



DISSERTATION | DOCTORAL THESIS

Titel | Title

Salivary hormone measurements in behavioural and cognitive biology – Advancing research in primates and corvids

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of
Doctor of Philosophy (PhD)

Wien | Vienna, 2024

Studienkennzahl lt. Studienblatt | Degree
programme code as it appears on the
student record sheet:

UA 794 685 437

Dissertationsgebiet lt. Studienblatt | Field of
study as it appears on the student record
sheet:

Biologie

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Chapter 1.

General introduction

The fields of behavioural and cognitive biology examine the complexity of behaviour exhibited by animals, including humans, and seek to understand the underlying processes, its development over an individual's life, its adaptive function and its evolutionary origins (compare Tinbergen, 1963). Researchers in these fields investigate how organisms perceive, learn, think, and behave, with a particular focus on the cognitive abilities and behavioural adaptations that contribute to their survival and reproduction.

Particularly throughout the past century, it has become clear that behaviour is regulated by physiological processes. These processes are governed by systems, such as the central nervous system, which is integral in processing information and coordinating behavioural responses, the autonomic nervous system, that plays a crucial role in controlling involuntary physiological functions, such as heart rate, digestion and respiratory rate, which can influence behavioural outcomes (see Porges, 2007), and the immune system, which can also affect mood and behaviour (see Dantzer et al., 2008). To illuminate the intricate connections between physiological processes and behaviour different physiological parameters, for instance heart rate variability and skin conductance, can be investigated (see appendix p. 129: (Massen et al., 2019)). In the case of another key regulator and modulator of behaviour, the endocrine system, hormones are a very important parameter providing information on such connections.

Hormones are chemical messengers that are produced by endocrine glands and released into the bloodstream, through which they travel to distant target tissues. Once hormones have reached their target cells, they initiate a series of specific cellular and physiological responses, that regulate growth, reproduction, energy homeostasis, and glucose, calcium and sodium levels, to name only a few (Hadley and Levine, 2007). As a consequence, hormones play an important role in regulating behavioural processes (see: Adkins-Regan, 2009; Oliveira, 2009; Soares et al., 2010), such as mating behaviour (e.g., Ouyang et al., 2014; Young et al., 2013), parental care (e.g., Crossin et al., 2012; Storey and Ziegler, 2016), territorial behaviour (e.g., Canoine and Gwinner, 2002) and cooperative behaviour (Massen et al., 2019; Soares et al., 2010). Understanding the endocrinological mechanisms underlying behaviour enhances our knowledge of how animals navigate their ecological niches and optimize their chances of survival and reproductive success.

Hormones affect cognitive processes such as learning and memory (e.g., Reich and Hölscher, 2022; Taxier et al., 2020; Wirth, 2015). Elevated glucocorticoid levels, for instance, as seen during periods of chronic stress, can impair working memory, attentional control, and the ability to retrieve and consolidate memories (see Wolf et al., 2016). By investigating such intricate interactions between hormones and cognitive abilities, such as spatial navigation (e.g., Blanchette et al., 2020), problem-solving (e.g., Bókony et al., 2014; Esch et al., 2019), and social cognition (see: Chang and Platt, 2014; Crespi, 2016; Platt et al., 2016), scientists can also gain a deeper understanding of the underlying mechanisms that shape cognitive processes in both animals and humans.

Furthermore, hormonal investigations have practical implications for animal welfare and conservation efforts (reviewed by Staley and Miller, 2020). Monitoring hormone levels helps assess the stress, reproductive health, and overall well-being of animals in various contexts, such as captive environments, conservation programs, and studies of wildlife populations. This knowledge enables scientists to design effective management strategies, develop appropriate conservation interventions, and improve the welfare of animals under human care.

The objective of the present doctoral thesis is to advance the (minimally invasive) collection and measurement of salivary hormones in non-human primates and corvids, animal groups that have captured the attention of scientists studying animal behaviour and cognition due to their remarkable cognitive abilities and social behaviours (see p. 16, *Salivary hormones in behavioural and cognitive biology – primates and corvids*). Ultimately, this thesis seeks to deepen our knowledge of the intricate relationship between hormones and the complex behaviours and cognitive processes exhibited by species of these animal groups.

In this introduction I will, first, provide a concise overview of biospecimens that are suitable for hormone analyses in the fields of animal behaviour and cognition, then I will introduce saliva as a medium for hormone measurements in more detail, including collection methods and analytes that can be measured. I will highlight the importance of method validation, explore existing research on salivary hormones in primates and corvids, addressing some currently existing gaps, and finally I will introduce the following chapters of this doctoral thesis.

Biospecimens for hormone analyses

Hormones can be measured in different biological substrates. However, when choosing the substrate to be collected for behavioural endocrinology, two factors in particular, must be taken into account, i.e., invasiveness and time scale. Blood and cerebrospinal fluid (CSF) sampling present invasive methods that generally require the animal to be restrained and sometimes even sedated. In many cases, this causes high levels of stress in the animals, which is rather undesirable

concerning animal welfare, and may pose a confounding factor for the study outcome; i.e. you are measuring the effect of the sampling method rather than that of the variable of interest. Stress may lead to increased glucocorticoid levels, which, if the timing of sampling is not well thought through or cannot be conducted according to plan, may not allow for the measurement of unaffected hormone concentrations, in this case glucocorticoid levels. This in turn, might even affect other hormones since elevated glucocorticoid levels can also influence the concentration of hormones, such as testosterone (Cumming et al., 1983). Thus, restraining animals to get biospecimen for hormone analyses is in many cases undesirable. In contrast, other biospecimens such as saliva, urine, faecal and hair/feather samples can be collected in a minimally or non-invasive manner and the collection is, therefore, usually less stressful for the animal, and as a result more sensitive to the variable of interest.

Besides invasiveness, the time scale of each biospecimen needs to be considered because there is a delay between the release of the hormone and the moment that it is detectable in a particular biospecimen (Fig. 1, reviewed by Gormally and Romero, 2020). Blood and CSF samples provide a snapshot of hormone levels at the time of sampling (Anestis, 2010; Gormally and Romero, 2020). Within a few minutes after the moment that hormonal changes can be detected in blood, they can be also measured in saliva (Gröschl, 2008). Therefore, these two biospecimens are especially, but not exclusively, suitable for the measurement of acute hormonal changes. Other excretions, such as urine and faeces represent an integrated measure of the amount of excreted hormones or their metabolites (Palme, 2019) since the previous urination or defaecation. Additionally, time-lags between the hormone release into the blood and the appearance of the hormone or its metabolites in the excretion differ between biospecimen (Fig. 1). For instance, due to the gut passage time, particularly metabolites in faeces exhibit a rather long time-lag that can range from hours to days, depending on the species (Möstl et al., 2002; Möstl and Palme, 2002). Though it is possible with urine and faeces to detect hormone peaks due to short stimuli, these biospecimen do not provide a high temporal resolution. Hence, it can be difficult to assign changes in hormone levels to specific short events. These biospecimen are, therefore, rather useful for studies interested in stimuli/situations that are longer-lasting (hours to days, Fig. 1). Further expanding the temporal spectrum, keratinized tissues such as hair, feathers, nails and even whale baleen offer insights into hormone profiles spanning periods of weeks to months (Gormally and Romero, 2020) (Fig. 1). This is due to the diffusion of hormones from the follicular capillaries into the developing tissue (Gormally and Romero, 2020). Keratinized tissue is, therefore, more suitable for studies investigating influences of stimuli/situations that are more chronic, such as housing conditions and seasons. However, there is also evidence that hair glucocorticoids are appropriate for quantifying short-term stressors (Kalliokoski et al., 2019).

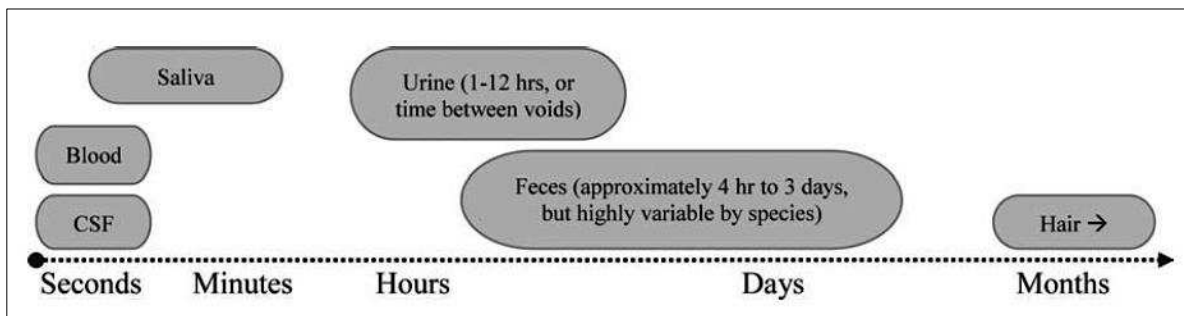


Figure 1. Biospecimen used for hormone analysis and their time depth. From (Anestis, 2010).

Concludingly, these differences in temporal resolution are one of the reasons why not all biospecimen are suitable for all research questions. Since in saliva hormonal changes can be detected within minutes and without major disturbance of the animal, saliva sampling is particularly suitable for studies that aim at measuring the immediate hormonal response to a certain (short) stimulus. Moreover, especially in trained animals (see p. 9: *Saliva sampling methods*), it allows for a high temporal resolution of hormonal changes because multiple samples can be collected over short time intervals as sampling is not restricted to, for example, defecation rates (Heintz et al., 2011). Besides, it avoids a number of ethical issues related to blood or cerebrospinal fluid sampling (Behringer and Deschner, 2017).

Saliva

Production and function

Saliva is a bodily fluid that is produced by the salivary glands, mainly through ultra-filtration (i.e., filtration at the molecular level) of the blood, but also through mechanisms, such as passive diffusion and active transport through salivary gland cells (Bellagambi et al., 2020; Laudenslager et al., 2006). Besides minor salivary glands, in humans, there are three major pairs of salivary glands – the parotid, the submandibular and the sublingual gland. Together, they produce more than 90 percent of the saliva (Porcheri and Mitsiadis, 2019). The number, size and location of salivary glands, however, vary between species (Gröschl, 2009; Tucker, 2010).

Saliva has three main functions, in some of which also hormones play an important role (Gröschl, 2009), i) it hydrates the oral cavity and lubricates food, which in turn increases taste as the food particles are brought to the tastebuds; ii) it protects against pathogens: Saliva is weaponed with several mechanisms that fight bacteria and/or viruses and aid the immune response; e.g., lysosomes, lactoferrin (Kell et al., 2020), IgA and cytokines, including the hormones leptin and adrenomedullin, which have an inhibitory effect on bacterial growth (Allaker and

Kapas, 2003; Gröschl, 2009; Gröschl et al., 2009; Slomiany and Slomiany, 2003); and iii) saliva is an important component of digestion as it contains, for example, amylase and lingual lipase, which break down carbohydrates and fat, respectively. Moreover, in some species, hormones in saliva act as pheromones and therefore play an important role in olfactory communication with, for instance, potential mates (Melrose et al., 1971; Smith and Block, 1991).

Hormones enter saliva either via diffusion (down a concentration gradient) through the acinar cells of the salivary glands or through paracellular transport (most likely ultra-filtration) between the acinar cells (Gröschl, 2009; Vining et al., 1983). A large amount of the hormones circulating in the bloodstream are bound to carrier proteins and are, therefore, not bioavailable to organs and tissue (see: Kirschbaum and Hellhammer, 1989; MacLean et al., 2019) (for more information see p. 12). However, this bound fraction of hormones cannot enter saliva. This means that in saliva only free/unbound and, therefore, bioavailable hormones can be measured (Vining et al., 1983). For a review on hormones in saliva see (Gröschl, 2009).

Saliva sampling methods

Saliva can be collected in several different ways. In humans, the most common methods to collect saliva samples are: the passive drooling method, in which a person has to wait briefly until some saliva has pooled in the person's mouth and then slowly let it flow into a collection tube by tilting the head forward (Padilla et al., 2020); and the oral swab method, in which a person has to put a saliva swab into the mouth until it is soaked with saliva and then place it in a collection vial. In research studies on humans, participants very often collect the samples themselves, since these procedures are relatively uncomplicated and, except for the small collection items, no special devices are required (Padilla et al., 2020).

Although animals usually cannot collect saliva samples themselves, saliva swabs can also be used to obtain saliva from various animal species (for a review on primates see Laudenslager et al., 2006). Domesticated animals, e.g., guineapigs (*Cavia porcellus*), dogs (*Canis lupus familiaris*), goats (*Capra aegagrus hircus*), pigs (*Sus scrofa domesticus*) and horses (*Equus caballus*), often tolerate saliva sampling with swabs without the need for (intensive) animal training (Aurich et al., 2015; Damián et al., 2018; Lürzel et al., 2020). Briefly, a saliva swab is placed in the mouth of an individual until the swab is soaked with saliva. This collection procedure is, however, not suitable for animals that do not tolerate the required handling (cf. adult male rhesus macaques, *Macaca mulatta*, Lutz et al., 2000). They would either have to be sedated (e.g., Rapp-Santos et al., 2017) or restrained, which likely causes elevated stress responses and often aggression. This, in turn, might affect biomarker concentrations, introducing a confounding factor (Cook, 2012), and, in case an animal becomes defensive/aggressive, it can even be dangerous for the experimenter.

(Lutz et al., 2000). Therefore, researchers resort to alternative tactics of obtaining saliva samples, be it opportunistically making use of the food-motivation of animals or through extensive training protocols. Oftentimes, stimulants are put on the swab to encourage the individuals to lick or chew on it and thereby deliver enough saliva for the analysis of the required biomarker (Damián et al., 2018). This is a practice that also allows for saliva sampling from free-ranging animals as it eliminates the need for any restraint or training of the animals (e.g., Broche et al., 2023; Higham et al., 2010). However, it's important to note that using certain stimulants can increase the saliva flow rate (Talge et al., 2005), resulting in sample dilution and, therefore, distorted analyte levels (Bellagambi et al., 2020). Depending on the assay method employed for analyte analysis, the use of stimulants can lead to higher or lower values than situations where stimulants are not used (Talge et al., 2005). This emphasises the importance of standardised collection and analysis regimes.

Through structured and species-specific animal training, sedation or restraint of the animal as well as saliva sample dilution through stimulants can be avoided and saliva samples can be acquired in a safe and positive way. With positive reinforcement training many animals across diverse settings, including laboratories, zoos, as well as agricultural animals and companion animals, can be trained to cooperate voluntarily with the experimenter and provide saliva samples (Behringer et al., 2022; Broche et al., 2019; Da Silva Vasconcellos et al., 2016; Rickert et al., 2021). Positive reinforcement training is a form of operant conditioning in which the trained individual receives a reward, often a small food item, from the trainer for responding appropriately to the trainers cues and commands and for showing the desired behaviour (see e.g., Behringer et al., 2014; Laule et al., 2003). Animal training using positive reinforcement can furthermore represent enrichment for the animals and strengthen the human-animal relationship, improving animal welfare (Rault et al., 2020; Sankey et al., 2010). In the studies presented in Chapter 2 and 4 of this doctoral thesis positive reinforcement training was used to train three different non-human primate species to provide saliva samples (Stocker et al., 2023, 2020), and in Chapter 3 we present a training protocol for saliva collection with positive reinforcement training in birds (Stocker et al., 2021).

After sample collection, one needs to take care of transportation to the laboratory and storage. For best practices and special consideration concerning saliva collection (including all sorts of contamination), handling, transport and storage, please see (Padilla et al., 2020).

Analytes of saliva samples – steroid and peptide hormones

Once saliva is collected, there is a whole range of genes and analytes that can be identified and measured. In the medical world, saliva has, therefore, become a very important tool for the

diagnosis of diseases (Javaid et al., 2016; Shah, 2018; Zhang et al., 2016), a recent example being the SARS-CoV-2/COVID-19 tests based on antigens in saliva. In the field of animal behaviour and cognition, salivary hormones, especially the class of steroid and peptide hormones, are of particular interest due to their important role in stress physiology, and sexual and social behaviour. Steroid hormones are derived from cholesterol and include the sex hormone groups produced by the gonads, i.e. androgens, estrogens and progestagens, and the hormones produced by the adrenal glands, the mineralocorticoids and glucocorticoids. Peptide hormones, on the other hand, consist of (rather short) aminoacid chains. Most peptide hormones are produced either in the central nervous system (e.g., oxytocin, vasopressin, corticotropin releasing hormone), the pituitary (e.g., adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH)) or the gastrointestinal tract (e.g., insulin, ghrelin, gastrin). Nonapeptides, such as the hormones oxytocin and vasopressin, are a particular group of peptide hormones that consist of nine aminoacids and play a crucial role in social behaviour.

In this doctoral thesis I focus on the glucocorticoid cortisol in mammals, as it is an important player in stress physiology, and on the nonapeptide mesotocin in birds, which is the avian homologue of oxytocin and very important in avian social behaviour.

Glucocorticoids

Glucocorticoids play an important role in the modulation of the immune system and the metabolism. In vertebrates, the release of glucocorticoids is an important part of the stress response as it helps the individual to restore balance after homeostasis has been disturbed by a noxious stimulus, a stressor (McEwen and Wingfield, 2010; Romero et al., 2015; Romero and Beattie, 2022). When an individual perceives a stressful stimulus, the hypothalamus-pituitary-adrenal (HPA) axis is activated and glucocorticoids (cortisol: major glucocorticoid in most mammal species; corticosterone: major glucocorticoid in birds and reptiles (Holmes and Phillips, 1976)) are released from the adrenal cortex into the blood stream. Glucocorticoids are then transported to target cells throughout the body, where they bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). While MRs have a high affinity for glucocorticoids and play a key role in regulating water balance (electrolyte excretion) and circadian rhythms, GRs have a lower affinity for glucocorticoids and are crucial in the stress response (De Kloet et al., 1998; Gomez-Sanchez and Gomez-Sanchez, 2014). Glucocorticoids, thus, trigger a cascade of physiological and behavioural changes (Sapolsky et al., 2000). They, for instance, influence appetite and feeding behaviour, reproductive physiology and behaviour, and memory formation (Sapolsky et al., 2000). However, whereas acute, short-term stress responses are generally aiding and optimizing survival, chronic, long-term stress responses have detrimental effects on an

individual's health and survival (Romero et al., 2015). Acutely high levels of glucocorticoids, for example, act on the immune system and can reinforce the inflammatory response (Busillo and Cidlowski, 2013; Cruz-Topete and Cidlowski, 2014; Dhabhar and McEwen, 1997; Duque and Munhoz, 2016). Further, acute increases in glucocorticoids lead to increased blood glucose levels, at least in fasted animals (see Romero and Beattie, 2022). The increased glucose appears to support the animal's recovery from, for example, a predator attack by replenishing energy stores depleted during the fight or flight response. This process, facilitating long-term energy maintenance, prepares the animal for potential future stressors (Romero and Beattie, 2022; Sapolsky et al., 2000). If, however, glucocorticoid levels become chronically elevated, they can disrupt the normal regulation of blood glucose levels, leading to persistent hyperglycemia (high blood sugar), which can increase the risk of developing insulin resistance (Beaupere et al., 2021), type 2 diabetes (Sharma et al., 2022), and other metabolic disorders. Further, chronically elevated glucocorticoid levels suppress the immune system, mainly by downregulating the expression of pro-inflammatory mediators (Cruz-Topete and Cidlowski, 2014; Dhabhar and McEwen, 1997; Duque and Munhoz, 2016). Although this is the reason why glucocorticoids are widely used to treat chronic inflammatory diseases, in healthy individuals chronic exposure to glucocorticoids increases the susceptibility to infections and decreases the ability to cope with them (Spencer et al., 2001).

In the behavioural sciences, however, researchers are particularly interested in how acute hormonal fluctuations elicit rapid, context-specific behavioural adjustments that enhance an organism's fitness and survival.

A large amount of glucocorticoids in blood is bound to corticosteroid-binding globulin, plasma proteins, albumin and platelets (Kirschbaum and Hellhammer, 1989; Laudenslager et al., 2006). This has two main reasons; i) glucocorticoids are lipid-soluble and therefore cannot be transported freely in the watery environment of the blood, and ii) these bindings regulate the bioavailability of the glucocorticoids to the tissue as only the free fraction of glucocorticoids can diffuse through the lipid bilayer cell membrane and exert their effects in the cells (for a review on the function of corticosteroid-binding globulin see (Rosner, 1990)). The latter is also the reason why only unbound glucocorticoids can pass (through passive intracellular diffusion) from the blood stream through the acinar cells of the salivary glands into saliva (Vining et al., 1983). This means, as mentioned earlier, that glucocorticoids in saliva only represent the bioavailable fraction of glucocorticoids. Either way, this fact again emphasizes that absolute values retained from (saliva) samples are not very informative, and that proper controls are needed to test the relative effect of specific stimuli.

Nonapeptides

The most famous member of the nonapeptide hormones is oxytocin, often termed the “love hormone”. Unlike glucocorticoids which are produced and released in the periphery (by the adrenal glands), oxytocin is produced mainly in the brain, particularly by the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus. It can be released centrally as a neurotransmitter, where it is involved in many social processes, such as social bonding (Bosch and Young, 2018, but see also Berendzen et al., 2023), trust (Kosfeld et al., 2005) and the regulation of stress (Dief et al., 2018), to name only a few, and it can be transported to the posterior pituitary where it is released via the bloodstream into the periphery. There, its most well known functions are to regulate smooth muscle contraction of the uterus during birth (Dale, 1906) and milk letdown during lactation (Nishimori et al., 1996; Young et al., 1996), for a review see (Russell and Leng, 1998).

It has long been debated whether oxytocin measured in the periphery also reflects central oxytocin concentrations. The current state of knowledge is that oxytocin release into the brain and into the periphery can occur independent of each other or in a coordinated manner (Jurek and Neumann, 2018; Landgraf and Neumann, 2004). Lefevre and colleagues (2017) found that in primates, simultaneously collected plasma oxytocin and cerebrospinal fluid oxytocin correlated positively, suggesting that peripheral oxytocin can be used as a proxy for central oxytocin. A meta-analysis on this topic revealed that such a correlation often depends on the experimental context; whereas significant associations between peripheral and central oxytocin were found after experimentally induced stress and intranasal oxytocin administration, no association was found under basal conditions (Valstad et al., 2017). Moreover, recent studies have identified a pathway through which peripheral oxytocin can cross the blood-brain barrier and thereby also affect social behaviour (Higashida et al., 2019; Yamamoto et al., 2019; Yamamoto and Higashida, 2020). To conclude, although there is evidence that peripheral oxytocin levels can be linked to central levels, more research is still needed to better understand under which conditions this is the case.

Even though it is still unclear how exactly oxytocin is entering saliva, the presence of oxytocin in saliva has been confirmed by studies using mass spectrometry (e.g., Franke et al., 2019; MacLean et al., 2018). When measuring oxytocin in the periphery there are several physiological and methodological aspects to consider. First, oxytocin has a very short half-life (in human blood 2-6 minutes (Fabian et al., 1969)) and undergoes rapid degradation as it passes through the liver and kidneys, owing to the activity of a variety of peptidases (MacLean et al., 2019). Second, like glucocorticoids, oxytocin easily binds to other molecules, e.g. plasma proteins and other oxytocin molecules, even after a sample has been collected (Avanti et al., 2013; Brandtzaeg et al., 2016; Liu et al., 2019; MacLean et al., 2019). Unfortunately, extraction of the hormone, which includes a variety of methods aimed at removing interfering substances from a

biospecimen, can result in substantial discard of bound oxytocin (Brandtzaeg et al., 2016; MacLean et al., 2017). In saliva, however, oxytocin occurs mainly in its unbound form, and since saliva is a relatively clean matrix compared to blood there are limited opportunities to bind to other molecules, which means that extractions before measurement are not always necessary (Jurek and Neumann, 2018; MacLean et al., 2019). Working with unextracted samples not only speeds up the analysis process, reducing the chances of additional human and technical errors, but also saves valuable time and labor costs (Gnanadesikan et al., 2021). If extractions are necessary largely depends on the assay kit used to analyse the samples (see López-Arjona et al., 2023). Therefore, it is crucial to rigorously test the need and kind of sample extraction for the assay that is going to be used in a study in advance (Gnanadesikan et al., 2021; López-Arjona et al., 2023).

While oxytocin only exists in mammals, a homologue of it, known as mesotocin is found in birds, marsupials, reptiles, amphibians and lung-fishes (for an overview see Donaldson and Young, 2008). It's worth noting, though, that a recent publication in *Nature* advocates for a universal nomenclature rooted in the genetic evolution of these nonapeptides (Theofanopoulou et al., 2021). In this framework, mesotocin is equivalently referred to as oxytocin. However, since this opens the door to potential confusion and controversy (Carter, 2022) and most of the present doctoral thesis was created before that time I will adhere to the term mesotocin. Research on mesotocin in birds has been relatively sparse compared to oxytocin in mammals, particularly concerning the measurement of peripheral concentrations. Studies employing manipulation techniques, such as administering mesotocin or oxytocin antagonists (Duque et al., 2020, 2018; Goodson et al., 2009, 2004; Kelly, 2019), as well as investigations into neural substrates using immunohistochemistry (Goodson et al., 2012), suggest that the mesotocinergic system plays a significant role in avian social behaviour, similar to mammals. This includes flocking behaviour (Goodson et al., 2012), pro-social behaviour (Duque et al., 2018), pair-bonding (Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012) and reproductive behaviour (Chokchaloemwong et al., 2013; Sinpru et al., 2017). There is, however, a scarcity of information regarding naturally occurring peripheral mesotocin levels in birds and their correlation with social behaviour in natural settings. With Chapter 3 of this thesis we start filling this gap by investigating salivary mesotocin in common ravens, *Corvus corax* (Stocker et al., 2021).

Overall, it is essential to be aware of the fact that hormone levels can be influenced by various factors, including age, sex, lactation, reproductive state, menstrual cycle, (socioeconomic) status, activity, food intake, illness/disease, and medication (Padilla et al., 2020). When planning a study it is therefore important to include these considerations in the study design. Within-subject study

designs, with, as mentioned before, proper controls, help standardize and contextualize measurements, allowing for more accurate and insightful relative assessments.

Validation

Before saliva can be used to reliably measure a certain analyte in a novel species, the applied methodology needs to be validated (which is also the case when other biospecimen are used). This is crucial because characteristics of salivary glands and of saliva itself as well as of other physiological aspects can differ between species (Tucker, 2010). An example of the latter is the difference in the major glucocorticoid i.e., cortisol or corticosterone, between animal taxa (Holmes and Phillips, 1976). There are three types of validation, the analytical validation (see (Andreasson et al., 2015; Möstl et al., 2005)), the physiological validation and the biological validation (Palme, 2019; Touma and Palme, 2005).

Typically, the validation process starts with an analytical validation, which serves the purpose of ensuring that the utilized assay effectively and consistently measures the target analyte in the relevant samples. This includes measures of assay precision, sensitivity, specificity and accuracy (for details see: Andreasson et al., 2015; Möstl et al., 2005; Palme, 2019). This step is followed by a physiological and/or biological validation, aiming i) to demonstrate that increases (or decreases) in circulating hormone levels are reflected in measured analyte concentrations, and ii) to get crucial information about temporal dynamics, i.e. the delay time between a certain stimulus and the detection of the following peak hormone concentrations as well as the clearance of the hormone (Palme, 2019).

Physiological validations involve stimulating the activity of a specific physiological pathway by administering an exogenous stimulant or suppressor. For example, administering adrenocorticotrophic hormone (ACTH) to an animal is anticipated to result in an elevated secretion of glucocorticoids by the adrenal glands (Möstl and Palme, 2002; Staley and Miller, 2020). Saliva samples are collected before and after the injection to assess if the concentrations of salivary glucocorticoids exhibit the expected increase (e.g., Aota et al., 2023; Heintz et al., 2011; Schwinn et al., 2018). Similarly, oxytocin can be administered, for example, subcutaneously (Kramer et al., 2004) or intranasally (Leeds et al., 2018), which is expected to result in increased peripheral oxytocin levels. After administering the exogenous stimulant, gathering multiple saliva samples can reveal the time delay between a stimulus and the subsequent peak hormone levels measured in saliva, along with how quickly the hormone clears.

Biological validations serve as an alternative or reinforcement to the findings from physiological validations (Staley and Miller, 2020). Instead of pharmacologically manipulating the physiological pathway, saliva samples can be collected before, during and after specific

situations that are expected to result in biologically meaningful hormonal changes. These situations include short events, but also circadian rhythms and different seasons. For glucocorticoids, this could be acutely stressful events such as transport (Behringer et al., 2014), social separation (Behringer et al., 2014; Pearson et al., 2008) or even the presence of a veterinarian (Verspeek et al., 2021), which are expected to activate the HPA axis and result in increased glucocorticoid concentrations. For sex hormones, such as testosterone, differences between sexes could be explored (Pribbenow et al., 2016). Note, though, that in some cases males and females have similar testosterone levels, depending on the male's reproductive state (Lipshutz and Rosvall, 2020). In the case of oxytocin, mating or play events could be investigated (Leeds et al., 2018).

In Chapter 2 (Stocker et al., 2023), 3 (Stocker et al., 2021) and 4 (Stocker et al., 2020) I present analytical and/or biological validations of salivary hormone measurements.

Salivary hormones in behavioural and cognitive biology – primates and corvids

Due to the short response time of salivary hormones, their measurement is particularly, but not exclusively, useful to determine short-term changes in hormone concentrations in a minimally/non-invasive way. This becomes especially advantageous in behavioural and cognitive research where scientists often want to investigate how hormone levels fluctuate in response to specific, often fleeting situations, both social and non-social, in order to understand the underlying mechanisms of certain behaviours. Experiments are, therefore, often designed in a way that saliva samples are collected before and after an individual is exposed to a certain experimental test condition (e.g., Pearson et al., 2015), taking into account the hormone's response time. By calculating the differences between these samples, researchers can gain insights into any ongoing changes in the hormone of interest. Since, as mentioned earlier, hormone levels do not only differ within an individual but also often vastly vary between individuals (see e.g., Ouyang et al., 2011), such a within subject study design in which an individual is its own control, helps to control for individual differences.

Non-human primates as well as corvids, a bird family that includes crows, ravens, jays and magpies, are fascinating study species for behaviour and cognition research due to their advanced cognitive abilities and complex behaviours. They exhibit remarkable problem-solving skills, tool use, and high levels of learning and memory (reviewed by: Emery and Clayton, 2004; Güntürkün and Bugnyar, 2016; Tomasello and Call, 1997), making them ideal candidates for investigating intelligence and cognitive evolution. Both primates and corvids live in complex social groups and engage in intricate social behaviours like cooperation, deception, and social learning (reviewed by: Güntürkün and Bugnyar, 2016; Hall and Brosnan, 2017; Whiten and van

de Waal, 2017). By studying their social interactions, researchers can gain insights into the evolution of social behaviour and the underlying cognitive processes involved. Moreover, comparing human behaviour and cognition with that of non-human primates and corvids provides insights into both the divergent and convergent evolutionary origins of human intelligence and social behaviour, and the potential interplay between intelligence and sociality (see for example: Burkart et al., 2014; Massen et al., 2010; Platt et al., 2016). Overall, research on primates and corvids contributes to our understanding of animal and human behaviour and cognition, with implications for fields such as neuroscience, psychology, and evolutionary biology.

Endocrinological research focusing on behaviour and cognition has garnered significant attention in both non-human primates (Anestis, 2010; Crockford et al., 2017; Trumble et al., 2015; Ziegler and Crockford, 2017) and corvids (de la Cruz et al., 2003; Duque et al., 2020, 2018; Munteanu et al., 2016; Stocker et al., 2016; Stöwe et al., 2008). However, despite the growing body of research utilizing non-invasive methods to monitor endocrine status in primates (Behringer and Deschner, 2017; Heistermann, 2010; Novak et al., 2013) and corvids (Munteanu et al., 2016; Stocker et al., 2016; Stöwe et al., 2008), salivary hormone analyses remain relatively limited.

A comprehensive literature search on PubMed (August, 2023) revealed 55 publications on salivary hormones in non-human primates (including the publications presented in Chapter 2 (Stocker et al., 2023) and 4 (Stocker et al., 2020)). To retrieve these publications I applied the non-human primate search string of Cassidy and colleagues (2021) and added various other search terms for “saliva” and “hormones” (see appendix p. 119: *Comprehensive literature search on PubMed*). Five more publications were found on Google Scholar, making a total of 60 (only research articles; see appendix p. 121: Table A1). These 60 studies were conducted in only 15 primate species, mostly in rhesus macaques, *Macaca mulatta* (e.g., Boyce et al., 1995; Higham et al., 2010; Lutz et al., 2000; Pfefferle et al., 2018) and the different ape species, and in only three different New World primate species – squirrel monkeys, *Saimiri sciureus* (e.g., Fuchs et al., 1997), common marmosets, *Callithrix jacchus* (e.g., Cross et al., 2004) and pied tamarins, *Saguinus bicolor* (Price et al., 2019). While 52 studies investigated cortisol levels (some not exclusively) (e.g., Behringer et al., 2013; Kutsukake et al., 2018; Pearson et al., 2015; Price et al., 2019), only five studies examined oxytocin levels (Festante et al., 2021; Heintz et al., 2019; Leeds et al., 2020, 2018; Simpson et al., 2014) and six testosterone (Arslan et al., 1984; Kutsukake et al., 2019, 2009; von Engelhardt et al., 2000; Wobber et al., 2013, 2010). This pattern also signifies the simplicity of cortisol measurement compared to, for example, oxytocin measurements. Nevertheless, as described earlier, assay validations are crucial to ensure accurate hormone measurement whenever a hormone is measured in a novel substrate or species. In non-human primates, methods to measure salivary cortisol have been (partly) validated in, for example, chimpanzees, *Pan troglodytes* (Heintz et al., 2011), gorillas, *Gorilla gorilla gorilla* (Leeds et al., 2018), rhesus

macaques, *Macaca mulatta* (Higham et al., 2010), common marmosets, *Callithrix jacchus* (Ash et al., 2018) and squirrel monkeys, *Saimiri sciureus* (Fuchs et al., 1997). Such validations establish a robust foundation for forthcoming studies to build upon and as such have the potential to guide and shape the trajectory of future research endeavours in this domain. While about one half of the 60 studies on salivary hormones in non-human primates focuses on method validation, stress physiology (Behringer et al., 2013; Cross and Rogers, 2004), and medical/pharmacological research, particularly in the realm of drug studies (McKenzie-Quirk and Miczek, 2008; Platt et al., 2007), the other half is dedicated to exploring animal behavior and cognition. This includes studies investigating social behaviour, for instance, the physiological response to inequity (Verspeek and Stevens, 2023) and competition (Wobber et al., 2010) and the influence of hormones on caregiver–infant interactions (Simpson et al., 2014). Furthermore, it includes studies on cognitive processes such as decision making (Bosc et al., 2017), and the effect of enrichment and training on salivary cortisol (e.g., Behringer et al., 2022, 2014; Fagot et al., 2014; Leeds et al., 2020).

In birds only four publications report to have measured salivary hormones, of which only two were conducted in corvid species. In 2011, the first study to attempt measuring corticosterone and also testosterone in birds' saliva (cockatiel, *Nymphicus hollandicus*; rose-ringed parakeet, *Psittacula krameri*) concluded that saliva does not appear to be a good medium for reliable determination of hormone concentrations (Hahn et al., 2011). However, a decade later, this concept has undergone re-evaluation, yielding significant advancements within the field. In fact, a pioneering paper authored by our team in 2021 (Stocker et al., 2021) presented the first measurement of mesotocin (the avian homologue to mammalian oxytocin) within the saliva of a bird species, the common raven, *Corvus corax*, as elaborated in Chapter 3. Currently, only one additional study pertains to the validation of the measurement of salivary corticosterone in large-billed crows, *Corvus macrorhynchos* (Aota et al., 2023), alongside a study discussing salivary corticosterone in wild common gulls, *Larus canus* (Carbillet et al., 2024).

While the current body of literature on salivary hormone analysis in primates and even more so in birds remains modest, its non-invasive nature and the growing interest in endocrinology suggest that this method holds significant potential for future research endeavours in understanding the hormonal regulation of behaviour and cognition in primate as well as avian species.

Outline of the thesis

This doctoral thesis endeavours to bridge some of the existing knowledge gaps by furthering the understanding of salivary hormone collection and measurement in both non-human primate and

corvid species, particularly for the use of behavioural or cognitive studies. Through targeted research that applies positive reinforcement training to acquire saliva samples (see p. 59 for more information on a training protocol), this thesis seeks to advance our capabilities in this critical area of investigation.

Chapters 2 and 3 of the doctoral thesis delve into the (partial) validation of salivary hormone measurements in two New World primate species and a representative of the corvid family, respectively, all species that are frequently studied in cognitive and behavioural biology.

Of the 60 studies that have included salivary hormone measurements in non-human primates only 25,8% were conducted in New World primates. To promote further (endocrinological) research in this primate group, in **Chapter 2**, we validated cortisol measurements in brown capuchin monkeys' (*Sapajus spp.*) and squirrel monkeys' (*Saimiri sciureus*) saliva using a specific enzyme immunoassay.

Chapter 3 takes a pioneering approach to exploring saliva sampling feasibility in birds, particularly the measurement of salivary mesotocin, the avian homologue of oxytocin. This novel endeavour involves positive reinforcement training in common ravens (*Corvus corax*) to obtain saliva samples. Our findings indicate that during the breeding season (spring), mesotocin levels surpass those observed in the mating season (winter), underscoring the ability to detect biologically significant fluctuations in mesotocin within raven saliva. In the field of behaviour and cognition, ravens are one of the most widely studied corvid species, and the possibility to measure salivary hormones in these birds opens many intriguing opportunities to study the link between their behaviour and cognitive abilities and hormones.

While Chapters 2 and 3 concentrate on implementing hormone measurements, **Chapter 4** expands on the application of these physiological measures in cognitive and behavioural studies to unravel the proximate mechanisms of animal social behaviour. In this chapter, the focus shifts to the relationship between cooperation, social bonds, and cortisol regulation in long-tailed macaques (*Macaca fascicularis*). This chapter uncovers how cortisol levels, indicative of stress, decrease during cooperation with closely bonded individuals, contributing to our understanding of the hypothalamic-pituitary-adrenal axis' role in cooperative behaviour.

Finally, in **Chapter 5**, I present a general discussion and conclusion of my PhD, followed by the **Appendix** which includes a review article, one additional research paper (*in prep*) related to Chapter 4, the list of publications investigating salivary hormones in non-human primates and corvids, and a general abstract.

Altogether, this doctoral thesis discusses saliva sampling in non-human primates and corvids as a tool for non-invasive monitoring of the endocrine status in the course of cognitive and behavioural studies, with the primary goal of fostering a deeper understanding of animal (social) behaviour and cognition.

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Chapter 2.

Published in Biology, 2023, 12(9), 1181.

Measurement of salivary cortisol in two New World primate species

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DOI: 10.3390/biology12091181

Simple Summary

Glucocorticoids (GCs), play a vital role in the body's response to stress. Therefore, scientists use GC-level measurements as a valuable tool to understand the well-being of animals in behavioral, conservation and welfare research. GC levels can be measured from different sample types, one of them being saliva. Saliva samples are especially useful because they can be collected rather easily and in a non-invasive manner. The aim of this study is to validate a method to measure salivary GCs, i.e., an enzyme immunoassay, in two monkey species: capuchin monkeys and squirrel monkeys. Individuals of both species were trained to provide saliva samples. Using high-pressure liquid chromatography, we confirmed that two main GCs, cortisol and cortisone, are present in saliva of these species and that they can be detected by the enzyme immunoassay. We further verified the biological validity of the assay in the two species by showing that it can track the decrease in cortisol levels throughout the day, which is typical in these monkeys. Our findings support the usefulness of the enzyme immunoassay to measure salivary cortisol levels in these non-human primate species and highlight its potential as a valid tool in research and welfare assessment.

Abstract

Glucocorticoids (GCs) are mammalian steroid hormones involved in a variety of physiological processes, including metabolism, the immune response, and cardiovascular functions. Due to their link to the physiological stress response, GC measurement is a valuable tool for conservation and welfare assessment in animal populations. GC levels can be measured from different matrices such as urine and faeces. Moreover, especially in captive settings, measuring GCs from saliva samples proved particularly useful as those samples can be collected non-invasively and easily from trained animals. Salivary GC levels can be measured using a variety of analytical methods, such as enzyme immunoassays. However, it is crucial to validate the analytical method for each specific application and species when using a new matrix. Using high-pressure-liquid chromatography and a cortisol enzyme immunoassay, we show that the main glucocorticoids secreted in the saliva of squirrel monkeys and brown capuchin monkeys are cortisol and cortisone. Our biological validation found the expected salivary cortisol level decline throughout the day. Our findings support the reliability of salivary cortisol measurements and their potential to be used as a valid tool in research and welfare assessment for these non-human primates.

Keywords

HPLC; enzyme immunoassay; saliva swab; glucocorticoid; steroid hormone; capuchin monkey; squirrel monkey; *Sapajus* spp.; *Saimiri sciureus*

Introduction

Glucocorticoids (GCs) are steroid hormones involved in a wide range of vital physiological processes in mammals (Hadley, 2000). In behavioural, welfare, and conservation research, monitoring changes in GC levels have received attention due to their involvement in the physiological stress response (e.g., Angelier & Wingfield, 2013; Sapolsky et al., 2000) and their association with emotional states (e.g., Erickson et al., 2003). When the hypothalamic-pituitary-adrenal (HPA) axis is stimulated by a stressor that threatens homeostasis, cortisol (the main GC in many mammals) is released into the bloodstream to restore it (Sapolsky et al., 2000). Therefore, an elevation in cortisol levels indicates a threat to homeostasis and the organism's effort to restore it (Karatsoreos & McEwen, 2010; Romero & Beattie, 2022). This adaptive process of maintaining homeostasis and the accompanying changes in cortisol levels can be activated by a multitude of diverse factors, including food intake, activity patterns (such as increased movement), mating, and external stressors like extreme ambient temperature, noise exposure and social conflicts (Romero & Wingfield, 2016). Additionally, cortisol levels are influenced by life-history factors such as reproductive periods and events, as well as environmental factors like season (Behringer et al., 2022; Crespi et al., 2013; Landys et al., 2006; Miller et al., 2016). Furthermore, pleasurable experiences can also cause an increase in cortisol levels (Ralph & Tilbrook, 2016).

Cortisol levels in the blood follow a typical fluctuation pattern over a 24-hour cycle, known as the diurnal rhythm of cortisol. In species that are active during the day, cortisol levels peak in the morning shortly after waking up, and then decrease rapidly in the hours that follow. During the first few hours of sleep, blood cortisol levels reach their lowest point and remain low throughout the night (Kirschbaum & Hellhammer, 2008; Pruessner et al., 1997; Ross et al., 2014; Weitzman et al., 1971). This diurnal pattern is observed in various diurnal species, including horses (Bohák et al., 2013), cetaceans (bottlenose dolphins and orcas Suzuki et al., 2003), elephants (Brown et al., 2010), and apes like bonobos (Verspeek et al., 2021). It can be detected not only in blood but also in other body fluids such as saliva (e.g., Bohák et al., 2013; Menargues et al., 2012; Verspeek et al., 2021) and urine (Brown et al., 2010). This rhythmic pattern has been associated with the functioning of various processes, including sleep-wake patterns (van de Werken et al., 2014), metabolism, learning, and memory (Law & Clow, 2020), and immune functions (Hucklebridge et al., 2005). Due to the involvement of GCs, their measurement has become a crucial conservation and welfare assessment tool for animals (Madliger et al., 2018).

Changes in levels of cortisol metabolites can be monitored in different organic matrixes such as urine and faeces (Sheriff et al., 2011). Non-invasive methods of GC measurement have been developed and validated as invasive blood sampling can be a stressor to the animal and

sometimes impractical in the field (Sheriff et al., 2011). The analysis of cortisol level changes from saliva samples is particularly convenient in captive settings, as individuals can easily be trained to provide saliva samples (e.g., Behringer et al., 2022; Stocker et al., 2020). Furthermore, this method allows frequent sampling of the same individual, in fast succession. Moreover, salivary cortisol levels are highly correlated with serum cortisol concentration at a given time point (e.g., Kahn et al., 1988; Kirschbaum & Hellhammer, 2008; Vincent & Michell, 1992).

For the assessment of steroid hormone concentration in organic substrates (saliva, milk, urine, faeces, hair, feathers), many excellent methods, such as enzyme immunoassays (EIAs) and mass spectrometry (Taylor et al., 2015) are available (e.g., reviewed in Behringer & Deschner, 2017; Heistermann, 2010; Palme, 2019). Differences in biochemical and structural characteristics of samples between substrates and species can have major implications for the suitability of an existing immunoassay to measure steroid hormone levels in a new substrate or species (Buchanan & Goldsmith, 2004; Palme, 2005). First, depending on sample type and species, the target molecule may differ: for instance, the primary glucocorticoid found in blood or saliva of many mammalian species is cortisol (e.g., Botía et al., 2023). In faeces of these species, native cortisol is usually not found (in large quantities) but only metabolized forms of glucocorticoids can be measured (Palme et al., 2005). Thus, an immunoassay with appropriate binding characteristics, that may differ depending on sample type, has to be selected. Secondly, other, non-target molecules in a sample may bind to the antibody (cross-reactions), which would lead to false results for the target hormone. Hence, before using an immunoassay for a new substrate and/or species, molecules that may cross-react with the binding agents have to be identified (Higham, 2016). Thirdly, species may differ in how they metabolize and excrete hormones (Palme et al., 2005). Examining which hormones or hormone metabolites should be targeted by an assay for a given sample substrate and species is of utmost importance to render biologically meaningful results. To summarize, it is crucial to validate EIAs when applied to new species and sample types to ensure accurate and reliable GC measurements (Buchanan & Goldsmith, 2004; Palme, 2019; Sheriff et al., 2011; Touma & Palme, 2005).

Capuchin monkeys (*Cebus* spp., *Sapajus* spp.) and squirrel monkeys (*Saimiri* spp.), both belonging to the family Cebidae, are intensively studied in biomedical research, neuroscience, cognitive biology, or metabolic disorders (Abee, 2000; Fragaszy, 1985; Fragaszy et al., 2004; Lankau et al., 2014). Furthermore, capuchin monkeys and squirrel monkeys are often exhibited in zoos. Therefore, assessment of GC levels as a metabolic or stress-related marker may be valuable. In capuchin monkeys, cortisol and/or its metabolites has already been measured in hair (Phillips et al., 2018) and faeces (Mendonça-Furtado et al., 2017; Wheeler et al., 2013). However, to our knowledge, there are no peer-reviewed studies published, which measured salivary cortisol in capuchin monkeys. In squirrel monkeys, at least six studies have investigated salivary

cortisol levels so far (Fuchs et al., 1997; Lee et al., 2003, 2004; McKenzie-Quirk & Miczek, 2008; Platt et al., 2007; Tiefenbacher et al., 2003). Most of those studies are based on a rather small sample size and only a single study reported having measured salivary cortisol levels in a female individual (one male and one female, Fuchs et al., 1997); the remaining published work has either only reported measurements in males, or sex has been unreported (Lee et al., 2003, 2004; McKenzie-Quirk & Miczek, 2008; Platt et al., 2007; Tiefenbacher et al., 2003).

To promote studies using cortisol measurements gained from non-invasively collected saliva samples in these two non-human primate species that are often kept under human care, the aim of the current study was to validate an in-house cortisol EIA (antibodies against cortisol-3-CMO:BSA; Palme & Möstl, 1997). To that end, we collected saliva samples of male brown capuchin monkeys (*Sapajus* spp.) and a group of female squirrel monkeys (*Saimiri sciureus*). We performed a high-performance liquid chromatography (HPLC) to confirm that the target molecule cortisol was indeed present in the saliva of both species and could therefore be detected by the chosen cortisol EIA and to detect molecules that may cross-react with the assay antibodies. To assess the reliability of cortisol measurements, we examined parallelism of serially diluted pooled saliva samples and the standard curves of the assay for each species. Finally, we quantified cortisol concentrations from a of total 455 saliva samples (114 capuchin monkey samples, 341 squirrel monkey samples) with the chosen cortisol EIA and tested the biological validity by investigating whether salivary cortisol concentrations decreased throughout the day, as would be predicted by the circadian cortisol secretion pattern shown in blood for both species (capuchin monkeys, Torres-Farfan et al., 2008, squirrel monkeys, Coe & Levine, 1995).

Materials and Methods

Animals and housing

Saliva samples were collected from 17 female squirrel monkeys, *Saimiri sciureus* (mean age = 6.69 years $\pm$ 2.62 SD) and eight male brown capuchin monkeys, *Sapajus ssp.* (mean age = 5.19 years $\pm$ 2.02 SD) housed at the Living Links to Human Evolution Research Centre at Edinburgh Zoo, UK. The two species were co-housed in the same enclosures and, thus, lived in two mixed-sex and mixed-species groups, the “East group” and the “West group”. At the time of testing the East group consisted of 13 capuchin monkeys (6 females and 7 males) and 19 squirrel monkeys (15 females and two males). The West group consisted of 16 capuchin monkeys (7 females, 7 males, and two unsexed infants) and 16 squirrel monkeys (14 females, 2 males). Each group had an indoor (31.50 m², 12h light-dark cycle 07:30-19:30) and outdoor area (approx. 900 m²) shared by all individuals of each group. Additionally, the squirrel monkeys of both groups had an indoor area (24.75 m²),

which was not accessible for the capuchin monkeys. Both species received main feeds in all indoor areas twice a day with a mix of vegetables and fruit (Leonardi et al., 2010). Three to four additional scatter feeds were provided at variable times throughout the day and water was available *ad libitum*.

Saliva collection

Using positive reinforcement training, monkeys were trained to chew on an oral swab (Salimetrics, SalivaBio Children's Swab) that was presented to them by the experimenter. Once an individual was able to provide saliva samples, data collection for the project started. Samples were collected within one minute, put in a labelled plastic vial, placed immediately on ice packs and stored in a freezer at -20°C within one hour after sample collection. To avoid hormonal contamination, the monkeys were trained not to touch the swabs with their hands and the experimenters wore gloves.

Saliva samples of the squirrel monkeys were collected between 28-01-2013 and 16-12-2013. The capuchin monkey sample collection took place between 16-09-2013 and 09-12-2013. All samples were collected between 11:00-13:00 and 14:00-16:00, as the study aimed to investigate the daily pattern of cortisol. Data collection times at Edinburgh Zoo were restricted, which did not allow for sample collection to cover the complete circadian cortisol excretion pattern. Saliva collection was conducted in separate research cubicles (49.5 cm x 52.1 cm x 51.4 cm; separate ones for each species) arranged in a 2x4 matrix that created a corridor connecting the indoor and outdoor enclosure (see Figure 1). Partitioning slides were inserted between cubicles to separate individual monkeys from their groupmates – a procedure these individuals were familiar with. Once an individual was isolated in a research cubicle and all other monkeys had left, the entrances to the cubicles were closed, and the saliva sample collection started, typically taking less than one minute. Participation of the monkeys was strictly voluntary. If an individual showed clear signs of discomfort, such as persistently touching the door leading to its enclosure, prior to or during saliva collection, the individual was promptly released, and the saliva collection session was terminated.

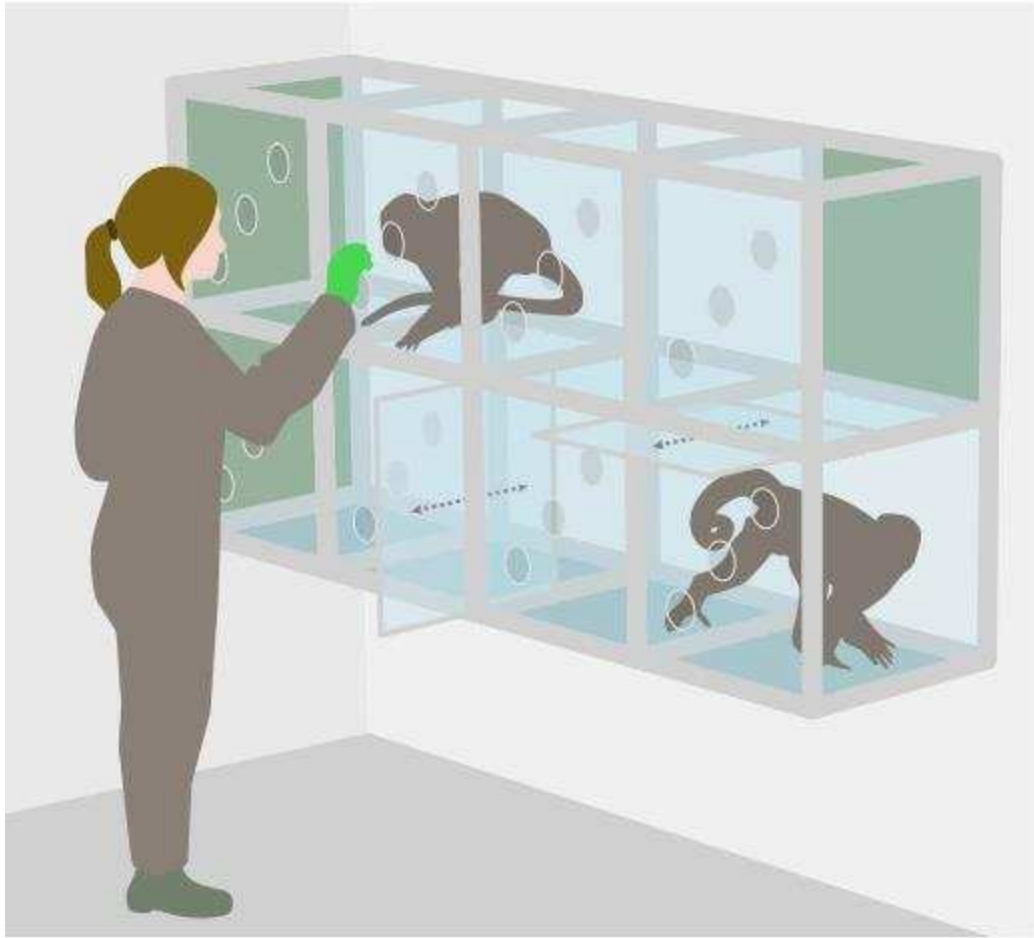


Figure 1. Research cubicles at Living Links Research Centre, Edinburgh Zoo. The green squares on the left indicate the exits towards the outdoor enclosure and on the right towards the indoor enclosure. All exits can be closed with sliding doors. Between each cubicle, partitioning slides can be inserted to separate individual monkeys.

Salivary cortisol analyses

Samples of both species were transported from Edinburgh to Vienna overnight on dry ice. In February 2014, the samples were analysed in the Endocrinology Laboratory of the Department of Behavioural Biology, University of Vienna, Austria. Prior to analysis samples were stored frozen at -20°C. In the laboratory, the saliva swabs were thawed, followed by centrifugation (3600 rpm, 10 min). The resulting saliva was then diluted with assay buffer using predetermined (through serial dilution of pooled samples) ratios (squirrel monkeys 1:400, capuchin monkeys 1:1000).

Some samples were not analysed due to contamination or insufficient amount of saliva. In total, 114 capuchin monkey saliva samples and 341 squirrel monkey saliva samples were analysed in duplicates using an in-house cortisol enzyme immunoassay (EIA) with an assay antibody against cortisol-3-CMO:BSA. This EIA demonstrated the following cross-reactions:

cortisol, 100%; cortisone, 9.6%; corticosterone, 6.2%; 5 α -dihydrocortisol, 4.6%; 5 α -tetrahydrocortisol, 0.8%; 5 β -tetrahydrocortisol, 0.1%; several other 5 β -reduced metabolites, <0.01%. For a more detailed description of the assay, please refer to Palme and Möstl (1997).

For the capuchin monkeys intra- and inter-assay coefficients of variation were 8.1% and 10.8%, respectively. In the squirrel monkeys, the intra-assay coefficient was 7.7% and the inter-assay coefficient 8.6%.

Biochemical validation

To characterize the substances measured using the selected cortisol EIA, we performed a reversed-phase high-performance liquid chromatography (RP-HPLC) on Novapak C18 columns (3.9 x 150 mm, Millipore Corporation, Milford, MA, USA) with a Mini-Guard column (C18 Guard-Pak TM, WAT085825, Waters Corp., UK) used at room temperature with the following linear gradient: 30% to 50% methanol/water over 30 min. The flow rate was 1 ml per min and three fractions per minute were collected (Keckeis et al., 2012). One pool sample (mix of aliquots of samples collected in 2013) for each species was used for this analysis. The immunoreactivity was measured in each fraction using the cortisol EIA (see Palme & Möstl, 1997). The RP-HPLC analysis was conducted at the Department of Biomedical Sciences of the University of Veterinary Medicine, Vienna, Austria.

Furthermore, we pooled 10 capuchin monkey and 10 squirrel monkey saliva samples and serially diluted the pools with assay buffer to assess parallelism. The capuchin monkey sample pool was diluted 1:10, 1:50, 1:100, 1:200, 1:300, 1:400, 1:500, 1:1000, 1:10000 and 1:50000. The squirrel monkey samples pool was diluted 1:100, 1:200, 1:300, 1:400, 1:450, 1:500, 1:550, 1:600, 1:700, and 1:800.

Statistical analyses

To investigate the daily variation pattern in cortisol secretion (within the two time frames of the research slots) we constructed linear mixed-effect models for each species. To achieve a more symmetrical distribution, we log-transformed the response variable cortisol concentration. Sampling time in minutes since midnight was included as predictor variable and the age of the individual in days at the time of sampling as control variable. As individuals may vary in regard to their salivary cortisol excretion profiles and potential group effects on salivary cortisol levels, random intercepts for individual (ID) and group were included in the models. However, due to a singularity issue resulting from the lack of variance accounted for by the group variable, group was excluded from the model in both species.

All statistical analyses were performed using R version 3.5.2 (R Core Team, 2018). The mixed models were constructed using the *lmer* function of the *lme4* package (Bates et al., 2015) and fitted with maximum likelihood estimation. P-values were generated using the *lmerTest* package (Kuznetsova et al., 2017). Figures were created using the *ggplot* package (Wickham, 2016). To ensure the absence of collinearity issues, we inspected variance inflation factors (VIF: squirrel monkeys = 1.015, capuchin monkeys = 1.010) using the *vif* function of the *car* package (Fox & Weisberg, 2019). Diagnostic plots of model residuals were examined to identify any potential violations of the normality assumption and homogeneity of variance. Initially, the residuals of the model for the capuchin monkeys were not normally distributed. To ensure normal distribution of the residuals, two data points with deviating residuals were removed from the data set.

Results

Biochemical validation (HPLC and parallelism)

Squirrel and capuchin monkeys showed very similar profiles of immunoreactivity. In both species, two main peaks were identified (Figure 2), which showed immunoreactivity with the cortisol EIA. The first peak eluted at the approximate position of the cortisone standard, the second at the position of cortisol. Unfortunately, the pool samples of the two species were analysed at different times and different HPLC columns were used for the separation. Therefore, the standards (and thus also the immunoreactivity) eluted at slightly different positions (later in case of capuchin monkeys). Serially diluted pooled saliva samples were parallel to the cortisol standard curve in both, capuchin and squirrel monkeys (Figure 3).

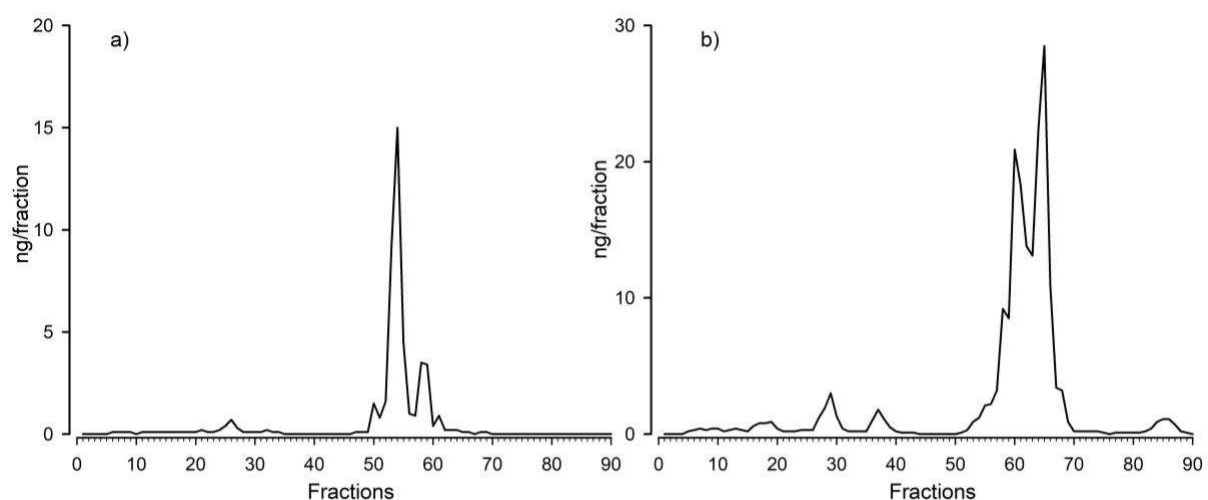


Figure 2. High-performance liquid chromatography (reverse phase) separation of immunoreactive substances in a salivary pool sample of a) squirrel monkeys and b) capuchin monkeys when measured with the cortisol EIA.

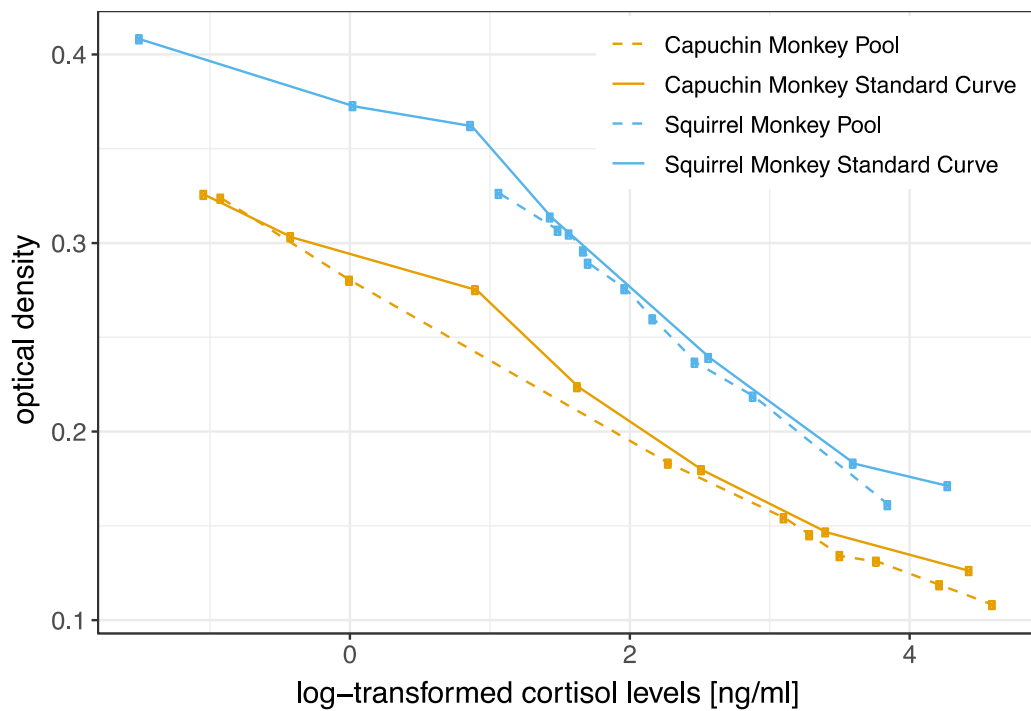


Figure 3. Parallelism between standard curve (solid lines) and serial dilutions of salivary cortisol (dashed lines) of capuchin monkeys (orange lines) and squirrel monkeys (blue lines). The y-axis shows the optical density, the x-axis shows log-transformed levels of cortisol [ng/ml]. For both species, standard curves and serial dilution curves are parallel to each other.

Daily pattern in cortisol

Overall, salivary cortisol levels in squirrel monkeys ranged between 2.3 and 1957.5 ng/ml and 3.0 and 454.8 ng/ml in capuchin monkeys. Table 1 gives an overview of salivary cortisol level measures by species. Linear mixed effect models found that in both species, cortisol concentrations declined in the course of the day (Table 2, Figure 4).

Table 1. Descriptive statistics of salivary cortisol levels in capuchin monkeys and squirrel monkeys.

	Sex	Individuals	Samples	Salivary cortisol [ng/ml]					
				Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
Squirrel monkeys	females	17	341	2.33	33.38	61.35	96.13	119.08	1957.52
Capuchin monkeys	males	8	114	3.00	60.38	95.25	123.77	179.18	454.80

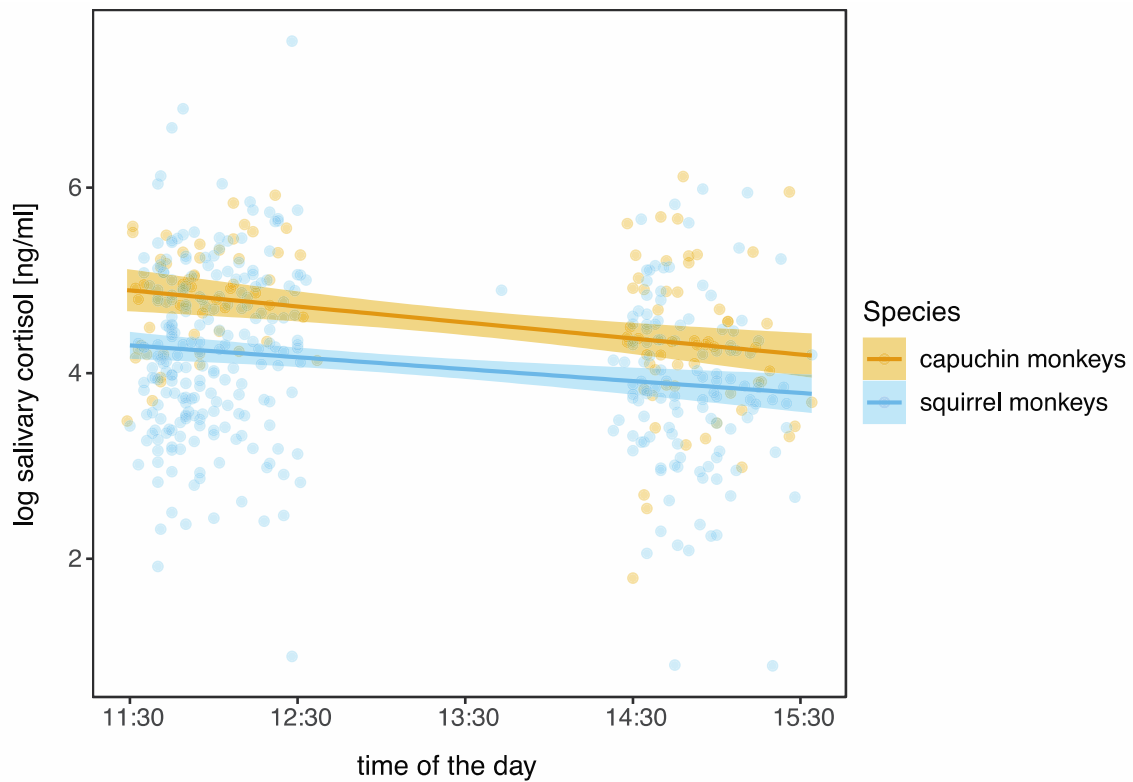


Figure 4. Diurnal patterns of salivary cortisol levels (log-transformed) plotted against time of the day for capuchin monkeys (orange) and squirrel monkeys (blue). Individual dots represent individual datapoints. The lines are linear regression lines with 95% confidence intervals as calculated from the model. Salivary cortisol levels decrease throughout the day in both, capuchin and squirrel monkeys, as expected due to the circadian rhythm in salivary cortisol excretion patterns.

Table 2. Results of the linear mixed effects model of the squirrel monkeys and the capuchin monkeys. Given are estimates, standard errors (SE), confidence intervals (CI), degrees of freedom (df), t-values and p-values.

	Parameter	Estimate	SE	CI	df	t	p
Squirrel monkeys	(Intercept)	5.996	0.516	(4.980, 7.041)	158.700	11.621	< 0.001 ***
	time of day	-0.002	0.001	(-0.003, -0.001)	328.000	-3.600	< 0.001 ***
	age (in days)	0.000	0.000	(0.000, 0.000)	13.140	-0.739	
Capuchin monkeys	(Intercept)	7.313	0.666	(5.978, 8.631)	68.700	10.987	< 0.001 ***
	time of day	-0.003	0.001	(-0.004, -0.002)	104.700	-4.488	< 0.001 ***
	age (in days)	0.000	0.000	(-0.001, 0.000)	7.999	-0.836	

Discussion

To the best of our knowledge, our study represents the first investigation to measure salivary cortisol in capuchin monkeys (*Sapajus spp.*) and a group of female squirrel monkeys (*Saimiri sciureus*). Additionally, it is the first study to utilize an EIA for the measurement of salivary cortisol in the two monkey species. Previous studies on salivary cortisol in squirrel monkeys (male or sex not reported) have employed different methods such as radioimmunoassays (RIAs; Lee et al., 2003, 2004; McKenzie-Quirk & Miczek, 2008; Platt et al., 2007; Tiefenbacher et al., 2003) or time-resolved fluorescence detection (DELFA; Fuchs et al., 1997) for cortisol determination. Our findings demonstrate that the two species have salivary GCs and that they can be reliably measured using a cortisol EIA.

The immunograms obtained from the HPLC and the EIA using antibodies raised against cortisol-3-CMO:BSA (Palme & Möstl, 1997) confirmed that saliva of both species contained cortisol. Besides the peaks of cortisol, the RP-HPLC immunograms showed another peak (even dominating in squirrel monkeys) appearing somewhat earlier that approximately corresponded with the position where cortisone eluted (cross-reactivity = 9.6 %). Cortisone was also detected previously in the faeces of capuchin monkeys (Mendonça-Furtado et al., 2017) or in the urine of marmoset monkeys (Pryce et al., 2002) using the same EIA, and in the blood of other non-human primates (e.g., Schmidt & Squires, 1951; Waddell et al., 1988). Due to its cross-reactivity with the cortisol EIA, the presence of cortisone in these New World primates' saliva should not be entirely overlooked. Several studies found cortisol as well as cortisone in human saliva (e.g., Katz & Shannon, 1964; Meulenberg et al., 1987; Morineau et al., 1997). Meulenberg and colleagues (1987) reported that cortisone and cortisol levels correlate in saliva as well as plasma in humans. Furthermore, cortisone follows a circadian pattern that runs parallel to the rhythm of cortisol and that can be detected in saliva, urine, and blood of human subjects (Morineau et al., 1997; Nomura et al., 1997). Cortisone levels in human saliva were higher than cortisol concentrations (Katz & Shannon, 1964; Morineau et al., 1997), which was attributed to the activity of an enzyme in the parotid glands that oxidizes cortisol to cortisone (Katz & Shannon, 1964; Meulenberg et al., 1987). Similar patterns and relationships between cortisone and cortisol levels were found in the saliva and might be expected in the blood of capuchin and squirrel monkeys. On the one hand, this calls for further studies exploring levels of cortisone (in relation to levels of cortisol) in salivary samples of these two primates. On the other hand, it suggests that cortisone should be considered in study designs and that measurement of cortisone might be an alternative in future studies (e.g., should cortisone levels indeed be higher but correlated to cortisol levels in saliva of these monkey species, required amounts of saliva could be reduced for instance).

Furthermore, we validated our assay by showing that concentrations of serially diluted pooled saliva samples were parallel to the cortisol standard curves in both species. This suggests that the assay reliably measures cortisol levels in the saliva of both New World monkey species. An ACTH (adrenocorticotrophic hormone) challenge, which is a commonly used physiological validation method (Palme, 2019), was not performed in the scope of the current study as no invasive procedures were performed. Therefore, we aimed to assess the biological suitability of the selected method for analysing salivary cortisol levels in the two New World primate species by determining relevant changes in salivary cortisol levels: we investigated whether salivary cortisol levels collected during the morning and afternoon research sessions corresponded to the species' diurnal secretion pattern of cortisol observed in blood samples (Coe & Levine, 1995; Torres-Farfan et al., 2008). Due to practical constraints, such as limited data collection times at Edinburgh Zoo, we were unable to examine the entire circadian cortisol excretion pattern. Nevertheless, our findings demonstrate that, as expected, daytime influenced salivary cortisol levels, with higher levels observed earlier in the day. This confirms that using the tested EIA allows for tracking biologically relevant changes in salivary cortisol levels in both species. Furthermore, many New World primate species were reported to have particularly high levels of (salivary) cortisol levels (Chrousos et al., 1986; Fuller et al., 2004), which was confirmed in our study: salivary cortisol levels in squirrel monkeys and capuchin monkeys were markedly higher than those reported for bonobos, gorillas, and orangutans in a study (Behringer et al., 2022) where saliva samples of these apes were measured with the same assay as employed in the current study.

Conclusion

In summary, we successfully validated an in-house cortisol enzyme immunoassay (EIA) for measuring salivary cortisol levels in capuchin monkeys and squirrel monkeys. Our results provide valuable information on the applicability of the employed method and can be informative for researchers interested in studying salivary cortisol in these New World primate species (e.g. Robinson et al., 2021). Further research exploring the relationship between cortisol and cortisone levels in the species' saliva is recommended to enhance our understanding of their hormonal profiles and stress responses.

Author Contributions: Conceptualization, M.S. and R.S.; Methodology, M.S., E.O., R.P. and R.S.; Validation, M.S., R.P. and R.S.; Formal Analysis, M.S. and R.S.; Investigation, M.S., E.O., R.P. and R.S.; Resources, E.O., R.P., E.M. and R.S.; Data Curation, M.S. and R.S.; Writing – Original Draft Preparation, M.S., R.P. and R.S.; Writing – Review & Editing, M.S., E.O., R.P., E.M. and R.S.; Visualization, M.S., R.P. and R.S.; Supervision, R.S..

Funding: R.S. was supported by the Austrian Science Fund (FWF, Young Independent Researcher Group (YIRG) grant; Grant Number ZK 66) and ERC Grant 230604 SOMACCA (to W. Tecumseh Fitch).

Institutional Review Board Statement: The study was approved by the scientific board of the Living Links and Budongo Research Consortium (Royal Zoological Society Scotland), the ethics board of Life Sciences of the University of Vienna and the University of Stirling Psychology Ethics Committee.

Data Availability Statement: Data are available on request.

Acknowledgments: The authors thank the animal keepers of Edinburgh Zoo and Living Links staff for their support on the research site, Luz Maria Guevara for her help with collecting saliva samples, Elisa Barnreiter and Samy El Makarem for their support in the endocrinology lab, Verena Behringer for constructive comments and Francisca van Hassel for the figure of the research cubicles.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3.

Published in Hormones and Behavior, 2021, 134, 105015.

Measuring salivary mesotocin in birds - Seasonal differences in ravens' peripheral mesotocin levels

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DOI: 10.1016/j.yhbeh.2021.105015

Abstract

Oxytocin is involved in a broad array of social behaviours. While saliva has been used regularly to investigate the role of oxytocin in social behaviour of mammal species, so far, to our knowledge, no-one has tried to measure its homolog, mesotocin, in birds' saliva. Therefore, in this study we measured salivary mesotocin in common ravens (*Corvus corax*), and subsequently explored its link to three aspects of raven sociality. We trained ravens (n=13) to voluntarily provide saliva samples and analysed salivary mesotocin with a commercial oxytocin enzyme-immunoassay kit, also suitable for mesotocin. After testing parallelism and recovery, we investigated the effect of bonding status, sex and season on mesotocin levels. We found that mesotocin was significantly more likely to be detected in samples taken during the breeding season (spring) than during the mating season (winter). In those samples in which mesotocin was detected, concentrations were also significantly higher during the breeding than during the mating season. In contrast, bonding status and sex were not found to relate to mesotocin detectability and concentrations. The seasonal differences in mesotocin correspond to behavioural patterns known to be associated with mesotocin/oxytocin, with ravens showing much more aggression during the mating season while being more tolerant of conspecifics in the breeding season. We show for the first time that saliva samples can be useful for the non-invasive determination of hormone levels in birds. However, the rate of successfully analysed samples was very low, and collection and analysis methods will benefit from further improvements.

Keywords

Common raven, *Corvus corax*, corvids, saliva, oral swab, enzyme immunoassay, nonapeptide, mesotocin, oxytocin, breeding season, animal training

Introduction

Behavioural endocrinology has long depended mainly on blood sampling techniques, which have advanced the knowledge in the field tremendously. However, if performed on untrained subjects, such invasive techniques are often associated with elevated stress levels and welfare issues, and can consequently lead to immediate modulation/change in hormonal concentrations. The consequent need for non-invasive methodologies has put forward alternative approaches such as quantifying hormones out of faeces, urine, saliva, milk, hair or feathers (reviews: Behringer and Deschner, 2017; Palme, 2019). In bird studies the most commonly selected matrices for hormone analysis are blood or droppings. Both techniques have their advantages and disadvantages: While the former entails the before-mentioned invasive approach, it does allow the detection of almost immediate hormonal changes. And while the latter is non-invasive, it does create a time-lag between the hormone release into the blood and the appearance of the hormone metabolites in the droppings, which results from gut passage time (Palme, 2019). Another limitation is that certain hormones, such as oxytocin (and its homologues), or their metabolites, cannot easily be measured in faeces. Consequently, the choice of the appropriate matrix depends on the hormone to be measured, the temporal scale of the research question and the feasibility of sample collection. So far, scientific progress in ornithology has been limited due to the lack of non-invasive techniques which are able to detect immediate or short-term changes in hormone concentrations.

Oxytocin (OT) is a nonapeptide that exhibits a broad range of central and peripheral effects, from the modulation of neuroendocrine reflexes to complex social behaviours (Gimpl and Fahrenholz, 2001). Depending on the species, the latter include social bonding, trust, maternal and alloparental care, cooperation, consolation, outgroup derogation and sexual behaviour (reviews: De Dreu and Kret, 2016; Goodson, 2013; Quintana and Guastella, 2020; Walum and Young, 2018). Whereas OT occurs mainly in mammals, a homologous form of it, mesotocin (MT), occurs in birds and reptiles. MT has so far, however, been much less investigated than OT, at least when it comes to measuring peripheral concentrations. Manipulation studies administering MT or OT antagonists (Duque et al., 2020, 2018; Kelly, 2019), or studies investigating neural substrates with immunohistochemistry (Goodson, 2013), however, do suggest that, similar to OT in mammals, MT plays an important role in social behaviour in birds. Nevertheless, there is not much information available on naturally occurring peripheral MT levels in birds and how they relate to social behaviour in natural contexts.

The first two goals of the present study are therefore to test the feasibility i) of training birds to voluntarily provide saliva samples and ii) of quantifying salivary MT in those samples. Since saliva samples allow a non-invasive assessment of the endocrine response to certain stimuli with a delay of only a few minutes, this would open up a suit of opportunities to examine the role

of MT in birds more directly. To do so, we tested common ravens (*Corvus corax*), which are well-suited for that endeavour: Because of their relatively large body size we expect them to produce more saliva than a small bird species, and their large beak facilitates saliva collection. Further, ravens have already been shown to be successful in an exchange paradigm (Massen et al., 2015; Müller et al. 2017), which could be used to collect saliva samples by exchanging swabs for a reward. Moreover, they have a relatively complex social life with individuals staying in non-breeder groups until they form pair bonds (Boucherie et al., 2019), and show marked seasonality with regard to breeding and mating, with the latter being accompanied with high levels of aggression (Braun and Bugnyar, 2012; Gwinner, 2003). In particular, in the present paper we make a clear distinction between the mating season, in winter, in which the birds are sexually highly active and the breeding season, in spring, which we refer to as the time period in which the birds usually lay eggs and care for their offspring.

Our third goal is, therefore, iii) to investigate whether salivary MT levels differ seasonally and/or are associated with the ravens' bonding status. In a comparative study on different sparrow species, Goodson and colleagues (2012) concluded that an increase in MT innervation in certain parts of the brain is important for flocking and may reduce aggression. Moreover, it was shown that the MTergic system is involved in reproductive behaviour (e.g. incubation) in Thai hens (*Gallus domesticus*) (Chokchaloemwong et al., 2013; Sinpru et al., 2017). We, therefore, expected ravens' MT levels to be higher in the breeding season (spring) than in the mating season (winter). Nonapeptides have also been shown to be involved in pair bonding. In zebra finches (*Taeniopygia guttata*) the administration of OT antagonists decreases pair formation (Pedersen and Tomaszycki, 2012) and influences pair maintenance behaviours (Kelly, 2019), and oxytocin-like receptors mediate pair bonding (Klatt and Goodson, 2013). We, therefore, expected pair-bonded ravens to have higher MT levels than group-living ones. Finally, nonapeptide effects are often sex-specific (Goodson, 2013; Kelly and Goodson, 2014), and MT levels seem to differ between males and females in some species. In White Leghorn chickens (*Gallus domesticus*), for instance, males have twice as much MT, at least in their neurohypophysis, as females (Robinson et al., 1990). Hence, we investigated the potential effect of sex on MT levels in ravens and predicted MT levels in males to be higher than in their female conspecifics.

Material and Methods

Animals and housing

The study was conducted on 13 ravens (7 males, 6 females; age: 2 to 6 years). They were housed at Haidlhof Research Station, Bad Vöslau, Austria. Towards the end of the study three pair-bonded

subjects were transferred to Cumberland Wildpark in Grünau, Austria. Six ravens were kept in a mixed-sex non-breeder group (aviary ~210m²) and seven were pair-bonded and kept in separate aviaries (~80m² each). All aviaries consisted of several compartments with sheltered areas for weather protection. The subjects were fed twice a day (meat, dairy products, vegetables, fruits and cereals), water was available ad libitum.

Saliva collection

We trained the ravens to take a saliva swab (~2.5 cm long piece of Salimetrics SalivaBio Children Swab) into their beak, place it in their throat pouch where saliva is accumulating, and return it on command. To achieve this, we followed a training protocol based on positive reinforcement (cf. Massen et al., 2015; see ESM). Saliva samples (max. three/subject/day) were collected opportunistically from whichever bird we could get them from on a given day, either before or at least 1h after the birds got fed. All samples were stored in Salimetrics Swab Storage Tubes at -20°C within 10 minutes after collection. Saliva samples were collected between May and June 2016 and in April and May 2017, representing the breeding seasons, and between November 2016 and January 2017, representing the mating season.

Hormone analysis

Salivary MT concentration was quantified using a commercially available enzyme-immunoassay (EIA) kit for oxytocin (Catalog No. K048-H1/H5, Arbor Assays, Michigan, USA), which has been used successfully to measure (salivary) OT in humans and other species (e.g. mice, *Mus musculus* (Ferrer-Pérez et al., 2019); dogs, *Canis familiaris* (Wirobski et al., 2021); gorillas, *Gorilla gorilla gorilla* (Leeds et al., 2018)). This assay has a cross-reactivity of 88.4% with MT and can therefore be used to measure MT. Saliva samples of the ravens were extracted following the instruction of the manufacturer and using the extraction solution provided in the kit. Briefly: To extract the samples the swabs were centrifuged at 1600g and 4°C for 20 min. Supernatant was pipetted into an Eppendorf tube, diluted with extraction solution (1:1.5), vortexed and incubated for 90 min at room temperature. After centrifugation (1600g at 4°C for 20 min), supernatant was pipetted into a glass tube and dried-down under a N₂-stream. Samples were then resuspended in 210µl assay buffer and processed following the producers EIA protocol. Final concentrations were corrected for dilution factor. All samples were analysed in duplicates. We calculated the intra-assay coefficient of variation (CV) from concentrations of duplicate aliquots. Samples with an intra-assay CV above 20% were excluded from our analysis and the mean CV of duplicates of all remaining samples was 5.9 ± 4.8% (mean ± SD). Inter-assay CV was calculated by comparing the optical density (OD) of two standards (i.e., at the high and low range of the curve) between assays

and were 10.3% and 11.4%, respectively. The detection limit reported by the manufacturer was 22.9 pg/ml.

Prior to analysing the individual samples, we successfully conducted a serial dilution of pooled raven saliva in triplicates (1:1 up to 1:8 dilution) to exclude any possibility of matrix effects (Tab. 1). The CV of corrected values of the serial dilution was 3.58%. Further, we tested the recovery of known amounts of MT in saliva samples. Recoveries were obtained by spiking a total of 16 aliquots of 50 µl of pooled raven saliva with two concentrations of standard mesotocin (Arbor Assay, Cat.nr. X127) and calculating the recovery in respect to the concentration of unextracted standards, after correcting for the concentration measured for the pooled saliva. Average recoveries for standards of 2000 pg/ml and 1000 pg/ml were 119% and 94%, respectively.

Table 1. Mesotocin concentrations in serially diluted pooled raven saliva.

Dilution	Mesotocin [pg/ml]	Upcalculated [pg/ml]
1:1	274.85	274.90
1:2	129.90	259.80
1:4	69.18	276.73
1:8	32.34	258.75
	mean	267.55
	SD	9.59
	CV%	3.58

Statistical Analysis

Since the MT concentration of many samples fell below the assay's detection limit, we first fitted a binomial model (*glmer*) to investigate if the detectability of MT within the samples depended on the ravens' bonding status, sex, season and/or on the saliva sample volume (prior analysis showed that sample volume correlated negatively with MT concentrations; see below). This model also comprised subject as random intercept effect and was compared to the null model only including this random effect. Subsequently we investigated the effect of the same factors on ravens' salivary MT concentrations (log-transformed). We computed a linear mixed-effects model (*lmer*), which included bonding status, sex and season as fixed effects, sampling time since sunrise as an offset, and subject as random intercept effect. This main-effects model was compared to the null model, which included only subject as random intercept effect and time since sunrise as an offset. We visually inspected whether the model residuals were normally distributed and homogenous. We detected no multicollinearity issues (max. variance inflation factor = 1.617). Effect sizes were estimated via partial omega squared. Prior to constructing the models, we found

that sample volume correlated negatively with MT concentrations ($n = 20$ samples, $r^2 = -0.49$, $p = 0.027$). Since including saliva volume as fixed effect into our main effects model resulted in singularity issues, we decided not to include this factor in the final model. Instead, we ran a post-hoc analysis, which indicated that sample volume was not driving our main results (see ESM, Post-hoc analysis, Tab. S1). Statistical analysis were conducted in R (version 3.5.2) (R Core Team, 2018). Further details and R-packages are reported in the ESM.

Results

We collected 151 saliva swabs ($n = 13$ subjects; 11.62 mean ± 2.45 SEM samples per subject). Collected saliva volume after centrifugation of the swabs ranged between $2 - 200$ μl with an average of 30.36 $\mu\text{l} \pm 37.70$ SD. 73 swabs did not contain saliva, 7 contained less than 5 μl and, thus, only the remaining 71 samples which contained at least 5 μl saliva volume ($n = 11$ subjects) were analysed. MT could be detected in 28 samples ($n = 9$ subjects), but only samples, which had a duplicate CV below 20% were considered in the statistical analysis. Consequently, for the MT concentration model we had 20 samples ($n = 7$ subjects; see ESM: Tab. S2), whereas for the MT detectability model we had data on 62 samples ($n = 10$ subjects).

Detectability of MT

Overall, we found a clear effect of season (Fig. 1a) and saliva volume on the detectability of MT concentrations (binomial full-null model comparison: $\Delta\text{AIC} = 4.147$, $\chi^2 = 12.147$, $\text{df} = 4$, $p = 0.016$). MT was less likely to be detected in samples collected during mating season (mean probability = 0.23 ± 0.17 SD) and in samples of low volume (not detected: mean = 28.00 $\mu\text{l} \pm 27.95$ SD) than in samples collected during breeding season (mean probability = 0.53 ± 0.12 SD) and in samples of high volume (detected: mean = 40.40 $\mu\text{l} \pm 46.84$ SD; Tab. 2).

Effects of bonding status, sex and season on MT levels

Season also had an effect on the ravens' salivary MT levels themselves (main effects-null model comparison: $\Delta\text{AIC} = 5.372$, $\chi^2 = 11.372$, $\text{df} = 3$, $p = 0.010$), with lower MT concentrations occurring during mating season (mean = 355.44 $\text{pg/ml} \pm 226.75$ SD) than during breeding season (mean $812.22 = \text{pg/ml} \pm 471.83$ SD; $p = 0.015$; Fig. 1b, Tab. 2 and ESM: Tab. S2). Neither the subject's bonding status nor sex had a significant impact on MT concentrations (Tab. 2).

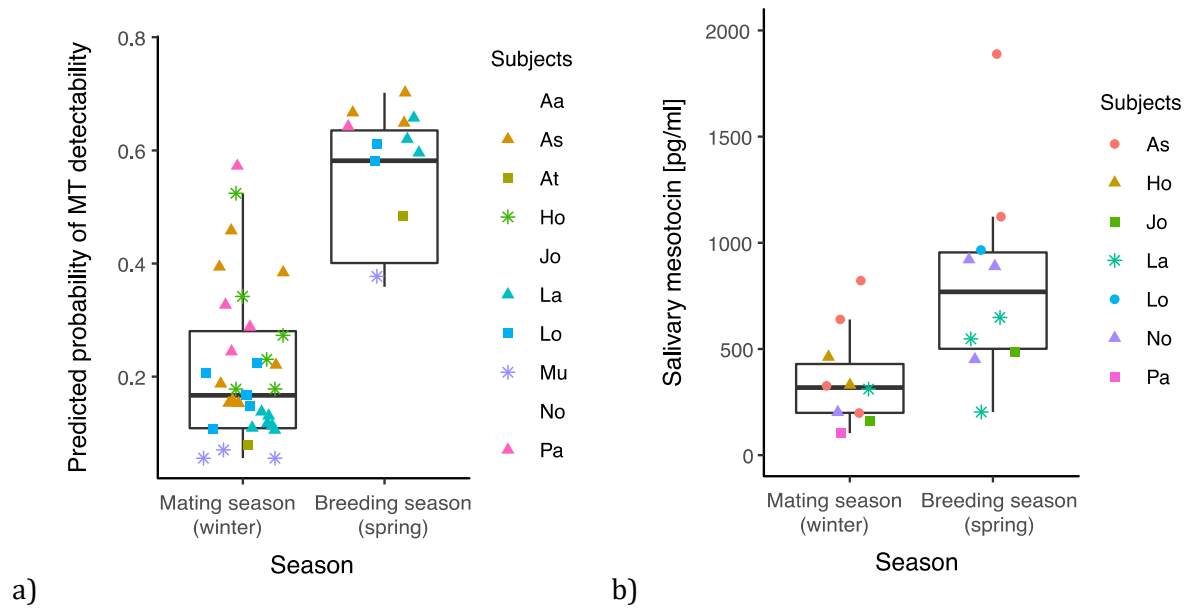


Figure 1. a) Predicted probability of MT detectability and b) salivary MT levels in mating (winter) and breeding season (spring). Boxplot: bold line represents the median, the boxes represent 1st and 3rd quartile, and the whiskers show the largest and smallest value within 1.5 times the interquartile ranges respectively.

Table 2. Given are estimates, standard errors (SE), confidence intervals (CI), degrees of freedom (df), z- (binomial model) or t-values, p-values, and partial omega squared (ω^2) for each parameter of the (linear) mixed-effects model. * $p \leq 0.05$, *** $p \leq 0.001$

Parameter	Estimate	SE	CI	df	z / t	p	ω^2 ^a
Binomial model:							
Detectability of MT							
(Intercept)	0.176	0.716			0.246	0.806	
Sex (male)	0.441	0.708			0.623	0.533	
Season (mating)	-2.281	0.789			-2.892	0.004**	
Status (non-breeder)	-0.811	0.737			-1.100	0.271	
Saliva volume	0.020	0.010			2.105	0.035*	
Main effects model:							
Effects on MT							
(Intercept)	6.664	0.392	(5.726, 7.539)	12.956	16.984	< 0.001***	0.94
Sex (male)	-0.349	0.271	(-1.037, 0.361)	1.215	-1.289	0.390	0.04
Season (mating)	-0.906	0.278	(-1.502, -0.236)	7.896	-3.260	0.012*	0.33
Status (non-breeder)	-0.334	0.287	(-0.972, 0.496)	1.586	-1.163	0.390	0.02
Time since sunrise	0.034	0.062	(-0.096, 0.168)	5.970	0.546	0.605	-0.05

Discussion

In the present study we could show i) that it is possible to collect saliva from birds in a non-invasive manner, based on the voluntary collaboration of the subjects with the experimenter, ii) that MT is detectable in ravens' saliva using a commercial enzyme-immunoassay, and iii) that ravens' salivary MT levels are linked with a biologically relevant parameter, i.e., mating vs. breeding season. This opens up new opportunities to study peripheral MT in birds. Measuring naturally occurring MT levels could particularly be useful in studies related to sociality or animal welfare and can be easily applied in research institutions and zoos.

Although we were able to measure ravens' salivary MT levels, the effectiveness rate shows that some improvements are needed. To begin with, it was difficult to estimate the saliva volume soaked up by the swab during the ongoing collection procedure, resulting in many empty swabs. Additionally, we encountered constraints due to the enzyme-immunoassays detection limit. In any immunoassay, low volume samples need to be diluted to reach the required assay volume. This causes a reduction of the hormone concentration, which can fall below the assay detection limit. In the present study, samples of low saliva volume and samples that were collected during the mating season, during which MT concentrations were generally lower, were thus more likely to have a MT concentration below the detectable limit. Another issue was that several samples had a variation coefficient (CV) of duplicates higher than 20 percent and hence could not be used for further analysis. Future studies, thus, should focus i) on increasing the saliva volume gained by improving and intensifying the training of the birds as well as the training of the trainers/experimenters, and ii) on developing more sensitive assays. Improving the methodology is unquestionably crucial for future applications.

The third goal of our study was to investigate whether we can detect biologically relevant differences in salivary MT levels. We found that MT concentrations differed between mating (winter) and breeding season (spring), but that there was no evidence for an effect of bonding status or sex. As expected, MT levels were higher in the breeding season, a time in which ravens are less aggressive than in the mating season (Braun and Bugnyar, 2012; Gwinner, 2003). A study on sparrow species suggests that an increase in MT innervation in certain parts of the brain plays an important role for flocking and might lower aggression (Goodson et al., 2012). Furthermore, studies on Thai hens (*Gallus domesticus*) suggest that the MTergic system is involved with reproductive behaviour (Chokchaloemwong et al., 2013; Sinpru et al., 2017). Therefore, MT in our ravens might have increased in preparation for breeding.

Although the seasonal difference in MT concentrations in our ravens could be associated with social factors (related to aggression in the mating season or behavioural changes associated with reproduction in the breeding season), it could also result from abiotic environmental factors, such as temperature. Several studies show that the MT system is involved in thermoregulation

(McConn et al., 2019; Robinson et al., 1988). In chicks (*Gallus domesticus*) central injections of MT led to increased cloacal temperature and reduced water and food intake, suggesting that MT plays an important role in avian metabolism (McConn et al., 2019). Accordingly, changes in MT levels, like the ones predicted by the present study may facilitate metabolic adaptations to the different seasons (accompanied by different environmental temperatures).

The effects of nonapeptides are often sex-specific (Goodson, 2013; Kelly and Goodson, 2014), and there is evidence for differences in nonapeptide levels between males and females in birds (Robinson et al., 1990). However, in our study MT levels did not differ between the sexes. Neither did we find evidence for MT levels to differ between pair-bonded and group-living ravens. These results parallel a recent study in another corvid species, pinyon jays (*Gymnorhinus cyanocephalus*), which showed that intranasally administered MT neither affects the formation of pair bonds nor their maintenance (Duque et al., 2020), although administration of MT in the same species has been found to increase pro-social food-donations (Duque et al., 2018). However, the effects of administered hormones rely heavily on the available receptors. Studies on zebra finches (*Taeniopygia guttata*) showed that oxytocin-like receptors do in fact mediate pair bonding (Klatt and Goodson, 2013) and that the administration of OT antagonists decreases pair formation (Pedersen and Tomaszewski, 2012). In the present study, it should be noted that the number of group-living subjects included in the model that investigated effects on MT concentrations was very low ($n = 2$), which may have hampered the detection of potential differences between them and the pair-bonded subjects. Moreover, the group-living subjects might have already started to form pair-bonds within their non-breeder group, which might have been reflected in similar physiological activity in the group-living birds as in the pair-bonded ravens. Finally, it is important to keep in mind that we explored bonding status as a general factor and did not investigate the effect of social interactions in a way that would allow us to ascribe causality to it (cf. e.g. Lürzel et al., 2020). To be able to study a causal link between MT and certain behaviours in a non-invasive way, we recommend specific sampling regimes as well as matched controls. The herein described procedure of salivary MT determination would allow for that more easily and precisely than existing methods, like analysing droppings.

We do acknowledge that it is debated whether peripheral OT/MT concentrations reflect central concentrations, which affect the above described social correlates (Neumann, 2008). Recent studies, however, have identified a pathway through which peripheral OT can cross the blood-brain barrier and thereby affect social behaviour (Higashida et al., 2019; Yamamoto et al., 2019; Yamamoto and Higashida, 2020). This possibly explains why many of the reported differences in peripheral OT/MT have been linked to social factors (Crockford et al., 2014). In line with this, Lefevre and colleagues (2017) found that in primates, simultaneously collected (peripheral) plasma OT and (central) cerebrospinal fluid OT correlated positively, suggesting that

peripheral OT can be used as a proxy for central OT. (For further discussion about the relationship between central and peripheral OT levels and challenges for measuring OT, please, see among others Grinevich and Neumann, 2021; Higashida et al., 2019; Lefevre et al., 2017; MacLean et al., 2019; Yamamoto and Higashida, 2020.)

In sum, using positive reinforcement, birds can be trained to voluntarily provide saliva samples, from which biologically meaningful variation in MT can be analysed.

Ethical statement

This study complies with the Austrian Animal Experiments Act (§ 2, Federal Law Gazette No. 114/2012).

Acknowledgements

We would like to thank Martina Schiestl for her help concerning animal training. This project was financed by the FWF (Stand-alone project P 26806 to JJMM and P 33960 to TB), the Ethologische Gesellschaft (Research Grant to JJMM) and the University of Vienna (Marie Jahoda grant to LH).

Supplementary materials

Saliva collection – animal training

Training protocol based on positive reinforcement training:

Training sessions started with the experimenter sitting down in front of the aviary and showing an object and a food reward – small pieces of dog food (Frolic) – until the animal approached. In the beginning the object usually was a stone that was just too big to fit through the wire mesh. The training consisted of multiple incremental steps: i) The experimenter held the object close to the fence. A reward was given when the bird touched the object with its beak. ii) The experimenter plugged the object halfway through the fence, which allowed the bird to easily push it back out by touching the object with its beak. A reward was given when the object dropped into the hand of the experimenter. In the following steps a reward was given when the object, by then usually a saliva swab, was dropped into the experimenter's hand after the bird had taken it iii) partly into the beak, iv) fully into the beak, and v) fully into the beak and waited (max. 10 sec) until the experimenter showed his open hand as a cue for the animal to give the object back. If during saliva collection the experimenter thought that the swab did not soak up enough saliva the swab was immediately given back to the bird and the procedure repeated. During the collection

procedure the experimenter was wearing gloves to avoid contamination. Using the same training protocol some of the ravens had already been trained to exchange tokens with an experimenter in an earlier study (Massen et al., 2015).

Statistical Analysis

All statistical analyses were conducted in R (version 3.5.2) (R Core Team, 2018). To investigate if the detectability of salivary mesotocin (MT) depends on the ravens' bonding status, sex, season and/or saliva sample volume, we first fitted a binomial model with a logit link with the function *glmer* of the package *lme4* (Bates et al., 2015). To estimate the effect of bonding status, sex and season on ravens' salivary MT concentrations we computed linear mixed-effects models with the function *lmer* of the package *lme4*. MT follows a circadian rhythm (Chaturvedi et al., 2001), like oxytocin (OT) (Perlow et al., 1982). Prior to fitting the model, we therefore calculated sampling time from sunrise from sampling dates and times, using the packages *lubridate* (Grolemund and Wickham, 2011) and *suncalc* (Thieurmél and Elmarhraoui, 2019). Sampling time was unavailable for one of the samples and we imputed the value with the mean sampling time for the respective season. Additionally, we log-transformed the MT concentration to approximate normal distribution. We visually inspected whether the model residuals were normally distributed (QQ plot) and homogenous (residuals plotted against fitted values). We detected no multicollinearity issues via inspecting the variance inflation factors (max. VIF of all models=1.617; function *vif*, package *car*; Fox and Weisberg, 2019). P-values were calculated via Satterthwaite's degrees of freedom method (package *lmerTest*; Kuznetsova et al., 2017) and effect sizes were estimated via partial omega squared $\omega^2_P = (SS_{\text{Effect}} - (df_{\text{Effect}})(MS_{S/\text{Cells}})) / (SS_{\text{Effect}} + ((N - df_{\text{Effect}})(MS_{S/\text{Cells}})))$ (Keppel, 1991; package *effectsize*, Ben-Shachar et al., 2020).

Post-hoc analysis

Prior to constructing the models, we found that sample volume correlated negatively with MT concentrations ($n = 20$ samples, $r^2 = -0.49$, $p = 0.027$) and that there is a significant difference in saliva volume between seasons (Welch Two Sample t-test, $n = 78$ samples, $t = -2.9756$, $df = 69.97$, $p\text{-value} = 0.004$, mean volume in mating season = 37.02 μl , breeding season = 13.41 μl). We have, therefore, included saliva volume as fixed effect in our binomial model (MT detectable yes/no). However, including saliva volume as fixed effect into our main effects model ($n = 20$ samples) resulted in singularity issues. To, nevertheless, investigate if our results were driven by the season or by differences in sample volume, we constructed a linear mixed-effects model ($n = 20$) with log-transformed mesotocin concentration as response variable, sample volume and season

as predictors and subject as random intercept. The results of this post-hoc analysis indicate that sample volume did not have an effect on MT levels (see table below).

Table S1. Given are estimates, standard errors (SE), confidence intervals (CI), degrees of freedom (df), t-values, p-values, and partial omega squared (ω^2) for each parameter of the linear mixed-effects model.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Parameter	Estimate	SE	CI	df	t	p	ω^2 ^a
(Intercept)	6.574	0.212	(6.104, 7.017)	12.059	30.978	< 0.001***	0.98
Season (mating)	-0.856	0.282	(-1.441, -0.249)	19.588	-3.041	0.007**	0.33
Sample volume	-0.002	0.003	(-0.009, 0.004)	17.229	-0.817	0.425	-0.02

Table S2. Subjects' MT levels in mating (winter) and breeding season (spring). Given are subject (ID), sex, bonding status, season, number of samples (N), mean MT concentration (Mean), and standard deviation (SD).

ID	Sex	Bonding status	Season	N	Mean MT [pg/ml]	SD MT [pg/ml]
As	female	pair-bonded	mating	4	496.27	285.32
			breeding	2	1506.05	541.64
Jo	female	pair-bonded	mating	1	160.07	
			breeding	1	484.71	
Lo	female	pair-bonded	mating	0		
			breeding	1	965.80	
Ho	male	pair-bonded	mating	2	396.37	93.44
			breeding	0		
Pa	male	pair-bonded	mating	1	103.52	
			breeding	0		
No	female	group	mating	1	202.23	
			breeding	3	753.69	262.58
La	male	group	mating	1	310.80	
			breeding	3	466.18	233.67

References – Chapter 3

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Chapter 4.

Published in Royal Society Open Science, 2020, 7 (5), 191056.

Cooperation with closely bonded individuals reduces cortisol levels in long-tailed macaques

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DOI: 10.1098/rsos.191056

Abstract

Many animal species cooperate with conspecifics in various social contexts. While ultimate causes of cooperation are being studied extensively, its proximate causes, particularly endocrine mechanisms, have received comparatively little attention. Here, we present a study investigating the link between the hormone cortisol, cooperation and social bonds in long-tailed macaques (*Macaca fascicularis*). We tested 14 macaques in a dyadic cooperation task (loose-string paradigm), each with two partners of different social bond strength and measured their salivary cortisol before and after the task. We found no strong link between the macaques' cortisol level before the task and subsequent cooperative success. In contrast, we did find that the act of cooperating in itself led to a subsequent decrease in cortisol levels, but only when cooperating with closely bonded individuals. Two control conditions showed that this effect was not due to the mere presence of such an individual or the pulling task itself. Consequently, our study shows an intricate way in which the hypothalamic-pituitary-adrenal axis is involved in cooperation. Future studies should reveal whether and how our findings are driven by the anxiolytic effect of oxytocin, which has been associated with social bonding.

Keywords

cooperation, loose-string paradigm, *Macaca fascicularis*, salivary cortisol, glucocorticoid hormone, social bonds

Introduction

Cooperation can be described as the voluntary acting together of two or more individuals, (potentially) leading to an end situation that benefits at least one of the participants (Brosnan and de Waal, 2002). A multitude of animal species, as diverse as chimpanzees, *Pan troglodytes* (Melis et al., 2006), lions, *Panthera leo* (Heinsohn and Packer, 1995), ravens, *Corvus corax* (Massen et al., 2015), cichlid fish, e.g. *Astatotilapia burtoni* (Weitekamp and Hofmann, 2017), or eusocial paper wasps, *Polistes dominula* (Field and Leadbeater, 2016) cooperate with conspecifics in various social contexts such as cooperative territory defence (Heinsohn and Packer, 1995; Weitekamp and Hofmann, 2017) and hunting (Bailey et al., 2013; Boesch, 1994). Apart from the evolutionary function of cooperation (i.e. fitness benefits (Bshary and Oliveira, 2015)), during the past decades the cognitive mechanisms underlying cooperative behaviour have received specific attention in behavioural and psychological research. Cooperating individuals may require a suite of cognitive traits, allowing them to, for example, recognize and assess their partners, to tolerate spatial proximity of others, to coordinate their actions with others to achieve a particular aim, and, at times, to accept lower immediate pay-offs (Soares et al., 2010). These cognitive abilities are tightly linked to physiological processes, including hormonal mechanisms modulating cooperative behaviour (Massen et al., 2019; Soares et al., 2010; Trumble et al., 2015) that have, however, received considerably less attention.

Glucocorticoid hormones help individuals to cope physiologically with fluctuating environmental factors (McEwen and Wingfield, 2003). Changes in glucocorticoid levels can lead to changes in cooperative behaviour (Dantzer et al., 2017; Soares et al., 2014), yet so far there is no clear picture of this relationship. In some cases, e.g. the cleaner wrasse, *Labroides dimidiatus*, the effect of glucocorticoids on cooperation was shown to be condition dependent (Soares et al., 2014). Individuals that were injected with cortisol were less cooperative (i.e. biting instead of cleaning) towards potentially dangerous large clients, whereas they were more cooperative towards relatively harmless small clients, suggesting that cortisol mediates cooperative flexibility (Soares et al., 2014). Altogether however, still relatively little is known about the link between cooperation and glucocorticoids.

The identity of the cooperation partner represents another very important component when it comes to the performance of cooperative actions. One of the most influential features is the social relationship two individuals share with each other. Particularly close social bonds, reflected by high grooming rates and the individuals often being in close proximity (Asakawa-Haas et al., 2016; Massen et al., 2011; Schwing et al., 2016; Silk et al., 2006), have been shown to promote cooperation in a broad range of species (e.g. kea, *Nestor notabilis* (Schwing et al., 2016); wolves, *Canis lupus* (Marshall-Pescini et al., 2017); chimpanzees (Samuni et al., 2018); Barbary macaques, *Macaca sylvanus* (Berghänel et al., 2011; Molesti and Majolo, 2016; Young et al.,

2014b)). Chimpanzees, for example, are more likely to share food with closely bonded conspecifics (Samuni et al., 2018), and in male Barbary macaques close social bonds facilitate cooperation in coalitions against other males (Berghänel et al., 2011; Young et al., 2014b).

Besides positively affecting cooperation, close social bonds can also buffer hypothalamic-pituitary-adrenal (HPA) axis activity, which means they can lower glucocorticoid levels (Cohen and Wills, 1985). This has also been found in several primate species, such as (female) Yellow baboons, *Papio cynocephalus* (Seyfarth et al., 2012; Wittig et al., 2008), Barbary macaques (Young et al., 2014a), chimpanzees (Wittig et al., 2016) and also humans (Heinrichs et al., 2003). While many studies have shown that this applies to stressful situations in which responses to stressors are alleviated by interactions with and also the mere presence of closely bonded individuals (social buffering hypothesis (Cohen and Wills, 1985; Young et al., 2014a)), there is also evidence that this is a general effect that applies to different contexts throughout daily life, independent of immediate stressors (main effects hypothesis (Cohen and Wills, 1985; Wittig et al., 2016)).

Here, we investigate the relation between cooperation, social bonds and endogenous glucocorticoid levels in long-tailed macaques, *Macaca fascicularis*. This macaque species is relatively despotic, lives in multi-male multi-female groups with a steep linear hierarchy (Thierry, 2000), and is well suited for this investigation for the following reasons: They cooperate through alliances in agonistic contexts (de Waal, 1977), they support each other in exchange for grooming (Hemelrijk, 1994), and also reciprocate grooming for itself (Gumert and Ho, 2008). Moreover, in experimental settings long-tailed macaques can show pro-social behaviour (Massen et al., 2010; Sterck et al., 2015). Since they are averse to unequal food distributions (Massen et al., 2012), they only do so as long as it does not come at a cost (Sterck et al., 2015). Long-tailed macaques' prosocial choices mainly depend on actor and receiver's rank position (Massen et al., 2011, 2010), although there are minor indications that also social bonds may play a role in prosocial choices (Massen et al., 2011). In addition, studies on a closely related, but more egalitarian species, the Barbary macaque showed that strong social bonds in fact drive the maintenance of cooperative interactions over time (Berghänel et al., 2011; Molesti and Majolo, 2016), and that affiliative interactions are related to lower cortisol levels (Rincon et al., 2019) as well as higher oxytocin levels (Rincon et al., 2020). Social bonds, like rank (Massen et al., 2011, 2010; Young et al., 2014b), thus, seem to play an important role in macaque cooperation.

Glucocorticoids (or their metabolites) in long-tailed macaques have so far mainly been measured from blood, urine or faeces (Bardi et al., 2017; Czoty et al., 2009; Girard-Buttoz et al., 2009; Shively, 1998; van Schaik et al., 1991). Saliva sampling, however, provides a non-invasive alternative that allows measuring short-term changes in unbound, hence biologically active cortisol (Kirschbaum and Hellhammer, 1994). Due to this advantage saliva sampling is being applied more and more frequently in non-human primates (Ash et al., 2018; Behringer and

Deschner, 2017; Davenport et al., 2003; Higham et al., 2010; Laudenslager et al., 2006; Petrullo et al., 2016; Pfefferle et al., 2018), and is also the method of choice in this study.

Here, we aimed to gain a better understanding of the potential endocrine and social mechanisms underlying cooperation. In particular, we want to address three questions; **Q1**: Is an individual's cooperative behaviour in an experimental task modulated by its cortisol level before the task and/or by the social bond with its cooperation partner? **Q2**: Are subsequent changes in the individual's cortisol level influenced by the performance in the cooperation task; i.e. do cortisol levels change due to cooperation, and/or due to other social factors, such as the social bond between participants, and, **Q3**: Does the test setting itself, or only the cooperative aspect of it affect subsequent cortisol levels?

We tested 14 long-tailed macaques in a dyadic loose-string task (Hirata, 2003) – a task that has already been used to test cooperative tendencies in primates (Hare et al., 2007; Hirata, 2003; Melis et al., 2006; Molesti and Majolo, 2016), other mammals (Ostojić and Clayton, 2014; Plotnik et al., 2011) and birds (Asakawa-Haas et al., 2016; Massen et al., 2015; Péron et al., 2011; Scheid and Noë, 2010; Schwing et al., 2016; Seed et al., 2008), and which requires two individuals to collaborate (i.e. simultaneously pulling on the two ends of a rope) to access a sharable reward. Before and after the task we measured their salivary cortisol levels. Individuals were tested twice with group members of varying social bond strength, ranging from closely bonded conspecifics to rather neutral ones. We predicted cortisol before the task to be negatively and social bond to be positively related to cooperative success, and that these effects would be independent of subject's rank (see Massen et al., 2010) (P1). In addition, we expected that more successful individuals would experience a decrease in cortisol during the task, yet that a more pronounced drop in cortisol would occur after cooperation with closely bonded group members, and that these effects would be independent of possible effects of sex and dominance rank on cortisol levels (see Cavigelli and Caruso, 2015; Gesquiere et al., 2011) (P2). Finally, we predicted that only cooperation and not the mere presence of a conspecific or the pulling task itself reduces cortisol (P3).

Methods

Subjects and Housing

We tested 14 long-tailed macaques (5 males, 3 – 5 y; 9 females, 4 – 22 y) living in a captive social group of 29 individuals at the Biomedical Primate Research Centre (BPRC) in Rijswijk, the Netherlands. Test subjects were above two years of age and passed the training criteria (see ESM 1: *Training Procedure*). The group was kept in an enclosure consisting of freely accessible indoor

(72 m², 2.85 m high, light/dark cycle 12 h) and outdoor compartments (208 m², 3.1 m high). Plenty of structures, e.g. platforms, tires and ladders, served as environmental enrichment. The animals were fed twice a day with monkey chow pellets (Sniff; 9 am), and either fresh fruit, vegetables or bread (3 pm). Water was available ad libitum.

Social measures

Dominance hierarchy

The dominance hierarchy within the group was determined by Landau's linearity index corrected for unknown relationships (h') using MatMan 1.1 (de Vries et al., 1993). The calculation of the index was based on 132 occurrences of submissive behaviour, i.e. bare teeth displays and being displaced, collected on an all occurrence basis (9am – 5pm, indoors and outdoors) throughout the study period ($h' = 0.338$, $n = 18$, without individuals < 2 years, $p = 0.02$, 54.25% unknown relationships).

Social bond

To determine the social bond between individuals, we used the frequency of them being observed in close proximity (within one arm length, including grooming incidents) during scan samplings. Since these relationships changed slightly during a testing break (1 ½ months: August 17 to October 2, 2016) we calculated the scores for the testing period before and after the break using scans taken during the respective period plus one month before (period 1: 61 scans from 29 days; period 2: 40 scans from 27 days; max. 3 scans/day, at least 45 min in-between). As the relationship of two partners might be different for each partner (whereas one might spend much more time with others, for the other the partner might be the one he/she shows most of the proximity occurrences with), we calculated the individuals' proportions of proximity with each other group member (>2y), based on the total proximity frequency of the respective individual, rather than on a group level. Proportions ranged from 0 to 0.39 (39% of this individual's total proximity occurrences took place with this partner). Test partners (see below) were matched in a way that each individual was tested with one conspecific with a high social bond proportion and one with a low proportion. We avoided testing individuals that showed high levels of aggression towards each other. Due to this logistically challenging matching procedure we did not always pick the individual with the highest social bond proportion as a strongly bonded partner. Out of 16 tested dyads, six had a maternal kin relationship with $r \geq 0.25$ (1 sibling dyad, 2 mother-daughter dyads, 3 aunt/uncle-niece/nephew dyads).

Experimental Setup

Subjects were tested inside a corridor of their indoor enclosure, which, for the experiment, was divided into two smaller compartments (I and II, each 110 cm × 75 cm × 80 cm) with a transparent slide in the middle and opaque slides on both ends (Fig. 1). During testing subjects were kept separately in the two compartments to avoid physical aggression and grooming interactions which might affect cortisol levels (Sonnweber et al., 2015). Depending on the condition (see *Conditions*), either a dyadic or an individual test apparatus was placed in front of the corridor.

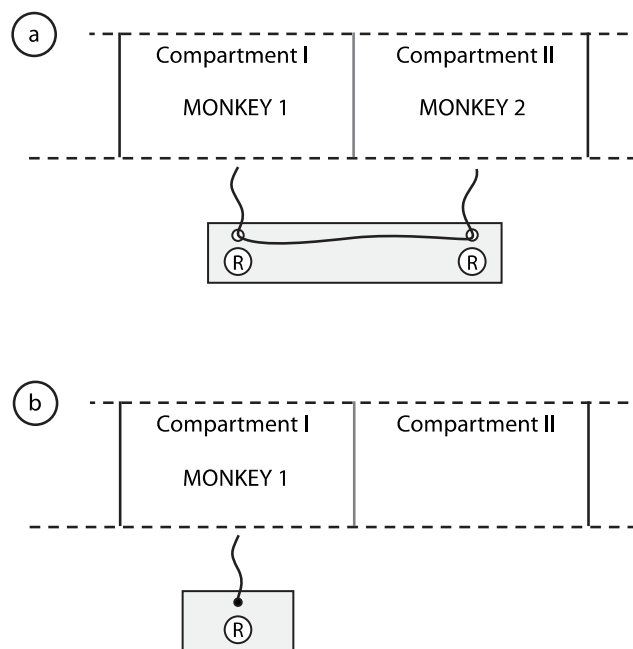


Figure 1. Test settings: a) Dyadic setting (loose string paradigm) and b) individual setting with fixed rope. The grey line between the two compartments represents a transparent slide, the grey rectangle the sliding platform and R the reward (peanuts).

a) Dyadic test setting

The dyadic test apparatus was based on the loose-string paradigm (Hirata, 2003) (Fig. 1a; for dimensions see ESM 1), and was used in the cooperation condition (see *Conditions*). In this set-up each macaque could reach just one end of a rope that was threaded through loops attached to a sliding platform. Only when the subjects pulled the ends simultaneously the platform moved within reach and the subjects could get the rewards (peanuts) that were placed on this platform. If only one individual pulled, the rope got disentangled and the dyad lost its chance to cooperate during that trial.

b) Individual test setting

The individual test apparatus (Fig. 1b; for dimensions see ESM 1) was used in the non-social control condition (see *Conditions*). It was presented in front of compartment I and differed from the dyadic apparatus in that the rope was not loose but fixed on the (smaller) sliding board. In this task one macaque alone had to pull the rope to bring the sliding board including a reward that was placed in the cup within reach.

Training

All subjects were familiar with coming into the corridor and being separated (physically, but not acoustically or visually) from their group. For the purpose of this experiment, we first trained the animals on the individual string-pulling task (Fig. 1b), and subsequently on the dyadic loose string task (Fig. 1a; for training procedure see ESM 1).

Conditions

The macaques were tested in three different conditions in a within-subject design (overview see Tab. 1). To examine the individuals' cooperative success and its relation to their hormone levels, we applied **i) the loose-string paradigm; i.e., cooperation condition** (Fig. 1a). Since changes in hormone levels could, however, not only arise from cooperation but due to other reasons, we implemented two controls; **ii) a social control condition**, controlling for the possible effect of a partner's presence, and **iii) a non-social control condition** to determine the effect of the task, hence the pulling (activity), itself. In the social control, the same dyads had to run through the same procedure as in the cooperation condition but were not presented with a task. In the non-social control, subjects were alone in the corridor and confronted with the individual test setting (Fig. 1b).

To test the effect the social bond with the cooperation partner may have on either the cooperation itself or on the mediating role of hormones, we tested subjects in the cooperation and social control condition with a closely bonded group member *and* with a neutral member (see *Social measures – Social bond*). Therefore, individuals were tested five times; twice in the cooperation test, twice in the social control and once in the non-social control.

Social conditions (i.e. cooperation and social control) as well as the social bond with the partner were counterbalanced over the individuals (see ESM 1: *Counterbalanced testing*), the non-social control was conducted after an individual was tested with one partner in both social conditions. Since for two subjects we lacked partners with appropriate social bond patterns who had not been tested yet, two already tested individuals had to be used as partners, and thus were

tested one additional time in both social control and cooperation test (for dyads see ESM 1: Tab. S1). The tests were conducted between June 30 and November 11, 2016.

Table 1. Test conditions including short description and purpose.

Condition	Description	Effect of...
Cooperation	Loose-string task with a partner	... cooperation?
Social control	Same partner, but no task	... conspecific's presence?
Non-social control	No partner, individual string-pulling task	...string-pulling/activity?

Experimental Procedure

At the beginning of each test session, the respective individual or dyad was led into the test compartments and separated from the rest of the group. Females with an infant (4 – 9 months old) were allowed to bring it to the test compartments (n=4). After 5 min of habituation they were confronted with one of the three conditions. Each session had 15 trials (á 35 sec) with an inter-trial interval of 40 sec, equalling exactly 10 min. In the cooperation and non-social control condition, an experimenter reset the apparatus after each trial (retracted platform and rope, baited cups, reoffered rope). In these conditions the monkeys thus got the opportunity to respectively cooperate to sequentially get 15 rewards (peanut halves) and to pull the tray on their own to get 15 rewards. Since we assumed that the subjects would not succeed in all 15 trials and to keep conditions as standardized as possible, we chose to simulate 12 successful cooperation trials in the social control condition. Therefore, in the social control condition the experimenter gave each individual one reward per trial (no task), except for trial 5, 10 and 15, equalling 12 successful cooperation trials. The whole study was conducted by the same person who was always present during the sessions. After the 10 min test sessions we took two saliva samples, the first one 5 min and the second one 10 min after the test (ESM 1: Fig. S1), always starting with the same individual. Prior to the experiments we performed a biological validation of salivary cortisol in long-tailed macaques (see ESM 2), which revealed that salivary cortisol has a time lag of approximately 15 min. Therefore, sample 1 should reflect what happened at the beginning of the test (“cortisol before”), and sample 2 represents the test situation. Following the last saliva sample, the macaques were released back into their group. To limit the variation in cortisol levels due to circadian rhythm (for rhesus macaques, *Macaca mulatta*, see Pfefferle et al., 2018), all test sessions were conducted between 1 and 3 pm. Individuals were never tested twice per day.

Salivary Cortisol

Sample collection procedure

Using positive reinforcement, the subjects were trained to give saliva samples by chewing on one end of a Salimetrics Children Oral Swab (previously validated for common marmosets, *Callithrix jacchus* (Ash et al., 2018)), which was held on the other end by the experimenter (wearing gloves). For each sample the animal was rewarded with a small piece ($\sim 1/8$) of peanut. The swabs were collected in 2 ml micro tubes and, after at most 90 min, frozen at -20°C until analysis (for general information on freezing and storage see (Laudenslager et al., 2006)).

Hormone analysis

The collected samples were thawed, centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge 5424, rotor FA-45-24-11), diluted 1:5 with diluent buffer (IBL International: KLZZ731; appropriate ratio determined beforehand by serial dilution of pooled saliva) and analysed with a commercial immunoassay kit (IBL International: Cortisol Saliva ELISA RE52611) following the instructions of the manufacturer, where also cross reactivities can be found (IBL International GmbH, 2015). For validation see ESM 2. The intra-assay coefficient of variance (CV) was 7.13 % and the inter-assay CV 9.99 %. Saliva samples were analysed in duplicates, however, in some cases the macaques did not provide sufficient saliva for cortisol analysis (sample size see Tab. 2), or the volume only sufficed for single analysis. These values were nevertheless included in the statistical analysis, since only one out of 165 duplicates (= 0.6%) had a CV above 10%, indicating that the assay was highly reliable. The analysis was conducted at the Biomedical Primate Research Centre within two months after sample collection.

Long-tailed macaques' salivary cortisol levels ranged from 1.45 to 43.55 ng/ml, with a mean of $5.67 \text{ ng/ml} \pm 4.35 \text{ SD}$. Three samples were excluded from further analysis, because they exceeded the individual's mean + 2.5 SD. This resulted in a total mean of $5.27 \text{ ng/ml} \pm 2.71 \text{ SD}$ and a maximal value of 16.85 ng/g.

Statistical analyses

All statistical analyses were conducted with R version 3.3.3 (R Core Team, 2017). Linear mixed effect models were calculated with the package *lme4* (Bates et al., 2014), except for the full model associated with Q1, which was a generalized linear mixed model (GLMM) with beta distribution constructed with the package *glmmADMB* (Bolker et al., 2012). In this case the response variable cooperative success (which is limited to max 15 trials) was better represented by a beta distribution than by a Poisson (open end distribution) or normal distribution and was therefore given as proportion instead of number of successful cooperation trials per session. All full models,

including data used, response variable and independent variables are listed in table 2 (for model summaries see Tab. S2). Due to the individual nature of cortisol levels and the fact that they are included in all models, we based our analyses on individuals and not dyads. All full models included the individual's identity as random factor. Model Q1 additionally included the partner's identity to account for the direct dependency of cooperative success of the two partners. Changes in cortisol levels (Δ cortisol) were calculated by subtracting the value of the first saliva sample from that of the second one. Positive values of Δ cortisol, therefore, represent an increase of cortisol. Since some of the participating females had an infant, which might have affected their cortisol levels, the presence of an infant was included as an independent variable in models with cortisol as response variable, as were the individuals' sex, dominance rank and kinship (Cavigelli and Caruso, 2015). We inspected diagnostic plots of the model residuals for homogeneity of variance, violation of normality assumptions or other departure from model assumptions and calculated the variance inflation factors (max. VIF across all models = 1.85), which indicated that the independent variables showed no significant collinearity.

We followed an information-theoretic approach (Burnham and Anderson, 2002) as this is suitable in case the number of subjects is limited and the model should incorporate potentially confounding variables (Mundry, 2011), such as the presence of an infant. We created a set of candidate models with all possible combinations of independent variables from the respective standardized full model. We ranked these models according to their AICc and selected the subset of models with $\Delta \text{AICc} \leq 2$ with respect to the best model (ESM 1: Tab. S3), as this gives substantial support that these models show an equally good fit. With the *MuMIn* package (Bartoń, 2017) the models of the respective subset were averaged to create model-averaged coefficients (Burnham and Anderson, 2002), which were ranked based on their relative importance (Tab. 3). The relative importance is the crucial value for further discussion of the independent variables (for details on this method see (Grueber et al., 2011)). If this subset included the null model (intercept only model) we do not report these results in detail since it supports the null hypothesis, i.e. none of the models explains the variance within the response variable better than the null model (Burnham and Anderson, 2002).

Table 2. Overview of the full models corresponding to the research questions Q1-3, including the data used (condition; sample size (number of observations)), dependent and independent variables. All models include individual ID as a random factor. Model Q1 also includes partner ID as random factor.

	Condition	N (Obs.)	Dependent v.	Independent variables					
Q1	CO	14 (29)	<i>coop. success</i>	cortisol before	soc. bond	rank			
Q2 a	CO	13 (27)	Δ cortisol	coop. success	soc. bond	rank	sex	infant	m. kin
Q2 b	SC	14 (27)	Δ cortisol		soc. bond	rank	sex	infant	m. kin
Q3	CO, SC, NS	14 (66)	Δ cortisol	condition		rank	sex	infant	

Condition: CO – cooperation, SC – social control, NS – non-social control; Δ cortisol – change in cortisol during the condition; coop. – cooperative; soc. – social; m. kin – maternal kin.

Results

Overall cooperation success rate ranged from 6 to 15 trials (40 - 100 %) per session, with a median of 12 trials. When considering this cooperative success, the subset of the best models from all candidate models contained the null model (Tab. S3), indicating that neither cortisol levels before the cooperation task, nor social bond or rank predict how successful individuals cooperated with each other (P1).

Changes in cortisol levels during the cooperation condition were also not affected by the individual's performance in the task (i.e. cooperative success; P2 a). An individual's dominance rank and the social bond it shared with the cooperation partner, however, did seem to have a strong effect on cortisol changes while cooperating (Tab. 3). Whereas higher-ranking individuals and individuals with a stronger social bond with their cooperation partner were more likely to experience a drop in cortisol, lower ranking individuals as well as weakly bonded partners were more likely to show stable cortisol levels or a slight increase (Fig. 2).

With regard to the social control, the subset of the best models from all candidate models included the null model (Tab. S3). Therefore, the individuals' social bond did not affect changes in cortisol in the social control (Fig. 2). This effect, hence, only occurs when individuals are actually cooperating and not while just sitting next to each other (i.e. during social control).

Whereas cooperation with closely bonded individuals led to a cortisol decrease, changes in cortisol were not affected by cooperation in general (irrespective of the bond with the partner), the mere presence of a conspecific, or the pulling task itself (P3). This was indicated by the fact that none of the models in the subset used for the model averaging contained the factor condition (ESM 1: Tab. S3). Instead, model-averaged results show that across all conditions (i.e. cooperation as well as both social- and non-social control) dominance rank and sex were the most important predictors of those changes (Tab. 3, Fig. 3).

Table 3. Model-averaged coefficients (full averaging from top-ranked models with standardized parameters) with adjusted standard errors, lower and upper confidence intervals and relative importance. For Q1 and Q2b model averaging was not conducted because the top-ranked models included the null model.

Model and Response variable	Parameter (level)	Estimate	Adjusted SE	Lower CI (2.5 %)	Upper CI (97.5 %)	Relative importance
Q2 a Change in cortisol during cooperation	Intercept	-0.36	0.31	-0.96	0.24	
	Rank	1.61	0.62	0.39	2.83	1.00
	Social bond	-0.89	0.60	-2.02	-0.15	0.82
	Sex (<i>male</i>)	0.35	0.58	-0.16	2.09	0.37
Q3 Changes in cortisol during cooperation, social control and non-social control	Intercept	-0.17	0.18	-0.52	0.17	
	Rank	1.07	0.37	0.36	1.79	1.00
	Sex (<i>male</i>)	0.62	0.48	0.06	1.55	0.77
	Infant (<i>yes</i>)	0.20	0.38	-0.26	1.47	0.33

SE – standard error; CI – confidence interval. Relative importance: bold – highly important.

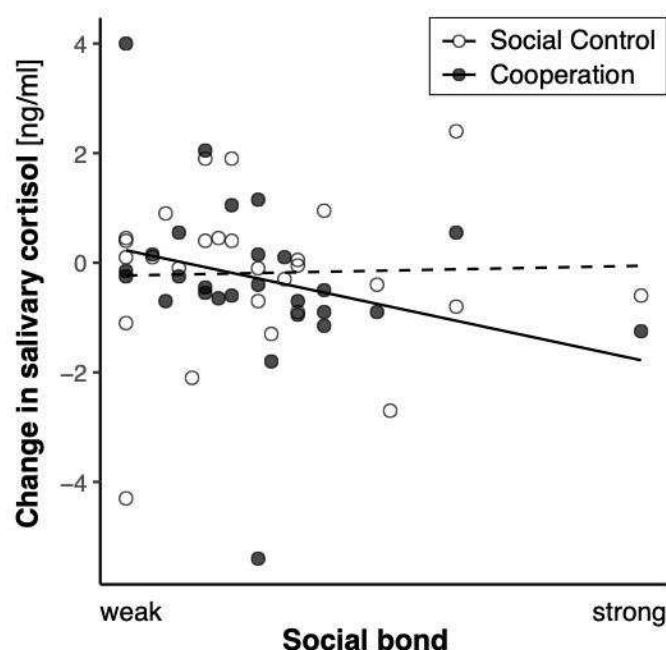


Figure 2. Relationship of changes in salivary cortisol levels during the social conditions (cooperation, social control; split for illustration) and social bond with the cooperation partner. Since individuals were tested with different partners, there are multiple points for each individual. Cooperation: filled circles, solid trend-line. Social control: open circles, dashed line.

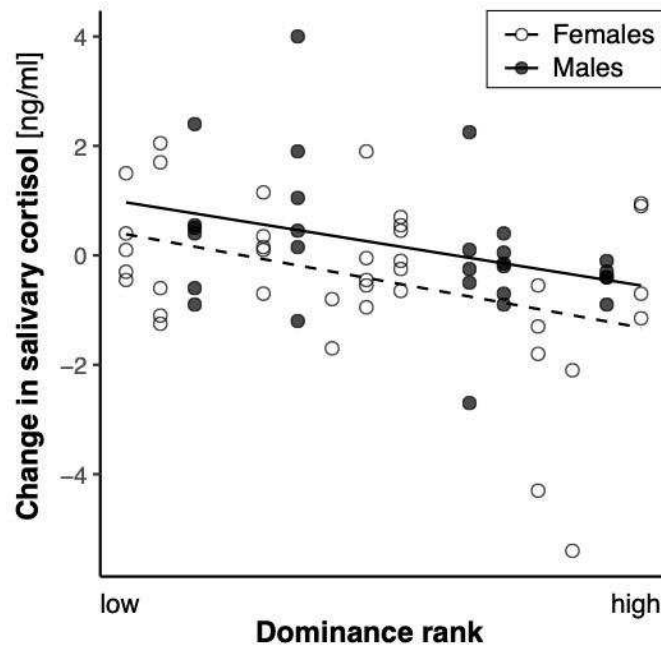


Figure 3. Relationship of changes in salivary cortisol levels across all conditions and individual dominance rank in males (filled circles, solid trend-line) and females (open circles, dashed line). As the figure presents data from all conditions, there are multiple data points for each individual.

Discussion

We investigated the possible link between cooperation and salivary cortisol in 14 long-tailed macaques. In contrast to our hypothesis, we found no evidence that cortisol levels at the beginning of a cooperation task or the strength of social bond with the partner affect an individual's subsequent cooperative behaviour. Also contrasting with our expectations, cooperative success did not affect subsequent cortisol levels. Hence, similar to recent findings in red-tailed monkeys, *Cercopithecus ascanius* (Jaeggi et al., 2018), the endocrine responses measured were not related to the *outcome* of the social interaction. Instead, two other factors that rather reflect the nature of the interaction, seemed to influence cortisol responsivity; i.e. dominance rank and social bond. First, during cooperation sessions salivary cortisol levels of higher ranking individuals dropped, whereas for lower ranking individuals it either stayed the same or even increased. Second, cortisol levels decreased more in animals that worked together with closely bonded individuals. The latter effect seemed specifically based on working together since we did not find a similar pattern in the social control.

Among the long-tailed macaques in this study there was no evidence for social bonds to influence cooperative behaviour. This contrasts with studies on, for example, chimpanzees

(Samuni et al., 2018) or the closely related, yet more egalitarian Barbary macaques (Berghänel et al., 2011; Molesti and Majolo, 2016; Young et al., 2014b), that did find a positive effect of social closeness on cooperation. It should be noted, however, that, unlike in these studies, that were conducted in the wild, in our experiment the subjects did not have free partner-choice, which is a very important factor in cooperative decision-making (Schino and Aureli, 2017). Restricting such partner-choice has been suggested to obscure the effects of social parameters (Suchak et al., 2014). Moreover, the macaques in our study performed generally very well in the cooperation task (mean 75.6 %, median 12 successful trials (indeed equalling the number of rewarded trials in the social control condition) and a range of 6 to 15 successful trials) leaving little variation to relate to social bond strength.

Irrespective of cooperative success, social bond seemed to have a large effect on cortisol changes during the cooperation test, with closer social bonds leading to a larger decrease in cortisol levels. This effect was not found in the social control, suggesting that it is actually the cooperative action with a closely bonded individual and not just its presence that is central to reducing cortisol. In wild chimpanzees close social bonds also have a downregulating effect on the HPA axis, though not just during cooperation but generally throughout their daily life (Wittig et al., 2016) (supporting the main effect hypothesis (Cohen and Wills, 1985)). Though in Barbary macaques affiliative interactions (between males) have been reported to reduce cortisol levels (Rincon et al., 2019), it has been shown that the stress buffering effect of strong social bonds depends on social and environmental factors and is therefore not always the same (Young et al., 2014a). The here demonstrated anxiolytic effect of cooperative interactions with closely bonded individuals could be the underlying proximate cause of the effect of close social bonds on the maintenance of cooperation found in macaques (Berghänel et al., 2011; Molesti and Majolo, 2016). One potential mechanism leading to a decrease in cortisol during cooperation of closely bonded individuals could involve oxytocin. Oxytocin plays a major role in facilitating the formation and maintenance of social bonds (Goodson, 2013; Insel, 2010; Rincon et al., 2020; Ziegler and Crockford, 2017) and mediates cooperative behaviour (Trumble et al., 2015) (equivalent mesotocin in birds (Duque et al., 2018)). Moreover, oxytocin possesses anxiolytic properties (Dief et al., 2018; Heinrichs et al., 2003; Ring et al., 2006) (for non-human primates see (Cavanaugh et al., 2016; Crockford et al., 2017)). Accordingly, we would expect oxytocin levels after socio-positive interactions with closely bonded conspecifics to be particularly high, and therefore the anxiolytic effect, eventually leading to decreased cortisol levels, to be strong. In wild chimpanzees, cooperative behaviours like grooming and food sharing were linked to higher peripheral urinary oxytocin levels as compared to after social feeding situations that do not involve cooperation (Wittig et al., 2014). However, unlike the effect of cooperation on cortisol levels in the present study and grooming on oxytocin in chimpanzees (Crockford et al., 2013;

Wittig et al., 2014), the effect of food sharing on oxytocin was independent of the social bond shared by the respective individuals (Samuni et al., 2018; Wittig et al., 2014). Also in the context of inter-group conflicts, which, too, require group members to cooperate, chimpanzees were found to have higher oxytocin levels compared with controls (Samuni et al., 2017). Though, the release of oxytocin was independent of cortisol activity (Samuni et al., 2019). Hence, whether the oxytocinergic system affects HPA axis activity during cooperation appears to depend on the specific type of cooperative action. To conclude, in our study closely bonded individuals did not cooperate more, but their cooperative interaction might have led to an increase in oxytocin, which in turn reduced cortisol levels. If by these means frequent cooperation counteracts chronically elevated glucocorticoid levels, it might promote the maintenance of cooperation over time (Berghänel et al., 2011; Molesti and Majolo, 2016), and improve the individuals' health status (Romero et al., 2015) (but see (Thompson and Cords, 2018)). This hypothesis, however, still remains to be tested in long-tailed macaques.

Dominance rank was found to predict changes in cortisol across all conditions (Fig. 3), i.e. in high ranking individuals the salivary cortisol level was more likely to drop, whereas in low ranking individuals it rather increased. This is in line with other studies on long-tailed macaques in which (experimentally manipulated) cortisol changes were rank dependent. Shively and colleagues (Shively, 1998; Shively et al., 1997) reported a less intense cortisol increase in response to a cortisol-enhancing factor (ACTH injection) as well as a stronger decrease in cortisol levels in response to a cortisol-suppressing factor (dexamethasone) in high-ranking females as compared to low-ranking ones. The latter was also found in rhesus macaques (Kohn et al., 2016). Accordingly, our finding might reflect a general pattern in macaques. One potential limitation of our study is that not all dominance relationships were known (see *Social measures - Dominance hierarchy*) as the determination of the social characteristics, i.e. social bonds and dominance rank, was based on a limited number of observations. Though this might be regarded as a major weakness, we do consider our rank calculation to be a reliable estimate of an individual's general positioning in the dominance hierarchy, indicating whether it is high or low ranking. Moreover, we used rank instead of rank differences between individuals in the statistical analyses because potential inaccuracies in the calculated ranking have major consequences for rank differences between individuals, though should be less impactful when it comes to assessing if an individual is high or low ranking. Furthermore, the fact that the link between rank and cortisol changes found in this study are in line with previous findings in macaques is supporting the validity of our rank data.

The test setting, i.e. the three conditions as such, did not influence subsequent cortisol levels (Q3), yet we did find an overall effect of sex. Regardless of the condition, males in our study were higher in the cortisol reactivity spectrum than females of similar rank, i.e. low-ranking males

showed a more pronounced cortisol increase than low-ranking females, and high-ranking females a stronger decrease than high-ranking males (Fig. 3). Similar sex differences in salivary free cortisol responses to acute psychological stressors were found in humans (Kudielka and Kirschbaum, 2005; Reschke-Hernández et al., 2017). These differences could result from the type of social context that causes the response, as different contexts might have different effects in males than in females (Byrd-Craven et al., 2015) and might engage different neuroendocrine pathways (Reschke-Hernández et al., 2017).

Conclusion

In summary, we investigated the relationship between glucocorticoids and cooperative behaviour in an experimental task in long-tailed macaques, while taking into account the individuals' social bonds. We found no evidence for cortisol or close social bonds to affect the macaques' cooperative performance (similar to Massen et al., 2011). Strong bond did, however, lead to decreasing glucocorticoid levels during cooperative interactions, irrespective of the performance. We propose that this could be mediated by the anxiolytic effect of oxytocin, which likely is up-regulated during cooperative interactions with a closely bonded individual, but not necessarily due to the mere presence of such an individual. Finally, we found that the macaques' cortisol responsivity was affected by their dominance rank and sex. This effect was, however, independent of the conditions and not specific to cooperation. Overall, these findings contribute to our understanding of the mediating role of cortisol in cooperation. However, to get a better understanding of the physiological processes involved in cooperation, we encourage future studies to investigate multiple biomarkers at once (Bardi et al., 2017; Jaeggi et al., 2018; Massen et al., 2019; Romero et al., 2015; Trumble et al., 2015).

Ethics

The study complied with the Association for the Study of Animal Behaviour / Animal Behavior Society Guidelines for the Use of Animals in Research (Animal Behaviour, 2018, 135, I-X), and was carried out after approval of the BPRC's Animal Welfare Committee (IvD 002a) and in accordance with the Dutch legal requirements. The macaques were never water or food deprived and training/testing was based on their voluntary participation.

Data accessibility

All data and R scripts are archived in Dryad: doi:10.5061/dryad.g6kh12j

Competing interests

We have no competing interests.

Authors' contributions

MS and JM conceived of the study and designed it; MS carried out animal training, data collection, hormone and data analysis and drafted the manuscript; MS and ML carried out the statistical analyses; ES and TB contributed to the interpretation of the findings; JM coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

Acknowledgements

We thank Annet Louwerse and Jan Langermans for allowing us to conduct the study at the BPRC, Caroline Olesen, Lisette van den Berg, Saskia Visser-van Soest and Willem van der Spek for supporting us in the work with the macaques, Daniella Mortier, Linda Hofman, Elisabeth Pschernig and Ruth Sonnweber for their help/advice concerning the hormone analyses, and Tjeerd Fluitsma for building the test apparatuses. Finally, we would like to thank Loeske Kruuk, Sarah Brosnan and the anonymous reviewers for their constructive comments on earlier versions of this manuscript.

Funding

MS and JM were supported by the FWF (Austrian Science Fund, P 26806 to JM) and the IPS (International Primatological Society, Research Grant to JM and MS), ML was supported by the FWF (Austrian Science Fund, P 29705) and by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 798091.

SUPPLEMENTARY MATERIAL - 1

Apparatuses

The dyadic test apparatus consisted of a large wooden base plate (125 cm × 60 cm × 1.2 cm) and a smaller sliding board (100 cm × 28 cm × 1.2 cm) that was attached to the base through rails and could be moved out of reach of the animals. Mounted on that board were two transparent cups that held food rewards (60 cm apart) and two metal loops with a 140 cm long rope threaded through them (60 cm apart). The apparatus for one individual was shorter (base: 43 cm × 65 cm × 1.2 cm), featured only one cup, and the rope was not loose but fixed on the sliding board.

Training procedure

Each individual had to pass 9 out of 10 trials in two consecutive sessions before advancing to the next training stage where macaques were presented with the dyadic loose string task. All subjects accomplished that within the first two sessions. In the loose string task dyads had to cooperate successfully in a total of 20 trials before both subjects could proceed to the test (for number of trials needed to reach criterion see Tab. S1). Note that these 20 successful trials did not have to be achieved within one training session and that training dyads were not used in test conditions. During the training individuals were rewarded with raisins, peanuts, sunflower seeds, dried corn and peas, whereas in the test sessions we only used peanuts to keep the rewards constant.

Counterbalanced testing

Social conditions (i.e. cooperation and social control) as well as the degree of affiliation of the partner were counterbalanced over the individuals, i.e. eight subjects were first tested with the partner with which they share a stronger social bond, of which four started with the cooperation condition. The other seven started with the rather neutral partner, of which four started with the cooperation condition. The non-social control was conducted after an individual was tested with one partner in both social conditions. When tested with the second partner six individuals started with the same condition as with which they started with their first partner and eight with the other condition.

Table S1. Subjects & Dyads. For each individual we listed the trainings partner (incl. trials needed to reach test criterium) and test partners, starting with the partner with which the individual shared a stronger bond. Individuals that were tested with an additional partner: After the control condition Nu did not go into the testing area together with Op (one of the 4 females with an infant). Therefore, we used Ta as rather neutral partner for Nu, however for Op we could not find an appropriate replacement for Nu. To was supposed to be the rather neutral partner for Ta, but eventually Ta's relationship with To was better than the one with Nu. Su, after being tested with two partners, was used as neutral partner for An. For Ch we could not find a second partner. Since the hormone analysis of one of Nu's and one of Az' samples did not work and we did not have enough material to repeat it, we tested the respective dyad (Nu-Dn, Az-Mc) again at the end of the study. When we first tested Az with Mc his relationship to her was rather neutral, but when tested again their bond was stronger than before. Note that social bonds are given in proportion and are not symmetric. As Wa did not provide saliva samples, she was not included in the statistical analyses but did act as partner for others.

Individual characteristics					Test partner					Trainings partner		
ID	Sex	Age	Rank	Carried infant	ID	Sex combi	Infant in dyad	Social bond	Coop. success	ID	Sex combi	Trials needed
An	f	12	14	yes	Bi	ff	yes	0.10	7	Dn	mf	26
Az	m	3	13	no	Su	ff	yes	0.02	6	Se	mf	39
Ba	f	10	11	yes	Mc	mf	no	0.00; 0.10	14; 12	Mc	ff	32
Bi	f	4	3	yes	Ta	mm	no	0.08	12	Op	ff	29
Ch	f	22	12	no	Wa	ff	yes	0.13	11	Ba	ff	32
Dn	m	4	16	no	Ka	ff	yes	0.06	12	Nu	mf	37
Ka	f	7	5	no	An	ff	yes	0.15	7	Ta	mf	38
Mc	f	4	17	no	Su	ff	yes	0.03	10	An	mf	26
Nu	m	3	8	no	Mc	ff	no	0.25	9	Op	ff	29
Op	f	11	6	yes	Nu	mm	no	0.25; 0.15	11; 14	Ba	ff	32
Se	f	21	18	no	Wa	mf	no	0.08	13	To	mm	26
Su	f	20	10	no	To	mf	no	0.10	11	Ka	ff	29
Ta	m	5	7	no	Ba	ff	yes	0.05	12	Az	mf	39
To	m	3	4	no	Ch	ff	no	0.39	9	Wa	ff	39
Wa	f	4	9	no	Az	mf	no	0.00; 0.06	14; 12	Ch	mf	38
					Dn	mm	no	0.20; 0.15	11; 14	Nu	mm	26
					Op	mf	yes	0.00	control	Su	ff	39
					Ta	mm	no	0.00	14			
					Se	ff	no	0.11	13			
					Nu	mf	yes	0.00	control			
					Op	ff	no	0.12	13			
					Su	ff	no	0.06	13			
					Se	ff	no	0.07	13			
					Bi	ff	yes	0.04	10			
					An	ff	yes	0.04	6			
					Az	mm	no	0.13	12			
					To	mm	no	0.13	15			
					Nu	mm	no	0.00	14			
					Ka	mf	no	0.19	11			
					Ta	mm	no	0.10	15			
					Ba	ff	yes	0.23	11			
					Dn	mf	no	0.08	13			

Sex combi. – sex combination; Infant in dyad – one or both individuals had an infant; Coop. success – cooperative success (number of successful trials); trials needed – to reach test criterion.

Table S2. Summary of the full models (Q1-3)

Parameter (level)	Estimate	SE	t value	p-value
Q1 - Cooperative success: Random effect variance: individual ID: 0.26; partner ID: 0.13				
Intercept	2.28	0.70	3.28	0.00
Cortisol before	-0.06	0.07	-0.96	0.34
Social bond	-2.56	2.46	-1.04	0.30
Rank	-0.03	0.05	-0.74	0.46
Q2 a - Change in cortisol during cooperation: Random effect variance: 0.42				
Intercept	-2.48	1.81	-1.37	0.18
Success	-0.02	0.12	-0.19	0.85
Social bond	-6.25	2.80	-2.24	0.04
Sex (<i>male</i>)	1.37	0.66	2.07	0.06
Rank	0.19	0.06	3.44	0.01
Infant (<i>yes</i>)	0.83	0.79	1.05	0.32
Maternal kin (<i>non-kin</i>)	0.29	0.49	0.59	0.56
Q2 b - Changes in cortisol during social control: Random effect variance: 0.42				
Intercept	-1.40	0.93	-1.51	0.15
Social bond	-0.31	2.49	-0.12	0.90
Sex (<i>male</i>)	1.09	0.66	1.65	0.12
Rank	0.09	0.06	1.47	0.17
Infant (<i>yes</i>)	0.73	0.75	0.98	0.34
Maternal kin (<i>non-kin</i>)	-0.50	0.51	-0.99	0.33
Q3 - Changes in cortisol across all conditions: Random effect variance: 0.03				
Intercept	-1.99	0.54	-3.70	0.00
Condition (coop)	-0.18	0.34	-0.55	0.59
Condition (non-social)	0.33	0.43	0.77	0.45
Sex (<i>male</i>)	0.96	0.38	2.56	0.03
Rank	0.12	0.04	3.42	0.01
Infant (<i>yes</i>)	0.63	0.44	1.44	0.18

Table S3. Subset of top-ranked models (with standardized parameters, $\Delta \text{AICc} < 2$) used for model-averaging (if null model is not included).

Explanatory variables of each model	df	logLik	ΔAICc	weight
Q1 - Cooperative success				
Social bond	5	18.00	0	0.43
(Null model)	4	16.33	0.40	0.35
Cortisol before	5	17.37	1.26	0.23
Q2 a - Changes in cortisol during cooperation				
Rank, social bond	5	-44.14	0	0.46
Rank, social bond, sex	6	-42.69	0.44	0.37
Rank	4	-46.59	1.87	0.18
Q2 b - Changes in cortisol during social control				
(Null model)	3	-45.85	0	0.43
Rank	4	-45.19	1.45	0.21
Maternal kin	4	-45.32	1.72	0.18
Sex	4	-45.35	1.78	0.18
Q3 - Changes in cortisol during cooperation, social control and non-social control				
Rank, sex	5	-109.94	0	0.44
Rank, sex, infant	6	-109.01	0.57	0.33
Rank	4	-111.76	1.31	0.23

df – degrees of freedom; logLik – log-likelihood; ΔAICc – differences of the second order Akaike information criterion between the best model and the other top-ranked models; weight – Akaike weight.

Additional figure

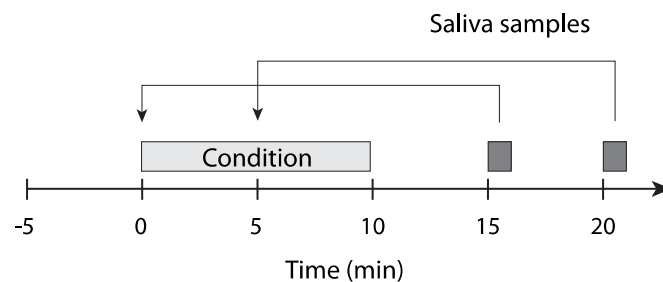


Figure S1: Procedure timeline. The macaques had 5 min to habituate to the situation before the test – one of the three conditions (light grey square) – started. After the test, the individuals stayed in the test compartment and gave two saliva samples (dark grey squares), each representing what happened approximately 15 min earlier (arrows).

SUPPLEMENTARY MATERIAL - 2

Salivary cortisol

Biological validation

Prior to the cooperation experiment, we conducted a “stress challenge” to determine how long it takes until a stressor is reflected in salivary cortisol concentrations in long-tailed macaques. Out of four tested individuals (two males, two females), only one very well-trained male (2.5 years old) provided all saliva samples needed.

We observed the male in the group for 30 min before starting the challenge to ensure that at least no obvious stressors occurred. Then we let the subject into the testing compartment and took 13 saliva samples in an interval of 2.5 min, hence for 30 min (approx. 1 min/sample, 1.5 min in-between). In the stress challenge, the experimenter left the room after collection of the first sample. An animal keeper (to the subject unfamiliar) immediately entered the room, stood in front of the subject and made noise with a large paper bag. We chose to present a paper bag because animal keepers have previously observed that this macaque group shows an alert/aversive, though not extremely intense responses to that. After one minute the animal keeper left and the experimenter returned and continued to collect saliva samples.

To determine the influence of the training itself and the presence of the experimenter, we conducted a control test employing the same procedure with the same individuals, but without presenting a stressor. The male was tested in June 2016, first in the control condition, and two days later in the stressor condition, both between 11 and 12 am.

The analysis of the saliva samples collected during the test situations was conducted as described in the *Salivary cortisol – Hormone analysis* section (IBL Cortisol Saliva ELISA, intra-assay CV: 1.56 %).

Results and discussion

The results show a time lag between the start of the stressor and the appearance of the cortisol peak of 15 min (Fig. SC). During that time, the male had a 2.6-fold increase (4.7 ng/ml) in cortisol (3.7-fold (5.6 ng/ml) from the lowest point). During the first half of the control condition the individual showed a highly varying and overall increasing cortisol level (sample 0 – 12.5: mean = 4.13 ± 1.86 SD), which might be due to interactions in the group or the experimenter starting the session. The level, however, stabilized 12.5 to 15 min after the session has started (sample 15 – 30: mean = 5.72 ng/ml ± 0.42 SD), indicating, first, that the stable cortisol level in the second half represents the stable situation during the control session, and second, that also training and test

sessions lead to an elevated level of arousal. In the stress condition the cortisol level finally (after 30 min) drops to the stable, but elevated level shown in the control.

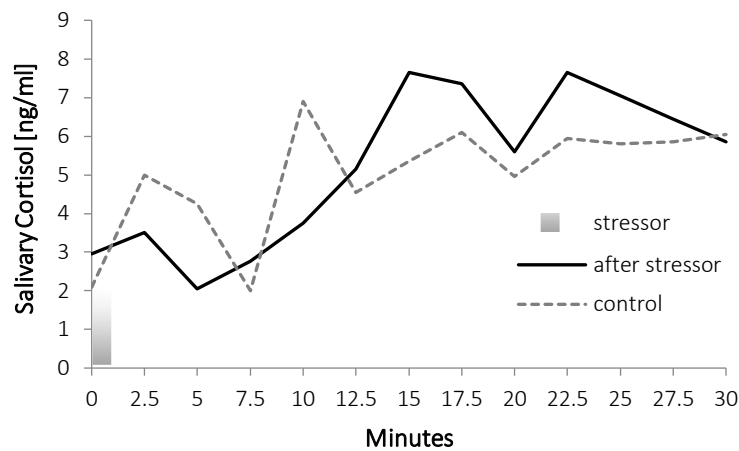


Figure SC. Salivary cortisol of a male long-tailed macaque after a stressor (solid line) and during a control condition (dashed line).

As a precaution regarding animal welfare, we did not keep the monkeys in the training compartment for more than 30 min. Therefore, we were not able to investigate full recovery time in the stress challenge and the second sample during test conditions already had to be taken 10 min after the end of the tests instead of 15 min later. We are aware that one sub-adult male is not necessarily representative for a whole species, but the time lag of 15 min in his salivary cortisol response corresponds with what was shown in blood samples after ACTH challenges in the same species (Czoty et al., 2009; Shively et al., 1997). Considering that the peak cortisol concentration in saliva follows the one in blood with a time lag of 2-3 min (Lutz et al., 2000) one might have expected the peak in our male to occur after 17.5 min. However, blood samples after the ACTH challenges were collected only at two time points (probably due to animal welfare concerns) – 15 and 30 min after ACTH administration (Czoty et al., 2009; Shively et al., 1997), whereas the sampling interval in our stress challenge was much higher, leading to a more detailed temporal resolution. Moreover, the response to ACTH is most likely much more intense than to the rather mild stressor we used in our study.

The male's elevated but stable cortisol level in the stress challenge control indicates that subjects might also experience slightly increased arousal/stress when interacting with an experimenter. However, the mean cortisol level of the second half of this control session (reflecting the subject-experimenter interaction), which coincides with the overall average level in this study, is somewhat lower than what was found in other studies investigating salivary

cortisol in macaques (e.g., long-tailed macaques, *Macaca fascicularis* (Rapp-Santos et al., 2017); rhesus macaques, *M. mulatta* (Pfefferle et al., 2018)). This suggests that, probably due to regular training with positive reinforcement, most subjects tested in this study did not experience extreme stress during training and test sessions. During the stress test, in which the subject not only showed an endocrine response, but also clear behavioural signs of arousal (piloerection, lip-smacking (Shively et al., 1997)) that were absent in the control test, salivary cortisol concentrations increased by 4.7 ng/ml. Accordingly, even though changes in cortisol during the stress challenge were rather small, we consider them meaningful.

References – Chapter 4

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Chapter 5.

General discussion

Summary of research

With this doctoral thesis, I aimed to promote saliva sampling for the purpose of endocrinological research in non-human primates and corvids and thereby to increase the knowledge about the link between physiology and behaviour as well as cognition in these animal groups. In pursuit of this objective, together with my colleagues, I first conducted two studies in which I validated methods to measure salivary hormones in i) two New World primate species i.e., brown capuchin monkeys, *Sapajus spp.*, and squirrel monkeys, *Saimiri sciureus* (Chapter 2); and ii) in common ravens, *Corvus corax* (Chapter 3). In both studies, we trained the individuals to provide saliva samples in the form of oral swabs using positive reinforcement training. Afterwards, we partially validated an enzyme immunoassay (EIA) for the measurement of salivary cortisol in the two primate species and salivary mesotocin in ravens, respectively. ‘Partially’ because we did not conduct all possible validation steps. In both cases, we did, however, show parallelism of the standard curves and serially diluted pooled samples, as part of the analytical validation.

In Chapter 2, we further conducted a (reversed-phase) high-pressure liquid chromatography (HPLC) and found that the main glucocorticoids that are detected by the chosen in-house EIA in these primate species’ saliva are cortisol and likely cortisone. Our biological validation, investigating the cortisol pattern from late morning till afternoon, further confirmed expected daily decreases in salivary cortisol concentrations, underscoring the utility of salivary glucocorticoid measurement as a valuable instrument for future research and welfare assessment in squirrel monkeys and capuchin monkeys.

In Chapter 3, we further tested the recovery of known amounts of mesotocin in saliva samples of ravens by spiking pooled raven saliva with two concentrations of (standard) mesotocin and calculating the recovery. In contrast to Chapter 2, this chapter also includes analyses of the detectability of mesotocin since numerous samples exhibited mesotocin concentrations falling below the assay's detection limit. These analyses revealed that the saliva volume as well as the factor season is connected with the detectability of mesotocin (detectable/not detectable), with higher chances of detecting mesotocin in samples of higher volume and in samples that were collected during breeding season (spring). Following this, we explored how the factors season, sex and bonding status (pair bond or group-living) influenced

salivary mesotocin concentrations (that could be detected) in the ravens. We found that in the breeding season mesotocin levels are higher than in the mating season (winter), which explains why the detectability was worse in the latter and serves as biological validation.

Finally, in Chapter 4, I focused on the link between physiology and behaviour as well as cognition by investigating the link between salivary cortisol, social bonds and cooperative behaviour in an Old World primate species, the long-tailed macaque, *Macaca fascicularis*. Like in the previous two chapters, I trained the individuals to provide saliva samples, however, in this case I applied a time-sensitive sampling regime. To investigate the time delay between a stressful stimulus and peak salivary cortisol levels, I carried out a small biological validation. Following this, I conducted a dyadic cooperation task (loose-string paradigm (Hirata, 2003)) involving 14 macaques, each paired on separate days with either of two partners of varying social bond strength, and measured salivary cortisol levels both before and after the task. While we did not find a strong connection between the macaques' cortisol levels before the task and their subsequent cooperative success, we made another intriguing discovery: the act of cooperation itself led to a decrease in cortisol levels, but this effect was observed only when the macaques were cooperating with closely bonded individuals. To rule out alternative explanations, we included two control conditions that demonstrated that this cortisol reduction was not due to the mere presence of a closely bonded individual or the nature of the pulling task itself. These findings contribute to our understanding of the mediating role of cortisol in cooperation and, hence, provides information on a proximate mechanism underlying cooperative behaviour.

Insights and Contributions

Animal training

A fundamental element of this doctoral thesis revolves around the acquisition of saliva samples utilizing (Salimetrics) oral swabs, primarily through positive reinforcement training and behavioural shaping techniques. Throughout this thesis, I have demonstrated the feasibility of obtaining saliva samples through the voluntary cooperation of the studied individuals, without the need for stimulants (e.g., sugar or citric acid) on the swabs. While this outcome may not be unexpected in apes, where similar methods have been previously applied (e.g. in chimpanzees, *Pan troglodytes* (Heintz et al., 2011) and bonobos, *Pan paniscus* (Verspeek et al., 2021)), it serves as compelling evidence that this approach is effective in three other non-human primate species for which it had not been previously established.

Chapter 2 is, to my knowledge, the first case in which salivary hormones were collected from brown capuchin monkeys. In squirrel monkeys, on the other hand, a few studies have

investigated salivary hormones. Fuchs and colleagues (1997) were the first ones to measure salivary cortisol in squirrel monkeys. However, although the paper is called “Salivary cortisol: a non-invasive measure of hypothalamo-pituitary-adrenocortical activity in the squirrel monkey, *Saimiri sciureus*”, saliva samples were collected from restrained individuals in a monkey chair. It is, therefore, not clear how much training and voluntary cooperation of the tested individuals was involved. Soon after, Lutz and colleagues (2000) presented a technique to collect saliva from awake, unrestrained, adult rhesus macaques, *Macaca mulatta*, that included the use of a stimulant (Cool-Aid) to motivate the primates to lick and chew on the braided cotton dental rope the stimulant was put on. A research group at the New England Primate Research Center (NEPRC), at Harvard Medical School, USA, thereafter, adapted this technique to the use in squirrel monkeys (Tiefenbacher et al., 2003), and used it in several studies (Lee et al., 2004, 2003; Platt et al., 2007). Further, there is one study from a different research group that used the same method (McKenzie-Quirk and Miczek, 2008) and one study that collected saliva samples from anaesthetised squirrel monkeys (Soltis et al., 2005). Also in long-tailed macaques, the, to my knowledge, only two studies that have investigated salivary hormones besides the study presented in Chapter 4 (Stocker et al., 2020), were conducted in anaesthetised individuals (Arslan et al., 1984; Rapp-Santos et al., 2017). Therefore, in all three non-human primate species included in this doctoral thesis, using behavioural shaping and (almost exclusively) positive reinforcement training to gain saliva samples is a new approach that motivates the individuals to cooperate with the trainer/experimenter. This method is, thus, a very promising tool for monitoring hormone concentrations in future studies on these species.

In birds, the use of this training and sampling method was a completely new approach, that, to our knowledge, no one has ever tried or at least not reported before (cf. Hahn et al., 2011). Following our publication in 2021, other researchers also started looking into avian salivary hormones as an alternative to hormone measurements such as blood (or faecal) sampling, though animal training did not play a role in the two studies deriving from that, and the birds were restrained instead (Aota et al., 2023; Carbillet et al., 2024). Both studies investigated salivary corticosterone, however, only one was conducted in a corvid species, the large-billed crow, *Corvus macrorhynchos*, in a lab setting (Aota et al., 2023), whereas the other study was conducted in wild common gulls, *Larus canus* (Carbillet et al., 2024), which renders restraint for saliva sampling necessary. Therefore, up to date, Chapter 3 remains to be the only study in which birds were trained to provide saliva samples for hormone analyses. This (in birds new) approach of training animals to provide saliva samples voluntarily offers numerous benefits inherent to saliva sampling, e.g., it is non-/minimally invasive, and measurements of immediate hormonal changes are possible, while allowing for multiple sampling and maintaining a positive relationship with the animal. In contrast, saliva sampling in restrained animals may not permit the collection of

multiple samples due to the potential influence of restraint on the endocrine system, likely distorting hormone values. Consequently, for behavioural and cognitive studies necessitating repeated sampling to explore the impact of specific stimuli, working with trained animals offers considerable advantages. To clarify, bird training is often used for scientific purposes other than saliva sampling. In fact, especially studies on avian behaviour and cognition often make use of bird training (e.g., Gruber et al., 2019; Levenson et al., 2015; Massen et al., 2015a; Schnell et al., 2021), since many bird species are highly trainable, with their intelligence and problem-solving abilities playing a significant role in their trainability. Even one of the renowned training programs in the field of animal training, developed by the animal trainer Bob Bailey, centres on the use of a (domesticated) bird species – chickens.

Validations as foundation

Besides showing that saliva collection in birds as well as non-human primates is feasible using animal training, a major contribution of this doctoral thesis to the scientific world is the (partial) validation of methods to measure salivary hormones. Validated hormone collection and measurement methods provide a foundation upon which researchers can build their investigations. This assurance of methodological soundness fosters scientific credibility and confidence in the results obtained, ultimately advancing the field of endocrinology and its connection to animal behaviour and cognition. Validations of the methods are, therefore, an important prerequisite for studies investigating salivary hormones.

As mentioned earlier, Chapter 2 represents the first study in which an EIA was (partially) validated for the measurement of salivary cortisol concentrations in capuchin monkeys and squirrel monkeys. Notably, the analysis of salivary hormones in capuchin monkeys is unprecedented in the existing literature, which has predominantly relied on endocrinological investigations utilizing faecal sampling (e.g., Mendonça-Furtado et al., 2017; Wheeler et al., 2013). Therefore, EIAs to measure faecal cortisol metabolites are already validated for brown capuchins, *Sapajus apella* (Wheeler et al., 2013) and bearded-, *Sapajus libidinosus*, and black capuchins, *Sapajus nigritus* (Mendonça-Furtado et al., 2017). However, due to the features of salivary hormones, particularly the short delay time between stimulus and rise in hormone concentrations in saliva, researchers working with capuchin monkey become more and more interested in collecting saliva samples for the analyses of hormones (e.g., Robinson et al., 2021). In the case of squirrel monkeys, prior studies have explored salivary hormones using different techniques to quantify hormones, predominantly employing radio-immunoassays (RIAs; Lee et al., 2004, 2003; McKenzie-Quirk and Miczek, 2008; Platt et al., 2007; Tiefenbacher et al., 2003). However, although RIAs often have a high sensitivity, they also have associated disadvantages,

notably the use of radioactive materials, posing potential hazards. In contrast, the EIA method, adopted in this study, eliminates the need for radioisotopes, enhancing safety for both laboratory personnel and the environment. Our findings conclusively confirm the expected presence of salivary glucocorticoids in both capuchin and squirrel monkeys and establish the reliability of their measurement through a cortisol EIA. For researchers studying these species, this presents new avenues for exploring the connections between acute HPA activity and behaviour (see O'Sullivan et al., *in prep*, a and b). Furthermore, Chapter 2 serves as a stimulus for scientists to validate salivary cortisol measurements in additional primate species.

Chapter 3 marks a significant contribution to the scientific field as it presents the first and only study on salivary mesotocin in birds. Notably, it revealed the impact of season (mating season/winter vs. breeding season/spring) on the concentration of mesotocin, showing that ravens' salivary mesotocin levels are linked with a biologically relevant parameter. That raven mesotocin concentrations are lower in the mating season (winter) than in breeding season (spring) was also corroborated by the finding that in the mating season more samples had mesotocin concentrations below the detection limit. Furthermore, based on research in other bird species indicating that the oxytocic/mesotocic system plays a major role in pair bonding and flocking (Goodson et al., 2009; Klatt and Goodson, 2013), we hypothesised that salivary mesotocin concentrations would differ between ravens with different social bonding status. This, however, was not the case, which is in line with a study on pinion jays, *Gymnorhinus cyanocephalus*, showing that exogenously introduced mesotocin does not affect same-sex social bond formation or maintenance (Duque et al., 2020). However, it is crucial to note that bonding status in our study was investigated as a general factor, and we did not delve into the impact of social interactions in a manner that would enable us to attribute causality to it (cf. e.g. Lürzel et al., 2020). Regarding the methodology for analysing salivary mesotocin in ravens, further refinement is certainly required to ensure the production of reliable results. To improve the overall detectability of birds' salivary mesotocin, larger saliva volumes, increasing the total concentration of a sample, and a more sensitive assay would be needed. This complexity seems to arise from the inherently delicate and often more challenging nature of mesotocin/oxytocin analysis (reviewed by MacLean et al., 2019) compared to the analysis of glucocorticoids. A recent study in large-billed crows, however, faced comparable challenges in the detectability of salivary corticosterone (Aota et al., 2023). Around 30% of the baseline samples in this study fell below the detection limit of the EIA (Aota et al., 2023). Interestingly, scientists studying salivary corticosterone in Common gulls using the same EIA did not encounter such issues (Carbillet et al., 2024). In conclusion, Chapter 3 presents a valuable first step towards the use of salivary mesotocin measurements as a tool to investigate naturally occurring peripheral mesotocin levels in birds in behavioural and cognitive sciences, a field, in which, so far, studies investigating avian

mesotocin were mainly using manipulation techniques (Duque et al., 2020, 2018; Goodson et al., 2009, 2004; Kelly, 2019).

As part of Chapter 4, we biologically validated an EIA for the analysis of salivary cortisol in long-tailed macaques. For this purpose, we took a baseline sample, exposed individuals to a brief stressor, and then collected saliva samples at 2.5-minute intervals over a thirty-minute period. Although this schedule did not allow us to determine the recovery time to baseline cortisol concentrations, the EIA was able to detect a salivary cortisol peak 15 minutes after exposure to the stressor. Unfortunately, the only other study investigating salivary cortisol in long-tailed macaques did not conduct a comparable stress challenge (Rapp-Santos et al., 2017). However, in another Old World primate, the chimpanzee, a similar 15-minute delay in salivary cortisol elevation was reported (Heintz et al., 2011), albeit with a lower temporal resolution due to samples being taken every 15 minutes. Acknowledging potential individual variations in the time lag, our study's high temporal resolution highlights the necessity for thorough testing, enabling more accurate application of saliva sampling for cortisol analyses in future studies. Concludingly, (despite the small sample size, see p. 102: *Limitations and future direction*) this biological validation revealed that the chosen cortisol EIA is able to detect biologically relevant changes in cortisol and can be applied in future studies on long-tailed macaques.

To date, the most common non-invasively collected biospecimen for hormone analyses used in behavioural and cognitive studies on animals are faecal samples. However, it is important to note that, besides the fact that they serve distinct research purposes compared to saliva samples, validating EIAs for measuring glucocorticoid metabolites in faecal samples (see Touma and Palme, 2005) often entails more extensive effort than validating EIAs for salivary cortisol. This is mainly due to the diverse array of glucocorticoid metabolites that exhibit significant variation across species (Palme, 2005; Palme et al., 2005). Hence, saliva sampling not only presents novel avenues for exploring acute fluctuations in hormone levels but also offers simpler validation processes.

In conclusion, the (partial) validations presented in this thesis serve as a critical step in ensuring the integrity and validity of subsequent research on the studied species, while also paving the way for further explorations of acute hormone level dynamics and their link to behaviour and cognition in these and other species.

Insights into animal cooperation

Cooperation is currently one of the hot topics in the field of animal behaviour and cognition. However, although there is an extensive body of literature on behavioural and cognitive processes behind cooperation (see Brosnan et al., 2010; Bugnyar and Massen, 2017; Cheney,

2011; Dale et al., 2020; Dugatkin, 2002; Massen et al., 2019; Melis and Raihani, 2023), the literature on other proximate mechanisms of cooperation, particularly hormonal mechanisms, is relatively scarce. It is, however, acknowledged that hormones modulate cooperative behaviour (e.g., Cardoso et al., 2015; Madden and Clutton-Brock, 2011; Scheid and Noë, 2010; Soares et al., 2014, 2010; Trumble et al., 2015). For example, in meerkats, *Suricata suricatta*, a cooperatively breeding species, cortisol administration causes adult female-, but not male helpers to spend less time on foraging and more time in close proximity to pups (Santema et al., 2013). In certain instances, such as with the cleaner wrasse, *Labroides dimidiatus*, glucocorticoids have been demonstrated to influence cooperation in a condition-dependent manner (Soares et al., 2014). Specifically, individuals injected with cortisol exhibited reduced cooperation towards larger, potentially threatening clients, yet displayed heightened cooperation towards smaller, less hazardous clients (Soares et al., 2014). This suggests that hormones play a mediating role in cooperation.

Moreover, one potential explanation for the evolution of cooperation is emotionally mediated reciprocity. This theory posits that decisions to cooperate or not are guided by long-term emotional evaluations of specific cooperation partners, which are formed through numerous interactions with those individuals (Schino, 2007; Schino and Aureli, 2009). Although promising, few studies have identified and measured the specific emotions involved. Measuring hormones, such as oxytocin and cortisol, could provide insights into these emotional states, enhancing our understanding of how emotions regulate cooperative behaviours in social species. See also the review (Massen et al., 2019) in the appendix p. 129.

In Chapter 4 (Stocker et al., 2020) we applied endocrinological work to further illuminate the proximate mechanism of cooperative behaviour in long-tailed macaques. Since the social bond between the cooperation partners i) is a crucial component influencing the execution of cooperative actions (e.g., Berghänel et al., 2011; Bhattacharjee et al., 2024; Marshall-Pescini et al., 2017; Molesti and Majolo, 2016; Samuni et al., 2018; Schwing et al., 2016; Young et al., 2014b) and ii) can affect hypothalamic-pituitary-adrenal (HPA) axis activity, influencing glucocorticoid levels, for example by stress buffering through social support (Cohen and Wills, 1985; in primates: e.g., Heinrichs et al., 2003; Seyfarth et al., 2012; Wittig et al., 2016, 2008; Young et al., 2014a), we specifically investigated changes in salivary cortisol in response to cooperative interactions with partners of different social bond strength.

Our study revealed that although the social bond strength between the cooperation partners did not influence the success rate in the experimental cooperation task, it did influence the cortisol response. Closer social bonds correlate with a more pronounced decrease in cortisol levels, a phenomenon not observed in the social control condition, suggesting that it is the cooperative engagement with closely bonded individuals (see also (Rincon et al., 2019)), rather

than mere presence, that is pivotal in cortisol reduction. Interestingly, in a study investigating inequity aversion, which enables individuals to avoid exploitation and, hence, aids cooperation, bonobos' salivary cortisol concentrations were more likely to increase when subjects were paired with closely bonded individuals (Verspeek and Stevens, 2023). The authors of this study argue that this might be because of the non-cooperative nature of the task, in line with the findings of our study showing that cortisol levels only decrease when closely bonded individuals engage in a cooperative task. Regarding the emotionally mediated reciprocity hypothesis (Schino, 2007; Schino and Aureli, 2009), decreasing cortisol levels indicate that an individual is becoming less aroused. In animals, emotions can be described using the two-dimensional valence-arousal spectrum, also known as core affect space (Mendl et al., 2010; Russell, 2003; Russell and Barrett, 1999). While low cortisol levels suggest lower arousal, they do not provide information on the valence of the experience. Although it is likely that a cooperative task with a closely bonded individual that involves food rewards is associated with positive valence, cortisol levels alone are insufficient to determine this (see e.g., Adriaense et al., 2020; Mendl et al., 2010 for more information and alternative approaches to investigate valence). Our finding, nevertheless, provides valuable insights into cooperation mechanisms and further suggests that the anxiolytic effect linked to cooperative interactions with closely bonded conspecifics may potentially serve as a proximate cause behind the observed impact of close social bonds on the maintenance of cooperation in macaques (Berghänel et al., 2011; Molesti and Majolo, 2016). A potential mechanism underlying this anxiolytic effect may involve oxytocin, which influences the HPA axis activity and thereby cortisol levels (Pati et al., 2020; Uvnäs Moberg et al., 2019). Oxytocin facilitates social bonding (Goodson, 2013; Insel, 2010; Rincon et al., 2020; Ziegler and Crockford, 2017), mediates cooperative behaviour (Duque et al., 2018; Trumble et al., 2015), and has anxiolytic properties (Cavanaugh et al., 2016; Crockford et al., 2017). Moreover, several studies on chimpanzees in the wild indicate that oxytocin is involved in primate cooperative behaviour (Crockford et al., 2013; Samuni et al., 2019, 2018, 2017; Wittig et al., 2014) (for further discussion see Chapter 4 – Discussion). In our study, the cooperative interactions between closely bonded individuals may have increased oxytocin and, therefore, reduce cortisol. Further studies are, however, required to corroborate this. Concludingly, Chapter 4 provides an important contribution to research on the link between physiology and cooperation, and as such to behavioural and cognitive sciences.

Limitations and future direction

Although this doctoral thesis expands the scope of salivary hormone research and provides the groundwork for future investigations in the species investigated i.e., in common squirrel

monkeys, brown capuchin monkeys, long-tailed macaques and common ravens, it also holds some limitations.

First of all, the validations conducted in this thesis are partial validations and could be more exhaustive (see Andreasson et al., 2015). In Chapter 2, for example, we investigated biologically meaningful changes in salivary cortisol concentrations, but we did not investigate the reaction time of the stress response. Therefore, we do not know exactly how long it takes to detect a rise and peak in cortisol after the exposure to a stressor. This, however, is information needed to properly design studies. Moreover, in both, capuchin monkeys and squirrel monkeys we only included one sex, since we did not have a representative number of participating individuals of the other sex. The same counts for the biological validation of Chapter 4. While this validation initially included four individuals – two males and two females – only one male provided all samples needed to properly visualize changes in cortisol in regular intervals. The other individuals were not willing to provide all samples needed after they were exposed to the stressor (animal keeper crackling with a large paper sack in front of them). This indicates that even if an individual has received thorough training in providing saliva samples, the reliability of obtaining (multiple) samples during stressful situations may be compromised. In general, the feasibility of saliva sampling varies across diverse situations and settings. Besides differences in cognitive capacities and stress responses, individuals differ in their hierarchical rank, which means that in group settings, lower-ranking individuals often face challenges in interacting with an animal trainer/experimenter to provide saliva samples (see also STRANGE animals: Webster and Rutz, 2020). Such individuals might, however, be able and willing to give samples (or participate in the training) when they are provided with a safe space in which they cannot be harassed or punished by higher-ranking individuals. This could be accomplished by either physically separating the animals, which could be stressful if they are not used to such separation, or alternatively, training higher-ranking group members to remain stationed at specific locations using target training while lower-ranking individuals engage with the trainer/experimenter. Therefore, I recommend, if feasible, assessing in advance of a study whether, under stressful test conditions, the animal remains willing to engage with the trainer or experimenter.

Further regarding validations, Chapter 4 lacks analytical validations of the EIA, such as parallelism and recovery (see Andreasson et al., 2015). Chapter 3 includes a partial assay validation, however, similar to Chapter 2, lacks a biological validation with a clear causal relationship between certain acute behaviours and immediate changes in salivary hormone concentrations that would allow for the determination of the reaction time as well as the clearance of the hormone. This would be an important next step to get a better understanding of the link between salivary mesotocin concentrations, reflecting naturally occurring peripheral

mesotocin levels, and behaviour, for example pair bonding and parental care behaviour, in bird species.

A second constraint regarding Chapter 3 concerns the limited number of samples in which salivary mesotocin could be reliably measured – only 20 out of 151 samples. Future investigations should prioritize two key aspects. The first one is enhancing the saliva volume obtained by refining and intensifying the training protocols for the birds, along with training for the trainers/experimenters. In the context of birds, the critical constraint in saliva sampling and subsequent hormone analysis lies in the size of the beak and, consequently, the volume of saliva that can be obtained. The analysis of a specific hormone requires a particular saliva volume/hormone concentration, contingent on the chosen analysis method and specific product, such as EIA (kit), LC-MS, and others. This limitation is underscored by a review of the few existing studies, all of which have been conducted on large bird species (Aota et al., 2023; Carbillet et al., 2024; Stocker et al., 2021). Additionally, the positioning of the swab in the beak is a crucial factor influencing the amount of saliva absorbed by the swab. Regarding the ravens, when the swab is positioned parallel to the length of the beak, allowing it to be fully inserted, it will absorb a greater amount of saliva compared to other swab positions. This, however, requires more detailed and professional training. Training an animal to provide saliva samples (and animal training in general) is in some ways more difficult to learn than, for example, drawing blood, because it is an interaction between the trainer and individuals that can differ largely in their cognitive capacities and behaviour. This asks a lot of practical skills, flexibility as well as the knowledge of different training strategies of the animal trainer. Hence, for efficient animal training it is crucial to invest in the professional training of the animal trainer. Another reason to do that is the following: Because it is hard to predict when all the study subjects will have learned to provide samples, training phases are often extended, and the start of studies postponed. This might lead to higher costs and to conflicts with other studies or colony management tasks planned afterwards. The better the animal trainer the faster the animals will learn, the more time will be saved. The second aspect future studies on salivary mesotocin should attend to is advancing the development of more sensitive assays. Undoubtedly, refining the methodology is crucial for future applications.

Another concern regards the influence of food rewards during positive reinforcement training on hormone measurements. Positive reinforcement training makes the use of stimulants superfluous, which is beneficial because it avoids potential dilution of saliva as well as interferences with the EIA (see Padilla et al., 2020). The use of, for example, citric acid dose-dependently increase measured salivary cortisol concentrations in dogs (Dreschel and Granger, 2009). In common marmosets, banana coating on different collection devices had varying effects on cortisol concentrations, which led the authors of the study to conclude that using Salimetrics Oral Swabs without fruit coating is the preferred method for salivary cortisol measurements (Ash

et al., 2018). Talge and colleagues (2005), however, investigated the effect sizes of the influence of stimulants on salivary cortisol measurements and concluded that as long as all samples of a study are collected in a standardised manner using the same small amount of a carefully chosen stimulant, the effects are neglectable. Positive reinforcement training, on the other hand, can also potentially introduce disturbing factors, such as food contamination through food rewards. Ideally, subjects are trained to chew for at least 30 seconds in one go to provide enough saliva without the experimenter having to reward the individual in between chewing bouts. Prolonging a trained behaviour is, however, often a task that requires a lot of time. In the studies presented in this thesis the food rewards the animals got during saliva sampling were very small (e.g., 1/8 of a peanut for the macaques) and standardized, limiting the potential disturbance. However, as part of another project, I am currently testing different training protocols for the collection of salivary hormones in rhesus macaques (*Macaca mulatta*) to enhance the efficiency of the training process, the duration of chewing bouts and the amount of saliva, while maintaining or even improving the positive relationship with the trained individual. Animal training protocols are not always “one size fits all” but they often require tailored adjustments for individual subjects and, therefore, investigating different training approaches would be highly beneficial for future studies on salivary hormones in animals.

Regarding cooperation, we have initiated a follow-up study on long-tailed macaques' cooperation with free partner choice, funded by the Marietta Blau Grant from the Austrian Federal Ministry of Education, Science, and Research. Previous experimental investigations revealed that tolerance for a partner's immediate proximity (potentially enabling physical contact) is the most common determinant of successful cooperation (Hare et al., 2007; Martin et al., 2021; Massen et al., 2015b; Melis et al., 2006; Molesti and Majolo, 2016; Seed et al., 2008); but see also (Scheid and Noë, 2010; Schwing et al., 2016). The ability or option, particularly in experimental setups, for individuals to freely choose a cooperation partner, typically someone with whom they share a high mutual level of social tolerance, promotes cooperation (Asakawa-Haas et al., 2016; Martin et al., 2021; Molesti and Majolo, 2016). Therefore, setups allowing a whole group, instead of artificial pairings (like in Chapter 4), access to the experimental apparatus, where any two individuals can potentially cooperate to solve a task, better reflect ecologically relevant situations and avoid potential social constraints on exhibiting cooperative behaviour. A recent study on long-tailed macaques in a group setting, for example, showed that, unlike what was found in Chapter 4, the quality of the social relationship of the cooperating individuals does influence the success rate (Bhattacharjee et al., 2024). While our previous investigation delved into the acute cortisol levels of long-tailed macaques in a dyadic cooperation task, the follow-up study aims to examine cooperative behaviour in a group setting in connection with (i) chronic levels of cortisol and testosterone in hair, since saliva sampling is rather difficult

in such a setting, and (ii) with prenatal testosterone exposure, measured by the monkeys' current second to fourth digit (2D:4D) ratio. However, the validity of the latter as a reliable indicator of prenatal androgen levels in long-tailed macaques appears to be questionable (see appendix p. 181: *Digit ratio (2D:4D) in long-tailed macaques – investigating sex differences and stability during growth.*). Although the data from this follow-up cooperation study is yet to be analysed, we hope it will contribute to a more detailed understanding of the link between hormones and cooperation in long-tailed macaques. In the meantime, to get a better understanding of the hormonal mechanisms of cooperation in a more natural social setting that allows for partner choice, future studies could focus on groups of less despotic macaque species (see Thierry, 2000). This approach would facilitate training all group members to provide saliva samples while minimizing dominance-related issues during training as well as data collection. However, the social style of the species, which indicates their level of social tolerance, will likely affect their cooperative behaviour (see also Kaigaishi et al., 2019). Differences in social bonds, which may lead to varying levels of HPA-axis activation during cooperation, are nevertheless expected to be present, though possibly less pronounced. Alternatively, other biospecimens, such as urine, could be used to investigate the link between physiology and cooperation (e.g., Samuni et al., 2018), though these samples represent a different time frame. For researchers not specifically interested in primate cooperative behaviour, domestic animals that live in social groups and that are known to cooperate in certain situations, such as pigs, *Sus scrofa (domesticus)*, (Focardi et al., 2015; Koglmüller et al., 2021), offer another viable option. In these species, it is easier to manage dominance-related issues during saliva sampling without extensive training. Furthermore, to find out if the anxiolytic effect of cooperating with closely bonded conspecifics is mediated through oxytocin, future studies could investigate salivary cortisol as well as oxytocin in a loose-string cooperation task. This would reveal how these two hormones interact with each other in this specific sort of cooperation, which would help to further illuminate the proximate mechanisms of cooperation.

Finally, further studies are needed to expand our understanding of salivary hormones in birds and to fully explore the range of insights that can be gained from this approach. I am delighted to see that we could lay the groundwork for other studies on salivary hormones in birds, such as the one by Aota and colleagues (2023), and I hope that in the future this thesis will inspire other researchers to further investigate this topic.

Conclusion

The exploration of salivary hormones proves to be an invaluable tool across various scientific domains, including animal- behaviour, cognition, welfare and conservation. This approach brings

forth numerous advantages, including the option for non-invasive sampling, which minimizes potential influences on test subjects, and the capability to detect short-term changes in hormone levels brought about by certain (experimental) stimuli. Additionally, the ease of collecting saliva enables a high temporal frequency of sampling, facilitating a detailed examination of hormonal fluctuations over time. This thesis leverages these advantages to advance behavioural and cognitive studies, particularly on corvid and primate species. It not only expands the scope of salivary hormone research but also lays the groundwork for future investigations, fostering a deeper understanding of the intricate links between endocrinology, behaviour, and cognition in corvids and primates.

Funding

This thesis and the projects presented in it were funded by the following grants and scholarships: FWF Stand Alone Grant to Jorg Massen (P 26806), a Research Grant from the International Primatological Society to Jorg Massen and Martina Stocker, and a short-term grant abroad (Kurzfristiges wissenschaftliches Auslandsstipendium – KWA) from University of Vienna (Edinburgh 2013), an Erasmus scholarship from the EU (Netherlands 2016), and a Marietta Blau Grant from the Austrian Federal Ministry of Education, Science, and Research (Netherlands 2018), all granted to Martina Stocker.

Acknowledgement of AI assistance

In the course of writing this thesis, I have utilized AI assistance provided by OpenAI's ChatGPT to refine the language. While AI tools have contributed to the formulation and articulation of various sections of this thesis to enhance clarity and coherence, all intellectual content presented herein is my own or properly cited from relevant sources/publications.

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Appendix

General abstract & deutsche Zusammenfassung

General Abstract

In the field of animal behaviour and cognition, researchers strive to unravel the complexities of the cognitive abilities and behavioural adaptations that contribute to the animal's survival and reproduction. Hormones act as chemical messengers orchestrating various physiological functions and behaviours. They play pivotal roles in regulating behaviours such as mating and territorial behaviour and are involved in cognitive processes such as memory and decision-making. Salivary hormone measurements can be very useful to non-invasively determine hormonal changes on a short time scale. This doctoral thesis endeavours to advance salivary hormone collection and measurement in non-human primate and corvid species, renowned for their highly developed cognitive abilities. By employing positive reinforcement training for saliva sample acquisition, the thesis aims to enable non-invasive monitoring of endocrine status during cognitive and behavioural studies. The thesis comprises three studies. The first study focuses on validating cortisol measurements in the saliva of brown capuchin monkeys and squirrel monkeys, with the aim of furthering endocrinological research in this primate group. The second study explores saliva sampling feasibility in birds, specifically measuring salivary mesotocin in common ravens, offering intriguing opportunities to investigate the interplay between behaviour, cognition, and hormones in corvids. Lastly, the third study delves into the relationship between cooperation, social bonds, and salivary cortisol levels in long-tailed macaques, shedding light on the role of the hypothalamic-pituitary-adrenal axis in cooperative behaviour. These studies hopefully facilitate future research in the investigated species and will thereby help getting a deeper understanding of the underlying mechanisms of their behaviour and cognition.

Zusammenfassung

Im Bereich der Verhaltens- und Kognitionsbiologie wird danach gestrebt, die Komplexität der kognitiven Fähigkeiten und Verhaltensanpassungen von Tieren (inkl. Menschen) zu entschlüsseln, die zum Überleben und zur Fortpflanzung der Tiere beitragen. Hormone wirken als chemische Botenstoffe, die verschiedene physiologische Funktionen und Verhaltensweisen orchestrieren. Sie spielen eine entscheidende Rolle bei der Regulierung von Verhaltensweisen wie Paarungs- und Territorialverhalten und sind an kognitiven Prozessen wie Gedächtnis und Entscheidungsfindung beteiligt. Die Messung von Speichelhormonen kann sehr nützlich sein, um hormonelle Veränderungen, die innerhalb kurzer Zeiträume stattfinden nicht-invasiv zu bestimmen. Diese Doktorarbeit zielt darauf ab, die Sammlung und Messung von Speichelhormonen bei nicht-menschlichen Primaten und Rabenvögeln, die für ihre hochentwickelten kognitiven Fähigkeiten bekannt sind, voranzutreiben. Durch den Einsatz von Tiertraining mit positiver Verstärkung zur Gewinnung von Speichelproben soll die Arbeit eine nicht-invasive Überwachung des Hormonstatus während Verhaltens- und Kognitionsstudien ermöglichen. Die Arbeit umfasst drei Studien. Die erste Studie konzentriert sich auf die Validierung von Messungen des Hormons Cortisol im Speichel von Braunen Kapuzineraffen und Totenkopffaffen, um die Hormonforschung in dieser Primatengruppe voranzutreiben. Die zweite Studie untersucht die Durchführbarkeit der Speichelentnahme bei Vögeln, speziell die Messung von Mesotocin im Speichel von Kolkraben, und zeigt interessante Möglichkeiten auf, das Zusammenspiel von Verhalten, Kognition und Hormonen bei Rabenvögeln zu erforschen. Schließlich befasst sich die dritte Studie mit der Beziehung zwischen Kooperation, sozialen Bindungen und Speichel-Cortisolspiegeln bei Javaneraffen, um die Rolle der Hypothalamus-Hypophysen-Nebennieren-Achse im kooperativen Verhalten zu beleuchten. Diese Studien sollen zukünftige Forschungen an den untersuchten Tierarten erleichtern und dadurch ein tieferes Verständnis der zugrunde liegenden Mechanismen ihres Verhaltens und ihrer Kognition ermöglichen.

Comprehensive literature search on PubMed

(August, 2023)

To retrieve research articles on salivary hormones in non-human primates I applied the non-human primate search string of Cassidy and colleagues (2021) and added various other search terms for “saliva” and “hormones” creating the following search string:

("catarrhini"[mh:noexp] OR "cercopithecidae"[mh] OR "gorilla gorilla"[mh] OR "haplorhini"[mh:noexp] OR "hominidae"[mh:noexp] OR "hylobatidae"[mh] OR "pan paniscus"[mh] OR "pan troglodytes"[mh] OR "platyrrhini"[mh] OR "pongo"[mh] OR "primates"[mh:noexp] OR "strepsirhini"[mh] OR "tarsii"[mh] OR "allenopithecus"[tiab] OR "allocebus"[tiab] OR "alouatta"[tiab] OR "alouattinae"[tiab] OR "angwantibo*"[tiab] OR "anthropoid"[tiab] OR "anthropoidea"[tiab] OR "anthropoids"[tiab] OR "aotes"[tiab] OR "aotidae"[tiab] OR "aotinae"[tiab] OR "aotus"[tiab] OR "ape"[tiab] OR "apes"[tiab] OR "arctocebus"[tiab] OR "ateles"[tiab] OR "atelidae"[tiab] OR "atelineae"[tiab] OR "avahi"[tiab] OR "aye-aye*"[tiab] OR "baboon"[tiab] OR "baboons"[tiab] OR "bonobo"[tiab] OR "bonobos"[tiab] OR "brachyteles"[tiab] OR "bushbabies"[tiab] OR "bushbaby"[tiab] OR "cacajao"[tiab] OR "callibella"[tiab] OR "callicebinae"[tiab] OR "callicebus"[tiab] OR "callimico"[tiab] OR "callithrichid*"[tiab] OR "callithrichinae"[tiab] OR "callithrix"[tiab] OR "callitrichid"[tiab] OR "callitrichidae"[tiab] OR "callitrichide"[tiab] OR "callitrichids"[tiab] OR "callitrichinae"[tiab] OR "capuchin"[tiab] OR "capuchins"[tiab] OR "carlito syrichta"[tiab] OR "catarrhine*"[tiab] OR "catarrhini"[tiab] OR "catarrhina"[tiab] OR "catarrhine*"[tiab] OR "catarrhini"[tiab] OR "cebid"[tiab] OR "cebidae"[tiab] OR "cebids"[tiab] OR "cebinae"[tiab] OR "ceboidea"[tiab] OR "cebuella"[tiab] OR "cebus"[tiab] OR "cephalopachus"[tiab] OR "cercocebus"[tiab] OR "cercopithecid*"[tiab] OR "cercopithecinae"[tiab] OR "cercopithecine*"[tiab] OR "cercopithecini"[tiab] OR "cercopithecoid"[tiab] OR "cercopithecoidae"[tiab] OR "cercopithecoids"[tiab] OR "cercopithecus"[tiab] OR "cheirogaleidae"[tiab] OR "cheirogaleus"[tiab] OR "cheracebus"[tiab] OR "chimp"[tiab] OR "chimpanzee"[tiab] OR "chimpanzees"[tiab] OR "chimps"[tiab] OR "chiromyiformes"[tiab] OR "chiropotes"[tiab] OR "chlorocebus"[tiab] OR "colobidae"[tiab] OR "colobinae"[tiab] OR "colobine*"[tiab] OR "colobini"[tiab] OR "colobus*"[tiab] OR "cynomolgus"[tiab] OR "daubentonia"[tiab] OR "daubentoniidae"[tiab] OR "douc"[tiab] OR "doucs"[tiab] OR "erythrocebus"[tiab] OR "eulemur"[tiab] OR "euoticus"[tiab] OR "euprimate*"[tiab] OR "galagid*"[tiab] OR "galago"[tiab] OR "galagoides"[tiab] OR "galagonidae"[tiab] OR "galagos"[tiab] OR "gelada"[tiab] OR "geladas"[tiab] OR "gibbon"[tiab] OR "gibbons"[tiab] OR "gorilla"[tiab] OR "gorillas"[tiab] OR "grivet"[tiab] OR "grivets"[tiab] OR "guenon*"[tiab] OR "guereza*"[tiab] OR "hapalemur"[tiab] OR "haplorhine*"[tiab] OR "haplorhini"[tiab] OR "haplorrhine*"[tiab] OR "haplorrhini"[tiab] OR "hominid*"[tiab] OR "hominin"[tiab] OR "homininae"[tiab] OR "hominine"[tiab] OR "hominines"[tiab] OR "hominini"[tiab] OR "hominins"[tiab] OR "hominoidea"[tiab] OR "hoolock"[tiab] OR "howler*"[tiab] OR "hylobates"[tiab] OR "hylobatidae"[tiab] OR "indri"[tiab] OR "indridae"[tiab] OR "indriid*"[tiab] OR "indris"[tiab] OR "kipunji*"[tiab] OR "lagothrix"[tiab] OR "langur"[tiab] OR "langurs"[tiab] OR "lemur"[tiab] OR "lemurid*"[tiab] OR "lemuriform"[tiab] OR "lemuriformes"[tiab] OR "lemuriforms"[tiab] OR "lemurinae"[tiab] OR "lemuroidea"[tiab] OR "lemurs"[tiab] OR "leontideus"[tiab] OR "leontocebus"[tiab] OR "leontopithecus"[tiab] OR "lepilemur"[tiab] OR "lepilemurid*"[tiab] OR "lesula*"[tiab] OR "lophocebus"[tiab] OR "loriform"[tiab] OR "loriformes"[tiab] OR "lorinae"[tiab] OR "loris"[tiab] OR "lorises"[tiab] OR "lorisid*"[tiab] OR "lorisiform*"[tiab] OR

"lorisinae"[tiab] OR "lorisoid*"[tiab] OR "lutung"[tiab] OR "lutungs"[tiab] OR "macaca"[tiab] OR "macaque's"[tiab] OR "macaque"[tiab] OR "macaques"[tiab] OR "malbrouck*"[tiab] OR "mandrill"[tiab] OR "mandrills"[tiab] OR "mandrillus"[tiab] OR "mangabey*"[tiab] OR "marmoset"[tiab] OR "marmosets"[tiab] OR "mico argentatus"[tiab] OR "mico chrysoleucos"[tiab] OR "mico emiliae"[tiab] OR "mico humilis"[tiab] OR "mico marcai"[tiab] OR "mico melanurus"[tiab] OR "mico rondoni"[tiab] OR "microcebus"[tiab] OR "miopithecus"[tiab] OR "mirza coquereli"[tiab] OR "mirza zaza"[tiab] OR "monkey"[tiab] OR "monkeys"[tiab] OR "muriqui*"[tiab] OR "nasalis larvatus"[tiab] OR "nomascus"[tiab] OR "nycticebus"[tiab] OR "oedipomidas"[tiab] OR "orang utan*"[tiab] OR "orang-utan*"[tiab] OR "orangutan*"[tiab] OR "oreonax"[tiab] OR "otolemur"[tiab] OR "pan paniscus"[tiab] OR "pan troglodytes"[tiab] OR "panin"[tiab] OR "panina"[tiab] OR "panins"[tiab] OR "papio"[tiab] OR "papionini"[tiab] OR "paragalago"[tiab] OR "perodicticinae"[tiab] OR "perodicticus"[tiab] OR "phaner"[tiab] OR "piliocolobus"[tiab] OR "pithecia"[tiab] OR "pithecidae"[tiab] OR "pitheciid*"[tiab] OR "pitheciinae"[tiab] OR "pithecinae"[tiab] OR "platyrhine*"[tiab] OR "platyrhini"[tiab] OR "platyrrhina"[tiab] OR "platyrrhine*"[tiab] OR "platyrrhini"[tiab] OR "plecturocebus"[tiab] OR "pongid*"[tiab] OR "ponginae"[tiab] OR "pongo"[tiab] OR "potto"[tiab] OR "pottos"[tiab] OR "presbytini"[tiab] OR "presbytis"[tiab] OR "primate"[tiab] OR "primates"[tiab] OR "procolobus"[tiab] OR "prolemur"[tiab] OR "propithecus"[tiab] OR "prosimian*"[tiab] OR "prosimii"[tiab] OR "pseudopotto"[tiab] OR "pygathrix"[tiab] OR "rhinopithecus"[tiab] OR "rungwecebus"[tiab] OR "saguinus"[tiab] OR "saimiri"[tiab] OR "saimiriinae"[tiab] OR "sapajus"[tiab] OR "sciurocheirus"[tiab] OR "semnopithecus"[tiab] OR "siamang"[tiab] OR "siamangs"[tiab] OR "sifaka"[tiab] OR "sifakas"[tiab] OR "simians"[tiab] OR "simias"[tiab] OR "simiiform*"[tiab] OR "strepsir*"[tiab] OR "surili*"[tiab] OR "symphalangus"[tiab] OR "talapoin*"[tiab] OR "tamarin"[tiab] OR "tamarins"[tiab] OR "tamarinus"[tiab] OR "tarsier"[tiab] OR "tarsiers"[tiab] OR "tarsiid*"[tiab] OR "tarsiiform*"[tiab] OR "tarsius"[tiab] OR "theropithecus"[tiab] OR "trachypithecus"[tiab] OR "uacari*"[tiab] OR "uakari"[tiab] OR "uakaris"[tiab] OR "varecia"[tiab] OR "vervet*"[tiab])

AND ("hormones" [MeSH] OR "hormones" [tiab] "glucocorticoid" [tiab] OR "glucocorticoids" [tiab] OR "cortisol" [tiab] OR "corticosterone" [tiab] OR "nonapeptide"[tiab] OR "oxytocin"[tiab] OR "mesotocin"[tiab] OR "androgens"[tiab] OR "testosterone"[tiab]))

AND ("saliva" [MeSH] OR "saliva"[tiab] OR "salivary"[tiab])

The resulting collection of publications was then reviewed and 55 of the studies actually measured salivary hormones in non-human primates. Five more studies were found on Google Scholar, making a total of 60 research articles.

For birds, unfortunately there was no pre-existing search string that includes different bird species. With the MeSH term "bird", however, all studies except for the ones published in the past one to two years should be found and the tiab terms should cover the rest:

((("birds" [MeSH] OR "bird" [tiab] OR "birds" [tiab] OR "avian" [tiab] OR "corvid" [tiab] OR "passerine" [tiab] OR "chicken" [tiab] OR "broiler" [tiab] OR "quail" [tiab] OR "fowl" [tiab] OR "pigeon" [tiab] OR "seagull" [tiab] OR "finch" [tiab] OR "sparrow" [tiab] OR "tit" [tiab] OR "starling" [tiab] OR "budgerigar" [tiab] OR "raven" [tiab] OR "crow" [tiab]))

AND ("hormones" [MeSH] OR "hormones" [tiab] "glucocorticoid" [tiab] OR "glucocorticoids" [tiab] OR "cortisol" [tiab] OR "corticosterone" [tiab] OR "nonapeptide"[tiab] OR "oxytocin"[tiab] OR "mesotocin"[tiab] OR "androgens"[tiab] OR "testosterone"[tiab]))

AND ("saliva" [MeSH] OR "saliva"[tiab] OR "salivary"[tiab])

With this search string I could find three research articles on salivary hormones in birds. Please, be aware that, despite my efforts to find all relevant studies for both, non-human primates and birds, there is a small chance that some studies could not be found with the comprehensive search on PubMed (and Google Scholar).

Table A1. Research articles including the measurement of salivary hormones in non-human primates and birds

Species	Hormone	Field	Study
<i>Apes</i>			
bonobo	cortisol	animal behaviour	(Hohmann et al., 2009)
bonobo	cortisol	animal behaviour	(Behringer et al., 2009)
bonobo	cortisol	animal behaviour, stress - method	(Verspeek et al., 2021)
bonobo	cortisol	animal behaviour	(Verspeek and Stevens, 2023)
bonobo, chimpanzee	cortisol, testosterone	animal behaviour	(Wobber et al., 2010)
bonobo, chimpanzee	testosterone	development	(Wobber et al., 2013)
bonobo, orangutan	cortisol	animal behaviour	(Behringer et al., 2014)
bonobo, orangutan, western lowland gorilla, chimpanzee	cortisol, alpha amylase	stress	(Behringer et al., 2013)
bonobo, Sumatran orangutans, Western lowland gorilla	cortisol	animal behaviour	(Behringer et al., 2022)
chimpanzee	cortisol, testosterone	method - validation	(Kutsukake et al., 2009)
chimpanzee	cortisol	method - validation	(Heintz et al., 2011)
chimpanzee	cortisol	animal behaviour	(Kano et al., 2016)
chimpanzee	cortisol	animal behaviour	(Kutsukake et al., 2018)
chimpanzee	cortisol, testosterone	animal behaviour	(Kutsukake et al., 2019)
chimpanzee	cortisol	animal behaviour	(Kutsukake et al., 2023)
western lowland gorilla	oxytocin	method - validation	(Leeds et al., 2018)
western lowland gorilla	cortisol, oxytocin	animal behaviour	(Heintz et al., 2019)

western lowland gorilla	cortisol, oxytocin	animal behaviour	(Leeds et al., 2020)
<i>Other Old World primates</i>			
Japanese macaques	cortisol	stress	(Kawakami et al., 2002)
Japanese macaques	cortisol, alpha amylase	animal behaviour, stress - method	(Broche et al., 2023)
long-tailed macaques	cortisol	animal behaviour	(Stocker et al., 2020)
long-tailed macaque, rhesus macaque	testosterone	method - validation	(Arslan et al., 1984)
rhesus macaques	cortisol	development, stress	(Boyce et al., 1995)
rhesus macaques	prolactin	serotonin	(Lindell et al., 1999)
rhesus macaques	cortisol	method	(Lutz et al., 2000)
rhesus macaques	cortisol	animal behaviour	(Lutz et al., 2003)
rhesus macaques	cortisol	stress	(Davenport et al., 2003)
rhesus macaques	cortisol	animal behaviour	(Lutz et al., 2004)
rhesus macaques	cortisol	medical	(Newman et al., 2007)
rhesus macaques	growth hormone, erythropoietin	medical	(Voutetakis et al., 2008)
rhesus macaques	cortisol	animal behaviour	(Davenport et al., 2008)
rhesus macaques	cortisol, alpha amylase, (immunoglobulin A)	method	(Higham et al., 2010)
rhesus macaques	oxytocin	animal behaviour	(Simpson et al., 2014)
rhesus macaques	cortisol, alpha amylase	development	(Petrullo et al., 2016)
rhesus macaques	cortisol, alpha amylase	animal behaviour	(Mandalaywala et al., 2017)
rhesus macaques	cortisol	cognition	(Bosc et al., 2017)
rhesus macaques	cortisol	animal behaviour, stress	(Pfefferle et al., 2018)
rhesus macaques	cortisol, oxytocin	development	(Festante et al., 2021)
rhesus macaques, long-tailed macaques, African green monkeys	cortisol	method - validation	(Rapp-Santos et al., 2017)
Guinea baboon	cortisol	animal behaviour	(Fagot et al., 2014)
hamadryas baboon	cortisol	method - validation	(Pearson et al., 2008)
hamadryas baboon	cortisol	animal behaviour	(Pearson et al., 2015)
Lemur catta	testosterone	animal behaviour	(von Engelhardt et al., 2000)

pied tamarins	cortisol	animal behaviour, stress	(Price et al., 2019)
<i>New World primates</i>			
common marmoset	cortisol	animal behaviour, stress - method	(Cross et al., 2004)
common marmoset	cortisol	stress	(Cross and Rogers, 2004)
common marmoset	cortisol	animal behaviour	(Cross and Rogers, 2006)
common marmoset	cortisol	medical	(Kuehnel et al., 2012)
common marmoset	cortisol	method - validation	(Ash et al., 2018)
common marmoset	cortisol	PTSD	(Philippens et al., 2021)
common marmoset	cortisol	animal behaviour, stress	(Ash et al., 2021)
common marmoset	cortisol	autism	(Nakamura et al., 2022)
squirrel monkeys	cortisol	method - validation	(Fuchs et al., 1997)
squirrel monkeys	cortisol	medical	(Lee et al., 2003)
squirrel monkeys	cortisol	method	(Tiefenbacher et al., 2003)
squirrel monkeys	cortisol	medical	(Lee et al., 2004)
squirrel monkeys	prolactin (not detectable)	animal behaviour	(Soltis et al., 2005)
squirrel monkeys	cortisol	medical	(Platt et al., 2007)
squirrel monkeys	cortisol	medical	(McKenzie-Quirk and Miczek, 2008)
squirrel monkeys, brown capuchin monkeys	cortisol	method - validation	(Stocker et al., 2023)
<i>Birds</i>			
cockatiel, rose-ringed parakeet	testosterone, corticosterone	method - validation	(Hahn et al., 2011)
common raven	mesotocin	method - validation	(Stocker et al., 2021)
large-billed crow	corticosterone	method - validation	(Aota et al., 2023)
common gull	corticosterone	method - validation	(Carbillet et al., 2024)

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Additional publication

A comparative approach to affect and cooperation

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DOI: 10.1016/j.neubiorev.2019.09.027

Abstract

A central premise of the science of comparative affect is that we can best learn about the causes and consequences of affect by comparing affective phenomena across a variety of species, including humans. We take as a given that affect is widely shared across animals, but a key challenge is to accurately represent each species' affective experience. A common approach in the comparative study of behavior and cognition is to develop standardized experimental paradigms that can be used across species, with the assumption that if the same task is being used, we can directly compare behavioral responses. This experimental approach rests on two underlying assumptions: first, that different species' perception of and affective response to these paradigms are the same; and second, that behavioral and physiological (including endocrine and neural) responses to these paradigms are homologous; if either of these assumptions is not true, then the comparison becomes much less straightforward. Our goal in the present paper is to summarize the dominant paradigms that have been used for such comparative research, with a particular focus on paradigms common in the cooperation literature, and to critically discuss dominant assumptions about what affective states these tasks can or should measure. We then consider the advantages and drawbacks of this experimental method, and consider alternatives that may improve our understanding. We hope that this will help scholars recognize and avoid pitfalls inherent in studying affect, and stimulate them to create novel, ecologically relevant paradigms for examining affect across the animal kingdom.

Keywords: *emotions, physiology, animal emotions, evolution, animal cooperation*

1. Introduction

The immense scale and plasticity of human cooperation is a defining feature of our species (Melis & Semmann, 2010). Human societies show a large variety of complex social configurations characterized by diverse cooperative relationships among multiple, often unrelated individuals (Hill et al., 2011; Kaplan, Hooper, & Gurven, 2009). In general, our cooperative abilities are considered to be a major force in explaining humans' ecological dominance and biodemographic success (Kramer, 2010; Roberts & Stewart, 2018), and some argue it may have been a strong force driving the evolution of our intelligence (Vygotskian intelligence hypothesis: Vygotsky, 1978; Moll & Tomasello, 2007; but see González-Forero & Gardner, 2018). However, while extensive progress has been made in explaining the evolution of cooperative behavior across taxa (West, Griffin, & Gardner, 2007), the evolution of complex human cooperation continues to pose explanatory challenges that, although debated for more than 50 years, remain unresolved (e.g. Burkart, Hrdy, & Van Schaik, 2009; Pinker, 2010; Silk & House, 2016; Taborsky, Frommen, & Riehl, 2016a,b)

Whereas definitions for cooperation vary substantially between authors and scientific fields (Noë, 2006; West, Griffin, & Gardner, 2007), all refer to benefits and costs for one or more social partners (often termed *players*). A key explanatory problem is that cooperation becomes prone to cheaters that reap the benefits, yet never pay the costs. Hamilton (1964) reconciled the problem of cooperation among genetically similar individuals with his inclusive fitness theory. Nevertheless, cooperation in animal societies often involves unrelated individuals, suggesting that direct fitness benefits must also play a central evolutionary role (Leimar & Hammerstein, 2010; Taborsky et al., 2016b). For example, cooperation between unrelated individuals may be explained when all parties immediately benefit from the action (i.e. byproduct mutualism, West et al, 2007), yet when possible, individuals are still expected to minimize their own cost at the expense of others (a.k.a. ‘the tragedy of the commons’, Lloyd, 1833) and cooperation can break down easily (e.g., Burton-Chellew & West, 2013; Kummerli, Burton-Chellew, Ross-Gillespie, & West, 2010).

This is particularly apparent for behaviors that do not reap immediate benefits for an actor. Trivers (1971) suggested that this could be resolved in situations in which individuals interact repeatedly and can differentiate cheaters from cooperators, as reciprocal altruism can evolve such that individuals engage in immediately costly behaviors that increase their probability of receiving future (greater) benefits from a partner. Originally, such iterated interactions were modeled using repeated prisoner’s dilemma games, which suggested that reciprocity can be maintained through the evolution of decision rules reliant on a ‘tit-for-tat’ or similar strategy (Axelrod, 1984). Whereas there is a considerable amount of evidence for long-term exchange of commodities in social mammals (e.g., Kern & Radford, 2018; Schino & Aureli, 2008; Schino, 2007), and even for long-term memory for reciprocal interactions in birds (Müller, Massen, Bugnyar, & Osvath, 2017), evidence for active ‘bookkeeping’ of the costs and benefits of reciprocity (a.k.a. calculated reciprocity, de Waal & Luttrell, 1988) is scarce (but see Dufour, Pele, Neumann, Thierry, & Call, 2009; Schweinfurth & Taborsky, 2018a). Furthermore, such calculated reciprocity may be too cognitive demanding for most animals (Stevens & Hauser, 2004).

Therefore, less cognitively demanding proximate mechanisms have been suggested to underpin reciprocity, such as symmetry-based reciprocity, attitudinal reciprocity and emotionally mediated reciprocity. Symmetry-based reciprocity describes simple decision rules reliant on symmetrical features of interaction partners: e.g. age, sex or mutual association (de Waal & Luttrell, 1988). Most relevant here, however, are attitudinal reciprocity, which describes decision rules based on short term attitudes towards specific cooperation partners, which in turn is based on recent interactions with that individual (Brosnan & de Waal, 2002), and/or emotionally mediated reciprocity, which describes decision rules based on long-term emotional appraisal of specific cooperation partners, which in turn is based on multiple interactions with

that individual (Aureli & Schaffner, 2002; Schino & Aureli, 2009; Schino, di Sorrentino, & Tiddi, 2007). Agent-based models (ABMs) have recently shown that over multiple generations, reciprocal altruism breaks down in populations that base partner choice solely on similarities with conspecifics, suggesting that symmetry-based reciprocity on its own is an evolutionarily unstable proximate mechanism for non-kin cooperation (Campenni & Schino, 2016). In contrast, several other studies using ABMs have generated evolutionary stable systems that represent actual social organizations (e.g. those of monkey groups) when individuals rely on emotionally mediated reciprocity (Campenni & Schino, 2014; Evers, de Vries, Spruijt, & Sterck, 2016; Puga-Gonzalez, Hoscheid, & Hemelrijk, 2015). Note, however, that emotionally mediated reciprocity (or attitudinal reciprocity), and the more cognitively complex calculated reciprocity need not be mutually exclusive. In fact, human brain imaging studies support the notion that complex cognitive mechanisms and more ‘basal’ hormonal mechanisms may exist in parallel, and show that their employment depends on familiarity (Krueger et al. 2007); i.e. more hormonal/emotional areas are activated when cooperating with familiar individuals (cf. *emotionally mediated reciprocity* (Schino, & Aureli, 2009); and/or *attitudinal reciprocity* (Brosnan, & de Waal, 2000)), whereas more cortical areas involved in mentalizing are activated while cooperating with unfamiliar individuals (cf. *calculated reciprocity* (de Waal, & Luttrell, 1988). Thus, the employment of these different mechanisms should be seen as a dynamic process that flexibly adjusts to the specific context in which it is needed (West, El Mouden, & Gardner, 2011), and where the different components are also not independent— i.e. cognition can affect subsequent emotional appraisal, and emotions can affect cognitive decisions (cf. the componential view of emotions; Mendl, Burman, & Paul, 2010; see below).

Given the ubiquity of cooperation in animals (Dugatkin 1997; Clutton-Brock, 2009), biologists traditionally adopt a phylogenetic approach to trace the evolutionary history of cooperation (Huxley, 1923; Tinbergen, 1964), comparing cooperation among different species. We now have evidence for non-kin cooperation in a growing number of species from different lineages, ranging from primates (e.g., Cronin, Kurian, & Snowdon, 2005; Melis, 2006; Mendres & de Waal, 2000; Miss & Burkart, 2018; Molesti & Majolo, 2016; Suchak, Eppley, Campbell, & de Waal, 2014) and other social mammals (e.g., Drea & Carter, 2009; Marshall-Pescini, Schwarz, Kostelnik, Virányi, & Range, 2017; Plotnik, Lair, Suphachoksahakun, & Waal, 2011; Schmelz, Duguid, Bohn, & Völter, 2017; Schweinfurth & Taborsky, 2018b) to birds (e.g., Massen, Ritter, & Bugnyar, 2015; Schwing, Jocteur, Wein, Noë, & Massen, 2016; Seed, Clayton, & Emery, 2008) and fish (e.g., Vail, Manica, & Bshary, 2013, 2014). However, while this approach is very useful for understanding how cooperation functions in response to a species’ ecology, it has been less useful for identifying common cognitive and/or affective mechanisms underlying cooperation across species. Relevant to the current paper, while there have been some studies testing broad

hypotheses about the evolution of cognition (Fitch, Huber, & Bugnyar, 2010; MacLean et al., 2012), fewer are explicitly comparative (but see Burkart et al., 2014) and even fewer have considered the emotional mediation of cooperation, despite its emerging prominence in explaining cooperation.

Emotional responses, specifically those of non-human animals, have been notoriously difficult to measure due to the subjective component of emotional experience, which per definition cannot be directly measured in another person, let alone in animals (Tinbergen, 1963). Yet, human emotions are considered to be multifaceted, comprising a subjective component as well as behavioral and physiological components (Izard, 1977). Indeed, most theoretical approaches to animal emotions assume that different components, including affect, interact and that emotions are either the sum of those parts (the componential view of emotions; Mendl, Burman, & Paul, 2010; Paul, Harding, & Mendl, 2005; 2019) or are an emergent phenomenon of these parts (the constructivist view: Bliss-Moreau 2017). While our message does not require discriminating further between these two views, we do emphasize the key point common to both for studying animals: emotions involve specific, measurable components and animal emotions, whatever they may be, should be studied by exploring each of these components and their interactions separately. Moreover, we support the view that, irrespective of whether animals can consciously experience feelings, it is important in this work not to make *a priori* assumptions about potential subjective experiences. Yet, to our knowledge, this approach has not been systematically imposed on questions regarding cooperation.

Therefore, in this review we aim to provide an overview of attempts to explore affective responses in the context of cooperation and consider ways in which related work informs our understanding of the affective components of cooperation. In particular, given the nascent state of the field, we will consider the limitations of experimental set-ups used thus far and how this constrains our understanding, and then provide our vision for future directions that the field can take.

2. Review of paradigms in light of “cognitive and/or emotional” cooperative decision making

2.1. Coordination paradigms

Animal cooperation studies have a long history, starting with the seminal work of Meredith Crawford in 1937 (Crawford, 1937). Crawford confronted two young chimpanzees with a tray filled with food with two ropes attached to it. The tray was too heavy for only one of the juvenile chimpanzees to pull into reach, so food could only be acquired when the two chimpanzees pulled the ropes simultaneously. Since then, several other studies have adopted this paradigm (Mendres

& de Waal, 2000) or variations thereof (Fady, 1972; Suchak et al., 2014, 2016; Suchak, Watzek, Quarles, & de Waal, 2018). Similar paradigms that rely on coordinated action have been developed as well (e.g.: Cronin et al., 2005; Drea & Carter, 2009; Eskelinen, Winship, Jones, Ames, & Kuczaj, 2016; Jaakkola, Guarino, Donegan, & King, 2018; Miss & Burkart, 2018; see Table 1 for a full overview), with the loose-string paradigm designed by Hirata (Hirata, 2003) being the most popular across a diversity of species (Table 1). Whereas much of human cooperation requires coordinated action, surprisingly few studies have experimentally tackled this phenomenon in humans, and those that do generally test children (Kagan & Madsen, 1970), or even infants (Ross & Lollis, 1987; Warneken, Chen, & Tomasello, 2006)

Most of the studies employing coordination paradigms have concentrated on the cognitive underpinnings of the task, examining whether particular species (or age groups) do or do not understand the contingencies of the specific task. Recently, however, focus has also shifted to examining the influence of social context on task performance. Generally, in those species in which it has thus far been tested, social tolerance and relationship quality seem to be important constraints on the emergence of cooperation. Interspecific comparisons reveal that more socially tolerant species cooperate more (easily) (e.g., Petit, Desportes, & Thierry, 1992; Hare, Melis, Woods, Hastings, & Wrangham, 2007; Joly et al., 2017). Intraspecific comparisons also show that populations with high tolerance outperform those with low tolerance on a cooperation task (Kaigaishi, Nakamichi, & Yamada, 2019), and further demonstrate that dyads within a group cooperate more often and more successfully when they have good relationships and/or are socially tolerant to each other (see Table 1). Such individuals even select each other more often as cooperation partners when given the choice (e.g. Asakawa-Haas, Schiestl, Bugnyar, & Massen, 2016).

For our purposes, this is important because these patterns are consistent with animals utilizing emotionally mediated decision rules, yet specific emotional mechanisms are rarely considered. Friends, for example, tend to have similar personalities (Massen & Koski, 2014; Youyou, Stillwell, Schwartz, & Kosinski, 2017; Ebenau, von Borell, Penke, Ostner, & Schülke, 2019), and it has been suggested that this phenotypic similarity increases cooperation through a better understanding of each other's behavior, subsequent increased trust, easier coordination of behavior, and the maintenance of a similar affective state while cooperating (Massen & Koski, 2014). Recent studies testing these hypotheses have indeed found that, at least among chimpanzees, friends tend to trust each other more than non-friends (Engelmann & Herrmann, 2016; but see Calcutt, Proctor, Berman, & de Waal, 2019), and that for Barbary macaques, cooperative success is enhanced by personality similarity in animals (Molesti & Majolo, 2016).

Table 1. Coordination paradigms used to study cooperation in animals, the species in which they were tested and several parameters tested

Paradigm	Species	Study	Understanding the need of partner	Tolerance as constraint	Reward Equity as constrain	Physiological Measures	Other (affective) measures
Cooperative pulling task	Primates						
	<i>Pan troglodytes</i>	(Crawford 1937; Suchak et al., 2014, 2016, 2018)	Y	Y	Y	-	-
	<i>Macaca Mulatta</i> & <i>M. Tonkeana</i>	(Petit, Desportes, & Thierry, 1992)	N	Y	-	-	-
	<i>Cebus Apella</i>	(de Waal & Berger, 2000; de Waal & Davis, 2003; Mendres & de Waal, 2000)	Y	-	Y	-	
Coordinated pulling/pushing	Primates						
	<i>Pan troglodytes</i>	(Chalmeau & Gallo, 1996)	Y	-	-	-	-
	<i>Pongo Pygmaeus</i>	(Chalmeau, Lardeux, Brandibas, & Gallo, 1997)	Y				
	<i>Cebus apella</i>	(Chalmeau, Visalberghi, & Gallo, 1997)	N	-	-	-	-
	<i>Saguinus oedipus</i>	(Cronin et al., 2005)	Y	-	-	-	-
	Other mammals						
	<i>Crocutta Crocutta</i>	(Drea & Carter, 2009)	Y	Y	-	-	-
	<i>Tursiops truncatus</i>	(Eskelinen et al., 2016; Jaakkola et al., 2018)	Y	-	-	-	Acoustic parameters: an increase in sound production while cooperating

Loose-string task	Primates						
	<i>Pan troglodytes</i>	(Hirata, 2003; Hirata & Fuwa, 2007; Melis, 2006; Melis, Hare, & Tomasello, 2006)	Y	Y	-	-	A preference for efficient partners
	<i>Pan paniscus</i>	(Hare et al., 2007)	-	Y	-	-	-
	<i>M. sylvanus</i>	(Molesti & Majolo, 2016)	Y	Y	-	-	Similarity in boldness-shyness increased cooperation
	<i>M. fascicularis</i>	(Stocker et al. <i>in review</i>)	-	N	-	Cortisol decreased while cooperating, but only when cooperating with a friend.	-
	<i>M. fuscata</i>	(Kaigaishi et al., 2019)	±; i.e. only one individual	Y	-	-	-
	<i>Callithrix jacchus</i>	(Martin et al. <i>in review</i>)	±; i.e., individual and/or dyadic learning was observed	Y	-	-	Prosociality increased cooperation
Other mammals							
	<i>Elephas maximus</i>	(Plotnik et al., 2011)	Y	-	-	-	-
	<i>Canis familiaris</i>	(Sarah Marshall-Pescini, Basin, & Range, 2018; Sarah Marshall-Pescini et al., 2017; Ostojić & Clayton, 2014)	Y/N	Y	-	-	-
	<i>Canis lupus</i>	(Sarah Marshall-Pescini et al., 2017)	Y	Y	-	-	-
	<i>Pteronura brasiliensis</i> & <i>Aonyx cinrea</i>	(Schmelz et al., 2017)	N	-	-	-	-

Birds						
<i>Corvus frugilegus</i>	(Scheid & Noë, 2010; Seed et al., 2008)	N	Y	-	Cortisol related to boldness-shyness; i.e. higher in the latter after stressful event	Temperament; i.e. Bold individuals performed better in the cooperation task
<i>Psittacus erithacus</i>	(Péron, Rat-Fischer, Lalot, Nagle, & Bovet, 2011)	±	-	-	-	-
<i>Corvus corax</i>	(Asakawa-Haas, Schiestl, Bugnyar, & Massen, 2016; Massen et al., 2015; in review)	Y/N	Y	Y	-	-
<i>Nestor notabilis</i>	(Heaney, Gray, & Taylor, 2017; Schwing et al., 2016)	Y/N	Y	Y	-	-
<i>Ara Glaucoocularis</i>	(Tassin du Montaigu, et al., <i>in press</i>)	N	N	-	-	-
Complementary tasks						
<i>Callithrix jacchus</i>	(Werdenich & Huber, 2002)	-	-	-	-	-
<i>Nestor notabilis</i>	(Tebbich, Taborsky, & Winkler, 1996)	-	-	Y	-	Dominant kea manipulated their partners to produce.
<i>Plectropomus leopardus</i>	(Vail et al., 2014)	Y	-	-	-	-
<i>Pan Paniscus</i>	(Engelmann, Herrmann, & Tomasello, 2015; Engelmann & Herrmann, 2016)	Y	Y	Y	-	-

Cheating, on the other hand, seems to have negative effects on subsequent cooperation, and all studies that examined this effect to date report a loss of interest in the paradigm by the animal that was cheated (de Waal & Davis, 2003; Engelmann et al., 2015; Massen et al., 2015; Schwing et al., 2016) (see also section 2.2.5 on inequity aversion). In human studies, participants are sometimes given the opportunity to punish defectors at a personal cost, and do so readily (Fehr & Gächter, 2002), although cultural differences in the tendency to punish are apparent (e.g., Henrich & Henrich, 2014). Interestingly, from our perspective, participants often report being angry when their partners ‘defect’, and this anger seems to mediate the amount of costly punishment in these games (Cubitt, Drouvelis, & Gächter, 2011; Seip, Van Dijk, & Rotteveel, 2014a). Moreover, when primed with anger prior to the experiment, humans punish defectors even more than normal (Seip et al., 2014a), again underlining the important mediating role of this emotion in dealing with defection. Whether the reported loss of motivation in animals that experience defection also reflects anger or is otherwise emotionally mediated remains unknown and is a promising avenue for further studies (see also section 2.2.5 on inequity aversion).

2.2 Economic games

Original work on cooperation focused on more ‘naturalistic’ paradigms, such as the aforementioned barpull task, but more recently there has been interest in using economic games to explore cooperative decision-making across species. Although these games lack ecological validity, they are highly standardized and easy to use in (what we hope are) very similar ways across a variety of species and contexts (see Interpretations section 4.1 for a further discussion of how animals interpret our tasks). This allows for developing and testing comparative hypotheses and deriving the underlying similarities and differences across species that can then be tested using more species-specific paradigms in more ecologically relevant contexts (Smith, Watzek, & Brosnan, 2018). These game scenarios are very simple. Two or more subjects are each given a choice, typically among only two options, and then rewards are distributed based on both what the subject and their partner(s) chose. The payoffs for outcomes can be adjusted so that researchers can test everything from coordination (Assurance game: both players benefit most if they choose the same outcome) to competition (Hawk Dove game: no coordinated outcome, players do best to play opposite their partner) and cooperation in the face of defection (Prisoner’s Dilemma)(Table 2).

Table 2. Economic games tested in animals, type of game, purpose, species, and basic results within primates. Note that there are also results in species outside the primate order, particularly involving the Prisoner's dilemma game (Milinski 1987; Dugatkin, 1991; Stephens et al. 2002; Wood, Kim, & Li, 2016), but for the sake of brevity and clarity chose to include only primates here.

Game	Purpose	Species	Studies	Result
Assurance Game (Stag Hunt)	Coordination game with two Nash equilibria (NE), one payoff dominant	Chimpanzees	(Brosnan et al., 2011; Bullinger, Wyman, Melis, & Tomasello, 2011; Duguid, Wyman, Bullinger, Herfurth-Majstorovic, & Tomasello, 2014; Hall et al., 2019)	Chimpanzees can coordinate, and show evidence of a strategy, but do not always do so. Evidence of a leader/follower dynamic whereby the first player's choice affects the second player's strategy
		Rhesus monkeys	(Brosnan, Wilson, & Beran, 2012; Parrish, Brosnan, Wilson, & Beran, 2014)	Rhesus coordinate whether or not they can see their partner's choice; mechanism appears to be <i>Stag</i> bias
		Capuchin monkeys	(Brosnan et al., 2011, 2012; Smith, Leverett, & Brosnan, 2019)	Capuchins coordinate, but only when they can see their partner's choice; mechanism appears to be matching. There is no impact of oxytocin on decisions.
		Squirrel monkeys	(Vale, Williams, Schapiro, Lambeth, & Brosnan, 2019)	Squirrel monkey females show a tendency to coordinate.
Snowdrift Game	Coordination game with a temptation to slack	Chimpanzees	(Sánchez-Amaro, Duguid, Call, & Tomasello, 2016)	Chimpanzees coordinate, and their choices are influenced by the identity of their social partner.
Chicken Game (Hawk Dove)	Anti-coordination game	Chimpanzees	(Hall et al., 2019)	There is little evidence of anti-coordination, although this is a population that did not coordinate, either, indicating disinterest in the task.
		Rhesus monkeys	(Brosnan et al., 2017)	Rhesus monkeys play the NE, but only when they can see what their partner plays.
		Capuchin monkeys	(Brosnan et al., 2017; Smith et al., 2019)	Capuchin monkeys play the NE, but only when they can see what their partner plays. The second mover's

		Squirrel monkeys	(Vale et al., 2019)	choice is impacted by the first mover's decision. No impact of oxytocin on decisions. No evidence of NE play.
Matching pennies	Zero sum Anti-coordination game	Chimpanzees	(Martin, Bhui, Bossaerts, Matsuzawa, & Camerer, 2014)	Chimpanzees play the NE, but are faster at matching than anti-matching.
Prisoner's dilemma	Cooperation with temptation to defect	Chimpanzees	(Hall et al., 2019)	Little evidence of a consistent strategy, but over time second movers were less likely to <i>Defect</i> if the first mover did, suggesting they learned to avoid the worst outcome.
		Rhesus monkeys	(Haroush & Williams, 2015)	Mostly mutual defection, but cooperation was more common on trials subsequent to mutual cooperation
		Capuchin monkeys	(Smith et al., 2019)	Both mutual cooperation and mutual defection in different pairs. Subjects were more likely to defect after the partner defected. No impact of oxytocin on decisions.
		Squirrel monkeys	(Vale et al., 2019)	No evidence of NE play.
Ultimatum Game	Bargaining/punishment game	Chimpanzees	(Jensen, Call, & Tomasello, 2007; Kaiser, Jensen, Call, & Tomasello, 2012; Proctor, Williamson, de Waal, & Brosnan, 2013)	No evidence of refusals, but donors make more equitable choices when partners have the opportunity to refuse.
Trust Game			(Calcutt et al. 2019)	Subjects were more averse to social risk than in a non-social control. No influence of relationship on decisions.

Thus far, these games indicate that both monkeys and apes can find the solutions to coordination games (Brosnan et al., 2011, 2012a). However, the mechanisms used to solve these games can differ across species. These mechanisms range from simple matching where the second player chooses the same option as their partner, a strategy that is often quite effective in these games even if it is not the highest paying outcome, to probability matching where the subjects play a strategy with the same frequency at which it is played in the environment, although not in a contingent manner (i.e., matching), to explicit strategy, in which the subject understands the rules of the game and makes decisions to maximize their overall benefit (Brosnan et al., 2011; Parrish et al., 2014). Early work also showed that chimpanzees used a leader-follower dynamic, whereby the second mover takes the first mover's choice into account (Bullinger et al., 2011), and recent studies suggest the same in at least some cases with monkeys as well (Smith et al., 2019; Vale et al., 2019).

In games that lack a mutually beneficial outcome, such as anti-coordination games, monkeys still find the Nash equilibrium (NE, or the choice for which they can do no better given what their partner is playing), although they find the NE in fewer contexts, indicating that it is either more difficult for them or that they only do so in specific circumstances (Brosnan et al., 2017). Intriguingly, for our current focus on affect and relationship quality, in the closely related snowdrift game, where players have a conflict of interest over freeriding and cooperation, chimpanzees' choices are influenced by the identity of their social partner (Sánchez-Amaro et al., 2016). Finally, the only game in which substantial variation has been found across species is the prisoner's dilemma game. Early work on macaques found little evidence of mutual cooperation, although choosing the cooperation option was more common after mutual cooperation, suggesting that individual choices were influenced by the payoffs (Haroush & Williams, 2015). In capuchins, there was a tendency to defect after a partner's defection, also suggesting that choices were influenced by the game payoffs. More interestingly for our purposes, however, there were also substantial differences among pairs, with some showing high levels of mutual cooperation, some high levels of mutual defection, and some showing no discernable strategy (Smith, Leverett, Wilson & Brosnan, 2019). This variability suggests that the Prisoner's Dilemma may be a valuable game in which to explore how individual and social factors influence decision-making, including looking at how affect influences decision-making (Smith, Leverett, Wilson & Brosnan, 2019).

2.3 Reciprocity paradigms

Much of the evidence suggestive of animal cooperation stems from patterns of commodity exchange among individuals (e.g., Carter & Wilkinson, 2013; Jaeggi & Gurven, 2013; Schino & Aureli, 2008; Schino, 2007). The reciprocal nature of many of these exchanges is indeed suggestive of reciprocal altruism (Trivers, 1971), yet most of these studies consider correlational

evidence only. Whereas some inferences about the nature of these exchanges can be made based on partner choice and the timeframe of reciprocity (Brosnan & de Waal, 2002; Schino, et al., 2007), contextualizing specific motivations for reciprocal exchanges remains difficult. Experimental studies of reciprocity so far have mixed results. The initial absence of proof for contingent reciprocity in several species, including great apes (Amici et al., 2014; Brosnan et al., 2009; Melis, Hare, & Tomasello, 2008; Stephens, McLinn, & Stevens, 2002), supported the claim that cognitive constraints prevent the emergence of reciprocity in non-human animals (Stevens & Hauser, 2004). Since then, however, several studies have demonstrated contingent tit-for-tat reasoning in non-human animals (Dufour et al., 2009; Schweinfurth & Taborsky, 2018a), appropriate inhibition with regard to temporal discounting (Hayden, 2016), and the necessary memory for the nature of exchanges (Müller et al., 2017).

Moreover, the original experimental studies on reciprocity considered reciprocal altruism from a more cognitively mediated cost-benefit perspective only (cf. calculated reciprocity: de Waal & Luttrell, 1988), whereas there is now considerable theoretical evidence that emotional mediation can facilitate the evolution of reciprocity (Campenni & Schino, 2014; Evers, de Vries, Spruijt, & Sterck, 2016; Puga-Gonzalez, Hoscheid, & Hemelrijk, 2015; see also attitudinal reciprocity: Brosnan & de Waal, 2002). These theoretical models make assumptions about emotional decision rules based on valence, arousal and the relatively slow feedback mechanisms of emotions (in comparison to purely calculated decision rules), yet do not precisely define what these emotions may be, nor how they directly influence decision rules. However, the timeframe of most reciprocal patterns (i.e. balanced over the long-term only), and the specificity of partner choice in reciprocal altruism, suggests that this phenomena is generally specific to long-term strong social bonds (Massen et al., 2010; Freiden, Carballo, & Bentosela, 2017), including in humans (Majolo et al., 2006). Consistent with this hypothesis, friendship has been found to increase trust in reciprocal interactions (Engelmann & Herrmann, 2016) and long-term stable relationships are correlated with a decrease in aversive reactions to inequity (Brosnan, Schiff, & de Waal, 2005; Clark & Grote, 2003).

2.4 Inequity aversion

Inequity aversion, whereby individuals respond negatively to receiving less than a social partner (Adams, 1963), has been argued to be a way by which subjects can judge the value of their cooperation partners; subjects who consistently get less than their partners should seek out a new one (Brosnan & de Waal, 2014; Brosnan, 2006; Fehr & Schmidt, 1999). Although the first evidence for inequity responses in a non-human species was found relatively recently (Brosnan & Waal, 2003), since then there has been quite a lot of work in primates and other species using paradigms in which subjects receive different rewards than their partners for completing a task.

Humans react very strongly when they receive fewer benefits than another for the same effort (Fehr & Schmidt, 1999), and tend to do so from an early age (Riedl, Jensen, Call, & Tomasello, 2015). Humans also even react negatively when they receive more than another, albeit not as often or as strongly as they react to being disadvantaged (Blake et al., 2015; Loewenstein, Thompson, & Bazerman, 1989).

However, not every species responds negatively in these tasks, even within primates (see Table in Brosnan & de Waal, 2014 for details). Among those that do, it seems that whether a species routinely cooperates with non-kin is a better predictor of whether they respond to inequity than factors such as brain size, group size, or phylogeny (i.e., it is not a homology in either the primates more generally or the great apes specifically). This is true in primates as well as in corvids (Wascher, & Bugnyar, 2013) and canids (Essler, Marshall-Pescini, & Range, 2017), and recently some tentative evidence has been reported in parrots as well (Laumer, et al., *in press*). There are also individual differences in responses to inequity, sometimes substantial, and it is not yet clear what is underlying this variation. Although some factors show up consistently (i.e., dominance rank), it is clear that we do not yet have a full understanding of what other mechanisms may be contributing. Relevant to the current paper, affect is likely to be a component that strongly influences this response, although this possibility also remains currently unexamined. Finally, there is evidence that even species that respond negatively to inequity only do so in conditions in which the partner played a role in generating the inequity. For instance, species that respond negatively to inequity when partners are given different rewards for the same task do not do so when the rewards are simply handed out for “free” (reviewed in Brosnan & de Waal, 2014). Moreover, chimpanzees are not spiteful when they lose their food to a partner unless the partner actively steals it (Jensen, Call & Tomasello, 2007) and will not block their partner from receiving food (Jensen et al, 2006), although, interestingly, capuchin monkeys do show some evidence of spite (Leimgruber, Rosati & Santos, 2016).

While the above findings support the link between cooperation and inequity at the phylogenetic level, there is also evidence that inequity directly impacts cooperative interactions within species. For instance, using the popular barpull task for cooperation (discussed in section 2.1), researchers have found that subjects refuse to cooperate when the rewards are monopolizable (de Waal & Davis, 2002), and that whether subjects cooperate for unequal rewards (i.e., slices of apple vs. a more preferred grape) depends on their partner’s behavior. If the partner dominates the better reward across most trials, cooperation ceases, whereas if subjects both benefit from the better reward on some trials, cooperation continues at high rates despite the short-term inequity (Brosnan, Freeman, & de Waal, 2006). There is also an impact of social relationships, as chimpanzees prefer cooperative partners who tolerantly share food with them in other contexts (Melis et al, 2006a & b).

Inequity aversion is a very likely candidate for being a behavior with a strong affective component. Responses to inequity are hypothesized to be driven by, at minimum, frustration, anger, and envy. In humans, negative emotions that have been consistently associated with inequity aversion are, among others, anger and guilt (Cubitt, Drouvelis, & Gächter, 2011; Fehr & Gächter, 2002; Seip, Van Dijk, & Rotteveel, 2014b), spite (Gummerum & Chu, 2014; McAuliffe, Blake, & Warneken, 2014), and jealousy (Matsuzawa & Tanimoto, 2018). Indeed, a very plausible hypothesis is that responses to inequity, in both humans and non-human animals, are emotionally mediated, rather than being underpinned by an explicit appraisal of their relative costs and benefits (even if such appraisal leads to an emotional response; Talbot, Price & Brosnan, 2015), which, as in the case of reciprocity, may be so cognitively demanding as to be impossible for many species. Indeed, it may even be more parsimonious to assume a general state of frustration across experimental trials rather than a series of specific comparisons within trials. This suggests that we should explore the endocrine and sympathetic nervous system responses underlying individuals' decision to reject, which may indicate shifts in their arousal and valence following inequity. For example, one possible explanation for the individual variability mentioned above is that different subjects show different levels of arousal to being treated inequitably, which leads to different behavioral responses.

2.5 Prosocial motivations for cooperation

Given that cooperators are prone to exploitation by defectors, what motivates individuals to nonetheless start a cooperative interaction? Prosociality, here defined as behavior that is intended to benefit someone else (Jensen, 2016), has been suggested as important (motivational) driver for cooperative interactions (Silk, 2007; Jaeggi, Burkhardt, & van Schaik, 2010). Yet, few studies have tried to directly link these specific motivations to cooperative behavior. Indirect support for this hypothesis has been found in cross-species comparisons that link the varying natural cooperative tendencies of different species with the results of experimental prosociality tests of those same species (for reviews see Cronin, 2012; Marshall-Pescini, Dale, Quervel-Chaumette, & Range, 2016). This work in turn motivated a central prediction of the cooperative breeding hypothesis (Burkart et al., 2014; Burkart, Hrdy, & Van Schaik, 2009; Burkart, Fehr, Efferson, & Schaik, 2007), which argues that prosocial motivations enhance the ability to coordinate and cooperate, particularly in species that engage in cooperative breeding. Direct experimental evidence supports this hypothesis, demonstrating that intraspecific variation in prosociality among common marmosets predicts cooperative success (Martin et al. *in review*). This work suggests that prosociality may have been an important driver for the evolution of cooperation in these cooperative breeders or vice versa, although the benefits of prosociality need not be restricted to cooperative breeders.

A major drawback of interspecific comparisons of prosociality is the diversity of paradigms and procedures used (see Cronin, 2012). This is even more problematic if we wish to compare animals with humans, whose prosocial tendencies are often inferred from complex game-theoretical paradigms. There have been several attempts to make the human studies more comparable to those on animals, yet these simplified and more playful games are generally only tested on children (e.g. Burkart & Rueth, 2013; Horn, Hungerländer, Windhager, Bugnyar, & Massen, 2018; House, Henrich, Brosnan, & Silk, 2012). Such discrepancies in the developmental stages of the participants, and most importantly, the variety of paradigms used, make direct comparisons practically impossible (Leavens, Bard, & Hopkins, 2017). In light of this limitation, Burkart and colleagues (Burkart et al., 2014) utilized the same experimental paradigm to study prosociality in 15 different primate species (including humans, albeit children only), finding that the amount of allomaternal care exhibited by a species predicts their prosocial tendencies. More recently, this paradigm has been converted to accommodate birds (Horn, Scheer, Bugnyar, & Massen, 2016), and subsequently validated (Martin et al. *in review*) in one of the primate species from the original study by Burkart and colleagues (2014). This work has demonstrated a similar relationship between prosocial tendencies and social structure, at least in the cooperatively breeding azure-winged magpies, *Cyanopica cyanus* (Horn et al., 2016).

What constitutes or triggers these prosocial preferences remains, unfortunately, relatively unknown. Some authors, for example, have argued that chimpanzee prosocial preferences are in fact self-serving preferences that come about as a by-product of task design (Tennie, Jensen, & Call, 2016), while others remain convinced that chimpanzees are truly willing to help others (Melis, Engelmann, & Warneken, 2018). One study has examined whether sympathy might motivate prosociality in great apes, as it does in humans (e.g. Vaish, Carpenter, & Tomasello, 2009), but did not find evidence for the role of sympathetic concern in the prosocial behaviours of these apes (Liebal, Vaish, Haun, & Tomasello, 2014). Nevertheless, amidst other contextual features, the strength and nature of the relationship between donor and recipient does seem to have a strong influence on prosocial preferences in various species (reviewed in Cronin, 2012), suggesting a mediating role of emotions, as in emotionally mediated reciprocity. Social bonding is in part mediated by oxytocin (see also section 3.5), so oxytocin levels may therefore be a valuable measure for further assessing the affective states involved in prosociality. In humans, the administration of oxytocin has been found to produce more generous donations to charity (Barraza, McCullough, Ahmadi, & Zak, 2011), and also more coordinated cooperation, albeit at the expense of an out-group (Zhang, Gross, De Dreu, & Ma, 2019). Studies using oxytocin in experimental prosociality paradigms in non-human animals are scarce and results are so far inconsistent. A recent study in Clark's nutcrackers, *Gymnorhinus cyanocephalus*, did corroborate a positive link between prosociality and the intranasal administration of mesotocin, the avian

homologue of oxytocin (Duque, Leichner, Ahmann, & Stevens, 2018), whereas a study on capuchin monkeys found that food-sharing was negatively impacted by the intranasal administration of oxytocin (possibly as a byproduct of oxytocin's anxiolytic effects; Brosnan et al., 2015). Nevertheless, this line of research seems to be a promising avenue into the potential affective mediation of cooperative behaviours.

3. Affective correlates of cooperation

Since most studies only measure subjects' behavioral responses during cooperation games, inferences can only be made about behavioral decision-rules and little remains known about the underlying mechanisms. To tap into the proximate mechanisms underlying these decision rules, we need to understand the affective correlates of cooperation (which ultimately will help us to understand what emotions non-humans may or may not experience and how they might relate to our own). For this reason, several scientists have highlighted the need to include physiological parameters in studies on cooperation (e.g., Brosnan & Bshary, 2010; Bshary, Oliveira, & Grutter, 2011). However, in animal research the ability to include physiological measuring techniques is dependent on factors such as the species and the individual's level of training. Nonetheless, new techniques have made studies investigating such parameters more frequent. Here, we provide a broad overview of the most frequently applied non- or minimally invasive physiological measuring techniques in cooperation studies, first in humans and then, if applicable, in other animal species. Subsequently, we will also review assessments of affect possibly involved in cooperative interaction. Note that although research on brain activity can provide valuable insights into emotions and cooperation (e.g. Pan, Cheng, Zhang, Li, & Hu, 2016; Prochazkova et al., 2018), brain scanning technologies generally restrict social interactions between participants (Dulleck, Schaffner, & Torgler, 2014) and are difficult to apply in animals, particularly in the context of cooperation. Therefore, we do not discuss such techniques below.

3.1. Physiological measures of affect

3.1.1. Cardiac function

Researchers have been using different parameters of cardiac function to get information about the effect of specific (affective) stimuli on an organism's autonomic nervous system. Heart rate (HR), the number of heartbeats within a given period of time (usually per minute), is regulated by the sympathetic (fight and flight) as well as the parasympathetic system (rest and relax) and therefore represents a net effect of both systems' activity (Brownley et al., 2000). In order to disentangle the effects of the two systems, it is useful to determine heart rate variability (HRV), which refers to the fluctuation of the time interval between successive heartbeats (Berntson et al.

1997). Most of the HRV computations, such as the high frequency HRV or the root mean square of successive difference (RMSSD) represent parasympathetic activity. Specifically, if the high frequency band of a heartbeat is used for HRV calculations, then we are referring to the high frequency HRV, also called respiratory sinus arrhythmia (RSA) as it is associated with the breathing cycle, and since RSA is primarily controlled by vagal efferent pathways it can be used as an index of parasympathetic activity (Berntson et al. 1997). The low frequency HRV, in contrast, is thought to represent the sympathetic branch of the autonomic nervous system (Berntson et al. 1997).

Whereas heart rate (HR) measures thus provide information on the net effects of vagal (rest and relax) and sympathetic (fight and flight) activity, heart rate variability (HRV) reflects the balance between the two, making it a good indicator of psychosocial stress (von Borell et al., 2007). Cardiac activity and respiration rate can be measured with a single telemetry device, which has, for example, been used for measuring emotion responses in goats (*Capra hircus*) (Briefer, Tettamanti, & McElligott, 2015). Such monitoring devices usually consist of a belt that is strapped around the thorax of the subject, which records the respective physiological signals, and a computer that receives these signals, making them both non-invasive and relatively easy to use for at least a subset of species (those without dexterous hands). Electrocardiograms (ECG) can also be recorded with electrodes attached to specific spots on the body, such as the inner forearm and inner leg. Using the latter method, Osumi and Ohira (2009) found that human participants' HR changes predicted their decision to accept or reject an offer in the ultimatum game, with initial HR decelerations preceding rejections. Note, however, that the act of measuring cardiac function in itself was found to promote giving in the trust game (Van Lange, Finkenauer, Popma, & van Vugt, 2011), suggesting that the method can influence the participant's behavior and the outcome of such a game, at least in humans.

Cardiac and respiratory parameters are often included in animal research because their respective measuring techniques can rather easily be adjusted to species-specific requirements (e.g. dogs, *Canis familiaris*, Kortekaas, Range, Virányi, & Kotrschal, 2013); sheep, *Ovis aries*, Reefmann, Bütikofer Kaszàs, Wechsler, & Gygax, 2009). Early work using subcutaneously implanted telemetry devices in rhesus macaques, showed marked increases in HR when the macaques were in a risky situation, and notably, a decrease in HR when they received grooming, suggesting a tension reduction function of this cooperative behavior (Aureli, Preston, & de Waal, 1999). Most of the more recent studies using the less invasive belts have not investigated cooperation per se, yet many have focused on animal emotions. Sheep, for example, exhibit higher heart and respiration rates in response to a negative situation (presentation of unpalatable food) as compared to neutral or positive food-related situations (presentation of a desirable food; Reefmann et al., 2009). As the sheep's HRV (RMSSD) was not significantly affected, Reefmann and

colleagues (2009) concluded that the sheep's reactions were mainly driven by the degree of sympathetic activity. Similar findings were reported for goats, who showed higher heart and respiration rates, yet also lower HRV (RMSSD) during situations that cause high arousal, irrespective of their valence (Briefer et al., 2015). However, using cardiac measures in rhesus macaques, Bliss-Moreau and colleagues (2013) did find differential responses of the sympathetic and parasympathetic system to the affective valence of social video stimuli. Due to the value of the information provided and the good acquisition possibilities in animals, we highly recommend greater use of cardiac measures for the study of cooperation in animals where it is feasible (review on the use of HRV in animals: von Borell et al. 2007).

3.1.2. Skin conductance

As the sympathetic tone briefly increases in response to most arousing stimuli, sweat glands are activated that produce more sweat, which reduces the electrical resistance of the skin and, hence, increases skin conductance (Boucsein, 2012). Electrodermal activity therefore reflects autonomic activity. In fact, it is often used to validate the success of a stimulus in eliciting the appropriate arousal response consistent with a specific affective valence or, in humans, emotion (e.g. fear, Phelps et al., 2001). Electrodermal activity can be measured by using a skin conductance amplifier, which amplifies the electric signal received from electrodes that are placed on specific spots of the subject's palm. Van 't Wout and colleagues (2006) were one of the first to include this parameter in the study of cooperation using an Ultimatum Game, demonstrating that participants' skin conductance activity was higher when they were confronted with an unfair offer. This suggests that unfair offers elicited stronger emotional arousal than fair offers (but see Osumi & Ohira, 2009). Elevated skin conductance levels have also been observed after opponent-directed negative emotions (e.g. anger) were induced in subjects who had to play a social decision-making game with the respective opponent (Eimontaite, Nicolle, Schindler, & Goel, 2013). Consequently, in the game itself, participants were more likely to defect against opponents they were angry at than against those whom they felt positive about (Eimontaite et al., 2013). Unfortunately, although skin conductance is a very informative parameter of affect in humans, it is quite difficult to measure in animals, which currently limits its applicability in the study of animal affect and cooperation.

3.1.3. Facial skin temperature

Another index of autonomic reactivity is the facial temperature, which shows specific patterns in response to different physical and psychological stimuli (see Panasiti et al., 2016). Facial skin temperature can be recorded with thermal infrared imaging. One major advantage of this method is that, in contrast to electrodermal and cardiovascular measurements, subjects are not

influenced by the measuring technique as no device needs to be attached to them. There is an additional advantage for non-humans, for whom it may be dangerous, difficult or impossible to use a device that attaches to them in some way (cf. Ioannou, Chotard, & Davilla-Ross, 2015). Changes of facial skin temperature are often measured in studies on deception, which generally counteracts any cooperation. Panasiti and colleagues (2016), for example, gave participants the choice of spontaneously deceiving someone in a setting where their reputation could potentially be harmed. Participants who deceived in the reputation-risk situation showed an increase in nose temperature (reflecting parasympathetic activation) during and after their decision to deceive (Panasiti et al., 2016). In another study, however, opposing results were found and nose and hand temperature decreased when participants called a significant person in their lives to tell an important lie (Moliné et al., 2017).

Facial temperature measurements can also be applied in a wide array of animal species, as the most frequently used target areas, the eyes and nose, are regions that are usually not covered by hair/feathers. The eye temperature and HR of dogs, for example, increases while individuals receive treats, a positively arousing situation (Travain et al., 2016). Proctor and Carder (2016) similarly investigated cows' (*Bos taurus*) responses using food rewards. Although the cows' nose temperature dropped when they experienced high arousal, they did not exhibit a change in temperature between positively and negatively valenced situations. Studies on rhesus macaques consistently show that a threatening stimulus induces a decrease in nasal temperature (Nakayama, Goto, Kuraoka, & Nakamura, 2005; Kuraoka, & Nakamura, 2011). Similarly, studies on chimpanzees show that playbacks of conspecifics fighting (Kano, Hirata, Deschner, Behringer, & Call, 2016) or, more generally, aversive vocalisations of conspecifics (Dezecache, Zuberbühler, Davilla-Ross, & Dahl, 2017) are associated with a decrease in nasal temperature. So far, facial skin temperature in animals has mostly been used as a tool to better understand whether individuals are experiencing heightened arousal, but there is also the potential for this technique to be very informative about more complex processes involved in cooperation. Given the small data set and relatively high inconsistency in responses, however, care will need to be taken until we have a better idea of what exactly these changes are measuring and are indicative of.

3.1.4. Pupil mimicry

Whereas facial skin temperature is regularly used in studies on deception, pupil mimicry is a physiological parameter that is of particular interest in the study of trust, a factor that promotes cooperation. Changes in pupil size are, like the parameters presented above, mediated by the autonomic nervous system, and can be measured with an eye-tracker. Recent findings suggest that the synchronized dilation of two persons' pupils recruits specific areas in the theory of mind network, resulting in individuals feeling 'mentally connected' and subsequently enhancing trust

(Prochazkova et al., 2018). Kret and De Dreu (2017) showed that in a trust game, men trusted partners with dilating pupils even more after oxytocin treatment as compared to after placebo administration. In female participants, in contrast, oxytocin blunted the effect of pupil dilation on trust (Kret & De Dreu, 2017). Since collecting pupillometry data requires the subjects to keep their head still, there are only a few studies on this parameter in animals. Kret and colleagues (2014), for example, managed to investigate pupil mimicry in chimpanzees (*Pan troglodytes*) by using a binocular eye tracker, which allowed for relatively large head movements of the animals. They reported that chimpanzees exhibit stronger pupil mimicry with (unfamiliar) conspecifics than with humans, and found that chimpanzee mothers and their offspring exhibited the strongest effect (Kret et al., 2014). Several researchers have begun using creative ways to keep subjects' heads still, such as presenting the stimuli behind a wall that only allows animals to peek through a small slit (Ryan et al., 2019). Creative solutions like these will be key to the measurement of pupil mimicry in a much wider array of species, making it a promising factor for better understanding animal cooperation.

3.1.5. Hormones

Hormones are involved in many cooperative processes (reviewed by Soares et al., 2010; Trumble, Jaeggi, & Gurven, 2015) and have a clear link to affect (Bos, Panksepp, Bluthé, & Honk, 2012). They can be measured endogenously or actively manipulated by, for example, administering hormones or blocking receptors (e.g., Bird et al., 2018; Bos, Terburg, & van Honk, 2010; Brosnan et al., 2015; Rilling et al., 2012; Soares, Cardoso, Grutter, Oliveira, & Bshary, 2014). Although this is a promising and popular technique, it is not without challenges. Not only circulating hormone levels, but also neural responses to them, can be influenced by several different factors (illustrated by Trumble et al., 2015), and most fluctuate substantially. Levels also differ depending on how they are measured. Endogenous hormones and/or their metabolites can be extracted from different organic materials, such as blood, saliva, urine, feces and hair/feathers. Which of these should be used depends on the hormone and the time frame to be investigated. All of these materials reflect hormone levels on different time scales, from minutes (e.g. in saliva) to months (in hair/feathers), hence not all of them are suitable for the investigation of emotions, which are considered to have a short time frame (for non-invasive monitoring of endocrine markers in primates see Behringer & Deschner, 2017). The hormones oxytocin and testosterone, as well as glucocorticoids such as cortisol, have been the most studied in relation to cooperation.

Oxytocin plays an important role in social bonding, trust and generosity. In preschoolers, for example, salivary oxytocin measured directly before a dictator game was associated with how much chocolate the children allocated to others (Fujii et al., 2016). In boys, oxytocin levels were negatively correlated with allocations; in girls, levels before and after the game were positively

correlated when playing with a class mate (ingroup member), but no effect was found when playing with an outgroup member (Fujii et al., 2016). This emphasizes an important emerging point—the effects of oxytocin are highly dependent on sex (see Feng et al., 2015) and on context (Shamay-Tsoory et al., 2009; Ne’eman, Perach-Barzilay, Fisher-Shofty, Atias, & Shamay-Tsoory, 2016), and there may be opposite effects for ingroup and outgroup members (De Dreu, Greer, Handgraaf, Shalvi, & Van Kleef, 2012; De Dreu & Kret, 2016; Zhang et al., 2019; for a review see Shamay-Tsoory, & Abu-Akel, 2016). Results are perhaps even more variable in non-human species, although this may be in part due to a combination of less research effort and less ability to control the parameters of the study. In general, endogenous oxytocin has been positively associated with food sharing, intergroup conflict, and cooperative hunting in chimpanzees (Samuni, Preis, Deschner, Wittig, & Crockford, 2019; 2018a; Samuni et al., 2017; 2018b; Wittig et al., 2014), grooming in capuchins (Benítez, Sosnowski, Tomeo, & Brosnan, 2018), and, more generally, to influence social cognition, behavioral synchrony, and prosociality across a variety of non-human primates (Chang & Platt, 2014; Finkenwirth, van Schaik, Ziegler, & Burkart, 2015; Jiang & Platt, 2018; Wittig et al., 2014; but see Staes et al., 2015). Recent work has also identified the importance of endogenous oxytocin in social bonding and cooperation among female bonobos (Moscovice et al., 2019). However, the effects of *exogenously* administered OT have been far less consistent (Brosnan et al., 2015; Duque et al., 2018; *in press*; Mustoe, Harnisch, Hochfelder, Cavanaugh, & French, 2016; Proctor, Calcutt, Burke, & de Waal, 2016). Note however, that as oxytocin is primarily involved in social bonding, the mediating effects of this hormone on cooperation may, as with humans, be highly dependent on the dyad tested, and this should be accounted for in future tests. Clearly more work is needed to determine the complex influences of oxytocin on behavior.

Popular belief dictates that steroid hormones should inhibit cooperation, yet recent research shows that the picture is far more intricate. Specifically, the hormones testosterone and cortisol seem highly dependent on each other with regard to behavior related to social status (cf. dual-hormone hypothesis: Mehta & Josephs, 2010; Mehta & Prasad, 2015), and as such may influence decision-making in economic games (Mehta & Prasad, 2015). Social status or rank, in turn, seem to have effects on cooperation (e.g. Horn et al., 2018; Massen et al., 2015; Suchak et al., 2014), thus making the interplay of these two hormones particularly interesting for the study of affect and cooperation. Unfortunately, little work has been done on cooperation while considering both hormones in parallel; generally, studies consider only one of these hormones. Testosterone is often associated with aggressiveness and competition (Eisenegger, Naef, Snozzi, Heinrichs, & Fehr, 2010), but recent results show that testosterone also mediates parochial altruism (Reimers & Diekhof, 2015) and prosociality (Dreher et al., 2016; Eisenegger et al., 2010). For example, male soccer fans who participated in a Prisoner’s Dilemma Game exhibited

increased salivary testosterone while cooperating with other fans of their team (Reimers & Diekhof, 2015). Cortisol, on the other hand, is often associated with stress. Social interactions can be very stressful, and indeed tend to lead to an increase in glucocorticoid hormones, such as cortisol and corticosterone. Jäschke and colleagues (2016), for instance, reported that playing a cooperative game generally seems to increase the participants' salivary cortisol levels, while playing with a cooperative partner of higher social status leads to a faster recovery of cortisol levels. Similarly, salivary cortisol in long-tailed macaques (*Macaca fascicularis*) has been found to decrease when participating in a cooperation task with closely bonded individuals, irrespective of the dyad's cooperative performance (Stocker, Loretto, Sterck, Bugnyar, & Massen, *in review*). Moreover, glucocorticoid excretion patterns have also been connected to personality traits, such as boldness, which in turn affect cooperative behavior (e.g. in rooks, *Corvus frugilegus*, Scheid & Noë, 2010). For non-invasive glucocorticoid measurements see Palme (2019). Despite the promise of endocrine measures as important indicators of affect, care is warranted, as these hormones interact intensively with each other; i.e. not only amongst other steroid hormones (Mehta & Josephs, 2010), but also with oxytocin (Ziegler & Crockford, 2017). Therefore, systematic studies that investigate cooperation and measure, at minimum, these three hormones in parallel, are needed.

3.2. Behavioral measures of affect

3.2.1. Cognitive Bias paradigms

One often used behavioral measure of affect is a cognitive bias paradigm. Cognitive bias paradigms (Harding, Paul, & Mendl, 2004; Mendl, Burman, Parker, & Paul, 2009; Paul, Sher, Tamietto, Winkielman, & Mendl, this issue) present an exciting and relatively untapped resource for measuring consistent individual differences in affect. Originally, these paradigms were mostly used to study the effect of husbandry procedures on animals' affective states (Bateson, & Matheson, 2007; Bateson, Desire, Gartside, & Wright 2011; Bethell, Holmes, Maclarnon, & Semple, 2012). Using this approach, however, Bateson and Nettle (2015) also provided tentative evidence for consistent moods in chimpanzees. Notably, Schino and colleagues (2016) showed that in tufted capuchin monkeys, *Sapajus [Cebus] sp.*, the amount of grooming received was positively correlated to 'optimistic' responses in this paradigm, albeit based only on overall grooming received and not on grooming received immediately before the cognitive bias test (Schino, Massime, Pinzaglia, & Addessi, 2016). This work, along with other studies using bias paradigms to investigate the effects of (chronic) stress in, for example, capuchin monkeys (Boggiani, Addessi, & Schino, (2018), rats (Chaby et al., 2013) and starlings (Gott, Andrews, Bedford, Nettle, & Bateson, 2018), suggests the feasibility of these methods for incorporating rigorous measures of affective dispositions into experimental studies of animal cooperation.

Importantly, inter-individual differences in cognitive bias need to be taken into account, in both these experimental and observational studies of cooperation. Therefore, some knowledge about individuals 'base-line' cognitive bias is required as a reference point for the cognitive bias of the same individual after the cooperation manipulation, be it experimental (cf. Adriaense, Martin, Schiestl, Lamm, & Bugnyar, 2019) or observed cooperative behaviors (cf. PC/MC method by de Waal, & Yoshihara, 1983).

3.2.2. Vocalizations

Communication can be an important aid in coordination while cooperating. Not surprisingly, humans cooperate more when they are able to converse with each other (Wichman, 1970; Cooper, DeJong, Forsythe, & Ross, 1992), and some argue that communication as a coordination tool has been the key selection pressure for the evolution of human language (David-Barret, & Dunbar, 2016). Cross-species comparisons have revealed that among non-human animals, cooperative breeding influences communicative complexity, namely the number and type of vocalizations in birds (Leighton, 2017) and mammals (Manser et al., 2014), which supports the idea that communicative complexity is predicted by social complexity and cooperation. Within a species and within an actual cooperative context, there is some evidence that animals use vocalizations to coordinate cooperation (e.g. lunge feeding in humpback whales, *Megaptera novaeangliae*: D'Vincent, Nilson, Hanna, 1985), recruit conspecifics to a food-source, either in the prosocial context of sharing food with fledged offspring (e.g. pied babblers, *Turdoides bicolor*: Radford, & Ridley, 2006), or friends in the group (e.g. chimpanzees: Schel, Machanda, Townsend, Zuberbühler & Townsend, 2013), or recruit befriended conspecifics whose help is needed to acquire a food source (e.g. common ravens: Sierro, Loretto, Szpl, Massen, & Bugnyar, in review). Animals may also communicate information about their inner state; for example, pied babblers negotiate how much time they will spend performing sentinel behavior based on their hunger state (Bell, Radford, Smith, Thompon, & Ridley, 2010), rats communicate their need to those who can provide (Schweinfurth, & Taborsky, 2018c), and many animal species react to increased begging from offspring with increased help (Bee, & Miller, 2016). Although vocalizations can inform the receiver about the inner state of the sender, it remains unclear whether and how that may be emotionally mediated.

A recent study showed that humans are able to detect arousal in the calls of animals from all classes of terrestrial vertebrates, suggesting that vertebrates, at least for contexts with high arousal, share fundamental mechanisms of vocal expression (Filippi, et al. 2017). Highly arousing states like being trapped do indeed elicit distress or alarm calls in animals which may lead to conspecifics helping their distressed group member (chimpanzees: Boesch, & Boesch, 1989; Amati, Babweteera, & Wittig, 2008; rats: Bartal, Decety, & Mason, 2011), although these instances

are often too rare to study systematically and unethical to invoke experimentally. What is needed is an extensive library of vocalizations characterized by both their valence and arousal, as well as the context, if known, to consider possible affectively mediated modulations that can be actively linked to cooperation and subsequently coded during cooperation experiments, or even manipulated in such an experiment. An experiment on humans found that priming participants with vocal expressions of joy increased their cooperative tendencies in an assurance game, whereas vocal expressions of anger decreased cooperative tendencies in comparison to a neutral vocal stimulus (Caballero Meneses, & Menez Díaz, 2017). To our knowledge, the only study that used a similar framework in a non-human animal is a study on rats by Łopuch and Popik (2011), which not only showed that allowing communication between rats benefits cooperation, but also that the number of 50kHz calls, a call-type that was previously validated as an indicator for positive emotions (Burgdorf, et al., 2008), positively influenced cooperative behavior (Łopuch, & Popik, 2011). Although studies on the role of vocalizations in cooperative interactions among animals are still rare, given the right framework this field may be very informative about the mediating role of affect on cooperation.

3.2.3. Facial displays and body language

Communication does not necessarily involve vocalizations. Many species, including most non-human primates, in fact seem restricted in their use of vocal signals (but see Fitch, de Boer, Mathur, & Ghazanfar, 2016). On the other hand, there are often consistent changes in facial displays, often interpreted as facial expressions, which seem very potent as emotional indicators, especially with regard to affect, something Darwin already noticed while describing homologies in the facial expressions of humans and other animals (Darwin, 1972; see also Kret, Prochazkova, Sterck, & Clay, this issue). Primates, for example, show clear, often ritualized, facial displays that have been linked to both negative and positive emotional states (e.g. van Hooff, 1971; 1976). These are often used in social contexts to display benign intent, or to appease prior conflicts (e.g. de Waal, 1988), and could thus potentially also be involved in initiating cooperative actions. The play-face, for example, is a clear example of an affective facial expression that also helps facilitating play between interaction partners (e.g. Waller, & Cherry, 2012). In humans, there are several studies showing that a smile can initiate cooperation and/or increase cooperative investments (e.g. Stouten, & de Cremer, 2010; Scharlemann, Eckel, Kacelnik, & Wilson, 2001), yet so far similar studies in non-human animals are missing. The development of the Facial Action Coding Systems (FACS) for multiple animal species (see Waller, Julle-Daniere, & Micheletta, this issue) allows for more rigorous testing of affective correlates in animal facial expressions, and would subsequently allow experimental testing of the effect of affective facial expressions on cooperation.

As with facial displays, many animal species also show clear, often ritualized, bodily displays to communicate with each other (e.g. van Hooff, & Wensing, 1987). Dominance and subordination displays are probably the best-known examples. Unfortunately, however, little work has been done on whether and how this ‘body-language’ relates to potential underlying affective responses. One exception are studies on displacement activities, which have now been consistently shown to indicate stress (e.g. Maestripieri, Schino, Aureli, & Troisi, 1992; Troisi, 2002) and as such are suggested to have a communicative function as well. Indeed, Barbary macaques do attend to scratching in others, yet this seems rather to avoid re-directed aggression from these others than to facilitate cooperative efforts (Whitehouse, Micheletta, Kaminski, & Waller, 2016). Nevertheless, informed decisions about, for example, with whom not to cooperate, can be as valuable as knowing with whom to cooperate. Therefore, assessing the body-language of test-subject during cooperation experiments/observations, as well as more generally, might be a valuable tool to investigate possible affective correlates.

4. Interpretation of results

4.1. How comparable are the paradigms?

There may be more paradigms to test for cooperation than there are species tested within those paradigms. More critically, perhaps, it is extremely difficult to compare human studies with those involving other species due to differences in procedure. Human paradigms largely consist of social dilemmas that often rely on elaborate explanations to the participants, whereas animals must learn the structure of the game through repeated exposure. Moreover, the often complex game structures used to study human economic decision-making may be too challenging for non-human animals. Aside from the fact that null results are not proof of absence, in some cases null results are a consequence of the experimental set-up used. To address this challenge, researchers often test a specific species in multiple different paradigms. As a consequence, particularly within the animal literature, the number of different paradigms employed has increased drastically. Even when the same paradigm is used (e.g. loose-string paradigm, see section 2.1 & table 1), the procedures used to test the specific paradigm may differ across labs, so that results may differ even within a species. Consequently, particular caution is needed in the interpretation of comparisons based on a different procedure, albeit using the same experimental set-up (cf. Massen et al. *in press*).

Recently, there have been several attempts to study animal decision-making using the same set-ups *and* procedures in multiple species (e.g. MacLean et al., 2014; see also Bohn et al., 2019), including studies on socio-cognitive traits like prosociality (Burkart et al., 2014). Moreover, the paradigms and procedures originally used to investigate prosociality in primates

have now been successfully translated to birds (Horn et al., 2016). These phylogenetically broad comparisons are key if we are to understand the core mechanisms driving decision-making across species, but they still rely on strong assumptions: firstly, that the perception of and response to these paradigms are the same for all species; and secondly, that behavioral and physiological (including endocrine and neural) responses to these paradigms are homologous or sufficiently analogous. If either of these assumptions is not true, then the comparison becomes much less straightforward.

Indeed, we know that in many cases these assumptions are false. For example, work on the assurance game finds that many primate species can find the payoff dominant coordinated outcome (coordinated Stag play; Brosnan et al., 2011; Brosnan, Wilson, & Beran, 2012). However, we also know that these primates are using different mechanisms to reach these outcomes (Parrish et al., 2014; reviewed in Smith et al., 2018). Capuchin monkeys, a New World monkey species, appear to be matching their partner's play, albeit with a (strong) bias towards the payoff dominant outcome. However, they cannot maintain it when they cannot see their partners' choices. Of course, capuchins also live in relatively small, cohesive groups, so there may have been little selection pressure to coordinate when they cannot see their partners. Humans probability match, but, interestingly, rhesus monkeys, who probability match in other contexts, do not do so in this task; instead they simply show a preference for the outcome that generally pays the best. Chimpanzees sometimes show evidence of understanding that there is a strategy to the game, extrapolating their choices from one situation to a new one. That being said, this is highly context dependent, as indicated by the fact that some chimpanzees play randomly despite hundreds of trials of exposure (Hall et al., 2019). This may be due to their level of experience and/or disinterest in the game, but in either case shows very clearly that even *within* the same species, using the same procedure (within practical constraints), there is substantial variability.

As this detailed summary shows, null results must be taken within the context of the study, and just because an animal is known to show some mechanism in one context does not mean that they will show it in another, even in a context in which other species are known to use that mechanism. What, then, do we do as researchers? First, we recognize that any research program will involve numerous studies, first documenting outcomes and then trying to tease apart underlying mechanisms. Second, we must keep in mind the distinction between function and mechanism, and be careful not to conflate the two, or assume one from the other (Tinbergen, 1963). Third, it is clear that a comparative program must start by testing multiple species using the same paradigm, or as close as we can get to the same paradigm, but we must also give every species multiple opportunities using different approaches. For instance, subjects who do poorly on a manual version of a task may do quite well on a computerized version (Brosnan, Wilson & Beran, 2012). Fourth, once we have some idea of how the species are similar and different, we

must “back test” those species on more species-specific paradigms to see if the predictions from our tightly controlled comparisons yielded correct hypotheses. If not, then we tweak the paradigms and iterate towards a better understanding of how species’ unique abilities and evolutionary history have influenced a behavior (Smith, Watzek & Brosnan, 2018).

In the case of comparative affective science, this presumably means starting by testing animals on situations in which we know affect influences decision-making in humans. Of course, it may not – and often will not – be the case that the same situations lead to the same responses, but it is a reasonable starting point (Williams, Brosnan & Clay, this issue). Next, we need to compare across a variety of species in order to determine which outcomes are species-specific and which are more general (Shettleworth, 2012; Andics & Miklósi, 2018; Kret & Tomonaga, 2016; MacLean et al., 2012). Finally, we should try, when possible, to simultaneously measure several physiological parameters to get a more nuanced view of animals’ responses (Dunn, Evans, Makarova, White, & Clark, 2012; Osumi & Ohira, 2009; Reefmann et al., 2009; Travain et al., 2016).

We also emphasize the importance of actually measuring affect, rather than simply assuming it after priming, or from subjects’ behavior. As these above examples illustrate, it is very common for different species to reach the same outcomes through entirely different mechanisms (and the same may be true for different animals within a species). Likewise, they may show different behavioral manifestations of the same affective response, or the same behavioral manifestation for different affective responses. It is critically important to provide further objective evidence of assumed similarities (or differences), in the form of a physiological parameter, endocrine measure, cognitive bias or, ideally, multiple responses taken together.

One final note is in order about the challenge of understanding animal decision-making from captive studies. While captive settings allow for tight experimental control, they are also an artificial environment for the development and expression of normal adaptive decision-making processes (Boesch, 2007), particularly in the context of cross-species comparisons (Leavens, Bard, & Hopkins, 2017). For instance, as previously mentioned, while prosocial behavior has been surprisingly difficult to demonstrate in captive studies of primates, field workers have consistently documented behaviors such as food sharing and group hunting in wild populations (Samuni et al., 2018). Even the presence of a physical barrier, comparable to barriers common in research with captive apes, impedes dogs’ success in an imperative pointing task (Kirchhofer, Zimmermann, Kaminski, & Tomasello, 2012), suggesting that work in apes may be underestimating their abilities. This difference between captive and wild studies could be particularly important in understanding affect. For instance, research has demonstrated increased pessimism biases in animals raised in impoverished captive environments (e.g., Harding, Paul, & Mendl, 2004).

Captive research clearly is important in determining underlying mechanisms, and there are a few steps researchers can take to ensure that results are as generalizable as possible. Animals should be socially housed, for example, and when feasible, testing can be done within social groups (e.g., Martin et al., in review; Massen et al., 2015). Unfortunately, although relatively non-invasive, many physiological measures require specific set-ups that often inhibit testing in ecologically relevant complex social configurations. Thus, if early experiments require more tightly controlled social situations, subsequent studies can be done in group situations, allowing for both the interpretation of messy results in the context of the more controlled studies, as well as the ability to document behaviors that may correlate with emotional responses that are impossible to measure in group situations. Similarly, tests with human experimenters as social partners should be avoided as much as possible as this is an artificial social situation without any ecological relevance and prohibits the measurement of the potentially intricate socio-emotional interplay between two cooperating conspecifics. Finally, ideally field experiments can also be used to validate laboratory results, as well as to better probe the developmental plasticity of the proximate mechanisms hypothesized to influence task performance (Morand-Ferron, Cole, & Quinn, 2016).

4.2. Impact of social and individual factors on decision-making

Another likely source of individual differences that is often ignored in experimental studies is social relationships. Humans can flexibly adjust among different proximate mechanisms to meet the requirements of specific cooperative contexts (Melis & Semmann, 2010; West, El Mouden, & Gardner, 2011). In particular, as previously noted, human brain imaging studies support the notion that complex cognitive mechanisms and more 'basal' hormonal mechanisms may exist in parallel, and show that their employment depends on familiarity (Krueger et al., 2007), such that more emotional areas are activated when cooperating with familiar individuals (i.e., emotionally based bookkeeping, Schino & Aureli, 2009; attitudinal reciprocity, Brosnan & de Waal, 2002), whereas more cortical areas involved in mentalizing are activated while cooperating with unfamiliar individuals (i.e., calculated reciprocity, de Waal & Luttrell, 1988). This is a key dichotomy that must be resolved, but studies examining this dualism in decision rules in non-human animals have so far been lacking, and consequently any conclusions about the uniqueness of this phenomenon for humans are premature.

These results also indicate that caution should be used in interpreting existing results. Human cooperation research using experimental games rarely takes such proximate mechanisms into account, and many behavioral economic studies rely on games played by anonymous dyads/duos (e.g. in Prisoner's dilemmas) and/or groups of individuals that did not know each other prior to the game (e.g. in Public goods games). Similarly, cooperation research in animals

generally tests experimentally determined dyads (Table 1), inhibiting subjects' capacity for partner choice. However, individuals of social species are typically surrounded by multiple individuals, and these multiple partners are generally *known* individuals that interact within their group. This suggests that we may be getting very different (affective) responses in the experiments than in wild observations, or their natural interactions, because we fail to take into account the impact of social context on their decision-making.

Moreover, while these experiments often focus on just two individuals, cooperation in the daily lives of most animals is a dynamic process within a multi-player field in which relationships differ substantially. Indeed, in such 'biological markets' (Hammerstein & Noë, 2016), individuals often have their choice of cooperative partner (Bull & Rice, 1991; Noë & Voelkl, 2013), and pay-off structures may differ dramatically among different partners (Taborsky, Frommen, & Riehl, 2016a). For example, long-term positive relations or 'friendships' can create mutual trust that increases the benefits of cooperation and the costs of partner-switching. Empirical evidence in chimpanzees indeed shows that friendship increases trust (Engelmann & Herrmann, 2016; but see Kaburu & Newton-Fisher, 2016), and studies allowing for partner choice find that animals prefer to cooperate with their friends (e.g. Asakawa-Haas et al., 2016; Massen et al., 2015; Molesti & Majolo, 2016). Additionally, interdependence of pair-bonds through investment in mutual offspring may also inhibit partner-switching for the parents during critical periods of offspring development (Johnstone & Rodrigues, 2016), as do other forms of interdependence, such as inter-household food sharing and commodity exchange in nonindustrial human societies (Ember, Skoggard, Ringen, & Farrer, 2018; Jaeggi, Hooper, Beheim, Kaplan, & Gurven, 2016). Much more attention needs to be paid to relationships and how they interact with the affective components of cooperation.

Personality, or consistent individual differences in behavioral responses (Gosling, 2001; Réale et al., 2007), also influences decision-making. Over a century of research has demonstrated the importance of human personality for determining physical and psychological health (Ozer & Benet-Martínez, 2006), social relationships (Asendorpf & Wilpers, 1998; Robins, Caspi, & Moffitt, 2002), and reproductive success (Alvergne, Jokela, & Lummaa, 2010; Berg, Lummaa, Lahdenperä, Rotkirch, & Jokela, 2014; Gurven, Von Rueden, Stieglitz, Kaplan, & Rodriguez, 2014), and recent work indicates that personality is influencing animals in similar ways. For instance, personality influences cooperation and responsiveness to punishment in public goods games (Schroeder, Nettle, & McElreath, 2015), sensitivity to inequity (Brosnan, et al., 2015), and patterns of social learning in experimental tasks (Barrett, McElreath, & Perry, 2017), which can lead to population-level inferences deviating appreciably from the behavior observed for most individuals (Barrett, McElreath, & Perry, 2017). There is less known about how personality is influenced by or influences affect, but this literature could help us understand the affective components of animal

cooperation. Monoamine systems, for instance, are highly conserved across animals (Edsinger & Dölen, 2018; Gruber, 2014) and have been linked to dispositional affect (Aluja, Balada, Blanco, Fibla, & Blanch, 2018; Chester et al., 2015; DeYoung, 2013), and variation in the functionality of these systems influences personality in rhesus macaques (McCormack, Newman, Higley, Maestriperi, & Sanchez, 2009), great tits (Fidler et al., 2007), and crickets (Abbey-Lee et al., 2018), among other taxa. Similarly, regulatory patterns of the HPA axis influence personality in numerous species (Carere, Caramaschi, & Fawcett, 2010). Given the deep homology of these systems, which play a crucial role in the adaptive regulation of social behavior, it is plausible that similar affective phenomena accompany their activity in non-human animals.

5. Future Directions and Conclusions

It is an exciting time in the study of affective science, as we are at a threshold where we can begin to get objective, consistent measures of affect that are comparable across multiple species. Technology has advanced to the degree that we can measure sympathetic and endocrine responses in a non-invasive way, often even in group situations. Given that cooperation research necessarily involves a group (of at least two!), these advances are essential to develop a comparative affective science of cooperation.

We have a few recommendations to allow us to fully benefit from our ability to measure affect. First, and most obviously, we need to be cautious in how we interpret what we measure. In most cases, we are not measuring affect *per se*. We are measuring physiological responses that we *interpret* as determining or being determined by affect. As we cannot ask our subjects how they feel, this is what we have. To its benefit, this objective measurement approach is arguably less biased than self-report and is directly comparable across species. Nonetheless, this caveat must be kept in mind when interpreting our results. We encourage researchers to not depend on just one measure, but to triangulate affect by looking at physiology, behavior and cognition (e.g. cognitive bias tests) in parallel whenever possible (cf. Izard, 1977; Mendl et al., 2010; Paul et al., 2005; this issue). Similarly, we encourage the incorporation of *multiple* physiological measures whenever possible, to see if they are consistent. Second, it is important to compare results across species, and when contradictions emerge, consider carefully what might have caused them. Our ability to interpret physiological responses is only increasing, but failing to recognize the intricacies of how these responses interact with each other and how they compare across species will slow the advancement of our knowledge.

Second, keeping animals in atypical social configurations or stressful circumstances will influence their affective state and alter results. Of course, captive work is important to perform the carefully controlled behavioral studies that are needed to determine the role of affect in decision-making. Nonetheless, it is important to keep animals in social groups that are as close to

species-typical as is possible, and moreover, to run studies that include more than two individuals. While we have gained immense knowledge from even dyadic studies of cooperation, extending these results to larger groups provides a better understanding of how social dynamics influence cooperation (Fruteau, van Damme, & Noë, 2013; Massen et al., 2015; Suchak et al., 2016).

Relatedly, we want to stress the importance of animal training. By designing and following training protocols using positive reinforcement training (e.g. Kemp et al., 2017), one can facilitate the use of measuring devices, reduce unwanted effects of testing procedures, which potentially influence the outcome of the study (e.g. Lambeth, Hau, Perlman, Martino, & Schapiro, 2006), and improve animal welfare (Prescott & Buchanan-Smith, 2003). Clearly, ecologically valid and psychologically informed experiments will be more instructive as to the proximate mechanisms and evolution of cooperation (McAuliffe & Thornton 2015).

Scientists have been discussing the possibility of emotions in animals since at least Darwin (1872), if not before. Scholars have, however, often rejected the idea, as they argued that it was solely based on anthropomorphism (but see de Waal, 1999; Williams, Brosnan & Clay, this issue), and that non-human species lack the phenomenological experience so typical of human emotions. Contemporary science, however, does not view emotions as solely based on subjective feelings, but considers it as a multifaceted construct that includes some combination of affect, behavior, physiology and cognitive elements (Mendl, Burman, & Paul, 2010; Paul, Harding, & Mendl, 2005; this issue; Bliss-Moreau 2017). With recent developments, we now finally also have the tools to be able to measure what is going on physiologically in ways that previous scientists could not, and sophisticated behavioral experiments are disclosing the underlying cognition. This provides us with the ability to more objectively validate conjectures about affect in general, but particularly in animals, and to explore influences that we may not have even considered, given that other species' affect may be considerably different from our own (Bliss-Moreau 2017). Aside from providing a better understanding of the evolution of affect, and, thereby, emotion, these new tools may also allow us to recognize new and exciting ways in which other species interact with the world. Much theoretical work has assumed a mediating role for affect in cooperation, yet this has so far mainly been based on assumptions. With the recent methodological developments reviewed above now in place, and the framework we aimed to provide here, we are at the dawn of gaining a much more comprehensive insight into the proximate, affective mechanisms underlying cooperation, as well as the evolutionary history of these mechanisms across the animal kingdom.

Acknowledgements

The authors thank the Lorentz Center of the University of Leiden, the Netherlands, for hosting a stimulating workshop that led to the development of many of the ideas in this paper, as well as Eliza Bliss-Moreau and Mariska Kret for co-hosting this workshop and for initiating and editing this special issue. During the writing of this manuscript SFB was funded by NSF grants SES 1658867, SES 1425216, and IBSS 1620391 and JJMM was funded by a Templeton Grant (# TWCF0267) to M.E. Kret. JSM would like to thank the Miami University Honors Department and Linda Marchant for supporting his involvement in the Comparative Affective Science workshop through the Joanna Jackson Goldman Prize.

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In preparation – first draft

Digit ratio (2D:4D) in long-tailed macaques – investigating sex differences and stability during growth

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Abstract

Prenatal androgen exposure has been associated to behavioural traits as well as health related issues in humans, yet the levels of such exposure are difficult to measure. The second-to-fourth digit ratio (2D:4D ratio), however, may function as a biomarker for this, reflecting the effects of prenatal androgen exposure in such a way that higher *in utero* concentrations of androgens cause low 2D:4D ratios. The digit pattern is generally found to be sexual dimorphic in humans (male 2D:4D < female 2D:4D), in line with the predicted effects of androgens, yet recent reports also show that it is relatively unstable during postnatal development, making it a less suitable biomarker at later stages in life. Comparative analyses on other primate species could shed light on the evolutionary history of this trait, and as a potential biomarker the 2D:4D ratio could also provide important insights regarding health issues in those primate species that are being used in biomedical research. Yet so far, primate 2D:4D ratio studies are less abundant. This study, therefore, seeks to validate the 2D:4D ratio as a biomarker for prenatal androgen exposure in long-tailed macaques – a primate species that is often used in biomedical research – by testing sex-differences and stability during development, using longitudinal data obtained from socially housed long-tailed macaques in 2016 and 2018. Remarkably, no sexual dimorphism was found. Moreover, the 2D:4D ratio appeared to change slightly during a period of growth. This study adds to the limited knowledge about 2D:4D ratios in primates and to the general understanding of the use of the 2D:4D ratio as a proxy for the effects of prenatal androgen exposure.

Keywords

Macaca fascicularis, crab-eating macaque, cynomolgus macaque, androgens, testosterone, second-to-fourth digit ratio, 2D:4D ratio, finger length, prenatal effect

Introduction

Androgens play crucial roles in the differentiation of the male phenotype. The organizational hypothesis states that permanent masculinization of brain structures and behaviour occurs under the influence of *in utero* androgens (Phoenix et al., 1959). Androgens also affect other tissues such as the liver, central nervous system, skeletal muscle, fat tissue, and beta cells of the pancreas (Navarro et al., 2015). The effects androgen levels have on these tissues include insulin production and sensitivity. Therefore, the influence of (prenatal) androgens and the androgen receptor are widely studied in epidemiological research. Yet studying prenatal androgen exposure is difficult as it happens in utero. The effect of prenatal androgen levels on adult phenotypical outcomes such as predispositions for androgen-related health conditions is, therefore, often studied using a biomarker for *in utero* exposure to prenatal androgens, which is the second-to-fourth digit ratio (2D:4D ratio) (Gámez et al., 2023; Talarovičová et al., 2009). Manning and colleagues (1998) were among the first to use this, now widely used, biomarker to study fertility related characteristics in adults. Lower female 2D:4D ratios compared to male 2D:4D ratios observed in 2-year-old children indicated a sexually dimorphic pattern, which led to the hypothesis that *in utero* conditions have an organizational role in the establishment of this sexual dimorphism. *In utero* concentrations of androgens are higher for male than for female fetuses. Zheng and Cohn (2011) indicated that a prenatal environment high in androgen concentration is essential for a low 2D:4D ratio. Already in 1888, observations suggested that the length of the human ring finger, the fourth digit (4D), tends to be longer in men than in women (Baker, 1888). A longer ring finger than index finger (2D) results in a low, often called “masculinized”, 2D:4D ratio. In women, the length of 2D generally approaches or resembles that of 4D, resulting in a “feminized”, high 2D:4D.

The relationship between 2D:4D ratio and muscular fitness (Pasanen et al., 2022), the ability to jump vertically (Disterhaupt et al., 2022), and obesity (Iljin et al., 2022) are examples of recent study topics examining the effect of prenatal androgen levels on physical traits using this biomarker. Similarly, the influence of prenatal androgen levels, by proxy of the 2D:4D ratio, on the development of behavioural patterns is studied. Research topics include prosocial choices (Horn et al., 2018), gender-typical play preference (Körner et al., 2020), as well as autism spectrum disorder (Kyselicová et al., 2021). Links are even drawn between 2D:4D ratio and pro-environmentalism (Pickering et al., 2022) and parental income inequality (Manning et al., 2021).

Although the 2D:4D ratio is widely used, it is, however, still a controversial biomarker, particularly for two reasons: Firstly, evidence for the temporal stability of it is sparse. Recent findings suggest that children’s 2D:4D ratios are significantly lower than those of adults (Kyselicová et al., 2021). In addition, significant increases in 2D:4D ratios of boys and girls between months 20 and 40 of life were reported (Körner et al., 2020). These findings contradict

an earlier claim that the 2D:4D ratio is established in the prenatal environment and subsequently stable through life (Manning and Fink, 2018), and instead suggest that postnatal influences also affect the digit ratio as the individual ages. Just a minority of 2D:4D ratio stability studies actually investigated the direct effect of age by studying individual changes in digit ratio over time. These longitudinal studies have accumulated more consistent results for humans compared to the cross-sectional studies which investigate the effect of age more indirectly. The ratio for humans between 0 and 30 years appears to increase with age, and thus can be considered unstable (Knickmeyer et al., 2011; Körner et al., 2020; Králík et al., 2017; McIntyre, 2006; McIntyre et al., 2005; Trivers et al., 2006; Wong & Hines, 2016b). However, stability of 2D:4D ratio ranks suggests stability on a relative scale (Králík et al., 2017; Trivers et al., 2006). This means that the individual ratios do change through development, but in similar directions and proportions for all individuals, resulting in a stable rank order of the ratio despite individual changes in 2D:4D ratio. Demonstration of 2D:4D ratio stability is essential for reducing the controversy of the use of this biomarker and thus for validation of the 2D:4D ratio as a proxy for the effects of prenatal androgen exposure. A lot of correlational studies have already been performed because digit ratio data can be obtained relatively easily and inexpensively. However, found correlations between 2D:4D ratio and phenotypical traits of adults cannot be solely linked to prenatal androgen exposure if the ratio appears to be influenced by postnatal factors as well.

Secondly, the controversy is fuelled by inconsistent study outcomes regarding the sexual dimorphism of the 2D:4D ratio. A recent study using a large sample size (3,802 men and 3,780 women) originating from Europe, East Africa and Central Asia found a sex difference (male < female) in left and right 2D:4D ratios (Butovskaya et al., 2021). In line with this, a significant sex difference (male < female) was found for 7-year-olds in a study from Jägetoft and colleagues (2022). The difference between the sexes has been found to be statistically significant for mean 2D:4D ratios, but also for left hand and right hand 2D:4D ratios separately (Buck et al., 2003; Ernten et al., 2021; Manning and Fink, 2018; Mitsui et al., 2016, 2015; Pruszkowska-Przybylska et al., 2018; Richards et al., 2019; Trivers et al., 2020, 2006; Wong and Hines, 2016). Often, however, researchers also observed non-significant sex differences between mean, left or right 2D:4D ratios only (Bloom et al., 2010; Crewther et al., 2015; Ernten et al., 2021; Körner et al., 2020; Manning and Fink, 2018; Spix and aan het Rot, 2021; Yamada et al., 2020), and others find no sexual dimorphism at all (Barrett et al., 2020; Yamada et al., 2020).

The second-to-fourth digit ratio is also studied in primate species other than humans, for example in rhesus macaques (*Macaca mulatta*) (Abbott et al., 2012; Baxter et al., 2018a; Nelson et al., 2010a; Paukner, 2019), baboons (*Papio spp.*) (Howlett et al., 2012a, 2015a; Huber et al., 2018; Roney et al., 2004a), titi monkey (*Plecturocebus cupreus*) (Baxter et al., 2020), bonobo (*Pan paniscus*) and chimpanzees (*Pan troglodytes*) (McIntyre et al., 2009). Studies on the 2D:4D ratio

in non-human primates seem to focus less on the link with physical traits, and more on links between 2D:4D ratio and (adult) behaviours, such as social bonding (Nelson and Shultz, 2010) and dominance (rhesus macaques: Baxter et al., 2018; Nelson et al., 2010; baboons: Howlett et al., 2012, 2014). In non-human primates, remarkably, varying patterns of the sexual dimorphism have been reported. Similar directionality in sexual dimorphism as humans (male 2D:4D < female 2D:4D) has been found in our closest living relatives, i.e., bonobos and chimpanzees (Mcintyre et al., 2009), but also, for example, in titi monkeys (Baxter et al., 2020). In some species no sexual dimorphism could be found, for instance in hamadryas baboons (*Papio hamadryas*) (Huber et al., 2017), and some studies show that, in contrast to humans, male baboons (Roney et al., 2004b) and male rhesus macaques (Abbott et al., 2012; Baxter et al., 2018b) have higher 2D:4D ratios than females. Wood and colleagues (2020), however, found this sexual dimorphism in adult rhesus macaques only in their left hand 2D:4D ratio, and in infants no difference was found (Paukner, 2019). The temporal stability of 2D:4D ratios in primates has not been studied extensively, and the only study to our knowledge that did so reported that for rhesus macaques the ratio is unstable between the ages 14 and 30 days (Paukner, 2019). To conclude, existing literature on non-human primates reveals similar inconsistencies as in humans regarding the presence of sexual dimorphism and its directionality as well as the temporal stability of the digit ratio, though not studied extensively.

Most of the existing studies on 2D:4D ratios in non-human primates were conducted on rhesus macaques, as this the number 1 model species for biomedical studies. They are therefore readily available, and if the 2D:4D ratio is indeed a validated proxy for prenatal androgen exposure, which may have potential long-lasting (health) effects, its applications in biomedical science could be plentiful. A close relative of the rhesus monkey, i.e., the long-tailed macaque (*Macaca fascicularis*), is also often used as model species in biomedical research, yet so far very little is known about their 2D:4D ratios. Nelson and Schultz (2010) have included 2D:4D data of long-tailed macaques in a broader analysis on primate family level but did not investigate sexual dimorphism on species level. The reported means and standard deviations do, however, indicate that there is no difference between sexes. Nevertheless, these data are based on a very small sample of individuals of this species (9 females, 6 males) and thus requires further attention.

The present study, therefore, investigates if long-tailed macaques' 2D:4D ratio is i) sexually dimorphic and ii) stable in the postnatal phase of life. Longitudinal data obtained from left and right hands in 2016 and 2018 from socially housed long-tailed macaques at the Biomedical Primate Research Center in Rijswijk, The Netherlands, was used for these investigations. Based on study results from Nelson and Shultz (2010), no sexual dimorphism is expected to be found, although results on the closely related rhesus macaque also remain inconclusive. Furthermore, it is expected that the 2D:4D ratio changes even after birth. The results

from this longitudinal study will add to the understanding of the evolutionary significance of the 2D:4D ratio, its temporal stability after birth and the discussion concerning the validity of this biomarker as a proxy for prenatal androgen exposure and its potential role in biomedical research.

Methods

Subjects and housing

Data for this study was collected from long-tailed macaques at the Biomedical Primate Research Centre (BPRC) in Rijswijk, the Netherlands. In 2016 2D:4D ratios were obtained from 131 individuals (87 females, 44 males) and in 2018 from 151 individuals (96 females, 55 males). 90 individuals are represented in both datasets. The macaques age ranged between 0.67 and 24.27 years and they were housed in eight social groups of max. 40 individuals, each with access to large indoor (72 m², 2.85 m high, light/dark cycle 12 h) and outdoor enclosures (208 m², 3.1 m high). They are fed twice daily, around 8:30 am they get monkey chow pellets (Sniff) and at 3 pm they get either fresh fruit, vegetables or bread. Water is available ad libitum.

Taking hand scans

To be able to measure the macaques' digit lengths, which are needed to calculate the 2D:4D ratio, we took digital scans of the ventral surface of both left and right hand. Hand scans were taken with a Canon CanoScan LiDE 220 digital flatbed scanner during annual health check-ups for which the individuals were anaesthetized. For this, one person was holding the monkey (on his lap), while the experimenter (always the same) was placing the monkey's hand on the scanner, covering it with a white underpad (Tena) to block the light (as the scanner could not be closed because of the hands), and holding the hand in place during the scanning procedure. The use of flatbed scanners yields the most precise measurements compared to direct measurements or measurements of photocopies (Neyse and Brañas-Garza, 2014). Visibility and the spreading of all digits, flatness of the hand, definition, and contrast of each scan were directly assessed by the experimenter. If, at a first glance, it seemed that the scan did not meet these requirements, the hand was scanned again. All scans were saved with unique ID labels. Date, subject ID, and hand were specified for each scan.

Digit measurement and 2D:4D calculation

For this study, second and fourth digits from scans containing either a left or right hand were measured by one experimenter using the ruler tool in Adobe Photoshop (Version 12.0). The scale

was calibrated to millimetres with recording to 0.1 mm. Contrast was adjusted if needed. Obtaining reliable data of 2D and 4D lengths relied on the application of a standardized method for all scans. Here, the methodology for digit length measurements using digital scans and Adobe Photoshop was based on a measurement guide designed by Neyse & Branas-Garza (2014). In preliminary observations, it became clear that the scans varied in clearness of the creases. 'Z' lines were observed from the creases between the finger and the hand palm (Figure 1). Some hands only had the 'upper' line of the 'Z', some only the 'lower' line. The crease between the digit and the remarkable circular lines on the skin of the hand palm will be considered the dividing line between digit and hand palm, and thus was used as 'starting point' of the digit.

All digits were measured twice and the mean of the two measurements was used to calculate the 2D:4D ratio. The first time the length between the crease between finger and hand palm to the tip of the finger was assessed. In second measurements the length between the tip and the crease was measured. All measurements were blind to the sex and physical information of the individual. Second measurements were performed on different days than the first measurements of the respective group. Also, they were done blind to the outcomes of the first measurement. The data was collected in a Microsoft Excel file. If the measurements differed more than 0.6 mm a third measurement followed. This way, measurement error was limited.



Figure 1. 'z-line' on long-tailed macaque's fourth digit (4D).

Data analysis

In some cases, at the time of data collection it was not clear whether the scan would meet the quality criteria and, therefore, immediately more scans were taken. This resulted in a total of 677 scans. At the time of the digit measurement the quality of the scans was assessed more thoroughly and often the first scan seemed to meet the criteria as well. In these cases, though, the mean of the 2D:4D ratio of all valid scans of one hand was calculated and used for further analyses.

Nevertheless, 42 scans had to be excluded due to bad quality, resulting in a data set of 635 valid hand scans and 531 2D:4D ratios (some averaged per hand, but not year) of 192 individuals.

Statistical analyses were conducted in R (version 3.5.2) (R Core Team, 2018). To determine if the 2D:4D ratio in long-tailed macaques is sexually dimorphic we computed a linear mixed-effects model (*lmer*, package *lme4*, (Bates et al., 2015)), which included the two-way interactions of sex, age and side (left or right) as fixed effects and year and ID as random intercept effects. Normal distribution and homogeneity of the model residuals were inspected visually. Variance inflation factors were calculated (*vif*, package *car*, (Fox and Weisberg, 2019)) and showed no multicollinearity issues (max. variance inflation factor = 4.11).

To test if the 2D:4D ratio is stable after birth, a data subset was formed that only contained 2D:4D ratios which were sampled in 2016 as well as in 2018. This resulted in longitudinal data of 165 2D:4D ratios (83 left hands, 82 right hands; 90 individuals: 21 males, 69 females). The interclass correlation of 2D:4D ratios of 2016 and 2018 was calculated using *ICC* (type ICC3, package *psych*, (Revelle, 2022)). Since the ICC was only moderate, we further investigated if this was due to i) young and still growing individuals in which the 2D:4D ratio might still change or ii) a systematic measurement error.

To test if the 2D:4D ratio is stable during growth, a data subset was formed that only contained individuals that were younger than 4 years at the day of the health check in 2016, and therefore still growing (44 individuals: 18 males: mean age in 2016 1.73 ($\pm$ 0.69) years, 26 females: mean age in 2016 2.22 ($\pm$ 1.03) years). This subset consisted of longitudinal data of 81 2D:4D ratios (42 left hands, 39 right hands). To determine if the 2D:4D ratio of growing individuals is increasing or decreasing over the years, we fitted a linear mixed-effects model (*lmer*, package *lme4*, (Bates et al., 2015)), which included the 2D:4D ratio as response variable, year as fixed effect and ID as random intercept effect to control for repeated sampling. Followingly, a second linear mixed-effects model was fitted to determine how the growth of the fingers caused the change in the 2D:4D ratio found in the previous model, i.e., if the change in finger length depends on the finger (2D or 4D). This model included the finger growth i.e., the difference in finger length between 2016 and 2018 as the response variable, the finger (2D or 4D) as fixed effect and ID as random intercept effect. Also for these models, normal distribution and homogeneity of the model residuals were inspected visually, and since there is only one fixed effect in each model collinearity tests are not required. To rule out a systematic measurement error, we also calculated the ICC for longitudinal data (70 2D:4D ratios: 34 left hands, 36 right hands) of individuals that in 2016 were six years and older and, therefore, not growing anymore (38 individuals: only 2 males due to structure of breeding group simulating male dispersal).

Results

The 531 2D:4D ratios from 192 individuals ranged between 0.693 and 0.932, with an overall mean of 0.818 ± 0.034 SD. However, we found no difference in 2D:4D ratios between males (mean 0.819 ± 0.032 SD) and females (mean 0.818 ± 0.035 SD), nor did we find any effect of (the interactions of) sex, age and side (left or right) (Tab. 1a, Fig 2a).

With regard to stability over time we found that overall, the 2D:4D ratios of 2016 were only moderately correlated with their counterparts of 2018 with an ICC of 0.66 ($F = 4.9$, $df = 165$, $p \leq 0.001$, see (Koo and Li, 2016)). Taking a closer look at individuals that in 2016 were younger than four years of age showed that the ICC was even lower in growing individuals (ICC = 0.63, $F = 4.5$, $df = 80$, $p \leq 0.001$), suggesting that the originally low ICC was not due to measurement error or external effects in adult life. Indeed, in the above six-year-old individuals' 2D:4D ratios we found good agreement between 2016 and 2018 (ICC = 0.76, $F = 7.2$, $df = 69$, $p \leq 0.001$). As for the cause of the low ICC in the younger individuals, in 2016 the mean 2D:4D ratio of the under four-year-old individuals was 0.8133 and in 2018 the 2D:4D ratio was 0.0076 higher than in 2016 (Tab. 1b, Fig. 2b), which is a very small increase of 0.94 %. Comparing growth among the two separate digits, the fourth digit (4D) was growing 0.5 mm more than the second digit (2D) (Tab. 1c).

Table 1: Presented are the models on a) sexual dimorphism in the 2D:4D ratio including all individuals, b) on differences between 2D:4D ratios measured in 2016 and 2018 using longitudinal data of individuals under the age of four years (in 2016), and c) on the finger growth of these young individuals. Given are estimates, standard errors (SE), lower confidence interval (CI), upper CI, degrees of freedom (df), t-values and p-values for each parameter of the linear mixed-effects models. * $p \leq 0.05$. *** $p \leq 0.001$.

	Estimate	SE	Lower CI	Upper CI	df	t value	p	
a) Model: sexual dimorphism [2D:4D]								
Intercept	0.8223	0.0056	0.8116	0.8332	20.14	146.316	< 0.001	***
sex (male)	-0.0056	0.0072	-0.0196	0.0087	247.30	-0.773	0.440	
age	-0.0006	0.0005	-0.0015	0.0004	251.10	-1.173	0.242	
side (right)	0.0005	0.0040	-0.0074	0.0084	331.60	0.124	0.901	
sex (male): age	0.0016	0.0010	-0.0003	0.0035	244.40	1.638	0.103	
sex (male): side (right)	-0.0024	0.0044	-0.0111	0.0063	334.60	-0.546	0.586	
age: side (right)	0.0001	0.0004	-0.0007	0.0008	333.40	0.198	0.843	
b) Model: difference between years [2D:4D]								
Intercept	0.8133	0.0048	0.8038	0.8226	53.52	170.786	< 0.001	***
year (2018)	0.0076	0.0034	0.0009	0.0143	114.90	2.242	0.027	*
c) Model: finger growth [mm]								
Intercept	3.4975	0.3763	2.7527	4.2419	45.61	9.294	< 0.001	***
finger (4D)	0.5020	0.1307	0.2448	0.7592	116.93	3.841	< 0.001	***

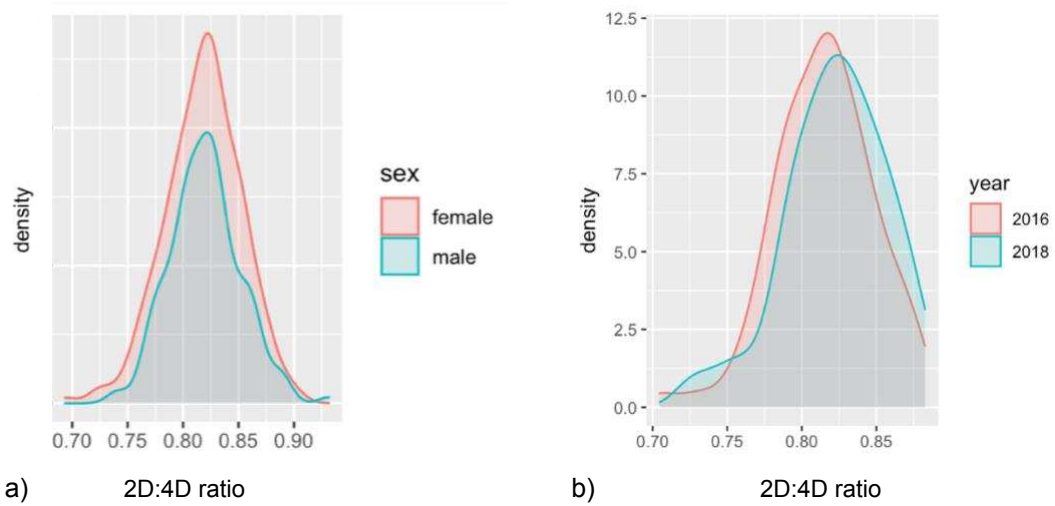


Figure 2. a) Density of male and female long-tailed macaques' 2D:4D ratios (all individuals), **b)** Density of growing individuals' 2D:4D ratios in 2016 and 2018. Since no difference in the 2D:4D ratio between left and right hands was found, left and right hand 2D:4D ratios were combined in these figures.

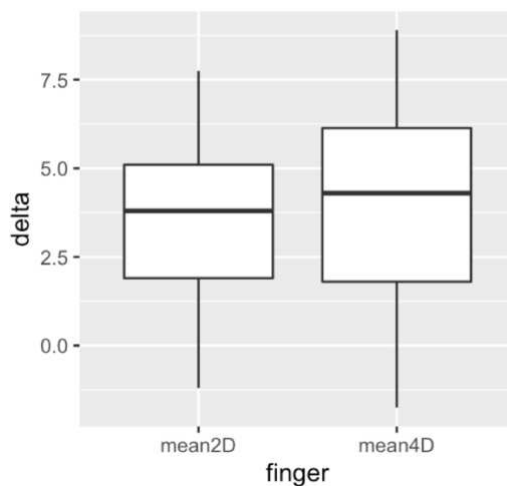


Figure 3. Growth of the second (2D) and fourth (4D) digit between 2016 and 2018 in long-tailed macaques that in 2016 were younger than 4 years of age and hence likely still growing.

Note that in 15 out of 162 measured fingers of growing individuals, hence less than 10 percent, the calculated finger growth between 2016 and 2018 resulted in negative values, meaning that the measured finger length appeared to have decreased (see Fig. 3). In the adult individuals (above 6 years of age in 2016), however, 80 out of 140 fingers, 57.14 percent, revealed negative differences in measured length. This distribution suggests a random occurrence, which aligns with the expectation that measurements taken two years apart will seldom yield identical results, with roughly half showing positive differences and the other half showing negative

differences. On a closer look at the growing population, negative values mostly appeared in individuals born in 2012 and in a few born in 2013, but not in individuals younger than that. This suggests that the fingers of these individuals might already have come to a growth stop in 2016.

Discussion

The 2D:4D ratio in primates, even more so long-tailed macaques, is not extensively studied. Hence, this study aimed to broaden the scientific knowledge about this allegedly valid biomarker for the effects of prenatal androgen exposure. This study focused on sexual dimorphism and temporal stability of 2D:4D ratio in long-tailed macaques during a period of growth using longitudinal data obtained in 2016 and 2018. Notably, no significant difference in the 2D:4D ratio was observed between male and female individuals. We did, however, find that the ratio changes during growth and that this is due to the fourth digit (4D) growing 0.5 mm more than the second digit (2D). In adult individuals, on the other hand, the 2D:4D ratio remains rather stable.

Our zero findings concerning sexual dimorphism align with expectations based on previous digit ratio research by Nelson and Schultz (2010), which suggested an absence of sexual dimorphism in long-tailed macaques. Although their study reported slightly higher mean 2D:4D ratios for both females and males compared to our findings, the consistent pattern of no discernible differences between sexes persisted. If the 2D:4D ratio indeed serves as a marker for prenatal androgen exposure, these results would imply similar androgen levels in male and female macaque fetuses. However, this raises intriguing questions about the mechanisms behind sexual differentiation in males in non-human primates, and how that relates to findings in humans. Consequently, the likelihood of 2D:4D being a reliable proxy for prenatal androgens, at least in this non-human primate species, appears questionable.

Regarding the temporal stability of the 2D:4D ratio it was expected that the digit pattern was, like observed in humans, changing during growth, but that the ranking of the 2D:4D ratios would persist, indicating relative stability (Králík et al., 2017; Trivers et al., 2006). Overall, the 2D:4D ratios of 2016 were only moderately correlated with their counterparts of 2018, meaning that there was poor (relative) stability. Splitting the dataset and taking a closer look at individuals that in 2016 were younger than four years of age showed that the measures of 2016 and 2018 were even less correlated in growing individuals. The fact that the 2D:4D ratios were not stable in long-tailed macaques, indicates that the 2D:4D ratio changes during growth and, therefore, is likely affected by postnatal factors. This further implies that the 2D:4D ratio may not serve as a reliable indicator of prenatal androgen levels in long-tailed macaques.

Over the two-year growth period, the 2D:4D ratio exhibited a subtle average increase of approximately one percent. Notably, similar studies in humans have reported significant increases in the 2D:4D ratio as well (Knickmeyer et al., 2011; Körner et al., 2020; Králík et al.,

2017; McIntyre et al., 2006, 2005; Trivers et al., 2006; Wong and Hines, 2016). To examine the underlying mechanism causing the slight increase in 2D:4D ratio, the digit growth was further investigated. This revealed that the fourth digit (4D) was growing 0.5 mm more than the second digit (2D). Intuitively, this would seem to decrease the ratio, and not increase, which is what we observed albeit only slightly. Note, however, that if both fingers were to grow at the same rate, the 2D:4D ratio would naturally increase, as the values approach each other more and more. Therefore, for the ratio to remain consistent, the fourth digit (4D) must undergo greater growth than the second digit (2D). Only if the 4D were to grow substantially more than the 2D would the ratio decrease. In this context, the observed increase in the ratio indicates that either the 2D experienced excessive growth or the 4D did not grow sufficiently (essentially the same scenario) to maintain the established ratio. The longitudinal data reveal that, over the years, the 2D:4D ratio grew larger, indicating that, relative to their initial lengths, the 2D exhibited more growth than the 4D. It is important to note that this does not imply greater absolute finger growth, as we actually found the opposite with the 4D growing more, but rather a disproportional growth in relation to the original length between the 2D and 4D, leading to an increased 2D:4D ratio during the postnatal period of the individuals under study.

We also investigated changes in 2D:4D ratios in the individuals that in 2016 were above six years old and therefore fully grown and found good agreement between 2016 and 2018. These results, thus, suggest that in adult long-tailed macaques the 2D:4D ratio is reasonably stable. However, it is questionable whether after the change during growth, adult 2D:4D is indicative of prenatal androgen levels. Nevertheless, this finding rules out that the instability found in the growing individuals is due to a standardized measurement error.

Despite strictly following the quality criteria of the scans and standardized measurement methods (including measuring all digits twice, followed by a third time if the first two measurements differed too much), this study has its limitations. Single measurement errors could have occurred due to the fact that some hands had multiple creases between the digit and the palm of the hand making it difficult to determine the proximal end of the digit. As already mentioned by Králík and colleagues (2017), obtaining two-dimensional scans of three-dimensional hands inescapably results in dependency on a standardized hand position during scanning. Despite our best efforts to place all individuals' hands on the scanner in the same way, there were some deviations. Time pressure during collection, due to temporally restricted sedation of the macaques, led to challenges in obtaining optimal scans for every hand. As a result, numerous scans had to be excluded from the analysis.

Moreover, due to colony management reasons, our data had a sex bias in terms of age. In the breeding colony of long-tailed macaques at BPRC, only females stay in the group beyond the age of four, whereas natal-born males are removed from the group to simulate male dispersal and

prevent inbreeding. Consequently, the sample of individuals older than six years is predominantly composed of females and a few breeding males, which may have hampered our analyses of sex differences.

To conclude, there is no sexual dimorphism in 2D:4D ratio in long-tailed macaques. Also, the 2D:4D ratio of growing long-tailed macaques has been found to increase slightly during the postnatal phase of life. This can be attributed to differential growth of 2D and 4D. To further investigate possible postnatal effects on the 2D:4D ratio, future studies could also take into account castrated/neutered animals. They can be used to rule out the influence of testicular testosterone on the 2D:4D ratio. Nevertheless, the herein found temporal instability of 2D:4D ratios casts doubt on the validity of the use of the 2D:4D ratio as a proxy for prenatal androgen exposure, at the very least in macaques.

Ethical statement

Ethical review and approval were not required for this study. All data were obtained from the annual health evaluations, which is part of BPRC's regular health program.

Acknowledgements

We thank Dian Zijlmans, Nina Stalknecht and Annet Louwerse for their help regarding the hand scans.

Funding

This project was financed by the FWF (Stand-alone project P 26806 Jorg Massen) and the Austrian Federal Ministry of Education, Science, and Research (Marietta Blau Grant 2018 to Martina Stocker).

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Acknowledgements

First and foremost, this doctoral thesis is a collective achievement, and I owe its success to my co-authors: **Jorg, Virginie, Thomas, Ruth, Matthias, Rupert, Eoin, Jonathan, Lisa-Claire, Leonie, Lisa, Liesbeth, Eva, Sarah, Jordan, and Friederike**. Their collaboration and contributions have been invaluable.

I extend my deepest gratitude to Jorg, Virginie, and Thomas for their supervision. **Jorg**, thank you for giving me the opportunity to pursue a PhD within your team and allowing me to explore my interests in behavioural endocrinology, even though this was not part of your initial plans. I greatly appreciate that you always took the time to give me critical and timely feedback. Most importantly, though, I want to thank you for introducing me to the Netherlands and the BPRC, as this has profoundly changed and enriched my life. **Thomas**, I am grateful for the opportunity to work with ravens and for your enthusiasm about animal behaviour, particularly corvid research. Your insights have improved my thesis, and your support and encouragement throughout this process have been very important to me. **Virginie**, your expertise in endocrinology has been invaluable, deepening my understanding of this complex field. Particularly in our study on salivary mesotocin in ravens, your effort and insight have been greatly appreciated.

To the other people who have accompanied my path in endocrinology: **Ruth**, you have been my "hormone buddy," sharing the same fascination with endocrinology. It has been incredible to have a friend to discuss hormone-related topics with, and I have learned so much from you. Thank you for all your help and support! **Elisa** and **Rupert**, everything I know about hormone analyses is thanks to both of you. Elisa, thank you for allowing me to work in your lab and thereby gaining more lab experience. Magical colour changes and sore arms from drying the microtiter plates!

Matthias, thank you for guiding me through the complexities of statistics and helping me find a solid solution for the long-tailed macaque paper. When I felt completely lost with my statistical challenges, your support, both technical and mental, was invaluable. I am deeply grateful for your assistance and encouragement.

Lisette, Caroline, Martina Sch., Marit and **Marlies**, thank you for teaching me so much about animal training and thereby helping me conduct my studies. The topic of animal training initially brought me to the study of animal behaviour and eventually led me to the job I am currently doing with pleasure.

I also wish to extend my heartfelt thanks to my colleagues at BPRC, particularly **Annet, Jan, and Dian**, alongside the diligent **animal caretakers**, whose support has been crucial in my studies on long-tailed macaques. Additionally, I am deeply grateful to **Eoin**, who afforded me the opportunity to collaborate with him at Edinburgh Zoo on one of his projects concerning capuchin monkeys. This experience marked my first venture into research with non-human primates, and for that, I am profoundly appreciative.

I would like to express my gratitude to my dear biology friends who, although not directly involved in my studies or thesis, have been pillars of support throughout. **Anita, Bella, Jessie, Juju, Marlen, Megan, Pavla, Rachel, Stephan, and Vedrana**, your camaraderie and companionship in the office, lab, conferences and beyond have enriched my journey immensely. To my fellow long-term PhD candidates and cherished friends, **Christian** and **Kim**, I always enjoyed hanging out with you and sharing PhD-related topics and frustrations. Your company along this long path made me feel understood and normal. **Michelle**, it is inspiring to see how you have become a true role model, leading your own research group while being a wonderful mother to three children and a supportive friend throughout my PhD journey.

Kathi, Linda, Willi, and Claus, I deeply appreciate your constant interest in my work and PhD life, despite not being biologists, and for always offering your support. **Riccardo**, thank you for your support during the first half of my PhD and for escaping with me to Spain to ease my despair when I had to retract a paper that had already passed the first round of reviews – one of the reasons why this thesis was almost titled “When the spit hits the fan” (idea by Wouter).

Most importantly, I want to thank my family. **Mama und Papa**, danke dafür, dass ihr all meine Entscheidungen unterstützt habt, einschließlich ausgefallener Ideen wie erst Modedesign und dann Verhaltensbiologie zu studieren und später ins Ausland zu ziehen. Danke, dass ihr mich auf diesem langen Weg unterstützt, mir nie Druck gemacht habt und immer eure Liebe und euren Stolz ausgedrückt habt. **Tjeerd and Ella**, my loves, I am incredibly grateful to have you both in my life. Your support throughout the concluding years of my PhD have meant the world to me. Juggling the demands of completing my PhD on weekends while prioritizing quality family time was often challenging. Your understanding and patience have been invaluable. Thank you, from the depths of my heart. I am looking forward to welcoming our next family member!

Finally, this research would not have been possible without the financial support of the **OeAD, Erasmus, IPS, Ethologische Gesellschaft, BPRC** and **Salimetrics**. Thank you!