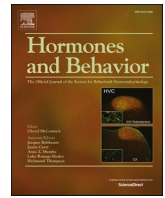


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Endocrine changes related to dog domestication: Comparing urinary cortisol and oxytocin in hand-raised, pack-living dogs and wolves

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ABSTRACT

Dogs are exceptionally well adapted to life close to humans, and alterations in their endocrine system during the domestication process may be an underlying mechanism. In particular, it has been suggested that low circulating cortisol concentrations in conjunction with simultaneously high oxytocin concentrations may have resulted in dogs' increased docility ('selection for tameness' hypothesis) and heightened propensity to interact and form relationships with humans ('hypersociability' hypothesis) compared to wolves. To investigate this, we analyzed cortisol and oxytocin metabolite concentrations from urine samples of hand-raised, pack-living domestic dogs and their non-domestic relatives, grey wolves. Based on the hypotheses outlined above, we predicted lower cortisol but higher oxytocin concentrations in dogs than wolves. In contrast to our prediction, we found higher cortisol concentrations in dogs than wolves. However, oxytocin concentrations were higher in dogs compared to wolves although the effect was relatively small. Indeed, male dogs had the highest oxytocin concentrations while female dogs' oxytocin concentrations were comparable to wolves'. Feeding status, reproductive phase, and conspecific social interactions also significantly affected cortisol and oxytocin concentrations. Furthermore, we compared two methods of correcting for variable water content of urine samples. We discuss our results in light of physiological and behavioral changes during domestication and highlight the importance of accounting for confounding variables in future studies.

1. Introduction

Animal domestication is a process comprising dramatic changes in morphology, physiology, and behavior ('domestication syndrome'; Wilkins et al., 2014). Domesticated animals show reduced fear responses and increased inclination to approach humans, in contrast to their wild-type, non-domestic counterparts (Belyaev, 1969; Trut et al., 2009; Wilkins et al., 2014). Changes in their endocrine profiles, in particular, dampened reactivity of the hypothalamo-pituitary-adrenal (HPA) axis and heightened oxytocinergic system activity, have been suggested as important underlying mechanisms (Künzl and Sachser, 1999; Zipser et al., 2014; Kaiser et al., 2015; Buttner, 2016; Herbeck et al., 2017). HPA axis activation results in glucocorticoid (GC) hormone release which physiologically prepares individuals to cope with challenging

conditions (McEwen and Wingfield, 2003). Oxytocin (OT) is a neuropeptide hormone that facilitates social approach and bonding (Young and Wang, 2004), and enhances the salience of social cues to promote context-appropriate behavioral responses (Oliva et al., 2015; Shamay-Tsoory and Abu-Akel, 2016). OT reduces fear and anxiety particularly in social contexts (Smith and Wang, 2014) by down-regulating parts of the HPA axis (Jurek et al., 2015; Winter and Jurek, 2019), and OT administration results in lowered circulating GC levels during physical and social challenges (Linnen et al., 2012; Cardoso et al., 2013).

Hormonal correlates of animal domestication have been researched in the past mainly using two slightly different approaches: 1) Comparisons of animals selected for tameness (i.e. reduced fear of humans, mimicking the behavioral phenotype of domesticated animals) and control groups (randomly selected or selected for increased fear and

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aggression towards humans) and 2) Comparisons of domesticated species and their wild-type, non-domestic counterparts (see Table 1 for an overview). To summarize evidence from the first line of research, in some species, individuals selected for tameness had lower basal GC concentrations and decreased GC reactivity than their aggressive or randomly selected counterparts (Albert et al., 2008; Trut et al., 2009) but in other species, no differences were found (Agnvall et al., 2015). Regarding the OT system, tame silver foxes had a higher number of OT neurons in the hypothalamus than farm-bred foxes (selected for fur quality) and foxes selectively bred for aggression towards humans (Herbeck et al., 2019), but to our best knowledge nothing is published on circulating OT levels in these animals yet. As regards the second line of research, comparing domesticated species to their wild-type forebears, baseline GC levels did not differ between the domestic and the wild form in several species (Künzl and Sachser, 1999; Künzl et al.,

2003; Ericsson et al., 2014; Fallahsharoudi et al., 2015) but in others, either the domestic (Martin, 1978) or the wild-type (Suzuki et al., 2012) had higher baseline GC levels. Thus, the available evidence regarding the role of the GC system in animal domestication is inconclusive. In relation to the OT system, a handful of genetic studies found evidence of selective pressure on the OT system over the course of domestication in several species (Oliva et al., 2016; Ruan and Zhang, 2016; vonHoldt et al., 2017; Fam et al., 2018; Herbeck et al., 2019) but further research on the role of OT during domestication is needed.

Particularly interesting models to study the domestication process are wolves and domestic dogs. It has been suggested that dogs' (compared to wolves') reduced fearfulness ('selection for tameness' hypothesis; Albert et al., 2008; Trut et al., 2009) and increased sociability towards humans ('hypersociability' hypothesis; vonHoldt et al., 2017) is facilitated by changes in their OT and GC systems (Buttner, 2016; Herbeck et al., 2017; Herbeck and Gulevich, 2018; Kikusui et al., 2019) however, comparative endocrine studies are sparse. Some data are available from early ecological field studies: McLeod et al. (1996) found a relationship between urinary glucocorticoid metabolite (uGCM) concentrations and dominance status in a captive pack of wolves, and noted that the uGCM concentrations observed appeared similar to those found in other studies on free-living wolves and pet dogs. Seal and Mech (1983) report serum GC concentrations of captive grey wolves similar to those of dogs. However, a more recent study compared human-socialized, pack living dogs' and wolves' behavioral and physiological reactions to positive reinforcement training and found that while both species' salivary glucocorticoid metabolite (GCM) concentrations dropped significantly during the training session, dogs' GCM concentrations were twice as high as wolves', both before and after the training (Vasconcellos da Silva et al., 2016), contradicting the idea of generally decreased GC system activity in domesticated animals.

As regards the OT system, a few studies have investigated differences between wolves and dogs on the genetic level: Oliva et al. (2016) found changes in dogs' OT receptor (OTR) genes compared to wolves' and vonHoldt et al. (2017) described a variation in the dog genome orthologous to humans affected by a condition characterized by reduced social anxiety and heightened OT system activity (Dai et al., 2012; Procyshyn et al., 2017), absent in the wolf genome. However, to date, only one study compared peripheral oxytocin metabolite (OTM) concentrations of dogs and wolves. Nagasawa et al. (2015) found an increase in urinary OTM (uOTM) concentrations after an interaction with a known human partner in a subset of pet dogs but not in wolves. The authors also reported pre-test uOTM concentrations three times higher in the wolves than dogs. While it is unclear if wolves' high, pre-test levels were caused by the experimental protocol and/or different socialization procedures of the dogs and wolves used in the study (see Fiset and Plourde, 2015, and Kekecs et al., 2016, for commentaries), or indeed accurately reflect baseline values, they are in contrast with the expected effect of domestication on OTM levels.

Conducting comparative endocrine studies between species requires careful consideration of confounding variables. Both the GC and OT system are involved in a number of physiological processes in which they fulfil roles that may differ with sex, metabolic state, reproductive phase, early-life experiences, and which can be affected by internal and environmental stimuli, both of social and non-social in nature, i.e., interactions with conspecifics or daytime (Gimpl and Fahrenholz, 2001; Sapolsky, 2002). For example, males and females may differ in their basal GC and OT concentrations in some but not all species and reports of such differences vary from study to study (dogs: Stephen and Ledger, 2006; Mongillo et al., 2014; MacLean et al., 2017; rodents: Kramer et al., 2004; humans: Weisman et al., 2013). Sex thus should always be considered when comparing circulating GC and OT levels in different species. In relation to this, researchers need to account for differences in gonadal steroid concentrations, which can affect GC and OT system activity (McLeod et al., 1996; Snowdon et al., 2010), and may fluctuate across sampling time in one species, but not the other (i.e., when lengths

Table 1

Overview of previous studies on the glucocorticoid (GC) and oxytocin (OT) systems in the context of animal domestication.

Study species	Basal GC/OT levels	GC/OT reactivity	Reference
<i>Selectively bred for tameness vs. control group</i>			
Rat	GC: Tame < aggressive	GC: Tame < aggressive	Albert et al., 2008
Silver fox	GC: Tame < control (selected for fur quality)	GC: Tame < control (selected for fur quality)	Trut et al., 2009
Red Jungle Fowl	–	GC: Low fear = high fear	Agnvall et al., 2015
<i>Domesticated vs. wild-type, non-domesticated relatives</i>			
Bengalese finch, White-backed munia	GC: Domesticated < wild-type	GC: Domesticated = wild-type	Suzuki et al., 2012
Domestic chicken, Red Jungle Fowl	GC: Domesticated = wild-type	GC: Domesticated < wild-type	Ericsson et al., 2014; Fallahsharoudi et al., 2015
Mallard, Pekin duck	GC: Domesticated > wild-type	GC: Domesticated < wild-type	Martin, 1978
Guinea pig, cavy	GC: Domesticated = wild-type	GC: Domesticated < wild-type	Künzl and Sachser, 1999; Künzl et al., 2003
Domestic dog, grey wolf	GC: Domesticated = wild-type	GC: Domesticated = wild-type	Seal and Mech, 1983
	GC: Domesticated = wild-type	GC: Domesticated = wild-type	McLeod et al., 1996
	OT: Domesticated < wild-type	OT: Domesticated > wild-type	Nagasawa et al., 2015
	GC: Domesticated > wild-type	GC: Domesticated = wild-type	Vasconcellos et al., 2016
<i>Additional evidence for oxytocin system involvement during animal domestication</i>			
Mice, Rats	Lab mice and rats have higher density of OT reactive neurons in certain brain areas than wild-type mice and rats.		Ruan and Zhang, 2016
Multiple domestic and wild species	Selective pressure on several genes of the OT/AVP ^a system in domesticated versus wild-type species.		Fam et al., 2018
Domestic dog, grey wolf	Differences in OTR ^b genes between wolves and dogs.		Oliva et al., 2016
Domestic dog, grey wolf	Structural variants in dogs' genome similar to Williams-Beuren-Syndrome in humans, a condition known for heightened OT system activity and 'hypersociability'.		vonHoldt et al., 2017
Silver fox	Higher number of OT reactive neurons in the hypothalamus of foxes selectively bred for tameness than randomly selected and aggressive foxes.		Herbeck et al., 2019

^a AVP = Arginine vasopressin.

^b OTR = Oxytocin receptor.

and frequencies of reproductive cycles differ). Grey wolves are strictly seasonal breeders with one estrus cycle per year, whereas domestic bitches have evolved the capacity for multiple estrus cycles per year, with considerable variation between and within individuals, and male dogs are constantly able to reproduce (Lord et al., 2013). To account for such differences, an individual's reproductive status should be monitored throughout the sampling period.

Similarly, species specific differences in social structure and mating systems (e.g., monogamous versus polygamous species) can also affect hormonal levels at a given time point. Indeed, OT's role in monogamous pair bonding is widely recognized (Insel et al., 1998; Carter and Keverne, 2002; Young and Wang, 2004) and species differences in plasma OT concentrations according to their mating system have been described (Kramer et al., 2004: monogamous female voles had higher plasma OT concentrations than female rats that do not pair bond). In accordance, a recent review (Kikusui et al., 2019) suggests that wolves may have higher basal OT concentrations than dogs because their mating system relies on monogamous pair bonds in contrast to the promiscuous dogs. Wolves, like most wild canids, form family packs and breed cooperatively, whereas dogs do not usually show alloparenting behavior (Lord et al., 2013; but see Paul and Bhadra, 2018). Free-ranging dogs may live solitarily, but many form packs of two or more (2–4: Daniels and Bekoff, 1989; 2–5: Krauze-Gryz and Gryz, 2014; 4–11: Bonanni et al., 2011) and even up to 42 individuals (Cafazzo et al., 2014), with a linear, hierarchical social structure (Cafazzo et al., 2014; Bonanni et al., 2017). The relationship between GC concentrations and cooperative breeding has received much attention. Elevated GC concentrations were reported in dominant compared to subordinate grey wolves (Sands and Creel, 2004), male Ethiopian wolves (Van Kesteren et al., 2012), and African wild dogs (Creel et al., 1997), but to date no such studies are published on (free-ranging) dogs.

Furthermore, early-life history and previous social experiences can impact GC and OT system activity later in life. Nursery-reared as compared to parent-reared macaques still have lower basal OT concentrations (measured in cerebrospinal fluid) at 3 years of age (Winslow et al., 2003) and living in a shelter compared to a private household correlates with increased basal salivary GCM concentrations in dogs (Sandri et al., 2015). Comparisons of pet dogs living with their human families from an early age and wolves housed in enclosures with considerably less human contact may thus be inherently biased (Fiset and Plourde, 2015; Kekecs et al., 2016). Other factors that potentially influence GC and OT concentrations include feeding (Mitsui et al., 2011; Aulinas et al., 2019), locomotor activity (Mitsui et al., 2011), body weight (Sandri et al., 2015; Lawson et al., 2020), and sampling time. Diurnal patterns of GC release have been described in some dog populations but not others (Beerda et al., 1996; Kolevská et al., 2003). OT is not known to follow a diurnal pattern, but previous research described light-dependent release in laboratory rodents (Devarajan and Rusak, 2004). Both GC and OT concentrations have also been associated with conspecific social interactions of affiliative (Crockford et al., 2013) and agonistic (Samuni et al., 2017) nature, which need to be taken into account.

Finally, sample collection and analysis require some thought. Both GCM and OTM can be measured in dog and wolf urine (Zeugswetter et al., 2013; Schaeb's et al., 2019). A major advantage of urine samples is their non-invasive (sample collection does not require physical restraint) and integrative nature: rather than depicting one point in time, they reflect a time period with events happening 30–90 min before sampling considered to affect subsequent urinary GCM and OTM concentrations (Beerda et al., 1996; Schatz and Palme, 2001; Seltzer and Ziegler, 2007; Mitsui et al., 2011; Crockford et al., 2013). Urinary hormone concentrations need to be corrected for urine concentration to control for varying amounts of water in the samples (White et al., 2010). This is usually done by measuring urinary creatinine (crea mg/ml) concentrations or specific gravity (SG) of each sample. Creatinine concentrations, however, can be affected by diet, sex, age, muscle mass and activity,

sampling time, and technical procedures such as repeated freeze-thaw cycles (summarized in White et al., 2010). Additionally, variations in food intake, in particular occurrence of fasting, are associated with fluctuation in urinary creatinine concentrations (Delgiudice et al., 1987; Darnell et al., 2005). SG, on the other hand, defined as the ratio of the density of the urine sample to that of distilled water, is more robust to dietary effects and considered a practical alternative (Miller et al., 2004; Anestis et al., 2009; White et al., 2010; Wauters et al., 2018). Thus, the latter method may be more appropriate when comparing species or populations with varying feeding regimes.

In the current study, we compared unstimulated, urinary GCM and OTM concentrations of pack-living dogs and wolves with similar experiences with humans. Based on the 'reduced fear and increased tameness towards humans'-hypothesis of domestication (Albert et al., 2008; Trut et al., 2009), we expected to find lower GCM concentrations in domestic dogs compared to wolves. However, given contrasting evidence from other studies (Seal and Mech, 1983; Vasconcellos da Silva et al., 2016), domestic dogs might have higher (or similar) GCM concentrations than wolves. Based on the 'hypersociability' hypothesis (Bentosela et al., 2016; vonHoldt et al., 2017) and previous genetic work suggesting an increase in OT system activity during domestication (Ruan and Zhang, 2016; vonHoldt et al., 2017; Fam et al., 2018), we predicted we would find higher urinary OTM concentrations in dogs than wolves. Additionally, based on the dampening effect of OT system activity on the HPA axis (Cardoso et al., 2013; Jurek et al., 2015), we expected to see a negative correlation between circulating GCM and OTM concentrations (high OTM in conjunction with low GCM).

2. Material and methods

2.1. Study site and animals

We sampled 25 adult animals (11 grey wolves: 6 males, 5 females; 14 mongrel dogs: 7 males, 7 females; details in Table 1, SI) housed in outdoor enclosures at the Wolf Science Center (WSC), Austria. All individuals were hand-raised by animal professionals at the WSC from 10 days of age until 5 months old (see Range and Virányi, 2014, for more details on their upbringing) and then integrated into existing packs. The wolves were kept in conspecific dyads ($N = 5$) or packs of 3 animals ($N = 2$). The dogs were kept in conspecific dyads ($N = 4$) or packs of 3–4 animals ($N = 3$). At the time of first sampling for the current study, the animals were between 2 and 9 years old. Ages ranged from 2 to 9 (mean (SD) 6.1 (2.7)) years in the wolves and 3 to 8 (mean (SD) 5.6 (2.2)) years in the dogs. There was no significant difference between wolf and dog age in our sample population ($t = 0.45$, $df = 19.14$, p -value = 0.66). All females were hormonally intact. Males were vasectomized (at the age of 6 months) and thus hormonally intact at the time of sampling. The dogs were provisioned daily with commercially available dry dog food, while the wolves were fed raw meat and carcasses of deer, rabbit, chicken, or beef, 3–4 times a week (resulting in 1–2 fasting days in between feedings). All animals had ad libitum access to drinking water in their home enclosures.

2.2. Behavioral data collection

To ensure samples represented unstimulated measures of the animals' urinary GCM and OTM concentrations, all animal keepers, trainers, and other staff members were instructed not to interact with, or clean enclosures of the focal pack during at least 2 h before sample collection. To record social interactions within the packs, all focal individuals were observed and filmed for 60 min before sample collection. This time window was chosen because urine samples provide an integrated measure over approximately 45–60 min for OTM (Mitsui et al., 2011) and 60–90 min for GCM (Beerda et al., 1996; for additional biological validation of the immunoassay used for this study, see SI) in dogs. Durations of locomotor activities, resting, and social interactions

(affiliative and agonistic) were subsequently coded from video (Solomon coding software, Version beta 17.03.22, copyright András Péter) (ethogram; Table 2, SI).

2.3. Urine sample collection

Samples of spontaneously voided urine were taken non-invasively during leashed walks using an expandable metal stick with a plastic cup attached to it (Fig. 1 a - b). All animals were habituated to this procedure beforehand and showed no signs of distress while donating urine. Within 15 min of urine collection, samples were split into a maximum of four 1 ml aliquots and transferred by pipette into 2 ml cryotubes. 100 μ l of 0.1% phosphoric acid per 1 ml sample volume was added to the first two aliquots to acidify and thus prevent OT degradation in the samples (Ziegler, 2018; Schaebs et al., 2019). Whenever enough sample volume was available, two aliquots were kept for creatinine and uGCM assessment. All samples were frozen at -20°C until extraction.

We collected urine samples of males and females during the non-heat period (anestrus and diestrus; assessed behaviorally by the animal keepers and visually during regular veterinary checks) to minimize the effect of fluctuating gonadal steroids (i.e. rising levels of estrogen and testosterone (T)). The wolf breeding season takes place from late December to the beginning of March in our population; hence wolf samples were collected from late March to early December only. Dog samples were collected throughout the year, but heat (proestrus and estrus) phases were avoided. As we sampled during anestrus and diestrus, we included reproductive phase as a factor with two levels into the

statistical models to control for subtle effects. All samples were collected between 7:00 am and 5:00 pm and sample time was included into the statistical models.

A total of 109 samples (52 dog and 57 wolf samples; mean number of samples per individual 4.4 (wolves: 5.2, dogs: 3.7)) of both sexes, collected on different days over the course of 2 years (2017–2019), were used for statistical analyses of uOTM concentrations. Thirteen of those samples could not be used for simultaneous uGCM measurement due to low sample volume resulting in a total of 96 samples (49 dog and 47 wolf samples; mean number of samples per individual 3.8 (wolves: 4.3, dogs: 3.5) for statistical analyses of uGCM concentrations and the correlation analyses of uGCM and uOTM concentrations.

2.4. Ethics statement

Urine sample collection was approved by the institutional ethics and animal welfare committee in accordance with Good Scientific Practice (GSP) guidelines and national legislation (approval number ETK-05/03/2017, University of Veterinary Medicine Vienna).

2.5. Extraction and hormone analyses

Solid-phase extractions (SPE; following the protocol described in Schaebs et al., 2019) for OT and diethyl-ether extractions for GC (Zeugswetter et al., 2013) were performed. Briefly, for OT extraction, samples were thawed, gently vortexed for 10 s (sec), and centrifuged (365g; 1 min; 4°C). SPE cartridges (Chromabond HR-X, 30 mg, 1 ml, Macherey-Nagel, Dueren, Germany) were conditioned with 1 ml methanol (100%, HPLC grade) followed by 1 ml HPLC water on a vacuum chamber (Chromabox, Macherey-Nagel). Cartridges were then loaded with 0.5 ml of the urine sample and diluted with 0.5 ml buffer solution (water, 0.1% trifluoroacetic acid (TFA)). The following washing step entailed adding 5 ml wash buffer (10% (vol/vol) acetonitrile (ACN) containing 1% TFA in water) to each cartridge. Afterwards, cartridges were sucked dry using the vacuum pump and samples were eluted with 1 ml 80% (vol/vol) ACN into clean glass tubes. Finally, eluted samples were evaporated until completely dry, at 50°C for 35 min using a gentle stream of compressed air, and reconstituted in 0.3 ml 100% ethanol. They were then capped, sealed, and stored at -20°C until shipment on dry ice to the Max-Planck-Institute for Evolutionary Anthropology, Leipzig, Germany, for analysis.

For GC extraction, samples were thawed and vortexed for 10 s before pipetting 0.5 ml of each sample into a clean 10 ml glass tube. To each tube, 5 ml diethyl-ether was added, vortexed again, and centrifuged (2,500g; 15 min; room temperature). Tubes were capped and frozen at -20°C for at least 3 h or overnight. Next, the supernatant organic phase was transferred into a new glass tube and evaporated until completely dry at 60°C for approximately 45 min using a gentle stream of compressed air. A total of 0.5 ml enzyme immunoassay (EIA) buffer (pH 7.5) was added to each tube, which were left uncapped at room temperature for 5–10 min and then vortexed until no residue was visible anymore. Tubes were capped, sealed, and stored at -20°C until analysis at the Unit of Physiology, Pathophysiology and Experimental Endocrinology, University of Veterinary Medicine, Vienna, Austria.

In order to measure uOTM concentrations, we analytically and physiologically validated (Wirobski et al., 2020) a commercially available EIA kit (Arbor Assays, Ann Arbor, Cat. No: K048-H5). The assay standard curve ranged from 16.38 to 10,000 pg/ml. Assay sensitivity was 17.0 pg/ml. Intra-assay coefficients of variation (CV) of high and low concentration quality controls (QCs) were 8.0% (high) and 16.6% (low). Inter-assay CVs were 12.2% (high) and 19.7% (low). For uGCM measurement, we used an in-house cortisol EIA (Zeugswetter et al., 2013; antibody B), with an antibody produced against cortisol-21-HS: BSA. We biologically validated the EIA for our purpose (see SI). The assay standard curve ranged from 2 to 200 pg/well. Assay sensitivity was 2 pg/well. Intra- and interassay CV were 5.3% and 7.5%,



Fig. 1. a - b: Non-invasive urine sampling of a dog (a) and a wolf (b) during a leash walk.

respectively. All samples were measured in duplicates and repeated if optical density (OD) values differed more than 10%.

2.6. Comparison of urinary specific gravity and creatinine

We measured specific gravity (SG) of each sample with a digital refractometer (TEC++, serial no. T6017) and urinary creatinine (crea) on a microtiter plate by means of the Jaffe reaction (Bahr et al., 2000). The SG correction formula was identical to the one used in Miller et al. (2004). SG corrected hormone concentrations are expressed as uOTM pg/ml SG and uGCM ng/ml SG, and crea corrected concentrations as uOTM pg/mg crea and uGCM ng/mg crea.

2.7. Statistical data analyses

2.7.1. Behavioral data analysis

Wolves and dogs were observed for an average of 60 min (range: 35–65 min) before being taken on urine collection walks. Out of the total observation time, wolves were in sight, i.e. visible on video, for an average of 46 min (range: 2.4–91 min) and dogs for 52 min (range: 3.3–65 min). Thus, all behaviors used for further statistical analyses were normalized for the total time the animal was in sight. To investigate whether wolves and dogs differed behaviorally, we fitted two mixed models with beta error structures (package glmTMBB; Brooks et al., 2017). The response variables were the normalized durations (i.e., proportions of total time in sight) of locomotor activity and social behaviors. Social behaviors were grouped into affiliative (comprising grooming, playing, social sniffing, body contact, greeting) and agonistic (comprising threatening, chasing, fighting) interactions (see Table 2, SI, for the ethogram). In the models, species was the test predictor and sex was included as a control predictor. Subject and pack were added as random intercept effects to control for repeated sampling and account for variation within packs. Full models were compared with a null model (Forstmeier and Schielzeth, 2011) lacking the test predictor ‘species’ but comprising the control predictors and complete random effects structure using a likelihood ratio test (Dobson, 2002). Collinearity was assessed using the function vif of the package car (version 3.0-0), applied to a model lacking the random effects, revealing no higher values than 1.0 (for ‘species’ and ‘sex’). The model for affiliative interactions was overdispersed (dispersion parameter 6.9). Overdispersion may result in distorted estimated standard errors and test statistics but given the full-null model comparison revealed non-significance ($\chi^2 = 0.04$, $df = 1$, $P =$

0.84), no further inferences were made from this model output.

2.7.2. Comparison of urinary specific gravity and creatinine

We plotted the effect of feeding on urinary creatinine and SG (Fig. 2 a-b) and fitted two linear mixed models (LMMs; Baayen, 2008) with Gaussian error structures and random effect of subject to investigate whether values differed between fasted and fed wolves. Finally, to check whether the two correction methods resulted in correlating within-hormone concentrations, we used the R package rmcrr (version 0.3.1; Bakdash and Marusich, 2017).

2.7.3. Urinary glucocorticoid and oxytocin metabolites

To investigate whether dogs and wolves have different unstimulated uGCM and uOTM concentrations, we fitted two separate LMMs (Baayen, 2008) with Gaussian error structure. The response variables (uGCM ng/ml SG and uOTM pg/ml SG) were log transformed to obtain normally distributed and homogenous residuals. We included the sex by species interaction since in some, but not all species, uGCM and uOTM concentrations may be affected by sex, and to investigate whether the domestication process has altered male and female endocrine profiles differently. Feeding status (i.e., factor ‘fed the day before’ with two levels ‘yes’ and ‘no’) and reproductive phase (factor with two levels ‘anestrus’ and ‘diestrus’) were accounted for in the models. In addition, we included ‘sample time’ (as a continuous co-variate) to account for daytime, and the normalized durations of locomotion, as well as affiliative and agonistic social interactions within the packs during the observation period. To investigate a potential effect of body weight on uGCM concentrations, we fitted a third model comprising all predictors mentioned above including body weight (in kg) as an additional covariate. All co-variates were z-transformed prior to model fitting to facilitate interpretation. Random effects of subject, pack, and assay plate were included to control for repeated sampling of the same individuals and to account for variation within packs and plates. To keep type I error rate at 5%, we included all theoretically identifiable random slopes components (Schielzeth and Forstmeier, 2009; Barr, 2013) which were manually dummy coded and centered (for reference levels – see Tables 2 and 3). We further compared the full models with a null model (Forstmeier and Schielzeth, 2011) lacking the test predictor ‘species’ but comprising the control predictors and complete random effects structure using a likelihood ratio test (Dobson, 2002). In case the interaction term (‘species’ and ‘sex’) did not reveal significance, a reduced model lacking the interaction but comprising both main effect terms was fitted. Model

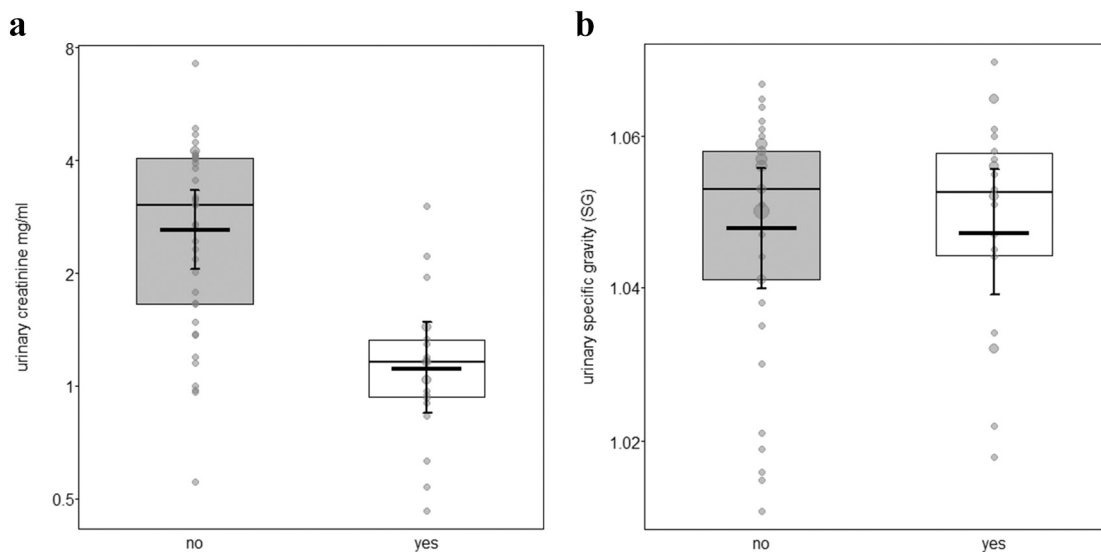


Fig. 2. a-b: Effect of feeding (‘no’ = fasted individuals, ‘yes’ = fed individuals) on urinary creatinine (mg/ml) (a) and specific gravity (b) in wolves. Indicated are medians and quartiles (horizontal lines with boxes) as well as the fitted model and its confidence limits (horizontal lines with error bars).

Table 2

Linear mixed model (LMM) output for fixed effects of full and reduced models (uGCM ng/ml SG).

Full model	Estimate	SE	Df	χ^2	P
(Intercept)	2.150	0.176	NA ^a	NA ^a	NA ^a
Species:sex ^b	0.152	0.208	1	0.451	0.502
Feeding status ^c	-0.492	0.137	1	11.964	0.001
Reproductive phase ^d	0.272	0.103	1	6.679	0.010
Sample time ^e	-0.049	0.057	1	0.736	0.391
Locomotion ^f	-0.051	0.045	1	1.261	0.262
Affiliative behavior ^f	-0.026	0.044	1	0.349	0.554
Agonistic behavior ^f	0.106	0.045	1	5.320	0.021

Reduced model	Estimate	SE	Df	χ^2	P	Lower CI	Upper CI	Min	Max
(Intercept)	2.087	0.157	NA ^a	NA ^a	NA ^a	1.776	2.393	1.899	2.154
Species ^b	-0.739	0.146	1	21.806	0.000	-1.043	-0.440	-0.811	-0.604
Sex ^b	0.109	0.109	1	0.979	0.322	-0.119	0.331	0.007	0.160
Feeding status ^c	-0.468	0.134	1	11.486	0.001	-0.732	-0.210	-0.532	-0.211
Reproductive phase ^d	0.273	0.103	1	6.695	0.010	0.063	0.484	0.229	0.333
Sample time ^e	-0.046	0.057	1	0.652	0.419	-0.157	0.069	-0.099	-0.016
Locomotion ^f	-0.047	0.045	1	1.064	0.302	-0.137	0.040	-0.066	0.000
Affiliative behavior ^f	-0.022	0.044	1	0.319	0.572	-0.108	0.068	-0.035	0.223
Agonistic behavior ^f	0.100	0.044	1	4.772	0.029	0.011	0.187	0.086	0.197

^a Not shown because of limited interpretation only.^b Reference levels for species and sex were 'dog' and 'female', respectively.^c Reference level was 'not fed'.^d Reference level was 'anestrus'.^e Co-variate (day time converted to decimals), z-transformed.^f Co-variate (normalized duration, i.e. proportion of total time in sight), z-transformed.**Table 3**

Linear mixed model (LMM) output for fixed effects of full and reduced models (uOTM pg/ml SG).

Full model	Estimate	SE	Df	χ^2	P
Intercept	5.759	0.141	NA ^a	NA ^a	NA ^a
Species:sex ^b	-0.200	0.143	1	1.480	0.224
Feeding status ^c	-0.367	0.090	1	14.886	0.000
Reproductive phase ^d	-0.125	0.073	1	2.312	0.128
Sample time ^e	0.033	0.045	1	0.487	0.485
Locomotion ^f	0.006	0.032	1	0.026	0.871
Affiliative behavior ^f	0.068	0.031	1	4.675	0.031
Agonistic behavior ^f	0.051	0.030	1	2.664	0.103

Reduced model	Estimate	SE	Df	χ^2	P	Lower CL	Upper CL	Min	Max
Intercept	5.818	0.133	NA ^a	NA ^a	NA ^a	5.556	6.094	5.727	5.907
Species ^b	-0.299	0.124	1	4.677	0.031	-0.571	-0.056	-0.463	-0.195
Sex ^b	0.063	0.092	1	0.427	0.513	-0.117	0.252	0.022	0.138
Feeding status ^c	-0.377	0.090	1	15.967	0.000	-0.558	-0.202	-0.451	-0.297
Reproductive phase ^d	-0.113	0.073	1	1.802	0.179	-0.252	0.031	-0.198	-0.031
Sample time ^e	0.029	0.045	1	0.375	0.540	-0.064	0.122	-0.011	0.063
Locomotion ^f	0.010	0.032	1	0.096	0.756	-0.054	0.075	-0.010	0.038
Affiliative behavior ^f	0.070	0.031	1	4.831	0.028	0.008	0.126	0.043	0.165
Agonistic behavior ^f	0.055	0.030	1	3.164	0.075	-0.003	0.115	0.035	0.112

^a Not shown because of limited interpretation only.^b Factor, reference levels for species and sex were 'dog' and 'female', respectively.^c Factor, reference level 'not fed'.^d Factor, reference level 'anestrus'.^e Co-variate (day time converted to decimals), z-transformed.^f Co-variate (normalized duration, i.e. proportion of total time in sight), z-transformed.

stability was assessed by comparing the estimates obtained from the model based on all data with those obtained from models with the levels of the random effects excluded one at a time. This revealed good model stability (Tables 2 and 3). All models were fitted in R (version 3.6.2; R Core Team, 2019) using the function lmer of the R package lme4 (version 1.1–21; Bates et al., 2015) with the optimizer 'bobyqa'. Parametric bootstrapping was performed to obtain confidence intervals (function bootMer of lme4). Collinearity was assessed using the function vif of the package car (version 3.0–0), applied to a model lacking the random effects, revealing no higher values than 2.9 (for predictor

'species'). Effect sizes (i.e., variance explained by entirety of fixed and random effects, or conditional R², Nakagawa and Schielzeth, 2013) of the full models were calculated using the function r.squaredGLMM of the R package MuMIn (version 1.43.15).

2.7.4. Correlation of urinary glucocorticoid and oxytocin metabolites

To investigate whether uGCM and uOTM concentrations correlate in dogs and wolves, we used the package rmcorr (version: rmcorr_0.3.1) to calculate correlation coefficients separately for each species, accounting for repeated measures.

3. Results

3.1. Behavioral data

On average, animals were in sight for 81% of the observation time (dogs: 88%, wolves: 75%). Behavioral video coding revealed that both wolves and dogs spent on average 12% of the time in sight moving around in their enclosures. There was no statistical difference between wolves and dogs regarding locomotor activity ($\chi^2 = 0.38$, $df = 1$, $P = 0.54$). Both wolves and dogs spent 88% of their time in sight resting, i.e. either standing, sitting or lying down. Wolves spent on average 0.8% and dogs 0.3% of the total time in sight performing affiliative behaviors and there was no statistical difference between them ($\chi^2 = 0.04$, $df = 1$, $P = 0.84$). Agonistic interactions were short and occurred only during 4 observations in wolves and 2 observations in dogs (wolves: 0.01%, dogs: 0.03% of the total time in sight), which prevented further statistical analysis of species differences. In wolves, observed agonistic interactions consisted only of low-level aggressive behavior such as threats and chases, whereas in dogs, on one occasion, physical fighting was recorded, and chasing on the other.

3.2. Comparison of urinary creatinine and specific gravity

Fasted wolves had significantly higher urinary creatinine concentrations than fed wolves (fasted: mean (SD) 2.88 (1.46) mg/ml; fed: mean (SD) 1.22 (0.57) mg/ml; linear mixed model with random effect of subject: $\chi^2 = 34.9$, $df = 1$, $P < 0.001$, Fig. 2 a). This was not the case for SG measurements (fasted: mean (SD) 1.048 (0.02) mg/ml; fed: mean (SD) 1.049 (0.01) mg/ml; linear mixed model with random effect of subject: $\chi^2 = 0.1$, $df = 1$, $P = 0.76$), which were not affected by feeding or fasting (Fig. 2 b). Further, to check within-hormone correlations using the two correction methods, uOTM corrected for SG and crea were correlated with each other (the same was done for uGCM concentrations). Specifically, uOTM pg/ml SG and uOTM pg/mg crea (and uGCM ng/ml SG and uGCM ng/mg crea) correlated strongly in dogs ($R = 0.84$, $P < 0.001$, $CI = 0.71$ – 0.92 for uOTM; $R = 0.88$, $P < 0.001$, $CI = 0.78$ – 0.94 for uGCM) and fed wolves ($R = 0.88$, $P < 0.001$, $CI = 0.64$ – 0.96 for uOTM; $R = 0.89$, $P < 0.01$, $CI = 0.42$ – 0.98 for uGCM), but not in fasted wolves ($R = 0.13$, $P = 0.52$ for uOTM, $CI = -0.28$ – 0.5 ; $R = 0.50$, $P < 0.05$, $CI = 0.09$ – 0.77 for uGCM). Thus, using urinary creatinine to correct for urine concentration in our wolf samples would bias results depending on their feeding status. We used the SG correction method for further analyses but we report creatinine corrected hormone values in the SI (Tables 1 and 3).

3.3. Urinary glucocorticoid metabolites

The full-null model comparison revealed significance (likelihood ratio test: $\chi^2 = 22.3$, $df = 2$, $P < 0.001$; variance explained by entirety of fixed and random effects: $R^2c = 0.39$; by fixed effects only: $R^2m = 0.36$). As the interaction between species and sex was not significant ($\chi^2 = 0.5$, $df = 1$, $P = 0.5$), we fitted a reduced model to investigate the main effects of species and sex separately. Dogs had significantly higher uGCM concentrations than wolves ($\chi^2 = 21.8$, $df = 1$, $P < 0.001$, Fig. 3, Table 2), but sex did not have an effect ($\chi^2 = 0.9$, $df = 1$, $P = 0.3$, Table 2). Feeding status ($\chi^2 = 11.5$, $df = 1$, $P < 0.01$), reproductive phase ($\chi^2 = 6.7$, $df = 1$, $P < 0.01$), and agonistic behaviors ($\chi^2 = 4.9$, $df = 1$, $P < 0.05$) were revealed to have significant impact on uGCM concentrations. Specifically, being fed the day before sampling led to lower uGCM concentrations in wolves than if they were not fed the day before and uGCM concentrations were higher in diestrus than anestrus samples. Agonistic interactions increased uGCM concentrations. Sample time did not have a significant effect on uGCM concentrations (Table 2). Body weight did not affect uGCM concentrations (Table 4, SI).

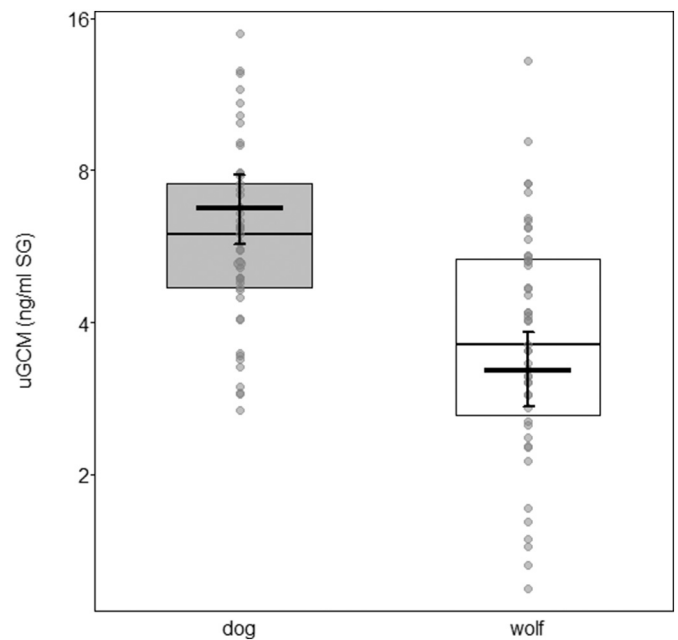


Fig. 3. Urinary glucocorticoid metabolite (uGCM) concentrations (ng/ml SG) by species (dog = grey boxes, wolf = white boxes). Indicated are medians and quartiles (horizontal lines with boxes) as well as the fitted model and its confidence limits (horizontal lines with error bars).

3.4. Urinary oxytocin metabolites

The full-null model comparison was significant (likelihood ratio tests: $\chi^2 = 6.2$, $df = 2$, $P < 0.05$; variance explained by entirety of fixed and random effects: $R^2c = 0.67$; by fixed effects only: $R^2m = 0.16$). The interaction between species and sex was not significant ($\chi^2 = 1.5$, $df = 1$, $P = 0.2$, Table 3, Fig. 4), thus we fitted a reduced model including the main effects of species and sex but lacking their interaction. This revealed significant effects of species ($\chi^2 = 4.7$, $df = 1$, $P < 0.05$; dogs higher than wolves), feeding status ($\chi^2 = 15.9$, $df = 1$, $P < 0.001$; not being fed led to higher uOTM concentrations), and affiliative interactions ($\chi^2 = 4.8$, $df = 1$, $P < 0.05$; uOTM concentrations increased with the normalized duration of affiliative behavior). A trend was found for agonistic interactions ($\chi^2 = 3.2$, $df = 1$, $P = 0.08$; uOTM levels increased with the normalized duration of agonistic behavior). Sample time and reproductive phase did not have significant effects on uOTM concentrations (Table 3).

3.5. Correlation of urinary glucocorticoid and oxytocin metabolites

Urinary GCM (ng/ml SG) and OTM (pg/ml SG) concentrations correlated significantly and positively in wolves ($R = 0.59$, $P < 0.001$, 95% $CI = 0.33$ – 0.77), but not in dogs ($R = 0.01$, $P = 0.95$, 95% $CI = -0.33$ – 0.35) (Fig. 1 a-b, SI).

4. Discussion

Overall, we found that urinary GCM concentrations were significantly higher in pack-living dogs than in similarly raised and kept wolves. Feeding status and female reproductive phase also affected this: wolves that were not fed the day before sampling had higher GCM concentrations than fed wolves and GCM concentrations were higher during diestrus than anestrus. Agonistic interactions such as threats, fighting, or chasing before urine collection, were associated with heightened GCM concentrations. Pack-living dogs also had higher urinary OTM concentrations than wolves, but the effect of species on OTM concentrations was relatively small and variance explained by random

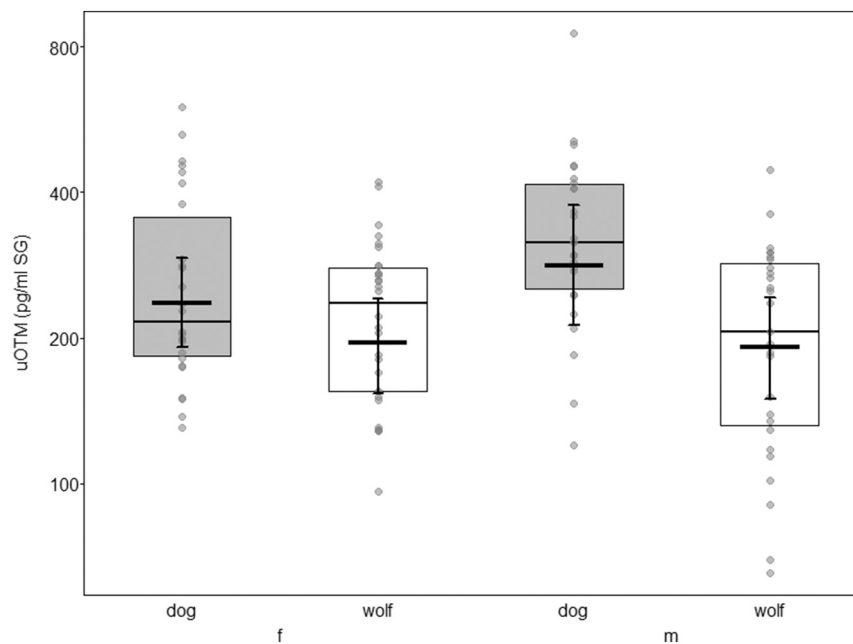


Fig. 4. Urinary oxytocin metabolite (uOTM) concentrations (pg/ml SG) by species (dog = grey boxes, wolf = white boxes) and sex (f = female, m = male). Indicated are medians and quartiles (horizontal lines with boxes) as well as the fitted model and its confidence limits (horizontal lines with error bars).

compared to fixed effects was high, suggesting large within-individual variation. Although the interaction between species and sex was not significant, the higher OTM concentrations in male dogs may be driving this effect. Urinary OTM concentrations were also strongly affected by feeding status with hungry wolves having higher values than individuals that had received food the previous day. Affiliative interactions, such as grooming, playing, or resting in body contact, before sampling positively affected OTM concentrations. Lastly, wolves showed a positive correlation of urinary GCM and OTM concentrations, while no such correlation appeared in the dogs.

With regard to uGCM concentrations, we found a clear species effect: Pack-living dogs had significantly higher concentrations than wolves. While this is in contrast with [McLeod et al. \(1996\)](#) and [Seal and Mech \(1983\)](#), who note similar concentrations of urinary and serum GCs in captive and free-ranging wolves and pet dogs, and to predictions based on studies of individuals selected specifically for increased tameness towards humans ([Albert et al., 2008](#); [Trut et al., 2009](#)), it is in line with another study previously conducted at the WSC, in which pack-living dogs had overall higher salivary GCM concentrations than pack-living wolves ([Vasconcellos da Silva et al., 2016](#)). There may be a number of possibilities for the higher uGCM concentrations in our dog population. Circulating GC levels are known to rise in response to increased physical activity ([Powell et al., 2015](#); but see [Mitsui et al., 2011](#)). Hence one possibility is that the dogs spent more time physically active than the wolves thereby resulting in higher uGCM concentrations. However, we carefully monitored the animals' behavior 60 min prior to urine collection and the animals were primarily resting during that time. Further, there was no difference between the proportion of time spent moving around in the enclosure between dogs and wolves and uGCM concentrations were not affected by locomotion ([Table 2](#)). It thus seems unlikely that the higher GCM concentrations in dogs were caused by increased physical activity. Another possibility is that our dogs' GCM concentrations are peculiarly high compared to other dog populations. However, GCM concentrations of the pack-living dogs were comparable to measures we obtained from ten pet dogs using the same test paradigm (i.e. urine samples taken following 60 min of resting in a familiar environment) and analytical methods (see [Table 3](#), SI).

Importantly, GCs are not only stress-responsive hormones, but also the main endocrine mediators of the glucose metabolism ([MacDougall-](#)

[Shackleton et al., 2019](#)) and thus should be interpreted with this in mind. An experimentally induced rise in metabolic rate (MR; i.e., by cold exposure) correlated strongly and positively with circulating GC and blood glucose concentrations in zebra finches, underscoring their function as primarily metabolic hormones ([Jimeno et al., 2018](#)). In a recent meta-analysis, [Haase et al. \(2016\)](#) describe associations of GC concentrations, body mass, and MR: Body mass and resting mass-specific MR (i.e., MR controlled for body mass at resting in a thermo-neutral state) explained 55% and 54% of variation in baseline GC concentrations across different species of mammals, respectively. While body mass was inversely related to GC concentrations, MR correlated positively with GC concentrations ([Haase et al., 2016](#)). Indeed, this pattern has previously been observed in dogs: Smaller dog breeds had higher resting MR ([Jimenez, 2016](#); [Middleton et al., 2017](#)) and salivary GCM concentrations compared to larger sized dog breeds ([Sandri et al., 2015](#)). In light of this, we note that the average body weights differed between wolves and dogs in our sample (dogs mean: 25.5 kg, range: 15.4–38.5 kg; wolves mean: 38.6 kg, range: 27.9–50.0 kg), which could potentially have affected uGCM concentrations. To investigate this further, we repeated the statistical analysis of uGCM concentrations with body weight (in kg) as an additional control predictor, but this did not affect the significance of species in explaining the response (for model output see [Table 4](#), SI). Similarly, no effect of body weight on resting heart rate in those same wolves and dogs was found in a recent study ([Kortekaas and Kotschal, 2019](#)). However, dogs had higher resting heart rates than wolves, which suggest higher resting MRs as well ([Green, 2011](#); [Malchaire et al., 2017](#)) and may reflect relaxed selection on metabolic efficiency in dogs compared to wolves, and more generally, in domesticated compared to wild-type animals. This has previously been shown in chicken selectively bred for tameness ([Agnvall et al., 2015](#)), but no wolf-dog comparisons with regard to MR exist to date. Yet, comparative locomotor analyses of three dog breeds (Northern breeds, retrievers, hounds) revealed optimized energy expenditure in the Northern breeds morphologically most similar to grey wolves ([Bryce and Williams, 2017](#)). Furthermore, alterations in environmental conditions, such as seasonal temperature changes or visitor presence in the wild park, may have affected wolves and dogs differently and influenced their metabolic demands. This could potentially be reflected in fluctuating GCM and OTM concentrations throughout the year. To avoid effects of rising

gonadal steroids on GCM and OTM concentrations, however, we did not sample the wolves during their breeding season, which takes place in the winter months (December – March), while dogs were sampled throughout the year (but still avoiding female proestrus and estrus periods). This sampling schedule prevented comparative analysis of seasonal effects on the species level and may have added to the relatively high within-individual variation in OTM concentrations as samples were collected across seasons. Nevertheless, as regards GCM concentrations, within-individual variation was small, and our results indicate that the domestication process has altered dogs' GC system compared to wolves. Additional environmental factors such as temperature and visitor presence may play a role and should be included in future studies.

Reproductive phase affected uGCM concentrations. In line with data presented by McLeod et al. (1996), we found that uGCM concentrations were higher during diestrus than anestrus. Diestrus defines the period of canid (pseudo)pregnancy and comprises the preparation of the den for potential pups (Lord et al., 2013) which results in increased metabolic demands for the parents associated with heightened GC concentrations. Furthermore, we found that being involved in agonistic interactions in the pack (i.e., threats, chasing, fighting) correlated with higher uGCM concentrations. This has been described before in wolves (McLeod et al., 1996; but see Sands and Creel, 2004) and other species, such as bonobos (Surbeck et al., 2012), and chimpanzees (Muller and Wrangham, 2004), and often been discussed in light of dominance hierarchies, group composition, and breeding season. In the current study, agonistic interactions happened very rarely (only twice in dog packs and 4 times in wolf packs) and group compositions remained stable throughout the sampling period. Since we did not include social status into our analysis, it is possible that subtle, ephemeral changes in group or dyad social dynamics could have affected daily hormone concentrations and contributed to within-individual variation. However, since average pack size and social structure (one dominant individual per pack or dyad) was comparable between wolves and dogs during our study, this seems unlikely to have caused the species difference in GCM concentrations. Nevertheless, it is important to note that several wolf packs in our sample population consisted of only two individuals, which is not representative for free-living wolves where average pack sizes have been reported to range from 3.4 to 4.2 in Europe (Mattioli et al., 2018). In North America, packs of up to 20 individuals have been recorded (Stenglein et al., 2011). Given the even larger variability found in free-ranging dogs' pack sizes (ranging from solitary animals to packs of 42 individuals; Cafazzo et al., 2014), our results may not be representative for packs found in the wild, however, the species-specific comparison of animals raised and housed at the WSC remains valid.

As regards the OT system, we found higher uOTM concentrations in pack-living dogs compared to wolves. However, it should also be noted that in our sample, male dogs had the highest uOTM concentrations. Although the species/sex interaction was not statistically significant, considering the higher concentrations of male dogs compared to all other groups (male dogs: 343 (147) pg/ml SG, female dogs: 275 (132) pg/ml SG, male wolves: 213 (96) pg/ml SG, female wolves: 234 (84) pg/ml SG) it is possible that it is in fact male dogs that are driving this result. Indeed in two other (yet unpublished) studies we found a consistent species/sex interaction indicating that it is indeed male dogs' higher uOTM concentrations that drive the wolf/dog difference. A possible explanation for this finding could be related to the different breeding physiology of wolves and dogs. While wolves (both males and females) are highly seasonal breeders sexually active only once per year (Seal et al., 1987), domestic dogs show increased reproductive activity, a common result of animal domestication. Female dogs may become receptive two to three times per year and male dogs are capable of reproducing all year round (Lord et al., 2013). Male dogs exhibit high levels of circulating testosterone (T) throughout the year, while male wolves' testes size and T levels fluctuate according to the breeding season (Haase, 2000). Gonadal steroids directly affect OT binding sites in the brain (Tribollet et al., 1990; Insel et al., 1993), and T specifically,

has been shown to enhance central OT receptor binding (Johnson et al., 1989), and inhibit OT synthesis in male rats (Okabe et al., 2013). The potential influence of breeding physiology and mating system on endogenous OT system activity in (male) dogs and wolves remains to be investigated. At present it is important to point out that our results support the idea that higher uOTM concentrations in dogs compared to wolves are likely linked to dogs' different breeding physiology (Kikusui et al., 2019), rather than to their increased capacity to bond with humans ('hypersociability hypothesis'; Bentsosela et al., 2016; vonHoldt et al., 2017) although further studies directly investigating the link between OT and the dog-human bond are needed to fully test this hypothesis.

Our results are in stark contrast with a previous study comparing OT concentrations in dogs and wolves. Nagasawa et al. (2015) report uOTM concentrations in pre-test wolf samples three times higher than pre-test samples of pet dogs. This may be explained by a number of reasons. First of all, it is not clear whether the pre-test levels in the study by Nagasawa et al. represent baseline values. For instance, OT system activity could have already increased due to the study design, which included the owner taking the wolves out from their enclosures and to the unfamiliar testing area before collecting the pre-test samples. At least in our wolves, familiar people approaching the animals in the enclosures usually elicit greeting behaviors. Thus, those samples might have already reflected increased uOTM concentrations caused by previous greeting of the owners. Pet dogs were already with their owners upon arrival at the test area and would not have shown this 'greeting effect'. Alternatively, as peripheral OTM concentrations may also rise in response to acute stressors (Neumann and Landgraf, 2012; Jong et al., 2015), it is possible, that the wolves were more aroused by the novel environment than the dogs. It is noteworthy that while the authors report no behavioral signs of distress in the dogs, the same was not reported for the wolves. Unfortunately, Nagasawa et al. (2015) did not measure simultaneous GC release, which could have helped to clarify this. Although a wolf-dog comparison of basal OTM concentrations was not their aim, the inaccuracy in the pre-test measures likely affected the subsequent post-measures by introducing a ceiling effect in wolves' OTM concentrations (Kekecs et al., 2016). This in turn, could have led authors to the conclusion that, whereas pet dogs' OTM concentrations increased following interaction with the owner, no such effect occurred in the wolves. Importantly, early-life experiences and socialization may also affect (baseline) OT concentrations later in life. Such 'rearing effects' have been demonstrated by Winslow et al. (2003), who compared mother-reared and nursery-reared rhesus monkeys and found significantly lower central OT concentrations in the nursery-reared animals. Comparing animals with different human exposure and experience could thus inadvertently bias results, even in adult animals. By using hand-raised dogs and wolves with comparable life-histories and previous experiences with humans, we were able to account for ontogenetic effects on the OT system as much as possible. Discrepancies between our findings and previous results (Nagasawa et al., 2015) likely reflect the different socialization of the animals used. While we did not include pet dogs for statistical comparison into this study, descriptively we found that pet dogs had even higher unstimulated, urinary OTM concentrations than the pack-living dogs and wolves (Table 3, SI), suggestive of rearing effects on the OT system. This highlights the importance of comparing similarly raised and housed individuals when conducting comparative studies.

Further, we found that rates of affiliative social interactions with pack mates (i.e., grooming, playing, social sniffing, body contact, and greeting) were comparable in wolves and dogs, and correlated positively with uOTM concentrations. This is in line with previous studies associating heightened uOTM concentrations with conspecific bonding activities such as grooming (Crockford et al., 2013) and body contact (Snowdon et al., 2010) in primates, and OT administration with increased motivation to play in dogs (Romero et al., 2015).

Lastly, we found that feeding status had strong effects on uGCM and

uOTM concentrations. Both uGCM and uOTM concentrations were significantly higher following fasting days in wolves. Indeed, serum GC concentrations initially increase strongly in response to caloric restriction but return to baseline after some weeks (Nakamura et al., 2016). In line with this, following long-term fasting due to food shortage in winter, raccoon dogs (Asikainen et al., 2005) and grey wolves (Delgiudice et al., 1987) did not have altered GC concentrations. Hence it is likely that the heightened uGCM concentrations we found after short-term fasting reflect the physiological response to food restriction and would decrease again after some time. We also found higher uOTM concentrations following fasting than feeding days which seems to contradict previous studies reporting OT release in response to food in dogs (Mitsui et al., 2011). OT, administered or released in response to feeding, in fact, dampens appetite and decreases subsequent feeding behavior (Leslie et al., 2018; Lawson et al., 2020) whereas experimental GC system stimulation mostly increases food intake. Thus, the GC and OT systems may interact to keep the body's physiological state in balance. On the other hand, one could speculate about a potential 'reward anticipation' effect in our sample: upon seeing the experimenter (or the testing equipment), the animals may have expected a food reward based on previous experiences taking part in similar studies. This could have caused a stronger reaction in hungrier animals, and uGCM and uOTM concentrations would have risen in response. Such anticipatory effects on GC release have previously been demonstrated for example in bonobos (Hohmann et al., 2009) and a recent paper discusses OT's regulatory role for metabolic stability through anticipation of environmental changes (Quintana and Guastella, 2020). In any case, further studies are clearly needed to clarify the link between feeding status and GC/OT release in dogs and wolves.

Until now, many studies have focused on measuring either GC or OT concentrations but considering the close interplay of the HPA axis and the oxytocinergic system (Jurek et al., 2015; Winter and Jurek, 2019), their effects are best discussed with regard to each other. The OT system has been described as the body's 'calming system' that antagonizes GC effects and may be the underlying mechanism of the stress-protecting effect of social support (DeVries et al., 2003). GCs and OT are co-released under acute stress (Neumann and Landgraf, 2012; Jong et al., 2015) and even in anticipation of a mildly stressful event (Brown et al., 2016) but the dampening effect of OT on the HPA axis (and eventually circulating GC levels) might only become evident after some time (Cardoso et al., 2013), hence simultaneously high GC and OT concentrations may be found. While we did not find a correlation between uGCM and uOTM concentrations in the dogs, we found a positive one in the wolves. In line with this, a recent study found a positive correlation of salivary GCM and OTM following a mildly stressful event (i.e., isolation in an unfamiliar room) in dogs (Ogi et al., 2020). In our study, it is possible, that the wolves perceived the presence of the experimenter at the enclosure differently than the dogs and perhaps experienced increased arousal. In any case, the co-activation of GC and OT systems remains to be investigated in more depth in our study population, in particular in response to different social and non-social stimuli.

Some important limitations to this study remain to be discussed. First, measuring systemic hormone levels may only tell half the story. To be really conclusive, one ought to know about (central) receptor density, distribution, and affinity, which vary considerably between even closely-related species and are associated with behavioral differences (Insel and Shapiro, 1992). However, given the highly invasive nature of this research, we can only report on systemic hormone levels. Second, the relevance of the measurement of peripheral OT has previously been called into question due to little evidence of a correlation between central and peripheral levels, insufficient validation of assay systems, and high variance introduced by the analytical methods (McCullough et al., 2013; Leng and Sabatier, 2016). However, previous studies indicate a link between central and peripheral OT release in a number of contexts (lactation and osmotic challenge: Neumann et al., 1993; swim stress: Wotjak et al., 1998; restraint: Lopes-Azevedo et al., 2019), and

recently a study described the mechanism underlying OT release during physical social touch (Tang et al., 2020), providing compelling evidence for simultaneous central and peripheral OT release. A thorough analytical and physiological validation has been carried out for the OT EIA used in this study (Wirobski et al., 2020). Although we found that the intra- and inter-assay CVs were still relatively high, they are comparable to what has been published in the field (e.g., Rincon et al., 2020), and given that we see consistent effects of test and control predictors across studies (unpublished data), we are confident that they reflect 'true' effects. Nevertheless, this source of variation needs to be kept in mind and warrants cautious interpretation of the OT results. Lastly, species- (and potentially, sex-) specific differences in biological clearance windows of circulating GC and OT molecules into urine as well as in retention times and accumulation of urine in the bladder may have contributed to the relatively high within- and between-individual variation.

Finally, we established that specific gravity (SG) is more robust than urinary creatinine to dietary changes in wolves and dogs. In particular, intermittent feeding schedules including fasting days (in accordance with the physiological needs of grey wolves) can heavily affect urinary creatinine concentrations, which in turn biases hormone ratios and leads to skewed results, especially in comparative studies. We thus only used uGCM and uOTM concentrations corrected for SG for all statistical analyses but report hormone concentrations as ratios to urinary creatinine in the supplementary material (Tables 1 and 3, SI).

In summary, we here report a comprehensive investigation of unstimulated, urinary GCM and OTM concentrations in wolves and dogs that accounts for important variables such as sex, breeding season, feeding status, early-life experiences and human socialization, sample time, and housing. Our results clearly show higher uGCM concentrations in comparably raised and kept dogs than wolves that might be a result of increased basal metabolic activity in dogs compared to wolves as a result of domestication, however this needs further investigation. Feeding status and reproductive phase affected uGCM concentrations as well as agonistic interactions within the packs. Urinary OTM concentrations were higher in dogs than wolves but the effect was rather small with high individual variation and considerable variability introduced by the analytical methods which warrant cautious interpretation. However, given consistent findings of male dogs having the highest uOTM concentrations across several studies (yet unpublished data) the effect may be driven by the species' specific breeding physiology. Feeding decreased and affiliative interactions increased uOTM concentrations. Based on the link between the oxytocinergic system and HPA axis activity, we predicted a negative correlation of uGCM and uOTM concentrations in dogs but we found a positive one in wolves. This may reflect simultaneous release of GC and OT in wolves in anticipation of the sample collection or species-specific differences in physiological clearance windows.

The current study represents a step towards understanding hormonal correlates of dog domestication, in particular regarding the activity of the GC and oxytocinergic systems. While it may be too early to conclude how exactly the domestication process has shaped dogs' hormonal profiles, our data suggest that a more general selection process on metabolic parameters rather than selection for tameness and sociability alone may have altered dogs' physiology compared to wolves'. Further studies will need to investigate GC and oxytocinergic system reactivity in response to human and conspecific social stimuli.

Authors' contributions

SMP, FR, TD secured funding; GW, SMP, FR, and TD conceived the study; GW collected the data; FSS validated the OT assay; FSS and GW conducted the OT extraction and analysis; RP provided lab space and material for OT and GC extraction and analysis; GW conducted the statistical analyses and wrote the manuscript. All authors contributed and approved the final manuscript.

Data accessibility

Raw data will be made available.

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Declaration of competing interest

The authors report no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2020.104901>.

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