Anatomical and histological comparisons of the epiglottis between brachycephalic, mesocephalic and doliocephalic dogs and puppies

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Statement of Originality

This is to certify that the content of this thesis is my own work. This thesis has not been submitted for any other degree or purpose.

I certify that the intellectual content of this thesis is the product of my own work, and that all assistance received in preparing this thesis and all sources have been acknowledged.

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Abstract

In Brachycephalic Obstructive Airway Syndrome (BOAS), anatomical crowding of structures lead to interlinked pathophysiology involving increased negative airway pressure, turbulence and irritation. The syndrome presents with variable functional severity and increased risk of airway maintenance, thermoregulation and anaesthesia. The contribution of head morphology, nares, nasopharynx, tongue, soft palate, palatine tonsils, larynx, trachea and lungs in the pathophysiology of the syndrome has been well characterised. However, the epiglottal component of the larynx of brachycephalic dogs has scant anatomical and histological evaluation for its role in BOAS pathophysiology. In comparison to mesocephalic and doliocephalic dogs, it is hypothesised that the epiglottis of dogs affected by BOAS could have gross morphometric variation (dimension, distortion and relative volume), micromorphometric composition variation (epithelium, lamina propria-submucosa, cartilage core, chondral cell/matrix, fibrous and adipose tissue), and histological variation (inflammation, hyperplasia, reactive and degenerative changes). This study represents the first to demonstrate micromorphometric evaluation of six tissue components using camera-imaging software followed by area calculations from canine epiglottises using a manual approach. Results of this anatomical and histological study showed chondral cell/matrix area and chondral cell numbers were significantly greater in distal, middle or proximal sections of the epiglottis in adult brachycephalic dogs compared to mesocephalic dogs. While not statistically significant, adult brachycephalic dogs had relatively larger epiglottal dimensions compared to similar sized mesocephalic dogs, and brachycephalic individuals displayed subtle tissue proportion, reactive and degenerative differences. Findings suggest that the epiglottis has nuanced structural and histological differences in brachycephalic dogs interlinked with the pathophysiology of BOAS.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BC	Brachycephalic group
BOAS	Brachycephalic obstructive airway syndrome
DC	Doliocephalic group
H&E	Haematoxylin and eosin
MC	Mesocephalic groups

Chapter 1: Literature Review

1.1 Brachycephalic obstructive airway syndrome

Brachycephalic obstructive airway syndrome (BOAS) is a condition frequently associated with brachycephalic breeds that has variable functional severity with increased risk of airway maintenance, thermoregulation and anaesthesia. The anatomical features at multiple levels of the airway in brachycephalic dogs and their role in BOAS pathophysiology has been well characterised, however there are still some aspects which have had minimal anatomical and/or histological evaluation. This study will focus on the role of the epiglottis in BOAS pathophysiology.

1.1.1 Brachycephalia in dogs

Brachycephalia in dogs is characterised by a reduced facial length and wider skull width compared to mesocephalic and doliocephalic dogs and with severe shortening of the muzzle and flattened facial conformation in extreme brachycephalic breeds.[1-4] The genetic origins of brachycephalia are associated with multiple different chromosomes linked with skull shape notably on quantitative trait loci found on chromosomes 1, 5, 18, 24, 30, 32 and X.[5-7] Popularity of brachycephalic breeds with increasing facial flattening has been postulated to be a combination of their cute, akin to a human baby, neotenic features leading to increased caretaking responses and additionally contagious conformity trends that occur in human society.[8,9] However, the ensuing functional pathology due to this conformation has led to serious health and welfare concerns related to airway pathophysiology.[3,4,10] In turn the pathophysiological consequences of BOAS has led to a variety of descriptive publications, surgical treatments, complex health management systems and campaigns with associated legislation.[3,4,10-12]

There are several conventions used to define brachycephalia in dogs. These include a skull width to length ratio of 0.81 or greater, cranial length to skull length range of 1.60-3.44, skull length to skull width ratio less than 1.44, facial skull length to cerebrum length less than or equal to 1.25, and a craniofacial angle between the base of the skull and facial skull of 9-14° in brachycephalic dogs compared to 19-21° and 25-26° in mesocephalic and doliocephalic dogs respectively.[13-16] There are at least 24 recognised brachycephalic dog breeds, and these include the French bulldog, pug, bulldog, boxer, cavalier King Charles spaniel, Boston terrier, bullmastiff, dogue de Bordeaux, Pekingese, shih tzu, affenpinscher, Fila Brasileiro, Brussels griffon, Japanese chin and Lhasa apso.[10,15,17]

1.1.2 Clinical consequence of brachycephalia

Brachycephalic obstructive airway syndrome is described as a group of conformation abnormalities that deform the upper airway tract leading to respiratory obstruction at multiple levels.[4,18-20] Clinical signs associated with BOAS include noisy breathing, snoring, snorting, coughing, gagging, dysphagia, vomiting, regurgitation, aerophagia, flatulence, sleep apnoea, inspiratory dyspnoea, exercise intolerance, heat stress, cyanosis and collapse with death.[3,4,9,10,18-22] Affected dogs typically present with clinical signs at 1- 4 years of age that are often exacerbated by elevated ambient temperature and exercise.[15,18,23] As the condition is progressive, affected dogs tend to deteriorate proportionate to the severity of BOAS lesions, and there have been reports of dogs as young as a few months of age presenting with life-threatening events.[18,23,24] Clinical evaluation requires a detailed assessment which includes a pre-evaluation protocol, a functional grading system, BOAS-specific systemic evaluation, clinical pathology investigations and diagnostic imaging.[3,17,18] A plethora of non-respiratory conditions are also associated with brachycephalia ranging from skin, ocular, oral, auditory, spinal,

sternal and gastrointestinal diseases, in addition to dystocia and abnormalities of the patella.[3,18,25-30]

1.1.3 Pathophysiology

The characteristic flattened face of brachycephalic dog breeds is due to selective breeding for abnormal cartilage and bone development associated with ossification centres (localised chondrodysplasia) which result in growth inhibition of the dermal bones of the midface, early ankylosis of basal cranial cartilages of the skull, and occasionally tracheal hypoplasia.[9,15,21,31] The shortened craniofacial features without concomitant reduction of nasal, nasopharyngeal and oropharyngeal tissues results in an array of abnormal features that include stenotic nares, nasal passage compression and rotation, turbinate crowding and malformation, soft palate hyperplasia and elongation, macroglossia, hyoid malformation, and glottis and cricoid cartilage narrowing.[3,10,15,17-22,31-33]

Systemic effects of these anatomical changes include chronic hypoxia, reduced respiratory evaporative cooling capacity, elevated mean arterial blood pressure, pulmonary hypertension, respiratory acidosis, hypercoagulability, sleep apnoea, subacute inflammatory states, and hepatic and splenic injury and fibrosis.[3,15,18,33-40] Secondary changes then variably manifest including obstructive nasopharyngeal tissue and collapse, tonsillar eversion and hypertrophy, pharyngeal collapse, epiglottal retroversion, laryngeal saccule eversion, laryngeal oedema and collapse, tracheal hypoplasia, tracheal and bronchial collapse, right sided heart failure, gastroesophageal reflux, and aspiration pneumonia, which can progress to life-threatening respiratory obstruction, cardiovascular or thermoregulatory failure culminating in death.[3,10,15,18-20,22,32,41,42]

Stenotic nares, elongate soft palate and tracheal hypoplasia are considered the three primary contributors to BOAS leading to increased inspiratory resistance, increased respiratory effort, and increased negative intraluminal pressure during inspiration.[3,10,15,17,18,21,22,43] Turbinate crowding with aberrant growth, comparatively increased tongue size, and soft palate hyperplasia with entrapment can also contribute to BOAS.[3,10,15,18,21,44,45] Chronic tissue contact irritation, increased inspiratory pressure, and inefficient thermoregulation efforts (panting and salivation) leads to inflammation and oedema of the nasopharyngeal mucosa, hyperplasia of the soft palate, which further alters pharyngeal anatomy and narrows the airway lumen with generalised upper airway tissue inflammation.[3,9,20-23,43,46]

Chronically high negative pressures within the pharynx and turbulent airflow can lead to laryngeal narrowing, stridor and collapse.[3,15,17,20,21,47,48] Increased respiratory effort to overcome airway resistance progressively alters and weakens the laryngeal structures, leading to laryngeal collapse that is often preceded by pharyngeal collapse.[3,9,10,15,17,18,20-22,49] Increased intraglottic luminal pressure and increased air velocity causes medial displacement of the rostral laryngeal structures with permanent cartilage deformation resulting in a gradual collapse of the rostral laryngeal aperture, culminating in airway collapse, which can result in syncope and even death by suffocation.[15,23,50]

Tracheal and bronchial collapse can also occur with the left cranial bronchus most commonly affected.[10,15,21] Tracheal and bronchial collapse have been significantly correlated to laryngeal collapse and can also be associated with dynamic pharyngeal collapse.[3,23,51] Tracheal hypoplasia when present contributes minimally to BOAS, despite the smaller diameter contributing to increased airway resistance, however it can delay or prevent resolution of pneumonia.[3,23,52] Bronchial collapse and variable lung lobe

atelectasis in BOAS-affected dogs is thought to be due to chronic airway obstruction, negative pressure gradients, turbulent air flow, early age onset, increased force expiration requirements, small breed size and barrel chest conformation.[53-55] Aspiration pneumonia occurs more frequently in brachycephalic dogs and at an earlier age with emergencies and surgery further increasing its likelihood.[17,20,41,56] Gastrointestinal and respiratory disease are often interlinked with the negative intrathoracic pressure generated by the increased inspiratory efforts considered to be a major contributor to gastroesophageal reflux.[3,22,41,57] Upon increasing negative pressure and degenerative changes, continued airway narrowing, collapse and regurgitation-aspiration events may lead to a viscous pathological cycle of deterioration.[3,15,17,21,22]

1.1.4 Risk factors

The main conformation risk factors for the development of BOAS are muzzle length less than half cranial length, closed nostrils, thicker neck girths, and obesity.[3,9,18,40] These features are typified in extreme brachycephalic breeds such as pugs, French bulldogs and bulldogs.[3,58] Additionally, in pugs, females are more commonly affected than males, as are those with a wider head and wider set eyes.[4] In bulldogs and French bulldogs, males are more commonly affected, and bulldogs with increased head width and thick necks and French bulldogs with short necks and short muzzles are more commonly affected.[4,9,18] Pugs have the lowest glottic width of the extreme brachycephalic breeds and a higher risk of respiratory disease.[57,59] English bulldogs have an increased prevalence of tracheal hypoplasia (males more likely) and caudal aberrant turbinates, while French bulldogs have higher prevalence of intramucosal contact points.[46,60,61] However, these external factors account for less than 50% of the variance of BOAS.[4]

Brachycephalic dogs also have a higher anaesthetic risk, especially English bulldogs, as anaesthetic agents relax upper airways whilst allowing diaphragmatic contraction resulting in an increased likelihood of collapse; the latter is exacerbated by increased respiratory effort during inspiration.[4,33,62-64] Lastly, the coexistence of respiratory and gastrointestinal disease has been identified with them being considered risk factors for each other.[41,57]

1.1.5 Epidemiology

Compared to non-brachycephalic breeds, brachycephalic have a lower health status demonstrated by an increased odds ratio (x 1.60) for upper respiratory tract disorders; lower median life span (8.6 years versus 12.7 years) for pugs, bulldogs and French bulldogs with a high proportion of deaths associated with upper respiratory disease (16.7%); and increased relative risk of BOAS for pugs (x 53.92), French bulldogs (x 30.89) and English bulldogs (x 19.20).[12,58,65-67] Brachycephalic dogs have a higher relative risk (3.77 times more) of aspiration pneumonia than other breeds, with a median age disease onset of 6 months (bulldogs), 8 months (French bulldogs) and 83 months (pugs).[41]

1.1.6 Treatment and prognosis

Various surgical treatments have been developed for BOAS that focus on removing airway obstructive tissues using traditional, endoscopic, laser, cautery and intervention techniques. These include stenotic nares expansion, aberrant nasal turbinate removal, elongate and thickened soft palate resection, enlarged and everted tonsillectomy, everted laryngeal saccule resection, arytenoid lateralisation, subtotal epiglottectomy, laryngeal collapse management, temporary or permanent salvage tracheostomy, and hiatal hernia corrective surgery.[3,10,15,17,47,50,68-74]

Critical to BOAS management are detailed anaesthesia and complication prevention guidelines given the increased risks associated with brachycephalic breeds.[3,17,47] Medical therapy includes obesity management, emergency respiratory distress management, gastric reflux management and advanced monitoring post-surgery.[3,15,22,68,75-77]

Life-threatening post-surgical complications include airway swelling resulting in dyspnoea, regurgitation that comprises airway patency and surgical sites, and aspiration pneumonia; other potential complications include pulmonary oedema and infection or dehiscence of surgical sites.[3,15,68,78,79] Long-term complications have been reported as dyspnoea, heatstroke and bronchopneumonia.[80] Approximately 70-90% of clinically affected BOAS dogs have significant respiratory and digestive clinical sign improvement with surgery; early intervention, more advanced soft palate and multi-level techniques lead to enhanced long-term improvement; and negative prognostic indicators include laryngeal collapse, younger age at diagnosis and normal body condition.[3,15,68,74,81-86]

1.1.7 Welfare considerations

Serious welfare concerns for brachycephalic dogs continue to grow based on the increasing prevalence of chronic respiratory breathlessness and distress, exercise and heat intolerance, digestive and sleeping problems, and other associated pathology such as ocular, ear, skin and spinal diseases.[4,17,23,87,88] Numerous epidemiological studies have provided strong statistical evidence of lower health status for brachycephalic breeds, particularly in the more severely affected group of pugs, bulldogs and French bulldogs.[11,17,65-67] Strategies to improve welfare of brachycephalic dogs include national awareness campaigns on facial flattening linked disorders, the disuse of images of brachycephalic breeds, independent inquiry commissions, national advisory panels, dedicated BOAS clinics, petitions to companies and clubs, and working with kennel clubs

and associations.[23,47,87,89-95] In 2019, the Dutch government passed legislation banning the breeding of dogs with a snout length shorter than a third of their skull, and in 2022, the Norwegian Society for Protection of Animals won a lawsuit that made it illegal to breed the King Charles Cavalier Spaniel dog breed in Norway.[96,97]

1.2 Anatomical and microscopic features associated with BOAS pathology

The role of the head, neck, nares, nasal cavity, nasopharynx, pharynx, tongue, palatine tonsils, soft palate, larynx, trachea, bronchi and lungs in BOAS has broad coverage in the literature, with reduced histological information on the palantine tonsils and bronchi.[98,99] However, there is a scarcity of information on both anatomical and histological features of the epiglottal cartilage of the larynx; this knowledge gap led to this research study.[23]

1.2.1 Head and neck

The main drivers for airway passage compression are skull changes associated with rostrocaudal shortening of the muzzle and mediolateral widening of skull shape coupled with rostral muzzle angle change and mandibular prognathism.[9,10,21,23] Whilst these changes are present at birth in brachycephalic breeds, external conformation accounts for less than 50% of BOAS clinical signs.[4,100] Anatomical and brain endocast studies have demonstrated the association between brachycephalia and progressive pitching of the brain on its longitudinal axis and ventral shifting of the olfactory bulb.[101,102] Olfactory bulb angle reduction and soft palate thickness have been shown to be significantly associated with the degree of brachycephalia.[103,104] Pugs, the breed associated with the most severe craniofacial dimensions, have dorsal rotation of the maxillary bone, largely absent frontal sinuses, and ventral orientation of the olfactory bulb.[3,10] In addition, thicker necks in bulldogs and shorter neck length in French bulldogs have increased correlation with BOAS clinical signs.[9,18]

1.2.2 Nares and nasal cavity

Stenotic nares have frequently been reported as a significant external predictor of BOAS as airflow through the nostrils and nasal cavity accounts for approximately 77% of airway resistance in normal dogs.[4,17,21,22] Closed nostrils result from malformation of nasal cartilages and medial collapse of the alae, which can converge into vertical slits and have immobile nostril wings, resulting in continual open-mouth breathing in some dogs.[3,4,9,15]

Postnatal inhibition of the turbinate skeleton results in a shortened nasal cavity, leading to nasal turbinate crowding, hyperplasia or extension of turbinates into the nasopharynx.[31,105,106] Intra-nasal contact points were observed in 87.0 to 91.7% of brachycephalic dogs.[3,46] The distribution of narrow and wide regions within the nasal cavity differs in brachycephalic breeds, although a smaller airway cross sectional area to total nasal cavity area ratio can be associated with more severe clinical signs.[45,107,108] Rostral aberrant turbinates were reported to be common in pugs (90.9%), less frequent in French (56.4%) and English (36.4%) bulldogs, while caudal aberrant turbinates causing nasopharyngeal obstruction were common in all brachycephalic breeds (66.7%).[3,17,109,110] Pugs are more affected by the presence of rostral turbinates and have deviated nasal septa (98.5%).[3,109] Histologically, hyperaemia, oedema and lymphoid hyperplasia may be present in the nares and nasal cavity due to the presence of anatomical contact points and increased inspiratory pressures.[20,111]

1.2.3 Nasopharynx and pharynx

Brachycephalic dogs commonly have hyperplastic nasopharyngeal mucosa and a smaller nasopharyngeal cross-sectional area and volume.[3,43,112] Pugs have a smaller nasopharyngeal cross-sectional area compared to French bulldogs.[45]

Pharyngeal collapse occurs more commonly in brachycephalic dogs (72%) compared to non-brachycephalic dogs (28%) with or without airway collapse.[113] As a consequence of chronic negative pressure and inspiratory resistance which may be exacerbated by obesity, it is suspected that the pharyngeal dilator muscles undergo pathological changes, predisposing to pharyngeal collapse.[43,51,113] Histologically, in English bulldogs affected by sleep apnoea, increased fast twitch muscle fibres alongside increased abnormal fibres and fibrosis was observed; features consistent with ongoing and prior injury.[114]

1.2.4 Tongue

Tongues of brachycephalic breeds, especially in French and English bulldogs, demonstrate relative macroglossia with larger and denser tongues, with the exception of relatively smaller tongues in pugs.[3,17,112,115] Macroglossia in brachycephalic breeds has also been associated with increased body condition score and sleep apnoea.[17] The thicker and longer tongue displaces the soft palate and reduces upper airway volumes and can significantly contribute to BOAS pathophysiology.[17,23]

1.2.5 Palatine tonsils

Eversion of the palatine tonsils is considered a secondary manifestation of chronic increased airway pressure associated with BOAS.[15,17,23] Brachycephalic dogs with everted tonsils are significantly more likely to also have stenotic nares and everted laryngeal saccules.[32] Apart from French bulldogs with clinical signs of BOAS, differences in tonsillar size were not seen between brachycephalic and non-brachycephalic dogs.[116,117] A smaller tonsillar crypt in brachycephalic dogs likely contributes to more prominent eversion of the tonsils which occupy airway space and are considered contributors to pharyngolaryngeal obstruction in BOAS-affected dogs.[32,117] In French bulldogs, higher tonsillar volume and cross-sectional area was correlated with BOAS clinical signs.[116]

Increased inflammation in BOAS-affected dogs likely occurs due to chronic irritation and negative pressures.[15,20,118] Histological changes are also seen in brachycephalic dogs including lymphocytic-neutrophilic tonsillar infiltrates within the epithelium, hyperplastic lymphoid tissue adjacent to the tonsils including secondary follicular development, plasmacytic infiltrates in the connective tissue stroma, and rarely oedema and infection.[98]

1.2.6 Soft palate

The soft palate in brachycephalic dogs is considered to be a primary contributor to BOAS.[3,10,17] It's more caudal position, elongation and thickening lead to displacement of the soft palate, up to 1-2 cm beyond the tip of the epiglottis, where it can flutter, obstruct during inspiration and when transiently inhaled into the larynx, produces the characteristic stertor sound.[3,10,17,20,23,32,47,103] Brachycephalic dogs have thicker soft palates and increased epiglottis-soft palate overlap compared to non-brachycephalic dogs.[3,17,23,103,119] A correlation also exists between more severely brachycephalic dogs, soft palate thickness and clinical signs.[119] Additional dorsal displacement of the soft palate may result from nasopharyngeal hyperplasia, tonsillar hypertrophy/eversion and macroglossia with contributions from the soft palate to nasopharyngeal obstruction, stertor sound generation and sleep apnoea.[3,23,43,47] Despite having more severe brachycephalic features, pugs have a shorter and thinner soft palate compared to French bulldogs.[45]

Histologically, degenerative and inflammatory changes considered secondary to BOAS have not been observed in neonatal brachycephalic dogs, suggesting they are acquired with chronic and increasingly obstructive airway breathing.[120] Changes of the soft palate in BOAS-affected dogs compared to mesocephalic dogs include epithelial hyperplasia and intracellular oedema; various changes to the lamina propria including oedema, increased connective tissue stroma, glandular hyperplasia and dilatation; and additionally myofiber

changes.[17,121-124] Atrophy, hypertrophy, increasing type I and II myofibers, muscle hyalinisation, sarcoplasm fragmentation, satellite cell activation, fibrosis and reduced peripheral nerve branches have been observed, the latter which could be interlinked with BOAS-escalatory damage.[17,121-125]

1.2.7 Larynx

Secondary pathophysiological effects of BOAS progression are seen in the larynx although these can be exacerbated by additional abnormalities within individual dogs. The larynx and respiratory tract are displaced caudally in brachycephalic dogs possibly secondary to tongue crowding due to reduced oral and pharyngeal space.[126] Changes to the larynx in BOAS include mucosal oedema, everted laryngeal saccules and laryngeal collapse.[3,23] Previous classification systems considered eversion of the laryngeal saccules as stage 1, medial displacement of the cuneiform processes of the arytenoid cartilages as stage 2, and collapse of the corniculate processes of the arytenoid cartilages with loss of the dorsal arch of the rima glottis as stage 3.[3,15,47,111] Laryngeal collapse occurs in 50-95% of BOAS-affected dogs and cases as young as 4.5 months in French bulldogs have been reported.[3,24,48] Pugs have smaller rima glottis and are more severely affected by both laryngeal and left bronchus collapse in comparison to French and English bulldogs.[3,54,111]

Other laryngeal changes in brachycephalic breeds include larger cricoid cartilage to tracheal ratios with the trachea having the smallest diameter.[127] Pugs have a more oval cricoid cartilage and narrower elliptical glottis compared to French and English bulldogs.[3,59,127] French bulldogs have a more acute curvature and increased ventrodorsal thickness of the basihyoid bone compared to mesocephalic dogs.[3,128] Congenital malformed hyoid conformation, narrowed cricoid cartilage, laryngeal vocal fold granulomas and polyps, epiglottal retroversion, epiglottal cysts and glossoepiglottic mucosal

enlargement have also been reported in brachycephalic breeds with contribution to BOAS clinical signs.[3,111,128-130]

Histologically, increased degenerative change has also been described in the arytenoid cartilage of brachycephalic dogs, as well as a reduced load to failure and stiffness compared to non-brachycephalic dogs.[131] Inflammatory and degenerative changes have also been demonstrated in resected laryngeal saccules from brachycephalic dogs.[132] The impact of BOAS associated with the epiglottis has not been previously described, which will be evaluated in this study.

1.2.8 Trachea, bronchi and lungs

Tracheal abnormalities including hyperaemia, polyps and tracheal secretions and less frequently tracheal hypoplasia, dorsal tracheal muscle thickening and tracheal collapse were reported in 64.2% of brachycephalic dogs.[111] Tracheal hypoplasia, defined as a tracheal diameter : thoracic inlet ratio of less than 0.16 (brachycephalic dogs) and less than 0.12 (English bulldogs, the breed which has the highest incidence of this feature), is seen in 13% of BOAS-affected dogs and more often in the screw-tail breeds.[3,127]

In tracheal hypoplasia, tracheal cartilages touch or overlap and the dorsal tracheal muscle may be thickened or thinned, resulting respectively in a variably rigid or flattened trachea.[23,24,99,133] Histologically, changes in the tracheal cartilage include reductions in chondrocytes, chondroitin, glycosaminoglycans, water content, fibrocartilage and rarely, loss of ciliated epithelium and mucus gland dysplasia.[23,24,99,133] These changes have been attributed to extrinsic compression (barrel chest conformation), chronic inflammation, and elastic fibre changes in the dorsal tracheal membrane.[99] Tracheal hypoplasia contributes minimally to BOAS but can delay or prevent resolution of pneumonia.[3,23,52] Studies have

shown that tracheal hypoplasia may partially improve with maturity, however it does not improve in adult dogs that undergo BOAS surgical treatments.[3,52,134]

Bronchial abnormalities include bronchial collapse (42-87% of BOAS-affected dogs), bronchial wall thickening and less frequently hyperaemia, stenosis and bronchial hypoplasia.[15,54,55,99,111] The left cranial bronchus is more commonly affected followed by the right middle bronchus.[53,55,99,135] Pugs are the most commonly affected breed followed by English and French bulldogs.[53,55,99,135] Bronchial wall thickening also occurs in brachycephalic dogs, most commonly in French bulldogs, and is significantly correlated with body weight.[55]

Brachycephalic dogs have an increased risk of developing aspiration pneumonia at an earlier age with emergencies and surgery further contributing to the risk.[17,41,56] Pugs have an increased frequency of left lung lobe torsion likely due to atelectasis subsequent to increased risk of left bronchus collapse.[54]

1.2.9 Grading schemes and systems for clinical, anatomical and histological evaluation of BOAS

Clinical schemes for the assessment of BOAS are the primary basis for veterinary management of the condition with several schemes that include combinations of functional, anatomical and physiological features. Anatomical schemes include those developed for individual anatomical components of brachycephalic dogs correlated to the degree of BOAS. Histological studies are limited to features of soft palate and arytenoid cartilage in BOAS-affected dogs. Table 1 details the various clinical, anatomical and histological schemes currently employed for BOAS assessment.

Table 1: Clinical, anatomical and histological schemes and systems for assessment of BOAS.[3,4,17,83,121,131,136-144]

Grading scheme or system	Components
Brachycephalic syndrome functional score	Six-minute walking test, upper airway noise auscultation, noise recording, and morphological parameters (height, body condition, nasal stenosis, muzzle length, cranial length, neck length, neck girth). Nasal stenosis accounts for 32% of variation in the score.
Cambridge respiratory functional grading scheme	Three-minute trot test, physical examination, pre- and post-evaluation of respiratory noise, inspiratory effort, dyspnoea, cyanosis and syncope.
Laryngeal collapse diagnostic test	Three-minute trot test and careful auscultation of the larynx.
Whole body barometric plethysmography	Air flow traces and generation of a BOAS index which includes severity, risk and effectiveness of surgery.
Brachycephalic risk score	Breed, temperature, body condition score, prior airway surgery, plan for surgery, upper respiratory noise and need for oxygen, sedation or intubation.
Conformational risk factor system	Respiratory grade, body condition score, nasal stenosis grade, cranial length, skull length, skull width, muzzle length, neck length, neck girth, chest girth and body length.
Anatomical schemes (individualised for each region)	Nasal stenosis, nasopharyngeal turbinate protrusion, soft palate elongation, pharyngeal changes (dorsoventral flattening, thickness at base of tongue, tonsillar protrusion and pharyngeal oedema), and laryngeal changes.

Table 1 – continued.

Grading scheme or system	Components
	(laryngeal hypoplasia, mucosal oedema, everted laryngeal saccules and laryngeal collapse).
Histological degenerative changes for arytenoid cartilage of the larynx	Total and degenerate chondrocyte numbers and degenerate cell definition, cartilage proteoglycan content (safarin O staining level and definition), and glycosaminoglycans and sulphur content (toluidine blue stain metachromasia levels and definition).
Histological changes for the soft palate	Mucosal hyperplasia and oedema, glandular appearance, hyperplasia, lobule numbers and mucin stasis, and palatine muscle appearance, fibre numbers, degeneration and fragmentation.

Schemes absent include walk/distance variation assessment. [3,4,17,83,121,131,136-144]

1.3 Anatomical and histological features of the epiglottis in dogs

The anatomical features at multiple levels of the airway in brachycephalic dogs and their role in BOAS pathophysiology has been well characterised. However, there is a relative dearth of information on anatomical and histological features of the epiglottis in brachycephalic dogs and any associated BOAS-related pathophysiology.

1.3.1 Form and function

The epiglottis is the most rostral of the laryngeal cartilages, consisting of a flexible stalk and leaflike blade, where the stalk is implanted between and with attachments to the root of the tongue, basihyoid bone, and thyroid cartilage (Figure 1).[145] The function of the epiglottis is to provide functional airway protection during swallowing. At rest, the blade lies dorsorostral to the soft palate and while swallowing, it is tilted caudally to partially cover the laryngeal opening.[145] Epiglottal structural detail relating to innervation, neurochemical structures, interaction with adjacent structures in live dogs, advanced imaging characterisation of composition in live dogs, and refinement of roles played in swallowing and panting, have more recently been reported.[146-151] The larynx receives blood supply primarily from cranial and caudal thyroid arteries, sensory innervation from cranial and caudal laryngeal nerves, and lymphatic drainage into the medial retropharyngeal lymph nodes.[13]

1.3.2 Dimensions and spatial relationships

Recent morphometric description of the larynx details 10 laryngeal measurements, including epiglottal width (17.79 to 32.76 mm) and length (17.35 to 30.42 mm), based on the evaluation of 13 larynges from unspecified dog breeds ranging from 3.95 to 21.6 kg.[152] Structure, weight and linear laryngeal parameters were

significantly associated with body weight; the highest variability was associated with width of the epiglottis and height of the thyroid cartilage, and lowest variability associated with the laryngeal inlet width.[152] Forming the rostral cartilage of the larynx, the epiglottis lies caudal to the oral cavity, palatine tonsils and soft palate in order of proximity, and ventral to the pharynx (Figure 1).[145] The larynx is suspended by the cranial base of the hyoid apparatus and is partially contained by the rami of the mandible and partially within the ventral soft tissue structures of the neck.[145]

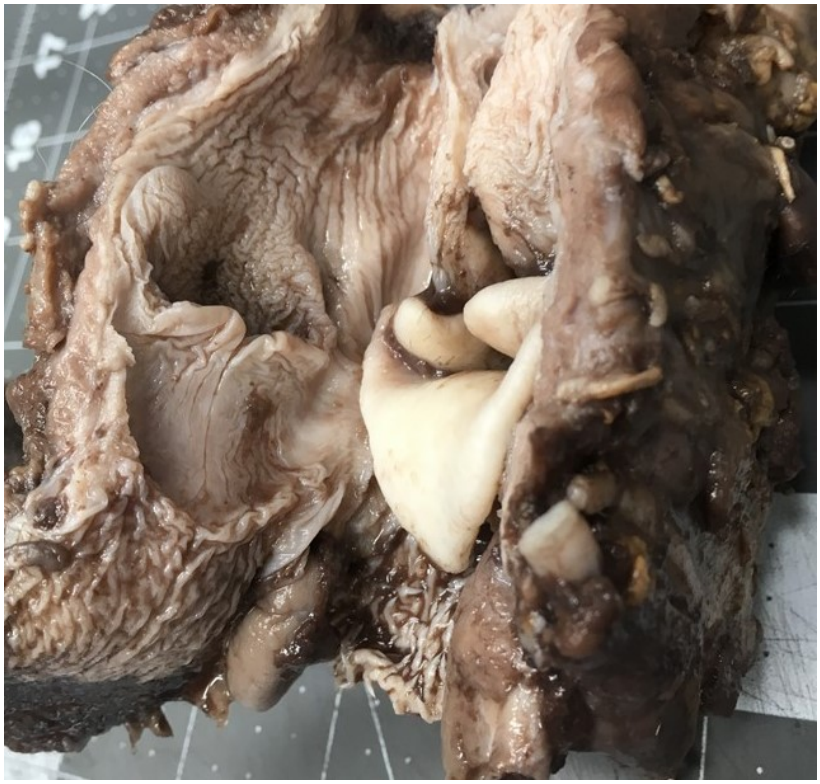

Figure 1: Fixed specimen of epiglottis in-situ with surrounding laryngopharyngeal tissue collected en bloc from a mesocephalic dog.

1.3.3 Histology

The epiglottis consists of a central elastic cartilage core covered by non-keratinised, stratified squamous epithelium that overlies a lamina propria-submucosa composed of dense, irregular connective tissue interspersed with seromucosal glands (Figure

2).[153,154] The elastic cartilage core consists of a peripheral cartilaginous wall enclosing areas of chondrocytes located within lacunae in small aggregates (isogenous clusters), avascular extracellular matrix, adipose tissue and interweaving elastic fibres.[153,155,156] The epiglottis has a rostral lingual surface and a caudal laryngeal surface with the laryngeal surface containing taste buds and sensory nerves.[146,147,152,153] Regarding the normal canine epiglottis, there is limited information on the proportion of epithelial, lamina propria-submucosa and cartilage tissue components, the typical distribution and proportions of various histologically distinct cellular components, and changes related to aging and breed.[150,152]

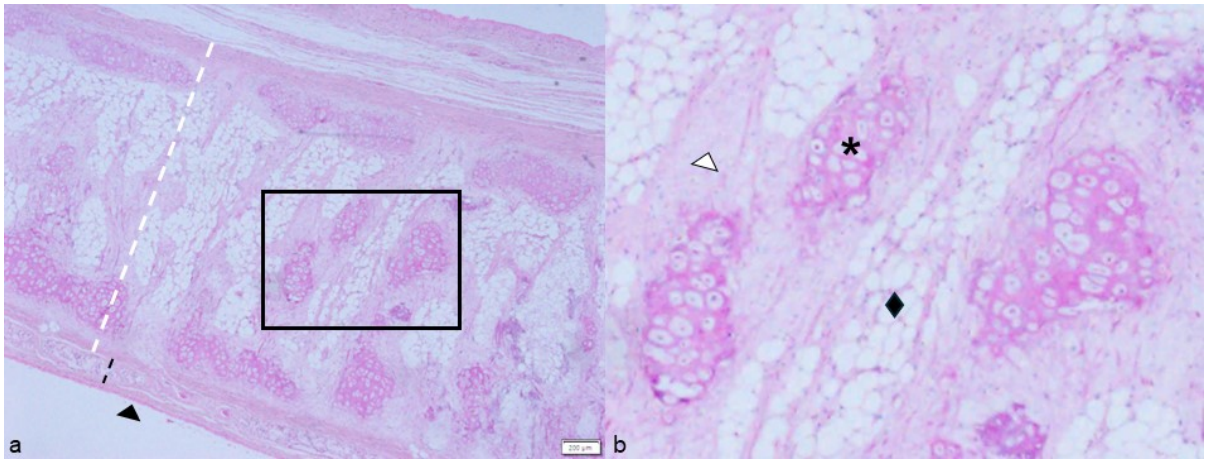

Figure 2: Photomicrographs of H&E sections of the middle third of the epiglottal cartilage from a representative section of a) mesocephalic dog; note the epithelium (black arrowhead), lamina propria-submucosa (dashed black line) and the cartilage core (dashed white line) and b) inset displaying chondral cells clusters (asterisk), fibrous tissue (white arrowhead) and adipose tissue (black diamond). Scale bar for 2a = 200 µm.

1.3.4 Pathological processes

There is a relative dearth of information on anatomical and histological features of the epiglottis in BOAS-affected dogs and limited histological information on epiglottal

retroversion and laryngeal collapse. Other epiglottis pathology in dogs include neoplasia including chondrosarcoma, plasmacytoma, rhabdomyoma, lipoma and malignant myoepithelioma, epiglottal abscesses, epiglottal trauma resulting in haemorrhage and inflammation, and experimental thermal and pressure animal models on injury using mesocephalic dogs.[157-168]

The role of the head, neck, nares, nasal cavity, nasopharynx, pharynx, tongue, palatine tonsils, soft palate, larynx, trachea, bronchi and lungs in the pathophysiology of brachycephalic obstructive airway syndrome and progressive deterioration has been well characterised.[3,4,9,10,15,17,18,21] In contrast, the role of the epiglottis in BOAS pathophysiology is little described and is the basis of this thesis.[98]

1.4 Thesis structure

1.4.1 Research objectives

The anatomical restrictions and crowding of structures in brachycephalic dogs are asserted to cause interlinked pathophysiology from increased negative airway pressure, turbulence, resistance and contact irritation. Additionally, BOAS severity has breed and sex trends within the extreme brachycephalic group of pugs such as French bulldogs and bulldogs. It is hypothesised that BOAS may also affect the epiglottis, a tissue previously not evaluated, and this will be reflected anatomically and histologically. The objectives of this study are to compare the epiglottal anatomical and histological features between brachycephalic and non-brachycephalic dogs (mesocephalic and doliocephalic) and evaluate for BOAS-associated changes which may be anatomical dimensional, compositional, inflammatory or degenerative.

1.4.2 Research questions and hypotheses

The following three research questions and associated hypotheses will be examined:

1) Does the epiglottis of brachycephalic dogs have anatomical dimensional variation that can be attributed to BOAS compared to mesocephalic and doliocephalic dogs? It is hypothesised that the epiglottis of brachycephalic dogs will have relatively increased length, breadth, height and volume in comparison to similar sized mesocephalic or doliocephalic dogs due to disproportionate reduction of internal soft tissue components.
2) Does the epiglottis of brachycephalic dogs have microscopic compositional changes that can be attributed to BOAS compared to mesocephalic and doliocephalic dogs? It is hypothesised that the epiglottis of brachycephalic dogs will have a differing percent composition of epithelium, lamina propria-submucosa, cartilage core, chondral cells plus matrix, fibrous tissue and adipose tissue that can be attributed to BOAS compared to mesocephalic and doliocephalic dogs.
3) Does the epiglottis of brachycephalic dogs have inflammatory and/or degenerative changes that can be attributed to BOAS compared to mesocephalic and doliocephalic dogs? It is hypothesised that the epiglottis of brachycephalic dogs will have increased oedema, increased inflammatory cell numbers, calcification/mineralisation, cartilage metachromasia, total chondral cells and degenerate chondral cells that can be attributed to BOAS compared to mesocephalic and doliocephalic dogs.

Chapter 2: Materials and Methods

2.1. Study design

This study utilised an observational, retrospective, case-control approach to compare epiglottal anatomy and histology in three head types (brachycephalic, mesocephalic and doliocephalic). Inclusion criteria for dogs enrolled were any breed, age or weight. Exclusion criteria were overt inflammatory, traumatic or neoplastic lesions involving the epiglottis, palatine tonsils and adjacent soft tissue. Conventions used were brachycephalic breeds having skull width:length ratio of 0.81 or greater or a craniofacial angle between the base of the skull and facial skull of 9-14° in brachycephalic dogs, 19-21° in mesocephalic dogs, and 25-26° in doliocephalic dogs.[15] Breeds were categorised into three head types with confirmation of skull dimensions and allocated to one of three groups: brachycephalic dogs (BC group), mesocephalic dogs (MC group) and doliocephalic dogs (DC group). Forty-one cadaver dogs were included in the study that comprised of brachycephalic dogs (BC group, n = 10 adults, n = 2 juvenile/puppy), mesocephalic dogs (MC group, n = 21 adults) and doliocephalic dogs (DC group, n = 3 adults, n = 5 puppies). Mixed breeds were allocated into a group based on skull dimensions.

2.2. Sample collection

Measurements and tissues were obtained from 41 euthanased dogs submitted to the University of Sydney Veterinary Teaching Hospital as part of the body donation scheme for which owners' consent was received. As such, animal ethics approval for this study was not required. Dog breed, age, sex, neuter status, body weight, body condition, fresh/frozen status and degree of autolysis level were recorded. When age was unknown, a morphological and dental assessment was used to

assign as juvenile/puppy or adult. Cadavers were categorised into one of the three groups (BC, MC or DC) based on breed and skull dimension confirmation. In addition to the examination undertaken for this study, standard necropsy was conducted in all cases including routine tissue collection, documentation of all concurrent pathological diagnoses, and any history of prior BOAS surgery.

2.3. Morphological gross assessment

Measurements (millimetres) of the following were taken from brachycephalic (n = 3 adults, n = 1 puppy), mesocephalic (n = 12 adults) and doliocephalic (n = 2 adults, n = 5 puppies): epiglottal length, breadth and width; and bilateral tonsillar length, breadth and width (epiglottal data located in Appendix B; tonsillar data located in Appendix C). Figure 2 displays an example of the epiglottis in-situ with surrounding tissues. Measurements of skull length, breadth and width were taken from brachycephalic (n = 3 adults, n = 1 puppy), mesocephalic (n = 9 adults) and doliocephalic (n = 2 adults) dogs (skull data located in Appendix B). In addition, measurement of the craniofacial angle (degrees) between the base of the skull and facial skull was taken from brachycephalic (n = 5 adults, 1 juvenile), mesocephalic (n = 8 adults) and doliocephalic (n = 1 adults, n = 5 puppies) dogs. Overall, variable combinations of epiglottal, skull and craniofacial angle measurements were not measured in 4 to 8 brachycephalic, 9 to 13 mesocephalic, and 1 to 6 doliocephalic dogs as samples were obtained retrospectively. Linear measurements of the skull and epiglottis were performed with an anatomical ruler with an accuracy of 0.5 mm. Angular measurements of the craniofacial angle were performed with a protractor with an accuracy of 0.5 °. The following measurements were carried out prior to formalin fixation:

- l_s – maximum length of skull
- w_s – maximum width of skull
- a_s – craniofacial angle between the base of the skull and facial skull
- l_e – maximum length of epiglottis
- w_e – maximum width of epiglottis
- h_e – maximum height of epiglottis

Figure 3 demonstrates the skull and epiglottal measurements symbolically portrayed as manipulation required for accurate measurements. Based on previous literature, the following formula was utilised to calculate for skull width:length ratio.[15]

- Skull width:length ratio of the head = w_s / l_s

A formula for epiglottal volume was not available in the literature, so epiglottal volume was estimated to be a rectangular pyramid based on the closest possible geometric shape that allowed a volume calculation using the following standard formula.

- Volume of epiglottis = 1/3 x l_e x w_e x h_e (mm^3)

Epiglottal dimensions of the three head type groups were compared relative to body weight and skull dimensions, based on previous reports that linear laryngeal parameters were significantly associated with body weight.[152]

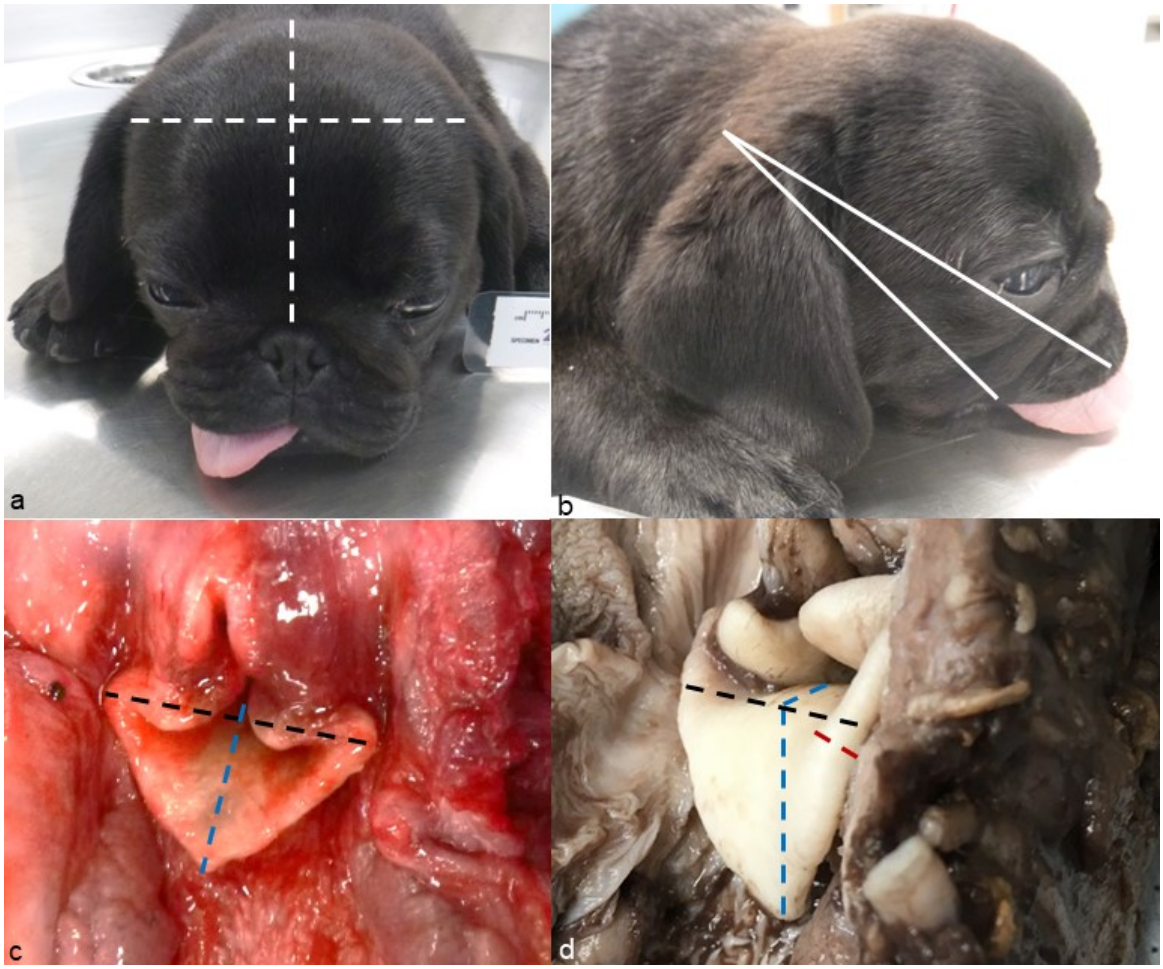

Figure 3: Skull and epiglottal measurements symbolically portrayed displaying a, b) frontal and lateral head views from a brachycephalic dog showing skull length (vertical white dashed line), skull width (horizontal white dashed line), and craniofacial angle between base of skull and facial skull (solid white line angle); c, d) unfixed dorsal and fixed side epiglottis views from a brachycephalic and mesocephalic dog respectively, showing epiglottal length (blue dashed line), epiglottal width (black dashed line), and epiglottal height (red dashed line).

2.4. Histological section preparation

Epiglottal tissues were obtained from 41 dogs comprised of brachycephalic dogs (n = 10 adults, n = 2 juvenile/puppy), mesocephalic dogs (n = 21 adults) and doliocephalic dogs (n = 3 adults, n = 5 puppies). Additionally, variable numbers of nares, palatine tonsils, soft palate, larynx, trachea and lungs were obtained from the

same cadavers as described in Appendix A. The larynx was collected en bloc within the surrounding proximal pharynx, proximal hard palate, palatine tonsils, soft palate, proximal tongue and proximal trachea to minimise fixation-distortion artefact and fixed in 10% neutral buffered formalin (Figure 1). Upon fixation, the central region of the epiglottal cartilage was dissected across its length to produce two to three samples of 2 to 4 mm thickness (Figure 4). The number of samples varied due to the size of available tissue. The fixed samples were processed by an automated tissue processor (Medite TPC15, Germany), paraffin-embedded, and 4 µm sections were obtained by microtome (Leica RM 2235, Switzerland). Sections were routinely stained with haematoxylin and eosin (H&E) by an automated slide stainer (Tissue-Tek Prisma, Japan) for the purposes of microscopic and histopathological description. Cartilaginous tissue was also evaluated with toluidine blue stain for degree of metachromasia. All H&E and toluidine blue sections were visually subdivided into proximal, middle and distal thirds on the slide (Figure 4).

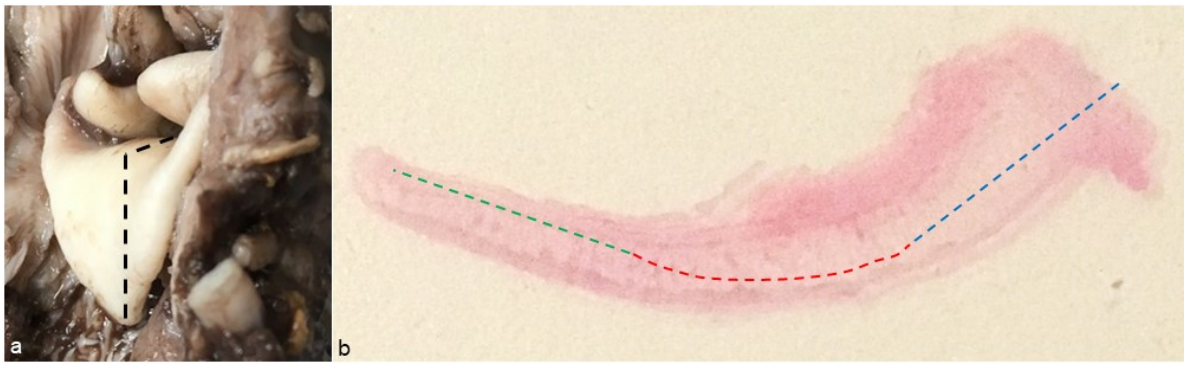

Figure 4: Epiglottal anatomical location of section and illustration of histological subdivisions, displaying a) anatomical location of epiglottal section indicating the plane of section (black dashed line) and b) representative H&E slide of epiglottis from a mesocephalic dog, indicating subdivision into proximal epiglottal third (blue dashed line), middle epiglottal third (red dashed line) and distal epiglottal third (green dashed line) used for micromorphometric evaluation.

Representative samples, when allowable were also taken in replicate of the nares, palatine tonsils, soft palate, mid-transverse larynx, proximal trachea, distal trachea and lungs to assess the entire respiratory tract for any concurrent pathology (Appendix A).

2.5. Micromorphometric evaluation

Using H&E sections, the proportions of epithelium, lamina propria-submucosa, cartilage and cellular components of the cartilage core (cartilage, adipose tissue, fibrous tissue and calcification) were evaluated within the proximal, middle and distal third of 41 epiglottal tissues that were blinded to head type. Tissue examination was performed blinded to the dogs' classification as brachycephalic, mesocephalic and doliocephalic. Measurements were undertaken at 12.5x magnification using microscope interphase camera-imaging software (Olympus BX53, Olympus DP80 Camera; Olympus cellSens Standard 3.1, Japan) based on the instructional manual, intuitive menu features, and instruction videos.[169, 170,171] As six tissue components required evaluation, the following area measurements were undertaken manually using the polygon area calculation tool and optimised contrast enhancement for each third of tissue.[170,171]

- TA = Total area
- LP_SM_CC_M = Lamina propria-submucosa + cartilage core (+ muscle if present)
- CC = Cartilage core
- M = Muscle if present
- CL = Chondrocyte/chondroblast clusters-cartilage (thence forth chondrocytes/chondroblasts referred to chondral cells collectively)

- F_CL = Fibrous tissue + chondral cells cluster
- C = Calcification if present

Figure 5 displays a representative section from the brachycephalic group with contrast enhancement and highlighted areas for manual measurements of tissue components captured using cellSens imaging software which allowed area analysis and comparison.

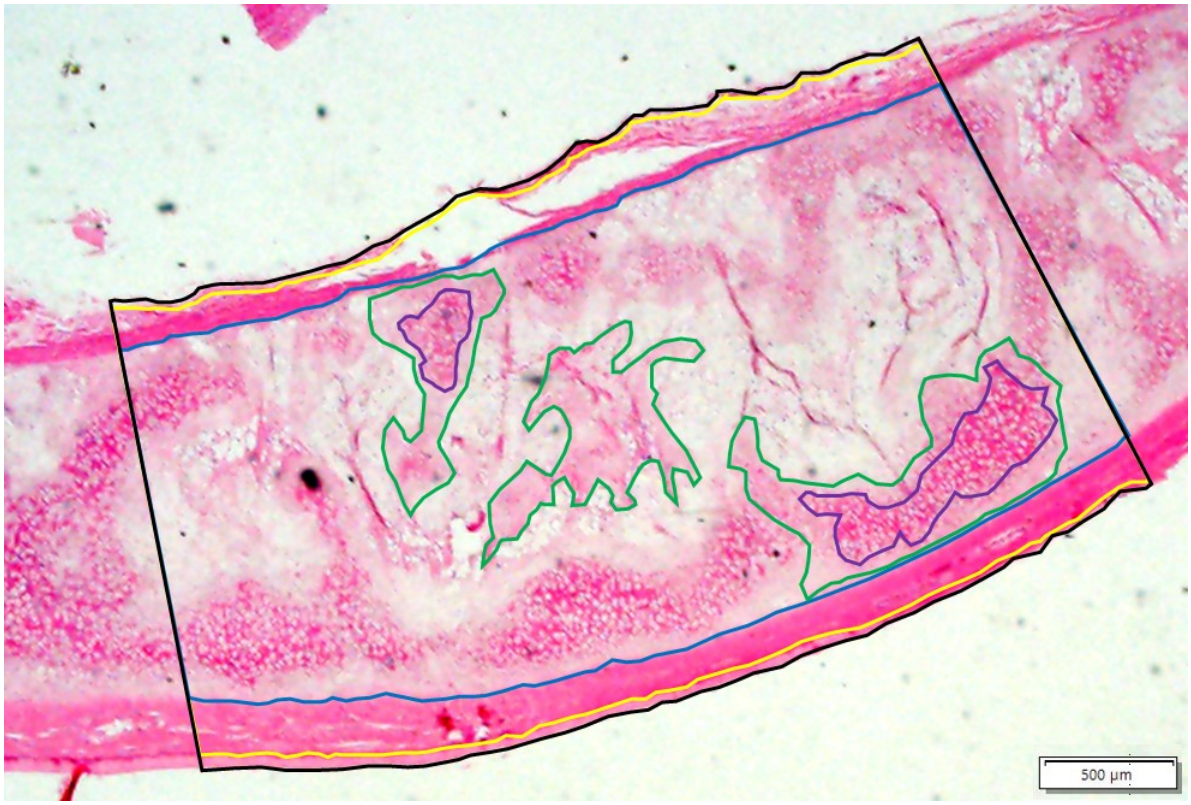

Figure 5: Photomicrograph of a contrast enhanced H&E section of the middle third of the epiglottal cartilage from a brachycephalic dog showing a schematic diagram of area measurements, which were used for calculation of proportions of epithelium, lamina propria-submucosa, cartilage and histologically distinct components of the cartilage core (chondral cell/matrix, adipose and fibrous tissues). Total area of middle third of epiglottis = black line, lamina propria-submucosa + cartilage core = yellow line, cartilage core = blue line, examples of chondral cell clusters-cartilage = purple line, examples of fibrous tissue + chondral cell clusters-cartilage = green line. Scale bar = 500 µm.

Using Microsoft Excel (Version 2402 Build 16.0.17328.20648), the following calculations for areas were undertaken:

- E = Epithelium = TA – (LP_SM_CC_M)
- LP-SM = Lamina propria-submucosa = (LP_SM_CC_M) – CC
- TCL = Total chondral cells clusters-cartilage = SUM(CL)
- TF_CL = Total fibrous tissue + chondral cells clusters-cartilage = SUM (F_CL)
- FT = Fibrous tissue = TF_CL – TCL
- A = Adipose tissue = CC – (TCL + FT)

Lastly, the above areas were used to calculate the following proportions:

- % epithelium = (E/TA)*100
- % lamina propria-submucosa = (LP-SM/TA)*100
- % cartilage core = (CC/TA)*100
- % muscle = (M/TA)*100
- % chondral cells clusters-cartilage within cartilage core = (TCL/CC)*100
- % fibrous area within cartilage core = (FT/CC)*100
- % adipose area within cartilage core = (A/CC)*100
- % calcification if present within cartilage core = (C/CC)*100

2.6. Evaluation of histopathological features

Using the same blinded H&E sections utilised in the micromorphometric study, the distal, middle and proximal sections were evaluated with light microscopy (Nikon Eclipse 50i) for the following histopathological features: oedema, inflammation, cartilage metachromasia, and cartilage mineralisation, median chondral cell number across five fields, and median degenerate chondral cell number across five fields

(Table 2). Note that chondrocytes and chondroblasts were referred to as chondral cells collectively. Grading schemes were developed for oedema, inflammation, calcification/mineralisation, and cartilage metachromasia (Table 2). Premised on previous literature, tissue samples were evaluated for oedema and inflammatory cell numbers and designated grade 0 to 3 dependant on the degree of change (0 = normal, 1 = mild, 2 = moderate, 3 = marked), and grade 0 designated as no change (Figures 6 and 7).[121-124] Evaluation of oedema was undertaken on the epithelium and lamina propria-submucosa, however in lieu of postmortem samples, the contribution of separation artefact was also present. Calcification/mineralisation was graded as 0: absent or 1: present (Figure 8).[172]

The total number of chondral cells and degenerative chondral cells were counted in five randomly selected fields of epiglottal cartilage at 400x magnification (total area 0.8 mm^2), and the median number of total chondral cells and degenerative chondral cells calculated for each group, as described previously.[131,144,173] Degenerative chondral cells were defined as those displaying characteristic cell shrinkage, cytoplasm density increase, chromatin condensation and nucleosomal fragmentation.[131,173,174] Replicate sections of epiglottal tissue were stained with toluidine blue for assessment of metachromasia and cartilage degeneration.[131,173] In accordance with previously employed histological grading scales, epiglottal sections stained with toluidine blue were graded as 0: normal, 1: mildly reduced, 2: moderately reduced, 3: markedly reduced metachromatic staining (Figure 9).[131,144,173] Grade 0 or normal toluidine blue staining of cartilage defined as intense purple metachromatic staining due to binding of glycosaminoglycans in cartilage

matrix; whereas Grades 1 to 3 were associated with corresponding increased depletion of glycosaminoglycans and cartilage degeneration.

Table 2: Criteria for categorising histopathological changes in the epiglottal tissue of brachycephalic (n = 10 adults, n = 2 juvenile/puppy), mesocephalic (n = 21 adults) and doliocephalic (n = 3 adults, 5 puppies) dogs premised on previous literature schemes. [121-124, 131, 144, 172, 173]

Oedema grade of epithelium and lamina propria-submucosa	
0	Normal appearance of epithelium and lamina propria-submucosa
I	Mild interstitial oedema with tissue separation and/or mild epithelial intracellular oedema
II	Moderate interstitial oedema with tissue separation and/or moderate epithelial intracellular oedema
III	Marked interstitial oedema with tissue separation and/or marked epithelial intracellular oedema
Inflammation grade	
0	Occasional inflammatory cells
I	Mildly increased inflammatory cells
II	Moderately increased inflammatory cells
III	Markedly increased inflammatory cells
Calcification/mineralisation grade of cartilage	
0	Absent
I	Present
Metachromasia grade of cartilage using toluidine blue stain	
0	Normal (intense purple metachromatic staining)
I	Mildly reduced metachromatic staining
II	Moderately reduced metachromatic staining
III	Markedly reduced metachromatic staining

Table 2 – continued.

Number of total chondral cells in cartilage	Median number of total chondral cells in 5 randomly selected fields at 400x magnification, (total area = 0.8 mm^2)
Number of degenerate chondral cells in cartilage	Median number of degenerate chondral cells in 5 randomly selected fields at 400x magnification, (total area = 0.8 mm^2)

Chondrocytes and chondroblasts collectively included in chondral cell counts.

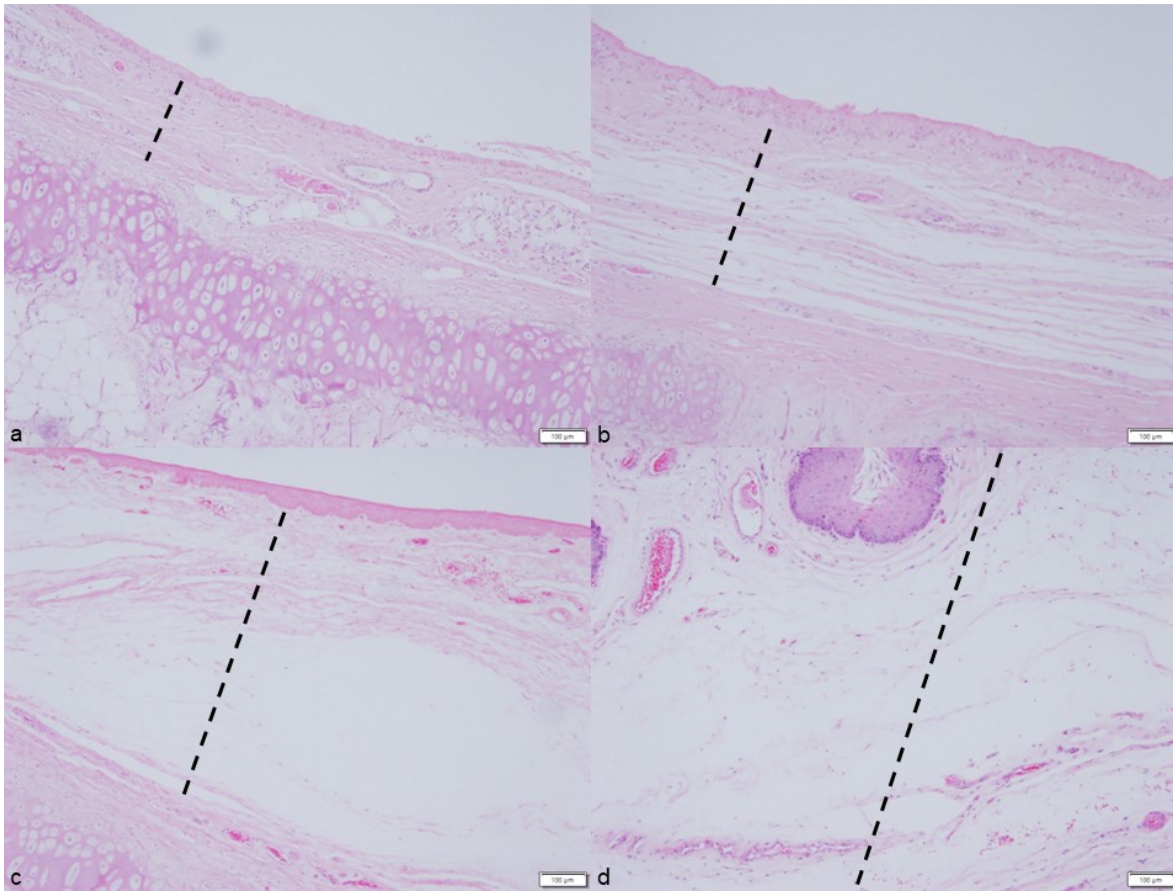

Figure 6: Photomicrographs of H&E sections of oedema grading in the epiglottal lamina propria-submucosa (dashed black line) displaying a) Grade 0 or no interstitial oedema, b) Grade 1 or mild interstitial oedema with tissue separation, c) Grade 2 or moderate interstitial oedema with tissue separation, and d) Grade 3 or marked interstitial oedema with marked separation of tissue and dilated lymphatics. Example of epithelial intracellular oedema only available for Grade 2 (not shown); however matched interstitial oedema grade in same section. Scale bar = 100 µm.

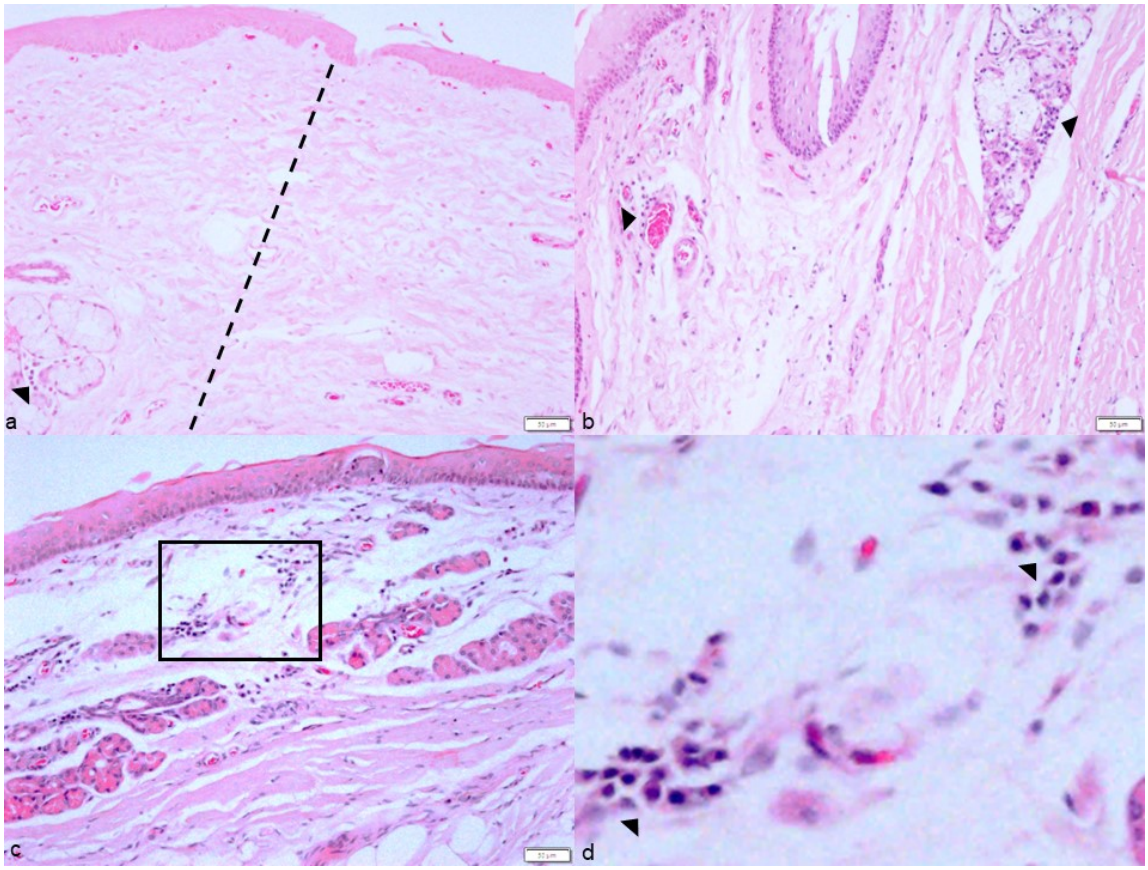

Figure 7: Photomicrographs of H&E sections of inflammation grading in epiglottal lamina propria-submucosa assessment displaying a) Grade 0 or no/few inflammatory cells, b) Grade 1 or mildly increased inflammatory cells, c) Grade 2 or moderately increased inflammatory cells, and d) inset from Grade 2. Lamina propria-submucosa denoted by dashed black line and inflammatory cells denoted by black arrowheads. Representative example of Grade 3 or markedly increased white blood cells not observed in viewed sections in this study. Scale bar = 50 μm.

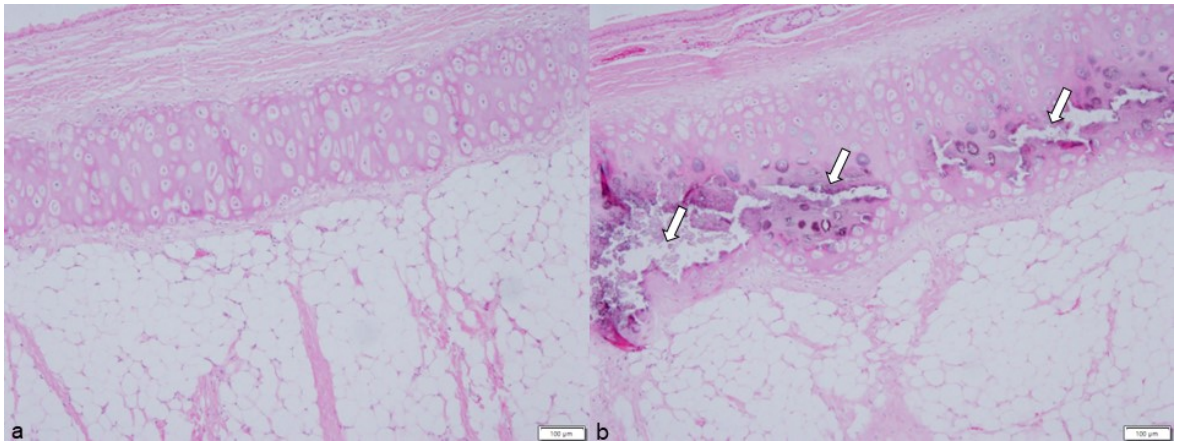

Figure 8: Photomicrographs of H&E sections of calcification/mineralisation grades for epiglottal cartilage displaying a) Grade 0 or absence of calcification/mineralisation, b) Grade 1 or presence of calcification/mineralisation (white arrow). Scale bar = 100 µm.

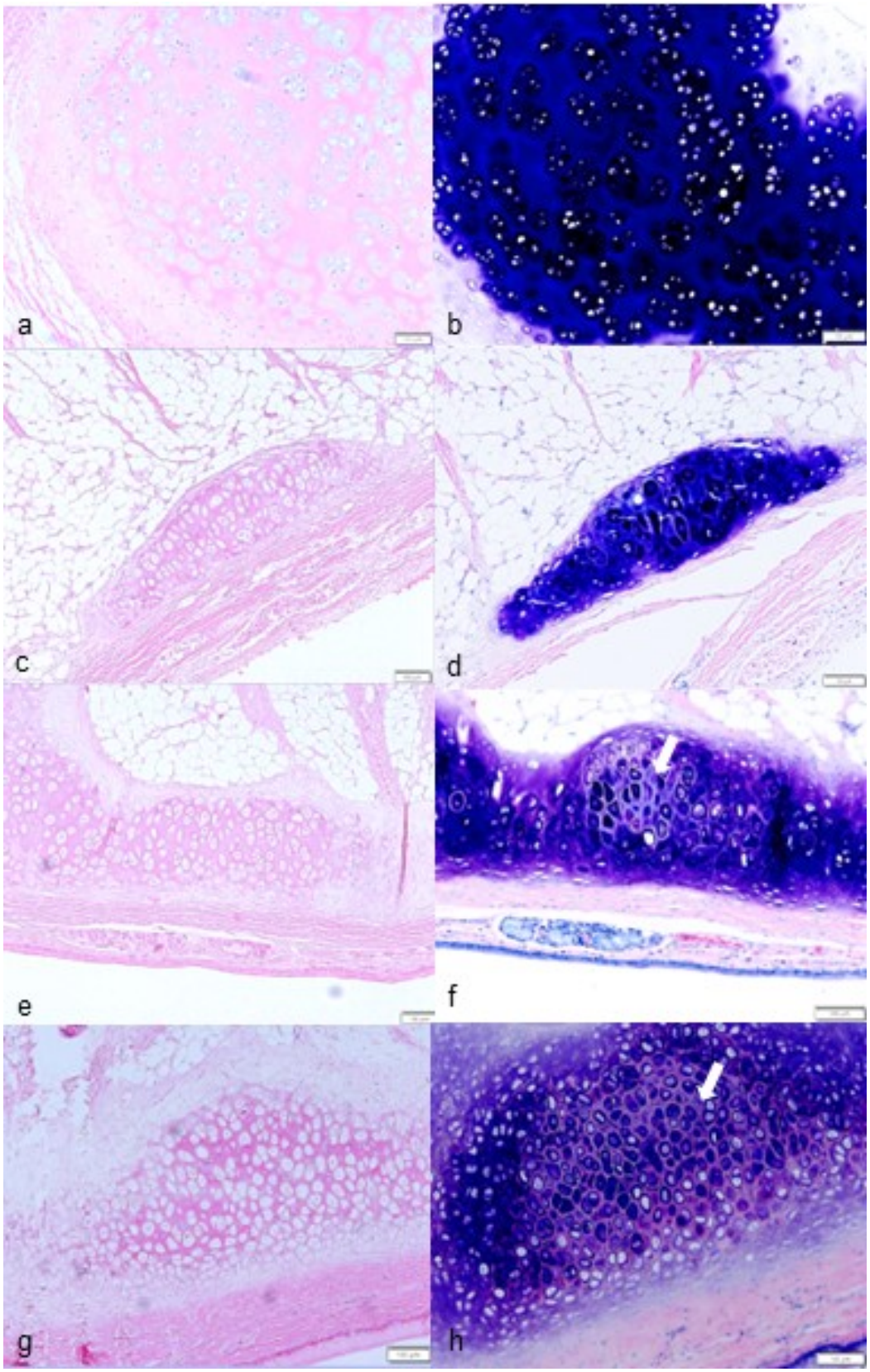

Figure 9: Photomicrographs of H&E and Toluidine Blue sequential sections for metachromasia grading displaying a, b) normal laryngeal cartilage and normal intense purple metachromatic staining with toluidine blue stain; in comparison to

Figure 9 – continued.
epiglottal cartilage sections c, d) Grade 0 or normal metachromatic staining; e, f) Grade 1 or mildly reduced metachromatic staining; and g, h) Grade 2 or moderately reduced metachromatic staining. Reductions in metachromatic staining denoted by pink to pale purple region (white arrows) in toluidine blue sections. Representative example of Grade 3 with marked reduction of metachromatic staining not observed in viewed sections in this study. Scale bar = 100 μm.

2.7. Statistical analysis

Descriptive statistics, median and observed range of values were determined for all gross morphometric, micromorphometric, binary and ordinal data (that is grading schemes) for each head group; brachycephalic, mesocephalic and doliocephalic using Microsoft Excel (Version 2402 Build 16.0.17328.20648).

Comparative statistics was undertaken on continuous micromorphometric, binary and ordinal data using Genstat (22nd Edition [64-Bit].lnk). For continuous micromorphometric data comparison, normality was checked using probability distribution plots or the Shapiro Wilk test with significance set at $p<0.05$. For continuous data or natural log transformed continuous data that was established to be normally distributed post natural log transformation, a two sample t-test was undertaken with significance set at $p<0.05$. For non-parametric continuous data, the non-parametric Mann-Whitney U test was undertaken with significance set at $p<0.05$. Where sample size was sufficient, continuous data compared were from the distal, middle and proximal epiglottal sections and included the following micromorphometric data: percent areas of epithelium, lamina propria-submucosa, cartilage core, chondral cells/matrix, fibrous tissue, adipose tissue, and histopathological changes data: total chondral cells and degenerate chondral cells.

Continuous data statistical comparison was undertaken on micromorphometric data of adult brachycephalic and adult mesocephalic dogs only as sample sizes of doliocephalic and juvenile/puppies groups were not sufficient for inclusion in the quantitative statistical analysis.

For binary data comparison, the Wald test for logistic regression was undertaken with significance set at $p<0.05$. Similarly, for ordinal data, the Wald test for ordinal regression was undertaken with significance set at $p<0.05$, and scores reduced to three categories: 0, 1 or 2 or more (that is scores 2 and 3 combined into one category). Binary and ordinal data compared were from the distal, middle and proximal sections and included oedema grade, inflammation grade, cartilage metachromasia grade and cartilage calcification/mineralisation grade. For binary and ordinal data, statistical comparison was undertaken on all three head groups of adult dogs only, as sample sizes of juvenile/puppies groups were not sufficient for inclusion in the quantitative statistical analysis.

Chapter 3: Results

3.1. Animals and head group assignment

Table 3 presents distribution of sex, age, body weight, skull width:length ratio and craniofacial angle of the 41 cadaver dogs included in the study. Dogs were assigned to the brachycephalic, mesocephalic and doliocephalic head group based on a combination of skull width:length ratio, craniofacial angle, reported breed and photograph evaluation.[13-16] This resulted in the following composition of study groups: brachycephalic dogs (BC group, n = 10 adults, n = 2 juvenile/puppy), mesocephalic dogs (MC group, n = 21 adults) and doliocephalic dogs (DC group, n = 3 adults, n = 5 puppies). Thirty-four dogs with available craniofacial angles and skull width:length ratios were assigned to a group were consistent with conventional definitions of brachycephalic, mesocephalic and doliocephalic heads.[13-16] A Staffordshire bull terrier (n = 1) with a craniofacial angle of 18° intermediate between the conventional definitions was assigned to the BC group based on its breed association with BOAS. A French bulldog crossbreed was assigned to the mesocephalic group based on a skull width:length ratio of 0.74. Six dogs lacking measured skull width:length ratio and craniofacial angle were assigned to head groups based on breed and photographs. Three cross breeds from the mesocephalic group displayed skull width:length ratio in the upper range reflecting the continuum of head shape within dogs. For dogs with unknown age, morphological and dental assessment assigned them to be adults, and these included brachycephalic (n = 3), mesocephalic (n = 6) and doliocephalic (n = 1) dogs.

Table 3: Distribution of adult dogs and juveniles/puppies categorised by head shape. Data for age and body weight provided as median and observed range of values in parenthesis. Skull width: length ratio and craniofacial angle provided as observed range of values and number of animals in parenthesis based on available data collection. Sex is denoted by M = male, MN = male neuter, F = female, FN = female neuter, IS = intersex and number of animals in parenthesis.

	Brachycephalic	Mesocephalic	Doliocephalic
Epiglottal measurements samples	3 (adults) 1 (juvenile/puppy)	12 (adults)	2 (adults) 5 (juvenile/puppy)
Tissue samples	10 (adults) 2 (juvenile/puppy)	21 (adults)	3 (adults) 5 (puppies)
Sex	M (3) MN (4) F (1) FN (3) IS (1)	MN (15) F (1) FN (5)	M (4) F (3) FN (1)
Age (y)	2.58 (0.31-12.0)	10.7 (1.33-14.5)	0.12 (0.12-7.66)
Body weight (kg)	11.4 (4.20-24.0)	14.3 (3.20-53.0)	2.95 (1.20-27.5)
Skull width:length ratio	0.80-0.89 (4)	0.47-0.78 (10)	0.45-0.46 (2)
Craniofacial angle (°)	11.0-14.0 (5)	20.0-22.0 (8)	25.0-26.0 (6)

The three head groups consisted of 25 reported breeds of dogs: the brachycephalic group included pugs (4), French bulldogs (3), English bulldog (1), American bulldog

(1), Staffordshire bull terrier (1), cavalier King Charles spaniel (1), and shih tzu (1); the mesocephalic group included German shepherds (3), Jack Russell terriers (2), beagle (1), springer spaniel (1), spoodle (1), border collie (1), golden retriever (1), Labrador retriever (1), bichon frise (1), fox terrier (1), miniature fox terrier (1), Labrador retriever crossbreed (1), kelpie crossbreed (1), dachshund crossbreed (1), Maltese crossbreed (1), mixed breed (2) and French bulldog crossbreed (1); and doliocephalic group included only one breed: greyhound (8).

3.2. Morphological gross assessment

During postmortem tissue collection or processing, gross evaluation of the epiglottis was undertaken on all 41 dogs, however epiglottal measurements were limited to 23 dogs depending on whether they were undertaken during the postmortem examination. Figure 10 presents photographs of the epiglottis and surrounding tissues from cases obtained both retrospectively and prospectively and were dependant on the necropsy scheme which required different collection of the postmortem sample. As such optimal retrospective gross photographs were not often possible to obtain. In addition, elongate/swollen soft palates or everted palatine tonsils, both lesions relatable to BOAS, frequently obscured or entrapped the epiglottis in BC group individuals (Figure 10).

Table 4 presents the distribution of epiglottal length, width, height and volume proportionate to body from available data that comprised of a total of 23 dogs including brachycephalic dogs (BC group, n = 3 adults, n = 1 juvenile/puppy), mesocephalic dogs (MC group, n = 12 adults) and doliocephalic dogs (DC group, n = 2 adults, n = 5 puppies). Individual measurements specific to breed, age, sex,

weight, craniofacial angle, skull dimensions and additional information relating cadaver condition, collected tissues, slide replicate samples, and relevant concurrent pathology or conditions are provided in Appendices A, B and C.

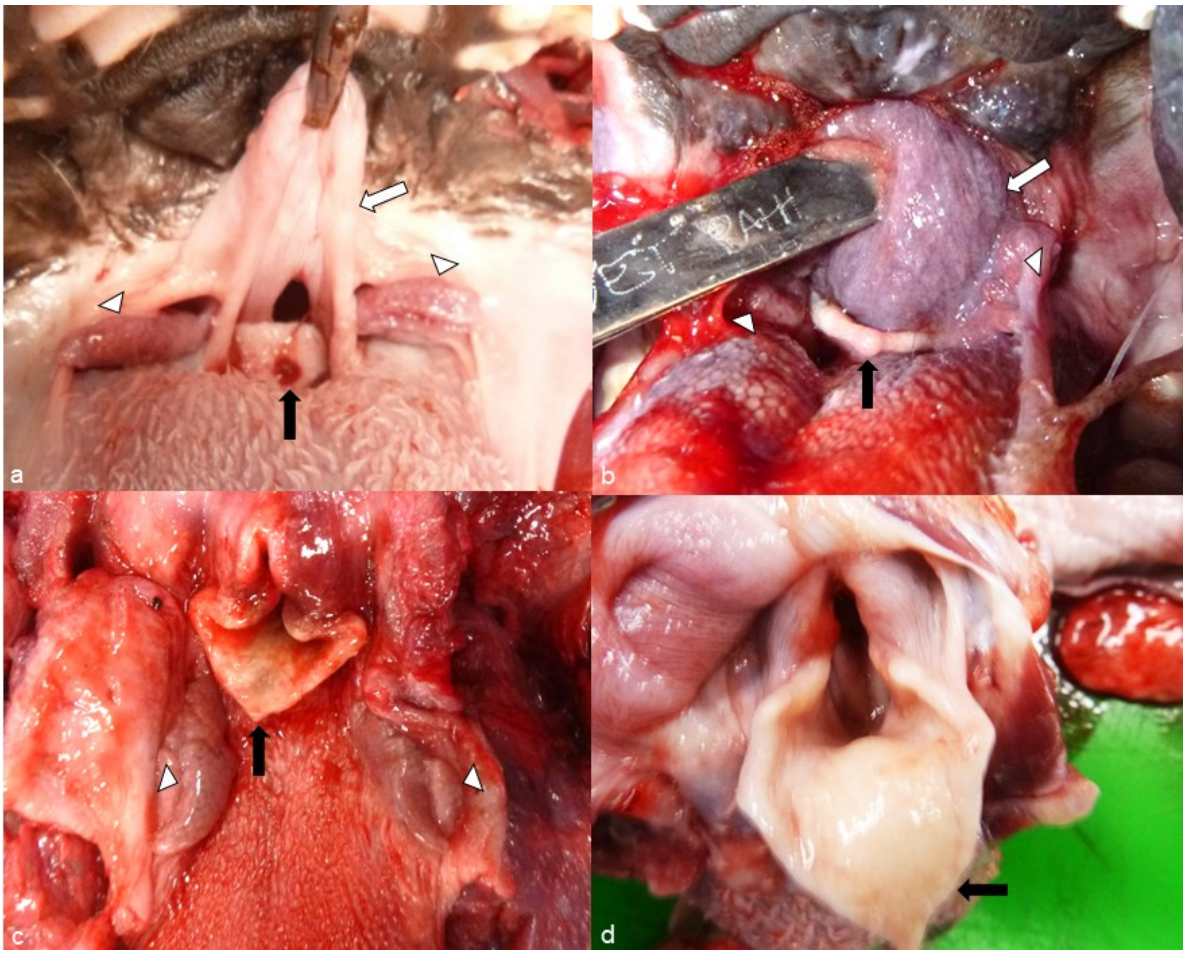

Figure 10: Gross images of epiglottises (black arrow) in brachycephalic (a-c) in-situ tissues with lesions relatable to BOAS displaying: a) elongate soft palate and everted palatine tonsils with epiglottis centrally, b) elongate markedly swollen soft palate resting on epiglottis and poorly visible everted palatine tonsils, c) epiglottis centrally and partially everted palatine tonsils, and d) epiglottis of a mesocephalic dog for comparison with no observable BOAS pathology. Note the obscuring or entrapping the epiglottis involving the soft palate (white arrow) and palatine tonsils (white arrowhead).

Table 4: Distribution of epiglottal length, width and height proportionate to body weight from brachycephalic (n = 3 adults, n = 1 juvenile/puppy), mesocephalic (n = 12 adults) and doliocephalic (n = 2 adults, n = 5 juvenile/puppy) dogs. Data provided as median and observed range of values in parenthesis, head shape denoted by BC = brachycephalic, MC = mesocephalic, DC = doliocephalic. Epiglottal volume estimated to be a rectangular pyramid, where volume equals 1/3 x length x width x height (mm^3).

Head shape	Epiglottal length/body weight (mm/kg)	Epiglottal width/body weight (mm/kg)	Epiglottal height/body weight (mm/kg)	Epiglottal volume (mm^3/kg)
BC adults	2.19 (1.80-2.60)	2.19 (1.97-2.60)	0.13 (0.12-0.14)	21.6 (17.3-25.2)
BC puppies	3.57 (3.57)	3.33 (3.33)	0.24 (0.24)	16.7 (16.7)
MC adults	1.35 (0.70-4.69)	1.35 (0.57-4.69)	0.11 (0.02-0.47)	18.1 (6.98-35.2)
DC adults	1.32 (1.27-1.36)	1.28 (1.20-1.36)	0.05 (0.05-0.05)	17.3 (13.6-21.0)
DC puppies	6.00 (4.69-13.3)	5.60 (4.69-11.7)	0.20 (0.16-0.42)	14.0 (11.7-31.1)

3.3. Gross morphological measurements of the epiglottis

Epiglottal measurement data was evaluated from the three groups (BC, n = 3 adults, n = 1 puppy; MC, n = 12 adults; DC, n = 2 adults, n = 5 puppies; total of 23 dogs). Overall, while statistical analysis was not undertaken due to insufficient sample numbers, brachycephalic dogs tended to have relatively larger epiglottal dimensions. Puppies (especially brachycephalic and doliocephalic) tended to have larger epiglottal proportions than adults except in volume. The brachycephalic group had

measurements that were similar or higher than the mesocephalic group in terms of length, width, height and volume; higher than the doliocephalic group for length, width and height; and similar with the doliocephalic group for volume. In the brachycephalic and doliocephalic groups, length, width and height measurements were higher in puppies compared to adults, however volumes were similar. The brachycephalic puppy had a higher length, width and height compared to the adult brachycephalic dogs, but a lower volume. The brachycephalic puppy also had lower length and breadth measurements in comparison to the doliocephalic puppies, but similar height and volume measurements. The greatest variation of measured values was observed in the mesocephalic group.

3.4. Micromorphometric measurements of the epiglottis

Histological assessment of the epiglottis was undertaken on all 41 dogs. Table 5 presents the distribution of micromorphological measurements from distal, middle and proximal epiglottis. The percentage of epithelium, lamina propria-submucosa, and cartilage core relative to total epiglottis, as well as the proportions of chondral cells plus matrix, fibrous tissue and adipose tissue relative to the cartilage core were calculated from individual dogs divided into their three head groups and further partitioned by age.[169,170,171] Individual measurements specific to breed, age, sex and weight are displayed in Appendix D.

Table 5: Distribution of percent areas (median and observed range of values) of tissue components from distal, middle and proximal epiglottis. Measurements include epithelium, lamina propria-submucosa, cartilage core relative to total epiglottis area, as well as chondral cells/matrix, fibrous tissue and adipose tissue relative to cartilage core area. Data presented from 41 dogs, including brachycephalic (n = 10 adults, n = 2 juvenile/puppy), mesocephalic (n = 21 adults) and doliocephalic (n = 3 adults, n = 5 juvenile/puppy) dogs. Head shape denoted by BC = brachycephalic, MC = mesocephalic, DC = doliocephalic, and histological section denoted as D = distal, M = middle, P = proximal.

Head shape		Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
BC adults	D	6.05 (3.53-9.73)	34.6 (23.8-51.3)	59.5 (44.2-72.7)	22.4 (4.02-31.2)	46.7 (35.3-73.9)	29.7 (9.36-52.9)
	M	3.35 (1.87-10.6)	43.9 (17.8-52.1)	49.7 (37.4-79.2)	15.2 (1.33-24.2)	38.3 (0.91-56.0)	46.4 (28.0-83.6)
	P	3.16 (1.63-6.49)	40.9 (24.0-48.7)	50.3 (37.9-65.5)	12.8 (0.88-30.7)	23.6 (12.9-57.7)	59.3 (24.4-86.2)
BC puppies	D	7.50 (3.47-11.5)	43.3 (42.0-44.6)	49.2 (43.9-54.5)	13.7 (3.01-24.3)	44.5 (40.3-48.8)	41.9 (35.5-48.2)
	M	6.10 (1.52-10.7)	32.9 (32.6-33.3)	52.1 (43.7-60.5)	4.98 (2.84-7.12)	30.4 (25.1-35.7)	64.6 (57.2-72.0)
	P	1.82 (1.06-2.57)	24.7 (21.6-27.8)	41.8 (35.9-47.6)	0.77 (0.60-0.93)	50.0 (34.4-65.6)	49.2 (33.4-65.0)
MC adults	D	6.22 (3.25-11.0)	37.4 (15.2-53.7)	56.4 (37.0-80.4)	14.5 (0.75-25.4)	52.0 (32.9-67.3)	33.3 (17.7-61.9)
	M	2.78 (1.45-9.63)	38.1 (22.0-57.5)	55.1 (35.1-75.5)	10.9 (1.89-20.0)	29.8 (19.1-50.2)	59.6 (31.6-79.0)

Table 5 – continued.

Head shape		Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
	P	2.57 (0.55-17.6)	34.1 (1.09-61.4)	45.9 (23.9-71.6)	3.70 (0.33-15.5)	23.8 (13.2-39.0)	72.1 (47.0-86.5)
DC adults	D	4.34 (4.32-4.80)	36.1 (34.5-47.8)	59.6 (47.9-60.7)	22.0 (19.2-22.2)	39.7 (37.1-57.6)	38.4 (20.2-43.7)
	M	4.99 (3.18-5.03)	35.5 (27.6-36.1)	61.3 (59.0-67.4)	15.2 (9.42-24.8)	35.9 (28.9-37.2)	54.7 (38.0-56.0)
	P	4.52 (2.01-4.94)	43.0 (31.6-43.7)	48.8 (46.9-58.9)	7.51 (6.56-8.07)	24.0 (20.4-25.8)	68.5 (66.2-73.0)
DC puppies	D	12.1 (10.7-13.4)	27.9 (20.6-32.7)	58.7 (55.5-67.4)	0.46 (0.16-24.6)	33.9 (0.98-56.7)	59.9 (42.6-98.9)
	M	8.62 (5.38-10.6)	30.9 (29.6-37.7)	53.7 (48.2-58.6)	0.65 (0.48-14.8)	28.1 (10.8-31.1)	71.4 (68.3-74.4)
	P	6.08 (4.15-11.4)	39.8 (19.1-51.0)	36.7 (34.7-39.7)	1.09 (0.12-17.0)	24.7 (16.0-32.7)	72.8 (50.3-83.9)

3.5. Qualitative assessment of epiglottal micromorphology

Chondral cells (chondrocytes/chondroblast clusters) and associated cartilaginous matrix were distributed in clusters primarily on the peripheries of the cartilaginous core, and they often coalesced on the ventral aspect or less frequently formed bridging structures through the centre (Figure 11). In contrast, fibrous and adipose tissue appeared to be distributed more centrally and adipose tissue appeared to increase from distal to proximal areas. Sectional artefacts were observed in 23/41 specimens, seen most commonly as uplifted soft tissue overlying the cartilage core or mineralised regions within the cartilage core where brittle tissue dropped off the section (Figure 11). Rarely sectional folding or complete loss of larger regions were observed.

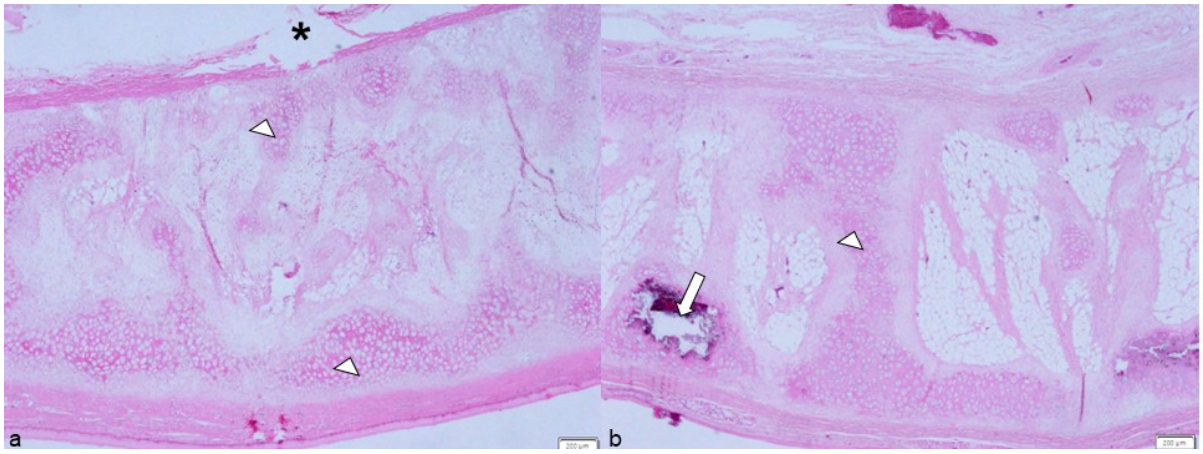

Figure 11: Photomicrographs of H&E sections of the middle third of the epiglottal cartilage from a) a brachycephalic dog and b) a doliocephalic dog displaying in chondral cells/matrix distribution (white arrowhead) on the dorsal and ventral borders and less frequently bridging. Also displayed are two most commonly seen sectional artefacts of uplifted soft tissue overlying the cartilage (asterisk), and mineralised region where brittle tissue has dropped off the section (white arrow). Scale bar = 200 µm.

3.6. Quantitative micromorphometric measurements

Overall for all dogs and puppies included in the study (n = 41), the median percent areas were as follows: epithelium (adult: 3.35%, puppy: 7.36%), lamina propria-submucosa (adult: 37.4%, puppy: 31.9%%), cartilage core (adult: 55.1%, puppy: 51.73%) relative to total epiglottis, and chondral cells/matrix (adult: 15.2%, puppy: 2.82%), fibrous tissue (adult: 35.9%, puppy: 36.3%) and adipose tissue (adult: 54.7%, puppy: 60.3%) relative to the cartilage core (Table 5). Figure 12 displays a middle section from a mesocephalic dog displaying the distinct tissues measured.

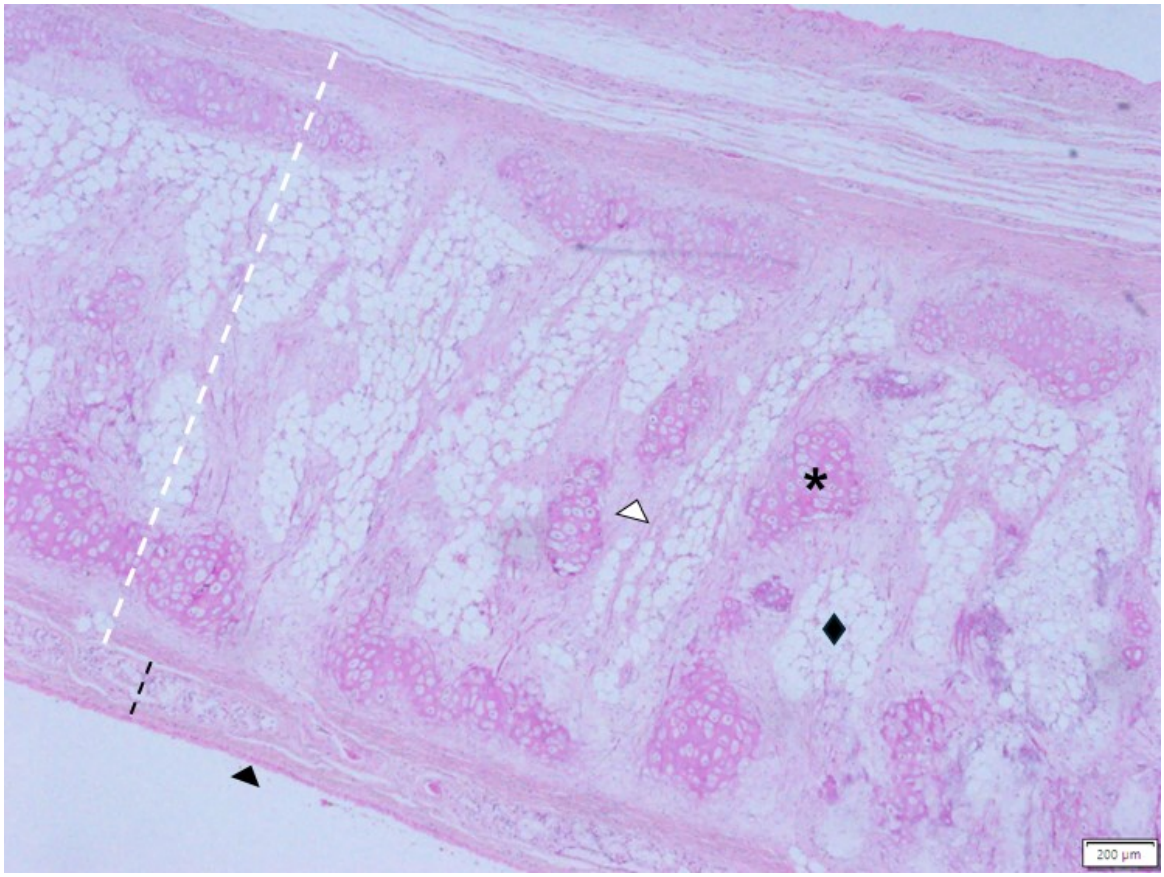

Figure 12: Photomicrograph of a H&E section of the middle third of the epiglottal cartilage from a representative section of a mesocephalic dog. Areas measured are epithelium (black arrowhead), lamina propria-submucosa (dashed black line), cartilage core (dashed white line), chondral cells clusters (asterisk), fibrous tissue (white arrowhead) and adipose tissue (black diamond). Scale bar = 200 µm.

Figure 13 presents the statistically significant comparisons of micromorphometric measurements observed in adult brachycephalic (n = 10) and mesocephalic (n = 21) dogs and relates to percent chondral cell/matrix area of the distal epiglottis and percent chondral cell/matrix area of the proximal epiglottis. With the exception of the chondral cell/matrix component, there were no statistically significant differences in micromorphometric measurements in the distal, middle and proximal sections when adult brachycephalic and mesocephalic dogs were compared. The percent area of chondral cell/matrix was significantly greater in the distal section for BC adults (P = 0.016, Figure 13) and also was significantly greater in the proximal section for BC adults (P = 0.043, Figure 13). Statistically significant differences were not observed in epithelium, lamina propria-submucosa, cartilage core, fibrous tissue and adipose tissue measurements in adult brachycephalic and mesocephalic dogs.

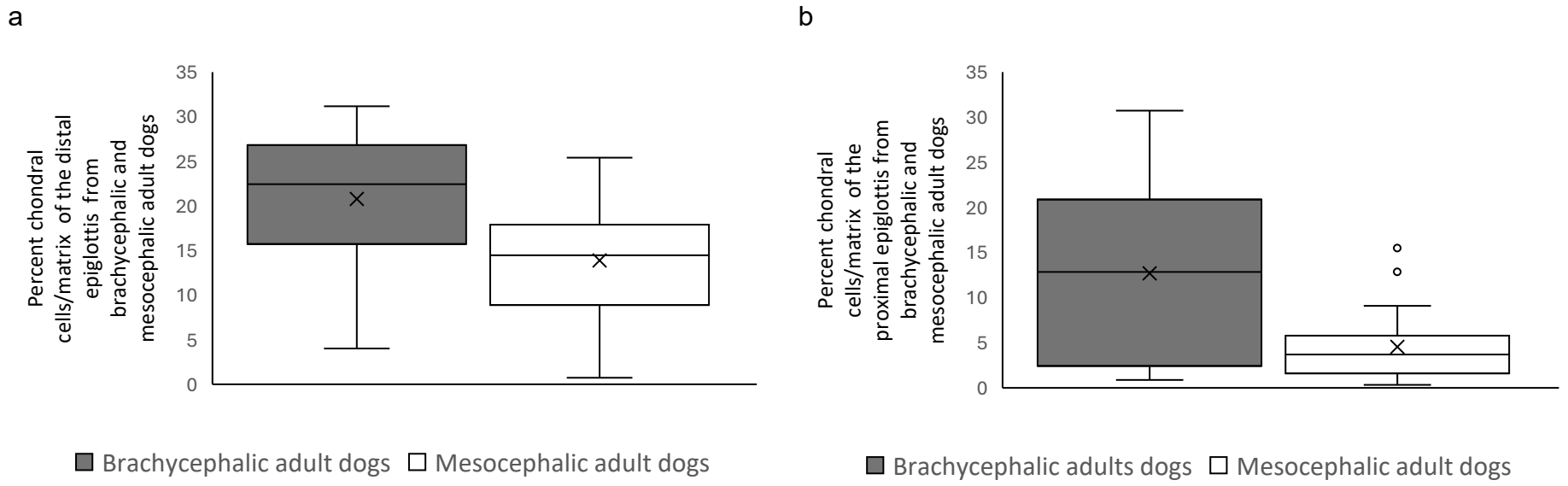

Figure 13: Comparison of micromorphometric measurements (two sample t-test) for adult brachycephalic (n = 10) and mesocephalic (n = 21) dogs, displaying a) percent chondral cell/matrix area of the distal epiglottis and b) percent chondral cell/matrix area of the proximal epiglottis. Data series components displayed as upper and lower quartiles relating to internal horizontal line corresponding to the median, with internal cross symbol corresponding to the mean, whisker lines extending parallel from the box indicating variability outside the upper and lower quartiles, and outliners plotted as individual dots in-line with the whiskers.

Subtle variations, whilst not compared statistically due to insufficient sample numbers, were also observed amongst the three head groups. Except for in DC adults, percent epithelium decreased from distal to proximal sections and doliocephalic puppies had a greater percent epithelium area than doliocephalic adults. Percent lamina propria-submucosa in the distal section was greater in doliocephalic puppies compared to doliocephalic adults. Percent cartilage core was similar across all groups and percent chondral cells/matrix was highest from distal to proximal sections across all groups with the exception of DC puppies. Percent fibrous decreased from distal to proximal sections except in brachycephalic dogs and puppies, while percent adipose tissue increased from distal to proximal sections in all groups with the exception of BC puppies.

3.7. Histopathological features

Table 6 presents the histopathological features of the distal, middle and proximal epiglottis from 41 dogs divided into their three groups (BC, MC and DC) partitioned by age. Individual measurements of the grading and median numbers specific to breed, age, sex and weight are displayed in Appendix E.

Table 6: Distribution of histopathological grading and changes from distal, middle and proximal epiglottis. Oedema, inflammation, cartilage metachromasia, and cartilage calcification/mineralisation evaluated in brachycephalic (n = 10 adults, n = 2 juvenile/ puppy), mesocephalic (n = 21 adults) and doliocephalic (n = 3 adults, n = 5 puppies) dogs. Data includes median values from five randomly selected fields (total area: 0.8 mm^2) for total chondral cells and degenerate chondral cells, and percent degenerate chondral cells. Data for age and body weight provided as median and observed range of values in parenthesis, head shape denoted by BC = brachycephalic, MC = mesocephalic, DC = doliocephalic, and histological section denoted as D = distal, M = middle, P = proximal.

Head shape		Oedema grade*	Inflammation grade	Cartilage metachromasia grade	Cartilage calcification/ mineralisation grade	Total chondral cells median (total area: 0.8 mm^2)	Degenerate chondral cells median (total area: 0.8 mm^2)	Percent degenerate chondral cells relative to total cells
BC adults	D	1 (0-3)	0.5 (0-1)	2 (0-2)	0 (0-1)	50 (31-102)	2 (0-9)	3.00
	M	2 (0-2)	1 (0-2)	2 (0-2)	0 (0-1)	59 (37-85)	2 (0-14)	2.56
	P	1 (0-2)	1 (0-1)	1.5 (0-2)	0 (0-1)	47 (19-81)	1 (0-15)	2.15
BC puppies	D	1.5 (1-2)	0 (0)	0.5 (0-1)	0 (0)	55 (41-70)	2 (0-3)	2.70
	M	1.5 (1-2)	0.5 (0-1)	0.5 (0-1)	0 (0)	46 (30-62)	0 (0)	0.00
	P	1 (1)	0.5 (0-1)	0 (0)	0 (0)	31 (29-32)	0 (0)	0.00

*It was not always possible to distinguish between oedema and separation artefact.

Table 6 – continued.

Head shape		Oedema grade	Inflammation grade	Cartilage metachromasia grade	Cartilage calcification/ mineralisation grade	Total chondral cells median (total area: 0.8 mm^2)	Degenerate chondral cells median (total area: 0.8 mm^2)	Percent degenerate cells relative to total cells
MC adults	D	2 (0-3)	0 (0-1)	2 (0-2)	0 (0-1)	41 (17-72)	2 (1-9)	4.88
	M	2 (0-3)	1 (0-2)	2 (1-2)	1 (0-1)	42 (23-65)	2 (0-8)	4.76
	P	1 (0-3)	0 (0-2)	1 (0-2)	0 (0-1)	35 (8-52)	2 (0-6)	5.71
DC adults	D	2 (1-2)	0 (0)	1 (1-2)	1 (0-1)	49 (46-65)	2 (2-4)	4.08
	M	2 (1-2)	0 (0-1)	2 (0-2)	1 (1)	48 (42-60)	2 (2-3)	4.17
	P	1 (1)	0 (0-1)	2 (1-2)	0 (0-1)	48 (33-52)	6 (2-6)	12.5
DC puppies	D	1 (1-2)	0 (0)	0 (0)	0 (0)	81 (0-97)	0 (0)	0.00
	M	2 (1-3)	1 (0-1)	0 (0)	0 (0)	77 (60-84)	0 (0-1)	0.00
	P	2 (2-3)	0 (0-1)	0 (0)	0 (0)	44 (20-65)	0 (0)	0.00

*It was not always possible to distinguish between oedema and separation artefact.

3.8. Qualitative evaluation of histopathological features

In all head groups, inflammatory cells observed were varying amounts of predominantly lymphocytes and plasma cells within the lamina propria-submucosa, tending towards peri-glandular or peri-vascular distribution in higher grades. Figure 14 shows a representative example of inflammation observed in a mesocephalic dog. Occasionally, associated with adult dogs in all head groups, chondral cell/matrix regions were mineralised or showed myxoid material deposition and more areas of fibrosis. Figure 15 shows representative examples of fibrosis and myxoid material deposition in brachycephalic dog and mineralisation in a doliocephalic dog. Furthermore, the majority of chondral cells in the 41 samples were chondroblasts versus chondrocytes and in some sections, features of degeneration were noted. Chondroblasts more reliably displayed primarily cytoplasmic eosinophilia and vacuolation associated with degeneration, and less frequently nuclear changes.

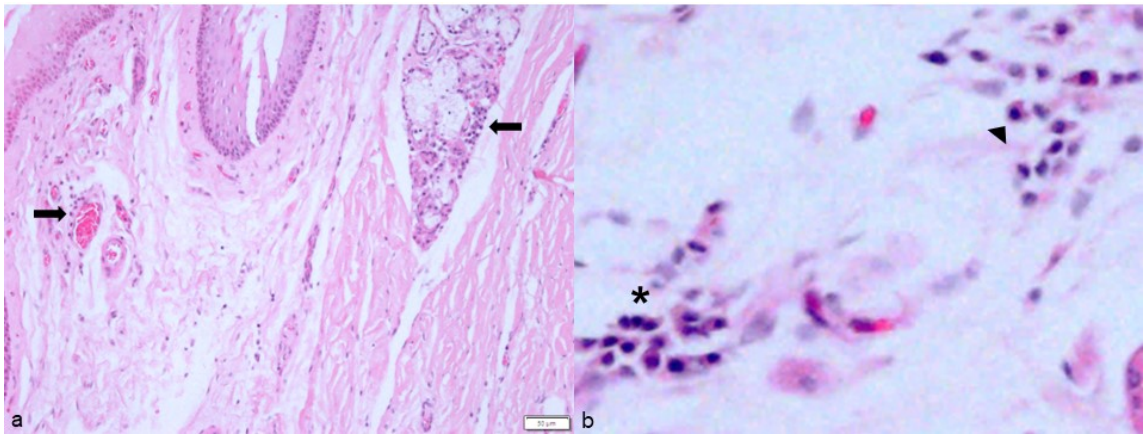

Figure 14: Photomicrographs of H&E sections of the epiglottis from a mesocephalic dog showing a) inflammation within the lamina propria-submucosa and inflammatory cells (black arrow) tending towards peri-glandular and peri-vascular distribution, and b) magnified view of lymphocytes (asterisk) and plasma cell (black arrowhead). Scale bar = 50 µm.

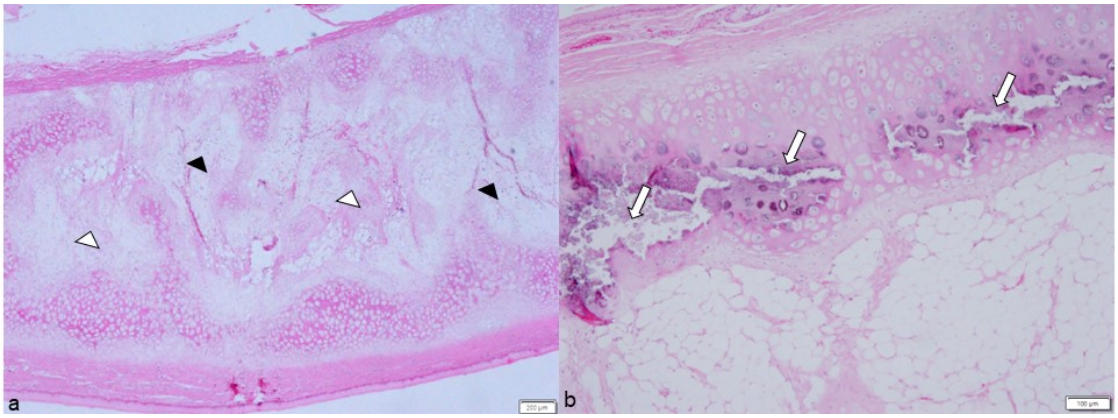

Figure 15: Photomicrographs of H&E sections from the middle third of epiglottal cartilage displaying a) fibrosis (white arrowhead) or myxoid material deposition (black arrowhead) in brachycephalic dog and b) mineralisation (white arrows) in a doliocephalic dog. Scale bar = 200 µm (a) and 100 (b).

3.9. Semi-quantitative evaluation of histopathological features

For all dogs and puppies included in the study, grading and medians ranged as follows: oedema (grades 0-3), inflammation (grades 0-2), cartilage metachromasia (grades 0-2), calcification/mineralisation (grades 0-1), total chondral cells (0-102), degenerate chondral cells (0-15), and percent degenerate cells relative to total chondral cells (0-12.5%) and summarised in Table 6. Figure 16 shows grade 0 versus representative examples for grade 2 oedema, inflammation, and cartilage metachromasia, and grade 1 calcification/mineralisation. Figure 17 shows viable chondral cells versus a representative example of degenerate chondral cells.

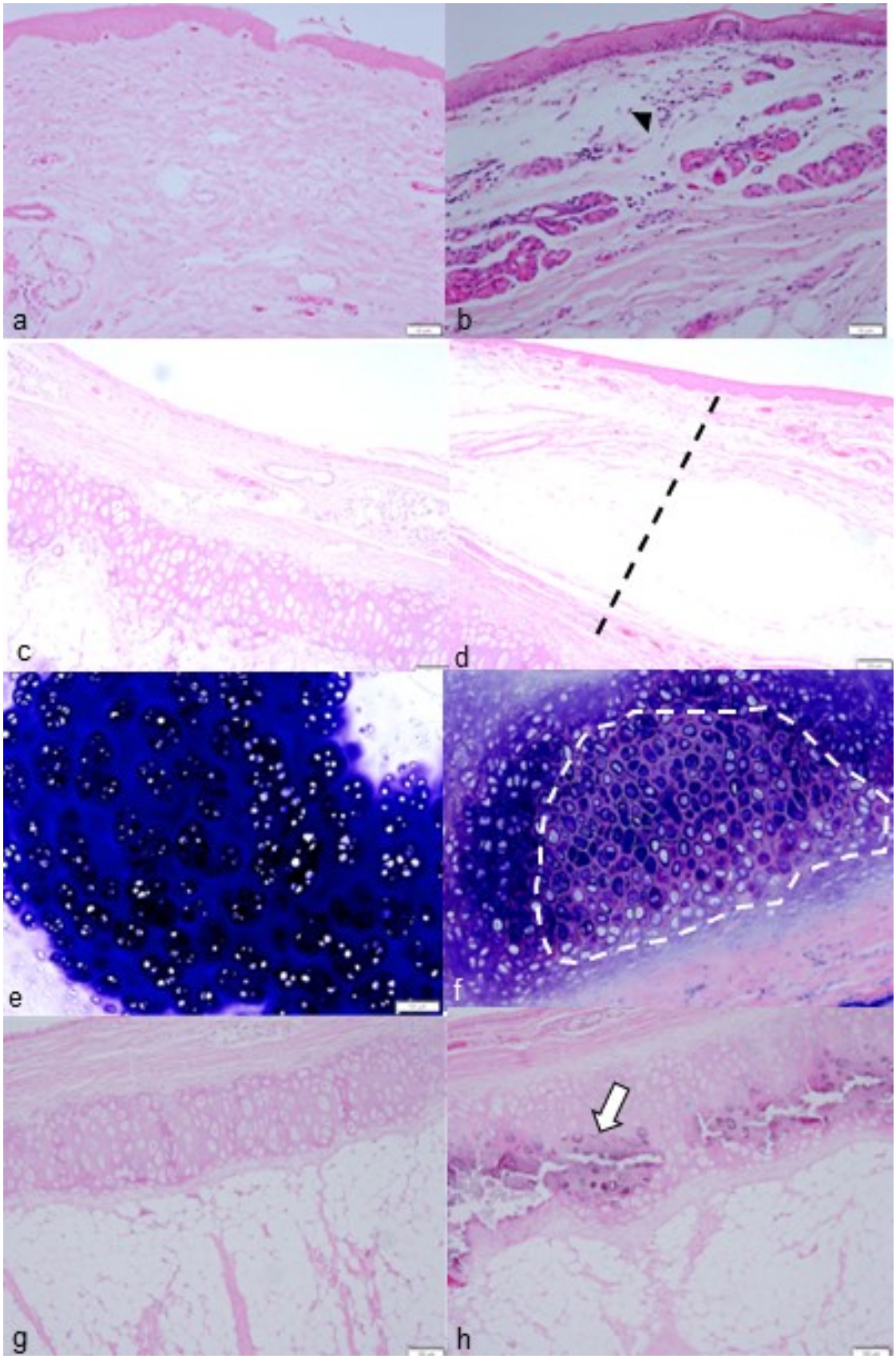

Figure 16: Photomicrographs of H&E and Toluidine Blue sections from a, c, e, g) normal epiglottal and laryngeal cartilage versus b) grade 2 inflammation, d) grade 2 oedema, f) grade 2 metachromasia and h) grade 1 calcification/mineralisation.

Figure 16 – continued.
Symbols denote inflammatory cells (black arrowhead), oedema (dashed black line), metachromasia (white dashed line) and mineralisation/calcification (white arrow). Scale bar = a, b) 50 µm and c, d, e, f, g, h) 100 µm.

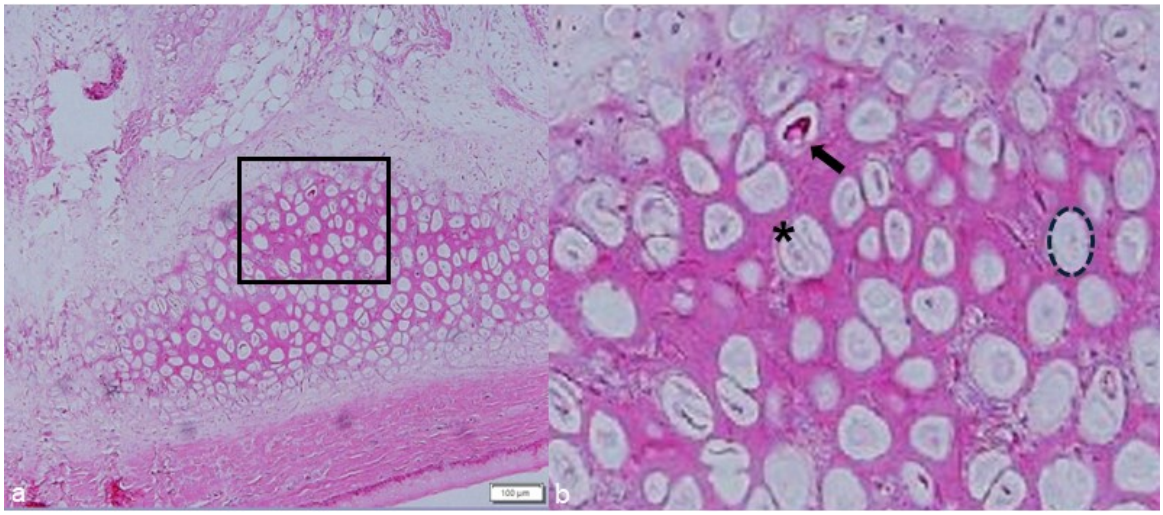

Figure 17: Photomicrographs of H&E sections from the epiglottal cartilage core displaying a) a peripheral chondral cells/matrix region, and b) inset showing chondral cells with central nuclei, pale staining eosinophilic cytoplasm (asterisk) within lacunae (dashed black line) and a degenerate chondral cell displaying cytoplasmic eosinophilia and vacuolation (black arrow). Scale bar = 100 µm.

Figure 18 presents the statistically significant comparisons of histopathological features observed in adult brachycephalic (n = 10) and mesocephalic (n = 21) dogs, and relates to total chondral cells of the distal epiglottis and total chondral cells of the middle epiglottis. With the exception of the total chondral cells component, there were no statistically significant differences in the semi-quantitative evaluation of histopathological features in the distal, middle and proximal sections when adult brachycephalic and mesocephalic dogs were compared. In the distal section, total chondral cells was significantly greater in BC adults (median 50, P = 0.046, Figure 18); as it was in the middle section for total chondral cell in BC adults (median 58, P

= 0.035, Figure 18). Statistically significant differences were not observed for oedema, inflammation, cartilage metachromasia, and cartilage calcification/mineralisation grading in BC and MC adult dogs.

Amongst all three head groups including puppies, whilst not compared statistically due to insufficient sample numbers, subtle variations were observed. The inflammatory cell grade was greater in middle and proximal sections for both adults and puppies, with the higher grade seen in brachycephalic adults, but was lower in the distal sections of brachycephalic puppies, and both puppy and adult doliocephalic dogs. Metachromasia was present in all sections of adults and the distal to middle section of brachycephalic puppies but absent in doliocephalic puppies. Mineralisation was present in all sections of adults and absent in all puppies. The median number of degenerative chondral cells was lower in puppies compared to adults in all groups except in the distal section of brachycephalic puppies. Percent degenerate cells was greater over distal to proximal sections in all adult dogs, absent in puppies, and lower in adult brachycephalic dogs compared to other adult dogs

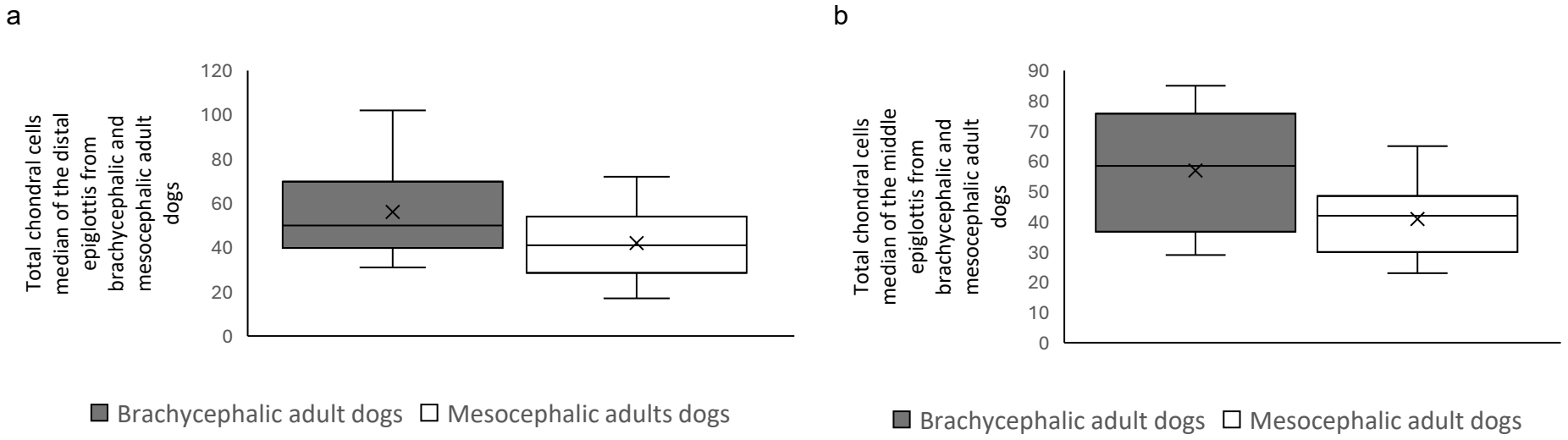

Figure 18: Comparisons of histopathological observations (two sample t-test) for adult brachycephalic (n = 10) and mesocephalic (n = 21) dogs, displaying a) total chondral cells median of the distal epiglottis, and b) total chondral cells median of the middle epiglottis. Data series components displayed as upper and lower quartiles relating to internal horizontal line corresponding to the median, with internal cross symbol corresponding to the mean, whisker lines extending parallel from the box indicating variability outside the upper and lower quartiles, and outliners plotted as individual dots in-line with the whiskers.

Chapter 4: Discussion

The anatomical restrictions and crowding of structures in brachycephalic dogs are asserted to cause interlinked pathophysiology from increased negative airway pressure, turbulence, resistance and contact irritation.[3,10,15] The anatomical features at multiple levels of the airway in brachycephalic dogs and their role in BOAS pathophysiology has been well characterised, however the epiglottis is a tissue that has had minimal evaluation.[3,10] Forty-one dogs and puppies from 25 reported breeds of dogs and crossbreeds ranging from six weeks to 14.5 years were divided into brachycephalic, mesocephalic and doliocephalic head groups. Micromorphometric and histopathological data was collected from all 41 dogs while gross morphological measurement was limited to a subset of 23 dogs. Based on the great variety of size and appearance of dog breeds, and in particular in the mesocephalic (preferred comparison group), gross morphological and microscopic characteristics reflected that variation.

4.1 Morphological gross assessment

Overall, brachycephalic dogs tended to have relatively larger epiglottal dimensions than mesocephalic and doliocephalic dogs, and brachycephalic and doliocephalic juveniles/puppies tended to have larger proportions than adults. However, the limited sample size of the brachycephalic juvenile/puppy group necessitates any trends to be interpreted with caution. Epiglottal length, width, height and volume relative to body weight was evaluated from the three head groups. Epiglottal volume was used as a substitute for epiglottal weight to elucidate any subtle increases collectively of all three linear dimensions that may not be overt when examined

individually. A formula for epiglottal volume was not available in the literature, so epiglottal volume was estimated to be a rectangular pyramid in this study based on the closest possible geometric shape which had a standard formula, given that determining the exact volume or weight was not possible for en bloc unfixed tissues.

A previous morphological study on the adult canine larynx detailed 10 laryngeal measurements including epiglottal length and width, based on the evaluation of 13 larynges from unspecified dog breeds ranging from 3.95 to 21.6 kg.[152] Linear laryngeal parameters and weight were significantly associated with body weight and the highest variability was associated with epiglottal width.[152] Epiglottal dimensions of adult dogs in this study followed the same trend in larger/heavier dogs, but as the previous study did not specify breed nor head shape, direct comparisons to head shape were not possible. Interestingly, in this study, puppies tended to have larger epiglottal linear dimensions than adults. This could correspond to the pattern of puppies having an increased percentile proportion of internal organs, such as lungs and liver, compared to adult dogs, however increased sample sizes would be beneficial to be certain.[175]

In this study, brachycephalic dogs tended to have relatively larger epiglottal dimensions than mesocephalic and doliocephalic dogs. As brachycephalic dogs have shortened craniofacial features without concomitant reduction of internal nasal, nasopharyngeal and oropharyngeal tissues, this can be reflected in reduced airway space or relative increase in soft tissue.[3,10] Disproportionate reduction of soft tissue leading to airway obstruction can be seen with stenotic nares, nasal passage compression and rotation, and turbinate crowding and malformation.[4,46,107] Airway

changes include nasopharyngeal volume reductions, laryngeal cricoid cartilage airway narrowing, and tracheal and bronchial diameter reductions. [43,55,127] Disproportionate reduction of soft tissue is similarly reflected in an elongate and thickened soft palate, macroglossia of the tongue and tonsillar volume increase secondary to eversion.[103,115,116]

Overarchingly, the gross morphological findings in this study lent support to the hypothesis that the epiglottal proportions in brachycephalic dogs were relatively increased in comparison to similar sized mesocephalic dogs due to the disproportionate reduction of internal soft tissue components.[3]

4.2 Micromorphological measurements methodology

In this study, micromorphometric evaluation was undertaken using microscope interphase camera-imaging software by manual individual six component area measurement followed by total tissue area calculations for epithelium, lamina propria-submucosa, cartilage core, chondral cells/matrix, fibrous and adipose tissue.[169,170,171] To the author's best knowledge, this study is the first to encompass tissue evaluation of six components from dog epiglottises using this manual approach. The manual micromorphological measurements of the epiglottis provided quantitative data and allowed for objective and reproducible comparison of tissue components in the context of brachycephalic breeds and BOAS pathology.

Micromorphometric evaluation of the epiglottis required manual area measurement for six component proportion determination for tissue types. However, manual micromorphometric area measurement is being superseded by automatic image

analysis which has advanced rapidly in the development of digital image analysis and deep machine learning where tissue classifications are being increasingly used in pathological diagnosis.[169, 176] Two component automatic analysis, that is region(s) of interest versus the other/normal region, is more commonly undertaken as demonstrated in cirrhosis subclassification where the proportionate area of collagen was able to demonstrate differences in liver biopsies from human patients.[177] Three component automatic analysis in combination with immunohistochemistry, pattern analysis and human-verification has also been shown to be able to classify neoplastic, stroma and necrosis components in a mouse xenograft tumour, and move towards classifying benign proliferation, ductal or lobular carcinoma in breast pathology.[176,178] Similarly, four component automatic analysis has been demonstrated to classify nuclei into epithelial, inflammatory, fibroblast and miscellaneous cells in colorectal adenocarcinoma slide images to allow for better understanding of the neoplastic microenvironment.[179] Six component automatic analysis has not been demonstrated in the literature.

In this study, the software's automatic image analysis was limited to two components, so manual six component measurement of tissues was undertaken using the image software followed by total area calculations. Proportions for epithelium, lamina propria-submucosa, cartilage core, chondral cells/matrix, fibrous and adipose tissue were generated for 41 dog epiglottal specimens, and this data now forms a human-verified dataset which can potentially be used in automatic image analysis and deep machine learning.

4.3 Qualitative micromorphometry

Chondral cells/matrix, fibrous and adipose tissue within the epiglottal cartilage core reflected subtle structural and functional distribution of tissue components in all head groups. Chondral cells and associated cartilaginous matrix were distributed in clusters primarily on the peripheries of the cartilaginous core, and they often coalesced on the ventral aspect or less frequently formed bridging structures through the centre. These findings align with a cartilaginous structure requiring rigidity ventrally for functionality.[145] Similarly, fibrous and adipose tissue were distributed more centrally in the cartilage core, and adipose tissue approximately doubled across distal to proximal areas, aligning with the elastic cartilage requiring increased flexibility at the base for swallowing and airway protection.[153]

Chondral cells observed were predominantly chondroblasts, which may have been included under the umbrella term chondrocytes in the literature.[153,154,180,181] Given the nature of elastic tissue and the ongoing requirement or flexibility, chondroblast-rich tissues could suggest dynamic replacement and remodulation.[153] Previously reported canine epiglottal histology has described the epiglottis to be composed of a main central elastic cartilage core with an overlying lamina propria-submucosa and non-keratinised, stratified squamous epithelium.[153,155,156] The elastic cartilage core was reported to be a peripheral cartilaginous wall enclosing areas of chondrocytes located within lacunae in small aggregates (isogenous clusters), avascular extracellular matrix, adipose tissue and interweaving elastic fibres.[153,155,156] Previous studies on the feline epiglottis confirmed similar findings and further characterised the development and tissue distribution within cartilage core highlighting myxoid tissue to also play a supporting role in cartilage structure.[180,181] Feline epiglottal

studies also proposed that the proportion of adipose tissue could change according to the metabolic situation; as such, a similar process relating to functional requirement could also be occurring in the epiglottis of canines.[180]

In this study, all head groups aligned largely with the reported canine descriptions and additionally demonstrated subtle structural and functional features such as non-uniform distribution of the chondral cells/matrix, fibrous and adipose tissue similar to reported feline descriptions.[153,155,156,180,181]

A previous study of the arytenoid cartilage in brachycephalic and non-brachycephalic dogs reported predominantly chondrocytes with increased extracellular matrix seen in hyaline cartilage, however distinction between chondrocytes and chondroblasts were not mentioned and increased extracellular matrix is consistent with hyaline cartilage that is rigid in comparison to elastic cartilage.[131] Detailed histological studies on canine corniculate and cuneiform elastic laryngeal cartilage are not available for comparison in the literature.

4.4 Quantitative micromorphometry

Chondral cells/matrix area were significantly greater in the distal and proximal sections of the epiglottis in adult brachycephalic dogs compared to mesocephalic dogs. A greater proportion of chondral cell/matrix area, versus softer tissue components such as adipose and fibrous tissue in brachycephalic dogs, suggest increased shape conservation and rigidity required for maintenance of the airway in a relatively reduced space with increased oropharyngeal soft tissue crowding and impedance.[3] Adjacent tissues lacking cartilage and rigidity such as the soft palate, tongue or palatine tonsils demonstrate more overt shape changes from

disproportionate reductions in soft tissue that include respectively elongation or thickening, enlargement, and eversion or enlargement.[103,115,116]

To date, no studies have examined the epiglottis in brachycephalic dogs with the same level as the current study. However, cartilage evaluation in anatomically related regions of the upper and lower airway provide context. Previous studies in brachycephalic dogs evaluating the arytenoid cartilage of the larynx did not include tissue component proportions but reported reduced chondrocyte numbers and matrix degeneration in brachycephalic dogs.[131] Similarly, proportions of tissue components were not included in feline epiglottal studies however detailed location, in-depth cellular characterisation, and interaction of tissue components was undertaken.[180,181] In addition, changes in the tracheal cartilage of brachycephalic dogs with tracheal hypoplasia have been reported as reductions in chondrocytes, chondroitin, glycosaminoglycans, water content and fibrocartilage.[23,24,99] Bronchial wall thickenening evaluated by computer tomography imaging has also been reported in brachycephalic dogs undergoing airway assessment, which could suggest cartilage tissue changes, however histological evaluation was not undertaken.[54,55]

Amongst all head groups including puppies, subtle observations were noted in tissue proportions. Percent cartilage core was similar across all groups suggesting a conservation of anatomical structure as a functional requirement. Percent chondral cells/matrix was highest from distal to proximal sections across all groups with the exception of doliocephalic puppies. This was consistent with the expectation for the physiological need for flexibility at the base of the epiglottis to allow for swallowing and breathing.[145,153,154] Doliocephalic puppies were six weeks old; the youngest of

all dogs in this study, and chondral cells/matrix was less clearly discernible due to the overall increased cellularity and immaturity of the tissue. This finding was similar to that reported in 8-week old kittens where the supporting tissue of the epiglottis was found to be mainly myxoid tissue with few interspersed islets of chondrocytes.[181] Percent fibrous tissue decreased from distal to proximal sections except in brachycephalic dogs and puppies, while percent adipose tissue increased from distal to proximal sections in all groups with the exception of brachycephalic puppies. Interestingly, this could translate to reduced flexibility in the proximal section in brachycephalic dogs suggesting increased shape conservation and rigidity required for maintenance of the airway, that is evident even in puppies.[3]

Regarding the subtilty of variation in epithelium, lamina propria-submucosa, cartilage core, fibrous and adipose tissues, alternatively this could support the epiglottis as being a more conserved structure as a critical requirement for breathing, versus other oropharyngeal soft tissues such as the soft palate and nasal turbinates that have a more disproportionate reduction of soft tissue and contribute to BOAS more overtly.[3,46,103,153,154] Hence, the increased similarity between the three head groups could suggest genetic selection pressure with positive selection for live puppies born and breeding lines.

Broadly, micromorphometric findings in this study suggest that rigid components in the epiglottal cartilage such as chondral cells, cartilaginous matrix and to some extent fibrous tissue, have an increased proportion in brachycephalic dogs. These findings supported the hypothesis that the epiglottis of brachycephalic dogs can have microscopic compositional changes that can be attributed to anatomical constraints

and compensatory tissue changes associated with their head shape and BOAS compared to mesocephalic dogs. Findings of this study also highlight the epiglottal elastic cartilage to be generally a more conserved structure as a necessity for the viability of breeds, but still having an element of subtle dynamic tissue distribution in response to airway maintenance.

4.5 Qualitative histopathological assessment

Histopathological changes observed were associated with inflammatory cells and the elastic cartilage core in all head groups. The predominant inflammatory cells observed in the lamina propria-submucosa were scattered lymphocytes and plasma cells in small numbers, tending towards a peri-glandular or peri-vascular distribution in higher grades. As mucous membranes are continuously exposed to antigens, variable numbers of lymphocytes and plasma cells infiltrate through the lamina propria and can have peri-vascular and peri-glandular distribution as a normal finding.[120,182] It is worth noting none of the dogs in this study had infiltrating neutrophils, nor diffuse aggregates of inflammatory cells or lymphoid follicles, all of which can be seen with an active inflammatory response in pharyngeal mucosa.[182]

In the elastic cartilage core of all head groups, adult dogs displayed mineralisation replacing chondral cells/matrix or rarely, myxoid material deposition associated with fibrous and adipose tissue. Premised on previous literature, mineralisation in cartilage can be a feature associated with degeneration of cartilage though is more commonly observed in articular hyaline cartilage.[172,173] The rarely observed myxoid material on H&E sections was not confirmed by special stain, but could represent functional or an age-related degenerative change.

Feline studies have reported myxoid tissue to be an additional supporting tissue and variable component of the epiglottal elastic cartilage using a combination of special stains, immunohistochemistry and electron microscopy.[180] In the feline epiglottis, myxoid cells were identified and were observed to have a close relationships with chondrocytes and myxoid material was demonstrated to coat all adipocytes and blend in at the boundaries of cartilage matrix.[180,181] By analysing the distribution and relationships of myxoid cells and tissue in embryo, newborn, kitten and adult cat stages, it was presumed a few myxoid cells within the centres of myxoid area transform into chondroblasts that continue to divide and differentiate into cartilage tissue types, and myxoid tissue to serve as a precursor of elastic and fibrous cartilage.[180,181] In this canine study, myxoid tissue when present could be playing a similar role, however special staining and further analysis would be required to understand it's contribution more fully.

As discussed previously, the chondral cells observed were predominantly chondroblasts, which may have been included under the umbrella term chondrocytes in the previous literature.[153,154,180,181] However, features of chondroblast cell degeneration were similar to the reported chondrocytes observed in the arytenoid hyaline cartilage with chondroblasts more reliably displaying cytoplasmic eosinophilia and vacuolation, and less frequently nuclear changes.[131,174] This suggests that characteristics of chondral cell degeneration is similar in both epiglottal elastic cartilage and arytenoid hyaline cartilage, however cartilage remodulation in epiglottal elastic cartilage could also function to increase airway protection.[3,153]

4.6 Semi-quantitative histopathological assessment

Total chondral cell numbers were significantly greater in the distal and middle sections of the epiglottis in adult brachycephalic dogs compared to mesocephalic dogs. This finding suggests that an increased cartilaginous architecture could provide additional stiffness and shape compensation to maximise airway maintenance with increased oropharyngeal soft tissue crowding and impedance.[3] In contrast, previous studies of the arytenoid cartilage in brachycephalic dogs showed reduced chondrocyte numbers and reduced stiffness, supportive of distinct degenerative changes in this region of the larynx.[131] Laryngeal collapse, which is a potential consequence of such structural weakening, has been reported in 50-90% of BOAS-affected dogs with cases as young as 4.5 months.[3,34,48] Mechanical evaluation of epiglottal stiffness was not undertaken in this study.

No statistically significant differences were observed for oedema, inflammation, cartilage metachromasia, calcification/mineralisation and degenerate chondral cells between adult brachycephalic and mesocephalic dogs. However, amongst all head groups, variations were observed with a caveat relating to the oedema parameter. Tissues from dogs in this study, many of which displayed clinical signs of BOAS, were obtained as postmortem samples. Frequently, the evaluation of oedema in the epiglottal epithelium and lamina propria-submucosa was impaired due to emergency treatments, variable postmortem intervals and processing artefact that artificially separated the overlying soft tissues from the cartilage core. However, previous studies of surgical biopsies from the soft palate of brachycephalic dogs with BOAS reported intracellular oedema of the epithelium, oedema within the underlying

lamina-propria with amplified myxoid matrix, and regional increased salivary tissue.[120,122]

In this study, although not a statistically significant finding, inflammatory cells (lymphocytes and plasma cells) within the lamina propria-submucosa were greater particularly in the middle and proximal sections of the epiglottis in all adults and puppies. Higher grades were more commonly observed in brachycephalic adults, whereas lower grades were noted in the distal sections of brachycephalic puppies, and in both puppy and adult doliocephalic dogs. Variable numbers of lymphocytes and plasma cells within the nasopharyngeal tissue have been reported in dogs without clinical respiratory disease.[182] However, previous studies of the nasal turbinates in brachycephalic dogs have reported increased contact points grossly and oedema and lymphoid hyperplasia histologically.[20,46,111] Increased contact points and subsequent irritation of oropharyngeal structures may result in greater inflammatory changes in brachycephalic dogs.[20,46,11] In this study, clinically significant and active inflammation was considered unlikely due to the absence of neutrophils, diffuse chronic inflammatory infiltrates, and lymphoid follicles in all dogs, however higher numbers of scattered inflammatory cells were still associated with brachycephalic adult dogs and could suggest an increased immunosurveillance requirement.[182]

Metachromasia was present in all sections of the epiglottal elastic cartilage in adult dogs in all head groups, and in the distal to middle section of epiglottal cartilage of brachycephalic puppies, but was absent in epiglottal cartilage of doliocephalic puppies. Metachromasia has been reported as a degenerative change in hyaline

cartilage and more commonly described in osteoarthritis in many species which is linked to wear and tear and age.[131,144,172,173] By comparison, metachromasia in the elastic cartilage of brachycephalic puppies could suggest that degenerative changes occur earlier in brachycephalic dogs, providing support for the hypothesis that such changes could be attributable to BOAS. Previous studies of the arytenoid cartilage in brachycephalic dogs also showed increased cartilage metachromasia in the hyaline cartilage with correlation to reduced stiffness in comparison to non-brachycephalic dogs.[131] Metachromasia in the epiglottal cartilage core in adults in this study was interpreted as a likely age-related change.

Mineralisation of the epiglottal cartilage was present in all three adult head groups with representation in all three sections but absent in all sections of brachycephalic and doliocephalic puppies. This change was also attributed to an age-related finding due to its presence in all adult head types.[173] It was likely absent from brachycephalic puppies as it would represent a more advanced cartilage degenerative change when compared to cartilage metachromasia. [144,172,173]

The proportion of degenerative chondral cells was lower in puppies compared to adults in all groups except in the distal section of brachycephalic puppies. The overall lower proportion of degenerative cells in puppies suggest reduced cartilage degeneration at a younger age.[3,131] Interestingly, the presence of degenerate cells specifically in the distal portion of the epiglottal cartilage of brachycephalic puppies could indicate that cartilage degeneration begins earlier in this head shape, consistent with the earlier onset of laryngeal collapse reported in this group.[3,24,48] This distal epiglottic vulnerability could reflect early mechanical impedance from

oropharyngeal soft tissues, supporting the hypothesis that such degenerative changes are associated with BOAS.[3] Notably, while percent degenerate cells was greater from distal to proximal sections in all adult dogs, and absent in doliocephalic puppies, it was lower in adult brachycephalic dogs compared to other adult dogs. This could suggest increased chondral cells maintenance for matrix production and stiffness in distal portions of the elastic cartilage with less requirement for puppies in general, but increased requirement in all sections for brachycephalic adults due to increased necessity for airway maintenance from soft tissue obstruction.[3,131,153,154]

The histopathological findings of an increased proportion of total chondral cells in brachycephalic dogs suggests cartilage preservation, increased shape compensation, and increased rigidity to prioritise airway maintenance. This finding refutes the hypothesis that the epiglottis of brachycephalic dogs will have increased degenerative changes such as reduced chondral cells and reduced stiffness associated with their head shape and BOAS compared to mesocephalic dogs. Other histopathological findings, such as cartilage metachromasia and degenerative chondral cells were more supportive of degenerative change occurring in brachycephalic dogs, including puppies, that could be attributed to their head shape and BOAS, whilst all adult dogs displayed cartilage mineralisation which was attributed to age.

Chapter 5: Limitations, Future Directions and Conclusions

The scarcity of information on both anatomical and histological features of the epiglottis in brachycephalic dogs in the literature resulted in this study into the detailed evaluation of epiglottal dimensions, microscopic components, and any associated histopathology relatable to the three head shapes of dogs.

5.1. Limitations

The main limitations of this study related to insufficient sample size, specifically of doliocephalic adult dogs, brachycephalic puppies and mesocephalic puppies; for robust statistical comparisons and grouping for statistical comparison was limited by diversity of breeds and age (including insufficient recording of age in the animal's history). Histological artefacts also impacted micromorphometric epithelial and lamina propria-submucosa area measurements and histopathological oedema and inflammation grading schemes.

The recruitment of dogs into the three head groups was solely dependent on the university necropsy scheme resulting in retrospective enrolment and suboptimal sample sizes. Delayed commencement of gross morphometric data collection resulted in epiglottal measurements collection of 23 samples from each head group. Retrospective sample availability (paraffin embedded blocks) allowed for inclusion of additional samples into the micromorphometric and histopathological changes data which resulted in increased numbers to 41 samples. This allowed for an improved statistical comparison ratio of greater 1:2; brachycephalic: non-brachycephalic dogs. Sample sizes of doliocephalic adult dogs and juvenile/puppies in all groups were n <

5, necessitating their exclusion from the statistical analysis. Statistically significant differences were demonstrated for chondral cells/matrix area and chondral cells numbers when adult brachycephalic dogs were compared to adult mesocephalic dogs. However, with respect to the other parameters and subtle observations between all head groups, the limited sample size necessitates any trends to be interpreted with caution.

The three head groups consisted of 25 reported breeds of dogs and crossbreeds with ages ranging from six weeks to 14.5 years. Based on the great variety of size and appearance of dog breeds, and in particular in the mesocephalic (preferred comparison group), gross morphological and microscopic characteristics likely reflect that variation, with the potential to reduce statistical significance of the analysis. Head groups were broadly subdivided into adults and juvenile/puppies, but given the large age variation, division into life stages (e.g. puppy, juvenile, subadult, adult, middle aged, aged, geriatric) would have been morphologically and microscopically more appropriate, however the reduced samples sizes prevented robust statistical analysis for the adult brachycephalic group.

Limitations and artefacts associated with the interpretation of the H&E and toluidine blue sections were observed during slide evaluation. Sectional and processing artefacts occurred in the majority of tissues (23/41 slides). This primarily affected the epithelium and lamina propria-submucosa which displayed uplifting, increased separation, partial and complete loss of one or both soft tissue components. As such, epithelial hyperplasia was not included in the analysis and epithelial comparisons were considered less reliable. Relating to oedema grading, it was not

always possible to distinguish between true oedema and sectional artefact; as such oedema grading was also interpreted with caution. Overall, postmortem autolysis, freeze-thaw changes and ante-mortem emergency treatments caused more epithelium sloughing and artificial separation mimicking interstitial oedema within the lamina propria-submucosa, however, did not impact intracellular oedema.

Manual evaluation for epiglottal epithelium, lamina propria-submucosa, cartilage core, chondral cells/matrix, fibrous and adipose tissue proportions was undertaken using microscope interphase camera-imaging software. This utility of this method allowed objective and reproducible data evaluation of the tissue components on a level of detail not previously seen in epiglottal tissue. However, manual area measurement and total area calculations were highly time intensive and while feasible for this study with a limited sample size, could be prohibitive in a study with greater sample size.

5.2. Future directions

A larger study confined to the same brachycephalic breed with a consistent age life stage and medium sized animal (e.g. French bulldog) with comparison to a mesocephalic breed of dog of similar age, life stage and size (e.g. beagle) would allow for improved comparison of gross morphometric, microscopic and histopathological changes associated with BOAS. Ideally, this should be extended to age-matched controls of all life stages with clinical evaluation, which would facilitate the determination of when and how the impact of BOAS occurs as brachycephalic dogs progress through life.

In this study, micromorphometric evaluation was undertaken using microscope interphase camera-imaging software followed by tissue area calculations for six tissues.[169, 170,171] To the author's best knowledge, this study represents the first study to encompass evaluation of six tissue components of dog epiglottises using a manual approach. This data now forms the human-verified dataset component that can be used for algorithm development, programming, automatic image analysis, and a deep machine learning study.

In previous studies of the feline epiglottis, using a combination of special stains, immunohistochemistry and electron microscopy, myxoid cells and matrix were identified to have a close relationship with chondral cells in support and development roles, and myxoid tissue was proposed to be a precursor of elastic and fibrous cartilage.[180,181] In this study, myxoid material was rarely observed in H&E canine epiglottal sections, and special staining for confirmation was not undertaken. Similar to the feline epiglottis, myxoid material could be more widespread with myxoid cells playing a similar role in the canine epiglottis. Alcian blue special staining and composition analysis of myxoid material, alongside with S-100 protein, glial fibrillary acidic protein, neuron specific enolase and neurofilament protein 200 immunohistochemistry for myxoid cells could constitute further study into the role, if any, of myxoid cells and myxoid material distribution in the canine epiglottis.[180,181]

Chondroblasts as opposed to chondrocytes, formed the majority of observed chondral cells in this study, and may have been included under the umbrella term chondrocytes in the original literature describing the elastic cartilage of the epiglottis.[153,154] However, chondroblasts and chondrocytes have differing cartilage

matrix production and maintenance, reflecting a constantly replacing and remodulating nature of elastic cartilage of the epiglottis versus the more commonly studied hyaline cartilage of the larynx and articular joints.[131,154] The role of chondroblasts versus chondrocytes and cartilage matrix production may provide further insight about the epiglottal elastic cartilage in dogs

Additional methods to evaluate degeneration in the elastic cartilage core of the epiglottis could include evaluation and change in fibrous tissue composition and elastic fibres. Polarisation, immunohistochemistry and elastic fibre staining techniques have been previously employed for characterisation of collagen subtypes.[183,184,186] As collagen subtype III and IV are involved more in repair and remodulation, including evaluation of collagen I-IV subtypes and distribution is a consideration for future studies.[185] Additionally, elastic fibres are a distinct feature of elastic cartilage, and evaluation of their distribution or change in the epiglottal cartilage core could be pursued using elastic fibre special stains.[153] The use of these methodologies was beyond the scope of the current study.

Lastly, the focus of the study was the epiglottis, but gross morphometric data and specimens of the palatine tonsils were collected in this study given their close proximity to the epiglottis. The existing palatine tonsils literature is limited, and future studies on this sample collection could provide additional gross morphometric, micromorphometric, inflammatory, degenerative and potentially lymphocyte immunohistochemistry information.[98, 116, 117] Collection of all respiratory system tissues was undertaken in this study as part of the necropsy scheme, and these included nares, soft palate, larynx, trachea and lungs. Comparison of

micromorphometric, inflammatory, reactive and degenerative changes on all levels of the respiratory system with correlation to BOAS may provide additional information into the development of the syndrome in individual dogs.

5.3. Conclusions

This study used multiple methodologies including gross morphological measurements, micromorphometric tissue proportion evaluation, camera interphase imaging software, and histopathological evaluation to evaluate for changes in the epiglottis of brachycephalic dogs and puppies in comparison to mesocephalic and doliocephalic dogs and puppies. Tissue components in the epiglottis were shown to have subtle proportion, reactive and degenerative changes in brachycephalic dogs and puppies that could be relatable to anatomical constraints, physiological requirements, and compensatory tissue changes associated with their head shape and BOAS. This study showed changes in the epiglottis were also more nuanced, variably constrained, age-related, and in particular within the elastic cartilage core.

Gross morphological findings in this study, while not statistical analysed due to insufficient sample numbers, showed brachycephalic dogs tended to have relatively larger epiglottal dimensions than mesocephalic and doliocephalic dogs. This lent support to the hypothesis that the epiglottal proportions in brachycephalic dogs were relatively increased in comparison to similar sized mesocephalic dogs due to the disproportionate reduction of internal soft tissue components due to brachycephalia.

Micromorphometric evaluation showed chondral cells/matrix area were statistically significantly greater in the distal and proximal sections of the epiglottis in adult

brachycephalic dogs compared to mesocephalic dogs. This finding supported the hypothesis that epiglottis of brachycephalic dogs can have microscopic compositional changes that lend themselves to increased shape conservation and rigidity to prioritised airway maintenance associated with their head shape and BOAS compared to mesocephalic dogs. Other qualitative micromorphometric such as chondral cells and cartilaginous matrix distribution alongside overall similarities of cartilage core, fibrous and adipose tissues may possibly indicate the epiglottis as a more conserved, critical structure related to viability of breeds.

Histopathological evaluation showed total chondral cell numbers were statistically greater in the distal and middle sections of the epiglottis in adult brachycephalic dogs compared to mesocephalic dogs. Such a finding could further support increased shape compensation and increased stiffness to prioritise airway maintenance but opposed aspects of the hypothesis that the epiglottis of brachycephalic dogs would have increased degenerative changes associated with their head shape and BOAS compared to mesocephalic dogs. Other histopathological findings such as cartilage metachromasia and degenerative chondral cells were more supportive of degenerative change occurring in brachycephalic dogs and in puppies compared to doliocephalic dogs, whilst all adult dogs displayed cartilage mineralisation which was attributed to age.

Future studies to further understand the role of the epiglottis in BOAS could utilise age-matched controls of life stages, automatic image analysis, myxoid tissue evaluation, chondral cell roles, fibrous tissue composition, elastic fibre evaluation, palatine tonsil evaluation, and multi-level comparison of the respiratory tract.

References

1. Bannasch D, Young A, Myers J, et al. Localization of canine brachycephaly using an across breed mapping approach. *PLoS One*. 2010;5(3):e9632. doi:10.1371/journal.pone.0009632

2. Geiger M, Schoenebeck JJ, Schneider RA, Schmidt MJ, Fischer MS, Sánchez-Villagra MR. Exceptional changes in skeletal anatomy under domestication: the case of brachycephaly. *Integr Org Biol*. 2021;3(1):obab023. doi:10.1093/iob/obab023

3. Krainer D, Dupré G. Brachycephalic obstructive airway syndrome. *Vet Clin North Am Small Anim Pract*. 2022;52(3):749-780. doi:https://doi.org/10.1016/j.cvsm.2022.01.013

4. Liu NC, Troconis EL, Kalmar L, et al. Conformational risk factors of brachycephalic obstructive airway syndrome (BOAS) in pugs, French bulldogs, and bulldogs. *PLoS One*. 2017;12(8):e0181928. doi:10.1371/journal.pone.0181928

5. Boyko AR, Quignon P, Li L, et al. A simple genetic architecture underlies morphological variation in dogs. *PLOS Biol*. 2010;8(8):e1000451. doi:10.1371/journal.pbio.1000451

6. Schoenebeck JJ, Hutchinson SA, Byers A, et al. Variation of BMP3 contributes to dog breed skull diversity. *PLoS Genet*. 2012;8(8):e1002849. doi:10.1371/journal.pgen.1002849

7. Marchant TW, Johnson EJ, McTeir L, et al. Canine brachycephaly is associated with a retrotransposon-mediated missplicing of SMOC2. *Curr Biol*. 2017;27(11):1573-1584. doi:10.1016/j.cub.2017.04.057

8. Teng KT, McGreevy PD, Toribio JA, Dhand NK. Trends in popularity of some morphological traits of purebred dogs in Australia. *Canine Genet Epidemiol*. 2016;3:2. doi:10.1186/s40575-016-0032-2

9. Packer RMA, Hendricks A, Tivers MS, Burn CC. Impact of facial conformation on canine health: brachycephalic obstructive airway syndrome. *PLoS One*. 2015;10(10):e0137496. doi:10.1371/journal.pone.0137496

10. Dupre G, Heidenreich D. Brachycephalic syndrome. *Vet Clin North Am Small Anim Pract*. 2016;46(4):691-707. doi:10.1016/j.cvsm.2016.02.002

11. Bannasch D, Famula T, Donner J, et al. The effect of inbreeding, body size and morphology on health in dog breeds. *Canine Med Genet*. 2021;8(1):12. doi:10.1186/s40575-021-00111-4
12. O'Neill DG, Pegram C, Crocker P, Brodbelt DC, Church DB, Packer RMA. Unravelling the health status of brachycephalic dogs in the UK using multivariable analysis. *Sci Rep*. 2020;10(1):17251. doi:10.1038/s41598-020-73088-y
13. Evans HE, Miller ME. *Miller's Anatomy of the Dog*. 3rd ed. Saunders; 1993.
14. Koch DA, Wiestner T, Balli A, et al. Proposal for a new radiological index to determine skull conformation in the dog. *Schweiz Arch Tierheilkd*. 2012;154(5):217-220.
15. Meola SD. Brachycephalic airway syndrome. *Top Companion Anim Med*. 2013;28(3):91-96. doi:10.1053/j.tcam.2013.06.004
16. Regodon S, Vivo JM, Franco A, Guillen MT, Robina A. Craniofacial angle in dolichocephalic, mesocephalic and brachycephalic dogs: radiological determination and application. *Ann Anat*. 1993;175(4):361-363. doi:10.1016/s0940-9602(11)80043-9
17. Phillips H. Updates in upper respiratory surgery. *Vet Clin North Am Small Anim Pract*. 2022;52(2):339-368.
18. Ladlow J. Brachycephalic obstructive airway syndrome: guide to the respiratory functional grading scheme. *In Pract*. 2021;43(10):548-555. doi:10.1002/inpr.149
19. Riecks TW, Birchard SJ, Stephens JA. Surgical correction of brachycephalic syndrome in dogs: 62 cases (1991-2004). *J Am Vet Med Assoc*. 2007;230(9):1324-1328. doi:10.2460/javma.230.9.1324
20. Lodato DL, Hedlund CS. Brachycephalic airway syndrome: pathophysiology and diagnosis. *Compend Contin Educ Vet*. 2012;34(7):E3.
21. Koch DA, Arnold S, Hubler M, Montavon PM. Brachycephalic syndrome in dogs. *Compend Contin Educ Pract Vet*. 2003;25(1):48-55.
22. Trappler M, Moore K. Canine brachycephalic airway syndrome: pathophysiology, diagnosis, and nonsurgical management. *Compend Contin Educ Vet*. 2011;33(5):E1-4.
23. Ekenstedt KJ, Crosse KR, Risselada M. Canine brachycephaly: anatomy, pathology, genetics and welfare. *J Comp Pathol*. 2020;176:109-115. doi:10.1016/j.jcpa.2020.02.008

24. Pink JJ, Doyle RS, Hughes JML, Tobin E, Bellenger CR. Laryngeal collapse in seven brachycephalic puppies. *J Small Anim Pract*. 2006;47(3):131-135. doi:10.1111/j.1748-5827.2006.00056.x
25. Freiche V, German AJ. Digestive diseases in brachycephalic dogs. *Vet Clin North Am Small Anim Pract*. 2021;51(1):61-78. doi:10.1016/j.cvsm.2020.09.006
26. Costa J, Steinmetz A, Delgado E. Clinical signs of brachycephalic ocular syndrome in 93 dogs. *Ir Vet J*. 2021;74(1):3. doi:10.1186/s13620-021-00183-5
27. Brown JD, Podadera J, Ward M, Goldsmid S, Simpson DJ. The presence, morphology and clinical significance of vertebral body malformations in an Australian population of French Bulldogs and Pugs. *Aust Vet J*. 2021;99(9):378-387. doi:10.1111/avj.13094
28. Sokolowski W, Barszcz K, Kupczynska M, et al. Morphometry and morphology of rostral cranial fossa in brachycephalic dogs – CT studies. *PLoS One*. 2020;15(10):e0240091. doi:10.1371/journal.pone.0240091
29. Regalado Ibarra AM, Legendre L. Anatomy of the brachycephalic canine hard palate and treatment of acquired palatitis using CO_2 laser. *J Vet Dent*. 2019;36(3):186-197. doi:10.1177/0898756419893127
30. Komsta R, Osiński Z, Dębiak P, Twardowski P, Lisiak B. Prevalence of pectus excavatum (PE), pectus carinatum (PC), tracheal hypoplasia, thoracic spine deformities and lateral heart displacement in thoracic radiographs of screw-tailed brachycephalic dogs. *PLoS One*. 2019;14(10):e0223642. doi:10.1371/journal.pone.0223642
31. Wagner F, Ruf I. "Forever young"-Postnatal growth inhibition of the turbinal skeleton in brachycephalic dog breeds (*Canis lupus familiaris*). *Anat Rec*. 2021;304(1):154-189. doi:10.1002/ar.24422
32. Fasanella FJ, Shivley JM, Wardlaw JL, Givaruangsawat S. Brachycephalic airway obstructive syndrome in dogs: 90 cases (1991-2008). *J Am Vet Med Assoc*. 2010;237(9):1048-1051. doi:10.2460/javma.237.9.1048
33. Hendricks JC. Brachycephalic airway syndrome. *Vet Clin North Am Small Anim Pract*. 1992;22(5):1145-1153. doi:10.1016/s0195-5616(92)50306-0
34. Canola RAM, Sousa MG, Braz JB, et al. Cardiorespiratory evaluation of brachycephalic syndrome in dogs. *Pesqui Vet Bras*. 2018;38(6):1130-1136. doi:10.1590/1678-5150-pvb-5376

35. Barker DA, Tovey E, Jeffery A, Blackwell E, Tivers MS. Owner reported breathing scores, accelerometry and sleep disturbances in brachycephalic and control dogs: A pilot study. *Vet Rec*. 2021;189(4):e135. doi:10.1002/vetr.135
36. Planellas M, Cuenca R, Tabar MD, et al. Evaluation of C-reactive protein, haptoglobin and cardiac troponin 1 levels in brachycephalic dogs with upper airway obstructive syndrome. *BMC Vet Res*. 2012;8:152. doi:10.1186/1746-6148-8-152
37. Facin AC, Uscategui RAR, Maronezi MC, et al. Liver and spleen elastography of dogs affected by brachycephalic obstructive airway syndrome and its correlation with clinical biomarkers. *Sci Rep*. 2020;10(1):16156. doi:10.1038/s41598-020-73209-7
38. Yamini SH, Kavitha S, Nambi AP. Blood gas analysis and management of canine brachycephalic airway syndrome (BAS) in brachycephalic dogs. *Intas Polivet*. 2016;17(2):506-508.
39. Dias MLD, Morris CFM, Moreti BM, et al. Anatomical, cardiovascular, and blood gas parameters in dogs with brachycephalic syndrome. *Acta Sci Vet*. 2016;44(1):1356.
40. Davis MS, Cummings SL, Payton ME. Effect of brachycephaly and body condition score on respiratory thermoregulation of healthy dogs. *J Am Vet Med Assoc*. 2017;251(10):1160-1165. doi:10.2460/javma.251.10.1160
41. Darcy HP, Humm K, ter Haar G. Retrospective analysis of incidence, clinical features, potential risk factors, and prognostic indicators for aspiration pneumonia in three brachycephalic dog breeds. *J Am Vet Med Assoc*. 2018;253(7):869-876. doi:10.2460/javma.253.7.869
42. Van Ginneken K, Van Goethem B, Devriendt N, Bosmans T, De Rooster H. Epiglottic retroversion in nine dogs. *Vlaams Diergeneeskundig Tijdschrift*. 2020;89(3), 152-158.
43. Kim YJ, Lee N, Yu J, et al. Three-dimensional volumetric magnetic resonance imaging (MRI) analysis of the soft palate and nasopharynx in brachycephalic and non-brachycephalic dog breeds. *J Vet Med Sci*. 2019;81(1):113-119. doi:10.1292/jvms.17-0711
44. Hostnik ET, Scansen BA, Zielinski R, Ghadiali SN. Quantification of nasal airflow resistance in English bulldogs using computed tomography and computational fluid dynamics. *Vet Radiol Ultrasound*. 2017;58(5):542-551. doi:10.1111/vru.12531

45. Heidenreich D, Gradner G, Kneissl S, Dupre G. Nasopharyngeal dimensions from computed tomography of pugs and French bulldogs with brachycephalic airway syndrome. *Vet Surg*. 2016;45(1):83-90. doi:10.1111/vsu.12418
46. Schuenemann R, Oechtering GU. Inside the brachycephalic nose: intranasal mucosal contact points. *J Am Anim Hosp Assoc*. 2014;50(3):149-158. doi:10.5326/jaaha-ms-5991
47. Packer RMA, Tivers MS. Strategies for the management and prevention of conformation-related respiratory disorders in brachycephalic dogs. *Vet Med Res Rep*. 2015;6:219-232. doi:10.2147/vmrr.S60475
48. MacPhail CM. Laryngeal disease in dogs and cats: an update. *Vet Clin North Am Small Anim Pract*. 2020;50(2):295-310. doi:10.1016/j.cvsm.2019.11.001
49. Harvey CE. Inherited and congenital airway conditions. *J Small Anim Pract*. 1989;30(3):184-187. doi:10.1111/j.1748-5827.1989.tb01531.x
50. White RN. Surgical management of laryngeal collapse associated with brachycephalic airway obstruction syndrome in dogs. *J Small Anim Pract*. 2012;53(1):44-50. doi:10.1111/j.1748-5827.2011.01156.x
51. Rubin JA, Holt DE, Reetz JA, Clarke DL. Signalment, clinical presentation, concurrent diseases, and diagnostic findings in 28 dogs with dynamic pharyngeal collapse (2008–2013). *J Vet Intern Med*. 2015;29(3):815-821. doi:10.1111/jvim.12598
52. Clarke DL, Holt DE, King LG. Partial resolution of hypoplastic trachea in six English bulldog puppies with bronchopneumonia. *J Am Anim Hosp Assoc*. 2011;47(5):329-335. doi:10.5326/JAAHA-MS-5596
53. Yoon H, Yu J, An G, et al. CT and radiographic evaluation of bronchial collapsibility at forced expiration in asymptomatic brachycephalic dogs. *Vet Radiol Ultrasound*. 2020;61(2):167-180. doi:10.1111/vru.12829
54. De Lorenzi D, Bertoncello D, Drigo M. Bronchial abnormalities found in a consecutive series of 40 brachycephalic dogs. *J Am Vet Med Assoc*. 2009;235(7):835-840. doi:10.2460/javma.235.7.835
55. Guillem JS, Schiborra F, Rossanese M, Maddox TW, Mortier JR. Prevalence of bronchial wall thickening and collapse in brachycephalic dogs with and without brachycephalic obstructive airway syndrome and in nonbrachycephalic dogs. *J Am Vet Med Assoc*. 2022;261(10):1-8. doi:10.2460/javma.21.10.0448

56. Ree JJ, Milovancev M, MacIntyre LA, Townsend KL. Factors associated with major complications in the short-term postoperative period in dogs undergoing surgery for brachycephalic airway syndrome. *Can Vet J*. 2016;57(9):976-980.
57. Petchell WHR, Noble PJM, Broome HAO, Burrow R. Incidence of alimentary and respiratory disease in brachycephalic dogs presenting to primary care veterinary practices participating in the SAVSNET project. *Vet Rec*. 2022; 191(7):e1685. doi:10.1002/vetr.1685
58. O'Neill DG, Jackson C, Guy JH, et al. Epidemiological associations between brachycephaly and upper respiratory tract disorders in dogs attending veterinary practices in England. *Canine Genet Epidemiol*. 2015;2:10. doi:10.1186/s40575-015-0023-8
59. Caccamo R, Buracco P, La Rosa G, Cantatore M, Romussi S. Glottic and skull indices in canine brachycephalic airway obstructive syndrome. *BMC Vet Res*. 2014;10(1):12. doi:10.1186/1746-6148-10-12
60. Vilaplana Grosso F, Haar GT, Boroffka SAEB. Gender, weight, and age effects on prevalence of caudal aberrant nasal turbinates in clinically healthy English bulldogs: a computed tomographic study and classification. *Vet Radiol Ultrasound*. 2015;56(5):486-493. doi:10.1111/vru.12249
61. Kaye BM, Boroffka SAEB, Haagsman AN, Haar GT. Computer tomographic, radiographic, and endoscopic tracheal dimension in English Bulldogs with Grade 1 clinical signs of brachycephalic airway syndrome. *Vet Radiol Ultrasound*. 2015;56(6):609-616. doi:10.1111/vru.12277
62. Downing F, Gibson S. Anaesthesia of brachycephalic dogs. *J Small Anim Pract*. 2018;59(12):725-733. doi:10.1111/jsap.12948
63. Gruenheid M, Aarnes TK, McLoughlin MA, et al. Risk of anesthesia-related complications in brachycephalic dogs. *J Am Vet Med Assoc*. 2018;253(3):301-306. doi:10.2460/javma.253.3.301
64. Oda A, Wang WH, Hampton AK, Robertson JB, Posner LP. Perianesthetic mortality in English Bulldogs: a retrospective analysis in 2010-2017. *BMC Vet Res*. 2022;18(1):198. doi:10.1186/s12917-022-03301-9
65. O'Neill DG, Sahota J, Brodbelt DC, Church DB, Packer RMA, Pegram C. Health of pug dogs in the UK: disorder predispositions and protections. *Canine Med Genet*. 2022;9(1):4. doi:10.1186/s40575-022-00117-6

66. O'Neill DG, Packer RMA, Francis P, Church DB, Brodbelt DC. French bulldogs differ to other dogs in the UK in propensity for many common disorders: a VetCompass study. *Canine Med Genet*. 2021;8(1):13. doi:10.1186/s40575-021-00112-3
67. O'Neill DG, Skipper A, Packer RMA, et al. English Bulldogs in the UK: a VetCompass study of their disorder predispositions and protections. *Canine Med Genet*. 2022;9(1):5. doi:10.1186/s40575-022-00118-5
68. Lodato DL, Hedlund CS. Brachycephalic airway syndrome: management. *Compend Contin Educ Vet*. 2012;34(8):E4.
69. Turkki OM, Bergman CE, Lee MH, Hoglund OV. Complications of canine tonsillectomy by clamping technique combined with monopolar electrosurgery – a retrospective study of 39 cases. *BMC Vet Res*. 2022;18(1):242. doi:10.1186/s12917-022-03342-0
70. Holloway GL, Higgins J, Beranek JP. Split staphylectomy to address soft palate thickness in brachycephalic dogs: 75 cases (2016-2018). *J Small Anim Pract*. 2022;63(6):460-467. doi:10.1111/jsap.13485
71. Collivignarelli F, Bianchi A, Vignoli M, et al. Subtotal epiglottectomy and ablation of unilateral arytenoid cartilage as surgical treatments for grade III laryngeal collapse in dogs. *Animals (Basel)*. 2022;12(9):1118. doi:10.3390/ani12091118
72. Dickerson VM, Dillard CMB, Grimes JA, Wallace ML, McAnulty JF, Schmiedt CW. Dorsal offset rhinoplasty for treatment of stenotic nares in 34 brachycephalic dogs. *Vet Surg*. 2020;49(8):1497-1502. doi:10.1111/vsu.13504
73. Gobbetti M, Romussi S, Buracco P, Bronzo V, Gatti S, Cantatore M. Long-term outcome of permanent tracheostomy in 15 dogs with severe laryngeal collapse secondary to brachycephalic airway obstructive syndrome. *Vet Surg*. 2018;47(5):648-653. doi:10.1111/vsu.12903
74. Trappler M, Moore K. Canine brachycephalic airway syndrome: surgical management. *Compend Contin Educ Vet*. 2011;33(5):E1-7.
75. Vangrinsven E, Broux O, Massart L, Claeys S, Clercx C, Billen F. Diagnosis and treatment of gastro-oesophageal junction abnormalities in dogs with brachycephalic syndrome. *J Small Anim Pract*. 2021;62(3):200-208. doi:10.1111/jsap.13279

76. Lindsay B, Cook D, Wetzel JM, Siess S, Moses P. Brachycephalic airway syndrome: management of post-operative respiratory complications in 248 dogs. *Aust Vet J*. 2020;98(5):173-180. doi:10.1111/avj.12926
77. Franklin PH, Liu NC, Ladlow JF. Nebulization of epinephrine to reduce the severity of brachycephalic obstructive airway syndrome in dogs. *Vet Surg*. 2021;50(1):62-70. doi:10.1111/vsu.13523
78. Fenner JVH, Quinn RJ, Demetriou JL. Postoperative regurgitation in dogs after upper airway surgery to treat brachycephalic obstructive airway syndrome: 258 cases (2013-2017). *Vet Surg*. 2020;49(1):53-60. doi:10.1111/vsu.13297
79. Hughes JR, Kaye BM, Beswick AR, Ter Haar G. Complications following laryngeal sacculectomy in brachycephalic dogs. *J Small Anim Pract*. 2018;59(1):16-21. doi:10.1111/jsap.12763
80. Carabalona JPR, Le Boedec K, Poncet CM. Complications, prognostic factors, and long-term outcomes for dogs with brachycephalic obstructive airway syndrome that underwent H-pharyngoplasty and ala-vestibuloplasty: 423 cases (2011-2017). *J Am Vet Med Assoc*. 2022;260(S1):S65-S73. doi:10.2460/javma.20.09.0534
81. Pohl S, Roedler FS, Oechtering GU. How does multilevel upper airway surgery influence the lives of dogs with severe brachycephaly? Results of a structured pre- and postoperative owner questionnaire. *Vet J*. 2016;210:39-45. doi:10.1016/j.tvjl.2016.01.017
82. Haimel G, Dupre G. Brachycephalic airway syndrome: a comparative study between pugs and French bulldogs. *J Small Anim Pract*. 2015;56(12):714-719. doi:10.1111/jsap.12408
83. Seneviratne M, Kaye BM, Ter Haar G. Prognostic indicators of short-term outcome in dogs undergoing surgery for brachycephalic obstructive airway syndrome. *Vet Rec*. 2020;187(10):403. doi:10.1136/vr.105624
84. Planellas M, Cuenca R, Tabar MD, et al. Clinical assessment and C-reactive protein (CRP), haptoglobin (Hp), and cardiac troponin I (cTnI) values of brachycephalic dogs with upper airway obstruction before and after surgery. *Can J Vet Res*. 2015;79(1):58-63.
85. Liu NC, Oechtering GU, Adams VJ, Kalmar L, Sargan DR, Ladlow JF. Outcomes and prognostic factors of surgical treatments for brachycephalic

obstructive airway syndrome in 3 breeds. *Vet Surg*. 2017;46(2):271-280. doi:10.1111/vsu.12608
86. Torrez CV, Hunt GB. Results of surgical correction of abnormalities associated with brachycephalic airway obstruction syndrome in dogs in Australia. *J Small Anim Pract*. 2006;47(3):150-154. doi:10.1111/j.1748-5827.2006.00059.x
87. Fawcett A, Barrs V, Awad M, et al. Consequences and management of canine brachycephaly in veterinary practice: perspectives from Australian veterinarians and veterinary specialists. *Animals (Basel)*. 2019;9(1):3. doi:10.3390/ani9010003
88. Beausoleil NJ, Mellor DJ. Introducing breathlessness as a significant animal welfare issue. *N Z Vet J*. 2015;63(1):44-51. doi:10.1080/00480169.2014.940410
89. Farnworth MJ. Rising ownership of brachycephalic dogs: what can we do to intervene? *Vet Rec*. 2022;190(11):459-461.
90. Holland KE. Acquiring a pet dog: A review of factors affecting the decision-making of prospective dog owners. *Animals (Basel)*. 2019;9(4):124.
91. Taking action on brachycephalic breeds in advertising. *Vet Rec*. 2018;183(4):122-122. doi:10.1136/vr.k3276
92. Honey L. Future health and welfare crises predicted for the brachycephalic dog population. *Vet Rec*. 2017;181(21):550-550. doi:10.1136/vr.j5429
93. Brachycephalic dogs: BVA calls for revised breed standards. *Vet Rec*. 2016;179(6):131-131. doi:10.1136/vr.i4283
94. RVC opens dedicated clinic for brachycephalic dogs. *Vet Rec*. 2014;175(1):6. doi:10.1136/vr.g4338
95. Bateson P. *Independent Inquiry into Dog Breeding*. University of Cambridge; 2010.
96. Limb M. Dutch crackdown on brachycephalic breeds. *Vet Rec*. 2019;184(23):693-693.
97. Full victory for the dogs! News release, Norwegian Society for Protection of Animals. Accessed January 20, 2023. https://kommunikasjon.ntb.no/pressemelding/17925382/full-victory-for-the-dogs?publisherId=16958427
98. Belch A, Matiasovic M, Rasotto R, Demetriou J. Comparison of the use of LigaSure versus a standard technique for tonsillectomy in dogs. *Vet Rec*. 2017;180(8):196. doi:10.1136/vr.103873

99. Della Maggiore A. An update on tracheal and airway collapse in dogs. *Vet Clin North Am Small Anim Pract*. 2020;50(2):419-430. doi:10.1016/j.cvsm.2019.11.003
100. Andreis ME, Polito U, Veronesi MC, Faustini M, Di Giancamillo M, Modina SC. Novel contributions in canine craniometry: Anatomic and radiographic measurements in newborn puppies. *PLoS One*. 2018;13(5):e0196959. doi:10.1371/journal.pone.0196959
101. Roberts T, McGreevy P, Valenzuela M. Human induced rotation and reorganization of the brain of domestic dogs. *PLoS One*. 2010;5(7):e11946. doi:10.1371/journal.pone.0011946
102. Selba MC, Bryson ER, Rosenberg CL, Heng HG, DeLeon VB. Selective breeding in domestic dogs: how selecting for a short face impacted canine neuroanatomy. *Anat Rec*. 2021;304(1):101-115. doi:10.1002/ar.24471
103. Barker DA, Rubinos C, Taeymans O, Demetriou JL. Magnetic resonance imaging evaluation of olfactory bulb angle and soft palate dimensions in brachycephalic and nonbrachycephalic dogs. *Am J Vet Res*. 2018;79(2):170-176.
104. Hussein AK, Sullivan M, Penderis J. Effect of brachycephalic, mesaticephalic, and dolichocephalic head conformations on olfactory bulb angle and orientation in dogs as determined by use of in vivo magnetic resonance imaging. *Am J Vet Res*. 2012;73(7):946-951. doi:10.2460/ajvr.73.7.946
105. Buzek A, Serwańska-Leja K, Zaworska-Zakrzewska A, Kasprowicz-Potocka M. The shape of the nasal cavity and adaptations to sniffing in the dog (*Canis familiaris*) compared to other domesticated mammals: A review article. *Animals (Basel)*. 2022;12(4):517.
106. Schuenemann R, Pohl S, Oechtering GU. A novel approach to brachycephalic syndrome. 3. Isolated laser-assisted turbinectomy of caudal aberrant turbinates (CAT LATE). *Vet Surg*. 2017;46(1):32-38. doi:10.1111/vsu.12587
107. Oshita R, Katayose S, Kanai E, Takagi S. Assessment of nasal structure using CT imaging of brachycephalic dog breeds. *Animals (Basel)*. 2022;12(13):1636. doi:10.3390/ani12131636
108. Khoa ND, Phuong NL, Tani K, Inthavong K, Ito K. Computational fluid dynamics comparison of impaired breathing function in French bulldogs with nostril stenosis and an examination of the efficacy of rhinoplasty. *Comput Biol Med*. 2021;134:104398. doi:10.1016/j.compbiomed.2021.104398

109. Oechtering GU, Pohl S, Schlueter C, et al. A novel approach to brachycephalic syndrome. 1. Evaluation of anatomical intranasal airway obstruction. *Vet Surg*. 2016;45(2):165-172. doi:10.1111/vsu.12446
110. Auger M, Alexander K, Beauchamp G, Dunn M. Use of CT to evaluate and compare intranasal features in brachycephalic and normocephalic dogs. *J Small Anim Pract*. 2016;57(10):529-536. doi:10.1111/jsap.12541
111. de Carvalho IC, dos Santos M, Hainfellner DC, et al. Brachycephalic syndrome in dogs - endoscopic findings in the airways. *Acta Sci Vet*. 2022;50:1869. doi:10.22456/1679-9216.122144
112. Siedenburg JS, Dupre G. Tongue and upper airway dimensions: a comparative study between three popular brachycephalic breeds. *Animals (Basel)*. 2021;11(3):662. doi:10.3390/ani11030662
113. Pollard RE, Johnson LR, Marks SL. The prevalence of dynamic pharyngeal collapse is high in brachycephalic dogs undergoing videofluoroscopy. *Vet Radiol Ultrasound*. 2018;59(5):529-534. doi:10.1111/vru.12655
114. Petrof BJ, Pack AI, Kelly AM, Eby J, Hendricks JC. Pharyngeal myopathy of loaded upper airway in dogs with sleep apnea. *J Appl Physiol*. 1994;76(4):1746-1752. doi:10.1152/jappl.1994.76.4.1746
115. Jones BA, Stanley BJ, Nelson NC. The impact of tongue dimension on air volume in brachycephalic dogs. *Vet Surg*. 2020;49(3):512-520. doi:10.1111/vsu.13302
116. Köhler C, Fromme V, Kohl S, Winter K, Weidauer J, Kiefer I. 3T MRI characteristics of the palatine tonsil in brachycephalic dogs. *Vet Rec*. 2022;190(5):e1077. doi:10.1002/vetr.1077
117. Ruiz-Drebing M, Dennis R, Sparkes A, Dominguez E. MRI features of presumed normal palatine tonsils in dogs. *J Small Anim Pract*. 2019;60(4):231-238. doi:10.1111/jsap.12967
118. Cook DA, Moses PA, Mackie JT. Clinical effects of the use of a bipolar vessel sealing device for soft palate resection and tonsillectomy in dogs, with histological assessment of resected tonsillar tissue. *Aust Vet J*. 2015;93(12):445-51. doi:10.1111/avj.12384
119. Grand JGR, Bureau S. Structural characteristics of the soft palate and meatus nasopharyngeus in brachycephalic and non-brachycephalic dogs analysed by CT. *J Small Anim Pract*. 2011;52(5):232-239. doi:10.1111/j.1748-5827.2011.01047.x

120. Pichetto M, Arrighi S, Gobbetti M, Romussi S. The anatomy of the dog soft palate. III. Histological evaluation of the caudal soft palate in brachycephalic neonates. *Anat Rec*. 2015;298(3):618-623. doi:10.1002/ar.23054
121. Temwichitr J, Pongchairerk U, Sutthiprapa W, Temwichitr P. Evaluation of pre- and postoperative clinical signs, endoscopic findings and histomorphology of caudal soft palates in French bulldogs with brachycephalic obstructive airway syndrome that underwent elongated soft palate surgery. *Vet Integr Sci*. 2019;17(1):87-99.
122. Crosse KR, Bray JP, Orbell GMB, Preston CA. Histological evaluation of the soft palate in dogs affected by brachycephalic obstructive airway syndrome. *N Z Vet J*. 2015;63(6):319-325. doi:10.1080/00480169.2015.1061464
123. Arrighi S, Pichetto M, Roccabianca P, Romussi S. The anatomy of the dog soft palate. I. Histological evaluation of the caudal soft palate in mesaticephalic breeds. *Anat Rec*. 2011;294(7):1261-1266. doi:10.1002/ar.21418
124. Pichetto M, Arrighi S, Roccabianca P, Romussi S. The anatomy of the dog soft palate. II. Histological evaluation of the caudal soft palate in brachycephalic breeds with grade I brachycephalic airway obstructive syndrome. *Anat Rec*. 2011;294(7):1267-1272. doi:10.1002/ar.21417
125. Arai K, Kobayashi M, Harada Y, et al. Histopathologic and immunohistochemical features of soft palate muscles and nerves in dogs with an elongated soft palate. *Am J Vet Res*. 2016;77(1):77-83.
126. Plotsky K, Rendall D, Chase K, Riede T. Cranio-facial remodeling in domestic dogs is associated with changes in larynx position. *J Anat*. 2016;228(6):975-983. doi:10.1111/joa.12452
127. Rutherford L, Beeverl L, Bruce M, ter Haar G. Assessment of computed tomography derived cricoid cartilage and tracheal dimensions to evaluate degree of cricoid narrowing in brachycephalic dogs. *Vet Radiol Ultrasound*. 2017;58(6):634-646. doi:10.1111/vru.12526
128. de Bruyn BW, Hosgood G. Abnormal hyoid conformation in French Bulldogs: case report and computed tomographic anatomical comparison. *Aust Vet J*. 2022;100(1-2):63-66. doi:10.1111/avj.13120
129. Skerrett SC, McClaran JK, Fox PR, Palma D. Clinical features and outcome of dogs with epiglottic retroversion with or without surgical treatment: 24 cases. *J Vet Intern Med*. 2015;29(6):1611-1618. doi:10.1111/jvim.13628

130. Schabbing KJ, Seaman JA. Resection and primary closure of edematous glossoepiglottic mucosa in a dog causing laryngeal obstruction. *J Am Anim Hosp Assoc*. 2017;53(3):180-184. doi:10.5326/JAAHA-MS-6423
131. Tokunaga S, Ehrhart EJ, Monnet E. Histological and mechanical comparisons of arytenoid cartilage between 4 brachycephalic and 8 non-brachycephalic dogs: A pilot study. *PLoS One*. 2020;15(9):e0239223. doi:10.1371/journal.pone.0239223
132. Cantatore M, Gobbetti M, Romussi S, et al. Medium term endoscopic assessment of the surgical outcome following laryngeal saccule resection in brachycephalic dogs. *Vet Rec*. 2012;170(20):518. doi:10.1136/vr.100289
133. Portier K, Viguier E. Fatal tracheal collapse in a dog with brachycephalic syndrome during gastric endoscopic examination. *Vet Rec*. 2009;165(14):412-414. doi:10.1136/vr.165.14.412
134. Regier PJ, Grosso FV, Stone HK, van Santen E. Radiographic tracheal dimensions in brachycephalic breeds before and after-surgical treatment for brachycephalic airway syndrome. *Can Vet J*. 2020;61(9):971-976.
135. Bernaerts F, Talavera J, Leemans J, et al. Description of original endoscopic findings and respiratory functional assessment using barometric whole-body plethysmography in dogs suffering from brachycephalic airway obstruction syndrome. *Vet J*. 2010;183(1):95-102. doi:10.1016/j.tvjl.2008.09.009
136. Ravn-Molby EM, Sindahl L, Nielsen SS, Bruun CS, Sandoe P, Fredholm M. Breeding French bulldogs so that they breathe well-A long way to go. *PLoS One*. 2019;14(12):e0226280. doi:10.1371/journal.pone.0226280
137. Riggs J, Liu NC, Sutton DR, Sargan D, Ladlow JF. Validation of exercise testing and laryngeal auscultation for grading brachycephalic obstructive airway syndrome in pugs, French bulldogs, and English bulldogs by using whole-body barometric plethysmography. *Vet Surg*. 2019;48(4):488-496. doi:10.1111/vsu.13159
138. Tarricone J, Hayes GM, Singh A, Davis G. Development and validation of a brachycephalic risk (BRisk) score to predict the risk of complications in dogs presenting for surgical treatment of brachycephalic obstructive airway syndrome. *Vet Surg*. 2019;48(7):1253-1261. doi:10.1111/vsu.13291
139. Liu NC, Adams VJ, Kalmar L, Ladlow JF, Sargan DR. Whole-body barometric plethysmography characterizes upper airway obstruction in 3 brachycephalic breeds of dogs. *J Vet Intern Med*. 2016;30(3):853-65. doi:10.1111/jvim.13933

140. Liu NC, Sargan DR, Adams VJ, Ladlow JF. Characterisation of brachycephalic obstructive airway syndrome in French bulldogs using whole-body barometric plethysmography. *PLoS One*. 2015;10(6):e0130741. doi:10.1371/journal.pone.0130741
141. Poncet CM, Dupre GP, Freiche VG, Estrada MM, Poubanne YA, Bouvy BM. Prevalence of gastrointestinal tract lesions in 73 brachycephalic dogs with upper respiratory syndrome. *J Small Anim Pract*. 2005;46(6):273-279. doi:10.1111/j.1748-5827.2005.tb00320.x
142. Aromaa M, Lilja-Maula L, Rajamaki MM. Assessment of welfare and brachycephalic obstructive airway syndrome signs in young, breeding age French Bulldogs and pugs, using owner questionnaire, physical examination and walk tests. *Anim Welf*. 2019;28(3):287-298. doi:10.7120/09627286.28.3.287
143. Lilja-Maula L, Lappalainen AK, Hyytiäinen HK, et al. Comparison of submaximal exercise test results and severity of brachycephalic obstructive airway syndrome in English bulldogs. *Vet J*. 2017;219:22-26. doi:10.1016/j.tvjl.2016.11.019
144. O'Driscoll SW, Marx RG, Beaton DE, Miura Y, Gallay SH, Fitzsimmons JS. Validation of a simple histological-histochemical cartilage scoring system. *Tissue Eng*. 2001;7(3):313-320. doi:10.1089/10763270152044170
145. Dyce KM, Wensing CJG. *Textbook of Veterinary Anatomy*. 4th ed. Saunders; 2010.
146. Yamamoto Y, Hosono I, Atoji Y, Suzuki Y. Morphological study of the vagal afferent nerve endings in the laryngeal mucosa of the dog. *Ann Anat*. 1997;179(1):65-73. doi:10.1016/s0940-9602(97)80138-0
147. Yamamoto Y, Atoji Y, Suzuki Y. Innervation of taste buds in the canine larynx as revealed by immunohistochemistry for the various neurochemical markers. *Tissue Cell*. 1997;29(3):339-46. doi:10.1016/s0040-8166(97)80009-2
148. Amis TC, O'Neill N, Van der Touw T, Brancatisano A. Control of epiglottic position in dogs: role of negative upper airway pressure. *Respir Physiol*. 1996;105(3):187-94. doi:10.1016/0034-5687(96)00052-7
149. Biewener AA, Soghikian GW, Crompton AW. Regulation of respiratory airflow during panting and feeding in the dog. *Respir Physiol*. 1985;61(2):185-95. doi:10.1016/0034-5687(85)90125-2

150. Perez B, Gomez M, Mieres M, Galecio JS, Yefi C, Aburto P. Computed tomographic anatomy of the larynx in mesaticephalic dogs. *Arch Med Vet*. 2010;42(1):91-99. doi:10.4067/s0301-732x2010000100013
151. Bray JP, Lipscombe VJ, White RAS, Rudorf H. Ultrasonographic examination of the pharynx and larynx of the normal dog. *Vet Radiol Ultrasound*. Nov-Dec 1998;39(6):566-571. doi:10.1111/j.1740-8261.1998.tb01653.x
152. Dzierzecka M, Charuta A. Morphometric description of the larynx in the dog (*Canis familiaris*). *Acta Sci Pol Zootech*. 2021;20(4):43-50.
153. Eurell JA, Frappier Brian L. *Dellmann's Textbook of Veterinary Histology*. 6th ed. Wiley; 2006.
154. Aughey E, Frye FL. *Comparative Veterinary Histology with Clinical Correlates*. 1st ed. Manson Publishing Ltd; 2001.
155. Shoieb A. Managing epiglottal chondrosarcoma of a dog: A case report. *J Interdiscip Histopathol*. 2014;12(2):168-173.
156. Bacha WJ, Bacha LM. *Color Atlas of Veterinary Histology*. 3rd ed. Wiley-Blackwell; 2012.
157. Mullins RA, Stanley BJ, Flanders JA, et al. Intraoperative and major postoperative complications and survival of dogs undergoing surgical management of epiglottic retroversion: 50 dogs (2003-2017). *Vet Surg*. 2019;48(5):803-819. doi:10.1111/vsu.13226
158. Mullins R, McAlinden AB, Goodfellow M. Subtotal epiglottectomy for the management of epiglottic retroversion in a dog. *J Small Anim Pract*. 2014;55(7):383-385. doi:10.1111/jsap.12207
159. De Lorenzi D, Bertoncello D, Dentini A. Intraoral diode laser epiglottectomy fortreatment of epiglottis chondrosarcoma in a dog. *J Small Anim Pract*. 2015;56(11):675-678. doi:10.1111/jsap.12356
160. Witham AI, French AF, Hill KE. Extramedullary laryngeal plasmacytoma in a dog. *N Z Vet J*. 2012;60(1):61-64. doi:10.1080/00480169.2011.621876
161. Dunbar MD, Ginn P, Winter M, Miller KB, Craft W. Laryngeal rhabdomyoma in a dog. *Vet Clin Pathol*. 2012;41(4):590-593. doi:10.1111/j.1939-165x.2012.00484.x
162. Carpenter M. Lipoma on the epiglottis of a dog. *Vet Rec*. 2012;171(9):226-226. doi:10.1136/vr.e5834

163. Flanders JA, Thompson MS. Dyspnea caused by epiglottic retroversion in two dogs. *J Am Vet Med Assoc*. 2009;235(11):1330-1335. doi:10.2460/javma.235.11.1330
164. Faustino AM, Pereira PD. A salivary malignant myoepithelioma in a dog. *Vet J*. 2007;173(1):223-226. doi:10.1016/j.tvjl.2005.08.018
165. Stockinger A, Fritz L, Hof S, Delfs J. Two case reports of epiglottic abscess in dogs-clinical signs, diagnosis and treatment. *Kleintierpraxis*. 2021;66(5):260-268. doi:10.2377/0023-2076-66-260
166. Ressel L, Hetzel U, Ricci E. Blunt force trauma in veterinary forensic pathology. *Vet Pathol*. 2016;53(5):941-961.
167. Wang C, Liu B, Wan J. Pathological changes of larynx in inhalational thermal injury causing laryngeal stenosis based on a canine model. 2020; *Research Square*; 2020. doi: 10.21203/rs.3.rs-25077/v1
168. Milo AG, Eliachar I, Lane CJ, Myles JL, Munoz-Ramirez H. Clinical and histologic evaluation of an indwelling, inflatable, long-term laryngeal stent in the canine model. *Otolaryngol Head Neck Surg*. 1999;121(3):195-202.
169. Olympus Coporation. *User Manual cellSens Life Science Imaging Software*. 2013; Version 510_UMA_cellSens19-Krishna-en_00_01August2013.
170. Sung, N. CellSens Entry 1.15 Measurement [Video]. YouTube. Published 2017. Accessed March 2024, <https://www.youtube.com/watch?v=xKLI8npQZVQ>.
171. Sung, N. CellSens Entry 1.15 Measurement excel [Video]. YouTube. Published 2017. Accessed March 2024, <https://www.youtube.com/watch?v=TvPVuV1co60>.
172. Nicolae C, Ko Y-P, Miosge N, et al. Abnormal collagen fibrils in cartilage of matrilin-1/matrilin-3-deficient mice. *J Biol Chem*. 2007;282(30):22163-22175.
173. Soliman NBE. Effect of intra-articular injection of high-molecular-weight versus low-molecular-weight hyaluronic acid on the articular cartilage structure in experimentally induced knee joint osteoarthritis in albino rats. *Egypt J Histol*. 2012;35(3):483-495.
174. Wyllie A. Apoptosis: cell death in tissue regulation. *J Pathol*. 1987;153(4):313-316.
175. Latimer, HB. Variability in body and organ weights in the newborn dog and cat compared with that in the adult. *Anat Rec.* 1967; 157(3):449-456.

176. Aeffner, F, Zarella, MD, Buchbinder, N et al. Introduction to digital image analysis in whole-slide imaging: a white paper from the digital pathology association. *J Pathol Inform.* 2019;10:9. doi: 10.4103/jpi.jpi_82_18.
177. Tsochatzis, E, Bruno, S, Isgro, G et al. Collagen proportionate area is superior to other histological methods for sub-classifying cirrhosis and determining prognosis. *J Hepatol.* 2014; 60(5):948-954.
178. Robertson, S, Azizpour, H, Smith, K et al. Digital image analysis in breast pathology – from image processing techniques to artificial intelligence. *Transl Res.* 2018; 194:19-35.
179. Sirinukunwattana, K, Raza, SEA, Tsang, YW et al. Locality sensitive deep learning for detection and classification of nuclei in routine colon cancer histology images. *IEEE Trans Med Imaging.* 2016; 35(5):1196-1206.
180. Egerbacher, M, Krestan R, Bock P. Morphology, histochemistry, and differentiation of the cat's epiglottic cartilage: a supporting organ composed of elastic cartilage, fibrous cartilage, myxoid tissue and fat tissue. *Anat Rec.* 1995;242(4):471-482.
181. Egerbacher, M, Bock, P. Myxoid tissue: its morphology, histochemistry, and relationship with other supporting tissues. *Arch Histol Cytol*. 1997; 60(2):121-131.
182. Billen, F, Peeters, D, Dehard, S et al. Distribution of leucocyte subsets in the canine pharyngeal tonsil. *J Comp Pathol,* 2006;135(2-3):63-73.
183. Bhutda S, Surve MV, Anil A, et al. Histochemical staining of collagen and identification of its subtypes by picrosirius red dye in mouse reproductive tissues. *Bio Protocol.* 2017;7(21):e2592-e2592.
184. Augsburger H, Henzi D. Immunohistochemical expression of collagen types I, III, IV and α-actin in the uterine horns of nulliparous and multiparous beagles. *Theriogenology*. 2008;69(9):1070-1076.
185. Singh, D, Rai, V, Agrawal, DK. Regulation of collagen I and III in tissue injury and regeneration. *Cardiol Cardiovasc Med.* 2023;7(1):5-16.
186. Kazlouskaya, V, Malhotra, S, Lambe, J et al. The utility of elastic Verhoeff-Van Gieson staining in dermatopathology. *J Cutan Pathol.* 2013;40(2):211-225.

Appendix A

Table A1: Breed, head group, cadaver condition, collected tissues, samples collected (no number = sample not taken, n = sample taken and n sections taken for slide preparation), numbers and relevant concurrent pathology or conditions from brachycephalic (n = 10 adults, n = 2 juvenile/puppy), mesocephalic (n = 21 adults) and doliocephalic (n = 3 adults, n = 5 puppies) dogs. Head shape denoted by BC = brachycephalic group, MC = mesocephalic group, DC = doliocephalic, and postmortem condition denoted by F = fresh, FR = frozen, A = moderate or severe autolysis.

	Breed	Cadaver condition	Nares	Epiglottis	Soft palate	Palatine tonsils	Larynx	Proximal trachea	Mid-trachea	Distal trachea	Lungs	Relevant concurrent pathology or conditions
BC	French bulldog	F		2	1	1	1		2		2	
	English bulldog	F		2	3	1	1	1		1	5	Bronchopneumonia Soft palate surgery
	Shih tzu	A	1	1	2	2	1	2			2	
	French bulldog	A, FR	1	1	1	1	1	1			3	Bronchopneumonia post bone-obstruction
	Pug	F	1	3	3	4	2	3			3	Aspiration pneumonia and swollen airways
	Cavalier King Charles spaniel	F		3	2	3 (left only)	1	3			4	Everted laryngeal saccules
	Staffordshire bull terrier	A	1	3	3	4	1	3			2	
	Pug	FR	1	3	2	4	1	3		3	3	
	Pug	F	1	3	2	4	1	3		3	3	
	American bulldog	A		3		3 (left only)	1	3				
	Pug	F	1	4	2	4	1	3		3	3	Pneumothorax
	French bulldog	F	1	3	3	6	1	3		3	3	Lymphoma with consolidated lungs

Table A1 – continued.

	Breed	Cadaver condition	Nares	Epiglottis	Soft palate	Palatine tonsils	Larynx	Proximal trachea	Mid-trachea	Distal trachea	Lungs	Relevant concurrent pathology or conditions
MC	Beagle	F		3	1	1					2	Metastatic lung neoplasia
	Springer spaniel	A	1	1	1	2	1	2			3	
	Kelpie crossbreed	F	1	3	2	2	3	3		3	2	
	Golden retriever	F	1	3	3	6	2	3		3	2	Ventilated
	Mixed breed	F	1	3	3	3 (left only)	1	3			3	Aspiration pneumonia
	Spoodle	A	1	3	3	6	1	3		3	2	
	Border collie	F		3	3	5	1	3		3	2	
	German shepherd	F		3	2	6	1	3	3		4	
	Miniature fox terrier	F		3	1		1	3			4	
	Bichon frise	A	1	3	2	4	1	3		3	3	
	German shepherd	F	1	2	2	6	1	3		3	3	Haemoabdomen
	Jack Russell terrier	FR	1	3	2	5	1	3		3	3	
	German shepherd	F	1	3	2	6	1	3		3	3	
	Labrador retriever crossbreed	FR	1	3	2	6	1	3		3	3	
	Dachshund crossbreed	F		3	3	5	1	3			4	
	Labrador retriever	A	1	3	2	6	1	1		1	3	
	Mixed breed	FR	1	3	2	6	1	3		3	3	Slack tracheal membrane

Table A1 – continued.

	Breed	Cadaver condition	Nares	Epiglottis	Soft palate	Palatine tonsils	Larynx	Proximal trachea	Mid-trachea	Distal trachea	Lungs	Relevant concurrent pathology or conditions
MC	French bulldog crossbreed	F	1	3	2	6	1	3		3	3	
	Maltese crossbreed	FR	1	5	2	6	1	3		3	4	
	Jack Russell terrier	FR	1	6	2	5	1	3		3	4	
	Fox terrier	FR	1	4	2	6	1	3		3	4	
DC	Greyhound	F		3	3	4	1	3			3	
	Greyhound	FR	1	2	2	4	1	3		3	4	Softer limb bones
	Greyhound	FR	1	2	2	4	1	3		3	4	Softer limb bones
	Greyhound	FR	1	2	2	4	1	3		3	4	Softer limb bones and sloped back
	Greyhound	FR	1	2	2	4	1	3		3	4	Softer limb bones
	Greyhound	FR	1	2	2	4	1	3		3	4	Softer limb bones
	Greyhound	FR	1	3	2	6	1	3		3	3	
	Greyhound	FR	1	3	2	6	1	3		3	3	

Appendix B

Table B1: Signalment, head group, morphometric measurements of skull and epiglottis, calculated skull width:length ratio, and calculated epiglottal volume of brachycephalic (BC, n = 10 adults, n = 2 juvenile/puppy), mesocephalic (MC, n = 21 adults) and doliocephalic (DC, n = 3 adults, n = 5 puppies) dogs. Ratio of the skull shape calculated as width:length ratio and volume of epiglottis estimated to be a rectangular pyramid, where volume equals 1/3 x length x width x height (mm^3). Sex denoted by M = male, MN = male neuter, F = female, FN = female neuter, IS = intersex.

	Breed	Age (yr)	Weight (kg)	Sex	Craniofacial angle (°)	Skull			Epiglottis			
						Width (mm)	Length (mm)	Ratio	Length (mm)	Width (mm)	Height (mm)	Volume (mm^3)
BC	French bulldog	1.92	12.7	MN	13.0							
	English bulldog	2.58	21.2	M	14.0							
	Shih tzu	8.33	7.60	FN	13.0							
	French bulldog	0.42	9.10	M	13.0							
	Pug	10.5	6.40	FN	11.0							
	Cavalier King Charles spaniel	7.58	15.1	MN								
	Staffordshire bull terrier	12.0	22.9	MN	18.0							
	Pug	Adult	7.70	MN		100	120	0.83	20.0	20.0	1.00	133
	Pug	Adult	10.5	FN		100	125	0.80	23.0	23.0	1.50	265
	American bulldog	1.75	24.0	M								
	Pug	0.31	4.20	IS		85.0	100	0.85	15.0	14.0	1.00	70.0
	French bulldog	Adult	12.2	F		120	135	0.89	22.0	24.0	1.50	264
MC	Beagle	10.6	14.3	MN	21.0							

Table B1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Craniofacial angle (°)	Skull			Epiglottis			
						Width (mm)	Length (mm)	Ratio	Length (mm)	Width (mm)	Height (mm)	Volume (mm^3)
MC	Springer spaniel	7.75	26.8	MN	20.0							
	Kelpie crossbreed	14.0	10.6	MN	20.0							
	Golden retriever	1.33	28.9	MN	20.0							
	Mixed breed	14.0	10.3	MN	21.0							
	Spoodle	11.8	19.3	MN	20.0							
	Border collie	13.5	24.1	MN	21.0				29.0	30.0	1.50	435
	German shepherd	10.7	33.8	MN								
	Miniature fox terrier	10.8	4.80	FN								
	Bichon frise	13.0	7.30	FN	22.0				17.0	20.0	2.00	227
	German shepherd	8.00	30.3	FN		150	240	0.63	35.0	35.0	1.00	408
	Jack Russell terrier	Adult	7.00	FN		85.0	140	0.61	20.0	19.0	1.00	127
	German shepherd	14.5	31.8	MN		140	230	0.61	35.0	30.0	1.50	535
	Labrador retriever crossbreed	Adult	53.0	MN		160	270	0.59	37.0	30.0	1.00	370
	Dachshund crossbreed	10.0	12.2	FN								

Table B1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Craniofacial angle (°)	Skull			Epiglottis			
						Width (mm)	Length (mm)	Ratio	Length (mm)	Width (mm)	Height (mm)	Volume (mm^3)
MC	Labrador retriever	10.0	36.2	MN					39.0	30.0	2.00	780
	Mixed breed	Adult	3.20	F		90.0	115	0.78	15.0	15.0	1.50	113
	French bulldog crossbreed	9.25	15.6	MN		115	155	0.74	21.0	21.0	2.00	294
	Maltese crossbreed	Adult	8.50	MN		100	140	0.71	19.0	19.0	1.00	120
	Jack Russell terrier	Adult	10.3	MN		100	160	0.63	20.0	20.0	1.50	200
	Fox terrier	Adult	7.80	MN		75.0	160	0.47	19.0	17.0	1.00	108
DC	Greyhound	7.66	22.8	FN	26.0							
	Greyhound	0.12	3.20	M	25.0				15.0	15.0	0.50	37.5
	Greyhound	0.12	1.20	M	26.0				16.0	14.0	0.50	37.3
	Greyhound	0.12	2.50	M	25.0				15.0	14.0	0.50	35.0
	Greyhound	0.12	1.80	F	26.0				15.0	13.0	0.50	32.5
	Greyhound	0.12	2.70	M	26.0				15.0	15.0	0.50	37.5
	Greyhound	Adult	22.0	F		100	220	0.45	30.0	30.0	1.00	300
	Greyhound	Adult	27.5	F		110	240	0.46	35.0	33.0	1.50	578

Appendix C

Table C1: Signalment, head group, morphometric measurements and palatine volume calculations of brachycephalic (n = 10 adults, n = 2 juvenile/puppy), mesocephalic (n = 21 adults) and doliocephalic (n = 3 adults, n = 5 puppies). Head shape denoted by BC = brachycephalic group, MC = mesocephalic group, DC = doliocephalic group. Sex denoted by M = male, MN = male neuter, F = female, FN = female neuter, IS = intersex. Tonsillar volume estimated to be an ellipsoid where volume equals 4/3 x π x (length/2) x (width/2) x (height/2) (mm^3).

	Breed	Age (yr)	Weight (kg)	Sex	Palatine tonsils							
					Length (mm)		Width (mm)		Height (mm)		Volume (mm^3)	Volume (mm^3)
					R	L	R	L	R	L	R	L
BC	French bulldog	1.92	12.7	MN								
	English bulldog	2.58	21.2	M								
	Shih tzu	8.33	7.60	FN								
	French bulldog	0.42	9.10	M								
	Pug	10.5	6.40	FN								
	Cavalier King Charles spaniel	7.58	15.1	MN								
	Staffordshire bull terrier	12.0	22.9	MN								
	Pug	Adult	7.70	MN	15.0	16.0	4.00	6.00	6.00	6.00	189	302
	Pug	Adult	10.5	FN	18.0	17.0	4.00	3.00	3.00	3.00	113	80.1
	American bulldog	1.75	24.0	M								
	Pug	0.31	4.20	IS	14.0	13.0	5.00	3.00	4.00	4.00	147	81.7
	French bulldog	Adult	12.2	F	16.0	19.0	7.00	4.00	6.00	3.00	352	119
MC	Beagle	10.6	14.3	MN								
	Springer spaniel	7.75	26.8	MN								
	Kelpie crossbreed	14.0	10.6	MN								
	Golden retriever	1.33	28.9	MN								
	Mixed breed	14.0	10.3	MN								
	Spoodle	11.8	19.3	MN								
	Border collie	13.5	24.1	MN	21.0	20.0	6.00	7.00	4.00	6.00	264	440

Table C1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Palatine tonsils							
					Length (mm)		Width (mm)		Height (mm)		Volume (mm^3)	Volume (mm^3)
					R	L	R	L	R	L	R	L
MC	German shepherd	10.7	33.8	MN								
	Miniature fox terrier	10.8	4.80	FN								
	Bichon frise	13.0	7.30	FN	15.0	15.0	3.00	3.00	3.00	3.00	70.7	70.7
	German shepherd	8.00	30.3	FN	30.0	29.0	10.0	10.0	9.00	10.0	1414	1518
	Jack Russell terrier	Adult	7.00	FN	15.0	14.0	10.0	10.0	1.00	1.00	78.5	73.3
	German shepherd	14.5	31.8	MN	25.0	27.0	7.00	7.00	7.00	9.00	641	891
	Labrador retriever crossbreed	Adult	53.0	MN	25.0	26.0	4.00	4.00	6.00	5.00	314	272
	Dachshund crossbreed	10.0	12.2	FN								
	Labrador retriever	10.0	36.2	MN	24.0	25.0	4.00	7.00	6.00	7.00	302	641
	Mixed breed	Adult	3.20	F	15.0	14.0	5.00	5.00	5.00	4.00	196	147
	French bulldog crossbreed	9.25	15.6	MN	20.0	20.0	9.00	6.00	10.0	4.00	943	251
	Maltese crossbreed	Adult	8.50	MN	15.0	16.0	3.00	5.00	3.00	3.00	70.7	126
	Jack Russell terrier	Adult	10.3	MN	18.0	19.0	3.00	3.00	2.00	1.00	56.6	29.9
	Fox terrier	Adult	7.80	MN	15.0	16.0	3.00	3.00	2.00	2.00	47.1	50.3
DC	Greyhound	7.66	22.8	FN								
	Greyhound	0.12	3.20	M	15.0	16.0	6.00	7.00	4.00	5.00	189	293
	Greyhound	0.12	1.20	M	13.0	14.0	6.00	5.00	5.00	4.00	204	293
	Greyhound	0.12	2.50	M	12.0	13.0	4.00	5.00	3.00	3.00	75.4	102

Table C1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Palatine tonsils							
					Length (mm)		Width (mm)		Height (mm)		Volume (mm^3)	Volume (mm^3)
					R	L	R	L	R	L	R	L
DC	Greyhound	0.12	1.80	F	14.0	12.0	4.00	6.00	5.00	5.00	147	189
	Greyhound	0.12	2.70	M	15.0	16.0	6.00	6.00	5.00	4.00	236	201
	Greyhound	Adult	22.0	F	19.0	18.0	4.00	5.00	6.00	7.00	239	330
	Greyhound	Adult	27.5	F	25.0	25.0	6.00	7.00	10.0	9.00	785	825

Appendix D

Table D1: Signalment, head group, micromorphometric measurements of percentage areas from distal, middle and proximal section of the epiglottis. Data includes the percentage epithelium, lamina propria-submucosa, cartilage core relative to total epiglottis area, as well as chondral cells (chondrocytes/chondroblasts) plus matrix, fibrous tissue and adipose tissue relative to cartilage core area from brachycephalic (n = 10 adults, n = 2 juvenile/puppy), mesocephalic (n = 21 adults) and doliocephalic (n = 3 adults, n = 5 puppies) dogs. Head shape denoted by BC = brachycephalic group, MC = mesocephalic group, DC = doliocephalic. Sex denoted by M = male, MN = male neuter, F = female, FN = female neuter, IS = intersex

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
BC	French bulldog	1.92	12.7	MN	Distal	6.74	29.5	63.7	19.8	39.4	40.8
					Middle	2.99	17.8	79.2	17.6	49.6	32.8
					Proximal	6.49	40.9	52.7	18.0	57.7	24.4
	English bulldog	2.58	21.2	M	Distal	3.53	23.8	72.7	31.2	35.3	33.5
					Middle	2.59	44.0	53.4	12.0	34.9	53.2
					Proximal	3.23	46.3	50.5	24.7	34.4	40.9
	Shih tzu	8.33	7.60	FN	Distal	8.24	27.8	64.0	12.8	44.3	42.9
					Middle	4.60	42.5	48.1	4.50	13.1	82.3
					Proximal	2.98	24.0	61.7	2.60	15.4	82.0
	French bulldog	0.42	9.10	M	Distal	3.47	42.0	54.5	24.3	40.3	35.5
					Middle	1.52	33.3	60.5	7.10	35.7	57.2
					Proximal	1.06	27.8	47.6	0.93	65.6	33.4
	Pug	10.5	6.40	FN	Distal	6.32	45.4	48.3	17.1	41.1	41.8
					Middle	4.59	49.3	45.3	6.22	26.1	67.6

Table D1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
BC					Proximal	3.17	48.6	37.9	1.91	24.7	73.4
	Cavalier King Charles spaniel	7.58	15.1	MN	Distal	9.70	46.1	44.2	29.8	51.6	18.7
					Middle	4.18	43.8	52.0	14.4	46.0	39.6
					Proximal	3.61	33.9	62.5	2.96	14.5	82.5
	Staffordshire bull terrier	12.0	22.9	MN	Distal	6.54	34.9	58.6	16.7	73.9	9.40
					Middle	3.67	43.4	44.6	16.1	56.0	28.0
					Proximal	1.92	33.8	41.8	30.7	22.5	46.8
	Pug	Adult	7.70	MN	Distal	3.67	51.3	45.0	4.02	43.1	52.9
					Middle	1.96	44.6	44.4	1.33	15.1	83.6
					Proximal	1.63	27.7	50.2	0.88	12.9	86.2
	Pug	Adult	10.5	FN	Distal	5.77	25.8	68.5	25.0	49.1	25.9
					Middle	10.6	52.1	37.4	23.0	0.91	76.1
					Proximal	3.48	48.2	44.9	8.65	19.7	71.7
	American bulldog	1.75	24.0	M	Distal	3.57	50.3	46.1	25.6	51.7	22.7
					Middle	1.87	46.7	51.4	21.4	41.6	37.0

Table D1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
BC					Proximal	3.15	48.7	48.2	19.6	37.8	42.6
	Pug	0.31	4.20	IS	Distal	11.5	44.6	43.9	3.01	48.8	48.2
					Middle	10.7	32.6	43.7	2.84	25.1	72.0
					Proximal	2.57	21.6	35.9	0.60	34.4	65.0
	French bulldog	Adult	12.2	F	Distal	5.39	34.2	60.4	25.8	52.4	21.8
					Middle	3.02	26.7	70.3	24.2	45.4	30.4
					Proximal	2.87	39.7	57.4	17.0	44.1	38.9
MC	Beagle	10.6	14.3	MN	Distal	3.85	32.5	63.7	17.0	42.6	40.4
					Middle	2.52	40.0	55.1	4.03	38.7	57.2
					Proximal	3.87	22.0	63.9	3.12	17.2	79.6
	Springer spaniel	7.75	26.8	MN	Distal	7.22	51.4	41.4	14.5	62.4	23.2
					Middle	1.45	31.8	53.4	11.2	44.7	44.1
					Proximal	17.6	1.09	45.9	1.89	34.4	63.7
	Kelpie crossbreed	14.0	10.6	MN	Distal	5.28	35.4	59.3	8.78	37.4	53.8
					Middle	2.18	36.1	46.6	4.86	20.9	74.3
					Proximal	1.23	8.35	71.6	0.33	13.2	86.5
	Golden retriever	1.33	28.9	MN	Distal	3.87	53.7	42.4	15.0	54.5	30.6

Table D1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
MC					Middle	1.76	32.8	55.5	13.7	41.7	44.6
					Proximal	1.88	38.1	37.2	4.93	20.6	74.5
	Mixed breed	14.0	10.3	MN	Distal	6.59	38.3	55.1	13.5	39.4	47.0
					Middle	4.63	38.1	57.3	1.89	19.1	79.0
					Proximal	2.09	35.0	62.9	1.43	21.4	77.2
	Spoodle	11.8	19.3	MN	Distal	6.36	29.4	64.2	7.85	38.6	53.6
					Middle	5.45	32.7	61.9	10.9	19.3	69.9
					Proximal	2.14	27.4	52.7	5.19	13.4	81.4
	Border collie	13.5	24.1	MN	Distal	6.22	37.4	56.4	20.6	49.8	29.6
					Middle	2.71	57.7	39.8	16.0	50.2	33.8
					Proximal	1.93	40.4	28.8	12.9	27.5	59.6
	German shepherd	10.7	33.8	MN	Distal	5.56	36.8	57.7	9.01	52.0	39.0
					Middle	2.70	22.9	74.3	11.8	41.7	46.5
					Proximal	2.43	57.8	23.9	3.92	39.0	57.1
	Miniature fox terrier	10.8	4.80	FN	Distal	10.3	42.8	47.0	18.7	62.1	19.2
					Middle	9.63	46.2	44.1	6.37	29.8	63.8

Table D1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
MC					Proximal	2.72	43.3	38.7	1.44	17.9	80.7
	Bichon frise	13.0	7.30	FN	Distal	3.25	22.1	74.7	12.7	45.2	42.1
					Middle	1.69	35.3	61.9	7.32	24.3	68.4
					Proximal	3.04	34.1	62.5	2.67	18.1	79.3
	German shepherd	8.00	30.3	FN	Distal	3.73	23.6	72.7	5.21	32.9	61.9
					Middle	2.52	22.0	75.5	13.2	19.3	67.5
					Proximal	0.55	28.4	45.8	3.21	37.7	59.1
	Jack Russell terrier	Adult	7.00	FN	Distal	11.0	52.0	37.0	10.6	52.1	37.3
					Middle	5.00	43.4	47.6	7.60	28.2	64.2
					Proximal	2.57	41.1	41.7	1.66	33.1	65.2
	German shepherd	14.5	31.8	MN	Distal	8.11	30.2	61.7	25.4	46.6	28.0
					Middle	3.33	39.7	57.0	18.7	49.7	31.6
					Proximal	3.23	49.7	47.1	15.5	37.5	47.0
	Labrador retriever crossbreed	Adult	53.0	MN	Distal	4.12	52.6	43.2	16.9	49.0	34.1

Table D1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
MC					Middle	2.04	47.5	35.1	9.34	37.0	53.7
					Proximal	1.91	15.9	60.3	1.55	18.9	79.5
	Dachshund crossbreed	10.0	12.2	FN	Distal	5.82	46.2	48.0	14.4	61.5	24.0
					Middle	4.01	35.9	60.1	9.07	25.1	65.8
					Proximal	3.17	29.3	67.6	4.02	18.9	77.1
	Labrador retriever	10.0	36.2	MN	Distal	5.72	33.6	60.7	25.1	53.7	21.2
					Middle	2.27	37.4	60.4	19.2	47.6	33.2
					Proximal	1.55	44.6	48.3	6.39	23.8	69.8
	Mixed breed	Adult	3.20	F	Distal	8.44	51.8	39.8	16.2	53.6	30.2
					Middle	5.54	42.5	46.3	5.52	21.0	73.5
					Proximal	3.11	31.7	41.4	3.72	28.3	68.0
	French bulldog crossbreed	9.25	15.6	MN	Distal	6.61	47.1	46.3	15.0	67.3	17.7
					Middle	6.59	49.0	44.4	18.7	39.1	42.2
					Proximal	4.47	54.5	41.0	9.09	34.1	56.8
	Maltese crossbreed	Adult	8.50	MN	Distal	10.1	29.6	60.3	19.1	63.0	18.0

Table D1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
MC					Middle	2.78	38.1	45.3	13.9	26.5	59.6
					Proximal	3.26	30.6	36.3	3.70	24.7	71.6
	Jack Russell terrier	Adult	10.3	MN	Distal	4.36	15.2	80.4	5.60	40.1	54.3
					Middle	4.18	40.3	55.6	8.65	22.9	68.5
					Proximal	1.53	24.7	50.8	1.00	26.4	72.6
	Fox terrier	Adult	7.80	MN	Distal	7.57	49.5	42.9	0.75	66.0	33.3
					Middle	4.89	57.3	37.8	20.0	31.7	48.3
					Proximal	4.60	61.4	34.0	7.90	20.0	72.1
DC	Greyhound	7.66	22.8	FN	Distal	4.32	36.1	59.6	19.2	37.1	43.7
					Middle	4.99	36.1	59.0	24.8	37.2	38.0
					Proximal	4.94	43.0	46.9	8.07	25.8	66.2
	Greyhound	0.12	3.20	M	Distal	11.9	32.7	55.5	0.76	56.7	42.6
					Middle	9.05	37.0	54.0	0.62	31.1	68.3
					Proximal	4.74	39.8	36.7	0.12	16.0	83.9
	Greyhound	0.12	1.20	M	Distal	10.7	26.1	63.3	24.6	15.5	59.9
					Middle	5.38	30.3	48.2	14.9	10.8	74.4
					Proximal	6.08	19.1	36.4	17.0	32.7	50.3

Table D1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Epithelium (%)	Lamina propria-submucosa (%)	Cartilage core (%)	Chondral cells/matrix (%)	Fibrous tissue (%)	Adipose tissue (%)
DC	Greyhound	0.12	2.50	M	Distal	12.1	32.5	55.5	0.16	0.98	98.9
					Middle	5.86	29.6	50.3	0.65	27.2	72.1
					Proximal	4.15	35.6	34.7	0.16	27.2	72.6
	Greyhound	0.12	1.80	F	Distal	13.4	27.9	58.7	0.46	46.9	52.6
					Middle	8.62	37.7	53.7	0.48	28.1	71.4
					Proximal	11.4	51.0	37.7	1.09	18.7	80.2
	Greyhound	0.12	2.70	M	Distal	12.1	20.6	67.4	0.23	33.9	65.9
					Middle	10.6	30.9	58.6	0.86	28.5	70.6
					Proximal	9.23	51.0	39.7	2.45	24.7	72.8
	Greyhound	Adult	22.0	F	Distal	4.80	34.5	60.7	22.0	39.7	38.4
					Middle	5.03	27.6	67.4	15.2	28.9	56.0
					Proximal	4.52	31.6	58.9	7.51	24.0	68.5
	Greyhound	Adult	27.5	F	Distal	4.34	47.8	47.9	22.2	57.6	20.2
					Middle	3.18	35.5	61.3	9.42	35.9	54.7
					Proximal	2.01	43.7	48.8	6.56	20.4	73.0

Appendix E

Table E1: Signalment, head group and histopathology features from the distal, middle and proximal epiglottis displaying grading for oedema, inflammation, cartilage metachromasia, and cartilage mineralisation. Medians from 5 random fields (total area: 0.8 mm^2) calculated for total chondral cells (chondrocytes/chondroblasts) and degenerate chondral cells. Total 41 dogs; brachycephalic (n = 10 adults, n = 2 juvenile/puppy), mesocephalic (n = 21 adults) and doliocephalic (n = 3 adults, n = 5 puppies) dogs. Grades for oedema, inflammation and metachromasia were 0 = normal, 1 = mild, 2 = moderate and 3 = marked. Grade for mineralisation were 0 = absence, 1 = presence. Median for total chondral cells and degenerate chondral cells undertaken over 5 fields (total area: 0.8 mm^2) . Head shape denoted by BC = brachycephalic group, MC = mesocephalic group, DC = doliocephalic. Sex denoted by M = male, MN = male neuter, F = female, FN = female neuter, IS = intersex *It was not possible to distinguish between oedema and separation artefact.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields (total area: 0.8 mm^2)	Degenerate chondral cells median of five random fields (total area: 0.8 mm^2)	Metachromasia grade	Mineralisation grade
BC	French bulldog	1.92	12.7	MN	Distal	0	0	102	0	2	0
					Middle	0	0	85	1	2	0
					Proximal	0	1	67	1	1	0
	English bulldog	2.58	21.2	M	Distal	0	1	67	9	2	0
					Middle	2	1	65	14	0	0
					Proximal	2	1	81	15	2	0
	Shih tzu	8.33	7.60	FN	Distal	0	0	52	1	2	0
					Middle	2	0	45	1	2	0
					Proximal	1	1	67	1	1	0
	French bulldog	0.42	9.10	M	Distal	1	0	70	3	1	0
					Middle	1	0	62	0	1	0
					Proximal	1	0	32	0	0	0
	Pug	10.5	6.40	FN	Distal	2	1	33	1	0	0

Table E1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields	Degenerate chondral cells median of five random fields	Metachromasia grade	Mineralisation grade
BC					Middle	2	1	29	0	1	0
					Proximal	0	0	19	0	1	0
	Cavalier King Charles spaniel	7.58	15.1	MN	Distal	3	0	48	1	2	1
					Middle	2	1	78	2	2	1
					Proximal	1	1	54	1	1	0
	Staffordshire bull terrier	12.0	22.9	MN	Distal	2	1	44	0	2	0
					Middle	1	2	67	0	2	0
					Proximal	0	1	59	0	2	1
	Pug	Adult	7.70	MN	Distal	2	0	31	2	1	0
					Middle	2	2	37	2	2	0
					Proximal	1	0	20	0	0	0
	Pug	Adult	10.5	FN	Distal	1	1	42	3	2	1
					Middle	1	2	36	5	2	1
					Proximal	1	1	39	3	2	1
	American bulldog	1.75	24.0	M	Distal	1	1	64	3	2	0
					Middle	2	0	75	5	2	0

Table E1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields	Degenerate chondral cells median of five random fields	Metachromasia grade	Mineralisation grade
BC					Proximal	1	1	22	6	2	0
	Pug	0.31	4.20	IS	Distal	2	0	41	0	0	0
					Middle	2	1	30	0	0	0
					Proximal	1	1	29	0	0	0
	French bulldog	Adult	12.2	F	Distal	1	0	78	3	1	1
					Middle	2	0	52	1	2	1
					Proximal	2	0	38	2	2	0
MC	Beagle	10.6	14.3	MN	Distal	2	0	30	3	0	0
					Middle	2	0	40	2	1	0
					Proximal	0	0	32	1	0	0
	Springer spaniel	7.75	26.8	MN	Distal	2	0	45	2	2	0
					Middle	1	0	47	1	2	0
					Proximal	1	0	37	2	0	0
	Kelpie crossbreed	14.0	10.6	MN	Distal	1	1	35	5	1	0
					Middle	1	1	30	3	1	0
					Proximal	0	1	15	1	0	0
	Golden retriever	1.33	28.9	MN	Distal	1	0	63	2	2	0

Table E1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields	Degenerate chondral cells median of five random fields	Metachromasia grade	Mineralisation grade
MC					Middle	0	0	53	1	2	0
					Proximal	0	0	39	0	2	0
	Mixed breed	14.0	10.3	MN	Distal	0	0	25	1	2	0
					Middle	2	1	30	0	2	0
					Proximal	1	0	28	0	1	0
	Spoodle	11.8	19.3	MN	Distal	2	1	46	2	2	1
					Middle	2	1	44	3	2	1
					Proximal	2	2	52	1	1	1
	Border collie	13.5	24.1	MN	Distal	2	0	60	1	2	0
					Middle	2	0	42	1	2	1
					Proximal	3	0	35	1	1	1
	German shepherd	10.7	33.8	MN	Distal	2	0	61	3	0	0
					Middle	2	1	65	2	1	1
					Proximal	1	1	48	4	0	0
	Miniature fox terrier	10.8	4.80	FN	Distal	2	1	27	3	2	1
					Middle	3	1	31	5	2	1

Table E1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields	Degenerate chondral cells median of five random fields	Metachromasia grade	Mineralisation grade
MC					Proximal	2	1	13	0	1	1
	Bichon frise	13.0	7.30	FN	Distal	0	1	25	4	2	0
					Middle	1	1	23	2	1	0
					Proximal	0	1	21	5	1	0
	German shepherd	8.00	30.3	FN	Distal	1	0	34	1	2	0
					Middle	1	2	45	1	2	1
					Proximal	1	2	38	1	2	0
	Jack Russell terrier	Adult	7.00	FN	Distal	2	0	46	5	1	0
					Middle	2	0	40	3	2	0
					Proximal	2	1	20	0	2	0
	German shepherd	14.5	31.8	MN	Distal	2	0	65	9	2	0
					Middle	1	1	53	7	2	0
					Proximal	0	0	35	3	2	0
	Labrador retriever crossbreed	Adult	53.0	MN	Distal	1	0	36	3	2	1
					Middle	2	0	37	4	1	1

Table E1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields	Degenerate chondral cells median of five random fields	Metachromasia grade	Mineralisation grade
MC					Proximal	0	0	41	3	1	0
	Dachshund crossbreed	10.0	12.2	FN	Distal	3	1	25	1	1	0
					Middle	2	2	29	3	2	1
					Proximal	2	1	33	2	2	0
	Labrador retriever	10.0	36.2	MN	Distal	1	0	72	6	2	1
					Middle	0	0	58	8	1	1
					Proximal	0	0	38	6	0	0
	Mixed breed	Adult	3.20	F	Distal	2	0	41	1	1	0
					Middle	2	1	44	2	1	1
					Proximal	2	0	46	3	0	0
	French bulldog crossbreed	9.25	15.6	MN	Distal	2	0	44	2	2	0
					Middle	2	0	50	4	2	0
					Proximal	2	0	37	2	1	0
	Maltese crossbreed	Adult	8.50	MN	Distal	2	0	17	1	2	1

Table E1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields	Degenerate chondral cells median of five random fields	Metachromasia grade	Mineralisation grade
MC					Middle	1	1	23	1	2	1
					Proximal	0	0	8	1	2	1
	Jack Russell terrier	Adult	10.3	MN	Distal	1	0	36	4	1	0
					Middle	0	0	29	3	1	0
					Proximal	0	1	18	3	0	0
	Fox terrier	Adult	7.80	MN	Distal	2	0	48	2	0	0
					Middle	3	0	47	1	1	1
					Proximal	2	1	48	3	1	1
DC	Greyhound	7.66	22.8	FN	Distal	1	0	49	2	2	0
					Middle	2	0	48	2	2	1
					Proximal	1	1	52	2	2	0
	Greyhound	0.12	3.20	M	Distal	2	0	97	0	0	0
					Middle	3	0	77	0	0	0
					Proximal	2	0	52	0	0	0
	Greyhound	0.12	1.20	M	Distal	1	0	40	0	0	0
					Middle	2	0	60	1	0	0
					Proximal	2	0	20	0	0	0

Table E1 – continued.

	Breed	Age (yr)	Weight (kg)	Sex	Section	Oedema grade*	Inflammation grade	Total chondral cells median of five random fields	Degenerate chondral cells median of five random fields	Metachromasia grade	Mineralisation grade
DC	Greyhound	0.12	2.50	M	Distal	2	0	84	0	0	0
					Middle	1	1	77	0	0	0
					Proximal	3	0	44	0	0	0
	Greyhound	0.12	1.80	F	Distal	1	0	0	0	0	0
					Middle	2	1	81	0	0	0
					Proximal	2	1	29	0	0	0
	Greyhound	0.12	2.70	M	Distal	1	0	81	0	0	0
					Middle	1	0	81	0	0	0
					Proximal	2	0	65	0	0	0
	Greyhound	Adult	22.0	F	Distal	2	0	46	4	1	1
					Middle	2	0	42	3	2	1
					Proximal	1	0	33	6	2	1
	Greyhound	Adult	27.5	F	Distal	2	0	65	2	1	1
					Middle	1	1	60	2	0	1
					Proximal	1	0	48	6	1	0