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Differential testosterone response to GnRH-induced LH release before and after musth in adult Asian elephant (*Elephas maximus*) bulls

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ABSTRACT

Bull elephants exhibit marked increases in testosterone secretion during musth, and studies have shown a heightened sensitivity of the testis to GnRH-stimulated testosterone production in musth compared to nonmusth males. However, activity of the hypothalamo-pituitary-gonadal axis before or soon after musth has not been studied in detail. The aim of this study was to evaluate LH and testosterone responses to GnRH challenge in nine adult Asian elephant (*Elephas maximus*) bulls during three periods relative to musth: premusth, postmusth, and nonmusth. Bulls were administered 80 μg of a GnRH agonist, and blood was collected before and after injection to monitor serum hormone concentrations. The same bulls were injected with saline 2 weeks before each GnRH challenge and monitored using the same blood collection protocol. All bulls responded to GnRH, but not saline, with an increase in LH and testosterone during all three periods. The mean peak LH (1.76 ± 0.19 ng/mL; $P < 0.001$) and testosterone (6.71 ± 1.62 ng/mL; $P = 0.019$) concentrations after GnRH were higher than the respective baselines (0.57 ± 0.07 ng/mL, 3.05 ± 0.60 ng/mL). Although basal- and GnRH-induced LH secretion were similar across the stages, evaluation of the area under the curve in GnRH-treated bulls indicated that the testosterone response was greatest during premusth (2.84 ± 0.76 area units; $P = 0.019$) compared to postmusth (2.02 ± 0.63 area units), and nonmusth (2.01 ± 0.46 area units). This confirms earlier reports that GnRH stimulates LH release and subsequent testosterone production in bull elephants. Furthermore, although the hypothalamo-pituitary-gonadal axis is active throughout the year, the testis appears to be more responsive to LH in terms of testosterone production in the period leading up to musth, compared to the nonmusth and postmusth periods. This heightened sensitivity, perhaps as a result of LH receptor up-regulation, may prime the testis for maximal testosterone production, leading to the physiological and behavioral changes associated with musth.

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1. Introduction

Musth is the circannual period of heightened sexual activity typically observed in mature Asian and African bull elephants. Musth comprises physiological, anatomical, and behavioral changes that play an important role in elephant society by helping ensure that dominant bulls produce the most offspring [1,2]. Musth is typically associated with increases in serum androgen concentrations and temporal gland secretions (TGS) and continuous urine dribbling (UD) [3–6]. Rajaram (2006) [7] described five stages of musth: premusth, earlymusth, mid-musth, postmusth, and nonmusth. In premusth, volatile compounds are released from the temporal gland. The early- and mid-musth stages are characterized by TGS, which becomes odorous as the period progresses. During mid-musth, TGS and continual UD are observed, and the bull often becomes very aggressive [7] in conjunction with a significant increase in circulating androgens [4,6,8–11]. These signs decrease during postmusth and disappear during the nonmusth period. In Asian range countries, most healthy adult bulls exhibit musth at a predictable time each year although the timing can vary between individuals [8]. In Thailand, musth is most often observed during the late rainy season or early winter (November–January) and lasts from 1 week to 3 months or more.

Musth in elephants appears to be under control of the hypothalamo-pituitary-gonadal (HPG) axis; indeed, exogenous GnRH induces a surge of LH secretion, followed by the release of testosterone [8,12]. Frequent sampling of Asian elephant bulls has further demonstrated that pulses of testosterone closely follow those of LH (~1 pulse/3 hours) [13]. In a long-term study (4 years) of one African bull, serum LH increased about 4 weeks before the onset of musth and was maintained for approximately 5 weeks and was considered likely to be responsible for triggering the rise in testosterone [14]. The temporal relationship between aggressive behaviors and androgen production during musth suggests that the two are linked although the regulatory mechanisms are unknown. Temporally, TGS and UD occur one to several weeks after the initial rise in testosterone, with TGS almost always observed before UD [4,5,8,10]. Aggressive behavior also appears to be more intense when testosterone begins to decline [8,12]. In short, there tends to be a lag between peak testosterone production and musth symptoms, not unlike that described for Soay sheep, where maximal aggression occurs after androgens begin to decrease [15]. Men also exhibit increased aggression, anger, and irritability after a temporary reduction in testosterone [16]. However, mahouts can often tell when a bull is coming into musth based on behavioral changes, which presumably is due to increasing testosterone concentrations.

Based on frequent blood sampling (every 15 minutes for 12 hours), little difference in LH and testosterone pulse frequency was observed between musth and nonmusth Asian bulls [13]; however, pulse amplitude and pulse area were significantly increased during musth. Likewise, in African elephants, the testes of musth bulls appeared to be hyper-responsive to LH with regard to testosterone secretion after GnRH injection, compared to nonmusth males [12]. There are, however, no reports on whether the sensitivity of the elephant HPG axis differs during the more discrete periods of pre and postmusth compared to nonmusth in Asian or African elephant bulls. The objective of this study was therefore to compare the LH and testosterone responses to GnRH administered during the nonmusth, premusth, and postmusth periods in captive Asian elephant bulls.

2. Materials and methods

2.1. Animals

Nine adult male Asian elephants (*Elephas maximus*) housed at the Ban Pang Lah rehabilitation area of the Thai Elephant Conservation Center (National Elephant Institute, Forest Industry Organization in Lampang, Thailand) were used in this study. The bulls (E1–E9) averaged 58.5 ± 8.5 years of age (range, 45–67 years) and had a history of regular, predictable annual musth periods occurring in late November to January, based on mahout interviews and facility records (Table 1). The musth period was defined as the period in which elephants exhibited both TGS and UD [5]. During this time, bulls were isolated from other elephants and tethered in a restricted forest area using a 20- to 25-m chain, within reach of a natural water supply. Food was provided by the mahout at least four times per day, and the bulls' behavior was observed until they came out of musth. During nonmusth, the bulls were tethered in the forest using a 25- to 30-m chain, and moved to a different location every 2 to 3 days. Nonmusth bulls were allowed to forage in the forest, and all bulls were additionally supplied with bana grass (*Pennisetum purpureum* × *Pennisetum americanum* hybrid), banana, sugar cane, grain, and elephant pellets (CPF Feed Marketing Bureau, Thailand); water was provided ad libitum. Elephant health was monitored daily by mahouts, and general health checks were conducted by veterinarians three times annually. The body condition of each bull was scored using an 11-point scale developed for Asian elephants [17] (Table 1). All bulls were privately owned and used in the

Table 1
Summary of Asian elephant bulls used to assess pituitary-gonadal responses to GnRH agonist injection during nonmusth, premusth, and postmusth periods.

Elephant number	Age (years)	BCS[c]	Previous musth duration (weeks)	GnRH challenge[d]		
				Non	Pre	Post
E1	45	7	2	+	+	+
E2	65	10	8	+	+	+
E3	67	8	2	+	+	+
E4[a]	54	9	8	+	+	−
E5[b]	60	7	2	+	+	−
E6[a]	45	7	2	+	+	−
E7	60	8	4	+	+	+
E8	65	9	20	+	+	+
E9	65	10	20	+	+	+

[a] Translocated before the third GnRH challenge.
[b] Died before the third GnRH challenge.
[c] Body condition score (BCS) based on an 11-point system [17].
[d] Bulls received (+) or did not receive (−) GnRH.

study at the owner's written request. All procedures were conducted by licensed veterinarians.

2.2. GnRH challenge

Elephants were injected intravenously with 80 μg of a GnRH agonist (Receptal; Intervet International B.V., Boxmeer, The Netherlands) on three occasions with the timing on the basis of historical, predetermined temporal relationships to musth: nonmusth (June–August; no TGS or UD); premusth (October; temporal gland swelling and enlarged opening); and postmusth (February; TGS and UD diminished). As a control, each bull received an injection of physiological saline 2 weeks before the GnRH agonist challenge. Because of anticipated aggression and the risk of human injury, bulls were not treated or blood sampled during the musth period. Two bulls were removed (at the owners' discretion) and one died from a venomous snake bite, before receiving the third (i.e. postmusth) GnRH challenge. The dose of GnRH selected was on the basis of what had previously been shown to stimulate the HPG axis in elephants [18,19]. Blood (5–7 mL) was collected from an ear vein at −15, 0, 30, 60, 90, and 180 minutes and 24 hours after GnRH administration via an indwelling catheter. Samples were allowed to clot at room temperature for 1 to 2 hours, before being centrifuged at 2000 × *g* for 5 minutes to separate serum from blood cells. Serum samples were stored at −20 °C until hormone analysis.

2.3. Hormone analysis

Serum testosterone was quantified using a single-antibody enzyme immunoassay (EIA) based on a rabbit anti-testosterone polyclonal antibody (R156/7) and a horseradish peroxidase–conjugated testosterone tracer obtained from Coralie Munro (University of California, Davis, CA, USA), that has been previously validated in elephants [20,21]. The testosterone antibody was reported to cross-react with testosterone (100%), 5α-dihydrotestosterone (57.4%), androstenedione (0.3%), androsterone (0.04%), Dehydroepiandrosterone (0.04%), cholesterol (0.03%), and β-estradiol (0.02%) [22]. The EIA was performed in 96-well plates (Nunc Maxisorp, Fisher Scientific, Pittsburgh, PA) coated 16 to 24 hours previously with antiserum (50 μL; 1:8500 dilution) in coating buffer (0.05-M $NaHCO_3$, pH 9.6). Standards (50 μL; range, 2.3–600 pg/well) diluted in assay buffer (0.1-M $NaPO_4$, 0.149-M NaCl, 0.1% BSA, pH 7.0) and samples (50 μL; diluted in 0.1-M $NaPO_4$, 0.149-M NaCl, pH 7.0) were combined with HRP (50 μL; 1:90,000 dilution) and incubated at room temperature (RT) for 2 hours. Plates were washed (Biochrom Anthos Fluido 2 microplate washer, Cambridge, UK) five times with wash buffer (0.1-M $NaPO_4$, 0.149-M NaCl, 0.05% Tween 20) before addition of 100-μL substrate (0.4-mM 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) to each well. After incubation for approximately 30 minutes, the absorbance was measured at 405 nM (TECAN Sunrise microplate reader; Salzburg, Austria) until the optical density approached 1.0. The EIA was validated for elephant serum by demonstrating parallelism between serial dilutions of bull elephant serum and the standard curve and greater than 90% recovery of testosterone standard added to samples before analysis. Assay sensitivity, based on 90% of maximum binding, was 0.047 ng/mL.

Serum LH was quantified with an EIA, previously validated for elephants, that used a mouse monoclonal anti-bovine LH antiserum (518-B_7 supplied by Dr. Jan Roser, University of California, Davis), biotin-conjugated ovine LH label (provided by Dr. Janine Brown), horseradish peroxidase-conjugated streptavidin (catalog #1089153; Roche Diagnostics, Indianapolis, IN), and bovine LH standards (NIH-LH-B10; provided by Dr. A. Parlow, National Hormone and Pituitary Program) [23]. The biotinylated LH was prepared using an EZ-Link Sulfo-NHS-LC-Biotinylation kit (catalog #21430; Pierce, Rockford, IL). The EIA was performed in 96-well plates coated 16 to 24 hours previously with affinity purified anti-mouse gamma globulin (250 μL; 1:8500 dilution; Sigma Chemical Company, Singapore) in coating buffer (0.05-M $NaHCO_3$, pH 9.6). On Day 2, plates were washed and 300 μL protein blocking buffer (0.02-M Trizma, 0.3-M NaCL, 1.0% BSA, 0.01% NaN_3, pH 7.5) was added. The plates were then covered with an acetate plate sealer and incubated for at least 12 hours at RT. Plates were used within 2 weeks of coating. On Day 3, plates were washed, and 50 μL of standards (1.95–500 pg/well), samples or controls were combined with 518-B_7 antiserum (50 μL; 1:600,000 dilution) and incubated for at least 12 hours at RT. On Day 4, biotinylated LH (100 μL; 1:250,000) was added, the plates were sealed and incubated for 4 hours at RT. After incubation, plates were washed and 200 μL streptavidin-peroxidase conjugate (1 μL in 30 mL assay buffer; Roche Molecular Biochemicals, Indianapolis, IN) was added to each well and incubated for 45 minutes at RT. Plates were washed and 200-μL substrate solution (500 μL of 0.016-M tetramethylbenzadine in dimethyl sulfoxide and 100-μL 0.175 M H_2O_2 diluted in 24-mL 0.01 M $C_2H_3O_2Na$, pH 5.0) was added and incubated for approximately 45 minutes at RT. The enzyme reaction was stopped with 50 μL of 3 M H_2SO_4 and the optical density measured at 430 nM. Sensitivity of the assay was 0.16 ng/mL.

For both assays, the intra-assay and interassay coefficients of variation for high-and low-concentration controls were less than 10% and 15%, respectively.

2.4. Statistical analysis

The data are presented as mean (±standard error of the mean) hormone concentrations. Analysis of variance followed by Least Significant Difference post hoc tests was used to determine differences in overall mean LH and testosterone concentrations in saline-treated bulls during the three different stages. For GnRH-treated bulls, peak hormone concentrations were identified as the highest point after GnRH injection for each bull. A repeated measures analysis was used to determine differences in hormone responses between nonmusth, premusth, and postmusth bulls after saline and GnRH treatment (SPSS 15.0; SPSS Inc., Chicago). Within each musth stage, a Dunnett's procedure was used to test for differences in mean serum LH and testosterone concentrations at time points after GnRH

compared to the baseline (15 and 0 minutes). Area under the curve (AUC; ng-h mL^{-1}) was calculated for individual serum LH and testosterone responses to GnRH or saline [24,25], and the data transformed to give the increase relative to baseline concentrations (fold change). Paired *t* tests were used to compare the AUC of hormone responses between control (saline) and treatment (GnRH) groups for the nonmusth, premusth, and postmusth periods. Pearson's correlation was used to examine relationships between age and BCS, BCS and previous musth duration, and age and previous musth duration. Differences were considered significant at $P < 0.05$.

3. Results

Body condition scores of elephants in this study averaged 8.3 ± 1.2 (range, 7–10) and did not change significantly during the study period. There was a positive correlation between BCS and the duration of previous musth ($r = 0.756$; $P < 0.05$). The relationship between BCS and age approached significance ($r = 0.639$; $P = 0.064$), but age was not significantly correlated with previous musth duration ($r = 0.491$). Bulls came into musth within the predicted time period, about 4 to 8 weeks after the premusth GnRH challenge.

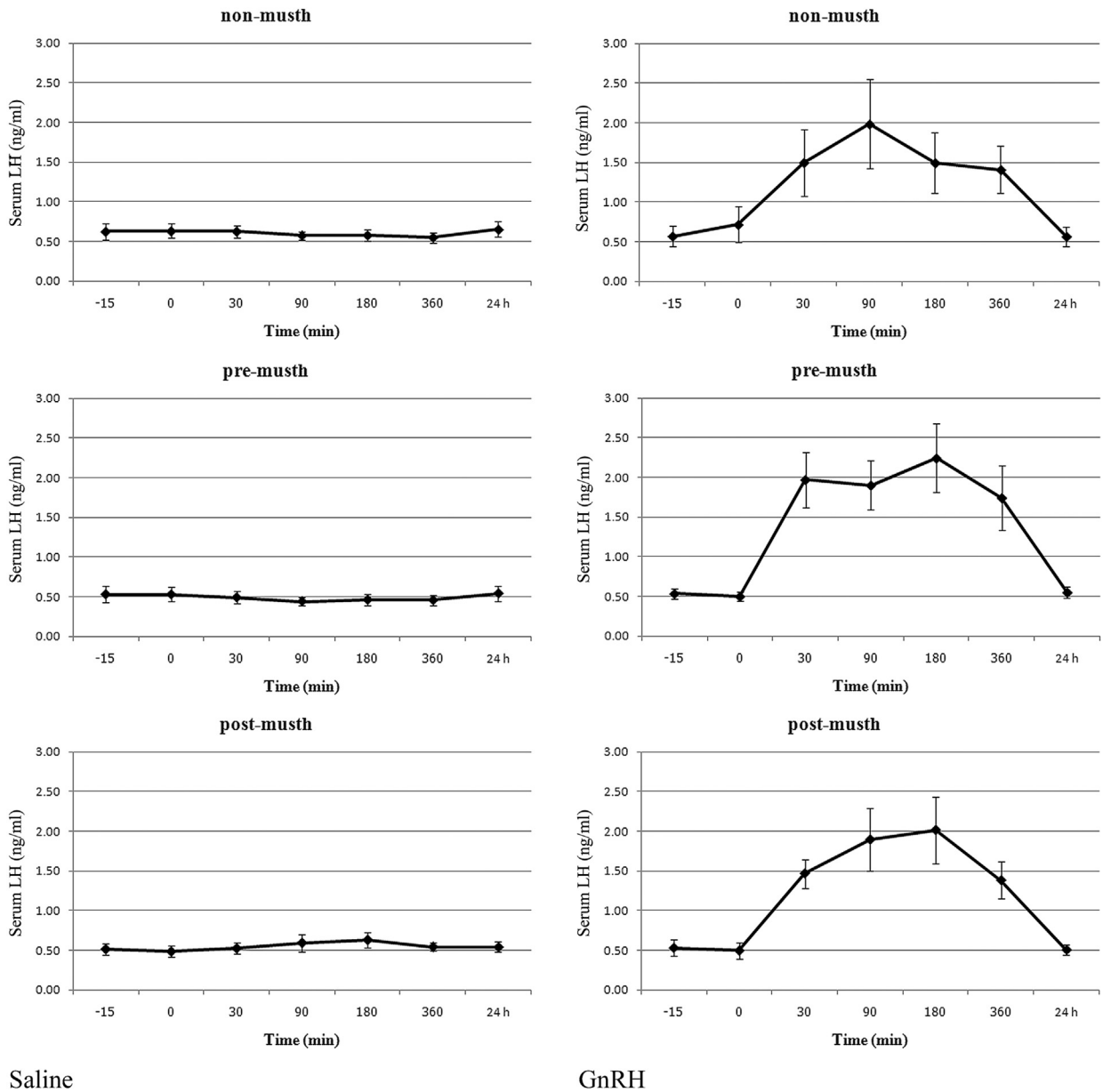

Fig. 1. Mean (±standard error of the mean) serum LH concentrations before and after intravenous injection of physiological saline (control) or a GnRH agonist (80-μg buserelin) at 0 minutes in Asian elephant bulls during the nonmusth (n = 9), premusth (n = 9), and postmusth (n = 6) stages.

Overall mean circulating hormone concentrations for the saline-treated bulls were 0.54 ± 0.02 ng/mL for LH and 5.10 ± 0.61 ng/mL for testosterone. Indeed, mean LH and testosterone concentrations after saline treatment were similar across the nonmusth (0.61 ± 0.05 ng/mL; 5.61 ± 1.14 ng/mL), premusth (0.50 ± 0.03 ng/mL; 5.13 ± 0.98 ng/mL), and postmusth (0.55 ± 0.03 ng/mL; 4.30 ± 0.95 ng/mL) periods, respectively, although testosterone concentrations did vary between bulls. All bulls responded to GnRH with an increase in serum LH (Fig. 1) that rose beyond the baseline within 30 minutes of injection. Concentrations remained elevated throughout the initial 360 minutes of sampling but had declined to baseline at 24 hours after GnRH injection (Fig. 1). Testosterone began to increase about 30 minutes after the initial rise in LH and remained elevated throughout the rest of the 360 minutes but had also returned to baseline at 24 hours (Fig. 2). No significant injection-related increases in LH or testosterone secretion were observed in any of the saline-treated animals.

When comparing responses between GnRH and saline treatment, the percentage AUC for LH was higher after GnRH than saline injection across all periods: nonmusth ($P = 0.015$), premusth ($P < 0.001$), and postmusth ($P = 0.007$; Fig. 3). The testosterone AUC after GnRH was significantly greater than after saline injection during the

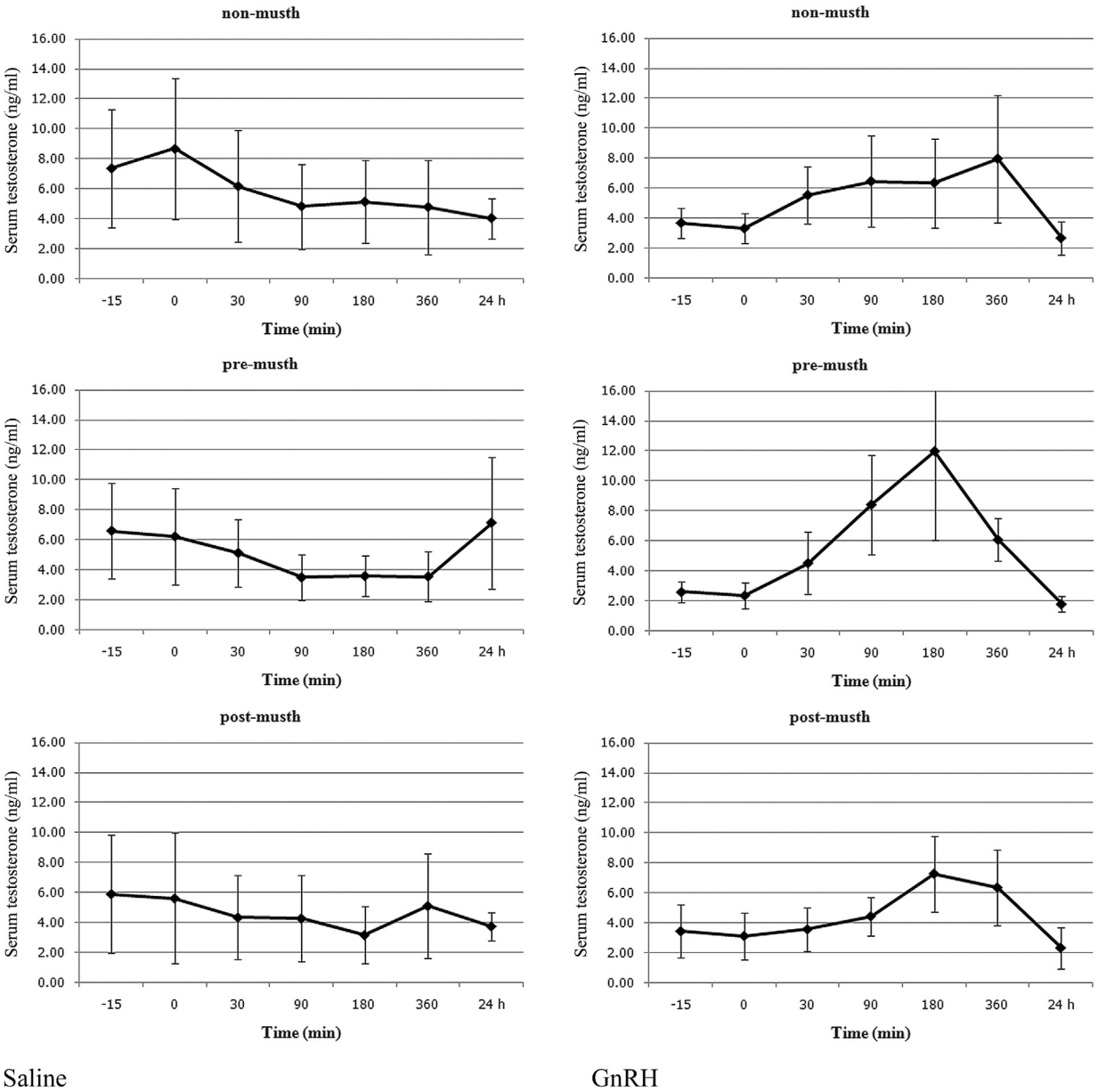

Fig. 2. Mean (±standard error of the mean) serum testosterone concentrations before and after intravenous injection of physiological saline (control) or GnRH at 0 minutes in Asian elephant bulls during nonmusth (n = 9), premusth (n = 9), and postmusth (n = 6) stages.

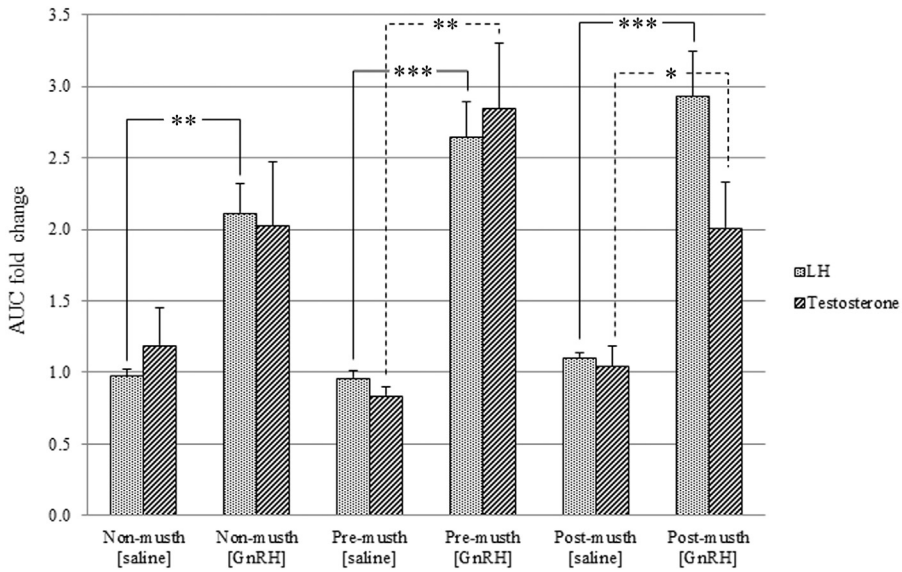

Fig. 3. Mean (±standard error of the mean) fold change in the area under the curve (AUC) of the LH and testosterone responses after administration of saline or GnRH to Asian elephant bulls during nonmusth(n = 9), premusth (n = 9), and postmusth (n = 6) stages. The percentage of AUCs was increased 2- to 3-fold after GnRH treatment but showed little response after saline. The star superscripts within treatments and within stages indicate differences: *(P = 0.07), **(P < 0.05), ***(P < 0.01).

premusth period (P = 0.019) but not during nonmusth (P = 0.245) or postmusth (P = 0.070; Fig. 3).

AUCs for LH after saline injection were similar for all three musth stages; LH AUCs between 30 and 180 minutes after injection were higher after GnRH administration (P < 0.05) but did not differ among nonmusth, premusth, and postmusth stages. By contrast, the AUC for testosterone after GnRH injection was higher during premusth than during the nonmusth and postmusth periods (P < 0.05; Fig. 3). Peak LH and testosterone concentrations were similar after GnRH injection across the nonmusth, premusth, and postmusth periods (Fig. 4).

4. Discussion

These results confirm earlier reports that exogenous GnRH stimulates pituitary LH release and subsequent testicular testosterone secretion in Asian bull elephants. Temporal patterns of GnRH-induced LH and testosterone secretion were comparable to previous reports in bull elephants, with serum LH increasing approximately 30 minutes after GnRH administration, followed by a rise in testosterone about another 30 minutes later. For example, in a study of captive Asian bulls in Sri Lanka, an increase in serum testosterone was observed 45 to 60 minutes after intravenous administration of 20-μg GnRH [8]. In wild African bulls, LH increased within 10 to 25 minutes after 300-μg GnRH, followed by testosterone elevations about 35 to 40 minutes postinjection [12]. Similar pituitary responses to GnRH also have been observed in female elephants, with LH increasing within a half hour of injection of varying doses of GnRH (80-μg Receptal, as used in this study, [19]; 500-μg–5-mg Cystorelin, [18]) and remaining elevated for 4 to 6 hours before returning to baseline.

Time series analyses indicated that bulls responded to GnRH with a similar, significant increase in LH during all of the nonmusth, premusth, and postmusth periods. However, further analyses using AUC measurements revealed that for testosterone, a more robust increase after GnRH occurred during the premusth period. This difference was observed despite similar GnRH-induced LH responses. It therefore appears that the HPG axis is more sensitive during the premusth period, with respect to the testicular response to LH. Niemuller and Liptrap (1991) [13] also noted similar mean LH concentrations between musth and nonmusth Asian elephant bulls, whereas testosterone responses to pulsatile LH were greater during musth. Likewise, African elephant bulls secreted more testosterone in musth than nonmusth after a GnRH challenge, although the LH response was lower [12]. Taken together, prior results and the current study suggest that the testes of musth bulls are more responsive to LH in terms of testosterone production. Although not examined in elephants, this sensitivity change could be receptor mediated. In other species, increased testosterone production is associated with higher concentrations of testicular LH receptors rather than changes in circulating LH [26–28]. In this regard, premusth bulls may be more similar physiologically to musth bulls than postmusthor nonmusth males in terms of HPG activity. Temporally, testosterone production increases before TGS and UD, whereas aggression tends to be greatest when testosterone is declining [4,5,8,10]. It follows that changes in testosterone concentrations may be causal in stimulating the onset of musth, at least in part, although it is important to point out that mahouts are often aware of the start of musth because of behavioral changes.

What triggers annual musth cycles in Asian and African elephant bulls is not clear. Although musth episodes often

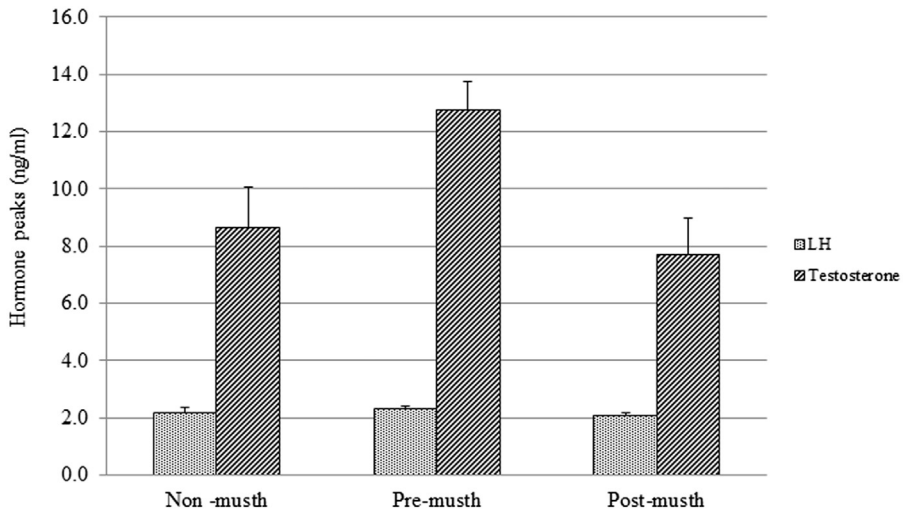

Fig. 4. Mean (±standard error of the mean) peak concentrations of LH and testosterone after administration of GnRH to Asian elephant bulls during nonmusth (n = 9), premusth (n = 9), and postmusth (n = 6) stages. There were no differences between stages for either hormone.

coincide with seasonal components (e.g., end of the rainy season), bulls can exhibit musth at other times of the year [8,10,13]. Nutrition has been linked to duration and intensity of musth episodes. Both wild [29] and domesticated [30] elephants fall out of musth in response to the loss of body condition. Consequently, one method of decreasing unwanted symptoms is to withhold food and water [8,31]. The bulls in this study all had relatively high BCSs, ranging from 7 to 10 on an 11-point scale; thus, all were above what might be considered normal (mid-score = 5.5) [17]. In fact, those that scored a 10 could be considered overweight. Musth lasted between 2 and 20 weeks, and was positively correlated with BCS, which adds to the evidence that high body condition can help sustain a bull in musth for longer. Dominance status and age can also affect the degree and intensity of musth [8,10,13,32,33]; however, animals in this study were not housed together or even in close proximity, so there was no dominance hierarchy per se. The bulls were all relatively old, ranging from 45 to 67 years of age, but still showed a positive relationship between age and previous musth duration.

In this study, mean LH in saline-treated controls did not differ among bulls in nonmusth, premusth, and postmusth. Other studies found no difference in mean LH between musth and nonmusth periods in Asian [13] and African [12] bull elephants. However, Kaewmanee et al. (2011) [14] noted a distinct increase in serum LH about 4 weeks before the onset of musth that persisted for about 5 weeks, declining to baseline when musth behaviors became more evident. As a result, LH concentrations were significantly higher during premusth, which was designated to occur around 6 weeks before the beginning of musth. In our study, the premusth period was not as clearly defined and was preemptively chosen on the basis of a bull's historical musth periods. For most bulls, the premusth period was considered to take place about 1 to 2 months before musth was observed. Thus, it is possible we conducted the GnRH challenge before the natural window of increasing LH. Then again, the study of Kaewmanee et al. (2011) [14] was conducted in a single African elephant bull, so additional studies are required to more fully describe LH secretory patterns in the period leading up to musth in both Asian and African elephants.

In summary, our results confirm earlier reports that GnRH stimulates LH release and subsequent testosterone production in Asian bull elephants. Furthermore, the HPG axis is active and capable of responding to a GnRH challenge throughout the year. However, the testis appears to be more responsive to LH secreted in response to GnRH challenge in terms of subsequent testosterone production in the period leading up to musth as compared to the nonmusth and postmusth stages. Such heightened sensitivity, perhaps through up-regulation of LH receptors, may prime the testes for maximal testosterone production, leading to the physiological and behavioral changes associated with musth.

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