Effect of time and temperature on stability of progestagens, testosterone and cortisol in Asian elephant blood stored with and without anticoagulant

Jaruwan Khonmee1,5, Janine L. Brown2, Mu-Yao Li3, Chaleamchat Somgird4,5, Khajohnpat Boonprasert5, Treeradab Norkaew5, Veerasak Punyaporwithaya6, Wei-Ming Lee3 and Chatchote Thitaram4,5, *

1Department of Veterinary Bioscience and Veterinary Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Canal Road, Chiang Mai 50100, Thailand
2Center for Species Survival, Smithsonian Conservation Biology Institute, Remount Road, Front Royal, VA 22630, USA
3College of Veterinary Medicine, National Chung-Hsing University, Xingda Road, Taichung, 40227, Taiwan, R.O.C.
4Department of Companion Animal and Wildlife Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Canal Road, Chiang Mai Thailand
5Center of Elephant and Wildlife Research, Chiang Mai University, Canal Road, Chiang Mai 50100, Thailand
6Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Canal Road, Chiang Mai 50100, Thailand

* Corresponding author: Department of Companion Animal and Wildlife Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand. Tel: +66 53 948 046. Email: chatchote.thitaram@cmu.ac.th

The value of biological samples collected in the field is compromised if storage conditions result in analyte degradation, especially in warmer climates like Thailand. We evaluated the effects of time and temperature on immunoactive steroid hormone stability in Asian elephant (Elephas maximus) blood stored with and without an anti-coagulant before centrifugation. For each elephant (5 male, 5 female), whole blood was aliquoted (n = 2 ml each) into 13 red top (without anticoagulant) or purple top (with anticoagulant) tubes. One tube from each treatment was centrifuged immediately and the serum or plasma frozen at −20°C (Time 0, T0). The remaining 12 aliquots were divided into stored temperature groups: 4°C, room temperature (RT, ~22°C), and 37°C, and centrifuged after 6, 24, 48 and 62 h of storage. Serum and plasma concentrations of progestagens in females, testosterone in males and cortisol in both sexes were quantified by validated enzyme immunoassays. Steroid concentration differences from T0 were determined by a randomized complete block ANOVA and Dunnett’s tests. The only evidence of hormone degradation was cortisol and testosterone concentrations in serum stored at 37°C. Testosterone concentrations declined by 34% at 48 h and 52% at 62 h, cortisol was decreased by 19% after 48 h and 27% after 62 h at 37°C, respectively. None of the other aliquots displayed significant changes over time at any temperature. In conclusion, steroids appear to be stable in blood for nearly 3 days at room or refrigeration temperatures before centrifugation; steroids in samples with ethylenediaminetetraacetic acid were particularly stable. However, warmer temperatures may negatively affect steroids stored without anti-coagulant, perhaps due to red blood cell metabolism. Thus, under field conditions with no access to cold or freezer temperatures, collection of plasma is a better choice for elephants up to at least 62 h before centrifugation.

Key words: Asian elephant, cortisol, hormone degradation, progestagens, testosterone
Introduction

Asian elephants (Elephas maximus) are endangered throughout most of their natural ranges (EN-A2c, ver. 3.1; IUCN Red list 2009), with several populations heading toward extinction unless mitigating efforts are successful in stemming population declines. From studies on captive animals, much is known about elephant biology, particularly through analyses of serum or plasma hormones (Brown, 2014). Assessments of progestagens are key to monitoring female reproductive condition (Brown, 2014), whereas testosterone is useful in studying musth, a period characterized by temporal gland secretions, urine dribbling and more antagonistic behaviors (Rasmussen et al., 1984; Dickerman et al., 1997). Cortisol increases under acute and chronic stress conditions (Woolley et al., 2008; Ghosal et al., 2013; Boyle et al., 2015; Moltesen et al., 2016; Benitez-Dorta et al., 2017) and if prolonged, can suppress reproductive function (Wayland et al., 2002; Wingfield and Sapolsky, 2003; Breuner et al., 2008), leading to irregular cycling and acyclicity (Fanson et al., 2014). Cortisol increases during normal physiological states as well, including the follicular phase of the estrous cycle (Fanson et al., 2014) and musth (Brown et al., 2007). Non-invasive steroid monitoring methods (urine, feces, saliva, milk and hair) have been developed (Verkerk et al., 1998; Brown et al., 2010; Marcilla et al., 2012; Mack and Fokidis, 2017; Pawluski et al., 2017; Rakotoniaina et al., 2017), with feces being particularly well suited for field studies. However, under some circumstances (e.g. collections under field anesthesia), measures of circulating hormones in serum or plasma are desired.

Stability of hormones in blood varies among species, sample types and preservation methods (Wiseman et al., 1983; Abal et al., 1996; Taylor and Schuett, 2004; Hegstad-Davies, 2006; Jones et al., 2007; Tahir et al., 2013). It is recommended that blood be centrifuged soon after collection to obtain serum or plasma, and frozen immediately. However, for samples collected in the field, it can take hours or even days to reach laboratory processing facilities. Little is known about how steroids degrade in elephant blood, so the goal of this study was to determine the effects of storage time and temperature on immunoactive stability of steroids in Asian elephant blood; progestagens in females, testosterone in males and cortisol in both sexes.

Materials and methods

Animals and sample collection

This study was approved by the Faculty of Veterinary Medicine, Chiang Mai University, Animal Care and Use Committee (FVM-ACUC; permit number S39/2559). Female (nt = 5; age range, 9–35 yr; mean, 19.6 ± 10.6 yr) and male (nt = 5; age range, 15–50 yr; mean, 26.2 ± 14.0 yr) Asian elephants were housed at the Baan Chang Elephant Camp in northern Thailand (latitude, 19°06′51.6″N; longitude 98°53′39.2″E). Elephants were fed primarily corn stalk, napier grass (Pennisetum purpureum) and hana grass (P. purpureum X, Petalophyllum americanum hybrid) with regular access to water. Elephants participated in tourist activities, including bareback riding, bathing and feeding, and were in good health at the time of the study based on physical examinations by elephant camp veterinarians. Blood samples were collected from an ear vein by elephant camp staff or Chiang Mai University veterinarians.

In Study 1, 30 ml of blood was collected from each elephant using a 22-gauge IV catheter and 50-ml syringe between 1000–1100 h. Blood in 2-ml aliquots was divided among 13 red top tubes without anticoagulant (serum) and was kept in a styrofoam box with ice (maintained ~4°C) for transportation to Chiang Mai University. Upon arrival at the laboratory (<4 h), one tube from each elephant was centrifuged at 1500×g for 10 minutes, representing Time 0 (T0). The other 12 tubes were centrifuged after 6, 24, 48 and 62 h of storage at 4°C, room temperature (~22°C), or in a 37°C controlled temperature chamber (typical ambient temperature in the warm season).

Study 2 was conducted 2 days later using the same elephants, with 30-ml of blood aliquoted into 13 purple top tubes with ethylenediaminetetraacetic acid (EDTA) anticoagulant (plasma), and the plasma harvested after 0, 6, 24, 48 and 62 h of storage at 4°C, RT or 37°C before centrifugation.

Serum and plasma samples (0.5–0.8 ml) were stored at −20°C until hormone analyses.

Hormone analysis

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated. Concentrations of
testosterone and cortisol in males, progestogens and cortisol in females were quantified by enzyme immunoassays (EIAs) validated for elephants using antibodies for progesterone (monoclonal, CL425; Brown et al., 2004), testosterone (polyclonal, R1567; Somgird et al., 2016) and cortisol (polyclonal, R4866; Somgird et al., 2016). The monoclonal progesterone antibody cross-reacts with reduced pregnanes present in elephant serum (Brown, 2014), and are herein referred to as ‘progestagens’. Briefly, 96-well plates (catalog no. 07–200-39; Fisher Scientific, Pittsburgh, PA, USA) were pre-coated with secondary antibody diluted in coating buffer (catalog no. X108, 20X; Arbor Assays, Ann Arbor, MI): 150 μl (10 μg/ml) goat anti-mouse IgG for progesterone, and goat anti-rabbit IgG (Arbor Assays) for cortisol and testosterone EIAs. Coated plates were prepared by incubating at RT for 15–24 h. Wells were emptied and blotted dry, followed by adding 250 μl blocking buffer (100 mM phosphate, 150 mM sodium chloride, 1% Tween20, 0.09% sodium azide, 10% sucrose, pH 7.5) and incubating for 15–24 h at RT. After incubation, wells were emptied, blotted and dried in a Sanpla Dry Keeper (Sanplatec Corp., Auto A-3, Japan) with loose desiccant in the bottom. After drying (humidity <20%), plates were heat-sealed in a foil bag with a 1-g desiccant packet, and stored at 4°C until use.

Samples or standards (50 μl) (progestagens: Sigma Diagnostics Cat. #P0130, range 0.78–200 pg/well; testosterone: Steraloids Cat. #A6930, range 2.3–600 pg/well; cortisol: Sigma Diagnostics Cat. #H4001, range 3.9–1000 pg/well) were added to appropriate wells. Next, 25 μl of steroid horseradish peroxidase conjugate (HRP; progestagens 1:40000 dilution; testosterone 1:10000 dilution; cortisol 1:16000 dilution) was immediately added to each well, followed by 25 μl of primary antibody (progestagens, 1:10000 dilution; testosterone, 1:8500 dilution; cortisol, 1:75000 dilution) added to all but non-specific binding wells and incubated at RT for 1 h. Plates were then washed four times with wash buffer (1:20 dilution, 20X Wash Buffer Part No. X007; Arbor Assays, MI) and 100 μl of TMB substrate solution was added, followed by incubation for 45–60 min at RT without shaking. The absorbance was measured at 405 nm by a microplate reader (TECAN). Assay sensitivities were 0.078, 0.047 and 0.078 ng/ml for progestagens, testosterone and cortisol, respectively. Samples were analyzed in duplicate; inter- and intra-assay coefficients of variation were <10% and <15% (n = 6 for progestagens, n = 5 for testosterone, n = 11 assays for cortisol), respectively.

### Statistical analyses

Aliquots from the same animals were assigned as a block following a randomized complete block design. Each aliquot was randomly assigned to a time and temperature treatment. Hormone concentrations were converted into percentages of T0 values by the following equation: [concentration Tx (x = 6, 24, 48, 62)] / [concentration T0]. Percentage data (n = 5 for testosterone and progesterone, n = 10 for cortisol; no missing data points) are presented as mean ± standard deviation (SD). The effect of time (0, 6, 24, 48 or 62 h) on hormone concentration was assessed using a randomized complete block ANOVA, with concentration as the dependent variable and time as a fixed effect. Separate models were run for each substrate, temperature and hormone combination. Normality of residuals was evaluated by plotting QQ graphs, and the homogeneity of variance assessed by plotting residuals and fitted values. Most models did not violate normality and homogeneity of variance assumptions; however, slight deviations from a normal distribution were observed in some models as evidenced by residuals deviating from a straight line. Results were still used because ANOVA is particularly robust to normality problems (Glass et al. 1972; Harwell et al. 1992; Lix et al. 1996; Khan and Rayner, 2003; Blanca et al., 2017). If time was significant at P < 0.05, a post hoc Dunnert’s test was used to compare differences in hormone concentrations between time points. Statistical significance was set as α = 0.05. All statistical analyses were performed using R version 3.4.4 (R Development Core Team, 2015).

### Results

#### Hormone concentrations

Descriptive data are presented in Table 1, highlighting the variability in mean and mean range progestagens, testosterone

| Hormone          | Number of elephants | Serum               | Plasma              |
|------------------|---------------------|---------------------|---------------------|
| Progestagens (ng/ml) | 5                   | 3.16 ± 2.20 (0.26–5.99) | 2.80 ± 1.79 (0.34–5.00) |
| Testosterone (ng/ml) | 5                   | 1.85 ± 1.19 (0.58–3.3) | 1.88 ± 1.48 (0.35–3.97) |
| Cortisol (ng/ml) | Male 5               | 1.12 ± 0.50 (0.65–1.73) | 0.97 ± 0.61 (0.33–1.88) |
|                   | Female 5             | 0.60 ± 0.28 (0.26–1.00) | 0.56 ± 0.35 (0.22–1.12) |
and cortisol values across individuals. Progestagen concentrations were above baseline (0.05 ng/ml) indicating females were in the luteal phase of the cycle (Brown et al., 2004). Bulls were not in musth, as reflected by testosterone values <5 ng/ml (Brown et al., 2007).

**Study 1**

Progestagen concentrations in female serum were not significantly affected by either storage time or temperature. Testosterone concentrations in male serum did not change significantly when stored at 4°C or RT. However, time did have a significant effect on testosterone concentrations when stored at 37°C ($F_{4,16} = 11.99$, $P = 0.0001$). Post-hoc comparisons indicated that testosterone concentrations were 34% lower than T0 after 48 h ($t_{16} = 4.16$, $P = 0.0027$), and 52% lower after 62 h ($t_{16} = 6.33$, $P = 0.0001$; Fig. 1).

In addition, cortisol concentrations in male and female serum did not change significantly when stored at 4°C or RT; however, time did have a significant effect on cortisol

**Figure 1:** Mean ± SD concentrations of progestagen ($n = 5$), testosterone ($n = 5$) and cortisol ($n = 10$) in elephant serum and plasma samples stored at 4°C, RT (~22°C) and 37°C for up to 62 h before centrifugation. Data are expressed as a percentage of T0 values. For each treatment, asterisks denote values that differ from the initial T0 concentration ($P < 0.05$).
concentrations when stored at 37°C ($F_{4,36} = 4.43, P = 0.0051$). Post-hoc comparisons indicated that cortisol concentrations were 19% lower than T0 after 48 h ($t_{36} = 2.996, P = 0.017$), and 27% lower after 62 h ($t_{36} = 3.624, P = 0.0003$; Fig. 1).

**Study 2**

There were no significant time and temperature of storage effects on concentrations of plasma steroids relative to T0 (Fig. 1).

**Discussion**

This study investigated the impact of temperature and time on steroid hormone (progestagens, testosterone and cortisol) degradation in blood of male and female Asian elephants stored with or without anticoagulant before centrifugation, and found that storage at 4°C or RT ($\sim 22^\circ$C) for at least 62 h had little impact on serum or plasma concentrations. All steroids in blood with anticoagulant were not significantly different from T0 when stored at 37°C for up to 62 h before plasma harvesting. By contrast, both testosterone and cortisol in serum stored at 37°C declined significantly within 48 h, $\sim 34\%$ for testosterone and 20% for cortisol, and by 62 h levels were only half to a quarter of original levels, respectively. These findings agree with reports in some species, but not others, and highlight species and steroid differences in hormone stability between sample types.

Studies on the stability of progesterone in blood have yielded mixed results. While some found progesterone to be quite stable, human serum unaltered after 48 h at 22°C (Wiseman et al., 1983; Diver et al., 1994; Jones et al., 2007) and dog serum and plasma (EDTA) stable for 2 weeks at 20–22°C (Tahir et al., 2013), others found that progesterone degrades rapidly. For example, progesterone in dog serum (2 h) declined more quickly than in dog plasma (heparin; 5 h) at 4°C (Volkmaan, 2006). In cows, serum or plasma (heparin) progesterone decreased 50% within 24 h at 22°C (Wiseman et al., 1983) and $> 70\%$ after 72 h at 22–26°C (Reimers et al., 1983). In another study, serum progesterone was only 40% of initial concentrations after 8 h and $< 10\%$ after 24 h at varying temperatures (De Castro et al., 2004). Declining progesterone concentrations in blood before centrifugation may be due to the presence of blood cells and effects on steroid metabolism (Ohtsuka and Koide, 1969; Vahdat et al., 1981, 1984). Cytochrome P-450 in lymphocytes and platelets also can metabolize steroids (Lemberg and Barrett 1973; Hodgson and Guthrie, 1980). However, in elephants, degradation of progestagens in serum or plasma was not observed at any storage temperature, at least up to 62 h, suggesting blood cell steroid metabolism of this steroid did not occur during that time. Perhaps this is related to $\alpha$-reduced pregnanes (e.g. 5α-pregnan-3,20-dione, 5α-pregnan-3-ol-20 one, 17α-hydroxyprogesterone) being the predominant luteal steroids, rather than progesterone (Hodges, 1998).

Testosterone concentrations in elephant bull samples stored at 4°C and RT were stable in serum and plasma for at least 62 h, but decreased within 48 h in samples stored at 37°C without anticoagulant. In goats, testosterone was stable in samples with fluoride-potassium oxalate for at least 24 h at 22°C (Fahmi et al., 1985). Similarly, in diamondback rattlesnakes, testosterone concentrations in plasma were unchanged during storage at 0°C for up to 24 h (Taylor and Schuett, 2004). However, concentrations in that study were equally stable at 40°C, and did not show the decline observed in elephants at an elevated temperature (37°C in our study). Testosterone in human blood exhibited no clinically relevant changes during storage at RT for 168 h (Diver et al., 1994). However, a more recent study found testosterone concentrations in human samples without anticoagulant actually increased within the first 48 h of storage at 22°C (Jones et al., 2007). Similarly, in dogs, testosterone in plasma stored at RT were unchanged for up to 144 h, but in serum, concentrations were increased at 48 h (Frankland, 1985). Thus, there can be differences in steroid immunoactive stability between samples stored with and without anticoagulant, with serum values being influenced more.

Cortisol concentrations in the blood of cows with and without anticoagulant were stable at 25°C for at least 62 h (Reimers et al., 1983), and at 4°C for up to 40 h in the blood of dogs with (EDTA, heparin) or without anticoagulant (Olson et al., 1981), and at RT in gray seals (Bennett et al., 2012), consistent with the results of this study. By contrast, cortisol in human blood stored in heparin was increased by $\sim 15\%$ in plasma samples stored at RT by 48 h, and at 4°C by 148 h (Diver et al., 1994).

**Conclusion**

Immonoactive concentrations of progestagens, testosterone and cortisol in blood stored with anticoagulant were not significantly different from T0 over time, and exhibited no significant changes when stored at 4°C, RT or 37°C for up to 62 h. For blood without anticoagulant, serum progestagens also were not significantly different from T0 across all temperatures and times of storage. However, serum cortisol and testosterone showed significant decreases in concentrations at 48 and 62 h of storage at 37°C.

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