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Variation in metabolic factors and gonadal, pituitary, thyroid, and adrenal hormones in association with musth in African and Asian elephant bulls

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ABSTRACT

Longitudinal analyses of serum testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, glucose, insulin, triglycerides, cholesterol, total and free thyroxine (T_4), total triiodothyronine (T_3), thyroid stimulating hormone (TSH), and cortisol were conducted to investigate pituitary, metabolic, and adrenal changes related to testicular function and musth status in zoo-housed elephant bulls. Blood samples were collected twice a month for 12 months from 14 African and 12 Asian bulls at 17 facilities in North America. Building on previous studies, our results show that musth is associated with increased testosterone, LH, FSH, and cortisol secretion, and a decrease in thyroid hormone (total and free T_4) production. In addition, glucose and triglycerides were higher during musth than non-musth periods, indicative of altered sugar and fat metabolism. There were significant differences associated with age for LH, FSH and testosterone, all increasing, whereas the glucose-to-insulin ratio (G:I) decreased with age. A species comparison found African and Asian elephants differed in measures of insulin, prolactin, cholesterol and the G:I. Across all hormones, high inter-individual variability was observed, making it difficult to define a general musth endocrine profile or to assess musth status from single samples. These results highlight the need for facilities hosting bulls to closely and consistently monitor each individual from an early age and throughout musth and non-musth periods to determine the pattern for each male.

1. Introduction

Historically, nearly all elephants imported into North American zoos were females: 80% of Africans (*Loxodonta africana*) and 87% of Asians (*Elephas maximus*) (Keele, 2014; Olson 2014). This was in part because they are easier to handle, but also because initially, few zoos were interested in captive breeding. With the development of artificial insemination techniques in the late 1990's (Brown et al., 2004) and increased numbers of facilities housing breeding bulls, males now make up 20% of the captive population, with 40 to 50% being less than 20 years of age (Keele, 2014; Olson, 2014; Prado-Oviedo et al., 2016). Because of their increased size and strength, adult bulls require specialized facilities, especially during musth. They also have more social needs than originally believed (Slotow et al., 2000; Rees 2004; Evans and Harris, 2008; Evans et al., 2013). This new generation of maturing bulls offers a unique opportunity to study male physiology and social behavior, and how facilities can improve management to accommodate health, social and welfare needs.

Musth is a phenomenon unique to African and Asian elephants, typically observed in sexually mature bulls in good physical condition (Poole and Moss, 1981; Lincoln and Ratnasooriya, 1996). Increased androgen production, urine dribbling (UD), temporal gland secretion (TGS) and more aggressive and unpredictable behaviors are all indicators of musth (Jainudeen et al., 1972; Poole and Moss, 1981; Hall-Martin and Van der Walt, 1984; Poole, 1987, 1989; Rasmussen and Schulte, 1998; Brown et al., 2007; Ganswindt et al., 2010), although occurrence, physical signs and intensity can be highly variable among individuals (Lincoln and Ratnasooriya, 1996; Ganswindt et al., 2005a). Understanding the physiological mechanisms associated with musth may help in predicting its onset, leading to better bull management.

Behavioral and physiological changes exhibited during musth are typically associated with increased androgen production (Jainudeen

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et al., 1972; Hall-Martin and Van der Walt, 1984; Rasmussen et al., 1984; Niemuller and Liptrap, 1991; Lincoln and Ratnasooriya, 1996; Ganswindt et al., 2002; Ganswindt et al., 2005a, Yon et al., 2007; Ganswindt et al., 2010). However, it appears there is not always a simple relationship between behavioral signs and endocrine changes. In fact, previous studies suggest that musth behaviors are more prevalent after the elevation in testosterone and when concentrations begin to decline (Lincoln and Ratnasooriya, 1996; Brown et al., 2007). The hypothalamic-pituitary–testicular axis regulates androgen production and spermatogenesis. In elephants, pulsatile secretion of luteinizing hormone (LH) stimulates testicular androgen production, and LH concentrations have been shown to increase during musth (Brannian et al., 1989; Niemuller and Liptrap, 1991; Yon et al., 2007). Follicle-stimulating hormone (FSH) plays a role in sperm cell maturation; however, only one study has described FSH variation during musth (Kaewmanee et al., 2011). Investigating these pituitary gonadotropins may help to better define musth and understand its regulation and physiological effects.

Another pituitary hormone, prolactin, is primarily known for its role in female reproduction, but it has a variety of other physiological effects that could have relevance to bull elephant physiology. In other species, prolactin regulates male fertility and androgen production via hypothalamic and pituitary regulatory factors and a prolactin-dependent regulatory mechanism for testosterone synthesis in the Leydig cells (Gill-Sharma, 2009). As reviewed by Torner (2016), prolactin also is associated with the stress response through a variety of mechanisms, including but not limited to activation of the hypothalamic–pituitary-adrenal (HPA) axis to trigger the release of corticotropin releasing hormone (CRH), which promotes the secretion of adrenocorticotropin (ACTH) from the pituitary that then causes the release of glucocorticoids from the adrenal glands. Stress is one physiological cause of hyperprolactinemia and infertility in women (Levine and Muneyyirci-Delale, 2018), while low prolactin has been associated with reduced ejaculate and seminal vesicle volume in infertile human males (Rastrelli et al., 2015). Finally, prolactin functions in energy balance and metabolism through the regulation of key enzymes and transporters associated with glucose and lipid metabolism in target organs (Petryk et al., 2000; Ben-Jonathan et al., 2006; Brandebourg et al., 2007). In adipose tissue, prolactin acts to suppress lipid storage and adipokine release, supports the growth and survival of islets, and stimulates insulin secretion in the pancreas (Ben-Jonathan et al., 2006). It can also affect food intake and body weight gain (Sauve and Woodside, 2000). So, it is possible prolactin plays multiple roles in the expression of musth in elephant bulls.

Within individual bulls, musth often occurs at a similar time each year (Poole, 1987, 1989), although this is not always the case (Brown et al., 2007). Musth bulls actively seek out estrous females, but sexual activity is not restricted to the musth condition (Ganswindt et al., 2005b). Therefore, musth is not considered to be strictly seasonal or essential for reproduction. During musth, food intake may decrease voluntarily (Brown et al., 2007), and elephants can lose body condition (Poole, 1989), often up to 10% of their total body weight (Rasmussen and Perrin, 1999). Both wild (Poole, 1989) and captive (Jainudeen et al., 1972) elephants have been shown to drop out of musth in response to loss of body condition. Species such as reindeer (*Rangifer tarandus tarandus*) (Barboza et al., 2004), impala (*Aepyceros melampus*) (Brooks, 1978) and red deer (*Cervus elaphus*) (Mitchell et al., 1976) undergo significant loss of body weight and condition during a similar, though not completely analogous, condition of rut, which is associated with an increase in testosterone, reduction in food intake, and increased activity. Rut is a metabolically challenging period for males, with active selection for those in good condition (Barboza et al., 2004). Thus, musth may be metabolically challenging for elephants as well, and result in altered levels of metabolic factors (glucose, insulin, triglycerides, cholesterol, thyroid) and stress-related hormones (cortisol).

Glucose is the major carbohydrate presented to the cell for energy production, and most tissues and organs require a constant supply of glucose. Therefore, blood glucose concentrations need to be maintained within narrow limits (Szablewski, 2011). Insulin plays a key role in the regulation of energy homeostasis, coordinating storage, mobilization, and utilization of free fatty acids and glucose in adipose tissue, liver, and muscle (Ruan and Lodish, 2003). Generally, extended food deprivation is characterized by suppressed insulin signaling (Viscarra et al., 2011), so reduced food intake during musth could be associated with a decrease in insulin concentration. The glucose-to-insulin ratio (G:I) is commonly used as a proxy to account for the effects of feeding status on glucose and/or insulin levels (Ralston, 2002), and so is an important consideration when fasting prior to sample collection is not feasible, as is often the case for wildlife. Cholesterol is a structural component of cell membranes, essential to maintain membrane structural integrity and fluidity. It is also a precursor in the synthesis of steroidal hormones (Kaneko, 1989; Silva and Dangolla, 2002). Triglycerides are the main constituents of body fat in mammals. They can be stored in adipose tissue or used to generate energy through gluconeogenesis (Guyton, 1986; Silva and Dangolla, 2002), and have been shown to increase during musth (Rasmussen and Perrin, 1999), so variation with musth status would be indicative of altered lipid metabolism. In most species, the hypothalamic-pituitary-thyroid axis is involved in metabolic activity regulation (Greenspan, 2004), and thyroid hormone activity decreases during fasting to lower metabolism and conserve energy (St. Aubin et al., 1996; Ortiz et al., 2001). So, assessing glucose and lipid metabolism, as well as thyroid hormone variation may elucidate metabolic changes associated with musth.

Glucocorticoids are essential to the stress response by mobilizing energy stores (Uchoa et al., 2014), releasing stored amino and fatty acids (promoting hyperlipidemia) (Miller and Chrousos, 2001), and stimulating gluconeogenesis (thus promoting hyperglycemia). Increased cortisol secretion during musth has been shown in elephant bulls (Wingate and Lasley, 2002; Brown et al., 2007; Yon et al., 2007), which could be related to stress or metabolic changes. In addition to its involvement in HPA stress responses, cortisol also is increased during periods of high metabolic demand and reduced food intake (Muller and Wrangham, 2004), and so may play a role in musth.

The objectives of this study were to assess individual and species variability in musth characteristics and associated physiological activity of zoo-housed Asian and African elephant bulls. Longitudinal analyses of serum testosterone, LH, FSH, prolactin, glucose, insulin, triglycerides, cholesterol, total and free thyroxine (T_4), total triiodothyronine (T_3), thyroid stimulating hormone (TSH), and cortisol were conducted to investigate metabolic and other hormonal changes related to testicular function and musth status.

2. Material and methods

2.1. Animals and sample collection

Male African and Asian elephants housed in AZA accredited-zoos [n = 26 at 17 zoos; 14 African (20 ± 11 years of age), 12 Asian (32 ± 15 years)] were included in this study (Table 1). Two African bulls that had been castrated before sexual maturity also were evaluated in comparison to intact bulls (Table 2). This study was approved by the Institution Animal Care and Use Committees of the Smithsonian National Zoological Park and Conservation Biology Institute and all participating zoos.

Blood was collected into 7- to 10-ml red top serum separator tubes from an ear vein approximately twice a month over a 12-month period (December 2011 to December 2012) by collaborating veterinarians at the facilities in which the animals resided. All elephants were conditioned to the blood sampling procedure, which was part of the normal management routine. Blood was maintained at ~ 4°C and centrifuged within a few hours of collection to separate serum. Serum samples were stored at −20°C or colder until analysis.

Table 1
List of Animal ID, age (at the start of the study), facility, and musth status for the African and Asian elephant bulls included in this study.

Animal ID	Species	Facility	Age	Exhibited musth
1	Asian	A	3	No
2	Asian	B	7	No
3	Asian	C	24	Yes
4	Asian	A	24	Yes
5	Asian	D	29	Yes
6	Asian	E	34	Yes
7	Asian	F	41	Yes
8	Asian	D	41	Yes
9	Asian	G	41	Yes
10	Asian	H	45	Yes
11	Asian	B	47	Yes
12	Asian	D	50	Yes
13	African	I	6	No
14	African	J	6	No
15	African	K	9	No
16	African	L	9	No
17	African	I	11	No
18	African	I	11	No
19	African	M	18	Yes
20	African	N	24	Yes
21	African	K	29	Yes
22	African	O	30	No (castrated)
23	African	I	31	No
24	African	H	32	Yes
25	African	P	33	Yes
26	African	Q	34	No (castrated)

Bi-monthly serum samples were analyzed for concentrations of testosterone, prolactin, glucose, insulin, triglycerides, cholesterol, and cortisol. Monthly samples were analyzed for LH, FSH, total and free T_4, total T_3, and TSH.

2.2. Immunoassays

Serum steroids (testosterone and cortisol) and thyroid hormones (total T_3, free and total T_4) were measured using solid-phase ^{125}I radioimmunoassays (RIA) (Coat-A-Count; Siemens Healthcare Diagnostics Inc., Los Angeles, CA, USA) following the methods of Brown et al. (2007). Serum prolactin, FSH and TSH were measured by validated heterologous ^{125}I double-antibody RIAs (Brown et al., 1991, 1993; Brown and Lehnhardt 1997; Brown et al., 1999, 2004). The prolactin assay employed an anti-human prolactin antiserum (NIDDK-anti-hPRL-3) and ovine prolactin label and standards (NIDDK-oPRL-I-2). The FSH assay employed an anti-ovine FSH antiserum (JADLER #178) and ovine FSH label and standards (NIDDK-FSH-S16). The TSH assay employed an anti-ovine TSH antiserum (NIDDK-anti-oTSH-1) and human TSH label and standards (NIDDK-hTSH-RP-2). Serum LH concentrations were measured using an enzyme immunoassay (EIA), that employed a monoclonal anti-bovine LH antiserum (518 B7), a biotinylated ovine LH tracer and streptavidin peroxidase, adapted from Brown et al. (2004). Serum insulin concentrations were measured using a solid-phase, two-site bovine insulin enzyme immunoassay (EIA) kit (10-1201-01, Mercodia Inc., Uppsala, Sweden) validated for elephants (Morfeld and Brown, 2016). All assays were previously validated for elephant serum by demonstrating: (1) parallelism between dilutions of pooled serum samples to the respective standard curve preparation and (2) significant (> 90%) recovery of exogenous standard hormone added to pooled samples before analysis. Assay sensitivities were: 0.08 ng/ml for testosterone, 0.25 ng/ml for cortisol, 20 ng/dl for total T_3, 0.25 ng/dl for free T_4, 1 µg/dl for total T_4, 0.63 ng/ml for prolactin, 0.95 ng/ml for FSH, 0.3 ng/ml for TSH, 0.3 ng/ml for LH, 0.050 µg/l for insulin. For all assays, intra- and inter-assay coefficients of variation were < 10% and < 15%, respectively.

2.3. Glucose, triglycerides and cholesterol analyses

Serum glucose was determined using an automated glucose analyzer (One Touch Ultra, LifeScan, Inc., Milpitas, CA, USA) and glucose strips (Unistrip 1 generic, code 49). Serum (20 µl) was added to each strip and analyzed in duplicate. The G:I was calculated following the method of Morfeld (2013), by dividing glucose concentration in mg/dl by insulin concentration in µg/l.

Triglycerides and cholesterol were quantitated on a RX Daytona automated clinical chemistry analyzer (Randox Industries-US Ltd., Kearneysville, WV, USA). Commercially available reagents (TR3823 and CH3810), calibrators (CAL2351) and two-level controls (HN1530 and HE1532) were all purchased from Randox Industries-US Ltd. (Kearneysville, WV, USA). The technical ranges were 0–12.8 mmol/l and 0–17.0 mmol/l, respectively. The analyzer was subject to routine quality control measurements throughout the study, with normal and elevated controls for each analyte maintained within 2 standard deviations (SD) of the respective target value.

2.4. Musth definition

Musth periods were identified based on serum testosterone levels (Brown et al., 2007). Baseline testosterone values were calculated for each individual using an established iterative process (Brown et al., 2004) conducted in R version 3.1.1 (R Core Team, 2014) using the package hormLong (Fanson and Fanson, 2014). For each animal, all data points with values above the mean plus 1.5 times the standard deviation (SD) were removed and the process repeated until all values exceeding the mean +1.5*SD had been removed. The remaining data points defined the baseline for that individual and the baseline cutoff was the highest value that remained after this iterative process. The baseline standard deviation (bSD) was calculated from all remaining points below that baseline cutoff.

Each musth period was defined by the following criteria: 1) the first musth sample was the first sample that exceeded an upper threshold, defined as baseline +1.5*SD; 2) musth continued for as long as testosterone concentrations remained above a lower threshold, defined as baseline +2*bSD for at least two consecutive points; 3) the first non-musth sample following a musth period was defined as testosterone concentration below the baseline +2*bSD for at least two consecutive points; 4) single point fluctuations above or below the defined thresholds were assigned to the same phase as the surrounding points; and 5) if a point was missing during a musth period, the bull was still considered in musth as long as the next point was above the lower threshold. If the next point was below the lower threshold, the bull was considered out of musth (Fig. 1).

For a subset of the elephants (N = 11), behavioral records during the sampling period were available and summarized to include observations of UD, TGS, increased aggression, and increased sexual activity, to aid in confirming musth periods (see Poole and Moss, 1981; Hall-Martin, 1987; Poole, 1987, 1989; Rasmussen and Schulte, 1998; Ganswindt et al., 2010). Among these bulls, the minimum testosterone concentration associated with musth behavior was 10 ng/ml, and was therefore considered the minimum concentration for a first musth point.

Bulls were subsequently assigned to one of two groups: Group 1 – intact bulls that exhibited musth during the study period, based on testosterone profiles and available behavioral data (n = 15); and Group 2 – intact bulls that did not exhibit musth during the study period, based on testosterone concentrations < 10 ng/ ml (n = 11).

2.5. Data analysis

Mean, minimum and maximum concentrations and SD in serum testosterone, LH, FSH, prolactin, glucose, insulin, G:I, triglycerides, cholesterol, total and free T_4, total T_3, TSH and cortisol were calculated

Table 2
Overall mean, range and standard deviation (SD) for serum testosterone, LH, FSH, glucose, insulin, glucose:insulin ratio (G:I), triglycerides, cholesterol, total and free thyroxine (t_4), total triiodothyronine (t_3), TSH, prolactin and cortisol in male Asian (*E.m*) and African (*L.a*) elephants. Values calculated during musth compared to non-musth periods in Group 1 bulls (intact bulls that did exhibit musth during the study period) and non-musth periods in Group 2 bulls (intact bulls that did not exhibit musth during the study period) and the two castrated bulls.

Analyte	Species	Musth Samples (Group 1, n = 15)				Non-musth Samples (Group 1, n = 15)				Non-musth Samples (Group 2, n = 11)				Non-musth Samples (castrated bulls, n = 2)			
		Mean	Min.	Max.	SD	Mean	Min.	Max.	SD	Mean	Min.	Max.	SD	Mean	Min.	Max.	SD
Testosterone	*E.m*	35.07†	0.12	153.19	30.46	4.76	0.08	45.33	7.09	2.20#	0.12	6.46	1.55				
(ng/ml)	*L.a*	23.79†	0.24	97.24	20.06	5.95	0.12	29.80	5.92	1.73#	0.08	6.79	1.58	0.74	0.08	3.63	1.10
Luteinizing hormone (LH)	*E.m*	2.61†	0.89	6.10	1.23	1.85‡	0.34	5.29	0.95	1.40#	0.99	2.19	0.33				
(ng/ml)	*L.a*	2.38†	1.14	6.77	1.59	2.25‡	0.68	8.13	2.15	1.14#	0.54	3.14	0.49	3.44	0.93	5.78	1.47
Follicle stimulating hormone (FSH)	*E.m*	6.69†	1.55	23.52	5.74	5.87‡	0.50	22.09	4.68	2.75#	1.18	4.92	1.03				
(ng/ml)	*L.a*	9.76†	2.49	18.70	5.97	5.91‡	1.58	14.26	3.60	3.11#	0.50	12.86	3.33	11.05	4.57	16.95	2.97
Glucose	*E.m*	75.90	20.00	120.50	25.02	71.95‡	20.00	117.50	21.15	104.48	77.00	147.50	17.31				
(mg/dl)	*L.a*	78.60	20.00	121.00	31.00	88.04‡	46.00	129.50	17.76	90.57	53.50	150.17	15.75	89.83	35.17	146.00	20.63
Insulin	*E.m*	0.89	0.05	5.28	1.12	0.75	0.03	5.17	0.86	0.75	0.14	2.45	0.52				
(ug/l)	*L.a*	0.29	0.05	0.79	0.21	0.30	0.06	1.30	0.21	0.30	0.05	1.87	0.32	0.64	0.09	1.92	0.43
Glucose:insulin ratio (G:I)	*E.m*	274.06	12.33	1228.57	278.30	277.15	17.25	1617.65	311.39	199.37	52.10	727.27	121.02				
	L.a	427.03	93.96	1204.23	305.08	414.47	84.59	1384.06	288.90	546.67	58.89	1920.00	349.19	207.63	56.80	704.17	130.16
Triglycerides	*E.m*	0.39	0.05	0.96	0.21	0.33	0.01	1.07	0.20	0.37	0.14	0.61	0.11				
(mmol/l)	*L.a*	0.22	0.04	0.61	0.14	0.29	0.04	0.64	0.16	0.30	0.07	0.580	0.082	0.37	0.02	1.25	0.305
Cholesterol	*E.m*	0.98	0.04	1.73	0.37	0.99	0.09	1.65	0.36	1.04	0.51	1.69	0.23				
(mmol/l)	*L.a*	1.16	0.21	2.03	0.52	1.54	0.43	2.33	0.47	1.58#	0.77	2.23	0.34	1.09	0.04	1.81	0.54
Total thyroxine (Total T_4)	*E.m*	5.31†	2.63	8.77	1.41	7.03‡	3.50	11.12	1.29	6.65	3.28	9.73	2.11				
(μg/dl)	*L.a*	4.70†	3.36	6.48	0.95	6.25‡	3.12	10.34	1.81	8.47	5.45	12.97	1.49	9.16	5.37	11.52	1.51
Free thyroxine (Free T_4)	*E.m*	0.40†	0.16	0.88	0.15	0.48‡	0.21	0.96	0.18	0.72	0.51	1.12	0.16				
(ng/dl)	*L.a*	0.37†	0.24	0.52	0.07	0.44‡	0.22	0.69	0.10	0.81	0.53	1.21	0.17	0.75	0.54	1.17	0.14
Total triiodothyronine (Total T_3)	*E.m*	82.02†	15.42	213.61	36.52	76.81‡	10.79	159.40	31.27	126.51	88.92	175.35	22.97				
(ng/dl)	*L.a*	72.27†	36.77	120.36	24.88	80.21‡	17.00	125.77	24.38	88.60	43.95	152.43	25.76	109.56	78.34	144.64	15.96
Thyroid stimulating hormone (TSH)	*E.m*	1.13	0.16	3.53	0.66	1.07	0.10	3.42	0.53	0.82	0.10	1.45	0.35				
(ng/ml)	*L.a*	0.73	0.10	1.17	0.30	0.77	0.10	2.09	0.39	0.85	0.10	1.80	0.30	0.82	0.49	1.11	0.121
Prolactin (PRL)	*E.m*	1.80	0.68	4.25	0.53	1.89	0.64	4.86	0.69	1.98	0.86	3.83	0.72				
(ng/ml)	*L.a*	2.58	0.98	5.59	1.12	2.45	1.25	5.43	0.88	3.53	0.69	10.32	1.59	4.87	1.45	8.74	2.44
Cortisol	*E.m*	30.81†	4.10	83.70	16.86	22.97‡	2.50	92.60	15.02	11.68	3.90	38.60	6.43				
(ng/ml)	*L.a*	19.67†	6.10	42.00	10.45	20.55‡	5.00	72.70	13.14	16.35	1.77	69.90	11.26	20.89	2.50	81.70	16.74

† Significant differences ($P < 0.05$) between musth and non-musth samples in Group 1 bulls.
‡ Significant differences ($P < 0.05$) in non-musth samples between Group 1 and Group 2 bulls.
# Significant differences ($P < 0.05$) in non-musth samples between Group 2 bulls and castrated bulls.

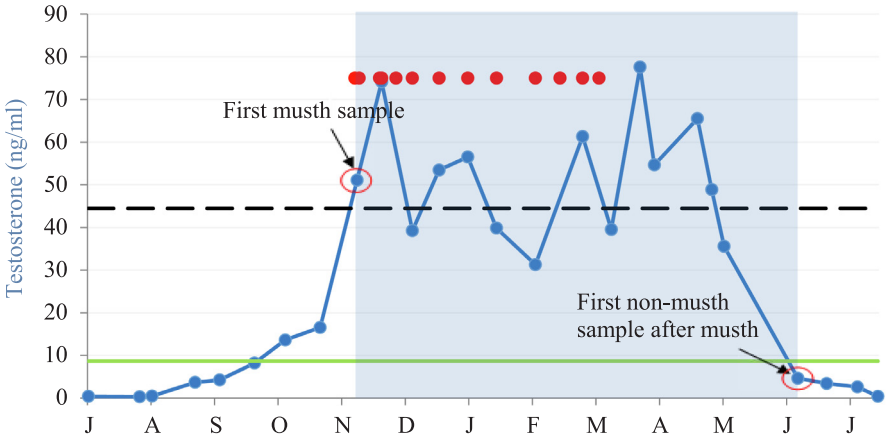

Fig. 1. Example profile demonstrating the first and last samples defined as musth according to the criteria used in this study. Serum testosterone concentration is in blue, and behavioral and/or physiological signs observed by keeper staff are represented by red circles. The upper threshold of baseline cutoff plus 1.5 times the overall standard deviation (SD) is denoted by the black dashed line; the lower threshold of the baseline cutoff plus 2 times the baseline standard deviation (bSD) is denoted by the solid green line. The blue shaded area is the proposed musth period for this bull following the established criteria.

for each species and each musth group.

For those bulls that exhibited musth (Group 1), each serum sample was assigned to one of four musth stage categories: 1) pre-musth – all non-musth samples within 2 months prior to the first defined musth sample; 2) musth – all samples between the first and last musth sample; 3) post-musth – all non-musth samples within 2 months following the last defined musth sample; and 4) non-musth – all other non-musth samples that were not included in pre- and post-musth categories.

To investigate differences in each hormone concentration according to musth status, serum samples were analyzed and compared using generalized linear mixed models (GLMM's) in MLwiN version 2.02 (Rashbash et al., 2005). GLMMs allow nested random effects to be incorporated into the model (Bolker et al., 2009) to control for non-independence of data, such as repeated serum samples per subject. Hormone data were transformed where necessary to improve the distribution of data. Testosterone, LH, FSH, prolactin, insulin, G:I, free T_4, and cortisol were transformed using a $\log_{10}$ transformation. Total T_3, TSH, triglycerides, cholesterol were transformed using a square-root (SQRT) transformation. Glucose and total T_4 did not require transformation.

Each dependent variable was then incorporated into a GLMM along with random (date of sample collection and subject ID) and fixed effects. Variation due to age and species was investigated by adding variables as continuous or categorical fixed effects, respectively. If significant, these remained in the model for addition of musth variables. Differences in hormones between non-musth, pre-musth, musth and post-musth were examined by entering musth status in the GLMM as a fixed effect.

Log_{10}-testosterone also was assessed individually with each dependent variable to investigate any relationship between androgen concentration and the hormone of interest in Group 1 and Group 2 bulls combined, and in Group 1 and Group 2 bulls separately. Because musth was categorized based on testosterone concentrations, this analysis was used to determine whether correlations between testosterone and other hormones might confound differences observed according to musth period. Finally, concentrations of each dependent variable were compared: 1) between musth and non-musth samples in Group 1 bulls; between non-musth samples in Group 1 versus Group 2 bulls; and between non-musth samples in Group 2 versus castrated bulls.

A normal error structure was used for all models of hormone concentration, and the significance of each fixed effect was determined using the Wald statistic and chi-squared (χ^2) distribution, with alpha set to 0.05. Only fixed effects that explained significant variation in hormone concentration were kept in the model, and all statistics reported are taken from this minimal model. Any non-significant terms of interest were re-entered individually into the minimal model to determine their level of non-significance.

3. Results

Five out of 14 African, and 10 out of 12 Asian bulls exhibited musth during the 12-month study period, on average lasting 3.2 ± 1.8 months in duration, and ranging from 1.0 − 7.8 months per episode. Mean, minimum and maximum concentrations and SD in serum testosterone, LH, FSH, prolactin, glucose, insulin, G:I, triglycerides, cholesterol, total and free T_4, total T_3, TSH, and cortisol during musth or non-musth periods in Asian and African bulls are presented in Table 2. Predictions for each hormone between musth periods, as obtained from the GLMMs, are presented in Table 3, with post-hoc comparisons presented in the text, where relevant.

3.1. Testosterone

Both peak and baseline testosterone concentrations were highly variable among bulls (Table 2). Peak concentrations ranged from 28.52 to 153.19 ng/ml in Asian, and 24.78 to 97.24 ng/ml in African elephants. By contrast, testosterone generally was < 10 ng/ml in bulls not exhibiting musth behaviors or during inter-musth periods (Table 2). Across all bulls, age was a significant predictor of serum testosterone concentration, with testosterone increasing with age (GLMM coefficient = 0.018, SE = 0.006, χ^2 = 8.68, df = 1, P = 0.003). There were no differences in testosterone concentrations between African and Asian bulls (P = 0.239).

Of the 15 bulls that exhibited musth during the study period, 12 had one musth episode and three bulls had two. Three Asian bulls housed at the same facility all exhibited musth during the 12-month study period. The 50-year-old bull was in musth from late-winter to mid-summer (16 weeks), followed by a 29-year-old, from mid-summer to late fall (8 weeks). Interestingly, the third bull (41 years old) exhibited musth during both periods. This was the only facility housing multiple bulls where each exhibited musth at some point during the study period. At the two facilities housing multiple African bulls, none exhibited clear musth during the study, although each has subsequently (Brown et al., *unpublished data*).

Physical and behavioral signs indicative of musth varied among individuals. TGS, UD, increased aggression, unpredictable behavior, sexual activity, and decreased appetite coincided with elevated testosterone concentrations. In general, signs indicative of musth were observed as testosterone began to increase or a few weeks after, although all signs were not always present simultaneously during musth. Two bulls exhibited clear musth periods based on testosterone, with concentrations elevated for 4 and 7 months, respectively. However, the physical and behavioral signs of musth were only observed for the first 18 days and 3.9 months, respectively.

Table 3
Predictions and effect size with standard error (SE), Wald statistic, degree of freedom (df) and P-value from the GLMM, for luteinizing hormone (Log_{10}-LH), follicle stimulating hormone (Log_{10}-FSH), glucose, triglycerides (SQRT-triglycerides), cholesterol (SQRT-cholesterol), total and free thyroxine (Total T4 and Log_{10}-free t4), total triiodothyronine (SQRT-total T3), and cortisol (Log_{10}-cortisol) in male elephants that exhibited musth during the study period. Each hormone was compared between non-musth, pre-musth, musth and post-musth periods, with covariates of age or species included in the GLMM, where relevant.

Dependent Variable	Fixed Effect	Model Prediction (SE)	Back-transformed Prediction (SE)	Effect Size (SE)	Wald	df	P
Log_{10}-LH (ng/ml)	Non-musth*	0.210 (0.054)	1.620 (0.087)		59.794	3	< **0.001**
	Pre-musth	0.219 (0.057)	1.655 (0.094)	0.009 (0.032)	0.084	1	0.772
	Musth	0.377 (0.054)	2.381 (0.128)	0.167 (0.028)	36.474	1	< **0.001**
	Post-musth	0.139 (0.060)	1.377 (0.083)	−0.071 (0.038)	3.388	1	0.068
Log_{10}-FSH (ng/ml)	Non-musth*	0.651 (0.073)	1.183 (0.086)		21.446	3	< **0.001**
	Pre-musth	0.730 (0.075)	1.187 (0.088)	0.078 (0.027)	8.170	1	**0.004**
	Musth	0.737 (0.073)	1.183 (0.086)	0.085 (0.024)	12.648	1	< **0.001**
	Post-musth	0.624 (0.077)	1.193 (0.091)	−0.027 (0.033)	0.693	1	0.405
Glucose (mg/dl)	Non-musth*		74.551 (4.892)		4.713	3	0.194
	Pre-musth		77.775 (5.070)	3.224 (2.342)	1.895	1	0.169
	Musth		78.605 (4.892)	4.055 (2.006)	4.087	1	**0.043**
	Post-musth		78.145 (5.256)	3.595 (2.734)	1.729	1	0.189
SQRT-triglycerides (mmol/L)	Non-musth*	0.519 (0.036)	0.269 (0.197)		8.328	3	**0.040**
	Pre-musth	0.547 (0.037)	0.299 (0.211)	0.028 (0.017)	2.798	1	0.094
	Musth	0.550 (0.036)	0.303 (0.209)	0.031 (0.015)	4.661	1	**0.031**
	Post-musth	0.568 (0.039)	0.323 (0.223)	0.049 (0.020)	6.252	1	**0.012**
SQRT-cholesterol (mmol/L)	Non-musth*	1.041 (0.049)	1.084 (0.462)		5.329	3	0.149
	Pre-musth	1.033 (0.051)	1.066 (0.467)	−0.009 (0.025)	0.121	1	0.728
	Musth	1.012 (0.049)	1.023 (0.449)	−0.030 (0.021)	1.938	1	0.164
	Post-musth	1.076 (0.053)	1.157 (0.496)	0.035 (0.029)	1.409	1	0.235
Total T_4 (μg/dl)	Non-musth*		6.822 (0.285)		85.851	3	< **0.001**
	Pre-musth		6.512 (0.315)	−0.310 (0.235)	1.738	1	0.187
	Musth		5.111 (0.283)	−1.711 (0.198)	74.325	1	< **0.001**
	Post-musth		6.574 (0.342)	−0.248 (0.273)	0.825	1	0.364
Log_{10}-free T_4 (ng/dl)	Non-musth*	−0.341 (0.030)	0.456 (0.014)		29.665	3	< **0.001**
	Pre-musth	−0.358 (0.032)	0.438 (0.014)	−0.017 (0.020)	0.716	1	0.397
	Musth	−0.431 (0.030)	0.371 (0.011)	−0.090 (0.017)	27.265	1	< **0.001**
	Post-musth	−0.371 (0.034)	0.425 (0.015)	−0.030 (0.024)	1.621	1	0.203
SQRT-total T_3 (ng/dl)	Non-musth*	8.626 (0.358)	74.415 (10.324)		17.575	3	< **0.001**
	Pre-musth	8.886 (0.385)	78.959 (11.030)	0.260 (0.245)	1.122	1	0.289
	Musth	8.971 (0.358)	80.470 (10.728)	0.344 (0.208)	2.745	1	0.097
	Post-musth	7.830 (0.409)	61.315 (10.014)	−0.796 (0.284)	7.836	1	**0.005**
Log_{10}-cortisol (ng/ml)	Non-musth*	1.282 (0.041)	19.129 (0.784)		19.815	3	< **0.001**
	Pre-musth	1.226 (0.046)	16.834 (0.772)	−0.056 (0.036)	2.392	1	0.122
	Musth	1.368 (0.041)	23.329 (0.957)	0.086 (0.031)	7.949	1	**0.005**
	Post-musth	1.240 (0.051)	17.362 (0.881)	−0.042 (0.042)	0.995	1	0.320

* denotes the reference category for GLMMs.

3.2. LH and FSH

There was an overall increase in LH and FSH with respect to age, with LH and FSH positively correlated with age (GLMM coefficient = 0.008, SE = 0.003, χ^2 = 9.92, df = 1, P = 0.001 and GLMM coefficient = 0.013, SE = 0.004, χ^2 = 12.03, df = 1, P < 0.001 respectively). There were no differences in either hormone between African and Asian bulls (P > 0.313).

After taking the effect of age into account, bulls that exhibited musth also exhibited significant variation in LH and FSH across time-periods (Fig. 2). In particular, LH was significantly increased during musth compared to non-musth (Table 3), pre-musth (χ^2 = 23.71, df = 1, P < 0.001) and post-musth (χ^2 = 39.45, df = 1, P < 0.001) periods. FSH was significantly increased in pre-musth and musth compared to non-musth (Table 3), and post-musth. (χ^2 = 8.40, df = 1, P = 0.004 and χ^2 = 11.86, df = 1, P < 0.001 respectively).

LH was positively correlated with testosterone, both when grouping Group 1 and Group 2 bulls together (GLMM coefficient = 0.141, SE = 0.010, χ^2 = 212.06, df = 1, P < 0.001) and when separated into those individuals that did (GLMM coefficient = 0.154, SE = 0.012, χ^2 = 178.29, df = 1, P < 0.001) or did not (GLMM coefficient = 0.067, SE = 0.014, χ^2 = 23.52, df = 1, P < 0.001) exhibit musth. FSH was positively correlated with testosterone (GLMM coefficient = 0.040, SE = 0.011, χ^2 = 13.31, df = 1, P < 0.001) in all bulls combined and those that did exhibit musth (GLMM coefficient = 0.064, SE = 0.012, χ^2 = 29.65, df = 1, P < 0.001). For bulls that did not exhibit musth, FSH was negatively correlated with testosterone (GLMM coefficient = −0.064, SE = 0.024, χ^2 = 6.94, df = 1, P = 0.008). LH and FSH values were significantly higher in the two castrated bulls than in intact bulls (Table 2).

3.3. Prolactin

Prolactin did not vary with age (P = 0.939), but was lower in Asian compared to African bulls (GLMM coefficient = −0.231, SE = 0.051, χ^2 = 20.183, df = 1, P < 0.001). In bulls that exhibited musth, prolactin did not vary according to musth status (P = 0.100). Prolactin also was not correlated with testosterone in all bulls combined (P = 0.154), bulls that did exhibit musth (P = 0.556), or bulls that did not exhibit musth (P = 0.949).

3.4. Glucose, insulin and G:I

There was an overall decrease in glucose with respect to age (GLMM coefficient = −0.604, SE = 0.203, χ^2 = 8.84, df = 1, P = 0.003), but not for insulin or G:I (P > 0.407). Insulin and G:I varied between

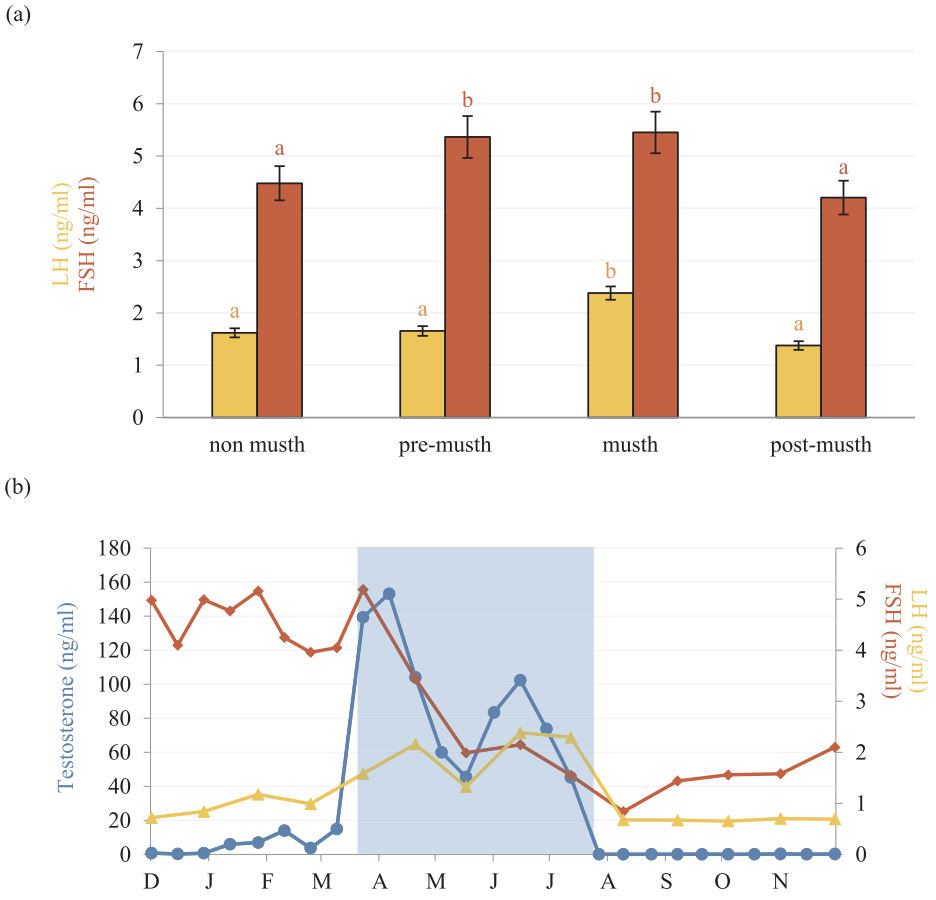

Fig. 2. (a) Prediction from the generalized linear mixed model (GLMM) for serum luteinizing hormone (LH; orange) and follicle stimulating hormone (FSH; red) concentration between non-musth, pre-musth, musth and post-musth time-periods in Group 1 bulls (those that did exhibit musth during the study period, n = 15), taking into account non-independence of data (error bars represent standard error of the prediction). Letters denote significant differences within predicted hormone concentrations across time-periods. (b) Representative profile of serum testosterone (blue) and pituitary hormones (luteinizing hormone (LH; orange) and follicle stimulating hormone (FSH; red) concentrations in an Asian elephant bull. The blue shaded area represents the musth period according to the definition used in this study.

species, with insulin concentrations higher and G:I lower in Asian compared to African bulls (GLMM coefficient = 0.281, SE = 0.117, $\chi^2 = 5.83$, df = 1, P = 0.016 and GLMM coefficient = −0.340, SE = 0.121, $\chi^2 = 7.85$, df = 1, P = 0.005 respectively). Glucose concentrations did not vary according to species (P = 0.708).

In bulls that exhibited musth, and controlling for age in the model, there was no difference in glucose concentration across the four musth stage categories (P = 0.194; Table 3). However, glucose was increased during musth compared to non-musth (P = 0.043, Table 3). There were no differences in either insulin (P = 0.703) or G:I according to musth status (P = 0.772).

Glucose, insulin and G:I were not correlated with testosterone in all bulls combined (P = 0.395, P = 0.366, P = 0.221, respectively) or bulls that did exhibit musth (P = 0.068, P = 0.804, P = 0.864, respectively). In bulls that did not exhibit musth, insulin was negatively correlated with testosterone (GLMM coefficient = −0.090, SE = 0.036, $\chi^2 = 6.39$, df = 1, P = 0.011) and G:I was positively correlated with testosterone (GLMM coefficient = 0.083, SE = 0.033, $\chi^2 = 6.31$, df = 1, P = 0.011). Glucose was not correlated with testosterone (P = 0.309).

3.5. Triglycerides and cholesterol

There were no differences in cholesterol or triglycerides with respect to age (P > 0.236). Cholesterol concentrations were lower in Asian compared to African bulls (GLMM coefficient = −0.194, SE = 0.061, $\chi^2 = 10.26$, df = 1, P = 0.001), but triglyceride concentrations did not vary according to species (P = 0.277).

In bulls that exhibited musth, and controlling for differences according to species, there was no overall difference in cholesterol concentration across the four musth periods (P = 0.149; Table 3). However, cholesterol was increased in post-musth compared to musth (P = 0.026). Triglycerides were increased during musth and post-musth compared to non-musth (Table 3).

Cholesterol and triglycerides were not correlated with testosterone when Group 1 and 2 bulls were combined (P = 0.378, P = 0.115 respectively) or in just those bulls that did exhibit musth (P = 0.149, P = 0.209, respectively). In bulls that did not exhibit musth, cholesterol and triglycerides were positively correlated with testosterone (GLMM coefficient = 0.024, SE = 0.012, $\chi^2 = 3.89$, df = 1, P = 0.048 and GLMM coefficient = 0.018, SE = 0.009, $\chi^2 = 3.84$, df = 1, P = 0.049, respectively).

3.6. Thyroid hormones

There was an overall decrease in total T_4, free T_4 and total T_3 concentrations with respect to age (GLMM coefficient = −0.059, SE = 0.021, $\chi^2 = 7.94$, df = 1, P = 0.005; GLMM coefficient = −0.007, SE = 0.002, $\chi^2 = 14.66$, df = 1, P < 0.001 and GLMM coefficient = −0.042, SE = 0.017, $\chi^2 = 5.73$, df = 1, P = 0.017, respectively), but not for TSH (P = 0.180). There were no differences in thyroid hormones according to species (P > 0.308), although there was a tendency for TSH to be higher in Asian compared to African bulls (P = 0.061). Total T_4 and free T_4 were negatively correlated with testosterone in all bulls (GLMM coefficient = −0.633, SE = 0.093, $\chi^2 = 46.41$, df = 1, P < 0.001 and GLMM coefficient = −0.028, SE = 0.007, $\chi^2 = 17.51$, df = 1, P < 0.001, respectively).

In bulls that exhibited musth, total T_4 and free T_4 were decreased during musth compared to non-musth (Table 3; Fig. 3), pre-musth ($\chi^2 = 35.13$, df = 1, P < 0.001 and $\chi^2 = 12.55$, df = 1, P < 0.001, respectively) and post-musth ($\chi^2 = 29.76$, df = 1, P < 0.001 and $\chi^2 = 6.60$, df = 1, P < 0.001, respectively). Total T_3 was decreased in post-musth compared to non-musth (Table 3; Fig. 3), pre-musth

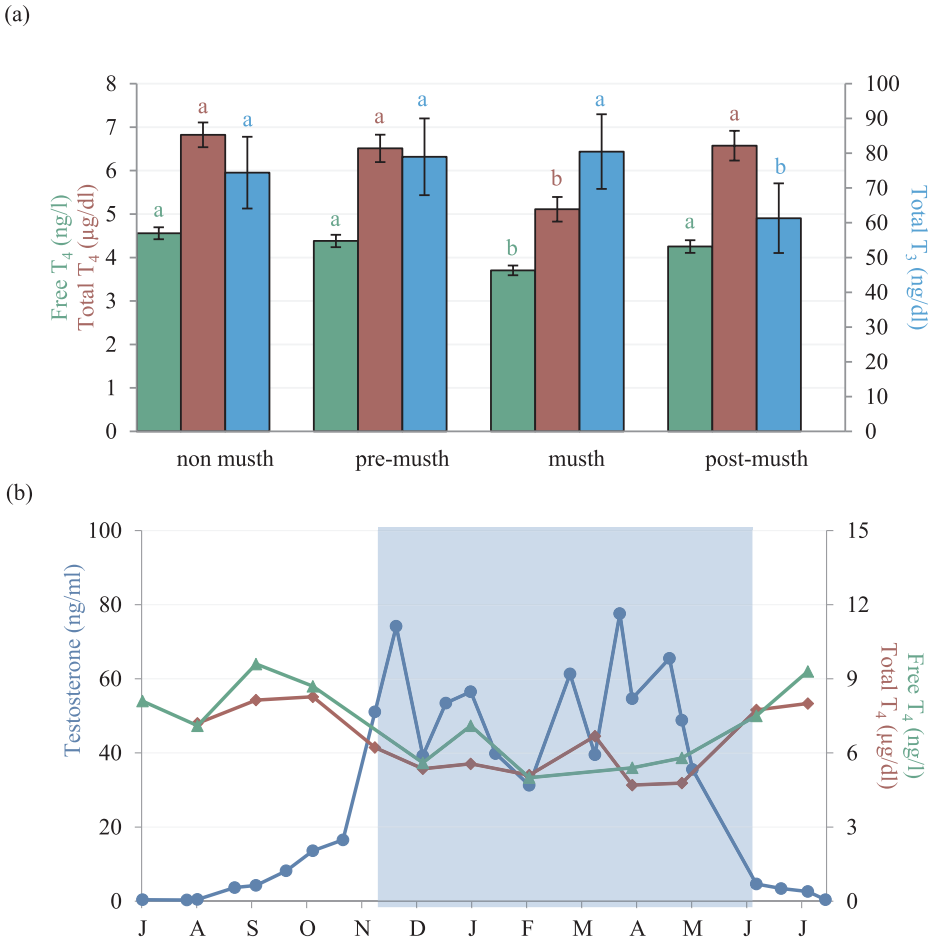

Fig. 3. (a) Prediction from the generalized linear mixed model (GLMM) for serum free (dark green) and total (dark pink) thyroxine (T_4) and total triiodothyronine (T_3; pale blue) concentration between non-musth, pre-musth, musth and post-musth time periods in Group 1 bulls (those that did exhibit musth during the study period, n = 15), taking into account non-independence of data (error bars represent standard error of the prediction). Letters denote significant differences within predicted hormone concentrations across time-periods. (b) Representative profile of serum testosterone (blue) and thyroid hormones (total thyroxine (T_4; pale green) and free thyroxine (dark pink) concentrations in an Asian elephant bull. The blue shaded area represents the musth period according to the definition used in this study.

(χ^2 = 11.02, df = 1, P < 0.001) and musth (χ^2 = 16.58, df = 1, P < 0.001). TSH did not vary with musth status (P = 0.995).

Both total and free T_4 were negatively correlated with testosterone, when grouping all bulls together (GLMM coefficient = −0.652, SE = 0.093, χ^2 = 49.04, df = 1, P < 0.001 and GLMM coefficient = −0.029, SE = 0.007, χ^2 = 18.93, df = 1, P < 0.001, respectively). Similarly, the two were negatively correlated in individuals that did exhibit musth (GLMM coefficient = −0.651, SE = 0.113, χ^2 = 33.46, df = 1, P < 0.001 and GLMM coefficient = −0.030, SE = 0.009, χ^2 = 10.98, df = 1, P < 0.001, respectively). In individuals that did not exhibit musth, total T_4 tended to be negatively correlated with testosterone (GLMM coefficient = −0.321, SE = 0.173, χ^2 = 3.46, df = 1, P = 0.062), although free T_4 was not (P = 0.427). Total T_3 was positively correlated with testosterone, both when grouping all bulls together (GLMM coefficient = 0.418, SE = 0.077, χ^2 = 29.29, df = 1, P < 0.001) and in Group 1 bulls (GLMM coefficient = 0.548, SE = 0.102, χ^2 = 28.84, df = 1, P < 0.001); however, it was not correlated with testosterone in Group 2 bulls (P = 0.506). TSH was not correlated with testosterone in all bulls combined (P = 0.864), bulls that did exhibit musth (P = 0.561), or bulls that did not exhibit musth (P = 0.506).

3.7. Cortisol

Across all bulls, there was an overall increase in cortisol with respect to age (GLMM coefficient = 0.007, SE = 0.002, χ^2 = 15.168, df = 1, P < 0.001), but no variation in concentrations with regards to species (P = 0.747). In bulls that exhibited musth, cortisol was significantly increased in musth compared to non-musth (Table 3), pre-musth (χ^2 = 15.20, df = 1, P < 0.001) and post-musth (χ^2 = 9.29, df = 1, P = 0.002) periods (Fig. 4).

Cortisol was positively correlated with testosterone in all bulls combined (GLMM coefficient = 0.052, SE = 0.013, χ^2 = 15.48, df = 1, P < 0.001) and those that exhibited musth (GLMM coefficient = 0.084, SE = 0.015, χ^2 = 30.02, df = 1, P < 0.001), but was not correlated in bulls that did not exhibit musth (P = 0.330).

4. Discussion

The present study is the most comprehensive assessment of physiological factors associated with musth in elephants to date, assessing 14 hormones and health biomarkers in 26 bulls across 17 facilities in North America. Results confirm that musth is associated with increased testosterone, cortisol, LH and FSH secretion, and a decrease in thyroid hormone (total and free T_4) production, similar to previous reports (Niemuller and Liptrap, 1991; Wingate and Lasley, 2002; Brown et al., 2007; Yon et al., 2007). In addition, an increase in triglycerides, consistent with Rasmussen and Perrin (1999), and glucose, during musth, was indicative of altered sugar and fat metabolism.

There were significant differences associated with age for LH, FSH, cortisol and testosterone, all increasing, whereas the G:I decreased with age. A species comparison found Asian and African elephants differed overall in measures of insulin, prolactin, cholesterol, and the G:I. Across all hormones, we observed a high inter-individual variability, making it difficult to define a general musth endocrine pattern or to assess musth status from single samples. These results highlight the need for facilities hosting bulls to closely and consistently monitor each individual from an early age and throughout every musth and non musth periods to determine the pattern for each male.

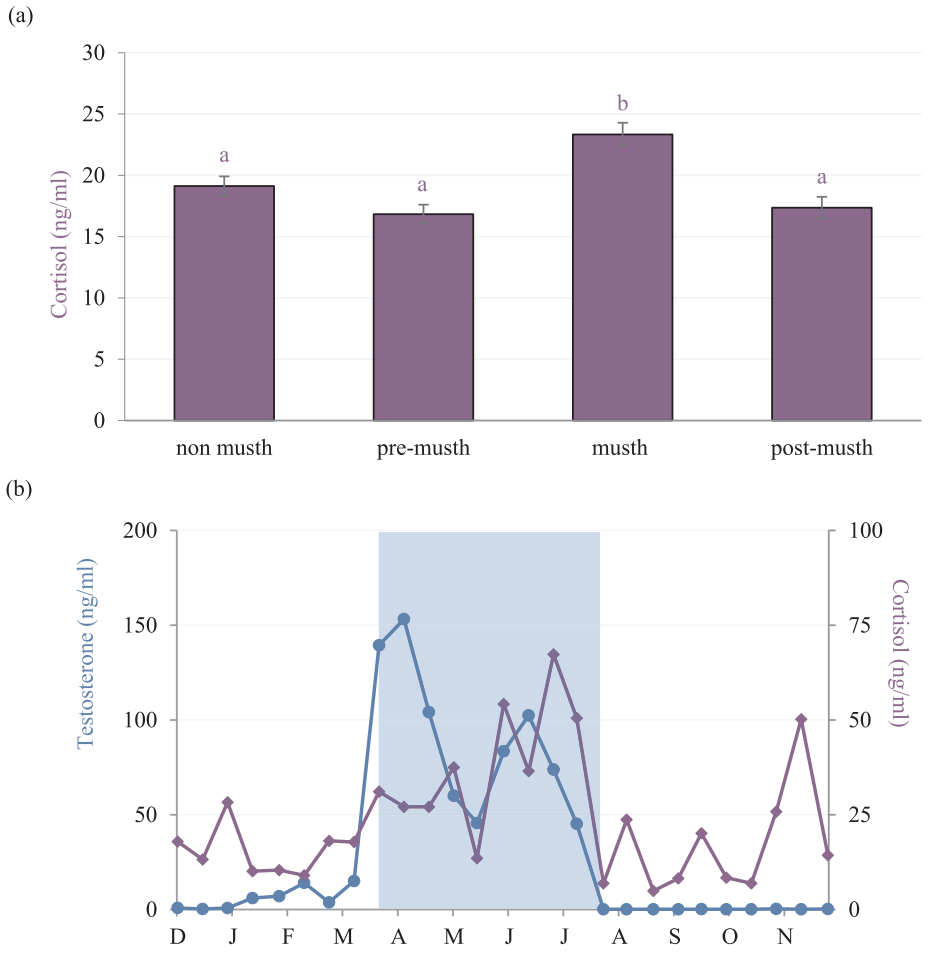

Fig. 4. (a) Prediction from the generalized linear mixed model (GLMM) for serum cortisol concentration between musth time-periods in Group 1 bulls (those that did exhibit musth during the study period, n = 15), taking into account non-independence of data (error bars represent standard error of the prediction). Letters denote significant differences within predicted hormone concentrations across time-periods. (b) Representative profile of serum testosterone (blue) and cortisol (purple) concentrations in an Asian elephant bull. The blue shaded area represents the musth period according to the definition used in this study.

4.1. Testosterone

The occurrence of musth varied among individuals in this study. Over the 12-month period, some bulls exhibited one musth period, some were in musth several times, while a number of others did not show any obvious physical (TGS and UD), physiological (increased testosterone) and/or behavioral (aggression, disobedience, spacey behavior) signs of musth, despite being of adequate age and physical maturity. Expression of musth can be affected by social environment (presence of cycling females or post-pubertal males) and dominance status (Poole, 1987, 1989; Ganswindt et al., 2005b), although not all dominant bulls in our study did so. It is also known to be affected by physiological condition, particularly observed in those that are healthy with good nutritional status (Poole, 1989).

In general, testosterone production and musth are related to advancing age. In the wild, musth does not occur until bulls reach their mid-20's, and bouts tend to be short and sporadic until the mid-30's (Poole, 1989). In captivity, however, bulls can exhibit musth earlier. Scott and Riddle (2003) demonstrated that captive African bulls exhibited first musth at between 10 and 19 years of age, while in Asian bulls it ranged from 12 to 20 years of age. Brown et al. (2007) also reported first musth in three Asian bulls occurring at 12, 15 and 21 years of age, while Cooper et al. (1990) recorded a first musth at 7 years of age for an Asian, and at 17 years of age for an African bull at the same zoo. During the first musth episode, not all physical and behavioral signs are present. Younger bulls generally exhibit shorter musth episodes (defined as "moda" or "honey" musth), intermediate testosterone concentrations, and mild signaling (Rasmussen et al., 2002). Most of the sub-adult males in this study did not meet our definition of experiencing a true musth and therefore were not included in the musth status comparisons conducted on Group 1 bulls.

In the wild, large and medium sized male African elephants in a given population tend to have non-overlapping musth periods, whereas they may overlap among younger bulls (Poole, 1987, 1989). Non-overlapping musth patterns were previously described for a group of three zoo-housed Asian elephants (Brown et al., 2007). The present study confirmed that two of those bulls continued to exhibit non-overlapping musth periods, whereas a third adult bull that joined the herd later exhibited musth that overlapped the other bulls.

In the 15 bulls that exhibited musth, periods ranged from less than 1 to nearly 8 months, consistent with the 1–10 month range reported previously (Brown et al., 2007). In a 5-year study of captive Asian elephants in Sri Lanka, Lincoln and Ratnasooriya (1996) described much shorter musth periods of only 2 to 10 weeks based on observations of at least two of the following signs: enlarged temporal glands with continuous secretion, urine dribbling, loss of appetite, or aggressive or restless behavior. Differences in musth definitions, based on behavioral signs versus testosterone levels, as well as climatic conditions, diet composition, body condition, and management conditions could explain variations in musth durations between captive elephants in Sri Lanka and U.S. zoos. In the wild, previous studies also describe much shorter musth periods than those observed in the present study

(Poole, 1989). This could be explained by differences in nutrition, no presence of adult bulls, or lack of environmental stressors in captivity (Brown et al., 2007).

Physical and behavioral signs indicative of musth varied among individuals, and few exhibited the full array. Interestingly, in two bulls, overt physical and behavioral signs ceased several months before testosterone returned to baseline. This could explain why some bulls remain unpredictable after they stop signaling musth, leading to accidents in Asian range countries during the post-musth period (E. Chave, *personal observation*). Of note was an inconsistency in how musth is recorded across U.S. zoos. Use of a uniform musth log-book across zoos would standardize the collection of this information and facilitate more rigorous studies of musth occurrence and variability. Bulls whose testosterone level did not vary during the study period were assigned to Group 2 and were used in hormonal comparisons (Table 2). Most of these bulls were young, although a few were adults that could have expressed musth. This categorical choice was made to simplify the model; however, different mechanisms might be involved among these groups that were beyond the scope of this study.

4.2. LH, FSH and prolactin

Increases in testosterone were associated with changes in gonadotropins during musth, which agrees with previous studies showing GnRH stimulates LH release and subsequent testosterone production in bull elephants (Brown et al., 1993; Lincoln and Ratnasooriya 1996; Somgird et al., 2016). Increased LH concentrations during musth also was comparable to findings of Niemuller and Liptrap (1991) and Yon et al. (2007), but contrasts with a long-term study of one African elephant bull (Kaewmanee et al., 2011) that described a sharp rise in LH initiated about 4 weeks before the onset of musth and lasting for roughly 5 weeks.

Ours is only the second study to describe FSH secretion in relation to musth. Kaewmanee et al. (2011) found an increase in FSH during pre-musth compared to musth and non-musth periods. Our data similarly demonstrate a rise in FSH concentrations during pre-musth; however, concentrations remained elevated during musth, and were lower only in the post-musth and non-musth periods. FSH plays a role in spermatogenesis in other species, and although sperm production in elephants is not restricted to the musth period (Hermes et al., 2013), an increase in FSH could be important for supporting normal spermatogenic activity. As sperm quality is typically low in captive elephants (Schmitt and Hildebrandt 1998; Kiso et al., 2011, 2012, 2013), longitudinal studies combining endocrine and semen evaluations temporally associated with musth would be of great interest. In castrated bulls, LH and FSH concentrations were higher than in intact males, which agrees with Yon et al. (2007) and other species (Galloway and Pelletier, 1975; Kitahara et al., 1990).

This was the first study to measure prolactin in bull elephants, and found no relation to musth, suggesting it plays a limited role in altered physiological function during this reproductive state. Evaluations of prolactin in relation to sperm and seminal quality are needed to understand if prolactin has effects on fertility in male elephants, as it does in other species (Gill-Sharma, 2009; Rastrelli et al., 2015).

4.3. Glucose, insulin and G:I

In bulls that exhibited musth, glucose, insulin and G:I were not correlated with testosterone, which was contrary to our expectations. Musth is a physiological phenomenon unique to elephants, often associated with a voluntary, partial decrease in food intake that can last for months. It therefore may not be appropriate to compare it with non-physiological short-term food restriction findings in domestic species. Northern elephant seals (*Mirounga angustirostris*) might be a more relevant model, as they endure a 2–3 month fast twice a year as a natural component of their life history (Keith and Ortiz, 1989, 1994; Viscarra et al., 2011). As with elephants, fasting elephant seals remain normothermic, and physiologically and metabolically active. They rely on the oxidation of extensive fat stores to maintain high levels of plasma glucose, with decreases in plasma glucose and insulin during fasting (Keith and Ortiz, 1989; Champagne et al., 2005). In the present study, no general metabolic adaptation to musth consistent across all elephant bulls was identified. Indeed, metabolic parameters were not significantly different according to musth status. Musth was variable in duration (1 to 8 months in this study) so the degree of voluntary nutritional restriction might not be the same among bulls. Moreover, body condition at the onset of musth could influence the way an elephant copes with the metabolic challenges of musth, and explain why different metabolic hormone patterns were observed among the bulls. Most zoos do not monitor feed intake closely or record regular body condition scores. Adding these parameters to a weekly musth log would help in better understanding metabolic challenges of musth, both within and among individual bulls.

Glucose concentrations were variable, but within the range previously described for elephants (60–116 ng/ml) (Mikota, 2006; Norkaew et al., 2019). Glucose concentration decreased with age; a result consistent with Nirmalan et al. (1967) who found higher glucose concentrations in juvenile Asian elephants (3–14 years of age) compared to adult males, and adult lactating and non-lactating females in India. Similarly, in a study evaluating metabolic status of captive bull elephants in Thailand, Norkaew et al. (2019) found higher glucose concentrations in young ($\leq$30 years of age) compared to older bulls. This might be due to differences in metabolism in growing animals, and warrants further investigation.

Insulin concentrations were slightly higher, and G:I values lower, than those described in bulls working in tourist camps in Thailand (Norkaew et al., 2019). This could be explained by differences in nutritional status and amount of exercise. Morfeld et al. (2016) showed that increased exercise in zoo elephants helped maintain a good body condition and metabolic health.

4.4. Triglycerides and cholesterol

Triglycerides were increased during musth and post-musth, compared to non-musth. These results are consistent with those of Rasmussen and Perrin (1999) and confirm that changes in fat metabolism occur during musth, presumably due to the mobilization of fat reserves. Furthermore, increased products from fat metabolism in circulation and used as an energy source can affect brain chemistry and function (Rasmussen and Perrin, 1999), so musth behaviors may be due to altered mentation. Cholesterol did not vary significantly according to musth status. Both triglyceride and cholesterol values in the present study were comparable to normal ranges reported for elephants in Sri Lanka (Ratnasooriya et al., 2006) and Thailand (Norkaew et al., 2019).

4.5. Thyroid hormones

Bulls that exhibited musth showed decreased concentrations of total and free T_4 during musth, which supports our hypothesis. Total T_4 and free T_4 were negatively correlated with testosterone secretion, as described in Brown et al. (2007). There was also a decrease in thyroid hormone (total and free T_4 and total T_3) concentration as a function of age, which agrees with findings in elephants (Brown et al., 2007) and other mammals (St. Aubin et al., 1996; Greenspan, 2004). Thyroid hormones are associated with reproduction regulation in seasonal breeders, and increased concentrations have been described during the transition from a reproductive to quiescent state (Ryg and Langvatn, 1982; Loudon et al., 1989; Shi and Barrell, 1994). This transition is shortened by thyroid hormone administration, whereas thyroidectomy can extend the duration of the breeding season (Parkinson and Follett, 1995; Zucker and Prendergast, 1999). Musth is not seasonal, and elephant reproduction is not limited to the musth period; however, thyroid

hormones may still play a role in altering testicular steroidogenesis through regulation of metabolic function. Because thyroid hormones are associated with an increase in metabolic rate, their concentrations are usually reduced during periods of food deprivation as a means to lower metabolism and conserve energy (St. Aubin et al., 1996; Ortiz et al., 2001). Musth is not usually associated with complete food deprivation, but it has been described as a period of decreased feed intake, leading to a loss of weight and body condition (Poole, 1987, 1989). A decrease in thyroid hormones might therefore help to cope with this metabolic challenge.

4.6. Cortisol

Our results showing increases in cortisol during musth are consistent with previous studies in Asian and African elephants (Wingate and Lasley, 2002; Brown et al., 2007; Yon et al., 2007) and support the idea that musth represents a form of physiological stress (Wingate and Lasley, 2002). In other species, reduced food intake or fasting can result in an increase in glucocorticoids (Bergendahl et al., 1996; Samuels and McDaniel 1997; Ortiz et al., 2001) that help maintain circulating glucose concentrations by increasing lipolysis (Bergendahl et al., 1996) and gluconeogenesis (Exton et al., 1972). Moreover, cortisol has been shown to increase during rut and in response to mating stimuli in domestic bulls, goats and boars (Liptrap and Raeside, 1978; Howland et al., 1985; Borg et al., 1991). Increased cortisol can also be associated with aggressive behavior in males (Sands and Creel, 2004). As musth is associated with increased activity (sexual activity and aggression), and reduced food intake, often leading to a progressive loss of condition (Poole, 1987, 1989), an increase in cortisol concentrations may help to cope with this challenging period. By contrast, Ganswindt et al. found a reduction in glucocorticoid metabolites excreted in feces during musth in captive (Ganswindt et al., 2003; Ganswindt et al., 2005a) and free-ranging (Ganswindt et al., 2005b, Ganswindt et al., 2010) African elephants. Still others report no significant changes in fecal glucocorticoid concentrations related to musth in free ranging African (Rasmussen et al., 2008) or Asian elephants (Ghosal et al., 2013). A number of explanations are possible for these apparent study differences. It may be related to measuring circulating (Brown et al., 2007; Yon et al., 2007) versus excreted (Ganswindt et al., 2003, 2005b, Rasmussen et al., 2008; Ganswindt et al., 2010; Ghosal et al., 2013) glucocorticoids (Brown et al., 2007) or due to different ecological and environmental settings, like wild versus captive. Cortisol levels also are affected by a wide range of extrinsic factors, and even under human care, social factors such as the presence of other males, dominance ranking, and the presence of estrous females could explain part of the cortisol level variation.

Although Brown et al. (2007) found significant correlations between circulating cortisol and testosterone in all bulls exhibiting musth, in the present study, only five out of 10 Asian (+one tendency) and one out of five African (+one tendency) bulls in musth did so. However, in bulls not showing musth, cortisol and testosterone levels were not correlated, similar to Brown et al. (2007).

5. Conclusion

The present study is the most comprehensive to date with regards to the biology of musth in bull elephants, and confirms that this is a complex phenomenon with regards to hormone and other biomarker variations, characterized by altered gonadal (testosterone), pituitary (LH and FSH), metabolic (glucose, triglycerides), adrenal (cortisol) and thyroid (total and free T_4) activity. LH concentrations were significantly higher during musth, although there was considerable individual variation, which limits its use as an indicator of impending musth. FSH variations with musth were even less clear, but remain of interest in addition to LH monitoring. Our results showed an increase in glucose and triglyceride concentrations during musth; however, individual profiles were less clear, suggesting there is inter-individual variability in how bulls cope with musth. Because metabolic parameters vary with food intake and body condition, frequent blood sampling, combined with regular assessments of body condition, weight and appetite, could help us better understand the metabolic challenges of musth. Thyroid hormone profiles showed a clear decrease in total and free T_4 during musth in most of the bulls, which was consistent with it being a period of partial food deprivation and increased physical/sexual activity. Cortisol increased during musth, suggesting that it represents a form of physiological stress.

As the number of maturing bulls in the captive population continues to increase, we will have continuing opportunities to build upon this research and conduct more targeted comparative research on the physiology of musth. Our results highlight the need for facilities housing bulls to closely and consistently monitor each individual from an early age and throughout every musth cycle. Longitudinal weekly or bi-weekly blood (preferred), urine or fecal sampling should be combined with weekly body condition scoring and weight, as well as physical (TGS, UD) and behavioral (increased aggression, unpredictable behavior, sexual activity, decreased appetite) signs indicative of musth and recorded using a standardized musth log. Regular information on the social circumstances of each bull (size and composition of the group) as well as health records would also be useful to understand variation in musth expression. Uniformity in data collection would help facilities define musth cycles of each bull, as well as facilitate more rigorous large scale studies of musth control in captivity. Furthermore, additional long-term studies of musth in wild bulls of both species could shed light on the natural expression of musth and mechanisms underlying this phenomenon.

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