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Cortisol secretion in an African elephant *Loxodonta africana* calf including evidence for a foetal surge

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Abstract

Cortisol is a useful tool for monitoring the well-being of elephants. In addition to its role in the stress response, this hormone is involved in regulating many natural metabolic reactions, including those associated with birth. However, very little is known about the production of cortisol from birth through the first few months of an elephant's life. Cortisol secretion in mammalian foetuses participates in preparation for extrauterine life. Precocial mammals, which need to be able to move and remain with the dam and the herd have a strong surge in foetal cortisol shortly before parturition. Elephants are precocial, but a foetal cortisol surge has not been previously documented in either species. Within 10 min of the birth of a male African elephant *Loxodonta africana* calf at the Louisville Zoo, a serum sample from the calf revealed a surge in cortisol ten-fold higher than subsequent samples from the calf and higher than samples from the dam. A urine sample from the calf within two hours of birth had a similarly high concentration of cortisol that was also well above that of samples from the days that followed. This appears to be the first direct evidence of the foetal cortisol surge in elephants. This study followed the serum and urinary cortisol of the calf for 18 months. Cortisol was also monitored in the dam and an adult female Asian elephant *Elephas maximus* herd-mate from one week prior to birth until 18 months after birth. This study documents natural changes in cortisol associated with birth and lactation.

Background

The mammalian nervous system responds to physiological and psychological stressors with activation of the hypothalamus-pituitary-adrenal (HPA) axis (Munck et al. 1984). Among other actions, HPA activity results in secretion of glucocorticoids, including cortisol, by the adrenal glands. Monitoring cortisol is a common strategy for gauging the well-being of animals (Busch and Hayward 2009). Spikes in cortisol levels may indicate that something is amiss in the animal's internal or external environment. However, some escalations in cortisol secretion are normal and necessary for typical development and physiological functions. Knowing when to expect small elevations and large surges in cortisol can help wildlife caretakers correctly identify hormonal signals of distress and well-being (Millspaugh and Washburn 2004).

Cortisol levels have been useful for identifying acute stressors in African elephants *Loxodonta africana* and Asian elephants and *Elephas maximus* (Brown et al. 2019). For example, loud noises and transportation are associated with abnormally high levels of glucocorticoid metabolites in adult elephants (Fanson et al. 2013; Millspaugh et al. 2007). However, concentrations of serum and urinary cortisol also covary with natural physiological changes, such as circadian rhythms (Bechert et al. 2021; White et al. 2019), ovarian cycling (Fanson et al. 2014), reproductive state and age (Glaeser et al. 2020). A recent large-scale study of cortisol secretion in zoo elephants identified several factors that correlate with normal and abnormal cortisol levels in elephants, including species differences, housing conditions, social interactions, joint health and individual variation (Brown et al. 2019). While this study included elephants aged 0–64 years, the results from younger elephants were not reported

separately from those of their older counterparts. Glaeser and colleagues (2020) report that elephants younger than 10 years old had lower cortisol when compared to older animals. Similarly, Dhairykar et al. (2020) found that elephants younger than five years old had significantly lower cortisol than subadults and adults. While informative, these group comparisons do not reveal details about normal cortisol fluctuations that may occur during an elephant's first years of life. In fact, the vast majority of research on adrenal activity has focused on older elephants, leaving little known about cortisol levels of elephants immediately prior to birth or during the first few years of life. For example in many mammalian species, the imminence of parturition is marked by an escalation in foetal adrenal activity, resulting in increased secretion of cortisol (Edwards and Boonstra 2018). In the foetus, cortisol induces maturation of organs that are necessary to sustain extrauterine life, such as the lungs, liver and gut (Fowden et al. 1998) and may also play a role in initiating parturition (Whittle et al. 2001). To date, there have been no reports of calf cortisol levels before or at the time of birth, so it is unknown whether a periparturition foetal cortisol surge occurs in elephants.

In the present study, an African elephant calf's serum and urinary cortisol was monitored from birth and throughout the first 18 months of life. Of particular interest was whether the calf showed a periparturition surge in cortisol. The dam and a female herd-mate (Asian elephant) were also monitored during the same time period to better elucidate the influence of environmental factors on cortisol levels.

Action

The primary subject was a male African elephant (#103708, FI) born on 2 August 2019 to a 32-year-old artificially inseminated cow (#101038, MI). The calf, dam and a 47-year-old female Asian elephant (#100479, PU) were housed in a barn and yard designed for restricted/protected contact management at the Louisville Zoo (detailed description of elephant management in White et al. 2019).

Sample collection and processing

Venous puncture blood samples were drawn from an auricular or saphenous vein, using a 5.0 mL vacutainer system or hand syringe. Weekly blood samples were drawn on Monday mornings (FI: mean=0926±SD=28 min, PU: 0845±40 min). MI's blood was sampled daily (MI: 0918±40 min). Whole blood samples were stored at room temperature for an estimated mean duration of 2 hr and centrifuged to separate the serum, which was frozen at -80°C until assayed for cortisol. Morning urine samples from FI (0904±56 min) and MI (0921±1 hr 54 min) were aspirated from the dry barn floor after it had been cleaned with detergent and bleach. PU's urine samples were free-caught during urination (0830±45 min). Specific gravity of the urine was measured with a veterinary refractometer (Vista, model A366ATC) before storage at -80°C. Urinary cortisol (UC) was normalised for variation in hydration, using norms established from each elephant's specific gravity history. Specific gravity was converted to whole numbers (SG=(specific gravity−1)×1000) and the mean iterative baseline of these values (repeated iterations eliminating values greater than the mean+2×SD) was established for each elephant and served as the respective norm (SGb). The radioimmune assay (RIA) result (UC) for each urine sample was corrected (UCSG) for hydration using the following formula:

$$UCSG = UC \times (SGb/SG)$$

A blood sample was obtained from the calf (FI) 10 min after birth and a urine sample within two hours. Weekly blood and daily urine sampling from FI continued over the following 18 months. To provide a herd context for interpretation of cortisol changes during this period, daily morning blood samples and weekly urine samples were collected from MI from a week prior to parturition and throughout the subsequent 18 months. Weekly blood and urine samples were also obtained from the Asian herd-mate. Ninety-four percent of planned samples were obtained.

Cortisol assay

Serum and urine samples were assayed for cortisol using the MP Biomedicals RUO cortisol radioimmunoassay kit (MP Biomedicals LLC, Orangeburg, NY, USA). The protocol followed the manufacturer's procedure with the exception of the additional standard of 0.25 µg/dL derived from dilution of the 1.0 µg/dL standard solution, providing an extension of the range of the standards. Urinary cortisol is often reported in µg/dL units as yielded by the MP Biomedicals assay. Elephant serum cortisol is reported in ng/mL units (Proctor et al. 2010), requiring a lower range of sensitivity from the assay. Further research is needed to understand differences in the concentration of cortisol in serum and urine. In the assays in this study, cortisol concentrations were read from an Isocomp-I gamma counter (MGM Instruments, Hamden, CT, USA).

Quality control

Intra-assay reliability for serum assays is reflected in the mean sample replicates CV of 3.13. A serial dilution test resulted in mean agreement of expected and obtained concentrations of -2.8%. Mean percentage recovery from serum samples spiked with moderate cortisol concentrations was 96.7%, with a range from 87 to 110. Aliquoted pooled samples were assayed across 16 runs (CV=17.6) to evaluate interassay reliability. The serum interassay CV was higher than preferred, however similar values have been reported in other elephant studies (Fanson et al. 2014; Proctor et al. 2010).

All urine samples were assayed in duplicate (mean replicate CV=2.03), using the MP Biomedicals cortisol assay kit described for serum. Samples repeated across different assay runs yielded a CV of 4.9 when the sample concentration was 1.0 µg/dL or higher, with lower concentrations showing more variability. The correlation of the two runs, including all samples, was 0.99 ($P<0.0001$). Serial dilutions were tested on three occasions with -2.5% mean agreement between expected and obtained concentrations. Samples spiked with known concentrations of cortisol resulted in a correlation of 0.87 ($P=0.001$) between spike concentration and recovered concentration with mean recovery of 111.9%.

Statistical analysis

Data manipulation and statistical analyses employed Statistix 9.1 (Analytical Software, Tallahassee, FL, USA). Pearson product-moment correlations were used to evaluate assay quality control tests. Cortisol results from individual elephants were analysed using an iterative baseline approach. In successive iterations a baseline was established by eliminating values that were two SD above the mean. This process continued until there were no values greater than two SD. The mean iterative baseline was the mean of the remaining values at the completion of the iterative process.

Consequences

African calf: Birth until 18 months

A blood sample taken from FI within 10 min of birth had a serum cortisol concentration of 40.2 µg/dL. The next serum sample at 11 days postpartum had a cortisol concentration of 2.99 µg/

dL (compared to serum mean iterative baseline of 4.12). The first urination occurred 1.9 hr after birth and had a cortisol concentration of 24.0 µg/dL, corrected with specific gravity. The subsequent urination occurred 36.5 hr later and cortisol was at 2.94 µg/dL (compared to urine mean iterative baseline of 1.43). Figure 1 (top panel) shows serum and urine samples that are two SD above the iterative baseline from birth throughout the following 18 months. The four vertical boxes that extend across each elephant's graph mark the significant events that were documented in keeper and veterinary records and are labelled on the horizontal axis. All data points in Figure 1 (both open and closed circles) represent values 2×SD. Mean serum baselines are represented by horizontal dashed lines, and mean urine baselines are solid horizontal lines near the zero point of each elephant graph.

In the initial samples from FI, both urine and serum cortisol concentrations were very high and at levels not repeated until FI's tooth eruption almost 13 months later. At approximately seven months, FI began a period of elevated urinary cortisol. This increase corresponded with the zoo's closure to the public in response to the COVID-19 pandemic. The elephant keepers were divided into two teams that worked distinct schedules that altered the pattern of individual keeper interactions with FI. The last event in FI's record is the marked elevation in urinary and serum cortisol at about 12.7 months, documented by veterinary and keeper records as an apparent tooth eruption.

African dam

For the week prior to the birth of FI, MI's mean morning serum cortisol (4.53 µg/dL) was near the mean iterative serum baseline (3.95). On the morning of the birth MI's serum cortisol was 4.31 and the morning after it was 4.55, with no indication of a birth-related surge. MI's urinary cortisol was elevated four days before birth (25.2) at about twice the mean urine iterative baseline (11.6), and there was a large peak (68.3) seen in Figure 1 (middle panel) three days after parturition. Urine samples from MI were collected on a weekly schedule, so the record is not as complete as the serum sampling. Both MI's urinary and serum cortisol show elevations at around six months after the birth. Records from elephant keepers and veterinary staff indicate this time period coincided with an episode of colic due to free gas bloat characterised as lethargy, low appetite and visually identified enlargement of the abdomen. The dam also had elevated urinary and serum cortisol at 12.7 months after birth when FI's odontiasis was recorded.

Asian herd-mate

Despite the human activity associated with the birth of FI, there was no elevation detected in PU's urinary or serum cortisol at this time (Figure 1, bottom panel). Note the scale change in the y-axis of Figure 1 due to PU's low cortisol baselines and low variability in cortisol concentrations. Preceding and during MI's bloating episode, PU had a series of elevated cortisol samples that may be weakly associated with the bloating. The mean iterative baselines for PU's serum (2.16 µg/dL) and urine (1.48) were lower than those of MI, but similar to FI's.

African calf cortisol

The present findings represent the first documentation of neonatal cortisol levels in elephants, providing previously missing insight into the HPA activity of foetal and neonatal development. In the first few minutes post-parturition, serum cortisol concentrations of the calf were very high then dropped substantially. Despite the presence of zoo staff, another serum sample was not obtained until several days after parturition. However, continuing oversight of the newborn allowed for nearly daily collection of urine, beginning on day one. This provided a record of cortisol excretion with minimal manipulation of the calf. Urinary cortisol revealed the same pattern as that of the serum, with a large spike in cortisol from the calf's first urination two hours after birth followed by very low daily concentrations. Although foetal cortisol was not directly measured in this study, the findings are consistent with reports of neonatal cortisol levels in species known to exhibit foetal cortisol surges shortly before birth (Fowden and Silver 1995; Heo et al. 2003; Ingram et al. 1999; Kattesh et al. 1990; Nagel et al. 2019). Therefore, it is likely that the high levels of serum and urinary cortisol seen in the first two hours after the birth reflect a prenatal increase in foetal HPA activity. It is unlikely that the elevation in neonatal cortisol was merely a result of the dam's HPA activity since placental transfer of cortisol is typically less than 20% (Barlow et al. 1974; Hennessy et al. 1982; Nathanielsz and Abel 1973) and the cow's serum cortisol on the morning of the birth and the morning after was substantially lower than that of the calf. The pattern of cortisol release seen in the newborn calf is consistent with Liggins' (1994) prediction that precocial species, such as elephants, will exhibit a spike in cortisol shortly before birth to facilitate maturation of organs necessary for extrauterine life. Additionally, the presence of high levels of cortisol at birth indicates that increased production of foetal cortisol may play a role in initiating parturition in elephants.

Within 38 hr of birth, the elephant calf's urinary cortisol concentration had decreased by 88% and remained low for several months. Cortisol measured in serum showed a similar pattern. After the birth-related surge, the calf's cortisol concentrations were substantially lower than those of the dam. In contrast, the calf's cortisol was similar to that of the female Asian herd-mate, PU. This was unexpected since Asian elephants typically have higher cortisol than African elephants (Brown et al. 2019) and adults are reported to have higher levels than calves (Dhairykar et al. 2020; Glaeser et al. 2020). However, PU's cortisol tended to be stable at a relatively low level throughout the study, indicating low stress reactivity to the arrival of the herd-mate's calf.

The cluster of the calf's urinary cortisol peaks during the months when the zoo was closed to the public suggests the calf was more affected by changes in keeper staffing patterns than the adults. The lack of serum peaks during the COVID-19 closure was likely due to staffing shortages, resulting in fewer serum samples.

An overt stressor occurred shortly before 13 months of age. At this time, keepers noticed inappetence and evidence of sore gums, which they attributed to odontiasis. Pain associated with tooth eruption likely activated the HPA axis leading to a spike in cortisol. From soon after birth, the calf was observed pushing against objects. Pushing with the side of the face may have stimulated sensitive tissue surrounding a developing tusk as it replaced a tush (Raubenheimer 2000). Presumably, odontiasis is a painful event but, to our knowledge, HPA response to teething/odontiasis has not been studied in this species. These results suggest that odontiasis may be a stressful event for the calf and the cow, who also showed elevated serum and urinary cortisol during this time. The dam may have been reacting to the distress of the calf and the interruption to nursing. It is worth noting that during the teething episode, the calf received rectally administered fluids and medication for the first time which along with a pause in nursing may have contributed to the spike in cortisol. Asian elephants have been shown to respond behaviourally to the distress of herd-mates (Plotnik and de Waal 2014).

African cow cortisol

Similar to the findings of Glaeser and colleagues (2020), cortisol secretion in the African cow was elevated in the months following birth of the calf, during lactation. An increase has also been observed in Asian elephants living in remnant habitat; faecal glucocorticoid metabolites (FGM) positively correlate with the

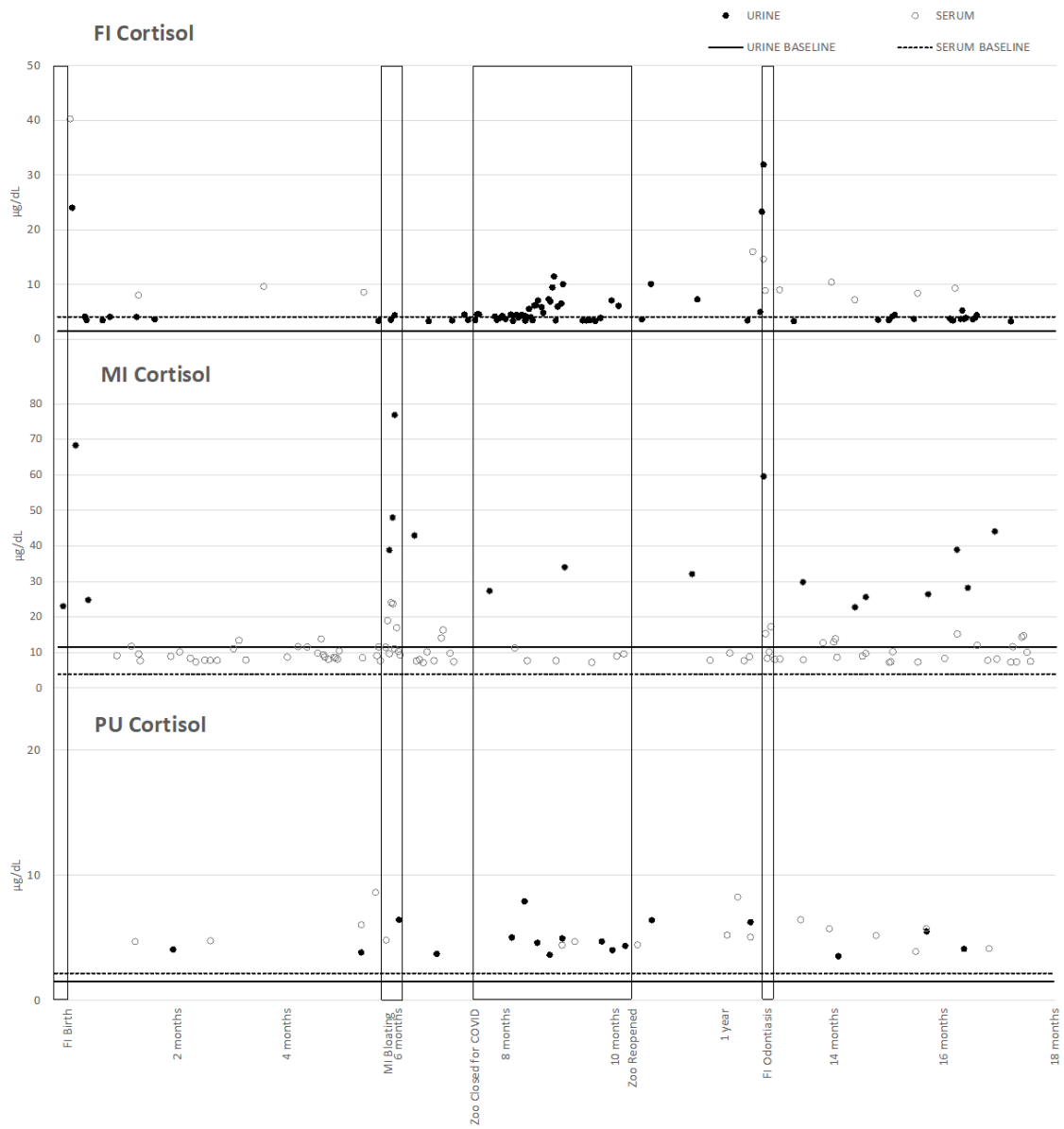

Figure 1. All urinary (closed circles) and serum (open circles) cortisol peaks that were at least two SD above the iterative baselines for the respective elephants, covering a week before the birth of FI and the 18 months following the birth. Mean baselines are represented by the solid (urine) and dashed (serum) horizontal lines near the base of each elephant's graph. Vertical boxes delineate four significant events defined by keeper and veterinary records: birth, MI bloat, zoo closure during COVID-19 pandemic and FI odontiasis.

number of calves and lactating females in a herd (Pokharel et al. 2019). This evidence does not necessarily suggest that lactation and parental care cause chronic physical and/or psychological stress for female elephants. Rather, it may reflect the role of glucocorticoids in lactogenesis (Chida et al. 2011).

Brown and Lehnhardt (1995) report a substantial peripartum increase in an Asian cow's serum cortisol along with an even larger elevation in urinary cortisol. Morning serum samples collected in the current study did not reveal a peripartum increase in cortisol. However, urinary cortisol sampled four days preparturition and three days postparturition shows a similar peripartum increase. The surge in urinary cortisol may represent the dam's physiological response to the birth process and the initiation of nursing.

The calf's bout of odontiasis provided evidence that parent-offspring interactions can induce an acute maternal stress response. The cow's cortisol concentrations in both serum and urine were elevated at the same time as those of the calf, which may represent the cow responding to the distress of the calf. The cow's elevated cortisol may have occurred because African elephants are sensitive to social stressors (Brown et al. 2019), which is consistent with the strong social bonds that develop in this species (de Silva and Wittemyer 2012).

Asian herd-mate cortisol

Following the birth, routine monitoring of the calf and the dam altered the morning activity routine for the Asian herd-mate. Serum samples were obtained in the morning when PU may have been responding to morning changes in the African cow's behaviour and interactions with keepers. However, PU's urinary cortisol does not appear to be elevated, suggesting no prolonged elevation. Glaeser et al. (2020) also did not find a significant cortisol response when Asian adult female elephants were in a group in which herd-mates gave birth.

PU has consistently had low serum and urinary cortisol compared to MI. Asian elephants have been reported to have higher basal serum cortisol (Brown et al. 1995) and, in a large study of North American zoo elephants, higher FGM (Brown et al. 2019), suggesting that species differences do not account for the current results. PU has dominated MI in shared feeding opportunities and in access to attractive enrichment items. PU is older and larger, characteristics associated with dominance in zoo-housed (Proctor et al. 2010) and free-ranging (Douglas-Hamilton and Douglas-Hamilton 1975) African elephants. However, the relationship between dominance status and cortisol is uncertain. Foley et al. (2001) found evidence of correlation in a large free-ranging group of African elephants, where subordinate animals had higher FGM than dominant animals. On the other hand, studies of zoo-housed African elephants found no correlation (Proctor et al. 2010) or a complex association involving a circular pattern of dominance (Kelling 2008). PU's low cortisol values may simply represent HPA reactivity unique to the individual or adjustment to the environment rather than a species-related attribute.

Many peaks greater than 2×SD were recorded outside of the boxed events identified in Figure 1. Keepers did not notice significant behavioural changes or abnormalities on the days surrounding these cortisol surges. This is not surprising since Brown et al. (2010) reported that >80% of fluctuations in cortisol were not associated with recordable events in keepers' daily records. Such elevations may correspond with normal developmental or physiological functions. Moderate increases in cortisol activity may indicate a minor stress response triggered by either external or internal factors that are not detected by human caretakers. In addition, the vast majority of serum and urine samples were collected at the beginning of the working day. Events triggering elevated cortisol secretion may have occurred when zoo personnel were not present.

The lack of a complete observational record of the elephants is a limitation to this study as is the small subject number. More complete sampling of blood and urine would allow for more definitive analyses, which is precluded by the cost of personnel and other resources. However, this study finds unique evidence for a foetal cortisol surge, which coupled with the absence of early elephant development reports justifies publication of this report.

In conclusion, the present findings provide indirect evidence for an active hypothalamus-pituitary-adrenal axis in foetal elephants. The resulting cortisol surge may play a role in initiating parturition. During the first 18 months of life, cortisol levels were usually low with occasional moderate increases that did not usually correspond with changes in behaviour that would indicate distress. However, the apparently painful experience of an emerging tooth may have caused both a significant elevation in cortisol and atypical behaviour (inappetence and localised sensitivity). It is unclear whether all young elephants would show an equivalent stress response to odontiasis. It is likely that the degree of HPA activation triggered by painful and stressful events varies from one elephant to the next. Further study of these animals in well-managed zoo settings will reveal not only when to expect elevated cortisol but also the range of variation between individuals' physiological and behavioural responses.

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