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Analysis of GnRH-1 and kisspeptin neuronal systems in the non-photoregulated seasonally breeding eastern rock elephant-shrew (*Elephantulus myurus*)

Running title: GnRH-1 and kisspeptin in elephant-shrews

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ABSTRACT

Of the eighteen sub-Saharan elephant-shrew species, only eastern rock elephant-shrews reproduce seasonally throughout their distribution, a process seemingly independent of photoperiod. The present study characterizes gonadal status and location/intensity of GnRH-1 and kisspeptin immunoreactivities in this polyovulating species in the breeding and non-breeding seasons. GnRH-1-immunoreactive (ir) cell bodies are predominantly in the medial septum, diagonal band and medial preoptic area; processes are generally sparse except in the external median eminence. Kisspeptin-ir cell bodies are detected only within the arcuate nucleus; the density of processes is generally low, except in the septohypothalamic nucleus, ventromedial bed nucleus of the stria terminalis, arcuate nucleus and internal and external median eminence. Kisspeptin-ir processes are negligible at locations containing GnRH-1-ir cell bodies. The external median eminence is the only site with conspicuously overlapping distributions of the respective immunoreactivities and, accordingly, a putative site for kisspeptin's regulation of GnRH-1 release in this species. In the non-breeding season in males, there is an increase in the rostral population of GnRH-1-ir cell bodies and density of GnRH-1-ir processes in the median eminence. In both sexes, the breeding season is associated with increased kisspeptin-ir process density in the rostral periventricular area of the third ventricle and arcuate nucleus; at the latter site this is positively correlated with gonadal mass. Cross-species comparisons lead us to hypothesize differential mechanisms within these peptidergic systems: that increased GnRH-1 immunoreactivity during the non-breeding season reflects increased accumulation with reduced release; that increased kisspeptin immunoreactivity during the breeding season reflects increased synthesis with increased release.

KEYWORDS: Afrotheria, anteroventral periventricular nucleus, arcuate nucleus, GnRH-1, kisspeptin, seasonal reproduction, RRID:AB_572248, RRID:AB_2631061, RRID:AB_2315026, RRID:SCR_014199, RRID:SCR_002865, RRID:SCR_003070

1. INTRODUCTION

Kisspeptin has been shown to regulate reproduction by direct actions on gonadotrophin-releasing hormone-1 (GnRH-1) neurons in mice, animals that breed non-seasonally (Clarkson, D'Anglemont de Tassigny, Moreno, Colledge, & Herbison, 2008; Kalló et al., 2011; Liu & Herbison, 2016; Messenger et al., 2005). It has also been implicated in the transduction of photoperiodic signals to GnRH-1 neurons in several species of seasonally breeding mammals (Ansel et al., 2010; Greives et al., 2007; Revel et al., 2007; Simonneaux et al., 2009; Smith, Clay, Caraty, & Clarke, 2007; Smith et al., 2008; Wagner, Johnston, Clarke, Lincoln, & Hazlerigg, 2008). However, relatively little is known about the seasonal regulation of kisspeptin and GnRH-1 of wild animals. We are aware of only two previous studies in which kisspeptin's involvement in seasonal reproduction has been investigated under well-nigh natural conditions; these involved two rodent species: jerboas (*Jaculus orientalis*) and Libyan jirds (*Meriones libycus*) (Janati et al., 2013; Talbi et al. 2016).

The present study has examined the reproductive organs and the GnRH-1 and kisspeptin neuronal systems in male and female eastern rock elephant-shrews (*Elephantulus myurus*) captured in South Africa during the breeding (October) and non-breeding (April) seasons. These small, non-rodent mammals are endemic to the African continent and also known as sengis (Skinner & Chimimba, 2005; Rathbun, 2009). They belong to the diverse superorder Afrotheria, which includes dugongs, manatees, sea cows, hyraxes, elephants, aardvarks, golden moles and tenrecs (Kuntner, May-Collado, & Agnarsson, 2011). Elephant-shrews are most closely related to aardvarks, golden moles and tenrecs; together they form the Afroinsectiphilia (Kuntner et al., 2011). These animals differ widely in their habitats, which range from entirely fossorial for golden moles to highly diverse (fossorial, terrestrial, arboreal or aquatic) for tenrecs. Eastern rock elephant-shrews are terrestrial; their diet consists primarily of termites and ants (Churchfield, 1987; Rathbun, 2009). They are socially monogamous and precocial, characteristics that are rare in small mammals (Rathbun, 1979, 2009), and particularly active at dawn and dusk, as confirmed under laboratory conditions (van der Merwe, Oosthuizen, Bennett, & Chimimba, 2012). Their habitat is open rocky land with limited hiding places (Rathbun, 2009). To offset the high predation pressure, elephant-shrews are remarkably agile, with a cursorial, saltatorial mode of locomotion (Rathbun, 2009).

The home range of a male eastern rock elephant-shrew usually overlaps with that of only one female or, in rare cases, two (Ribble & Perrin, 2005). They reproduce from August until January, the spring and summer months in the southern hemisphere, and are reproductively inactive during the cold, dry autumn and winter months from February until July (Medger, Chimimba, & Bennett, 2012). Of the 18 elephant-shrew species that live in southern or eastern Africa, only eastern rock elephant-shrews reproduce seasonally throughout their distribution (Skinner & Chimimba, 2005). Prominent seasonal changes affect their habitat; food availability is especially low during the winter (Andrews & O'Brien, 2000). We have recently reported (Medger, Chimimba, & Bennett, 2016) that males of this species do not appear to use photoperiod to regulate reproductive onset. Thus, males exposed for 3 to 4 months to either a short- or long-day photoperiod show no difference in the size of the testes or the diameter of the seminiferous tubules (Medger et al., 2016). These findings suggest that environmental factors other than photoperiod regulate their reproduction.

Eastern rock elephant-shrews are polyovulators (Tripp, 1971). Polyovulation is a rare feature in mammals and involves the production of many more eggs than are implanted (Weir & Rowlands, 1973). It occurs in four species of elephant-shrews (Tripp, 1971) and in the plains viscacha (*Lagostomus maximus*), a rodent for which 200-800 eggs per ovulatory cycle have been reported despite a litter size of only 1-3 (Weir, 1971a, 1971b). Between 23 and 89 corpora lutea per ovary have been counted in eastern rock elephant-shrews (Tripp, 1971). Most eggs are fertilized, but only the first to reach each uterine horn is able to implant, usually resulting in a litter of two (Weir & Rowlands, 1973).

The unusual characteristics of non-photoregulated seasonal reproduction and polyovulation make eastern rock elephant-shrews especially interesting for reproductive neuroendocrine research. The present study has characterized the gonadal status and the distribution and intensity of GnRH-1 and kisspeptin immunoreactivity in females and males of this small, non-rodent species and compared the findings between the breeding and non-breeding seasons. This is the first study that has investigated the GnRH-1 and kisspeptin systems in (1) an Afrotherian species, (2) a polyovulating species and (3) a non-photoregulated seasonally breeding species captured in the wild. The findings are discussed in comparative terms, with particular reference to studies on photoregulated seasonally breeding species.

2. MATERIALS AND METHODS

2.1. Animals

Eastern rock elephant-shrews of both sexes were captured in a private game reserve (Goro Game Reserve; 22°58'S, 22°57'S; 29°25'E, 29°24'E) in the Soutpansberg, Limpopo Province, South Africa, during October (in the breeding season) and April (in the non-breeding season) in 2008 to 2009, under permit CPM-002-00002 issued by the Department of Economic Development, Environment and Tourism, Limpopo Province, South Africa. The animals were trapped overnight with Sherman live-traps (HB Sherman Traps Inc., Tallahassee, Florida, USA) baited with peanut butter, oats and fish. Five animals of each sex were caught during the non-breeding season; six males and five females were caught during the breeding season. The animals were weighed to the nearest 0.1 g with a digital balance (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, USA) and housed individually in polyurethane cages furnished with wood shavings during transportation and for no more than 48 hours under 12/12 hours light/dark conditions in the laboratory. They were fed with canned dog food (Proméal Ltd., Dassenberg, South Africa) and pronutro, a high protein cereal (Pioneer Foods Ltd., Bokomo Foods, Cape Town, South Africa), with additional grated apples and carrots. Water was provided in an open dish. All experimental procedures were approved by the animal ethics committee of the University of Pretoria, Pretoria, South Africa (ethics clearance number: EC037-08).

2.2. Perfusion and histology

Within 48 hours after arrival at the laboratory, the animals were anaesthetised with halothane and perfused transcardially with 100 ml of 0.9 % saline, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). Brains were removed and stored in 4% paraformaldehyde at 4 °C until subsequent processing. Females were examined for current pregnancy and for placental scars in the uterine horns, evidence of previous pregnancies. Testes and ovaries were cleaned of fat and connective tissue and weighed to the nearest 0.1 mg with a high precision scale (Ohaus Corp. Pine Brook, NY, USA). The average testicular or ovarian mass was calculated per male or female, respectively. The ovaries together with the uterine horns or the testes were post-fixed in Bouin's solution for 20 hours and then stored in 70% ethanol. They were

cut at 7 μm , stained with Ehrlich's haematoxylin and counter-stained with eosin (Drury & Wallington, 1967).

The follicular stages were identified and classified according to Bloom and Fawcett (1964) at a magnification of $\times 200$ (Vickers Instruments, UK). The total numbers of primary, secondary, tertiary and Graafian follicles as well as corpora lutea and corpora albicantia were determined through each ovary and expressed as the mean per animal. Testicular sections were examined for seminiferous tubules with a circular profile. The diameter of 100 of these profiles (50 in each testis) was measured to the nearest 0.1 μm , as described by Medger et al. (2012), and expressed as the mean diameter for each male.

2.3. Immunohistochemistry and quantification

A cryostat (Bright Instruments, Huntingdon, UK) was used to cut coronal sections (25 μm); these were collected into 6 rostrocaudal series, starting at the olfactory bulbs rostrally and extending through the hypothalamus caudally. Of these series, two were processed immunohistochemically for GnRH-1 and kisspeptin immunoreactivity; in addition, sections at the rostrocaudal level of the paraventricular nucleus of the hypothalamus were processed for oxytocin-neurophysin immunoreactivity. The tissue was pre-treated with 0.5% Triton X-100 (30 minutes) and 2% H_2O_2 (30 minutes) and then exposed to 2% normal donkey serum (Sigma-Aldrich, U.K.) for 1 hour. The primary antisera (Table 1) were rabbit anti-GnRH-1 (1:10,000; RRID:AB_572248; Incstar Corporation, Stillwater, MN, USA), rabbit anti-kisspeptin (1:300; JLV1, RRID:AB_2631061) and mouse anti-oxytocin-neurophysin (1:500; PS-38, RRID:AB_2315026; a gift from Dr H. Gainer). The distributions of the immunoreactivities revealed in the present study did not differ qualitatively from those reported for other mammals (see Discussion). Increasing dilutions of each of the primary antisera led to a commensurate attenuation of the immunoreactive signal. No immunoreactivity was observed when the primary or secondary antiserum was omitted. These primary antisera have been used extensively and their specificity has been demonstrated (Ben-Barak et al., 1985; Desroziers et al., 2010; Du Toit et al., 2006; Kalamatianos et al., 2010; Mikkelsen & Simonneaux, 2009; Whitnall, Key, Ben-Barak, Ozato, & Gainer, 1985). The rabbit GnRH-1 antiserum was raised against mammalian GnRH-1 conjugated to bovine thyroglobulin with carbodiimide. When the antiserum was

pretreated overnight with GnRH-1 (5 µg/ml; Phoenix Pharmaceuticals, Peterborough, UK) immunoreactive signals were blocked. The rabbit kisspeptin antiserum (JLV-1) was raised against full-length rat/mouse kisspeptin-52 conjugated to keyhole limpet hemocyanin with carbodiimide; the serum was subsequently affinity-purified on a column with the natural peptide (Davids Biotechnologie, Regensburg, Germany). JLV-1 does not cross-react with the arginine-phenylalanine (RF)-amides RFRP-1, RFRP-1, NPFF, 26RFa or PrRP; pretreatment with 0.1 µM rat/mouse kisspeptin-52 prevented its immunoreactivity in accord with previous results (Desroziers et al., 2010). The mouse oxytocin-associated neurophysin antibody was raised against extracts of rat posterior pituitary coupled to keyhole limpet hemocyanin. Extensive characterization, by liquid- and solid-phase immunoassays, epitope analysis, and immunohistochemistry (Ben-Barak et al., 1985; Whitnall et al., 1985), indicates that this antibody binds specifically to rodent oxytocin-associated neurophysin and does not cross-react with vasopressin-associated neurophysin.

The sections were incubated in the primary antiserum at 4°C for 72 hours for the rabbit antisera and for 5 days for the mouse monoclonal antibody. Subsequently, biotin-SP conjugated AffiniPure donkey anti-rabbit IgG or sheep anti-mouse IgG (1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was applied for 2 hours. After it had been washed in PBS, the tissue was incubated in an avidin-biotin peroxidase complex (1:1000 Elite Kit; Vector Laboratories, Peterborough, UK). The immunoreactivity for GnRH-1, kisspeptin or oxytocin-associated neurophysin was visualised with 0.05% 3'-3 diaminobenzidine plus 0.15% ammonium nickel sulphate and 0.005% H₂O₂. A separate series of sections was Nissl-stained (0.5% cresyl violet acetate) to visualize the cytoarchitecture.

Brain sections from elephant-shrews are a scarce and valuable resource. These animals must be captured under permit in the wild, where they are not common; furthermore, they are not prolific breeders. Consequently, it was not possible to assign full sets of brain sections for serial reconstruction or sets of paired contiguous sections for the physical di-sector method (Coggeshall & Lekan, 1996). Each series of sections processed for GnRH-1 or kisspeptin immunoreactivity was selected at random, in accordance with the equal opportunity rule (Coggeshall & Lekan, 1996), and the counting was undertaken blind. The number of GnRH-1-ir cell bodies found in every sixth section between the confluence of the two hemispheres rostrally and the posterior

limit of the hypothalamus caudally was determined bilaterally. An Abercrombie correction (Abercrombie, 1946) was used to correct for oversampling the number of cell bodies. The corrected numbers of cell bodies were multiplied by six to estimate the total number for each brain. Thus, the total numbers presented here form a dataset that is suitable for intergroup comparisons (Guillery & Herrup, 1997). These findings were also expressed as the number rostral to or at the rostrocaudal level of the suprachiasmatic nuclei and the number caudal to the suprachiasmatic nuclei. The presence of kisspeptin-ir cell bodies was assessed throughout the rostrocaudal series. The only site at which such cell bodies were observed was the arcuate nucleus; their total number could not be determined because of the density of the enveloping kisspeptin-ir processes. Image analysis software (ImageJ; National Institutes of Health, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>, RRID:SCR_003070) was used according to the method described by Kriegsfeld, Ranalli, Bober, & Nelson (2000) and Janati et al. (2013) to quantify the density of GnRH-1-ir processes in the median eminence and kisspeptin-ir processes in the septohypothalamic nucleus, rostral periventricular area of the third ventricle (RP3V), which comprises the anteroventral periventricular nucleus (AVPV) plus adjacent medial preoptic nucleus (MPO), and arcuate nucleus. Because of the frequent loss of tissue at the head of the pituitary stalk in the material collected for this species, the analysis of GnRH-1-ir processes was restricted to the caudal median eminence. The density of kisspeptin-ir processes was determined in anatomically matched levels at the mid rostrocaudal points of the septohypothalamic nucleus, RP3V and arcuate nucleus. The mean of the bilateral data was calculated after subtraction of the background signal. To prepare the images for publication, the original TIFF files were imported into Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA, <https://www.adobe.com/products/photoshop.html>, RRID:SCR_014199) for minor adjustments to contrast and brightness and for cropping, composing into plates and labelling.

2.4. Statistical analysis

All analyses were performed using Generalized Linear Models (GZLM) in recognition of the small sample sizes. Ovarian and testicular mass and seminiferous tubule diameter were compared between the breeding and non-breeding seasons; body mass was used as a covariate to correct for any confounding effects. Numbers of primary, secondary

and tertiary follicles were compared between the breeding and non-breeding seasons. Body mass, numbers of immunoreactive cell bodies and densities of immunoreactive processes were compared between the sexes and between the seasons. Tertiary follicle numbers, GnRH-1-ir cell body numbers caudal to the suprachiasmatic nuclei and the density of GnRH-1-ir processes were analyzed using gamma distributions with log-link function; for all other parameters linear distributions were fitted to the GZLMs. Least significant difference (LSD) pairwise comparisons were performed for each model. Correlations between gonadal mass and either GnRH-1-ir or kisspeptin-ir process density were evaluated with the Pearson or Kendall's Tau test according to the distribution of the data. All statistical analyses were performed using IBM SPSS 20 (IBM Corp., <http://www-01.ibm.com/software/uk/analytics/spss/>, RRID:SCR_002865). Results are presented as mean \pm 1 standard error.

3. RESULTS

3.1. Body mass and reproductive parameters

Body mass did not differ significantly between the female and male elephant-shrews (females: 53.3 ± 1.2 g; males: 53.6 ± 2.2 g; Wald $\chi^2 = 0.02$; $df = 1$; $P = 0.89$) or between the non-breeding season (53.1 ± 2.0 g) and the breeding season (53.8 ± 1.5 g; Wald $\chi^2 = 0.13$; $df = 1$; $P = 0.72$). Furthermore, there was no interaction between sex and season (Wald $\chi^2 = 0.42$; $df = 1$; $P = 0.52$). Testis mass was found to be positively correlated with body mass (Wald $\chi^2 = 6.32$; $df = 1$; $P = 0.01$); this is taken into account for seasonal comparisons by using body mass as a covariate in the GZLM. In contrast, there was no significant correlation between body mass and seminiferous tubule diameter or ovarian mass (Wald $\chi^2 < 1.99$; $df = 1$; $P > 0.16$). Testicular mass and seminiferous tubule diameter were significantly greater during the breeding season than during the non-breeding season (Wald $\chi^2 \geq 17.58$; $df = 1$; $P < 0.001$; Fig. 1a,b). Similarly, during the breeding season ovarian mass was greater (Wald $\chi^2 = 4.34$; $df = 1$; $P = 0.04$; Fig. 1c) and there were more primary, secondary and tertiary follicles (Wald $\chi^2 \geq 4.52$; $df = 1$; $P \leq 0.03$; Table 2). Graafian follicles, corpora lutea and corpora albicantia were detected only during the breeding season (Table 2); all of the females caught during that season were pregnant. Of the females caught during the non-breeding season, two had placental scars in their uterine horns indicating a previous pregnancy.

3.2. Distribution of GnRH-1-ir cell bodies and processes

In sengis, GnRH-1-ir cell bodies in eastern rock elephant-shrews are predominantly detected in the region of the diagonal band, in the medial septum and in the medial preoptic area (Fig. 2a1-e1). Some are also found in the region of the supraoptic nuclei (Fig. 2f1). Caudal to the suprachiasmatic nuclei, GnRH-1-ir cell bodies are seen in low numbers lateral to the retrochiasmatic area (Fig. 2g) and lateral to the arcuate nucleus (Fig. 2h). GnRH-1-ir processes are notably sparse in the region of the diagonal band, in the medial septum and in the medial preoptic area (Fig. 2a1-f1); they are not detected in the AVPV or the MPO (Fig. 2e1), but present at a moderate density within the organum vasculosum of the lamina terminalis (OVLT; Fig. 2c1,d1). In this species the OVLT (Fig. 2c1 inset) is present not only immediately rostral to the preoptic recess of the third ventricle but also, more caudally, ventral to that ventricle (Fig. 2d1 inset). Subchiasmatic GnRH-1-ir processes are detected at the point at which the optic nerves begin to converge (Fig. 2c inset). GnRH-1-ir processes are absent from the region of the suprachiasmatic nucleus (Fig. 2f1), which lies immediately above the bifurcated base of the third ventricle (Fig. 2f1). At the rostrocaudal retrochiasmatic level, GnRH-1-ir processes are at a moderate density dorsal to the optic tract lateral to the retrochiasmatic area (Fig. 2g). Adjacent to the third ventricle and within the mediobasal hypothalamus GnRH-1-ir processes are diffuse (Fig. 2h). Because of the frequent loss of tissue at the head of the pituitary stalk in the material collected for this species (Fig. 2g,h), the intense GnRH-1 immunoreactivity within the median eminence was observed only in its caudal region; at that site, its distribution is predominantly within the external zone (Fig. 2i). No differences in the distribution of GnRH-1-ir cell bodies and processes were observed between the sexes.

3.3. GnRH-1-ir cell body numbers and density of GnRH-1-ir processes in the median eminence

The mean total number of GnRH-1-ir cell bodies detected across all of the groups studied for this species was 412.1 ± 36.0 . There was no significant difference in the total number of cell bodies between males (434.0 ± 57.4) and females (388.0 ± 43.5 ; $t_{19} = -0.63$, $P = 0.54$). However, at sites that are rostral to or at the rostrocaudal level of the suprachiasmatic nuclei, males displayed more GnRH-1-ir cell bodies during the non-breeding season than during the breeding season (LSD: $P < 0.02$; Fig. 3a). No such

seasonal difference was observed in the females; however, outside the breeding season, the females showed fewer GnRH-1-ir cell bodies than the males (LSD: $P < 0.03$; Wald $\chi^2 = 5.31$; $df = 1$; $P < 0.02$; Fig. 3a). The considerably smaller number of GnRH-1-ir cell bodies detected caudal to the suprachiasmatic nuclei showed no sex- or season-related differences (Wald $\chi^2 < 0.32$; $df = 1$; $P > 0.57$; Fig. 3b). In the caudal median eminence, the density of GnRH-1-ir processes was greater during the non-breeding season than during the breeding season in the males (LSD: $P = 0.046$; Fig. 4); no such seasonal tendency was observed in the females (LSD: $P = 0.45$; Fig. 4). There was no significant correlation between the density of GnRH-1-ir processes in the median eminence and gonadal mass in either the males ($r = -0.33$; $P = 0.18$) or the females ($r = -0.42$; $P = 0.26$).

3.4. Distribution of kisspeptin-ir cell bodies and processes

Cell bodies immunoreactive for kisspeptin are apparent at a very low incidence within the arcuate nucleus (Fig. 5i,j) in both sexes and both seasons; it was not possible to determine their total number because of the density of the enveloping kisspeptin-ir processes. No kisspeptin-ir cell bodies were detected at more rostral sites.

The most rostral kisspeptin-ir processes are found in the septohypothalamic nucleus (Fig. 5a1-c1) and ventromedial division of the bed nucleus of the stria terminalis (Fig. 5b1,c1). At these sites, they are present at a high density. Such processes are found only rarely in the region of the diagonal band or medial septum (Fig. 5a1,b1) or in the rostral medial preoptic area (Fig. 5c1), the sites that contain most of the rostral population of GnRH-1-ir cell bodies in this species (Fig. 2a1-e1). Kisspeptin-ir processes are not detected in the median preoptic nucleus (Fig. 5b1). More caudally, the septohypothalamic nucleus continues to exhibit a high density of kisspeptin-ir processes (Fig. 5d1); they are also found, but at a low density, in the anteromedial division of the bed nucleus of the stria terminalis (Fig. 5d1). The AVPV and adjacent MPO, collectively called the RP3V, contain only a moderate density of these processes (Fig. 5e1). Caudal to this region (Fig. 5f1), the paraventricular nucleus of the hypothalamus, as identified in adjacent sections by oxytocin-neurophysin immunoreactivity (Fig. 5g,g1), displays a moderate density of kisspeptin-ir processes. At this rostrocaudal level, only diffuse kisspeptin-ir processes are present in the anterior hypothalamic area, in the perifornical region and dorsal to the optic chiasm; they are

absent from the suprachiasmatic nucleus (Fig. 5f1). At the retrochiasmatic level, kisspeptin-ir processes are found at a moderate density dorsal to the optic tract and at a low density periventricularly (Fig. 5h). The highest density of kisspeptin-ir processes is seen in the arcuate nucleus (Fig. 5i,j). Given the loss of tissue at the head of the pituitary stalk (Fig. 5h,i), kisspeptin immunoreactivity within the median eminence was observed only in its caudal region; at that site, it is intense throughout the internal and external zones (Fig. 5j). Kisspeptin-ir processes were detected in the amygdala in only one of the elephant-shrews, a male in the breeding season. In that animal, no more than a single immunoreactive process was detected within the medial amygdala in any section.

3.5. Density of kisspeptin-ir processes

In the septohypothalamic nucleus, there was no significant difference in the density of kisspeptin-ir processes between the breeding and non-breeding seasons (Wald $\chi^2 = 1.01$; $df = 1$; $P = 0.31$; Fig. 6a-c) or between the males and females (Wald $\chi^2 = 1.02$; $df = 1$; $P = 0.31$; Fig. 6A); there was no interaction between the seasons and the sexes at this site (Wald $\chi^2 = 0.97$; $df = 1$; $P = 0.33$; Fig. 6a). In contrast, the density of kisspeptin-ir processes was substantially greater in both sexes during the breeding season in the RP3V (Wald $\chi^2 = 16.47$; $df = 1$; $P < 0.001$; Fig. 6d-f) and arcuate nucleus (Wald $\chi^2 = 60.89$; $df = 1$; $P < 0.001$; Fig. 6g-i). Within each season, there was no significant sex difference in the density of kisspeptin-ir processes in the RP3V (Wald $\chi^2 < 1.04$; $df = 1$; $P > 0.31$; Fig. 6d); but in the arcuate nucleus during the breeding season the density was 8% greater in the females than in the males (LSD: $P = 0.003$; Wald $\chi^2 = 6.41$; $df = 1$; $P = 0.01$; Fig. 6g). A positive correlation was observed between the density of kisspeptin-ir processes in the RP3V and gonadal mass in the females ($r = 0.76$; $P = 0.01$), but not in the males ($r = 0.39$; $P = 0.24$). In contrast, both sexes showed a positive correlation between the density of kisspeptin-ir processes in the arcuate nucleus and gonadal mass (males $r = 0.47$; $P = 0.001$; females $r = 0.31$; $P = 0.01$).

4. DISCUSSION

4.1. Seasonal effects on gonads

The annual development of the reproductive system in eastern rock elephant-shrews starts around June (mid-winter) in the males, about two months before the females;

April and October are the months in which reproductive activity is, respectively, lowest and highest (Medger et al., 2012). Plasma testosterone in males is low from November until April, but considerably increased from May until October; in females, plasma progesterone is elevated from August until November, but low for the remainder of the year (Medger et al., 2012). The research undertaken to assess plasma sex steroid levels across the seasons (Medger et al., 2012) was unable to detect estradiol (assay sensitivity: 0.54 pg/ml) in the 39 females sampled during the non-breeding season from December to July and in 15 out of the 19 females sampled during the breeding season from August to November (unpublished findings). Given that the largest embryos were found in the animals that had detectable estradiol and the highest levels of plasma progesterone, current evidence suggests an association between raised estradiol and the later stages of pregnancy. In keeping with our earlier findings, the present study found greater testicular and ovarian mass and increased seminiferous tubule diameter in the breeding season, the only time at which Graafian follicles, corpora lutea and corpora albicantia were seen. All females captured during the breeding season were pregnant.

4.2. Distribution of GnRH-1-ir cell bodies and processes

In eastern rock elephant-shrews, GnRH-1-ir cell bodies are numerous in the diagonal band, medial septum and medial preoptic area at the rostrocaudal level of the OVLT. More caudally, smaller numbers are observed near the supraoptic nuclei and at sites lateral to the retrochiasmatic area and to the arcuate nucleus. In mammals, GnRH-1 cell bodies migrate caudally from the olfactory placode during development (Schwanzel-Fukuda & Pfaff, 1989). In eastern rock elephant-shrews, most of these cell bodies (~91%; Fig. 3) complete their migration within the diagonal band, medial septum or medial preoptic area. This is consistent with reports that few GnRH-1-ir cell bodies migrate caudal to those sites in opossums (*Monodelphis domestica*), ewes, springboks (*Antidorcas marsupialis*) or in rodents such as rats, mice and Syrian (*Mesocricetus auratus*; golden) and Siberian (*Phodopus sungorus*; Djungarian) hamsters (King & Anthony, 1984; Lehman et al., 1997; Merchenthaler, Göracs, Sétáló, Petrusz, & Flerkó, 1984; Robinson, Skinner, Skinner, & Haupt, 1997; Schwanzel-Fukuda, Fadem, Garcia, & Pfaff, 1988; Witkin, Paden, & Silverman, 1982; Yellon, Lehman, & Newman, 1990). Similarly, in most African mole-rat rodents (naked, *Heterocephalus glaber*; Cape,

Georychus capensis; Damaraland, *Fukomys damarensis*; Highveld, *Cryptomys hottentotus pretoriae*; Natal, *Cryptomys hottentotus natalensis*) 65-95% of GnRH-1-ir cell bodies are found in the diagonal band, medial septum or medial preoptic area (Du Toit et al., 2006; Molteno et al., 2004; Oosthuizen, Bennett, & Coen, 2008; Zhou et al., 2013). However, in common mole-rats (*Cryptomys hottentotus hottentotus*) and Cape dune mole-rats (*Bathyergus suillus*), equivalent proportions are found in those rostral sites and in the mediobasal hypothalamic regions (Du Toit et al., 2006; Hart et al., 2008). In mink (*Mustela vison*), in contrast, approximately 80% of the GnRH-1-ir cell bodies complete their migration in the mediobasal hypothalamus (Toumi, Martinet, & Peytevin, 1992), which is also the location for most of these cell bodies in humans, monkeys, bats and ferrets (King, Anthony, Fitzgerald, & Stopa, 1985; King & Anthony, 1984).

The present findings indicate that the distribution of GnRH-1 neurons in the eastern rock elephant-shrew is similar to that in opossums, bovids and most rodents. The total number of GnRH-1-ir cell bodies detected in eastern rock elephant-shrews (~400) is at the high end of the range found in Siberian hamsters, Syrian hamsters, Cape mole-rats and naked mole-rats (~200-400; Yellon et al., 1990; Urbanski et al., 1991; Oosthuizen et al., 2008; Zhou et al., 2013), but low in comparison with that in mice, Damaraland mole-rats, Natal mole-rats and common mole-rats (~600-700; Molteno et al., 2004; Du Toit et al., 2006; Herbison et al., 2008; Oosthuizen et al., 2008) and markedly smaller than that in rats, Cape dune mole-rats, Highveld mole-rats, sheep, springboks and rhesus monkeys (~1000-2000; Silverman et al., 1982; Lehman et al., 1986; Wray and Hoffmann, 1986; Robinson et al., 1997; Du Toit et al., 2006; Hart et al., 2008).

In comparison with the other species that have been investigated, eastern rock elephant-shrews possess notably sparse GnRH-1-ir processes in the region of the diagonal band, in the medial septum and in the medial preoptic area. Unlike springboks and naked mole-rats, these animals show no such processes in the median preoptic nucleus (Robinson et al., 1997; Zhou et al., 2013). They do, however, possess GnRH-1-ir processes in abundance in the external zone of the median eminence and also, but at a lower density, in the OVLT, a structure that extends ventral to the third ventricle in this species (Fig. 2D' inset), unlike its more restricted location at the rostral end of the ventricle's preoptic recess in rats, mice and mole-rats (Paxinos & Franklin, 2001; Paxinos & Watson, 2007; Zhou et al., 2013). In sheep, rhesus monkeys and humans,

GnRH-1-ir processes are also at a higher density in the median eminence than in the OVLT (Caldani, Batailler, Thiéry, & Dubois, 1988; King & Anthony, 1984). In contrast, in rats, springboks and Cape, Damaraland, Highveld, Natal, common and naked mole-rats, their density is similarly high at the two sites (Du Toit et al., 2006; Hart et al., 2008; King & Anthony, 1984; Molteno et al., 2004; Oosthuizen et al., 2008; Robinson et al., 1997; Zhou et al., 2013); but in ferrets and bats, the GnRH-1 immunoreactivity in the OVLT is exceptionally sparse (King & Anthony, 1984). As reported for rats (Hoffman & Gibbs, 1982), GnRH-1-ir processes can be seen passing under the optic chiasm in eastern rock elephant-shrews; such processes are also present within the vestigial optic chiasm in Damaraland, common, Highveld, Cape and naked mole-rats (Du Toit et al., 2006; Molteno et al., 2004; Oosthuizen et al., 2008; Zhou et al., 2013). At the rostrocaudal levels of the preoptic area and suprachiasmatic nucleus, the base of the third ventricle is bifurcated in eastern rock elephant-shrews; this unusual feature has also been seen in naked mole-rats (Zhou et al., 2013). In summary, although the distribution of GnRH-1-ir processes in eastern rock elephant-shrews is similar to that found in other mammalian species, the density is relatively low, other than in the median eminence.

The southern African eastern rock elephant-shrew shares certain reproductive characteristics with the South American plains viscacha. They both breed seasonally (Branch, 1993; Jackson, Branch, & Villarreal, 1996; Medger et al., 2012) and show a discrepancy between their high rate of ovulation (polyovulation) and the small litter size (Tripp, 1971; Weir & Rowlands, 1973). The full distribution and regional density of GnRH-1-ir processes have not yet been described for the plains viscacha. However, noteworthy differences exist between these species in the distribution of the GnRH-1-ir cell bodies: in the plains viscacha they are present not only in the preoptic area but also in the arcuate nucleus and septohypothalamic nucleus (Dorfman, Fraunhofer, Inserra, Loidl, & Vitullo, 2011). The latter location has not been reported for other species apart from Syrian hamsters (de la Iglesia, Blaustein, & Bittman, 1995).

4.3. Distribution of kisspeptin-ir cell bodies and processes

The arcuate nucleus is the only location in which kisspeptin-ir cell bodies were seen in the present study; the incidence was very low. This is the site at which kisspeptin cell bodies have been detected in all mammalian species investigated thus far. Studies on

other non-rodent species, using immunohistochemistry or *in situ* hybridization histochemistry (ISHH), have found most kisspeptin cell bodies in the caudal part of this nucleus, considerably fewer being identified in the rostral hypothalamus (Lehman, Merkley, Coolen, & Goodman, 2010). Thus, in colchicine-treated ewes, kisspeptin-ir cell bodies were found predominantly in the arcuate nucleus, with smaller populations in the dorsomedial hypothalamic nucleus and medial preoptic area (Franceschini et al., 2006). In orchidectomized rhesus monkeys, the arcuate/infundibular nucleus is the only site at which they were detected (Ramaswamy, Guerriero, Gibbs, & Plant, 2008); this is the location for the majority of the neurons immunoreactive for kisspeptin in men and women (Hrabovszky et al., 2010) and those expressing kisspeptin mRNA in premenopausal and postmenopausal women and in intact and ovariectomized cynomolgus monkeys (Rometo, Krajewski, Voytko, & Rance, 2007). In gonad-intact pony mares, kisspeptin-ir cell bodies were found primarily in the arcuate nucleus, with a few scattered cell bodies in the dorsomedial hypothalamic nucleus (Decourt, Tillet, Caraty, Franceschini, & Briant, 2008). In orchidectomized Shiba goats and in male and female red deer (*Cervus elaphus*) during the breeding and non-breeding seasons, the arcuate nucleus is the only site at which kisspeptin-ir cell bodies were detected (Ohkura et al., 2009; Barrell et al., 2016). Thus, there are precedents in non-rodent species for the absence of immunohistochemically detectable kisspeptin cell bodies in the rostral hypothalamus as reported here.

Various rodent species possess a notable kisspeptin cell body population not only in the arcuate nucleus but also, more rostrally, in the RP3V (AVPV plus adjacent MPO); projections from the latter neurons to rostrally located GnRH-1 cell bodies play a key role in the generation of the preovulatory surge of luteinizing hormone in rats and mice (Campbell & Herbison, 2014; Clarkson et al., 2008). These RP3V/AVPV kisspeptin cell bodies can be readily detected by immunohistochemistry in mice (~40-90 per section in females; Clarkson and Herbison, 2006), in jerboas (~35 per section in females; Janati et al., 2013), in Siberian hamsters (a total of ~50 from 1 in 4 sections in females; Mason et al., 2007) and, at the highest incidence reported thus far, in naked mole-rats (a total of ~650 in breeder “queens”; Zhou et al., 2013). In rats, the RP3V kisspeptin cell bodies are revealed by ISHH; but their detection by immunohistochemistry requires intracerebroventricular treatment with colchicine (Adachi et al., 2007; Bentsen et al., 2010; Desroziers et al., 2010; Kinoshita et al., 2005; Overgaard et al., 2013). These

findings indicate reduced retention of the peptide in RP3V cell bodies in rats compared with mice; this is believed to reflect a species difference in synthesis, transport and/or release (Desroziers et al., 2010). Whether the low incidence of kisspeptin-ir cell bodies observed in the arcuate nucleus of eastern rock elephant-shrews reflects a similarly reduced storage of the peptide near sites of synthesis remains to be determined.

The present study on a non-rodent species found no evidence for kisspeptin-ir cell bodies in the RP3V during the breeding or non-breeding season in either sex. As previously discussed (Lehman et al., 2010), the question of whether certain non-rodent mammals lack a rostral hypothalamic kisspeptin cell population remains unresolved. The possibility that the detection of kisspeptin cell bodies in that region depends on the endocrine milieu has not been systematically examined in those species. In contrast, gonadal steroids have been shown to increase the number of detectable kisspeptin-ir and kisspeptin mRNA-expressing cell bodies in the RP3V in rats, mice and Siberian hamsters (Adachi et al., 2007; Clarkson et al., 2012; Greives et al., 2008; Smith, Dungan, et al., 2005; Smith, Cunningham, Rissman, Clifton, & Steiner, 2005; Vida et al., 2010). Nevertheless, no such cell bodies were seen in elephant-shrews during the breeding season either in males, when plasma testosterone is raised (Medger et al., 2012), or in females; a possible explanation for the latter absence may lie in the uniformly pregnant state of those animals. Further studies involving colchicine treatment or ISHH are needed to establish whether the failure to detect kisspeptin-ir cell bodies in the rostral hypothalamus in the present study was due to a low rate of synthesis and/or a high rate of transport for release or whether kisspeptin-synthesizing cell bodies are indeed absent from that region. Such studies will be necessary to begin to elucidate the sources of the kisspeptin-ir processes identified in this study. Given the discovery of a major projection from RP3V kisspeptin neurons to the arcuate nucleus in mice (Yeo & Herbison, 2011), it should not be assumed that kisspeptin-ir processes in the arcuate nucleus derive exclusively from local cell bodies.

In eastern rock elephant-shrews, kisspeptin-ir processes are found at their greatest densities in the arcuate nucleus and in the internal and external zones of the median eminence. Elsewhere their density is high in the ventromedial subdivision of the bed nucleus of the stria terminalis and in the septohypothalamic nucleus, but only moderate in the RP3V, in the paraventricular nucleus and dorsal to the optic tract lateral to the retrochiasmatic area. They are at a lower density in the anteromedial subdivision of the

bed nucleus of the stria terminalis and even more diffuse in the anterior hypothalamic area and perifornical region and dorsal to the optic chiasm. The suprachiasmatic nucleus lacks kisspeptin-ir processes, as in mice, guinea pigs and naked mole-rats (Bosch et al., 2012; Clarkson, d'Anglemont de Tassigny, Colledge, Caraty, & Herbison, 2009; Zhou et al., 2013) but not rats (Desroziers et al., 2010). Although present in the medial amygdala in mice (Clarkson et al., 2009), kisspeptin-ir processes were not detected at this site in eastern rock elephant-shrews other than at a minimal incidence in a single male in the breeding season.

In summary, kisspeptin-ir processes in eastern rock elephant-shrews are present at a high or moderate density at a small number of sites. Their overall distribution lacks the notable rostrocaudal contiguity seen in mice (Clarkson et al., 2009), in rats (Desroziers et al., 2010), in guinea pigs (Bosch et al., 2012) and, at a particularly high density, in naked mole-rats (Zhou et al., 2013). In those species, there is a moderate or high density of kisspeptin-ir processes at sites extending caudally from the ventrolateral and/or medial septal nuclei to the arcuate nucleus and median eminence. The relatively low density of kisspeptin-ir processes at many of those sites in eastern rock elephant-shrews, including the RP3V, draws attention to the isolated high density in the ventromedial subdivision of the bed nucleus of the stria terminalis and the septohypothalamic nucleus, regions which lie within an intense immunoreactive continuum in rats, mice and guinea pigs (Clarkson et al., 2009; Desroziers et al., 2010; Bosch et al., 2012). Although kisspeptin's functions at those particular sites are unknown, it should be noted that the ventromedial subdivision of the bed nucleus of the stria terminalis projects to the RP3V and to the preoptic location of GnRH-1-ir cell bodies in rats (Hahn & Coen, 2006) and that electrochemical stimulation of that subdivision induces luteinizing hormone release in estrogen-primed ovariectomized rats (Beltramino & Taleisnik, 1980). It should also be noted that the septohypothalamic nucleus contains a high density of androgen receptor-expressing neurons in rats (Bakker, Pool, Sonnemans, Van Leeuwen, & Slob, 1997) and is implicated in their stress response to a predator's odor or intense auditory stimulation (Campeau & Watson, 1997; Day, Masini, & Campeau, 2004). Comparison with naked mole-rats prompts further questions about the role of kisspeptin in the ventromedial subdivision of the bed nucleus of the stria terminalis and in the septohypothalamic nucleus: thus,

although kisspeptin-ir processes in naked mole-rats are exceptionally dense elsewhere, they are present at no more than a low density at those two sites (Zhou et al., 2013). In the median eminence, eastern rock elephant-shrews display a dense plexus of kisspeptin-ir processes in both the internal and external zones, as is the case in naked mole-rats and guinea pigs (Bosch et al., 2012; Zhou et al., 2013), two species that share remote ancestry (Kalamatianos et al., 2005). In rats, sheep, goats and rhesus monkeys, kisspeptin-ir processes are also present in both zones, but more abundant in the internal zone (Desroziers et al., 2010; Franceschini et al., 2006; Ohkura et al., 2009; Ramaswamy et al., 2008). In contrast, kisspeptin-ir processes are found only in the internal zone in mice (Clarkson et al., 2009). These differences may reflect species differences in the sites at which kisspeptin neurons exercise their GnRH-1-releasing actions (as discussed below).

4.4. Associations between the distributions of GnRH-1 and kisspeptin immunoreactivity

The distributions of kisspeptin-ir processes and GnRH-1-ir cell bodies show negligible overlap in eastern rock elephant-shrews. In contrast, their distributions in mice display a high level of concurrence within the rostral preoptic area, where synaptic relations are found between kisspeptin-ir terminals and GnRH-1-ir dendrites (Kalló et al., 2011). Such direct synaptic contacts have also been identified in the preoptic area, anterior hypothalamus and mediobasal hypothalamus in sheep (Lehman, Coolen, Goodman et al., 2010; Smith et al., 2008). Among the various species known to have overlapping distributions of kisspeptin-ir processes and GnRH-1-ir cell bodies, naked mole-rats show a particularly marked concurrence (Zhou et al., 2013). The dearth of such concurrence in eastern rock elephant-shrews seems to preclude regulation of GnRH-1 release at perikaryal or proximal dendritic sites by kisspeptin. In this species, the distribution of immunoreactivity for these peptides is conspicuously coextensive only in the external zone of the median eminence. There is also an overlapping distribution, at a considerably lower density, dorsal to the optic tract lateral to the retrochiasmatic area; however, this appears to involve fibers of passage following parallel routes. *In vitro* studies have shown that kisspeptin can induce GnRH-1 release from ovine median eminence explants and from rat mediobasal hypothalamic explants that lack GnRH-1 cell bodies but contain GnRH-1 terminals in the median eminence (d'Anglemont de

Tassigny, Fagg, Carlton, & Colledge, 2008; Smith et al., 2011). Conditional viral tract-tracing studies in mice have identified kisspeptin processes that originate in the arcuate nucleus and form close appositions with GnRH-1 processes in the median eminence (Yip, Boehm, Herbison, & Campbell, 2015). Optogenetic stimulation of these kisspeptin projections induces luteinizing hormone release (Han, McLennan, Czielesky, & Herbison, 2015). The present study has detected an unusually high density of kisspeptin processes in the external zone of the median eminence; this has been previously observed only in naked mole-rats and guinea pigs (Bosch et al., 2012; Zhou et al., 2013). The abundant overlapping distribution of kisspeptin-ir and GnRH-1-ir processes in the external zone of the median eminence identifies a possible site for the regulation of GnRH-1 release by kisspeptin in eastern rock elephant-shrews.

4.5. Sex differences in GnRH-1 and kisspeptin neuronal systems

During the non-breeding season, the rostral population of GnRH-1-ir cell bodies in eastern rock elephant-shrews was found to be more numerous in males than females; but no other sex difference was detected in the distribution or density of GnRH-1-ir cell bodies or processes. For kisspeptin, the relatively low density of immunoreactivity in the RP3V did not differ between the sexes. In contrast, the relatively high density of kisspeptin-ir processes in the RP3V in mice and rats is greater in females (Overgaard et al., 2013) and the number of kisspeptin-ir or kisspeptin mRNA-expressing cell bodies at that site has also been found to be greater in female mice, rats and jerboas (Adachi et al., 2007; Clarkson & Herbison, 2006; Janati et al., 2013; Kauffman et al., 2007; Overgaard et al., 2013). In female sheep and humans, kisspeptin-ir cells are more numerous in the preoptic/periventricular region and in the arcuate/infundibular nucleus (Cheng, Coolen, Padmanabhan, Goodman, & Lehman, 2010; Hrabovszky et al., 2010). In the present study, it is the arcuate nucleus that shows a sex difference for kisspeptin: a greater density of kisspeptin-ir processes in the females during the breeding season. This site has been found to contain more kisspeptin-ir cell bodies in females than males during the breeding season in sheep (Cheng et al., 2010) and during the breeding and non-breeding seasons in red deer (Barrell et al., 2016). Furthermore, in non-seasonally breeding mice, rats and humans, there are more kisspeptin-ir cell bodies and a greater density of kisspeptin-ir processes in the arcuate/infundibular nucleus in females (Hrabovszky et al., 2010; Overgaard et al., 2013; Overgaard, Ruiz-Pino, Castellano,

Tena-Sempere, & Mikkelsen, 2014). Interpretation of the sex difference in arcuate nucleus kisspeptin in the breeding season in the present study is confounded by the pregnant state of the females captured at that time. Thus, the slightly greater density of kisspeptin-ir processes in the females may have been due to raised circulating progesterone (Medger et al., 2012); but this seems unlikely given that the limited evidence about kisspeptin expression in response to progesterone, with or without estrogen, indicates inhibition within the arcuate nucleus (Alçin et al., 2013; Smith et al., 2007). The sex difference identified here is small; in contrast, the notable finding for the arcuate nucleus in the present study is the greatly elevated density of kisspeptin-ir processes during the breeding season in both sexes (as discussed below).

4.6. Seasonal effects on GnRH-1 neurons

GnRH-1 immunoreactivity has been shown to increase in response to a photoperiod that simulates the non-breeding season in various mammals, such as white-footed mice (*Peromyscus leucopus*; Glass, 1986), Syrian hamsters (Ronchi, Aoki, Krey, & Pfaff, 1992), deer mice (*Peromyscus maniculatus*; Korytko et al., 1995), prairie voles (*Microtus ochrogaster*; Kriegsfeld and Nelson, 1999), Siberian hamsters (Bernard, Abuav-Nussbaum, Horton, & Turek, 1999) and jerboas (El Qandil et al., 2005). In keeping with those findings, the present study has identified an increase in the rostral population of GnRH-1-ir cell bodies and in the density of GnRH-1-ir processes in the median eminence in the non-breeding season in males. Such responses were first observed in female white-footed mice by Glass (1986): gonadal regression induced by a short photoperiod was associated with an increase in the number of GnRH-1-ir cell bodies in the preoptic area and anterior hypothalamus and in the density of GnRH-1-ir processes in the median eminence. In the present study, those increases were found in the non-breeding season in males, but not in females. We speculate that the absence of such seasonal differences in females is due to the absence of a significant depletion of GnRH-1 from the cell bodies and from the median eminence during the breeding season and that this is a consequence of inhibited GnRH-1 release during pregnancy, a state that applied to all the females captured during the breeding season. GnRH-1 immunoreactivity has also been quantified in various species in relation to the effects of social status, rather than season, on reproduction. Thus, in subordinate, reproductively suppressed male and female Natal mole-rats and female Damaraland and Highveld

mole-rats, the density of GnRH-1-ir processes in the median eminence is greater than in their dominant, reproductive counterparts (Du Toit et al., 2006; Molteno et al., 2004; Oosthuizen et al., 2008).

These instances of increased GnRH-1 immunoreactivity, accompanying suppressed reproduction in response to either seasonal or social cues, are believed to reflect increased GnRH-1 accumulation due to reduced release, rather than increased synthesis. Studies on GnRH-1 expression support this hypothesis: the expression of GnRH-1 mRNA remains unchanged in photoperiodically regressed male prairie voles (Kriegsfeld, Trasy, & Nelson, 2000) despite increases in the number of GnRH-1-ir cell bodies in the preoptic area and anterior hypothalamus and in the density of GnRH-1-ir processes in the median eminence (Kriegsfeld & Nelson, 1999). Thus, the present findings and the weight of evidence from studies on other species suggest that the non-breeding season in male eastern rock elephant-shrews is characterized by inhibited GnRH-1 transport from cell bodies in the preoptic area and anterior hypothalamus and inhibited GnRH-1 release from the median eminence.

In contrast to the changes in the rostral population of GnRH-1-ir cell bodies observed here, there were no seasonal differences in the small number of detectable GnRH-1-ir cell bodies in the mediobasal hypothalamus in either sex. In white-footed mice and prairie voles, it is also the case that changes in GnRH-1-ir cell body number in response to photoperiod are seen only in the preoptic area and anterior hypothalamus (Glass, 1986; Kriegsfeld & Nelson, 1999; Kriegsfeld, Ranalli, et al., 2000).

4.7. Seasonal effects on kisspeptin neurons

Research on several species indicates that reproductive development, whether the single irreversible process of puberty or the annual process seen in seasonal breeders (Ebling, 2010), is associated with activation of kisspeptin systems in both sexes (Barrell et al., 2016; Bentsen et al., 2010; Boufermes et al., 2014; Clarkson & Herbison, 2006; Greives et al., 2007; Han et al., 2005; Jafarzadeh Shirazi et al., 2014; Janati et al., 2013; Mason et al., 2007; Revel et al., 2007; Simonneaux et al., 2009; Smith et al., 2007; Takase et al., 2009; Talbi et al., 2016; Wagner et al., 2008). Under natural conditions, eastern rock elephant-shrews show seasonal development of the gonads and, as previously reported (Medger et al., 2012), an associated rise in circulating testosterone and progesterone in, respectively, males and females. Reproduction in this

species does not appear to be a photoregulated phenomenon: neither the testes nor seminiferous tubules differ in size between short- and long-day housed eastern rock elephant-shrews (Medger et al., 2016). Nevertheless, the large increases in the density of kisspeptin-ir processes in the RP3V and arcuate nucleus in the breeding season and the positive correlations between the density of those processes and gonadal mass seem in keeping with changes previously reported for reproductively photoresponsive species, namely increases in: kisspeptin mRNA expressing cell bodies in the AVPV and arcuate nucleus in male and female Syrian hamsters (Ansel et al., 2010; Revel et al., 2007), kisspeptin mRNA in tissue containing the AVPV and arcuate nucleus in male Libyan jirds (seasonally reproducing desert rodents; Boufermes et al., 2014), kisspeptin-ir process density, kisspeptin-ir cell bodies and kisspeptin mRNA expression in the arcuate nucleus in wild-captured male jerboas (results for the AVPV were not reported; Janati et al., 2013; Talbi et al., 2016), kisspeptin-ir cell bodies in the AVPV (but not in the arcuate nucleus) in male and female Siberian hamsters (Greives et al., 2007; Mason et al., 2007) and kisspeptin-ir cell bodies in the arcuate nucleus in ewes, female Abedeh goats (an Iranian seasonally breeding ecotype) and male and female red deer (Barrell et al., 2016; Jafarzadeh Shirazi et al., 2014; Smith et al., 2008). This peptide has also been studied in relation to the effects of social status, rather than season, on reproduction; in dominant, reproductively active female naked mole-rats, the RP3V contains a greater number of kisspeptin-ir cell bodies than in subordinate, reproductively suppressed animals of either sex (Zhou et al., 2013). Thus, studies on reproductive regulation in response to either seasonal or social cues have found an increase in immunoreactivity or mRNA for kisspeptin in the AVPV and/or arcuate nucleus following reproductive activation.

If photoperiod is not a seasonal signal for eastern rock elephant-shrews, other environmental factors, such as rainfall, ambient temperature and/or food quantity and quality may be salient. Medger et al. (2012) found that male reproductive development was positively related to rainfall and negatively related to ambient temperature. Although the link between climate and female reproduction was weaker (Medger et al., 2012), the current evidence suggests that the substantial seasonal differences in rainfall and their effect on food availability and quality may play a significant role in regulating seasonal breeding in this species. The effects of food availability on kisspeptin expression have been examined in other species. Thus, a fasting regimen that

suppresses luteinizing hormone pulses in female rats in an estrogen-dependent manner also causes an estrogen-dependent suppression of kisspeptin expression in the AVPV, but not in the arcuate nucleus (Kalamatianos, Grimshaw, Poorun, Hahn, & Coen, 2008). In contrast, in male Siberian hamsters food restriction to 90% of the *ad lib* diet for 6 weeks followed by 80% for 5 weeks reduces kisspeptin expression in the arcuate nucleus, but not in the AVPV (Paul, Pyter, Freeman, Galang, & Prendergast, 2009). The relevance of food availability to seasonal reproduction in eastern rock elephant-shrews remains to be elucidated.

5. CONCLUSIONS

Although the distribution of GnRH-1 and kisspeptin immunoreactivity in the polyovulating eastern rock elephant-shrew is qualitatively similar to that found in other mammals, the immunoreactive processes are generally less dense and less widespread. The minimal overlap in the distributions of kisspeptin-ir processes and GnRH-1-ir cell bodies prompts questions about the sites at which kisspeptin may be regulating GnRH-1 release in this species. The high abundance of processes immunoreactive for both peptides within the external zone of the median eminence, a feature previously seen only in naked mole-rats and guinea pigs (Bosch et al., 2012; Zhou et al., 2013), identifies this as a possible site for the regulation of GnRH-1 release by kisspeptin. Quantification of the immunoreactivity for GnRH-1 and kisspeptin has uncovered differences between the breeding and non-breeding seasons in these wild-captured, reproductively non-photoresponsive animals. In the non-breeding season, there is an increase in the number of GnRH-1-ir cell bodies in the diagonal band, medial septum and medial preoptic area and in the density of GnRH-1-ir processes in the median eminence in males. For kisspeptin, the breeding season is associated with a large increase in the density of the immunoreactive processes in the RP3V and arcuate nucleus in both sexes. As reviewed here, these findings show similarities to those obtained in photoregulated seasonally breeding species. Studies in which the photoperiodically increased immunoreactivity for GnRH-1 or kisspeptin has been compared with the expression of the corresponding mRNA have found no change for GnRH-1 mRNA, but a significant increase for kisspeptin mRNA. Thus, whether or not the seasonal breeding is photoregulated, comparative studies provide grounds for hypothesizing that the increase in GnRH-1 immunoreactivity during the non-breeding

season reflects an accumulation of the peptide associated with reduced GnRH-1 release and, in contrast, that the increase in kisspeptin immunoreactivity during the breeding season reflects increased synthesis associated with increased kisspeptin release. These putative differences in the seasonal synthesis, transport and release of GnRH-1 and kisspeptin may reflect differences in the respective neuroendocrine and neurotransmitter actions of these neurons. Further studies involving ISHH will be needed to test and refine these hypotheses.

CONFLICT OF INTEREST STATEMENT

The authors declare no actual or potential conflict of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: KM, CWC. Acquisition of data: KM, MKO. Analysis and interpretation of data: KM, CWC. Writing the manuscript: KM, CWC. Finalizing the manuscript: NCB, CTC, MKO, JDM. Obtained funding: NCB, CWC.

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Figure legends

Fig. 1. Mean \pm SEM (a) testicular mass (mg), (b) seminiferous tubule diameter (μm) and (c) ovarian mass (mg) in the eastern rock elephant-shrew during the non-breeding and breeding seasons. *** $P < 0.001$; * $P < 0.05$

Fig. 2. GnRH-1 immunoreactivity in darkfield and brightfield photomicrographs of representative coronal sections (most rostral, a; most caudal, i) through the brain of a female eastern rock elephant-shrew during the breeding season. The white-framed areas in the darkfield photomicrographs (a-f) are shown in brightfield (a1-f1). The white-framed areas in the brightfield photomicrographs (a1, b1, c1, g) are shown at a higher magnification (a2, b2, c2, g1). Insets (c, c1, d1, f1) show a higher magnification image of the corresponding black-framed area (c, c1, d1) or a Nissl-stained image at a corresponding rostrocaudal level (f1), identifying the location of the suprachiasmatic and supraoptic nuclei. Arrows indicate selected GnRH-1-immunoreactive (-ir) cell bodies; arrowheads indicate isolated GnRH-1-ir processes. AC, anterior commissure; HDB, horizontal limb of the diagonal band; ME, median eminence; MnPO, median preoptic nucleus; MPA, medial preoptic area; MS, medial septum; OCh, optic chiasm; OT, optic tract; OVLT, organum vasculosum of the lamina terminalis; RCh, retrochiasmatic area; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; VDB, vertical limb of the diagonal band. Scale bars = 300 μm for a (applies to a-f); 100 μm for a1 (applies to a1-f1, g-i).

Fig. 3. Mean number (\pm SEM) of GnRH-1-ir cell bodies located either (a) rostral to or at the rostrocaudal level of the suprachiasmatic nucleus or (b) caudal to that nucleus in female and male eastern rock elephant-shrews during the non-breeding and breeding seasons. * $P < 0.05$

Fig. 4. Mean percentage density (\pm SEM) for GnRH-1-immunoreactivity (GnRH-1-ir) in the median eminence of female and male eastern rock elephant-shrews during the non-breeding and breeding seasons. * $P < 0.05$

Fig. 5. Kisspeptin immunoreactivity in darkfield and brightfield photomicrographs of representative coronal sections (most rostral, a; most caudal, j) through the brain of a

female eastern rock elephant-shrew during the breeding season. Oxytocin-neurophysin immunoreactivity is shown at a single rostrocaudal level in darkfield and brightfield photomicrographs (g, g1). The white-framed areas in the darkfield photomicrographs (a-g) are shown in brightfield (a1-g1). The white-framed areas in the brightfield photomicrographs (a1-g1, h) are shown at higher magnification (a2-g2, h1). Arrows indicate selected kisspeptin-ir cell bodies; arrowheads indicate isolated kisspeptin-ir processes. AC, anterior commissure; AHA, anterior hypothalamic area; Arc, arcuate nucleus; AVPV, anteroventral periventricular nucleus; F, fornix; ME, median eminence; MnPO, median preoptic nucleus; MPA, medial preoptic area; MPO, medial preoptic nucleus; MS, medial septum; OCh, optic chiasm; OT, optic tract; OVLT, organum vasculosum of the lamina terminalis; PVH, paraventricular hypothalamic nucleus; RCh, retrochiasmatic area; SCN, suprachiasmatic nucleus; SHy, septohypothalamic nucleus; STMA, anteromedial division of the bed nucleus of the stria terminalis; STMV, ventromedial division of the bed nucleus of the stria terminalis; VDB, vertical limb of the diagonal band. Scale bars = 300 μm for a (applies to a–f); 200 μm for a1 (applies to a1–f1); 500 μm for g; 200 μm for g1; 100 μm for h (applies to h–j).

Fig. 6. Mean relative density (expressed as a percentage of the density in the non-breeding females \pm SEM) for kisspeptin-immunoreactivity (kisspeptin-ir) in (a) the septohypothalamic nucleus (SHy), (d) the rostral periventricular area of the third ventricle (RP3V, anteroventral periventricular nucleus plus medial preoptic nucleus) and (g) the arcuate nucleus (Arc) in female and male eastern rock elephant-shrews during the non-breeding and breeding seasons. Kisspeptin immunoreactivity in photomicrographs of representative coronal sections illustrating (b, c) the SHy, (e, f) the RP3V and (h, i) the Arc in male eastern rock elephant-shrews during the non-breeding (b, e, h) and breeding (c, f, i) seasons. Kisspeptin-immunoreactivity was similar in the females and is not shown here. AC, anterior commissure; MS, medial septum; OCh, optic chiasm. Scale bars = 300 μm for b (applies to b, c, e, f) and h (applies to h, i). ** $P < 0.01$

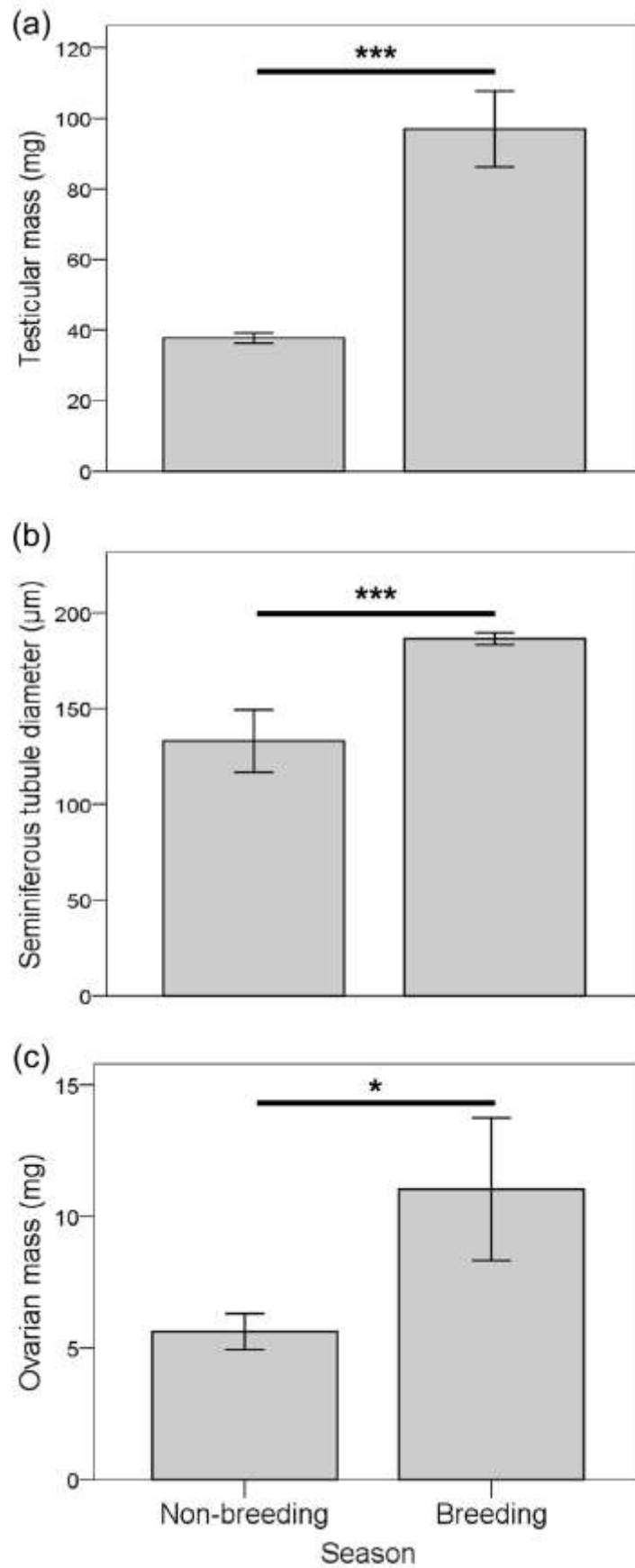


Figure 1

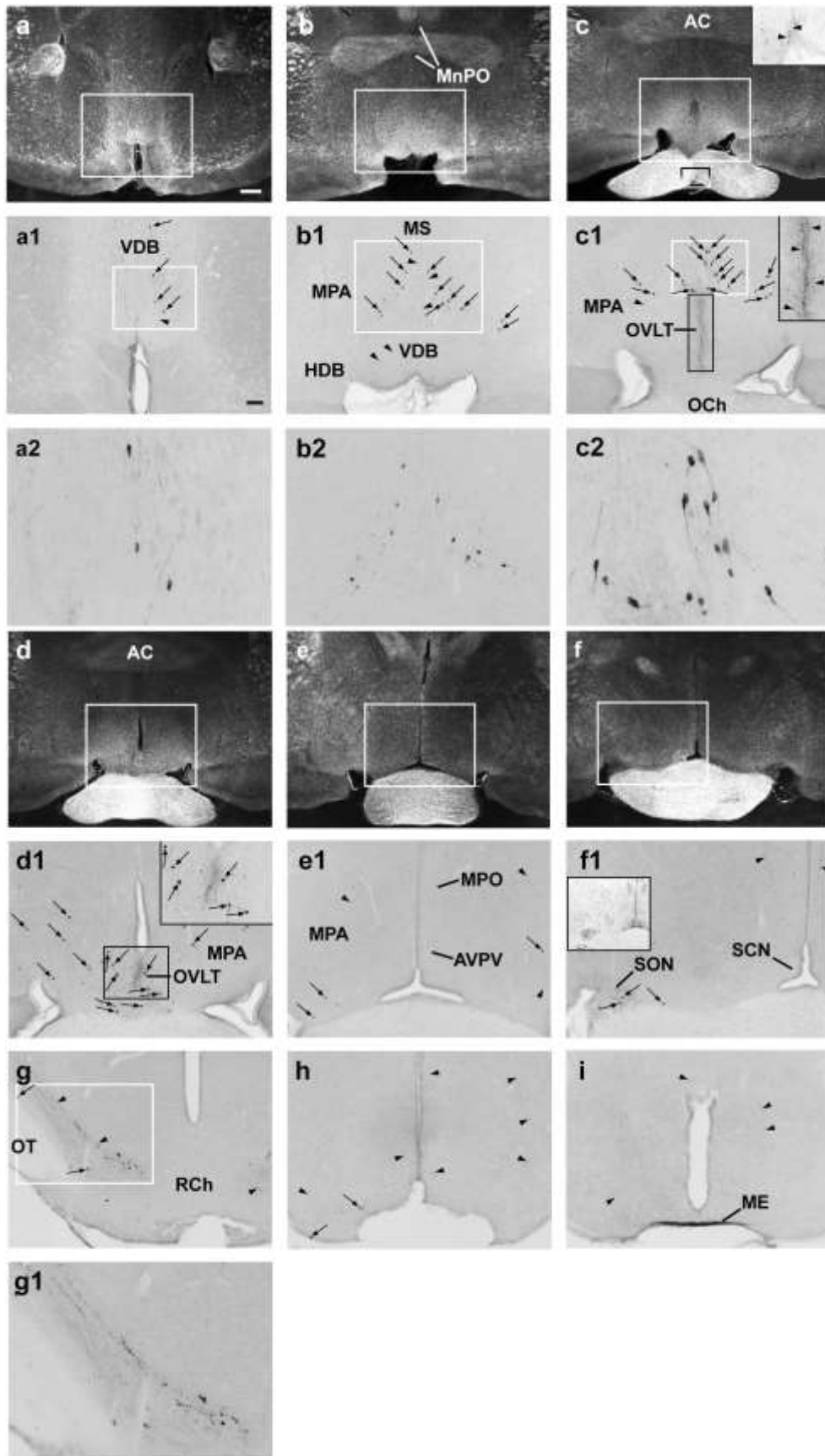


Figure 2

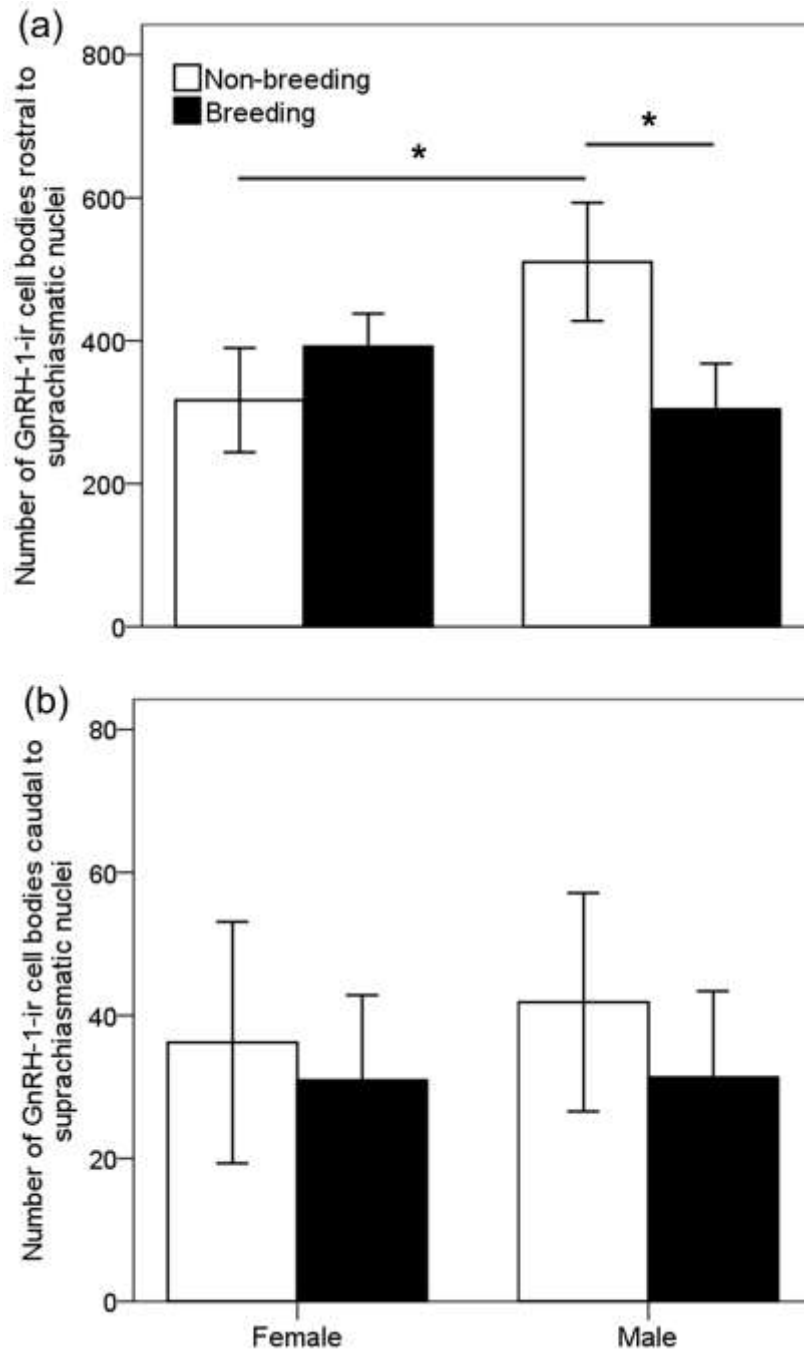


Figure 3

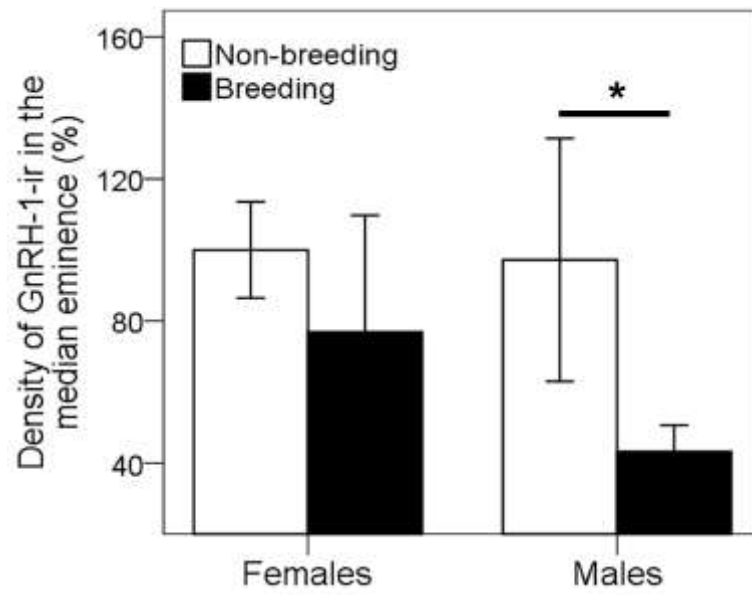


Figure 4

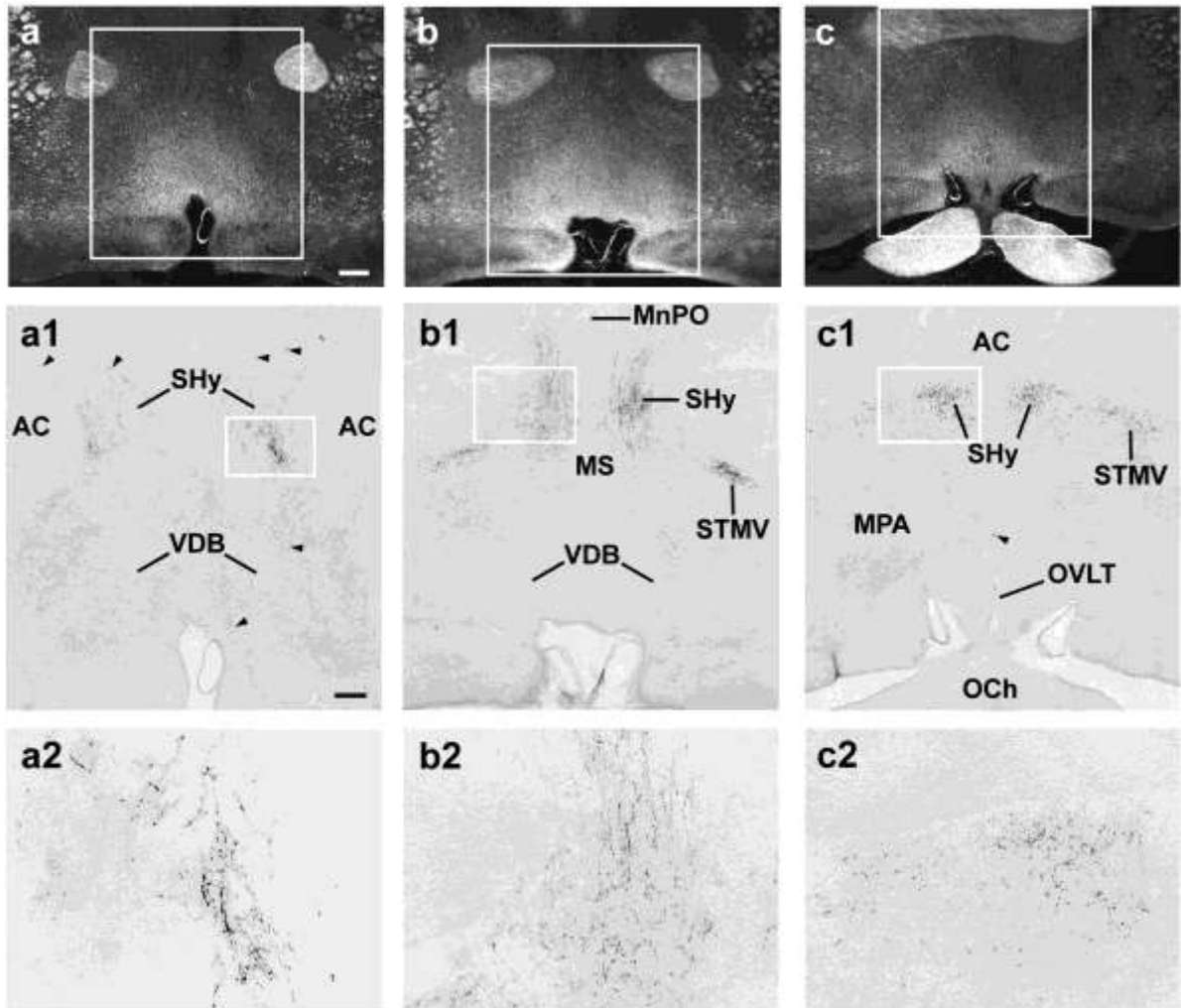


Figure 5_1

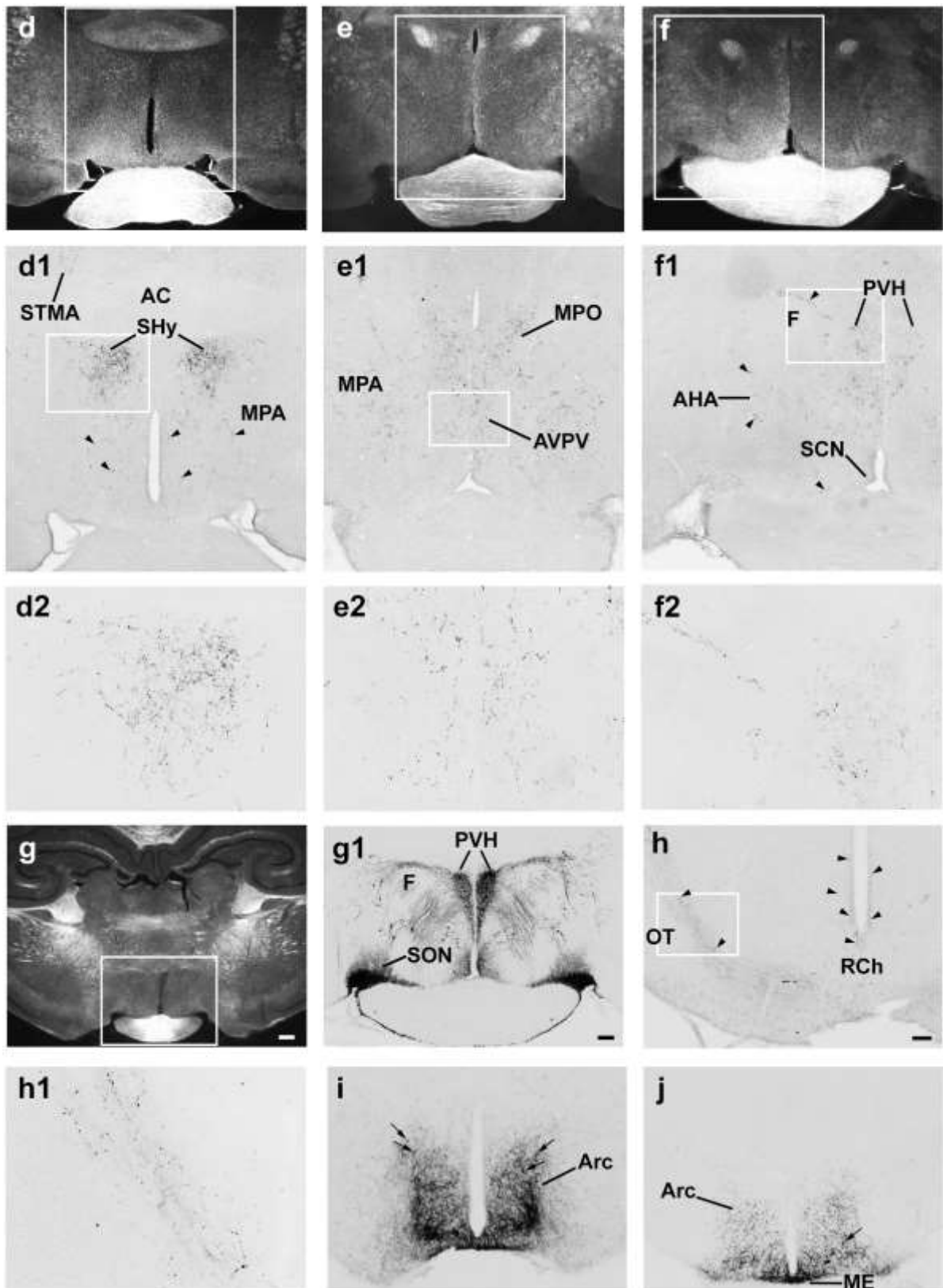


Figure 5_2

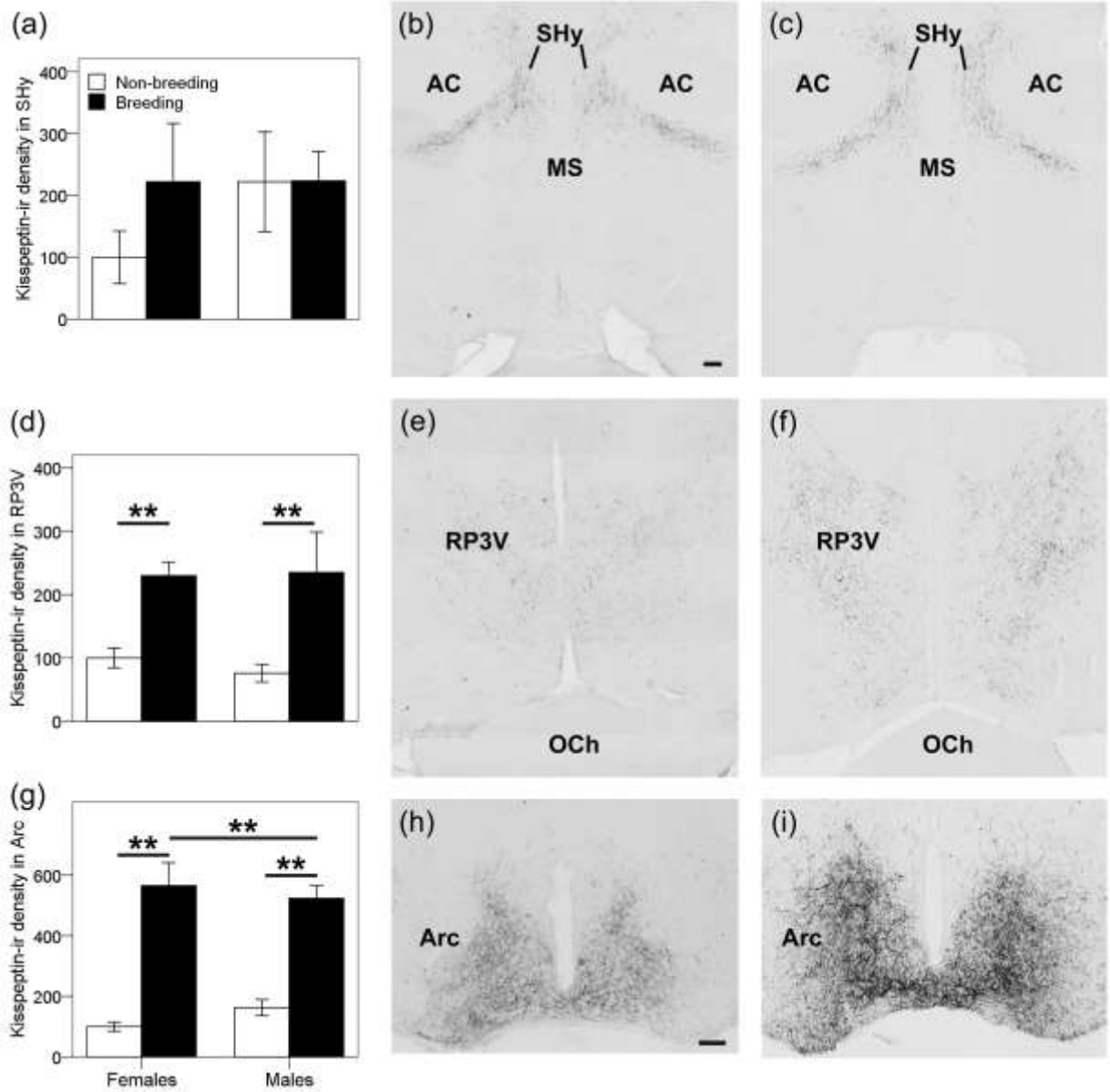


Figure 6