Species differences in the ovarian distribution of 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow 4$ isomerase (3 β -HSD) in two marsupials: the brushtail possum *Trichosurus vulpecula* and the grey, short-tailed opossum *Monodelphis domestica*

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The ovarian distribution of the steroidogenic enzyme 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow 4$ isomerase (3 β -HSD) was investigated by immunocytochemistry in two marsupial species throughout the reproductive cycle, using a rabbit polyclonal antibody raised against human placental 3 β -HSD. In the polyoestrous and polyovular South American opossum *Monodelphis domestica*, immunostaining was positive for 3 β -HSD in the adrenal cortex, the ovarian interstitial tissue, the corpus luteum and the granulosa cells of antral and atretic follicles. The theca interna was weakly positive for 3 β -HSD, but only in late preantral to early antral stages of follicular development. The adrenal medulla and smaller preantral follicles were completely negative for 3 β -HSD. In contrast,

in the polyoestrous and monovular Australian brushtail possum *Trichosurus vulpecula*, immunostaining showed a strong positive reaction for 3 β -HSD in the theca, whereas the granulosa layer remained predominantly negative for 3 β -HSD except in the largest follicles. The atretic follicles were completely negative for 3 β -HSD. The ovaries of pregnant animals contained grossly enlarged, persistent, antral follicles, which reacted positively for 3 β -HSD. The function of these follicles in *T. vulpecula* and the 3 β -HSD-positive atretic follicles in *M. domestica* has not been determined. The differences between the two marsupials represent species variations. The situation in *M. domestica* does not represent a marsupial–eutherian dichotomy as previously conjectured.

Introduction

According to the ‘two-cell hypothesis of ovarian steroidogenesis’, androgens of thecal origin must diffuse through the basement membrane separating granulosa and thecal layers of the follicle; the aromatase enzymes then convert the androgens into oestrogens (Gore-Langton and Armstrong, 1994). 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow 4$ isomerase (EC1,1,1,145; 3 β -HSD) is an essential enzyme in the biosynthesis of the sex steroids (Johnson and Everitt, 1997). Histochemical studies have shown indirectly that 3 β -HSD is present in the ovaries of eutherian mammals after birth (Wattenberg, 1958; Levy *et al.*, 1959; Ferguson, 1965; Pupkin *et al.*, 1966; Müller, 1975), but the results of these studies have indicated that the presence of 3 β -HSD in the granulosa cells differs among species (Goldman and Kohn, 1970; Hoyer and Anersin, 1977).

Immunocytochemistry allows direct detection of enzyme location in tissue sections and anti-3 β -HSD antibodies have been produced for this purpose by a number of research groups. A polyclonal rabbit anti-human placental 3 β -HSD antibody has been used on adrenal glands (humans: Doody *et al.*, 1990; Dupont *et al.*, 1990a; Sasano *et al.*, 1990a; rodents: Dupont *et al.*, 1990b,c; cows: Ishimura *et al.*, 1988; Conley *et al.*, 1992) and ovaries (humans: Sasano *et al.*, 1990b; rodents: Dupont *et al.*, 1990b,c; pigs, sheep, cows: Conley *et al.*, 1995; bears: Tsubota *et al.*, 1994). All these studies on adrenal glands demonstrate the presence of 3 β -HSD exclusively in the cortex of the organ; however, observations on the ovary demonstrate species differences.

Apparently contradictory results have been reported regarding the presence of 3 β -HSD in the granulosa tissue when different techniques were used in the same species. In a histochemical study of the rat ovary, Zoller and Weisz (1979) demonstrated the presence of 3 β -HSD

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only in the preovulatory follicle and found activity at its highest in the periphery of the follicle, reaching a peak at pro-oestrus. However, Dupont *et al.* (1990b) were unable to detect the enzyme in the granulosa cells using immunocytochemistry, and Juneau *et al.* (1993), although confirming the above negative results, were able to show the presence of 3 β -HSD mRNA by *in situ* hybridization in this tissue. Finally, Teerds and Dorrington (1993), using the same antibody as in the previous studies, succeeded in demonstrating the presence of 3 β -HSD, but as reported by Zoller and Weisz (1979), only in preovulatory follicles. Evidently steroidogenic capability in rat granulosa cells develops progressively and is stage-dependent.

Although the steroidogenic abilities of marsupial ovaries are well established (Tyndale-Biscoe and Renfree, 1987), histochemical studies are lacking. The steroid hormone content of the gonads has been investigated by radioimmunoassay in the Virginia opossum *Didelphis virginiana* (George *et al.*, 1985) and the tammar wallaby *Macropus eugenii* (Renfree *et al.*, 1992). As immunocytochemistry offers a direct method of enzyme detection, this approach has been used to locate the cellular distribution of 3 β -HSD.

The initial observations made by Ullmann *et al.* (1995) were on *Monodelphis domestica*, a small, pouchless, South American opossum which, since its introduction into the laboratory in 1978, has attained the status of a biomedical model (VandeBerg, 1990). *M. domestica* belongs to the family Didelphidae and is regarded as more primitive than the Australian marsupials. It is polyovular, polyoestrus and, unlike most other marsupials studied, is an induced ovulator (VandeBerg, 1983; Fadem and Rayve, 1985; Hinds *et al.*, 1992). Male pheromonal stimuli are necessary for maturation of the reproductive organs and initiation of the first oestrus in pubertal females (Stonerook and Harder, 1992). Once a female has entered oestrus, ovulation can occur in the absence of copulation (Baggot *et al.*, 1987) but some physical contact with the male still appears to be necessary (Stonerook and Harder, 1987). Contrary to the observations of other authors on isolated females (Maliniak and Tuft, 1981; Fadem, 1985), Butcher (1995) found that in the Glasgow colony, female *M. domestica* maintained repetitive cycles of follicle growth and regression, although these females were anovular when males were absent.

Ullmann *et al.* (1995) performed pilot immunocytochemical studies on the ovaries of *M. domestica* and obtained surprising results: the steroidogenic enzyme 3 β -HSD, which is generally present in the theca interna of eutherian mammals, was not detected in this tissue. It was speculated that the situation in *M. domestica* either reflected a species difference or a marsupial–eutherian dichotomy in the ovarian distribution of 3 β -HSD. A similar investigation of 3 β -HSD in the ovaries of another, unrelated marsupial, the brushtail possum *Trichosurus*

vulpecula was carried out to distinguish between these two possibilities.

T. vulpecula belongs to the family Phalangeridae and, as all Australian marsupials, is pouched. It is polyoestrous, monovular and a spontaneous ovulator (Kean *et al.*, 1964). Since its introduction into New Zealand in the 19th century, *T. vulpecula* has become a major economic and ecological pest there. In this species the localization of 3 β -HSD was similar to that in eutherians, in that the theca interna reacted positively. Thus, it was concluded that the failure to detect 3 β -HSD in the theca interna of *M. domestica* reflects a species difference rather than a marsupial deviation from the eutherian pattern of enzyme distribution. These results were reported in an abstract (Ullmann *et al.*, 1996).

The preliminary observations on *M. domestica* were based on few specimens, the exact reproductive status of which had not been determined (Ullmann *et al.*, 1995): animals with large antral follicles were presumed to be in oestrus, but may just have been undergoing an anovular cycle (Butcher, 1995). As ovulation in *M. domestica* appears to require the presence of a male, the preovulatory stage may differ physiologically from antral follicles in anovular cycles. As 3 β -HSD in the granulosa cells may depend not only on methodology but also on the stage of the reproductive cycle examined, it is possible that our anomalous results in *M. domestica* were due to similar factors. Therefore, the aim of this study was to repeat and extend these observations on animals of known reproductive status.

Materials and Methods

Animals

The adult opossums *M. domestica* were routinely kept in separate cages. On the 3 days before the opossums were caged together, male bedding was placed in the cage of the female, to expose the female to male pheromones and render the female receptive to the male. Animals were killed by an i.p. injection of 4% (w/v) sodium pentobarbitone (Sagatal: Rhone Merieux Ltd, Hounslow). All procedures were carried out with the approval of the University of Glasgow Ethics Committee under a Home Office Licence.

The tissues from the brushtail possums *T. vulpecula* were obtained during culls near Christchurch, Masterton and Palmerston North, New Zealand, in 1994, 1995 and 1999 during the breeding season (April to September). Adult female brushtail possums are of approximately 3.0–3.5 kg body weight and adult female *M. domestica* in the Glasgow colony were an average of 75 g body weight.

Specimens were obtained from representative stages of the reproductive cycle in both species. The ovaries of five non-pregnant and 14 pregnant *T. vulpecula* and

five non-pregnant and three pregnant *M. domestica* were examined.

Assessment of reproductive status

Butcher (1995) showed that both urogenital smearing and visual assessment of the urogenital opening were unreliable predictors of reproductive status in the Glasgow colony of *M. domestica*; therefore, mating was monitored by continuous time-lapse video recording, using a Panasonic NV-DS 150B camcorder and a Philips RT24A video recorder. The stage of pregnancy was calculated from the time of the first copulation, using the developmental table of Mate *et al.* (1994).

The reproductive status of specimens from *T. vulpecula* captured in the wild was assessed by examination of the pouch immediately after the animals were killed by anaesthesia. Oestrus or pregnancy was assumed if the pouch was pink, flushed and moist. The presence of pouch young indicated lactation. The ovaries were dissected out and examined to determine the size of the antral follicles: follicle > 2 mm in diameter indicated oestrus and follicles of between 4 and 5 mm in diameter indicated late oestrus. The presence of a new bouchon was indicative of a recent ovulation, whereas a corpus luteum was indicative of the luteal phase of the reproductive cycle. Pregnancy was determined by removal of the reproductive tract on the side on which the ovary contained a corpus luteum. The oviduct was flushed with PBS and the uterine contents everted into PBS. Any oocytes collected were examined to determine, by the presence of numerous spermatozoa in the inner egg coats and the polarized appearance of the oocyte, whether they had been fertilized, or the stage of pregnancy was assessed. Procedures were carried out in New Zealand, following Australian National Health and Medical Research Council Guidelines for the care and use of animals in research.

Tissue preparation

After the animals were killed by anaesthesia, tissues (reproductive tracts, embryos and ovaries) were dissected out immediately and immersion-fixed in 10% (v/v) neutral buffered formalin or 4% (w/v) paraformaldehyde overnight. The tissues were then dehydrated through an ascending series of ethanol, embedded in paraffin wax at 57°C, cut into serial sections of 6 μ m in thickness and mounted on poly-L-lysine coated slides. Relevant sections for light microscopy were stained with either Mayer's or Ehrlich's haematoxylin and eosin, or with Masson's trichrome. Ovarian follicles were staged according to the classification used by Pedersen and Peters (1968) in mice.

Antibodies

The polyclonal 3 β -HSD antibody (S683) was obtained by immunizing rabbits with purified human placental 3 β -HSD suspended in Freund's adjuvant. Details of antibody characterization and preparation are described by Doody *et al.* (1990).

Immunocytochemistry

The adrenal glands of the rat and *M. domestica* and the ovaries of the two marsupial species were examined for the presence of 3 β -HSD using immunocytochemistry. Sections were deparaffinized, hydrated and incubated in 1.5% (v/v) hydrogen peroxide (H₂O₂) for 15 min to eliminate endogenous peroxidase activity. After rinsing in 0.01 mol PBS l⁻¹ (3 $\times$ 5 min), sections were treated with 20% (v/v) normal goat serum in PBS with 3% (v/v) Triton-X100 for 1 h to clear background staining. The tissues were then incubated in a humidity chamber overnight at 4°C with the primary antibody (S683), which was diluted to 5 μ g ml⁻¹ in PBS containing 1% normal goat serum + 0.3% (v/v) Triton-X100. The rest of the procedure was carried out at room temperature. After rinsing in PBS (3 $\times$ 5 min), a biotinylated anti-rabbit secondary antibody raised in goat (1 : 500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and then an avidin-biotin complex (1 : 50; Vector Laboratories, Inc., Burlingame, CA) were used to label the bound primary antibody. The location of the antigen-antibody complex was visualized by incubating sections in a medium containing 0.05% (v/v) 3,3'-diaminobenzidine, 0.01% (v/v) H₂O₂ and 0.02% (w/v) nickel chloride in 0.01 mol PBS l⁻¹ (2–5 min). After dehydration, sections were mounted in Histomount (Hughes and Hughes Ltd, Wellington).

Rat adrenal glands, known to be positive for 3 β -HSD, were used as positive controls. Negative controls included substitution of preimmune goat or rabbit serum for the primary antibody. These controls were always processed simultaneously with tissue exposed to S683 and were run for a sample of each tissue at each stage studied. No preadsorption controls were carried out as the original peptide used to raise antibody to S683 was unavailable. Photographs were taken on a Leitz Varior-Orthomat photomicroscope.

Results

Adrenal glands

Both the rat and opossum adrenal glands reacted intensely with the 3 β -HSD antibody, but only in the cortical layers of the gland.

Ovaries

In the disc-shaped ovaries of *M. domestica*, the preantral follicles (up to type 5a) did not stain for 3 β -HSD.

Fig. 1. Sections through the ovaries of the grey, short-tailed opossum (*Monodelphis domestica*), showing 3 β -hydroxysteroid dehydrogenase/ $\Delta^{5\rightarrow4}$ isomerase (3 β -HSD) immunostaining. (a) Granulosa cells of antral (GF) and atretic (AF) follicles and interstitial cells (I) immunostained for 3 β -HSD. (b) Note the light immunostaining for 3 β -HSD of the theca interna (T) in the two large preantral follicles (PF) and the lack of immunostaining in the granulosa layer (G). The interstitial tissue (I), atretic follicles (AF) and corpus luteum (CL) are also immunopositive for 3 β -HSD. (c) Portions of two Graafian follicles (GRF) and interstitial tissue (I) showing strong immunostaining for 3 β -HSD. The theca interna (T) remains completely unreactive. (d) The atretic follicles (AF) and degenerating granulosa cells dispersed in the antrum are intensely immunoreactive. Scale bars represent (a) 600 μ m, (b) 100 μ m, (c) 60 μ m, and (d) 120 μ m.

In older preantral (type 5b) and antral follicles (types 6–8), the granulosa cells gave a positive reaction that increased in intensity with the development of the follicles (Fig. 1a,c), but its presence was variable at earlier stages. Occasionally, a light granulation was detected over the theca interna cells, but this was observed only in follicles (types 5b–6) that were on the threshold of antrum formation (Fig. 1b). In older follicles, the theca remained completely unreactive for 3 β -HSD (Fig. 1a,c). Close below the basement membrane of the granulosa layer there was an extensive capillary network, which was particularly well developed in the largest follicles.

The embedded corpora lutea in *M. domestica* and the enlarged, luteinized, polygonal granulosa cells comprising them (Fig. 1b) gave a positive reaction for 3 β -HSD, as did the interstitial tissue, which consisted of groups of scattered cells between the follicles (Fig. 1a–c).

However, the most intense reaction for 3 β -HSD was shown by the granulosa cells of atretic follicles. The nuclei of cells nearest the antrum became pycnotic first and gave a strong, positive reaction for the enzyme. This process then progressed towards the basement membrane, while the degenerating cells became dispersed in the antrum until all that remained was a single layer of strongly staining cells (Fig. 1a,b,d).

The corpus luteum in the elongate, flattened ovary of *T. vulpecula* projected prominently from its surface (Fig. 2a). The granulosa cells of all but the large follicles (stage 8) remained substantially unreactive for 3 β -HSD (Fig. 2b). A weak reaction appeared in the granulosa cells of medium-sized (stage 7) antral follicles and gradually increased with the development of these follicles. The steroidogenic cells of the theca interna formed an intermittent layer of largely flattened, deeply staining cells, about three to five cells deep, enclosing

Fig. 2. Sections through the ovaries of a brushtail possum (*Trichosurus vulpecula*) showing 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow 4$ isomerase (3 β -HSD) immunostaining. (a) The large, protruding corpus luteum (CL) and abundant interstitial tissue (I) are intensely immunoreactive for 3 β -HSD. (b) Follicles at various stages of development. Only the theca interna (T) and interstitial tissue (I) appear immunopositive for 3 β -HSD. Note that in the smallest follicle, the theca is only partly reactive. (c) A portion of ovary showing the luteinising follicle (LF) collapsing around the antrum (A) and the ovulation site (OS). Note the peripheral location and circular appearance of the interstitial tissue (I) and a supernumerary follicle (SF). (d) Note the two supernumerary follicles (SF). The larger one is almost as big as the corpus luteum (CL) and lacks a granulosa layer. In the smaller SF the granulosa is attenuated. There is no theca interna associated with the CL. AF: atretic follicle; G: granulosa; GRF: Graafian follicle; O: oocyte. PF: preantral follicle; S: stroma. Scale bars represent (a) 1.1 mm, (b) 250 μ m, (c) 600 μ m and (d) 1.6 mm.

the granulosa cells of large preantral (stage 5b) and antral follicles (Fig. 2a,b).

A single corpus luteum was present in the active ovary of this monovular species and it reacted positively for 3 β -HSD (Fig. 2a). The earliest stages of the formation of the corpus luteum were observed in three specimens in which the ovulation sites were still visible. In each case, the large, collapsed, luteinizing follicle reacted positively for 3 β -HSD; and some of the transforming granulosa cells had spread out on to the ovarian surface, around the putative ovulation site, forming a bouchon epithelium (Fig. 2c).

By the time the embryo had attained the 16-cell stage, the corpora lutea had developed into a large, solid structure, infiltrated by connective tissue trabeculae, carrying blood vessels into the gland. However, hardly

a trace of the 3 β -HSD positive cells of the theca interna could be discerned, either within or around the corpus luteum (Fig. 2a). This lack of positive cells was apparent even at early stages of corpus luteum formation, such as when the ovulated follicle was luteinizing (Fig. 2c).

The interstitial tissue was represented by variably sized groups of steroidogenically active cells and was present in the stroma at all stages of development investigated. In the early stages of pregnancy, these groups tended to be smaller and more peripherally distributed (Fig. 2c), but by the trilaminar embryonic stage the interstitial tissue was abundant throughout the ovarian stroma. The larger groups of these interstitial cells often presented a ring-like appearance in sections, the cells being arranged around a stroma-filled central space (Fig. 2c). The genesis of these structures, the largest of which

Table 1. Results of immunostaining for 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow 4$ isomerase (3 β -HSD) in the ovaries of the brushtail possum (*Trichosurus vulpecula*) and the grey, short-tailed opossum (*Monodelphis domestica*)

Species		Follicular stages							Corpus luteum	Interstitial tissue
		Preantral		Antral			Atretic			
		Type 3–5a	5b	6	7	8				
<i>Monodelphis domestica</i> ^a	G	—	±	+	++	++	+++			
	T	—	±	±	—	—	—	++	+++	
<i>Trichosurus vulpecula</i> ^b	G	—	—	—	+	++	—			
	T	—	++	++	++	+++	±	++	+++	

^a $n = 3$ pregnant, $n = 5$ non-pregnant.

^b $n = 14$ pregnant, $n = 5$ non-pregnant.

G: granulosa; T: theca interna. +: positive reaction; –: negative reaction; ±: occasional weak reaction.

in the present study measured 550 μm in diameter, is unclear.

A noteworthy feature of the ovaries of *T. vulpecula* was the persistence of supernumerary antral follicles of various sizes (Fig. 2c,d) and up to eight of these structures were located within one ovary. The sizes of these follicles of pregnancy were equal to the size of the corpus luteum and they were observed in both the active and inactive ovaries. In the larger follicles, the granulosa cells and, in all of the follicles, the theca interna cells were steroidogenically active. In one animal, one of two large follicles completely lacked a granulosa layer, whereas the other follicle appeared normal and the attenuated mural granulosa layer was steroidogenically active (Fig. 2d).

Atretic follicles were present at all stages investigated (Fig. 2a). They were identified in stained histological sections by an irregular granulosa layer, numerous pycnotic nuclei and frequently a blood-filled antrum. Immunostaining showed a weak positive reaction for 3 β -HSD in the theca interna, but no reaction in the granulosa layer. These results are summarized (Table 1).

Discussion

Several studies have demonstrated the importance of knowing the stage of the oestrous cycle reached when enzyme experiments are carried out, so that correct interpretation of ovarian dynamics can be made (Pupkin *et al.*, 1966; Zoller and Weisz, 1979; Teerds and Dorrington, 1993).

Oestrus in the opossum *M. domestica* is induced by tactile and pheromonal cues from the male (Fadem, 1985, 1987; Baggott *et al.*, 1987), but it is not detected as easily by vaginal or, rather, urogenital smearing as in rodents. Moreover, the changes in colour and enlargement of the urogenital opening at oestrus, described by Hinds *et al.* (1992), were not observed in

our colony (Butcher, 1995). Although there is evidence that opossums are reflex ovulators (Hinds *et al.*, 1992), they continue to cycle even when isolated from males (Butcher and Ullmann, 1993).

The 24 h time-lapse video recordings in the present study allow observation of mating and determination of the stage of pregnancy with accuracy. However, obtaining an animal in oestrus proved more problematic, as animals can take between 3 and 11 days after being caged together to mate (Fadem, 1985, 1987, 1989; Stonerook and Harder, 1992). In our colony, mating usually occurred within 3–4 days after the animals had been caged together. Once mating has occurred, ovulation takes place within a day (Mate *et al.*, 1994). The preovulatory animal used in the present study had been exposed to male pheromones for 3 days before being caged with the male and mating had not occurred when the female was killed, 18 h later. Thus, the female must have been in or near oestrus and this was confirmed by the presence of large Graafian follicles in the ovaries.

Ideally, more animals should have been used in the present study but, as our small colony of *M. domestica* appears now to be the only one in the UK, we were anxious to use the minimum number of animals necessary. However, the results of the present study proved largely consistent with those reported by Ullmann *et al.* (1995).

The ovaries and adrenal glands are important steroidogenic organs in all mammals, but there are variations in the biochemical pathways and tissues involved in the production of these hormones. As already noted, histological localization of 3 β -HSD has been demonstrated in the ovaries and adrenal glands of a number of eutherian species. However, the present study is the first investigation in marsupial mammals, and indicates not only that eutherian antibodies directed against 3 β -HSD can crossreact with marsupial tissues

but also that the localization of the enzyme is species-specific.

The reaction of the adrenal glands in the opossum to 3 β -HSD antibody conformed to the pattern known to occur in eutherians, namely, the cortex showed intense staining, whereas the medulla remained unreactive. Thus, 3 β -HSD is strictly localized to the adrenal cortex in both groups of mammal.

The acquisition of steroidogenicity by the granulosa cells in both of these marsupials appeared to be gradual. Moreover, the corpus luteum in both marsupials contained granulosa–lutein cells of one type only. The unusual corpus luteum formation in *T. vulpecula* – by follicular collapse – observed by O'Donoghue (1916) has been confirmed and the granulosa–lutein cells became strongly positive for 3 β -HSD soon after ovulation. However, in contrast, only traces of the previously positive steroidogenic cells within the theca interna were detectable around the corpus luteum: the fate of these cells is perplexing.

An unusual finding reported in the present study is that the theca interna in *M. domestica* remained largely negative for 3 β -HSD. A light, positive reaction was detected fleetingly only in late preantral–early antral stages and this is why it was not detected in the preliminary investigation by Ullmann *et al.* (1995). The reaction differed from that observed in *T. vulpecula* in as much as it was diffuse and continuous throughout the theca interna. The presence of 3 β -HSD has been shown to be variable in the granulosa layers of eutherian mammals but, as far as we are aware, it is always present in the theca interna; and has also been demonstrated in lower amniotes, such as fowl (Wells and Gilbert, 1984; Nitta *et al.*, 1993). However, with reference to the situation in *M. domestica*, it is noteworthy that although the theca interna is generally well developed in the marsupials investigated (Sharman, 1959), it is inconspicuous in the native cat *Dasyurus viverrinus* (Sandes, 1903).

The very low expression of 3 β -HSD in the theca interna of *M. domestica* may resemble that found in the mature follicles of primates (Doody *et al.*, 1990) and cows (Bao and Garverick, 1998) in which a principal steroidogenic role of the theca interna is to produce the C19 steroids (androstenedione, testosterone, dehydroepiandrosterone and 5-androstenediol) (Conley *et al.*, 1995; Conley and Bird, 1997). In these species, unlike in rodents, pigs and horses, the conversion of 17 α -hydroxy-progesterone to androstenedione by 17 α -hydroxylase/17,20-lyase is very inefficient. Too much 3 β -HSD in the theca of these species would result in significant accumulation of 17 α -hydroxy-progesterone. This potential problem is averted in humans, primates and cows by the relatively low expression of 3 β -HSD in the theca when compared with the granulosa.

Teerds and Dorrington (1993) reported that in rats, 3 β -HSD persists in the thecal cells of preantral and

antral follicles that are undergoing atresia. The granulosa cells disappear, and the thecal cells undergo hypertrophy and invade the space vacated by the granulosa cells. Throughout the entire process of atresia the thecal cells retain 3 β -HSD. Similarly, the results of the present study demonstrate the presence of 3 β -HSD in atretic follicles of *M. domestica*. Here, however, it is the disintegrating mural granulosa, rather than the theca interna, which reacts intensely for the enzyme. In contrast, in *T. vulpecula*, the atretic follicles were not detectable by immunostaining alone, as the granulosa cells were completely negative for 3 β -HSD.

The interstitial tissue in mammals is variable in form and origin, and Mossman and Duke (1973) list several possible origins for it, including atretic follicles. The presence of 3 β -HSD in the granulosa cells of atretic follicles in *M. domestica* would thus support such a provenance for the interstitial tissue of this species, but the dispersion of these cells in the antrum makes this unlikely. O'Donoghue (1916), who found interstitial tissue present in ten Diprotodont and absent in six Polyprotodont marsupials, argues convincingly that it 'be regarded as a tissue sui generis'. However, Eckery *et al.* (2002) derive the interstitial tissue in *T. vulpecula* from the fetal medullary cords, the tissue which also appears to give rise to the granulosa cells of the first formed follicles (Ullmann, 1996). The interstitial tissue in both *M. domestica* and *T. vulpecula* is abundant and scattered. In contrast in *M. eugenii*, the interstitial tissue forms a discreet structure within the ovary, and Renfree *et al.* (1984) were able to isolate the structure and demonstrate 3 β -HSD within it biochemically; however, they state that its physiological significance in steroid metabolism is uncertain.

In mammals large antral follicles are generally absent from the ovary during pregnancy. However, in *T. vulpecula*, antral follicles were observed at all stages investigated. The reason for the persistence of these follicles, which could be numerous and greatly enlarged, and which have been reported by Kean *et al.* (1964), is enigmatic. Although these follicles generally stained positively for 3 β -HSD, the reaction was light and the attenuated granulosa layer could not have been responsible for much steroidogenesis; indeed, this layer could even be lacking altogether, as shown by this study. Perhaps the clue to this phenomenon lies in the contents of the follicular fluid, which should be investigated.

The present study included marsupials of two species that were at representative stages of the reproductive cycle; however, no stage-specific differences for 3 β -HSD were observed in any of the tissues monitored, as have been reported for rats (Zoller and Weisz, 1979; Teerds and Dorrington, 1993).

In conclusion, the situation in *M. domestica* does not represent a marsupial–eutherian dichotomy, as previously conjectured. The results of the present study demonstrate that the differences between *M. domestica*

and *T. vulpecula* are due to species variations. Moreover, the present study draws attention to several interesting features, notably the function of the follicles of pregnancy in *T. vulpecula*, the negligible production of 3 β -HSD by the theca interna and its abundant expression in atretic follicles in *M. domestica*, the significance of which remains to be elucidated. All these unresolved factors warrant further investigation.

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