Effect of oestradiol treatment on mast cell populations and microflora in the vaginal cul-de-sac of seasonally anoestrous brushtail possums (*Trichosurus vulpecula*)

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Mast cell populations in the vaginal cul-de-sac of female brushtail possums do not appear to be related to microbial invasion but changes in their density occur at oestrus, indicating a hormonal influence. The present study examined the effect of treatment with oestradiol on microflora and on mast cell numbers and their spatial location in cul-de-sac tissue of seasonally anoestrous brushtail possums. Tissue was collected from seasonally anoestrous brushtail possums ($n = 6$ per group) that were either untreated (anoestrous group) or were subjected to 6 days of treatment with oestradiol (oestradiol group) administered via subcutaneous implants or with the oil vehicle alone (control group). Tissue was collected aseptically

for microbiological procedures and the fractionator and optical disector were used to quantify mast cell populations. Microflora populations were low ($< 4.0 \times 10^4$ organisms g^{-1}) and numbers of mast cells were similar in all groups. Mast cell density was greatest in epithelial and connective tissues from seasonally anoestrous and control animals and lowest in oestradiol-treated possums, in which there was a significant increase in cul-de-sac mass and volume. There is an inverse relationship between circulating oestrogen concentrations and mast cell density in possum cul-de-sac tissue, which is probably the result of an increase in tissue volume.

Introduction

The vaginal cul-de-sac complex is a unique feature of the female reproductive tract in marsupials (Kean *et al.*, 1964). It is lined with a single layer of epithelial cells and has underlying connective tissue with interspersed muscle tissue (Crawford *et al.*, 1999). The median cul-de-sac does not open directly to the outside environment, but lateral canals open superiorly into the median cul-de-sac and join inferiorly to form a common urogenital tract with an external opening (Kean *et al.*, 1964). The reproductive tract of the brushtail possum (*Trichosurus vulpecula*) undergoes extensive hypertrophy during the oestrous cycle, with the vaginal cul-de-sac increasing in volume 10–20-fold, accompanied by widespread tissue vascularization and copious mucus production (Pilton and Sharman, 1962; Crawford *et al.*, 1997, 1999; Mahoney *et al.*, 2002), when circulating oestrogen concentrations are maximal (Curlew and Stone, 1987). This finding indicates that there is a direct influence of oestradiol on the female reproductive tract in the possum, as has been shown

for several eutherian species (Souza *et al.*, 1997). Moreover, exogenous oestradiol increases vaginal cul-de-sac mass in ovariectomized brushtail possums in a dose-dependent manner (Hughes and Rodger, 1971); and steroid receptors with a high affinity for oestradiol are present in the vaginal cul-de-sac complex in large numbers at the time of oestrus and in small numbers during seasonal anoestrus, when plasma oestradiol concentrations are low (Young and McDonald, 1982; Curlew, 1986).

Specific histological stains show that the epithelial lining and underlying connective tissue of the vaginal cul-de-sac contain mast cells (Mahoney *et al.*, 2002). These cells exhibit two aspects of heterogeneity: (i) they are situated predominantly in the epithelial tissue or at the connective tissue–epithelium boundary, and (ii) they may occur as aggregations of three or more cells per unit volume (average disector volume, $4.80 \times 10^4 \mu\text{m}^3$) at those sites (Mahoney *et al.*, 2001, 2002). These aggregations of mast cells may be indicative of a chemotactic response to a foreign stimulus from microbial invasion (Conti *et al.*, 1995). However, it was shown that microflora populations in the possum vaginal cul-de-sac are typically very low (often undetectable),

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and are not correlated with the number of mast cells (Mahoney *et al.*, 2002).

There may be an inverse relationship between oestrogen concentration and number of mast cells in the vaginal cul-de-sac of cyclic female brushtail possums, since mast cell density is significantly lower in the follicular than in the luteal phase of the oestrous cycle or in lactationally anoestrous possums (Mahoney *et al.*, 2002). Furthermore, the decrease in mast cell density may be due to cellular degranulation, after which the cells would no longer be distinguishable by histological staining. Mast cell degranulation under the influence of elevated oestrogen may result in histamine release from the cell granules (Krishna and Terranova, 1985; Krishna *et al.*, 1986; Wilhelm *et al.*, 2000). For example, a correlation between oestrogen concentration and histamine production by mast cells has been reported during embryo implantation in rats (Ferrando and Nalbandov, 1968). Similar relationships between reproductive status and numbers of mast cells in reproductive tissues have been reported in eutherian species, in which degranulation is associated with increased vascularization of the tissue (Jones *et al.*, 1980; Krishna and Terranova, 1985; Krishna *et al.*, 1986, 1989).

The objective of the present study was to determine the influence of exogenous oestrogen on the number of mast cells and microflora populations in vaginal cul-de-sac tissue, using seasonally anoestrous brushtail possums as the experimental model and stereological methods to objectively quantify changes in cell populations.

Materials and Methods

Brushtail possums (*Trichosurus vulpecula*) were live-captured and group-housed in the possum facility at AgResearch Invermay (McLeod *et al.*, 1997), as described by Mahoney *et al.* (2002). Only adult animals of mass ≥ 2.0 kg were used in this study. Possums kept in this facility are routinely given a prophylactic antibiotic injection (3 ml Penodure S, Boehringer, Ingelheim (NZ) Ltd, Auckland) at the time of capture. As this may influence microflora populations, a further six animals from the wild included in the study were not subjected to this antibiotic treatment.

All experimental procedures had prior approval from the AgResearch Invermay Animal Ethics Committee under the *Animal Welfare Act 1999*.

Experimental design

Groups of six seasonally anoestrous, female possums were either untreated (seasonal anoestrus group, no antibiotic treatment) or received Silastic implants (25 mm, 3.35 mm i.d., 4.65 mm o.d., Dow Corning, MI) that were left *in situ* for 6 days; and contained either oestradiol (0.125 mg; Sigma, St Louis, MO) in sesame oil

(oestradiol group) or the sesame oil vehicle alone (control group).

For implant insertion, the animals were anaesthetized by halothane inhalation (Fluothane: ICI New Zealand Ltd, Lower Hutt), with local anaesthetic (0.5 ml lignocaine hydrochloride, BP (British Pharmacopeia) 2% w/v; Techvet Laboratories Ltd, Auckland) administered to the incision site, which was secured by sutures (Mersilk 3/0, Ethicon, Johnson and Johnson International, Brussels), and liberally dusted with Megasant Antibiotic powder (Bayer New Zealand Ltd).

Blood sampling and oestradiol assay

A single blood sample (1 ml) was collected by jugular venepuncture (26-G needle) from animals in the exogenous oestradiol and vehicle control groups, immediately before they were killed under halothane-induced anaesthesia. The samples were centrifuged at 1000 g for 15 min, and the plasma was withdrawn and stored at -20°C . Plasma oestradiol concentrations were determined in a single assay by double-antibody radioimmunoassay (Lun *et al.*, 1998), a method recently adapted for possum plasma (D. Eckery and N. Dryden, personal communication). The intra-assay coefficient of variation was $< 6\%$ and the limit of detection (equivalent dose at 80% binding) was 0.49 pg ml^{-1} .

The animals were killed by an intra-cardiac injection of barbiturate (4–8 ml, EuthalTM, Delta Veterinary Laboratories Pty Ltd) under halothane-induced anaesthesia, weighed and a mid-line and two lateral incisions made in the abdominal wall to expose the reproductive tract. The status of the ovaries, uteri and cul-de-sac was recorded before the reproductive tract was removed under aseptic conditions.

Tissue collection for microbiology and stereology

All tissue collection procedures for microbiological and stereological analysis and for tissue Gram staining were as described by Mahoney *et al.* (2002).

Microbiological procedures

On the basis of the mass of the cul-de-sac tissue recovered, either brain heart infusion broth (BHI, Difco, BD, Palo Alto, CA) was added to the whole piece of tissue in a homogenizer tube to give a 1:10 dilution (w/v), or 0.5 g piece of tissue was placed in a homogenizer tube and 1:10 dilution (w/v) made in BHI. Samples were homogenized and diluted in tenfold dilution steps to 10^{-3} . Aliquots (100 μl) of the 10^{-1} dilution were spread on to either Rogosa (Difco), Sabouraud (Difco), MacConkey (Difco) or blood agar plates. In addition, 10 μl of the 10^{-3} , 10^{-2} , and 10^{-1} dilutions were plated to sectors of blood agar (3) and brain heart infusion plus 0.5% yeast extract (BHIYE) (Difco) plates. One blood

Fig. 1. Mast cells aggregated in the connective tissue (Ct) of the vaginal cul-de-sac of a brushtail possum treated with exogenous oestradiol. An unbiased counting frame with exclusion lines (—) and inclusion lines (---) is shown. Mast cells (arrows) that are captured within the frame or that transect the exclusion line but not the inclusion line are counted. A mast cell (arrowhead) that transects the inclusion line is not counted. Ep: epithelial tissue. Scale bar represents 10 μm .

agar and one BHIYE plate (which had been pre-reduced) were incubated under anaerobic conditions in a glove-box (Forma Scientifica, Marietta, OH) at 37°C for 5–7 days. One Rogosa plate and one blood agar plate were incubated in 5% CO_2 at 37°C; Sabouraud plates were incubated at 30°C and the remaining plates were incubated aerobically at 37°C.

A single smear of the initial homogenate was made onto a microscope slide, stained with Gram's stain and examined by light microscopy.

Stereological analysis and tissue Gram staining

The remaining cul-de-sac tissue from each animal was fixed in 4% (w/v) paraformaldehyde in PBS (8.8 g NaCl, 1.5 g Na_2HPO_4 , 0.4 g KH_2PO_4 ; pH 7.2–7.4; osmolarity approximately 600 mosmol kg^{-1}) for 18–24 h as described by Mahoney *et al.* (2002), fractionated into pieces of similar size (Gundersen *et al.*, 1988), arranged linearly and then random-systematically selected tissue blocks were embedded in Technovit (Technovit 7100, Kulzer and Co., Werheim). One block of cul-de-sac tissue from each animal was embedded in paraffin wax, and sections cut at 4 μm thickness (Leica Rotary Microtome 2050, Labsupply Pierce, Nussloch) for tissue Gram staining (Bancroft and Stevens, 1990).

Random-systematic sampling (Gundersen and Jensen, 1987) was further applied to select those blocks to be

sectioned exhaustively at 30 μm thickness and every tenth section was stained with Giemsa histological stain (Giemsa (Azur Eosin), George T. Gurr Ltd, London) for identification of mast cells. The optical disector (Gundersen, 1986) was used to estimate the total number of mast cells in epithelial and connective tissues of the cul-de-sac, and total reference volume of the component tissues was estimated using the Cavalieri method (Cavalieri, 1635, cited by Gundersen and Jensen, 1987). Cell density was defined as the number of mast cells in the component reference volume (epithelium or connective tissue), and was expressed for all groups as the number of mast cells that occupied $1 \times 10^6 \mu\text{m}^3$ of cul-de-sac tissue. This was determined by the use of a disector with final x and y dimensions of $71 \mu\text{m} \times 50 \mu\text{m}$ (Fig. 1), and z was the mean disector depth that, in conjunction with x and y , provided the measure of disector volume.

Statistical analyses

Comparison of plasma oestradiol concentrations between groups was made by ANOVA. Data for cul-de-sac mass, epithelial and connective tissue volume, total cell number and density were log-transformed to overcome heterogeneity of variance and comparisons between groups were made by ANOVA. The distribution of mast cells in the cul-de-sac tissue did not fit a

Table 1. The mean numbers [range] and description of micro-organisms cultured from vaginal cul-de-sac tissue from untreated (seasonally anoestrous) brushtail possums, and from possums treated with oestradiol or with the oil vehicle (control)

Group	<i>n</i>	pH range	Number of colonies	Gram stain morphology of types of colony isolated
Seasonal anoestrus	6	7–9	12.04 × 10 ³ [3.1 × 10 ² –4.0 × 10 ⁴] (3/6) 9 types of colony	2 × G+ cocci 1 × G+ rod (coryneform) 4 × G+ rod 2 × G– rod
Oestradiol implant	6	6–8	2.32 × 10 ³ [< 10 ² –5.9 × 10 ³] (4/6) 13 types of colony	5 × G+ cocci 1 × G+ cocci (chains) 1 × G+ rod (lactobacilli) 1 × G+ rod 5 × G– rod
Control	6	7–8	1.07 × 10 ³ [< 10 ² –8.6 × 10 ³] (5/6) 35 types of colony	10 × G+ cocci 5 × G+ cocci (chains) 4 × G+ cocci (clusters) 1 × G+ rod (coryneform) 1 × G+ rod (lactobacilli) 8 × G+ rod 7 × G– rod

The detection limit for bacterial isolation was 100 micro-organisms g⁻¹. The numbers in parentheses indicate the proportion of animals from which micro-organisms were isolated out of the total number sampled. G+: Gram positive; G–: Gram negative.

Table 2. Mean (± SEM) masses and volumes of vaginal cul-de-sac tissues in untreated (seasonally anoestrous) brushtail possums, and from possums treated with implants containing oestradiol or with the oil vehicle (control)

Group	<i>n</i>	Cul-de-sac mass (g)	Tissue volume (× 10 ¹¹ μm ³)	
			Epithelial tissue	Connective tissue
Seasonal anoestrus	6	0.46 ± 0.04 ^a	0.22 ± 0.07 ^a	3.71 ± 0.75 ^c
Oestradiol implant	6	3.89 ± 0.38 ^b	2.12 ± 0.37 ^b	9.04 ± 2.08 ^d
Control	6	0.86 ± 0.19 ^a	0.32 ± 0.09 ^a	3.27 ± 0.64 ^c

Within columns, means with different superscripts are significantly different: ^{a,b}(*P* < 0.001); ^{c,d}(*P* < 0.05).

Poisson distribution. Consequently, statistical analysis of distribution of cells per disector volume was made by frequency distribution.

Results

Microbial populations

The numbers of microbial colonies isolated from cul-de-sac tissue, and their Gram stain morphologies are summarized (Table 1). Lactobacilli were not isolated from the seasonal anoestrus group but were present in one of six animals from both the oestradiol-treated and control groups. Bacteria were not isolated from all animals in any one group (Table 1). Bacteria were isolated from more animals (five of six) in the control group and these bacteria demonstrated the largest diversity of types of colony (35) compared with those from seasonally anoestrous (9) and oestradiol-treated (13) groups. More bacteria were isolated from

tissue of seasonally anoestrous possums. Gram stain morphologies were similar for all groups with Gram positive cocci and rods predominating.

The pH of luminal contents varied among individual possums (pH 6–9), but in all groups the mean pH was in the neutral range (Table 1).

Tissue Gram stain sections

Few bacteria associated with the tissue surface were observed, and there was no evidence of bacterial invasion into the epithelial cell layer.

Vaginal cul-de-sac mass and volume

The mean masses of the vaginal cul-de-sac of seasonal anoestrus and control animals were both significantly less (*P* < 0.001) than for animals treated with oestradiol (Table 2). These differences were paralleled

Table 3. Mean ($\pm$ SEM) numbers and densities of mast cells in epithelial and connective tissues of the vaginal cul-de-sac from untreated (seasonally anoestrous) brushtail possums, and from animals treated with oestradiol or oil vehicle implants (control)

Group	n	Number of mast cells ($\times 10^6$)		Mast cell density (cells per $10^6 \mu\text{m}^3$)	
		Epithelial tissue	Connective tissue	Epithelial tissue	Connective tissue
Seasonal anoestrus	6	1.06 ± 0.43^a	4.17 ± 1.50^a	48.64 ± 6.10^a	$10.05 \pm 3.76^{a,c}$
Oestradiol implant	6	1.59 ± 0.65^a	2.83 ± 0.90^a	8.31 ± 3.40^b	3.34 ± 0.85^b
Control	6	1.16 ± 0.46^a	4.18 ± 0.61^a	$36.61 \pm 7.14^{a,c}$	13.50 ± 1.73^a

*Within columns, means with different superscripts are significantly different: $^{a,b}P < 0.001$; $^{b,c}P < 0.01$.

Table 4. Frequency distribution of mast cells (number of cells per disector) in epithelial and connective tissue of the vaginal cul-de-sac from untreated (seasonally anoestrous) brushtail possums, and from animals treated with oestradiol or with the oil vehicle implants (control)

Group	n	Total*	Frequency distribution							
			Epithelial tissue				Connective tissue			
			0 cells	1 cell	2 cells	≥ 3 cells	0 cells	1 cell	2 cells	≥ 3 cells
Seasonal anoestrus	6	3332	118	65	80	43	2457	297	161	111
Oestradiol implant	6	6812	1304	115	71	21	4687	407	148	59
Control	6	3054	221	63	86	44	2042	290	197	111

*Indicates the total number of disectors viewed.

by differences in total cul-de-sac volume, which was approximately threefold greater in the oestradiol-treated group (Table 2). The ratio of epithelial:connective tissue in the cul-de-sac was also higher in animals treated with oestradiol than in the other two groups. The volume of both epithelial ($P < 0.001$) and connective tissue ($P < 0.05$) in the cul-de-sac was also greatest in oestradiol-treated animals.

Number and density of mast cells

The mean total number of mast cells present in the cul-de-sac was $5.23 \pm 1.92 \times 10^6$, $4.42 \pm 1.58 \times 10^6$ and $5.34 \pm 1.13 \times 10^6$ for seasonally anoestrous, oestradiol-treated and control animals, respectively. The mean number of mast cells and mean mast cell density for both epithelial and connective tissues of the cul-de-sac are summarized (Table 3). Neither the total number of mast cells present nor their spatial location in epithelial and connective tissues differed significantly among groups.

Mast cell density was not significantly different between seasonally anoestrous and control animals in either epithelial or connective tissues. However, in animals treated with oestradiol, there were fewer mast cells per unit volume of tissue ($10^6 \mu\text{m}^3$) in both epithelial ($P < 0.001$ seasonal anoestrus group; $P < 0.01$ control group) and connective tissues ($P < 0.01$ seasonal anoestrus group; $P < 0.001$ control group).

The frequency distribution of mast cells (number of mast cells observed per disector) is shown (Table 4). For all groups, the majority of optical disector frames did not detect any mast cells. In all groups, there were examples of ≥ 3 cells per disector and these were defined as mast cell aggregations (Figs 1 and 2). Analyses of the frequency distribution of mast cells per disector in the epithelial or connective tissue revealed no significant differences among groups.

Plasma concentration of oestradiol

Mean ($\pm$ SEM) plasma oestradiol concentrations were significantly higher ($P < 0.001$) in oestradiol-treated possums ($35.33 \pm 3.74 \text{ pg ml}^{-1}$) than in control animals ($9.58 \pm 1.29 \text{ pg ml}^{-1}$).

Discussion

Microflora were present in very low numbers in the vaginal cul-de-sac of all groups of female brushtail possums ($< 4.0 \times 10^4 \text{ g}^{-1}$). This corroborates our earlier study in which there were very low numbers ($< 5 \times 10^5 \text{ g}^{-1}$) of micro-organisms present in the vaginal cul-de-sac of adult and juvenile brushtail possums during the breeding season (Mahoney *et al.*, 2002). Nevertheless, the number of micro-organisms in the female

Fig. 2. Mast cells (arrows) are found in both epithelial (Ep) and connective tissue (Ct) of the vaginal cul-de-sac of the seasonal anoestrus brushtail possum. Cells with nuclei and clear cytoplasm (*) were also found in the epithelium among this group of animals. Scale bar represents 10 μ m.

reproductive tract of possums in all of these reproductive states is much lower than those recorded in the vaginal tract of eutherian mammals (for example humans, Tannock, 1999). Furthermore, in possums in the present study very few bacteria were observed in Gram stained tissue sections and micro-organisms could not be cultured from six of 18 animals investigated. Antibiotic powder was applied to the surgical incision made for implants as it was considered that this method would have minimal impact on microbial populations in the body compared with antibiotics given orally. No marked difference in the microbiology of the cul-de-sac was observed between seasonally anoestrous animals that were newly caught and not subject to the prophylactic antibiotic treatment and the control group of animals housed at the Invermay research facility, although only nine colonies were isolated from three of six newly caught animals compared with 35 colonies from five of six control group animals.

Although the numbers of bacteria isolated from tissue collected from brushtail possums during seasonal anoestrus in the present study were similar to those present at different stages of the oestrous cycle (Mahoney *et al.*, 2002), there were differences in the morphological

types of the bacterial colonies isolated. Gram stain examination showed that Gram positive cocci, followed in magnitude by Gram positive rods, predominated in all treatment groups. This is similar to the pattern of a predominance of staphylococci and streptococci observed in microflora in the vaginal tract of cows (Oterero *et al.*, 2000), in which the highest numbers of micro-organisms were recorded at the time of oestrus (Oterero *et al.*, 1999). Lactobacilli have also been identified as the main type of micro-organism in the vaginal tract of premenopausal women (Pfau and Sacks, 1977). In the present and an earlier study (Mahoney *et al.*, 2002), very few lactobacilli could be cultured from the vaginal cul-de-sac of brushtail possums.

It was previously noted that the pH within the vaginal cul-de-sac changed little with stage of the oestrous cycle and ranged between pH 6 and pH 9, although in the follicular phase a slightly lower pH was recorded (Mahoney *et al.*, 2002). If circulating oestrogen does influence luminal pH, it might be expected that treatment with exogenous oestrogen would also increase hydrogen ion concentration in the cul-de-sac. Low pH values in the female reproductive tract are thought to be attributed to lactic acid, the metabolic product of lactobacilli. Lactic

acid is also produced by vaginal epithelial cells when the glycogen content within the cells is increased by high concentration of oestrogen (Eschenbach *et al.*, 2000). A reduction in luminal pH was not evident in samples collected, although it is possible that pH values did fall at some stage during the 6 days that the subcutaneous implants were *in situ*.

In the present study, mast cells were distributed anisotropically within vaginal cul-de-sac tissues. This characteristic was identified by the fact that (i) most optical disectors captured no mast cells (a zero count), and (ii) most mast cells were located either within the epithelial tissue, or near the epithelium–connective tissue border. The aggregations of mast cells observed in the present study were similar to those recorded in cyclic animals (Mahoney *et al.*, 2002), indicating that mast cells may be induced to form aggregations by some chemical attractant either secreted from the epithelium or absorbed from the lumen (Conti *et al.*, 1995). Such a chemotactic compound and its function is yet to be identified, but it may involve synergy with changing concentrations of circulating hormones or with some cellular metabolic activity. For example, mast cell secretory products may influence a specific cellular mechanism such as histamine release (Ferrando and Nalbandov, 1968) or heparin release (Nakamura *et al.*, 1987).

Mast cells in the vaginal cul-de-sac tissue from brushtail possums may release histamine, and studies in other marsupial species have identified very high concentrations of histamine of mast cell origin (Haynes, 1991). Histamine is known to act as a vasodilator of blood vessels and contractor of smooth muscle tissue, changes which are typically associated with copious mucus production (Brock and Madigan, 1991). A possible role for heparin produced by mast cells in the possum cul-de-sac is yet to be established, but it may act synergistically with oestrogen to influence such mechanisms as cell growth and cell proliferation (Gunin and Sharov, 1998). An alternative possibility is that mast cells in the cul-de-sac tissue do play a major role in the control of pathogenic invasion and that the efficiency of this immunological defence mechanism is reflected in the very low numbers of micro-organisms found within cul-de-sac tissues.

The presence of mucus in the cul-de-sac of brushtail possums has been described by Pilton and Sharman (1962), Kean *et al.* (1964), Hughes and Rodger (1971) and Crawford *et al.* (1997). Copious secretion of mucus is coincident with oestrus and therefore likely to have a role in sperm survival. Hughes and Rodger (1971) suggested that the carbohydrate and protein content of mucus may be associated with a sperm reservoir, and although subsequent studies have found that spermatozoa do not remain for long in the cul-de-sac after mating (Jungnickel *et al.*, 2000), it may be that an interaction of spermatozoa

and mucus is necessary for sperm maturation and/or capacitation.

The decreasing density of mast cells in epithelial and connective tissues in response to circulating oestrogen concentrations may be a result of increased tissue volume and/or of mast cell degranulation. The presence of structures with the appearance of mast cell shells, or a cell nucleus surrounded by clear cytoplasm as found in the present study, might be indicative of the degranulation process; however, it should be noted that such structures were observed in cul-de-sac tissue from seasonally anoestrous animals, but not in the tissue from the oestradiol-treated animals. This finding indicates that changes in mast cell density are more likely to be due to an increase in tissue volume than to an increase in mast cell degranulation.

Plasma concentrations of oestradiol in the oestradiol-implanted possums (35.3 ± 3.74 pg ml⁻¹) were much higher than endogenous oestradiol concentrations reported for the brushtail possum, with maximum concentrations near the time of oestrus of 14.4 pg ml⁻¹ (53.3 pmol l⁻¹) (Curlewis *et al.*, 1985). Although the assay method used in this study is still under development for possum plasma, and only a limited number of samples have been analysed to date, the validation criteria were sufficiently robust to verify the wide differences in plasma concentrations between treatment groups.

Despite the high circulating oestradiol concentrations, the mass and volume of the vaginal cul-de-sac in oestradiol-implanted possums were much lower than those recorded in the late follicular phase of the oestrous cycle. For example, the mean mass and volume of the vaginal cul-de-sac was 3.89 ± 0.38 g and 1.12×10^{12} μm^3 respectively in the present study, compared with 11.00 ± 2.10 g and $5.46 \pm 0.77 \times 10^{12}$ μm^3 in the follicular phase (Mahoney *et al.*, 2002). It should be noted that there would be profound differences in hormonal patterns between the oestradiol-treated anoestrous possums in the present study and those that occur during the follicular phase of the oestrous cycle. The increase in plasma oestradiol concentration would have occurred abruptly after the subcutaneous implantation, compared with the gradual increase in oestradiol secretion associated with the preovulatory follicle. In addition, increased oestradiol secretion at the time of oestrus follows an extended period of increased progesterone concentration, which animals in the present study were not exposed to. It is likely that there would also be significant differences in gonadotrophin concentrations between the oestradiol-implanted anoestrous possums and those possums in the oestrous cycle, indicating that oestradiol alone is not sufficient to promote cul-de-sac hypertrophy or for the recruitment of full complement of mast cells as occurs during the follicular phase of the oestrous cycle (Mahoney *et al.*, 2002). It is also likely that the priming effect of progesterone before the increase in oestradiol

concentration that would occur during the oestrous cycle would affect cul-de-sac hypertrophy.

In conclusion, this study has shown that changes in mast cell populations in the vaginal cul-de-sac comparable to those seen in the late follicular phase of the oestrous cycle, can be induced in seasonally anoestrous possums by treatment with exogenous oestradiol. However, whether the specific role of mast cells in the cul-de-sac of the brushtail possum involves protection against colonization by micro-organisms or some direct action on reproductive processes remains to be determined.

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