Evaluation of the pituitary–gonadal response to GnRH, and adrenal status, in the leopard (*Panthera pardus japonensis*) and tiger (*Panthera tigris*)

J. L. Brown*, K. L. Goodrowe†‡, L. G. Simmons§, D. L. Armstrong§ and D. E. Wildt†‡

Departments of *Obstetrics and Gynecology, and †Physiology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; ‡National Zoological Park, Smithsonian Institution, Washington, DC 20008; and §Henry Doorly Zoo, Omaha, NE 68107, U.S.A.

Summary. Frequent blood samples were collected to study hormonal responses to GnRH in male and female leopards and tigers. Animals were anaesthetized with ketamine–HCl and blood samples were collected every 5 min for 15 min before and 160 min after i.v. administration of GnRH (1 µg/kg body weight) or saline. No differences in serum cortisol concentrations were observed between sexes within species, but mean cortisol was 2-fold greater in leopards than tigers. GnRH induced a rapid rise in LH in all animals (18·3 ± 0·9 min to peak). Net LH peak height above pretreatment levels was 3-fold greater in males than conspecific females and was also greater in tigers than leopards. Serum FSH increased after GnRH, although the magnitude of response was less than that observed for LH. Basal LH and FSH and GnRH-stimulated FSH concentrations were not influenced by sex or species. Serum testosterone increased within 30–40 min after GnRH in 3/3 leopard and 1/3 tiger males. Basal testosterone was 3-fold greater in tiger than leopard males. LH pulses (1–2 pulses/3 h) were detected in 60% of saline-treated animals, suggesting pulsatile gonadotrophin secretion; however, in males concomitant testosterone pulses were not observed. These results indicate that there are marked sex and species differences in basal and GnRH-stimulated hormonal responses between felids of the genus *Panthera* which may be related to differences in adrenal activity.

Keywords: GnRH, leopard, tiger, LH, cortisol, testosterone

Introduction

Basic endocrine characteristics have been studied in a number of species of wild felids including the lion (*Panthera leo*), cheetah (*Acinonyx jubatus*), tiger (*Panthera tigris*), clouded leopard (*Neofelis nebulosa*), leopard (*Panthera pardus*) and puma (*Felis concolor*) (Schmidt *et al.*, 1979; Wildt *et al.*, 1984a, 1986a, 1987a (review); Seal *et al.*, 1985). These investigations confirmed that taxonomically related felids exhibit species-specific endocrine patterns. Circulating cortisol concentrations are greater in North Chinese (*P. p. japonensis*) and clouded leopards than in tigers or pumas, and the concentrations in tigers and pumas are greater than those in cheetahs or domestic cats (Carter *et al.*, 1984; Wildt *et al.*, 1987a, b). Serum testosterone concentrations are similar among male tigers, pumas and clouded leopards but are lower in cheetahs (Wildt *et al.*, 1984a, b, 1987a, b). Serum LH concentrations appear less species-dependent, but can vary considerably within individual animals presumably due to episodic release (Wildt *et al.*, 1984a, b, 1987a; Goodrowe *et al.*, 1985). The infrequent blood-sampling protocols used in these earlier studies, however, precluded identification of pulsatile hormone secretion.

Administration of GnRH has been useful for evaluating pituitary–gonadal function in many mammalian species, including the domestic cat (Chakraborty *et al.*, 1979; Goodrowe *et al.*, 1985), as well as for studying the mechanisms and regulation of fertility (Sandow, 1982). To date, the effects of GnRH on wild felids have been limited to the cheetah (Wildt *et al.*, 1984a) and clouded leopard (Wildt *et al.*, 1986a). As propagation of captive animals continues to play a role in preserving rare species, it becomes increasingly important to establish a data base of hormonal norms for understanding the basic biology and evaluating the reproductive potential of these animals.

In this study the hormonal responses of male and female Chinese leopards and tigers to exogenous GnRH were compared while simultaneously assessing species variability in adrenal activity. A frequent blood sampling protocol was used to monitor closely exogenous GnRH-stimulated hormonal responses and enhance the probability of detecting pulsatile hormonal patterns.

Materials and Methods

Animals and treatments. Three male and three female adult North Chinese leopards (*Panthera pardus japonensis*) and tigers (*P. tigris*) were maintained in indoor–outdoor enclosures at the Henry Doorly Zoo (Omaha, NE, U.S.A.). Mean ages were 11.0 ± 0.7 years for leopards and 8.8 ± 1.1 years for tigers. Body weights averaged 56 ± 2 and 33 ± 2 kg for male and female leopards and 184 ± 9 and 102 ± 5 kg for male and female tigers, respectively. Sampling was conducted in April, corresponding to mid- to late-breeding season for both species (Seager & Demorest, 1978; Seal *et al.*, 1985).

Blood samples were collected from animals anaesthetized with ketamine–HCl (13–17 mg/kg i.m.) delivered via a darting rifle. An attempt was made to minimize disturbance of the animal before darting. Generally, the cage room was entered and the ketamine–HCl injection was successfully administered within 2–5 min. A light surgical plane of anaesthesia was maintained for 3–3·5 h with supplemental ketamine–HCl injections (100–300 mg i.v.). Animals were immobilized and sampled twice, 48 h apart (bleeding Periods I and II), so that hormonal responses to saline (0·14 M-NaCl, pH 6·9) and GnRH (Gonadorelin; Abbott Labs, Chicago, IL, U.S.A.) could be evaluated in each animal. Because tigers often experience brief (15–90 sec) episodic convulsions under ketamine–HCl anaesthesia (Wildt *et al.*, 1987a) a low dose of diazepam (Valium: Hoffman LaRoche, Inc., Nutley, NJ, U.S.A.; 0·5–2·5 mg i.v.) was administered after the first seizure to minimize their intensity. All tigers convulsed during Period I (1–5 seizures/tiger); however, only one female experienced a single seizure during Period II. Leopards did not convulse and received no diazepam. Treatments (GnRH or saline) were assigned at random and stratified by species and sex between bleeding Periods I and II. Animals were weighed immediately before Period I. Indwelling catheters with obturators (Becton-Dickinson, Rutherford, NJ, U.S.A.) were placed into a saphenous or lateral coccygeal vein and blood sampling was begun 15–35 min after the initial injection of ketamine–HCl. Blood samples (3–4 ml) were collected and placed on ice at 5-min intervals for 15 min before and 160 min after i.v. administration of GnRH (1 μg/kg body weight) or saline vehicle. Blood samples were centrifuged and the sera stored at −20°C until subsequent hormonal analyses.

Radioimmunoassays. A heterologous double-antibody radioimmunoassay validated for the domestic cat (Chakraborty *et al.*, 1979) was used to measure serum LH. Rabbit anti-bovine antiserum was provided by Dr J. J. Reeves (JJR No. 5), highly purified ovine LH (LER-1056-C2) used as iodinated tracer and canine LH (LER-1685-1) used as standard were provided by Dr L. E. Reichert, Jr. Recovery was determined by adding increasing amounts of canine LH to 100 μl pooled serum from each species. Recovery estimates after subtracting endogenous serum LH from the assay of 0·2, 0·4, 0·8, 1·6 and 3·2 ng LH were 0·26, 0·47, 0·91, 1·65 and 3·08 ng ($y = 0.94x + 0.11$; $r = 0.99$; $P < 0.001$) for leopard serum and 0·23, 0·42, 0·82, 1·64 and 2·82 ng ($y = 0.87x + 0.11$; $r = 0.99$; $P < 0.001$) for tiger serum, respectively. Inhibition curves for serum pools were parallel to the canine standard. Sensitivity was 0·5 ng/tube. Inter- and intra-assay coefficients of variation were 7·5 and 5·9%, respectively.

A double-antibody radioimmunoassay previously developed for measuring bovine FSH (Acosta *et al.*, 1983) was validated for use with leopard and tiger serum. Rabbit anti-ovine FSH was provided by Dr J. A. Dias (JAD-LER 178), highly purified ovine FSH (LER-1976-A2) used as the iodinated tracer was provided by Dr L. E. Reichert, Jr and ovine FSH (NIH-FSH-S8) served as the standard. Cross-reactivity was <3% for 200 ng LH (NIH-LH-S18), growth hormone (NIH-GH-S11), prolactin (NIH-PRL-S12) and GnRH. Upon addition of 5, 10, 20 and 40 ng ovine FSH to 100 μl of pooled serum from each species, and after subtracting endogenous hormone, 5·2, 10·9, 22·5 and 41·5 ng ($y = 1.03x + 0.64$; $r = 0.99$; $P < 0.001$) and 4·6, 9·0, 21·6 and 44·0 ng ($y = 1.14x + 1.59$; $r = 0.99$; $P < 0.001$) were recovered for leopard and tiger serum, respectively. Inhibition curves of NIH-FSH-S8 and the serum pools tested were parallel. Sensitivity was 1·5 ng/tube and inter- and intra-assay coefficients of variation were 9·5 and 7·2% respectively.

Testosterone was measured in male felid sera using a double-antibody radioimmunoassay ^{125}I kit (Radioassay Systems Laboratory, Carson, CA) as described previously by Howard *et al.* (1983). Sensitivity was 0·01 ng/tube. Inter- and intra-assay coefficients of variation were 5·5 and 4·6%, respectively.

Cortisol was measured using a ^{125}I radioimmunoassay kit (New England Nuclear, N. Billerica, MA) (Carter *et al.*, 1984). Because of high cortisol concentrations, leopard serum was diluted 1:1 with Gel-PBS buffer (0·01 M-PO_4,

0·14 M-NaCl, 0·1% gelatin, pH 7·2) before analysis. Sensitivity was 0·2 ng/tube. Inter- and intra-assay coefficients of variation were 5·8 and 5·8%, respectively.

Serum oestradiol-17β (Korenman *et al.*, 1974) was quantified in a single serum pool from each saline-treated female. Recovery of [³H]oestradiol-17β from pooled samples after extraction with diethyl ether was >90%. Sensitivity was 3 pg/tube. Oestradiol-17β concentrations were quantified in a single assay and the intra-assay coefficient of variation was 5·6%.

Data analysis. Data were analysed using a split-plot analysis of variance for repeated measures in a factorial arrangement with treatment (GnRH or saline), sex and species as main effects. Gonadotrophin responses to GnRH and testosterone responses to LH were evaluated as net peak height (greatest post-treatment value minus mean pre-treatment level) and as net area under the response curves, using analysis of variance. Net area, expressed as mm²/120 min (LH and FSH) or 160 min (testosterone), was determined using a planimeter with a 3% coefficient of variation. A paired *t* test was used to determine differences in cortisol levels for individual animals between bleeding Periods I and II. Data are presented as means ± s.e.m.

Basal serum LH and FSH concentrations were determined by an iterative process similar to that used by Melson *et al.* (1986) in which high values were excluded if they exceeded the mean plus two standard deviations (s.d.) of the remaining values. Fluctuations in serum hormones were determined to be pulses if: (1) the amplitude was at least 2 s.d. greater than mean basal levels, (2) the peak occurred within two samples of a previous nadir value and, (3) the peak was followed by at least three successive values that were declining or represented basal levels. Pulse amplitude was defined as the highest point associated with the peak minus the mean basal concentration.

In males, the increase in serum testosterone after GnRH was considered significant if the rise was greater than 2 s.d. above pretreatment levels.

Results

Serum LH

Serum LH concentrations peaked within 15–25 min after GnRH administration in all animals. Mean LH concentrations remained elevated above baseline for ∼120 min in males and 75–90 min in females (Fig. 1). Peak height above pretreatment levels was approximately 3-fold greater ($P < 0.05$) in males than conspecific females for leopards and tigers (Figs 1a, b). Similarly, net area under the LH curve was also greater ($P < 0.05$) in GnRH-challenged male than female leopards (139 ± 5; 47 ± 13 mm²/120 min) and tigers (195 ± 36; 64 ± 10 mm²/120 min), respectively. Within sex, net LH peak height was nearly 2-fold greater ($P < 0.05$) in tigers than leopards.

There were no species or sex differences ($P > 0.05$) in mean basal LH concentrations in saline-treated animals (Figs 1c, d). During the control bleeding period, LH pulses (1–2 pulses/3 h) were detected in 3/3 leopard females, 0/3 leopard males, 2/3 tiger females and 2/3 tiger males (Fig. 2). Pulse amplitudes ranged from 0·7 to 7·2 ng/ml and averaged 2·6 ± 0·7 ng/ml.

Serum FSH

Because there were no effects of gender ($P > 0.05$) on quantitative or temporal FSH patterns, data were pooled within species (Fig. 3). Serum FSH concentrations increased in all animals within 15–30 min after GnRH; however, the magnitude of the increase was much less than that observed for LH. Net area under the FSH response curve was not significantly different ($P > 0.05$) between species (99 ± 20 mm²/120 min and 82 ± 8 mm²/120 min for leopards and tigers, respectively). Mean FSH concentrations remained elevated for ∼45 min before declining to baseline by 90–120 min after GnRH.

There were no differences ($P > 0.05$) in mean basal FSH concentrations among saline-treated leopards and tigers. Pulsatile FSH secretion was not apparent in any animal sampled during the 3-h control period.

Serum testosterone

Increases in serum testosterone occurred within 30–40 min of the GnRH injection in all leopard males and values remained elevated 2–3-fold over baseline concentrations for the entire bleeding
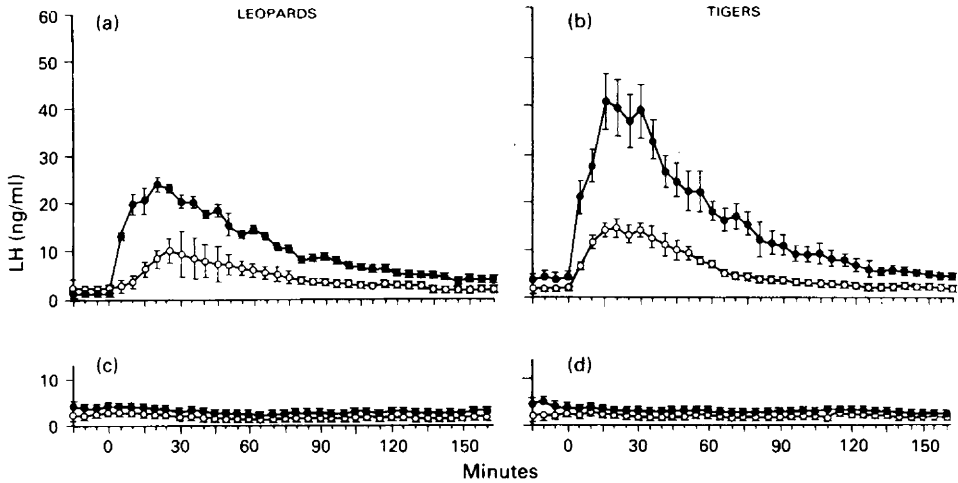
Fig. 1. Mean ± s.e.m. serum LH concentrations after injection of GnRH (a, b) or saline (c, d) at 0 min in male (●) and female (○) leopards and tigers.
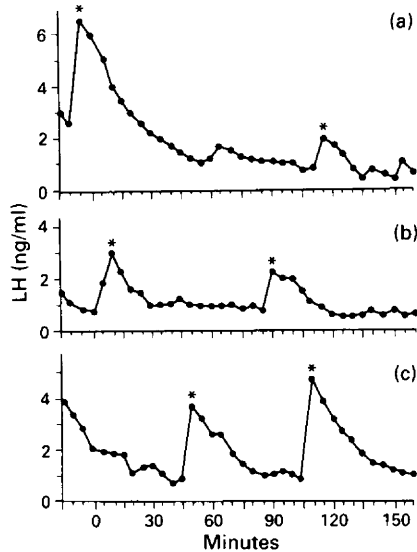

Fig. 2. LH pulses (*) during the 175-min control bleeding period from an individual female leopard (a), female tiger (b) and male tiger (c). No LH pulses were detected in male leopards.

interval (Fig. 4). Only one tiger male responded with a significant increase in testosterone over pretreatment levels after GnRH. There was no apparent effect of bleeding period (I *vs* II) or diazepam (used during Period I but not Period II) on the testosterone response to GnRH. Individual testosterone profiles for a leopard male and tiger males that did and did not respond to GnRH treatment are shown in Fig. 5.

In saline-treated males, overall basal testosterone concentrations were 3-fold greater ($P < 0.05$) in tigers than leopards (Fig. 4). Episodic testosterone secretion was not evident (Fig. 5).
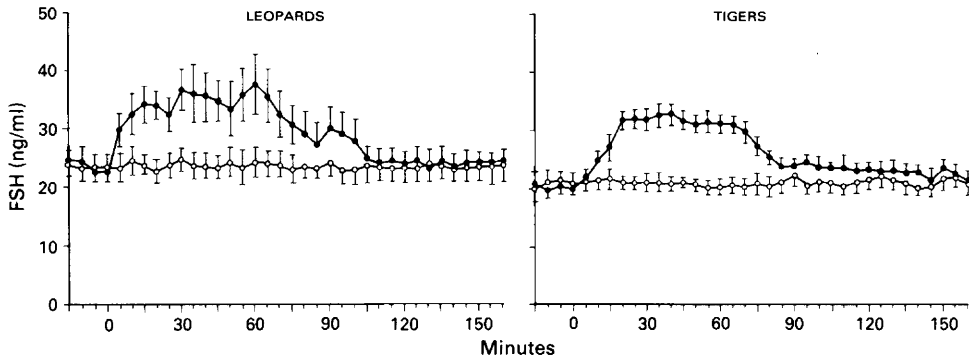
Fig. 3. Mean ± s.e.m. serum FSH concentrations in leopards and tigers after injection of GnRH (●) or saline (○) at 0 min. Values are pooled within species.
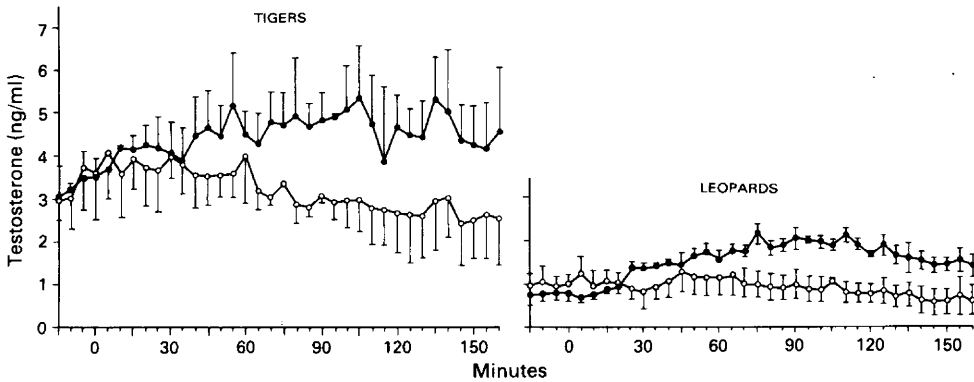

Fig. 4. Mean ± s.e.m. serum testosterone concentrations in male leopards and tigers after injection of GnRH (●) or saline (○) at 0 min.

Serum cortisol and oestradiol-17β

There was no effect ($P > 0.05$) of GnRH treatment or gender on serum cortisol concentrations; therefore, data were pooled within species (Fig. 6). Mean serum cortisol concentrations for the 175 min collection period were 2-fold greater ($P < 0.05$) in leopards than tigers. In tigers, mean cortisol concentrations remained stable over time while in leopards, cortisol rose ~50–100 ng/ml during the first 60 min of blood sampling. Secretory patterns of cortisol varied within and between animals (Fig. 7) and were highly variable in about half and nearly static in the remaining bleeding periods. There were no apparent relationships between bleeding period, use of diazepam, sex or species and the type of temporal secretory pattern observed. However, in all leopards, serum cortisol concentrations were 10–35% (average 19 ± 4%) lower ($P < 0.05$) in Period II compared to Period I, an effect not observed in tigers.

Although oestradiol-17β concentrations varied considerably among animals, no differences ($P > 0.05$) between leopard (8·8 ± 1·1; range 6·7–12·9 pg/ml) and tiger (9·4 ± 1·5; range 6·4–14·8 pg/ml) females were observed.
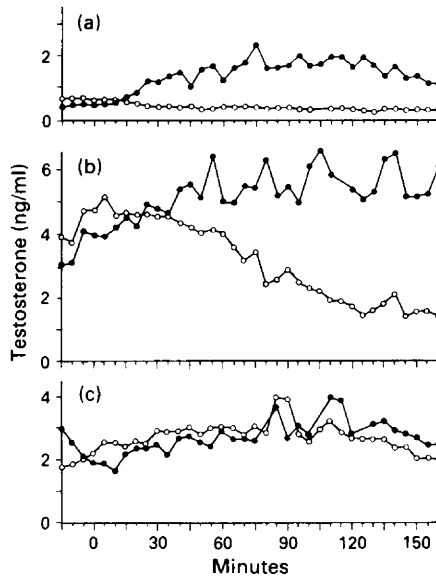
Fig. 5. Individual serum testosterone profiles after injection of GnRH ($\bullet$) or saline ($\circ$) at 0 min for a responding male leopard (a), responding male tiger (b), and non-responding male tiger (c).
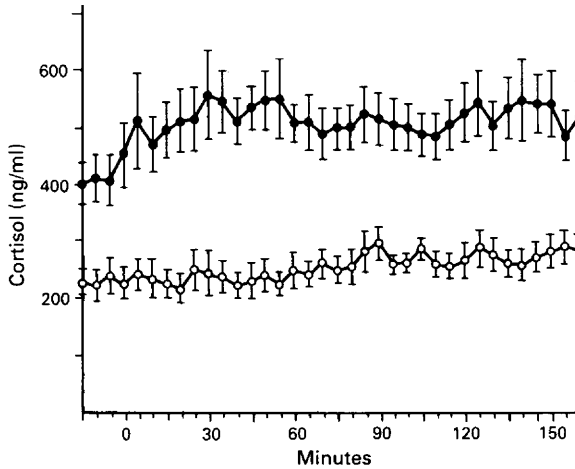

Fig. 6. Mean $\pm$ s.e.m. serum cortisol concentrations in leopards ($\bullet$) and tigers ($\circ$). Values are pooled within sex and treatment (GnRH or saline) for each species.

Discussion

The present study is the first to identify pulsatile LH secretion in wild felids and emphasizes the need for frequent blood sampling to assess episodic activity. Several low-amplitude pulses would not have been identified had the sampling interval been increased from 5 to 10 min. However, because ketamine anaesthesia can suppress episodic LH secretion in domestic species (Johnson &
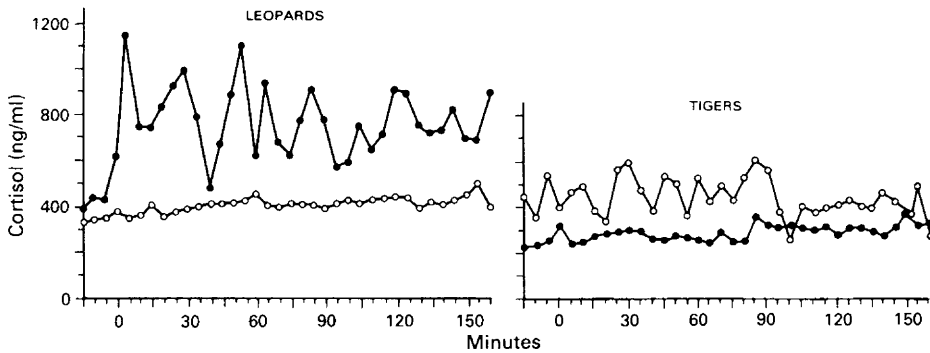
Fig. 7. Individual serum cortisol profiles for male (●) and female (○) leopards and tigers showing episodic and non-episodic secretory patterns.

Gay, 1981; Clarke & Doughton, 1983), the pulsatile activity measured in this study may not accurately reflect the patterns present in unanaesthetized, non-manipulated animals. The lack of pulsatile LH release in some animals suggests individual variation in sensitivity to the inhibitory effects of anaesthesia. Anaesthetics, including ketamine–HCl, do not alter GnRH-stimulated gonadotrophin responses (Hobson & Fuller, 1977; Lewis *et al.*, 1985). Therefore, although possibly inhibiting tonic hypothalamic GnRH secretion, anaesthetics do not appear to alter pituitary responsiveness to exogenous stimulation.

All animals responded to GnRH with a sharp increase in serum LH similar to that observed in other carnivores including the dog (Chakraborty & Fletcher, 1977), ferret (Donovan & ter Haar, 1977) and domestic cat (Chakraborty *et al.*, 1979; Johnson & Gay, 1981; Goodrowe *et al.*, 1985). In the leopards and tigers examined, the GnRH-stimulated LH response was greater in males than conspecific females although basal levels were not different. A similar divergence in sex-related responses has also been reported for the cheetah (Wildt *et al.*, 1984a), in which basal LH values were also greater in males than females. It is possible that some of these sex differences are attributable to endocrine status since pituitary responsiveness (LH release) to GnRH is dictated, in part, by the physiological condition of the animal. In intact females, GnRH-stimulated LH release is considerably greater during oestrus compared to other stages of the oestrous cycle or during the anoestrous season (Reeves *et al.*, 1971; Chakraborty *et al.*, 1979). Leopard and tiger females demonstrated no overt signs of oestrous behaviour and serum oestradiol concentrations were in the range of interoestrous values reported for other wild felids during the breeding season (Wildt *et al.*, 1984a; Seal *et al.*, 1985). Although it is possible that sex differences may have been reduced if females had been challenged during oestrus, it is evident from this and a previous study (Wildt *et al.*, 1984a) that gender variability in GnRH response is observed in randomly sampled felids.

No data are available on circulating FSH concentrations in any wild felids. The relative magnitude of the increase in serum FSH after GnRH administration (0·4–1·4-fold) was considerably less than the GnRH-induced increase in LH (6–25-fold). In comparison to LH, attenuated FSH responses to GnRH have been demonstrated in several domesticated species including the ferret (Donovan & ter Haar, 1977), bull (Schanbacher & Echternkamp, 1978) and ewe (Wheaton *et al.*, 1982). In contrast to LH, no species or sex differences in GnRH-stimulated FSH responses were observed and there was no evidence of pulsatile FSH release. Divergence in LH and FSH secretion further confirms that different control mechanisms for the release of these two gonadotrophins exist (Savoy-Moore & Schwartz, 1980). Peak levels of FSH were sustained for a longer period compared to LH, presumably due to the longer half-life of FSH in circulation (Niswender *et al.*, 1974).

The magnitude of GnRH-stimulated testosterone secretion in male leopards was comparable to that observed in the cheetah (Wildt *et al.*, 1984a), domestic cat (Goodrowe *et al.*, 1985) and clouded leopard (Wildt *et al.*, 1986a); however, the tiger response either was muted or non-existent.

Inexplicably, this decreased testicular response in tigers occurred in the face of greater overall GnRH-induced LH responses.

Basal testosterone concentrations in leopards were about 30% those in tigers, and these concentrations, in turn, were much lower than values previously reported for the domestic cat, clouded leopard and puma (Goodrowe et al., 1985; Wildt et al., 1986a, 1987a). The lower basal testosterone concentrations of North Chinese leopards cannot be explained by inadequate gonado-trophin stimulation since LH concentrations were similar to those observed in the tiger, as well as values previously reported for other felids (Wildt et al., 1984a, b, 1987a; Goodrowe et al., 1985).

Taken together, these data serve to identify marked functional differences in the hypothalamo-pituitary–gonadal axis of the North Chinese leopard and tiger. This striking variability between two taxonomically related species may be related, in part, to (1) differences in the biological activity of LH (Beitins et al., 1981); (2) altered steroidogenic response of Leydig cells to LH (Purvis & Hansson, 1978; Catt et al., 1979); or (3) depressions in genetic diversity, since increased genetic monomorphism has been associated with compromised basal testosterone secretion in cheetahs and lions (O'Brien et al., 1985; Wildt et al., 1984b, 1986b).

The extreme individual variability in temporal cortisol profiles was inexplicable. The literature has not addressed the question of cortisol release patterns in anaesthetized mammals. In leopards, the consistently lower overall cortisol concentrations detected during the second bleeding period suggests rapid adrenal adaptation to repeated manipulation, similar to that observed in domesticated species (Riegle, 1973; Amario et al., 1984). In contrast, no differences in serum cortisol concentrations between bleeding periods in tigers were observed, suggesting that the adrenal response to handling and/or anaesthetic stressors differs amongst species.

The relationship between increased adrenal activity and reproductive function is complex and not well understood. Administration of ACTH, cortisol or dexamethasone has been shown to attenuate GnRH-stimulated LH release in the cow and ram (Matteri & Moberg, 1982; Matteri et al., 1984). Glucocorticoids have been shown to reduce bioactive and immunoactive LH concentrations (Chantaraprateep & Thibier, 1978; Mann et al., 1982; Sapolsky, 1985), suppress basal and LH-stimulated testosterone secretion (Thibier & Rolland, 1976; Doerr & Pirke, 1976) and directly inhibit testicular function by reducing the concentrations of Leydig cell LH receptors (Bambino & Hsueh, 1981). However, in other studies, serum testosterone and/or LH are either increased or unchanged depending upon the stressor (Pollard et al., 1980; Liptrap & Raeside, 1983; Lescoat et al., 1984). The cortisol concentrations measured in the North Chinese leopard are 2- to 15-fold greater than values reported for other felids (Carter et al., 1984; Wildt et al., 1984b, 1987a, b). It is therefore possible that the hyperadrenal activity of the North Chinese leopard may be related to the comparatively depressed pituitary and testicular responsiveness observed in this study.

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