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Provocative tests with Kisspeptin-10 and GnRH set the scene for determining social status and environmental impacts on reproductive capacity in male African lions (*Panthera leo*)

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ABSTRACT

Understanding the hypothalamic factors regulating reproduction facilitates maximising the reproductive success of breeding programmes and in the management and conservation of threatened species, including African lions. To provide insight into the physiology and pathophysiology of the hypothalamic-pituitary–gonadal reproductive axis in lions, we studied the luteinising hormone (LH) and steroid hormone responses to gonadotropin-releasing hormone (GnRH) and its upstream regulator, kisspeptin. Six young (13.3 ± 1.7 months, 56.2 ± 4.3 kg) and four adult (40.2 ± 1.4 months, 174 ± 6 kg) male lions (Ukutula Conservation Centre, South Africa) were used in this study. Lions were immobilised with a combination of medetomidine and ketamine and an intravenous catheter was placed in a jugular, cephalic or medial saphenous vein for blood sampling at 10-min intervals for 220 min. The ten-amino acid kisspeptin which has full intrinsic activity (KP-10, 1 μg/kg) and GnRH (1 μg/kg) were administered intravenously to study their effects on LH and steroid hormone plasma concentrations, measured subsequently by ELISA and liquid chromatography tandem mass spectrometry (LC-MS/MS), respectively. Basal LH levels were similarly low between the age groups, but testosterone and its precursor levels were higher in the adult animals. Adult lions showed a significant LH response to KP-10 (10-fold) and GnRH (11-fold) administration ($p < 0.05$ and $P < 0.001$, respectively) whereas in young lions LH increased significantly only in response to GnRH. In adults alone, testosterone and its precursors steadily increased in response to KP-10, with no significant further increase in response to GnRH. Plasma levels of glucocorticoids in response to KP-10 remained unchanged. We suggest that provocative testing of LH and steroid stimulation with kisspeptin provides a new and sensitive tool for determining reproductive status and possibly an index of exposure to stress, environmental insults such as disease, endocrine disruptors and nutritional status.

272 words.

1. Introduction

The African lion population has declined about 40 % during the last two decades and is currently listed as vulnerable by the IUCN, with<40,000 individuals and a decreasing population trend (Bauer et al., 2016). The main threats to lions are indiscriminate killing, trophy hunting, prey depletion and habitat loss and conversion, which have led to a number of subpopulations becoming small and isolated (Bauer et al., 2015; Craigie et al., 2010; Lindsey et al., 2013; Scholte, 2011). In addition, the effects of other factors such as disease on reproductive capacity have contributed to the decline of lion populations (Adams et al., 2009; Broughton et al., 2021; De Vos et al., 2001; Michel et al., 2006; Munson et al., 2008; Trinkel et al., 2011; Viljoen et al., 2015).

Understanding the role of hypothalamic factors regulating reproduction is key to maximising the reproductive success of breeding programmes and in the management and conservation of threatened species (Brown, 2006). Basic endocrine characteristics have been studied in several species of large wild felids including the lion, tiger, leopard,

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cheetah, clouded leopard and puma (Andrews et al., 2020; Barone et al., 1994; Brown et al., 1988; Seal et al., 1985; Wildt et al., 1987a; Wildt et al., 1986; Wildt et al., 1987b).

A number of external and internal inputs such as photoperiod, metabolic products and nutrients, infection/inflammation and hormones regulate the hypothalamic-pituitary–gonadal (HPG) axis control of reproduction (Millar and Newton, 2013). These inputs are integrated in the brain and hypothalamus to regulate the biosynthesis and secretion of the trophic peptide hormone gonadotropin-releasing hormone (GnRH). GnRH in turn stimulates the biosynthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH then stimulate gametogenesis and steroid/peptide hormone production and secretion in the ovaries and testes. GnRH and its analogues have been extensively employed in treating hormone-dependent diseases and assisted reproductive technologies in humans (Anderson and Millar, 2021; Kotlyar et al., 2021; Wu et al., 2021) and many species of domestic and wild animals, including lions. For example, Callealta and colleagues recently used artificial insemination in combination with systemic administration of a GnRH analogue to induce ovulation in lionesses presenting natural oestrus (Callealta et al., 2019).

A family of hypothalamic neuropeptides, the kisspeptins, have recently emerged as a key central regulator of GnRH secretion. Kisspeptins (which are all products of cleavage of a common prepro-kisspeptin precursor), together with their receptor, are required for normal functioning of the HPG axis (Abbara et al., 2021; Pinilla et al., 2012; Roseweir and Millar, 2009). Kisspeptin stimulation of gonadotropins is ablated by co-administration of a GnRH antagonist, demonstrating that kisspeptin acts through the stimulation of GnRH secretion (Shahab et al., 2005). In humans and mice, loss-of-function mutations in genes encoding prepro-kisspeptin or the kisspeptin receptor lead to isolated hypogonadotropic hypogonadism caused by deficient GnRH secretion and consequently to defective pituitary secretion of FSH and LH (d'Anglemont de Tassigny et al., 2007; de Roux et al., 2003). This results in the impairment of both pubertal maturation and reproductive function (Seminara et al., 2003; Tng, 2015). In addition to regulation of gonadotropin secretion by gonadal hormones, kisspeptins are involved in sexual differentiation of the brain, the timing of puberty and the control of fertility by metabolic and environmental cues (Roa et al., 2011).

All biologically active kisspeptins have the same carboxyl terminal ten-amino acid sequence. The shortest kisspeptin with full intrinsic bioactivity, KP-10, potently stimulates LH, FSH and gonadal steroid secretion when administered both centrally and systemically and has been utilised in laboratory rodents, humans and wild and domestic species (Akhtar et al., 2017; Albers-Wolthers et al., 2014; Caraty et al., 2012; George et al., 2011; Magee et al., 2009; Pottapenjera et al., 2018; Scott et al., 2019; Suzuki et al., 2008; Tena-Sempere, 2006; Ullah et al., 2019), but not in lions to date. To provide insight into the physiology of the hypothalamic-pituitary reproductive axis in lions, we studied the LH and steroid response to KP-10 and GnRH in anaesthetised male animals using enzyme-linked immunosorbent assay (ELISA) and liquid chromatography tandem mass spectrometry (LC-MS/MS).

2. Materials and methods

2.1. Study animals

Ten male lions (Ukutula Conservation Centre, South Africa) were used in this study: six young (age 13.3 ± 1.7 months) and four adults (age 40.2 ± 1.4 months). The lions selected for the procedure were separated from the rest of the pride into a smaller management camp. Lions were immobilised with a combination of medetomidine and ketamine (2–2.5 mg plus 60–80 mg for young, 9.5 mg plus 280 mg for adults, Kyron Laboratories, South Africa) by remote injection with darts (Daninject, Austin, TX, USA). After moving to a surgical theatre, they were maintained under light surgical anaesthesia throughout the experimental procedure with follow-up injections of a combination of medetomidine and ketamine as needed. On completion of the experiment the lions were returned to the management camp and anaesthesia reversed with either atipamezole (2.0 mg/mg medetomidine used; Alphanil, Wildlife Pharmaceuticals, South Africa) or 125 mg/kg yohimbine (Kyron Laboratories, South Africa), administered by hand with a syringe, either intravenously (iv) or intramuscularly. Each lion was observed until the veterinarian was satisfied that it had completely recovered before allowing it to re-join the pride. The veterinarian administered drugs and oversaw the welfare of the animals. Ethical clearance and permission for experimentation were obtained from the University of Stellenbosch and the University of Pretoria animal ethics committees, permission for transportation of samples from Ukutula to the University of Pretoria was obtained from the Department of Environmental Affairs (No: 28600) and for transportation to the UK for assay was obtained by a CITES permit (No: 206263).

2.2. Experimental protocol

Before commencement of the experiment each lion was weighed. A 14-gauge intravenous catheter was placed in a jugular, cephalic or medial saphenous vein for the intermittent collection of samples. Altogether, twenty-two blood samples (5 ml each) were taken at 10 min intervals from each lion into EDTA blood collection tubes. After 70 min of baseline sampling, kisspeptin (KP-10, 1 μg/kg, EZBiolab Inc., Parsippany, NJ, USA) was given intravenously (iv) after sample seven and GnRH (1 μg/kg, EZBiolab Inc., Parsippany, NJ, USA) after sample sixteen. One adult lion did not receive KP-10 at any point and received only GnRH after sample sixteen. The GnRH dose selected was in line with the 1 μg/kg dose previously used for lions (Brown et al., 1991; Brown et al., 1993). Since no previous studies have used KP-10 to interrogate lion neuroendocrine function, guidance was taken from our human studies that found a 1 μg/kg dose of KP-10 was the minimum required dose for a maximal LH response (George et al., 2011).

All blood samples were immediately centrifuged for 10 min at 2000 *g*. The plasma was then removed and aliquoted into 1.5 ml Eppendorf tubes and frozen at −20 °C until the time of further analyses.

2.3. LH measurements

The plasma samples were processed by ELISA to measure LH, as reported previously (Ivanova et al., 2021; Steyn et al., 2013). The capture antibody (monoclonal antibody, anti-bovine LHβ subunit, AB 2665514) was purchased from the Department of Animal Science at the University of California, Davis. The LH standard (AFP-5306A) and primary antibody (polyclonal antibody, rabbit LH antiserum, AB 2665533) were obtained from Harbor-UCLA. The secondary antibody (horseradish peroxidase-linked donkey anti-rabbit IgG polyclonal antibody, AB 772206) was purchased from VWR International (Leicestershire, UK). The sensitivity of this ELISA is 10 pg/ml with an intra-assay coefficient variation of 4.9 %. All samples were assayed in a single batch.

2.4. Steroid hormone measurements

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was adapted from the recently described assay (Denham et al., 2022) to measure eighteen steroid hormones in 200 μL aliquots of the plasma samples. We successfully quantified eleven steroids which are highlighted in Fig. 1 (Progesterone (P), 17-Hydroxyprogesterone (17-OHP), 11-Deoxycorticosterone (11-DOC), 11-Deoxycortisol (11S), Androstenedione (A4), Testosterone (T), Cortisol (F), Cortisone (E), Corticosterone (B), 11-dehydrocorticosterone (A), Aldosterone (Aldo)). The method also included mass transitions and internal standards for 8 other steroids, but levels were below the level of detection in these male lion samples (21-Deoxycortisol, 17-Hydroxypregnenolone,

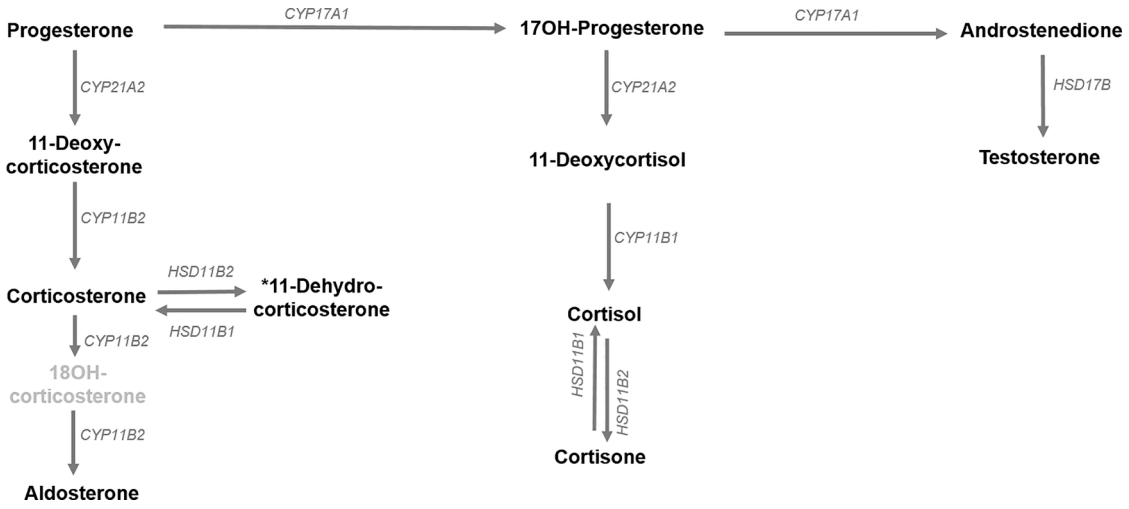

Fig. 1. Steroid biosynthetic pathway. Steroids that were measured in lion plasma samples by LC-MS/MS and could be confidently analysed are shown in black.

Dehydroepiandrosterone, Pregnenolone, 5α-Dihydrotestosterone, Estrone, Estradiol, Estriol). The sensitivity of this LC-MS/MS method ranges between 2.5 pg/ml for aldosterone and 5 ng/ml for cortisol with an intra- and inter assay coefficient variation of 6.7–12.2 %.

2.4.1. Chemicals, reagents and consumables

Water (LC-MS grade), acetonitrile (LC-MS grade), methanol (LC-MS grade), dichloromethane (HPLC grade) and propan-2-ol (HPLC and LC-MS grade) were from VWR, Lutterworth, UK. Formic acid (LC-MS grade) and ammonium hydroxide (35 %) were from Fisher Scientific, Loughborough, UK). Certified reference materials (1 mg/ml in methanol or acetonitrile) for A4, T, DHT, P, 17-OHP, F, B were supplied by Cerilliant/Sigma-Aldrich, Dorset, UK, as were certified reference materials (100 µg/ml in methanol or acetonitrile) for isotopically labelled internal standards; 2,2,4,6,6,17α,21,21,21-[$^{2}H_{9}$]-progesterone (d9P4), 2,3,4-[$^{13}C_{3}$]-testosterone ($^{13}C_{3}$-T), 2,3,4-[$^{13}C_{3}$]-androstenedione ($^{13}C_{3}$-A4), 2,3,4-[$^{13}C_{3}$]-cortisol ($^{13}C_{3}$-F), and 2,3,4-[$^{13}C_{3}$]-cortisone ($^{13}C_{3}$-E). 2,3,4-[$^{13}C_{3}$]-dihydrotestosterone ($^{13}C_{3}$-DHT) was from IsoSciences/QMX laboratories, Thaxted, Essex, UK. E and A were from Steraloids Inc, Newport, Rhode Island, USA. S, 11-DOC, pregnenolone (Preg), 17-hydroxypregnenolone (17-OHPreg), Aldosterone (Aldo) were provided as powders from Sigma-Aldrich, UK. Reference standard solutions and powders were stored as directed by the manufacturers. ISOLUTE®, SLE + 400 µL Supported Liquid Extraction Plates were from Biotage (Uppsala, Sweden) and 2 ml collection plates from Waters (Wilmslow, UK).

2.4.2. Extraction of steroids from plasma samples and analysis by LC-MS/MS

Steroid analysis was performed by extraction of samples through automated supported liquid extraction (SLE) on an Extrahera liquid handling robot (Biotage, Uppsala, UK) followed by targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). Calibration curves covered the range 0.0025 – 100 ng/ml. Analysis was performed on an I-Class Acquity UPLC (Waters, Wilmslow, UK) interfaced to a QTRAP 6500+ (AB Sciex, Warrington, UK) mass spectrometer. Instrument control and data acquisition were achieved using Analyst® 1.6.3 Software. Data were integrated and evaluated using MultiQuant® 2.3.1 (AB Sciex, Warrington, UK). Chromatographic separation was achieved on a Kinetex C18 reverse phase (2.1 × 150 mm; 2.6 µm particle size), column fitted with a KrudKatcher Ultra In-Line Filter (0.5 µm porosity) both from Phenomenex, UK. The mobile phase system was water (A) and methanol (B). both with ammonium fluoride (50 µM) as modifier at a flow rate of 0.3 ml/min over 16 min, starting at 55 % B for 2 min, rising to 100 % B over 6 min, held for 2 min, before returning to 55 % B over 0.1 min and equilibrating for 4.9 min, all held at a temperature of 50 °C. The solvent flow was diverted to waste from 0 to 2 min and 11–16 min. The mass spectrometer was operated in electrospray ionisation mode with polarity switching using a TurboIonSpray source and data were collected in unit resolution (0.7 *m/z* full width at half maximum). The source was operated at 600 °C with an IonSpray voltage of 5.5 kV/-4.5 kV, a Curtain Gas of 30 psi, nitrogen nebulizer ion source gas 1 (GS1) and heater ion source gas 2 (GS2) of 40 psi and 60 psi, respectively. Multiple reaction monitoring transitions were monitored for each compound and isotopically labelled internal standard as described previously (Ostinelli et al., 2022). Calibration curves for each steroid were plotted as the peak area ratio of the analyte divided by the internal standard versus amount of steroid and amounts of each steroid were calculated by linear regression. Calibration lines of best fit were considered acceptable if the regression coefficient, r, was > 0.99, with 1/x weighting. Human quality controls from Chromsystems were used to confirm calculated concentrations of 8 steroids in each batch.

2.5. Statistical analysis

Statistics were carried out using Prism, version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Data were analysed by two-way ANOVA followed by Fisher's least significant difference test. LH and steroid responses to KP-10 and GnRH were evaluated in the baseline samples before treatment. The evoked LH release was calculated by subtracting the release under baseline conditions (mean of the five samples preceding KP-10 injection and mean of the two samples preceding GnRH injection) from that observed in the six samples collected after the respective injections. The strength of correlation between testosterone, age, testicular size and body weight was tested using Pearson's correlation coefficient. All values are expressed as means + SEM, and differences were considered significant at $p \leq 0.05$.

3. Results

The weights of the six young and four adult lions were 56.2 ± 4.3 kg and 174 ± 6 kg, respectively. The diameter of the testes in the young lions, measured using a calliper, was 1.68 ± 0.2 cm or unmeasurable in two of the youngest lions (10-month-old) as one of the testes was undescended, and in the adults was 3.84 ± 0.6 cm (average combined from both testicles).

Fig. 2 shows the release profile of LH in response to KP-10 and GnRH. Basal LH levels were not significantly different between the young and adult groups (average of 0.62 ± 0.01 ng/ml and 0.48 ± 0.01 ng/ml for adults and young lions, respectively, during the first seven samples, p =

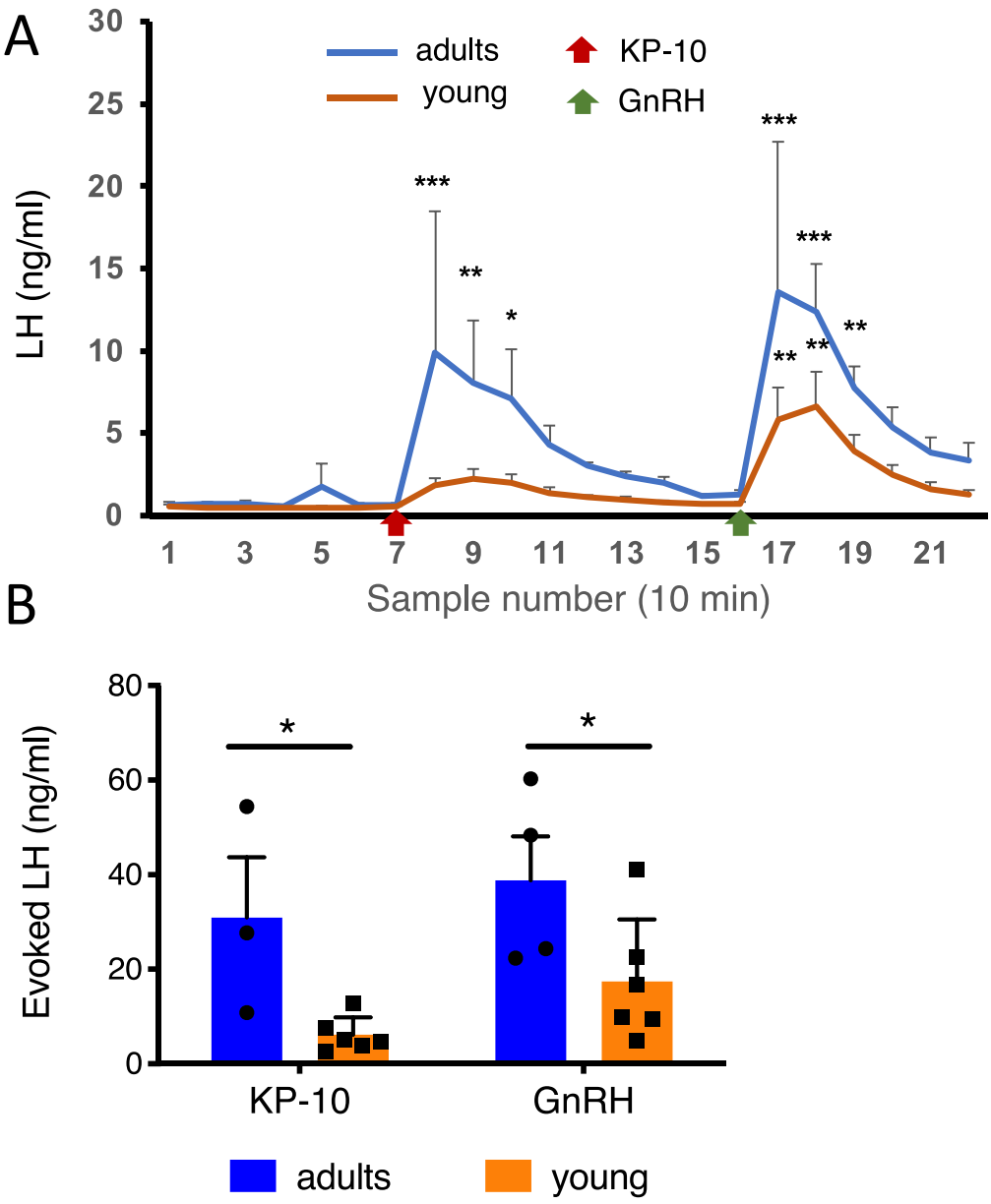

Fig. 2. LH release in response to KP-10 and GnRH. A) Release profiles and B) evoked release of LH in plasma samples in response to KP-10 and GnRH in adult (n = 3 for KP and n = 4 for GnRH) and young lions (n = 6). Means + SEM, (A) **p ≤ 0.01, ***p ≤ 0.001 compared sample before KP-10 (sample 7) and GnRH administration (sample 16). (B)*p ≤ 0.05 compared between groups.

0.22). The two-way ANOVA revealed a significant effect of treatment ($F(21, 153) = 7.908$, $P < 0.0001$), age ($F(1, 153) = 36.27$, $P < 0.0001$) and interaction ($F(21, 153) = 1.825$, $P = 0.0205$). The individual significances of the post-hoc comparisons are shown in Fig. 2. Adult lions showed a significant response to both KP-10 and GnRH administration for 30 min after drug administration. The greatest LH response to KP-10 and GnRH stimulation was observed in the most dominant adult lion who also had the highest body weight, testicular size, and basal testosterone level. The response in young lions did not show a significant difference after KP-10 but there was an increase in LH in every individual and there was a significant increase in the 20 min after GnRH administration (Fig. 2A). The individual significances of the post-hoc comparisons are shown in Fig. 2.

The two-way ANOVA revealed that the evoked LH responses to KP-10 and GnRH showed an age effect, being significantly higher in adult lions compared to young lions ($F(1,15) = 11.9$, $p = 0.0035$). The response to KP-10 was slightly less than to GnRH in both groups but there was no significant treatment ($F(1, 15) = 2.053$, $P = 0.1724$) or interaction effect ($F(1, 15) = 0.06687$, $P = 0.7995$, Fig. 2B).

Basal levels of the major male androgen, testosterone (T), were significantly higher in adults when compared to the young, with the highest levels being in the most dominant individual in the enclosure. There was a moderate correlation between T and age ($R = 0.572$), T and body weight ($R = 0.585$) and T and testicular size ($R = 0.586$). The release profiles of the steroid hormones in response to KP-10 and GnRH are shown in Figs. 3 and 4.

Testosterone (T), and its precursors, androstenedione (A4) and 17-hydroxyprogesterone (17-OHP), show a steady increase in response to KP-10 in the adults, but no or very little increase in young lions (Fig. 3). In contrast to LH levels, which returned to basal, the plasma levels of these steroid hormones remained high and plateaued or peaked just before GnRH administration. This is expected as the steroid hormone metabolic clearance is lower and the half-life longer for steroid hormones when compared to LH. After GnRH administration, the steroid

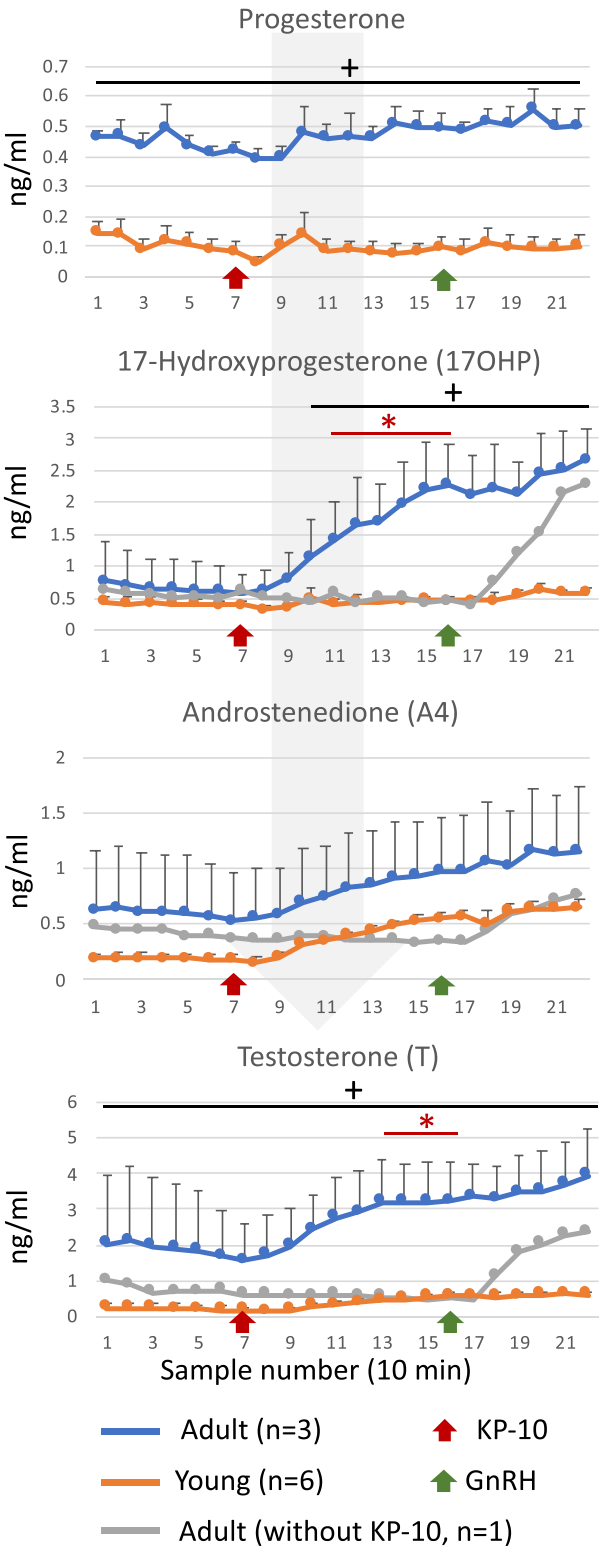

Fig. 3. Testosterone release in response to KP-10 and GnRH. Release profiles of testosterone and its precursors in plasma samples in response to KP-10 and GnRH in adult (n = 3) and young lions (n = 6). The grey arrow in the background indicates the direction of the biosynthetic pathway (see Fig. 1) Means + SEM, *p ≤ 0.05 compared to sample before KP-10 (sample 7) administration, +p ≤ 0.05 compared to young lions.

hormone levels continued to rise steadily at the same rate and this increase was not significant when compared to the levels before GnRH (sample 16). The single lion that did not receive KP-10 showed a rise in these three steroids in response to GnRH. The two-way ANOVA revealed a significant effect of interaction (17OHP: $F(21, 153) = 5.181$, $P < 0.0001$), treatment (17OHP: $F(21, 153) = 7.103$, $P < 0.0001$; A4: $F(21, 153) = 2.055$, $P = 0.0068$; T: $F(21, 153) = 1.68$, $P = 0.0395$) and age (17OHP: $F(1, 153) = 236.8$, $P < 0.0001$; A4: $F(1, 153) = 51.96$, $P < 0.0001$; T: $F(1, 153) = 221.7$, $P < 0.0001$). Progesterone levels were not affected either by Kp-10 or by GnRH but were significantly higher in adult lions throughout the sample period (age effect $(1, 153) = 835.4$, $P < 0.0001$). The individual significances of the post-hoc comparisons are shown in Fig. 3.

The analysed glucocorticoids and their precursors and metabolites are shown in Fig. 4. The precursor for cortisol (11-deoxycortisol (11S)) and precursor for corticosterone (11-deoxycorticosterone (11DOC)) show very similar release profiles in response to KP-10 and GnRH as those seen for the androgens A4 and T; a steady increase in response to KP-10 and no significant further rise in response to GnRH in the adults only. The two-way ANOVA revealed a significant effect of interaction (11S: $F(21, 151) = 7.14$, $P < 0.0001$); 11DOC: $F(21, 153) = 2.953$, $P < 0.0001$) treatment (11S: $F(21, 151) = 7.55$, $P < 0.0001$; 11DOC: $F(21, 153) = 2.58$, $P < 0.0001$) and age (11S: $F(1, 151) = 246.7$, $P < 0.0001$; 11DOC: $F(1, 153) = 176.1$, $P < 0.0001$).

The release profiles for cortisol, cortisone, corticosterone and 11-dehydrocorticosterone (11DHC) show no response to KP-10, but (besides cortisone) appear to have a small response to GnRH which reaches significance for corticosterone and 11DHC in adults when compared to the young (age effect corticosterone: $F(1,153) = 19.7$, $P < 0.0001$ and 11DHC: $F(1,153) = 79.14$, $P < 0.0001$, Fig. 4). A similar small increase in these steroid hormones after GnRH was also seen in the one adult lion that did not receive KP-10. Also, for the two precursors shown (11S and 11DOC), the data from this single lion showed a strong response to GnRH. The levels of cortisol were significantly higher throughout the sample period in the young lions when compared to the adults (age effect: $F(1, 153) + 46.52$, $P < 0.0001$, Fig. 4). For the mineralocorticoid aldosterone there was no response to KP-10 and GnRH and no age difference (data not shown).

4. Discussion

Our studies suggest that provocative tests of kisspeptin and GnRH in stimulating LH and steroid hormones provide a tool to determine the reproductive capacity of lions and may be a sensitive test to reveal the impacts of stress, nutrition and infection on the reproductive health of adult lions as well as the reproductive status (eg. puberty and dominance). Kisspeptin appears to be a more sensitive test as it distinguishes between young and adult lions in both LH and steroid hormones and may similarly distinguish between exogenous and endogenous impacts of reproduction in male lions.

In all animals, basal serum LH concentrations were stable over time and all responded to GnRH with an increase in serum LH concentration peaking within 30 min after administration, similar to that previously shown in male and female lions (Brown et al., 1991; Brown et al., 1993) and other wild carnivores including leopards, tigers and cheetahs (Brown et al., 1988; Wildt et al., 1984). The GnRH-stimulated LH response was greater in adults than in young lions although basal levels were not different. The latter may be expected as the anaesthetics ablate activity in the HPG axis. Similar age-related differences have previously been reported with respect to LH and testosterone responses (Brown et al., 1991).

Previously, pulses of LH secretion (1–3/4h) were observed in about half of the male and female lions studied in the wild (Brown et al., 1991; Brown et al., 1993). The lack of pulsatile LH release in the animals in our study over the course of the study (almost 4 h) suggests a continuing inhibitory effect of anaesthesia. Brown and colleagues used Telazol

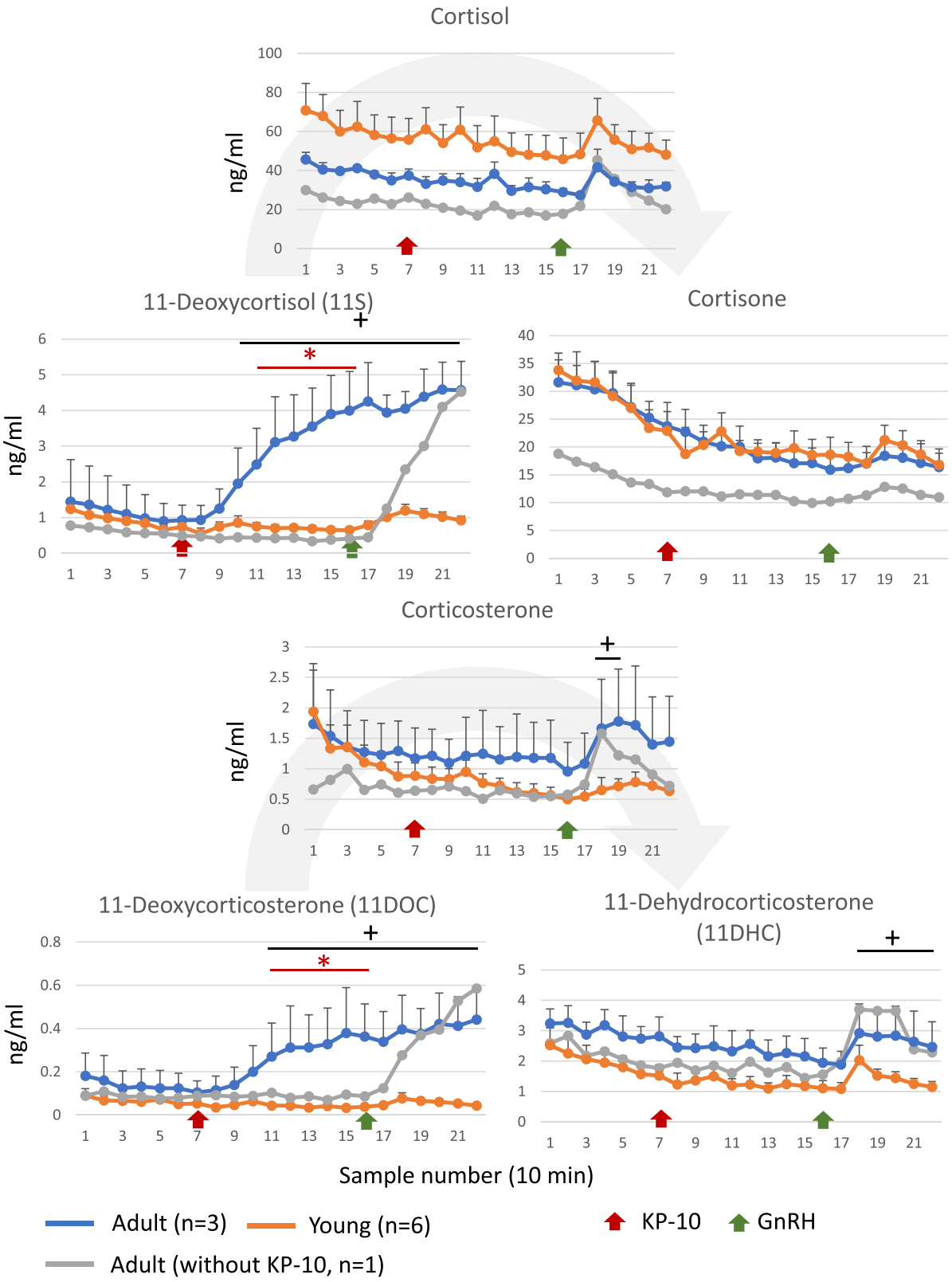

Fig. 4. Glucocorticoid release in response to KP-10 and GnRH. Release profiles of glucocorticoids and their precursors in plasma samples in response to KP-10 and GnRH in adult (n = 3) and young lions (n = 6). Grey arrows in background indicate direction of biosynthetic pathways (see Fig. 1) Means + SEM, *p ≤ 0.05 compared to sample before KP-10 (sample 7) administration, +p ≤ 0.05 compared to young lions.

(tiletamine-HCl + zolazepam-HCl) to anesthetize the animals whereas we used a combination of medetomidine and ketamine. Some anaesthetics, including ketamine, appear to inhibit tonic hypothalamic GnRH secretion (Clarke and Doughton, 1983; Johnson and Gay, 1981), but do not alter GnRH-stimulated gonadotrophin responses (Hobson et al., 1977; Lewis et al., 1985), giving a 'clean slate' on which to observe the response of the HPG axis to exogenous KP-10 and/or GnRH without variable endogenous input, and providing a powerful technique for determining the reproductive status of the animals under controlled conditions.

The Kiss 1 gene and kisspeptin neurons have been identified in domestic cats and wild felids (Amelkina et al., 2019; Rumpler et al., 2020) and kisspeptin agonists and antagonists are powerful investigative tools for extensive physiological and pathophysiological studies as well as therapeutic intervention (Millar et al., 2010). Here we demonstrated a rapid increase in LH release in adult lions in response to KP-10, like that seen after GnRH. In the young lions KP-10 did not significantly stimulate LH release and did not produce a marked rise in testosterone, indicating that the GnRH neurons in these lions may not be fully functional. Indeed, a variety of mammalian models have shown that the kisspeptin system plays an integral role in sexual maturation and the onset of puberty. Thus, progression into puberty is associated with increased kisspeptin receptor expression and kisspeptin production, increasing concentrations of kisspeptin neuron fibres around GnRH neurons, increased electrical response duration of GnRH neurons after kisspeptin receptor activation and/or increased sensitivity of the kisspeptin receptors in GnRH neurons (Clarkson et al., 2010; Pinilla et al., 2012; Roseweir and Millar, 2009). Although much of our knowledge of the postnatal development of the kisspeptin system is derived from rodent studies (Clarkson et al., 2010; Pinilla et al., 2012) studies in other species have highlighted species differences in pubertal development of the kisspeptin and its receptor system and for the neuroendocrine mechanisms in puberty onset (Pinilla et al., 2012). An immature kisspeptin system may explain the lack of a significant increase in LH release in response to acute exogenous KP-10 in sexually immature lions. Whilst the onset of puberty occurs earlier in captive-born male lions (<1.2 years of age) compared to their wild-born counterparts (<2.5 years of age) (Putman et al., 2019), the young lions used in this study were around 13 months of age and thus are likely to still be prepubertal.

In the adult lions, the KP-10 stimulation of LH release was only slightly lower than the GnRH response. By contrast, the kisspeptin stimulation of LH in humans and various other animal species is much less (10-fold lower in humans) than that of GnRH, and, unlike GnRH antagonists, kisspeptin antagonists only partially suppress LH, which suggests that a kisspeptin-independent component of LH secretion exists (Millar et al., 2010) but this does not appear to be the case in lions.

The testosterone response curves to KP-10 of the adult lions were similar to the GnRH response curves described previously (Brown et al., 1991). The adult males had higher baseline testosterone concentrations than the young lions and exhibited a steadily increasing testosterone response to KP-10 that was barely detectable in the young males. It appeared that GnRH is also eliciting a testosterone response, but this was masked by the high circulating levels of testosterone before GnRH in response to KP-10. The single lion that received GnRH but without KP-10 administration, showed a robust response to GnRH as seen before in other wild felids (Brown et al., 1991; Brown et al., 1993; Brown et al., 1988; Wildt et al., 1984).

KP-10 had no effect on the release of the stress hormones cortisol and corticosterone (and their metabolites cortisone and 11-DHC), but GnRH stimulated the release of the two glucocorticoids, suggesting that the increase in endogenous GnRH elicited by KP-10 is less than the level of GnRH administered by us exogenously. Cortisol and corticosterone are produced mainly in the adrenal cortex, stimulated by the release of adrenocorticotropic hormone from the pituitary, and their release is increased in response to stress. In the current study, the lions were deeply anaesthetized with the resultant ablation of the hypothalamic pituitary adrenal stress axis as reflected by the low levels of cortisol and corticosterone. Thus, the response of these hormones and their precursors to GnRH suggests a direct effect of GnRH on the pituitary corticotropes or on the adrenals. GnRH receptor mRNA and protein has been shown in fetal and adult adrenal glands (Kakar et al., 1994; Xing et al., 2009). Interestingly, only levels of cortisol were significantly higher in the young lions throughout the sample period when compared to the adults. There is evidence from humans and rodent studies that cortisol rhythmicity and plasma levels are blunted with age (Moffat et al., 2020; VanCauter et al., 1996; Zambrano et al., 2015). As an alternative explanation, the stimulation of the glucocorticoids may be due to high levels of LH elicited by GnRH (Alevizaki et al., 2006; Bernichtein et al., 2008; Schoemaker et al., 2008).

Immunoassays for steroid measurements are notoriously nonspecific, especially for steroid hormones that circulate at low levels and with metabolites that can lead to cross-reactivity. LC-MS/MS is a highly specific and sensitive technique for the quantification of the full range of steroids. It avoids the issue of antibody cross-reactivity and separation by time increases selectivity and enables the simultaneous analysis of multiple steroids, which in turn reduces the demand of multiple sample aliquots. Since the sample volume is often limited when working with wild animals, it is important to extract as much information as possible from each sample (Andrew and Homer, 2020; Shi et al., 2021). Here we analysed 10 different steroids which revealed some additional interesting observations. For example, the precursors of both cortisol and corticosterone (11S and 11DOC, respectively), were significantly increased in response to KP-10 in the adult lions whereas cortisol and corticosterone were unaffected. The ratio of cortisol:cortisone was found to be lower (~2) in the adult lions than is seen in healthy adult humans (~5) (Banker et al., 2021; Hirtz et al., 2022), suggesting a high 11β-hydroxysteroid dehydrogenase (HSD11B2) activity. 17-hydroxyprogesterone (17OHP, see Fig. 1), the precursor of 11-deoxycortisol (11S) is also a precursor in the androgen biosynthetic pathway. The accumulation of 11S suggests low activity of the 11b-hydroxylase (CYP11B1) that converts it to cortisol. These observations merit further investigation but are beyond the scope of this study.

Previous studies have used GnRH to interrogate the HPG axis function in lions (Brown et al., 1991). However, kisspeptin neurons are upstream of GnRH neurons and are responsive to many more inputs than GnRH neurons (e.g., reproductive and stress steroid hormones, inflammatory and growth factors) (Roseweir and Millar, 2009). Therefore, as the data presented here suggest, a provocative kisspeptin test might be more revealing than a GnRH test for investigation of the HPG axis.

Although GnRH and its analogues have been extensively employed in treating hormone-dependent diseases and assisted reproductive technologies in humans (Anderson and Millar, 2021; Kotlyar et al., 2021; Wu et al., 2021) and many species of animals, including lions (Callealta et al., 2019), GnRH agonists are not always recommended for induction of follicular maturation and ovulation in felids, due to the unreliable effect of this drug in some feline species (Goodrowe and Wildt, 1987; Pelican et al., 2006).

From a translational point of view, in veterinary medicine and wildlife conservation, the demonstration that kisspeptin not only stimulates gonadotropin secretion but also accelerates follicular growth and advances the time of ovulation in many species (Caraty et al., 2012) offers interesting alternatives to design new protocols aimed at improving fertility in mammals (Burke et al., 2022). These alternatives include stimulation of the quiescent gonadotropin axis (prepubertal, anoestrous periods, negative energy balance) or optimization of artificial insemination (Callealta et al., 2019; Caraty et al., 2012). Neutralisation of kisspeptin activity through antagonists or antibodies also have application in wildlife and livestock management. A recent development of a dual kisspeptin/GnRH immunogen shows promise as a single immunisation to induce infertility in pubertal female rats for at least 300 days (Junco et al., 2021). In this study we also demonstrate the wealth of data that multi-steroid profiling by LC-MS/MS generates and

suggest that this approach can be applied to other settings across species to investigate steroid changes in development, growth and reproduction.

In conclusion, we have established a provocative test of the hypothalamic-pituitary–gonadal axis in lions using kisspeptin. Future studies using this test will allow interrogation of the functioning of the hypothalamic-pituitary–gonadal axis as a sensitive surrogate index of reproductive status, management of breeding programmes and for detection of disease, and the nutritional and environmental impacts on lion reproduction.

CRediT authorship contribution statement

Mike Ludwig: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Claire Newton:** Methodology. **Ané Pieters:** Methodology, Writing – review & editing. **Natalie Z.M. Homer:** Formal analysis, Funding acquisition. **Xiao Feng Li:** Formal analysis, Writing – review & editing. **Kevin T. O'Byrne:** Formal analysis, Funding acquisition. **Robert P. Millar:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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