Spontaneous Pathology of Endangered Geriatric Julia Creek Dunnarts (*Sminthopsis douglasi*, Archer 1979) with Emphasis in Reproductive Pathology

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Simple Summary: This study focuses on the spontaneous pathology observed in a captive colony of geriatric Julia Creek dunnarts, an endangered carnivorous marsupial. Despite their conservation status, no prior research has focused on spontaneous pathology affecting captive individuals attaining senescence. We examined 36 captive-born, mostly elderly dunnarts that failed to reproduce over several breeding periods. From these, ten dunnarts had normal findings. Among females, common issues included cystic endometrial hyperplasia (7 cases), cystic ovaries (2 cases); cutaneous lesions were infrequent (2 cases). Males showed testicular degeneration (3 cases), aspermatogenesis, or atrophy. Cutaneous lesions compatible with epitheliotropic T cell lymphomas, were more frequent in males (3 cases). We speculate that male gender and hormonal dysregulation could be used as a predictor for lymphoid neoplasia, particularly in animals with testosterone-luteinizing hormone imbalances, although the role of an underlying oncogenic viral etiology is also possible. This is the first study that documents spontaneous diseases in aging Julia Creek dunnarts, shedding light on geriatric diseases within a conservation context.

Abstract: Julia Creek dunnarts are an endangered species of carnivorous marsupials and the focus of multiple conservation strategies involving significant resources such as captive breeding programs. Despite the relevance for conservation, no study to date has focused on evaluating geriatric diseases in dunnarts. This study describes the pathology findings in a group of 36 captive-born, mostly geriatric Julia Creek dunnarts that failed to produce offspring over multiple breeding periods. A total of 20 females and 16 males were submitted for a postmortem examination, with ages ranging from 9-42 and 12-42 months for females and males, respectively. Of these, 10 had unremarkable findings. The most common condition in females was cystic endometrial hyperplasia (n=7) and cystic ovaries (n=2), typical of hormonal dysregulation profiles in senescence, particularly hyperestrogenism. Rarely, cutaneous disease represented by unidentified dermal round cell infiltrates was observed in females (n=2). Primary reproductive hormonal dysregulation was also suspected in males diagnosed with testicular degeneration, necrosis and aspermatogenesis and/or atrophy (n=3). Cutaneous round cell infiltrates, possibly compatible with epitheliotropic lymphomas were more common in males (n=3), and 2/3 affected males also had concurrent testicular degeneration or necrosis, indicating male sex could be a predictor for lymphoid neoplasia in aged dunnarts especially in individuals with concurrent testosterone-luteinizing hormone dysregulation as it occurs in gonadectomized animals. The role of an underlying viral etiology is also explored. This study is the first to describe major spontaneous diseases in endangered aged Julia Creek

dunnarts, providing an important understanding of senescence and geriatric diseases within a conservation context.

Keywords: aging; captive breeding; Dasyuridae; lymphoma; marsupial; senescence; reproductive pathology; *Sminthopsis douglasi*

1. Introduction

Captive breeding programs have been used to counteract wildlife population declines, maintain gene pool diversity, achieve threat mitigation and reintroduce future self-sustaining populations. Such programs have been key in the recovery of multiple species such as the Arabian oryx (*Oryx leucoryx*), California condor (*Gymnogyps californianus*) and the golden lion tamarin (*Leontopithecus rosalia*), amongst others [1–3]. However, captive breeding is considered a last resort conservation tool due to the inexorable behavioral, phenotypical and genetic challenges faced by wildlife in captive environments. Successful establishment of captive breeding populations is challenging and requires careful planning to evoke an appropriate environment to achieve successful reproduction, major logistics, long-term financial and administrative continuity as well as avoiding domestication. These conditions are necessary to secure the maintenance of healthy individuals and ensure successful reintroductions and long-term maintenance of genetically viable populations.

Captive breeding has been one of the conservation tools used to secure healthy breeding stocks of Julia Creek dunnarts (*Sminthopsis douglasi*), a small nocturnal Australian carnivorous marsupial of the family Dasyuridae endemic to the state of Queensland. Longevity is similar to other small marsupials; wild male and female Julia Creek dunnarts live up to 12 and 24 months, respectively [4], with records extending to three years in captivity. Typically they are found in low densities [5] and their habitat includes grassland ecosystems with heavy clay soils and a rich diversity of invertebrate and vertebrate prey [4]. The species was considered extinct in the 1980s and was re-discovered from owl pellets, cat remains and by the capture of live specimens a decade later [6,7]. Currently, Julia Creek dunnarts are listed as vulnerable under the Environment Protection and Biodiversity Conservation Act 1999 and Endangered under the Nature Conservation Act 1992 in Queensland [5], with feral cats (*Felis catus*), invasive weeds (e.g. prickly acacia, *Vachellia nilotica*) outcompeting understory vegetation and heavy livestock grazing as important threats [8]. Additional threats include extreme weather from climate change, as Julia Creek dunnarts abundance patterns are positively correlated to rainfall which directly influences understory cover growth which provides shelter from potential predators [4]. Significant resources have been invested to bring forward a conservation plan for the species [9] which does not include disease management despite the documented predisposition of dasyurids to proliferative diseases [10]. Thus, diseases are not currently contemplated as a threat to the species and thus no active measures to investigate or prevent the occurrence of common diseases have been proposed.

As other wildlife managed in captive environments, Julia Creek dunnarts are vulnerable to potential disease outbreaks and age-related diseases as they easily outlive wild conspecifics. Due to the direct impact of spontaneous geriatric diseases on their conservation, this manuscript focuses on documenting the reproductive and non-reproductive lesions observed in geriatric Julia Creek dunnarts held in a captive breeding colony.

2. Materials and Methods

Population and Data Collection

The colony was initially formed in the 1990s following the capture of eight wild-caught founder individuals that were held at La Trobe University prior to their transfer to the Pearcedale Conservation Park (PCP) in Victoria [6,9], where an additional eight wild-caught dunnarts from Bladensburg National Park were transferred to the captive colony in April 2001 [6]. In August 2002,

12 dunnarts (3M:9F) were transferred from PCP to David Fleay Wildlife Park (DFWP) on the Gold Coast in Queensland, Australia. In August 2006 a male was introduced into the captive colony and was the last wild caught dunnart to be introduced into the colony. The captive colony was transferred from DFWP to The University of Queensland (UQ) Native Wildlife Teaching and Research (NWT&R) Facility on the Gatton Campus in March 2009 (Animal Ethics permits SAS/930/08/BREED, SAFS/441/11/BREED; Queensland Government permit WIEP05809909).

Reproductive management of the captive colony at UQ involved monitoring female dunnarts for signs of estrus (increased presence of epithelial cells in urine, running wheel activity and feed residue) and were paired for breeding. Following the placement of the male in the female's enclosure (n=298), mating occurred in approximately one third of the introductions from 2009 to 2014.   A total of 11 litters (47 joeys) were produced at the NWT&R Facility with 98% surviving to weaning.

The colony in the UQ NWT&R Facility rapidly attained senescence with multiple individuals reaching up to 3.5 years (lifespan 1-2 years) failing to produce offspring, despite observed matings across multiple breeding seasons. The absence of new genetics into the colony contributed to inbreeding depression resulting in the disbanding of the colony in early 2014. Dunnarts eligible for inclusion in this study were found dead aged > 1 year or euthanized due to age and/or signs of disease (e.g. progressive loss of body condition, abdominal distension, lethargy, alopecia) which failed to reproduce upon reaching senescence and received post-mortem examination. There were two subsets of dunnarts. The first subset were those that had received a post-mortem examination and follow-up histopathology at the time of death/euthanasia (1991 – 2014, n = 17).   Detailed post-mortem examinations were performed in ten cases and histopathology was conducted in sections of formalin-fixed tissues received in an additional seven cases from offsite autopsies. Relevant case material was procured from archived samples from the UQ Pathology Department. The second subset were those that had died/been euthanised between 2009 and 2012 and preserved at -20°C (n = 19) and autopsied after a slow thawing process at 4°C in April 2023. Details about origin, postmortem workup, broad lesion category and demographics are included in Supplementary Table 1.

Gross examination, Sample collection and Processing

A detailed post-mortem examination was conducted in each dunnart on the first subset following UQ's standard operating procedures. Representative samples of main organs were collected and fixed in 10% buffered formalin prior to routine histopathology processing, to be paraffin-embedded, sectioned at 4 um, mounted on glass slides, and stained with hematoxylin and eosin (H&E) stain. Due to the expected severe freezing artefact and lack of funding to cover for histopathology of all main organs, histopathology was only carried out in the reproductive tract and skin in the second subset of dunnarts showing gross lesions. Immunohistochemistry (IHC) was done in samples from individuals of both subsets showing cutaneous round cell infiltrates.

Ancillary Testing

IHC was carried out using DAKO OMNIS Automatic Stainer with 4 um sections and 30-minute heat fixation for tissue adhesion at 70°C following published protocols [11]. Variations to the protocol for each IHC marker (CD3, CD20, CD79a and PAX5) were carried out to follow internal validation protocols for quality control and institutional standard operating procedures.

3. Results

Main demographic data, reproductive tract and significant non-reproductive tract morphological diagnoses are included in Tables 1 and 2 and in Supplementary Table 1. A total of 36 dunnarts from the colony were submitted for a post-mortem examination, 20 females and 16 males from 2009 through 2022. From these, five geriatric males, one adult non-geriatric male and four adult, non-geriatric females were excluded from analyses as their gross and histopathological examinations were unremarkable or had non-reproductive lesions (e.g. cryptococcosis in male dunnart 127) or their histopathology examination was outsourced (fibrosarcoma in the hindlimb of male dunnart 176).

Few dunnarts had good to excellent (n=5) or adequate/moderate body condition (n=6). Poor body condition was recorded in 9 dunnarts. Body condition was not disclosed in the clinical history of 5 dunnarts. Weight was recorded in the second subset of autopsies with dunnarts being in normal to excellent body condition when weighing around >52 g (n=9), whilst those in poor body condition weighing often <50 g (n=10).

Table 1. Female reproductive and non-reproductive tract lesions in endangered geriatric Julia Creek dunnarts from a captive breeding colony.

Diagnoses	Dunnart ID	Age (months)
Ovaries		
Multilocular follicular cyst	2	24
Cystic ovary	6	25
Ovarian cystadenoma	3	27
Oviduct		
Cystic oviduct	10	42
Uterus		
Cystic endometrial hyperplasia/dysplasia, squamous metaplasia	7	25
Cystic endometrial hyperplasia with mucin, anovulatory ovaries	8	25
Cystic endometrial hyperplasia	10	42
Cystic endometrial hyperplasia	24	24
Cystic endometrial hyperplasia, polyp formation	27	24
Cystic endometrial hyperplasia, squamous metaplasia	11	20
Cystic endometrial hyperplasia, squamous metaplasia, polyp	14	42
Endometrial polyp	23	12
Endometrial polyp, cystic	30	24
Endometrial polyp, cystic	32	24
Endometrial adenoma (mixed type)	13	42
Cervix and vagina		
Neutrophilic cervicitis and vaginitis with fibrosis	8	25
Neutrophilic vaginitis	10	42
Skin		
Round cell infiltrates, superficial dermis, non-epitheliotropic	30	24
Round cell infiltrates, mid-to-deep dermal, epitheliotropic	33	12

For the second subset of dunnarts in the present study (n=14), there was a similar mating success (33.33% from 168 introductions) to the colony. Only two litters (6 joeys) were produced resulting in a lower birthing success; however, all six offspring survived to weaning.

Table 2. Male reproductive and non-reproductive tract lesions in endangered geriatric Julia Creek dunnarts from a captive breeding colony.

Diagnoses	Dunnart ID	Age (months)
Testes		
Testicular degeneration and necrosis with aspermatogenesis	4	42
Testicular degeneration and necrosis with aspermatogenesis and metastatic lymphoma	5	42
Testicular atrophy	9	42
Prostate		
Prostatic mineralization, multifocal	9	42
Lymphoplasmacytic prostatitis	12	42
Skin		
Cutaneous epitheliotropic lymphoma (mycosis fungoides)	1	24
Cutaneous epitheliotropic lymphoma (mycosis fungoides), with splenic and pulmonary metastasis	4	42
Cutaneous epitheliotropic lymphoma (mycosis fungoides), with splenic, pulmonary, and testicular metastasis	5	42
Cutaneous round cell infiltrates, telogen alopecia	25	48
Cutaneous round cell infiltrates	31	48
Telogen alopecia, hyperkeratosis	20	36
Telogen alopecia	28	24
Telogen alopecia	29	24

3.1. Gross Pathology - Female Reproductive Tract

Lesions were often found affecting the uteri, mainly represented by cystic endometrial or adenomatous hyperplasia (CEH, Figure 1A-C), and less frequently by cysts affecting other parts such as ovarian follicles (Figure 1D). Reproductive inflammation was uncommon and included most often vaginitis (Figure 1E). No cases of malignant neoplasms were observed. Grossly, when the reproductive tract was affected by cystic disease, lesions were more often evident in the endometrium (dunnarts 7-8, 10-11, 14, 27), rarely accompanied by cystic expansion of adjacent anatomy such as the oviduct (dunnart 10). In three cases, cystic disease affected the ovary only (dunnart 2-3, 6); one of these represented by a cystadenoma (dunnart 3). Cystic ovaries were described as having unilateral severe distension, and the oviducts as having one enlarged 5-7 mm diameter fluid filled cyst each mostly in the proximal end. No macroscopic lesions were observed in the external genitalia in any of the females in this study. In general, and for those individuals for which information was recorded, disease of the reproductive tract in females was more often unilateral (n=14) in comparison to bilateral (n=3).

Figure 1. Spontaneous Gross Female Reproductive Pathology in geriatric Julia Creek dunnarts. A) Cystic endometrial hyperplasia (CEH), dunnart 14. A large, 2.5 cm diameter abdominal cyst filled by a pale tan fluid is expanding the cavity and displacing abdominal viscera. B) CEH, dunnart 11. The right uterus measures 3.5 cm diameter and is expanding the abdominal cavity displacing and compressing the abdominal viscera cranially as well as compressing the thoracic cavity causing pulmonary collapse. The uterine mass is filled with approx. 5 mL of dark red fluid. C) CEH and endometrial adenoma, dunnart 13. Rarely, a single uterus was expanded by two separate masses; the largest mass measures 2.5 cm diameter (arrowhead) and corresponds to CEH. The second mass (asterisk) is an endometrial adenoma of a mixed type, measuring about 2 cm diameter and filled by viscous dark brown-orange material. D) Cystic ovary, dunnart 6. The ovary is expanded by a single, unilocular mass filled by light yellow-pink fluid; bilaterally the uteri are appreciated below the mass. Photo credit: Peter Moore. E) Neutrophilic vaginitis, dunnart 10. Grossly, the lateral vaginas (arrowhead) were discolored off-white and moderately expanded. This dunnart also had CEH and a cystic oviduct (not on image).

3.2. Histopathology - Female Reproductive Tract

In the ovary, a cystadenoma was diagnosed (Figure 2A, dunnart 3) causing complete effacement of the ovarian architecture. Additionally, in one of the dunnarts with CEH, an anovulatory ovary was found (Figure 2B, dunnart 8). A cystic oviduct was observed (Figure 2C, dunnart 9) causing luminal expansion and degeneration of the oviductal mucosa. Most cases in which the endometrium was affected, corresponded to CEH (Figure 2D, dunnart 7), displaying the typical disruption of the endometrial mucosa due to irregularly-dilated and cystic endometrial glands. An endometrial adenoma was observed (Figure 2F-G, dunnart 12) and was diagnosed as a mixed mucinous and squamous type, resulting in the production of large amounts of mucin. Lesions in the cervix and vagina were infrequent (two cases) and were represented by acute, neutrophilic infiltrates or late-stage inflammation with fibrosis (Figure 2H, dunnart 10), without cystic formations or neoplasms as in the other portions of the reproductive tract.

3.3. Additional significant non-reproductive disease - Female Dunnarts

From the second subset, two dunnarts presented cutaneous round cell infiltrates, dunnart 30 and 33. In detail, dunnart 30 had a cystic uterine polyp and also presented with bilaterally, markedly thickened skin with alopecia and with focal, asymmetrical/unilateral erosions grossly, which histologically corresponded to a moderate proliferation of round cells lacking epitheliotropism but infiltrating and separating the dermal collagen in an equivocal sheet pattern. Dunnart 33 with did not have concurrent reproductive lesions and was diagnosed instead with deep to mid-dermal round cell infiltrates that were not immunolabeled by any of the immunohistochemical markers used, as well as with cutaneous erosions and severe alopecia.

3.4. Gross Pathology - Male Reproductive Tract

Lesions were only grossly visible in the testes and scrotum. No lesions were observed for the epididymis, ducts deferens, bulbourethral glands or external genitalia. Main lesions observed were primarily degenerative, represented by testicular atrophy (Figure 3A) causing a dramatic reduction in the length of the inguinal canal compared to a control (Figure 3B). Asymmetrical testes were noted in two cases, one of which was confirmed as unilateral testicular degeneration and contralateral testicular necrosis (Figure 3C). For those individuals in which information was recorded, reproductive disease was more often unilateral (n=3) in comparison to bilateral (n=1) as seen in females.
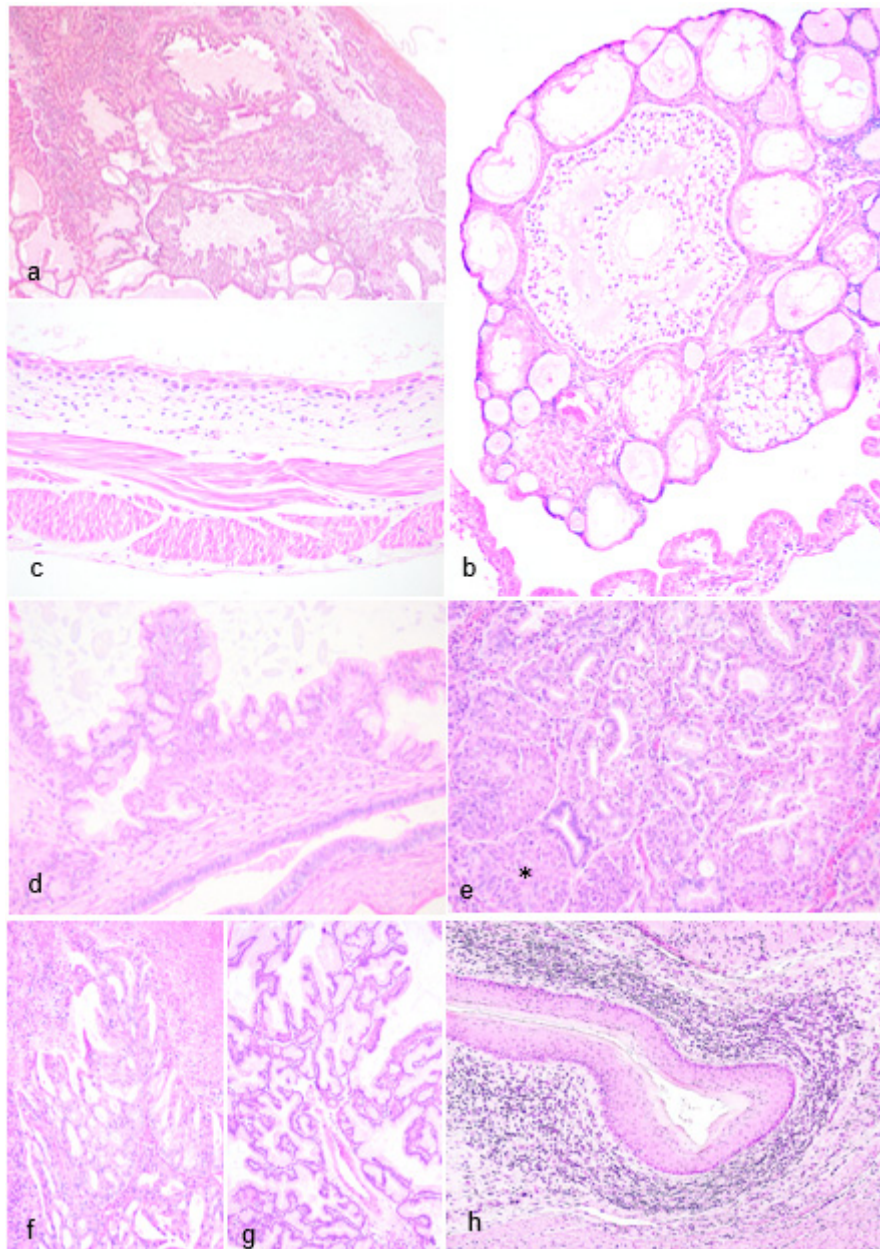
Figure 2. Female Reproductive Pathology in Geriatric Julia Creek Dunnarts. **A) Ovarian cystadenoma, dunnart 3**. A large encapsulated neoplasm causes complete effacement of the ovarian architecture, formed by haphazardly arranged nests and tubulo-papillary structures projecting into the cystic lumen. Hematoxylin and eosin (H&E) stain, 4X. **B) Anovulatory ovary, dunnart 8**. Low numbers of primordial or primary follicles, few secondary follicles and 2 corpora albicans are observed. No corpora lutea are present, potentially indicating acyclic state. H&E stain, 10X. **C) Cystic oviduct, dunnart 9**. The oviduct is expanded by multilocular cysts lined by a single layer of ciliated attenuated columnar epithelium, H&E stain, 40X. **D) Cystic endometrial hyperplasia (CEH) with mucin, dunnart 7**. High magnification view into one of the cystic structures found expanding the endometrium formed by anastomosing trabeculae of thin fibrous connective tissue septae lined by a single line of ciliated columnar secretory epithelium, with abundant goblet cells, H&E stain, 20X. **E) CEH with squamous metaplasia, dunnart 11**. The endometrium is expanded by numerous islands and anastomosing trabeculae forming cysts filled by fluid and necrotic debris lined by tall columnar cells. Foci of squamous metaplasia are observed (asterisk). H&E stain, 20X. **F-G) Endometrial adenoma, mixed type (mucinous and squamous), dunnart 12**. Two proliferating cell populations are observed; the predominant population is represented by stratified and pseudostratified non-keratinising epithelium (Figure F) lining fine fibrovascular cores forming fronds, covered by large amounts of necrotic debris. H&E stain, 20X. The second population (Figure G) corresponds to

columnar, mucus secreting epithelial cells. H&E stain, 10X. **H) Suppurative vaginitis, dunnart 10**. Florid neutrophilic inflammation is observed multifocally exocytosing into the vagina mucosa and infiltrating the lamina propria, H&E stain, 10X.
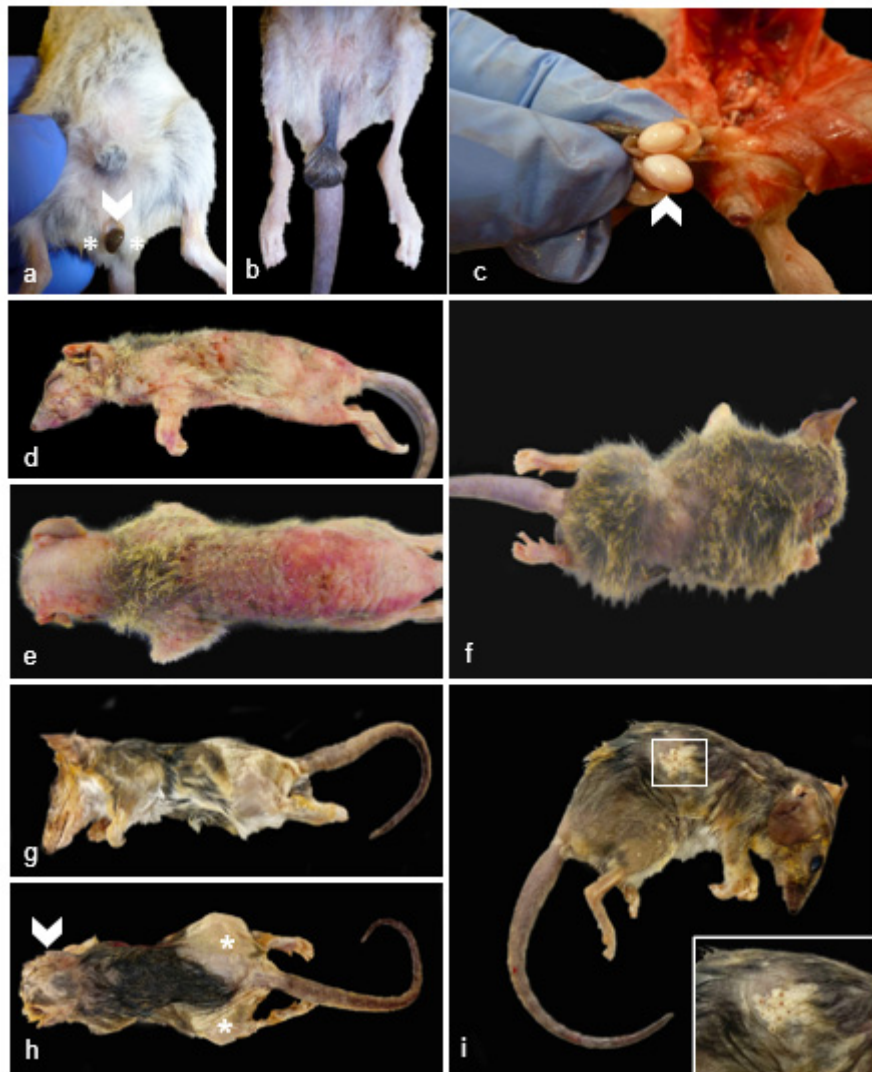
Figure 3. Gross Male Reproductive and Non-reproductive Pathology in Geriatric Julia Creek Dunnarts. A) Testicular atrophy, dunnart 9. The scrotal sac has a markedly shortened inguinal canal turning it into a sessile structure with no obvious palpable testes, easily exposing the urogenital sinus (arrowhead) and bulbourethral glands (asterisks). B) Normal testes and scrotum, control dunnart. Scrotum contains palpable testes and is suspended from the abdomen by a regular stalk with the inguinal canal containing the spermatic cord, completely covering the urogenital sinus and partially the bulbourethral glands. C) Testicular degeneration and necrosis, dunnart 4. The right testicle (arrowhead) is degenerated and is about 1.5 times larger than the left testicle, which is globally smaller and unevenly discolored pale yellow, indicating necrosis. D-E) Cutaneous epitheliotropic lymphoma, dunnart 4. Extensive areas of alopecia affecting 90% of the body with diffusely markedly thickened skin that is mottled pink, red and with multiple scabs and flaking in the caudo-dorsal region. The coat is maintained in the distal cervical and proximal dorsal and flank region. F) Dermatitis with alopecia, dunnart not included in study included for comparison. An approx. 2 cm band of circumferential alopecia is observed in the cranial abdomen. Follicular atrophy was noticed on histopathology. Note the skin is smooth, not crusty, ulcerated nor thickened as in the epitheliotropic lymphoma cases. G) Telogen alopecia, dunnart 25. Lateral view. Regionally extensive areas of alopecia are observed predominantly affecting the lateral thighs and rump region in this view. The skin appears thickened

and focally wrinkly.   H) Telogen alopecia, dunnart 25. Dorsal view. About 50% of the body presents a bilaterally symmetrical alopecia, particularly focused in the head, lateral thighs and rump region. I) Telogen alopecia, dunnart 31. There is bilaterally symmetrical alopecia affecting the lateral thighs, rump and cranio-dorsal abdominal region. Inset: Alopecia was occasionally observed along comedone formation. The right pinna has been removed post-mortem from all dunnarts in the photos for DNA analysis.

3.5. Histopathology - Male Reproductive Tract

Testicular degeneration and necrosis with aspermatogenesis (Figure 4A-B, dunnart 4) were diagnosed in two geriatric males, one of which also had a primary cutaneous epitheliotropic lymphoma resulting in splenic, pulmonary and unilateral testicular metastasis (Figure 4C, dunnart 5). Severe testicular atrophy was diagnosed in one case in which no intrascrotal testicular tissue was identified (Figure 4D, dunnart 8). Lesions in the prostate were mild and represented by interstitial inflammatory infiltrates (Figure 4E, dunnart 12) or intratubular calcified concretions (Figure 4F, dunnart 9).

3.6. Additional significant non-reproductive disease - Male Dunnarts

In the first subset, significant non-reproductive disease in males was represented by three cases (dunnarts 1, 4, and 5) of primary cutaneous epitheliotropic lymphoma resulting in severe and extensive gross cutaneous lesions (Figure 3D-E) compared to a lymphoplasmacytic dermatitis of unknown origin (Figure 3F). Telogen alopecia was also observed in four geriatric males (dunnarts 20, 25, 28 and 29; Figure 3G-H) occasionally along comedone formation (Figure 3I). In the second autopsied subset of dunnarts, two additional male dunnarts (dunnarts 31 and 33) were found with round cell infiltrates in the dermis, all manifesting in a clear sheet pattern but with dunnart 31 lacking epitheliotropism. On histopathology, the cutaneous epitheliotropic lymphoma cases presented with a densely-cellular neoplasm arranged in sheets (Figure 5A, dunnart 5) expanding the dermis, reaching the subcutis and multifocally infiltrating follicular epithelium (Figure 5B, dunnart 4) extending to the epidermis filling vesicles (Figure 5C, dunnart 5).

3.7. Histochemistry and Immunohistochemistry (IHC)

Toluidine blue was run in the second group of autopsied dunnarts that had cutaneous round cell infiltrates as frequently there were larger, rounded cells accompanying the primary infiltrating population. All samples were negative. IHC was ran in a lymph node as control tissue for leukocytic markers and in skin and intestine samples to test pancytokeratin (AE1/AE3) labelling in 6 dunnarts (dunnarts 20, 25, 30-33) belonging to the second subset of autopsies. Nodal control tissue had adequate immunolabeling pattern of paracortical T (CD3) and cortical B cell (CD20, CD79a, Pax-5) markers. However, there was minimal panleukocytic (CD68), histiocytic (Iba-1), or haemopoietic tumour markers (CD45 LCA) staining in control tissue. Pancytokeratin labelling was adequate in control tissue, and negative in the cells inducing the cutaneous round cell infiltrates.
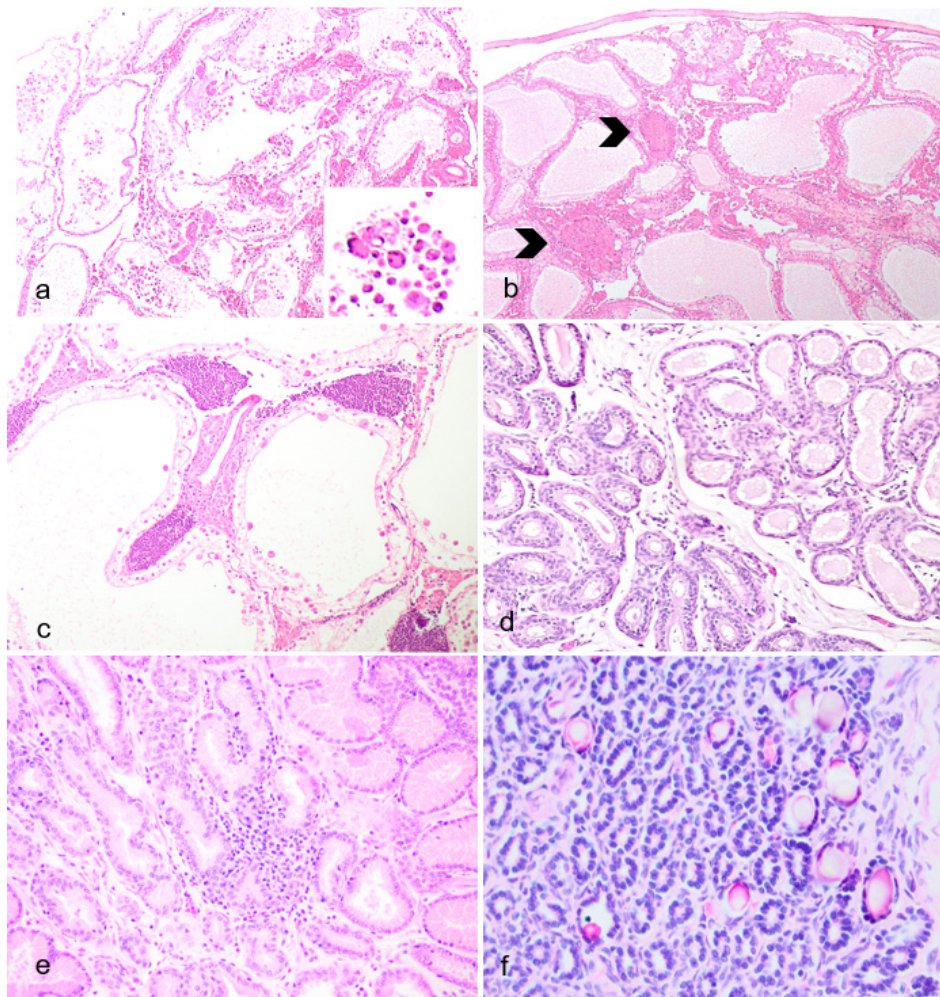
Figure 4. Male Reproductive Pathology in Geriatric Julia Creek dunnarts. A) Testicular necrosis with azoospermia, left testis, dunnart 4. Seminiferous tubules are almost completely devoid of germinal and the highly-resistant Sertolli cells with only the denuded basement membrane remaining. The interstitium is edematous. Hematoxylin and eosin (H&E) stain, 4X. Inset: Small aggregates of occasionally multinucleated spermatid multinuclear giant cells. B) Testicular degeneration with azoospermia, right testis, dunnart 4. The seminiferous tubules are devoid of early and late spermatids; no spermatogenesis is observed. Supporting basement membrane is wavy, buckled and thickened. Surrounding interstitium is multifocally expanded by moderate non-compressive hyperplasia of interstitial (Leydig) cells and mild edema. Few granulomas are observed replacing seminiferous tubules (arrowheads), likely resulting from tubular shrinkage and collapse and/or spermiostasis. H&E stain, 4X. C) Metastatic testicular lymphoma, dunnart 5. The interstitium is unilaterally expanded by dense aggregates of lymphocytes, mainly within vasculature. H&E stain, 10X. D) Testicular atrophy, dunnart 8. No identifiable testicular tissue was found within the scrotum, only a portion of the epididymis. Some of these tubules contain protein however no sperm is visualised. H&E stain, 20X. E) Prostatitis, dunnart 12. The interstitium is multifocally expanded by small aggregates of lymphocytes and occasional plasma cells. H&E stain, 20X. F) Calcified prostatic concretions, dunnart 9. Multifocally within glandular lumina are variably-sized, irregularly-shaped mineral deposits. H&E stain, 40X.
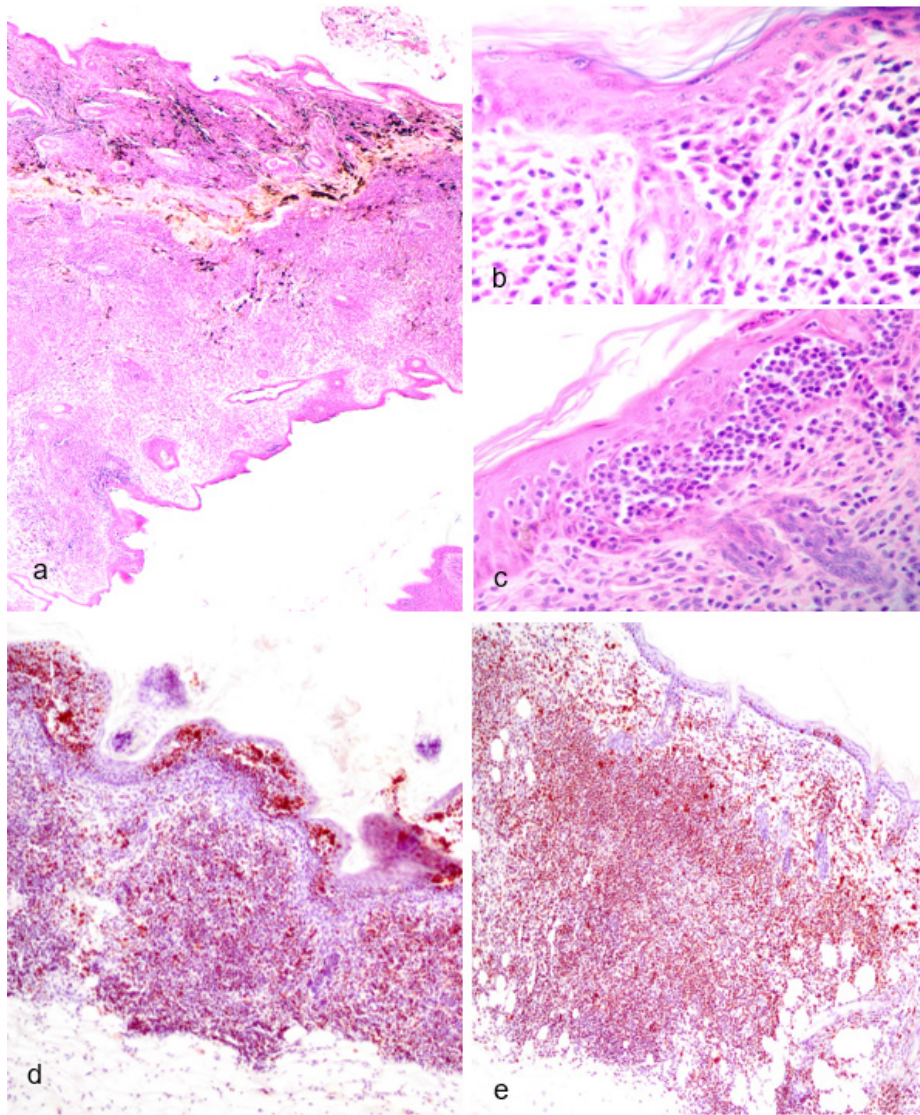
Figure 5. Non-Reproductive Pathology of Geriatric Male Julia Creek Dunnarts. A) Primary cutaneous epitheliotropic lymphoma (mycosis fungoides), dunnart 5. The dermis is infiltrated and replaced by a non-encapsulated and densely cellular neoplasm arranged in sheets. Hematoxylin and eosin (H&E) stain, 4X. In multiple areas, the subcutis is also infiltrated. B) Primary cutaneous epitheliotropic lymphoma (mycosis fungoides), dunnart 4. Dermal infiltrate is predominantly comprised of lymphocytes with hyperchromatic, convoluted or cerebriform nuclei admixed with occasional histiocytes and granulocytes. Multifocally, hair follicles are effaced by the infiltration of low numbers of neoplastic cells within the outer root sheath. H&E, 40X. C) Primary cutaneous epitheliotropic lymphoma (mycosis fungoides), dunnart 5. Multiple intraepidermal vesicles are filled with pleomorphic lymphoid cells (Pautrier's microabscesses) and occasionally by solitary cells surrounded by a clear halo. H&E, 40X. D) Peripheral diffuse cutaneous epitheliotropic lymphoma, dunnart 1. Sheets of neoplastic T-cell lymphocytes are observed expanding the dermis, displacing adnexa and infiltrating and expanding the epidermis forming discrete and often coalescing Pautrier's microabscesses. CD3 immunohistochemistry – IHC. 10X. E) Peripheral diffuse cutaneous epitheliotropic lymphoma, dunnart 1. Neoplastic T-cell lymphocytes also reached into and isolated adipocytes. CD3 IHC. 10X.

3.8. Non-reproductive, non-clinically significant findings - Geriatric Male and Female Dunnarts

In the first subset, non-reproductive pathology was also observed but was generally considered of little or with no clinical significance (e.g. mild inflammatory infiltrates in stomach, intestine, kidneys, and/or liver - 5 cases combined, mild hepatocellular lipidosis - 2 cases, or mild hepatic glycogen storage - 1 case); associated with senile changes (e.g. multifocal mild renal mineralization or fibrosis - 3 cases, hepatic lipofuscinosis - 1 case), or euthanasia artefacts (e.g. pulmonary hemorrhages, congestion and edema - 7 cases).

4. Discussion

In captivity, female Julia Creek dunnarts become sexually mature between 17-27 weeks and males between 28-31 weeks [4]. Similar to other dasyurids, dunnarts have evolved reproductive strategies to maximize fitness within their short life spans (2-3 years) such as: being seasonally polyestrous, avoidance of semelparity (rapid senescence and death after first breeding season as in Antechinus sp.), having a spontaneous ovulation, multiparity and the ability to store sperm; unique amongst marsupials [12,13]. In addition, in situ surveys have also found that Julia Creek dunnarts can raise two litters in an extended breeding season, which is a common reproductive trait in species inhabiting semi-arid ecosystems where food availability is unpredictable [4]. Despite these adaptations, captive dunnarts unable to breed rapidly attain senescence, increasing their vulnerability to aging-associated conditions as observed in this study. Photoperiod cues could offer some insight into reproductive failure particularly in male daryurids. As photoperiod increases in the southern hemisphere, plasma cortisol levels do so as well, negatively influencing spermatogenesis [14]. Mortality coincides in certain dasyurids with elevated post-mating plasma cortisol levels as well as a testosterone-dependent, post-mating decrease in plasma corticosteroid-binding globulin. Such reproductive strategy might bring positive, short-term reproductive effort benefits for species evolved to have few mating encounters, via increasing energy mobilisation. However, it can also induce fatal syndromes resulting in anaemia, anorexia, hippocampal neuronal apoptosis, increased vulnerability to parasitic and bacterial infections, decreased splenic follicle size, amongst other issues. The role of reproduction in post-mating morbidity and mortality is further supported by the significant increase in lifespan upon castration or pre-mating removal of males [14].

Susceptibility to age- and non-age-related hyperplasias, benign and malignant tumors vary considerably between marsupials or dasyurids. Interestingly and according to the literature, dasyurids are overrepresented in terms of neoplastic disease compared to other mammals, with one study reporting an incidence of 46% in this family [10] compared to 2-10% reported for other mammals [15]. None of the reported tumors of high incidence in dasyurids or other marsupials such as adenocarcinomas, mammary carcinomas, trichoepitheliomas, squamous cell carcinomas, fibrosarcomas, hemangiomas or hemangiosarcomas, and hepatic adenomas were observed in this study, with the exception of lymphoma [14,16]. Neoplasia in Julia Creek dunnarts was only represented by multiple cutaneous lymphomas, an uterine adenoma, and an ovarian cystadenoma. Additionally, the literature reports degenerative conditions in geriatric dasyurids primarily affecting nervous or musculoskeletal system. Aged quolls (Dasyurus sp.) and Tasmanian devils, (>3 years old) acquire progressive leukoencephalopathies, myelopathies and intervertebral disc disease [17]; these were not observed in this study.

It is important to note that the present study had a relatively small sample size and unfortunately not all dunnarts received a thorough postmortem examination with histopathology. These factors could have negatively influenced our ability to have a more accurate demonstration of geriatric dunnart diseases. Nonetheless, the literature on dunnart diseases is very limited compared to other marsupials. This fact is not surprising as diseases are not considered a significant threat to dunnart conservation, compared to other dasyurids with well-studied diseases such as the case with Tasmanian devils, Sarcophilus harrisii [18] or other marsupials, such as koalas, Phascolarctos cinereus [19]. This fact has understandably re-directed research efforts into mapping conservation-priority habitat or answering ecology or biology questions [4,7,12,20]. A few studies regarding naturally-occurring disease in captive fat-tailed dunnarts (S. crassicaudata) documented dermal

spindle cell tumors, splenic lymphomas, squamous cell carcinomas and round cell sarcomas of the upper limb [10], whilst other stressed the vulnerability of captive dunnarts to reverse transmission of zoonotic pathogens, when human-derived Helicobacter pylori infection resulted in an outbreak of gastric bleeding and weight loss [21]. The cause of the high incidence of neoplasia is currently unknown but genetic predisposition to lymphoma has been suggested in fat-tailed dunnarts, which grants further exploration of this question in dunnart species [10].

Little information is available in peer-review literature on ovarian tumors in marsupials or more specifically, dasyurids, with scattered reports of ovarian hemangiomas and adenocarcinomas in quolls and koalas [22,23]. Most of the lesions found in the female reproductive tract of these dunnarts are considered degenerative changes, namely CEH and cystic ovarian follicles and oviduct which, as in other species, is associated to long-term exposure to female reproductive hormones in non-castrated animals [24]. Interestingly, this prolonged exposure is also a risk factor for mammary neoplasia, which was not observed in this study despite being reported in other marsupials [16,25]. The reasons behind this discrepancy are unknown. It is worth noting that in our study, there were 5 of 11 female dunnarts that did not meet the inclusion criteria (autopsy at NWT&R with no histopathology) also had gross ovarian lesions (dunnart 134, 159, 170, 200), illustrating the high frequency of reproductive lesions in this colony.

As reported elsewhere in possums [16,23], CEH was the most common disorder of growth in aged female dunnarts, as in aged rabbits, mice, rats and dogs [26–28]. Uterine cysts and uterine metaplasia has also been reported in mature grey short-tailed possums (Monodelphis domestica) [29]. Malignant neoplasia appears to be of common occurrence in marsupial literature [10,30] and from these, carcinomas occur but are more often associated to other tissues (e.g. pulmonary, mammary gland) rather than endometrium [16,30–33]. Interestingly, primary malignant reproductive neoplasia was not observed in this study and likewise, it was found to not be the major cause of death in a captive colony of endangered mountain pygmy-possum (Burramys parvus), the report focusing instead on progressive renal disease [25]. Endometrial carcinomas are uncommon in animals except for the rabbit, cattle and certain rat strains. Multiple parallels regarding reproduction have been identified between rabbits and dunnarts which might provide insights into the pathogenesis of reproductive disorders in dunnarts. The two most common uterine disorders in rabbits are adenocarcinomas – affecting rabbits at any age and commonly resulting in pulmonary metastases - and CEH, with endometrial inflammation being uncommon [34,35]. CEH was the only entity frequently observed in this study. Notably, vaginal serosanguineous discharge and abdominal distension were frequent clinical findings in captive Julia Creek dunnarts with CEH and are also observed in rabbits with adenocarcinoma [35,36]; thus, it is recommended that any future captive breeding attempt in the species incorporates frequent checkups for either of these two clinical signs, with culling being a suitable endpoint. Ovariohysterectomy is the only successful treatment to date, with an overall post-surgical survival of 80% in rabbits [35]; however, this would not be a viable treatment option for a captive breeding colony, where fertility is important. In rabbits with endometrial adenocarcinoma, the non-carcinomatous foci in the endometrium have evidence of CEH, suggesting an association with hyperestrogenism. As induced ovulators, both rabbits and dunnarts can have quite prolonged estrus and experience little luteal activity, bringing a persisting estrogen:progesterone ratio favoring estrogens [37]. At least in the rabbit, there is evidence of CEH evolving to malignant anaplastic carcinoma [36] so it is unknown if given sufficient time, the CEH dunnart cases would have turned malignant. Future research in this area evaluating the degree of hormone dependence of endometrial carcinomas and CEH could help in understanding the pathogenesis of spontaneous proliferative reproductive disorders in dunnarts.

Both cases of vaginitis and/or cervicitis were predominantly neutrophilic, suggesting an underlying acute ascending bacterial infection, despite bacteria not observed on histopathology. Vaginal and cervical inflammation have been reported before in marsupials, mostly due to ascending bacterial infections from the cloaca (site of convergence for urogenital and alimentary tract) or due to tears during mating with an overly aggressive male [38].

Cases of testicular degeneration are consistent with advanced age as reported elsewhere in other aged male marsupials [29]. In one case (dunnart 9), the left testicle was unilaterally enlarged, necrotic, and with a diffusely edematous tunica albuginea, suggesting that the necrosis was secondary to impaired thermoregulation [39]. Testicular atrophy is seen as an advanced state of degeneration, with mineralization and fibrosis. Causes are multiple, however intrinsically, aging is a well-recognized predisposing factor in all species, probably due to degenerative vascular lesions in testis or pampiniform plexus [39]. Interstitial (Leydig) cell hyperplasia was also observed in one case of testicular degeneration and is a recognized phenomenon in aged animals with or without atrophied testes. These multifocal proliferations were interpreted as hyperplasia rather than adenoma as the diameter of the hyperplastic foci was less than three seminiferous tubules [40].

Prostatitis has been reported in sugar gliders (Petaurus breviceps) and Virginia opossums (Didelphis virginiana) mostly associated with urinary tract infection resulting in hematuria, constipation and local pain, none of which were observed in this study perhaps due to the mild nature of the inflammation observed in dunnart 11 [31,38]. Venerally-transmitted, cytomegalovirus-associated prostatitis has been reported in other Australian dasyurids, the brush-tailed phascogale (Phascogale tapoatafa), the brown antechinus (Antechinus stuartii), and the dusky antechinus (A. swainsonii; [41,42]). As with other cytomegaloviruses, lesions were most frequent during stressful periods. If the inflammation observed in dunnart 11 was virally-induced, it is unlikely that viable virions would still be observable in the tissue due to chronicity. Nonetheless, future studies should consider a potential cytomegalovirus infection in dasyurid cases showing prostatic compromise. Prostatic mineralization is considered a senile change lacking clinical significance.

Cutaneous lymphoma was frequent in male dunnarts. There were two females in which both gross and histopathology findings could support a putative diagnosis of lymphoma; however, this diagnosis could not be confirmed due to the severe artefact present in the tissue impeding further testing. Thus, we will only discuss cutaneous epitheliotropic lymphoma in the male cohort. Dunnarts with cutaneous epitheliotropic lymphomas presented with extensive alopecia with crusting and ulcerative lesions in the skin. Interestingly, lesions were remarkably similar in distribution and severity, which could indicate a viral etiology. Oncogenic viruses, specifically those of the retroviral lineage, such as the Koala Retrovirus and Gunnison's Prairie Dog Retrovirus are associated with lymphoid neoplasia in wildlife in Australia and elsewhere [43,44]. As males were slightly overrepresented with lymphoid neoplasia in this study, it is less likely that a viral entity is involved although it might be worth exploring this hypothesis in future studies. Cutaneous epitheliotropic T-cell lymphoma (ETL) is primarily a disease of aged animals and has been reported in a variety of marsupials [45,46] and dasyurids, including Tasmanian devils [47]. In dogs, lesions develop from a patch displaying significant epitheliotropism into a tumour stage with metastasis in as little time as 5.5 months [48] with cutaneous forms (as the dunnarts in this study) considered independent predictors of poor survival compared to mucocutaneous/mucosal forms [49]. The ETL disease spectrum is divided into mycosis fungoides (MF; most common form, strong tropism for epidermis and adnexa), pagetoid reticulosis (MF with lymphadenopathy and circulating neoplastic lymphocytes) and Sézary syndrome (solitary plaque; [50]). According to this classification, the three ETL cases in this study correspond to MF due to the presence of: 1) intraepidermal vesicles containing pleomorphic neoplastic lymphocytes (Pautrier's microabscesses), 2) infiltration of follicles and adnexa (epitheliotropism), 3) pleomorphic infiltrate predominantly comprised of lymphocytes with occasional histiocytes and granulocytes. Predisposing factors to cutaneous ETL are not known, however, chronic inflammation and immune dysregulation, as it occurs in atopic dermatitis cases, is noted as a risk factor for dogs [51]. It remains to be determined if chronic dermatitis, as diagnosed in one male excluded from the study, predisposes aged dunnarts to cutaneous ETL. Another possible risk factor for dunnart lymphoma occurrence is the male sex, as indicated by previous studies in neutered dogs, and humans [52,53] suggesting an endocrine dysregulation component. Interestingly and perhaps unsurprisingly, 2 of the 3 dunnarts with ETL also had concurrent testicular degeneration and necrosis, which would lead to low circulating testosterone levels and subsequently, supraphysiologic luteinizing hormone (LH) levels due to the absence of negative feedback to the

hypothalamus and anterior pituitary axis as would happen in gonadectomized animals. LH has receptors in reproductive and non-reproductive tissues such as lymphocytes and lymphoid tissue (thymic medulla); the function of these receptors is still not fully understood but appears to relate to induction of cell division, amongst others [54]. High LH would lead to a constant activation and magnification of LH effects in non-reproductive tissues, including T lymphocytes [55]. LH-receptor positive T lymphocytes have been shown to circulate in higher numbers in gonadectomized animals compared to intact individuals [56] plausibly stimulating cell division through T-lymphocyte bound LH receptor activation. It is possible that dunnarts with testicular degeneration and necrosis have comparable endocrine profiles to gonadectomized dogs leading to similar pathophysiological effects of supraphysiologic LH levels.

The insufficient leukocytic marker immunolabeling observed in nodal control tissue hampered our ability to run IHC to definitively classify the cutaneous lymphomas observed. Possible issues that could have negatively impacted IHC immunolabeling quality include poor preservation of sample prior to freezing, age of samples (nearly a decade old), preservation artefact (freezing), lack of proper validation procedures in the species, and prolonged or variable formalin fixation time between samples. It is worth noting that proper pancytokeratin immunolabeling was observed in control tissue that faced the same preservation and fixation challenges; thus, it is still possible that the minimal immunolabeling observed was, although greatly reduced, targeting the correct cell populations.

Hair loss has been extensively documented in captive wildlife and can be caused by stress, excessive grooming, parasites, integument diseases, dietary deficiencies, and infections (fungal and bacterial) amongst others. The alopecia observed could have some similarity with telogen effluvium, a well-described condition in domestic animals in which there is pelage loss associated with extrinsic physical, physiological, or mental stressors or improper diets. With telogen effluvium, there is excessive generalised hair shedding with reduction in hair volume that does not render complete baldness. The pattern of alopecia observed in the three males affected were similar to what has been reported in macaques [57] and bats [58] where stress has been detected as an important cause. Further, comedone formation, although infrequently observed, could support underlying stress or endocrinopathies as it has been documented in small animals [59].

Barbering – the act of plucking whiskers and fur in mice due to excessive grooming – would be an important differential diagnosis in these cases if it is confirmed that captive dunnarts can replicate such behaviour responding to social dominance [60]. Excessive hair pulling (trichotillomania) is different and has also been observed in macaques and can be partially corrected with environmental enrichment [61]. Despite whiskers being intact in this study, males were overrepresented with alopecia which may grant the exploration of hierarchical conflicts with females as a cause, as female mice are more likely to apply this behavior than males. As chronic stress is a well-recognized issue in captive wildlife and has been found to be associated with conditions observed in this study, future attempts at establishing captive breeding dunnart colonies could benefit from exploring environmental enrichment as well as the incorporation of non-invasive fecal glucocorticoid metabolite testing or hair cortisol levels as biomarkers to monitor stress levels [62].

Senescence is a risk factor for cancer development. Both mature age and cancer share disease mechanisms such as genomic instability, epigenetic changes, altered nutrient sensing metabolism and are fundamentally divergent in others, such as telomere attrition in geriatric cells in contrast to the ability to perform rapid cell division and high energy consumption of neoplastic cells [63]. The lifespan of captive Julia Creek dunnarts was increased by approximately one year and so the molecular mechanisms associated with aging are suggested as the underlying mechanisms for the interconnectedness of aging and neoplasia observed in this study. Nonetheless, future studies should aim at investigating other potential etiologies including oncogenic viruses.

Reproductive aging is a crucial phenomenon of concern in any conservation breeding program, particularly for genetically-valuable wildlife as research has shown long-term captivity can lead to prolonged exposure to endogenous sex steroids and long non-reproductive intervals resulting in asymmetric reproductive aging and low reproductive performance [64]. In asymmetric reproductive

aging, females in which first breeding attempts and pregnancies are substantially delayed result in frequent cyclic fluctuations of estrogen subsequently causing reproductive disease as tested in cheetahs (Acinonyx jubatus) [65]. The theory is interesting and could be applied to dasyurids although its veracity remains to be tested. To avoid reduced fertility and irreversible acyclicity especially in short-lived wildlife, captive breeding programs can benefit from pioneered assisted reproductive technologies to induce pregnancies as early as possible and avoid asymmetric reproductive aging [64].

5. Conclusions

From the findings reported in this study, it can be inferred that captive breeding colonies of dunnarts can benefit from including health examinations into their program, particularly upon reaching geriatric age. This study is the first to document a comprehensive list of spontaneous reproductive and significant non-reproductive conditions affecting geriatric dunnarts to improve the paucity of dasyurid disease literature. These results paired with effective management prescriptions including captive breeding, maintenance and restoration of land with essential structure for the species are vital for the long-term conservation success for Julia Creek dunnarts.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., Table S1: Pathology examination and patient outcomes of Julia Creek dunnart colony at The University of Queensland, School of Veterinary Science.

Author Contributions: Conceptualization, R.A. and V.G.A.; methodology, R.A., V.G.A., A.S.W; formal analysis, R.A., V.G.A.; investigation, R.A., V.G.A., T.B.; resources, P.O., P.M.; data curation, V.G.A; writing—original draft preparation, V.G.A.; writing—review and editing, V.G.A., R.A., A.S.W., L.N., P.O., P.M., T.B.; supervision, R.A., V.G.A.; project administration, R.A.; funding acquisition, V.G.A. All authors have read and agreed to the published version of the manuscript.

Funding: Immunohistochemistry was partially funded by a 2022 small research grant from the Wildlife Disease Association – Australasia section.

Institutional Review Board Statement: Ethical review and approval were waived for this study due to utilization of archived and/or donated samples.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to inclusion of personal data from submitters.

Acknowledgments: We appreciate the work of the professional and technical staff involved with caring for the dunnart colony over the years contributing with data record input and keeping. We thank Jo Gordon and previous histology technicians at The University of Queensland, School of Veterinary Science for providing histopathology support.

Conflicts of Interest: The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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