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# The immune tissues of the endangered red-tailed phascogale (*Phascogale calura*)

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## Abstract

The lymphoid tissues of the red-tailed phascogale (*Phascogale calura*) were examined using histological and immunohistochemical techniques. The distribution of immune cells in the tissue beds was documented using antibodies to surface markers CD3 and an MHC Class II antigen (equivalent to HLA DRII). Spleen, gut-associated lymphoid tissues (GALT), lung, bronchus-associated lymphoid tissue (BALT) and liver were examined. The spleen had defined areas of red and white pulp, with follicles containing tingible-bodied macrophages. Anti-CD3 and anti-HLA DRII antibodies revealed the presence of T cells in areas of white pulp and around the peri-arterial lymphatic sheaths. GALT and BALT were detected and appeared as scattered areas of lymphocytes in the tissues beds. This is the first study to report on the lymphoid tissues of this endangered species of marsupial and the first report of the capacity of anti-human antibodies to a surface MHC molecule to react with Dasyurid cells.

**Key words** dasyurid; endangered species; marsupial; phascogale.

## Introduction

The red-tailed phascogale (*Phascogale calura*), commonly called the wambenger, is an arboreal, insectivorous Dasyurid (order Dasyuromorphia) that is listed as endangered by the IUCN (Jackson, 2003). Their habitat is currently restricted to south-western Western Australia in areas vegetated with *Eucalyptus wandoo* and *Casuarina huegeliana* (Bradley, 1997). The animal itself weighs 39–68 g, has ash-grey fur with a cream or white belly, whilst the ears and the proximal portion of the tail are red-brown and the distal portion of the tail has long black brush-like hairs (Strahan, 2002). Currently, 12 of the 55 species within the Dasyuridae family are listed as endangered or vulnerable with a further six species not classified by the IUCN (Jackson, 2003).

Very little is known of the immunology of members of the order Dasyuromorphia, the carnivorous marsupials of Australia, New Guinea and the surrounding islands. Their members include the quolls (*Dasyurus* species), the dunnarts (*Sminthopsis* species), *Antechinus* species, the *Phascogale* species and the Tasmanian devil (*Sarcophilus harrisii*). The Tasmanian devil has been recently afflicted with a fatal facial tumour, which makes an understanding of their immune system an important management issue. To date, studies of members of the Dasyuromorphia have been limited to documenting anatomical position and cellular structure, including involution of the thymus (Johnstone, 1898; Symington, 1898; Hill & Hill, 1955; Yadav, 1973; Poskitt et al. 1984a; Haynes, 1991). Most recently we have documented the development of the lymphoid tissues in the small dasyurid *Sminthopsis macroura* (Old et al. 2003a, 2004). However, no studies have been undertaken on red-tailed phascogales. No doubt this is in part due to the limited availability of tissues from this endangered species for study. This paper, based on opportunistic access to tissues sample, provides our first insight into the structure of the lymphoid tissues in this animal.

## Materials and methods

### Animals

A total of 11 juvenile and adult animals were used in this study. All samples were collected opportunistically

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from an established colony at Alice Springs Desert Park, NT, Australia, as part of normal husbandry of the colony (culling). All males were reproductively senescent ($n$ = 3) and no females ($n$ = 8) were caring for young at the time of death. Animals were housed in groups in a row of discrete enclosures measuring 2700 mm (l) × 1800 mm (w) × 2100 mm (h) (10.2 $m^3$), or as single males in enclosures 490 mm (l) × 430 mm (w) × 665 mm (h) (13.7 $m^3$) and fed crickets, cockroaches, mealworms, insects and baby mice. Their diet in captivity was supplemented with calcium and vitamin E.

### Tissue collection, preservation, histology and examination

Immediately after death the thorax and abdomen were pierced to allow penetration of the fixative and the whole animal was immediately placed in 10% neutral buffered formalin. Preserved specimens were then transported to Macquarie University for processing.

Upon arrival in the laboratory, the spleen, intestine, liver and lung were dissected from each animal and placed in fresh 10% neutral buffered formalin. All the tissue samples were found in similar locations to that of stripe-faced dunnarts (*Sminthopsis macroura*) (Old et al. 2003a). Not all gut sections were examined as they were technically difficult to cut due to insect exoskeletal remains throughout the gastrointestinal tract. In addition, some lung and liver samples were unable to be processed due to inadequate fixation. In total, ten liver, ten spleen, 11 intestinal (mostly ileal but including one entire gut) and seven lung samples were examined. All samples were located in similar positions to that of the stripe-faced dunnart and other marsupials. Samples of lymph nodes and thymus were not dissected from animals because of the difficulty of locating them among adipose tissue.

All tissues were routinely processed, using graded alcohol steps and xylene before being embedded in paraffin wax. All tissue blocks were cut at 7 μm. Sections were placed on precoated 3-aminotriethoxysilane (Sigma-Aldrich, St Louis, MO, USA) slides and dried in an oven overnight at 37 °C. All slides were stained using standard haematoxylin and eosin techniques (Bancroft & Stevens, 1982). Slides were dehydrated in graded alcohol steps, cleared in xylene and coverslipped with Entellan (Merck, Whitehouse, NJ, USA).

All slides were examined using an Olympus CX40 compound light microscope and representative photomicrographs taken using an SC35 camera.

Immunohistochemistry was conducted on spleen sections as described previously (Old & Deane, 2002a) using anti-CD3 (1 : 500 and 1 : 1000), anti-mouse anti-human MHC Class II β chain (1 : 50 and 1 : 100) and anti-human CD79a (1 : 100 and 1 : 1000). All antibodies used in this study were purchased from DAKO Cytomation (Carpentaria, CA, USA) with the exception of anti-human CD79a, which was purchased from DBS (Pleasanton, CA, USA). Appropriate negative controls were included as described previously (Old & Deane, 2002a).

## Results

### Spleen

The spleen was a Y-shaped organ located in a similar anatomical position to that of other mammals. Histologically, it contained areas of both red and white pulp (Fig. 1).

Most red pulp areas contained large numbers of blood cells, particularly erythrocytes. Many small trabeculae were observed transversing the red pulp areas (Fig. 1). In some sections the trabeculae appeared to enclose the areas of white pulp and some trabeculae merged with the connective tissue capsule.

Periarterial lymphatic sheaths were prominent in white pulp. Follicles and germinal centres were also observed in the white pulp areas of some spleen sections. A spleen section from one animal contained tingible-bodied macrophages within the follicles.

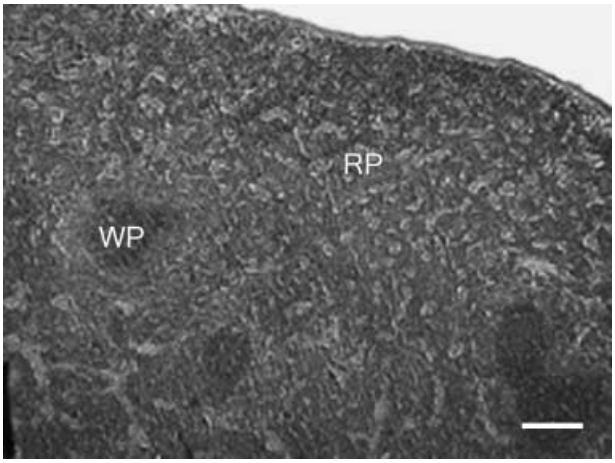


**Fig. 1** Phascogale spleen section showing defined white pulp (WP) and red pulp (RP). The red pulp is transversed by light-staining trabeculae. Scale bar, 20 μm.

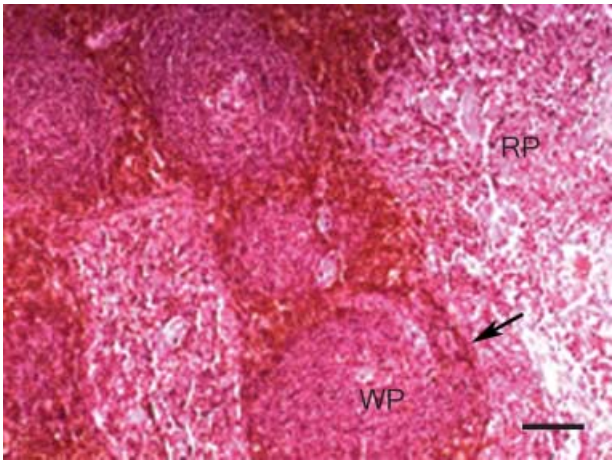


**Fig. 2** Phascogale spleen section stained with anti-CD3 with positively stained cells (arrow). Cells are stained in the white pulp areas (WP). A few scattered cells are stained in the red pulp (RP) areas. Scale bar, 80 μm.

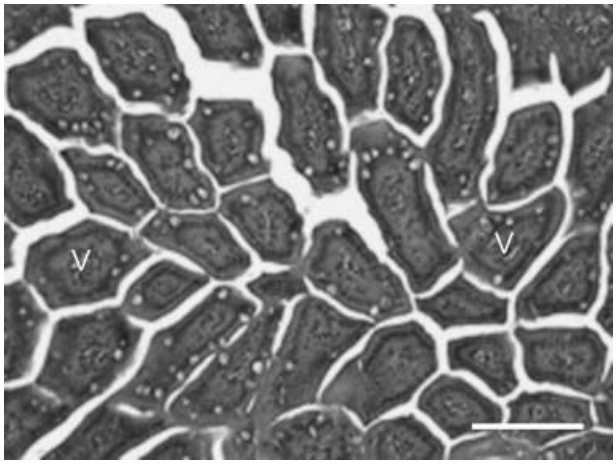


**Fig. 4** Phascogale gut section showing many villi (V) with goblet cells (round, clear circles) interspersed among epithelial cells. Lymphocytes are also seen. Scale bar, 19 μm.

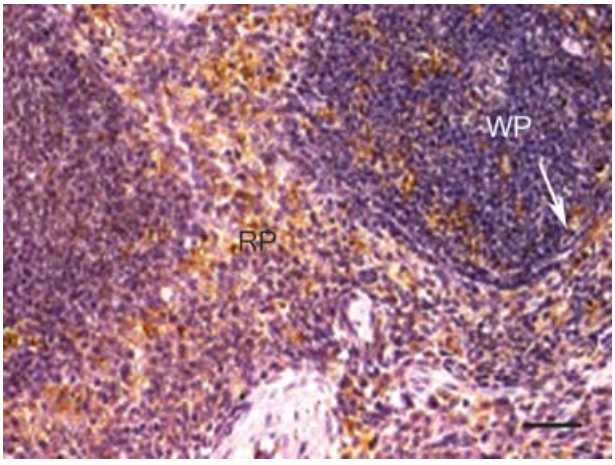


**Fig. 3** Phascogale spleen section stained with anti-HLA-DR. Positively stained cells are seen (arrow) scattered throughout the white pulp (WP) and red pulp (RP). Scale bar, 80 μm.

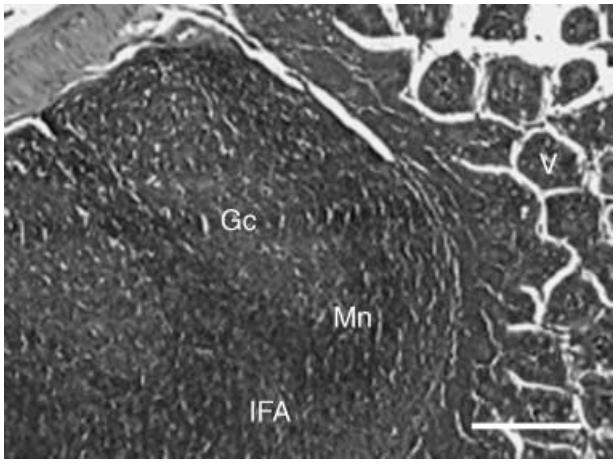


**Fig. 5** Phascogale gut section showing a Peyer's patch and villi (V). Note the germinal centre (Gc) with the defined mantle (Mn) and interfollicular areas (IFA). Scale bar, 19 μm.

Anti-CD3 antibody stained cells in the T-cell areas of the spleen tissue, such as those surrounding the peri-arterial lymphatic sheaths (Fig. 2). Anti-MHC antibody stained cells in the white pulp as well as scattered cells in the red pulp (Fig. 3). All negative control slides were unstained. No cells were stained using anti-human CD79a.

### Gut-associated lymphoid tissues (GALT)

The macroscopic appearance of the intestine revealed no caecum or appendix.

The ileal tissue had villi, with some villi appearing to be rich in lymphocytes whereas others appeared to have sparse numbers of lymphocytes distributed throughout the villous epithelium (Fig. 4). Likewise, not all villi had goblet cells.

GALT was observed in four of the 11 animals examined, but only one animal had its entire gastro-intestinal tract examined for lymphoid tissue and the majority of animals had only their ileum examined. Another animal had a lymphoid tissue accumulation, whereas another had a large area of lymphoid tissue (Fig. 5). One of the animals had two Peyer's patches (Fig. 6). Some of the Peyer's patches had domes without villi (Fig. 3) above them, whereas the other two animals with lymphoid tissue accumulations had villi above their lymphoid tissue accumulations. Another

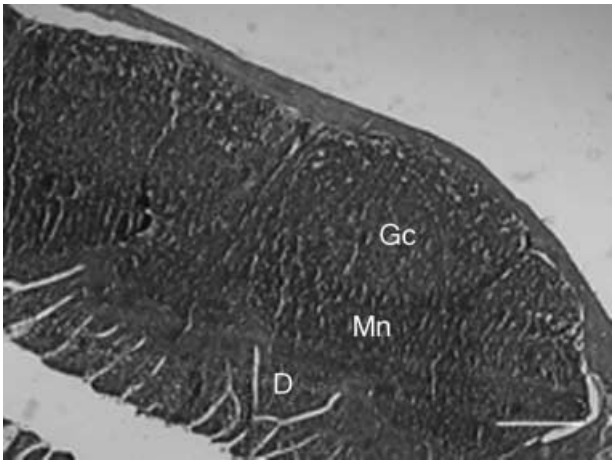


**Fig. 6** Phascogale gut section showing a Peyer's patch with an obvious dome (D) structure and no villi above. The villi are present above the other Peyer's patches. The patches have a germinal centre (Gc) with a well-defined mantle (Mn). Scale bar, 20 μm.

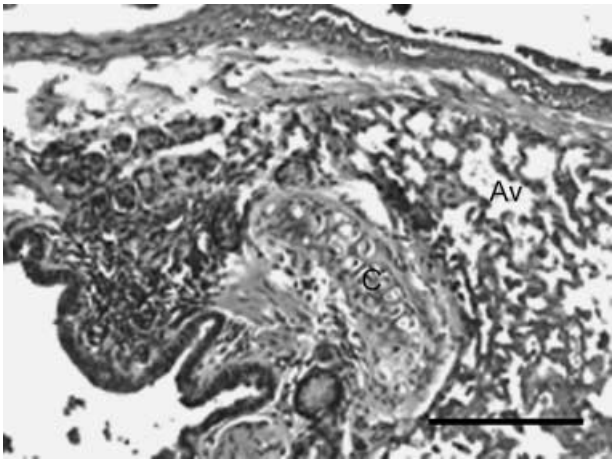


**Fig. 7** Phascogale lung section showing an accumulation of lymphocytes (darkly staining area) surrounding a bronchus. The bronchus lumen is lined by epithelial cells. Note the cartilage ring (C) and alveoli (Av). Scale bar, 75 μm.

animal had four Peyer's patches, with two having domes above them without villi, and two with no domes and villi above them. The villi above the lymphoid tissue accumulations and Peyer's patches had goblet cells.

Some follicles had germinal centres observed with distinct mantles (Fig. 5). No tingible-bodied macrophages were observed in any of the follicular sections.

### Lung

The lung tissue was mature with many alveoli observed in the peripheral tissue. Of the seven lung sections observed, only one had an accumulation of lymphoid tissue surrounding the primary bronchus (Fig. 7).

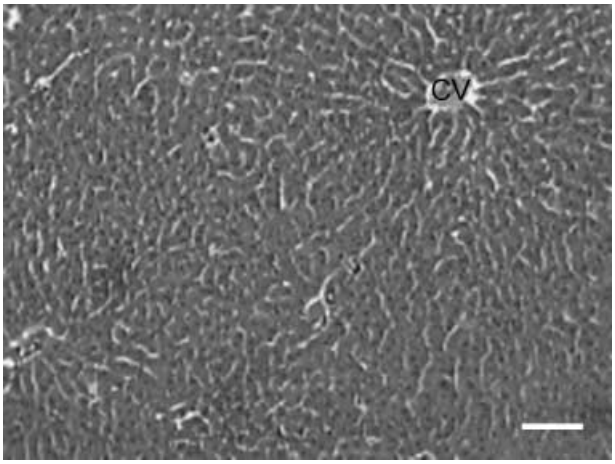


**Fig. 8** Phascogale liver showing a central vein (CV). Hepatic cords and sinuses radiate out from the central vein. Scale bar, 32 μm.

### Liver

All liver samples were mature in appearance and no haematopoiesis was observed in any of the samples (Fig. 8). The liver consisted primarily of hepatocytes forming a hepatic plate structure. Blood cells were evident within the hepatic sinuses and all samples had prominent central veins. Two animals had what appear to be many large adipocytes (hepatic stellate cells) scattered throughout the liver section, and the hepatic cords were very thin.

## Discussion

The histological appearance of spleen, gut-associated lymphoid tissues, bronchus-associated lymphoid tissues and liver tissues of phascogales are similar to those of other Dasyurids studied to date (Poskitt et al. 1984a,b,c; Old et al. 2003a) as well as those of other eutherian and metatherian mammals.

The spleen in the red-tailed phascogale was similar in appearance to that described for the stripe-faced dunnart (Old et al. 2004) with clearly defined red and white pulp areas. The amount of white and red pulp appears to differ in some marsupial species and has been suggested to be age-related in the pouchless opossum (*Marmosa mitis*) based on a decrease in the amount of white pulp in older animals (Bryant & Shifrine, 1972). By contrast, the tammar wallaby (*Macropus eugenii*)

spleen contains mainly red pulp at 60 days and the amount of white pulp increases as the animal matures (Basden et al. 1996). The amount of white and red pulp areas may be age related. To date, no studies have been conducted on the lymphoid tissues of aged marsupials, but studies on developing marsupial spleens suggest that the amounts of red and white pulp may be dependent on the level of antigenic challenge as younger animals mature (Old & Deane, 2002a; Old et al. 2004).

One spleen sample in this study contained follicles with tingible-bodied macrophages. The presence of tingible-bodied macrophages suggests that the animal had active turnover of lymphoid cells in response to antigenic stimulation. We have previously reported the presence of tingible-bodied macrophages in follicles within the gut-associated lymphoid tissues and lymph nodes of a northern brown bandicoot (*Isoodon macrourus*) (Old & Deane, 2002b).

The anti-CD3 antibodies used in this study have been well documented to cross-react with marsupial T cells previously. In the red-tailed phascogale, these antibodies appeared to stain cells in similar locations to those of T cells observed in other marsupials and eutherians. Anti-CD3 did not appear to stain cells in suspected B-cell areas.

The anti-MHC Class II β chain antibody (M0775) used in this study has been used previously on 10% neutral buffered formalin-fixed koala (*Phascolarctos cinereus*) spleen (Canfield & Hemsley, 2000) with positive cells detected in the periarterial lymphatic sheaths, follicular mantles and primary follicles. These HLA-DR- (MHC Class II equivalent) positive cells appeared to include B cells, antigen-presenting cells, macrophages and some activated T cells (Canfield & Hemsley, 2000). Similarly, Coutinho et al. (1995) found anti-HLA-DR-stained cells in the germinal centre of follicles or scattered throughout the red pulp splenic cords. A similar positively stained pattern appears in the spleen of red-tailed phascogales, but note that no follicles were observed in the spleen sections on which immunohistochemistry was conducted. In the red-tailed phascogale, follicles were observed in histology sections only. MHC Class II β chain genes have been cloned from the brushtail possum (*Trichosurus vulpecula*), red-necked wallaby (*Macropus rufogriseus*) and tammar wallaby (Schneider et al. 1991; Lam et al. 2001; Belov et al. 2004) but not from any dasyurid species. Our results, however, suggest that as the antibody recognized cells in comparable regions of phascogale spleen sections, comparable surface motifs can be found in a Dasyurid. The use of these antibodies will allow further comparative studies to be conducted and has expanded the range of animals in which these antibodies can be used.

No cells were stained using anti-human CD79a, and may suggest that the epitope sequence of the CD79a molecule is not conserved among mammals. Previous studies using monoclonal antibodies have shown mixed results in marsupials. Hemsley et al. (1996) found monoclonal anti-human CD79a to cross-react with cells in brushtail possum tissues but not tammar wallabies or koalas. Cisternas & Armati (2000) found the same antibody had weak cross-reactivity with cells in northern brown bandicoot lymph nodes. By contrast, Old & Deane (2002b) did not observe cross-reactivity of the monoclonal anti-human-CD79a with cells of the gut-associated lymphoid tissues from the same bandicoot species, or tissues in the tammar wallaby (Old & Deane, 2002a) or stripe-faced dunnart (Old et al. 2003a). Jones et al. (1993), however, stated that a polyclonal anti-human CD79a did cross-react with cells in grey short-tailed opossum (*Monodelphis domestica*) tissues and Coutinho et al. (1993) in the white-bellied opossum (*Didelphis albiventris*). The molecular or peptide identity of the CD79a molecule in marsupial tissues may provide further answers to the questions surrounding cross-reactivity of anti-human CD79a in some species.

The structure of the gut appears to differ from one marsupial species to another, although, as with all Dasyurids described to date, the red-tailed phascogale does not have a caecum or appendix (Poskitt et al. 1984c; Old et al. 2004). In addition, the structure of the GALT appears to differ among species. The Dasyurids studied to date, *Antechinus* species (Poskitt et al. 1984a,b,c) and the stripe-faced dunnart (Old et al. 2003a; Old et al. 2004), and the American Polyprotodont, the white-bellied opossum (*Didelphis albiventris*) (Coutinho et al. 1993, 1994), all lack dome regions above the follicles. Although these lack dome regions, *Antechinus* species, opossums, dunnarts and phascogales may or may not have villi above the follicles. The presence of villi above the follicles in Dasyurids may suggest the presence of M cells similar to those observed in opossums (Coutinho et al. 1990). By contrast, tammar wallabies lack defined Peyer's patches but do have accumulations of lymphocytes (Basden et al. 1997; Old & Deane, 2002a); rufous hare-wallabies (*Lagorchestes hirsutus*) appear to have Peyer's patches only when in diseased

states (Young et al. 2003) and northern brown bandicoots appear to have several different types, including lymphocyte aggregations distributed along the length of the intestine, clumps of lymphocyte aggregations and Peyer's patches (Old & Deane, 2002b). Koalas have one large lymphoid aggregation rather than small scattered accumulations (Hanger & Heath, 1994). However, Hemsley et al. (1996) described koala and brushtail possum Peyer's patches as well defined, and caecolic lymphoid patches as similar in appearance. The differences in appearance of the GALT in marsupials may be due to differences in diet, herbivorous (e.g. the koala), carnivorous (e.g. Dasyurids) or omnivorous (e.g. the brushtail possum); and level and nature of antigenic challenge. However, further investigations are required to determine why these differences in structure exist.

Like *Antechinus* species, phascogales have a male post-mating mortality and semelparity (Bradley, 1997). Males in this study were, however, reproductively senescent and no females were supporting young. It is therefore unlikely that the animals in this study were stressed due to active involvement in reproduction. In addition, these animals were from a captive colony, were fed regularly and were treated routinely with anti-parasite treatments, and therefore were unlikely to be suffering from the same stressors as wild caught animals.

Small amounts of BALT were found in one lung section, appeared to be non-compartmentalized and contained no follicles or germinal centres. All the animals in this study appeared to be healthy at the time of death so it is unlikely that the presence of BALT in these animals was dependent on antigenic challenge as has been reported in some eutherian mammals and other marsupials. In guinea-pigs and domestic pigs, BALT is sometimes present regardless of antigenic challenge, and a similar situation appears to occur in the dunnart (Old et al. 2004). Similarly, the tammar wallaby appears to lack BALT; however, all rufous hare-wallabies studied had BALT but were in a diseased state (Young et al. 2003). There appears to be no consistent pattern to BALT detection and distribution in marsupials and it is reasonable to suggest that the presence of BALT could be due to low-level antigenic stimulation and need not be associated with overt infection.

This study has documented the histological structures of the spleen, GALT, BALT and liver of the red-tailed phascogale. The histological appearance of these tissues was similar in that described for other Dasyurid species and comparable with other marsupials. This study does, however, highlight the variable structures of the lymphoid tissues of the gut. Not only do different species appear to have different patterns of cellular distribution but individual members of a species appear to differ. The roles of diet, antigenic challenge and gut microflora need to be investigated to come to a better understanding of the structure and function of gut and other mucosal immune tissue. This study has laid the foundations for such work in Dasyurids.

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