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Immunosenescence in a captive semelparous marsupial, the red-tailed phascogale (*Phascogale calura*)

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Abstract

Background: The red-tailed phascogale is a 'Near Threatened' dasyurid marsupial. Males are semelparous and die off shortly after the breeding season in the wild due to a stress-related syndrome, which has many physiological and immunological repercussions. In captivity, males survive for more than 2 years but become infertile after their first breeding season. Meanwhile, females can breed for many years. This suggests that captive males develop similar endocrine changes as their wild counterparts and undergo accelerated aging. However, this remains to be confirmed. The health status and immune function of this species in captivity have also yet to be characterized.

Results: Through an integrative approach combining post-mortem examinations, blood biochemical and hematological analyses, we investigated the physiological and health status of captive phascogales before, during, and after the breeding season. Adult males showed only mild lesions compatible with an endocrine disorder. Both sexes globally maintained a good body condition throughout their lives, most likely due to a high quality diet. However, biochemistry changes potentially compatible with an early onset of renal or hepatic insufficiency were detected in older individuals. Masses and possible hypocalcemia were observed anecdotally in old females. With this increased knowledge of the physiological status of captive phascogales, interpretation of their immune profile at different age stages was then attempted. During the breeding season, males developed a stress leukogram characterized by a marked lymphopenia, further aggravated by a severe leukopenia after the breeding season. To determine whether these changes were limited to the peripheral blood or had more profound implications, histopathology of the spleen was performed opportunistically. Adult males showed white pulp atrophy, at various degrees. The atrophy was mainly lymphoid and more severe in 1.5-year-old males than in 3.5-year-old females. These results suggest that captive males undergo accelerated immunosenescence.

Conclusions: Functional studies are now needed to characterize the underlying mechanisms leading to immunosenescence in marsupials. Semelparous dasyurids present great potential for studying the effects of sex and stress on immunity in marsupials. Characterization of these immune-endocrine interactions may help refine veterinary treatment plans, husbandry protocols and conservation programs to maintain the health of captive and wild populations.

Keywords: Marsupials, Dasyurid, Semelparity, Aging, Immunosenescence, Spleen

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Background

The red-tailed phascogale (*Phascogale calura*) is a small Australian dasyurid marsupial. Males are semelparous; they die off after a short, highly synchronous breeding season at around 11.5 months of age, while females can breed once a year for two to three years [1, 2]. The total mortality of red-tailed phascogale males has been shown to be stress-related [1], as reported for other semelparous dasyurids – the brush-tailed phascogale (*Phascogale tapoatafa*) [3] and several *Antechinus* species [4–6]. The dasyurid breeding season is characterized by an extremely intense level of sexual activity and aggressive interactions [2, 7]. Although Barnett [8] was unable to demonstrate accelerated aging of brown antechinus (*Antechinus stuartii)* males compared to females of the same chronological age, Bradley [2] suggested an "adaptive stress-senescence hypothesis". He proposed that males make major physiological adjustments to increase the success of mating, possibly at the longer-term cost of hormonally accelerated aging and senescence. In fact, a failure of glucocorticoid feedback has been shown to occur in red-tailed phascogale males within a few weeks after the completion of the breeding season [9]. This lack of regulation causes male tissues to be exposed to markedly elevated levels of biologically active glucocorticoid, along with a high plasma testosterone concentration [1]. Consequently, many physiological and immunological changes occur in the last weeks of life of the males; a loss of body condition, a marked lymphopenia and neutrophilia, reduced splenic size, low antibody titers in response to a humoral immune challenge, and eventually gastrointestinal ulcerations and hemorrhages leading to death [1, 2, 10].

The red-tailed phascogale was formerly very widespread across Australia, but the species is now restricted to captive colonies and the southern wheat belt of Western Australia, occupying less than 1% of its former range [11]. It is currently listed as "Vulnerable" under the Commonwealth *Environment Protection and Biodiversity Conservation Act* [12] and "Near Threatened" on the IUCN Red List of Threatened Species [13]. The species suffers from habitat loss and fragmentation, climate change, frequent intense fires and introduced predators. The single annual breeding attempt and post-mating mortality of males also place further constraints on the species' ability to recover from population declines [11, 14]. Captive breeding programs have therefore been implemented since 2001 with the end-goal of re-establishing wild populations [14]. Captive breeding colonies have provided excellent opportunities to study the unique biology of the red-tailed phascogale in regards to its nutrition [15–17], metabolism [18, 19], reproduction [20–22], pouch young growth and development [14], nervous tissues [23], and immune tissues and molecules [24–30]. While most findings are likely to reflect the natural physiology of the red-tailed phascogale, wild and captive populations strikingly differ when it comes to male longevity; in the absence of external stressors, adult phascogale males can survive for more than 2 years in captivity but become infertile after their first breeding season [1, 22]. Interestingly, a study with captive brush-tailed phascogales revealed that males that survive beyond the breeding season in captivity do not display the characteristic hormonal changes (stress response) exhibited by their wild counterparts [31]. Similar studies have yet to be conducted in captive red-tailed phascogales.

Here, we aimed to characterize the physical, hematological and immunological changes that occur during the life history of the red-tailed phascogale in captivity. Post-mortem examinations, blood biochemical and hematological analyses, as well as histopathology of the spleen were performed on opportunistically obtained samples. Comparisons were made between different age-sex groups. We found that, while captive males do not develop the fatal endocrine disorder experienced by their wild counterparts during the breeding season, they still undergo accelerated immunosenescence relative to females as they age in captivity.

Results

Captive males show only mild lesions compatible with an endocrine disorder during the breeding season

Post-mortem examinations were conducted to detect potential manifestations of an endocrine disorder, during or after the breeding season. The occurrence of all findings was recorded for each age-sex group (Table 1). However, as data were obtained opportunistically from animals euthanized for humane reasons or population management control, the incidence of these conditions is likely to be biased and their association with certain groups was not tested. The most common condition observed in males during the breeding season was alopecia (hair loss). The alopecic areas were multifocal (Fig. 1a) to extensive (Fig. 1b) and mostly centered on the tail and lower back. This distribution was similar to the endocrine alopecia previously described in the short-tailed opossum (*Monodelphis domestica*) [32]. Interestingly, the occurrence of alopecia substantially subsided after the breeding season. Meanwhile, only a few old females showed alopecia. This pattern and profile of alopecia in the colony are consistent with an endocrine disorder, although some non-endocrine factors (seasonality, aging, vitamin and mineral imbalances, immunologic diseases, genetic mutations, etc.) cannot be entirely excluded. No ectoparasites were visualized and no erythema or evidence of self-trauma suggesting pruritus were observed. Lesions compatible with conspecific aggression (tail lesions, skin wounds) were present in six males (19%) during the breeding season. Finally, some additional conditions with potential endocrine implications were detected in a few

Table 1 Post-mortem findings compatible with an endocrine disorder

	Juvenile females	Breeding females	1+ year females	Juvenile males	Breeding males	1+ year males
Alopecia	–	–	2/26 (8%)	–	10/31 (32%)	1/18 (6%)
Pododermatitis	–	1/10 (10%)	–	–	1/31 (3%)	–
Hepatomegaly	–	–	–	–	1/31 (3%)	1/18 (6%)
Obesity	–	–	–	–	1/31 (3%)	1/18 (6%)

individuals: two adult males showed obesity (Fig. 1c) and another two had a diffusely enlarged liver (hepatomegaly) with icterus. Localized inflammation of the skin of the feet (pododermatitis) was also seen in two individuals (Fig. 1e). However, no gastrointestinal ulcers were detected by macroscopic examination of tissues and no acute mortalities were recorded in the male population during or immediately following the breeding season.

Male body weight and scrotal width fluctuate during and after the breeding season

Males were significantly ($p < 0.005$) heavier than females throughout their lives (Fig. 2a). They also maintained an average body condition score (BCS) of 3.4 ± 0.1 throughout the breeding season and adulthood, while the females' average score was 2.9 ± 0.1 in both age groups and tended to further decrease in the oldest individuals (Additional file 1: Table S1). Two adult males were also clearly obese (BCS = 5) (Figs. 1c, 2a - outliers), as mentioned previously. Interestingly however, when these outliers were excluded from the analysis, the body weight of males was found to decrease significantly ($p = 0.007$) at the end of the breeding season, from a maximum of 60.8 ± 2.3 g in May, to a minimum of 53.9 ± 1.2 g in August (Fig. 2a). The average body weight of males later progressively increased again and reached, between November and April, similar values as those observed before the breeding season.

The scrotal width also appeared to fluctuate with season (Fig. 2b). After reaching a maximum of 12.4 ± 0.2 mm in May, the scrotal width decreased significantly ($p < 0.005$) to 10.0 ± 0.2 mm in July, and then returned in November–December to its previous size (12.0 ± 0.3 mm, $p < 0.005$), thus suggesting a potential reproductive recovery following the breeding season. However, the scrotal width started decreasing again before the second breeding season, until reaching a minimum of 9.0 mm in September (Additional file 1: Table S1).

Hematology analyses reveal possible hemoconcentration during the breeding season

The packed cell volume (PCV) was relatively high as it ranged from an average of 43.0 to 56.0% in females, and

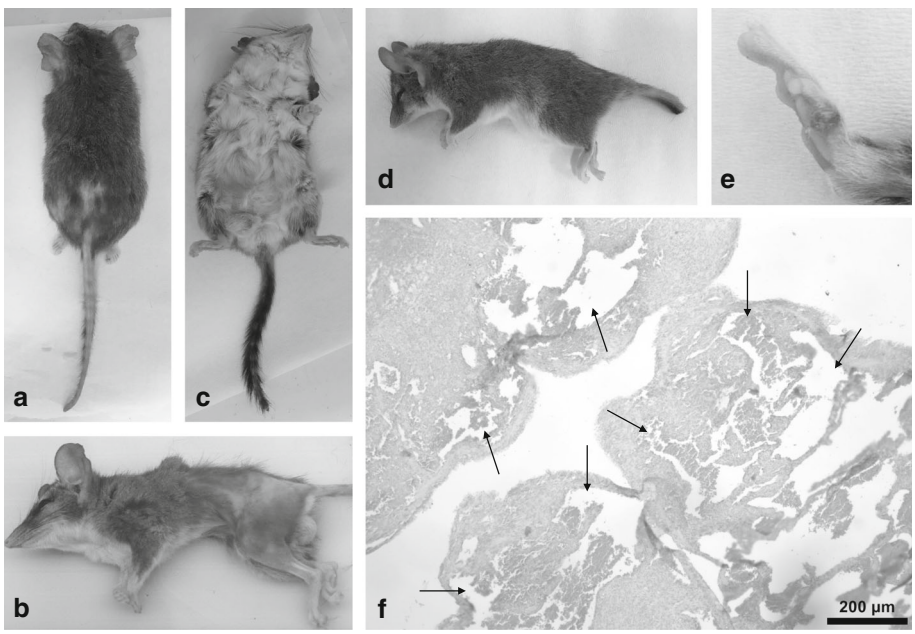

Fig. 1 Post-mortem findings in euthanized captive red-tailed phascogales. Representative photos of **a** multifocal alopecia, **b** extensive alopecia, **c** obesity, **d** tail lesions, **e** pododermatitis, and **f** a 9-µm section (40X) of a splenic tumor of benign appearance observed in a 5-year-old female. Arrows show large and well-defined vascular spaces. Scale bar represents 200 µm

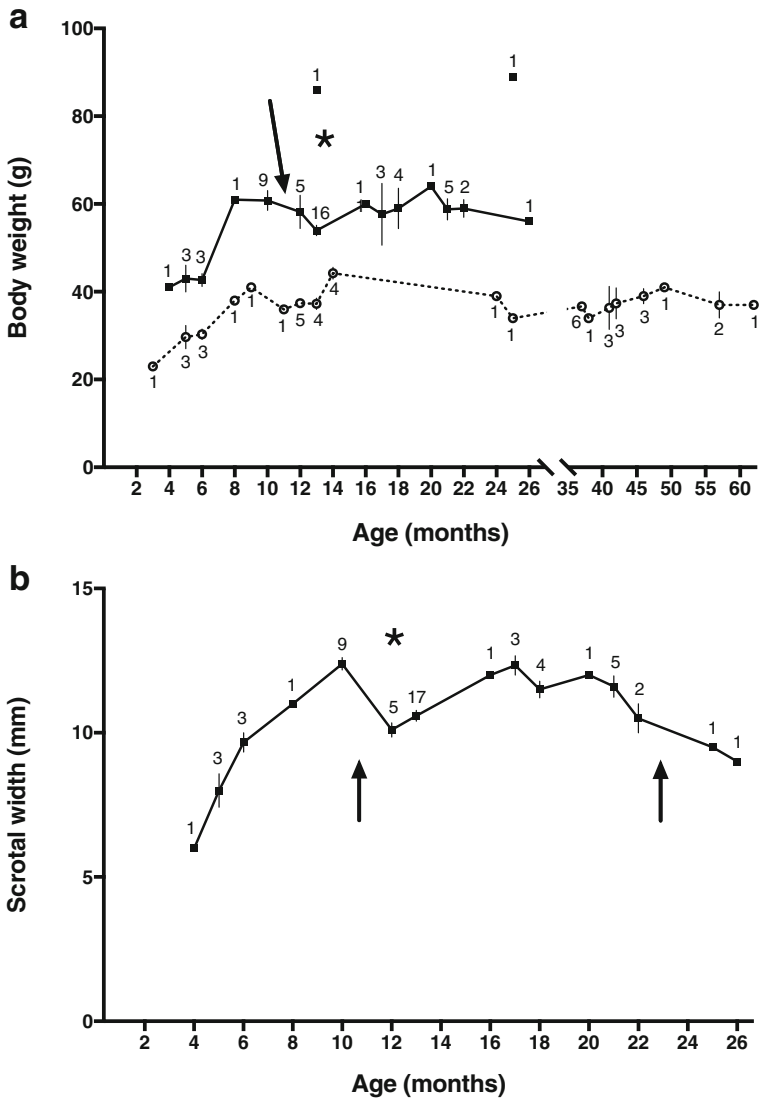

Fig. 2 Biometric measurements of males fluctuate seasonally. **a** Body weight of males (solid line) and females (dotted line) and **b** scrotal width were measured opportunistically on animals euthanized during routine population management procedures. Numbers indicate the number of individuals examined (n). Data are expressed as mean ± SEM. Arrows indicate the timing of the breeding season in the colony. Asterisks (*) indicate statistically significant differences ($P < 0.05$)

53.7 to 64.0% in males (Additional file 1: Table S2). The PCV varied between age groups ($p = 0.011$) and reached its maximal values in both sexes during the breeding season (Fig. 3a). Total protein (TP) concentrations varied in a similar pattern, although the effect of age did not reach statistical significance ($p = 0.068$) and ranged from an average of 5.5 to 6.3 g/dL in females and 6.2 to 7.0 g/dL in males (Fig. 3b). Both PCV and TP levels were significantly higher in males than in females ($p < 0.0005$). The fact that both parameters varied together suggests that the elevation in PCV and TP levels recorded during the breeding season was due mainly to hemoconcentration.

Old animals have a low blood urea nitrogen: Creatinine ratio (BUN: CRE)

As aging doesn't always lead to changes that can be detected in a post-mortem examination, blood biochemistry analyses were performed to help detect subclinical liver or kidney malfunction, two common conditions of geriatric animals. Serum samples from healthy, juvenile animals were used as a reference. Samples from individuals with abnormal post-mortem findings were also included to ensure that biochemical changes consistent with the lesions observed could be detected; A 9-month-old male ( ) and a 5-year-old female (▼) with active tail lesions (Fig. 1d) showed a marked creatine

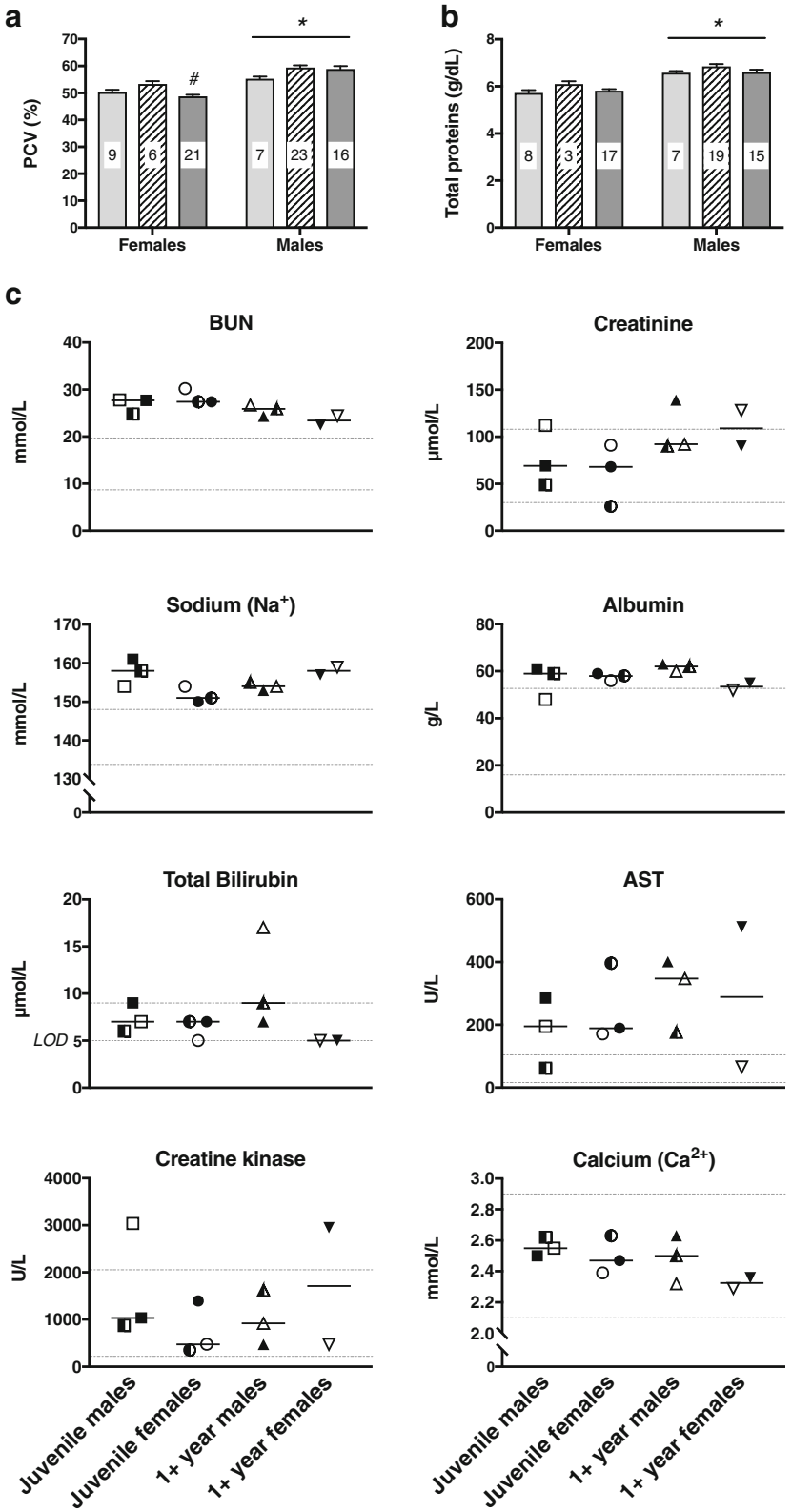

Fig. 3 (See legend on next page.)

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Fig. 3 Hematological and biochemistry results vary across different age-sex groups. **a** Microhematocrit values (packed cell volume, PCV) and **b** serum total protein (TP) concentrations are presented for each age-sex group (*Juveniles:* light gray; *Breeding:* hatched fill; *1+ year:* dark gray). Numbers indicate the number of individuals examined (n). Data are expressed as mean ± SEM. Asterisks (*) indicate a statistically significant difference between males and females ($P < 0.05$). # indicates a statistically significant difference between 1+ year females and breeding females ($P < 0.05$). In **c**, biochemistry analysis of serum was performed for selected individuals ($n = 2–3$ per group). Individual data are plotted and the median for each group is illustrated. Data from a particular individual is represented using the same symbol for all biochemical markers. Dashed lines represent reference intervals proposed by Stannard, et al. [33] for captive Tasmanian devils. BUN: blood urea nitrogen, AST: aspartate aminotransferase, LOD: limit of detection

kinase (CK) elevation, which is typically associated with muscle damage. Meanwhile, a 21-month-old male (△) with an enlarged liver showed high total bilirubin and aspartate aminotransferase (AST) concentrations (Fig. 3c). AST is known to be a fairly non-specific enzyme and its elevation can be attributed to hemolysis, liver or muscle damage, amongst other things. The sample showed no hemolysis and no elevation of CK, thereby making a pre-hepatic or muscular cause less probable.

The BUN, sodium and albumin levels were globally elevated for all individuals tested, when compared to normal values reported for captive Tasmanian devils (*Sarcophilus harrisii*), a closely related carnivorous marsupial [33] (Fig. 3c). Although no statistical analyses could be performed due to the small sample size, some parameters also tended to vary between groups. In fact, potential trends in the data suggest that old individuals have slightly elevated creatinine levels along with a low BUN, thus yielding a low BUN:CRE ratio compared to juvenile animals. This is more obvious when comparing juvenile females with older females (Fig. 3c). Although modest, these changes could be compatible with an early onset of renal disease or liver insufficiency.

Masses and possible hypocalcemia detected in old females, but no anemia

During post-mortem examinations, two masses were observed in old females. This type of pathology has never been described in red-tailed phascogales before. The first mass, a 3-mm smooth spherical cutaneous nodule, was located at the tip of the tail of a 4-year-old female. An incision confirmed the absence of pus or any fluid. No histopathology was performed on this sample. The second mass was found at one extremity of the spleen of a 5-year-old female that showed no clinical signs of disease. All the blood parameters and the rest of the post-mortem examination were within normal limits. The mass consisted of a 6-mm dark red, spherical nodule. It was relatively firm and raised above the capsular surface, and adherent to the parenchyma. The spleen also showed other multifocal dark spots, although these were not elevated. Histopathology revealed large and very well-defined vascular spaces (Fig. 1f and Additional file 1: Figure S1) compared to the rest of the parenchyma, and the endothelial cells were well-differentiated, uniform in size and shape with no obvious malignity criteria. The mass could thus be compatible with a benign tumor, likely a hemangioma.

Old females did not seem to develop anemia as their PCV (average of 49.1 ± 0.8%) remained similar to those of juvenile females. However, compared to the value obtained for females during the breeding season, this value still represented a statistically significant decrease of 4.2 ± 1.7% ($p = 0.037$) (Fig. 3a). Finally, in spite of dietary calcium supplementation, old females showed relatively low ionized calcium levels compared to juvenile animals (Fig. 3c).

White blood cell (WBC) counts decrease with age in males, with an altered neutrophil: Lymphocyte ratio (N:L)

While the WBC count remained relatively stable in females throughout their lives with an average of 4.7 ± 0.3×10^9 cells/L (Additional file 1: Table S3), this parameter varied with age in males; it decreased from 3.4 ± 0.2×10^9 cells/L during the breeding season to 2.1 ± 0.1×10^9 cells/L after the breeding season ($p = 0.007$) (Fig. 4a). When all age groups were combined, males had lower WBC counts than females ($p = 0.003$).

Differential WBC counts were also performed and revealed a clear variation of the neutrophil: lymphocyte ratio in males (Fig. 4b and c). While juvenile males and females displayed similar proportions of lymphocytes (Females: 84 ± 1%, Males: 83 ± 2%), this number dramatically dropped to 51 ± 2% for males during the breeding season and remained relatively unchanged in females at 77 ± 2% (Fig. 4c). The drop in the percentage of lymphocytes for breeding males led to significant changes in their N:L ratio, compared to juvenile males ($p < 0.0005$) and females during the breeding season ($p < 0.0005$) (Fig. 4b). This lymphopenia was also maintained in adult males (61 ± 2%), although not to the same severity as for the breeding males (variation of the N:L ratio: $p < 0.0005$). Meanwhile, the proportion of lymphocytes remained unchanged in older females (76 ± 1%) (Fig. 4c).

Small, medium and large lymphocytes were seen in varying proportions, as well as lymphocytes with an indented nucleus. A few annular leukocytes were visualized routinely, even in animals otherwise presumed to

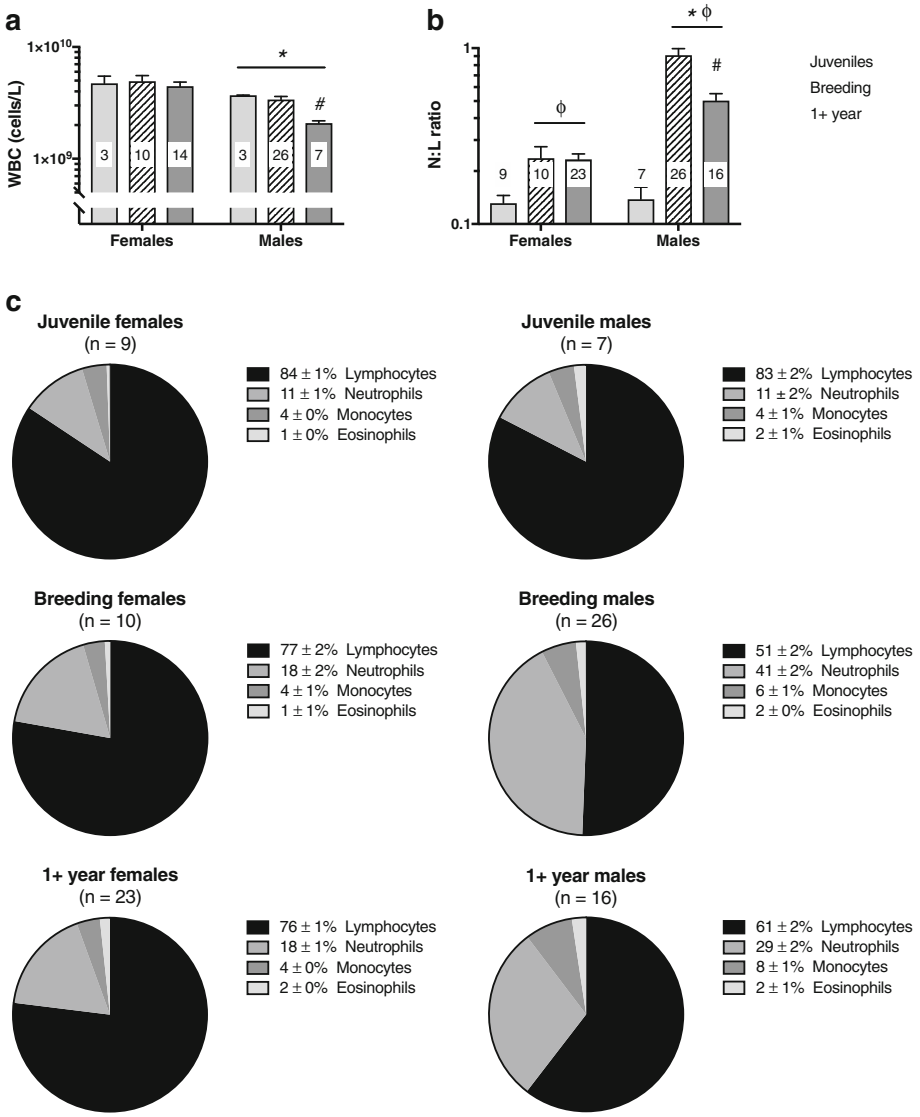

Fig. 4 Total and differential white blood cell (WBC) counts vary across different age-sex groups. **a** Total WBC counts, **b** neutrophil to lymphocyte (N:L) ratio and **c** the proportion of each cell type are presented for each age-sex group (*Juveniles:* light gray; *Breeding:* hatched fill; *1+ year:* dark gray). In **a** and **b**, data are log-transformed and expressed as mean ± SEM. In **c,** data are expressed as mean ± SEM. Numbers indicate the number of individuals examined (n). Asterisks (*) indicate a statistically significant difference between males and females ($P < 0.05$). # indicate a statistically significant difference between 1+ year males and breeding males ($P < 0.05$). Φ indicate a statistically significant difference compared to juveniles ($P < 0.05$)

be healthy. Low eosinophil counts were obtained for all age-sex groups and no basophils were identified. No hemoparasites or other anomalies were detected during the examination of the blood smears.

Adult males display splenic lymphoid depletion

Histopathological analyses revealed changes in the spleen of adult males compared to other age-sex groups ($n = 6$; Fig. 5 and Additional file 1: Figure S2). The scores for the size of the white pulp were significantly different ($p = 0.010$) between adult males (mean rank = 19.00) and male and female juveniles (mean ranks = 8.00). A higher score represented a smaller proportion of white pulp (white pulp atrophy). There was also a statistically significant difference ($p = 0.018$) between the scores for the size of the follicles of adult males (mean rank = 5.08) and male and female juveniles (mean ranks = 16.33), thus reflecting the smaller size of the follicles in adult males. The scores for the presence of germinal centers were significantly different ($p = 0.002$) between adult males (mean rank = 6.50) and juvenile females (mean rank = 20.6). Similarly, a significant difference ($p = 0.006$) was observed for this parameter between adult females (mean rank = 7.67) and juvenile females (mean rank = 20.6). No difference was

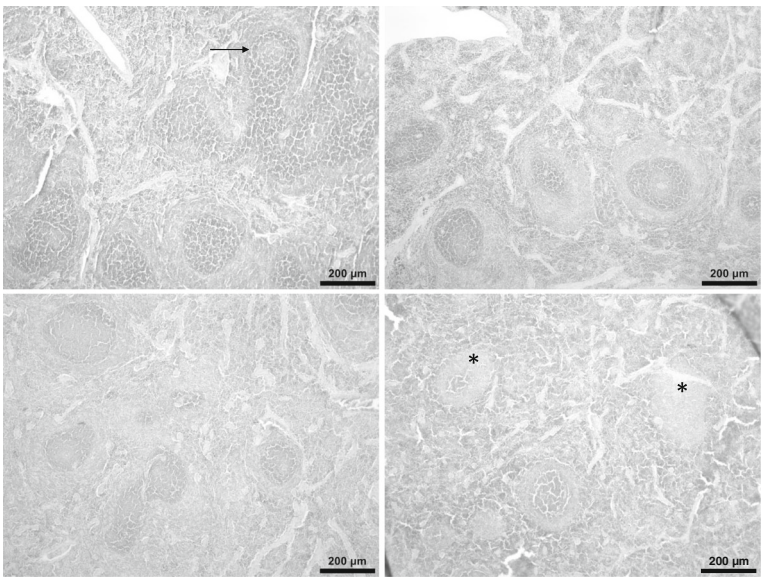

Fig. 5 Representative spleen sections ($n = 6$) of the splenic white pulp across different age-sex groups (*Juvenile females:* top left; *Juvenile males:* top right; *Adult females:* bottom left; *Adult males:* bottom right). The arrow shows a germinal center and the asterisks (*) mark areas of lymphoid depletion. Scale bars represent 200 µm

detected between any age-sex groups in the scores for the width of the marginal zone ($p = 0.164$), an antigen presenting cell-rich zone [34]. In fact, the white pulp atrophy seemed to affect more particularly the B and T cell-rich areas (lymphoid depletion).

Discussion

This study aimed to characterize the physical, hematological and immunological changes that occur during the life history of the red-tailed phascogale in captivity. An integrative approach combining post-mortem examinations, blood biochemistry, hematology and spleen histopathology was used to detect potential endocrine or geriatric disorders, as well as signs of immunosenescence. Juvenile animals were used as a baseline for comparison with individuals euthanized during or after their first breeding season. However, as the red-tailed phascogale is a 'Near Threatened' species [13], samples could only be obtained opportunistically and the low sample size sometimes limited our statistical power and the hypotheses that could be tested. While these data should obviously be interpreted cautiously, this paper nevertheless offers an insight in the physiological changes undergone by the red-tailed phascogale in captivity and will help define the normal blood values of this species.

Juvenile males and females presented no macroscopic lesions at post-mortem examination and both sexes shared comparable values of PCV, TP, total and differential WBC counts. The values were similar to previously published data obtained from red-tailed phascogales captured in the wild before the breeding season [10]. Histopathological analyses of the spleen also showed that juvenile males and females have well-defined areas of white pulp; a well-developed periarteriolar lymphatic sheath (PALS), numerous large follicles that occasionally harbor germinal centers, and a clearly defined marginal zone, as previously described by Old, et al. [24] and Borthwick and Old [29].

The body condition and health status of males going through their first breeding season was of particular interest, given the dichotomy in the survival of wild and captive males beyond this period. In the wild, males lose body weight, shed fur, and eventually die from gastric ulcerations and hemorrhages at the end of the breeding season due to an excess of circulating glucocorticoids [1, 2]. In the present study, only limited signs of hypercortisolism (or any other potential endocrine disorder) could be detected. A large number of males did present multifocal to extensive alopecia during the breeding season, but the incidence of fur loss subsided later on. A loss of body weight was also detected towards the end of the breeding season. However, males quickly regained weight after the reproductive period, sometimes even reaching obesity levels. Other non-specific signs like pododermatitis and hepatomegaly have also been observed in adult males, but only anecdotally. Therefore, captive males do not seem to develop a marked endocrine disorder like their wild counterparts during the breeding season. Woods and Hellgren [35] similarly evaluated physiological, morphological and immunological changes of male Virginia opossums (*Didelphis virginiana*) during the mating season and concluded that they differed from the semelparous dasyurid physiology.

Reproductive senescence due to spermatogenic failure has been reported previously in other semelparous dasyurids; a marked level of degeneration of the seminiferous epithelium and a senescent state of the testes have been described in a second-year brush-tailed phascogale [36] and in the brown antechinus [37]. The spermatogenic failure is thought to be permanent, with no possibility of recovery in future breeding seasons. In the present study, the scrotal width was found to decrease at the end of the breeding season, similar to what has been reported for wild red-tailed phascogales [2]. However, the scrotal width increased again temporarily during the males' second summer (November – March). It remains unknown how this affects their testosterone production capacity as captive males become infertile after their first breeding season [22]. Further studies are required to characterize potential sexual hormone fluctuations and testicular histological changes in this species.

Until now, there were limited or no reference intervals available for the red-tailed phascogale for most of the blood biochemical markers tested in this study. The red-tailed phascogale is carnivorous, and a high dietary protein intake is known to alter some parameters like the BUN and albumin levels [38]. We therefore compared our data with the values reported for another carnivorous marsupial in captivity, the Tasmanian devil [33]. Interestingly, the BUN, sodium and albumin levels of the red-tailed phascogale appeared relatively elevated compared to those for the Tasmanian devil, even in juvenile individuals that would otherwise be regarded as healthy. While these changes could be compatible with pre-renal azotemia (reduced kidney perfusion), dehydration seems unlikely as the animals had unlimited access to water and room temperature was maintained at 22 ± 4 °C. These changes most probably reflect physiological adaptations of the red-tailed phascogale to life in semi-arid and arid regions [39], as described for the cactus mouse (*Peromyscus eremicus*) in captivity [40]. Alternatively, the use of a reagent rotor designed for an herbivore species (VetScan® Equine Profile Plus) might have biased the levels of some of the biochemical parameters tested in our samples. Hemoconcentration nevertheless seems to occur during the breeding season as the PCV and TP both reached their highest values in both sexes during this period. Animals might be less prone to drink during the breeding season given the increased animal density and number of stressful social interactions. Water obtained through drinking has been reported to account for more than 1/3 of the total water intake in captive Tasmanian devils and dusky antechinus (*Antechinus swainsonii*) [41, 42], thus suggesting the importance of this source of water for these dasyurids in captivity. However, the energy and water requirements of wild red-tailed phascogales have been suggested to be lower than those of other dasyurid marsupials [18, 43].

Regular access to a high quality diet (nutritionally balanced and highly digestible as described by Stannard and Old [16]) is likely to improve the overall health and longevity, compared to an opportunistic diet in the wild or ad hoc diets often fed in captivity [15]. High quality nutrition in a controlled environment may thus explain why captive males and females globally maintained a good body condition throughout their lives. It may also explain why older females had a PCV comparable to that of juveniles, while nutritional anemia was reported for adult females in the wild [10]. The PCV values should however be interpreted with caution, as CO_2 euthanasia could have led to artificially elevated values [44]. Moreover, it is interesting to note that, in spite of calcium supplementation in the diet, the two 5-year-old females that were examined here had relatively low free calcium levels. Hypocalcemia is associated with conditions like age-related osteoporosis and chronic kidney disease [45]. The low BUN:CRE ratio observed in old individuals could also suggest an early onset of renal disease or hepatic insufficiency, two relatively common conditions of geriatric animals [46]. Urinalyses and a complete liver function test on more individuals would nevertheless be required to ascertain that. Alternatively, the altered BUN:CRE ratio could reflect a change in the kidney filtration rate due to endocrine changes. The filtration rate has been shown to decrease seasonally in brown antechinus males for example, and the modulation was found to be mainly driven by testosterone [47, 48]. Finally, the increased longevity of captive red-tailed phascogales might explain the incidence of masses detected in this study. Dasyurids appear to be prone to developing spontaneous proliferations, compared to other orders of marsupials [49–51]. Genetic predispositions, diet, breeding and environmental factors have all been proposed as potential causes for the high susceptibility of these species to proliferative disorders [49]. Hyperplastic and benign masses have been described in a large variety of dasyurid tissues, including lymphoid tissues, such as the spleen [49, 50]. However, this is the first time that a tumor is reported in the red-tailed phascogale.

With this increased knowledge of the physiological status of male and female red-tailed phascogales in captivity, interpretation of their immune profile at different age stages can now be attempted. As mentioned above, the proposed pathogenesis for the mortality of male dasyurids at the end of the breeding season is a failure of glucocorticoid feedback, which leads to exposure of tissues to deleterious amounts of biologically active cortisol [1, 9]. Increased concentrations of glucocorticoids can cause a "stress leukogram", which includes hematological changes

such as neutrophilia and lymphopenia and variable concentrations of eosinophils and monocytes depending on the species [52]. Wild red-tailed phascogale [10] and brown antechinus males [53], for instance, have been found to display a marked lymphopenia and neutrophilia towards the end of the breeding season. These changes are consistent with the development of a stress response. Similarly, in the northern quoll (*Dasyurus hallucatus*), total WBC counts have been shown to decrease over the breeding season [54]. In the present study, captive red-tailed phascogale males developed a marked lymphopenia and neutrophilia (stress leukogram) during the breeding season, with no variation of total WBC counts. Meanwhile, females maintained relatively stable N:L ratio and total WBC counts throughout their lives. Readers are invited to refer to Clark [52] for a detailed comparison of hematological values available in dasyurids. Comparisons between studies should however be made carefully as the use of different methods for blood collection can affect hematology results; sex differences in differential WBC counts have been detected in mice (*Mus musculus:* C57BL/6) when blood was obtained via cardiac puncture, but not with peripheral blood [55], and exposure to CO_2 has been associated with leukocytosis and lymphocytosis in rats (*Rattus norvegicus*: Sprague-Dawley) [44]. Morphological analysis of blood cells and manual differential counts also pose limitations in terms of precision and accuracy when compared to electronic differential counts [56].

After the breeding season, the lymphopenia exhibited by males became aggravated by a severe leukopenia. To determine whether these changes were limited to the peripheral blood or had more profound implications, we performed histopathological analyses of the spleen. We adapted the scoring system to red-tailed phascogale tissues, based on lesions described in rodents [57]. While these scores remain preliminary, results suggest that white pulp atrophy was present at various degrees in all adult males. The atrophy was mainly lymphoid; the marginal zone remained unchanged while the PALS and follicles were of reduced size. Only a limited number of germinal centers could be visualized in adults. Lymphocyte numbers have been reported to decrease with age in the white pulp of the spleen of many mammalian species and the absence of germinal centers is also a common finding in adult animals [34, 57]. However, the white pulp atrophy was found to be globally more severe in males than in females in this study. In fact, three out of the six 1.5-year-old males evaluated showed lesions that were much more pronounced than those presented by the six 3.5-year-old females examined. These results support the hypothesis that captive red-tailed phascogale males undergo accelerated immunosenescence compared to females.

Conclusions

No definitive conclusions can be drawn yet regarding the underlying mechanisms that lead to immunosenescence in the red-tailed phascogale. One or many mechanisms may be involved: testosterone is known to exert mainly immunosuppressive properties, in contrast to estradiol [58]; acute or chronic renal and liver disease have both been linked to a state of immune dysfunction [59, 60]; and hypercortisolism and obesity could be associated with advanced biological aging of the immune system [61, 62]. Future studies should investigate in vitro the role of these factors on the aging process using lymphoid cells derived from juvenile red-tailed phascogales. Alternatively, the use of specific inhibitors could potentially rescue cell function in adult males. Functional work with red-tailed phascogale cells shows great potential, particularly as the species can now breed successfully in captivity and tissues can be obtained opportunistically [20, 22].

In humans, many aging processes, including immunosenescence, are known to be expressed differently between males and females and to be further accelerated by stress [63, 64]. However, there are still very limited studies on the aging of the marsupial immune system. While the extreme reproductive strategy of semelparous dasyurids might be atypical among marsupials, it provides a unique opportunity for investigating the effects of sex and stress on the aging of the immune system [63]. Sex hormones are suspected to play a role in the immune susceptibility to devil facial tumour disease (DFTD) in the Tasmanian devil [65], and environmental and anthropogenic stressors have been shown to compromise the immune response and disease resistance of many marsupial species [66–68]. Characterization of these immune-endocrine interactions may help refine veterinary treatment plans in captivity, husbandry protocols and conservation programs to optimize wild populations' health and fitness in this increasingly challenging environment [69, 70].

Methods

Animals and tissues

Red-tailed phascogales used in the present study were sourced from a captive colony breeding annually since 2008 in the Western Sydney University (WSU) School of Science and Health Native Mammal Teaching and Research Facility, Richmond, NSW, Australia. Animals were initially sourced from the Alice Springs Desert Park captive colony established in 2001. During the breeding season, phascogales were housed in groups of up to five females with one male. After the breeding season, adult males were housed individually, while each mother and her pouch young were kept as a group in one enclosure. Enclosures ($1 \times 1 \times 0.58$ m^3) were made of wood, lined with glass, with a glass sliding door [22]. Animals were

provided with a wooden nest box lined with shredded paper. Breeder's Choice kitty litter (Fibre Cycle Pty Ltd., Queensland) served as substrate and native plant branches were provided for behavioral enrichment. A natural photoperiod was supplied through a window and skylight. Ambient room temperature was maintained at 22 ± 4 °C. Adults were fed daily 15 g of their scheduled diet (either adult mouse, crickets, mealworms, cockroaches, or neonate mice with calcium and vitamin E supplementation), with ad libitum access to water, as described previously [17]. Animal care was in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and the New South Wales Animal Research Act and its Regulations. All protocols and standard operating procedures were approved by the WSU Animal Care and Ethics Committee (A11197).

All samples were collected opportunistically from animals euthanized during routine population management procedures between November 2016 and October 2017. Animals were captured by hand from their nest box using a calico bag and were euthanized using carbon dioxide (CO_2) asphyxiation and cervical dislocation. Animals were classified as juveniles until 9 months of age, and animals from 10 to 13 months of age (May to August) were designated in the "breeding" category, as males were introduced to females in early May [20, 22] and as physiological and hematological parameters are known to be altered until at least late July in wild populations [10]. Unfortunately, data from older animals could not be further divided per season due to the uneven distribution of animals euthanized over the year. All animals older than 13 months were therefore grouped in the "1+ year" category. Altogether, 45 females (9 juveniles, 10 breeding and 26 adults [1+ year]) and 57 males (8 juveniles, 31 breeding and 18 adults [1+ year]) were included in this study. The age of the animals ranged from 3 months to 5 years for females, and 4 months to 2 years for males.

Blood and spleen samples were collected immediately after death, and a postmortem examination was conducted for each animal to identify any potential sign of disease. Animals presenting an inflammatory condition were excluded from the analyses, unless otherwise indicated. Body weight and biometric measurements were obtained from all animals. Body condition was assessed according to a scoring system (scale, 1 to 5; normal, 3) adapted from Ullman-Cullere and Foltz [71]. In males, the scrotal width was determined to the nearest 0.5 mm using Vernier calipers. Blood analyses were performed opportunistically depending on the availability of blood. Whole blood was aseptically collected via heart puncture and placed into dry tubes for serum biochemistry or sodium EDTA-coated tubes for other types of analyses. Blood was kept on ice and immediately transported to the laboratory. For spleen histopathological analyses, juvenile males and females (5–6 months old), and adult males (1.5 years old) and females (3.5 years old) were used ($n = 6$ per group). The spleen was fixed in 10% neutral buffered formalin for at least 48 h before further processing.

Microhematocrit and blood biochemistry

The microhematocrit (packed cell volume, PCV) and total proteins were evaluated to help detect, among other things, hemoconcentration, anemia, intestinal malabsorption or protein-losing pathologies. The microhematocrit was measured using glass capillary tubes (Hirschmann Laborgerate 75 mm X 1 mm) that were filled with approximately 70 μl of EDTA-blood and spun at 10,000 rpm for 5 min. Plasma total proteins were determined using a hand-held refractometer (American Optical) for samples that showed no hemolysis. In addition, biochemistry analyses were performed on serum from 8 randomly selected healthy animals, and 3 animals showing abnormal post-mortem findings to validate the test. 100 μl of serum collected after centrifugation (10 min at 5000 rpm) were analyzed using the VetScan® Equine Profile Plus reagent rotor (Abaxis, California, USA) to determine albumin (ALB), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (CA++), creatine kinase (CK), creatinine (CRE), gamma glutamyl transferase (GGT), glucose (GLU), total bilirubin (TBIL), total protein (TP), potassium (K+), sodium (Na+), and total carbon dioxide (tCO_2) levels. Biochemistry values were compared with the reference values available for the red-tailed phascogale [10] and captive Tasmanian devils, another carnivorous dasyurid marsupial [33]. GGT values obtained were below the detection limit and were thus excluded, and so were the values for glucose, K+ and tCO_2 as euthanasia with CO_2 is known to alter these parameters [72, 73].

Total and differential white blood cell (WBC) counts

Total leukocytes counts were performed on EDTA-blood samples diluted 1 in 10 with Turk's solution (0.01% crystal violet, 1% glacial acetic acid), a solution that lyses red blood cells and stains leukocytes to assist counting [74]. Cell counts were determined microscopically using an improved Neubauer chamber hemocytometer under 200X magnification and expressed as total cells/mL of blood. For differential WBC counts, blood films were prepared according to the method described in Clark [52] and were stained with Diff-Quik differential stain (Bacto Laboratories, Australia) as per the manufacturer's instructions. Cells were inspected at 1000X magnification for general morphology, nuclear structure, staining properties and characteristic attributes such as nuclear to cytoplasm ratio and the presence or absence of granules [75]. Differential WBC

Table 2 Scoring system for the histopathological analysis of spleen sections

Score	Size of the white pulp (atrophy)	Presence of germinal centers	Size of the follicles	Width of the marginal zone
1	> 40% of field	1–9% of follicles	< 15% of field	3–6 cells
2	26–40% of field	10–19% of follicles	16–30% of field	7–10 cells
3	11–25% of field	20–29% of follicles	31–50% of field	11–15 cells
4	< 10% of field	> 30% of follicles	> 50% of field	> 15 cells

counts are expressed as mean percentages of each cell type (lymphocyte, neutrophil, monocyte, eosinophil, basophil). The neutrophil to lymphocyte ratio (N:L) corresponds to the quotient of these two percentages.

Spleen histopathology

The spleens were processed and embedded in paraffin wax as described in Slaoui and Fiette [76]. Tissues were cut longitudinally at 9 μm, and sections of each sample were mounted on StarFrost silane-coated slides (Knittel Glass, Germany). The slides were dried at 40 °C on a slide dryer. Sections were dewaxed in HistoChoice (Sigma, Australia) and rehydrated in graded ethanol steps. The slides were stained with Mayer's Hematoxylin solution (Sigma) and Eosin Y-solution 0.5% alcoholic (Merck, Germany). The sections were then dehydrated in progressive ethanol steps, cleared in HistoChoice, and mounted with Entellan (ProSciTech, Australia). The slides were viewed with an Olympus BX60 compound light microscope. Images were acquired and prepared with the software Image-Pro Premier (v. 9.0). Histopathology scoring on a 4-grade scale (grade 4 being the highest) was developed based on lesions described in rodents [57] and principles detailed in Gibson-Corley, et al. [77]. Scorings were performed according to the criteria presented in Table 2. The size of the white pulp can be defined as the proportion of white pulp in a field of view (periarteriolar lymphatic sheaths [PALS], follicles and marginal zones; evaluated at 100X, median of 3 fields); the highest scores correspond to progressive degrees of "white pulp atrophy". The proportion of follicles that contain a germinal center was also evaluated (at 100X, median of 3 fields), as well as the size of the follicles and the width of the marginal zone (evaluated at 400X, median of 5 fields). Masking and randomization of samples were done prior to scoring.

Statistics

Two-way ANOVAs were conducted to examine the effects of age and sex on body weight, PCV, TP, WBC counts and N:L ratio. Residual analysis was performed to test for the assumptions of the two-way ANOVA. Normality was assessed using Shapiro-Wilk's normality test for each cell of the design and homogeneity of variances was assessed by Levene's test. Variables that proved to be non-homogeneous were log transformed before analysis. All parameters with unequal sample sizes respected the equality of variances assumption, thus allowing for the use of ANOVA. All pairwise comparisons were run, and *p*-values were Bonferroni-adjusted.

Paired comparisons for seasonal variations of body weight and scrotal width in males were analyzed using the student's T test. Outliers were assessed by inspection of a boxplot. Normal distribution of data and homogeneity of variances were confirmed by Shapiro-Wilk's test and Levene's test, respectively.

For comparison of the histopathological scores between groups, a Kruskal-Wallis H test was run for each parameter. Distributions of scores differed between groups, as assessed by visual inspection of a boxplot. Pairwise comparisons were performed using a Bonferroni correction for multiple comparisons. Values are mean ranks, unless otherwise stated, and adjusted *p*-values are presented.

All statistical analyses were performed using the IBM SPSS Statistics software (v. 24). A $p < 0.05$ was considered as statistically significant. Data are expressed as mean ± SEM, unless otherwise stated.

Additional file

Additional file 1: Table S1. Biometric measurements. **Table S2.** Microhematocrit and plasma total protein concentration. **Table S3.** Total WBC counts and WBC differential counts. **Figure S1.** Splenic tumor of benign appearance in a 5-year-old female; 9-μm section (100X). Section shows large, dilated vascular spaces filled with RBC. Scale bar represent 100 μm. **Figure S2.** Histological scorings of the splenic white pulp across different age-sex groups ($n = 6$). Spleens were evaluated using a 4-grade scale for four parameters: size of the white pulp (atrophy), presence of germinal centers, size of the follicles and width of the marginal zone. Individual data are plotted and the median for each group is illustrated. # indicate a statistically significant difference compared to juvenile animals ($P < 0.05$). (DOCX 310 kb)

Abbreviations

AST: Aspartate aminotransferase; BCS: Body condition score; BUN: Blood urea nitrogen; CK: Creatine kinase; CRE: Creatinine; N:L ratio: Neutrophil: lymphocyte ratio; PALS: Periarteriolar lymphatic sheaths; PCV: Packed cell volume; TP: Total proteins; WBC: White blood cells

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Authors' contributions
CL and JMO designed the research; CL and ES performed the experiments; CL, LJY and JMO analyzed the data, CL and JMO wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Animal care was in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and the New South Wales Animal Research Act and its Regulations. All protocols were approved by the WSU Animal Care and Ethics Committee (A11197).

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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