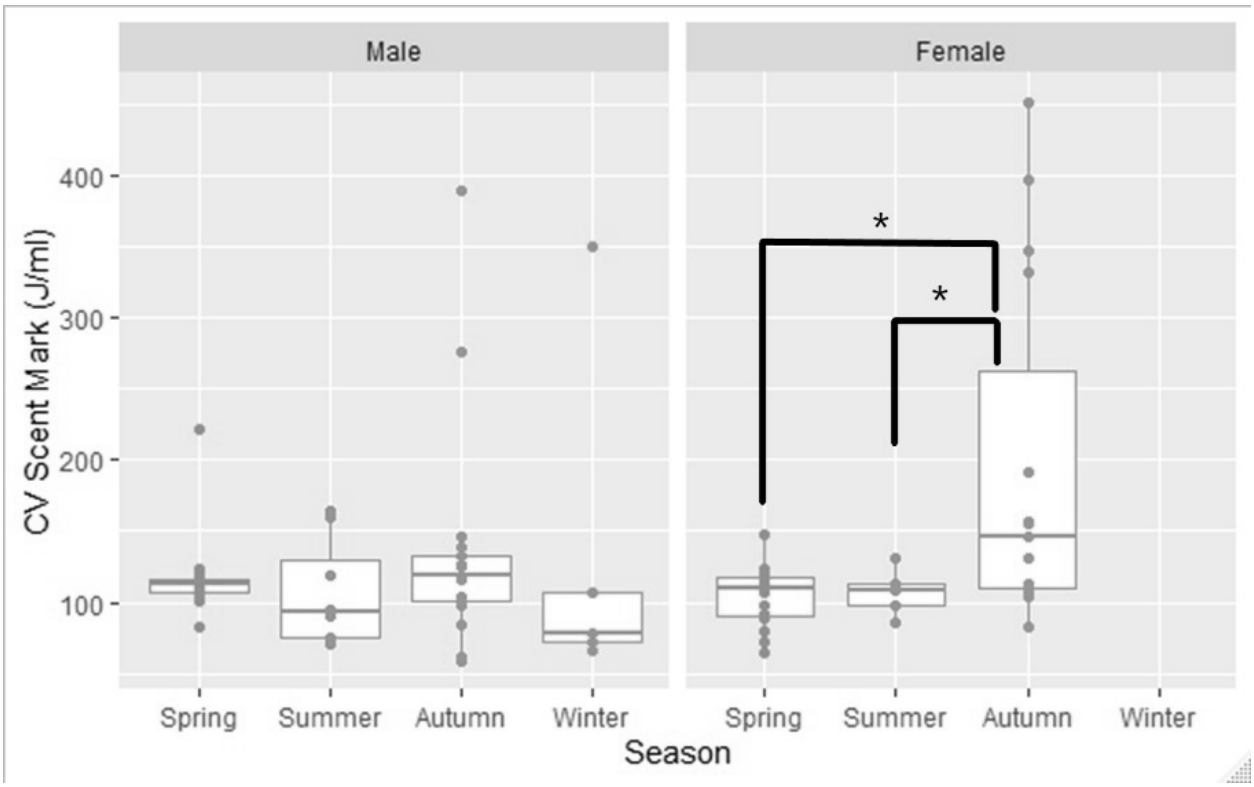


Figure 5: *Seasonal variation in male and female* $CV_{scentmark}$*. Asterisks denote significance.*

Discussion

Most behavioural studies investigating scent-marking behaviour within social contexts assume that scent-marking poses a considerable energetic cost, particularly for specially-produced olfactory secretions (Brown & Macdonald, 1985; Buesching & Stankowich, 2017; Gassett *et al.*, 1996). These presumed energetic costs lead to the interpretation of scent-marking functioning as honest fitness advertisement because only individuals with positive body condition can invest in scent secretion production (Gosling *et al.*, 2000). Here we present the first energetic measurements of a dedicated scent secretion: the subcaudal gland secretion of European badgers. Our study demonstrates bomb calorimetry's usefulness in quantifying the minimum energetic costs involved in producing olfactory secretions (additional "production costs" may result from biosynthesizing more complex high-energy molecules; Bauman *et al.*,

2006). Our results show considerable individual and seasonal variation in SGS calorific value. Although bomb calorimetry measures only the calorific value of freeze-dried secretion, other measures recorded during animal handling allowed us to calculate additional metrics that animals may use when decoding fitness-relevant biological information: The calorific value of natural (i.e., not freeze-dried) secretion, the energetic investment into stored secretion available for scent-marking, and the calorific value of each scent mark.

Bomb calorimetry as an analytical method

Most analytical methods are specific to chemical component classes (Soso *et al.,* 2014). Glandular secretions, however, contain a complex mixture of volatile organic compounds (VOCs) and non-volatile organic compounds (non-VOCs; including proteins; Apps *et al.,* 2015) which necessitates pre-processing extraction techniques. One exception is the proximate-composition analysis (PCA) that measures the percentages of water, ash, proteins, lipids, and carbohydrates (AOAC, 1990), but requires five different, consecutive processes (where, based on the compound combustion equivalents absolute energetic content can be calculated: Doyle *et al.,* 2007). Another exception is high-performance liquid chromatography (Nice & Aguilar, 2004) that – without any further processing - only determines presence/absence of different compounds. Quantification results differ considerably between methodologies (Riond *et al.,* 2014), and, importantly, all traditional methods measure relative rather than absolute compound amounts, thus requiring further statistical data normalisation and analysis (Noonan *et al.,* 2018).

Bomb calorimetry accounts for the absolute energy content of VOCs and non-VOCs (including proteins) simultaneously. Sample pre-processing – and thus chemical alteration and/or bias of compounds – is minimal. For behavioural and evolutionary biologists, the biggest

advantage of bomb calorimetry over compound-specific separation techniques is that it can be used to determine the energetic content of complex chemical mixtures, such as animal scent secretions without the need for sample pre-processing (Soso *et al.,* 2014), where biases towards single compounds/chemical classes (lipids: Asa, 1993; VOCs: Raymer *et al.,* 1985) can skew biological inferences. Proximate-composition analysis remains the only other method capable of measuring the energetic content of complex chemical mixtures, and Doyle *et al.'s* (2007) calorific analysis of jellyfish showed that bomb calorimetry and proximate-composition analysis returned similar results. Bomb calorimetry, however, is considerably less time-consuming than proximate-composition analysis: We freeze-dried samples in large batches for 24h before sample combustion which lasted ca. 10 minutes per sample. This greatly contrasts with proximate-composition analysis where determining crude fat alone via Soxhlet extraction can take 24 hours (Bhattacharjee *et al.,* 2013), VOCs an additional 2-3 hours using the gravimetric method, and non-VOCs an additional 24 hours (using the modified Fehling solution method; AOAC Official Methods of Analysis, 1990). Furthermore, standard bomb calorimeters can analyse samples as small as 1g (while micro-bombs/semimicro calorimeters can combust samples as small as 25 mg; Parr Instrument Company, 2019) whereas proximate-composition analytical stages (moisture, crude fibre, crude protein, crude fat) each require minimally 2 to 3 grams (Bhattacharjee *et al.,* 2013).

Nevertheless, to ensure accurate results, bomb calorimetry requires (freeze-)dried samples. Furthermore, samples with high fat content must be flattened during the freeze-drying process to improve water extraction. Secretion moisture content, however, can vary considerably between individuals and seasons; ranging from 41% to 88% in our SGS samples. Thus, even seemingly large natural secretion samples risk drying to below the minimum threshold analytical

sample weight. Consequently, we could sample only badgers that produced more than 1.5 ml of secretion (i.e., ca. 1g dry weight). This restricted our ability to analyse small (< 1ml) secretion samples – typically produced by juveniles, a proportion of females (Buesching *et al.*, 2002a), and reproductively senescent individuals (Sugianto *et al.*, 2020). Caution must therefore be applied when interpreting the biological relevance of methodologically biased sample classes.

What is the cost of scent-marking for badgers?

In our population, badgers feed predominantly on earthworms (*Lumbricus sp*), consuming ca. 175 worms per night (Kruuk, 1978). Each worm weighs ca. 2.5g (Daniel *et al.*, 1996), of which ca. 0.5g (20%; Bernard & Allen, 1997; French *et al.*, 1957) is dry matter equalling between 2.2 kcal (French *et al.*, 1957) and 4.65 kcal (Bernard & Allen, 1997). Ultimately, 2.2-4.65 kcal equals ca. 9.2-19.45 kJ (9200-19450 J).

If we use the lower end of these values, producing 1 ml of SGS ($CV_{natural}$) requires that badgers consume minimally (i.e., neither accounting for biosynthesis costs nor for incomplete nutrient absorption during the digestion of worms) between 0.3 and 3 worms. As males produce up to 3.5 ml of secretion per night (Buesching *et al.*, 2002a), calculations indicate that this represents the energetic equivalent of ca. 1-10.5 worms. Thus, SGS production requires between 0.4-6% of their daily energy budget (510 kcal/day or 2134000 J/day for basal metabolism of an average 10kg badger; Iversen, 1972). Nevertheless, this neither accounts for biosynthesis costs nor for the likely incomplete nutrient absorption during digestion. For example, American badgers (*Taxidea taxus*) have been shown to absorb between 67% and 75% of the energy content of a mixed dog food diet (Harlow, 1981), indicating that the CV of 1 ml of SGS is likely equivalent to nearer 0.4-4 worms. As a result, this drives male investment into scent production

in our example (above) up to nearer 0.8-8% of their daily energy budget (where we still do not account for currently unknown biosynthesis costs). This is particularly pertinent because males produce the most secretion – and engage in most frequent scent-marking – during the winter mating season when food is scarce and earthworms are hard to come by due to frost (Macdonald *et al.,* 2010).

Yet animals can, hypothetically, "stretch" secretion by diluting them with water, enabling them to increase scent-marking frequency with little additional energetic investment. We found support for this strategy by comparing CV_{dry} (a reflection of the SGS chemical composition) with $CV_{natural}$ (a reflection of the SGS moisture content), proving that some individuals with high CV_{dry} content had low $CV_{natural}$ content and vice-versa. Females in particular varied their secretion CV whereas males simply varied amounts produced.

Surprisingly, body condition only approached significance for one energetic metric: $CV_{natural}$ in females. Previous studies have shown that body condition significantly affects secretion volume (Buesching *et al.,* 2002a) and chemical composition (Buesching *et al.,* 2002b). Nevertheless, it should be noted that a positive correlation between body condition and secretion volume was only observed during winter for both sexes and during summer for males (Buesching *et al.,* 2002a). Furthermore, a strong correlation between body condition and chemical composition was only evident during winter and summer (Buesching *et al.,* 2002b). The seasonal variations found in our proof-of-concept study, however, were only apparent during autumn (for females) and spring (for males). Future work could correlate SGS composition with energetic content to determine if these findings are due to a seasonal abundance or lack of particular compounds.

The biological relevance of SGS energetic content to conspecifics was assessed in a study done in conjunction with this one (Allen *et al.*, submitted). Our study found that while energetic content alone did not significantly influence conspecific responses, the interaction between energetic content and donor age group as well as energetic content and donor sex influenced response likelihood and countermarking likelihood. Since badgers adjusted their behaviour according to both SGS energetic content and donor age group/sex, future longitudinal work could be done to assess how energetic investment in SGS production affects lifetime reproductive success.

In conclusion, bomb calorimetry provides a time-effective way to quantify the energetic content of specialised scent secretions. We found that some individuals produced secretions with high $CV_{natural}$ but low CV_{dry} and vice-versa, suggesting that animals can stretch secretions by varying their water content. Thus, it is important to calculate all four energetic metrics presented here rather than relying on CV_{dry} alone to avoid false/biased interpretation. Additionally, our results emphasise the importance of including energetic quantification in future research that relies on the underlying presumption that specialised secretions are honest fitness advertisement signals.

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Chapter 3:
Energetic content of specialised scent secretions in European badgers (*Meles meles*) influences behavioural responses of conspecifics[2]

Abstract

In many mammals, production and chemical composition of specialised scent secretions shows high inter-individual differences linked to body condition, where theory predicts that signalling costs help maintain signal honesty. This is because only fit individuals are likely to possess enough energy to produce costly high-quality signals while balancing their somatic requirements. Nevertheless, this has never been tested empirically. We used wild European badgers (*Meles meles*) as a model to investigate in scent-provisioning experiments if conspecifics modify their response behaviour to scent-marks based on secretion energy content. Controlling for donor sex, group membership, and sample collection year, seasonally contemporaneous subcaudal gland secretion samples from 84 individuals (n = 51 males, 33 females) of known calorific content quantified by bomb calorimetry were provided in pairs of a "cheap" (i.e., low-energy) secretion with an "expensive" (i.e., high-energy) secretion, and placed along frequently travelled paths in the vicinity of 3 focal setts. Responses were recorded with motion detector activated wildlife cameras (Crenova 2.4" LCD Display [12MP and 1080P HD] and Stealth Cam Model STC-G45NG [14MP and 1080P HD]). Energy content alone did not influence response likelihood, but low-energy secretions from older donors and high-energy secretions from younger donors elicited more responses. Adult responders also countermarked high-energy

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secretions from same-sex donor more often than low-energy secretions from same-sex donors. Meanwhile, adults countermarked low- and high-energy secretions from opposite-sex donors at similar rates. We discuss our findings within the context of reproductive advertisement and intra-sexual competition.

Introduction

In most mammals, olfactory communication plays an important role in resource acquisition and defence, mate selection, and competitor assessment (Brown & Macdonald, 1985). In particular, secretions from specialised scent glands function as olfactory signals (*sensu* Maynard Smith & Harper, 1995) to conspecifics and can convey information about physiological (e.g., age, sex, and reproductive condition) and social status (Wyatt, 2014; e.g., musteloids reviewed in Buesching & Stankowich, 2017). Thus, receivers (i.e., conspecifics sniffing these scent-marks) can draw inferences about the signaller's (i.e., the scent-marking individual`s) state relative to their own, which in turn helps shape appropriate response behaviour (e.g., Tinnesand *et al.*, 2015).

Signals can incur two types of energetic costs (Maynard Smith & Harper, 1995): efficacy costs (i.e., the base costs for conveying information unambiguously) and strategic costs (i.e., added costs evolved to ensure signal honesty *sensu* Zahavi's handicap hypothesis: Zahavi 1975, 1977). Therefore, two types of costly signals exist: Minimal signals accurately convey information while incurring no additional energetic costs beyond the signal itself, even if the signal is costly to produce (e.g. bird display song; Maynard Smith & Harper, 1995; Számadó, 2011); and cost-added signals, which, according to Handicap Theory, are thought to incur additional costs when conveying information, where Handicap Theory predicts that signal

quality corresponds with fitness of the signaller because only fit, high-quality individuals are likely to be able to generate high-quality (i.e., energy-expensive) signals, ensuring honest fitness advertisement (Grafen, 1990; Zahavi, 1975; though Számadó, 2011 and Penn & Számadó, 2020 question the Handicap Theory).

Therefore, two fundamental assumptions underlie the concept that signalling costs help with maintaining signal reliability/honesty: i) that signal production requires quantifiable energetic costs that vary with sender quality, and ii) that conspecifics can detect these qualitative differences and modify their behavioural responses accordingly, where high signal costs elicit responses that increase signaller fitness, resulting in evolutionary advantage (Maynard Smith & Harper, 1995).These assumptions, however, have proven difficult to investigate and have never been tested in the field.

Here, we use European badgers (*Meles meles*) as a model in scent-provisioning experiments in the wild to investigate if animals can indeed detect differences in calorific content of scent secretions and use this information to modify their behavioural response to these signals. Badgers rely heavily on olfactory signals for intra-specific communication (Buesching and Stankowich, 2017). In the UK, they are group-living and share communal dens – or 'setts' – (Macdonald *et al.,* 2015) situated within the centre of a group range demarcated by latrine boundaries (Buesching *et al.,* 2016a), where they deposit urine, faeces and secretions from specialised scent glands. Badgers convey individual- and group-specific information via paired anal glands (Noonan *et al.,* 2019) and a specially evolved subcaudal gland (Buesching *et al.,* 2002a). The subcaudal gland produces secretion high in short-, medium- and long-chained carboxylic acids (Buesching *et al.,* 2002b; Buesching *et al.,* 2002c; Buesching *et al.,* 2016b) and proteins (Gorman *et al.,* 1986), which is stored in a pouch lined with sebaceous and apocrine

cells that opens in a 30-62 mm long slit situated between anus and tail (Stübbe, 1971; unpublished data). Badgers deposit subcaudal gland secretions by pressing the pouch opening against substrates ('object-marking': Buesching & Macdonald, 2004) and conspecifics (termed 'allo-marking': Buesching *et al.*, 2003). The produced quantity and the chemical composition of subcaudal gland secretions (SGS) vary seasonally and with sex, age, reproductive status and body condition. SGS composition also encodes group membership (Buesching *et al.*, 2002b), information that can likely be deciphered by conspecifics who modify their behaviour accordingly (as previously shown for anal gland secretions: Tinnesand *et al.*, 2015).

Compared to other signal modalities (i.e.., auditory, visual, tactile), olfactory signals – particularly those resulting from specialised glandular secretions – allow for direct energetic measurements because secretion volume and composition can be easily quantified. However, these energetic measurements only represent minimum costs as production of complex compounds likely incurs additional biosynthesis costs (e.g., proteins; Pontes *et al.*, 2015). Badger SGS has been shown in bomb calorimetric analyses to differ considerably between individuals, ranging almost 10-fold, from ~3,700s-27,000 J/ml (Allen *et al.*, submitted).

Here we used scent-provisioning experiments to determine how badger responses vary with SGS energetic content, and how response behaviour is moderated by (and the interactions of) individual characteristics of sender and responder such as sex, age and calorific SGS value. If SGS energy content does indeed reflect the signaller's condition, and SGS can thus function as an honest fitness advertisement, we predict that:

i) Overall, high-energy secretions should elicit more responses and be investigated more thoroughly (i.e., sniffed for longer) than low-energy SGS.

ii) In the context of mate choice, sexually mature adults should show more interest (i.e., respond more often, sniff for longer, and counter-mark more frequently) in high-energy SGS than low-energy SGS from opposite-sex donors.

iii) In the context of intra-sexual competition, same-sex responders should investigate high-energy SGS more thoroughly to appraise themselves of high-fitness competitors, but low-energy SGS should be counter-marked more frequently to ascertain superiority/dominance.

iv) In the context of group cohesion, sexually immature responders should over-mark same-sex high-energy SGS more frequently than low-energy SGS.

Methods

Study population and sample collection

We collected 84 SGS samples (n = 51 males, 33 females) from three neighbouring social groups in a high-density badger population (Macdonald *et al.*, 2009) between 13 January 2010 and 25 November 2017, inhabiting the 424-ha of semi-natural mixed woodlands in Wytham Woods, Oxfordshire, UK (GPS reference: 51° 46’26”N; 1° 19’19”W; for details of study site see Savill *et al.*, 2011). Following the methodology in Macdonald *et al.* (2015), cage traps baited with peanuts were placed overnight near sett entrances and along well-used paths.

Badgers were sedated with intramuscular injection of 0.2 ml ketamine hydrochloride/kg body weight (McLaren *et al.*, 2005; Thornton *et al.*, 2015). Unique tattoo numbers on the left inguinal region (given during first capture, typically as cubs; Sun *et al.*, 2015) allowed for individual identification. All individuals within this dataset were first captured as cubs, and thus exact age was known. Badgers were categorised as cubs (< 1 year old), yearlings (1-2 years old),

young adults (2-7 years old), or old adults (8+ years old; Buesching *et al.*, 2009; Sugianto *et al.*, 2019).

Badgers were categorised as sexually immature (≤ 2 years old) and sexually mature (2+ years old: Buesching *et al.*, 2009; Sugianto *et al.*, 2019). Body condition was categorised on a 1-3 scale (Speedy, 1980): 1 = emaciated, 2 = intermediate, or 3 = corpulent. All adults were given unique clip marks (Stewart & Macdonald, 1997) for individual identification during scent-provisioning experiments, but cubs were not clip-marked to ensure thermal integrity.

SGS secretion was gently scooped out of the subcaudal pouch with a rounded, stainless steel spatula sterilised with ethanol between individuals. Secretion volume was estimated to the nearest 0.1 ml (Buesching *et al.*, 2002a). Samples were subdivided into glass vials with Teflon-coated screw lids (Sigma-Aldrich, Dorset, UK) for bomb calorimetry analysis (1 subsample; ca. 1 ml) and scent-provisioning experiments (4 subsamples; ca. 0.1 ml each) before freezing them immediately at -20°C to prevent bacterial decay from potentially altering scent profiles and/or energetic content (Buesching *et al.*, 2016b; Sin *et al.*, 2012).

Ethical review and approval was obtained for all procedures, and performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 currently under Home Office licence PPL 30/3379 and Natural England licence 2019-38863-SCI-SCI.

Bomb calorimetry analysis

All calorimetric analyses were conducted by Sciantec Analytical Services, Selby, UK. After over-night freeze-drying using a ScanVac CoolSafe (LaboGene ApS, Allerød, Denmark), energetic content (in MJ/kg) was determined using a 6100 Parr bomb calorimeter (Parr Instrument Company, Illinois, USA; for details see Allen *et al.*, subm.). Samples were weighed

before ('weight of natural SGS') and after ('weight of dried SGS') freeze-drying. The calorific value was calculated for dried SGS (CV_{dry}; in J/g) as well as for natural SGS ($CV_{natural}$; in J/ml).

Scent-provisioning experiments

Scent-provisioning experiments were performed at three focal setts (one sett per sampled social group) between 5th June 2018 and 15th June 2018. SGS samples collected in spring 2010, 2011, 2016, and 2017 were used because the experiment required seasonally contemporaneous samples with known energetic content. Following the rules in Sugianto *et al.* (2019), SGS donors were categorised as 'residents' (i.e., member of the respective focal sett; n = 26) or 'neighbours' (i.e., residing in a social group adjacent to the focal sett; n = 7).

Samples were paired based on sex and energy content (CV_{dry}). Low-energy female samples (n = 3; range: 6.64-7.22 MJ/kg) were paired with high-energy female samples (n = 3; range: 9.66-12.83 MJ/kg), and low-energy male samples (n = 5; range: 6.0-8.27 MJ/kg) were paired with high-energy male samples (n = 5; range: 9.42-10.45 MJ/kg).

To control for familiarity, samples from neighbours (n = 3 low-energy females, 3 high-energy females, 3 low-energy males, 3 high-energy males) were paired separately from samples from residents (n = 2 low-energy males, 2 high-energy males). Samples from 2010-2011 (n = 2 low-energy males, 2 high-energy males, 1 low-energy female, 1 high-energy female; all still alive at the time of the experiments) were paired together to further control for familiarity as group odour composition may have changed between 2010-2011 and 2018.

SGS samples were defrosted immediately before use. New latex gloves were worn when handling each sample to prevent cross-contamination between scents. Two SGS samples (1 low-energy, 1 high-energy) were distributed about 1-2 hours before badger emergence. Samples were

placed 50 cm apart (i.e., the head-body length of an adult badger) on opposite sides of frequently travelled paths near (ca. 2-8 m) each focal sett. An identical sample pair was positioned near the original pair (ca. 4-8 m) to maximise encounter frequency. Vial lids were removed for scent exposure, and the vials were pushed to rim level in the soil. All vials were removed the following morning. New samples were repositioned ca. 50 cm from the previous pair with the relative positions being switched (e.g., low-energy sample on right or left of the path) to prevent familiarisation.

Three infrared Crenova 2.4" LCD Display (12MP and 1080P HD) camera traps and three infrared Stealth Cam Model STC-G45NG (14MP and 1080P HD) were positioned on nearby trees (ca. 1.5-3 m from each sample pair) around 1.5 m high, and programmed to record 60-second videos with 15 frames per second when activated by motion sensors. Footage was downloaded each morning.

Videos were analysed 'blind' (i.e., sample identity and energy content were unknown to the observer) to reduce observer bias using the Behavioral Observation Research Interactive Software (BORIS; version 6.3.9; Friard & Gamba, 2016). Following the methodology used by Tinnesand *et al.* (2015), badgers were considered as showing 'no response' when they did not approach either sample within a 50 cm (i.e., 1 head body length). A 'potential response' was recorded if a badger came within a 10-50 cm radius of either sample and an 'actual response' was recorded if an individual approached either vial within 10 cm. For all potential and actual responses, the occurrence (categorised as yes/no) and the frequency of subcaudal countermarking (i.e., scent-marking with subcaudal secretion either directly on a vial ['over-marking'] or within a 15 cm radius ['proximity marking']) were recorded and sniffing duration was measured in seconds.

Statistical analysis

All statistical analyses were carried out with R (R Core team, 2020; version 3.6.3) and R Studio (version 1.2.1335). Mixed models with binomial distributions (fitted with the *glmer* function in the *lme4* package; Bates *et al.,* 2015) were used when analysing response likelihood (proportion of 'actual' responses to total responses) and countermarking likelihood (proportion of proximity marking/over-marking to actual responses), while F-tests were used when analysing sniffing duration (proportion of time onscreen spent sniffing SGS samples). Significance tests for binomial models were carried out using likelihood ratio tests (LRTs) with the *drop1* function. Model convergence was checked using the *performance* package (Ludecke *et al.,* 2020), and statistically significant model effects were plotted with the *effects* package (Fox *et al.,* 2003).

When analysing response likelihood, a binomial model was used where only observations with a potential or actual response were included. Analyses for sniffing duration and countermarking likelihood included only observations with actual responses. Proximity marking and over-marking were combined owing to their low frequency of occurrence. Sniffing duration was analysed as a continuous response (with the default gaussian family), and square-root transformed due to skewed distributions. Separate models were used to account for various interactions: between CV_{dry} ('low' or 'high') and responder age group, between CV_{dry} and donor age group, and between CV_{dry} and relative sex ('same' or 'different'). Observation ID and responder ID were added as random effects to account for individual variation in behavioural responses. A separate analysis was conducted for donor ID to assess if behavioural responses differed due to donor-related variations not accounted for in the experimental set-up.

Two SGS samples had low CV_{dry} content but high $CV_{natural}$ content (2 males), while 1 SGS sample had high CV_{dry} content but low $CV_{natural}$ content (1 male). However, the CV_{dry} and $CV_{natural}$ values of the provisioned SGS samples had a correlation of 0.93. Due to the high correlation, all analyses were performed with CV_{dry} values.

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Results

204 videos were recorded during the study period, providing a potential maximum of 408 proximity observations (204 for high-energy samples and 204 for low-energy samples). Badgers were not observed within 50cm of either sample in 113 videos (28.0%) – these were not considered in subsequent analyses.

Response likelihood

When all individuals (identifiable adults, unidentifiable adults, and cubs) were analysed, badgers had a 60.0% (90/151) response likelihood towards low-energy samples and a 60.0% (87/144) response likelihood for high-energy samples. There was evidence that donor ID affected response likelihood (LR chi-squared = 27.47, df = 14, $P = 0.016$).

246 observations remained when considering only identifiable badgers. These observations included 26 adult badgers (12 males and 14 females) and one female yearling. Specific individuals contributed between one and 27 observations. When analysing only observations where responders were identifiable, there was no evidence that energy content affected response likelihood (LR chi-squared = 0.06, $P = 0.80$). There was no evidence for interactions between CV_{dry} and responder age group (interaction term: LR chi-squared = 0.005, *P*

= 0.99), responder sex (interaction term: LR chi-squared = 0.006, *P* = 0.94), donor sex (interaction term: LR chi-squared = 0.29, *P* = 0.58), and relative sex (interaction term: LR chi-squared = 1.09, *P* = 0.29).

Badgers were more likely to respond to female SGS samples than male SGS samples (LR chi-squared = 6.2, *P* = 0.012; Fig. 1). Badgers were also more likely to respond to same-sex SGS samples than to opposite-sex SGS samples (LR chi-squared = 3.33, *P* = 0.07; Fig. 2). However, this trend was not attributable to responder sex-specific differences as responder sex alone did not affect response likelihood (LR chi-squared = 0.09, *P* = 0.76).

Overall, badgers responded more towards high-energy SGS from old donors and low-energy SGS from young donors (interaction term for donor age*energy content: LR chi-squared = 3.90, *P* = 0.049; Fig. 3). There was no evidence for a main effect of age (LR chi-squared = 0.24, *P* = 0.62).

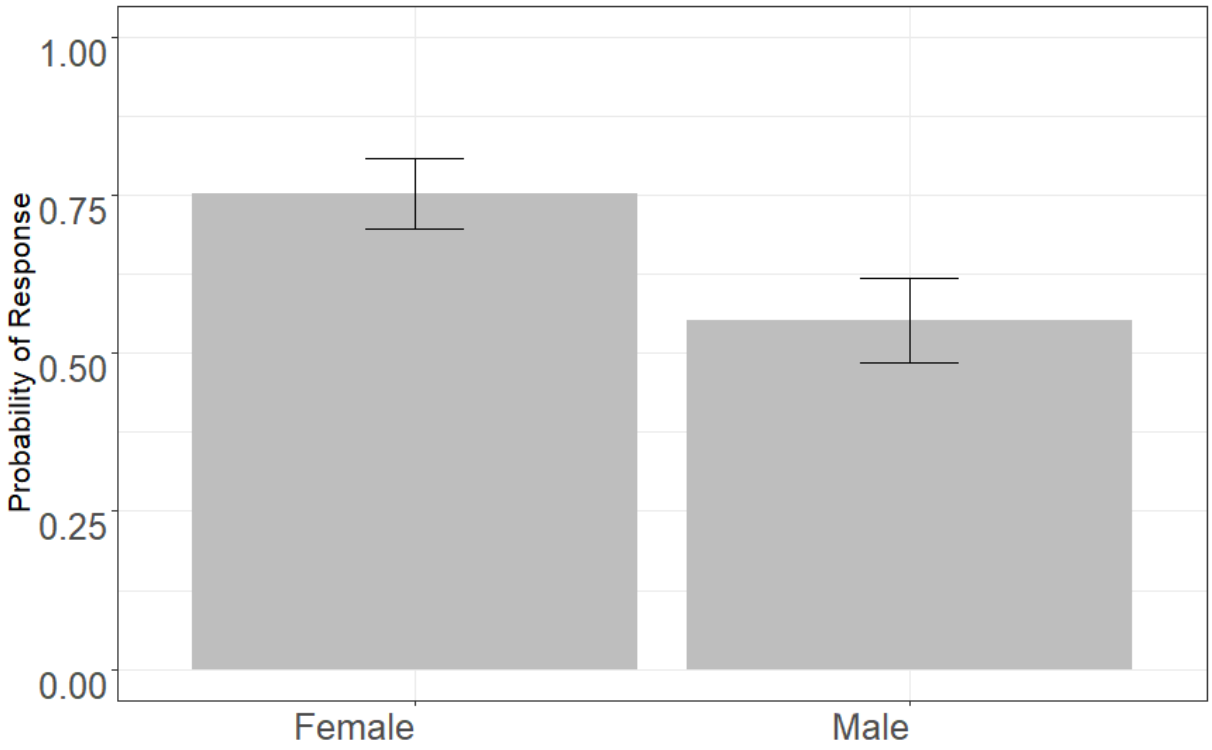


Figure 1: *When considering only identifiable adult responders, badgers were more likely to respond to female SGS than male SGS. Error bars in this and other figures represent a standard error of ± 1 unit.*

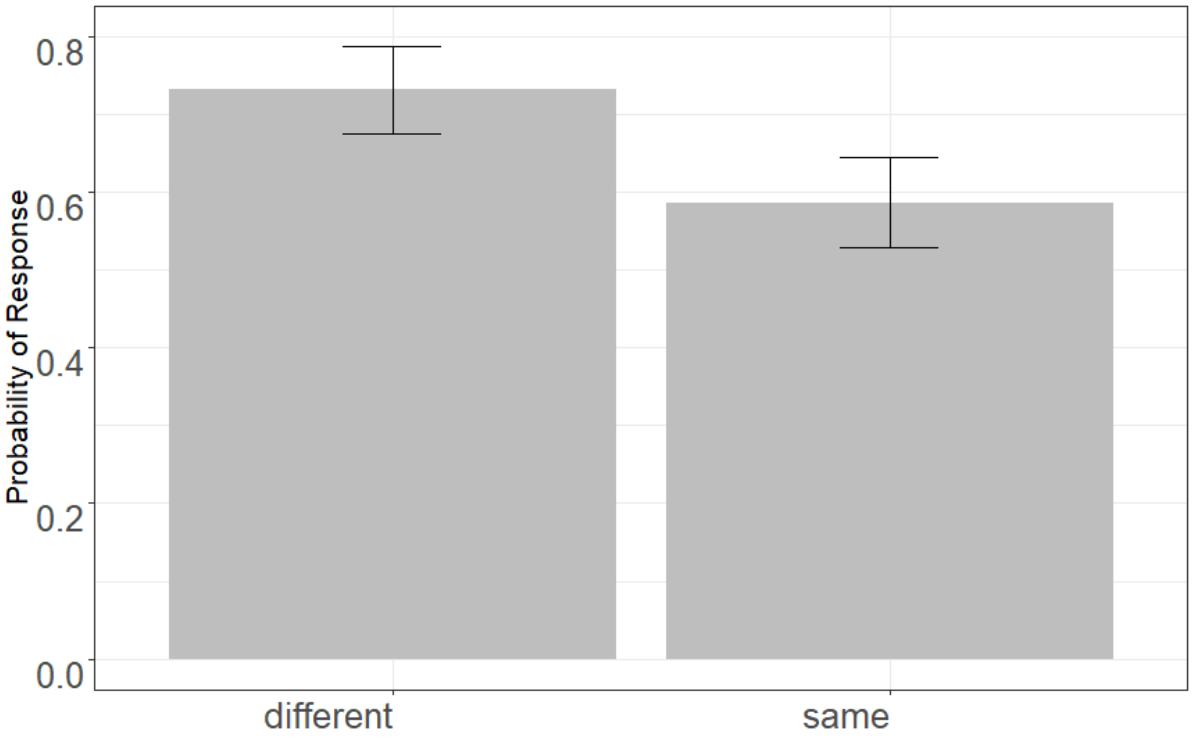


Figure 2: *When only identifiable responders were analysed, badgers were more likely to respond to SGS from opposite-sex donors.*

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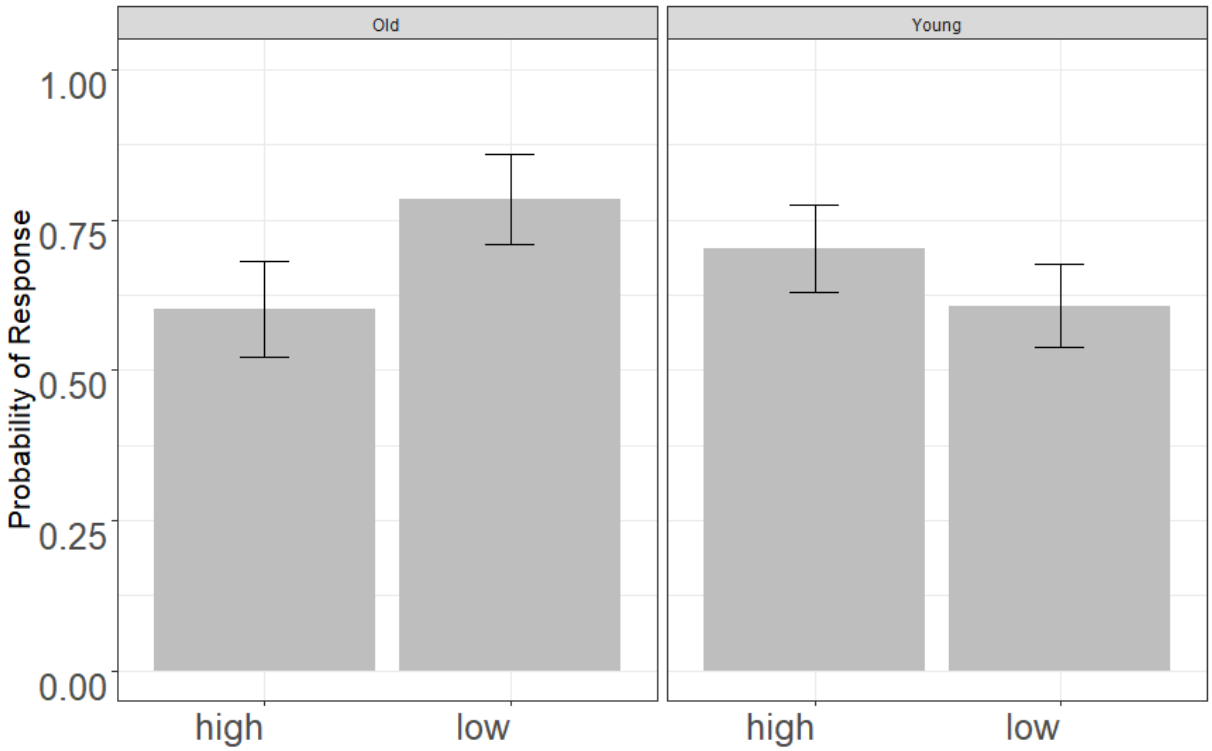


Figure 3: *Identifiable adult badgers responded more often towards low-energy secretions from older donors and high-energy secretions from younger donors.*

Sniffing behaviour

177 observations were recorded of identifiable and unidentifiable responders coming within 10 cm of either SGS sample. Within these observations, low-energy samples were sniffed for 61.0% of occasions (55/90) while high-energy samples were sniffed for 62.0% of occasions (54/87). The mean sniffing time for low-energy samples was 3.13s ± 0.38s (n = 90), and 4.07s ± 0.49s (n = 87) for high energy samples. There was no evidence that energy content alone affected sniffing duration (LR chi-squared = 1.02, df = 1, $P = 0.31$). When adjusted for observation time, badgers spent 1.28% ± 0.15% (n = 90) of their time sniffing low-energy samples and 1.69% ± 0.20% (n = 87) of their time sniffing high-energy samples. However, there was no evidence that these proportions were significantly different (LR chi-squared = 1.09, df = 1, $P = 0.30$). There

was evidence that donor ID affected whether or not a sample was sniffed (LR chi-squared = 33.82, df = 14, $P = 0.002$), but no evidence that it affected how long samples were sniffed (LR chi-squared = 7.25, df = 14, $P = 0.92$). Subsequent analyses considered only identifiable badgers (n = 156 observations).

There was no evidence that the interactions between energy content and responder age group ($F_{2,65} = 0.02$, $P = 0.97$), donor age group ($F_{1,109} = 0.02$, $P = 0.88$), responder sex ($F_{1,82} = 0.03$, $P = 0.95$), donor sex ($F_{1,88} = 1.01$, $P = 0.32$), and relative sex ($F_{1,82} = 0.47$, $P = 0.49$) affected sniffing duration. There was also no evidence that responder age group, responder sex, relative sex, and donor age group alone affected sniffing duration. Donor sex approached significance with badgers sniffing male SGS samples for a longer proportion of time than they sniffed female SGS samples ($F_{1,91} = 3.50$, $P = 0.06$; Fig. 4).

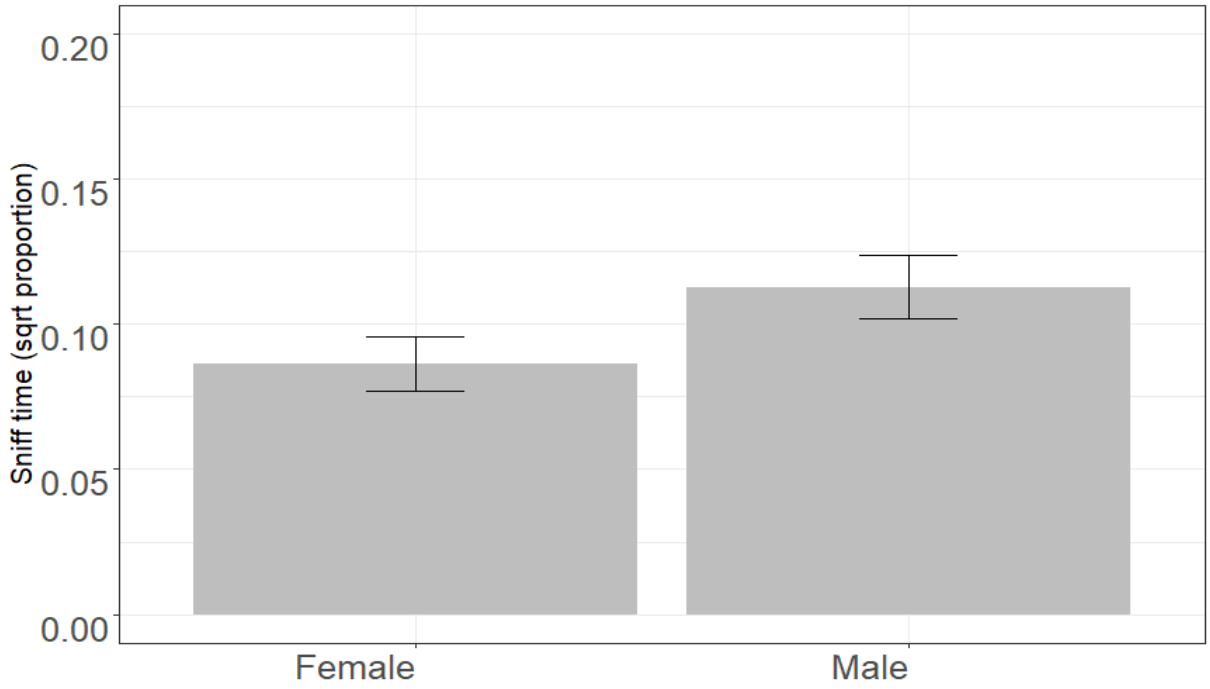


Figure 4: *When considering only identifiable responders, badgers sniffed male SGS longer than they sniffed female SGS. However, this effect only approached significance.*

Countermarking likelihood

When considering both identifiable and unidentifiable responders, marking was recorded during 16 out of 90 observations (18.0%) for low-energy samples and during 24 out of 87 observations (28.0%) for high-energy samples. There was no evidence that donor ID affected countermarking likelihood (LR chi-squared = 14.22, df = 14, $P = 0.43$).

When only identifiable responders were considered (n = 156 total observations; 35 marking occasions), energy content alone did not significantly influence countermarking likelihood (LR chi-squared = 1.36, $P = 0.25$). Furthermore, interactions between energy content and responder sex, donor sex, responder age group, and donor age group proved insignificant.

Badgers marked high-energy samples from same-sex donors (15.8%; 12/76) more often than low-energy samples from same-sex donors (7.50%; 6/80). However, secretions from

opposite-sex donors were marked at similar rates regardless of energy content as badgers marked low-energy secretions 11.25% of the time (9/80) and high-energy samples 10.5% (8/76) of the time (interaction term LR chi-squared = 4.60, $P = 0.03$).

Discussion

Targeted studies investigating the effects of signalling costs on responder behaviour are rare, particularly in the context of olfactory communication. Here, we investigated how European badgers vary their behavioural responses towards low- and high-energy scent secretions. We found that although energy content alone did not affect response likelihood, badgers responded significantly more towards low-energy secretions from older donors and towards high-energy secretions from younger donors. Furthermore, when energy content was considered alongside relative donor/responder sex, we found that badgers countermarked high-energy secretions from same-sex donors more than they countermarked low-energy secretions from same-sex donors. We could only measure minimum signal costs in terms of energetic content and could not account for additional biosynthesis costs (e.g., milk; Bauman *et al.,* 2006), so we have a conservative estimate of the energetic cost of producing the gland secretion. Our results support our hypothesis that costly signals do attract more attention from conspecifics.

Glandular secretions typically contain high molecular weight compounds which act as fixatives (Apps *et al.*, 2015; Hayes *et al.*, 2003; Hradecký, 1986; Raymer *et al.*, 1985). These fixatives facilitate the retention of volatile organic compounds, prolonging the duration for which the scent mark remains discernible in the environment (Hayes *et al.,* 2003). Subcaudal gland secretions contain a high number of proteins and fatty acids which delay the release of volatile components (Buesching *et al.*, 2002b; Buesching *et al.*, 2002c). Since synthesizing proteins and

fatty acids is energetically costly (Cui *et al.,* 2016; Pontes *et al.,* 2015), it is likely that these high-energy secretions contained more proteins and fatty acids. This in turn likely affected how long the volatile compounds would remain perceptible. Yet energetic content appeared to affect the secretion's detectability and/or attractiveness only when donor age was considered. Buesching *et al.* (2002b) showed that the chemical profile of subcaudal gland secretions varies significantly with age. Additionally, the chemical profile deteriorates (i.e., the loss of volatile organic compounds) only slowly over 48h (where the samples in this study were defrosted for a maximum of only 12h before response behaviour was observed), preventing the scent mark from losing encoded individual-specific information (Buesching *et al.,* 2002c). Age-related variations in composition may influence the fixative nature of proteins and fatty acids within secretions, thus affecting how discernible low- or high-energy secretions are within the environment. However, further research would be needed to confirm this. It should be noted that notwithstanding the large sample size of video observations, low donor sample sizes (resulting from methodological limitations of bomb calorimetry; Allen *et al.*, subm.) introduced some effects of individual variation other than those which were part of the experimental design.

Adult males countermarked high-energy male secretions significantly more than low-energy male secretions. In other group-living carnivore species, adult males exhibit strict territoriality and maintain dominance hierarchies (De Villiers *et al.,* 2003; Müller & Manser, 2008). In this context, intrasexual overmarking often occurs to signal territorial ownership/dominance, particularly when ownership/dominance gives males access to sexually receptive females. However, along with their lack of strict territoriality, badgers in our study population show little evidence of dominance hierarchies (Hewitt *et al.,* 2009; Macdonald *et al.,* 2002) and have a promiscuous, polygynandrous mating system in which all males and nearly all

females mount/are mounted (Dugdale *et al.,* 2011). As such, countermarking high-energy male secretions might be more likely to emphasise some dimension of priority within a social group and its range than posing a challenge. Potential intrasexual competition was also observed among females with adult females countermarking high-energy female secretions more than low-energy female secretions. Female signalling and signal perception are heavily influenced by endocrinological levels (Lynch, 2017; Petrulis, 2013). While hormonal changes (particularly during the breeding season) increase female attention and arousal towards male signals (Lynch, 2017; Petrulis, 2013), they can also increase competitiveness towards other females (Stockley *et al.,* 2013). For female badgers, setts are an essential, but limited (Macdonald *et al.,* 2004) resource during gestation and lactation (Kaneko *et al.,* 2010; Tsunoda *et al.,* 2018). Thus, some females may choose to emphasize their priority access to a sett by increasing their energetic investment in scent secretions.

In our study, varying conspecific responses towards specialised scent secretions with quantified energetic content supports the underlying assumptions regarding costly signalling: olfactory signal production does incur a cost, and conspecifics may at least sometimes alter their behavioural responses towards signals according to their calorific cost. Further work on quantifying energetic investment in secretion production (i.e., biosynthesis costs: Bauman *et al.,* 2006) – and possibly other signal modalities – may provide insight on the classification of specific signals (i.e., minimal signal, cost-added signal, index) and how conspecifics respond to varying levels of perceived cost.

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Section III: Olfactory advertisement via metabolic by-products



"The Forest Floor" by Imogen Thomas

Chapter 4:
Encoded information within urine influences behavioural responses among European badgers (*Meles meles*)[3]

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 Eppendorf 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);

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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 specially evolved subcaudal gland secretions.

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/km^2), 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, interdigital 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-provisioning 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.

Methods

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 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; but see da Silva & Macdonald, 1989 for limitations):

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

To identify individuals during scent-provisioning 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-provisioning 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.

Table 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

Scent-provisioning experiments and behavioural observations

Scent-provisioning 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 provisioning 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.

Videos were analysed using the BORIS software (version 6.3.9; Friard & Gamba, 2016). 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:

- Sniffing duration (in seconds)
- Subcaudal over-marking (directly over the tube) and proximity marking (≤ 50 cm of a vial)

<u>Enzyme immunoassay (EIA) analyses of urinary oestradiol and testosterone metabolites</u>

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). ß-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 µl aliquots were used.

Oestrogen EIA (ng/ml)

Total oestrogen analyses were carried out using a polyclonal antibody raised in rabbits immunised against 1,3,5(10)-oestratrien-3,17ß-diol-17-HS-BSA and 1,3,5(10)-oestratrien-3,17ß-diol-17-HS-peroxidase. Cross-reactivities to oestrogens were as follows: 1,3,5(10)-oestratrien-3,17b-diol (17ß-oestradiol), 100%; 1,3,5(10)-oestratrien-3,17-one (oestrone), 100%; 1,3,5(10)-oestratrien-3,17α-diol (17α-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.

Testosterone EIA (ng/ml)

Antibodies (provided by Prof. Meyer, Weihenstephan, 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-17ß-ol-3-one (testosterone), 100%; 5a-androstan-17ß-ol-3-one (dihydrotestosterone [DHT]), 13.6%; 5a-androst-2-en-17ß-ol (1.5%), and < 0.1% for 5a-androstan-3ß-ol-17-one (epi-androsterone), 5a-androstan-3a-ol-17-one (androsterone), and 5a-androst-2-en-17-one.

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:

$$Raw\ hormone\ concentration\ x\ \frac{(SGtarget - 1.0)}{(SGsample - 1.0)}$$

Statistical analysis

Behavioural observations with scent-provisioning 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 ± standard error (SE). To analyse if the duration that 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 permutated 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).

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).

Results

Behavioural observations with scent-provisioning 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-squared = 0.34, df = 1, p = 0.56) for both urine and water. Regardless, badgers sniffed urine (n = 40, 5.02 ± 1.58s) significantly longer than water (n = 23, 3.05 ± 0.49 s; estimate = 0.95, $F_{1,77} = 4.71$, 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-squared = 14.08, df = 1, p = < 0.001). Badgers demonstrated no significant variation in sniff duration regardless of sample freezing duration (estimate = -0.19, $F_{1, 38} = 1.09$, Pr(Prob) = 0.46).

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-squared = 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 (Tables 2 and 3, Fig. 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. 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-squared = 1, df = 1, p = 0.32) and countermarking responses (Tables 2 and 3) towards adult and cub urine. Sniffing duration, however, differed significantly between cub and adult urine samples (all donors: estimate = -1.78, $F_{1, 38} = 4.32$, Pr(Prob) = 0.03; unfamiliar donors only: estimate: -1.78, $F_{1, 38} = 4.42$, 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. 3).

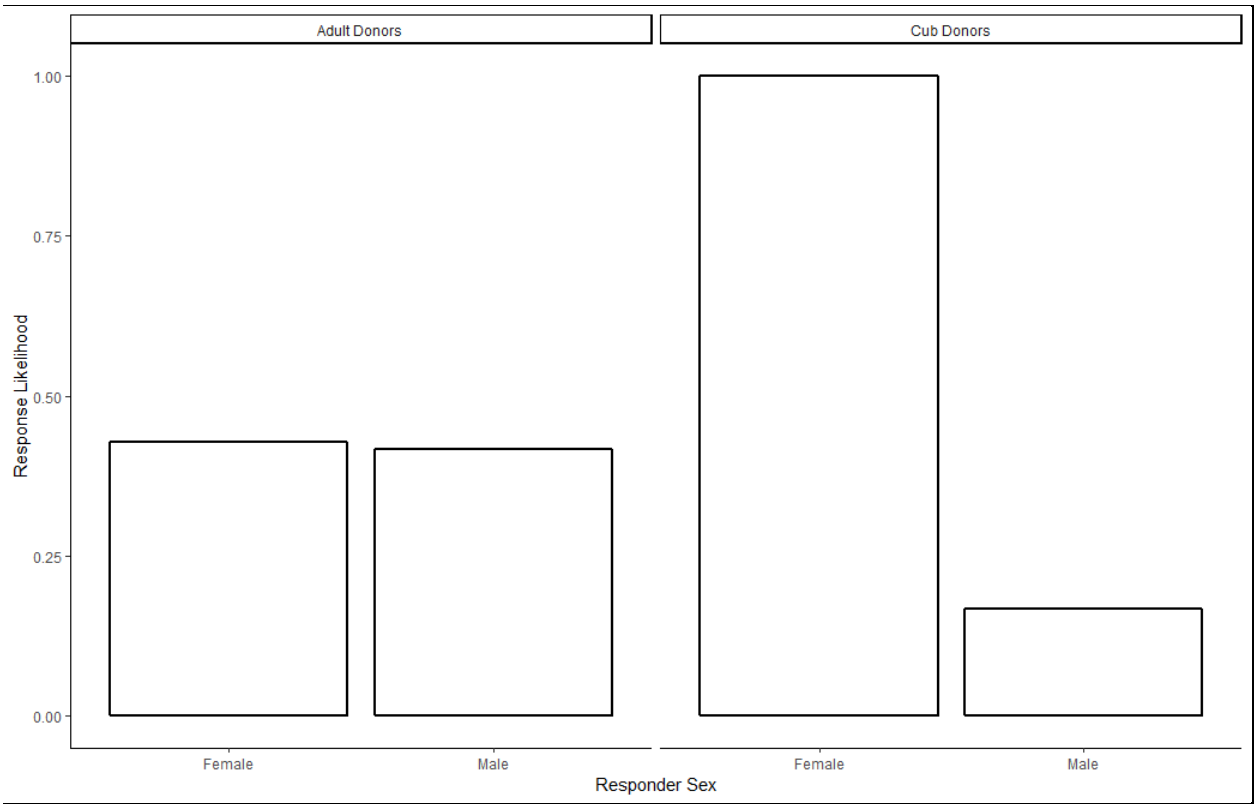
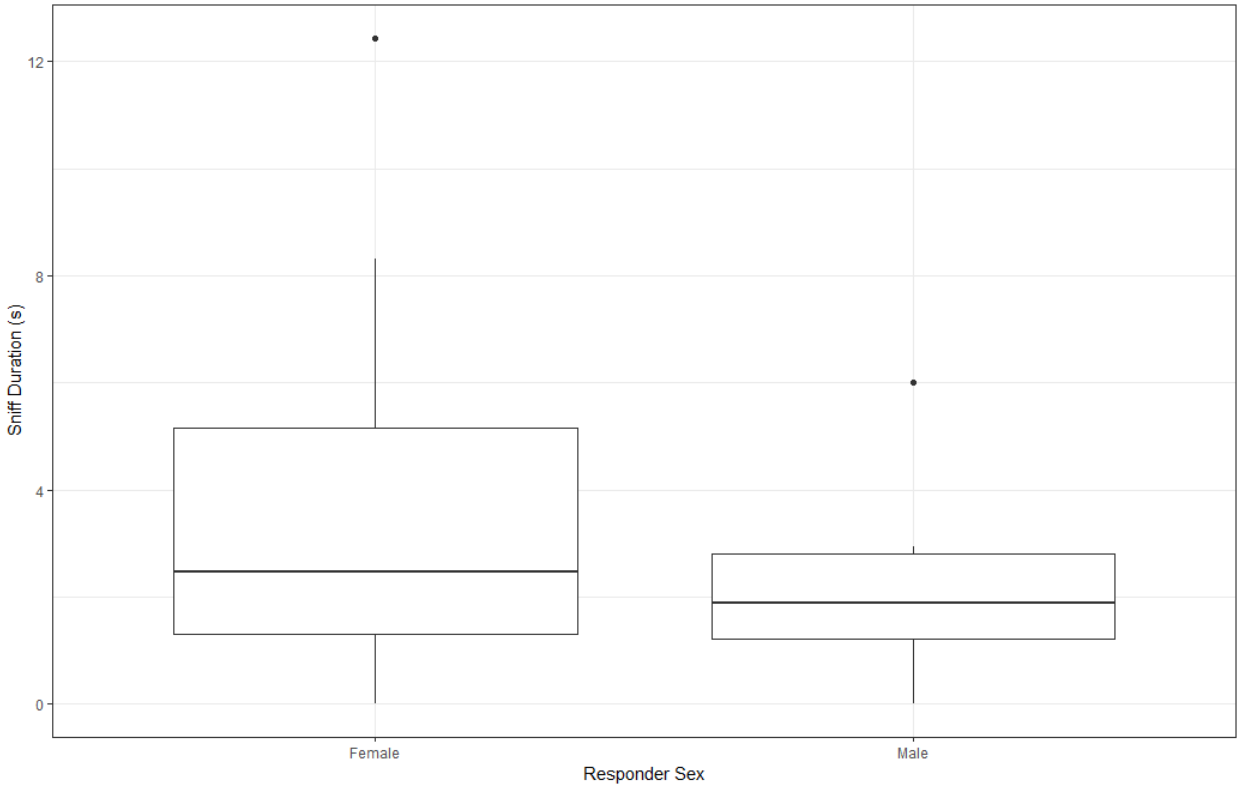


Figure 1: *Interactions between donor age and responder sex for response likelihood. When controlling for donor familiarity, female adults responded more often towards cub urine than adult urine.*



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Figure 2: *Urine sniffing 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).*

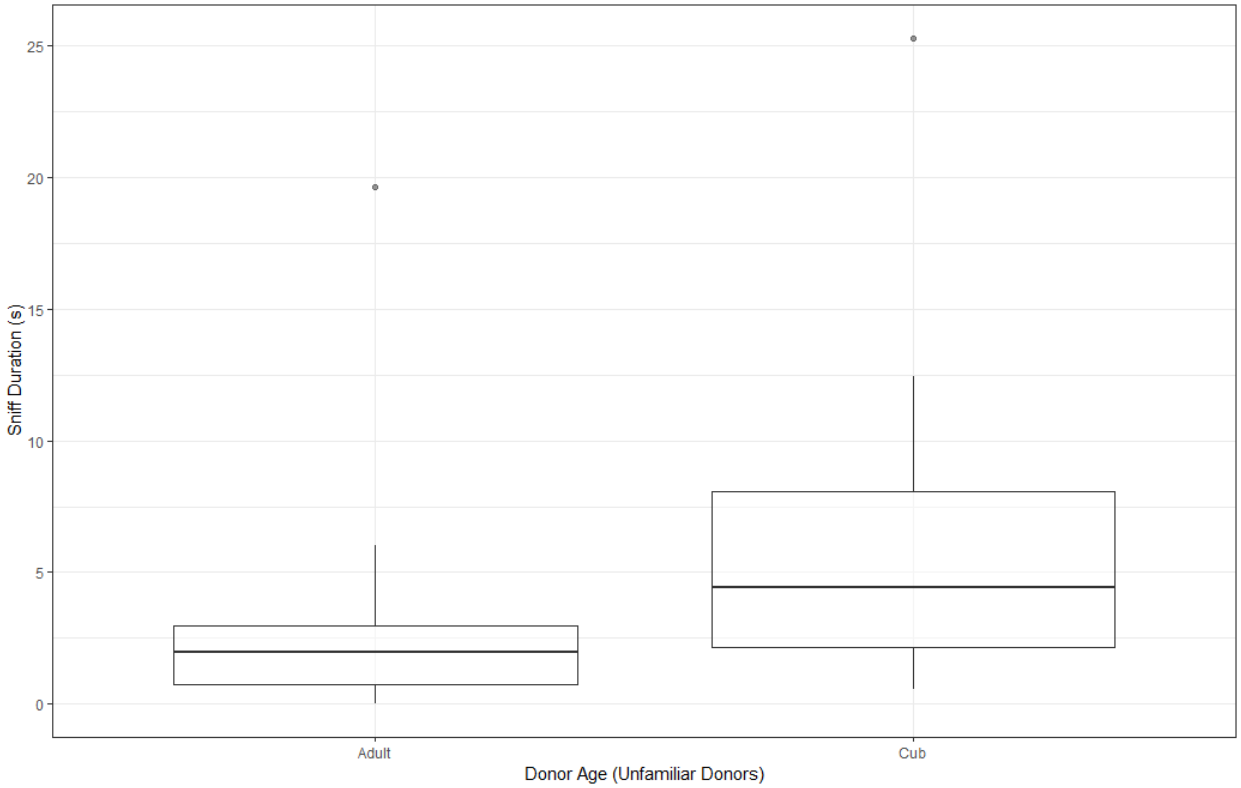


Figure 3: *Sniff duration of unfamiliar adult urine and cub urine by adult responders. When accounting for familiarity, adult badgers sniffed unfamiliar cub urine significantly longer (n = 13, 6.46 ± 1.86 s) than they did unfamiliar adult urine (n = 17, 3.05 ± 1.11 s; estimate: -1.78, $F_{1, 38} = 4.42$, Pr(Prob) = 0.02).*

Effects of sex-related information on behavioural responses

Overall, males and females were equally likely to respond to conspecific urine (response likelihood: chi-squared = 1, df = 1, p = 0.32) and did not differ in their propensity to countermark (chi-squared = 1.70, df = 1, p = 0.19) or sniffing duration (estimate = 0.86, $F_{1,13}$ = 0.96, 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-squared = 1, df = 1, p = 0.32; countermarking responses: chi-squared = 2.67, df = 1, p = 0.10; sniffing duration: estimate = 1.62, $F_{1, 29}$ = 2.34, Pr(Prob) = 0.19).

However, when responder sex was considered, adult females responded more to male donors (response likelihood = 0.80; Tables 2 and 3; Fig. 4) than to female donors (response likelihood = 0.36), whereas adult males responded more to female donors (response likelihood = 0.36; Fig. 4) than to male donors (response likelihood = 0.20), although sniff duration was not affected by the interaction between donor-responder sex dyads (Tables 2 and 3).

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. 5), with all donor-responder combinations differing significantly from each other (Tables 2 and 3).

Responder sex, donor age, and their interaction all significantly affected response likelihood (Tables 2 and 3). Females responded more often than males to adult and to cub donors (Fig. 4). Responder sex and donor age, but not their interaction, also significantly affected sniffing duration, with females (n = 4; 5.37 ± 2.39 s) investigating urine longer than males (n = 6; 2.35 ± 0.86 s; Fig. 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. 6).

Countermarking responses were affected by responder sex, donor age, and their interaction (Tables 2 and 3).

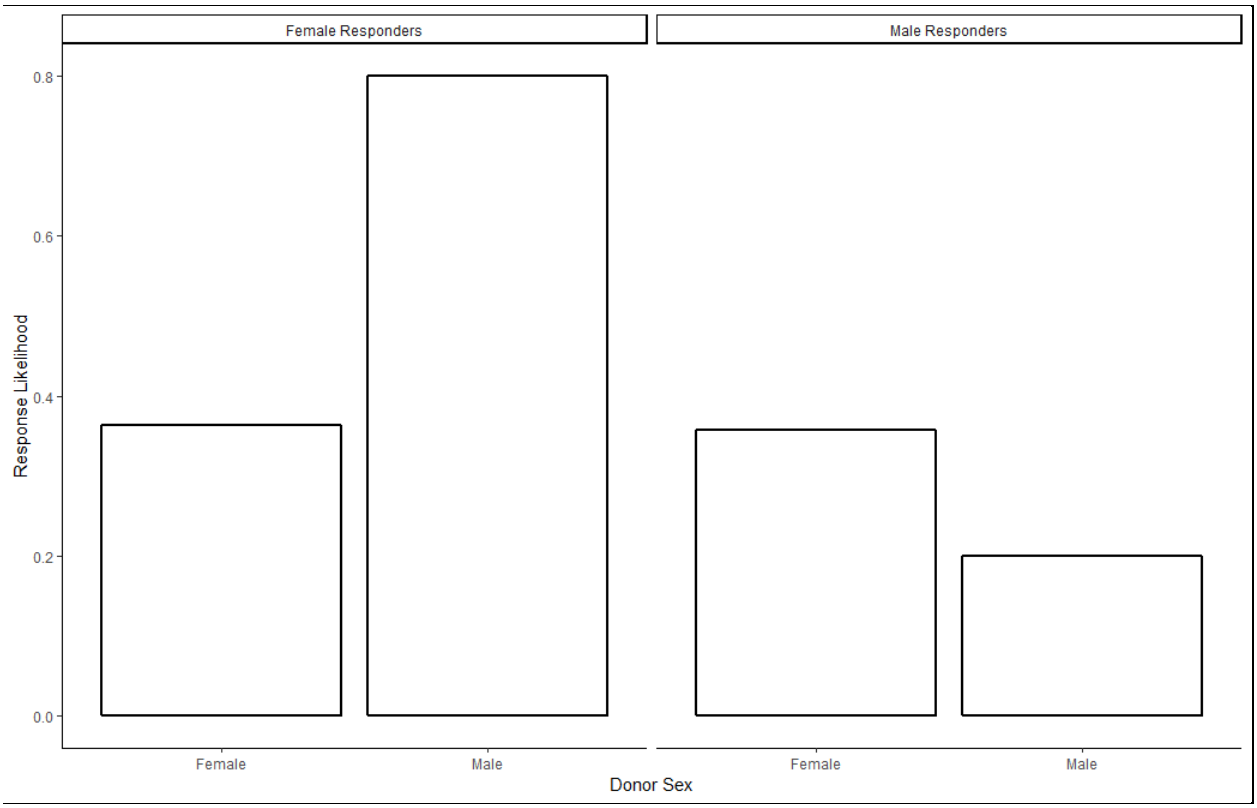


Figure 4: *Interactions between donor sex and responder sex for response likelihood. Adult females showed more actual responses towards male urine than female urine, while adult males showed more actual responses towards female urine than male urine.*

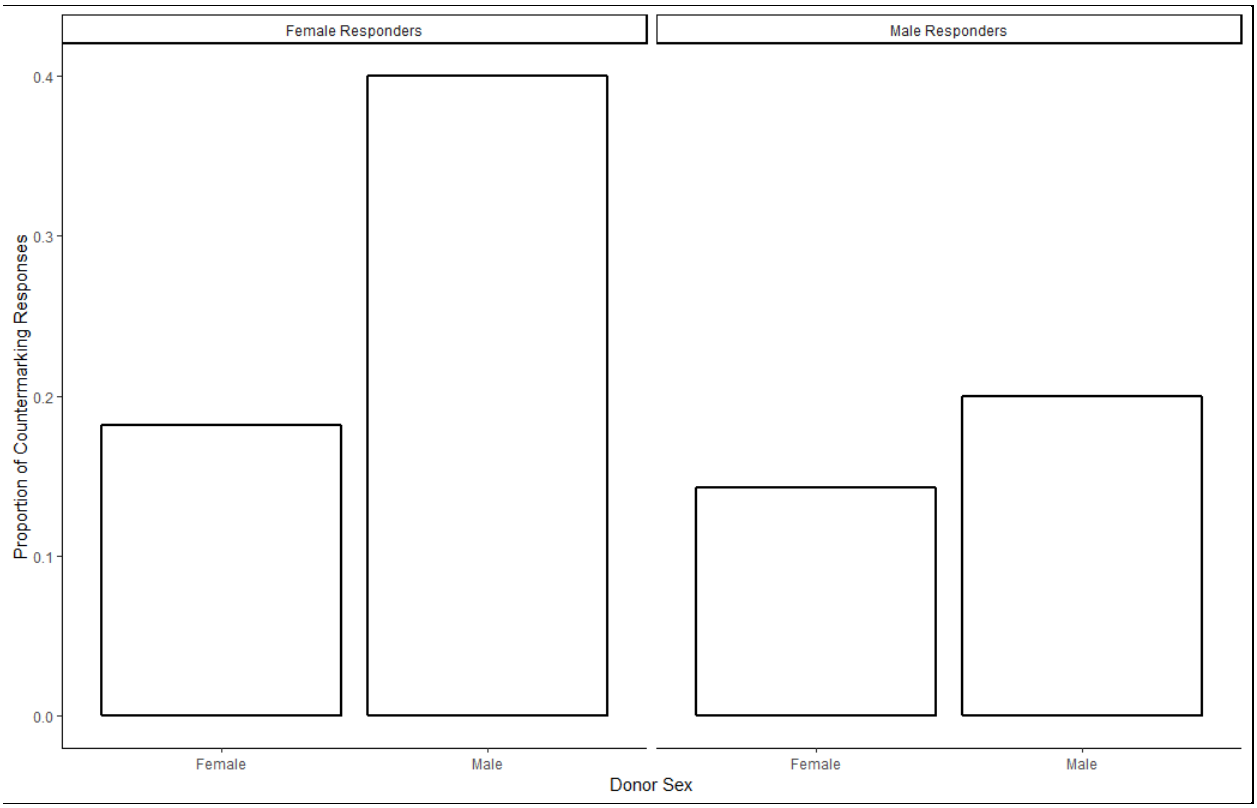


Figure 5: *Interactions between donor sex and responder sex for countermarking responses. Urine from male donors elicited more countermarking responses than urine from female donors, particularly amongst adult female responders.*

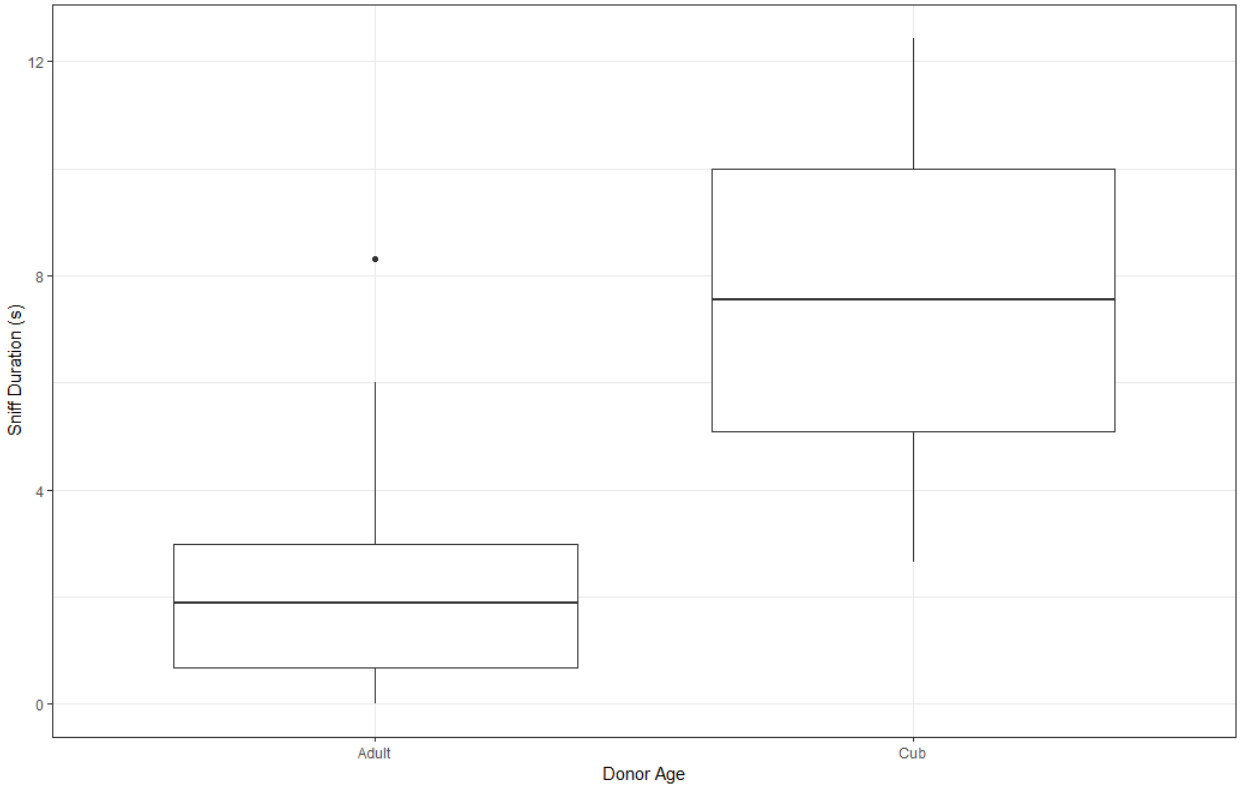


Figure 6: *Urine sniff duration in relation to donor age and responder sex. When analysed alongside responder sex, cub urine (n = 2; 7.54 ± 4.89 s) was sniffed longer than adult urine (n = 8; 2.56 ± 0.67 s). However, only two individuals sniffed cub urine.*

Effects of familiarity on behavioural responses

Overall, donor familiarity did not affect behavioural responses (response likelihood: chi-squared = 1, df = 1, p = 0.32; sniffing duration: estimate = -0.55, $F_{1,13}$ = 0.33, Pr(Prob) = 1; countermarking responses: chi-squared = 1.70, df = 1, p = 0.19). All adults responded more to unfamiliar male donors than to familiar females, unfamiliar females, and familiar males (Tables 2 and 3).

Correlating behavioural observations with sex steroid levels

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. 7). There were also no effects on countermarking.

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.

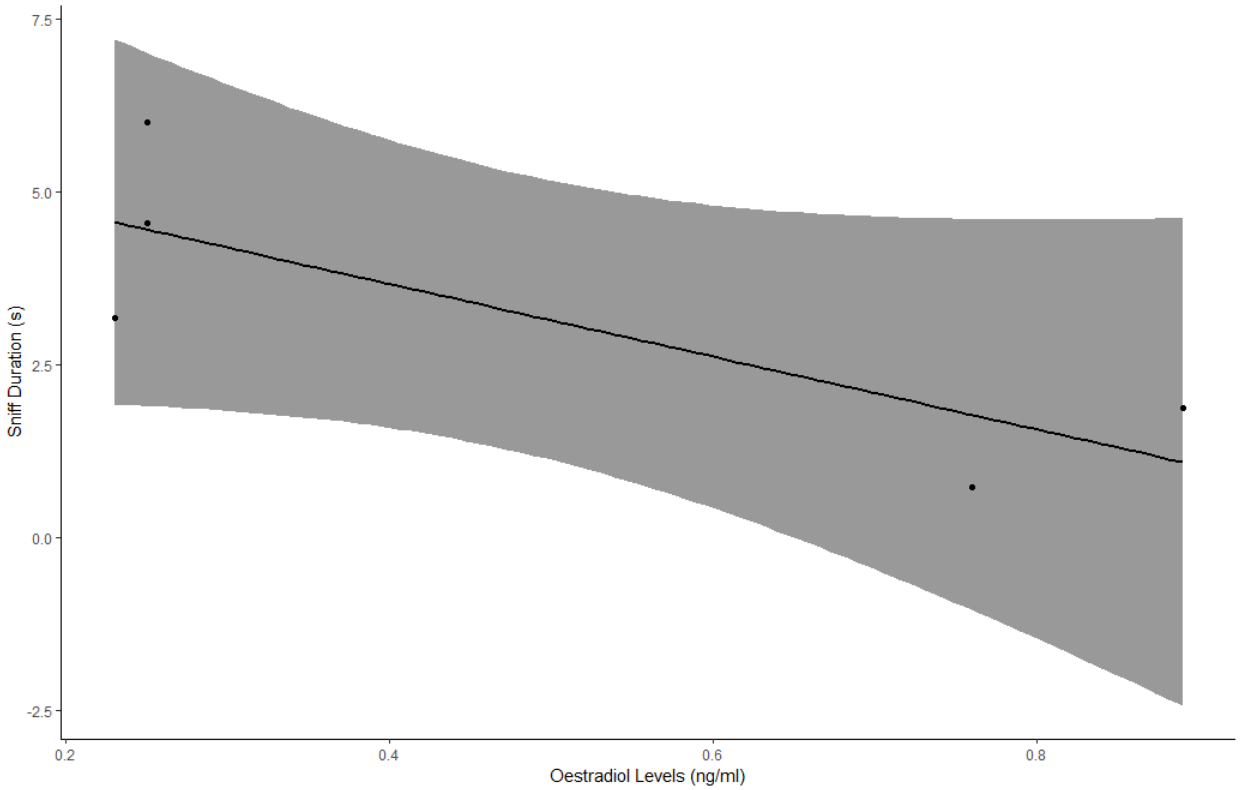


Figure 7: *Sniffing duration of adult male responders towards urine with oestradiol. Male responders spent significantly longer time investigating urine with lower oestradiol levels.*

Table 2: *Pairwise tests comparing behavioural responses based on donor sex / age and responder 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 *

Table 3: *Behavioural responses based on the interaction between donor-related traits and responder-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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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 sniffing 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 (Tinnesand *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-osmatic mammal species.

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Chapter 5:
Seasons of love: Seasonal reproductive contexts and individual traits affect reactions to urine marks among European badgers (*Meles meles*)[4]

Abstract

Metabolic by-products (i.e., urine and faeces) used in scent-marking include volatile organic compounds (VOCs), non-volatile organic compounds (non-VOCs), and excreted sex steroid metabolites. Urine mark composition can vary depending on spatial and temporal factors; and amongst mammals these factors can be indicative of social group membership/familiarity, sex, age, and sexual maturity. However, few studies have investigated i) how these spatial (indicative of group membership) and temporal (season, sex, age, sexual maturity) factors influence conspecific responses towards urine marks, and ii) how behavioural responses vary depending on the interaction between donor- and responder-related traits (sex, sexual maturity). Our study addressed these questions by conducting scent-provisioning experiments on a wild, high-density population of European badgers (*Meles meles*) located in Wytham Woods, Oxfordshire, U.K. We collected 110 urine samples during from spring 2017 to spring 2018 in order to encompass an entire year of seasonal cycles in sexual receptivity. Eppendorf tubes were filled with 0.5 ml of urine and frozen before use. Urine samples and a control sample (0.5 ml of water) were placed in the soil at rim level near four focal setts along commonly used paths, and motion-activated camera traps were situated on trees near the samples. We analysed response likelihood, countermarking likelihood, and sniffing behaviour for identifiable (via clip marks) adults, unidentifiable adults, and cubs. Mixed models analysing interactions between season,

[4] This chapter is the basis of a paper to be submitted to ***Behaviour,*** co-authored by Tanesha M. Allen, Paul Johnson, Chris Newman, David W. Macdonald & Christina D. Buesching

donor familiarity, donor/responder sex, and donor/responder sexual maturity indicated that year-round, badgers responded significantly more to urine from neighbouring conspecifics compared to resident or strange badgers. Adult females were also more likely to respond to urine than were adult males, while reproductively senescent badgers countermarked urine less often than sexually immature and reproductively active badgers. Furthermore, we found a weak seasonal effect on sniffing behaviour. We discuss our findings within the context of temporal differences in social interactions, particularly in regards to seeking mating opportunities and the 'Dear Enemy Phenomenon.'

Introduction

Mammalian chemical communication often occurs via scent-marking with metabolic by-products (i.e., urine and faeces; reviewed in Albone, 1984; Wyatt, 2014). These metabolic by-products contain both volatile and non-volatile organic compounds (VOCs and non-VOCs, respectively; Apps *et al.*, 2015). When an individual investigates a deposited scent mark, VOCs bind with receptors within the olfactory epithelium while non-VOCs bind with sensory receptors within the vomeronasal organ (Cherry & Baum, 2019). The behavioural responses triggered by these bound odorants depends on an individual's innate (Cox *et al.*, 2010) and/or learned knowledge (Laundré *et al.*, 2010) about how specific scent mark compositions correspond with a particular scent donor's identity or certain fitness-related traits.

However, these compositions can vary depending on the environment and time. Spatial variations in urinary mark composition can inform responding individuals about the scent donor's familiarity. For example, individuals occupying the same environment often share a more similar odour than they do with individuals from different environments, usually due to

sharing similar microbiota (Ezenwa & Williams, 2014; Theis *et al.*, 2013). Meanwhile, endocrinological changes from puberty (Courant *et al.,* 2010) and senescence (Lu *et al.,* 1979; Sugianto *et al.,* 2020b) produce fluctuations in sex-steroid levels (e.g., oestradiol, testosterone). Once these sex-steroids fulfil their function, they undergo filtration through the kidneys and are ultimately excreted via urine (Campbell *et al.,* 2001; Snoj *et al.,* 2014), thereby providing information about temporal variations in a donor's age and sexual maturity. Seasonality – yet another temporal factor – can influence compositional changes within urine marks, especially when individuals experience seasonal changes with sexual receptivity (i.e., oestrous cycles; Amendolagine *et al.,* 2018).

Previous studies have investigated conspecific responses to urine marks in regards to these aforementioned traits as well as how responses vary among different responders. Among group-living African lions (*Panthera leo*), adult females were less likely to respond to urine marks from resident females than from non-resident females. However, social group membership did not influence response likelihood from adult males towards urine marks (Gilfillan *et al.,* 2017). Among Indian blackbucks (*Antelope cervicapra L.*), adult males displayed more courtship and reproductive behaviours (e.g., Flehmen response) than adult females when exposed to urine from oestrous females. Males also displayed more courtship and reproductive behaviours towards urine from oestrous females than urine from proestrous and dioestrous females (Archunan & Rajagopal, 2013). Since mammalian individuals are sexually receptive at different times, it is expected that conspecific investigation of urine marks would increase during these periods. This should be especially true among sexually mature individuals when they encounter urine marks from the opposite sex. However, few studies have directly compared conspecific responses towards urine marks during and outside mating seasons. Even fewer have analysed

how seasonality influences conspecific responses alongside i) spatial variations indicative of group membership/familiarity, ii) temporal variations indicative of age and sexual maturity, and iii) the interaction between donor and responder traits (e.g., age, sexual maturity).

European badgers (*Meles meles*) are group-living, macrosmatic mammals which rely predominantly on olfactory signals/cues due to their adaptation to nocturnality (Buesching & Macdonald, 2001; Buesching & Stankowich, 2017). Although numerous studies have focused on how badgers utilise faeces (Buesching *et al.*, 2016; Buesching & Jordan, 2019; Stewart *et al.*, 2002) and subcaudal gland secretion (Buesching *et al.*, 2003; Buesching & Macdonald, 2004) in olfactory communication, urine-marking among European badgers (*Meles meles*) remains relatively understudied. Gas chromatography-mass spectrometry (GC-MS) analyses have demonstrated variation within the VOC composition of urine based on season and sex (Service *et al.*, 2001). Like other species, badger urine contains excreted sex-steroids which typically reflect circulating sex-steroid levels within the plasma (Sugianto *et al.*, subm.). Badgers can detect encoded information within urine about sex and reproductive status; this was demonstrated during a pilot study in which individuals responded more often towards, and countermarked, urine from opposite-sex conspecifics than urine from same-sex conspecifics (Allen *et al.*, 2019). The same pilot study – conducted during the primary mating season in spring (Sugianto *et al.*, 2020a) – indicated that badgers can detect familiarity from urine marks, as evidenced by the fact that urine from unfamiliar males elicited more responses than did urine from unfamiliar females, familiar males, and familiar females (Allen *et al.*, 2019). These studies indicate that urine does encode fitness-related information, and that badgers can decipher this information and adjust their behaviour accordingly.

Our study investigates how the interaction between spatial (familiarity) and temporal (age, sexual maturity, seasonality) variation influences conspecific responses towards urine marks in a wild badger population. In particular, we investigate:

1. If behavioural responses towards urine marks from neighbouring and unfamiliar individuals vary between seasons.
2. If behavioural responses vary based on the responder's sex and sexual maturity (i.e., sexually immature, reproductively active, and reproductively senescent).
3. If behavioural responses depend on how the donor's characteristics (sex, sexual maturity) relate to the responder's characteristics.

Methods

Study population and sample collection

As part of a long-term (1987-2019) capture-mark-recapture research project, 110 urine samples were collected from a high-density (44.55 ± 5.37 badgers/km^2) badger population in Wytham Woods, Oxfordshire, UK – a semi-natural mixed woodland encompassing 424-ha (GPS reference: 51° 46'26"N; 1° 19'19"W; additional details regarding the badger population in Macdonald *et al.,* 2009; Savill *et al.,* 2011). Cage traps were baited with peanuts and placed near sett entrances following the live-trapping methodology described by Macdonald *et al.* (2015). Trapping occurred during three seasons: once cubs have been fully weaned after the primary mating season in spring (May-June), in summer (August-September) when additional mating occurs during a second minor peak in receptivity observed in some populations (Corner *et al.,* 2015; but see Sugianto *et al.,* subm.), and during reproductive quiescence prior to embryo implantation in autumn (November).

Traps were checked daily between 7:00-7:30 am. Captured individuals were transferred to holding cages, transported to a central field station, and sedated with an intramuscular injection of 0.2 ml ketamine hydrochloride/kg body weight (Vetalar, Upjohns Ltd., Pontypool, Gwent, UK; McLaren *et al.*, 2005). Badgers were identified by their unique tattoo number given during their first capture (usually as cubs; Annavi *et al.*, 2014a) on their left inguinal region. Sex, weight (in kg to the nearest 100 g), and age were then recorded. Ages were recorded for individuals initially captured as cubs (n = 96). Meanwhile, estimated ages based on toothwear (categorised on a 1-5 scale with 1 = low and 5 = high) were calculated for individuals first captured as adults (n = 14) based on the below formula (Macdonald *et al.*, 2009; for limitations, see da Silva & Macdonald, 1989):

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

Individuals were then categorised into age groups: cubs (≤ 1 year old), yearlings (1-2 years old), and adults (2+ years old). Sexual maturity (Table 6) was assigned to individuals based on age (Sugianto *et al.*, 2019; Sugianto *et al.*, 2020a): sexually immature (≤ 2 years old), reproductively active (2-8 years old), and reproductively senescent (9+ years old).

Badgers were assigned to social groups according to their capture history (based on residency rules described by Annavi *et al.*, 2014a and Sugianto *et al.*, 2019). Assigning residency allowed us to provision scents from familiar (individuals residing in the same social group as the responder), neighbouring (individuals residing in adjacent social groups relative to the responder), and strange (individuals residing in other social groups) donors.

For identification during scent-provisioning experiments, adults from the four focal setts were given unique clip marks by trimming patterns into the black guard hair to expose the white under-fur (Stewart & Macdonald, 1997). Cubs were not clipped to prevent thermal loss.

Urine samples (Table 1) were collected via gentle manual palpation of the bladder (Vicente *et al.*, 2007) and placed into glass vials with Teflon-coated screw lids (Sigma-Aldrich, Dorset, UK). Plastic pipettes (one per urine sample) were used to divide samples between 5-10 Eppendorf tubes (2 ml), each filled with 0.5 ml of urine. Subsamples were frozen at -20°C until further use.

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.

Scent-provisioning experiments and behavioural observations

Contemporaneous urine samples were used for scent-provisioning experiments during spring (24 May 2017 to 12 June 2017; 23 May 2018 to 3 June 2018), summer (17 September 2017 to 30 September 2017), and autumn (18 November 2017 to 27 November 2017). Experiments were conducted to span an entire year.

About 1-2 hours before each experiment, urine samples were defrosted and the Eppendorf tube lids were removed, releasing the scent. Latex gloves were changed when handling each sample to prevent cross-contamination. Between 2-8 m from each focal sett, a urine sample and a control sample (an Eppendorf tube filled with 0.5 ml of deionised water) were positioned 50 cm (one head-body length of an average badger) apart along either side of a

badger path around 1-2 hours before badger emergence. Eppendorf tubes were pushed into the ground to level the rim with the surrounding soil, thereby minimising the likelihood of the samples being removed. Eppendorf tubes were removed each morning, and fresh samples were deployed in the afternoon. Following each trial, sample placements were adjusted by 50 cm and the relative positions of each sample (i.e., urine placement on right or left side of badger path) were alternated to reduce familiarisation. Sample presentation was randomised in relation to donor sex, age, and familiarity to reduce potential temporal and positional biases.

Behavioural observations were remotely recorded with Infra-red Crenova 2.4" LCD Display 12MP 1080P HD camera traps activated by three motion sensors between 5:30 pm and 7:00 am. Camera traps were positioned on nearby trees about 1.5 m above the ground and between 1.5-3.0 m away from the samples. Video length was set at 60 seconds with 15 frames per second, and videos recorded from the previous night were uploaded each morning.

Videos were analysed 'blind' (vial content was unknown to the observer) using BORIS (version 6.3.9; Friard & Gamba, 2016). We used body size as an indicator when estimating an individual's proximity to the samples. Badgers were considered to have no response if they did not come within a 50 cm radius (i.e., one head-body length) of either sample. "Potential" responses were recorded when badgers came within 10-50 cm of either sample since individuals likely detected the scent (Tinnesand *et al.*, 2015). "Actual" responses were recorded when badgers came within 10 cm of the urine sample and investigated the contents more closely by sniffing, licking, or direct contact.

The following were recorded for potential and actual responses:

- <u>Sniffing duration (in seconds):</u> When the badger's nose touched the urine vial.

- Subcaudal over-marking (≤ 10 cm from either vial) and proximity marking (10-50 cm from either vial): When the badger pressed its subcaudal pouch against a substrate.

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Statistical analysis

Statistical analyses were conducted in R (version 3.6.3) and R Studio (version 1.2.5033). Generalised linear mixed models (GLMMs) with binomial distributions were used to analyse response likelihood and subcaudal countermarking likelihood. For the former, any occasion
195 where a badger was observed within 50 cm of a sample was considered to be an observation (a 'potential' response). Observations were considered an 'actual' response when the badger subsequently approached within 10 cm of a sample (an 'actual' response). When a badger did not go within 10 cm, the observation was considered to be a 'non-response'. Only 'actual responses' were used when analysing countermarking and sniffing. Sniffing behaviour was analysed as both
200 a binary variable (sniffing either did or did not occur when a badger was close (< 10cm) to a sample), and as a continuous variable where sniffing occurred. Subcaudal over-marking and proximity marking were combined into one category ('countermarking') due to their low frequency. A square root transformation was applied to sniffing duration to correct skew and to improve model fit. Residual plots were used to assess model fit for all models, and using
205 surrogate residuals for binary responses (*sure* package; Greenwell *et al.*, 2018).

The analysis was carried out in two stages. We first tested if badgers were more likely to respond to urine compared with water, and if they were more likely to sniff and mark urine. These models included responder ID and observation ID (video file number) as random effects. Next, we carried out further analyses with only urine samples to observe if/how the response
210 variables were affected by season, donor familiarity, donor sexual maturity, and donor/responder

sex. Because samples were not paired, these models included only responder ID as a random effect.

Several interaction terms were included to test *a priori* hypotheses concerning moderating effects. These were added one at a time to the main effects model to avoid convergence failure of models, and were not retained where non-significant. Interaction terms and main effects were tested with likelihood ratio tests (LRTs) using the 'drop1' option in the *lme4* package (Bates *et al.,* 2015). The interaction terms were: season*donor familiarity, donor sex*responder sex, and donor sexual maturity*responder sexual maturity. Sett ID was included as a fixed effect, but was not retained in the final models to assist convergence and because there was no evidence for variation among setts. Initially, yearlings were grouped into the 'adult' age group due to their limited observations and to improve model fit. Furthermore, cubs tended to produce very little urine, and there were no cub donors during summer 2017, autumn 2017, and spring 2018 (Table 1). Model convergence was checked with the *performance* package (Lüdecke *et al.,* 2020). Statistically significant effects were visualised using the *effects* package (Fox, 2003).

Table 1: *Distribution of urine samples based on donor sex, age, and residency. 'Non-resident' refers to neighbours and strangers.*

Spring 2017 (n = 51)				
	Adults and Yearlings (n = 42)		Cubs (n = 9)	
	Females (n = 24)	Males (n = 18)	Females (n = 5)	Males (n = 4)
Resident (n = 17)	8	9	NA	NA
Non-resident (n = 34)	16	9	5	4
Summer 2017 (n = 16)				
	Adults and Yearlings (n = 16)		Cubs (n = 0)	
	Females (n = 7)	Males (n = 9)	Females (n = 0)	Males (n = 0)
Resident (n = 6)	3	3	NA	NA
Non-resident (n = 10)	4	6	NA	NA
Autumn 2017 (n = 18)				
	Adults and Yearlings (n = 18)		Cubs (n = 0)	
	Females (n = 9)	Males (n = 9)	Females	Males
Resident (n = 7)	3	4	NA	NA
Non-resident (n = 11)	6	5	NA	NA
Spring 2018 (n = 15)				
	Adults and Yearlings (n = 15)		Cubs (n = 0)	
	Females (n = 8)	Males (n = 7)	Females	Males
Resident (n = 7)	4	3	NA	NA
Non-resident (n = 8)	4	4	NA	NA

230

Results

Comparing behavioural responses to urine versus water

356 observations were recorded of badgers approaching within 50 cm of either the urine sample or the control water sample ('potential responses'). From these observations, 163 (46.0%) involved badgers approaching within 10 cm ('actual responses'). Badgers had 'actual responses' to urine during 86 out of 185 observations (46.0%), and 'actual responses' to water during 77 out of 171 (45.0%) observations. Response likelihood did not differ between urine and water (LR chi-squared = 0.06, $P = 0.079$).

However, badgers that showed 'actual responses' were more likely to sniff urine samples (56 out of 86 observations; 65.0%) than water samples (37 out of 77 observations; 48.0%; LR chi-squared = 6.76; $P = 0.009$; Fig. 1). Furthermore, among individuals that sniffed samples, badgers sniffed urine samples significantly longer (4.34s ± 0.47s; LR chi-squared = 6.30, $P = 0.01$) than water (2.65s ± 0.30s; Fig. 2).

Urine samples were also countermarked significantly more than water (LR chi-squared = 13.76, $P = 0.0002$; Fig. 3). Badgers countermarked urine samples during 25 of the 86 actual responses for urine (20.0%) and countermarked water samples during 6 of the 77 actual responses for water (8.0%).

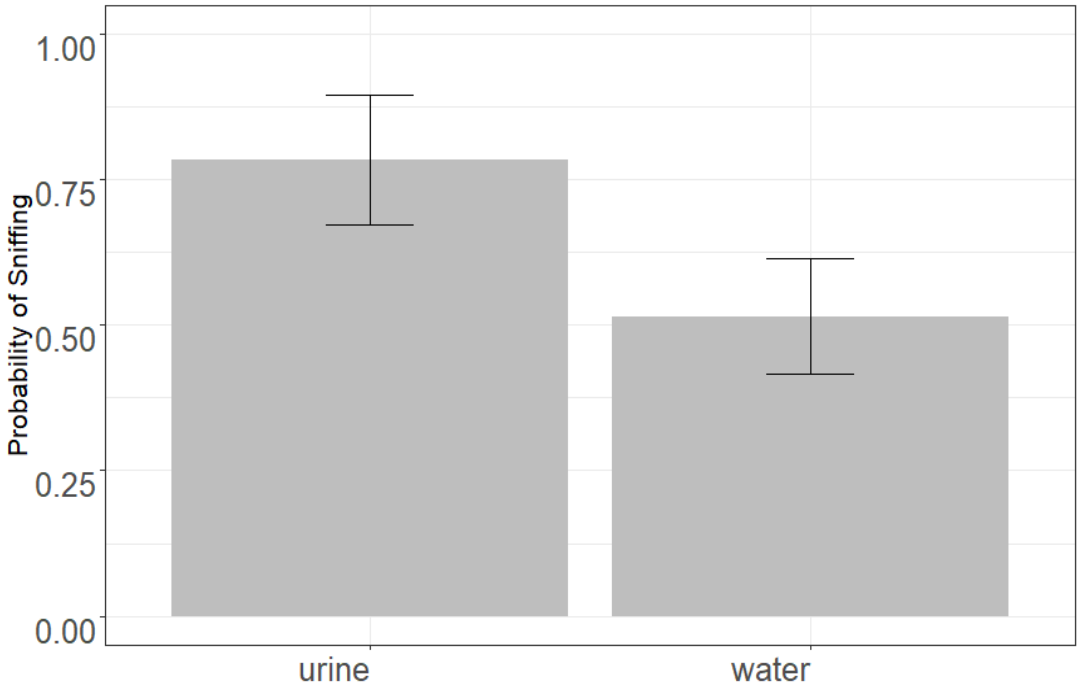


Figure 1: *Badgers were more likely to sniff urine samples compared to the control water samples (± 1 standard error).*

255

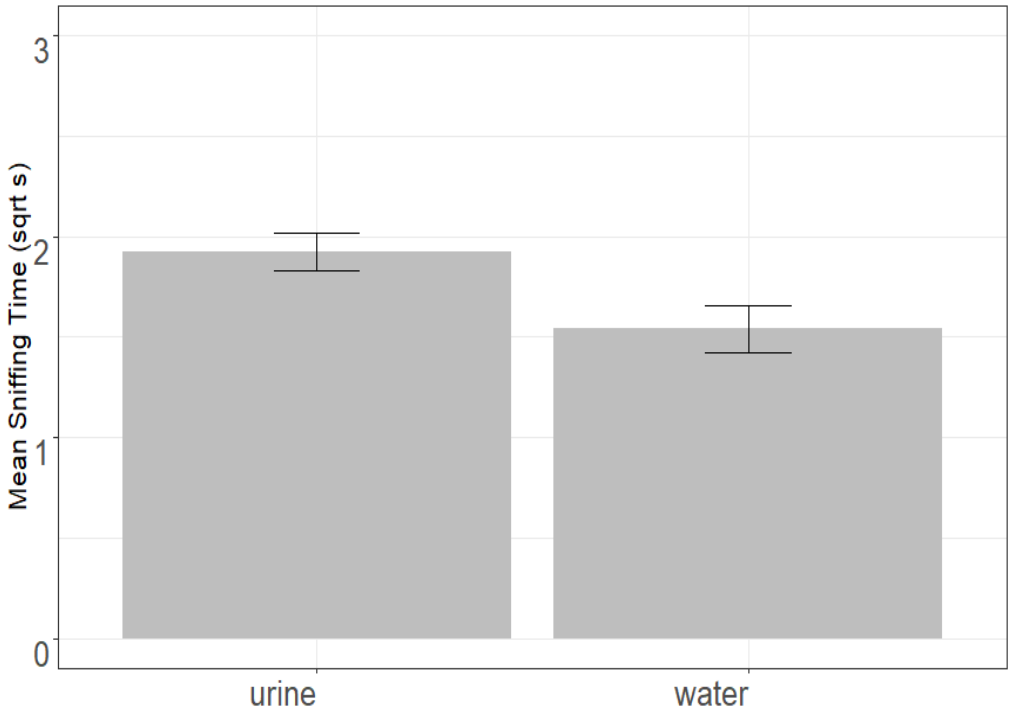


Figure 2: *Badgers sniffed urine samples for significantly longer durations than the control water treatment (± 1 standard error).*

260

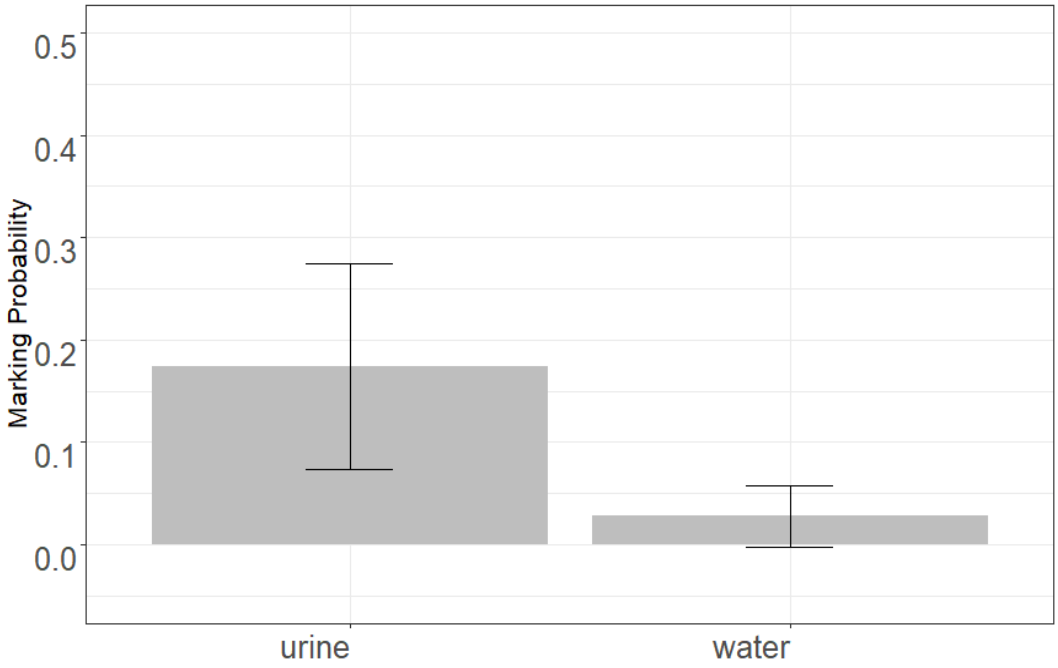


Figure 3: *Badgers were significantly more likely to countermark urine samples than the control water treatment (± 1 standard error).*

Comparing attributes of urine samples

Response likelihood

When identifiable responders were considered, response likelihood was not affected by any parameter interactions (all LR chi-squared ≤ 3.84, $P > 0.05$). However, responder sex did significantly affect response likelihood with adult females responding more often (34 actual responses out of 58 observations; 59.0%) than adult males (52 actual responses out of 127 observations; 41.0%; Table 2; Fig. 4).

Donor familiarity also significantly affected response likelihood (Table 2; Fig. 5). When only identifiable responders were considered, badgers were more likely to respond to urine from neighbouring donors (18 actual responses out of 28 observations; 64.0%) than to urine from familiar (28 actual responses out of 71 observations; 39.0%) or strange badgers (40 actual responses out of 86 observations; 47.0%).

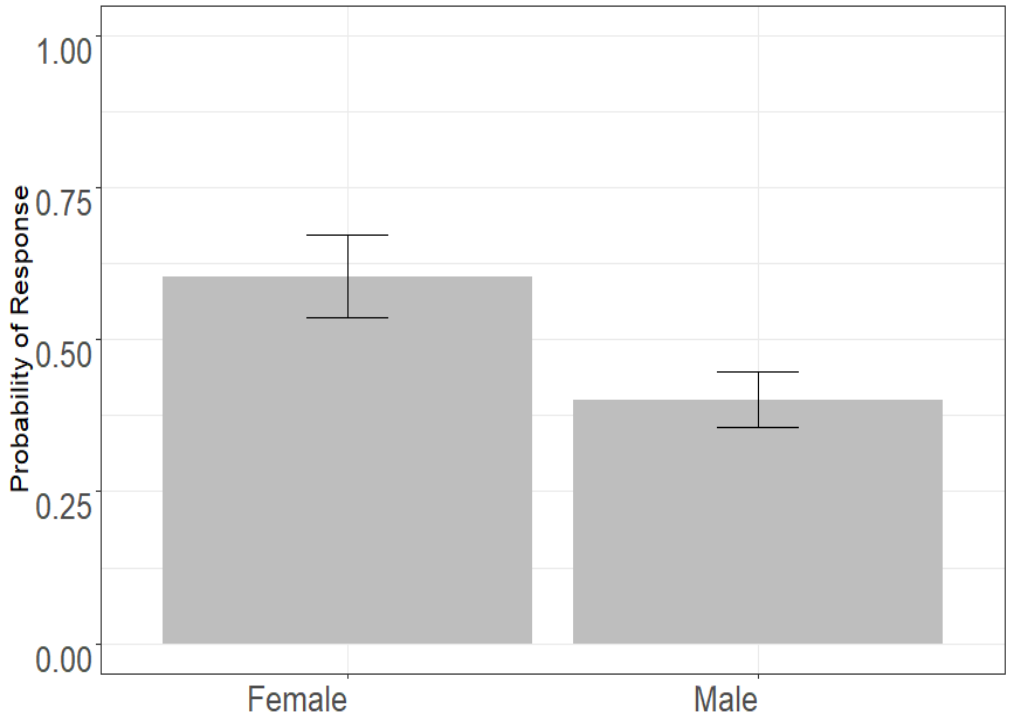


Figure 4: *When considering only identifiable responders, adult females were more likely to respond to urine samples than adult males (± 1 standard error).*

280

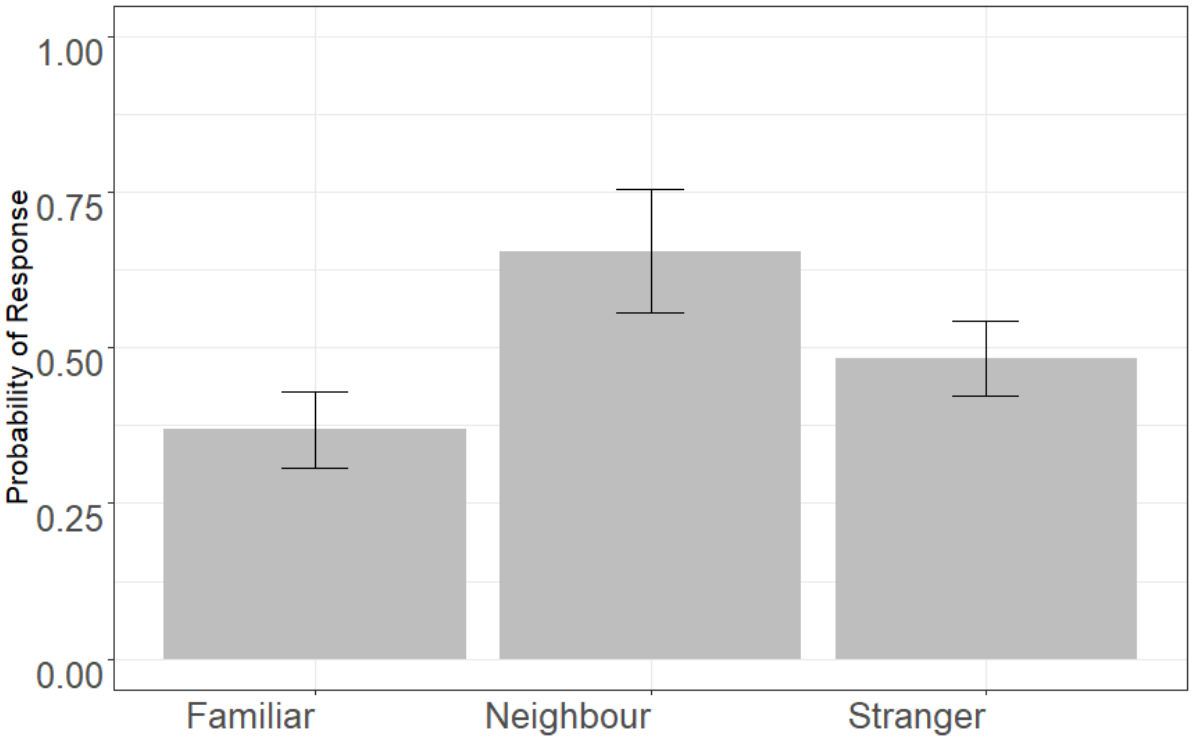


Figure 5: *When considering only identifiable responders, badgers were more likely to respond to urine samples from donors from neighbouring setts (± 1 standard error).*

285

Table 2: *Model output for response likelihood. Asterisks denote significance.*

	Npar	**AIC**	**LRT**	**Pr(Chi)**
Season	2	262.55	2.8263	0.24338
Donor familiarity	2	265.57	5.8434	0.05384*
Donor sex	1	261.84	0.1111	0.73887
Responder sex	1	267.48	5.7522	0.01647*
Responder sexual maturity	2	259.96	0.2297	0.89148
Donor sexual maturity	2	260.13	0.4004	0.81855

290

Countermarking likelihood

Only 25 countermarking events were recorded during actual responses. Countermarking likelihood was not significantly affected by any parameter interactions (all LR chi-squared ≤ 3.84, $P > 0.05$). However, responder sexual maturity did significantly affect countermarking likelihood (Table 3; Fig. 6) as reproductively senescent responders countermarked urine samples less often (1 countermarking event out of 13 actual responses; 8.0%) than did sexually immature badgers (12 countermarking events out of 21 actual responses; 57.0%) and reproductively active badgers (12 countermarking events out of 52 actual responses; 23.0%).

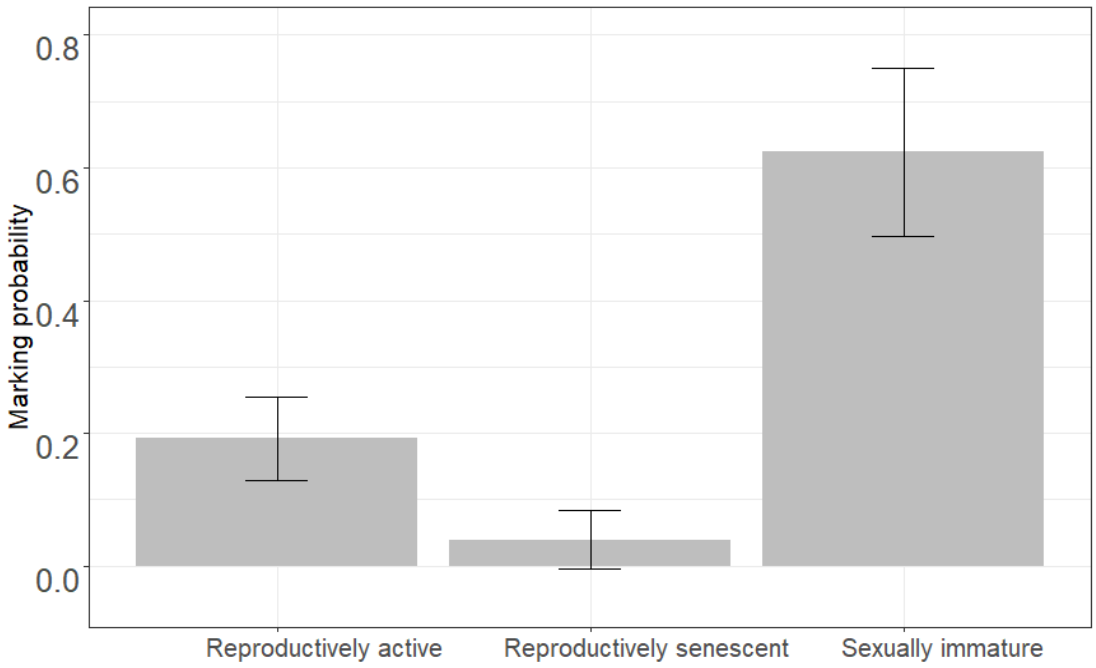


Figure 6: *Reproductively senescent responders were significantly less likely to countermark urine samples than sexually immature or reproductively active responders (± 1 standard error).*

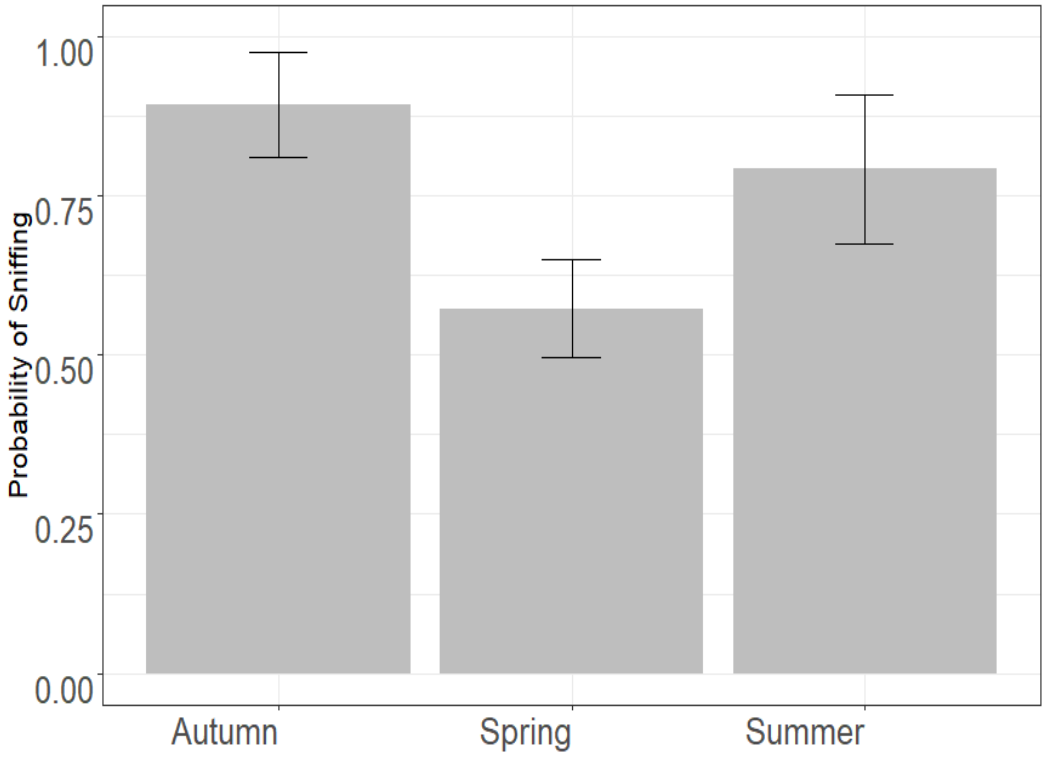
Table 3: *Model output for countermarking likelihood. Asterisks denote significance.*

	Npar	AIC	LRT	Pr(Chi)
Donor familiarity	2	104.59	4.1830	0.123503
Donor sex	1	103.56	1.1512	0.283289
Responder sex	1	105.04	2.6380	0.104336
Responder sexual maturity	2	110.75	10.3454	0.005669*
Donor sexual maturity	2	102.89	2.4831	0.288937

Sniffing behaviour

56 occurrences of sniffing behaviour were recorded during actual responses. Sniffing behaviour was not significantly affected by any parameter interactions (all LR chi-squared ≤ 3.84, $P > 0.05$). However, the effect of seasonal variation approached significance (Table 4; Fig. 7) with badgers sniffing urine samples less often during spring (25 events out of 59 actual responses; 59.0%) compared to summer (10 events out of 14 actual responses; 71.0%) and autumn (11 events out of 13 actual responses; 85.0%).

Sniffing duration was not significantly affected by any parameter (all LR chi-squared ≤ 3.84, $P > 0.05$).



320

Figure 7: *Badgers were less likely to sniff urine samples during spring than during other seasons (± 1 standard error).*

Table 4: *Model output for sniffing behaviour.*

	Npar	AIC	LRT	Pr(Chi)
Season	2	**121.78**	**4.8304**	**0.08935**
Donor familiarity	2	**117.43**	**0.4842**	**0.78497**
Donor sex	1	**121.64**	**2.6957**	**0.10062**
Responder sex	1	**120.02**	**1.0736**	**0.30013**
Responder sexual maturity	2	**121.29**	**4.3456**	**0.11386**
Donor sexual maturity	2	**119.44**	**2.4913**	**0.28775**

325

Table 5: *Distribution of identifiable and unidentifiable responders based on season, sex, and age.*

Spring 2017			
	Adults and Yearlings (n = 18)		Cubs
	Females	Males	
Identifiable	8	10	
Unidentifiable	n = 22 observations		n = 9 observations
Summer 2017			
	Adults and Yearlings (n = 15)		Cubs
	Females	Males	
Identifiable	5	10	
Unidentifiable	n = 24 observations		n = 17 observations
Autumn 2017			
	Adults and Yearlings (n =10)		Cubs
	Females	Males	
Identifiable	3	7	
Unidentifiable	n = 49 observations		n = 24 observations
Spring 2018			
	Adults and Yearlings (n = 24)		Cubs
	Females	Males	
Identifiable	11	13	
Unidentifiable	n = 13 observations		n = 13 observations

Table 6: *Distribution of donors and identifiable responders based on sexual maturity.*

Spring 2017		
	Donors	Responders
Sexually immature (n = 22)	18	4
Reproductively active (n = 40)	28	12
Reproductively senescent (n = 6)	5	1
Summer 2017		
	Donors	Responders
Sexually immature (n = 3)	2	1
Reproductively active (n = 23)	12	11
Reproductively senescent (n = 5)	2	3
Autumn 2017		
	Donors	Responders
Sexually immature (n = 2)	2	0
Reproductively active (n = 22)	14	8
Reproductively senescent (n = 4)	2	2
Spring 2018		
	Donors	Responders
Sexually immature (n = 8)	3	5
Reproductively active (n = 22)	7	15
Reproductively senescent (n = 9)	5	4

Discussion

European badger urine contains VOCs and excreted sex-steroid metabolites which encode fitness-related information (e.g., group membership, sex, age, sexual maturity; Service *et al.*, 2001; Sugianto *et al.*, submitted). We recorded conspecific behaviour towards simulated urine marks both during and outside the primary mating season, and we analysed how behavioural responses vary based on i) temporal variations in urine composition (indicative of

age and sexual maturity), and ii) spatial variations in urine composition (indicative of group membership). We found that responder sex, responder sexual maturity, and donor familiarity significantly affected response likelihood and countermarking, while sniffing behaviour was somewhat affected by season.

Statistically significant differences in responses at least confirm that badgers can distinguish different scents, and that different categories of badger make that discrimination differently. We expect such discriminations to have adaptive significance, but the discriminations themselves do not reveal their biological significance. That interpretation can be deduced from circumstances. For example, regardless of season, badgers responded to urine from neighbouring individuals significantly more than they responded to urine from familiar and strange individuals. Neighbouring setts share boundary latrines that are regularly visited by individual residents from both of a pair of neighbouring setts (Stewart *et al.*, 2001). The interpretation of badger society is not straightforward, but the greater 'interest' shown in the scent of immediate neighbours may be revealing. Since these regular visits often involve considerable time sniffing deposited faeces, urine, and AGS (Stewart *et al.*, 2002), badgers have the opportunity to 'scent match' (Gosling, 1982) conspecifics based on the individual- and group-level information encoded within these deposits. We demonstrate that badgers distinguish group members from non-members, especially non-members from stable, neighbouring groups. This discrimination can, as a general phenomenon, lead to less agonistic behaviour towards neighbours compared to strangers, a reaction known as the "Dear Enemy Phenomenon" (DEP; Fisher, 1954). The DEP has been previously evoked to explain the observation among the Wytham Woods badgers that individuals dug up samples of anal gland secretion (AGS) originating from unfamiliar donors more often than they did AGS samples from neighbouring

donors (Tinnesand *et al.,* 2015). The Wytham Woods badgers are also known to enter the ranges of neighbouring social groups (Ellwood *et al.,* 2017) and to display high levels of extra-group paternity (EGP) rates (Dugdale *et al.,* 2007). The presence of neighbouring urine within the vicinity of the sett (rather than at the boundary latrines at the edge of the group's boundary) doubtless contributed to the attention paid contextually to the odour by recipients. The question of how duration of sniffing relates to communicative function of a social odour is not settled and is further discussed in Allen *et al.* (2019); here, we interpret observations on the assumption that a more protracted duration of sniffing is indicative of more 'interesting' message content.

Seasonality approached significance for only one behaviour during our study – sniffing behaviour. In our high-density badger population, the peak post-oestrous mating season occurs during January-May (Sugianto *et al.,* 2020a). Furthermore, badgers have a polygynandrous mating system (Dugdale *et al.,* 2011b; Sugianto *et al.,* 2020a) in which most individuals mount/are mounted (Dugdale *et al.,* 2011a). Because encounters with potential mates are likely commonplace within this high-density population (as distinct to a low-density situation likely more characteristic of the badger's evolutionary past until recent centuries), sniffing urine marks during spring may be helpful but less essential, especially if other longer-lasting sources of olfactory information are available (e.g., boundary latrine deposits; Stewart *et al.,* 2002). Despite there being a peak mating season, mating (as inferred from observations of mounting) occurs amongst Wytham's badgers year-round. Some males prolong testicular descent (Woodroffe & Macdonald, 1995) and have higher testosterone levels (Buesching *et al.,* 2009) throughout the summer, perhaps as a strategy to procure additional mating opportunities. Females are also able to procure additional mating opportunities during summer by coupling delayed implantation with superfoetation (Yamaguchi *et al.,* 2006). Extending their sexual receptivity beyond the peak

mating season while acquainting themselves with the scents of non-resident individuals for future scent-matching – as seen by the increased sniffing likelihood during summer and autumn – may be amongst the mechanisms whereby badgers seek to maximise their reproductive output. In the context of seasonality in response to scent marks, we mention for completeness a further, and generally neglected factor, namely that the micro-climatic conditions occurring in each season may affect the detectability of scent.

Although badgers in high-density populations have a polygynandrous mating system, only 31% of males and 29% of females successfully produce cubs in a given year (Carpenter *et al.,* 2005). Furthermore, female badgers in Wytham generally reach their peak reproductive output at around 7 years old while male badgers reach their peak output at around 8 years old (Dugdale *et al.,* 2011). In comparison, younger badgers (2 years old) and older badgers (8+ years old) of both sexes have lesser reproductive output (Carpenter *et al.,* 2005; Dugdale *et al.,* 2011). Because badgers reach sexual maturity at 2 years old (Sugianto *et al.,* 2019) and undergo reproductive senescence around 7-8 years old, individuals have about 5-6 years to produce offspring. This provides the context in which to evaluate the adaptive significance of the tendency of reproductively senescent badgers to emphasise the deposition of faeces/anal gland secretion at latrines rather than urine; as mentioned, latrine deposits are more durable and are frequently investigated by conspecifics (Stewart *et al.,* 2002). Although both sexes are constrained by a limited reproductive time frame, older females face the additional pressure of securing shelter for cub-rearing and lactation. It is in this context that we observe that adult females responded more often towards urine marks than did adult males. This observation is a step towards interpreting communication within badger society, but it does not in itself provide that interpretation.

Nonetheless, we have demonstrated for urine marks that an individual's familiarity, sex, and sexual maturity – as perceived by olfactory signals – along with the seasonal context in which these scent marks are encountered can, amongst badgers, be discriminated. These are important elements of their communication which, in turn, is a step towards elucidating their social system.

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Chapter 6:
Don't pee afraid: Differing behavioural responses in olfactory landscapes of fear among European badgers (*Meles meles*)[5]

Abstract

Spatial and temporal risk perception influences how animals express certain behaviours (e.g., movement, communication, foraging) that are required for survival. Animals utilise various sensory modalities when assessing risks, but most 'landscape of fear' (LOF) research focuses predominantly on visual and auditory cues. Unlike visual/auditory cues, olfactory cues persist longer in the environment and can be investigated in the predator's absence. Nevertheless, olfactory LOFs remain understudied, particularly within the Carnivora to which humans function as apex predators. Our study addressed if/how a wild population of European badgers (*Meles meles*) – a common mesocarnivore species in the U.K. – assess perceived levels of risk in response to urine from other badgers, horses, dogs, and humans (vegetarian adults, non-vegetarian adults, and children). Urine samples were collected from healthy individuals of different ages, and were mixed proportionally in regards to sex and age to create a composite scent thereby controlling for individual variation that may impact response behaviour. For our scent-provisioning experiments, conducted in June-August 2019, four Eppendorf tubes filled with 0.5 ml of urine were placed around a bucket filled with sand and peanuts to stimulate foraging behaviour. A similar set-up was positioned at the opposite end of the sett. Infra-red Crenova 2.4" LCD Display 12MP 1080P HD motion-activated camera traps were used to observe latency to feed, countermarking frequency, sniffing duration, and foraging duration.

[5] This chapter is the basis of a paper to be submitted to ***Behavioural Ecology,*** co-authored by Tanesha M. Allen, Paul Johnson, Chris Newman, David W. Macdonald & Christina D. Buesching

Cubs foraged for significantly longer than adults while adults countermarked urine samples significantly more than did cubs. Furthermore, all badgers spent a shorter proportion of time sniffing urine from dogs and non-vegetarian humans, and also countermarked less often in the presence of dog and non-vegetarian human urine. This suggests that badgers may assess potential predation risk via olfactory cues. We discuss our results within the context of conspecific recognition and perceived levels of risk among suburban/urban wildlife.

Introduction

Perceived risks within the environment can affect spatial and temporal patterns of foraging (Brown *et al.,* 1999), movement (Brown *et al.,* 1999) and communication (Tsunoda *et al.,* 2019). This 'landscape of fear' (LOF; Laundré *et al.,* 2001; Laundré *et al.,* 2010) can thus impact somatic fitness and reproductive output as animals balance vigilance (sometimes at the cost of missing opportunities to gain resources) with seeking out resources (albeit increasing their risk of predation).

Most LOF research focuses on visual (e.g., Bates *et al.,* 2007) and auditory cues (e.g., Clinchy *et al.,* 2015). Nocturnal species, however, rely primarily on olfactory cues when detecting predators (e.g., deposited scent marks: Rosell & Sanda, 2006; 'whole body odours': Leo *et al.,* 2015), and many mammals have been shown to be able to distinguish other species based on their odour (Wyatt, 2014), either through learned (e.g., elk (*Cervus elaphus*): Laundré *et al.,* 2010; African elephants (*Loxodonta africana*): Bates *et al.,* 2007) or innate knowledge (e.g., goats (*Capra hircus*) and eastern grey kangaroos (*Macropus giganteus*): Cox *et al.,* 2010; mice (*Mus musculus*): Roberts *et al.,* 2002).

Compared to visual and auditory cues, olfactory cues persist for longer in the environment (Hayes *et al.,* 2003). This allows animals to investigate scent marks when the marker is not present, giving information about the past presence of predators even if they are elsewhere in the habitat at that moment. Furthermore, olfactory cues encoding information on the age of the scent mark (e.g., Buesching *et al.,* 2002b) can help assess the likelihood of the predator returning – or still being nearby (Bytheway *et al.,* 2013) – providing a proxy in the assessment of risk (Bates *et al.,* 2007). This olfactory-based information then enables individuals to avoid high-risk areas (Kats *et al.,* 1998) and/or risky times (e.g., Tsunoda *et al.,* 2019) and modify their own scent-marking behaviour (Roberts *et al.,* 2002; Rosell & Sanda, 2006; Tsunoda *et al.,* 2019).

In many ecosystems, humans have become the apex predator (Darimont *et al.,* 2015), and even species with no or few natural predators have learned to avoid predation by humans (Ordiz *et al.,* 2011). For example, Bates *et al.* (2007) found that African elephants (*Loxodonta africana*) presented with garments recently worn by Maasai tribe members (whose young men tend to spear elephants) travelled farther and faster than they did when presented with garments worn by Kamba tribe members (pastoralists who pose little risk to elephants). The fact that these elephants travelled downwind from the garments suggests that olfactory cues were used independently from/in addition to visual cues when informing this behaviour. Yet, whether or not other species that tend to experience negative human interactions (e.g., hunting, culling) can use olfactory cues in human-related risk perception remains under-researched.

European badgers (*Meles meles*) are nocturnal macrosmatic mustelids. Although solitary or pair-living in some parts of their geographic range, badgers are group-living in many habitats in the UK (Johnson *et al.,* 2000; Roper, 2010). They dig extensive underground tunnel systems

that connect a number of different sleeping chambers, and these communal ‘setts’ are used by all individuals sharing the surrounding home range marked with composite latrines (Buesching & Jordan, 2019). Badgers are opportunistic foragers (Robertson *et al.*, 2014) feeding predominantly on earthworms (*Lumbricus terrestris*), but supplementing their diet with seasonal crops (e.g., apples, wheat, sweetcorn) and leatherjackets (*Tipula spp.*; Neal & Cheeseman, 1996; Robertson *et al.*, 2015). Therefore, their home ranges can encompass farm yards (Robertson *et al.*, 2015), livestock barns (particularly cattle and horses: White *et al.*, 1993), gardens, and urbanised neighbourhoods (Huck *et al.*, 2008). The agricultural and lawn/garden (Roper, 2010) damage caused by foraging badgers in addition to their implied role as a wildlife host in the transmission of bovine tuberculosis (bTB; White *et al.*, 1993; Woodroffe & Donnelly, 2017) have resulted in heavy mortality by humans through hunting (Cassidy, 2012), governmental culling programmes (using either traps in the UK or stop-snares in the Republic of Ireland; Woodroffe & Donnelly, 2017) in the context of bTB management and agriculture (Bielby *et al.*, 2014), and badger baiting with specially bred terriers or dachshunds (reviewed in Roper, 2010). Unsurprisingly, therefore, badgers appear to have learned to associate human voices with high threat levels, resulting in increased vigilance and decreased foraging time when human voices (but not vocalisations from dogs, wolves, bears, or sheep) were played near their foraging site (Clinchy *et al.*, 2016). Because badgers rely predominantly on olfactory cues and signals for communication and orientation (reviewed in Buesching & Stankowich, 2017), we posit that olfactory cues may also be important when assessing risk. Here, we test in scent-provisioning experiments:

1. If wild badgers can detect olfactory, species-specific differences in urine from other badgers, humans (vegetarian adults, non-vegetarian adults, and non-vegetarian children),

horses, and dogs. Do badgers modify their vigilance, feeding pattern and response behaviour according to the potential threat level each species might pose?

2. If badgers learn to associate olfactory cues with (potential) threats posed by other species (and thus adults and juveniles differ in their reaction to urine from different species due to past experience), or if this is an innate reaction (and thus there is no age-related difference in response behaviours)?

Methods

Sample collection and study population

The study was carried out at a badger sett in Stansfeld Park, Oxfordshire, UK (GPS reference: 51°45'7"N; 1°12'2"W) – an 18-acre woodland with meadows, ponds, and marshland near a residential area. For our scent provisioning experiments, urine samples were collected from four mammal species that frequently use this area: domestic dogs (*Canis lupus familiaris*), horses (*Equus caballus*), humans, and other badgers.

Dog (n = 2 males, 1 female) and horse (n = 1 male, 1 female) urine was collected in plastic containers while the animals urinated naturally from local owners within Oxfordshire. Human urine (n = 18) was collected from vegetarian (n = 1 male, 1 female) and non-vegetarian (n = 8 males, 8 females) adults, and 2 non-vegetarian children (1 male, 1 female). Non-vegetarian donors were asked to consume a protein-heavy diet for two days prior to sample collection.

Badger urine (n = 6 males, 8 females) was collected from adults belonging to an independent badger population (Wytham Woods, Oxfordshire, UK; GPS reference: 51° 46’26”N; 1° 19’19”W), 15.7 km northwest of the study site in the context of a long-term

population study (Macdonald *et al.*, 2015). These badgers were trapped overnight in cage traps baited with peanuts and moved to a central handling unit between 7:00 am and 8:00 am. After sedation by intramuscular injection of 0.2 ml ketamine hydrochloride/kg body weight (McLaren *et al.*, 2005), urine was expressed by gently palpating the bladder (Vicente *et al.*, 2007), and collected in glass vials with Teflon-coated screw lids (Sigma-Aldrich, Dorset, UK).

Initially, all urine samples were frozen after collection and stored at -20°C. To create a composite species-specific urine mixture (thus controlling for individual-specific variation in odour profiles), urine samples were thawed at room temperature prior to being combined in species-specific, and – in the case of human urine – also diet- and age-specific mixtures. All samples were mixed proportionally to prevent sex biases within the composite scent. For each species and human diet category, 5-10 Eppendorf tubes (2 ml) were filled with 0.5 ml of the urine mixture using plastic pipettes (one pipette per urine sample) and centrifuged in an Eppendorf Centrifuge 5810 R (Eppendorf UK Limited, Stevenage, UK) for 3 minutes at 2000 rpm to remove any impurities. The supernatant was removed and transferred to new Eppendorf tubes before being frozen at -20°C.

Ethical review and approval were obtained for all badger 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. Ethical review and approval were obtained for human sample collection, and were performed in accordance with the Central University Research Ethics Committee (CUREC) under approved procedure AP24. Written consent was obtained from all adult volunteers, parental consent was obtained for both children to participate in this study, and all horse- and dog-owners were informed about the study's purpose.

Scent-provisioning experiments and behavioural observations

To determine the group size, and sex/age composition of the focal badger group, 10 primary and secondary school students placed 8 Infra-red Crenova 2.4" LCD Display 12MP 1080P HD camera traps in the vicinity of the sett prior to the experiment (8th - 15th May 2019) as part of a citizen science project funded by a Royal Society Partnership Grant.

Scent-provisioning experiments were conducted between 8th June to 1st August 2019. Experiments were set up at a minimum distance of 20 m from all badger setts in accordance with the Protection of Badgers Act (1992). Two 5 L buckets filled with sand mixed with 1 cup of peanuts to stimulate foraging behaviour were placed at opposing sides of the sett in specially dug holes so that the bucket rims were level with the surrounding soil. Urine samples were defrosted within 1-2 hours before the scent playback experiments. Latex gloves were used at all times when handling the samples, and changed between each sample to prevent cross-contamination with other scents. Four Eppendorf tubes were positioned in a circle at a distance of 10 cm from the bucket rim, their lids removed to expose the scent, and pushed into the ground so that their rim was level with the soil. Samples remained in place for 3 days before being removed and replaced with fresh samples and a new cup of peanuts was mixed into the bucket to replenish the supply. Urine samples were presented in random order regarding species, and the same species was never presented in two succeeding trials to avoid scent habituation. All urine mixtures were presented twice at each of the two feeding sites, with both sites having the same urine mixture. Baseline observations using Eppendorf tubes filled with 0.5 ml of water as control samples were recorded between 8th - 11th June 2019.

Behavioural observations were recorded remotely using Infra-red Crenova 2.4" LCD Display 12MP 1080P HD camera traps activated by three motion sensors. Camera traps were

positioned on nearby trees at a height of 1.5 m, about 1.5-3.0 m away from the sample. Videos were recorded for 1 minute with 15 frames per second. Footage from SD cards was downloaded every 3 days.

Videos were analysed 'blind' (i.e., relative locations of urine and water samples were unknown to the observer) with the Behavioral Observation Research Interactive Software (BORIS; version 6.3.9; Friard & Gamba, 2016) to minimise observer bias. Responding badgers were categorised by age as adult or cub. Badger proximity to the samples was estimated in number of body lengths, where one body length of an active adult badger equals ca. 50cm. Individuals were recorded as showing "no response" if they remained at a distance of ≥ 50 cm (i.e., ≥ 1 body length) of any sample. For all individuals approaching any sample within 50 cm, we recorded:

- Social patterns: If badgers were observed in the field of view alone or in pairs/groups.
- Latency to feed (in seconds): Elapsed time between a badger approaching the 50 cm radius from the samples (and therefore likely able to detect the scent) and starting to feed.
- Feeding duration (in seconds): Time period from when the badger's nose touched the sand/peanuts in the bucket or sand/peanuts displaced during previous foraging activity until the last time.
- Vigilance (duration in seconds): Defined as a badger holding its head up (i.e., long axis of its face not perpendicular to the ground) any time after an individual approached the 50 cm radius from the samples and ending when an individual exited from the field of view (to accommodate for the fact that badgers likely retain a memory of the scent: Gosling & Roberts, 2001).

- Sniffing (duration in seconds): Defined as the badger's nose approaching the vial within 10cm.
- Subcaudal marking (within 50 cm of any sample): Defined as a badger pressing its subcaudal pouch opening against the ground (Buesching & Macdonald, 2004).

Statistical analysis

All statistical analyses were performed in R (version 3.6.3) and R Studio (version 1.2.5033). The "survival" (Therneau, 2020) and "survminer" (Kassambara *et al.*, 2020) packages were used to test hypotheses concerning latency to feed, allowing for observations where no feeding had occurred by the end of the observation period to be properly accounted for.

Generalised linear models (GLMs) with a binomial distribution were used to test if the likelihood of observing foraging, sniffing, vigilance or countermarking was affected by treatment or badger age. Time spent near urine mixtures, average foraging bout length, average vigilance duration, average time spent sniffing, proportion of observation time spent foraging, proportion of observation time spent being vigilant, and proportion of observation time spent sniffing urine mixtures were treated as continuous responses. Proportion of time spent foraging, proportion of time spent being vigilant, proportion of time spent sniffing were square root transformed. Log transformation was applied to time spent near urine mixtures, average foraging bout length, average vigilance duration, average time spent sniffing due to skewed distributions. A survival analysis was used for latency to feed using the R 'survival' package.

Preliminary analyses include interaction terms between treatment and age group to test the hypothesis that effect of treatment differed between adults and cubs, these were all non-significant and removed from the final models. Previous scent-marking by conspecifics within 4

days and social patterns (whether the badger was observed alone or in a group) were added as fixed effects. However, as these parameters proved insignificant, they were removed from the final analysis.

When treatment or age group was statistically significant, post-hoc Dunnett's Tests were conducted to compare behavioural responses to the water control. Model fit was assessed with residual plots to assess residual normality and homogeneity of variance. Binomial models were assessed using plots of surrogate residuals (*sure* package; Greenwell *et al.,* 2018). Poisson and binomial models included an offset term to adjust for observation time. Results are reported as mean ± standard error.

Results

1338 video clips collected before the experiment revealed a minimum of 9 individuals to reside at the Stansfeld Park sett: 6 cubs, 2 adult females, and 1 adult male. Estimates were based on multiple individuals being seen simultaneously. However, since the average litter size in the UK is 2-3 cubs (Roper, 2010) and because badgers often remain in their natal territory (Macdonald *et al.,* 2008), it is highly likely that the number of adults is an underestimate. Additional adults as well as a number of pre-reproductive yearlings also likely share the site.

85 observations were recorded during the experiment's duration where badgers were observed within 50 cm of a sample.

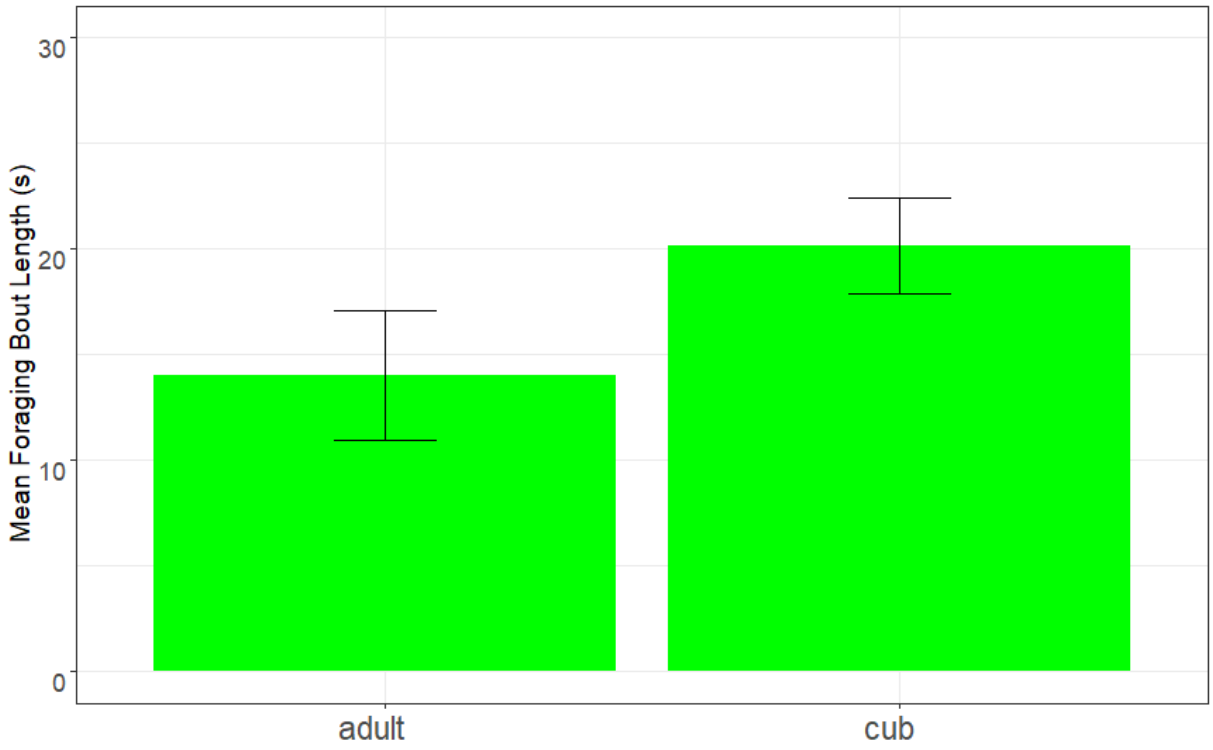
Latency to feed

From our 85 observations, we recorded 70 instances of foraging (adults = 25, cubs = 45). For the remaining 15, foraging was not observed by the end of the observation period. There was no evidence that treatment (log rank test: $p = 0.62$) or age group (log rank test: $p = 0.45$) significantly affected latency to feed.

Foraging

There was no evidence that treatment (df = 6, Pr(>Chi) = 0.29) or age group (df =1, Pr(>Chi) = 0.16) affected the likelihood of a badger foraging.

Treatment also did not affect the average length of foraging bouts ($F_{6,62} = 1.49$, $p = 0.19$) or the proportion of time spent foraging ($F_{6,77} = 0.99$, $p = 0.44$). However, age group did significantly affect average foraging bout length ($F_{1,62} = 6.61$, $p = 0.01$) with cubs (29.07 ± 41.73s) foraging for longer than did adults (13.93 ± 15.74s; Fig. 1). Cubs also spent a larger proportion of their time foraging (39.3% ± 27.0%; Fig. 2) than did adults ($F_{1,77} = 7.90$, $p = 0.006$; 22.6% ± 20.8%).



255 ***Figure 1:*** *Cub foraging bout lengths were longer than adults. Error bars in this and other figures are ± 1 standard error.*

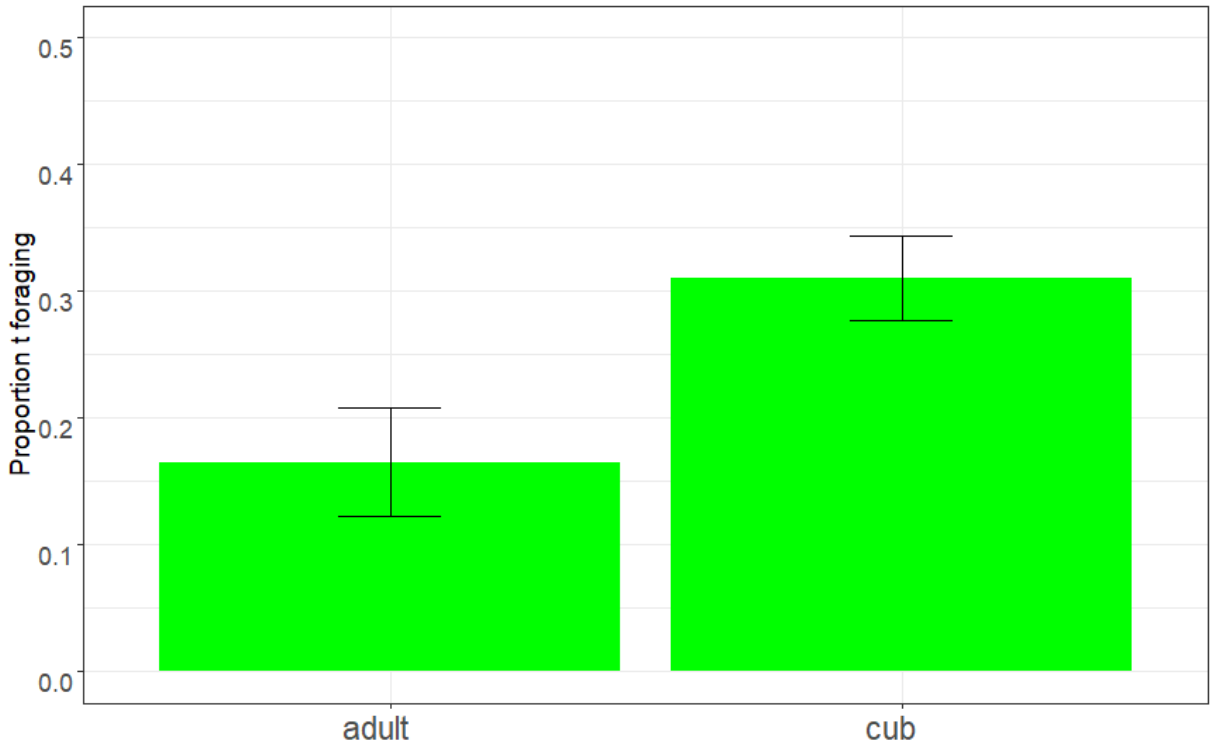


Figure 2: *Cubs spent a significantly higher proportion of their time foraging than adults.*

Sniffing

There was no evidence that age group affected the likelihood that badgers sniffed the urine mixtures (df = 1, Pr(>Chi) = 0.29); nor did it affect the average time spent sniffing urine mixtures ($F_{1,56}$ = 0.14, p = 0.71) or the proportion of time sniffing urine mixtures ($F_{1,77}$ = 0.39, p = 0.53).

Treatment did not affect sniffing likelihood (df = 6, Pr(>Chi) = 0.16) or the average time spent sniffing urine mixtures ($F_{6,56}$ = 0.83, p = 0.55), but there was evidence that it affected the proportion of time spent sniffing urine mixtures ($F_{6,77}$ = 2.98, p = 0.01). Compared to water (16.3% ± 12.5%; n = 4; Fig. 3), badgers spent shorter proportions of time sniffing dog urine mixtures (4.32% ± 3.65%; Dunnett's test: estimate = -0.18, p = 0.03; n = 11) and non-vegetarian human urine mixtures (3.08% ± 2.14%; Dunnett's test: estimate = -0.18, p = 0.02; n = 18).

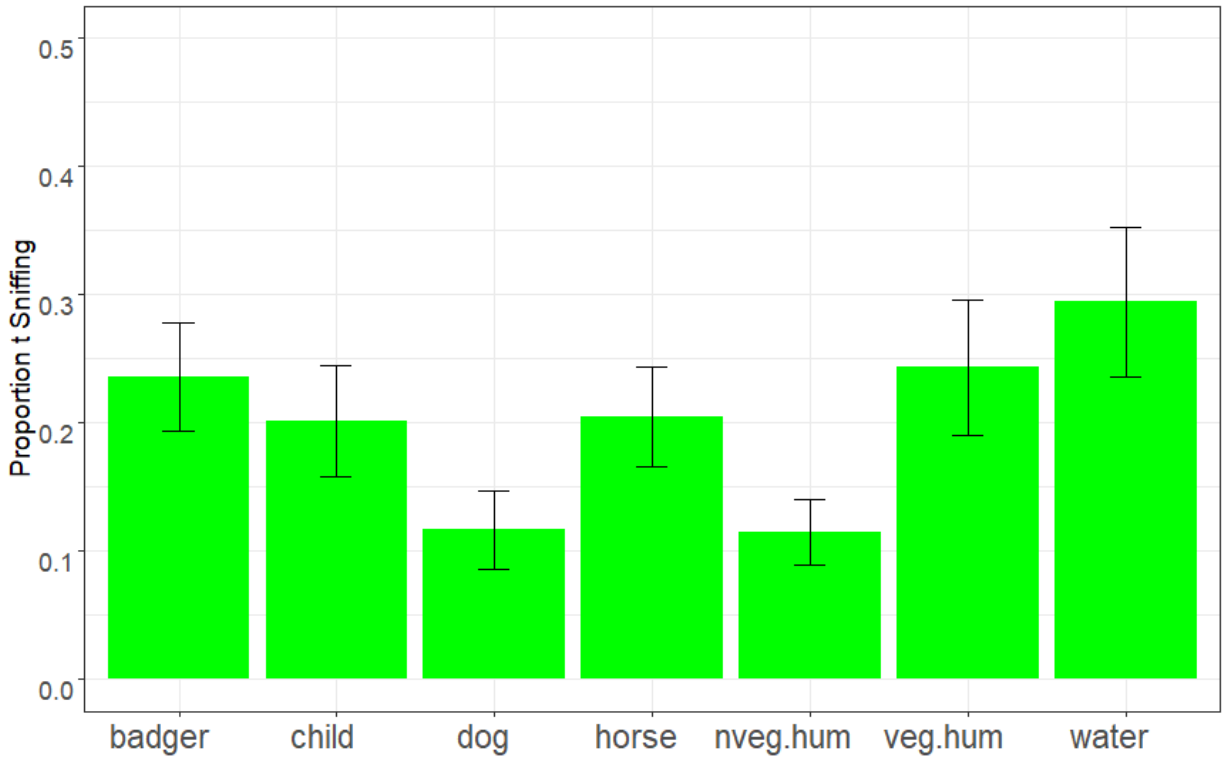


Figure 3: *When compared to water, all badgers spent a shorter proportion of time sniffing urine mixtures from dogs (Dunnett's test: estimate = -0.18, p = 0.03) and non-vegetarian humans (Dunnett's test: estimate = -0.18, p = 0.02).*

Vigilance

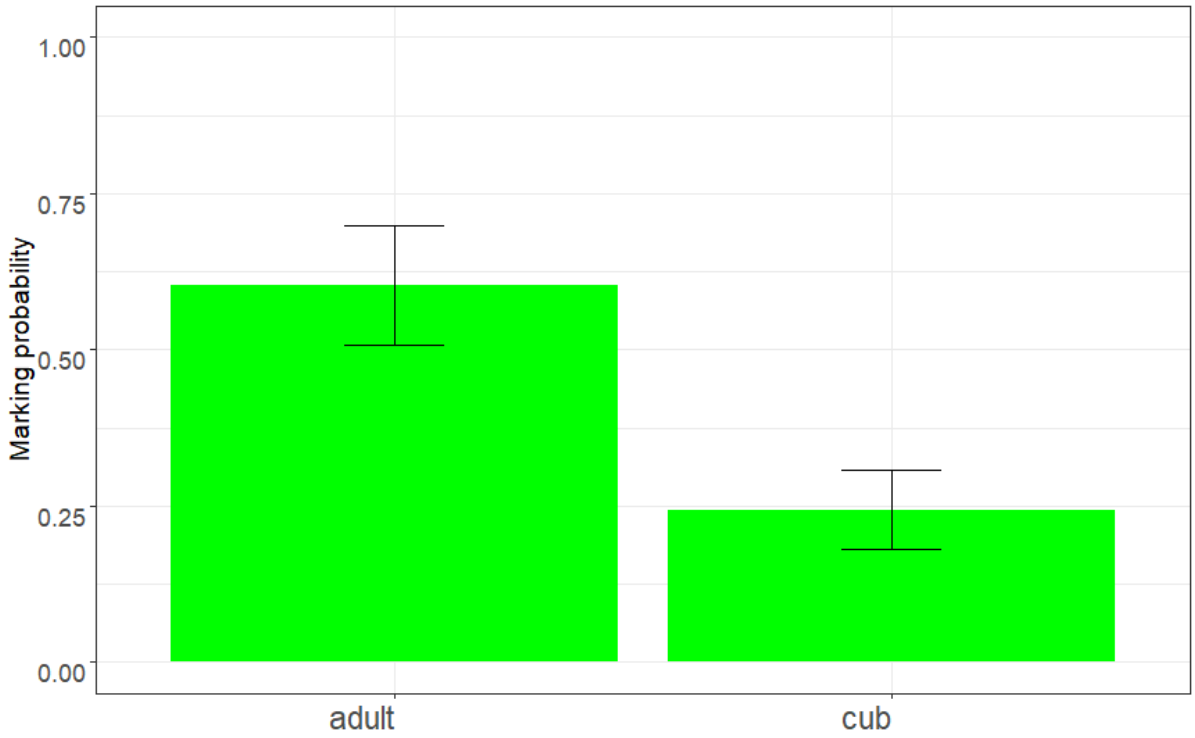
There was no evidence that either treatment or age group affected the likelihood of badgers displaying vigilance behaviour (treatment: df = 6, Pr(>Chi) = 0.25; age group: df = 1, Pr(>Chi) = 0.19), average length of time being vigilant (treatment: $F_{6,33} = 0.89$, p = 0.51; age group: $F_{1,33} = 2.64$, p = 0.11), or the proportion of time being vigilant (treatment: $F_{6,77} = 1.13$, p = 0.35; age group: $F_{1,77} = 2.61$, p = 0.11).

290 Countermarking likelihood and frequency

There was evidence that age group (df = 1, p = 3.28 x 10^{-4}), but not treatment (df = 6, p = 0.15), significantly affected countermarking likelihood with adults being more likely to mark urine mixtures than cubs (Fig. 4).

Adults also countermarked more frequently (n = 36; df = 1, Pr(>Chi) = 7.79 x 10^{-4}) than

295 cubs (n = 21). Countermarking frequency was also affected by treatment (df = 6, Pr(>Chi) = 0.003; Fig. 5) with badgers marking dog urine mixtures (n = 8; Dunnett's test: estimate = -1.74, p = 0.006) and non-vegetarian human urine mixtures (n = 9; Dunnett's test: estimate = -2.38, p = <0.001) significantly less than water.



300

Figure 4: *Regardless of treatment, adult badgers were significantly more likely to countermark urine mixtures than cubs.*

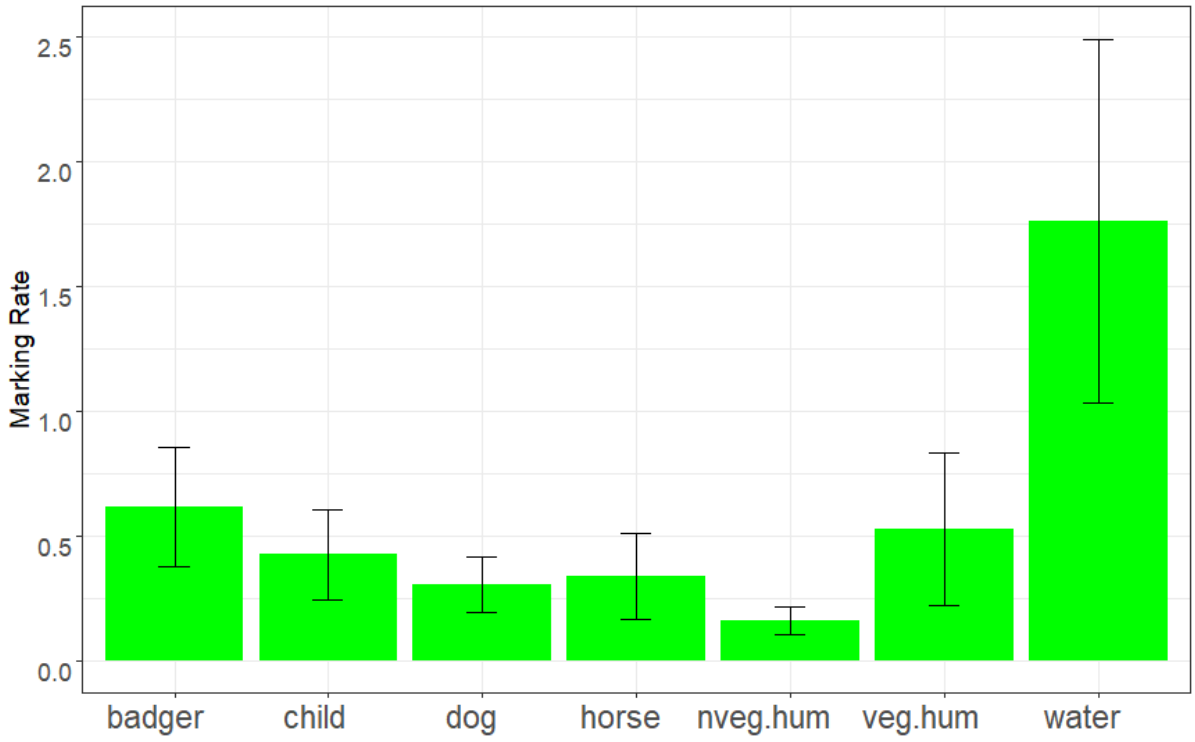


Figure 5: *When compared to water, all badgers countermarked dog urine mixtures (Dunnett's test: estimate = -1.74, p = 0.006) and non-vegetarian human urine mixtures (Dunnett's test: estimate = -2.38, p = <0.001) significantly less often.*

Time spent near the sample

There was no evidence that either treatment ($F_{6,77}$ = 1.16, p = 0.33) or age group ($F_{1,77}$ = 2.46, p = 0.12) affected how long an individual spent near urine mixtures.

Discussion

Humans function as apex predators within many ecosystems inhabited by the Carnivora. However, few studies have analysed if/how olfactory LOFs function among carnivore species experiencing negative interactions with humans (e.g., hunting, culling). We tested whether there was a detectable difference in the badgers' responses to urine samples of different provenances,

and there was. Our scent-provisioning experiments demonstrated that both adult and juvenile badgers i) spent a significantly shorter proportion of time sniffing urine mixtures from domestic dogs and non-vegetarian humans, and ii) countermarked samples from dogs and non-vegetarian humans less often than other samples. Meanwhile, age-specific differences in foraging duration (significantly longer among cubs) and countermarking frequency (more common among adults) were also observed, although these differences were not attributed to odour treatments.

While it is important to have demonstrated the existence of these discriminations, their functional significances are not obvious. One line of enquiry lies in the way that digestive processes influence the metabolite composition within the urine of heterotrophic species (Playdon *et al.,* 2016). Protein-heavy diets produce sulphurous metabolites (Nolte *et al.,* 1994) which have relatively high volatility compared to other organic compounds (Cannon & Ho, 2018). This high volatility enhances the ability of sulphurous volatile organic compounds (VOCs) to vaporise and bind with receptors within the olfactory epithelium, allowing them to be readily detectable by prey species (Kats & Dill, 1998; Nolte *et al.,* 1994) and competitors (Banks *et al.,* 2016). Perhaps the urine samples of dogs and non-vegetarian humans shared a common feature of relatively high levels of sulphurous VOCs? Domestic dogs generally consume protein-heavy diets (Bhadra & Bhadra, 2014) and our non-vegetarian human participants confirmed that they consumed protein-heavy diets prior to donating urine. We hypothesise that the presence of sulphurous VOCs within both of these two categories of urine may be associated with the badgers in our study population spending shorter proportions of their time sniffing these urine mixtures: a possible functional explanation is that these signals are strident and easily interpreted; a non-exclusive additional possibility is that they are somehow of limited interest. The latter possibility raises a general interpretative dilemma with respect to durations of sniffing:

is an odour sniffed only fleetingly indicative that the message is instantly clear and unambiguous, or that it is inconsequential. The answer may lie in how the recipient responds subsequently to circumstances (for example, a readily interpreted signal of dangerous portent might be expressed in the recipient subsequently increasing vigilance or readiness to flee), but our methodology could not disclose such second-order effects.

Quick detection of olfactory cues from potential predators could enhance an individual's fitness by allowing it to efficiently assess risk levels, perhaps informing future decisions about remaining in a particular area. However, although our results suggested that badgers easily detected urine from dogs and non-vegetarian humans, treatment showed no significant influence on the amount of time, in the immediate aftermath, that badgers spent near the samples, their latency to feed, likelihood of foraging, or their vigilance duration. We cannot exclude the possibility of longer-term impacts on subsequent behaviour (further, the depression of feeding observed by Clinchy *et al.* (2016) to human voices, which we did not observe in response to these scent signals, may reflect the immediacy of the threat). Furthermore, because we did not have a Home Office licence to conduct live-trapping at Stansfeld Park and therefore clip-mark individuals (Allen *et al.*, 2019; Tinnesand *et al.*, 2015), this prevented the inclusion of individual identity as a parameter in our analysis. This thwarted our attempt to account for individual variation in behaviour and limited our ability to prevent pseudoreplication. Our inferences are therefore confined to the observed individuals. Adults foraged for shorter durations and spent a shorter proportion of their time foraging compared to cubs. However, another and non-exclusive explanation may be that adults – with their presumably increased foraging experience – maximised their foraging efficiency by venturing to other food sources whenever there was increased conspecific competition around the buckets. Meanwhile, cubs may have tended to

remain at the buckets longer since it was a convenient food source at a period of their lives when they were less willing to stray far from the sett.

Despite the insignificant variation in vigilance behaviour, or in time spent near the samples, badgers may have been exhibiting risk averse behaviour by countermarking less frequently around dog and non-vegetarian human urine. Other species have demonstrated similar adjustments in their scent-marking behaviour when exposed to predator odours, interpreted as being due to the increased risk of 'eavesdropping' from predators (Roberts *et al.,* 2002; Rosell & Sanda, 2006). Additionally, although Clinchy *et al.* (2016) did not measure scent-marking behaviour when assessing badger behavioural responses to species-specific auditory cues, they did observe other aversive behaviours – in the forms of increased vigilance, reduced foraging durations, and decreased number of visits – when human noises (but not dog noises) were played near foraging sites. Framing our results within the context of these previous LOF studies might suggest that the badgers in our study population intentionally avoided advertising their presence via olfactory cues to potential predators (this raises the interesting possibility that the vegetarian eating habits of some of our human donors was associated with the badgers reacting to them as less of a risk – insofar as short sniff duration is indicative of less ambiguous response to danger). Our finding that adults countermarked more frequently than cubs regardless of treatment accords with the fact that adults produce more subcaudal gland secretion than cubs (Buesching *et al.,* 2002a). A conventional interpretation of the greater rate of marking amongst adult mammals is that for sexually mature adults, this signals priority access to mating opportunities/territorial resources in ways that are essential for increasing their reproductive output and inclusive fitness (Cresswell *et al.,* 1992). Yet despite the adults in our study population countermarking more frequently than cubs, the interaction between treatment and age group proved insignificant in our

preliminary analyses (thus leading to their removal from our final analyses). This indicates that even badger cubs respond quickly to the urine odours of domestic dogs and (non-vegetarian) humans, a rapidity of response that might be interpreted as aversive insofar as it signals clear cut danger.

Semio-chemicals and their impact on LOFs in human-dominated environments serve as an interesting avenue for further research and useful applications. Our findings that badgers modify their sniffing and countermarking behaviour around certain scents suggest that uncovering how different species respond to interspecific odours may assist in the development of wildlife management practices (e.g., odour repellents; Cox *et al.*, 2010), perhaps in the context of minimising human-wildlife conflict. Moreover, additional investigation into interspecific chemical communication between wild populations and domesticated animals could not only be an avenue leading to eased human-wildlife interactions, but also to better understanding of the dynamics of ecological communities.

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Section IV: The rules of effective advertisement for European badgers



"Badgertising" by Millie Scott

Chapter 7:
General discussion: The rules of effective advertisement for European badgers (*Meles meles*)[6]

Redundancy in olfactory communication

In the vocabulary of animal communication research, 'redundancy' refers to the use of a diversity of signals – sometimes from different modalities – to convey a single message (Breed & Moore, 2012). Redundancy is thought to assist communication by ensuring that information transmission between conspecifics occurs one way or another, and is therefore interpreted by researchers as an evolutionary mechanism to enhance message transfer (as opposed to wasteful duplication). Olfactory communication offers opportunities for redundancy to occur through the same signal being encoded in various specialised glandular secretions (e.g., anal gland secretions) and metabolic by-products (i.e., urine and faeces). For example, in the case of European badgers, *Meles meles*, social odours of both these provenances have been demonstrated to encode similar information regarding individual fitness and social group membership (Buesching & Stankowich, 2017).

Although these two provenances of olfactory information transmission encode information that is, at the least, similar, their deployment and behavioural consequences are rather little compared in the literature. However, that comparison sparks interest due to the likely different production costs of specialised glandular secretions and metabolic by-products. While specialised glandular secretions often contain considerable amounts of high molecular weight compounds (i.e., proteins, short- and long-chained fatty acids; Apps *et al.,* 2015) which are

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considered energetically costly to synthesise (Bauman *et al.,* 2006), metabolic by-products are likely less costly to produce since they arise naturally as by-products of heterotrophic diets and contain relatively few high molecular weight compounds (e.g., urine; Rivera-Vélez & Villarino, 2018).

The communicative significance of specialised glandular secretions versus metabolic by-products

European badgers (Meles meles) as a study species

European badgers are macrosmatic mammals which live in pairs or small groups throughout most of their geographic range in Europe and Asia (Roper, 2010). However, some high-density populations where badgers live in larger social groups do exist, especially in southern England (e.g., Wytham Woods, Oxfordshire; Macdonald *et al.,* 2015). Because badgers are nocturnal, and generally forage alone even when denning communally, olfactory signals and cues predominate over visual, auditory, or tactile signals/cues in their communications.

Scent-marking allows badgers to mark the intersections of group ranges (classically interpreted as territories) arranged around more or less centrally placed communal dens or ‘setts’ (Buesching *et al.,* 2016a; Stewart *et al.,* 2002), mark recently used resources (Buesching & Stankowich, 2017), and advertise themselves in the context of mating opportunities within their polygynandrous mating system (Buesching *et al.,* 2003; Buesching & Macdonald, 2004). Various glandular secretions – anal gland secretions, interdigital gland secretions, and subcaudal gland secretions (Buesching & Stankowich, 2017) – have communicative roles alongside urine and faeces (Buesching *et al.,* 2016a; Stewart *et al.,* 2002) when scent-marking. Here, in our comparison of the use of subcaudal gland secretion (SGS) and urine within olfactory advertisement, we focus on the high-density badger population living in Wytham Woods,

Oxfordshire, U.K. (GPS reference: 51° 46’26”N; 1° 19’19”W; Macdonald *et al.,* 2015) with some supplemented information from other badger populations in southern England.

A comparison of these two categories of social odours, specialised glandular secretions versus metabolic by-products, can insightfully be framed within the context of Daniel Starch’s (1923) marketing research, which outlines the five traits of effective advertisements. According to Starch, an effective advertisement has i) ‘to be seen’, ii) ‘to be read’, iii) ‘to be believed’, iv) ‘to be remembered’, and v) ‘to be acted upon.’ It is within this framework that we compare olfactory fitness advertisement via specialised glandular secretions (i.e., subcaudal gland secretions) and metabolic by-products (i.e., urine) among badgers.

i) <u>‘To be seen’: Spatial and temporal patterns of scent deposition</u>

Only badgers of the genus *Meles,* and their Asiatic congeneric kin – hog badgers, genus *Arctonyx* (*A. alboguralis, A. collaris,* and *A. hoevenii*; Zhou *et al.,* 2017), have this specialised gland subcaudal gland between their tail and anus (Buesching & Macdonald, 2004; Buesching & Stankowich, 2017). Unlike other species which deposit glandular secretions with additional movements such as handstands (e.g., giant pandas (*Ailuropoda melanoleuca*): White *et al.,* 2002) or scraping surfaces (e.g., jaguars (*Panthera onca*) and pumas (*Puma concolor*): Harmsen *et al.,* 2010), badgers deposit SGS by briefly pressing their subcaudal pouch against the substrate (Buesching & Macdonald, 2004). Common substrates include frequently used paths, tree trunks, latrines, and conspecifics (Buesching *et al.,* 2003; Buesching & Macdonald, 2004). Conspecific marking – or ‘allo-marking’ – takes two forms: sequential allo-marking where one badger marks another individual, and mutual allo-marking where two badgers press their subcaudal pouches against each other (Buesching *et al.,* 2003; Buesching & Stankowich, 2017). Both forms occur

between group members for social appeasement (Buesching *et al.*, 2003), maintaining and sharing a consistent group odour by exchanging microbiota (Buesching *et al.*, 2016b; Sin *et al.*, 2012), and for individual advertisement by using conspecifics as 'mobile billboards' (Buesching *et al.*, 2003; Buesching & Stankowich, 2017).

Subcaudal gland scent-marking behaviour and secretion production (Buesching *et al.*, 2002a; Buesching & Macdonald, 2004) increases during the post-oestrous mating season in late winter/early spring (January-May; Sugianto *et al.*, 2018; Sugianto *et al.*, in prep.). Cubs begin producing minimal amounts of SGS at around 4-6 months old (Buesching *et al.*, 2002a). Perhaps associated with the insufficiency of their own SCG secretion, they engage in 'scent theft' – a behaviour in which cubs crawl underneath an adult, thus forcing the adult to scent mark them (Fell *et al.*, 2006). As they gradually reach sexual maturity at around 2 years old (Sugianto *et al.*, 2019), badgers increase their investment in olfactory advertisement with peaks occurring during late winter and spring (especially among males with descended testes and oestrous females; Buesching *et al.*, 2002a; Buesching *et al.*, 2003).

Latrines provide foci of olfactory information for badgers during these peak marking periods, as evidenced by the increase in latrine usage during this time (Buesching & Jordan, 2019). Boundary latrines, evenly distributed along the perimeters of social group ranges, are used by badgers from neighbouring setts (and more often by males), while hinterland latrines within sett borders are used predominantly by group members (and more often by females and cubs; Buesching *et al.*, 2016a). Badgers spend, on average, about 23 seconds investigating latrine deposits; about 54% of that time is spent sniffing while 16% of that time is spent crouching during defecation or subcaudal marking (Stewart *et al.*, 2002). Latrines can contain a mixture of SGS, faeces, anal gland secretion, and urine (Buesching & Jordan, 2019). However, in regards to

urine, studies conducted across various counties in southern England found that badgers living in lower-density populations deposit more urine in contexts away from latrines (Hutchings *et al.,* 2002) and that about 48% of urination events occur outside latrines (White *et al.,* 1993). Furthermore, females and cubs appeared to urinate at a wider diversity of locations, perhaps more randomly positioned, compared to males (White *et al.,* 1993).

Overall, the spatial and temporal distribution of SGS appears to be more purposeful, allowing more reliable opportunities for conspecifics to investigate SGS marks. In contrast, the spatial and temporal distribution of urine appears relatively indiscriminate, thus making conspecific encounters with urine marks less dependable.

ii) <u>'To be read': Assessing the chemical composition of substances used for scent-marking</u>

SGS has a margarine-like texture, and its coloration varies between pale white to dark brown (Buesching *et al.,* 2002a). Water comprises around 41-88% of the composition (Allen *et al.,* in prep. c), while proteins as well as short- and long-chained fatty acids comprise the rest (Buesching *et al.,* 2002b; Buesching *et al.,* 2002c). Gas chromatography-mass spectrometry (GC-MS) analyses identified 110 different compounds within SGS, 21 of which were found in all samples (Buesching *et al.,* 2002b; Buesching *et al.,* 2002c). These GC-MS profiles indicated significant differences in SGS composition based on individual identity, sex, season, age, body condition, reproductive status, and social group membership (Buesching *et al.,* 2002b; Buesching *et al.,* 2002c). Individual profiles remained relatively consistent throughout the year (Buesching *et al.,* 2002b; Buesching *et al.,* 2002c).

Because SGS contains a considerable amount of proteins and fatty acids, the synthesis of this secretion has been considered energetically costly (e.g., protein synthesis; Pontes *et al.,* 2015). Recent work (Allen *et al.,* in prep, c) testing the use of bomb calorimetry in quantifying the minimum energetic investment of SGS (not accounting for the additional biosynthesis costs; Bauman *et al.,* 2006; Pontes *et al.,* 2015) calculated four energetic metrics: the calorific value of freeze-dried secretion (CV_{dry}; Joules/$gram_{secretion}$), the calorific value of natural secretion ($CV_{natural}$; Joules/$ml_{secretion}$), the calorific value of the total secretion stored in the subcaudal pouch (CV_{pouch}; Joules), and the calorific value of each scent mark ($CV_{scentmark}$; Joules). Similar to the aforementioned GC-MS variations, these four metrics significantly varied with season, sex, body condition, and reproductive life stage (as indicated by age).

GC-MS analyses have also been performed on urine samples from a wild badger population in Woodchester Park, Gloucestershire (Service *et al.,* 2001). This work identified 300 volatile organic compounds (VOCs) and analysed 33 common GC-MS peaks, mostly containing aldehydes, ketones, aromatics, and pyrazines. GC-MS profiles displayed prominent seasonal trends (presumably associated with diet) with sex-specific trends more evident during summer and autumn (Service *et al.,* 2001). Within our Wytham Woods population, seasonal trends in urine composition were observed via enzyme immunoassays (EIAs) measuring sex steroid metabolites (Sugianto *et al.,* submitted). Population-wide analyses showed correlations between sex steroid metabolite (testosterone and oestrogen) levels within urine and circulating plasma sex steroid levels which corresponded with seasonal reproductive patterns. A similar correlation was observed on an individual-level in males, but not in females. Furthermore, GC-MS analyses found that urine from reproductively active males and oestrous females had different VOC

compositions than urine from cubs and anoestrous females; meanwhile, VOC composition significantly varied between the sexes (Tinnesand *et al.,* unpublished data).

Overall, both SGS and urine encode similar information regarding season, sex, and reproductive condition. However, unlike urine, SGS encodes supplementary information about individual identity, body condition, and social group membership alongside whatever additional information that SGS energetic content may convey.

iii) ‘To be believed’: Assessing the biological relevance of the chemical composition

Although numerous methods can assess the chemical variations in scent-marking substances, analysing conspecific responses towards these variations requires a different method – scent-provisioning experiments. By presenting individuals with scent-marking substances and measuring their behavioural responses (e.g., response likelihood, countermarking frequency/likelihood, sniffing behaviour, avoidance behaviour), we can determine the biological significance of these chemical compositions and their encoded information.

One scent-provisioning experiment (Allen *et al.,* submitted) presented badgers with SGS paired according to energy content (while controlling for sex, familiarity, and sample collection date). While encoded information regarding body condition did not significantly affect how badgers responded to SGS samples, badgers did alter their behaviour (response likelihood, sniffing duration, and countermarking likelihood) based on encoded information regarding sex and age. In particular, badgers responded more often towards low-energy SGS samples from older (8+ years old) donors and high-energy SGS samples from young (2-7 years old) donors while spending a longer proportion of time sniffing male SGS samples compared to female SGS. Furthermore, badgers countermarked high-energy SGS samples from same-sex donors more than

they countermarked low-energy SGS samples from same-sex donors. However, regardless of energetic content, countermarking rates towards SGS samples from opposite-sex donors showed no difference.

Similar scent-provisioning experiments (Allen *et al.,* 2019; Allen *et al.,* in prep., a.; Allen *et al.,* in prep., b) indicated that encoded information within urine also carries biological relevance to badgers. For example, sex steroid metabolite levels significantly influenced sniffing duration with adult males spending a longer time sniffing female urine with lower oestradiol levels (Allen *et al.,* 2019). Furthermore, seasonal variations in chemical composition (Service *et al.,* 2001) likely played a factor in sniffing behaviour – in this case, sniffing likelihood – as badgers were less likely to sniff conspecific urine during spring (Allen *et al.,* in prep., b). Badgers also altered their behaviour based on encoded sex- and age-specific information within urine. In particular, a pilot study indicated conspecifics sniffed cub urine longer than adult urine, and that adult badgers countermarked male urine more often than female urine (Allen *et al.,* 2019).

Overall, scent-provisioning experiments indicate that encoded information regarding sex and age are biologically relevant in both SGS and urine. Seasonal information within urine also appears to have biological relevance, but no direct comparison between this and seasonal information within SGS can be made as season-specific responses towards SGS have not been analysed yet. Moreover, the energetic content within SGS appears to have biological relevance, but future work is needed to fully ascertain what this relevance is.

iv) ‘To be remembered’: Factors influencing olfactory signal memorability and discernment

Microbial communities within subcaudal pouches metabolise primary gland products into secondary odorants (Ezenwa & Williams, 2014; Sin *et al.,* 2012). The resulting signal may have attributes that have been selected for memorability and discriminability. Subcaudal pouches mostly contain Actinobacteria and Firmicutes (mainly found in cubs) along with numerous other – but less abundant – bacterial species (Sin *et al.,* 2012). These microbial communities vary between social groups (Buesching *et al.,* 2016b) as well as between adults and cubs (Sin *et al.,* 2012). Because these microbes metabolise primary gland products such as long-chained fatty acids, the group- and age-related variations in microbial communities directly relate to chemical composition variations within SGS (Buesching *et al.,* 2002b; Buesching *et al.,* 2002c), thereby allowing badgers to discern between conspecifics based on familiarity and age. Energetic content, when combined with these aforementioned variations, appears also to be a discriminable characteristic in SGS; this is evidenced by the significant influence that the interactions between energetic content and donor age group as well as between energetic content and donor sex had on behavioural responses (Allen *et al.,* submitted).

Because subcaudal pouches are specific to only the genera *Meles* and *Arctonyx*, SGS provides a genus-specific signal unlike urine. However, badgers were able to discern between urine mixtures from different species (badgers, domestic dogs, horses, vegetarian humans, non-vegetarian humans, and children; Allen *et al.,* in prep., a). In particular, badgers displayed reduced countermarking around urine mixtures from domestic dogs and non-vegetarian humans, prompting the hypothesis that this is because scents of these two provenances are aversive. Badgers also spent a shorter proportion of time sniffing the urine from these two sources. Possible VOC composition variation relating to the protein-heavy diets (Nolte *et al.,* 1994) consumed by these urine donors (Allen *et al.,* in prep., a) explains how badgers were able to

make this discrimination. With regards to badger urine, discrimination between conspecifics based on social group membership, donor sex, and donor age (Allen *et al.,* 2019; Allen *et al.,* in prep., b) can also be attributed to relevant compositional differences (as mentioned in previous sections). However, another factor comes from the fact that setts can remain active for generations. This results from two interconnected factors: space availability for the construction of new setts (Macdonald *et al.,* 2004), and limited dispersal from natal setts (Macdonald *et al.,* 2008). Because group ranges and the latrine-marked intersection between them can remain stable for years, this gives badgers the opportunity to learn individual- and group-specific odours; thus, allowing them the ability to discriminate between familiar and unfamiliar individuals and adjust their behaviour accordingly ('Dear Enemy Phenomenon'; Fisher, 1954).

Overall, both SGS and urine appear equally effective in terms of memorability and discriminability, particularly when combined with the long-term stability of group ranges and latrine-marked intersections.

v) 'To be acted upon': Behavioural responses towards conspecific scent marks

As previously mentioned, badgers modify their behavioural responses based on the energetic content within SGS and encoded information regarding sex and age (Allen *et al.,* submitted). Responder-related traits (age group, sex) alone did not influence how badgers responded towards SGS samples. Instead, conspecific behaviour was more influenced by donor-related traits (age group, sex) as well as the interaction between donor and responder traits (relative sex; results detailed in previous sections).

Donor-related traits (familiarity, age group, sex, and sexual maturity) and interactions between donor and responder traits exhibited similar effects on conspecific responses to urine

samples (Allen *et al.*, 2019; Allen *et al.*, in prep., b). However, in contrast to the findings of the scent-provisioning experiments conducted with SGS, responder-related traits alone also exhibited a significant effect with respect to urine. In particular, adult females responded more often towards urine samples than did adult males (Allen *et al.*, 2019; Allen *et al.*, in prep., b), while reproductively senescent badgers countermarked urine samples less often than did sexually immature and reproductively active responders (Allen *et al.*, in prep., b).

Overall, conspecific responses towards SGS and urine marks both appear relatively complicated in that interactions between various traits (donor-related characteristics and responder-related characteristics) influence how badgers respond. However, with SGS, the energetic content of the scent-marking substance and its interactions with donor/responder traits provides another layer of complexity.

Conclusion and future directions

Both subcaudal gland secretion (SGS) and urine are 'cheat-proof' (i.e., an 'index'; Wyatt, 2014), unambiguous signals which encode biologically relevant information that conspecifics can detect and use to adjust their behaviour accordingly. Nevertheless, badgers appear to utilise SGS as their preferred primary source for conspecific-related information based on i) the more purposeful distribution of SGS, ii) the encoding of information not yet found in urine, iii) the relatively higher energetic investment in producing SGS, and iv) the effects that this energetic investment has on conspecific responses. Meanwhile, urine appears to function in a subsidiary role, providing a method for safeguarding information and for increasing the chances that conspecifics will encounter and investigate such information.

Future work could usefully focus on longitudinal studies regarding how investment in olfactory advertisement (e.g., energetic content within SGS and scent-marking rates) is associated with individual life-history trajectories, especially in regards to reproductive success and residual reproductive lifespan. Such work could be undertaken in conjunction with research into how conspecific responses towards particular scent marks correspond with mating preferences. Additionally, analysing the use of specialised glandular secretions versus metabolic by-products can shed light on the spatial and physiological factors that are necessary for individuals within group-living species to mediate various social interactions (e.g., mate selection, territorial/resource competition) that influence individual fitness and, ultimately, population processes.

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