Gene expression and secretion of LH and FSH in relation to gene expression of GnRH receptors in the brushtail possum (*Trichosurus vulpecula*) demonstrates highly conserved mechanisms

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Abstract

In eutherian mammals, the gonadotrophins (LH and FSH) are synthesized and stored in gonadotroph cells under the regulation of multiple mechanisms including GnRH. Very little is known about the regulation of gonadotrophin secretion and storage in pituitary glands of marsupials. This study revealed, using quantitative PCR and heterologous RIA techniques, that *LHB* mRNA expression levels remained constant over the oestrous cycle, regardless of the presence of a preovulatory LH surge, which is characteristic of a hormone secreted under regulation. Our sampling regime was unable to detect pulses of LH during the follicular phase, although *GNRHR* mRNA levels had increased at this time. Pulses of LH were, however, detected in the luteal phase of cycling females, in anoestrus females and in males. There was a positive correlation between gene expression of *FSHB* and plasma levels of FSH at different stages of the oestrous cycle and no pulses of FSH were detected at any time; all characteristics of a hormone secreted via the constitutive pathway. Using *in situ* hybridisation and immunohistochemistry methods, we determined that mRNA expression of *LHB* and *FSHB*, and protein storage of gonadotrophins exhibited a similar pattern of localisation within the pituitary gland. Additionally, sexual dimorphism of gonadotroph populations was evident. In summary, these findings are similar to that reported in eutherians and considering that marsupial evolution diverged from eutherians over 100 million years ago suggests that the regulation of gonadotrophins is highly conserved indeed.

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Introduction

The gonadotrophins, LH and FSH, are dimeric protein hormones composed of a common α -gonadotrophin subunit (α GSU) and a unique β -subunit (LH β and FSH β respectively). Regulation of these hormones is facilitated through a complex interplay of multiple mechanisms including a direct action of hypothalamic GnRH, and both direct and indirect actions of gonadal-derived steroids and peptides (Farnworth *et al.* 1988, Carroll *et al.* 1989, Gharib *et al.* 1990, Wang *et al.* 1990a, 1990b, 1990c, Mann *et al.* 1992, Hamernik 1995, Besecke *et al.* 1996, Burger *et al.* 2001). These regulatory compounds act on gonadotroph cells to influence the differential synthesis, packaging, trafficking, storage and secretion of gonadotrophins, despite both often being present within the same specialized anterior pituitary cells (Childs *et al.* 1987, Lloyd & Childs 1988, Crawford & McNeilly 2002, Crawford *et al.* 2002). Additionally, a distinct pattern of localisation of gonadotroph subpopulations has been reported within the pituitary gland of rats

(Tixier-Vidal *et al.* 1975, Denef *et al.* 1978, Dada *et al.* 1983, 1984a,b, Childs *et al.* 1987), sheep (Taragnat *et al.* 1998, Tortonese *et al.* 1998, McNeilly *et al.* 2003), mice (Crawford *et al.* 2002), horses (Eagle & Tortonese 2000) and humans (Pelletier *et al.* 1976, Newman & Williams 1989), which may reflect concentration in areas with increased vascularisation. Whilst the pattern of secretion of gonadotrophins in brushtail possums in different physiological states has been defined (Crawford *et al.* 1999), the regulation and localisation of gonadotrophins in any marsupial species is not well understood.

The brushtail possum is a monovular, polyoestrous seasonal breeder with an oestrous cycle of ~26 days, consisting of a shorter follicular phase (~10 days) and a luteal phase similar in length to their gestation period (~18 days; Fletcher & Selwood 2000). There are some major differences in ovarian function in the possum compared with mono-ovulatory eutherian species. In particular, the steroidogenic capability (Whale *et al.* 2003), gonadotrophin dependency and the expression of LH receptors (Eckery *et al.* 2002) of granulosa cells occurs

in follicles at a much earlier stage of development in the possum. Expression of LH receptors was first observed in theca interna at the time of antrum formation in possums (Eckery *et al.* 2002) and many eutherian mammals (Bao & Garverick 1998, McNatty *et al.* 1999), and is thought to be indicative of steroidogenic capability. However, the expression of LHR in granulosa cells, an event signifying a dominant follicle capable of ovulation and limited to the later stages of antral follicular growth in eutherian mammals (Bao & Garverick 1998, McNatty *et al.* 1999), occurs at the beginning of antrum formation in possums (Eckery *et al.* 2002).

The development and maturation of ovarian follicles in eutherian species is dependent upon the successive actions of gonadotrophins. Upon stimulation of immature antral follicles by FSH, there is an upregulation of expression levels of both aromatase and LH receptor mRNA (Adashi & Resnick 1986, Richards 1994). Subsequently, LH acts directly or indirectly through the actions of growth factors (Park *et al.* 2004) on the FSH-stimulated follicle to facilitate steroid production, and induce luteinisation and ovulation (Adashi & Resnick 1986, Richards 1994). These events require a distinct pattern of gonadotrophin secretion.

Whilst the overall secretory pattern of the gonadotrophins has been established in the brushtail possum over the oestrous cycle (Crawford *et al.* 1999), the intrapituitary site(s) and levels of mRNA expression, and the storage pattern of gonadotrophins, as well as the parameters of pulsatile secretion are unknown. The aim of this study was to determine in the brushtail possum: the localisation and levels of mRNA expression of LH β -subunit (*LHB*), FSH β -subunit (*FSHB*) and GnRH receptor (*GNRHR*) within the pituitary gland; the frequency and amplitude of pulsatile gonadotrophin secretion in males and in females at different stages of the oestrous cycle; and the number and localisation of gonadotrophs within pituitary glands of females and males.

Results

Quantification of gene expression levels of LHB, FSHB and GNRHR

Confirmation of oestrous cycle stage

Confirmation of the correct stage of the oestrous cycle was validated by observation of ovarian structures either by laparoscopic examination or by gross examination at the time of tissue collection, along with the weight of the cul-de-sac and uteri. The cul-de-sac was pale and small at the time of pouch young removal (RPY) but continually increased ($P < 0.001$) in weight and apparent vascularisation throughout the follicular stage, peaking at the late follicular stage and declining to a nadir ($P < 0.001$) in the mid-luteal stage (Fig. 1A). The uteri were also pale and small at the time of RPY but continually increased ($P < 0.05$) in

Figure 1 Wet weight (mean $\pm$ S.E.M.) of (A) cul-de-sac and (B) both uteri (pooled weights) in adult female brushtail possums at different stages of the oestrous cycle. Different letters denote significant differences ($P < 0.05$) between stages of the oestrous cycle.

weight as the oestrous cycle progressed from the follicular to luteal stages, and peaked during the mid-luteal stage of the oestrous cycle (Fig. 1B). There were no significant differences between the weight of the left and right uteri at the time of RPY, during the early follicular stage, or between the ipsi- and contralateral uteri at all other stages of the oestrous cycle, therefore weights were pooled (Fig. 1B). A large (> 3.5 mm) presumptive preovulatory follicle, a rupture (ovulation) point on the dominant follicle and a large (> 5 mm) vascularized corpus luteum was observed in all animals during the late follicular, early luteal and mid-luteal stages respectively. Animals defined as in the early luteal stage were estimated to have undergone ovulation within 0–48 h before the time of tissue collection.

LHB, FSHB and GNRHR mRNA expression levels

The comparison of mean quantified mRNA expression levels of *LHB*, *FSHB* and *GNRHR* from pituitary glands of animals at different stages of the oestrous cycle are shown in Fig. 2. There were no differences ($P = 0.063$) in the mean expression levels of *LHB* mRNA at any stages of the oestrous cycle. In contrast, in comparison with the time of RPY, the mean expression levels of *FSHB* mRNA were lower ($P < 0.0005$) in the late follicular stage, and higher

Figure 2 Expression levels (mean $\pm$ S.E.M.) of *LHB* (top), *FSHB* (middle) and *GNRHR* (bottom) mRNA in adult female brushtail possums at different stages of the oestrous cycle. Different letters denote significant differences ($P < 0.05$) between stages of the oestrous cycle. RPY, removal of pouch young.

($P < 0.0005$) in the early luteal stage. Mean mRNA expression levels of *GNRHR* were elevated ($P < 0.05$) during the follicular stages, compared with the time of RPY and during the mid-luteal phase of the oestrous cycle.

Patterns of gonadotrophin secretion

Mean daily concentrations of plasma LH were elevated only at the time of the preovulatory LH surge (Fig. 3A–E) in those female possums that ovulated, and were continually

low in female possums that did not ovulate following RPY (Fig. 3F) and in male possums (Fig. 3G). In females that ovulated, mean daily FSH levels were high at the time of RPY but progressively declined to negligible levels during the late follicular stage (Fig. 3A–E). However, in most animals, mean FSH concentrations were elevated at the time of the preovulatory LH surge and variable during the luteal stages of the oestrous cycle (Fig. 3A–E). In those animals that did not ovulate following RPY (Fig. 3F) and in male possums (Fig. 3G), mean FSH concentrations were elevated throughout the sampling period.

Representative profiles of LH and FSH during the intensive sampling period (every 20 min for 6 h) are shown in Fig. 4. During the 6-hour intensive blood sampling interval, no pulses of LH release were detected in the early (Fig. 4A) and late (Fig. 4B) follicular stages or near the end of the preovulatory LH surge (Fig. 4C). One animal out of seven in the early luteal stage (Fig. 4D) and all animals (6/6) in the mid-luteal stage (Fig. 4E) exhibited pulses of LH secretion. A small proportion of both females that failed to ovulate after RPY (Fig. 4F) and males (Fig. 4G) exhibited LH pulses. Pulse amplitudes of LH in animals in the early and mid-luteal stage, in females that failed to ovulate and in males were 0.94 , 1.76 ± 0.30 , 1.88 ± 0.12 and 1.82 ± 0.67 ng/ml. No pulses of FSH release were observed at any time in either female or male possums.

Quantification of pituitary gland attributes and gonadotroph populations

Pituitary gland volume and total cell number, the numbers of LH- and FSH-positive cells, and the proportion of LH-positive cells were not different between females and males. There was a higher ($P < 0.005$) proportion of FSH-positive cells and a lower ratio of LH:FSH-positive cells in pituitary glands from male possums compared with that from females (Table 1).

Localisation of gonadotrophin gene expression in the pituitary gland

Representative micrographs of the localisation of *LHB* and *FSHB* mRNA in cross sections through the mid-sagittal region of a pituitary gland are shown in Fig. 5A and B respectively. Expression of both *LHB* and *FSHB* mRNAs were evident in cells throughout the cross section of the pituitary gland, but both appeared to be more concentrated in the lateral (outer) regions of the pars distalis and in particular, in those cells juxtaposed to the basement membrane and the pars intermedia (Fig. 5A and B).

Localisation of gonadotrophin protein storage in the pituitary gland

Representative micrographs of the localisation of LH and FSH protein in cross sections through the mid-sagittal

Figure 3 Profiles of plasma FSH (○) and LH (●) concentrations (mean $\pm$ S.E.M.) in groups of either female possums over the oestrous cycle relative to either the time of the preovulatory LH surge (day 0; A–E) or the day of pouch young removal (day 0; F), or male possums (G) over a defined time period. The shaded areas denote the day that a 6-hour period of frequent (20 min) blood samples were collected (see Fig. 5) to gather information on gonadotrophin secretory parameters in either female possums (A) in the early and (B) late follicular stages of the oestrous cycle, (C) immediately after the preovulatory surge, (D) in the early and (E) late luteal stages of the oestrous cycle, (F) that did not respond following removal of pouch young and in (G) male possums.

plane of a pituitary gland are shown in Fig. 6A and B respectively. For LH, immunostaining appeared punctate throughout the gland indicating that very few LH-positive cells were clustered together but were rather individual, or

in small groups of two to three (Fig. 6A). The population of both LH- and FSH-positive cells appeared to be at a higher density in the lateral regions of the pars distalis, particularly in those cells positioned adjacent to the basement

Figure 4 Representative profiles of plasma FSH (○) and LH (●) concentrations in female possums (A) in the early and (B) late follicular stages of the oestrous cycle, (C) at the end of the preovulatory surge, (D) in the early and (E) late luteal stages of the oestrous cycle, (F) that did not respond following removal of pouch young and (G) in male possums. Note the different scale in (B) and (G) for LH and FSH respectively. Asterisks denote a pulse of LH.

Table 1 Comparisons between volume and total cell number, and number, proportion and ratio of LH- and FSH-positive cells of pituitary glands of adult female and male brushtail possums.

Attributes	Female	Male
Pituitary volume (mm ³)	2.97 ± 0.64 ^a	3.41 ± 0.35 ^a
Total cell number (×10 ⁶)	3.17 ± 1.00 ^a	3.29 ± 0.21 ^a
LH-positive cell number (×10 ⁶)	0.24 ± 0.08 ^a	0.27 ± 0.04 ^a
FSH-positive cell number (×10 ⁶)	0.17 ± 0.06 ^a	0.31 ± 0.03 ^a
Proportion of LH-positive cells (%)	7.50 ± 0.86 ^a	8.26 ± 0.91 ^a
Proportion of FSH-positive cells (%)	5.50 ± 0.51 ^a	9.53 ± 0.23 ^b
LH:FSH-positive cell ratio	1.36 ± 0.03 ^a	0.86 ± 0.08 ^b

Different letters denote a significant difference ($P < 0.005$) between female and male possums.

membrane and pars intermedia (Fig. 6A and B). A proximal area immediately anterior to the pars nervosa was virtually devoid of immunopositive LH and FSH cells (Fig. 6A and B).

Discussion

This study has established for the first time, in a marsupial species, the relationship between levels of mRNA expression and protein secretion of gonadotrophins over the oestrous cycle. Additionally, changes

in *GNRHR* mRNA expression levels over the oestrous cycle reveal potential alterations in responsiveness of gonadotroph cells to GnRH in this species.

In eutherians, GnRH is released in pulses from the hypothalamus and acts directly on gonadotroph cells via the portal blood system to stimulate both the biosynthesis and secretion of LH and FSH. Alterations in levels of circulating gonadal steroids (e.g. oestradiol and progesterone; *Clarke et al. 1988, Gregg & Nett 1989, Sealfon et al. 1990, Brooks et al. 1993, Yasin et al. 1995*), growth factors (e.g. activin and inhibin; *Gregg et al. 1991, Braden & Conn 1992*) and GnRH itself (*Turzillo et al. 1995, 1998*) during the oestrous cycle modulates the sensitivity of gonadotroph cells to GnRH through the regulation of expression and hence numbers of GnRH receptors. Changes in GnRH pulse frequencies are capable of differentially regulating *LHB* and *FSHB* transcription and mRNA expression levels (*Dalkin et al. 1989, Burger et al. 2002*), as well as LH and FSH secretion throughout the oestrous cycle. Generally, a higher pulse frequency promotes upregulation of the α GSU (*CGA*) and *LHB* genes, and LH secretion, whilst lower frequencies increase *FSHB* mRNA expression and FSH secretion (*Wildt et al. 1981, Savoy-Moore & Swartz 1987, Kaiser et al. 1997, Burger et al. 2002*). In particular, during the late follicular phase, the GnRH surge from the hypothalamus combined

Figure 5 Bright-field (left; top and bottom inset) and dark-field (middle inset) views of a pituitary gland from an adult female possum showing expression of (A) *LHB* and (B) *FSHB* mRNA by immunohistochemistry and *in situ* hybridisation. Corresponding bright-field (top inset) and dark-field (middle inset) images at higher magnification show specific labelling of silver grains. Note a higher density of silver grains around lateral (outer) regions of the pars distalis whilst the proximal area immediately anterior to the pars nervosa was virtually devoid of silver grains. A negative control (bottom inset) of hybridisation of sense probe shows no labelling of silver grains.

Figure 6 Pituitary gland from an adult female possum labelled for (A) LH and (B) FSH. High magnification (top inset) reveals specific cytoplasmic labelling. Note a higher proportion of labelled cells around the lateral (outer) regions of the pars distalis whilst the proximal area immediately anterior to the pars nervosa was virtually devoid of labelled cells. No labelled cells were observed after the primary antiserum was pre-incubated with antigen (negative control; bottom inset) prior to incubation.

with the increased responsiveness of gonadotroph cells to GnRH provokes a cascade of intracellular events including potentiation of inositol 1,4,5-triphosphate, intracellular calcium mechanisms and protein kinase C (Mitchell *et al.* 1988, Johnson *et al.* 1992, Ison *et al.* 1993, Simpson *et al.* 1993, Haisenleder *et al.* 1997, Washington *et al.* 2004), as well as the rearrangement of microfilaments and LH-positive secretory granules relative to the plasmalemma (Pickering & Fink 1979, Lewis *et al.* 1985, Currie & McNeilly 1995, Crawford *et al.* 2000, 2001) to facilitate the preovulatory LH surge. In the subsequent luteal phase, rising progesterone levels cause a reduction in *GNRHR* mRNA expression levels (Wu *et al.* 1994, Nett *et al.* 2002) and numbers (Laws *et al.* 1990) and pulse frequency of GnRH and LH (Chabbert-Buffeta *et al.* 2000), as well as an increase in the LH pulse amplitude (Chabbert-Buffeta *et al.* 2000, McCartney *et al.* 2007). It would appear that similar regulatory mechanisms exist in the possum as *GNRHR* mRNA expression levels increased approximately twofold in the follicular phase after RPY, and declined in the mid-luteal phase.

In this study and as previously reported in female possums (Crawford *et al.* 1999, 2006), mean daily LH concentrations over the oestrous cycle are usually low with the exception of the preovulatory LH surge during the late follicular stage. The disparity in changes in steady-state levels of *LHB* mRNA and plasma LH concentrations

over the entire oestrous cycle in this study is characteristic of regulated secretion. This is due to the storage and subsequent release of LH in response to ligand (i.e. GnRH) stimulation without the necessity for *de novo* synthesis (McNeilly *et al.* 2003) and enables its pulsatile secretion. Interestingly, neither pulses nor rising circulating levels of LH were observed during the follicular phase despite a presumptive increase in gonadotroph responsiveness to GnRH at this time. In fact, pulses of LH were only detected in females in the luteal stages when *GNRHR* mRNA levels were depressed, and in females that remained anoestrous after RPY, and in males. In eutherians, pulses of LH are more frequent and lower in amplitude in the follicular, compared with the luteal stage of the oestrous cycle (Baird *et al.* 1976, Baird 1978, Wallace *et al.* 1988). The lack of frequent pulses of LH release in the follicular phase of possums is at odds with that observed in eutherians. It is possible that the 20-min sampling regime was not frequent enough to detect pulses of LH and/or that LH pulse amplitude during the follicular phase in the possum is lower than the detection limit of the RIA. Another possibility, considering that LH receptors are present in granulosa cells of follicles at a much earlier stage of development in the possum (Eckery *et al.* 2002), is that the regulatory mechanism(s) of LH is different in this species.

Conversely, *FSHB* mRNA expression levels reflected a similar pattern to plasma FSH secretion, which is characteristic of a protein that is predominantly secreted in a constitutive manner (McNeilly *et al.* 2003). This parallel, if somewhat delayed, pattern between gene expression and plasma levels of FSH in possums is similar to that observed in most eutherian species studied (Crawford & McNeilly 2002, Crawford *et al.* 2002), whereby levels decline during the late follicular stage of the oestrous cycle presumably due to the increasing negative feedback effects of oestradiol (Baird *et al.* 1981) and inhibin-A (Mann *et al.* 1992, Padmanabhan & McNeilly 2001) from the growing preovulatory follicle, which also subsequently suppresses the activin gene (Gregg *et al.* 1991, Nett *et al.* 2002). Elevation of gene expression levels and secretion of FSH observed in possums in the luteal stages of the oestrous cycle were probably due to the removal of these negative feedback effects (Wallace & McNeilly 1986, Wallace *et al.* 1988, Brooks *et al.* 1992), together with augmented progesterone levels (Mann & Barraclough 1973, Rao & Mahesh 1986, Attardi & Fitzgerald 1990, Crawford *et al.* 2006) and possibly a reduced GnRH pulse frequency (Molter-Gérard *et al.* 1999). The absence of pulses of FSH in this study is similar to that observed in other mammalian species and reinforces the dogma that FSH is mainly released via a constitutive pathway.

The pattern of gonadotroph localisation is sexually dimorphic in rats where, in males, gonadotrophs were more evenly distributed dorsoventrally within the pars distalis but exhibited a more pronounced anteroposterior polarity compared with that in females (Dada *et al.* 1984a,b). Whilst in this study we did not compare sex differences of the pattern of gonadotroph distribution, gonadotroph density as detected by both gene expression and protein storage of LH and FSH appeared to be higher in the lateral regions of the pars distalis, whilst a proximal area immediately anterior to the pars nervosa was virtually devoid of gonadotrophs. This may be directly proportional to the vascularisation pattern of the pars distalis as gonadotrophs are commonly located adjacent to blood vessels. It should be noted that in this study localisation of gonadotrophs was only investigated in mid-sagittal cross sections and not the entire pituitary gland.

In the rat, distinct populations of mono- and bi-hormonal gonadotrophs have been characterised by size (Denef *et al.* 1980) and reportedly exhibit differences in their storage and secretory responses to GnRH (Lloyd & Childs 1988), as well as shift in proportions during the oestrous cycle (Childs *et al.* 1987). This study did not address colocalisation of LH and FSH within the same gonadotrophs but it would seem sensible to assume that in the brushtail possum at least a proportion of gonadotroph cells contained both LH and FSH. Similar to those of the rat (Dada *et al.* 1984a,b), pituitary glands of lactating female possums showed a trend of containing more LH-positive and

fewer FSH-positive cells than in male possums, resulting in a markedly higher ratio of LH:FSH-positive cells in the pars distalis of female, compared with male, possums. This is consistent with the daily hormone profiles of gonadotrophins where male possums have an overall higher secretory rate of FSH, compared with the female. Additionally, there are some reports in rats that the proportions of LH and FSH-positive cells change over the oestrous cycle and an increase in LH-positive cells were observed at dioestrus (Childs *et al.* 1987).

In summary, in cycling female possums, *LHB* mRNA expression levels remained constant over the oestrous cycle, regardless of the presence of a preovulatory LH surge which is a characteristic of a hormone under regulated secretion. Despite an increase in mRNA expression levels of *GNRHR*, no pulses of LH secretion were detected during the follicular phase which is probably due to sampling or measurement deficiencies. Pulses of LH were, however, detected in the luteal phase, in those females that remained anoestrus after RPY and in male possums. There was a positive correlation between gene expression and plasma levels of FSH at different stages of the oestrous cycle, which is indicative of a hormone secreted via the constitutive pathway. No pulses of FSH were detected at any time. Sexual dimorphism of gonadotroph populations was evident where proportionately more FSH-positive and fewer LH-positive cells were observed in male compared with female possums, which was consistent with the daily secretory profile of these hormones. Gonadotrophs appeared to be concentrated in the lateral regions of the pars distalis, whilst a small region in the middle of the pars distalis, immediately anterior to the pars nervosa, was nearly devoid of gonadotrophs. These findings are similar to that reported in eutherians and considering that marsupial evolution diverged from eutherians over 100 million years ago suggests that the regulation of gonadotrophins is highly conserved indeed.

Materials and Methods

Animals and management

All possums were live-captured in the Wellington region (latitude 41°S) of New Zealand. At the time of capture, each animal was screened for health status and only outwardly healthy animals were included in this study. Animals were housed in the AgResearch Wallaceville possum facility under a group housing system (McLeod *et al.* 1997). A mixed diet of fresh fruits and vegetables, bread- and cereal-based pellets was provided along with selective browsing of *Pinus radiata* branches. Fresh water was always available.

At the end of an experiment, all possums were killed by asphyxiation using CO₂. All experimental procedures were approved by the AgResearch Wallaceville Animal Ethics Committee in accordance with the Animal Welfare Act of New Zealand, 1999.

Experimental design

To determine the levels of mRNA expression of *LHB*, *FSHB* and *GNRHR* at different stages of the oestrous cycle, 25 adult female possums with pouch young were divided randomly into five groups. Stages of the oestrous cycle were determined by infrequent laparoscopic examination (one to four examinations every ~2–7 days) of the reproductive tract to observe initial enlargement of the cul-de-sac (early follicular stage), presence of a 'presumptive' preovulatory follicle >4 mm in diameter (late follicular stage), or presence of an ovulation point on the preovulatory follicle or corpus luteum (luteal stage) as described previously in detail (Crawford *et al.* 1997). Pouch young were removed (and killed) from all animals in the late breeding season and five animals were immediately killed (RPY stage). Remaining animals were killed at the early follicular stage ($n=5$), late follicular stage ($n=6$), early luteal stage ($n=4$; 1–2 days after observation of an ovulation point) and mid-luteal stage ($n=5$; 8–10 days after observation of an ovulation point). Within 5 min of death, reproductive tracts were removed, dissected and weighed, and pituitary glands were removed, snap frozen in dry ice and stored at -80°C until required for processing for quantitative PCR.

To determine the parameters (i.e. pulse frequency and amplitude) of gonadotrophin secretion, a group of 33 lactating female and 12 adult male possums were studied over four breeding seasons (1998–2001). All animals had an indwelling jugular cannula inserted as described previously (Curlewis *et al.* 1985). Pouch young were removed from female possums, and return to oestrus was monitored daily in individuals by vaginal cytology (influx of epithelial cells and/or sperm in urine; Curlewis *et al.* 1985) and was retrospectively confirmed by examination of the ovaries and reproductive tract at time of death. Daily blood samples were collected in females from 2 to 4 days prior to RPY till 10–22 days following RPY, and in males over a 26-day period. To determine the pulse frequency and amplitude of gonadotrophin secretion, frequent blood samples (every 20 min) were collected over a 6-hour interval (0000–0600 h) during the early ($n=7$) and late ($n=3$) follicular stages of the oestrous cycle, near the end of the preovulatory LH surge ($n=3$), during the early ($n=7$) and mid- ($n=6$) luteal stages, and in females whose ovaries remained inactive after 7 days post-RPY ($n=7$) and in adult males ($n=12$).

The pattern of localisation of mRNA expression and protein storage of gonadotrophins was determined using pituitary glands from three anoestrous female possums. Pituitary volume, and the numbers of total and immunopositive LH and FSH cells, was

calculated from measurements of pituitary glands from three lactating females and three males. After euthanasia in all animals, pituitary glands were dissected and fixed in 4% (w/v) phosphate-buffered paraformaldehyde and embedded in paraffin wax until processing for *in situ* hybridisation and immunohistochemistry.

Quantitative PCR

All reagents used and procedures performed were according to the manufacturer's instructions. Expression levels of *LHB*, *FSHB*, *GNRHR* and *ACTB* (β -actin; housekeeping gene) mRNAs were determined using a quantitative Taqman PCR method. Frozen pituitaries were transferred directly to TRIzol reagent (Invitrogen New Zealand Ltd, 1006) and total RNA (tRNA) was extracted. To remove any genomic DNA, 3000 ng of each tRNA sample in 15 μl of di-ethyl-propyl carbonate-treated water was exposed to DNase from the TURBO DNA-free kit (Ambion Inc., Austin, TX, USA). An aliquot (600 ng in 6 μl) of DNase-treated tRNA from each sample was reverse transcribed using reagents including oligo(dT)₂₀ from the SuperScript III First-Strand Synthesis Super-Mix kit (Invitrogen New Zealand Ltd).

Primers and Taqman probes (Table 2) were designed using the computer package 'Beacon Designer' (Premier Biosoft International, Palo Alto, CA, USA) and were manufactured by Sigma–Proligo (supplied by either Proligo-France SAS 1, Paris France and Proligo-Singapore Pte Ltd, Helios, Singapore).

For quantification of expression levels of *LHB* and *FSHB* mRNA, a quadruplex reaction mix was prepared containing primers and Taqman probes (Table 2) for possum *LHB*, *FSHB*, *ACTB* and *PRL* (prolactin; data not presented in this study) genes at validated concentrations (Table 3; excluding prolactin) and reagents supplied in the 'Brilliant Multiplex QPCR Master Mix' kit (Stratagene, La Jolla, CA, USA). For quantification of *GNRHR* mRNA expression levels, a duplex reaction mix was prepared containing primers and Taqman probes (Table 2) for possum *GNRHR* and *ACTB* mRNA at validated concentrations (Table 3) and reagents supplied in the 'Brilliant Multiplex QPCR Master Mix' kit (Stratagene). Samples were prepared in duplicate by adding the cDNA sample (10.4 ng in 1.04 μl) into the prepared reaction mix (total volume of 52 μl) and then transferring two 25 μl aliquots containing 5 ng cDNA/aliquot to adjacent wells in an ABI PRISM Optical 96-well reaction plate (Applied Biosystems, Foster City, CA, USA), covered with an ABI PRISM Optical Adhesive Cover (Applied Biosystems). The amplification reaction was run on an MX3000P real-time PCR system (Stratagene) using the

Table 2 Sequence information of primers and TaqMan probes for real-time Taqman PCR and the resultant product size (bps) for possum *LHB* (accession no: AF090388), *FSHB* (accession no: AF008550), *GNRHR* (accession no: AF032379) and *ACTB* (β -actin, accession no: AF076190) mRNA.

Gene	Taqman probe (5'–3')	Primers (5'–3')	Product (bp)
<i>LHB</i>	(HEX)ACAGTGCTGCTGCTGCTGCTGCTGC(BHQ1)	F-GCAGGATGGAGAGATACCAGGAG R-ACACTGGGCAGGCATCGC	144
<i>FSHB</i>	(ROX)CTTGGTGCTCTGGTTACTGCCATACACGG(BHQ2)	F-AATTCTGCATCAGCATCAACACC R-GGTCTGATCGGGTCTTATATACC	82
<i>GNRHR</i>	(6-FAM)TTGGTCCTCTCTCATCATGCTGGTGTGC(BHQ1)	F-TCTTGACCTTTAGCTGCCTCTTC R-CCCTTGGGATATTGTTCTTTGATCG	144
<i>ACTB</i>	(CY5)AACACAGCTTCTCCTTGATGTCACGCACGATT(BHQ3)	F-GTGATCTGACGGACTACCTCATG R-CCATCTCCTGCTCGAAGTCTAGG	135

Hex, Rox, 6-Fam and Cy5 are fluorophores, and Bhq1–3 are quenchers from Sigma–Proligo.

Table 3 Final concentrations of primers and TaqMan probes for real-time PCR for possum *LHB*, *FSHB*, *GNRHR* and *ACTB* (β -actin) mRNA transcripts.

Gene	Forward primer (nM)	Taqman probe (nM)	Reverse primer (nM)
<i>LHB</i>	200	200	200
<i>FSHB</i>	100	100	100
<i>ACTB</i> ^a	200	200	200
<i>GNRHR</i>	150	100	200
<i>ACTB</i> ^b	150	150	200

^aDenotes the concentrations of primers and probe for *ACTB* mRNA used in a quadruplex reaction, together with primers and probes for possum *LHB*, *FSHB* and *PRL* (prolactin) mRNA (data not presented in this study). ^bDenotes the concentrations of primers and probe for *ACTB* mRNA used in a duplex reaction along with primers and probes for possum *GNRHR* mRNA.

following conditions: 1 cycle of 95 °C for 10 min; 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Controls included samples that underwent RT-PCR with the exclusion of Superscript III/RNaseout enzyme mix to check the effectiveness of DNase treatment, and reactions that omitted addition of template. Quantification of samples was performed by $\Delta\Delta C_T$ method (User Bulletin No 2, Applied Biosystems). Before analysis, serial dilutions (1:1–1:64) of a sample were made in both singleplex and quadruplex reactions to validate PCR efficiency. This included the calculation of the line of best fit (slope ± 0.1) for *LHB*, *FSHB* and *GNRHR* mRNA when $\Delta\Delta C_T$ was plotted against log (input RNA), as well as comparing C_T values (<0.65 cycles different) for identical samples for all mRNA transcripts in singleplex and multiplex reactions.

Hormone RIAs

Plasma concentrations of LH and FSH were measured by heterologous RIA using procedures previously described for the possum (Moore *et al.* 1997a, 1997b). Standards used for these RIAs were purified possum LH and FSH respectively. Within this study, the limits of detection (80% of zero binding) were 0.3 and 0.7 ng/ml for LH and FSH respectively. The intra- and interassay coefficients of variation (CV) in the LH RIA for the medium (2 ng/ml) controls were 15.7 and 16.1% respectively, and high (5 ng/ml) controls were 14.0 and 14.8% respectively. The intra- and interassay CVs in the FSH RIA for the medium (2 ng/ml) controls were 7.1 and 11.0% respectively, and high (5 ng/ml) controls were 6.6 and 7.3% respectively.

Cloning of possum LH β -subunit and FSH β -subunit cDNAs

Total RNA was isolated from frozen pituitaries using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. These tissues were collected from untreated possums being killed for other approved experiments at Wallaceville Animal Research Centre. For the generation of *LHB* and *FSHB* cDNAs, first-strand cDNA was produced from 5 μ g of tRNA using SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen) in accordance with the manufacturer's instructions. Complementary cDNAs encoding a section of possum *LHB* (bases 33–379 of AF090388, GenBank accession no.) and

possum *FSHB* (bases 105–402 of AF008550, GenBank accession no.) genes were produced using standard RT-PCR techniques. Resulting PCR products were ligated into pGEM-Teasy vector (Promega, *In Vitro* Technologies), and the sequences of resulting plasmids were verified.

In situ hybridisation

Cellular localisation of *LHB* and *FSHB* mRNAs was determined using an *in situ* hybridisation protocol described previously (Tisdall *et al.* 1999), with minor modifications. Sense and antisense RNA probes were generated from cDNA encoding the genes of interest with T7 and SP6 RNA polymerase using the Riboprobe Gemini system (Promega). In brief, 4–6 μ m tissue sections were incubated overnight at 55 °C with 45 000 cpm/ μ l of ³³P-labelled antisense RNA. Removal of non-specific hybridisation of RNA was achieved by RNase A digestion followed by stringent washes (2 $\times$ SSC and 50% formamide at 65 °C and 0.2 $\times$ SSC at 37 °C). Following washing, sections were dehydrated, air-dried, coated with autoradiographic emulsion (LM-1 emulsion; Amersham Pharmacia Biotech New Zealand) and exposed at 4 °C for 2 weeks (*LHB*) or 3 weeks (*FSHB*) and developed for 3.5 min in D10 developer (Kodak). Development was stopped with 1% acetic acid, and slides were fixed in ILFOFIX II (Ilford, Cheshire, UK) for 10 min. Sections were stained with haematoxylin and then viewed and photographed using both light- and dark-field illumination with a Leica DM6000 microscope (Leica Biosystems Ltd, Victoria, Australia). Hybridisation in adjacent serial sections with sense [³³P]UTP-labelled possum *LHB* and *FSHB* probes (negative controls) resulted in non-specific signal comparable with the background.

Immunohistochemistry

Immunohistochemistry was used to localize the storage of gonadotrophins in the pituitary gland and confirm the presence of LH and FSH proteins in those cellular types containing corresponding mRNA. As described previously, immunohistochemistry was performed using a pressure cooker antigen retrieval method with minor modifications (Tisdall *et al.* 1999, Quirke *et al.* 2001). In brief, LH and FSH proteins were localized in cross sections of pituitary glands using rabbit anti-porcine LH (No. 19526, kindly supplied by Dr Combarous, INRA, France; Dacheux & Dubois 1978) and rabbit anti-human FSH (M91, kindly donated by Prof AS McNeilly, MRC, Edinburgh, UK) antisera respectively, at final dilutions of 1:300 (counting LH-positive cells) or 1:1000 (visualising localisation of storage of LH) for LH, and 1:200 for FSH, in Tris–HCl buffer containing 0.1% (w/v) BSA (reagent grade; Immuno-chemical Products Ltd, Auckland, New Zealand). Following incubation with the swine anti-rabbit purified IgG secondary antibody (diluted 1:500; DAKO, DAKO Corporation, Carpinteria, CA, USA), staining sensitivity was increased using Tyramide Signal Amplification–Biotin System (New England Nuclear; Perkin–Elmer Life Sciences, Boston, MA, USA). The chromagen used was 3,3'-diaminobenzidine tetrahydrochloride and sections were counterstained with haematoxylin. Negative controls for LH consisted of pre-absorption of the LH antibody with purified possum LH (1 mg/ml) prior to the antibody step, which resulted in abolition of

immunostaining of pituitary cells. At the time of this experiment, there was no purified possum FSH available, therefore non-specific staining was determined by replacing the primary antibody with an equivalent amount of non-immune IgG. Recent experiments have proven that the pre-absorption of this FSH antibody with purified possum FSH resulted in abolition of immunostaining of pituitary cells.

A projection microscope (NeoPromar; Leitz) was used to observe and count nuclei in the cells that were immunolabelled for LH and FSH by the presence of brown cytoplasmic immunostaining. The number of total cells in each pituitary was estimated in the same manner, using sections stained with haematoxylin.

Stereology

Pituitary volumes (V_o) were estimated by the Cavalieri principle using the formula $V_o = \Sigma ah$, where a , determined by point counting, is the area in μm^2 of every 20th section ($5\text{ }\mu\text{m}$ thickness) and h is the distance in μm between the sections used to determine a (Gundersen 1986).

The number of cells, N , was determined using the nuclear disector method (Gundersen 1986) using the formula:

$$N = \frac{\Sigma C \times V_o}{\Sigma a_{(c)} \times h_{(c)}}$$

where $h_{(c)}$ is the thickness of adjacent sections in μm ($5\text{ }\mu\text{m}$) and C is the number of cells exclusive to one section in a known area, $a_{(c)}$.

Statistical analysis

All reproductive tract weights, quantified gene expression levels, volumes, numbers and ratios of total or specific pituitary cells and concentrations of plasma gonadotrophins are reported as mean $\pm$ S.E.M. Data were log transformed and analysed using one-way ANOVA and where a significant ($P < 0.05$) difference was calculated, and a *post hoc* Student's *t*-test was performed using the Minitab 15 statistical package (Minitab Pty Ltd, Sydney, Australia).

The detection of LH and FSH pulses was based on parameters used in the Munro programme, which originated from the pulsar algorithm of Merriam & Wachter (1982). The mean of the peak value and the following sample value was at least twice that of the mean of the two sample values previous to the peak value. Pulse amplitude was measured as the area under the curve (sum of concentration values above baseline).

Declaration of interest

There is no conflict of interest that would prejudice the impartiality of this study.

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