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Potential of a gonadotropin-releasing hormone vaccine to suppress musth in captive male Asian elephants (*Elephas maximus*)

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ABSTRACT

Musth in adult bull elephants is a period of increased androgen concentrations ranging from a few weeks to several months. For captive elephant bull management, musth presents a serious challenge because of the aggressive behavior of musth bulls toward people and other elephants. Commercially available GnRH vaccines have been shown to suppress testicular function by interrupting the hypothalamo-pituitary-gonadal (HPG) axis in many species. The aim of this study was to test the efficacy of a GnRH vaccine in elephant bulls for suppressing the HPG axis and mitigating musth-related aggressive behavior. Five adult Asian elephant bulls (22–55 years old) were immunized with a GnRH vaccine starting with an initial injection 2–4 months before the predicted musth period, and followed by three boosters at approximately 4-week intervals. Blood samples were collected twice weekly for hormone and antibody titer analysis. An increase in GnRH antibody titers was observed in all bulls after the second or third booster, and titers remained elevated for 2–3 months after the final booster. Musth was attenuated and shortened in three bulls and postponed completely in two. We conclude that GnRH vaccination is capable of suppressing symptoms of musth in adult bull elephants. With appropriate timing, GnRH vaccination could be used to control or manage musth and aggressive behavior in captive elephant bulls. However, more work is needed to identify an optimal dose, booster interval, and vaccination schedule for complete suppression of testicular steroidogenesis.

1. Introduction

The phenomenon of musth has been recognized for centuries in Asian elephants (*Elephas maximus*) (Darwin, 1871; Eisenberg et al., 1971; Jainudeen et al., 1972a; Lincoln and Ratnasooriya, 1996), and since the 80s in African elephants (*Loxodonta africana*) (Hall-Martin and van der Walt, 1984; Poole and Moss, 1981; Poole, 1989).

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In general, musth is a period of heightened aggressive and sexual behavior associated with temporal gland swelling (TG), temporal gland secretion (TGS), urine dribbling (UD) and elevated androgen (e.g., testosterone, dihydrotestosterone, androstenedione) production (Brown et al., 2007; Ganswindt et al., 2005; Lincoln and Ratnasooriya, 1996; Poole et al., 1984; Yon et al., 2008). A temporal relationship between aggressive behavior and increased androgen secretion during musth suggests that the two are linked. Bull elephants advertise their musth status via a variety of chemicals excreted in the TGS and urine. In young Asian bulls between ~8 and 13 years of age, the TGS consists of sweet odors; namely acetates, an alcohol derivative (3-hexen-2-ol) and ketones (acetophenone and 2-heptanone), and is associated with erratic and unpredictable behaviors expressed at comparatively low androgen levels (Rasmussen et al., 2002; Riddle et al., 2006). Older bulls produce malodorous compounds, such as carboxylic acids, ketones, frontalin, alkan-2-ones and alkan-2-ols in association with a more regular, annual musth cycle (Goodwin et al., 2012). Elephants have well-developed primary and secondary (vomeronasal) olfactory systems (Lazar et al., 2004), and both sexes respond to these chemical signals. Estrous females seek out musth bulls, and sub-adult males (especially non-musth) exhibit avoidance behavior when exposed to musth semiochemicals (Goodwin et al., 2012; Riddle et al., 2006). Ultimately, being in musth confers an advantage to adult bulls, which experience a temporary rise in dominance rank that ensures better access to estrous females and increases their likelihood of paternity, at least in African elephants (Hollister-Smith et al., 2007). Although not absolutely necessary for breeding, musth is regarded as an important reproductive strategy for both elephant species (Hollister-Smith et al., 2007; Sukumar, 2003).

From a management standpoint however, musth bulls create serious problems because of the associated increase in aggressive and unpredictable behavior (Eisenberg et al., 1971; Jainudeen et al., 1972a,b). Captive elephants in musth have severely injured and even killed handlers, and free-living musth bulls often threaten human life and property (Eisenberg et al., 1971; Lincoln and Ratnasooriya, 1996). In tourist camps in Thailand, musth bulls are isolated from other elephants and people, generally by tethering in a restricted area. One factor linked to the occurrence and/or intensity of musth is nutritional status. Both wild (Poole, 1989) and 'domesticated' (Jainudeen et al., 1972a) elephants drop out of musth in response to a loss of body condition. Consequently, one method of decreasing musth symptoms is to decrease food intake, especially of feedstuff high in energy (Cooper et al., 1990; Lincoln and Ratnasooriya, 1996). As well as raising ethical concerns, it is not clear what effect this food restriction has on the health and fertility of the bulls, and there is therefore considerable interest in identifying other means of suppressing the unwanted behaviors associated with musth.

Musth appears to be related to elevated circulating androgen concentrations (Cooper et al., 1990; Jainudeen et al., 1972b; Hall-Martin, 1987; Hall-Martin and van der Walt, 1984; Rasmussen et al., 1984); therefore, any therapy that suppresses pituitary LH release and subsequent testosterone secretion for a reasonable duration might attenuate or even alleviate behavior problems until the musth cycle ends. Castration offers a permanent solution, but the surgery is difficult, because of the intra-abdominal location of the testes, and would be unacceptable if a bull is later needed for breeding. The GnRH agonist leuprolide acetate (Lupron®; Tekada, Abbott) (Brown et al., 1993; de Oliveira et al., 2004) has been shown to reduce testosterone secretion during musth, but requires repeated injections. More recently, the use of GnRH vaccines has been described in numerous domestic animal species. These are designed to stimulate the production of anti-GnRH antibodies that block the binding of endogenous GnRH to gonadotrophin receptors in the pituitary gland (Stout and Colenbrander, 2004), which leads to a reduction in the release of LH from the anterior pituitary and, in turn, reduced stimulation of gonadal steroidogenic activity. Immunization against GnRH has been used successfully to suppress gonadal function in a number of laboratory, wildlife, and livestock species (Goodloe et al., 1996; Kirkpatrick et al., 2011), including elephants to control testosterone driven behaviors (Bertschiger et al., 2004; De Nys et al., 2010; Lueders et al., 2014). The advantages of a GnRH vaccine are that it is low cost, easy to use, safe, and at least after short-term use, it appears to be reversible (Bertschiger et al., 2004; De Nys et al., 2010; Kirkpatrick et al., 2011). Long-term use could, however, impair fertility based on a study of one young Asian elephant bull, in which multiple booster injections led to irreversible testicular, accessory gland and penile atrophy over a period of years (Lueders et al., 2014).

Bulls in Thailand typically enter musth after the rainy season, November–February, which is the high season for tourists. Thus, bulls in musth create both safety and economic concerns for elephant tourist camp managers. Additionally, current methods to mitigate musth behavior, such as social isolation, short chaining and food restriction, can have negative consequences for animal welfare. The objectives of this study were to assess the ability of a commercially available GnRH vaccine to suppress pituitary–testicular activity, and mitigate aggressive behaviors associated with musth in Thailand tourist camp elephants.

2. Materials and methods

2.1. Animals

Five adult male Asian elephants (*E. maximus*) maintained at three elephant camps in Thailand, one in the south ($n = 3$ bulls) in Suratthani province and two in the north ($n = 2$) in Chiang Mai and Lampang provinces, were used in this trial. All bulls were privately owned and used in this study on the basis of informed written consent by the owner. The bulls were 22–55 years of age and had experienced regular annual cycles of musth for at least 3 years prior to the study (Table 1). Bulls were observed daily for signs of musth, which was defined as exhibition of both TGS and UD (Ganswindt et al., 2005) and were assigned a musth score based on these signs and levels of aggressive behaviors (Table 2). Bulls were fed natural grasses (bana grass; *Pennicetum purpureum* × *P. americanum* hybrid, nepia grass; *P. purpureum*),

Table 1
GnRH vaccination schedule for five adult male Asian elephants, and the effect on subsequent musth cycles.

Bulls	Musth score (non-musth/musth)	BSC[b]	Age (years)	Days between vaccinations				Previous musth[c] (days)	Actual musth[e] (days)	Vaccine effects on musth	Musth signs[f]			
				1	2	3	4				TG	TGS	UD	Agg
1	1/2	8	35	0	30	60	75	Nov–Jan (60)	Nov (21)	Musth duration decreased	+	+	–	+
2	2/4	7	22	0	30	60	85	Nov–Dec (30)	–	Musth postponed	–	–	–	–
3	1/4	8	45	0	30	60	70	Nov–Feb (120)	Nov (17)	Musth duration decreased	+	+	–	+
4	5/5	7	50	0	29	57	86	Jan–Dec[d]	Nov (3)	Musth duration decreased	+	+	–	+/–
5[a]	1/4	8	55	0	32	59	89	Jul (30)	–	Musth postponed	–	–	–	–

[a] Bull was vaccinated and boosted with 1200 μg GnRH peptide.
[b] Body condition score based on criteria of Wemmer et al. (2006).
[c] Historical annual musth period; months that musth was observed with approximate length (days) in parentheses.
[d] Bull was in a nearly constant state of musth before vaccination.
[e] Post-vaccination musth period; months that musth was observed with approximate days in parentheses.
[f] Based on TG = temporal gland swelling, TGS = temporal gland secretion, UD = urine dribbling and Agg = aggression; + increased, – no change.

supplemented with banana, sugar cane and fruits, and provided with water *ad libitum*. Bulls in the southern camp participated in elephant riding in the jungle for about 3–4 h/day, and were bathed and fed by tourists. When not working, elephants were chained in a holding area with visual, auditory and olfactory contact with other elephants at a nearby Para rubber plantation. Bulls in the north were housed at an elephant rehabilitation center, where they interacted minimally with the public: mostly for viewing, and sometimes for feeding. Whenever possible, and safety permitting, bulls were allowed tactile contact with conspecifics several times a week. All bulls were in good health, which was monitored daily by the mahouts in addition to health exams performed approximately quarterly by veterinarians. Body condition of each bull was scored using an 11-point scale developed for Asian elephants (Wemmer et al., 2006) by a veterinarian (Table 1). A musth/behavior index also was developed (Table 2) based on interviews with mahouts. Each bull was assessed several times throughout the study, including determining a score for previous musth behaviors as a baseline.

2.2. Vaccination and sample collection

Bulls were vaccinated with a commercial GnRH vaccine (Improvac®; Pfizer Animal Health, Australia) as described for African elephants (Bertschiger et al., 2004). For Bulls 1–4, the initial injection consisted of a 600 μg dose (3 ml) given about 2 months before their expected musth (Table 1), followed by three booster vaccinations of 600 μg at approximately 4-week intervals. For two bulls (Bulls 1, 3), the third booster was given only 2 weeks after the second because they were beginning to enter their musth cycle based on the observation of increasing TGS. One bull (Bull 5) was administered a higher dose of 1200 μg GnRH peptide 4 months prior to his expected musth period, followed by three monthly boosters at the same higher dose. Each vaccination was administered aseptically, cleaning the skin thoroughly with alcohol, and then hand injecting deep intramuscularly into the cervical trapezius muscle with a 5 cm, 16-gauge needle. All animals were monitored for any vaccine site reactions for 24 h after injection.

Blood samples (10 ml) were collected twice weekly from an ear vein using a 2.5 cm, 20-gauge needle, beginning on

Table 2
Chart for scoring behavior and musth signs in Asian elephant bulls based on mahout interviews.

Score 1	Score 2	Score 3	Score 4	Score 5
1. Normal behavior[a] 2. Elephant puts or touches trunk to a stranger[b] [gentle] 3. Stranger can touch the bull's body	1. Ear extended and stiff 2. Stops activity and stares when stranger approaches 3. Stranger can touch the bull's body 4. Grumbles softly 5. Stranger can approach the bull	1. Ear extended and stiff 2. Stops activity and stares when stranger approaches 3. Hits trunk on ground when approached 4. Grumbles softly 5. Stranger cannot approach the bull	1. Ear extended and stiff 2. Stops activity and stares when stranger approaches 3. Hits trunk on ground or stamps foot when approached 4. Grumbles vigorously 5. Swings trunk at person 6. Charges at approaching stranger	1. Ear extended and stiff 2. Stops activity and stares when stranger or mahout approaches 3. Hits trunk on ground or stamps foot when approached 4. Grumbles or roars 5. Swings trunk at person 6. Charges at approaching person 7. Temporal gland swelling and secretions 8. Urine dribbling

[a] Relaxed ear flapping, trunk/tail movements, eating.
[b] Person or elephants that the bull is not familiar with.

the date of first vaccination (Day 0) through to Day 144 (Bull 1), Day 133 (Bulls 2 and 3), Day 143 (Bull 4) and Day 149 (Bull 5). Blood was allowed to clot for ~1 h before the serum was separated by centrifugation (1500 g) and stored at −20 °C until hormone and antibody titer analysis.

2.3. Hormone analyses

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 (HRP)-conjugated testosterone tracer obtained from Coralie Munro (University of California, Davis, CA, USA), and described previously for elephants (Thongtip et al., 2008; Mouttham et al., 2011). The testosterone antibody is reported to cross-react with testosterone (100%), 5α-dihydrotestosterone (57.4%), androstenedione (0.3%), androsterone (0.04%), DHEA (0.04%), cholesterol (0.03%) and β-estradiol (0.02%) (Gudermuth et al., 1998). The EIA was performed in 96-well plates (Nunc Maxisorp, Fisher Scientific, Pittsburgh, PA, USA) coated 16–24 h 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% bovine serum albumin [BSA], pH 7.0) and samples (50 μl) were combined with HRP (50 μl; 1:90,000 dilution) and incubated at room temperature (RT) for 2 h. 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 [ABTS]) to each well. After incubation for ~30 min, 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 >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 using an EIA that employed a mouse monoclonal anti-bovine LH antiserum (518-B_7 supplied by Dr. Jan Roser, University of California, Davis, USA), biotin-conjugated ovine LH label (provided by Dr. Janine Brown), HRP-conjugated streptavidin (catalog #1089153; Roche Diagnostics, Indianapolis, IN, USA) and bovine LH standards (NIH-LH-B10; provided by Dr. A. Parlow, National Hormone and Pituitary Program) (Graham et al., 2002); the assay was validated previously for elephant serum (Brown et al., 1991). The biotinylated LH was prepared using an EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (catalog #21430; Pierce, Rockford, IL, USA). The EIA was performed in 96-well plates coated 16–24 h 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, 0.30 M NaCL, 1.0% BSA, 0.01% NaN_3, pH 7.5) was added, the plates covered with an acetate plate sealer and incubated for at least 12 h 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 antisera (50 μl; 1:600,000 dilution) and incubated for at least 12 h at RT. On Day 4, biotinylated LH (100 μl; 1:250,000) was added, the plates sealed and incubated for 4 h at RT. Next, the plates were washed and 200 μl HRP-conjugated streptavidin (1 μl in 30 ml assay buffer) was added to each well before further incubation for 45 min at RT. Plates were washed and 200 μl substrate solution (500 μl of 0.016 M tetramethylbenzadine (TMB) 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 min 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- and inter-assay coefficients of variation (CV) for high and low concentration controls were <10 and <15%, respectively.

2.4. GnRH antibody titer analysis

An indirect, non-competitive EIA developed for swine (Zamaratskaia et al., 2008) was used to assess GnRH antibody titers in elephant serum. Ninety-six well plates were coated with GnRH (L7134, Sigma Chemical Co., Singapore; 100 μl of 10 mg/ml GnRH at 1:1000 in 1 M sodium carbonate buffer, pH 9.6) and incubated overnight at 2–8 °C. Before use, plates and reagents were equilibrated to RT. Plates were washed five times, blocked with a protein buffer (0.01 M Na_2HPO_4, 0.001 M KH_2PO_4, 0.05 M thimerosal, 5% casein, 0.149 M NaCl, 0.003 M KCl) and incubated for 1 h at RT. Plates were washed five times and 100 μl each of a control (from a vaccinated animal with Improvac®) or diluted serum sample (1:10–1:50) were incubated for 1 h at RT followed by addition of 100 μl of a rabbit anti-elephant gamma globulin (provided by A. Silimalaisuwan, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand) for 1 h at RT. After washing, 100 μl conjugate (goat anti-rabbit IgG-HRP, A6154, Sigma Chemical Co., Singapore, reconstituted with 1 ml 50% glycerol, diluted 1:5000 in buffer) was added and the plates were incubated for 1 h at RT, followed by a further wash, addition of 100 μl TMB substrate and incubation for 15 min at RT. The colorimetric reaction was stopped by adding 100 μl stop solution (0.6 M sulfuric acid) to all wells and reading the absorbance at 450 nm.

2.5. Data analysis

Data were tested for normality using a Shapiro–Wilk test for goodness-of-fit, and those that were not normal were log transformed. Titer and hormone data were subjected to statistical time series analysis and were modeled using the ASTRA program in SPSS 16.0 (SPSS Inc., Chicago, USA) for simple descriptive statistics. Baseline hormone and antibody titer values for each individual were determined using an iterative process (Brown et al., 2004) where the mean and standard deviation (SD) were calculated followed by removal of all values above the mean plus two times the SD. This process was repeated until only those values that no longer exceeded the mean plus 2

SD remained. The mean of the remaining values was considered baseline. Values above the mean plus 2 SD were considered elevated. Linear relationships between antibody titer, LH and testosterone profiles of individual bulls and all bulls combined were determined using Pearson Correlation Coefficient analyses. Data were compared between two time periods: before (Time Period 1) and after (Time Period 2) the third GnRH vaccine injection, and differences examined using *t*-tests. Data are presented as means ± SD. Significance was set at $P < 0.05$.

3. Results

The body condition score (BCS) of vaccinated elephants was 7–8 (Table 1), and did not change during the study period. There were no local or systemic adverse reactions to GnRH vaccination in any of the study animals. All bulls responded after the second or third booster with an increase in GnRH antibody titers (Fig. 1), although the magnitude of the response varied between individuals. Overall, antibody titers were higher in Time Period 2 than during Time Period 1 ($P < 0.001$). The clearest responses were observed in Bulls 1, 3 and 5, where GnRH antibody titers peaked at around 1:6000 to 1:7000, compared to the other two bulls in which they peaked at 1:4000 to 1:3000. Antibody titers remained elevated above initial values throughout the study period in all but Bull 4. In that animal, titers were only modestly increased and returned to baseline values about 85 days post initial vaccination. Bull 5, who received twice the vaccine dose (1200 μg vs. 600 μg), exhibited a robust antibody titer response, but titers were only elevated for about 60 days and were near baseline by the end of the study period. Although still elevated above baseline at the end of the study period, antibody titers were decreasing in all of the remaining bulls.

For all bulls combined, there was a negative correlation between anti-GnRH antibody titers and serum LH ($r = -0.284$; $P = 0.043$), and a positive correlation between serum LH and testosterone ($r = 0.328$; $P = 0.019$), but no correlation between anti-GnRH antibody titers and testosterone ($r = 0.039$; $P = 0.780$) throughout the study period. Examining the data by time relative to the third booster, there were no correlations between hormone concentrations and antibody titers during Period 1, whereas in Period 2, correlations were evident between anti-GnRH titer and serum LH ($r = -0.568$; $P = 0.003$) and serum LH and testosterone ($r = 0.395$; $P = 0.050$), but not between anti-GnRH titer and testosterone concentration ($r = -0.071$; $P = 0.736$). There were no differences in overall serum LH or testosterone concentrations between Periods 1 and 2 for all bulls combined ($P = 0.397$ and 0.546, respectively), although individual patterns of hormone secretion varied (Fig. 1).

Table 1 summarizes the vaccination schedule, BCS, and effects of vaccination on musth duration and behavior (musth score). In Bull 1, LH and testosterone were correlated ($r = 0.683$; $P = 0.030$), and concentrations of both increased temporarily as the male entered into an attenuated musth cycle (Fig. 1A). The bull exhibited TG swelling and TGS, but no UD and little aggressive behavior compared to his normal musth cycle (Table 1), and the episode lasted only 21 days, which was less than the previous true musth cycle that lasted 60 days. In Bull 2, both hormones were low, but correlated during the study period ($r = 0.819$; $P = 0.007$); Bull 2 exhibited no signs of musth and only limited aggressive behavior, compared to a 30-day musth the previous year (Fig. 1B; Table 1). A follow up with the mahout revealed that this bull did not exhibit a true musth until the following year. Bull 3 exhibited fluctuating concentrations of LH and testosterone that were correlated ($r = 0.806$; $P = 0.009$), especially during the first half of the study (Fig. 1C). However, after the third GnRH vaccine booster, when antibody titers were elevated markedly, LH and testosterone concentrations declined to baseline and remained low throughout. This bull had previously exhibited musth periods that lasted >100 days with a musth score of 4; after GnRH vaccination however, TG swelling, TGS and attenuated aggression were observed for only 17 days. Overall, his score during the following musth was reduced to a 2, compared to 4 during his previous, normal musth periods. Bull 4 had the highest concentrations of LH (0.72 ± 0.11 ng/ml) of all the study animals, compared to the LH mean range for the other four bulls (0.31–0.39 ng/ml). By contrast, testosterone was at baseline except for one point that was associated with a 3-day period of TG swelling and TGS (Table 1). In previous years, this bull exhibited musth-like symptoms throughout the year, and was particularly aggressive toward elephants and people. There was no correlation between LH and testosterone in Bull 4 ($r = 0.501$; $P = 0.116$). Bull 5 exhibited baseline concentrations of both LH and testosterone that were not correlated ($r = 0.318$; $P = 0.314$), and no signs of musth during the study period (Table 1). According to his mahout, Bull 5 did not show musth until the following year. Thus, altogether musth was attenuated and shortened in three bulls (Bulls 1, 3, 4) and postponed completely in two (Bulls 2, 5). Decreases in musth scores between prior musth episodes and post-GnRH vaccination musth were observed in all five treated bulls (Table 1).

4. Discussion

All elephant bulls exhibited an immune response to GnRH vaccination as indicated by increases in GnRH antibody titers after injection. As a result, musth was postponed or attenuated in all five elephants, two of which skipped the musth cycle entirely for that year. The results therefore suggest that GnRH vaccination could be a valuable method for controlling the negative effects of musth in tourist camp elephant bulls. In the two bulls in which musth was not completely suppressed, the intensity of symptoms was diminished as evidenced by the lack of an important musth indicator, UD, in these individuals. These results are similar to a study in Sri Lanka where in three of six bulls vaccinated with 600 μg GnRH (Improvac®) about 2 months before the expected musth, serum testosterone was reduced, musth was postponed for 2–6 months and was of a shortened duration (Rajapaksa et al., 2010). However, musth occurred on schedule in three of the elephants in Sri Lanka because they came into musth before the vaccination protocol had been completed; as a result the bulls in question received only two boosters at the time that musth started (Rajapaksa et al., 2010). In the current study, all elephants received

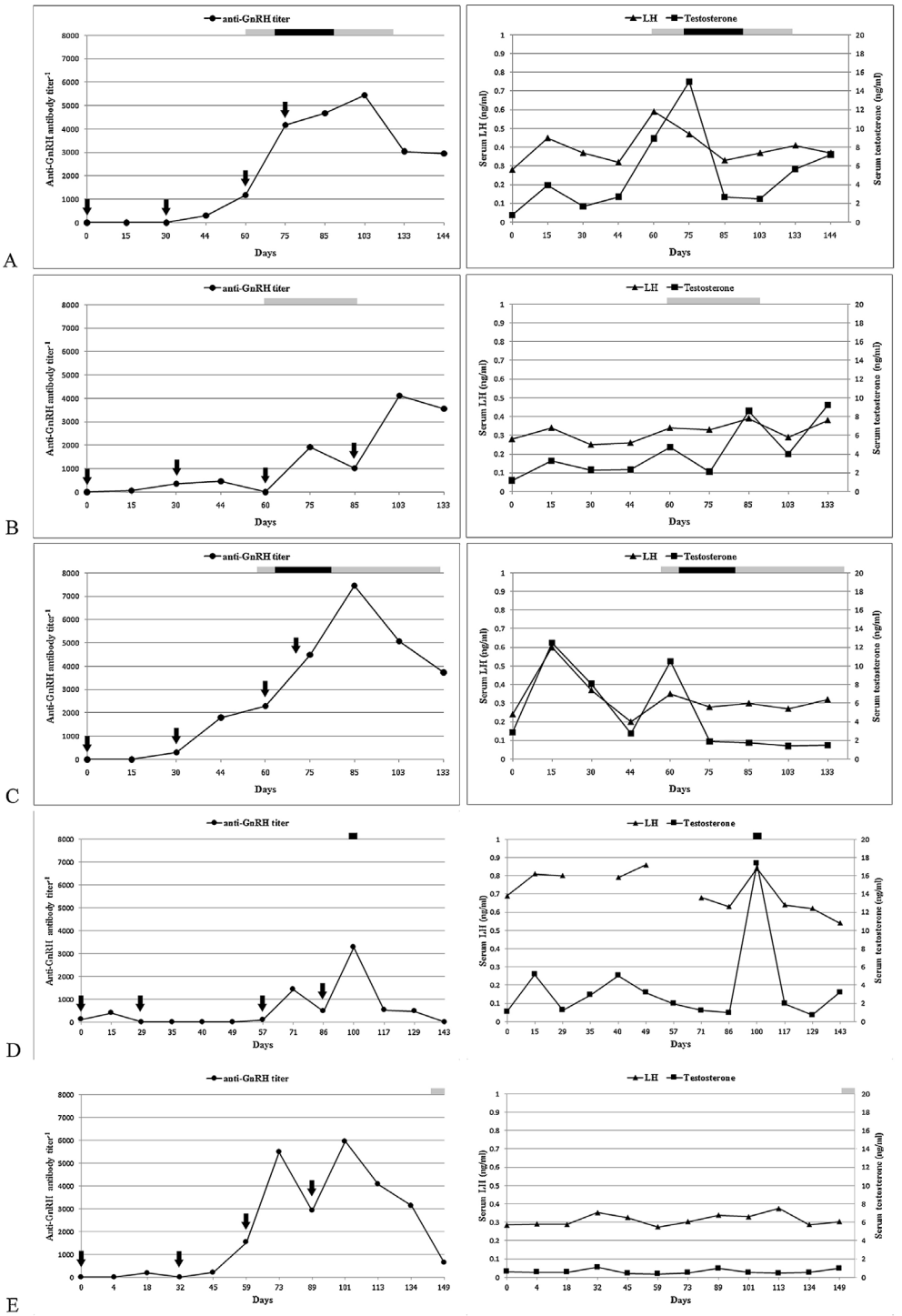

Fig. 1. GnRH antibody titer, LH and testosterone profiles of Asian elephant Bulls 1–5 (A–E) treated with a GnRH vaccine (arrows). Black bars indicate periods of TGS, compared to previous musth durations that included both TGS and UD (gray bars).

all three boosters; however, two began exhibiting musth-like symptoms earlier than expected and their third booster was therefore administered only 2 weeks after the second. The vaccination protocol for Bull 5 was modified based on the somewhat equivocal results for Bulls 1, 3 and 4, and involved a doubling of the vaccine dose, and an initial injection starting 4 rather than 2 months before the expected musth period. Bull 5 did not exhibit musth during the study period, and according to a follow up with the mahout, skipped musth for that season.

Similar to our study, GnRH vaccination has, after several boosters, also proven effective for suppressing musth behavior and reducing fecal androgen metabolite concentrations in African elephant bulls (Bertschiger et al., 2004; De Nys et al., 2010; Stout et al., 2007). In general, the effects of GnRH vaccination on gonadal steroidogenesis have been temporary, presumably related primarily to the duration of elevated antibody titers. There is only one published report of GnRH antibody titers in elephants; an Asian female boosted with increasing doses of Repro-BLOC® to successfully resolve reproductive tract pathologies through HPG downregulation (Boedeker et al., 2012). The rise in GnRH antibody titer in our study was similar to responses in other species, which lasted a few months to a year after the last booster (Janett et al., 2012; Levy et al., 2004; Miller et al., 2008). Because different methodologies were used to determine antibody titers, it is not possible to compare our GnRH antibody binding to that of other studies. Nevertheless, the response appeared to be adequate to attenuate musth in the treated bulls, indicating a sufficient titer had been reached. In one case report, GnRH vaccination appeared to result in an irreversible suppression of testicular function; beginning at the age of 7 years, a male Asian elephant was treated with two commercial GnRH vaccines (Improvac® and Equity®) monthly for 6 months and then every 12–24 months for 6 years. This male underwent significant changes in the size of the reproductive organs and penis, testosterone secretion, sperm production and body weight compared to an age-matched herdmate (Lueders et al., 2014). The authors concluded that GnRH vaccination is a noninvasive and inexpensive method of immune-castration, and resulted in a bull that was very easy to manage because of the absence of musth cycles. That bull did exhibit reduced weight gain compared to an age-matched untreated herdmate; however, BCS of the bulls in our study were not altered, presumably because of the short-term treatment period. If re-immunization is to be considered for long-term musth management, effects on body condition and fertility status should be monitored as it is possible that permanent sterilization might result.

Factors determining if or when a bull comes into musth are related to age and nutrition, and perhaps social status (Cooper et al., 1990; Lincoln and Ratnasooriya, 1996) and driven by changes in the hypothalamo-pituitary-gonadal (HPG) axis. As in other mammals, GnRH stimulates LH followed by subsequent secretion of testosterone, although in elephants, the testes of musth bulls appear to be hyper-responsive to LH (Brown et al., 1993; Lincoln and Ratnasooriya, 1996). Frequent sampling of Asian bulls demonstrated that androgen secretion is under control of LH, with pulses of testosterone closely following those of LH (~1 pulse/3 h), although there was little difference in hormone pulsatility between musth and non-musth (Niemuller and Liptrap, 1991). Based on a study of one African elephant bull, serum LH increases about 4 weeks before musth begins and is maintained for approximately 5 weeks; thus, it may be the trigger for the rise in testosterone (Kaewmanee et al., 2011). However, in our study, a clear increase in LH preceding musth was not observed, and neither were consistent changes in LH and testosterone after GnRH vaccination, perhaps because of the more limited sampling regimen. Bulls 1–4 in this study were initially vaccinated GnRH vaccine 2 months prior expected musth, when LH was expected to be increased. The bulls demonstrated that LH elevated after Day 0 and then reduced when the GnRH antibody titer increased (Fig. 1). During this period, LH elevation was interrupted, and fluctuated with contrast to antibody titer by GnRH neutralization. Serum LH was obviously suppressed in Bull 5, whom initial vaccination was performed with during 4 months before musth and LH elevation period. Musth may also be partially under thyroid control, with serum thyrotropin-stimulating hormone (TSH) concentrations being positively correlated, and thyroid hormones (T_3, T_4) negatively correlated to testosterone secretion (Brown et al., 2007). Positive correlations have also been found between serum cortisol and testosterone concentrations in Asian and African elephants, with both being elevated during musth (Brown et al., 2007; Yon et al., 2007). Elevated glucocorticoids have also been reported in musth Asian elephant bulls based on fecal analyses (Claassens, 2010), although no corresponding increases in fecal glucocorticoid metabolites were observed during musth in African elephants (Ganswindt et al., 2003, 2005). Although not all studies agree completely, evidence suggests that hormonal changes in association with musth may be necessary to increase metabolic activity in preparation for the physical and physiological changes associated with an enhanced sexual state.

One interesting finding in both the Sri Lankan (Rajapaksa et al., 2010) and Thai (present study) studies was a lack of UD in association with TG swelling and TGS in bulls that only partially responded to GnRH vaccination. The significance of this finding is not clear, but may indicate a differential physiological response to HPG and testosterone suppression in relation to musth. Also, lower concentrations of testosterone did not always equate to mild behavior. Bull 4 in particular was very aggressive, even after vaccination, although his testosterone values were not different overall from those in the other bulls. This suggests that aggression may not always be purely testosterone driven, but could in part be a learned behavior, or that bulls individual differences in threshold for the response to testosterone. Bull 4 was the dominant bull in the facility, so his aggressive behavior could stem from individual experiences, past management or training, and/or hierarchy. Thus, just reducing testosterone production through GnRH vaccination might not resolve all aggression problems, and handlers must continue to be diligent in handling these potentially dangerous animals. Rather, GnRH vaccination should be considered a tool to aid elephant bull management through improvements in animal welfare and human safety.

Earlier attempts to suppress the HPG axis and control musth involved the use of GnRH analog injections (leuprolide acetate depot; Lupron®) in captive Asian (de Oliveira et al., 2004) and wild African (Brown et al., 1993) elephant bulls. This high affinity analog binds to pituitary GnRH receptors and initially induces LH release, but then eventually results in pituitary desensitization or downregulation (or both), leading to suppressed circulating levels of gonadotropins and testosterone (Plosker and Brogden,

1994). In the study of de Oliveira et al. (2004), multiple Lupron injections were administered to an Asian elephant bull over a 7-year period, and appeared to cause a permanent suppression of testosterone in the final years of the study. However, the bull was 52 years of age at onset of the study, and some of the reduction in testicular function could therefore have been age-related. For the free-ranging African elephant study, after a transitory increase in LH and testosterone, basal concentrations were reduced over a 20-day study period. Interestingly, however, although the LH response to subsequent GnRH challenge was attenuated, testosterone release was markedly increased, suggesting that the bulls had become hyper-responsive to small increases in LH, not unlike the response observed in beef bulls treated with another GnRH analog, nafarelin acetate (Melson et al., 1986). In other species, for GnRH analog treatment to be successful, continued injections are required (Vickery, 1985, 1986). Therefore, by stimulating an endogenous immune response, GnRH vaccines have the advantage of potentially inducing a longer effect with fewer treatments. Both methods, however, aim to reduce aggression in musth bulls by suppressing LH and subsequent testosterone production.

Studies on the efficacy of GnRH vaccines to suppress the HPG in African male (De Nys et al., 2010), Asian male (Lueders et al., 2014) and Asian female (Boedeker et al., 2012) elephants indicate the need for multiple injections before an effect is observed. An attempt to use a single booster GnRH vaccination protocol to suppress ovarian cyclicity in female African elephants was not successful (Valades et al., 2012). This agrees with the study of a young Asian elephant bull where multiple boosters were needed to suppress gonadal function (Lueders et al., 2014). Vaccination protocols for other agents also often rely on re-immunization; for example, the multi-vaccination protocol to produce effective anti-inhibin antibody titers in cattle (Medan et al., 2004). In our study, antibody titers did not increase until after the second or third booster. Interestingly, the dose did not appear to have a significant effect, because the antibody titer response for Bull 5, which received twice the dose at each injection, was similar to that of the other treated bulls.

Numerous determinants modulate the intensity of a vaccine response, including antigen immunogenicity, type of adjuvant, interval between booster injections, animal age and health, body condition, and perhaps even environmental factors (Siegrist, 2008). All of the elephants in this study were healthy, with good body condition, although there was a wide range in age (22–55 years). Bull 2 was the youngest, and had one of the weaker antibody responses, although still sufficient to prevent musth for that cycle. Perhaps this is related to the fact that younger bulls have lower testosterone levels and exhibit shorter musth periods than older bulls (Brown et al., 2007; Lincoln and Ratnasooriya, 1996; Rajaram, 2006), and so may be more easily suppressed. By contrast, the other weak responder was a 50-year old bull (Bull 4), also in good health with good body condition, but unusual in historically tending to exhibit an almost constant state of musth. A study in horses found that young animals in general had better responses to GnRH vaccination than older ones (Stout and Colenbrander, 2004). And in previous studies, most elephants were young when they were treated and responded to a GnRH vaccine (Bertschiger et al., 2004; De Nys et al., 2010; Lueders et al., 2014; Stout et al., 2007). Nevertheless, responses to GnRH vaccination, at least using the formulations described in this and other studies, appear to be relatively short-lived, lasting only 2–3 months. Thus, the goal should be to time the vaccination protocol so that high antibody titers are attained before the onset of a musth cycle. This appeared to be the case for Bull 5, who was vaccinated initially about 4 months before his predicted musth, and responded with the complete abrogation of musth for that year. Therefore, elephant age and health should be considered in addition to vaccine dose and vaccination intervals, as each of these can effect individual response. Furthermore, a targeted vaccination program might be a better strategy than using regular boosters, e.g. every 6 months or more, to continually suppress the HPG axis, which could result in permanent problems with sperm production or breeding. Future studies should therefore be conducted to better define ideal doses, booster intervals, and vaccine formulas (Levy et al., 2004; Turkstra, 2005) to achieve efficacy in both the short- and long-term.

5. Conclusions

This study demonstrated the efficacy of GnRH vaccination to suppress musth symptoms in adult Asian bull elephants in tourist camps. All bulls responded after the second or third booster with an increase in GnRH antibody titers, and lower testosterone in the final phase of the study. Overall, musth was attenuated and shortened in three bulls and postponed in two, and aggression scores were lower than in previous musth episodes in all animals. The key appears to be administering the vaccine so that antibody titers are elevated before the beginning of a musth cycle. Given appropriate timing, GnRH vaccination could be used to control or manage aggressive musth behavior in captive elephant bulls. However, more work is needed to identify the optimal dose, booster interval, and vaccination schedule for maximum effectiveness.

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