A PHYSIOLOGICAL EVALUATION OF SOCIAL BONDING IN WESTERN LOWLAND GORILLAS (*GORILLA GORILLA GORILLA)*

by

CHARLES AUSTIN LEEDS

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Department of Biology
CASE WESTERN RESERVE UNIVERSITY

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CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We herby approve the dissertation of Charles Austin Leeds,

candidate for the degree of Doctor of Philosophy*

Kristen E. Lukas, Ph.D.

Committee Chair

Mandi W. Schook, Ph.D.

Committee Member

Patricia M. Dennis, DVM, Ph.D.

Committee Member

Mark A. Willis, Ph.D.

Committee Member

Tara S. Stoinski, Ph.D.

Committee Member

Date of Defense: 22 Month 2019

*We also certify that written approval has been obtained for any proprietary material contained therein.

Dedicated to:

Washoe, Dar, Tatu and Loulis – you four changed my life and perspective for the better. I will forever be grateful for knowing you.

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A Physiological Evaluation of Social Bonding in Western Lowland Gorillas (*Gorilla gorilla gorilla*).

By

CHARLES AUSTIN LEEDS

Abstract

The strength of one's social bonds has direct links to fitness, health and welfare. Evaluations of social bond strength are relatively straightforward for gregarious species through direction observation of affiliative behavior, but can prove difficult for less gregarious species. The western lowland gorilla (WLG: *Gorilla gorilla gorilla*) is one such social species that engages in low rates of affiliative behavior. Assessing variation in the neuroendocrine hormone oxytocin (OXT), which is the physiological driver of social bonds, in relation to social demographic variables may prove useful to better understand how gorilla form bonds and how these bonds vary in the zoo environment. The overall objective of this dissertation was to evaluate variation in WLG salivary and urinary OXT. Biological samples were collected from 143 gorillas living at 25 zoos. Using both experimental and exploratory methodology, this dissertation successfully validated the measurement of OXT in unextracted WLG saliva and urine. Specifically, significant changes in both salivary and urinary OXT were detected following an intranasal challenge and spontaneous social events. This methodology was then used to assess variation in OXT following interactions with animal care staff (ACS) and in baseline samples. Following positive reinforcement training with ACS, salivary OXT

concentrations did not change, but OXT did increase following play with ACS. This indicates that relationships between WLG and ACS in zoos, at least in certain contexts, are viewed as positive and affiliative by WLG. In a large-scale evaluation of OXT, multiple demographic and husbandry factors were associated with variation in OXT. Notably, OXT was greater in males living in bachelor groups compared to mixed-sex groups, providing evidence that male WLG form comparatively stronger bonds with other males than with females. This is supported by field studies of WLG that demonstrate males form dispersed, affiliative social networks with related males. Greater OXT concentrations were also associated with lactation in female WLG. This finding was expected based on the natural functioning of OXT and further validated our measurement of OXT in unextracted urine. Lastly, ACS perceptions of bonding were assessed in relation to OXT. Interestingly, ACS ratings of social bonding did not differ between mixed-sex and bachelor groups, indicating ACS perceive both group types to provide meaningful social environments for WLG. ACS also identified peripheral group members within their respective WLG groups. Peripheral status was negatively associated with OXT concentrations, indicating more socially distant group members maintained comparatively weaker social bonds than more integrated group members. Overall, these findings have improved our understanding of how WLG form social bonds and how social bonds vary in relation to husbandry and management practices. Of specific importance, this study further demonstrates the significant social value of bachelor groups for male WLG.

CHAPTER ONE: Introduction

Social Relationships and Individual Fitness and Welfare

Similar to other drives for survival, social species have a strong inherent need to seek out positive social interaction with conspecifics (Taylor, 2006). Particularly in primates, maintaining strong and healthy social relationships are important for individual welfare and fitness. In humans, the long-term benefits of positive social relationships are well documented. In one of the first studies of this phenomenon, Berkman and Syme (1979) found, when controlling for age and initial health status, having a stronger social network, including marriage, close family ties, and non-familial group membership, resulted in individuals having lower mortality rates than those who were less socially connected. Maintaining stronger social networks is also positively correlated with survival following myocardial infarction (Berkman et al., 1992) and in those with coronary artery disease (Brummett et al., 2001) and acquired immune deficiency syndrome (Patterson et al., 1996). Furthermore, a study of 503 women with coronary artery disease found that individuals with greater social networks had reduced risk factors including lower blood glucose concentrations, and lower rates of diabetes and hypertension (Rutledge et al., 2004). Interestingly, Frasure-Smith et al. (2000) found that depressive symptoms, and not a lack of social support, was linked to mortality following a myocardial infarction. However, individuals who had more social support had decreased depressive symptoms over 1-year of study. A recent meta-analysis of 148 studies (308, 849 subjects) found that strong social support was associated with a 50% decreased mortality risk across subjects regardless of age, sex, initial health status, and cause of death (Holt-Lunstad et al., 2010). In addition to mitigating impacts of disease,

social support is also associated with more optimal health factors, particularly heart rate, blood pressure and cortisol concentrations (Thorsteinsson and James, 1999).

Long-term studies of non-human primates are similarly examining the effects of social support on fitness and wellbeing. In a study of chacma baboons (*Papio cynocephalus ursinus*), Silk et al. (2009) compared the relationship strength of adult females and the survival rate of their offspring. Females who maintained strong social bonds with conspecifics had greater infant survival compared to females who maintained weaker bonds. Female chacma baboons with stronger social bonds also lived longer than females who had weaker social bonds (Silk et al., 2010b) and females who are more solitary and less affiliative towards other females had significantly higher glucocorticoid levels than more social females (Seyfarth et al., 2012). Similarly, female savannah baboons (*Papio cynocephalus*) with stronger social bonds had greater infant survival (Silk et al., 2003). Interestingly, strong, but inconsistent, social bonds over time were associated with greater mortality risk in blue monkeys (*Cercopithecus mitis stuhlmanni*; Thompson and Cords, 2018). Providing evidence that not only bond strength but the context of one's relationships are significant. Beyond the primate order, strong social bonds have also been shown to improve fitness. In feral horses (*Equus ferus caballus*), strong relationships between unrelated females are correlated with increased birthing frequency, foal survival, and reduced aggression from males (Cameron et al., 2009).

In non-human primates, female social relations have been a primary focus of study, however, studies investigating male social bonds are beginning to demonstrate similarly beneficial outcomes. Male Assamese macaques (*Macaca assamensis*) living in multi-male groups benefit from strong male-male social bonds. Males who maintained

strong social bonds with other males, as measured through exchange of affiliative behavior and coalition formation, had improved social dominance, reproductive success (Schülke et al., 2010) and lower glucocorticoid concentrations (Ostner et al., 2008). Coalition support between males has also reported to increase dominance rank and reproductive output in chimpanzees (*Pan troglodytes*; Gilby et al., 2013). Furthermore, socially peripheral male yellow baboons (*Papio cynocephalus*) have higher baseline cortisol concentrations than more socially integrated males (Sapolsky et al., 1997). Beyond male-male interactions, dominant male olive baboons (*Papio anubis*) who engage in more copulations and more frequent social affiliation with non-estrus females and infants have lower baseline glucocorticoid concentrations compared to male conspecifics (Ray and Sapolsky, 1992).

Social Environment and Zoo Animal Welfare

The term animal welfare is difficult to define and has been the focus of extensive discussion (Botreau et al., 2007; Broom, 2001; Broom, 1988; Broom, 1991; Carenzi and Verga, 2009; Fraser, 2009; Fraser et al., 1997). The Association of Zoos and Aquariums (AZA) definition is the most all encompassing and states "Animal welfare refers to an animal's collective physical, mental and emotional states over a period of time, and is measured on a continuum from good to poor" (AZA Animal Welfare Committee, 2018). In zoos, many inputs contribute to an animal's overall welfare state including enclosure design and complexity, diet and nutrition, opportunities to engage in species typical behavior, such as foraging and locomotion, and one's social environment.

In evaluating the social environment of animals in zoos, group size has been a major focus of attention. Given resource factors determine group size in free-range

settings, animals in zoos can generally have more flexible social groupings given there are fewer resource constraints (Price and Stoinski, 2007). For example, naturally solitary species such as snow leopards (*Panthera uncia*) can be housed socially, with no overall changes in behavior compared to solitarily housed leopards (Macri and Patterson-Kane, 2011). Socially housing the naturally solitary tamandua (*Tamandua tetradactyla*) results in increases in species-typical behaviors such as foraging and increased behavioral diversity, which has been associated with positive welfare (Catapani et al., 2018). In contrast, maned wolves (*Chrysocyon brachyurus*) naturally live in male-female pairs. In zoos, females are often housed together to manage breeding. This strategy has been found to increase cortisol concentrations, a physiological indicator of arousal or stress, and decrease ovarian activity, indicating this social grouping may have negative impacts to the wolves' overall welfare state (Jones et al., 2018). The solitary housing of social species, in contrast, has been documented to generally be negative. For example, solitary male western lowland gorillas (*Gorilla gorilla gorilla*) have significantly greater corticoid concentrations than socially housed males (Stoinski et al., 2002). It should be noted that free-ranging males can choose to be solitary, but this is likely an adaptation to resource availability that likely results in similarly negative physiological consequences for free-ranging gorillas. In addition, the solitary housing of male gorillas is also associated with stress-induced behaviors such as self-mutilation (Pizzutto et al., 2007). Not only does isolation have immediate effects on one's welfare, but isolation early in life, followed by group housing through adulthood is associated with decreased life expectancy and immune suppression in laboratory rhesus macaques (*Macaca mulatta*, Lewis et al., 2000).

Inappropriately large groups can also affect the welfare of individuals. In primates for example, when group size is large in relation to living space, a depression in rates of affiliative behavior is often observed. For example, in chimpanzees increased social density reduces rates of grooming between adults in both laboratory (Aureli and De Waal, 1997) and zoo populations (Koyama and Aureli, 2018). In zoo gorillas, increased social density was found to increase time spent sitting alone and avoidance of other group members (Cordoni and Palagi, 2007). Affiliative behavior maintains group cohesion and is generally associated with positive welfare given play is associated with positive affective states (Held and Špinka, 2011), thus decreases in affiliative behavior should be associated with negative welfare. Inappropriately large or dense social groupings have been associated with indicators of negative welfare in non-primate species as well. In giraffe (*Giraffa camelopardalis*) and okapi (*Okapi johnstoni*), increased social density is positively correlated with rates of stereotypic licking, which may be a sign of frustration (Bashaw et al., 2001). Interestingly, housing of duiker (*Cephalophus spp.*) in groups of five or more, decreases life expectancy by 50% compared to housing the species in smaller groups (Barnes et al., 2002).

Not only does group size affect welfare, but the social demographics of groups can as well. Play behavior is considered a positive indicator of welfare in animals because it generally does not occur when resources are limited and is associated with positive affective states (Held and Špinka, 2011). Younger animals generally play more than adults, thus it has been thought that the presence of young can contribute to the positive welfare of a group. In the only empirical evaluation of this hypothesis, Cronin et al. (2016) compared the behavior of adult female chimpanzees in groups with and

without young. Overall, no difference in behavior was found, particularly, no difference in rates of play were observed indicating that the presence of offspring does not significantly impact rates of play among adult female chimpanzees. In social groups, agonism is inevitable, however, minimizing injurious agonism is a goal, given physical injuries can have negative physical and emotional consequences, ultimately affecting welfare. Patterns of injurious aggression in zoo species, and the associated demographic factors that contribute to increased injury, are well documented in chimpanzees (Ross et al., 2009), western lowland gorillas (Leeds et al., 2015), and hamadryas baboons (*Papio hamadryas*; Wiley et al., 2018).

One aspect of group living that has not been directly evaluated in relation to animal welfare is the quality of the social bonds within groups. In contrast to zoo populations where humans manage the composition of social groups, individuals in free-ranging populations have some choice in terms of their social partners. For example social preferences contribute to fission-fusion decisions in spider monkeys (*Ateles geoffroyi*, Busia et al., 2017). As a result, the strength of social bonds between individuals in zoos, and ultimately the quality of the social environment, may vary more than free-ranging populations. Assessments of affiliative relationships, and ultimately bonding, may provide important insights into factors contributing to the overall welfare of animals in zoos.

The Assessment of Affiliative Relationships

The study of social bonding consists of assessing the exchange of affiliative interactions between individuals over time (Seyfarth and Cheney, 2012). While

behavioral measures are useful for gregarious species, the reliance on studying affiliative behavior in less gregarious species is insufficient to determine the strength of social bonds. As the study of social bonds expands across species, physiological measures may prove useful for assessing bonds in species that engage in low rates of affiliative behavior.

To date, physiological indicators of social relationships and social environments have improved our understanding of primate social relationships, and how these relationships may affect their welfare in human care. Aureli et al. (1999) implanted two adult female rhesus macaques with heart rate monitors. Following approaches from more dominant individuals, their heart rates increased, possibly due to anticipated dominance-related aggression. No increase in heart rate was observed when kin or subordinate group members approached them. Lastly, heart rate decreased when grooming, an affiliative behavior. During periods of male social instability in chacma baboons, rates of agonistic behavior and infanticide increase. During these periods female glucocorticoid concentrations increase, but females who had and maintained consistent grooming partners experienced less of an increase in concentrations (Wittig et al., 2008). Similarly, it is well documented across species that less socially bonded and more peripheral group members have greater cortisol concentrations than more socially integrated individuals (Abbott et al., 2003; Sapolsky et al., 1997; Zhang et al., 2018). In chimpanzees, increased cortisol concentrations are associated with not only rates of receiving aggression, but cortisol is also higher in those who have unbalanced relationships where they engage in affiliative behavior at much greater rates than they receive (Yamanashi et al., 2018). In one of the few evaluations of positive social behavior and a physiological measure,

cortisol concentrations were negatively associated with rates of affiliative behavior in rhesus macaques (Wooddell et al., 2017). To improve our understanding of the positive influences of social relationships, further investigation of physiological measures of affiliation are needed. The mammalian neuroendocrine hormone oxytocin may prove useful in this endeavor.

Introduction to Oxytocin

Oxytocin (OXT) is a mammalian neuroendocrine hormone produced in the brain. Central OXT is primarily secreted in the brain from magnocellular neurosecretory neurons that make up the supraoptic (SON) and paraventricular (PVN) nucleus within the hypothalamus (Poulain and Wakerley, 1982). Additional production sites of OXT include the anterior hypothalamus, bed nucleus of the stria terminalis, medial reoptic area, and medial amygdala (Gimpl and Fahrenholz, 2001). It was originally hypothesized that once released into the periphery, OXT could not re-cross the blood-brain barrier, but recent exogenous administration studies demonstrate that OXT can re-enter the brain from the periphery (Lee et al., 2018; Striepens et al., 2013). In the brain and in the periphery, OXT can only bind to OXT receptor sites (OXTR). Brain regions containing OXTR's include the cortical areas, basal ganglia, limbic system thalamus, hypothalamus, brain stem and pituitary gland. Peripheral OXTR sites include both the male and female reproductive system, kidneys, heart, vascular endothelium, thymus, adipocytes, adrenal gland, osteoblasts, and prostate gland (Gimpl and Fahrenholz, 2001).

Circulating Oxytocin

Limited information is available on the circadian variation of circulating OXT concentrations. Amico et al. (1983) collected plasma samples from six adult males and

females every hour for 24 hrs. No diurnal variation in OXT was found. Six of the subjects also were utilized to collected cerebrospinal fluid samples at 0600, 1200, 1800 and 2400. A peak in OXT was found at 1200, but no corresponding peak was found in the plasma samples. Levels of OXT in the cerebrospinal fluid were significantly higher than plasma concentrations at each time point. Parker et al. (2010b) collected plasma samples every hour from 1800 to 0900 in adult men and women and found little variation, though a slight peak was observed around 2300. Similarly, salivary OXT levels were not found to differ prior to and following sleep, or 2, 4, 6, and 8 hrs into a nights sleep in 20 adult men and women (Blagrove et al., 2012). In contrast, Forsling et al. (1998) collected 10 plasma samples over 24 hr from 15 adult males (22 – 40 yrs) and 9 elderly adult males (60 – 75 yrs). OXT concentrations were lowest in the afternoon and evening, with a steep increase in concentrations after midnight, with a peak at 0200. Age had no influence on OXT concentrations or circadian variation. Further research is needed to improve our understanding of circadian variation in OXT.

Measuring Oxytocin

Measuring central OXT concentrations is highly invasive and often terminal (Neumann et al., 2000b; Nishioka et al., 1998), which makes the practicality of such methods, especially for non-laboratory subjects, difficult if not impossible. As a result, the measurement of peripheral concentrations of OXT in plasma, urine and saliva has become common; however, there are concerns over the measurement of peripheral OXT, particularly relating to assay methodology and biological significance.

Across all peripheral matrices, assay interference from other molecules has been a major concern when measuring OXT (McCullough et al., 2013). As a result, some studies

utilize an extraction procedure to minimize the influence of non-OXT molecules (e.g. (Holt-Lunstad et al., 2014; Huffmeijer et al., 2011; Wittig et al., 2014a) see McCullough et al. (2013) for discussion of prevalence). However, extraction procedures may also remove OXT from biological media. Brandtzaeg et al. (2016) developed a novel nano liquid chromatography-mass spectrometry method to assess OXT in human plasma. It was found that plasma OXT levels were "startlingly" high compared to previous assessments of plasma OXT. Likely as a result of OXT having a high affinity for bonding with proteins. The authors concluded that the extraction steps commonly utilized in traditional assays remove the majority of OXT in plasma (as a result of removing the proteins OXT is bound to), leaving behind only a small amount for measurement via assay. The removing of bound OXT could be a major confounding variable given that the amount of bound and unbound hormone varies considerably between individuals (Bryson, 1983) and may result in reducing OXT to undetectable levels. This latter point may explain the difficulties previous studies have had validating the measurement of OXT when utilizing extraction procedures (i.e. Horvat-Gordon et al., 2005; Szeto et al., 2011). As a result, Brandtzaeg et al. (2016) concluded that the measurement of total OXT, in contrast to the OXT remaining following extraction, may often be the better option for assessing OXT concentrations. Extracted and unextracted OXT do have strong correlations in findings, indicating both can be useful in the measurement of peripheral OXT (MacLean et al., 2018). As a result, a growing number of studies are assessing peripheral OXT concentrations without extraction procedures (i.e. Carter et al., 2007; MacLean et al., 2018; MacLean et al., 2017). With this in mind, whether extracted or unextracted samples are utilized, studies of peripheral OXT should be accompanied by a

disclaimer that assays are measuring immunoreactive OXT and metabolites, given that assays still are likely measuring precursor, degraded and bound OXT (Brandtzaeg et al., 2016; MacLean et al., 2018). Assay specificity is further complicated between the use of radio immunoassays (RIA) and enzyme immunoassays (EIA), which have reported varying levels of OXT, though this has been almost exclusively reported in plasma, likely as a result of variation in plasma proteins between subjects (Leng and Sabatier, 2016). Urine and saliva provide more consistent matrices to measure OXT in (Leng and Sabatier, 2016), though comparison between assay type is difficult since such samples have been almost exclusively measured via EIA.

The measurement of salivary OXT in particular has received additional scrutiny. One study reported salivary OXT to be in undetectable concentrations and provided inconsistent parallelism to the standard curve (Horvat-Gordon et al., 2005). The authors of this study hypothesized that such low levels were correlated with low plasma levels of OXT and that OXT, due to its large molecular size, may have trouble transferring from plasma to saliva. Recent research, however; has demonstrated the OXT levels, as a result of binding to proteins, are actually higher in plasma than previously thought (Brandtzaeg et al., 2016), indicating that salivary OXT concentrations should be in measurable quantities in saliva if they are reflective of plasma concentrations. Furthermore, multiple studies have successfully reported strong parallelism in their assay use (Carter et al., 2007; Daughters et al., 2015; MacLean et al., 2018). As a result, with further attempts recent studies have successfully validated the measure of OXT in human (Carter et al., 2007; Daughters et al., 2015) and non-human saliva (MacLean et al., 2018).

In relation to biological significance, several studies have improved our understanding of how peripheral OXT relates to central concentrations, as well as how peripheral levels relate to behavior. Initial studies found no relationship between peripheral OXT concentrations and cerebrospinal fluid OXT concentrations (Kagerbauer et al., 2013; Striepens et al., 2013); however; more recent research showed a positive correlation (Carson et al., 2015). Similar findings have been found when comparing OXT concentrations between different peripheral mediums. Salivary and plasma OXT concentrations have been positively correlated when concurrently collected in humans (Feldman et al., 2011; Grewen et al., 2010); but see (Javor et al., 2014). Though urinary OXT has not been positively correlated to blood and saliva concentrations (Feldman et al., 2011), it may be a result of urine concentrations representing an accumulation over time rather than a point in time sample as collected in plasma and urine. In a recent meta-review of 17 studies Valstad et al. (2017) found no correlation between central and peripheral OXT during baseline conditions, however; during induced stress or following intranasal OXT administration a positive correlation was observed. Further evaluation is needed to understand how production of OXT in the brain is related to concentrations throughout the body as this field of study is still in its early stages.

Oxytocin and Social Behavior

OXT is associated with a variety of functions including lactation (Nishimori et al., 1996), metabolism and energy use (Yang et al., 2013), sleep regulation (Blagrove et al., 2012) and stress regulation (Babygirija et al., 2012). Most notably and studied, OXT is associated with social behavior and bonding. The OXT system within mammals is high conserved; yet a wide variety of social groupings, sociosexual behaviors and affiliative

behaviors exist. These differences are likely influenced by species differences in circulating OXT concentrations and OXTR location and density (Goodson and Thompson, 2010; Insel, 2010). Two strong examples exist exemplifying this, one in rodents and in primates.

Insel and Shapiro (1992) found that OXTR site location and density varied between monogamous and polygamous vole species. Prairie voles (*Microtus ochrogaster*) are highly selective in mate choice, form long-term pair bonds, share parental responsibilities and have a high density of OXTR in the nucleus accumbens and prelimibic cortex, brain centers influential in the neuro reward circuitry. In contrast, montane voles (*Microtus montanus*) live in solitary burrows, are polygamous, and have a low density of OXTR in the brain centers that regulate the neuro reward circuitry. Similarly, Rosenblum et al. (2002) found differences in OXT levels between two closely related macaque species. Bonnet macaques (*Macaca radiata*), who are characterized as more gregarious and socially stable, were found to have greater OXT concentrations than pigtail macaques (*Macaca nemestrina*), a species described as volatile and unsociable. These studies, in addition to further study of OXT and OXTR, demonstrate that the variation in social behavior seen is likely heavily influenced by the oxytocinergic system.

Not only do differences in the oxytocinergic system exist between species, but there is also evidence of epigenetic influences on the system within species. Opacka-Juffry and Mohiyeddini (2012) found that higher rates of early life stress, such as serious arguments with parents, correlated with low OXT levels in adult men. Similarly, women who experienced early life stress in the form of child abuse had lower OXT levels as an adults compared to women who reported no such abuse (Heim et al., 2009). Animal

models have similarly demonstrated the effects of early life experience on OXT concentrations. In rats (*Rattus norvegicus domesticus*), pups who had more attentive mothers had higher concentrations of OXTR sites in the amygdala than pups with less attentive mothers (Francis et al., 2000). Additionally, Winslow et al. (2003) found that hand-reared rhesus macaques had significantly lower OXT concentrations than mother-reared macaques. Hand-reared macaques also less frequently exhibited affiliative behavior and had increased rates of agonistic and stereotypic behavior.

Exogenous Oxytocin

Most of our understanding of the relationship between OXT and social behavior comes from the study of exogenous administration of OXT and observation of behavior following administration. Exogenous OXT has been shown to increase affiliative behavior in a variety of species. While many investigations were conducted for clinical use, the results nonetheless demonstrate the unique influences OXT has on affiliative behavior.

Exogenous Oxytocin Administration Promotes Affiliative Behavior and Bonding in Nonhuman Animals

Studies of exogenous OXT administration have primarily focused on the prairie vole and various marmoset species (*Callithrix spp.).* These species have been studied due to their natural reproductive strategies of forming monogamous pair bonds. Given this unique social structure and the strong social bonds that support these pairings, evaluating the effects of OXT on social behavior in these two species were hypothesized to provide insights into how OXT facilitates affiliative behavior and bonding across mammals.

Cho et al. (1999) found that the administration of OXT increased social contact with a familiar partner in male and female prairie voles. Additionally, Cushing and Carter (2000) found that female voles who received multiple doses of OXT preferred to spend more time in proximity to an established partner than with an unknown male. Control females showed no preference and spent equal time with the known and unknown partner, indicating OXT promotes fidelity with a bond partner. The genetic manipulation of OXTR density in the brain, which increases the sensitivity to naturally occurring OXT levels, also increased alloparenting and partner preference in female voles (Keebaugh and Young, 2011). OXT administration has also been shown to increase affiliation between female voles (Beery and Zucker, 2010), and to facilitate the formation of pair bonds (Bales and Carter, 2003; Williams et al., 1994). While the administration of OXT has been shown to increase alloparenting behavior, a core feature of pair bonded monogamy, in female prairie voles (Bales et al., 2007), OXT had no effect on male vole alloparenting behavior (Bales et al., 2004). However, administration of OXT antagonist did reduce male parenting behavior and increased aggression towards pups, demonstrating OXT is a main physiological driver of these behaviors, though there are varying response strengths to OXT between the sexes. Interestingly, while short-term exposure to exogenous OXT has demonstrated an increase in affiliative behavior, the long-term administration of OXT in male prairie voles caused a decrease in physical contact with a familiar female partner indicating that long term elevated levels of OXT may impair social functioning (Bales et al., 2013).

In a recent study, OXT administration increased partner fidelity in pair bonded common marmosets (*Callithrix jacchus*) compared to control individuals (Cavanaugh et

al., 2014). Interestingly, the specific effect of OXT differed between the sexes. Females chose to spend more time interacting socially with their mate and had decreased rates of sexual solicitation towards the unknown male. Males chose to spend less time in close proximity with both their mate and the unknown female but did have a reduction in sexual solicitation towards the unknown females. The administration of OXT has also been shown to increase paternal food sharing (Saito and Nakamura, 2011) and mate guarding in common marmosets (Cavanaugh et al., 2018b), and social huddling in black-tufted marmosets (*Callithrix penicillata*, Smith et al., 2010). In contrast, the administration of OXT antagonists has decreased social proximity and social huddling, and eliminated food sharing between pair bonded black-tufted marmosets (Smith et al., 2010) and reduced overall trends in affiliative behavior in common marmosets (Cavanaugh et al., 2018a).

Collectively, these studies of pair bonded rodents and primates have provided significant understanding of how affiliative behavior and social bonding are facilitated by OXT. Given the uniqueness of monogamy among mammals, the study of exogenous OXT has expanded to other species to better evaluate how OXT facilitates social behavior and bonding in species with more variable social structures. Rodents have continued o be a popular model due to their ease of manipulation and accessibility. Calcagnoli et al. (2013) administered either OXT or an OXT antagonist to male rats, and then introduced them to an unknown conspecific. Subjects who were administered OXT had reduced aggression and increased social examination towards the unknown conspecific compared to those who received the antagonist. Interestingly, the reduction of aggression in those who received OXT was greater in rats who were more aggressive

during baseline conditions. In female mice, OXT administration reduced aggression towards an unknown conspecific in individuals with no genetic manipulation and in individuals breed for low anxiety. Interestingly, high anxiety bred females were unaffected by OXT administration (De Jong et al., 2014). These studies provide evidence that OXT may reduce social vigilance, ultimately facilitating affiliation and bonding over time by allowing for social interaction with novel conspecifics. Similarly, the administration of an OXT antagonist to male rats and mice decreased rates of social approaches towards a novel conspecific, while male rats and mice who had previously suffered a social defeat to a conspecific increased their social approaches to that individual following administration of OXT (Lukas et al., 2011). In a two-part experiment Mooney et al. (2014) examined the effects of OXT on naked mole rat (*Heterocephalus glaber*) social behavior. In part one of this experiment adult non-breeding individuals were injected with either OXT or a saline control and then returned to their colony. Individuals who received OXT had an increased frequency of huddling behavior in the 30 min following injection compared to those who received the placebo. In part two, subjects were injected with either OXT or an OXT antagonist and placed in a chamber with either another group member or week old pups from their colony. No effect was observed in behaviors directed towards the pups, but increased investigation of and time spent in close contact to the adult group mate was observed in those who received OXT compared to subjects who received the antagonist.

The administration of exogenous OXT has been well studied in rodents. While the effects of OXT on social behavior may be less nuanced in rodents compared to other species (Curley and Keverne, 2005), rodents still represent a useful model for the study

OXT and its influences in regulating social behavior. That being said research on the influences of OXT on the social behavior of non-rodents is rapidly increasing and has provided important insights into the relationship between OXT and individual social relationships.

OXT likely facilitates affiliative behavior through a variety of mechanisms; one possible mechanism is that OXT reduces social vigilance towards conspecifics who may be a threat, ultimately facilitating affiliation and bond formation. This theory has been well evaluated in rodents and is now being testing in rhesus macaques, a species that is strongly hierarchical and agonistic. The administration of OXT to rhesus macaques was found to decrease social vigilance in a variety of experimental tasks involving eye gaze and facial expressions (Ebitz et al., 2013). To further support this, functional magnetic resonance imaging (fMRI) studies of rhesus macaques following OXT administration demonstrate decreased activation of brain areas associated with vigilance (Liu et al., 2015; Parr et al., 2018). Studies of rhesus macaques have also focused on more general actions of OXT. In a study of infant macaques, the administration of OXT was found to increase rates of affiliative behavior directed towards human caregivers (Simpson et al., 2014). Chang et al. (2012) evaluated the behavior of rhesus macaques participating in cognitive testing that provided participants the opportunity to share or not share their food rewards. OXT administration increased rates of reward sharing between macaques compared to control conditions.

In the only exogenous OXT administration study of a non-human ape, no change in social behavior was found in a single female chimpanzee following OXT administration (Proctor et al., 2016). Given the potential invasiveness of exogenous OXT administration

and the removal of most chimpanzees from research laboratories, the study of exogenous OXT in apes will likely not continue further than this initial case study.

To better understand the scope of influence of OXT within mammals, studies beyond the primate order are also becoming common. Madden and Clutton-Brock (2011) administered OXT to free-ranging meerkats (*Suricata suricatta*). Subjects who received OXT increased their contributions to cooperative group activities, such as pup guarding, and decreased rates of aggressive behaviors compared to controls. Romero et al. (2014) found that OXT facilitates affiliative behavior in dogs (*Canis lupus familiaris*). Dogs who were administered OXT had higher rates of affiliative behavior and were more socially oriented towards their owners than dogs who received a placebo. In addition, dogs who received OXT had increased rates of approach behavior towards other dogs. In vampire bats (*Desmodus rotundus*), a communal roosting species, OXT increased allogrooming and food donations, a species-specific altruistic behavior, but did not increase the number of social partners they engaged with under baseline conditions (Carter and Wilkinson, 2015). In zebra finches (*Taeniopygia guttata*), a monogamous bird species, both males and females who received a mesotocin antagonist (the avian homologue to OXT) decreased pair bond stability, increased latency to form pair bonds, and decreased the percentage of allopreening (Klatt and Goodson, 2013). In another investigation of zebra finches, OXT antagonist administration was associated with a decrease in male courtship behavior (Pedersen and Tomaszycki, 2012). Overall, these studies continue to demonstrate the relationship between affiliation and OXT is strong across species and the behavioral responses to OXT are often observed most clearly in species-specific social behaviors.

Exogenous Oxytocin Administration Promotes Affiliative Behavior and Bonding in Humans

While the study of exogenous OXT administration has primarily been studied in animal models, human studies are becoming more frequent and are demonstrating similar effects on social behavior. Ditzen et al. (2009) administered OXT or a placebo to 47 couples. Couples who received OXT exhibited an increase in positive communication compared to couples that received the placebo in a discussion of a previous conflict. Naber et al. (2010) administered either OXT or a placebo to adult fathers prior to interaction sessions with their children. Fathers who received OXT were more playful and less hostile with their children than fathers who received a placebo. Studies like these have focused on general behavior changes in specific social interactions, but studies have also evaluated the effect of OXT and specific behavioral components that ultimately facilitate affiliative behavior and bonding.

OXT administration has been shown to heighten an individual's social cognitive abilities. Enhanced social cognition provides an increased ability for individuals to connect with conspecifics, facilitating positive social affiliation. Guastella et al. (2008) examined the effects of intranasal OXT administration on eye gaze in 52 adult males. Eye gaze can be predictive of an individual's ability to interpret social interactions and the behavior of conspecifics, and is also important for regulating social interactions (Garrett et al., 2004; Klin et al., 2002; Spezio et al., 2007). Individuals who were administered OXT had an increased frequency of stares and total time spent gazing towards neutral images of human faces compared to those who received a placebo. Additionally, children diagnosed with autism, a condition that impairs an individuals ability to understand and

engage socially with conspecifics, who were administered OXT increased their gaze towards the face of interaction partners (Andari et al., 2010), and improved their ability to interpret emotional states through images of eyes (Guastella et al., 2010).

Social perception, such as viewing an unknown conspecific as friendly or aggressive, is an important factor in facilitating affiliative behavior. While reactions to basic fight or flight stimuli are easily examined in nonhuman animals, more nuanced behaviors such as subjective descriptions of personality are not possible to examine. The study of humans however is now allowing for this to be explored. Theodoridou et al. (2009) asked men and women to rate images of random men and women on trustworthiness and attractiveness following either an intranasal dose of OXT or placebo. Individuals who received OXT rated the images as more attractive and trustworthy, demonstrating that OXT may enhance social affiliation towards unfamiliar conspecifics. Additionally, Buchheim et al. (2009) reported that men classified as insecure who received OXT described pictures depicting possibly insecure scenarios more positively than individuals who received a placebo. Administration of OXT has also been shown to increase trust involving money and personal information with unfamiliar partners compared to individuals who received a placebo (Baumgartner et al., 2008; Kosfeld et al., 2005; Mikolajczak et al., 2010a; Mikolajczak et al., 2010b).

Human and non-human primates conform their behavior to that of their social group (Cialdini and Goldstein, 2004; Whiten et al., 2005), likely as an adaptive strategy for survival (Henrich and Boyd, 1998). OXT can bias an individual's behavior towards group conformity, even when these groups are random aggregations of individuals with no previous history. Stallen et al. (2012) administered either OXT or a placebo to adults

and then arbitrarily placed subjects into groups. Individuals were then asked to rate the attractiveness of stimuli in the absence of their group, but were told that their group rated the stimuli in a certain direction and that another group rated the stimuli differently. Subjects who received OXT rated the stimuli more closely to their group, while subjects who received the placebo showed no pattern of conformity to any group. Additionally, De Dreu et al. (2010) found that adult males who were administered OXT in a prisoners dilemma task showed more in group favoritism to randomly assigned group mates the subjects of competing groups.

Empathy is the ability to understand and share the emotional state of another and is an important facilitator of social affiliation and bonding. Though early in its evaluation, OXT appears to increase empathetic response in humans. Shamay-Tsoory et al. (2013) found that OXT can increase empathy towards out-group members. The emotional reactions of Jewish Israeli's towards other Israeli's (in-group), Europeans (neutral outgroup) and Palestinians (adversary out-group) were evaluated under the influence of OXT and a placebo. Individuals who were administered OXT had increased empathy towards Palestinians, compared to the in-group empathy bias of individuals who received the placebo. Bartz et al. (2010) evaluated the empathetic response of 27 men to videos of strangers sharing emotional stories following administration of both OXT. Empathetic responses were greater in the OXT condition but only in men who were categorized as less socially proficient. In addition, Krueger et al. (2013) found that OXT administration increased the perception of harm to victims of crime in unaffected third party males who were read transcripts of fictional criminal events. The authors concluded that OXT

increased empathetic response, which likely facilitates a perception of bonding with the imaginary victims.

Oxytocin Release Following Spontaneous Affiliative Behavior

The relationship between social behavior and OXT release is beginning to occur in natural contexts, expanding on the studies of exogenous OXT administration. These natural studies are similar to exogenous evaluations in that they are providing additional context for understanding the role of OXT in influencing social behavior by measuring natural changes in OXT following specific events. Initial research focused on mother-offspring interactions. This relationship is hypothesized to be the evolutionary starting point for OXT and social bonding. OXT increases in mothers prior to and following nursing across multiple mammal species (rats, Juszczak and Stempniak, 1997; Neumann et al., 2000a; dogs, Uvnas‐Moberg et al., 1985; pigs (*Sus scrofa domesticus*, Uvnas-Moberg et al., 1985; humans, Carter et al., 2007; White‐Traut et al., 2009). In addition, rates of nursing are associated with greater OXT concentrations in rhesus macaques (Maestripieri et al., 2009) and even the act of nipple stimulation alone can increase OXT in humans (Christensson et al., 1989). More broadly, baseline OXT concentrations are positively associated with rates of positive maternal behavior across mammalian species (human, Feldman et al., 2010; common marmosets, Finkenwirth et al., 2016; rhesus macaques, Maestripieri et al., 2009; grey seals, *Halichoerus grypus*, Robinson et al., 2015). Even more subtle parent-offspring interactions can cause the release of OXT in humans such as positive communication between mothers and daughters (Seltzer et al., 2010) and shared eye gaze between mother and infant (Kim et al., 2013).

Physical contact between individuals is another well-studied trigger of OXT release. Body stroking (petting) increases the OXT concentrations of rats (Stock and Uvnäs-Moberg, 1988) and dogs following interactions with both owners (Handlin et al., 2011) and familiar individuals (Mitsui et al., 2011). In humans, OXT can increase following massage by a stranger in adult females with no relationship anxiety but does not change in those who have relationship anxiety (Turner et al., 1999). In couples, following a period of "warm contact" OXT increase is positively associated with relationship quality (Grewen et al., 2005). In chimpanzees, following grooming with a bond partner, as assessed through dyadic rates of other affiliative behavior, OXT increased, but not following grooming with a non-bond partner (Crockford et al., 2013b). In tamarins, rates of grooming were positively associated with OXT concentrations (Snowdon et al., 2010) and in chacma baboon consort pairs, OXT was positively associated with time spent in close proximity (Moscovice and Ziegler, 2012). OXT also increases following sexual contact and stimulation across species (rat, Sansone et al., 2002; sheep, *Ovis aries*, Kendrick et al., 1986; human, Carmichael et al., 1987; Ogawa et al., 1980).

OXT also increases following more broad affiliative interactions. In humans, OXT increases in adult females following gossip but not following emotional, non-gossip conversations (Brondino et al., 2017). Engaging in experimental situations that involve trust but no physical contact also elicit OXT increases (Kéri and Kiss, 2011; Zak et al., 2005), as do interactions as simple as maintaining eye-contact with dogs (Nagasawa et al., 2009). In chimpanzees, OXT increases following food-sharing, regardless of bond-strength between conspecifics (Samuni et al., 2018; Wittig et al., 2014), as well as

following reconciliation and bystander affiliation following aggression (Preis et al., 2018). OXT also increases in chimpanzees prior to territory boundary patrols, an intensive, species-specific cooperative behavior (Samuni et al., 2017).

Oxytocin and Social Bonding

Social bonding is facilitated by the exchange of affiliative behavior. OXTR share many brain site locations associated with reinforcement and reward (Baskerville and Douglas, 2010). As a result, OXT release following affiliative interactions with conspecifics likely stimulates this neural network, resulting in the reinforcement of affiliative social interactions (Love, 2014). Over time, the repeated positive interaction between individuals, and the subsequent increases in OXT, results in the formation of social bonds. Examining overall relationships between individuals and the corresponding OXT concentrations have provided additional information into how OXT drives social bonding.

In cotton top tamarins (*Saguinus oedipus*) urinary OXT varies considerably between bonded pairs. Greater OXT concentrations are correlated with rates of affiliation and sexual behavior, though it differs between the sexes. OXT increased with rates of grooming and physical contact in females and rates of sexual behavior in males (Snowdon et al., 2010). This study also indicates that the specific affiliative behaviors that facilitate bonding may be sex specific. Additionally, Moscovice and Ziegler (2012) found a positive correlation between OXT levels and time spent in close proximity to mating partners in female chacma baboons. In humans, the OXT concentrations of married couples are positively correlated with their relationship quality (Holt-Lunstad et al., 2014). Furthermore, social isolation of social species results in significant decreases

in OXT (guinea pigs, *Cavia aperea f. porcellus,* Machatschke et al., 2004); cotton top tamarins, Snowdon et al., 2010).

Evolutionarily, bond formation initially arose in the context of mother-infant attachment and was evolutionarily co-opted to facilitate social bonding in other contexts (Wittig et al., 2014). As a result, there is significant data demonstrating major changes in OXT during pregnancy, parturition, and postpartum. Maternal OXT increases are observed in pregnant women from the first through third trimester (Dawood et al., 1978; Levine et al., 2007) (but see Giraldi et al., 1990). Interestingly, ratings of maternal-fetal attachment of pregnant females are positively associated with their OXT concentrations, indicating these increases in OXT over the course of pregnancy may be associated with initial stages of bond formation between mother and offspring (Levine et al., 2007). In the initial stages of labor, the fetus begins to excrete OXT (Dawood et al., 1978; Giraldi et al., 1990) contributing to contractions and also likely further strengthening mother-infant attachment. Postpartum affiliation between mother and offspring continues to facilitate mother-infant attachment via increases in OXT associated with rates of maternal care (Feldman et al., 2010; Finkenwirth et al., 2016; Maestripieri et al., 2009; Robinson et al., 2015). Interestingly, in the only study of human infant OXT, there was a positive association between baseline OXT levels and infant rates of soliciting attention from their mothers (Clark et al., 2013), indicating that the changes observed in mothers are likely occurring in infants as well. Nursing is a primary OXT eliciting interaction between mother and offspring. OXT not only facilitates milk production (Nishimori et al., 1996) but the act of nursing itself also causes spikes in maternal OXT (Carter et al., 2007; White-Traut et al., 2009). Thus the physiology of lactation and the act of nursing itself

both stimulate OXT release, further strengthening the bond between mother and offspring.

Oxytocin, Welfare, and Western Lowland Gorilla (*Gorilla gorilla gorilla*) Sociality

Given the relationship between welfare and social environment, the measurement of OXT to assess social bonds has significant applied value. OXT may be particularly useful for species that engage in little social behavior. Western lowland gorillas (WLG) are one species that may benefit from the study of OXT and social bonding. WLG are a critically endangered subspecies within the Genus Gorilla native to West-Central Africa, specifically Angola, Cameroon, Central African Republic, Republic of Congo, Gabon, and Equatorial Guinea (Maisels et al., 2018). Free-ranging populations often devote less than 1% of their time to affiliative behavior (Masi et al., 2009; Stokes, 2004), and zoo populations, that benefit from less resource limitations than free-ranging populations, still often spend less than 5% of their time engaged in affiliative behavior (Hoff et al., 1997; Ross et al., 2010). This is in stark contrast to more gregarious primate species such as chimpanzees that often spend almost 20% of their day engaged in affiliative behavior (Pomerantz and Terkel, 2009; Ross et al., 2010). This low rate of affiliative behavior makes the assessment of WLG social bonds and the quality of their social environment difficult. Studies up to this point have focused on specific social behaviors that contribute to WLG welfare, such as injurious (e.g. Leeds et al., 2015) and social behavior (e.g. Stoinski et al., 2013), but no studies have tried to evaluate bonding within WLG groups and how that may relate to their welfare status. Assessing OXT variation within WLG will be significant to identifying social factors that contribute to the formation of strong social bonds, and ultimately to the positive welfare of WLG in zoos. More broadly the

study of WLG OXT will also improve our understanding of their social relationships, which will add to our understanding of their biology and evolution.

In North America, WLG are well represented with over 350 individuals living in 49 AZA zoos (Lukas et al., 2017). WLG in zoos are primarily housed in mixed-sex breeding groups, comprised on average of one adult silverback, three adult females, and offspring. This social grouping mimics what is commonly observed in the wild (Parnell, 2002; Stokes, 2004), encourages species-typical behavior, and provides a portrait of typical gorilla life for zoo guests (Lukas et al., 2014; Stoinski et al., 2001). Due to an even gorilla birth sex ratio, the majority of male gorillas born in North American zoos are unable to be housed in mixed-sex groups. The long-term management of male gorillas not immediately needed or destined for breeding groups represents a unique challenge for North American zoos (Stoinski et al., 2001; Stoinski et al., 2004b). A solution to this is the formation of all-male (bachelor) groups.

Historically, bachelor groups were more commonly observed in mountain gorillas (*Gorilla beringei beringei*) (Robbins, 1995; Yamagiwa, 1987), but bachelor groups have been observed in western lowland gorilla populations (Gatti et al., 2004; Hagemann et al., 2018; Robbins et al., 2004). In zoos, bachelor groups provide male gorillas the benefits of socialization through a species appropriate social grouping. In addition, placing males in bachelor groups provides a more complex housing option than the alternative option of solitary housing. Solitary housing additionally has negative welfare implications as males who are housed solitarily have significantly higher levels of urinary corticoid concentrations compared to males in bachelor and mixed-sex groups (Stoinski et al., 2002) and have been observed to engage in behaviors indicative of a negative welfare

state such as self-mutilation (Pizzutto et al., 2007). Currently, 24 North American zoos house 79 males in 28 bachelor groups, and as the overall WLG population continues to grow, the number of zoos housing bachelor groups will need to increase (Lukas et al., 2017).

Despite the empirical evidence to support the cohesiveness of bachelor groups in zoos (Leeds et al., 2015; Stoinski et al., 2002; Stoinski et al., 2001; Stoinski et al., 2004a; Stoinski et al., 2013; Stoinski et al., 2004b), some zoo managers still question the social value of housing males in bachelor groups. The evaluation of OXT in relation to group-type and demographic variation within group-types will provide meaningful information on the social bonds formed by male gorillas in zoos, specifically for those in bachelor groups. This will significant to improving our understanding of how bachelor groups function in zoos and how management in this group-type relates to individual WLG welfare. In addition, this data will provide important information to supplement our understanding of free-ranging male WLG social dynamics that indicates males form meaningful relationships with other males (Bradley et al., 2004; Magliocca and Gautier-Hion, 2004), albeit in a dispersed and species-specific manner.

The study of female WLG OXT will also be significant to understanding how social factors contribute to their welfare in zoos. Free-ranging females regularly engage in secondary and tertiary group transfer (Bradley et al., 2007; Stokes et al., 2003) facilitated by perceptions of male (Breuer et al., 2012) and group quality (Stokes et al., 2003). Interestingly, despite regular group transfer, there is evidence that female WLG live with female kin more often than by chance (Bradley et al., 2007). In zoos, females do not exercise the same reproductive and social choices they would in free-range settings,

thus it is unknown how this affects the quality of their social environment. Evaluating female WLG OXT concentrations in relation to demographic factors will be informative to understanding what shapes female gorilla social bonds in zoos, and how this contributes to their overall welfare.

Study Objectives

The overall objective of this dissertation was to evaluate variation of OXT in WLG living in zoos. This was done to improve our understanding of social bonding in the species and how the social bonds of WLG may vary in relation to management in zoos. Using a primarily exploratory study design, OXT was evaluated across a large subset of the AZA WLG population. In addition, two case studies evaluating WLG OXT were conducted. This dissertation will provide insight into the biology of WLG social bonding and their care and welfare in zoos.

The first experimental study, presented in chapter two, discusses the techniques used to validate the measurement of OXT in both saliva and urine. The validation was primarily focused on a single male WLG that participated in a voluntary intranasal challenge. Saliva and urine samples were collected prior to and following this challenge. In addition, we evaluated diurnal variation in OXT across several subjects, and changes in OXT following specific events: play, breeding and the death of a conspecific. Collectively this study provided evidence that OXT can be reliably measured in the saliva and urine of WLG.

Following the validation, and in support of pushes to measure changes in OXT following normal social interactions, changes in both OXT and cortisol (CORT) concentrations were assessed following positive reinforcement training with a caregiver.

Training is an important part of WLG husbandry and care, and is a consistent, daily interaction between gorilla and caregiver. The animal-caregiver relationship is a major component of the daily lives of animals in zoos, so evaluating physiological changes following these interactions will provide insight into how animals in zoos perceive the relationships with their caregivers. In chapter three, we discuss our findings from this case study involving two male WLG.

To begin to better understand social bonding and how it relates to gorilla biology and husbandry, a large-scale study was conducted. In chapter four findings from the largest hormonal study of WLG to date are discussed. A total of 143 gorillas from 25 AZA zoos (50% of AZA zoos who care for gorillas) participated in this study. OXT was evaluated in these participants in relation to a variety of factors include, age, sex, group-type, reproductive state and group demographics.

In the final chapter of this dissertation, we evaluate animal care staff (ACS) perceptions of social bonding and OXT. We were interested in testing if ACS perceptions of bonding correlated with a physiological measure. ACS continually demonstrate a detailed and nuanced understanding of their charges' behavior. In this chapter, we expand this evaluation to a hormonal measure of social bonding in WLG. Specifically, we surveyed ACS about the quality of the social environment within each group and asked if ACS could identify less bonded, and thus more peripheral, group members.

CHAPTER TWO: Validating the use of a commercial enzyme immunoassay to measure oxytocin in unextracted urine and saliva of the western lowland gorilla (*Gorilla gorilla gorilla*).

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Introduction

Oxytocin (OXT) is a neuroendocrine hormone primarily synthesized in the paraventricular nucleus and supraoptic nuclei of the hypothalamus (Gainer 2012). OXT is released into the brain and body by the posterior pituitary where it binds to OXT receptor sites, which are widely distributed throughout the body as well as brain areas that regulate social behavior, emotional processing and stress regulation (Freeman and Young, 2016; Gimpl and Fahrenholz, 2001). The release of OXT accompanies prosocial and sociosexual behaviors, in addition to social and physical stress (for review see (Crockford et al., 2014; Olff et al., 2013). OXT has been primarily studied for its role in regulating mother-infant attachment, nursing, and childbirth (Feldman et al., 2010; Gordon et al., 2010; Levine et al., 2007). However, OXT is also important in the regulation of social behavior including visual attention and engagement (Graustella and MacLeod, 2012), social memory (Rimmele et al., 2009), trust (Kosfeld et al., 2005), generosity (Zak et al., 2007) and empathy (Shamay-Tsoory et al., 2013). In addition, OXT has important roles in maintaining one's health, including immune system function

and metabolism and energy use (Yang et al., 2013), as well as sleep regulation (Blagrove et al., 2012).

Measuring central OXT concentrations is highly invasive and often terminal (Neumann et al., 2000a; Nishioka et al., 1998), which makes the practicality of such methods, especially for non-laboratory subjects, difficult if not impossible. As a result, the measurement of peripheral concentrations of OXT in plasma, urine and saliva has become common; however, there has been a concern over the measurement of peripheral OXT, particularly relating to assay methodology and biological significance.

Across all peripheral matrices, assay interference from other molecules has been a major concern (McCullough et al., 2013). As a result, some studies utilize an extraction procedure to minimize the influence of non-OXT molecules (e.g. Holt-Lunstad et al., 2014; see McCullough et al. 2013 for discussion of prevalence). However, extraction procedures may also remove OXT from biological media. Brandtzaeg et al. (2016) developed a novel nano liquid chromatography-mass spectrometry method to assess OXT in human plasma. It was found that plasma OXT levels were "startlingly" high compared to previous assessments of plasma OXT as a result of OXT having a high affinity for bonding with proteins. The authors concluded that the extraction steps commonly utilized in traditional assays remove the majority of OXT in plasma (as a result of removing the proteins OXT is bound to), leaving behind only a small amount for measurement via assay. The removing of bound OXT could be a major confounding variable given that the amount of bound and unbound hormone varies considerably between individuals (Bryson, 1983) and because it may result in OXT being in undetectable levels which may explain the difficulties previous studies have had validating the measurement of OXT

(Horvat-Gordon et al., 2005; Szeto et al., 2011). As a result, Brandtzaeg et al. (2016) concluded that the measurement of total OXT, in contrast to OXT remaining following extraction, might often be the better option for assessing OXT concentration. Recent comparisons of extracted and unextracted OXT have found strong correlations between the two processes, indicating both can be useful in the measurement of peripheral OXT (MacLean et al., 2018). As a result, a growing number of studies are assessing peripheral OXT concentrations without extraction procedures (i.e. MacLean et al., 2018; MacLean et al., 2017). With this in mind, whether extracted or unextracted samples are utilized, studies of peripheral OXT should be accompanied by a disclaimer that assays are measuring immunoreactive OXT and metabolites, given that assays still are likely measuring precursor, degraded and bound OXT (MacLean et al 2018; Brandtzaeg et al. 2016). Assay specificity is further complicated between the use of radio immunoassays (RIA) and enzyme immunoassays (EIA), which have reported varying levels of OXT, though this has been almost exclusively reported in plasma, likely as a result of variation in plasma proteins between subjects (Leng and Sabatier, 2016). Urine and saliva provide more consistent matrices to measure OXT in (Leng & Sabatier 2016), though comparison between assay type is difficult since such samples have been almost exclusively measured via EIA.

The measurement of salivary OXT in particular has received additional scrutiny. One study reported salivary OXT to be in undetectable concentrations and provided inconsistent parallelism to the standard curve (Horvat-Gordon et al. 2005). The authors of this study hypothesized that such low levels were correlated with low plasma levels of OXT and that OXT, due to its large molecular size, may have trouble transferring from

plasma to saliva. Recent research, however; has demonstrated the OXT levels, as a result of binding to proteins, are actually higher in plasma than previously thought (Brandtzaeg et al. 2016), indicating that salivary OXT concentrations should be in measurable quantities in saliva. Furthermore, multiple studies have successfully reported strong parallelism in their assay use (i.e. Carter et al., 2007; Daughters et al., 2015). As a result, with further attempts recent studies have successfully validated the measure of OXT in human (Carter et al. 2007; Daughters et al. 2015) and non-human saliva (MacLean et al. 2018).

In relation to biological significance, several studies have improved our understanding of how peripheral OXT relates to central concentrations, as well as how peripheral levels relate to behavior. Initial studies found no relationship between peripheral OXT concentrations and cerebrospinal fluid OXT concentrations (Kagerbauer et al., 2013); however; more recent research showed a positive correlation (Carson et al., 2015). Similar findings have been found when comparing OXT concentrations between different peripheral mediums. Salivary and plasma OXT concentrations have been positively correlated when concurrently collected in humans (Grewen et al., 2010) though additional research contradicts these findings (Javor et al., 2014). Though urinary OXT has not been positively correlated to blood and saliva concentrations (Feldman et al., 2011), it may be a result of urine concentrations representing an accumulation over time rather than a point in time sample as collected in plasma and urine. In a recent meta-review of 17 studies Valstad et al. (2017) found no correlation between central and peripheral OXT during baseline conditions, however; during induced stress or following intranasal OXT administration a positive correlation was observed. Further evaluation is

needed to understand how production of OXT in the brain is related to concentrations throughout the body as this field of study is still in its early stages.

Despite the variation observed between different sample media and sampling techniques, peripheral OXT has been shown to positively correlate with behavior, indicating that there might be a relationship between peripheral concentrations and brain activity. In humans, salivary and plasma OXT concentrations are positively correlated with relationship quality in married couples (Holt-Lunstad et al., 2014). In addition, salivary OXT increased following cooperative hunting episodes among Tsimane' men (Jaeggi et al., 2015) and following social interactions involving gossip (Brondino et al., 2017), while urinary OXT increased following verbal reassurance from mother to daughter during a stress test (Seltzer et al., 2010).

In non-human primates, peripheral OXT has been correlated with a variety of social phenomenon. Chacma baboons (*Papio hamadryas ursinus*) live in multi-male multi-female promiscuous breeding groups. When cycling, a female forms a consortship with a male that generally lasts 3-4 days. During this period mating bouts occur frequently and the pair maintains close social proximity to one another. Moscovice and Ziegler (2012)found that among females that engaged in consortships, urinary OXT positively correlated with proximity to consort partner. This provides evidence that OXT is associated with close social bonds and the formation of short-term exclusive social relationships. Snowdon et al. (2010) found a similar positive relationship between urinary OXT and affiliative behavior in cotton-top tamarin (*Saguinus oedipus*) pairs. In chimpanzees (*Pan troglodytes*), urinary OXT increased following grooming bouts with a bonded partner compared to controls. Additionally, no difference was observed between

control samples and grooming with a non-bonded partner (Crockford et al., 2013). Similar to Moscovice and Ziegler and Snowdon et al. this provides evidence that OXT release may be dependent on the entirety of the social context and not just the social behavior itself. Interestingly, food-sharing events amongst chimpanzees resulted in increased urinary OXT for both receivers and donors compared to social feeding and control periods, but bond status had no effect on OXT concentrations (Wittig et al., 2014). This study, which was of the same chimpanzee population as Crockford et al. provides evidence that OXT is involved in facilitating social behavior and bonding across different social relationship contexts. Most recently, Samuni et al. (2017) reported that chimpanzee urinary OXT concentrations were elevated in anticipation of and during border patrols and intergroup encounters compared to control periods. OXT concentrations were correlated with cohesion during the events and not by potential threats posed during the events. This is the first evidence from a non-experimental study of non-human and human primates that OXT is involved in group cohesion and cooperation in competitive contexts.

The study of peripheral OXT has made important contributions to our understanding of primate social behavior. The genus *Gorilla* represents an important taxonomic group of study for two primary reasons. First, due to their genetic relatedness to chimpanzees and humans (Scally et al., 2012), gorillas represent an important group within great apes, the study of which will provide further context to the evolution of the OXT system within the primate order. Second, while chimpanzee sociality is described as cooperative, gorilla social relationships are described as tolerant (Harcourt and Stewart, 2007). OXT is an important facilitator of social behavior and the study of OXT in a

species that is markedly less affiliative and cooperative than previously studied species represents an important contribution to what mediates social behavior in primates. In order to facilitate such research, the measurement of peripheral OXT needs to be properly validated for this species.

The purpose of this study was to biologically validate the measure of OXT concentrations in the urine and saliva of western lowland gorillas (*Gorilla gorilla gorilla*) via EIA. The validation procedure consisted of several distinct steps serving together to validate the use of this assay to measure biologically relevant concentrations of OXT in western lowland gorillas. The first portion of this study was technical and conducted to assess assay validity through parallelism, sample recovery, and a comparison of extracted and unextracted samples. The second portion of the validation procedure was an intranasal OXT challenge for the measurement of OXT in saliva and urine. Intranasal challenges have successfully been used to validate the measurement of human salivary OXT (Daughters et al., 2015; Huffmeijer et al., 2011; Van Ijzendoorn et al., 2012; Weisman et al., 2012), urinary OXT (Francis et al., 2016), and OXT in other mediums (Dal Monte et al., 2014; Gossen et al., 2012). In addition, the above studies of humans and one involving a chimpanzee (Proctor et al., 2016) reported no adverse side effects of such methods. Given the history of intranasal OXT challenges as a means by which to validate OXT and its ability to permeate both central and peripheral fluids, it was hypothesized that this would be a successful method by which to validate OXT in urine and saliva of western lowland gorillas and that elevated levels would be observed in both peripheral fluids following the challenge. Third, the diurnal variation of urinary and salivary OXT was evaluated to determine the need to control for diurnal variation in

future studies. Finally, validation procedures were conducted in relation to distinct opportunistic events, 1) saliva samples were collected following breeding bouts between two adult gorillas and compared to match control samples, 2) saliva samples were collected from a single subject prior to and following two back-to-back spontaneous play bouts, and 3) urine samples were collected from a single subject prior to and following the death of his group mate.

Methods

Subjects

Two adult male western lowland gorillas, Mokolo and Bebac, living as a bachelor group at Cleveland Metroparks Zoo, Cleveland, OH, USA, participated in this study. Both subjects were born in the same natal group and transferred to Cleveland as part of a 4.0 bachelor dyad in 1994. The dyad's two other groupmates died in 1997 and 2005, respectively. Mokolo was 28 years of age at the beginning of this study, and Bebac was 31 years of age. Bebac was euthanized on January 6, 2017, due to declining health. Post mortem evaluation by Cleveland's veterinary team revealed an area of discospondylitis via cervical CT scan. Samples from Bebac were collected at least one month before initial symptoms of his illness were observed. At the time of Bebac's death, this group was the longest tenured bachelor gorilla group in North America. In a multi-institution study of gorilla wounding rates, this dyad had the third lowest rate of wounding out of 21 bachelor groups (Leeds et al., 2015) and was considered affiliative and cohesive by animal care staff.

Urine and Saliva Collection

Urine samples were collected following voiding from a clean (free from food, feces, and urine) surface with a syringe and transferred to a 15ml conical centrifuge tube (Falcon, Corning, NY). Saliva samples were collected by providing the subject with a salivette, a swab designed for the collection of saliva (Salimetrics, State College, PA), to chew, which was then provided back to the researcher and placed in a saliva collection vial (Salimetrics, State College, PA) (details of this trained behavior can be found in Kuhar et al. 2005). All samples were immediately frozen at -20 °C, and then transferred on ice to a -80 °C freezer. To minimize degradation, all samples were assayed within two months of collection and samples were aliquoted to avoid multiple freeze thaw cycles. Prior to preparation for assay, saliva samples were thawed and centrifuged at 2500 rpm for 15 min to separate saliva from salivette.

Initial Assay Validity

The study of peripheral OXT has utilized both extracted and unextracted samples. Extraction procedures may minimize assay interference but may also remove OXT from samples. We sought to compare how this assay quantified immunoreactive OXT in both extracted and unextracted samples. Parallelism between optical densities of serial dilutions (n = 6, 1:1 – 1:32) and the standard curve were tested for both extracted and unextracted urine and saliva. Parallelism was determined by both visual inspection of the curves and linear regression. Recovery of samples spiked with known amounts of OXT standard where also conducted for extracted and unextracted urine and saliva **(**high = 1600 pg/ml; low = 40 pg/ml). Finally, we sought to compare how OXT concentrations varied in urine and saliva samples collected from Mokolo that were both extracted and unextracted. The extraction procedure used was provided by the commercial kit described

below and followed their described procedure. Unextracted samples were similarly dried and reconstituted in assay buffer. Samples assayed were collected as part of an in-house hormone monitoring project. All available urine (n = 26) and saliva (n = 17) samples collected within two months of assay were used. Samples were simultaneously pulled from a single aliquot that had not been thawed since their initial collection. Sample concentrations were then compared using Wilcoxon Signed Ranks Test to determine if concentrations differed between paired samples. To further compare how samples were related, a two-tailed Pearson's correlation was utilized to assess the relationship between extracted and unextracted samples.

Intranasal Challenge Validation

Mokolo received an intranasal spray of OXT (Cat# O6379 Sigma-Aldrich, St. Louis, MO, dissolved in saline solution), consisting of a 40IU dose divided into a single 20IU dose in each nostril. Human studies frequently utilize 20-40IU doses of intranasal OXT for study, thus 40IU was selected for this study based on the previous success of this dosage in humans and because the larger dose may help account for size difference of humans and gorillas. The subject was trained to present his nose for the voluntary administration of the spray via positive reinforcement. Baseline urine and saliva samples were collected once daily for six days prior and seven days following the intranasal spray but seven of the samples (one urine, 72 hr prior; six saliva, 48, 96, 120, 144 hr prior/120, 144 hr post) were lost in processing. All of these baseline and post-intranasal challenge day saliva samples were collected between 0720 and 0800, and urine samples were collected between 0720 and 0927. The intranasal challenge occurred at 0730. On the day of the intranasal challenge, urine samples were collected 5 min prior to as well as 25 and

87 min following the challenge. Saliva was collected 15, 30, 60, 90 and 120 min following the challenge. Mokolo remained with Bebac during the entirety of this procedure. Following the nasal spray, no social interactions were observed between the two that would have impacted the samples collected the day of the challenge. Due to the difficulty of this procedure, this phase was limited to a single subject, which has similarly occurred in other ape challenge validations (Heintz et al., 2011).

For the intranasal challenge, baseline urinary and salivary OXT concentrations were separately calculated using an iterative process (Brown et al., 1999). All collected samples were averaged to generate an overall mean. If a sample exceeded the mean of all samples plus 1.5 standard deviations (SD), the sample was temporarily removed from the data set for baseline calculation purposes. The mean was then recalculated and this process continued until no sample concentration exceeded the mean plus 1.5 SD. The remaining samples were used to calculate a baseline. Once the baseline was calculated, all samples were returned to the data set for the study. Any sample concentration exceeding the baseline plus 1.5 SD was considered elevated (Heintz et al. 2011).

Diurnal Variation Evaluation

To assess diurnal variation in urine, urine samples were collected from Mokolo between 0700 and 1730 over a three-month period (n = 45). Urine collection was not a trained behavior, but rather relied on opportunistic collection when a sample could be reached immediately after voiding by AL, resulting in an uneven distribution of sample times collected. A two-tailed Spearman's correlation was used to assess the relationship between OXT concentration and time of day. In addition, on 10 days a urine sample was collected in both the morning (0718-0940) and again in the afternoon (1145-1710). These

matched samples were additionally compared using the Wilcoxon signed rank test to evaluate diurnal variation. This method of analysis has previously been used to assess diurnal variation of androgens and corticoids in matched western lowland gorilla urine samples (Stoinski et al., 2002).

To assess diurnal variation in saliva, saliva samples were collected from both Mokolo and Bebac at 0800, 1000, 1200, 1400, 1600, and 1700. Unlike urine collection, the collection of saliva was a trained behavior which allowed for systematic collection at set time points. For each subject, three saliva samples were collected per time point (18 total samples per gorilla). Samples were collected for no more than two time periods per day due to the gorilla's willingness to participate in sample collection. Samples were collected Monday-Friday for one month. To assess diurnal rhythm, the same iterative process described for the intranasal challenge was used. In addition, Friedman's ANOVA was additionally used to compare the changes in mean OXT concentrations between time points for each gorilla individually.

Post Event Evaluation

Breeding

Five breeding events occurred between Mokolo and an adult female gorilla Kebi Moya (one of two female gorillas brought to Cleveland Metroparks Zoo to provide appropriate socialization for Mokolo following Bebac's death). Saliva samples were collected approximately 15 min following each breeding bout to test for detectable short-term changes in salivary OXT. A match-control saliva sample was then collected within 3 days ($\mu = 1.6$ days) of each breeding event at the same approximate time of day (within 5 min) following a period where no social interaction occurred for 30 min to serve as a

control for OXT concentrations. Similar match-control methods have been used in the study of OXT in chimpanzees (Crockford et al., 2013). All breeding events and subsequent saliva sample collection occurred between 0700 and 0750. Post-breeding samples were compared to each respective match-control sample using the Wilcoxon signed rank test.

Social Play

Saliva samples were opportunistically collected following a spontaneous play interaction between an adult male gorilla, Bebac, and a keeper, and then the same adult male gorilla with his group mate, Mokolo, to test for detectable short-term changes in salivary OXT. A baseline sample from Bebac was collected at 0847 prior to the events. At 0855 a play session between Bebac and his primary keeper began. The play session was centered on the keeper spraying warm water through a hose into his off-exhibit area. Bebac played in the water and directed water back at the keeper (play is rare for this subject but when it does occur this represents a regular play style of Bebac). This play session occurred for approximately 10 min. Saliva samples were then collected approximately 15 and 30 min following the play session. At 0940 (35 min post play with keeper) Bebac initiated a play session with Mokolo. The pair engaged in low arousal wrestling on and off until 0953 when Mokolo ended the session by walking away from Bebac. Saliva samples were again collected from Bebac 15 and 30 min following the end of this play bout. Similar, single social events have been used as part of a larger validation for OXT in other primate species (Snowdon et al. 2010). Here, OXT concentrations were compared descriptively due to the small number of samples collected.

Conspecific Passing

Following the death of Bebac, urine samples were opportunistically collected from Mokolo in the morning between 0700 and 0800, to test for detectable long-term changes in urinary OXT. The death of Bebac occurred in the Sarah Allison Steffee Center for Zoological Medicine at Cleveland Metroparks Zoo, but his body was brought back to the group's off exhibit space where Mokolo was allowed to view and physically inspect Bebac for approximately 30 min. Urine samples were collected the day after Bebac's death as well as 6, 7, 8, 11, 13, 17, 20, 25, 27, 33, 34 and 39 days after. These 13 samples were then compared to 10 samples collected for the intranasal challenge (samples that were within baseline OXT range) when the dyad was together. The Mann Whitney U test was used to compare samples collected between these two independent conditions: samples collected following the death of Bebac and baseline samples collected for the intranasal challenge when the group was together and stable.

OXT Enzyme Immunoassay

Mean inter-assay (n = 12 assays) coefficients of variation (CV) for controls and standards were below 10%. Mean intra-assay (n = 2 per sample) CV for study samples, spiked samples, controls and standards were below 10%. Controls were a low (40pg/ml) and high concentration (1600 pg/ml) of OXT standard mixed in assay buffer. Urinary and salivary OXT concentrations were determined using a commercial EIA kit (Arbor Assays, Ann Arbor, MI). Manufacturer instructions were followed except samples were dried using an Evaporack (Cole Parmer, Vernon Hills, IL) and not a Speedvac, inside a warm water bath (32^0 C). Dried samples were then reconstituted in assay buffer to the original (neat) volume. In analysis all samples were adjusted for dilution factor. To

control for urine concentration, all urine samples were indexed by creatinine concentration. Creatinine concentration was determined following the procedure of Brown (1998)**.** Samples were read using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT) at 450-nm wavelength using Gen5 software (BioTek Instruments, Inc.).

All previously described statistical analysis was conducted using SPSS Version 24 (Chicago, IL). Inferential statistics can be useful for studies with small sample sizes, specifically validation procedures (e.g. Brown, 1999). However, there are strong arguments for the use of statistical tests when possible. The diurnal rhythm evaluation, breeding and conspecific passing study phases allowed for the use of statistical tests and thus were used. To improve the strength of these tests, the test statistics were determined using the Monte Carlo method (10,000 permutations) per the recommendation of Plowman (2008). This method calculates the mean significance value and confidence intervals around a distribution that is representative of the sample (Field, 2009), focusing the analysis on the individual and not a population.

Results

Initial Assay Validity

For urine samples, serial dilutions of both extracted ($y = 0.98x – 74.38$, $R^2 = 0.99$, $F(1,3) = 2427.60$, $P < 0.001$) and unextracted ($y = 3.29x – 114.65$, $R2 = 0.99$, $F (1,3) = 721.62$, $P < 0.001$) samples generated a displacement curve that were parallel to the standard curve and were highly correlated (Figure 1). Recovery of samples spiked with known amounts of low and high standard were high for both extracted (range: 94.54% - 116.98%) and unextracted samples (range: 92.38% -119.11%). For saliva samples, serial dilutions of both extracted ($y = 1.01x + 0.01$, $R^2 = 0.99$, $F(1,3) = 905.44$, $P < 0.001$) and

unextracted (y = 1.09x -0.01, R^2, = 0.99, F (1,4) = 1324.50, P < 0.001) samples generated a displacement curve that were parallel to the standard curve and highly correlated (Figure 2.1). Recovery of samples spiked with known amounts of low and high standard were high for both extracted (range: 89.1%-102.9%) and unextracted samples (range: 91.3% - 99.5%).

There was a significant difference in urinary OXT concentration between extracted and unextracted samples (z = -3.390, P = 0.001). On average, extracted urine OXT concentrations were 74.7% (SE = 10.2%) of the unextracted concentration. Pearson's correlation found a significant positive correlation between samples (r = 0.91, P < 0.001; Figure 2.2). There was no significant difference between extracted and unextracted salivary OXT concentrations (z = -0.781, P = 0.435). The mean difference between unextracted and extracted salivary OXT was 2.44 pg/ml. Given that (1) extracted and unextracted urine and saliva sample had equally successful parallelism and sample recovery (2) extracted and unextracted OXT concentrations were highly correlated in urine and not significantly different in saliva, the remaining data presented here were assayed as unextracted.

Intranasal Challenge

For urine, mean baseline OXT concentration was 1501 pg/mg CR (range 803-2118). Urinary OXT concentrations had approximately a nine fold increase 24 hrs post challenge and remained elevated 48 hrs post challenge, returning to baseline 72 hrs post challenge (Figure 2.3). No change was observed approximately 30 and 90 min following the challenge. An elevated OXT concentration was also observed two days prior to the

challenge. That morning, the subject's group mate was observed to have a quarter-sized bite wound on the back of his left leg, which was not observed the previous day.

For saliva, mean baseline OXT concentration was 105 pg/ml. Salivary OXT concentrations were observed to have a 100-fold increase compared to baseline 15 min post challenge. Concentrations then gradually decrease but remained elevated above baseline through 24 hrs post challenge (Figure 2.4). Concentrations returned to baseline 48 hrs post challenge.

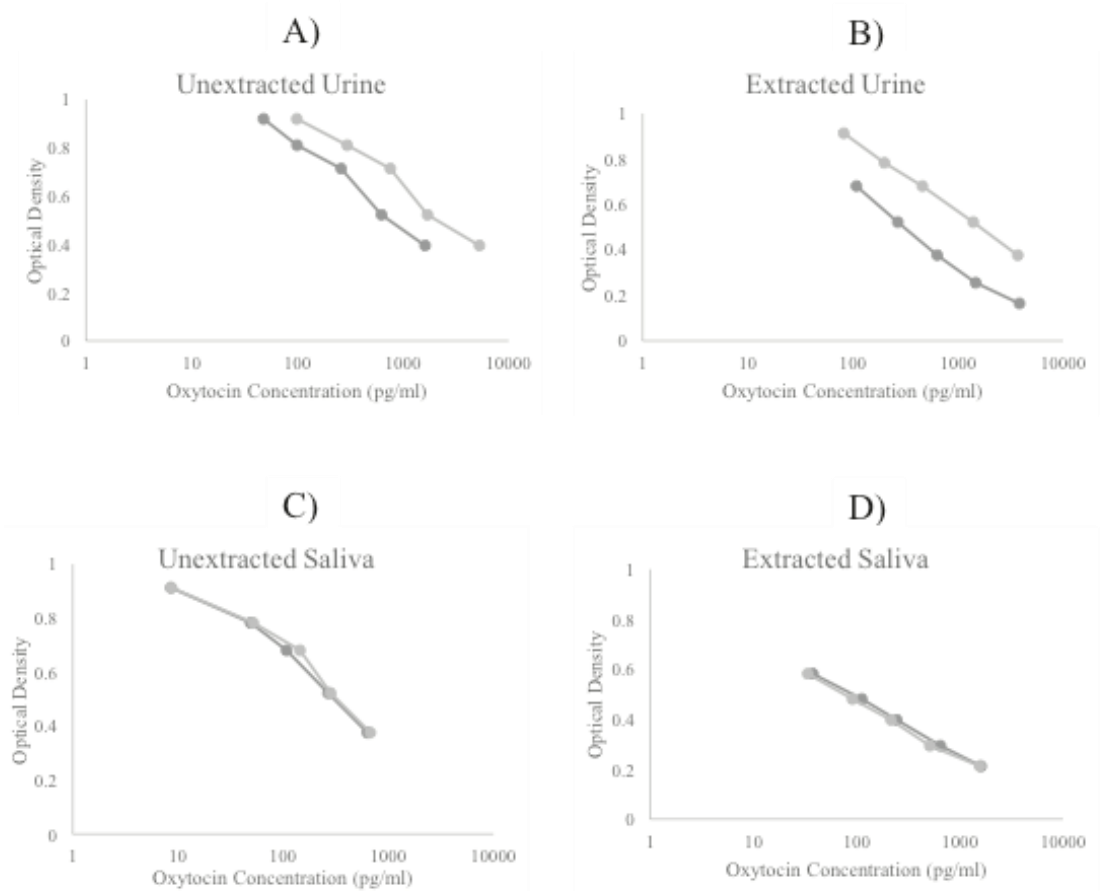

Figure 2.1: Parallelism of oxytocin standards and pooled samples from (A) unextracted urine, (B) extracted urine), (C) unextracted saliva and (D) extracted saliva. Dark grey lines represent oxytocin standard and light grey lines represent pooled samples.

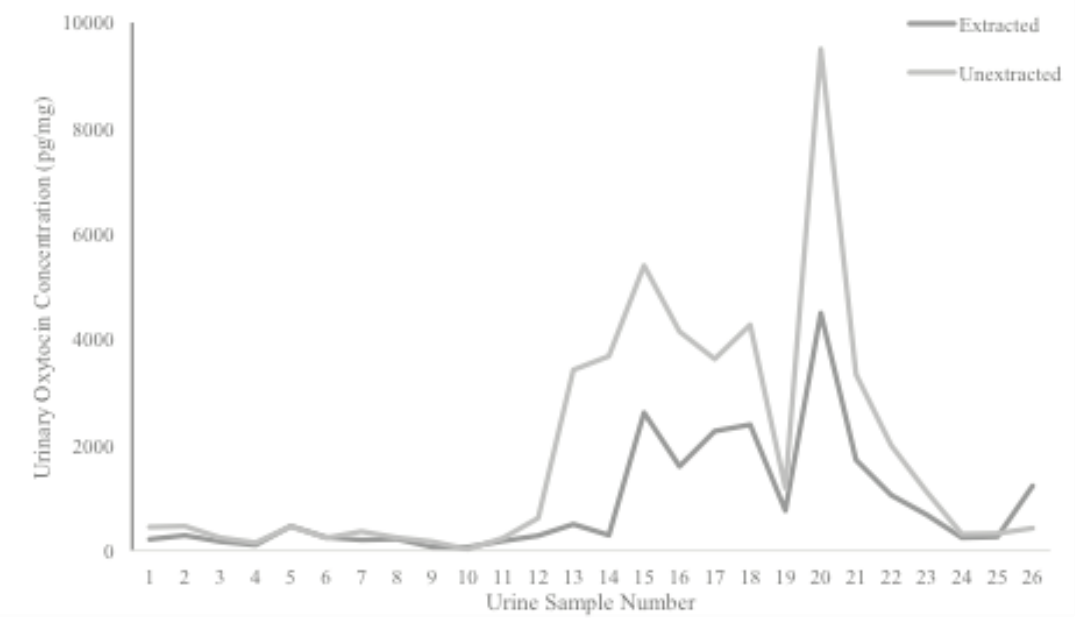

Figure 2.2: Urinary oxytocin concentrations for samples that were simultaneously assayed using both an extraction process and without. Pearson's correlation found that extracted and unextraced samples were highly correlated ($r = 0.91$, $P < 0.001$).

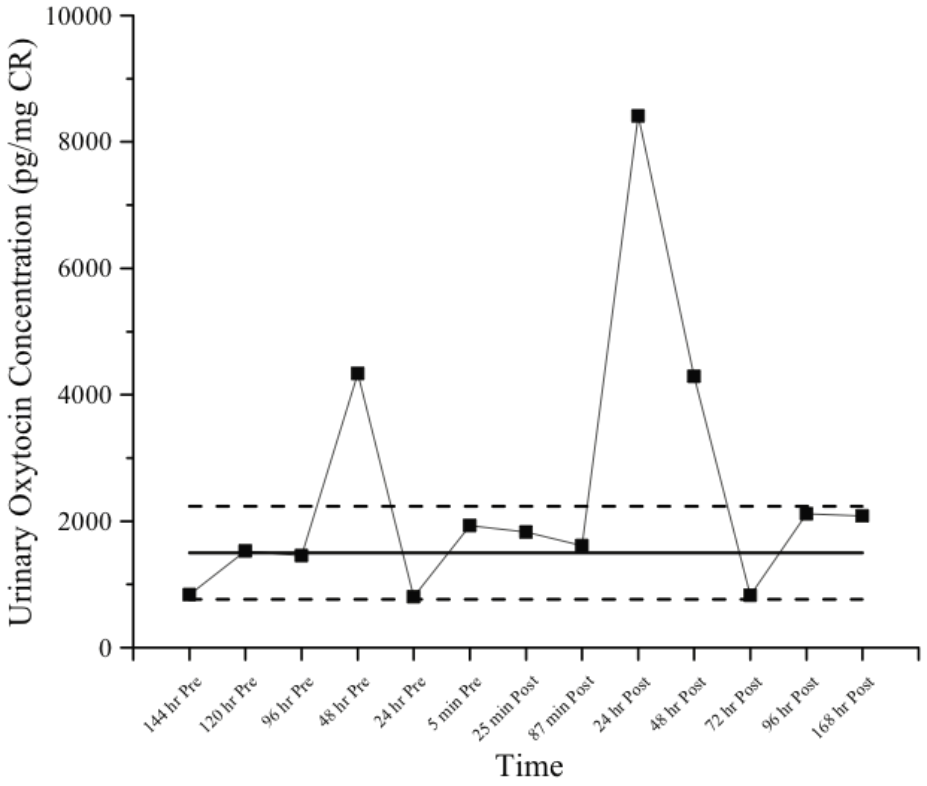

Figure 2.3: Urinary oxytocin concentrations prior to and following the intranasal challenge. Solid line represents baseline urinary oxytocin concentrations and the dashed lines represent elevated/depressed concentrations (baseline ± 1.5 SD).

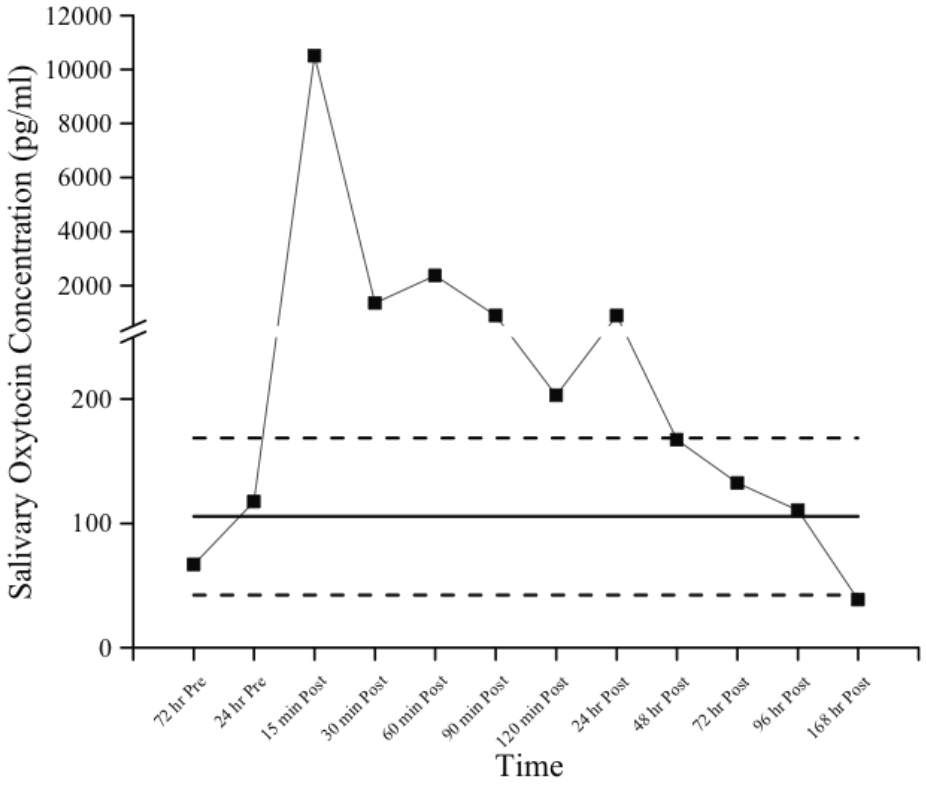

Figure 2.4: Salivary oxytocin concentrations prior to and following the intranasal challenge. Solid line represents baseline salivary oxytocin concentrations and the dashed lines represent elevated/depressed concentrations (baseline ± 1.5 SD).

Diurnal Variation

There was a statistically significant negative correlation between urinary OXT and time of day, with lower OXT concentrations observed later in the day compared to earlier (r = -0.462, P = 0.001). Same day matched morning and afternoon urine samples (n= 10 days) similarly had significantly lower OXT concentrations in the afternoon compared to morning (z = -2.293, P = 0.022) (Figure 2.5).

There was no significant difference in salivary OXT concentrations between time points (Bebac, χ^2 (5) = 6.619, P = 0.280; Mokolo, χ^2 (5) = 4.143, P = 0.589). However, using an iterative process to determine baseline and elevated sample concentrations,

samples collected at 1400 for both subjects were elevated 1.5 SD above baseline, and remained elevated for Bebac at 1600 (Figure 2.6).

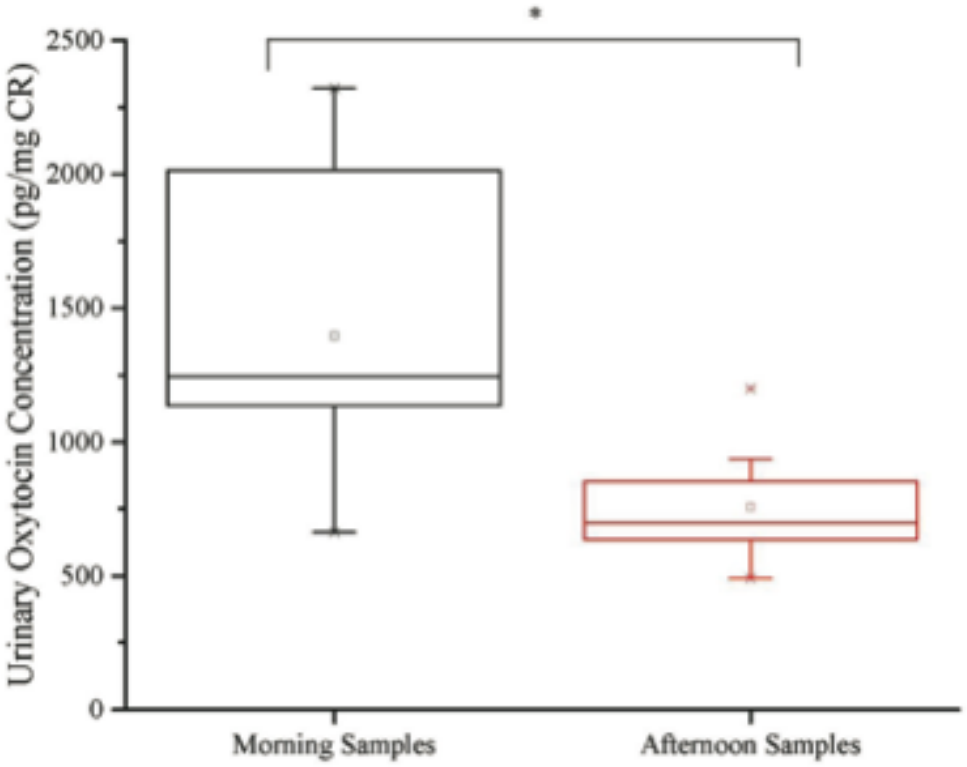

Figure 2.5: Urinary oxytocin concentrations for ten matched morning (opportunistically collected between 0718-0940) and afternoon samples (opportunistically collected between 1145-1710). Morning samples had significantly higher oxytocin concentrations than afternoon samples ($z = -2.293$, $P = 0.022$).

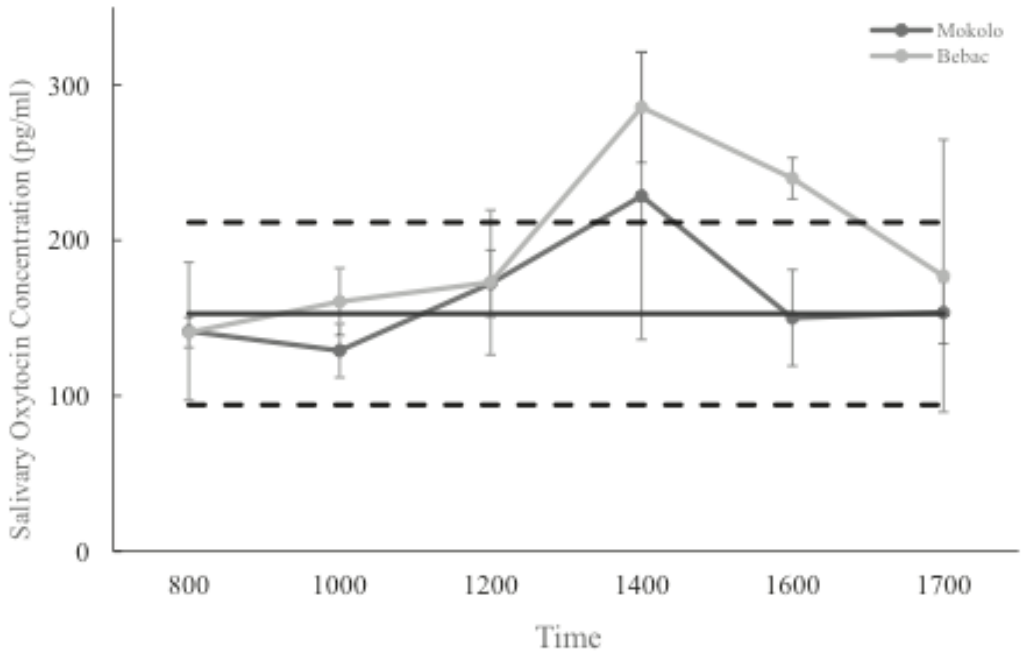

Figure 2.6: Diurnal variation in mean salivary oxytocin concentration (± SE) per gorilla (n =3 saliva samples per time point/gorilla). Solid line represents baseline salivary oxytocin concentrations and the dashed lines represent elevated/depressed concentrations (baseline ± 1.5 SD).

Post Event – Breeding

Following breeding events, salivary OXT concentrations were greater than (μ = 128.297 pg/ml, SE = 23.5) match-control samples (μ = 60.95 pg/ml, SE = 5.6; z = -2.023, P = 0.043) (Figure 2.7).

Post Event - Play

Prior to the two play bouts, Bebac's salivary OXT concentration was 28 pg ml. Following the first play bout with the animal keeper, his mean OXT concentration increased approximately two fold to 78 (SE = 7.3) pg/ml. His salivary OXT concentrations remained elevated compared to baseline following the second play bout with a conspecific, with a mean concentration of 62 (SE = 3.4) pg/ml (Figure 2.8).

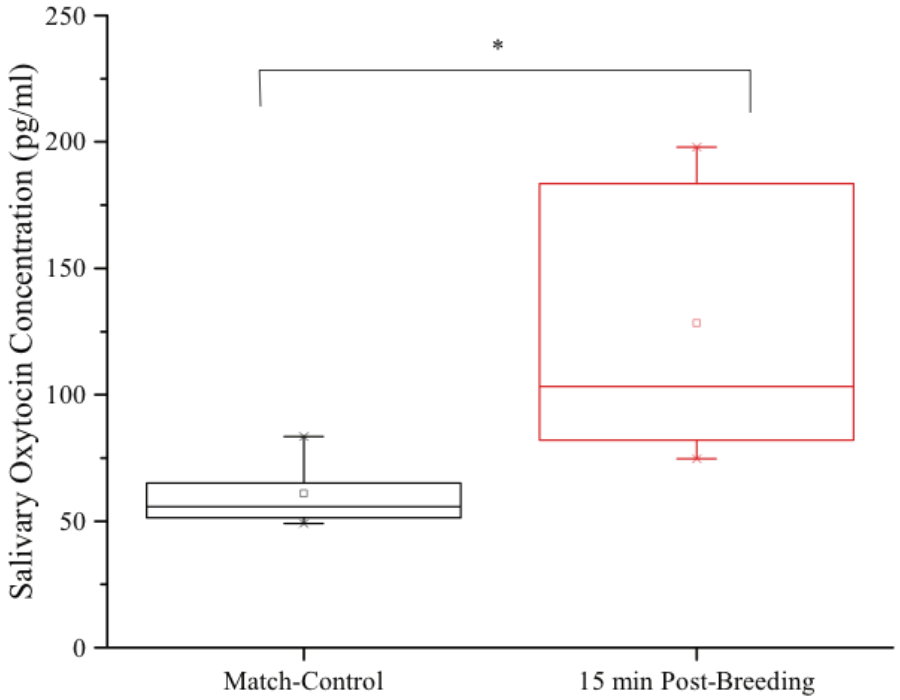

Figure 2.7: Salivary oxytocin concentrations of Mokolo were compared 15 min post-breeding with an adult female gorilla to a match-control sample for each respective post-breeding sample (n = 5). Match-control samples were collected within 3 days at the same approximate time (within 5 min) of the post-breeding sample where no social interaction between Mokolo and the female gorilla was observed for 30 min prior to sample collection. Post-breeding samples had significantly higher oxytocin concentrations than match-control samples (z = -2.023, P = 0.043).

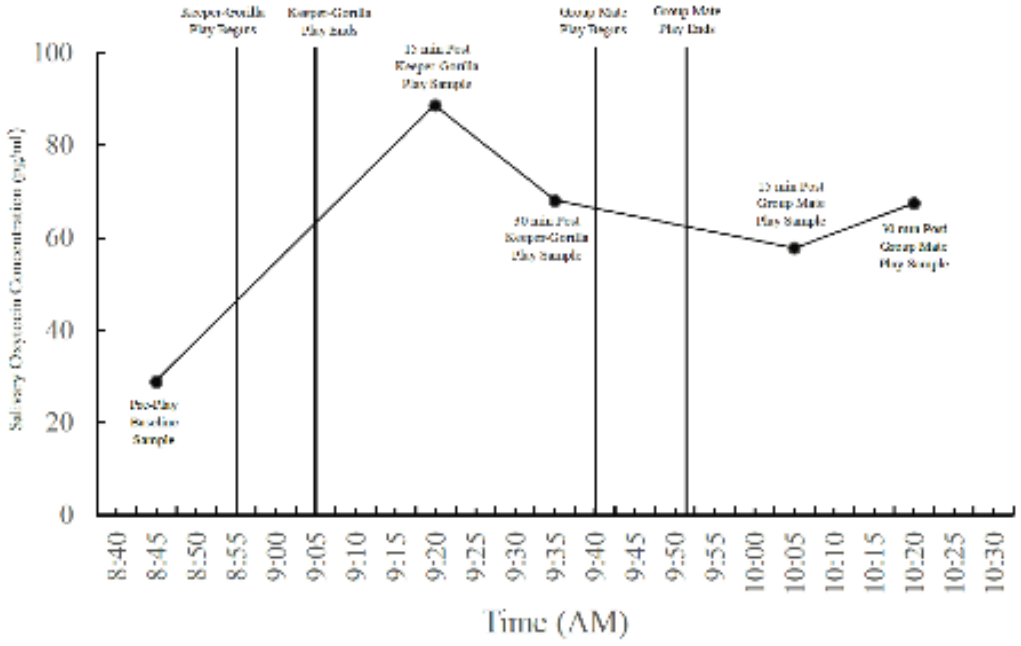

Figure 2.8: Salivary oxytocin concentrations following two consecutive play bouts. The first bout occurred between the gorilla Bebac and his primary animal keeper. The second bout occurred between Bebac and his group mate Mokolo.

Post Event - Conspecific Passing

Mean urinary OXT concentration for Mokolo following the passing of Bebac was 892 pg/ml CR. Mean OXT concentrations from samples collected when the dyad was together was 1501 pg/ml CR (mean of samples that fell into "baseline" from intranasal challenge). This difference between conditions was statistically significant (U = 18.00, z = - 2.769, P = 0.004) (Figure 2.9).

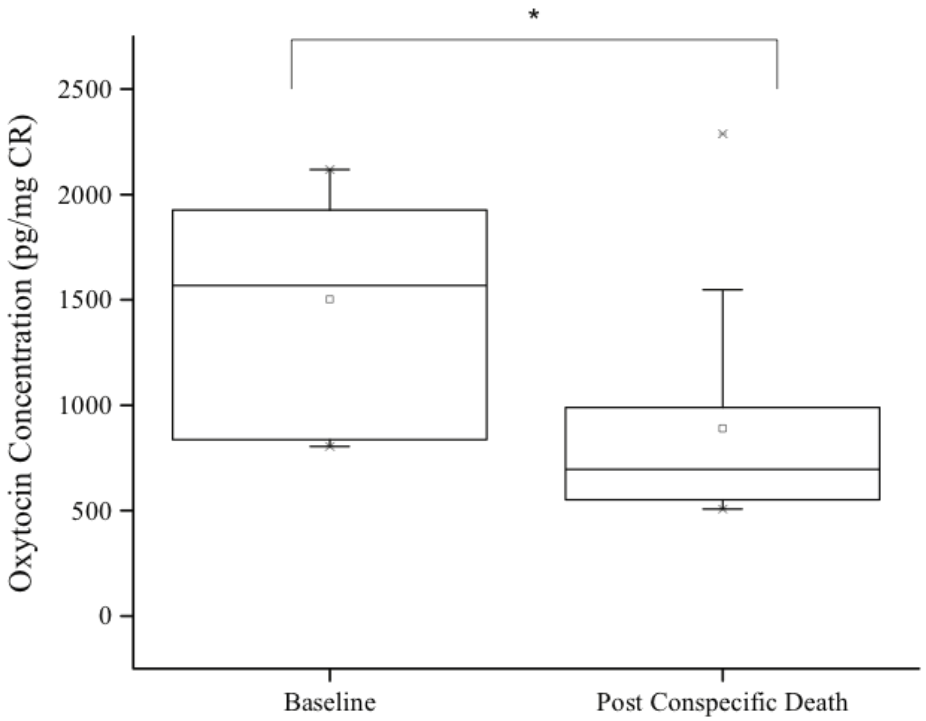

Figure 2.9: Urinary oxytocin concentrations of Mokolo. Baseline samples were collected during a period when he was housed with his group mate Bebac (n = 10). Post conspecific death samples were collected over 39 days following the death of Bebac (n = 13). Mokolo's oxytocin concentrations were significantly lower following Bebac's death compared to baseline (z = -2.769, P = 0.004).

Discussion

The purpose of this study was to biologically validate the measurement of the neuroendocrine hormone OXT in the urine and saliva of western lowland gorillas by EIA. Our validation demonstrated that a commercial EIA was successful in detecting OXT concentrations in both extracted and unextracted and that both sample types demonstrated strong spiked sample recovery and parallelism. There was a significant difference in the urinary OXT concentration between extracted and unextracted samples, though samples were highly correlated. OXT breaks down relatively quickly and regularly binds to other

compounds (Brandtzaeg et al. 2016), the extraction procedure thus may remove degraded and bound OXT from the media, lowering detectable concentrations. Interestingly, no difference was observed between extracted and unextracted saliva samples. This may be due to lower levels of protein within saliva (Leng and Sabatier, 2016), resulting in less bound OXT lost through extraction. Therefore, unextracted samples were presented for the remainder of this study. It is unclear which procedure type, extracted or unextracted, provides the most absolute measurement of OXT concentration, since both may over or under estimate OXT concentration. However, this data supports other recently published data that either form can detect meaningful changes in peripheral OXT concentrations (Cater et al. 2007; MacLean et al. 2018).

An intranasal challenge, similar to those that have been successfully conducted in human subjects (Daughters et al. 2015; Van Ijzendoorn et al. 2012; Weisman et al. 2012) resulted in elevated salivary OXT concentrations as early as 15 min post-challenge that remained elevated up to 24 hr later. In humans, peak salivary OXT concentrations have also been measured as early as 15 min (Weisman et al. 2012) and have remained elevated for more than 90 min post-challenge (Daughters et al. 2015; Huffmeijer et al. 2011; Weisman et al. 2012), with elevated concentrations reported as long as 7 hrs (Van Ijzendoorn et al. 2012). In humans mucociliary transport, the moving of material from the nasal cavity to the throat, takes approximately 12 minutes (Marttin et al., 1998). Thus it is possible that the initial peak at 15 min was the result of unabsorbed OXT dripping into the back of the throat. Given that material continues to move into the esophagus and stomach following mucociliary transport (which is further sped up by swallowing), the observed increase in salivary OXT at 30 min and beyond are likely independent of such

drip contamination. However, it should be noted that increases in OXT concentrations have been observed in as early as 10 min in plasma following intranasal administration (Andari et al., 2010), and thus 15 min post-event may still be an important time frame for salivary OXT measurement (see additional discussion below on post breeding samples).

Urinary OXT concentrations were found to be elevated 24 and 48 hrs following the intranasal challenge. No increase was observed in samples collected 25 and 87 min post intranasal challenge. A peak in urinary OXT may have occurred between the 90 min and 24 hr post challenge samples, but unfortunately due to husbandry constraints, samples were unable to be collected during this time period. Common marmosets (*Callithrix jacchus*) who were injected with radiolabeled OXT were found to excrete 38% of the sample within the first 30 min and an additional 29.3% between the end of the first and second hour (Seltzer and Ziegler, 2007). Similar urinary OXT clearance rates following injection have been reported in mice (30-90min; Polito Iii et al. 2006), dogs (15-45min; Mitsui et al., 2011), and humans (60-90 min; Amico et al., 1987). Given these findings it is likely that the excretion rate for gorillas is less than 24 hrs, however; this finding does provide evidence that urinary samples collected the day after a specific event can be informative and representative of OXT in relation to significant events occurring in the previous 24-48 hours.

An elevation in urinary OXT was observed 48 hrs prior to the intranasal challenge. At the time of sample collection a quarter sized bite wound was observed on the back of Bebac's left leg. This wound was not observed prior to the animal care staff leaving the previous evening. In a multi-institution study of gorilla wounding, this dyad was reported to have only 7 wounding events over a 26 mo period, and had the third

lowest wounding rate for a bachelor gorilla group (Leeds et al., 2015). In addition to facilitating social behavior and bonding, OXT is linked to the physiologic stress response, mainly as an internal means by which the body attenuates the stress response, which has been experimentally evaluated in humans (Ditzen et al., 2009) and squirrel monkeys (*Samiri sciureus*, Parker et al., 2005). As a result of this function of OXT, an increase in OXT concentrations is observed following exposure to stressors (Babygirija et al., 2012). Given the rarity of such events for this dyad and the particular severity of the wound, the event that resulted in this wound can be considered a strong stressor. Thus the increase in OXT observed following this event provides an additional validation for the measurement of urinary OXT in gorillas.

Baseline urinary and salivary OXT concentrations were assessed for the first time in gorillas, with observed mean values of 1501 pg/mg CR and 105 pg/ml, respectively. The measurement of OXT in other species has shown that OXT concentrations vary considerably by species in both urine and saliva. In humans, for example, urinary OXT concentrations are generally reported at approximately 10 pg/mg CR (Feldman et al., 2010) and similar levels are also reported in chimpanzees (Crockford et al. 2013). This is significantly lower than mean concentrations reported in other species, including mice (250 pg/ml; Polito et al. 2006), chacma baboons (146 and 428 pg/mg CR; (Moscovice and Ziegler, 2012), and tamarins (16 and 23 pg/mg CR; Snowdon et al. 2010). Similarly, in humans mean salivary OXT concentrations are reported to be approximately 10 pg/ml (McCullough et al. 2013), though some studies have reported higher mean concentrations in the 50-60 pg/ml range (Daughters et al. 2015). Salivary OXT has yet to be assessed in other primate species but has been reported on in dogs, with a mean concentration of 41-

260 pg/ml, depending on assay used (MacLean et al. 2018). It is premature to speculate what is the cause of these observed differences, but likely factors include differences in antibody and general assay design, which have been shown to impact reported OXT concentrations (MacLean et al. 2018), as well as biological differences among species. For saliva, though to some degree this applies to urine as well, Daughters et al. (2015) pointed out that there is no universal, or general, guidelines for saliva collection that exist for plasma. This may also be a contributor to differences observed in OXT concentrations both between and within species. However, since saliva collection and processing was consistent throughout our study and we collected match-control samples within individuals, we expect that observed changes in relative OXT and OXT metabolite concentrations in relation to the observed events (breeding, play, loss of conspecific) to be valid.

Diurnal variation of the hormone OXT has received limited study with conflicting results. In human cerebrospinal fluid (CSF), OXT peaks at approximately 1200 compared to 0600, 1800 and 2400. Interestingly, a peak in plasma OXT was not observed at this time (Amico et al., 1983). Similarly, circadian rhythms have been reported in the cerebrospinal fluid of rhesus macaques (Perlow et al., 1982; Reppert et al., 1983) but both studies only reported overall differences between day and night, and not hourly variation between and within subjects. A peak in salivary OXT was observed in the afternoon for both subjects in the current study. This provides evidence that there may be an afternoon spike in salivary OXT in gorillas, though additional study is needed. Specifically, more research is needed to understand how specific events within the gorilla's day, such as feeding, crowd size, activity level, and keeper presence, may impact OXT concentrations.

However, for most of the day, specifically in the morning, little variation was observed. Similar findings have been observed in humans (Blagrove et al., 2012; Weisman et al., 2013; White-Traut et al., 2009). Studies of human plasma OXT concentrations have similarly found stable concentrations over time (Parker et al. 2010; Weisman et al. 2013), which correlate with salivary concentrations (Feldman et al. 2011; Grewen et al. 2010). We found evidence of diurnal variation in urinary OXT, with higher levels observed in the morning compared to the afternoon. Similar patterns have been observed in both urinary testosterone and cortisol in western lowland gorillas (Stoinski et al. 2002). However, in a study of chimpanzee urinary OXT time of day was not a significant factor (Crockford et al. 2013). Morning urine samples are often considered a representation of hormone accumulation from the previous day, which was also corroborated in this study by the intranasal challenge. This variation, along with what was observed in the saliva, raises the important point that time of day should be a consideration when measuring peripheral OXT.

Salivary OXT was observed to significantly increase 15 min following breeding events between Mokolo and an adult female gorilla. Salivary OXT has similarly been observed to increase 10 min following sexual stimulation in humans (De Jong et al., 2014) and within 1 min in plasma (Blaicher et al., 1999). This data provides evidence that meaningful changes in gorilla OXT can be detected in saliva and further supports our observation of increased salivary OXT 15 min post-intranasal spray. Given the quickness that OXT changes can be detected in plasma (Blaicher et al. 1999), and that in humans salivary OXT levels change within 10 min of known events, it is possible that gorilla

salivary OXT changes can be detected sooner than the 15 min observed in this study and additional study is needed to better understand the timing of salivary OXT release.

Following two spontaneous and consecutive play bouts, Bebac's salivary OXT was observed to be elevated compared to a baseline sample collected before play began. This is further evidence that OXT changes following specific events can be measured in gorilla saliva. Similar findings have been reported in studies of urinary OXT in other primate species (baboons, Moscovice and Ziegler 2012; chimpanzees, Crockford et al. 2013; Wittig et al. 2014). Interestingly, observed increases have been associated with close social bonding. For example, urinary OXT increased in chimpanzees following grooming with closely bonded individuals (as measured by frequency of affiliative and cooperative interactions) but not following grooming with less bonded individuals (Crockford et al. 2013). Given that the observed change of OXT in this study followed two consecutive interactions, it is impossible to know if the elevation following the group mate play bout was due to maintained elevated samples from the keeper-gorilla bout or that the group mate play bout maintained the elevated concentrations. For the latter, this may provide preliminary physiological evidence of adult male-male bonding in gorillas, which has been described behaviorally in mountain gorillas (*Gorilla beringei beringei*) (Rosenbaum et al., 2016a), though further study is needed to clarify what caused the increase observed in this study. This also provides evidence that interactions with animal care staff can impact gorillas on a physiological level. This is interesting because gorilla-human interactions have received significant behavioral investigation (Carrasco et al., 2009; Chelluri et al., 2013; Leeds et al., 2016), but to date no evaluations have included a hormonal component. Future research on gorilla OXT should include studies of gorilla-

human interactions as this has implications for the welfare and management of gorillas in zoos and sanctuaries.

Mokolo's OXT concentrations were reduced by nearly half following the death of his groupmate, Bebac. Common marmosets (*Callithrix jacchus*) who were socially isolated had lower OXT concentrations compared to when they were provided social contact (Seltzer and Ziegler 2007). This finding was similarly found in singly and socially housed guinea-pigs (*Cavia aperear f. porcellus*, Machatschke et al., 2004). Given this it is not surprising that Mokolo's OXT levels decreased following the death of Bebac, whom he had lived with his entire life (approx. 29 years). However, female, but not male, prairie voles (*Microtus ochrogaster*) have been reported to maintain elevated OXT levels following 4 weeks of isolation (Grippo et al., 2007). More research is needed to understand what contributes to the differences observed across species. In addition, more research is needed in understanding OXT's role with both acute and chronic stress, as research has primarily focused on acute stressors, while situations such as the loss of a social partner are more representative of a chronic stress since isolation can occur for long periods of time. This result may also provide some additional insight into western lowland gorilla social bonding.

Western lowland gorilla social structure is based around male-female bonds, with groups often dissolving when the silverback dies (Parnell, 2002; Stokes, 2004), and where multi-male groups are rare (Robbins et al., 2004). In North American zoos, males are often housed in all-male or bachelor groups. Among zoo managers, the functionality and social significance of bachelor groups has been questioned. Functionally, repeated study has shown that bachelor groups are generally cohesive and can be managed over

the long-term, despite their rarity in the wild (Leeds et al., 2015; Stoinski et al., 2001; Stoinski et al., 2004a; Stoinski et al., 2013; Stoinski et al., 2004b). However, the social significance that such groups provide residents is still under studied. Stoinski et al. (2002) found that cortisol levels did not differ between males housed in mixed-sex or bachelor groups, but both social groupings had significantly lower levels than singly housed male gorillas. While this demonstrated that bachelor groupings are likely no more stressful than mixed-sex groupings, it did not provide evidence on whether individuals in these groups are bonded. Given that in humans and cotton-top tamarins OXT levels positively correlate with the quality of one's social environment (Holt-Lunstad et al. 2015; Snowdon et al. 2010), and that Mokolo's OXT concentrations significantly decreased following Bebac's death and remained low over 39 days, these data preliminarily suggest that social bonds form amongst male gorillas in bachelor groups and that OXT is part of the physiological process. If the group was not bonded, or the social environment did not provide a positive experience, it would be unlikely that OXT levels would decrease following the loss of a conspecific. This finding is significant to improving our understanding of gorilla social behavior and bonding given that this species has an understated expression of sociality compared to other primates. More research is needed to better understand the relationship between OXT and social bonding in bachelor groups, in addition to what ecological factors contribute to the general absence of bachelor groups in the wild despite their functionality in zoos.

This study provides a validation for the measurement of OXT in the urine and saliva of western lowland gorillas using a commercially available EIA. Using an intranasal challenge, initial peaks in OXT were observed within 15-30 min in saliva. In

addition, changes in salivary concentrations were observed 15 min following natural social interaction providing evidence that mucociliary transport was not the only cause of the intranasal spike, ultimately demonstrating that salivary OXT represents a useful point in time measurement of OXT. This was further supported by samples collected opportunistically following two spontaneous social interactions. The day following the challenge, OXT concentrations were elevated in the first morning urine void of the subject, while no change was observed 90 min following the challenge, though it should be noted that no samples were collected between the 90 min and 24 hr post sample, and thus true excretion rates are still unclear. This does provides evidence that for gorillas, urine may be a better measurement of baseline or aggregate OXT concentrations, representative of an accumulation of OXT over time rather than a point in time sample. This finding was further supported by an observed decrease in urinary OXT concentrations following the loss of a group mate. This validation procedure will allow researchers to move forward with evaluating OXT in gorillas. It should be noted that this study relied on data collected from two gorillas only. Given the protected status of gorillas and their availability in only non-laboratory settings, sample size will continue to be an issue in evaluating OXT in gorillas. Multi-institutional research will help alleviate some issues related to small sample sizes, but conducting manipulative research such as what was conducted in this study will continue to prove challenging and should be considered when pursuing further research. This study will not only help facilitate future research of gorilla biology, but will also aid in evaluating the welfare of gorillas in human care. Research has found that OXT is a noninvasive measure of positive emotion in domestic dogs (Mitsui et al., 2011) and a measure of social bond strength in multiple

primate species (Crockford et al. 2013; Holt-Lunstad et al. 2015; Snowdon et al. 2010). Evaluating OXT concentrations in gorillas may help improve their welfare by identifying management and husbandry factors that promote both positive emotion in gorillas and strong social bonds between both conspecifics and gorillas and their caregivers.

CHAPTER THREE: Evaluating changes in salivary oxytocin and cortisol following positive reinforcement training in two adult male western lowland gorillas (*Gorilla gorilla gorilla).*

Introduction

Positive reinforcement training (PRT) is a behavior shaping principle used primarily to train animals in human care to participate in their own husbandry (Laule and Whittaker, 2007). Rates of affiliative behavior between conspecifics increased following PRT in western lowland gorillas (WLG; *Gorilla gorilla gorilla*; Carrasco et al., 2009) and chimpanzees (*Pan troglodytes*; Pomerantz and Terkel, 2009) and rates of stereotypical and/or abnormal behavior have decreased following PRT in WLG (Carrasco et al., 2009; Leeds et al., 2016; Pizzutto et al., 2007), wild dogs (*Lycaon pictus*, Shyne and Block, 2010), olive baboons (*Papio hamadryas anubis*, Bourgeois and Brent, 2005), and rhesus macaques (*Macaca mulatta*, Baker et al., 2009; Coleman and Maier, 2010). The social interaction with animal care staff (ACS) may facilitate these behavioral changes. PRT is not a species-specific social interaction, but the active learning, problem solving, and social exchange required to participate are the building blocks of any species-specific social interaction. PRT may then be viewed as an affiliative and/or cooperative interaction and may stimulate responses from endocrine systems the same way interactions with conspecifics do.

The hormone oxytocin (OXT) increases following species-specific affiliative and cooperative interactions in primates (Crockford et al., 2013c; Leeds et al., 2018; Wittig et al., 2014d). Furthermore, the exogenous administration of OXT increases affiliative

behavior (Simpson et al., 2014), and OXT can have anxiolytic effects (Parker et al., 2005). Thus if OXT increases following PRT, this could explain increases seen in affiliative behavior and decreases in stress-related behaviors post-PRT. The hormone cortisol (CORT) is produced following a stressor, or a disruption to homeostasis (Cockrem, 2013), and is generally associated with physical and psychological arousal. CORT levels are lower following affiliative interactions, such as grooming, with a closely bonded social partner in chimpanzees (Wittig et al., 2016). Similar to OXT, a reduction in CORT post-PRT, independent of the oxytocinergic system, may result in a more relaxed state, ultimately facilitating an increase in affiliative behavior and reduction in stress-related behavior.

CORT, but not OXT, has been evaluated in relation to PRT. O'Brien et al. (2008) evaluated salivary CORT levels in male hamadryas baboons (*Papio hamadryas*). No difference in pre- and post-PRT CORT concentrations was found, however; both pre- and post-PRT concentrations were lower than concentrations collected prior to the PRT program beginning, indicating PRT may have a broad stress reduction effect. Similarly, a study of wolves (*Canis lupis*) and dogs (*Canis lupus familiaris*) found salivary CORT decreased in both species following PRT. In contrast, Behringer et al. (2014) found no difference in salivary CORT between pre- and post-PRT samples in seven orangutans (*Pongo abelii*) and ten bonobos (*Pan paniscus*). The purpose of this case study was to evaluate WLG salivary OXT (sOXT) and salivary CORT (sCORT) concentrations following PRT. This is the first evaluation of changes in sOXT following PRT in any species and the first evaluation of changes in sCORT following PRT in gorillas. Since it is well documented that PRT has effects on both affiliative and stress behavior of

participants, it was hypothesized that concentrations of OXT would increase and concentrations of CORT would decrease following PRT.

Methods

Subjects

Mokolo and Bebac, adult male WLG living at Cleveland Metroparks Zoo (CMZ), Cleveland, OH, USA, participated in this study. Bebac died in the early stages of this study. Because baseline and post-PRT samples were analyzed as matched samples from the same day, data collected from Mokolo before and after Bebac's death were included in this study.

Data Collection

PRT sessions occurred four times per week at approximately 0830 in the participant's off-exhibit space. PRT was conducted by the gorillas' primary ACS. PRT session length ranged from 10 to 11 min to control for any duration effect. Sessions focused on maintenance husbandry behaviors such as measuring heart rate, the presentation of body parts, and the shaping of behaviors that contributed to a novel blood collection behavior (description of these behaviors are described in Good et al. 2017).

Saliva samples were collected by providing the participant with a salivette, a swab designed for the collection of saliva (Salimetrics, State College, PA), to chew, which was then provided back and placed in a saliva collection vial (Salimetrics, State College, PA; Kuhar et al., 2005). Samples were immediately frozen at -20°C, and then transferred on ice to a -80°C freezer. Samples were collected at three time points relative to each PRT session: prior to PRT (range 2-10 min), 15 min post- and 30 min post-PRT. Prior to assay, saliva samples were thawed and centrifuged at 2500 rpm for 15min to separate

saliva from salivette. Mokolo participated in ten PRT sessions. Due to Bebac's death, only five PRT sessions occurred. Due to sample volume, only OXT was assayed for Bebac. If any social interaction occurred between participants post-PRT, the session was not utilized in this study.

OXT and CORT Assay and Analysis

sOXT and sCORT were assayed in the endocrinology laboratory of CMZ. The measurement of both hormones were validated prior to initiating this study (OXT, Leeds et al., 2018; CORT, Browning, 2013). Mean inter-assay coefficients of variation (CV) for controls and standards were below 15% for OXT ($\mu = 8.60$) and 10% for CORT ($\mu = 7.62$). Samples were run in duplicate and intra-assay CV's for samples, controls and standards were below 10%. OXT was assayed using a commercially available enzyme immunoassay (EIA; Arbor Assays, Ann Arbor, MI). Manufacturer instructions were followed except samples were dried using an Evaporack (Cole Parmer, Vernon Hills, IL) and not a Speedvac, inside a warm water bath (32°C). In addition, the extraction procedure was not utilized (Leeds et al., 2018). Dried samples were reconstituted in assay buffer to the original (neat) volume. CORT was assayed using the EIA described in Wark et al. (2016). Samples were run at a 1:4 dilution in assay buffer. For analysis, all samples were adjusted for dilution factor and reported as pg/ml (OXT) and ng/ml (CORT).

Non-parametric Friedman's ANOVA ($\alpha = 0.05$) with post hoc Wilcoxon Signed Ranks Tests (Bonferonni adjustment, $\alpha = 0.017$) were used to compare samples by condition for each subject individually (SPSS V.25, IBM, Chicago, IL). The test statistics were generated using the Monte Carlo method (10,000 permutations) per the recommendation of Plowman (2008) for small sample size studies.

Results

No difference in pre and post-PRT sOXT was observed for either Bebac (χ^2 <0.001, df = 2, p = 1.000) or Mokolo (χ^2 = 0.857, df = 2, p = 0.651; Table 3.1; Figure 3.1). For Mokolo, there was a significant difference in sCORT concentration by condition (χ^2 = 9.556, df = 2, p = 0.008; Table 1). Post hoc comparisons found sCORT was significantly lower both 15 min (z = -2.547, p = 0.011) and 30 min post-PRT (z = -2.380, p = 0.017; Figure 3.2) compared to pre-PRT concentrations.

Table 3.1. Comparison of salivary oxytocin and cortisol concentration by subject and condition.

Hormone	Participant	Mean Concentration By Sample Point (SE)			Statistics		
		Pre-PRT	15min Post PRT	30min Post PRT	χ^2	df	P
Oxytocin (pg/ml)	Mokolo	105.7 (9.6)	149.44 (28.2)	103.76 (12.1)	0.086	2	0.651
	Bebac	95.09 (16.4)	88.49 (14.0)	121.10 (12.9)	<0.001	2	1.000
Cortisol (ng/ml)	Mokolo	3.68 (0.4)	2.21 (0.1)	2.08 (0.2)	9.556	2	0.008*
	Bebac	-	-	-	-	-	-

*Statistically significant ($P \leq 0.05$).

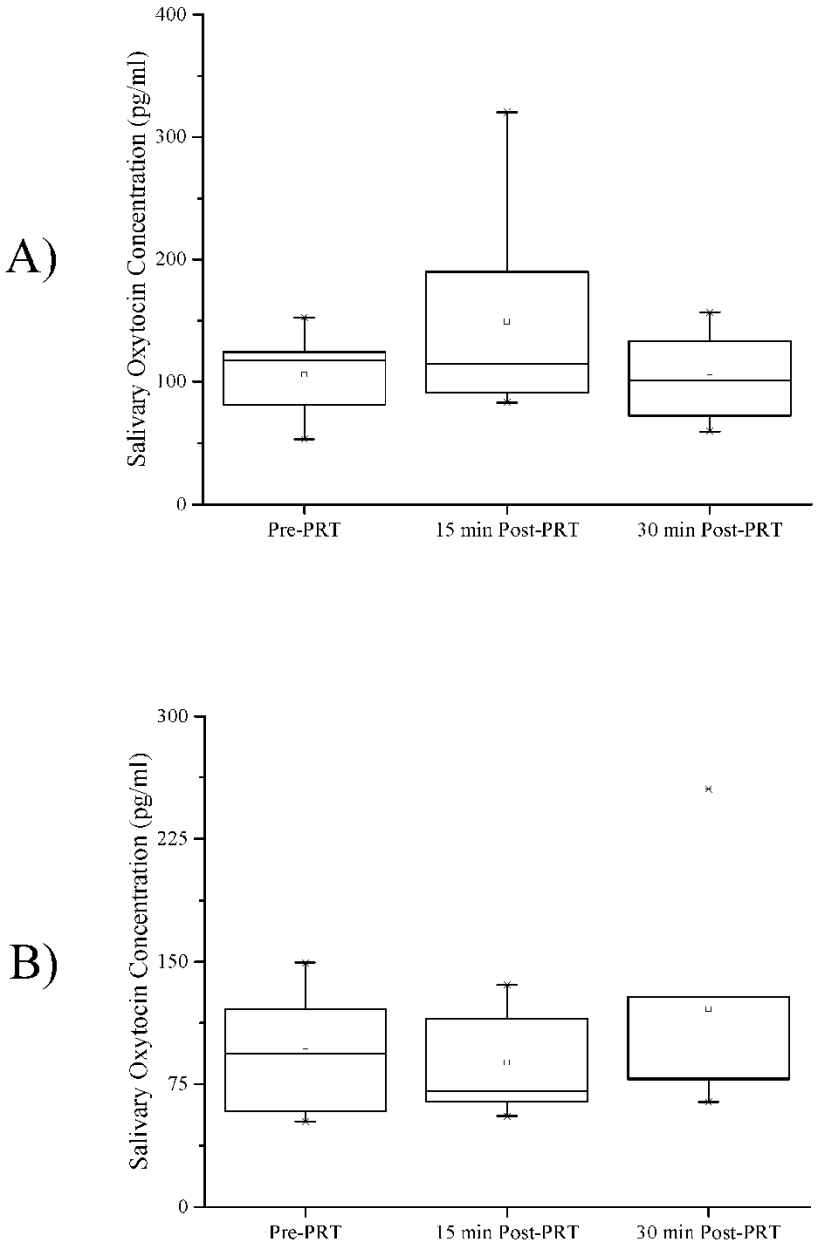

Figure 3.1. Salivary oxytocin concentrations (pg/ml) for Mokolo (A) and Bebac (B) before and after positive reinforcement training.

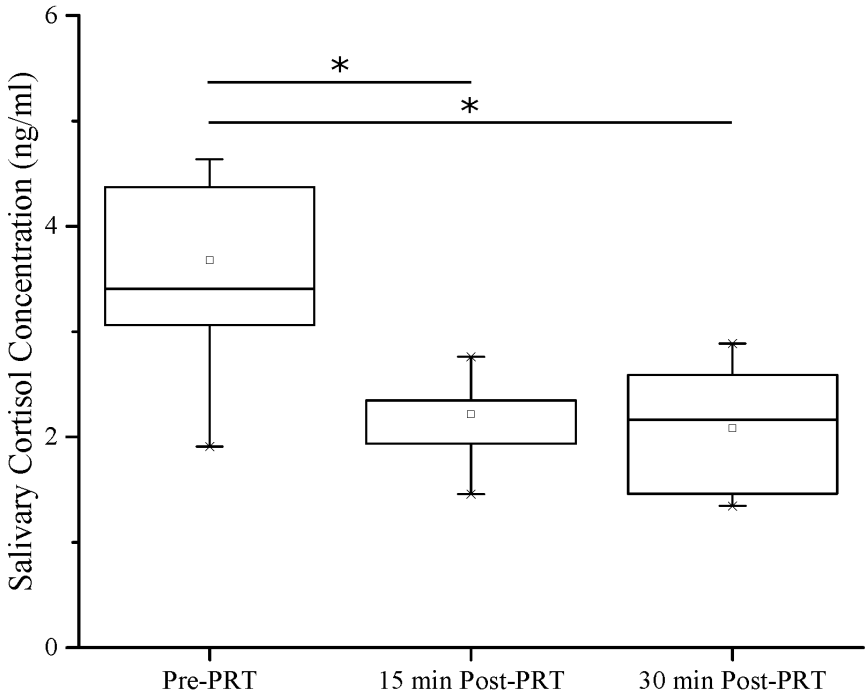

Figure 3.2. Salivary cortisol concentrations (ng/ml) for Mokolo before and after positive reinforcement training. Cortisol varied by condition ($\chi^2 = 9.556$, P = 0.008). Post-hoc comparisons found that concentrations were significantly lower 15 ($z = -2.547$, $p = 0.011$) and 30 min post-PRT ($z = -2.380$, $p = 0.017$) compared to pre-PRT concentrations.

Discussion

Despite the cooperative nature of PRT, no change in sOXT was detected following PRT. PRT is a transactional interaction where the ACS request a behavior, the participant executes that behavior, and then receives some form of reward if the behavior meets predetermined criteria. Affiliative and cooperative behavior can be transactional, such as reciprocity associated with grooming (Schino, 2006). However, the transactional nature of PRT, in the absence of any evolutionary significance, may not stimulate the oxytocinergic system the way species-specific affiliative and cooperative behavior does. sOXT can elevate following play and mating in WLG (Leeds et al., 2018), but these are high-arousal and evolutionarily significant behaviors, unlike PRT which is low-arousal and not tied to WLG evolutionary history.

sCORT was observed to decrease following PRT, indicating PRT may have stress reducing properties. PRT provides an opportunity to interact with ACS in a cooperative way (albeit not in a species-typical way). Such social interaction likely has positive psychological benefits. In zoos, ACS become social partners and PRT may be a way to facilitate regular and consistent interaction where expectations and outcomes are known similar to species-typical interactions with conspecifics. The change in sCORT may also be the result of anticipation, which has been associated with increased sCORT in zoo-living bonobos (Hohmann et al., 2009). PRT occurred at the same time and days, and had so for over a year. Often Mokolo would be sitting in his PRT station position when the primary trainer arrived, indicating anticipation of PRT. The decrease in sCORT following PRT may also have been reflective of elevated levels in anticipation of PRT rather than a

stress reduction effect. Evaluating sCORT in unscheduled interactions would control for this.

Another important consideration is that this study included only one ACS. In chimpanzees, changes in OXT and CORT following social interaction have shown to vary in relation to the conspecific one is interacting with (Crockford et al., 2013c; Wittig et al., 2016). Changes in wolf CORT following PRT have also been reported to vary in relation to specific ACS (da Silva Vasconcellos et al., 2016). Due to staff limitations at the time of this study, only one ACS, the gorillas' primary trainer, was included. PRT is a dyadic interaction and physiologic responses following PRT should vary based on the individuals involved. To better understand overall patterns of how animals in zoos respond to PRT (or any form of ACS interaction), multi-zoo assessments need to occur given even multiple comparisons within a single zoo still only represent a case study of that particular zoo's animal-ACS relationships.

This was a case study involving two WLG, with one passing away in the initial stages of this study, and one ACS. Thus the applicability of these findings are limited to these two dyads. The saliva collection methods described in this paper required extensive training, and thus it was not possible to include participants from other zoos. Salivary hormone measures provide an immediate, event specific measure of physiological change. Despite the difficulty of training for saliva collection, training this behavior should be encouraged given its usefulness in assessing specific hormone changes non-invasively that are beneficial for general hormone monitoring as well as research.

CHAPTER FOUR: Urinary oxytocin concentrations vary by social demographic, reproductive and developmental factors in western lowland gorillas (*Gorilla gorilla gorilla*).

Introduction

Western lowland gorillas (WLG; *Gorilla gorilla gorilla*) are generally described as living in reproductive groups composed of one adult male, three adult females, and their offspring (Gatti et al., 2004; Parnell, 2002; Robbins et al., 2004). WLG engage in little affiliative behavior, possibly as a result of their dispersal patterns resulting in limited long-term social associations among individuals. Stokes (2004), who observed nine WLG groups at Mbeli Bai, Republic of Congo, for 802 observation hours observed a total of eight affiliative interactions between gorillas. Six interactions were sexual interactions between a group silverback and an adult female and no grooming was observed between adults. Additional field study has found WLG spend as little as 0.5% of their time engaged in affiliative behavior (Masi et al., 2009). Agonisic behavior between males and females is common, with two-thirds of all intergroup aggression occurring between males and females, and rates of male-female agonism have been reported to be twenty two times greater than rates of male-female affiliative behavior (Stokes, 2004). Studies of the social behavior of WLG in zoos similarly report low levels of affiliative behavior, often accounting for less than 5% of activity budgets (Hoff et al., 1997; Ross et al., 2010). Due to WLG engaging in low rates of affiliative behavior, it is difficult to study what contributes to social bonding in the species, given affiliative behavior is a primary measure of social bond (Berghänel et al., 2011; Schülke et al.,

2010; Silk et al., 2009). The absence of affiliative behavior may demonstrate that strong social bonds do not shape gorilla relationships. Rather gorilla relationships may be shaped via functional benefits that result from group living, specifically females seek optimal mates and groups and males seek to maximize mating opportunities.

Female WLG seek the best social environment for themselves, regularly transferring between groups, often multiple times in one's lifetime (Robbins et al., 2004; Stokes et al., 2003). Transfers are likely associated with the perceived quality of the male, data suggest females prefer males with larger sagittal crests and gluteal muscles (Breuer et al., 2012), and the perceived quality of the group, females appear to prefer smaller groups, in reference to the number of adult females, possibly due to costs associated with intergroup feeding competition (Stokes et al., 2003). Interestingly, there is genetic evidence that despite secondary and tertiary group transfer, female gorillas live in groups with related females more often than by chance, indicating female gorillas may maintain kin associations over time (Arandjelovic et al., 2014; Bradley et al., 2007).

Male WLG use coercion and herding to maintain females in their respective groups (Breuer et al., 2016). Particularly, males use more intense and lengthy bouts of contact aggression with newer female group members (Stokes, 2004) and preferentially direct agonistic behavior towards females when potential rival males are in their proximity rather than towards the competitor male (Magliocca and Gautier-Hion, 2004). Furthermore, males specifically direct aggression towards females most likely to emigrate based on their current reproductive status (Breuer et al., 2016). In contrast to their relationships with females, males, despite their secondary sexual characteristics that indicate some level of male-male competition in their evolutionary past (Harcourt et al.,

1981), demonstrate some affinity to affiliate with other males. Though not abundant, bachelor groups are observed in the wild (Arandjelovic et al., 2014; Gatti et al., 2004), and in the absence of mate and food competition, are a stable and long-term management strategy for WLG in zoos (Stoinski et al., 2001; Stoinski et al., 2004a; Stoinski et al., 2013b). Interestingly, related free-ranging WLG males have been reported to maintain overlapping home ranges, forming dispersed networks of related males within a particular area (Bradley et al., 2004). In addition, encounters between male WLG are generally peaceful, even in the presence of receptive females (Magliocca and Gautier-Hion, 2004). In mountain gorillas, males also show an affinity for other males, but instead regularly form multi-male, multi-female groups (Gray et al., 2013). Bachelor groups have historically been observed in mountain gorilla populations (Robbins, 1996) but are not regularly observed now. What drives males to affiliate is unknown, but it may by a function of kin selection, or more simply long-term familiarity that is a proximate mechanism facilitating kin selection in the absence of true genetic relatedness (Waldman, 1987).

The reproductive tactics of male and female WLG likely make it difficult, nor advantageous, to form strong, long-term bonds typical of other old world primates (Seyfarth and Cheney, 2012). However, there are some data that indicate male gorillas may have a tendency to form relationships with other males, though in a dispersed and species-specific way. Given WLG engage in low rates of social behavior, evaluations of physiological variation amongst WLG may improve our understanding of bonding in this species. Oxytocin (OXT) is a mammalian neuroendocrine hormone synthesized in the paraventricular nucleus and supraoptic nuclei of the hypothalamus (Gainer, 2012). Most

notably, OXT is studied for its regulation of child rearing behavior, particularly nursing and mother-infant attachment (Carter et al., 2007; Grewen et al., 2010; Neumann et al., 2000a; Uvnas‐Moberg et al., 1985; White‐Traut et al., 2009). It has been hypothesized that OXT was evolutionarily co-opted to serve as a means to facilitate affiliation and bonding beyond the mother-infant relationship (Wittig et al., 2014).

In humans, OXT levels increase following affiliative behavior including brief physical contact (Feldman et al., 2010), massage (Holt-Lunstad et al., 2008), verbal reassurance (Seltzer et al., 2010), play (Fries et al., 2005) and even interactions with pets (Nagasawa et al., 2009; Odendaal and Meintjes, 2003). In non-human primates, OXT has similarly been observed to increase following grooming (Crockford et al., 2013), food sharing (Finkenwirth et al., 2016; Samuni et al., 2018; Wittig et al., 2014), mating (Snowdon et al., 2010), and spending time in close proximity to mating partners (Moscovice and Ziegler, 2012). As a result of OXT concentrations increasing following affiliative or cooperative interaction, baseline levels of OXT have been positively associated with the strength of one's social bonds. In married couples, higher baseline OXT levels are associated with a stronger social bond as assessed by the Dyadic Adjustment Scale, the most commonly used measure of relationship quality (Holt-Lunstad et al., 2014). Additionally, higher baseline OXT levels were associated with couples who hugged and massaged each other more frequently (Light et al., 2005). In non-human primates, OXT is correlated with affiliative and sexual behavior in pair-bonded tamarins (*Saguinus Oedipus*, Snowdon et al., 2010). Similarly, more strongly bonded marmoset (*Callithrix jacchus*) dyads living in family groups were found to have synchronized fluctuations in OXT concentrations over time (Finkenwirth et al., 2015).

The relationship between OXT and social bond was further strengthened by a study of chimpanzee (*Pan troglodytes*) grooming. Following grooming with a bonded partner, as defined by the frequency and direction of affiliative behavior, OXT significantly increased compared to baseline conditions, but OXT did not increase following grooming with a non-bond partner, further indicating variation in OXT is directly related to the strength of ones social bonds (Crockford et al., 2013).

Baseline levels of OXT have also been reported lower in individuals with weaker social bonds. In humans, OXT levels are lower in distressed married couples compared to more stable couples (Holt-Lunstad et al., 2014). In non-human primates, social isolation has been shown to decrease OXT levels compared to when they were socially housed (Leeds et al., 2018; Snowdon et al., 2010). In addition, rhesus macaques (*Macaca mulatta*) who were nursery reared in a laboratory by humans, an atypical social upbringing for their species, had lower OXT levels compared to mother reared individuals (Winslow et al., 2003).

Assessing OXT variation following specific events, primarily following a dyadic social interaction, has become increasingly common, but evaluations of baseline population level are limited. Population level studies of OXT may improve our understanding of what contributes to social bond formation at the group and population level, placing the study of OXT changes at the dyadic level, in addition to behavior studies at the dyadic level, in a broader context and vice versa. In non-human primates the study of OXT to date has focused exclusively on highly social and affliative primates (e.g. titi monkeys, *Callicebus cupreus*, Witczak et al., 2018; capuchin monkeys, *Cebus apella*, Brosnan et al., 2015; chimpanzees, Crockford et al., 2013; tamarins, Snowdon et

al., 2010; baboons, Moscovice and Ziegler, 2012). Evaluating OXT in species that live in less affiliative social groupings, such as the WLG, can help broaden our understanding of primate sociality both at the species and order level. To date, no large-scale evaluation of OXT in WLG has occurred. Given the difficulty of collecting biological samples from WLG in range countries, studying zoo populations of WLG provide a unique and practical opportunity to study OXT variation in the species. Sample collection and storage is relatively easy and reliable, and the known histories of each individual within a zoo population allows for more experimental control than studying free-ranging populations. Previous study of urinary corticoids and androgens in zoo WLG greatly improved our understanding of the sociality and development of this species (Stoinski et al., 2002). The purpose of this study was to explore variation of OXT concentrations in the Association of Zoos and Aquariums (AZA) population of WLG. The primary goal of this study was to compare OXT concentrations between males in bachelor and mix-sex groups, with the goal of comparing bond strength between male gorillas and between male and female gorillas. We predicted that male gorillas form stronger bonds, measured via greater OXT concentration, with other males than with females based on the cohesiveness and longevity of bachelor gorilla groups in zoos (Stoinski et al., 2004a; Stoinski et al., 2004b), evidence from free-ranging populations indicating males form relationships with other males (Bradley et al., 2004), and the coercive mating tactics males use towards females (Breuer et al., 2016).

Additional goals of this study were to add to the understanding of gorilla social bonding and biology, as well as inform managers of gorillas in human care how specific husbandry and management practices affect gorillas on the physiological level.

Specifically, OXT variation was explored in relation to age, sex, reproductive state, rearing history, social group type, and social group demographics. Given the importance of OXT and mother-infant attachment (Feldman et al., 2011; Gordon et al., 2010), it was predicted that infant gorillas would have greater OXT concentrations than older age classes. Furthermore, given evidence that atypical upbringings can decrease OXT concentrations in primates (Winslow et al., 2003), it was predicted that OXT concentrations would be greater in mother reared gorillas compared to gorillas who were not mother reared. The study of OXT often controls for sex, but few studies have explicitly reported on sex differences in OXT concentrations and when discussed, mixed findings have been reported (e.g. in chimpanzees (Crockford et al., 2013; Preis et al., 2018) and humans (Miller et al., 2013; Motoki et al., 2016). For this study we predicted that no difference would be found between males and females. OXT and reproductive status are intimately linked. Based on this knowledge we predicted that OXT concentrations in gorillas would reflect those observed in humans, specifically, OXT concentrations would be greater in lactating than non-lactating adult females (Carter et al., 2007; White-Traut et al., 2009) and would be greater in adult females on hormonal contraception (HC) compared to adult females not on HC (Silber et al., 1987). The process of childbirth involves changes in OXT (Fuchs et al., 1984; Fuchs et al., 1991), but it is unclear how baseline OXT varies between parous and nulliparous females, thus we predicted that no difference would be observed in our study population between these two categories of female.

When free-ranging males are unable to acquire females to form a mixed-sex group, bachelor groups can be formed instead (Bradley et al., 2004; Gatti et al., 2004) but

males generally choose a solitary lifestyle. Solitary life is likely adaptive to finding mates, but may not be not an optimal social environment for gorillas. A case study found that OXT significantly decreased in an adult male gorilla in a zoo following the death of his male group mate, and his OXT remained low for the five-months he was solitary (Leeds et al., 2018). In addition, a study of male gorillas in zoos found solitary males had greater cortisol concentrations, an indicator of stress, than group-living males. Collectively this indicates that there may be negative physiological consequences to the solitary lifestyle of male gorillas. We predicted that males living in groups would have greater OXT concentrations than solitary males. Furthermore, female gorillas are not observed as solitary individuals in the wild (Robbins et al., 2004), but are temporarily housed solitarily in zoos for management reasons, primarily related to quarantine immediately following transfer between zoos. As a result we additionally predicted that female gorillas in groups would have significantly greater OXT concentrations than solitary females. Multiple additional demographic factors related to group-living gorillas were tested and are described below. Given the limited knowledge of how these factors relate to OXT or bonding, predictions were not developed. To this point, it was an additional goal of this study to provide new data on WLG to initiate future research.

Methods

Study Participants

The methods of this study were approved by the animal care and use committees (ACUC) of all 25 participating zoos. Urine samples were collected from 143 WLG (71.72) living at 25 AZA zoos. Participants lived in a variety of contexts including outdoor exhibits with indoor holding, indoor-outdoor exhibits and indoor only exhibits.

Gorillas lived in mixed-sex groups (n = 27 groups; n = 31.65 gorillas), bachelor groups (n = 16 groups; n = 37.0 gorillas), all-female groups (n = 2 groups; n = 0.4 gorillas), and as solitary individuals (n = 3.3 gorillas). Group size varied by group type (mixed-sex, μ = 4.56; bachelor, μ = 3.05). Two zoos managed gorillas in a fission-fusion style (n = 5.5 gorillas). At one zoo, three males were alternated between living singly and as part of a mixed-sex group (with 0.5 gorillas). One of the three males alternated between group types on his own, the other two were alternated between group types together but remained solitary when not with the females. The second zoo managed a bachelor group that spent days (or parts of a day) together and apart. For analysis, these 5 males from two zoos were categorized as living in fission-fusion groups, given all males spent time as a solitary male and as a group-living male. The five females were categorized as living in a mixed-sex group because they were always housed in a mixed-sex group. The four females in two all-female groups were both unique. One group was a geriatric dyad and the other was an adult female and a surrogate infant. Variation between group types for female gorillas was only assessed between group-living and solitary individuals, and thus mixed-sex and all-female groups were combined for analysis.

Overall, age ranged from 0.17 to 59.25 yrs (μ = 25.2 yrs). For males, age ranged from 0.17 to 42.58 yrs (μ = 24.5 yrs). For females, age ranged from 0.67 to 59.25 yrs (μ = 26.1 yrs). To allow for comparison between sexes, sex-specific age classes developed for previous WLG studies (Breuer et al., 2009; Gatti et al., 2004; Stoinski et al., 2013) were combined and adjusted to create general age classes that could be uses for both sexes. Gorillas were categorized as infants (0.00-3.99 yrs; n = 2.6), juveniles (4.00-7.49 yrs; n = 4.3), subadults (7.50-13.99 yrs; n = 8.9), adults (14.00-34.99 yrs; n = 50.39) and geriatric

(≥ 35 yrs; n = 5.14). The geriatric category is not an age-class utilized in free-ranging gorilla studies. Based on reproductive function of female gorillas in zoos, 30-35 yrs has been determined as when females can be considered geriatric, and generally non-reproductive (Atsalis and Margulis, 2006; Margulis et al., 2007). The median life expectancy of male western lowland gorillas in AZA zoos is 31.7 yrs (Lukas et al., 2017). Given this combined information, the overall geriatric category for this study was established as ≥35 yrs.

Data Collection and Enzyme Immunoassay

A total of 566 urine samples (clean from food, contaminant urine, feces) were collected from participating gorillas. Urine samples were collected opportunistically once per day between 0700-1000 to control for diurnal variation (Leeds et al., 2018). Excluding 1.2 gorillas who participated in a long-term OXT monitoring study, of which data were included in this study, participating gorillas provided on average 2.98 urine samples (range: 1-8). For the 1.2 gorillas participating in a long-term study, 113, 38, and 13 samples were collected respectively. Samples were kept frozen in manual defrost freezers or -80°C freezers (depending on institution) inside 15-ml conical centrifuge tubs (Falcon, Corning, NY). Samples were shipped frozen overnight on dry ice to the endocrinology laboratory of Cleveland Metroparks Zoo (CMZ) where multiple aliquots were immediately prepared, to avoid multiple freeze-thaw cycles, and then stored at -80°C until assay. Samples from all participating zoos were assayed within 6 mo of collection (μ = 188.89 days).

Urinary OXT (uOXT) was measured using a commercially available enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI). Manufacturer instructions were

followed except samples were dried using an Evaporack (Cole Parmer, Vernon Hills, IL) and not a Speedvac, inside a warm water bath (32°C). In addition, following the methods of Leeds et al. (2018) that validated the measurement of WLG uOXT using this assay, urine samples were not extracted. Dried samples were reconstituted in assay buffer to the original (neat) volume. To control for urine concentration, all urine samples were indexed by creatinine concentration, using the procedure of Brown (1998). Samples were read using a spectrophotometer (Biotek Instruments, Inc., Winooski, VT) at 450-nm wavelength using Gen5 software (Biotek Instruments, Inc.). Mean inter-assay coefficients of variation (CV) for controls and standards were below 15% ($\mu = 8.60$). Mean intra-assay ($n = 2$ per sample) CV for study samples, controls and standards were below 10%. Controls were a low (40 pg/ml) and high concentration (1600pg/ml) of OXT standard in assay buffer.

Data Analysis

Samples were log transformed to approximate normality. Preliminary analysis identified 18 samples (3.2% of all samples) as outliers, as they were greater than 2.5SD above the mean of all samples, and thus were removed from analysis (a slightly more conservative procedure than 2.0SD utilized in the study of chimpanzee uOXT by (Crockford et al., 2013) and (Wittig et al., 2014). This outlier removal resulted in the complete removal of 0.1 gorilla from analysis (aged 1.0 yr; samples, $n = 4$).

Variation in uOXT was assessed using linear mixed models (MIXED; SPSS V. 20, IBM, Chicago, IL). Specifically, models were used to assess uOXT within the entire population (Model 1), the entire female study population (Model 2), the adult female study population (Model 3), the entire male study population (Model 4), the bachelor

group male study population (Model 5) and the mixed-sex group adult male study population (Model 6). Gorilla identity was included as a random factor in all models using an identity covariance structure. Models were built using a bottom-up approach where fixed factors were added and removed using maximum likelihood approximation. The lowest -2 log likelihood (-2LL), Akaike information criterion (AIC), and Bayesian information criterion (BIC) were used to assess model fit. Non-significant fixed factors ($P > 0.05$) were removed unless the factor contributed to a significantly better model fit. Degrees of freedom for fixed effects were calculated using the Satterthwaite approximation. Pairwise comparisons of significant fixed effects with multiple comparisons utilized a Bonferroni confidence interval adjustment to account for the multiple comparisons. Final models are presented using restricted maximum likelihood. To assure that the residuals of all models met the assumption of normal distribution, residuals were visually checked via P-P plots and histograms. No deviations from normality were found (except for one explained in detail below). To test for multicollinearity of fixed factors, a linear regression for each model was run (without the random factor). Variance inflation factors and tolerance statistics were checked for each factor, with no factors approaching or exceeding threshold values (for summary see (Field, 2009). The analyzed hormone data are presented as back-transformed estimated marginal means (EMM) ± 95% CI (rounded to the nearest whole number). For additional detail on all factors tested in model, see Table 4.1.

Table 4.1. Fixed factors tested in LMM's.

Name	Description	Coding
Sex	Gorillas were coded as either male or female	Categorical
Age Class	Gorillas were categorized into generic age classes. Details of age classes can be found in methods section.	Categorical
Group Type	Gorillas were categorized as living in fission-fussion groups (where individuals were moved regularly in and out of groups), bachelor groups (groups containing only males), or mixed-sex groups (groups containing one adult male, females and offspring) or solitarily.	Categorical
Rearing History	Gorillas were classified as being hand-reared (raised, at least in part, by humans), surrogate reared (reared by a female gorilla who is not their biological mother) or mother-reared (raised by their birth mother).	Categorical
Lactation	Female gorillas were classified as either lactating (producing milk) or non-lactating (not producing milk).	Categorical
Hormonal Contraception (HC)	Female gorillas were classified as either on HC or not on HC.	Categorical
Reproductive status	Female gorillas were categorized as nulliparous (had not had offspring) or parous (had offspring).	Categorical
Conspecific Offspring Presence	Female gorillas were categorized as living in groups where either another female had or did not have an offspring present.	Categorical
Sibling Presence	Gorillas lived or did not live with a sibling (full, shared both parents or half, shared one parent). Tested both categorically (yes/no) and continuously (number present).	Categorical/Continuous
Group Size	Number of individuals in a social group.	Continuous
Offspring Presence	An adult gorilla had an offspring present in the group. Tested both categorically (yes/no) and continuously (number).	Categorical
Group Age Demographics	Bachelor groups were categorized as mixed generation, at least one individual in the group was in a different age class then the others, or single generation, all group members were in the same age class.	Categorical

Young Silverback Present	Bachelor groups either contained a young silverback (male 14-20 yrs old) or did not.	Categorical
Length of Group Tenure	Time (years) that a group was together. Measured from the date that the most recent social addition occurred to the median date of all individual urine samples.	Continuous
Tenure as Dominant Male	Time (years) an adult male was in a mixed-sex group, regardless of group make up. Measured from the date he entered a mixed-sex group to the median date of all individual urine samples.	Continuous
Number of Adult Females Present	Count of the number of adult female gorillas in a group.	Continuous
Females Off HC	Tested both categorically and continuously as either a yes/no of at least one adult female not using HC or a count of the number of adult females not using HC.	Categorical/Continuous

Results

OXT Variation in Study Population

For the entire study population, social grouping ($F_{1,465.120} = 4.794$, $P = 0.029$), sex ($F_{1,\ 155.525} = 0.001$, $P = 0.981$), age class ($F_{4,150.043} = 9.060$, $P < 0.001$), and the interaction of age class and sex ($F_{4,148.509} = 3.145$, $P = 0.016$; Model 1, Table 4.2) were the best predictors of uOXT. Gorillas living in a social group (mixed-sex, all-female, bachelor, fission-fusion) had on average 19% greater uOXT concentrations (μ = 1072 pg/mg CR, 95% CI: 994-1213) than gorillas who were solitary (μ = 904 pg/mg CR, 95% CI: 745-1096, P = 0.029).

For age class, infant gorillas (μ = 2009 pg/mg CR, 95% CI: 1321-3055) had significantly greater uOXT than subadult (μ = 773 pg/mg CR, 95% CI: 614-973; P = 0.001), adult (μ = 659 pg/mg CR, 95% CI: 585-741; P < 0.001) and geriatric gorillas (μ = 774 pg/mg CR, 95% CI: 607-986; P = 0.001). Juvenile gorillas (μ = 1161 pg/mg CR,

95% CI: 824-1637) had significantly greater uOXT then adult gorillas (P = 0.016). No other pairwise comparisons were significant (P >0.05; Figure 4.1). The interaction between age class and sex revealed variation within each age class by sex and within each sex by age class. For subadult and adult gorillas, females (μ = 1014 pg/mg CR, 95% CI: 743-1384; μ = 729 pg/mg CR, 95% CI: 624-855) had significantly greater uOXT than males (μ = 589 pg/mg CR, 95% CI: 426-812, P = 0.015; μ = 594 pg/mg CR, 95% CI: 518-685, P = 0.031). This trend approached significance for geriatric gorillas (Female, μ = 968 pg/mg CR, 95% CI: 759-1233; Male, μ = 618 pg/mg CR, 95% CI: 409-935; P = 0.065). There was no difference in uOXT between juvenile female (μ = 973 pg/mg CR, 95% CI: 585-1618) and juvenile male gorillas (μ = 1384 pg/mg CR, 95% CI: 883-2173; P = 0.300). Infant male gorillas (μ = 3090 pg/mg CR, 95% CI: 1507-6324) had significantly greater uOXT than infant female gorillas (μ = 1309 pg/mg CR, 95% CI: 859-1991, P = 0.041). For female gorillas, there were no significant differences between age classes (P > 0.05), though a trend for infant females to have greater uOXT than adult females was observed (P = 0.092). For male gorillas, infants had significantly greater uOXT than subadult (P < 0.001), adult (P < 0.001), and geriatric gorillas (P = 0.002). Juvenile males had greater uOXT than subadult (P = 0.022) and adult males (P = 0.04), but not infant (P = 0.612) or geriatric males (P = 0.094). There was no difference between subadult, adult and geriatric males (P > 0.05; Figure 4.1).

Sex was the only included non-significant factor (Males, μ = 984 pg/mg CR, 95% CI: 798-1216; Females, μ = 982 pg/mg CR, 95% CI: 828-1164). Rearing history (hand reared, mother reared, surrogate reared) did not predict uOXT nor did it improve model fit (P >0.05).

Table 4.2. Linear mixed model output for evaluations of uOXT in the entire study population (Model 1), the female study population (Model 2), the adult female study population (females 14.00-34.99yrs; Models 3a & 3b), the entire male study population (Model 4), the bachelor group study population (Model 5) and the adult male in mixed-sex groups study population (Model 6).

Model 1 – Population Model								
Predictor Variable	**F**	**D.F.**	**P**	**Condition**	**Estimate**	**S.E.**	**t**	**P**
Intercept	8879.280	1,235.538	<0.001	-	2.733	0.077	35.610	<0.001
Sex	0.001	1,155.525	0.981	Female	0.236	0.096	2.475	0.015
				Male	0	0	0	0
Social Grouping	4.794	1,465.120	0.029	Social Group	0.074	0.034	2.190	0.029
				Solitary	0	0	0	0
Age Class	9.060	4,150.043	<0.001	Geriatric	0.022	0.114	0.193	0.847
				Adult	0.005	0.074	0.064	0.949
				Subadult	0	0	0	0
				Juvenile	0.372	0.119	3.121	<0.001
				Infant	0.720	0.172	4.195	0.002
Age Class*Sex	3.145	4,148.509	0.016	Geriatric (Female)	-0.042	0.141	-0.299	0.765
				Geriatric (Male)	0	0	0	0
				Adult (Female)	-0.147	0.104	-1.417	0.159
				Adult (Male)	0	0	0	0
				Subadult (Female)	0	0	0	0
				Subadult (Male)	0	0	0	0
				Juvenile (Female)	-0.389	0.175	-2.221	0.028
				Juvenile (Male)	0	0	0	0
				Infant (Female)	-0.610	0.205	-2.971	0.003
				Infant (Male)	0	0	0	0
Model 2. Female model								
Predictor Variable	**F**	**D.F.**	**P**	**Condition**	**Estimate**	**S.E.**	**t**	**P**
Intercept	4017.566	1,142.826	<0.001	-	2.888	0.094	30.571	<0.001
Age Class	2.986	4,68.949	0.025	Geriatric	-0.017	0.085	-0.199	0.843
				Adult	-0.142	0.073	-1.942	0.056
				Subadult	0	0	-	-

				Juvenile	-0.017	0.129	-0.132	0.896
				Infant	0.110	0.113	0.979	0.331
Group Type	5.303	1,187.035	0.022	Social	0.155	0.067	2.303	0.022
				Solitary	0	0	-	-
Model 3a. Adult female model								
Predictor Variable	**F**	**D.F.**	**P**	**Condition**	**Estimate**	**S.E.**	**t**	**P**
Intercept	5224.752	1,33.328	<0.001	-	2.621	0.093	28.224	<0.001
Lactating	8.506	1,34.636	0.006	Lactating	0.213	0.073	2.916	0.006
				Non-lactating	0	0	-	-
Birth Control	4.241	1,36.125	0.047	No	0.144	0.070	2.059	0.047
				Yes	0	0	-	-
Nulliparous	1.929	1,35.668	0.173	No	0.119	0.085	1.389	0.173
				Yes	0	0	-	-
Conspecific Offspring Present	3.865	1,37.728	0.057	No	0.124	0.063	1.966	0.057
				Yes	0	0	-	-
Model 3b. Adult female model with 3 outliers removed.								
Predictor Variable	**F**	**D.F.**	**P**	**Condition**	**Estimate**	**S.E.**	**t**	**P**
Intercept	7032.059	1,33.813	<0.001	-	2.623	0.080	32.642	<0.001
Lactating	13.392	1,35.755	0.001	Lactating	0.231	0.063	3.660	0.001
				Non-lactating	0	0	-	-
Birth Control	4.544	1,37.213	0.040	No	0.130	0.061	2.132	0.04
				Yes	0	0	-	-
Nulliparous	1.197	1.36.573	0.281	No	0.081	0.074	1.094	0.281
				Yes	0	0	-	-
Conspecific Offspring Present	6.788	1,38.513	0.013	No	0.142	0.054	2.605	0.013
				Yes	0	0	-	-
Predictor Variable	F	D.F.	P	Condition	Estimate	S.E.	t	P
Model 4. Male gorilla model								
Predictor Variable	**F**	**D.F.**	**P**	**Condition**	**Estimate**	**S.E.**	**t**	**P**
Intercept	4009.027	1,96.327	<0.001	-	2.648	0.084		
Social Grouping	6.033	3,144.864	0.001	Bachelor	0.154	0.051	3.028	0.003
				Fission-Fussion	0.301	0.085	3.554	0.001
				Mixed	0.027	0.038	0.700	0.485
				Solitary	0	0	0	0
Age Class	9.682	4,83.805	<0.001	Geriatric	0.139	0.111	1.252	0.214
				Adult	0.032	0.070	0.455	0.650
				Subadult	0	0	0	0
				Juvenile	0.500	0.115	4.332	<0.001
				Infant	0.764	0.177	4.308	<0.001
Model 5. Bachelor group model								
Predictor Variable	**F**	**D.F.**	**P**	**Condition**	**Estimate**	**S.E.**	**t**	**P**

Intercept	3355.959	1,23.740	<0.001	-	2.840	0.054	52.248	< 0.001
Mixed Generation Group	11.660	1,24.759	0.002	No	-0.318	0.093	-3.415	0.002
				Yes	0	0	-	-
YSB Present	4.155	1,24.829	0.052	No	0.193	0.095	2.038	0.052
				Yes	0	0	-	-
Tenure	5.580	1,21.341	0.028	-	0.012	0.005	2.362	0.028
Model 6. Mixed-sex adult male model								
Predictor Variable	**F**	**D.F.**	**P**	**Condition**	**Estimate**	**S.E.**	**t**	**P**
Intercept	5608.392	1,21.451	<0.001	-	2.773	0.037	74.889	<0.001

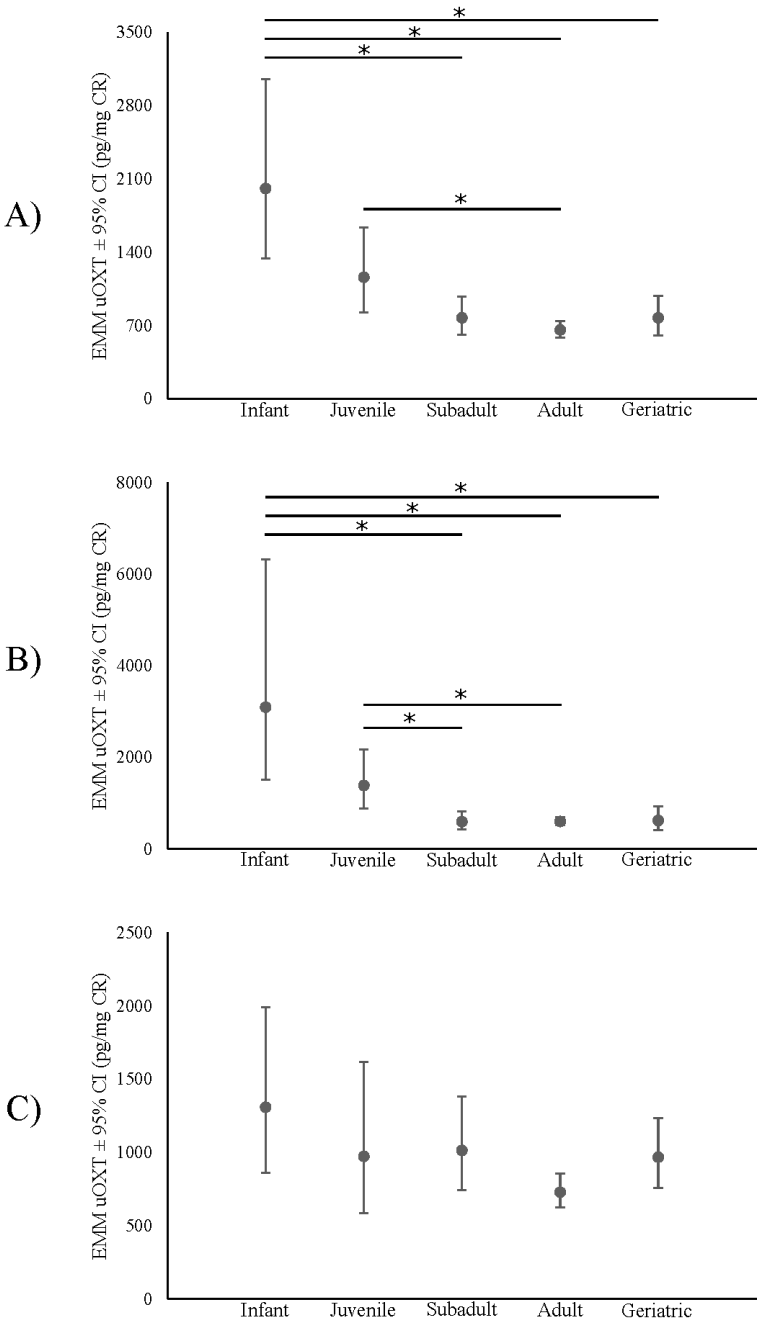

Figure 4.1. Estimated marginal mean (EMM) urinary oxytocin (uOXT) concentration varied by age class (A; $F_{4,150.043} = 9.060$, $P < 0.001$) and by the interaction of sex and age class (B, males; C; females; $F_{4,148.509} = 3.145$, $P = 0.016$). Significant pairwise comparisons ($P \leq 0.05$) are denoted by asterisks.

OXT Variation in Female Gorillas

For female gorillas, group type ($F_{1,187.035} = 5.303$, $P = 0.022$) and age class ($F_{4,68.949} = 2.986$, $P = 0.025$) significantly predicted uOXT concentration (Model 2, Table 4.2). Female gorillas that lived in social groups had on average 43% greater uOXT (μ = 1069 pg/mg CR, 95% CI: 914-1250) compared to females who lived solitarily (μ = 748 pg/mg CR, 95%CI: 536-1047). Overall, age was found to be a significant predictor of OXT, but all pairwise comparisons were non-significant ($P > 0.05$). Interactions between other factors were not significant and not retained in the model ($P > 0.05$).

For subsequent analysis of female variation, uOXT variation was assessed in adult females (14.00-34.99 yrs; Model 3a, Table 1). Overall, lactation ($F_{1,34.636} = 8.506$, $P = 0.006$) and use of HC ($F_{1,36.125} = 4.241$, $P = 0.047$) predicted uOXT. A female's reproductive state (nulliparous vs. parous; $F_{1,35.668} = 1.929$, $P = 0.173$) and the presence of a conspecific's offspring ($F_{1,37.728} = 3.865$, $P = 0.057$) both were non-significant predictors of uOXT but were retained due to improved model fit. Sibling presence, offspring presence, group size, the number of adult females present or any interactions between factors were not significant ($P > 0.05$) and were not retained in the model.

Upon analysis of model 3a's residuals, three outliers (2.5% of all adult female samples) were identified via histogram and P-P plot. Removal of these three outliers significantly improved model fit and produced the results for model 3b (Table 4.2). The only difference in result, other than improved model fit, was the presence of a conspecific's offspring became a significant contributor to uOXT ($F_{1,38.513} = 6.788$, $P = 0.013$), where in model 3a, it approached significance ($P = 0.057$). Given the improved model fit, details of the findings of model 3b are provided below.

Lactation ($F_{1,35.755} = 13.392$, $P = 0.001$), use of HC ($F_{1,37.213} = 4.544$, $P = 0.040$) and the presence of a conspecific's offspring ($F_{1,38.513} = 6.788$, $P = 0.013$) significantly predicted uOXT concentration. A females reproductive state did not predict uOXT concentration ($F_{1,36.573} = 1.197$, $P = 0.281$) but did contribute to improved model fit and was retained in the model. Sibling presence, offspring presence, group size, number of adult females present, or any interactions between factors were not significant and were not retained in the model ($P > 0.05$).

Lactating females had on average had 70% greater uOXT concentrations (μ = 1074 pg/mg CR, 95%CI: 820-1403) than non-lactating females (μ = 631 pg/mg CR, 95% CI: 540-736). Females not using HC had 35% greater uOXT concentrations (μ = 955 pg/mg CR, 95% CI: 778-1172) than females using HC (μ = 708 pg/mg CR, 95% CI: 565-889). Females that lived in groups where at least one conspecific was with their offspring had 28% lower uOXT (μ = 698 pg/mg CR, 95% CI: 581-841) than females in groups where conspecifics did not have offspring (μ = 968 pg/mg CR, 95%CI: 764-1213; Figure 4.2).

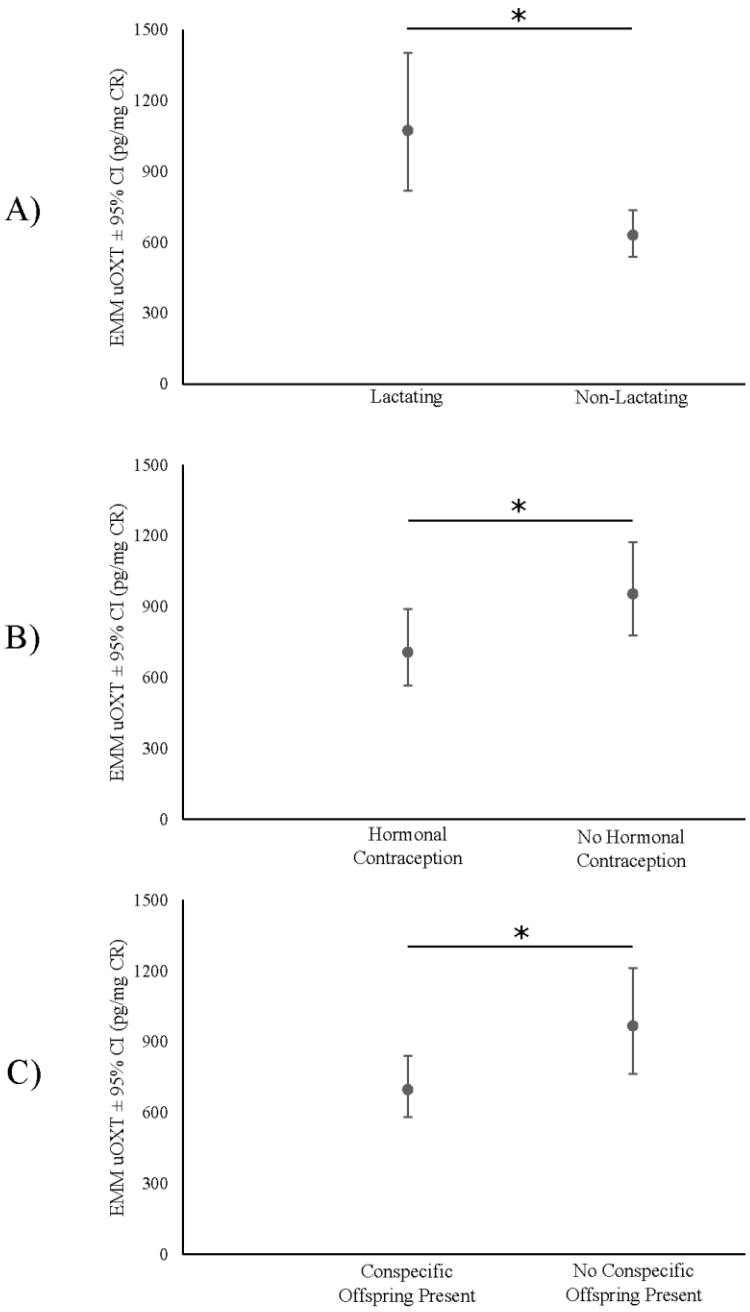

Figure 4.2. Comparison of estimated marginal mean (EMM) urinary oxytocin (uOXT) of adult female (14.00-34.99 yrs) gorillas. Lactating females had significantly greater uOXT than non-lactating females (A; $F_{1,35.755} = 13.392$, $P = 0.001$). Females not using hormonal contraception (HC) had significantly greater uOXT than those using HC (B; $F_{1,37.213} = 4.544$, $P = 0.040$). Lastly, females in groups containing an adult conspecific raising an offspring had significantly lower uOXT than females in groups without an adult conspecific raising an offspring (C; $F_{1,38.513} = 6.788$, $P = 0.013$).

OXT Variation in Male Gorillas

For male gorillas, age class ($F_{4,83.805}$, P <0.001) and group type ($F_{3,144.864}$ = 6.033, P = 0.001) were significant predictors of uOXT concentration (Model 4, Table 4.2). Interactions between factors were not significant and not retained in the model (P >0.05). Infant (μ = 3459 pg/mg CR, 95% CI: 1607-7447) and juveniles male gorillas (μ = 1879 pg/mg CR, 95% CI: 1208-2931) had significantly greater uOXT concentrations than subadult (μ = 589 pg/mg CR, 95% CI: 422-824; P < 0.001; P < 0.001), adult (μ = 637 pg/mg CR, 95% CI: 555-729; P < 0.001; P < 0.001), and geriatric male gorillas (μ = 820 pg/mg CR, 95%CI: 543-1239; P = 0.007; P = 0.037). No other differences between age groups were observed. Males in bachelor (μ = 1250 pg/mg CR, 95% CI: 984-1585) and fission-fusion groups (μ = 1746 pg/mg CR, 95% CI: 1156-2636) had significantly greater uOXT concentrations than males in mixed sex groups (μ = 920 pg/mg CR, 95% CI: 746-1138; P = 0.0011; P = 0.006) and solitary males (μ = 867 pg/mg CR, 95% CI: 668-1125; P = 0.016; P = 0.004). No difference between bachelor and fission-fusion males (P = 0.359), and mixed-sex and solitary males were observed (P = 1.00; Figure 4.3).

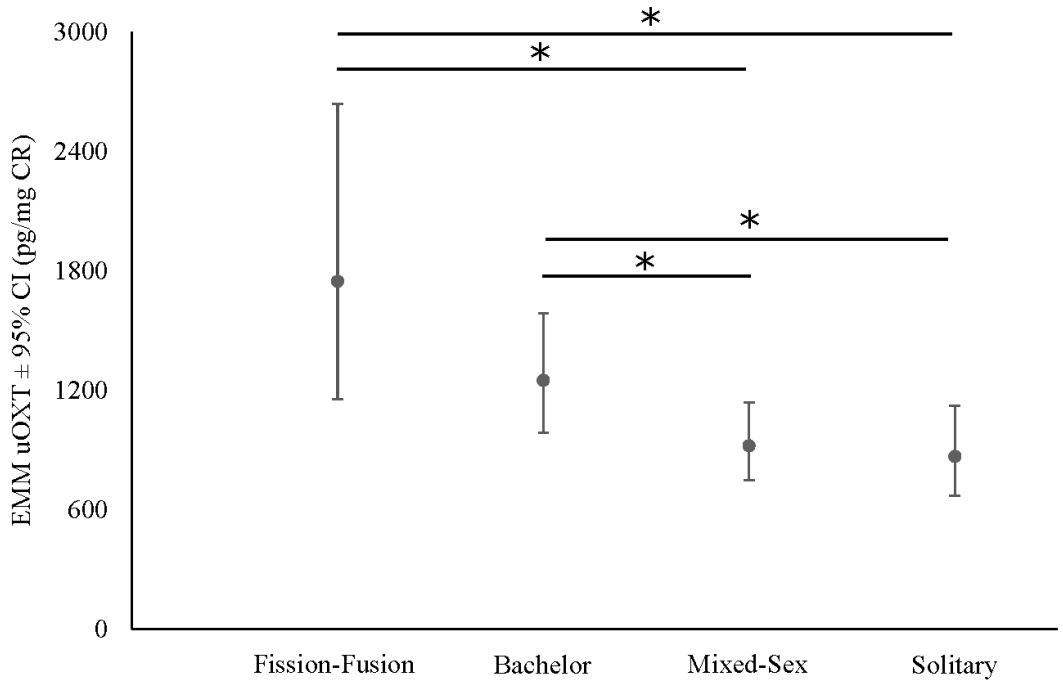

Figure 4.3. Group-type was a significant predictor of male urinary oxytocin (uOXT) concentration ($F_{3,144.864} = 6.033$, P = 0.001. Parwise comparisons found Estimated marginal mean (EMM) concentrations were greater in males living in fission-fusion and bachelor groups than males living in mixed-sex groups or as solitary individuals ($P \leq 0.05$).

Additional variation of uOXT within males living in bachelor groups (model 5, Table 1) and adult males in mixed-sex groups were assessed (Model 6, Table 4.2). For males in bachelor groups, group member age demographics ($F_{1,24.759}$, P = 0.002), the presence of a young silverback (YSB, male 14-19.99 yrs; $F_{1,24.829}$, P = 0.052), and length of group tenure (yrs, $F_{1,21.341}$, P = 0.028) significantly predicted uOXT concentration. Group size, age class, presence of mixed-sex groups or any interactions between factors were not significant and were not retained in the model (P > 0.05). For group age demographics, groups were classified as either single-generation, that is all males were of

the same age class, or as mixed-generation, that is at least one group member was in a different age class than the remaining group members, as defined and described by Leeds et al. (2015). Male gorillas in mixed generation bachelor groups on average had 108% greater uOXT concentrations (μ = 1076 pg/mg CR, 95% CI: 789-1466) than single generation groups (μ = 518 pg/mg CR, 95% CI: 414-647; Figure 4). Male gorillas in bachelor groups without a YSB had 56% greater uOXT concentrations (μ = 931 pg/mg CR, 95% CI: 684-1271) compared to groups with a YSB (μ = 597 pg/mg CR, 95% CI: 474-753; Figure 4.4). Length of group tenure was positively associated with uOXT. On average uOXT concentrations increased 33% in groups with more than a 10-year tenure.

For adult males in mixed-sex groups no fixed factors were significant or contributed to an improved model fit compared to a null model ($P > 0.05$; Model 6, Table 4.2). Specific factors tested were: group size, tenure as the dominant male (yrs), length of existing group tenure (yrs), presence of offspring, number of offspring present, number of adult females in group, presence of adult females off birth control and number of adult females off birth control.

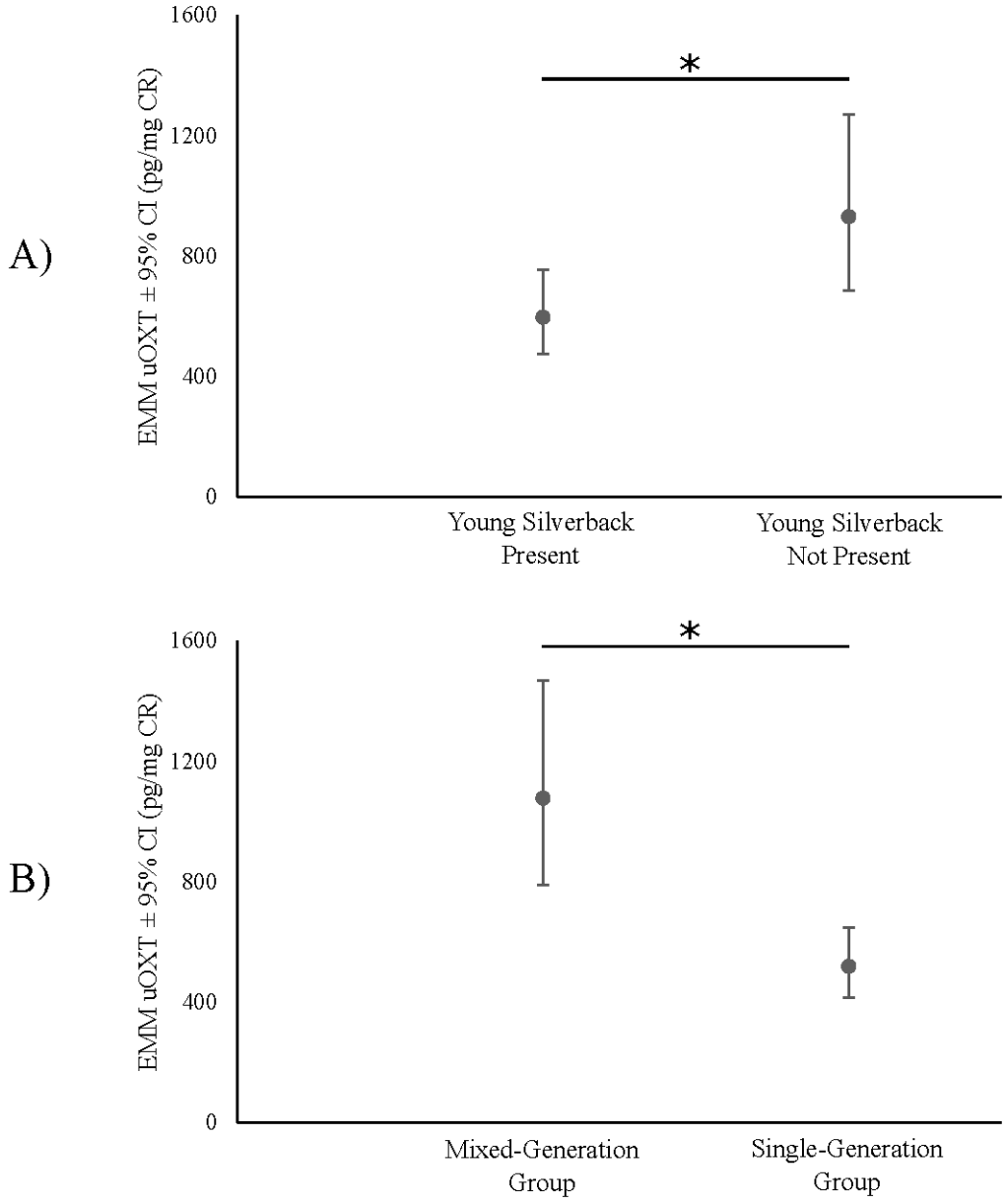

Figure 4.4. Estimated marginal mean (EMM) urinary oxytocin (uOXT) variation in male gorillas living in bachelor groups. Male gorillas in groups containing a young silverback (14.00-19.99 yrs; YSB) had significantly lower uOXT than males in groups without a YSB (A; $F_{1,24.829}$, P = 0.052). Males in mixed-generation groups, groups containing at least one male in a different age class than the rest of the group, had significantly greater uOXT than males in single-generation groups, groups containing males all in the same age class (B; $F_{1,24.759}$, P = 0.002).

Discussion

Our study found uOXT to vary in relation to a variety of group demographic and biological factors in WLG. Of particular note, uOXT was hypothesized to be greater in males living in bachelor groups than males living in mixed-sex groups. This was hypothesized due to the reproductive tactics and life history parameters of male WLG and was supported by our data. Furthermore, significant variation of OXT was found within bachelor groups, demonstrating a variety of social demographic factors relate to bond formation. Interestingly, no factors were found to predict uOXT in males in mixed-sex groups, further supporting that the bonds betweens male and female gorillas are comparatively weak. In addition, variation was found within adult female WLG, possibly a reflection of their competition with other females in their group, and across development of both sexes.

In male gorillas, fission-fusion and bachelor groups had significantly greater uOXT concentrations than males in mixed-sex groups and solitary individuals, and fission-fusion males had the largest uOXT concentrations on average. High uOXT concentrations in these males may reflect spiking of OXT following periods of social separation and reunion with conspecifics. Cotton top tamarins housed alone experience on average a four-fold increase in uOXT following pairing with a mate (Snowdon et al., 2010). OXT also increases synchronously with cortisol following acute stressors, such as during temporary isolation from a bond partner in male titi monkeys (Hinde et al., 2016). Stoinski et al. (2002) found that solitary male WLG had great corticoid concentrations than males in bachelor and mixed-sex groups. It is likely that reunion with social partners following social separation resulted in the observed elevated uOXT levels, and associated

variability, within fission-fusion males. Also of note, when socially housed and not solitary, four of the five fission-fusion males had an adult male companion. The uOXT concentrations observed in fission-fusion males may also be associated with male-male bonds that were also observed in bachelor groups and discussed in detail below.

This study provides evidence that male-male bond strength is comparatively stronger than between males and females due to greater uOXT concentrations observed in bachelor males than mixed-sex group males. Affiliation is strongly associated with kinship in non-human primates (Silk, 2002). Recent genetic evidence has found male gorillas form loose associations with related males by forming overlapping home ranges (Bradley et al., 2004). This pattern of dispersal is not true patrilineality but may be a species-specific form for WLG, demonstrating an innate ability for male gorillas to form bonds. Male mountain gorillas, instead of dispersing, commonly continue to reside in their natal group or transfer to new mixed-sex groups when they are young, with 61% of groups in the Virunga Massif containing more than one adult male (Gray et al., 2013). Thus there is significant evidence that males within the genus Gorilla form meaningful relationships with other males.

An alternative explanation is that in zoos, where male gorillas in bachelor groups do not range far distances for food nor compete for food and/or mates, the ability for males to form relationships is artificially boosted. In addition, it may be more difficult for adult males and females in mixed-sex groups to form relationships. In free-ranging populations females compete with other females and evidence suggests they do not form strong-bonds (Stokes, 2004). Male gorillas help manage the social dynamics of adult females (Breuer et al., 2016), but ultimately females are able to emigrate to new groups if

social tensions are high (Stokes et al., 2003). In a zoo environment, females do not have this option and males may spend more time managing females then forming relationships with them. To fully understand this, uOXT concentrations from free-ranging males are needed for comparison, as well as continued study of WLG social behavior and social dynamics.

Urinary OXT was positively associated with length of group tenure in bachelor group males, providing further evidence that male bonding is facilitated by kin recognition or long-term familiarity underlying kin recognition (Waldman, 1987). To our knowledge, these are the first data demonstrating that strength of social bonds, as measured by physiological changes, can increase with time. Data from humans has found that OXT is elevated, compared to controls, in the initial stages of romantic relationships (Schneiderman et al., 2012), but how this continues over the course of a relationship, and how human romantic relationships compare to kin-based relationships in non-human primates needs further investigation. This study provides evidence of a physiological mechanism that contributes to the well-documented behavioral cohesiveness of bachelor gorilla groups in zoos (Leeds et al., 2015; Stoinski et al., 2001; Stoinski et al., 2004a). In addition, these data further emphasize that bachelor groups provide a meaningful and important social grouping for young male gorillas in zoos that reflect evolved patterns of association between adult males.

Male gorillas in bachelor groups that contained a YSB had lower uOXT concentrations than males in groups without a YSB. The YSB developmental period is associated with major changes in hormones, specifically androgens (Stoinski et al., 2002), that result in behavior changes such as increased rates of initiating agonism

(Stoinski et al., 2013) and increased group rates of wounding compared to younger and older males (Leeds et al., 2015). This makes the social dynamics of bachelor groups with a YSB unstable or unpredictable compared to groups without a YSB. This instability likely leads to a more difficult time maintaining social bonds. For WLG in zoos, the YSB period is an unavoidable developmental period, but one that is relatively short (~6 yrs). Through proper behavioral management, modern zoo professionals relatively easily manage the YSB period. The intensity of this developmental period may also be a contributing factor to why bachelor groups are generally fleeting in free-ranging populations. Further study evaluating this unique developmental stage is needed; particularly more data from free-ranging populations will be informative as zoo populations have been the focus of research for some time (Leeds et al., 2015; Stoinski et al., 2002; Stoinski et al., 2013). Interestingly, mixed-generation bachelor groups had greater uOXT concentrations than single generation groups which was not in support of our initial hypothesis. In a multi-zoo study of wounding rates, mixed-generation groups, even in the absence of a YSB, had significantly greater wounding rates than single-generation groups (Leeds et al., 2015). If mixed-generation groups have greater wounding rates, then it is surprising that such groups have greater uOXT, since increased wounding should make social bonds less stable. It could be that the increase in uOXT may be the result of increased affiliative behavior if younger males are in mixed groups (Stoinski et al., 2013). The mean age of single generation groups was 21.98 yrs compared to 18.34 yrs in mixed-generation groups. The benefit of forming single-generation groups proposed by Stoinski et al. (2004b) was to minimize injury inducing agonsim by having similarly sized individuals in one group and to maximize affiliative behavior by forming

groups of young males that then grow up together. To date, only this study and Leeds et al. (2015) have empirically evaluated differences between these group types. More research is needed to evaluate social demographic factors within bachelor groups to better inform their respective husbandry and welfare.

The combined reproductive tactics of WLG, that include regular group transfer by females (Hagemann et al., 2018; Robbins et al., 2004), sexual coercion of females by males (Breuer et al., 2016) and potentially short dominance tenures for males (Breuer et al., 2010), minimize the opportunity for the exchange of affiliative and cooperative behavior, that ultimately facilitates bond formation (Seyfarth and Cheney, 2012). It is thus not surprising that male uOXT was relatively low in mixed-sex groups, given the limited affiliative behavior males and females engage in. In the closely related mountain gorilla, meaningful relationships between adult males and infants in mixed-sex groups have recently been described (Rosenbaum et al., 2011; Rosenbaum et al., 2018). There are data to support male parental behavior in WLG (Enciso et al., 1999; Tilford and Nadler, 1978) but empirical data to the degree that are available for mountain gorillas on WLG adult male-infant relationships are not available. This study found no effect of infant presence on adult male gorilla uOXT, indicating that this relationship may also be weak in terms of bond formation. The role of adult males may strictly be that of protector from predation and infanticide, rather than meaningful social partner, though additional study is needed to better understand this relationship. Most interesting was that uOXT did not differ between males in mixed-sex groups and solitary males. This provides further comparative evidence that male and female gorillas do not form strong bonds. In zoos, solitary males are rare due to concerns over animal welfare. Further study of free-ranging

solitary male WLG behavior and physiology may help improve our understanding of this life stage. Given the rarity of solitary male gorillas in zoos, further evaluation of the adaptiveness of this life stage and the associated physiological consequences are likely best studied in free-ranging populations.

This is the first study of any species, to directly assess differences in OXT related to age across all developmental stages in a large population. In general uOXT concentrations were greater in infant and juvenile WLG compared to subadult, adult and geriatric WLG. Oxytocin has been documented to decrease with age in both naturally and artificially, via ovariectomy, aged mice (Elabd et al., 2008; Elabd et al., 2014), but the data from non-human primates is less clear (Parker et al., 2010a; Winslow et al., 2003). Further data are needed to understand how OXT concentrations change in relation to infant development, but given infancy is the bonding period between both parent and offspring, it is not surprising that elevated uOXT levels were observed in infant WLG. Specific to WLG, infants spend a large proportion of their time clinging to their mother, this increased time spent in body contact may increase OXT concentrations, similar to how affiliative interactions between human parents and offspring increase infant OXT (Clark et al., 2013).

Subadult, adult and geriatric female gorillas had significantly greater uOXT than males of those age classes. This difference may be reflective OXT spikes as a result of female reproductive physiology, specifically ovulation and lactation. OXT concentrations have been documented to fluctuate during ovulatory cycling across primate species including humans (Mitchell et al., 1981; Salonia et al., 2005), rhesus macaques (Falconer et al., 1980), and baboons (Moscovice and Ziegler, 2012). Fluctuations caused by

ovulation, in addition to fluctuations caused by lactation (White-Traut et al., 2009), if lactating, may be proximate causes for mature and maturing female gorillas to have greater baseline uOXT concentrations than male gorillas. We initially did not predict to see any differences between the sexes due to the ambiguity of findings from previous research evaluating sex and OXT in primates (Crockford et al., 2013; Imamura et al., 2017; Miller et al., 2013; Motoki et al., 2016; Preis et al., 2018). This is an area of study that needs further evaluation and clarification.

Infant male gorillas had significantly greater uOXT concentrations than infant female gorillas. Maestripieri and Ross (2004) studied the development of play in infant WLG and found that males initiated play behavior significantly more than infant females, and infant males initiated play with a wider range of social partners including the silverback and unrelated adult females than infant females. This difference in play behavior may contribute to the differences observed in uOXT. Conversely, or in addition, given the frequency of interaction between infant and parent it may be that younger infants in general have greater OXT as a result of the increased attention mothers provide when infants are younger and less independent. Maestripieri et al. (2002) found that time in proximity between mother and infant had a significant linear decrease over the first four years of an infant's life, indicating that younger infants may receive and/or solicit more attention from their mother than older infants. Interestingly, the same study also found that older mothers spent more time in proximity to their offspring than younger mothers, indicating mother experience may also be a significant factor in the social experience of infant gorillas. In our study, infant males were on average 1.00 yr of age and female infants 1.76 yr of age. This age difference may have contributed to the

observed difference of uOXT between infant male and female gorillas if parental investment decreased with age. It should be noted that in the preliminary data processing, a 1.0 yr old infant female was removed from analysis, as all four of her urine samples were detected as outliers greater than 2.5SD above the mean of all samples (and were the highest sample concentrations of all other samples). If her samples had not violated assumptions of our statistical tests and had been retained, this difference between male and female infants may not have been observed. Overall, it is likely that infancy is associated with significant changes in OXT related to developing social bonds with one's mother and group members. This likely contributes to the observed elevated levels in all infants as well as the variation observed between infants. Future research evaluating OXT in infancy (of any species) would improve our understanding of developmental biology and prove to be a useful endeavor. Utilizing primate populations in AZA zoos, similar to this study, may be particularly informative given AZA's commitment to maintaining species-typical social groupings and providing complex and enriching environments necessary for species-typical infant development, while simultaneously allowing for non-invasive sample collection (i.e. saliva or urine) that would be difficult, if not impossible, in free-range settings.

Human studies have found an increase in salivary OXT prior to and following nursing (Carter et al., 2007; White-Traut et al., 2009). Given these spikes in OXT related to nursing, and other possible fluctuations in OXT associated with infant care and bonding, it makes sense that uOXT levels of lactating gorilla females, which represent an accumulation of OXT build up over time, were greater than non-lactating females. The use of HC was also associated with decreased uOXT concentrations in female gorillas. In

humans, HC use has been associated both with increased plasma OXT concentrations (Silber et al., 1987; Stock et al., 1989) and no change in plasma OXT (Feldman et al., 2011). Recent experimental evidence in humans suggests that HC may disrupt natural OXT function (Scheele et al., 2015). Given limited study, general patterns of HC effect on OXT is unclear and in need of further investigation. The use of HC is necessary for the management of zoo populations, particularly in relation to population and inbreeding control. It is unknown if this change in OXT is significant enough to change behavior, but future evaluations of behavioral differences of gorillas on and off HC would be informative to the care of zoo populations.

The presence of a conspecific's offspring was associated with lower uOXT levels in adult female gorillas. In mountain gorillas, the presence of an offspring was the strongest predictor of a females time spent in close proximity to the silverback (Harcourt, 1979) and females have been reported to compete for proximity towards the silverback (Watts, 1992). A trend for adult female WLG to spend more time in close proximity to the silverback than other groups members has been observed as well (Stokes, 2004). In addition, association indexes in mountain gorillas are stronger between adult males and infants than adult males and adult females (Rosenbaum et al., 2016b). Combined this can be interpreted that females, and offspring, compete for social interaction from the silverback. An increase in competition for social interaction may ultimately result in lower uOXT concentrations if females are out competed, the odds of which increase if infants are present. This competitive aspect of gorilla behavior has been primarily studied in the closely related mountain gorilla. More evaluation of this behavior in WLG would be useful to improving our understanding of gorilla social dynamics.

No difference in uOXT was found between mother-reared gorillas and gorillas who experienced any form of an atypical upbringing. Previous study of laboratory macaques found that OXT did differ between mother-reared and those not mother-reared (Winslow et al., 2003), however; allostatic load, a physiological measure of stress, was not associated with rearing in a study of gorillas in zoos (Edes et al., 2016). For gorillas, this may mean that an atypical upbringing in zoos, may not have a negative effect on the oxytocinergic system. That being said, behavioral differences between mother-reared and non-mother reared gorillas have been noted (Meder, 1989; Ryan et al., 2002; Stoinski et al., 2004a) indicating there are likely negative effects of an atypical upbringing on gorilla development.

This study provides data that further support the theory that male WLG form bonds with other males, but for the first time provides evidence that these bonds may be comparatively stronger than bonds formed with females. Reproductive tactics of WLG that include high rates of group transfer and agonism likely ultimately contribute to weak bond formation between males and females. However, the alternative explanation that these findings may be an artifact of zoo-living are still in need of study and clarification. It is still unclear what directly contributes to bond formation between males, both proximately and ultimately, though kin selection is likely one primary proximate mechanism. This was a novel study of WLG social dynamics from a physiological perspective that has improved our understanding of what shapes social bonds, as measured by uOXT, in a species that engages in little social behavior. The study of OXT in primates has drastically improved our understanding of primate sociality and evolution. The continued study of zoo populations has many benefits including ease of

access to both species and biological samples, reliable storage of samples, and known histories of individuals. Scientists interested in studying OXT in primates, or any other species, should consider zoo populations as study participants.

CHAPTER FIVE: Evaluating the association between urinary oxytocin and animal care staff perceptions of bonding in western lowland gorillas (*Gorilla gorilla gorilla*) living in zoos.

Introduction

The regular interactions animal care staff (ACS) have with their respective animal charges in zoos provide unique and meaningful insights into zoo animal behavior that ultimately guide animal care (Whitham and Wielebnowski, 2009). The inclusion of ACS knowledge into the study of zoo animal behavior has recently included quantifying the prevalence of abnormal behavior in primates (Cassella et al., 2012; Jacobson et al., 2016) and describing patterns of temperament (Hopper et al., 2018; Pederson et al., 2005). By utilizing ACS knowledge, study sample size can increase compared to studies that utilize more traditional single-observer techniques. Increased sample size allows for more reliable, reproducible and meaningful findings that can be used to inform husbandry and management decisions within zoos.

When compared to more traditional data collection methods, ACS knowledge of animal behavior has been validated in certain contexts, but not all. A study of elephant (*Loxodonta africana*) social behavior found that the social rank of adult females correlated well between direct observation and ACS ratings. Comparison of specific affiliative behavior associated with bonding had mixed reliability. Observed rates of body movement behaviors such as approaches and displacements correlated with ACS ratings, but no association was found between observed rates of conspecific trunk touching behaviors and ACS ratings of these behaviors (Freeman et al., 2010). Similarly, a study

of black rhinoceros (*Diceros bicornis*) behavior found multiple correlations between ACS ratings of behavior and behavioral responses of the rhinoceros in experimental novelty tests (Carlstead et al., 1999). Less et al. (2012) conducted a multi-zoo assessment of inactivity in western lowland gorillas (WLG; *Gorilla gorilla gorilla*) comparing both systematically collected behavior observations and ACS ratings of inactivity. Both time spent immobile (stationary) and time spent idle, (not engaging in any active behavior) positively correlated with ACS ratings of inactivity. In the only study comparing ACS perceptions of animal behavior to a physiological measure, Wielebnowski et al. (2002) found that clouded leopards (*Neofelis nebulosa*) rated by ACS to engage in or exhibit behaviors associated with stress had greater fecal corticoid concentrations, a physiological measure of stress.

Providing appropriate social environments for animals in zoos is significant to their psychological and physiological well-being (for review see Price and Stoinski, 2007). In contrast to zoo populations where humans manage the composition of social groups, individuals in free-ranging populations have some choice in terms of their social partners. For example social preferences contribute to fission-fusion decisions in free-ranging spider monkeys (Busia et al., 2017). Due to a lack of social choice, the strength of social bonds between individuals in zoos, and ultimately the quality of the social environment, may vary more than free-ranging populations. Both formal data collection and monitoring by ACS are utilized to monitor the social environment of animals in zoos to ensure groups are functioning well as a unit. For certain species quantifying the strength of social bonds, and ultimately the quality of the social environment, is relatively easy by assessing the exchange of affiliative behaviors between individuals (Seyfarth and

Cheney, 2012; Silk et al., 2010a). For species that engage in little affiliative behavior assessing the quality of the social environment can be a challenge given such behaviors are viewed rarely and exchanges are often unclearly distributed. A prime example of such a species is the WLG given they devote less than 1% of their time in free-range settings (Masi et al., 2009; Stokes, 2004) and less than 5% in zoo settings engaging in affiliative behavior (Hoff et al., 1997; Ross et al., 2010).

Given WLG engage in low rates of affiliative behavior, evaluation of variation in physiological indicators of bonding may improve our understanding of WLG bonding. Oxytocin (OXT) is a mammalian neuroendocrine hormone synthesized in the paraventricular nucleus and supraoptic nuclei of the hypothalamus (Gainer, 2012). Most notably, OXT is studied for its regulation of child rearing behavior, particularly nursing and mother-infant attachment (Carter et al., 2007; Grewen et al., 2010; Neumann et al., 2000a; Uvnas-Moberg et al., 1985; White-Traut et al., 2009). It has been hypothesized that OXT was evolutionarily co-opted to serve as a means to facilitate affiliation and bonding beyond the mother-infant relationship (Wittig et al., 2014). In humans, OXT levels increase following affiliative interactions, and is predominately studied following interactions between bonded pairs such as married couples and parents and children (Feldman et al., 2010; Fries et al., 2005; Holt-Lunstad et al., 2008; Seltzer et al., 2010). In non-human primates, OXT has similarly been observed to increase following affiliative interaction including food sharing (Finkenwirth et al., 2016; Samuni et al., 2018; Wittig et al., 2014), and time spent in close proximity to mating partners (Moscovice and Ziegler, 2012).

Since OXT concentrations increase following affiliative or cooperative interaction, baseline levels of OXT have been positively associated with the strength of one's social bonds. In married couples, higher baseline OXT levels are associated with a stronger social bond as assessed by the Dyadic Adjustment Scale, the most commonly used measure of relationship quality (Holt-Lunstad et al., 2014). Additionally, higher baseline OXT levels were associated with couples who hugged and massaged each other more frequently (Light et al., 2005). In non-human primates, OXT is positively associated with affiliative and sexual behavior in pair-bonded tamarins (*Saguinus oedipus*, Snowdon et al., 2010). Similarly, more strongly bonded marmoset (*Callithrix jacchus*) dyads living in family groups were found to have synchronized OXT fluctuations over time, further indicating baseline OXT concentrations are representative of social bond strength and can be used to differentiate the quality of social relationships (Finkenwirth et al., 2015). The relationship between OXT and social bonding was further strengthened by a study of chimpanzee (*Pan troglodytes*) grooming. Following grooming with a bonded partner, as defined by the frequency and direction of affiliative behavior, OXT significantly increased compared to baseline conditions, but OXT did not increase following grooming with a non-bond partner, further indicating variation in OXT is directly related to the strength of the dyad's social bond (Crockford et al., 2013). Baseline levels of OXT have also been reported to be lower in individuals with weaker social bonds. In humans, OXT levels are lower in distressed married couples compared to more stable couples (Holt-Lunstad et al., 2014). In non-human primates, social isolation has been shown to decrease OXT levels compared to when they were socially housed (Leeds et al., 2018; Snowdon et al., 2010). In addition, rhesus macaques (*Macaca mulatta*) who

were nursery reared in a laboratory by humans, an atypical social upbringing for their species, had lower OXT levels compared to mother reared individuals (Winslow et al., 2003).

Little is known about what social demographic factors contribute to social bond strength in WLG. Utilizing ACS perceptions of bonding in conjunction with measurements of OXT may prove useful to identify significant group-level factors associated with bonding in gorillas. The purpose of this study was to evaluate if ACS perceptions of bonding predicted concentrations of OXT, a physiological indicator of bond strength, in WLG. ACS were surveyed about the bond strength of the gorilla groups in their care. Survey questions targeted the overall quality of the groups' social environment and also focused on two behaviors related to social bonding: social proximity and behavioral synchrony. Time spent in close proximity has been used as a species-specific indicator of social tolerance in gorillas and is considered a contributor to group cohesiveness (Nakamichi and Kato, 2001; Nakamichi et al., 2014; Stoinski et al., 2004a). Behavioral synchrony has been associated with social bond strength (Fichtel et al., 2011; Lakens and Stel, 2011), but not specifically in WLG. Furthermore, both close social proximity (Moscovice and Ziegler, 2012) and behavioral synchrony have been documented to covary with OXT in humans and marmosets (Feldman et al., 2011; Finkenwirth et al., 2015). These behavior patterns should be more easily visible to ACS than behaviors such as play and grooming that occur at low frequencies, if at all, in WLG groups, and thus represent useful behaviors for this assessment.

Lastly, we asked if ACS could identify a peripheral group member. Social bond strength varies between group members, resulting in individuals being more or less

integrated within their respective group. A meta-analysis found that primates in social groups who are less socially integrated, in that they receive or engage in less affiliative behavior, have greater cortisol concentrations than individuals who are more socially integrated (Abbott et al., 2003). Thus it is likely that being less socially integrated, or a peripheral group member, may have effects on the oxytocinergic system as well, given this system is related both to social relationships and the stress response (Parker et al., 2005). Here, we hypothesized that more peripheral group members would have lower OXT concentrations as a result of maintaining weaker social bonds with conspecifics.

Methods

Survey

Surveys were completed for 38 gorilla groups (mixed-sex, n = 24; bachelor, n = 14; n = 63.62 gorillas) at 22 Association of Zoos and Aquariums (AZA) institutions. Surveys were completed by animal caregivers and animal care managers familiar with the gorillas (collectively referred to as ACS). On average 3.34 staff completed surveys for each individual gorilla group (range 1-7). Surveys contained five statements that ACS were asked to respond to using a five point Likert scale and one question identifying if there was a peripheral member (Table 1). For this final peripheral question, answers were included only if all completed surveys for each zoo identified the same gorillas (no zoo listed more than one peripheral member per group). In addition, this question was only asked for groups that had at least three members, due to difficulty of identifying a peripheral member of a social dyad. There was not 100% agreement among surveyees for six group surveys (mixed-sex, n = 5; bachelor, n = 1) and thus these groups were removed from analysis for this question. Final sample size for the peripheral group

member question was 29 social groups (mixed-sex groups, n = 23; bachelor, n = 6; n = 34.48 gorillas).

Table 5.1. Survey question information.

Question Label	Question	Answer Type	Answer Choices
Appropriate Social Environment (ASE)	This group provides an appropriate social environment for its members.	Likert	Strongly Agree Agree Neutral Disagree Strongly Disagree
Cohesion (COH)	This gorilla group is cohesive.	Likert	Strongly Agree Agree Neutral Disagree Strongly Disagree
Proximity (PROX)	Group members, by choice and not husbandry restraint, spend time together in close proximity (within 5m).	Likert	Always Often Sometimes Rarely Never
Tolerance (TOL)	Group members are tolerant of each other when in close proximity (within 5m).	Likert	Always Often Sometimes Rarely Never
Behavioral Synchrony (SYNC)	Group members engage in behaviors synchronously (i.e. feed at the same time, rest at the same time).	Likert	Always Often Sometimes Rarely Never
Peripheral Status	Is there an individual(s) in this group that is less bonded and/or more peripheral compared to other group members?	Yes/No	Yes/No If yes, include gorilla ID.

Urine Collection and Enzyme Immunoassay

Urine samples (n = 453) were collected from gorillas (n = 63.62) living at the 22 participating institutions. Excluding gorillas from one zoo participating in a long-term study of OXT, an average of 2.92 urine samples (range 1-8) were collected for each gorilla. For 1.2 gorillas participating in the long-term study 83, 6 and 10 samples were collected, respectively. To control for diurnal variation of OXT (Leeds et al., 2018), urine

samples were collected opportunistically from 0700-1000, once per day. Samples were stored in 15-ml conical centrifuge tubes (Falcon, Corning, NY) and kept frozen in manual defrost freezers or -80°C freezers (depending on institution). Samples were shipped overnight frozen on dry ice to the endocrinology laboratory of Cleveland Metroparks Zoo (CMZ) where samples were thawed and separated into multiple aliquots, to avoid multiple freeze-thaw cycles, and then stored at -80°C until assay. Time from collection to assay varied by institution. Samples were assayed on average within 6 mo of collection (μ = 188.89 days).

Samples were assayed for OXT using a commercially available enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI). Manufacturer instructions were followed except samples were dried using an Evaporack (Cole Parmer, Vernon Hills, IL) and not a Speedvac, inside a warm water bath (32°C). In addition, samples were not extracted (Leeds et al., 2018). Briefly, dried samples were reconstituted in assay buffer to the original (neat) volume. To control for urine concentration, all urine samples were indexed by creatinine concentration, using the procedure of Brown (1998). Concentration of OXT was measured in the samples using a spectrophotometer (Biotek Instruments, Inc., Winooski, VT) at 450-nm wavelength using Gen5 software (Biotek Instruments, Inc.). Mean inter-assay coefficients of variation (CV) for controls and standards were below 15% (μ = 8.60). Mean intra-assay (n = 2 per sample) CV for study samples, controls and standards were below 10%. Controls were a low (40 pg/ml) and high concentration (1600pg/ml) of OXT standard in assay buffer.

Data Analysis

Inter-rater reliability across all five questions for each gorilla group was assessed using mean percent agreement. For groups assessed by two or more raters, the highest and lowest score for each Likert question were subtracted from each other. The resulting value for each of the five questions were then summed and divided by the total number of questions (five) to generate a mean difference score between raters (range: 0.0-1.2). This mean difference score was then divided by five (each question was out of five) to generate a mean disagreement percentage across all questions. No raters had a mean disagreement greater than 25% (i.e. greater mean agreement of >75%).

For analysis, survey scores were averaged across raters for each group to generate a numeric value for each question for each group. Descriptive statistics in conjunction with t-tests assuming unequal variance were utilized to assess differences in survey answers between subsets of data ($\alpha = 0.05$). Specifically, variation in survey answers was assessed by group-type (mixed-sex or bachelor) and by variation within each respective group type. For mixed-sex groups, the gorilla SSP recommends that groups be composed of one adult male and at least three adult females. Given this recommendation is well known amongst participating institutions, variation in survey responses were assessed between groups with at least (n = 7) and less than three adult females (n = 17). Adult females in this study were categorized as ≥14 yrs, this differs slightly from the SSP recommending females ≥10 yrs for breeding, and thus considering them adults. However, given the mean age of female gorillas in the SSP with breeding recommendations (n = 52) is 19.5 yrs, and a previous evaluation of gorilla uOXT used 14 yrs as the beginning age for adults, we continued with the classification of 14 yrs for adult females to be able

to control for known age related variability in uOXT. Answers were further compared between mixed-sex groups with (n = 12) and without an infant or juvenile in the group (n = 12) to evaluate how their presence affected survey scores. For bachelor groups, the presence of a young silverback (YSB; male aged 14-20 yrs) can increase rates of wounding (Leeds et al., 2015) and agonistic behavior (Stoinski et al., 2013b). Thus survey answers were compared between groups with (n = 8) and without (n = 6) a YSB. In addition, group size (dyads, n = 8 or > two males, n = 7) was evaluated and if bachelor groups were composed of males all of the same age class (single generation group, n = 8) or males of at least two different age classes (mixed-generation group, n = 6). Mixed-generation groups have increased wounding rates compared to single generation groups (Leeds et al., 2015) and formation of single-generation groups are generally recommended (Stoinski et al., 2004b).

Variation in survey answers were used to frame linear mixed models (LMM; MIXED; SPSS V. 20, IBM, Chicago, IL) that evaluated uOXT variation in subjects as an outcome measure. Models were built using a top down approach with a maximum likelihood approximation, where survey scores, group factors identified in the descriptive analysis, and factors with known uOXT variability were included as fixed factors in the LMM. Non-significant fixed factors ($P > 0.05$) were removed unless they were related to the research question (survey scores or group factor with associated survey score variability) or if the non-significant fixed factor contributed to improved model fit. The lowest -2 log likelihood (-2LL), Akaike information criterion (AIC), and Bayesian information criterion (BIC) were used to assess model fit. Gorilla identity was included as a random factor in all models using an identity covariance structure. Degrees of

freedom for fixed effects were calculated using the Satterthwaite approximation. To assure that the residuals of all models met the assumption of normal distribution, residuals were visually checked via P-P plots and histograms. No deviations from normality were found. To test for multicollinearity of fixed factors, a linear regression for each model was run (without the random factor). Variance inflation factors and tolerance statistics were checked for each factor, with no factors approaching or exceeding threshold values (Field, 2009).

Results

Appropriate Social Environment (ASE)

ASE scores averaged 4.12 (SE = 0.10; range 2-5), and scores did not differ between mixed-sex and bachelor groups ($t = 0.88$, $df = 34$, $P = 0.39$; Table 2) or within bachelor groups for any of the factors tested (Table 3). Across all group types, after controlling for age ($F_{4,141.946} = 9.319$, $P < 0.001$), the interaction of age and sex ($F_{5,143.055} = 5.295$, $P < 0.001$) and group type ($F_{1,262.146} = 7.027$, $P = 0.009$) ASE score did not predict uOXT ($F_{1,178.891} = 0.353$, $P = 0.533$).

ASE score variation occurred within mixed-sex groups. Groups with three or more adult females had significantly higher ASE scores than mixed-sex groups with less than three adult females ($t = -3.14$, $df = 21$, $P = 0.005$). No other within group type variation was observed (Table 4).

To evaluate this difference in ASE score, a LMM assessing uOXT for mixed-sex groups that had at least or less than three adult females were run. For groups with less than adult three females, ASE score did not significantly predict uOXT ($F_{1,84.763} = 0.540$, $P = 0.465$), when controlling for age ($F_{4,61.095} = 3.251$, $P = 0.018$), lactation state ($F_{2,54.930} =$

3.130, P = 0.052) and the interaction of age and sex ($F_{3,66.385}$= 2.104, P = 0.108). For groups with at least three adult females, ASE score did not significantly predict uOXT ($F_{1,33.871}$=0.639, P = 0.430), when controlling for age ($F_{4,33.088}$= 2.932, P = 0.035) and the interaction of age and sex ($F_{3,34.759}$= 6.211, P = 0.002).

Table 5.2. Comparison of mean survey scores by group type.

Survey Question	Mean Score (SE)		Statistics		
	Mixed-Sex Group	Bachelor Group	t	df	P
ASE	4.06 (0.14)	4.28 (0.14)	0.88	34	0.39
COH	4.05 (0.15)	4.12 (0.17)	0.96	31	0.34
PROX	3.69 (0.13)	3.34 (0.22)	-1.26	23	0.22
TOL	3.84 (0.10)	3.62 (0.14)	-1.07	25	0.30
SYNC	4.09 (0.08)	4.15 (0.11)	0.62	25	0.54

Table 5.3. Comparison of mean survey scores within bachelor groups.

Survey Question	Mean Score (SE)			Mean Score (SE)			Mean Score (SE)		
	Dyad	≥3 Males	P	YSB Present	No YSB Present	P	Mixed Generation	Single Generation	P
ASE	4.22 (0.22)	4.34 (0.19)	0.69	4.11 (0.22)	4.51 (0.11)	0.13	4.20 (0.29)	4.34 (0.13)	0.68
COH	4.11 (0.27)	4.04 (0.23)	0.86	3.86 (0.25)	4.36 (0.16)	0.13	3.87 (0.31)	4.23 (0.18)	0.35
PROX	3.06 (0.28)	3.55 (0.30)	0.26	3.24 (0.30)	3.39 (0.31)	0.73	3.47 (0.24)	3.10 (0.38)	0.44
TOL	3.58 (0.20)	3.61 (0.22)	0.90	3.42 (0.19)	3.83 (0.18)	0.15	3.36 (0.25)	3.78 (0.14)	0.18
SYNC	4.26 (0.20)	4.05 (0.08)	0.36	3.95 (0.05)	4.44 (0.20)	0.06	3.98 (0.02)	4.29 (0.18)	0.12

Cohesion (COH)

COH scores averaged 3.86 (SE = 0.15, range 2.0 -5.0). COH scores did not differ between mixed-sex and bachelor groups (t = 0.96, df = 31, P = 0.34; Table 2). or within either mixed-sex (Table 4) or bachelor groups (Table 3). When controlling for age ($F_{(1,142.821)}$ = 10.131, P < 0.001), group type ($F_{1,343.269}$ = 9.827, P = 0.004) and the interaction of age and sex ($F_{5,144.316}$ = 5.744, P < 0.001), COH score did not predict uOXT ($F_{1,167.709}$ = 0.470, P = 0.494).

Proximity (PROX)

PROX scores averaged 3.59 (SE = 0.11; range 2.0 - 4.5. PROX scores did not differ between mixed-sex and bachelor groups (t = -1.26, df = 23, P = 0.22; Table 2), and no variation was observed within mixed-sex (Table 4) or bachelor groups (Table 3). When controlling for age ($F_{4,142.859}$ = 10.278, P < 0.001), group type ($F_{1,356.209}$ = 8.399, P = 0.004) and the interaction of age and sex ($F_{5,144.077}$ = 5.741, P < 0.001), PROX scores did not predict uOXT ($F_{1,135.041}$ = 0.804, P = 0.372).

Table 5.4. Comparison of mean survey scores within mixed-sex groups.

	Mean Score (SE)			Mean Score (SE)		
Survey Question	≥3 Adult Females	<3 Adult Females	P	Infant Present	No Infant Present	P
ASE	4.60 (0.14)	3.90 (0.17)	0.005*	4.32 (0.25)	3.90 (0.14)	0.15
COH	4.30 (0.19)	3.67 (0.19)	0.11	3.80 (0.25)	3.91 (0.19)	0.74
PROX	3.91 (0.06)	3.73 (0.01)	0.47	3.72 (0.17)	3.84 (0.11)	0.52
TOL	3.89 (0.34)	3.50 (0.17)	0.08	3.82 (0.13)	3.40 (0.21)	0.10
SYNC	4.34 (0.11)	3.97 (0.08)	0.02*	4.11 (0.01)	4.06 (0.06)	0.70

*Statistically significant

Tolerant (TOL)

TOL scores averaged 3.78 (SE = 0.08; range 2.0 - 4.5. TOL scores did not differ between mixed-sex and bachelor groups (t = -1.068, df = 25, P = 0.30; Table 2), and no variation in TOL scores were found within either mixed-sex (Table 4) or bachelor group-types (Table 3). When controlling for age ($F_{4,141.607}$ = 10.726, P < 0.001), group-type ($F_{1,339.836}$ = 8.292, P = 0.004) and the interaction of age and sex ($F_{5,142.777}$ = 5.776, P < 0.001), PROX score did not predict uOXT ($F_{1,128.361}$= 2.999, P = 0.086).

Synchrony

SYNC scores averaged 4.10 (SE = 0.06; range 3 – 5). There was no difference between the SYNC scores of mixed-sex and bachelor groups (t = 0.621, df = 25, P =

0.54; Table 2) and no variation in SYNC scores were observed within bachelor groups (Table 3).. When controlling for age ($F_{4,141.905} = 9.264$, $P < 0.001$), group-type ($F_{1,183.706} = 5.052$, $P = 0.026$) and the interaction of age and sex ($F_{5,139.895} = 4.702$, $P = 0.001$), it was found that SYNC score did not predict uOXT ($F_{1,176.318} = 1.123$, $P = 0.291$).

Variation in SYNC scores were observed in mixed-sex groups; specifically, groups containing at least three adult females had significantly greater mean synchrony score than groups containing less than three adult females ($t = -2.58$, $df = 12$, $P = 0.02$; Table 4). To test this variation, a LMM was run for both group types. For groups with at least three adult females, SYNC score did not predict uOXT ($F_{1,36.633} = 2.154$, $P = 0.151$), when controlling for age ($F_{4,34.907} = 2.034$, $P = 0.111$). For groups with less than three adult females, SYNC score did not predict uOXT ($F_{1,63.910} = 1.606$, $P = 0.210$) when controlling for age ($F_{4,56.274} = 6.012$, $P < 0.001$).

Peripheral Status

Only 6 males were described as peripheral (n = 2 in mixed-sex groups; n = 4 in bachelor groups). For the two mixed-sex group males, one had been with females for several years and the second was in a recently formed mixed-sex group for the first time in his adult life. For the bachelor males, no clear descriptive pattern emerged. Two groups were composed of three males and the other two groups composed of four males. Ages for all peripheral males, and their respective group mates varied, as did their length of group tenure.

A total of 27% (n = 13) of females were classified as peripheral. Only reproductively mature (≥14 yrs) were described as peripheral. Of the females described as peripheral, 15% (n = 2) were lactating with dependent offspring (both aged 4 yrs,

respectively) and 85% (n = 11) were non-lactating without dependent offspring. Of the non-lactating peripheral females 55% (n = 6) were considered breeding age (14-34.99 yrs) and 45% (n = 5) were past breeding age (≥35 yrs).

An initial overall model found when controlling for age ($F_{4,91.806} = 3.389$, P = 0.012) and the interaction of age and sex ($F_{5,88.419} = 3.045$, P = 0.014) peripheral status significantly predicted uOXT ($F_{1,106.401} = 4.019$, P = 0.048). Specifically, peripheral gorillas had 22% lower uOXT (μ = 828 pg/mg CR, 95% CI: 634-1084) than non-peripheral gorillas (μ = 1054 pg/mg CR, 95% CI: 833-1259). As a result, models were then run separately for male and female gorillas to examine peripheral status and uOXT. Only adult gorillas of each sex were identified as peripheral and thus younger age groups were removed from analysis.

A model examining mature females found that when controlling for age ($F_{1,53.148} = 3.982$, P = 0.051), peripheral status of a female significantly predicted uOXT ($F_{1,49.842} = 4.547$, P = 0.038). Peripheral females had lower uOXT (μ = 721 pg/mg CR, 95% CI: 601-1005) than non-peripheral females (μ = 1054 pg/mg CR, 95% CI: 861-1288; Figure 1). Lactation state, use of HC and the presence of a conspecific's offspring were not significant and removed to improve model fit. Because 85% of peripheral females were non-lactating, an additional LLM examining only non-lactating females was run. When controlling for age ($F_{1,38.745} = 4.483$, P = 0.041), peripheral status significantly predicted uOXT ($F_{1,36.109} = 5.871$, P = 0.021) . Peripheral females had lower uOXT (μ = 721 pg/mg CR, 95% CI: 548-946) than non-peripheral females (μ = 1057 pg/mg CR, 95% CI: 836-1334; Figure 1). Use of HC and the presence of a conspecific's offspring were not significant (P > 0.05) and removed to improve model fit.

For male gorillas peripheral status did not predict uOXT concentration ($F_{1,31.291}$= 0.491, P = 0.489). Group type and age class were not significant (P > 0.05) and removed to improve model fit.

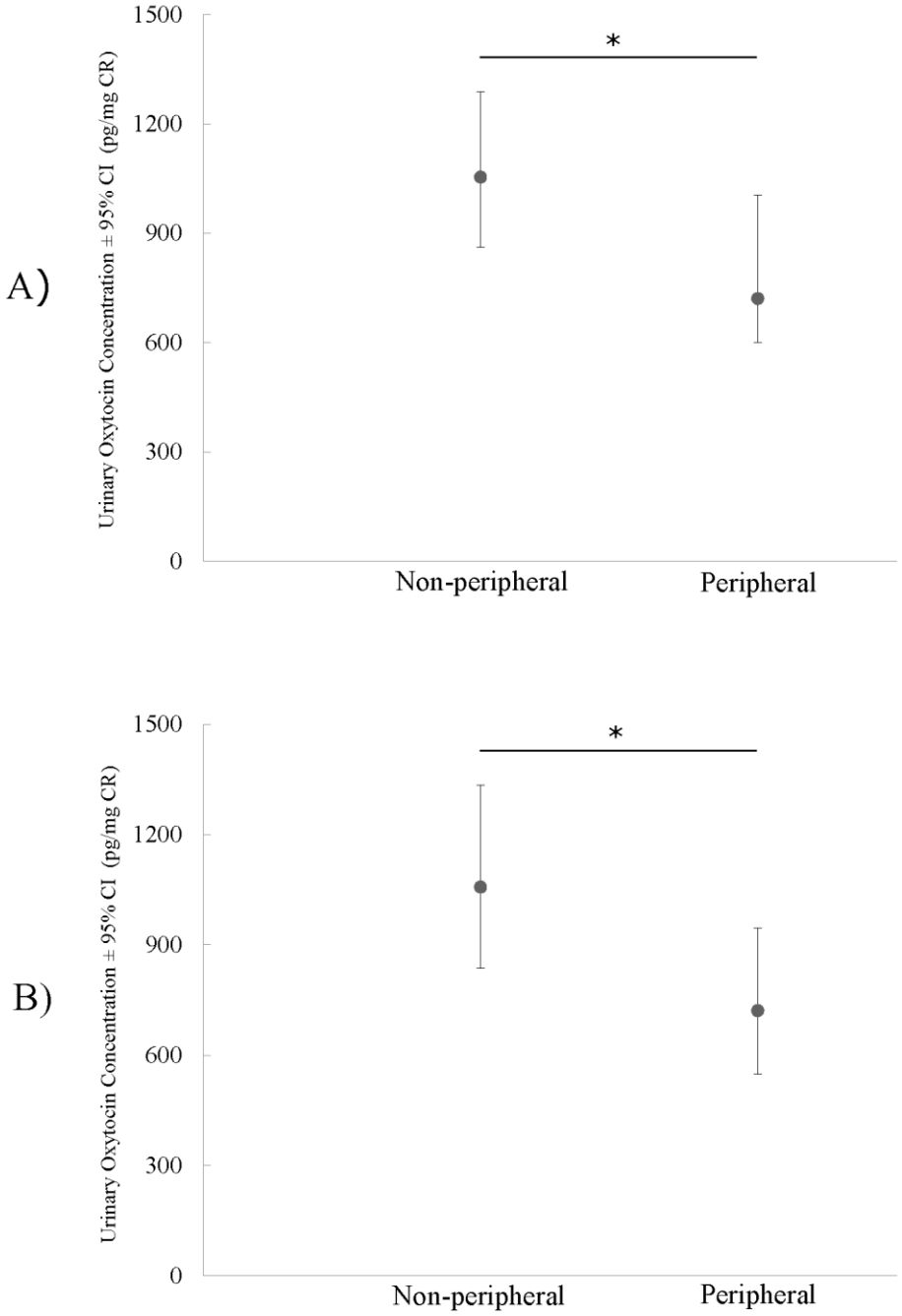

Figure 5.1. Peripheral status was associated with significantly lower urinary oxytocin (pg/mg CR) concentrations in all mature (≥14 yrs) females (A; $F_{1,106.401}$= 4.019, P = 0.048) and all non-lactating, mature females (B; $F_{1,36.109}$ = 5.871, P = 0.021).

Discussion

The purpose of this study was to assess if ACS perceptions of social bonding were associated with uOXT concentrations, a physiological indicator of social bond. Across all survey questions, the quality of most groups was viewed as positive, with minimal variation in scores between group types. This may be reflective of the expertise in gorilla husbandry that has been developed within AZA over the last several decades, supported by a significant investment in research by zoos managing gorillas and by dedicated professionals that organize regular workshops and advisory groups to share information and best practices with stakeholders in gorilla care. However, no relationship between uOXT and ACS perceptions of group-level bonding was observed. This does not mean that ACS cannot assess the quality of the social environment for gorillas, but more likely indicates that social bonding within gorilla is complicated and in need of further study. It is also likely there was a disconnect between the physiological measure and survey questions. However, keeper identification of peripheral individuals within specific groups was negatively associated with uOXT concentrations, providing evidence that keepers can identify behavior patterns related to bonding and social dynamics in WLG.

Social bonding is driven by the exchange of affiliative behavior over-time (Seyfarth and Cheney, 2012). WLG engage in little affiliative behavior (Masi et al., 2009; Stokes, 2004), which makes the behavioral assessment of social bonding difficult. ACS perceptions of social bonding may not have predicted uOXT concentrations because the behavioral mechanisms that facilitate social bonding in WLG are difficult to observe. There is evidence that social proximity is an indicator of social cohesiveness in gorillas (Nakamichi and Kato, 2001; Nakamichi et al., 2014; Stoinski et al., 2004a), but

quantitative patterns are normally described in terms of time spent within 5m of a conspecific (the same threshold used in this study). This is a relatively easy behavior pattern to distinguish but this may not be a specific enough indicator to relate directly to social bond formation. Furthermore, given the reproductive tactics of gorillas that include sexual coercion by males (Breuer et al., 2016) and secondary and tertiary group transfer in females (Arandjelovic et al., 2014; Stokes et al., 2003), adult male and female gorillas likely have not evolved to form strong social bonds. Rather, gorillas have evolved to live in functionally cohesive social groups where males maintain access to females for reproduction and females mate with preferred males in preferred groups. Our limited understanding of which behaviors specifically contribute to bond formation in WLG and the relatively weak bonds WLG form together likely mean the ACS responses we received are more representative of the social cohesion of groups, or the ability of group members to function as a unit, rather than the social bonds of the group. It also appears that the wording of our survey questions were more representative of group cohesion, rather than social bonding, further guiding ACS responses away from bonding and towards cohesion. Particularly the first two questions surveying ACS on the *appropriateness of the social environment* and the *cohesion* of the group were not worded to appropriately address our outcome measure of OXT in relation to social bonding, and were rather focused on the functional cohesiveness of the group. Furthermore, while proximity has been used to infer group stability and cohesion, it has never been directly tied to particular social bonds between gorillas, meaning our questions focused on proximity may also have been cohesion focused rather than bonding focused. Thus the inability of ACS to predict uOXT is likely more methodological rather than reflective of

ACS ability to describe their charges' behavior. Since the keeper surveys are more representative of how well each group functions as a cohesive unit, a better physiological measure may be gluccocorticoids (Cockrem, 2013) or allostatic load (Edes et al., 2016), which can provide a measure of physiological arousal and/or stress. It would be hypothesized that groups viewed as more cohesive would have lower concentrations of these physiological measures.

Based on both out-dated anecdotes of zoo gorilla behavior and premature conclusions of field researchers (Parnell, 2002) there have been perceptions within the zoo community that bachelor groups do not provide an appropriate social environment for male gorillas and that these groups are not cohesive or functional over the long-term. This study found no difference in the ACS survey scores of bachelor and mixed-sex groups. Decades of research (Leeds et al., 2015; Stoinski et al., 2002; Stoinski et al., 2001; Stoinski et al., 2004a; Stoinski et al., 2013; Stoinski et al., 2004b) have demonstrated that bachelor groups are functional and cohesive. The survey collected here demonstrates that the working knowledge of bachelor group management in zoos is in alignment with this research, despite earlier concerns to the contrary.

In mountain gorillas (*Gorilla beringei beringei*), the dominant male is the social center of the group and maintains strong associations with offspring (Rosenbaum et al., 2016b). Furthermore, in mountain gorillas the presence of an offspring is the strongest predictor of a female's time spent in close proximity to the silverback (Harcourt, 1979). Data from free-ranging populations of WLG demonstrate a trend for females to spend more time in close proximity to the silverback, but infant presence was not part of this evaluation (Stokes, 2004). In a case study of a single WLG group in a zoo, the entire

group spent more time in close proximity following the birth of an offspring, with no particular dyad having a significant influence on the whole groups' proximity measures (Kurtycz et al., 2014). A study of six post-partum zoo WLG found that new mothers spent more time in close proximity to juveniles and other females, rather than the silverback (Stoinski et al., 2003). Together this shows, despite species differences, offspring presence has important social implications for gorillas. Specifically lactating females, or females with young offspring, maintain a socially central role in mixed-sex groups. With few exceptions, identification as a peripheral group member in this study was specific to reproductively mature, non-lactating females, which matches well with what is known about gorilla social dynamics. It also makes biological sense that peripheral members of the group have lower uOXT than more central group members, since more central members would likely maintain stronger bonds and/or engage in affiliative interactions more with the rest of the group.

The identification of peripheral females by ACS may be helpful in identifying gorillas for transfer to other zoos for the purposes of population management. Free-ranging WLG females regularly engage in secondary and tertiary transfer, thus transfer is regular part of female natural history (Arandjelovic et al., 2014; Stokes et al., 2003). Transfer of females between social groups may be minimally invasive, both to the individual and group, if females who are less central to the social core of their existing group are preferentially selected. In contrast, if genetic matches allow, selecting peripheral females for breeding may naturally minimize their peripheral status within the group. However, it is unknown if that may cause another female in the group to become peripheral. In primates, varying social relations result in individuals being more or less

central to the core social group. Being more peripheral, or receiving less social engagement, can have negative physiological consequences (Abbott et al., 2003). For primates in human care, if there are opportunities to minimize this through husbandry and management practices, they should be considered since primates in human care have limited opportunities to change or remove themselves from these social situations the way they would in free-ranging settings (e.g. Busia et al., 2017). That being said, not all individuals in free-ranging populations can remove themselves from less optimal social environments either, thus being peripheral and the associated physiological consequences are a natural aspect of primate sociality.

Overall this study did not find a relationship between ACS perceptions of group-level bonding and individual levels of uOXT. However, a negative relationship between identification as a peripheral member of the social group by ACS and uOXT was found. This provides further validation that ACS can provide insightful and quantitatively important measures of zoo animal behavior. As zoos continue to improve welfare monitoring practices, the utilization of ACS in welfare evaluations will be integral to improving our understanding of zoo animal behavior and ensuring animals in zoos are experiencing optimal welfare. Lastly, this study provides understanding of how social status within a group can affect WLG on a physiological level. Understanding how changes in social status affect primates is important to improving our understanding of primate sociality.

CHAPTER SIX: General Discussion and Future Directions

Western Lowland Gorillas and Oxytocin

This project evaluated, for the first time, oxytocin (OXT) in western lowland gorillas (WLG; *Gorilla gorilla gorilla*). WLG are unique in that they live in social groups but appear to form weak social bonds with conspecifics. Bond strength between conspecifics is generally considered weak because of the limited amount of affiliative behavior exchanged between conspecifics (Stokes, 2004) and due to the reproductive tactics of males and females by which males use sexual coercion to maintain females in their respective groups (Breuer et al., 2016) and female mate choice drives secondary and tertiary group transfer by females (Arandjelovic et al., 2014; Stokes et al., 2003). OXT is a physiological measure of bond strength and was useful in providing additional context for the study of WLG social relationships. The measurement of OXT concentrations in WLG across a large population and in case studies following specific events in this project provided insight into what shapes bonds in WLG that would otherwise be difficult to assess.

A significant outcome of this dissertation was the validation of measuring OXT in WLG saliva and urine. The validation utilized unextracted saliva and urine samples. The use of extracted vs. unextracted samples is a major point of discussion in the study of OXT (McCullough et al., 2013). A few early studies into peripheral measures of OXT reported that unextracted samples may be unreliable (Horvat-Gordon et al., 2005). As our understanding of peripheral OXT has improved it has been discovered that extracted samples strip peripheral media of OXT (Brandtzaeg et al., 2016), significantly changing

the measurable concentrations of OXT. This project's validation provides new data demonstrating the effectiveness of utilizing unextracted samples in the study of peripheral OXT. This validation also utilized a commercially available assay. This will allow any interested scientist to study OXT in WLG. It is hoped that this project spurs additional inquires into the social bonding of WLG, both in zoos and free-ranging populations.

Western Lowland Gorilla Social Bonds

This project provides evidence that male WLG in bachelor groups form comparatively stronger bonds with other males than males with females in mixed-sex groups. This is supported by data from free-ranging studies of WLG indicating males form loose, affiliative networks (Bradley et al., 2004; Magliocca and Gautier-Hion, 2004) and from zoo studies demonstrating the cohesiveness of bachelor groups (Leeds et al., 2015; Stoinski et al., 2002; Stoinski et al., 2001; Stoinski et al., 2004a; Stoinski et al., 2013; Stoinski et al., 2004b). Longitudinal studies of how social bonds are shaped over time and how they vary in relationship to specific social demographic variables in bachelor groups will improve our understanding of WLG biology and inform the care and welfare of WLG in zoos. In addition, it is unclear if this finding is a result of zoo living that may allow for the development of male-male bonds and make male-female bonds more challenging to maintain. Comparative evaluation of free-living populations will help put these findings in a stronger context.

The longitudinal study of bachelor groups in conjunction with OXT will also improve our understanding of what behavioral exchanges shape bonding in WLG.

Grooming is a primary driver and maintainer of social bonds in other primate species (Schino, 2006; Wittig et al., 2008). WLG rarely engage in grooming (Stokes, 2004), thus identifying specific behaviors that drive social bonding is significant to better understanding WLG social dynamics. Play behavior is likely a contributor, but even in cohesive groups this can be a rare behavior that decreases with age (Stoinski et al., 2013b). Further evaluating how more subtle behaviors, such as social proximity, body posture, body orientation and eye gaze, facilitating social bonding will improve our understanding of how social bonds are developed and maintained amongst male WLG. From an applied perspective, this type of study will continue to provide checklist items that animal care staff (ACS) can use to monitor the formation and maintenance of bachelor groups in zoos and possibly identify social concerns before they become too serious.

OXT concentrations of males in mixed-sex groups did not differ from solitary males, further demonstrating, from a male perspective, male and female gorillas do not form strong bonds. Data from free-ranging populations already points to the limited opportunity for males and females to form strong social bonds given each sexes respective reproductive tactics (Arandjelovic et al., 2014; Breuer et al., 2016; Stokes et al., 2003). However, in this study there were no strong comparative opportunities to assess the strength of social bonds between males and females from a female perspective. OXT was lower in solitary females compared to group-living females, but no comparison amongst mixed-sex group females in specific relation to adult males was possible. Free-ranging studies of WLG demonstrate female choice is a major contributor to group membership. Particularly, females prefer males with large sagittal crests and gluteal

muscles (Breuer et al., 2012; Caillaud et al., 2008). It would be of value to compare these male secondary sexual characteristics in relation to female OXT concentrations. It would be hypothesized that females would form stronger bonds, or have stronger feelings of attachment, to males with features they find attractive, especially if these features are indicative of one's fitness.

Human-Gorilla Interactions

Relationships are formed between two individuals following repeated interaction (Hinde, 1976), thus animals in zoos form relationships with many different humans including guests, non-ACS, and ACS. Assessing how animals in zoos view these relationships is key to understanding how these relationships contribute to an individual's overall welfare state (Hosey, 2008; Hosey and Melfi, 2012). Arguably the relationship between animals in zoos and ACS has the greatest influence on an individual animal's welfare given the frequency with which the two interact. It is generally unknown how animals view their ACS, thus it is of significant importance to study this relationship. One difficulty in assessing this relationship, like any relationship, is that interactions between individuals need to be assessed not only across each dyad but also across all social contexts for each dyad. For animals in zoos this can mean during both non-structured interactions, such as greeting and checking in on animals when ACS arrive for their shift, and structured interactions, such as shifting procedures and positive reinforcement training (PRT). Given the prevalence of PRT in zoos, PRT has been a frequent interaction of study for the assessment of human-animal relationships. Research has indicated that PRT can improve the relationship between an animal and ACS (Leeds

et al., 2016; Savastano et al., 2003) and is associated with positive changes in animal behavior following PRT (O'Brien et al., 2008; Pomerantz and Terkel, 2009; Shyne and Block, 2010). Overall this indicates that PRT can be a positive interaction for participating animals, but these findings do not fully address how animals view ACS.

This project evaluated OXT changes in WLG following positive reinforcement training (PRT) with their primary animal care staff (ACS). Increases in OXT following a social interaction can be indicative of the strength of one's social bond (Crockford et al., 2013c) and thus may be a critical component in understanding how animals in zoos view their ACS. This is the first study to assess OXT change in a non-domesticated animal following an interaction with a human. In this study no change in OXT was observed following PRT, however, a decrease in cortisol (CORT), a physiological indicator of arousal and/or stress, was observed. Interestingly, in a case study part of our validation chapter, OXT did increase in a gorilla following play with ACS. It should be noted that PRT, unlike play, is not a spontaneous and naturally affiliative social interaction, thus the formation of a social bond over a transactional interaction like PRT may be unlikely. Though it is also worth noting that the cooperation and trust that accompanies PRT may override the transactional nature of PRT, creating an opportunity to form a social bond.

Ultimately, this was a case study that only evaluated changes in OXT in two male WLG following interaction with one ACS. To better understanding if WLG, and more broadly all animals in zoos, form strong bonds with their ACS, larger sample sizes of both WLG and ACS are needed. It is interesting that a decrease in CORT was observed. This may be a proximate mechanism facilitating the behavior changes noted in previous studies. Further evaluations of the human-animal relationship in the context of PRT

should utilize multiple measures to encompass all possible responses, given responses may differ in relation to the type of interaction occurring.

Another caveat of understanding the human-animal relationship is that every dyadic relationship is unique. Summarizing patterns in relationships is useful to provide a broader context, but ultimately every relationship is specific to two individuals. As the in-house monitoring and assessment of zoo animal welfare continues to expand, creating tools that quantify this relationship on a daily basis will provide information by which ACS can assess the overall welfare status of their charges. Initial steps may be to create a scale of how ACS feel any interaction with an animal went, on a scale ranging from a negative to neutral to positive. Following every interaction a score can be generated, that when added up across the day can create an overall metric for the day's interactions, both in terms of quality and quantity. This can then be tracked over time to see how each relationship is developing or maintaining and how it may be contributing to an individual animal's welfare.

Given the genetic relatedness of humans and non-human apes, and the similarities in social behavior that accompany them, research has focused on how ACS and non-human apes in zoos interact and form relationships (Behringer et al., 2014; Carrasco et al., 2009; Chelluri et al., 2013; Leeds et al., 2016; Pizzutto et al., 2007; Pomerantz and Terkel, 2009). The genetic relatedness may also predispose the two to interact more readily and/or form stronger relationships than ACS and other taxa. The expansion of study to species beyond non-human apes is of significant importance because one, more species in zoos are non-ape than ape, and two, given the more distant relatedness, the stimulus of interacting with ACS may be more novel and thus stronger, ultimately having

a greater influence on an individual animal's welfare. It is also worth noting that the context of how ACS and non-apes interact in zoos can significantly differ from how ACS and non-human apes interact. For example, non-human apes do not participate in ambassador animal programming. Such programs involve interacting in free contact, or without a protective barrier between individuals, and include significant handling of the animal by ACS. This intensive form of interaction likely contributes greatly to how relationships are formed between ACS and their charges and is in need of further study.

Animal Care Staff Perceptions

When it comes to identifying and addressing factors that affect an individual animal's welfare, ACS are on the front lines. This study has provided additional evidence that ACS can identify subtle differences in animal behavior that may relate to an individual's overall welfare status. In this project, ACS were able to identify less socially integrated, or peripheral, group members. This identification was associated with significantly lower OXT concentrations compared to more socially integrated individuals. For social species, being less socially integrated can have negative consequences (Abbott et al., 2003) thus these observations can then be used to develop husbandry and management protocols to mitigate these effects.

Projects such as this ensure that front line staff are accurately observing and reporting on their charges' behavior. In addition it provides credibility to the welfare processes of zoos as a whole by providing quantifiable evidence that those working in zoos have a strong understanding of how animals in zoos are adapting to their environment. Future research evaluating ACS perceptions of animal behavior should

continue to include novel measures, such as OXT, to ensure the welfare of animals in zoos is being assessed appropriately and to identify areas for improvement in the care of animals in zoos.

Future Directions

The study of OXT in primates has focused exclusively on highly gregarious species, for example chimpanzees (*Pan troglodytes*; Crockford et al., 2013; Preis et al., 2018; Samuni et al., 2017; Wittig et al., 2014). The study of OXT in less gregarious species, such as WLG, provides the opportunity to better evaluate social bonding, and sociality in general, within the order primates. Continual expansion of study species in relation to OXT will provide additional context to understand how differing social groups function within the order and how they have contributed to the evolution of primate social systems as a whole. Given the extensiveness of study in humans and chimpanzees, the expansion of OXT research across non-human apes will provide unique insights into our closest living relatives, informing on their own unique evolutionary histories as well as providing addition comparative context for understanding and appreciating our own evolution. Studies of bonobo (*Pan paniscus*) OXT are only just beginning (Boose et al., 2018). Given their intensely gregarious social system facilitated by extensive and variable affiliative behaviors (Hare and Yamamoto, 2017), the study of bonobo OXT will likely be highly fruitful and informative.

The study of OXT in orangutans (*Pongo spp*.) has yet to occur. Similar to gorillas, the study of social bonding in orangutans is interesting because of their unique social structure. Historically, orangutans were viewed as solitary, but similar to male gorillas,

female orangutans appear to form dispersed affiliative social networks (van Noordwijk et al., 2012). Also, recent historical data suggest that orangutans lived in much greater social densities prior to human exploitation, indicating how we view their social dynamics now may not be reflective of their most recent evolutionary history (Meijaard et al., 2010). The study of OXT in orangutans may provide insights into how social relationships and bonds are maintained in a dispersed social network while also possibly providing insights into their social dynamics prior to human disturbance.

For a more distant comparison, the study of hamadryas baboons may be another interesting primate species to study OXT variation in. Like WLG, both sexes of hamadryas disperse at maturity and both sexes are social in that they live in large groups but, similar to WLG, exchange little affiliative behavior with conspecifics (Matsuda et al., 2012; Swedell, 2002). Male hamadryas also use sexual coercion to maintain females in their harem groups like WLG males (Polo et al., 2014; Swedell and Schreier, 2009). Similar to female WLG, there is evidence that female hamadryas maintain associations with kin, despite regular group transfer (Städele et al., 2016). However, in hamadryas it is not clear how this occurs given, in contrast to WLG, female transfer between groups appears to be more strongly influenced by male coercion rather than female choice (Polo et al., 2014; Swedell and Schreier, 2009). The study of social bonds through a physiological measure in hamadryas may similarly provide additional insights and context to our understanding of what drives sociality in the order primates. Studying bonding in less gregarious species like WLG and hamadryas provide unique data points to better understand primate sociality as a whole. From an applied perspective, the study of social bonds in hamadryas may similarly improve the care and welfare of hamadryas

in zoos. Zoos are in early attempts of forming bachelor hamadryas groups. Similar patterns of affiliation (Koot et al., 2016) and wounding (Wiley et al., 2018) are being observed in hamadryas bachelor groups that have been observed in WLG bachelor groups. The study of OXT in hamadryas groups may similarly provide important insights into what contributes to stronger social bonding, and ultimately welfare, in this species.

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