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## The remarkable vocal anatomy of the koala (Phascolarctos cinereus): insights into low-frequency sound production in a marsupial species

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1	The remarkable vocal anatomy of the koala (Phascolarctos
2	cinereus): insights into low-frequency sound production in
3	a marsupial species
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10	Key words: marsupials, mating calls, bellow vocalisation, sexual selection, descended
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12	vocal folds, laryngeal vocal folds

#### 14 Abstract

Koalas are characterised by a highly unusual vocal anatomy, with a descended larynx 15 and velar vocal folds, allowing them to produce calls with disproportionately low 16 frequencies. Here we use advanced imaging techniques, histological data, classical 17 macroscopic dissection and behavioural observations to provide the first detailed 18 description and interpretation of male and female koala vocal anatomy. We show that 19 both males and females have an elongated pharynx and soft palate, resulting in a 20 permanently descended larynx. In addition, the hyoid apparatus has a human-like 21 configuration in which paired dorsal, resilient ligaments suspend the hyoid apparatus 22 from the skull, while the ventral parts tightly connect to the descended larynx. We also 23 show that koalas can retract the larynx down into the thoracic inlet, facilitated by a 24 dramatic evolutionary transformation of the ventral neck muscles. First, the usual 25 retractors of the larynx and the hyoid have their origins deep in the thorax. Second, 26 three hyoid muscles have lost their connection to the hyoid skeleton. Third, the 27 genioglossus and geniohyoid muscles have greatly increased in length. Finally, the 28 digastric, omohyoid and sternohyoid muscles, connected by a common tendinous 29 intersection, form a guiding channel for the dynamic down-and-up movements of the 30 ventral hyoid parts and the larynx. We suggest that these features evolved to 31 accommodate the low resting position of the larynx and assist in its retraction during 32 call production. We also confirm that the edges of the intra-pharyngeal ostium have 33 specialised to form the novel, extra-laryngeal velar vocal folds, which are much larger 34 35 than the true, intra-laryngeal vocal folds in both sexes, but more developed and specialised for low frequency sound production in males than in females. Our findings 36 illustrate that strong selection pressures on acoustic signalling not only lead to the 37

specialisation of existing vocal organs, but can also result in the evolution of novel
vocal structures in both sexes.

#### 40 Introduction

Examining vocal anatomy is an important step towards understanding the 41 proximate factors that shape the vocal signals of a given species. The link between 42 vocal anatomy and vocal output has been thoroughly investigated in humans (e.g. 43 speech production and operatic singing; Némai & Kelemen, 1933; Doscher, 1994; 44 Titze, 1994; Lieberman et al, 2001; Dayme, 2009; Fitch, 2010; Koda et al, 2012). 45 However, over the last 25 years the generalisation of the source-filter theory has also 46 allowed researchers to explicitly link vocal anatomy and acoustic output in non-human 47 mammals. For example, studies combining behavioural, morphological and acoustic 48 49 data have revealed that red deer (Cervus elaphus) and fallow deer (Dama dama) have a descended and retractable larynx that lowers vocal tract resonances in their 50 respective sexual calls (Fitch & Reby, 2001, McElligott et al 2006), that male saigas 51 (Saiga tatarica) and southern elephant seals (Mirounga leonina) extend the nasal tract 52 to lower resonance frequencies in their rutting calls (Frey et al, 2007, Sanvito et al 53 54 2007) and have also provided key insights into sound production in Savanna elephants (Loxodonta africana - Herbst et al 2012, 2013), North American wapitis (Cervus 55 canadensis - Reby et al, 2016), and koalas (Phascolarctos cinereus - Charlton et al 56 57 2011, 2013).

According to the source-filter theory, mammals produce vocal signals via a twostage process (Fant, 1960): firstly, a source signal is generated as air expelled from the lungs passes through the larynx, causing the vocal folds to open and close, and breaking up the airstream to produce a sound wave. The rate that the vocal folds open and close determines the fundamental frequency (F0), responsible for the perceived

pitch of the vocalisation. The supra-laryngeal vocal tract then acts as a resonator, selectively diminishing certain frequencies of the source signal and enhancing other frequencies termed formants before the filtered signal radiates out through the mouth or nostrils. Because source and filter characteristics of mammal vocal signals are produced by the larynx and vocal tract, respectively; variation in these features can be directly predicted from the laryngeal and vocal tract anatomy of the caller (for a review see: Taylor et al 2016).

The koala (*Phascolarctos cinereus*) is a specialized arboreal folivore that inhabits 70 the open forests and woodlands of eastern and southeastern Australia dominated by 71 trees of the genus *Eucalyptus* (Lee and Carrick, 1989). Koalas are the only mammals 72 that consume eucalypt foliage as a dominant component of their diet (Tyndale-Biscoe, 73 2005). This relatively low-energy diet means that koalas spend a large proportion of 74 their time resting, only coming to the ground for brief periods in order to move between 75 trees (Martin and Handasyde, 1999). During the annual breeding season, however, 76 both sexes roam over much larger areas in order to locate a suitable mating partner 77 and vocal activity increases (Ellis et al, 2009). In particular, males produce a high rate 78 of extremely low-pitched bellow vocalizations when most copulations are predicted to 79 occur (Ellis et al, 2011), indicating that these calls are important in reproductive 80 contexts. Bellows are produced in a characteristic two-stage calling posture, gradually 81 changing from pronounced extension to marked flexion of the head and neck region 82 (Charlton et al, 2011; Fig. 1). 83

Recent studies examining the information content and function of male koala bellows have shown that formants are important cues to male identity and size that are used in both inter- and intra-sexual contexts (Charlton et al, 2011a; Charlton et al, 2011b; Charlton et al, 2012c; Charlton et al, 2012a; Charlton et al, 2012b; Charlton et

al, 2013). Taken together, these findings indicate that identity- and size-related formant 88 89 information is functionally relevant to male koalas in their natural environment, and that sexual selection favours males able to produce lower formants in their bellows. 90 Interestingly, the formant pattern of male bellow exhalation phases and this species' 91 well-developed sternothyroid muscle indicate that koalas extend the vocal tract just 92 prior to call production by lowering the larynx (Charlton et al. 2011b). In addition, 93 anatomical studies have revealed that male koalas have an additional set of vocal folds 94 outside of the larynx, the 'velar vocal folds' (VVFs), which are used instead of the intra-95 laryngeal vocal folds to produce the extremely low F0 of bellow vocalisations (Charlton 96 97 et al, 2013). As the VVFs are much larger than the laryngeal vocal folds they can oscillate periodically at much lower frequencies (Charlton et al, 2013). 98

Female koalas produce bellow vocalizations when they are in oestrous (Smith, 99 1980) and it has been shown that they also possess adaptations of the soft palate 100 101 (velum) similar to those found in males (Curry et al 2016). Female koala bellows have a mean F0 of 31.3 Hz (Charlton, 2015) which suggests that female koalas, like males, 102 use VVFs to produce disproportionately low-pitched calls, although not as low as those 103 of males. The formant spacing of the inhalation sections of female bellows is 423.5 Hz 104 (Charlton, 2015). Applying the following equation  $eVTL = c/2\Delta F$ , in which eVTL is the 105 estimated vocal tract length of the caller, c = the speed of sound in warm air (350 m/s)106 and  $\Delta F$  is the formant spacing, yields an estimated vocal tract length of 41.3 cm. This 107 is clearly a much longer vocal tract than expected for an animal the size of a koala, 108 109 and indicates that female koalas, like males, possess adaptations that allow them to elongate their vocal tract. Female koalas also produce individually distinctive squawks, 110 screams, squeaks and wails (collectively termed "rejection calls") when they rebuff 111 112 male copulation attempts (Charlton, 2015). The mean F0 of these calls (792.1 Hz) is

substantially higher than the mean F0 of the inhalation sections of female bellows (31,3
Hz – Charlton 2015), indicating that the rejection calls are more likely to be produced
by the laryngeal vocal folds.

In this study, we combined histological investigations with measurement data derived 116 from dissections and computed tomography (CT) imaging to comprehensively 117 document male and female koala vocal anatomy. Our specific aims were to: 1) provide 118 a detailed description of the koala's vocal anatomy; 2) identify key differences in 119 laryngeal and vocal tract anatomy between the sexes; 3) examine the tissue 120 composition of the koala's remarkable velar vocal folds; and 4) determine in situ 121 whether male koalas can retract their larynx into the thoracic inlet. Our findings will 122 provide additional insights into the production mechanisms of male and female koala 123 vocal signals. 124

125

## 126 Material and Methods

## 127 **Specimens**

Whole-body specimens: One adult female and one adult male koala (weighing 128 5.9 and 7.6 kg, and having overall body lengths of 680 and 750 mm, respectively) were 129 dissected. The animals were euthanized as part of normal hospital practice at Moggill 130 Koala Hospital, Bellbowrie, Queensland, Australia. A second adult male was used for 131 demonstrating the 'retracted' larynx in a virtual 3D reconstruction based on a CT scan 132 (S-Tab. 1). The specimens were stored deep-frozen (-20° C) before shipment to the 133 Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany, for anatomical 134 investigations. No animals were killed expressly for the purpose of this study. 135

Excised larynges: A total of 18 excised larynges (9 male, 9 female), including the 136 part of the soft palate with the intra-pharyngeal ostium, were collected from dead and 137 euthanized individuals at Moggill Koala Hospital during the 2011 breeding season 138 (October-December). The specimens were stored deep-frozen (-20° C) before 139 shipment to the Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany, 140 for anatomical investigations. In addition, the larvnges of the two dissected whole body 141 specimens (1 male, 1 female) were included in this series. For measurements, the 142 larynges were individually defrosted and mediosagittally cut into two halves. Larynx 143 and vocal fold dimensions were measured in the half-larynx specimens whereas velar 144 145 vocal fold dimensions were measured in the undivided soft palate parts. Overall dimensions of the larynx and velar vocal folds were documented photographically. 146

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## Computer tomographic (CT) and anatomical investigations

To obtain as much information as possible about skeletal and soft tissue prior to 149 our macroscopic dissections, the male and female deep-frozen specimens were 150 scanned using a 64-slice spiral Computer Tomograph Aquilion CX (Toshiba Medical 151 Systems Corp., 1385 Shimoishigami, Otawara-shi, Tochigi 324-8550, Japan) at the 152 IZW. The post-mortem in situ positioning of the vocal anatomy was documented using 153 virtual serial sections (MPRs obtained with the settings: 120.0 kV, 120.0 mA, slice 154 155 thickness 0.6mm) and 3D-reconstructions established by Vitrea 2 software. A second male was slowly defrosted in a refrigerator at +5° C over a period of 48 hours. Once 156 the specimen had fully thawed, its larynx was pushed back into the thoracic inlet by 157 158 external manipulation and fixed using a plastic strap. It was then refrozen and the plastic strap was removed in order to CT scan with the larynx in a fully retracted state 159 (Fig. 2). 160

For all anatomical dissections conducted at the IZW the specimens were thawed 161 at +5°C for a period of 24-48 hours and then submersed in water to facilitate 162 macroscopic dissection and photographic documentation. This technique improves the 163 diagnosis of structural details whilst also allowing for the dissection of non-preserved 164 fresh specimens over a comparably long time period (cf. Frey et al 2007, 2008, 2011). 165 The water in the dissection tank was continuously replaced by cool water (about 16°C) 166 between consecutive dissection steps. Photographs were taken whilst the specimen 167 was placed in a larger tank (using a Nikon D70s digital camera, Nikon Corp., Tokyo, 168 Japan) and transferred to a computer for image processing (Adobe Photoshop 5.5 and 169 CS4). Specimens were kept overnight in water at temperatures ranging between +2 170 and +4°C (refrigerator: Philipp Kirsch GmbH, Okenstrasse 103, 77652 Offenburg, 171 Germany). 172

The specimens were dissected starting from the left side, and the anatomical 173 structures were removed layer by layer. The right side was dissected in an identical 174 manner. The larynges, head-and-neck glands, most muscles, hyoid apparatus, soft 175 palate, tongue, ears and the trachea of the specimens were all fixed in 4% formalin 176 and stored in the IZW morphological collection. The skeletal parts were cleaned and 177 also stored in the IZW morphological collection. Photos of consecutive dissection steps 178 were taken with a Nikon D70S digital camera (Nikon Corp., Tokyo, Japan) on a CF 179 card, fed to a PC and processed with Adobe Photoshop 5.5 and CS4 (Adobe Systems 180 Inc., San Jose, CA, USA). 181

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## 183

## Micro-CT investigation of excised larynges

184 In order to obtain *in-situ* information on laryngeal cartilages and soft tissues, one 185 male and one female excised larynx (both earlier cut into two halves for

measurements) were subjected to Micro-CT investigation prior to invasive 186 macroscopic dissection. The deep-frozen larynges were slowly defrosted at about 5° 187 C for approximately 24h, and put into 4% formalin for 3 days to achieve soft fixation 188 and avoid autolysis during the long scanning procedure. Subsequent storage in 189 Phosphate Buffered Saline (PBS) for 5 days served to wash out the formalin. 190 Immediately before scanning, a larynx was transferred to a 50 ml falcon tube filled with 191 FomblinY®Perfluoropolyether lubricant (Solvay GmbH, Hannover, Germany. This oil 192 is MR-inert, thus giving contrast only to the tissue, and avoids dehydration of the 193 specimen during scanning. The scanning was conducted using a 7T PharmaScan 194 70/16 US (Bruker BioSpin, Ettlingen, Germany) with Bruker software Paravision 5.1 195 and a linear 38 mm volume resonator (Bruker BioSpin). Scanning was performed with 196 the following settings: T2\_TurboRARE\_3D with TR/TE = 1500/32.5ms, rare factor 6, 197 198 averages 6; FOV (Field of View) =  $46 \times 30 \times 25.8$ mm; Matrix size =  $306 \times 200 \times 172$ ; image resolution = 150  $\mu$ m x 150  $\mu$ m x 150  $\mu$ m; Scan time for the male and female 199 200 larynx: 14h11m24s, respectively.

201

## 202 *Histology*

Following *in-situ* dissection in water, the VVFs of the whole body male specimen were excised and preserved in 10% neutral buffered formalin. Longitudinal and cross sections (5  $\mu$ m in thickness) of the VVFs were then stained with haematoxylin-eosin for a general overview, Masson/Goldner Trichrome as a collagen fibre stain, and Elastica-Van Gieson as an elastic fibre stain. Photographs of representative histological slides were taken with a Keyence VHX 1000 Digital Microscope (Keyence Deutschland, Neu-Isenburg, Germany) using a VH-Z00R, RZ  $\approx$  - 50, Zoom 5-50x lens

and a VH-Z20R, RZ x20-x200 lens. Photoshop version CS4 was used to process theimages.

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## 213 Laryngeal retraction

At key stages of the dissection, the larynx was manually retracted to document laryngeal mobility and extension of the pharynx, including the soft palate and the velar vocal folds. Retraction was achieved by fastening a string to the caudal end of the trachea, manually pulling the string through an opening in the abdominal wall, and attaching the string to the wall of the dissection tub using a clamp. Simultaneously, the head and neck were extended and pulled forward by a string fastened to an incisor tooth and attached to the opposite wall of the dissection tub by using another clamp.

Once the dissections were complete, the specimens were subjected to bacterial 221 skeletonization. For this purpose, the skeletal parts were first put into appropriately 222 223 sized tubs filled with cold water for several days to wash the blood out. The specimens were then transferred to a heated tank filled with water and kept at 38°C until the soft 224 parts had been fully decomposed by bacteria. The bacterial skeletonization process 225 took approximately one month. After this, the remaining bones were kept in the same 226 tank at the same temperature in a concentrated solution of washing powder for an 227 228 additional month before the skeletal parts were cleaned, dried, and used for graphic 2D-reconstructions. 229

Anatomical terms are in accordance with Nomina Anatomica Veterinaria (Schaller 2012; NAV, 2005, 2012).

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## Abbreviations

234	Adit. lar.	=	laryngeal entrance
235	App. hyo.	=	hyoid apparatus
236	Arc. pal. phar.	=	palatopharyngeal arc
237	Arc. zyg.	=	zygomatic arc
238	Basih.	=	basihyoid
239	Bifurc.	=	tracheal bifurcation
240	Bul. tymp.	=	tympanic bulla
241	Cart. aryt.	=	arytenoid cartilage
242	Cart. cost. IV	=	4 <sup>th</sup> costal cartilage
243	Cart. cric.	=	cricoid cartilage
244	Cart. postaryt.	=	postarytenoid cartilage
245	Cart. proaryt.	=	proarytenoid cartilage
246	Cart. thyr.	=	thyroid cartilage
247	Cart. trach. I, IV	=	1 <sup>st</sup> , 4 <sup>th</sup> tracheal cartilage
248	Cav. nasi	=	nasal cavity
249	Cav. or.	=	oral cavity
250	Ceratoh.	=	ceratohyoid
251	Choan.	=	choanae (internal nares)
252	Clav.	=	clavicle
253	Cont. Mand.	=	contour of lower jaw
254	Cor	=	heart
255	Corn. caud.	=	caudal horn of thyroid cartilage
256	Corn. rostr.	=	rostral horn of thyroid cartilage
257	Cost. I , III, IV	=	1 <sup>st</sup> , 3 <sup>rd</sup> , 4 <sup>th</sup> rib
258	Crist. med.	=	dorsomedian crest of soft palate
259	C1, C7	=	1 <sup>st</sup> , 7 <sup>th</sup> cervical vertebra
260	Diaphr.	=	diaphragm
261	Epigl.	=	epiglottis
262	Intsect. tend.	=	tendinous intersection

263	IPO	=	intra-pharyngeal ostium
264	Lar.	=	larynx
265	Lig. hyo:	=	hyoid ligament
266	Ling.	=	tongue
267	LVFs	=	laryngeal vocal folds
268	Man. sterni	=	sternal manubrium
269	Meat. ac. ext.	=	external acoustic meatus
270	M. ceratoh.	=	ceratohyoid muscle
271	M.cricphar.	=	cricopharyngeus muscle
272	M. digastr.	=	digastric muscle
273	M. digastr. vent. caud.	=	caudal belly of digastric muscle
274	M. digastr. vent. rostr.	=	rostral belly of digastric muscle
275	M. genioh.	=	geniohyoid muscle
276	M.geniogllar.	=	genioglossolaryngeal muscle
277	M. hyoceph.	=	hyocephalic muscle (united M. styloh.,
278			M. stylphar.caud., M.hyophar.)
279	M. hyogl. (sin.)	=	(left) hyoglossus muscle
280	M. hyophar.	=	hyopharyngeus muscle
281	M. omoh.	=	omohyoid muscle
282	M. parotaur.	=	parotidoauricularis muscle
283	M. sternceph.	=	sternocephalic muscle
284	M. sternoh. (sin., dex.)	=	(left, right) sternohyoid muscle
285	M. sternthyr. (sin., dex.)	=	(left, right) sternothyroid muscle
286	M. stylogl.	=	styloglossus muscle
287	M. styloh.	=	stylohyoid muscle
288	M. stylphar. caud.	=	caudal stylopharyngeal muscle
289	M. thyroh.	=	thyrohyoid muscle
290	M. thyrphar	=	thyropharyngeus muscle
291	M <sup>4</sup>	=	4 <sup>th</sup> upper molar
292	Nar. (sin.)	=	(left) nostril
293	Nasal vt.	=	nasal vocal tract

294	Nasophar.	=	nasopharynx
295	Oesoph.	=	oesophagus
296	Oral vt.	=	oral vocal tract
297	Orophar.	=	oropharynx
298	Os pteryg.	=	pterygoid bone
299	Os rostr.	=	rostral bone
300	Palat. mol.	=	soft palate
301	Phar.	=	pharynx
302	Plic. voc.	=	vocal fold
303	Proc. parac.	=	paracondylar process
304	Proc. voc.	=	vocal process of arytenoid cartilage
305	Proc. xiph.	=	xiphoid process
306	Pulm.	=	lungs
307	Scap.	=	scapula
308	Stern.	=	sternum
309	Sulc. med.	=	dorsomedian trench of soft palate
310	Thyroh.	=	thyrohyoid
311	Trach.	=	trachea
312	T1, T4	=	1 <sup>st</sup> , 4 <sup>th</sup> thoracic vertebra
313	Vest. lar.	=	laryngeal vestibule
314	Vest. nasi	=	nasal vestibule
315	VTL	=	vocal tract length
316	VVF(s) (dex.)	=	(right) velar vocal fold(s)
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## 318 **Results**

## 319 Nostrils, nasal vestibulum and nasal cavity

The nostrils of both sexes are obliquely oriented, from laterodorsal to ventromedial in frontal view. In lateral view, starting from the nares, the narrow, tube-like nasal vestibulum first courses 80° dorsally towards the nasal dorsum before it bends sharply in a caudal direction to connect to the larger volume of the nasal cavity proper. Caudally, the upward portion of the nasal vestibulum is supported by a small rostral bone (*Os rostrale*) at the rostral end of the nasal septum, resting mediosagittally on the incisive bone (Fig. 3, 8). From the most dorsal point of the nasal vestibulum, at eye level, the air passage runs slightly oblique caudoventrally (10°-15°) and almost straight, along the ventral nasal meatus and the nasopharyngeal meatus up to the choanae.

## 329 <u>Choanae</u>

330 The osseous choanae for both sexes are located comparatively far rostrally, at a level just caudal to the last molars (M<sup>4</sup>/M<sub>4</sub>). In lateral view, the lateral osseous laminae of 331 the palatine and pterygoid bones have a very oblique ventral edge (caudally inclined 332 333 by about 45°) as a foundation for anchoring the thick soft palate. As a consequence of the great dorsoventral height of the soft palate at this level, the nasopharyngeal meatus 334 is narrow dorsoventrally and transversely and courses horizontally along the skull 335 base, passing by the long pterygoids and the auditory bulla, before sharply bending 336 ventrally towards the nasopharynx at a level between the auditory bulla and the 337 338 paracondylar processes.

## 339 Cheek pouches

Cheek pouches are present in both the male and the female (S-Fig. 1). They are positioned immediately caudal to the upper lip and the mouth angle, between the nostril and the eye. Their opening is obliquely oriented, from the small upper canine rostrodorsally, to the first premolars  $P^3/P_3$  caudoventrally, following the contour of the upper lip. The dimensions of the cheek pouches for the female and male were: rostrocaudal length ~28/~35, dorsoventral height ~20/~25 and transverse width

~15/~20 mm, respectively (S-Table 2). The caudoventral half of the cheek pouches
 contains a layer of buccal salivary glands on its lateral surface and is covered by the
 buccinator muscle.

349 Pharynx

The upper pharynx is very spacious and extensible (S-Fig. 2; S-Video 1). It connects 350 the caudal ends of the nasal and oral cavities to the larynx and oesophagus. The 351 resting position of the head is approximately at a right angle to the longitudinal axis of 352 the body. This results in a pronounced bending of the upper pharynx, the oropharynx 353 354 in particular, while the head is kept in its typical resting position. In contrast, neck extension, as observed in the initial phase of call production, will straighten the 355 pharynx. Due to the short snout and the short hard palate, the choanae are positioned 356 357 rather far rostrally, between the upper last molars (M<sup>4</sup>), and about half way along the length of the head from a lateral view. The rostroventral, lateral and caudoventral parts 358 of the pharyngeal wall receive terminating fibres from the genioglossolaryngeal muscle 359 (see below and S-Tab. 7). The mucosa of the nasopharynx resembles the respiratory 360 mucosa in the respiratory region of the nasal cavity. The nasopharyngeal mucosa is 361 362 finely plicated, pliable and highly elastic. The mucosa of the oropharynx resembles the cutaneous mucosa of the oral cavity. The oropharyngeal mucosa is corrugated and 363 also pliable and highly elastic. Internally, the oropharynx is completely separated from 364 365 the nasopharynx, except for one connection just rostral to the laryngeal entrance, the intra-pharyngeal ostium (IPO). The soft palate is large, thick, and of triangular shape 366 when viewed from a mediosagittal perspective. Thickness is maximal close to the 367 368 choanae and tapers caudally towards the IPO. Inside the triangular space, between the roof of the oropharynx and the floor of the nasopharynx, the palatopharyngeal 369

muscle surrounds the IPO on both sides (S-Tab. 7). When the pharynx is manually extended (Fig. 4, 5) the IPO remains close to the epiglottis and the laryngeal entrance.

372 Soft palate

The overall dimensions of the soft palate in the female and male were: resting length 373 - 50/70, extended length - 75/100 and dorsoventral thickness at the choanae 15/20 374 mm, respectively (S-Tab. 3). The entire soft palate is remarkable: its roof (= the mucosa 375 of the dorsal surface of the soft palate) has a deep (~ 2 mm) median trench along its 376 entire length in both sexes, although it is less prominent in the female. In the male, its 377 378 transverse width in the resting position is only 1-2 mm, i.e. it is almost closed, whereas in the bilaterally extended state it is 6-8 mm wide, and becomes progressively wider 379 towards the IPO. There is a median, serrated crest along the trench that terminates 380 381 about 10 mm rostral to the IPO (Fig. 6A). The rostrocaudal length of the crest is ~ 20 mm and tapers rostrally. Maximum dorsoventral height of the crest is ~ 3 mm. Lateral 382 to the median trench, the mucosa is flat and smooth and the two halves of these 383 elevated surfaces of the floor of the nasopharynx are both ~ 5 mm wide (transversely). 384 The surface of the median trench is covered with small knobs or caruncles. The lateral 385 386 flat surfaces are a light whitish/greenish colour, whereas the mucosa of the median trench is dark reddish/brownish. The mucosa of the median trench is supplied with 387 388 many tiny blood vessels.

In addition, the male and female IPO is not a simple opening between the dorsal and ventral compartments of the pharynx, as it is in other mammals (S-Fig. 3). Instead, its medial edges form two large, longitudinally oriented, voluminous folds, which protrude ventrally into the caudal part of the oropharynx (laryngopharynx) (Fig. 6B). These folds are termed 'velar vocal folds' (VVFs - Charlton et al 2013).

The mucosa of the ventral surface of the soft palate possesses a shallow median 394 depression, about 9 mm wide rostrally, that narrows to 3 mm halfway along the 395 rostrocaudal length. Along its caudal third, the depression widens to form a triangle 396 that merges with a broad, caudally open horseshoe-shaped fold, which encompasses 397 the velar vocal folds. Its longitudinal limbs, 6 mm wide rostrally, taper in caudal direction 398 and fuse with the ventrocaudal parts of the velar vocal folds (VVFs) (Fig. 6B). The 399 palatopharyngeal arc consists of a narrow fold surrounding the IPO, the VVFs, and the 400 horseshoe-shaped fold, before fusing with the dorsocaudal wall of the pharynx caudal 401 to the VVFs. On each side there is a rostrally closed blind space of about 15 mm in 402 403 rostrocaudal length (destroyed on the left side when cutting into the pharynx), between the horseshoe-shaped fold medially and the palatopharyngeal arc laterally. The entire 404 mucosa of the ventral surface of the soft palate is finely plicated transversely (Fig. 6B). 405

## 406 Velar vocal folds

VVFs occur in the male and the female. Rostral to the IPO, the lateral flat ventral 407 surfaces of the soft palate rise up to form two longitudinally oriented, voluminous folds 408 medially along the edges of the IPO (Fig. 6B). These velar vocal folds are clearly 409 distinct from the surrounding soft palate. They narrow the IPO considerably, restricting 410 it to a slit-like opening that is slightly wider rostrally than caudally. Laterally, the velar 411 vocal folds are encompassed by a horseshoe-shaped fold and, further laterally, by the 412 palatopharyngeal arc. Caudally, each VVF fuses with the respective limb of the 413 horseshoe-shaped fold. The velar vocal folds protrude ventrally into the 414 laryngopharynx (Fig. 3). The dimensions of the VVFs for female and male (n = 10, 415 416 respectively) are: resting length - 26.2±2.59 versus 32.5±2.8, resting depth - 11.7±1 versus 14.9±2.13, resting width - 7.6±0.74 versus 10.7±1.32 and maximally extended 417 length – 43.6±5.1 versus 51.4±6.29 mm. (S-Tab. 4). 418

Histological examination of the VVFs reveals that they are differentiated from the 419 surrounding soft palate. Longitudinal and cross sections (5 µm thickness) show a three 420 layered structure. The superficial thin layer consists of a cornifying stratified squamous 421 422 epithelium. The intermediate layer comprises two sublayers: a subepithelial thin layer of collagenous and elastic fibres running mostly parallel to the surface of the VVFs; 423 and a thicker layer of very loosely organised short and mostly single collagenous fibres. 424 intermingled with few, mostly single elastic fibres. This sublayer contains numerous 425 small, thin-walled vessels, most likely venules or lymphatic vessels and nerves. The 426 deepest layer, constituting the base of the VVFs, is composed of a scaffolding of larger 427 bundles of collagenous and elastic fibres, and striated muscle fibre bundles 428 interspersed with groups of mucinous glands, larger blood vessels and nerves. The 429 collagen and elastin fibres within this deep layer are mostly longitudinal in 430 431 arrangement. The basal deep layer makes the transition to the larger muscle bundles, the larger, mostly transversely arranged, scaffolding of collagenous and elastic fibres, 432 larger aggregations of mucinous glands, and larger blood vessels and nerves of the 433 soft palate proper (Fig. 7). 434

Although the structure of the VVFs is broadly similar in males and females, there are 435 a few key differences. The VVFs of the dissected male specimen are larger than those 436 of the female (S-Tab. 4), and the second (b) sublayer of the intermediate layer appears 437 to contain a higher overall density of collagenous fibres, which are organised in thicker 438 bundles in the male than in the female. In the male there is also a large, longitudinally 439 oriented bundle of elastic fibres, running parallel to the free edge of the VVFs in the 440 intermediate layer. This bundle appears to be lacking in the female VVFs. The deep 441 layer of the VVFs also appears to be more muscular in the male than in the female. 442

## 443 Hyoid apparatus

The koala's hyoid apparatus is suspended from the skull base by a pair of highly 444 resilient hyoid ligaments. Each of these hyoid ligaments consists of several sub-445 ligaments that run parallel to one another. The dorsal attachment to the skull base is 446 447 located between the auditory bulla and the paracondylar process. This attachment is supported by a small occipitohyoid muscle. Ventrally, the hyoid ligaments connect to 448 the lateral ends of the ceratohyoids. Other parts of the suspension apparatus, for 449 example, the tympanohyoids, stylohyoids, and epihyoids, are lacking. Interestingly, the 450 hyoid ligaments of the male appear to be more elastic than those of the female: resting 451 length versus maximally extended length in the female 30/60 mm,(100% extension) 452 453 and 40/100 mm in the male, (150% extension) (S-Tab. 5). The ceratohyoids are short, cartilaginous, and fused rostrolaterally to the transversely oriented osseous basihyoid. 454 Caudolaterally, the basihyoid connects to two thyrohyoids that are directed 455 456 caudodorsally (S-Fig. 4). The larger rostral part of the thyrohyoids is ossified whereas the caudal ends are cartilaginous. The cartilaginous part of the thyrohyoids connects 457 to the rostral horn of the thyroid cartilage (Fig. 8). 458

## 459 <u>Thyrohyoid membrane</u>

460 The thyrohyoid membrane extends between the thyroid cartilage and the thyrohyoid laterally, and the thyroid cartilage and the basihyoid ventrally. It is short and rather 461 tough, with a ventral length that exceeds its lateral length. Rostrocaudal resting length 462 for the female and the male are 2-3/3-4 mm laterally and 6-7/10-11 mm ventrally; 463 maximally extended lengths are ~4/~5 mm laterally and 8-9/12-13 mm ventrally (S-464 Tab. 6). Due to the stiffness of the thyrohyoid membrane, when the larynx is manually 465 466 retracted the ventral parts of the hyoid apparatus (ceratohyoids, basihyoid, thyrohyoids) stay close to the larynx as it descends down the neck towards the thorax. 467 The highly elastic hyoid ligament permits the larynx to be fully retracted into the thoracic 468

inlet. The laryngeal entrance and the ventral parts of the hyoid apparatus are thenlocated at the level of the manubrium.

471 <u>Musculature</u>

The results of the ventral neck musculature dissection, including presumed muscle functions, are summarized in S-Table 7. Instances where muscles deviate greatly from the typical mammalian pattern are shortly listed below:

- Three muscles, the digastric, the omohyoid and the sternohyoid, share a
  common tendinous intersection. In the koala the omohyoid and sternohyoid
  muscles have entirely lost their connection to the hyoid apparatus (Fig. 9).
- The caudal fibres of the genioglossus muscle are of considerable length and
  do not terminate on the basihyoid. Instead they terminate on the lateral wall
  of the oropharynx and on the thyrohyoid membrane close to the rostral edge
  of the thyroid cartilage. As a consequence of this, it is henceforth called a
  genioglossolaryngeal muscle (Fig. 5).
- 3) The geniohyoid muscle is greatly extended in length and bunches up to form
  undulating reserve bends during the resting state of the larynx. It passes
  through a guiding arch provided by the hyoglossus muscle (S-Fig. 5).
- 486
  4) The sternohyoid and sternothyroid muscles are deeply anchored in the thorax.
  487 The truly intrathoracic origins of both muscles arise from the dorsal surface of
  488 the sternum and the first few costal cartilages (Fig. 10).
- 5) The mylohyoideus and hyoglossus muscles have completely lost their
  attachment to the hyoid apparatus. In the koala the hyoglossus muscle forms
  a type of guiding arch for the long geniohyoid muscle (S-Fig. 5).
- 492 6) The stylohyoid, caudal stylopharyngeus, and hyopharyngeus muscles are493 difficult to compare with the corresponding muscles of placental mammals

because an osseous suspension of the hyoid apparatus to the skull is lacking
in the koala. MacAlister (1872) and Young (1882) realised this and combined
them as the styloid group. We found that the main portion of this muscle sheet
originates from the thyrohyoid, and terminates on the base of the skull,
caudally adjacent to the attachment of the hyoid ligament. We therefore
suggest that these three muscles are grouped tougher under a new name: the
hyocephalic muscle (Fig. 11).

501 Larynx

## 502 Larynx dimensions

503 Overall measurements of the larynx for female (n = 9) and male (n = 10) koalas are: 504 maximal rostrocaudal length  $25.2\pm1.66$  versus  $28.2\pm2.68$  mm, maximal transverse 505 width  $22.0\pm2.13$  versus  $25.2\pm2.68$  mm and maximal dorsoventral height  $16.7\pm0.78$ 506 versus  $19.5\pm1.62$  mm (S-Table 8).

## 507 Laryngeal cartilages

508 In both sexes, the cartilages of the larynx comprise the epiglottis, the thyroid cartilage, 509 the cricoid cartilage, the two arytenoid cartilages, the proarytenoid cartilage, resting on the rostral commissure of the medial processes of the arytenoid cartilages, and a small 510 511 postarytenoid cartilage located caudal to the commissure of the medial processes of the arytenoid cartilages (Fig. 12). The thyroid and cricoid cartilages are fused 512 ventromedially (as previously noted, e.g., by Symington 1898; Schneider 1964 - S-Fig. 513 6). The vocal process of the arytenoid cartilage is crescent-shaped, rostrally directed, 514 and protrudes considerably in a ventral direction towards the laryngeal lumen. The 515 516 vocal process extends far rostrally, up to the level of the proarytenoid cartilage and approaching the laryngeal entrance (Fig. 12). On either side of the cricoid, the paired 517

dorsal cricothyroid connection is established by a synchondrosis, which is located dorsal to the caudal laryngeal nerve. Laterally, the proarytenoid, the arytenoids, the postarytenoid, and the cricoid cartilage(s) are covered by the thyroid lamina and the broad rostral and long caudal horns of the thyroid cartilage.

The epiglottis is spatula-shaped in dorsal view. Medioventrally, its base 522 connects to the rostral edge of the thyroid cartilage. Its laryngeal surface faces the 523 laryngeal vestibule whereas the ventral third of its lingual surface serves as the origin 524 of the hypepiglottic muscle. The dimensions of the epiglottis for female (n = 8) and 525 male (n = 9) koalas were: rostrocaudal length  $10.1\pm0.92$  versus  $11.0\pm0.77$  mm and 526 transverse width 12.2±1.08 mm versus 14.8±1.85 mm (S-Table 9). The colour of the 527 epiglottic cartilage is yellow, suggesting a composition of elastic cartilage (Fig. 12A). 528 Cuneiform processes are lacking in both sexes. 529

The thyroid cartilage has a short, ventrally-curved rostral horn that establishes the cartilaginous connection to the thyrohyoid of the hyoid apparatus. In contrast, the caudal horn is long and straight, and protrudes caudally in parallel to the longitudinal axis of the larynx. The laminae of the thyroid cartilage are of trapezoid shape in lateral view, their ventral rostrocaudal length exceeding the dorsal by about one third. A large fat pad is sandwiched between the thyroid lamina laterally and the thyroarytenoid and ceratocricoarytenoid muscles medially. A thyroid bulla is not observed in the koala.

The cricoid cartilage has almost the same rostrocaudal dimensions dorsally and ventrally, i.e. the dorsal cricoid lamina is very short. In the dorsal third, the left and right rostral edges of the cricoid cartilage bulge out rostrally and form the articular surfaces for connection with the arytenoid cartilages. Together with the ventrally and dorsally fixed connections to the thyroid cartilage, the bilateral bulging creates a boomerangshaped opening between the rigidly coupled thyroid and cricoid cartilages, its convex

shape being directed rostrally. In lateral view, the caudal edge of the cricoid cartilage,
to which the first tracheal cartilage connects, is of concave shape.

The arytenoid cartilages are comparatively large and articulate with the 545 dorsorostral bulges of the cricoid cartilage. Rostrally, the arytenoid cartilages extend 546 upwards to form the caudal boundary of the laryngeal entrance. Each arytenoid 547 cartilage possesses three well-developed processes: a lateral muscular process, a 548 (dorso)medial process, and a ventrally directed vocal process. Corniculate processes 549 of the arytenoid cartilages are lacking, and left and right arytenoid cartilage are linked 550 to each other via their medial processes by connective tissue. The dorsoventral extent 551 of the arytenoid cartilage is considerable. The vocal process protrudes widely in a 552 ventral direction, and causes a slight prominence of the laryngeal mucosa at its ventral 553 tip, to which the dorsal end of the vocal fold attaches. 554

The proarytenoid cartilage, which is also termed procricoid cartilage in the older literature, is a homologue of the interarytenoid cartilage in placental mammals (Symington 1898; Schneider 1964, p. 42). It is located rostral to where the medial processes of the arytenoid cartilages connect and its caudal surface is intimately linked to this transverse junction by connective tissue.

560 Intrinsic laryngeal muscles

The <u>cricothyroid muscle</u> is lacking, possibly as a result of the ventral fusion of the thyroid and cricoid cartilages, and the concomitant restriction in mobility between these two cartilages. The thyroarytenoid muscle is a homologue of the corresponding muscle of placental mammals (Schneider 1964, p. 34). It originates medioventrally from the thyroid lamina and its fibres course laterodorsally, before terminating on the muscular process of the arytenoid cartilage ventrally. The thyroarytenoid muscle does not connect to the vocal process of the arytenoid cartilage.

The <u>lateral cricoarytenoid muscle</u> is also a homologue of the corresponding muscle of placental mammals (Schneider 1964, p. 34). It originates from the laterodorsal surface of the cricoid cartilage, where it is partly covered by the ceratocricoarytenoid muscle. Its fibres course obliquely rostrodorsally and terminate lateroventrally on the muscular process of the arytenoid cartilage.

The <u>aryprocricoid muscle</u> in the koala is a homologue of the transverse arytenoid muscle of placental mammals (Schneider 1964, p. 35). It originates from the arcuate crest of the arytenoid cartilage and most of its fibres terminate on the proarytenoid cartilage, whereas the most rostral fibres fuse with those of the contralateral muscle.

577 The cricoprocricoarytenoid muscle originates from the dorsal edge of the cricoid 578 cartilage and terminates laterocaudally on the procricoid cartilage.

The koala's <u>ceratocricoarytenoid muscle</u> is a homologue of the dorsal cricoarytenoid muscle of placental mammals (Schneider 1964, p. 44). It originates from the medial surface of the caudal horn of the thyroid cartilage, the lateral surface of the cricoid cartilage, and the laterodorsal parts of the cricoid lamina. The muscle terminates dorsally on the muscular process and the arcuate crest of the arytenoid cartilage. We assume that the function of the <u>ceratocricoarytenoid</u> muscle is to dilate the glottis.

## 585 Laryngeal entrance and cavity

The laryngeal entrance is surrounded by the epiglottis rostrally, the aryepiglottic folds laterally, and the arytenoid cartilages caudally. A large pad of small glands is laterally attached to each of the aryepiglottic folds, and mostly covered by the thyroid laminae. The laryngeal cavity comprises a vestibule extending from the entrance of the larynx to the vocal folds, the glottis between the vocal folds, and an infraglottic space, extending from the vocal folds to the exit of the larynx at its junction with the trachea.

As a consequence of the large vocal process of the arytenoid cartilages and the relatively short vocal folds, the ratio of the intercartilaginous to intermembraneous portions of the glottis is approximately 50:50.

#### 595 Laryngeal vocal folds

The laryngeal vocal folds (LVFs) are rostrally inclined against the longitudinal axis of 596 the larynx by about 30°, from craniodorsally to caudoventrally. Ventrally, the LVFs 597 attach to the dorsal surface of the fused thyroid and cricoid cartilages, close to the 598 cranial edge of the cricoid arch in a paramedian plane. Dorsally, the LVFs attach to the 599 600 tip of the vocal process of the arytenoid cartilage. The dimensions of the LVFs for female (n = 9) and male koalas (n = 10) respectively are: dorsoventral resting length 601 602 8.0±0.57 versus 9.8±0.88mm, rostrocaudal resting length 0.8±0.1 versus 1.0±0.13 603 mm, transverse resting width 0.5±0.12 versus 0.8±0.16 mm and maximally extended dorsoventral length 9.8±0.39 versus 11.8±0.94 mm (S-Tab. 10). 604

## 605 Laryngeal mobility

The resting position of the larynx is approximately at the level of cervical vertebrae 4-606 6 in an adult male koala (Fig. 8). The larynx can be manually retracted down into the 607 thoracic inlet in both sexes. At maximal retraction, the ceratohyoids and the basihyoid 608 are close to the level of the first rib, and the maximally extended hyoid ligament runs 609 610 more or less parallel to the ventral edge of the hypaxial neck musculature. Larynx movements down and up the ventral neck region are facilitated by a highly flexible 611 double gliding layer of connective tissue that dorsally separates the pharynx, larynx 612 613 and oesophagus from the hypaxial neck musculature (longus capitis, longus colli muscles) (S-Fig. 7). The medial retropharyngeal lymph nodes are located on the dorsal 614 wall of the pharynx at the level of the first tracheal rings, contacting the dorsal gliding 615

layer. The lateral retropharyngeal lymph nodes are located a bit more cranially, at the 616 617 level of the osseous hyoid apparatus in its resting position, and in the groove between the nasopharynx and the neck musculature. Ventrally, the muscular channel or 618 guidance for any potential craniocaudal movements of the pharynx, larynx and 619 oesophagus, provided by the inter-linked digastric, omohyoid and sternohyoid 620 muscles, is lined with a connective tissue layer. In addition, there is a fat cushion 621 between the styloglossus muscle laterally and the genioglossolaryngeal muscle 622 medially, and a short connective tissue canal at the thoracic inlet, through which the 623 larynx can move. This canal is attached to the clavicula, the carotid artery, the neck 624 625 fascia and the sternal manubrium. This gliding space continues inside the thorax, dorsal to the heart and the large blood vessels at the base of the heart, which are both 626 covered by the pericardial pleura. Movement of the larynx, oesophagus and trachea 627 628 inside the thorax is further guided by the thoracic portions of the hypaxial neck musculature (longus colli muscle) dorsally and the sternohyoid and sternothyroid 629 muscles ventrally. A gliding space for minor cranio-caudal movements of the 630 intrathoracic portions of the sternohyoid and sternothyroid muscles along the 631 craniodorsal surface of the sternum is provided by the common connective tissue 632 sheath interlinking these muscles, ventrally, and by the dorsally adjacent pericardial 633 pleura. 634

635

## 636 Vocal tract length

Oral vocal tract length (hereafter VTL) was taken as the distance from the lips to the LVFs, and the nasal VTL was measured from the tip of the nostrils to the LVFs. The dimensions of the koala's nasal and oral vocal tract lengths during different stages of the dissection of one female and one male koala are: resting nasal vtl with angled head

160-165 versus 185-190 mm, maximally extended nasal vtl with angled 185-190 641 versus 230-240, maximally extended nasal vtl with head and neck extended (male only 642 255-260 mm; resting oral vtl with angled head 140-145 versus 160-170 mm, maximally 643 extended oral vtl with head and neck extended (male only) 210-220 mm (S-Tab. 11). 644 The laryngeal vocal folds are not involved in the production of male koala bellow 645 vocalizations, and it is presumed that the glottis is open during call production (Charlton 646 et al 2013). Accordingly, we also provide additional measurements of vtl from the 647 nostrils and lips to the VVFs (a) and from the nostrils and lips down to the tracheal 648 bifurcation (b) at maximal extension in the male specimen: nasal vtl ~220 mm (a) and 649 650 ~ 295 mm (b), oral vtl ~190 mm (a) and ~270 mm (b) (S-Tab. 11).

651

## 652 <u>Nerves</u>

The nerve supply to the pharynx, larynx, and associated muscles did not appear to 653 654 differ between the male and female adult koala specimens. The hypoglossal nerve emerges from the skull base ventral to the external acoustic meatus, between the 655 auditory bulla rostrally and the paracondylar process caudally. This nerve curves 656 laterally around the hyoid ligament obliquely in a rostral direction, and along the ventral 657 edge of the styloglossus muscle. It then enters the hyoglossus muscle laterally before 658 subdividing into 4 branches: three branches curve dorsally along the rostral edge of 659 this muscle to innervate the tongue and supply the intrinsic tongue muscles. The most 660 ventral of these branches supplies the rostral part of the genioglossolaryngeal muscle. 661 662 This branch is undulating, while the dorsal two are not. The most ventral branch of the hypoglossal nerve is also an undulating nerve that supplies the geniohyoid muscle. 663

664 Motor nerve supply to the omohyoid, sternohyoid and sternothyroid muscles is 665 provided by the ventral ramus of the first cervical nerve (N. cervicalis I). As a

consequence of the long sternohyoid and sternothyroid muscles, the associated nerve
fibres are of corresponding length. The lengths of the more or less straight branches
to the caudal, intrathoracic parts of the sternohyoid and sternothyroid muscles are
around 150 mm in the female and around 200 mm in the male. In contrast, branches
to the cranial parts of the sternohyoid and sternothyroid muscles form caudally convex
reserve loops that are slightly undulating.

The glossopharyngeal nerve emerges from the skull base close to the 672 paracondylar process. It traverses the caudal stylopharyngeal muscle in a rostral 673 direction and provides a small branch to this muscle. The pharyngeal branches of the 674 glossopharyngeal nerve to the dorsal wall of the pharynx were not dissected. The 675 larger lingual branch of the glossopharyngeal nerve runs ventrally along the rostral 676 border of the caudal stylopharyngeal muscle and medial to the hyoid ligament before 677 sharply bending rostrally towards the lateral wall of the oropharynx and the tongue. On 678 679 the way it subdivides into a dorsal branch leading to the soft palate and a ventral branch leading to the root of the tongue. All of these branches of the glossopharyngeal nerve 680 are strongly undulating nerves. 681

682 The vagus nerve emerges from the skull base together with the glossopharyngeal nerve. The oesophageal branch of the vagus nerve is a strongly 683 undulating nerve and courses caudally along the nasopharynx towards the caudal 684 685 constrictor muscles of the pharynx and the oesophagus. At the level of the thyroid cartilage it subdivides into three branches supplying the thyro- and cricopharyngeal 686 muscles and the vestibulum of the oesophagus (Fig. 13). A further branch of the vagus 687 688 nerve, the cranial laryngeal nerve, is also strongly undulating. It courses caudally along the caudal edge of the hyocephalic muscle and pierces the thyropharyngeal muscle 689 before entering the larynx with its ventral branch. The strongly undulating lingual 690

branch of the glossopharyngeal nerve, the cranial laryngeal nerve, and the
oesophageal branch of the vagus nerve appear to be enclosed in hose-like connective
tissue sheaths (Fig. 13).

694 Trachea

In both sexes all the tracheal cartilages between the cricoid cartilage and the bronchial cartilages are dorsally separated by a ~ 5 mm wide gap that is bridged by elastic connective tissue i.e. they do not touch dorsally. This membranous wall continues dorsally onto the main bronchi. The number of tracheal cartilages (from cricoid to bifurcation) was 17 in the female and 18 in the male. Both the resting and the maximally extended lengths of the trachea did not differ appreciably between male and female: ~ 40 mm and ~ 70 mm, respectively.

702 Sexual dimorphism of vocal anatomy and acoustic values

In order to evaluate the measured differences of vocal features and acoustic values
between males and females, intersexual body size differences have to be considered
(Tab. 1)

706

## 707 **Discussion**

This study provides the first comprehensive description of the koala's vocal anatomy. Although anatomical peculiarities of the vocal organs and the associated musculature in koalas were noted by the classical morphologists (MacAlister 1872; Young 1882; Symington 1898; Sonntag 1921), they were mostly considered in a taxonomic context. By combining detailed anatomical data obtained using modern imaging and histological techniques, systematic dissections, behavioural observations, and

published acoustic data, we are now able to provide a comprehensive and functional
account of the koala's unusual vocal anatomy.

716 A simple, but descended larynx and a specialised hyoid apparatus

Although most features of the koala larynx correspond to those of other marsupials, a 717 rostral thyroid bulla, generally present in marsupials, is lacking in the koala (Schneider 718 719 1964, p. 9). One of the key differences between the marsupial larynx and the larynx of placental mammals is the characteristic ventral fusion of the thyroid and cricoid 720 cartilages observed in the former. This longitudinal cartilaginous connection is derived 721 and arises postnatally (Symington 1898; Schneider 1964, p. 8, 14). The greatly 722 restricted mobility between both cartilages is likely to explain why the marsupial larynx 723 lacks a cricothyroid muscle (Schneider 1964, p. 26). In placental mammals, the mutual 724 mobility of thyroid and cricoid cartilages and coordinated activities of the thyroarytenoid 725 and cricothyroid muscles represent a major means of tensioning the laryngeal vocal 726 727 folds (LVFs), thereby increasing the range of producible fundamental frequencies (F0) (Titze 1993; Titze et al 2016). As a consequence of the stiffened thyroid/cricoid 728 connection and the lacking cricothyroid muscle in the marsupial larynx, the potential 729 F0 range and control over F0 is either reduced or differently achieved in marsupials. 730

The 50:50 ratio between the intercartilaginous and intermembraneous portions 731 of the koala glottis indicates a large vocal process of the arytenoid cartilage, which 732 733 widely protrudes rostroventrally along the inner laryngeal wall (Schneider 1964, p. 17). In our specimens we found that male LVFs were ~ 10 mm and female LVFs were ~ 8 734 mm long. Considering the LVFs and their predicted F0 frequency of ~ 400 Hz (Charlton 735 736 et al 2013), the LVFs are probably only involved in the production of the higher F0 vocalizations produced by this species. In addition, both male and female koalas the 737 LVFs are rostrally inclined, and dorsally attach to the rostrally directed vocal process. 738

A comparable rostral inclination occurs in wild boar (*Sus scrofa*); however, in this placental species the vocal process is caudally directed (Schneider 1964, p. 64f, fig. 66). The koala larynx does not possess lateral laryngeal ventricles and, as a result, vestibular folds are also lacking (Fig. 12). The lack of a thyroid bulla and lateral laryngeal ventricles means that there are virtually no intra-laryngeal resonance spaces.

744 Interestingly, in the koala, all the dorsal suspensory elements of the hyoid apparatus (except the ceratohyoids) have been evolutionarily transformed into a highly 745 resilient hyoid ligament. Due to the short cartilaginous connection between the 746 thyrohyoid and the larynx and the short and tough thyrohyoid membrane, it is likely that 747 the ventral cartilaginous and osseous parts of the hyoid apparatus, i.e. the 748 ceratohyoids, the basihyoid and the thyrohyoids, are retracted together with the larynx 749 towards the thoracic inlet during call production (see 'Laryngeal mobility, calling 750 posture and the VVFs'). Because the sternohyoid muscles have lost their attachment 751 752 to the hyoid apparatus, they cannot support the sternothyroid muscles in retracting the ventral parts of the hyoid apparatus and larynx towards the sternum (see 'Muscles and 753 nerves of the vocal tract'). 754

#### 755 <u>A novel organ for low frequency production</u>

The most spectacular evolutionary transformation of the koala's vocal organs has 756 occurred in the region of the intra-pharyngeal ostium (IPO). In both sexes, the lateral 757 758 edges of the IPO have been transformed into two large, longitudinally oriented, voluminous folds that protrude ventrally towards the laryngeal entrance and medially 759 towards the midline (cf. McCurry et al 2016 Fig. 6; S-Fig. 3). These extra laryngeal 760 761 folds have previously been termed 'velar vocal folds' or VVFs because they look remarkably similar to large laryngeal vocal folds (Charlton et al 2013). This previous 762 study also confirmed that VVFs function as extra-laryngeal acoustic sources in male 763

koalas (Charlton et al 2013), allowing them to produce the extremely low F0 of bellow
vocalisations (circa 27 Hz). While we confirm here that female koalas also possess
VVFs, in our specimens the male has larger VVFs (Tab. 5), which protrude further into
the laryngopharynx than in the female, indicating that these structures are significantly
more developed in males than in females.

769 Owing to their position and orientation, the VVFs can only function as a sound source during nasal inhalation phases of vocal production. This function requires 770 tensioning and closure of the IPO, which can be described as a 'velar glottis'. When 771 we extended the pharynx in our specimens the IPO remained close to the laryngeal 772 entrance, confirming that the VVFs are ideally located for sound production during 773 nasal inhalations. The VVFs are also heavily wrinkled in their resting (relaxed) state, 774 which might provide the reserve tissue necessary for allowing the dramatic length 775 changes accompanying larynx retraction and tensioning of the soft palate, including 776 777 the VVFs and the closure of the IPO prior to vocal production. Functionally, this would correspond to the tensioning and bringing together of the LVFs by muscle-induced 778 movements of the arytenoid cartilages inside the larynx. 779

780 It is also worth noting that narrowing of the IPO by the VVFs is expected to impede nasal respiration. Therefore, except prior to vocal production, the VVFs should 781 narrow the IPO as little as possible. As the VVFs cannot be pivoted laterally by 782 cartilage-muscle-induced pulling, the change between respiratory and phonatory 783 position must be achieved by a different mechanism. Shrinking of the VVFs after vocal 784 production might be facilitated by their distinct wrinkles in the resting position. 785 786 Additionally, expansion of the dorsal trench and the longitudinal plication of the soft palate, and relaxation of the palatopharyngeal muscle (the constrictor of the IPO) might 787 be involved in keeping the IPO open. These features may also play a role in bellow-788

synchronous pharynx expansion (S-Fig. 2; S-Video 1). Opening and closing of the IPO
might also be regulated by coordinated contractions of the strap muscles (inducing
length changes) and palatopharyngeal muscle (inducing width changes).

Our histological examination reveals that the VVFs are organised into three 792 discrete layers, approximating the structure of placental mammal LVFs (Hirano et al 793 794 1981; Kurita et al 1983; Hirano & Kakita 1985), although on a larger scale. The structure of the VVFs can be interpreted as a body-cover complex, in which the third, 795 deep layer (muscle bundles and connective tissue scaffolding) represents the 'body' 796 and the upper two layers (epithelium and sub-epithelial loosely organised connective 797 tissue) the cover. Hence, the VVFs consist of a robust basal region near their transition 798 to the soft palate and a soft and deformable apical region close to their free edge 799 flanking the IPO. Accordingly, the VVFs, like the LVFs, can be described as a layer-800 structured oscillator (Hirano & Kakita 1985). 801

In addition, we found that male VVFs contain more collagen fibres than female 802 VVFs, and have a large bundle of elastin fibres in the lower portion of the second layer 803 of the VVFs. In humans, significantly higher levels of collagen are found in the male 804 805 vocal fold (Chan et al 2007) and testosterone is known to increase the collagen/elastin ratio of tissue (Fischer & Swain 1980). If we consider that the levels of collagen and 806 elastin could contribute differentially to VVF elasticity, testosterone exposure during 807 808 development, or the dramatic increase in testosterone that is known to occur in male 809 koalas just prior to the breeding season (Mitchell 1990; Allen 2010), could facilitate low frequency, periodic oscillation of the VVFs during call production by increasing the 810 811 stiffness of these structures, allowing males to produce their extremely low F0 bellow vocalisations. 812

## 813 Muscles and nerves of the vocal tract

Several muscles of the koala's throat and ventral neck region deviate considerably 814 from the typical mammalian pattern (cf. Nickel et al 1979). Although this has been 815 noted previously, to our knowledge, no functional explanation has been proposed so 816 far for this unique arrangement in the koala (MacAlister 1872; Young 1882; Sonntag 817 1921, 1922). Recent findings (Charlton et al 2013), along with observations of vocal 818 behaviour, now allow us to propose a functional explanation. We suggest that the 819 unusual, muscular organisation of the koala's throat and ventral neck region facilitates 820 the dynamic movement of the larynx-hyoid complex during production of bellow 821 vocalisations. The main retractor of the larynx and the ventral hyoid parts, the strap-822 823 like sternothyroid muscle, has elongated and extended its origin deep into the thorax. Pronounced contractions of this muscle would produce a strong pull on the larynx and 824 ventral hyoid parts, resulting in the caudal movement of these structures down into the 825 826 thorax. In the koala, unlike other mammals, the caudal movement of the larynx-hyoid complex is not restricted by connections to the tongue and lower jaw because the 827 mylohyoid and hyoglossus muscles are not attached to the hyoid apparatus, and 828 because the genioglossolaryngeus and geniohyoid muscles have been greatly 829 elongated (Fig. 14). 830

We suggest that the return of the larynx and ventral hyoid parts to their resting 831 position is achieved by the resilience of the hyoid ligament and of the pharynx, which 832 become heavily extended during larynx and ventral hyoid retraction, and the constrictor 833 muscles of the pharynx, the hyocephalic muscle, and strongly elongated extrinsic 834 835 tongue muscles. Concomitant shortening of the pharyngeal walls would be supported by contractions of the middle and caudal pharyngeal constrictors and the hyocephalic 836 muscle. all of which are elongated during the extension 837 phase. The 838 genioglossolaryngeus muscle, which extends from the tongue down to the thyrohyoid

membrane and the rostral edge of the thyroid cartilage, would then be able to contract 839 and return the larynx to its resting position (around the 4<sup>th</sup> cervical vertebra). The 840 prominent, and greatly extended strap-like geniohyoid muscle, would additionally 841 support the mobility of the larynx and ventral hyoid parts down and up the ventral neck 842 region. For example, during the retraction phase, i.e. during contraction of the 843 sternothyroid muscle, the undulations of the geniohyoid muscle will be stretched and 844 contraction of the geniohyoid muscle during the protraction phase will restore the 845 undulations. In addition, because the fibres of the hyoglossus muscle diverge and 846 connect to the tendinous intersection between omohyoid, sternohyoid and digastric 847 muscle, it forms an arch around the long geniohyoid muscle. This arrangement 848 ensures, analogous to the retinacula of certain tendons of limb muscles (cf. Clavero et 849 al 2005; Numkarunarunrote et al 2007; Robertson et al 2007), that the geniohyoid 850 851 muscle is kept in the correct position as the larynx is maximally retracted and then returned to its resting position. Protraction of the larynx and ventral hyoid parts will 852 853 further be assisted by contraction of the hyocephalic muscle, which suspends these elements from the skull base (Fig. 14). 854

The three muscles that are connected to each other by a tendinous intersection 855 (digastric, omohyoid and sternohyoid muscles), together with their contralateral 856 counterparts, appear to function as a longitudinal muscular guiding channel, along 857 which the pharynx, the ventral hyoid parts, and the larynx and trachea are able to glide 858 in a rostrocaudal direction during their dynamic down and up movements. As the 859 860 koala's omohyoid and sternohyoid muscles have lost their connection to the hyoid apparatus, they would not be involved in moving the ventral hyoid parts. In contrast, 861 the sternohyoid muscle is likely to be very effective as a lateral guide bar because it 862 863 extends dorsally from the sternum and costal cartilages up to the rostral portion of the

digastric muscle. Strong extension of the head and neck, as observed during bellow 864 865 production, probably facilitates this guiding by stabilising, tensioning, and extending the channel along a straight line from the angle of the lower jaw down into the thorax. 866 867 Rostrocaudal gliding movements of the ventral hyoid parts and the larynx are further facilitated by the double connective tissue layer intercalated between the hypaxial neck 868 musculature and the cervical vertebrae dorsally and the pharynx, larynx and 869 oesophagus ventrally (S-Fig. 7). In addition, because the digastric muscles terminate 870 on the lower jaw, they are likely to act as an anchor for the guiding channel. However, 871 in contrast to laryngeal movements in ruminants with a descended larynx (Frey et al 872 873 2008; 2011; 2012), the short and weak thyrohyoid muscle of koalas cannot assist in returning the larynx to its resting position because the ventral parts of the hyoid 874 apparatus, including the thyrohyoid, descend together with the larynx during the 875 876 retraction phase. Hence, the most parsimonious explanation for the profound evolutionary transformation of the koala's throat and ventral neck musculature is that 877 878 it facilitates the retraction and return of the larynx and ventral parts of the hyoid apparatus during the production of this species' bellow vocalisations. The phylogenetic 879 distance between koalas and ruminants (marsupial vs. placental species) suggests 880 881 independent evolution of a descended and mobile larynx and the respective muscular organisation of these species reinforce this notion. 882

As the muscles involved in extensive rostrocaudal excursions of the larynx elongated and underwent considerable length changes during protraction and retraction of the larynx over evolutionary time, the nerves supplying these muscles had to elongate correspondingly. In order to accommodate the length changes of their target muscles these nerves evolved two different modes of adjusting to shortly and profoundly changing muscle lengths. First, the nerves to the rostral portions of the

ribbon-like strap muscles (M. sternohyoideus, M. sternothyroideus) evolved large, 889 890 caudally sagging loops, which can be stretched during muscle extension (e.g. by head and neck extension, or by strong muscle contraction during larynx retraction). Second, 891 the nerves to the muscles in the region of the pharynx evolved strongly undulating 892 branches, which can also be stretched during extensions of the pharynx. Additionally, 893 the strongly undulating nerve branches evolved hose-like connective tissue sheaths to 894 facilitate stretching and rewinding of the nerves (Fig. 13). Similar undulating nerves 895 have been documented in ruminants with a descended and mobile larynx, e.g. Iberian 896 red deer (Cervus elaphus hispanicus) and goitred gazelle (Gazella subgutturosa) (Frey 897 et al 2011, 2012). 898

# 899 Laryngeal mobility, calling posture and the VVFs

In ruminant species with a descended and mobile larynx (e.g. red deer, fallow deer, Mongolian gazelle, goitred gazelle) the larynx is retracted, thereby elongating the pharynx (the acoustic filter), and the LVFs (the acoustic source) produce the fundamental frequency at exhalation. In koalas the low fundamental frequency is produced by the pharyngeal VVFs, and hence, laryngeal retraction must have a different function.

In species that produce low fundamental frequencies using the LVFs, e.g. 906 elephants (Herbst et al 2012) sound production requires adduction of the arytenoid 907 cartilages, i.e. closure of the glottis, and tensioning of the LVFs by means of intrinsic 908 909 laryngeal muscle contractions. Then, and only then, can sustained periodic oscillations of the LVFs be achieved by an air stream (typically produced by exhalation from the 910 911 lungs). While the structure of the VVFs clearly differs from that of the LVFs, the glottis-912 like cleft in between these novel structures also has to be closed, and tension applied to the VVFs before periodic oscillations can be induced (by an inhalatory airstream in 913 37

koala bellows (Charlton et al 2011b, 2013). Since the VVFs are not directly connected
to any skeletal parts, closure of the 'velar glottis' and tensioning of the VVFs must be
achieved in a quite different way from that of the LVFs.

The koala's VVFs are part of the pharynx. Therefore, to produce a bellow 917 vocalisation both vocal tract elongation and tension on the VVFs have to be achieved 918 919 simultaneously by appropriate adjustment of the pharynx. The lowering of the formants is produced by larynx retraction (as in ruminants with a descended and mobile larynx, 920 for example: Frey et al 2008, 2011, 2012). In contrast to these ruminants, however 921 (see above), the tension on the VVFs that is required to produce a source signal is 922 effected by the same action. Strong extension of the pharynx will tend to bring the 923 median edges of the VVFs together, thereby closing the 'velar glottis'. Simultaneously, 924 strong extension of the pharynx, including the soft palate, will set the VVFs under 925 tension. As such, laryngeal retraction leading to the extension of the pharynx seems to 926 927 be the most likely mechanism for producing low F0 and formants in the koala (Charlton et al 2013). As a consequence of this coupled mode of sound production and formant 928 lowering, and in contrast to ruminants with a descended mobile larynx and an intra-929 laryngeal sound source, production of the lowest frequencies in koalas requires 930 maximal vocal tract extension, i.e. maximal extension of the acoustic filter. At low 931 pharynx tension, the 'velar glottis' would not close and the VVFs would not be 932 sufficiently tensed for producing the very low extra-laryngeal source frequencies. 933

As in male ruminants with a descended larynx, the extension of the pharynx in koalas is supported by a characteristic calling posture involving pronounced head and neck extension (Fig. 1 – for ruminants cf. Frey et al 2008, 2011, 2012). However, in contrast to ruminants, male koalas produce their mating calls in a characteristic twostage posture, gradually changing from pronounced extension (position 1) to distinctive

flexion (position 2) of the head and neck region (Charlton et al, 2011). In the first 939 position, the neck is extended and the head turned upward so that the longitudinal axis 940 of the head is held in parallel to the longitudinal axis of the body (Fig. 1A). In the course 941 of the call, along with numerous vocal in- and exhalations, the head is successively 942 lowered until, as the very low F0 and formants are produced at the end of the call, it 943 reaches the second position (Fig. 1B). Here, the longitudinal axis of the head is either 944 945 held at an obtuse angle relative to the longitudinal axis of the body, or the chin is pulled a bit more ventrally towards the sternum (Charlton 2011b). Presumably, position 1 946 involves pronounced larynx retraction down to the thoracic inlet, thereby effecting 947 948 longitudinal extension of the pharynx. In position 2, the angular position of the head will raise the base of the skull, to which the rostral portion of the pharynx is attached, 949 thereby achieving the utmost of pharynx extension. During previous experiments on 950 951 koala cadavers we found that position 2 was best for setting the VVFs under appropriate tension to produce this species' characteristically low F0 (Charlton et al 952 2013). 953

## 954 Sexual dimorphism of vocal anatomy versus acoustics

955 Using head length as a proxy for overall body size, male Queensland koalas are around 9% larger than females (Ellis & Bercovitch 2011). In the current study we found 956 that male head length (measured from the occipital ridge to the tip of the nasal bone: 957 958 Charlton et al 2011) was around 10% greater than female head length (Tab. 1), which accords well with the findings of Ellis and Bercovitch on 35 male and female koalas 959 (2011), and suggests that our relatively small sample of six males and females was 960 961 representative of the Queensland koala. Male body length was around 4% greater than female body length in our sample (Tab. 1). Accordingly, if we assume that male 962

koalas are between 4-10% larger than females, then the size dimorphism in vocalanatomy is greater than expected (Tab. 1).

When we consider how size dimorphism in vocal anatomy relates to acoustic 965 differences between male and female koalas, we find that the 15-16% longer male 966 vocal tract corresponds extremely well to the documented 16% lower  $\Delta F$  of male 967 koalas when compared to females (Tab. 1) (Charlton et al 2011, Charlton 2015). Since 968 differences in male and female vocal tract length closely track differences in male and 969 female formant spacing, these findings lend further support to the notion that the 970 broadband frequency components that have been identified in previous studies 971 (Charlton et al 2011, Charlton 2015) are indeed formants. The production of the 972 extremely low formant frequencies in male and female bellows remain unexplained. 973 974 We suggest that koalas use the VVFs to simultaneously excite resonances in the oral and nasal vocal tract, including "sub-laryngeal" spaces like the trachea and the main 975 976 bronchi. Future studies that use precise geometrical data to predict the centre frequencies of the oral and nasal vocal tracts (including the sub-laryngeal air spaces) 977 are now required to confirm how koalas produce such low formant frequencies for their 978 size. 979

Because koalas produce the exceptionally low F0 of bellow vocalisations using 980 their VVFs (Charlton et al 2013), we would also expect bellow F0 dimorphism to track 981 VVF dimorphism. We found that male VVFs were 24% longer than female VVFs, yet 982 male bellow mean F0 (of 28.7 Hz) is only 8% lower than the female bellow mean F0 983 (of 31.3 Hz) (Tab. 1). However, much closer correspondence between VVF sexual 984 985 size dimorphism and differences in male and female minimum bellow F0 was found (Tab. 1), illustrating that the minimum producible F0 is ultimately constrained by the 986 length of the oscillating structure generating the sound (in this case the VVFs). 987

Interestingly, male LVFs are 22% longer than females, which is far above the ~4-10% size difference expected from overall body size differences between the sexes. Data on male and female F0 from the same call type that is likely to be produced using the larynx are now required to determine how this large difference between male and female LVF length corresponds to acoustic differences.

993

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### 1183 Tables

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LVFs =	laryngeal vocal f	olds, VVFs = velar v	ocal folds, VTL =	vocal tract lengt	ì
oral and nasal VTL meas	ured from the li	ps and nostrils to th	e glottis, respe	ctively, with the l	arynx in its
resting state, head angle	ed and pharynx	opened. ^ denotes	values taken as	the midpoint bet	ween two
measures. Acoustic valu	es from Charlto	n et al (2011) and Cl	narlton (2015). I	n all cases sexual	dimorphism is
calculated by dividing m	nale over female	e measures.			
	Male (n)	Female (n)	Male	Female	Dimorphism
Anatomy					
Body length (mm)	6	6	685	657	1.04
Head length (mm)	6	6	139	127	1.10
LVFs (mm)	10	9	9.8	8	1.22
VVFs (mm)	10	10	32.5	26.2	1.24
oral VTL	1	1	165	142	1.16
nasal VTL	1	1	187	162	1.15
Acoustics					
Bellow mean F0	20	23	28.7	31.3	0.92
Bellow minimum F0	20	23	9.9	12.3	0.80
	20	23	354	423	0.84

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### 1188 Figure legends

Fig. 1: Two-stage calling posture of an adult male koala while producing a bellow 1189 vocalisation. Initial stretched posture, head, neck, and thorax extended (A); flexed 1190 1191 posture towards the end of the call, head angled, neck and thorax less extended (B). Landmarks used for the red longitudinal head line are the mouth opening and the 1192 visible ear base, which is close to the atlantooccipital articulation. Landmarks used for 1193 the red longitudinal body line are the visible ear base and (less reliable) the assumed 1194 position of the cranial edge of the pelvis. In A the head line is in straight continuation 1195 of the body line (modified from Charlton et al, 2011) 1196

**Fig. 2:** Initial calling posture and full larynx retraction demonstrated by a CT-based 3D reconstruction of the skeleton in an adult male specimen. Prior to CT scanning, the larynx had been externally fixed in a maximally retracted position. Left lateral view.

**Fig. 3:** The nasal cavity of an adult male koala. 3D reconstruction of the skeleton of the upper body; left foreleg, clavicle and shoulder removed; virtual sagittal section of the skull and lower jaw; background: a photo of the same specimen. Tongue, soft palate and hyoid ligament manually reconstructed for full larynx retraction. Left lateral view. Note the presence of a small rostral bone riding on the incisive bone and lending support to the tip of the nose (similar to European wild boar, *Sus scrofa*).

**Fig. 4:** Fully extended pharynx in an adult male koala. Multi Planar Reconstruction (MPR) based on a CT scan of an adult male, in which the larynx had been externally pushed back and fixed to simulate maximal larynx retraction. The ventral parts of the hyoid apparatus and the larynx are located at the thoracic inlet. Note the capacious naso- and oropharynx and the long soft palate. The IPO (indicated by the red ellipse) is also situated far caudally, opposite the laryngeal entrance.

**Fig. 5:** Dissection stage at which full retraction of the ventral hyoid parts and the larynx down into the thoracic inlet had been simulated. Maximal extension of the pharynx; the hyoid ligament, and the genioglossolaryngeal muscle are exposed. Scale bar 10 mm

Fig. 6: The excised soft palate of an adult male koala: dorsal view with part of right lateral wall of nasopharynx (A), and ventral view (B). A prominent feature of the dorsal surface is the narrow median trench passing caudally into the IPO. A prominent feature of the ventral surface is the IPO with the large VVFs protruding into the laryngopharynx. The asterisk marks a steel pin inserted into the right blind space lateral to the VVFs. Scale bar 10 mm

**Fig. 7:** <u>Upper panel</u>: Histological sections of the male VVFs. The cornifying stratified squamous epithelium and the underlying, parallel network of elastic fibres (black) and collagenous fibres (pink) of the upper intermediate layer (A); high density of thicker bundles of collagenous fibres in the lower intermediate layer (B); longitudinally oriented large bundle of elastic fibres, parallel to the free edge of the VVFs in the lower intermediate layer (C); longitudinal muscle fibre bundles of the deep layer near the laterodorsal base of the VVFs (D).

A, B and D: transverse sections, C: sagittal section. A, C and D: Elastica van Gieson
staining, D: Masson/Goldner Trichrome staining. In each subfigure the arrow points
towards the highlighted feature.

1231 Fig. 7: Lower panel: Histological sections of the female VVFs. The cornifying stratified 1232 squamous epithelium and the upper intermediate layer of collagenous and elastic fibres (A); the mostly longitudinally arranged muscle fibre bundles, collagenous and 1233 elastic fibres inside the VVF stand out against the mostly transversely oriented thick 1234 muscular bundles of the soft palate in the region of the VVFs (B); Detail of the deepest 1235 layer of the VVF (C), illustrating the scaffolding of collagenous and elastic fibres 1236 1237 interspersed with muscle fibre bundles and mucinous glands; soft palate immediately caudal to the VVFs containing transversely arranged larger bundles of elastic fibres 1238 1239 (D).

A, D: transverse sections; B, C: sagittal sections. A, B, C, D: Elastica van Gieson staining. In each subfigure the arrow points towards the highlighted feature.

**Fig. 8:** CT-based 3D reconstruction of the hyoid apparatus in an adult male koala. The position of the left hyoid ligament is indicated by the yellow bar. The left clavicle, ribs and shoulder girdle have been removed virtually to expose the *in situ* position of the

hyoid apparatus and larynx. The white asterisk marks the cartilaginous connection
between the hyoid apparatus and the larynx. The black asterisk marks the cartilaginous
longitudinal ventral fusion of thyroid and cricoid cartilages that is typical for marsupials.
The resting position of the larynx is approximately at the level of the cervical vertebrae
4-6.

**Fig. 9:** Superficial muscular triangle covering the ventrolateral neck region, consisting of the two bellies of the digastric muscle rostrally, the sternohyoid muscle ventrally, and the V-shaped omohyoid muscle laterally. A tendinous intersection links these three muscles. Position of lower jaw indicated by white contour. In the koala the omohyoid and sternohyoid muscles have completely lost their connection to the hyoid apparatus. The three muscles are laterally flanking the pharynx, the ventral hyoid parts, the larynx, trachea and oesophagus. Scale bar 10 mm

Fig. 10: Intra-thoracic origin of the sternohyoid and sternothyroid (strap) muscles in a 1257 female (A) and a male (B) adult koala. (A) shows the resting position of the larynx 1258 whereas in (B) the neck has been extended and the larynx pulled caudally (red arrows) 1259 to simulate its retraction down into the thoracic entrance. The origins of the two 1260 muscles extend down to the level of the 3<sup>rd</sup> and 4<sup>th</sup> costal cartilage. The sternohyoid 1261 muscle courses ventrally, contacting the inner thoracic wall, and is covered dorsally by 1262 the sternothyroid muscle. In situ the intrathoracic portions of both muscles are covered 1263 1264 by a sheath of connective tissue. The sternothyroid muscle is the main retractor of the larynx while the sternohyoid muscle, due to its tendinous connection to the digastric 1265 and omohyoid muscles, can guide the extensive up-and-down movements of the 1266 1267 larynx, the ventral hyoid parts and the pharynx during bellow production. Scale bar 10 1268 mm, respectively

Fig. 11: The hyocephalic muscle in an adult female koala. Left lateral view. The 1269 hyocephalic muscle (probably emerging from the fusion of the stylohyoid muscle 1270 rostrally, the caudal stylopharyngeal muscle (middle), and the hyppharyngeal muscle 1271 1272 caudally) originates from the cerato-, basi- and thyrohyoid and rostrodorsally from the thyroid cartilage, and mainly terminates on the base of the skull, caudally adjacent to 1273 the attachment of the hyoid ligament. As a result of this topographical relationships the 1274 1275 hypocephalic muscle can assist in protraction of the larynx subsequent to momentary retraction during bellow production. Scale bar 10 mm 1276

**Fig. 12:** Right half of the larynx of an adult male koala (A) and an adult female koala (B). Medial view. Dissection photo, showing the mucous membrane relief of the laryngeal cavity, including the laryngeal vocal fold (LVF), and the relative positions of the laryngeal cartilages. The thyroid and cricoid cartilages are ventrally fused by a longitudinal cartilaginous keel (A); translucent specimen (B) prepared by using the Spalteholz technique (Spalteholz 1914; Peters 1961; Piechocki 1967). + = rostral horn, o = caudal horn of thyroid cartilage. Scale bars 10 mm, respectively

**Fig. 13:** Undulating nerves supplying the musculature of the pharynx, which is subjected to considerable length changes during extensive rostrocaudal excursions of the ventral hyoid parts and the larynx. The undulating nerve branches are ensheathed by flexible hose-like connective tissue tubes (three red arrows), which facilitate stretching and rewinding. Asterisks mark two steel pins used for separating the nerves. Left lateral view. Scale bar 10 mm

Fig. 14: Reconstruction of the vocal anatomy of an adult male koala in six layers while producing the very low frequencies during the nasal inhalatory sections of the final stage of bellow vocalisations. The larynx is maximally retracted down into the thoracic inlet; the hyoid ligament is maximally extended and the sternothyroid muscle maximally 55

contracted. Left lateral view. Parotidoauricularis muscle terminates on sternal 1294 manubrium (A); Y-shaped tendinous intersection connects digastric, omohyoid and 1295 sternohyoid muscle, the latter with intrathoracic origin (B); the genioglossolaryngeal 1296 muscle terminates on pharynx wall, thyrohyoid and thyrohyoid membrane (C); the 1297 geniohyoid muscle is markedly elongated and kept in place by passing through an arch 1298 formed by the hyoglossus muscle and the rostral branch of the Y-shaped tendinous 1299 intersection; the stylohyoid muscle connects the thyrohyoid to the skull base; the 1300 sternothyroid muscle has an intrathoracic origin (D); the hyopharyngeus muscle and 1301 the caudal constrictors of the pharynx connect the thyrohyoid and the larynx to the 1302 1303 pharynx; first rib removed (E); nasal and oral vocal tract, naso- and oropharynx and soft palate maximally extended; VVFs opposite to the laryngeal entrance; complete 1304 1305 (longitudinally compressed) trachea from the larynx to the bifurcation (F).