

Development of the skin in the eastern quoll (*Dasyurus viverrinus*) with focus on cutaneous gas exchange in the early postnatal period

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Abstract

A morphological and morphometric study of the skin development in the eastern quoll (*Dasyurus viverrinus*) was conducted to follow the transition from cutaneous to pulmonary gas exchange in this extremely immature marsupial species. Additionally, the development of the cardiac and respiratory system was followed, to evaluate the systemic prerequisites allowing for cutaneous respiration. The skin in the newborn *D. viverrinus* was very thin ($36 \pm 3 \mu\text{m}$) and undifferentiated (no hair follicles, no sebaceous and perspiratory glands). Numerous superficial cutaneous capillaries were encountered, closely associated with the epidermis, allowing for gaseous exchange. The capillary volume density was highest in the neonate (0.33 ± 0.04) and decreased markedly during the first 4 days (0.06 ± 0.01). In the same time period, the skin diffusion barrier increased from $9 \pm 1 \mu\text{m}$ to $44 \pm 6 \mu\text{m}$. From this age on the skin development was characterized by thickening of the different cutaneous layers, formation of hair follicles (day 55) and the occurrence of subcutaneous fat (day 19). The heart of the neonate *D. viverrinus* had incomplete interatrial, inter-ventricular, and aortico-pulmonary septa, allowing for the possibility that oxygenated blood from the skin mixes with that of the systemic circulation. The fast-structural changes in the systemic circulations (closing all shunts) in the early postnatal period (3 days) necessitate the transition from cutaneous to pulmonary respiration despite the immaturity of the lungs. At this time, the lung was still at the canalicular stage of lung development, but had to be mature enough to meet the respiratory needs of the growing organism. The morphometric results for the skin development of *D. viverrinus* suggest that cutaneous respiration is most pronounced in neonates and decreases rapidly during the first 3 days of postnatal life. After this time a functional transition of the skin from cutaneous respiration to insulation and protection of the body takes place.

KEYWORDS

cutaneous gas exchange, marsupials, morphology, skin development

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1 | INTRODUCTION

Newborn marsupials and newly hatched monotremes are distinguished from eutherian mammals by their extremely small size and high immaturity at birth. Marsupial neonates are always highly altricial compared to eutherian neonates and most of their growth and development occur postnatally, supported by a prolonged lactation period (Renfree, 2006; Ferner and Mess, 2011; Ferner *et al.*, 2017). Corresponding to the altricial-precocial spectrum of eutherians, also marsupial neonates can be sorted into three grades of developmental complexity (G1, G2, G3). These grades are based on size variation and the developmental degree of their organ systems (Hughes and Hall, 1988): G1 (Dasyuridae) being the least developed, G2 (Peramelidae, Phalangeridae, Didelphidae) being intermediate developed and G3 (Macropodidae, Phascolarctidae) being the most developed marsupial neonates.

The eastern quoll (*Dasyurus viverrinus*) at birth it is one of the smallest newborn mammals known. After a gestation of 19 days, the newborn is 5.5 mm long and weighs ~12.5 mg (Hill and Hill, 1955; Tyndale-Biscoe and Renfree, 1987). The skeleton is entirely cartilaginous and the internal organs are visible through the transparent skin. The lungs are represented by a small number of air sacs of spherical shape on either side of the heart.

Increasing evidence suggests that the immaturity of the respiratory system of dasyurid neonates necessitates recruitment of an alternative organ system such as the skin for gas exchange at least for a restricted time period (Frappell and Mortola, 2000; Frappell and MacFarlane, 2006; Ferner, 2018).

In the neonates of two dasyurid species, the fat-tailed dunnart (*Sminthopsis crassicaudata*; Frappell and MacFarlane, 2006; Simpson *et al.*, 2011) and Julia creek dunnart (*Sminthopsis douglasi*; Frappell and Mortola, 2000), no thoracic movement and pulmonary ventilation was observed. The gas exchange has been conducted solely via cutaneous respiration for a number of days and the onset of pulmonary ventilation was delayed in these species (Mortola *et al.*, 1999).

But even in comparatively well-developed marsupial neonates, such as the tammar wallaby (*Macropus eugenii*), gas exchange through the skin accounts for 33% of the total oxygen uptake and is therefore an important complement to pulmonary ventilation (MacFarlane and Frappell, 2001; MacFarlane *et al.*, 2002). Emerging from these physiological studies, the role of cutaneous respiration for newborn marsupials, particularly in dasyurids, seems to be highly important. For this reason, it is necessary to examine the morphological basis for cutaneous gas exchange.

Earlier morphological studies of skin development in marsupials (Henrikson, 1969; Lyne, 1970; Lyne *et al.*, 1970; Krause *et al.*, 1978; Pralomkarn *et al.*, 1990) neglected parameters important for cutaneous respiration (capillary density or air-blood diffusion distances). Studies to the gas transfer of pouch young tammar wallabies largely dismissed the possibility that the skin may be an accessory, or a major site of gas exchange in marsupial pouch young (Randall *et al.*, 1984; Baudinette *et al.*, 1988). The comparison to the lung revealed that the skin had a poor vascularity and greater blood-air diffusion

distance. However, these studies used 3 days old young of a marsupial species where the neonates are relatively large (*M. eugenii*, 370 mg). A study on another macropodid species, the newborn quokka wallaby (*Setonix brachyurus*, 350 mg), reported a skin diffusion barrier between 30 and 35 μm and suggested the possibility that the skin participates in gaseous exchange (Makanya *et al.*, 2007). Only one recent study investigated the skin structure of neonates of several marsupial species with respect to diffusion distance and capillary density (Ferner, 2018). The thickness of the entire skin and its different layers varied among the marsupial species and reflected the differences in size and developmental degree of the neonates (G1-G3). In the skin of all marsupial neonates and a monotreme hatchling, numerous superficial cutaneous capillaries were encountered, but the highest capillary volume density and thinnest skin diffusion barrier of all examined marsupial neonates were found in the skin of the newborn *D. viverrinus*. This suggests that skin gas exchange might be most pronounced in the dasyurid neonate.

Following the physiological studies on other dasyurid species (Mortola *et al.*, 1999; Frappell and Mortola, 2000; Frappell and MacFarlane, 2006), cutaneous respiration in the newborn *D. viverrinus* might be crucial for survival. Gas exchange through the skin seems not simply an option, but a requirement because of the inefficient pulmonary system of newborn dasyurids. This raises the question of how long cutaneous respiration might persist during the postnatal development of *D. viverrinus* before pulmonary gas exchange takes over entirely.

The current study focuses on the structural development of the skin of *D. viverrinus* during the postnatal period and investigates also structural changes in the circulatory system related to the transition from cutaneous to pulmonary gas exchange.

2 | MATERIAL AND METHODS

2.1 | Animal provenance

Skin development was examined in the eastern quoll (*D. viverrinus*), a carnivorous marsupial that was formerly found across much of southeastern mainland Australia, where it became extinct in 1960 s, but it remains widespread in Tasmania and is recently reintroduced on the mainland (Burbidge and Woinarski, 2016). Within Tasmania, eastern quolls prefer dry grassland and forest mosaics, bounded by agricultural land (Jones and Barmuta, 2000). The average body weight of an adult female is about 1350 gm. The gestation period is approximately 19 days. The female has six teats and since the number of young born exceeds this number (up to 20 will be born), all become occupied after parturition and a litter size of 6 results (Hill and Hill, 1955; Tyndale-Biscoe and Renfree, 1987; Van Dyck and Strahan, 2008). The young remain attached to the teats in a quite rudimentary pouch for 49–56 days, begin to develop fur at around 51 days, open their eyes at about 79 days, and are fully weaned at 135–140 days (Tyndale-Biscoe and Renfree, 1987; Jones and Rose, 2001).

2.2 | Tissues

The samples of *D. viverrinus* were available from the Hubrecht & Hill collection, which is a part of the embryological collection of the Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung, Berlin. These specimens included serially sectioned as well as whole-mount fixed samples.

All samples used for the morphological analysis of the skin structure had been prepared for light microscope studies before (e.g., serially sectioned, transferred to glass slides, stained) and were already available for light microscopic investigation. The specimens from the Hubrecht & Hill collection were collected around 1900 in Australia (between 1897 and 1906) from James Peter Hill (Klima and Bangma, 1987). The exact date of sampling, age, crown-rump length (CRL), and head length (HL) of the specimen, as well as information about staining and section thickness was documented for most specimens. In addition, a staging system for the development of *D. viverrinus* (stage A-M) was provided by James Peter Hill. The number of animals investigated per age stage varied depending on the availability in the Hubrecht & Hill collection. The specifics and numbers of the specimens used for light microscopy and morphological description are summarized in Table 1.

The process of preservation is unknown, but it is most probable that the material was fixed with Bouin's solution and preserved in 70% ethanol as state of the art at that time. The fixative Bouin's solution (picric acid, acetic acid and formaldehyde in an aqueous solution) and the entire embedding process in paraffin can cause some shrinking of the material (~30%, see Ross, 1953). Considering the unavoidable shrinking during the processing of the material, the obtained absolute values might be underestimated, but comparable to each other due to the same treatment of the material. However, the morphometric measurements of capillary density are volume fractions and, thus, unaffected by volume changes.

2.3 | Measurements and skin morphometry

Serial histological sections were investigated by light microscopy using a stereomicroscope (Leica MZ 12; Wildt, Switzerland). Subcutaneous capillaries were present in the skin of the ventral, the dorsal, and the lateral sides of the trunk (Figure 1). A preceding study suggested the highest volume density of cutaneous capillaries ($V_{vc} = 0.33 \pm 0.05$) in the dorsolateral side of the trunk (Ferner, 2018). The volume density of cutaneous capillaries was lower in the ventral side of the trunk ($V_{vc} = 0.14 \pm 0.02$). Considering the lack of a pouch, the curled-up position of the neonates, and the close proximity to litter mates, the dorsolateral side of the trunk is the only body region freely exposed to the air and seems to be the most important site for cutaneous gas exchange. Therefore, the measurements of the skin were conducted on the dorsolateral side of the trunk.

To ensure that selected tissue samples represent the whole, all parts of the dorsolateral side of the trunk should have equal

probability of being sampled (Hsia *et al.*, 2010). This requirement is met by introducing randomness into the sampling process. For this reason, the Fractionator method was applied. This is a comprehensive sampling approach that is based on serial sectioning through the entire specimen (already done with the specimens from the Hubrecht & Hill collection), followed by systematic selection of a known fraction of the whole. Since the section thickness (usually 10 μm) was known, the total number of sections resulted in the total length of the specimen. The total length divided by 10 gave the sampling thickness for the fractionator. Ten sections per specimen were selected for measurement. In order to choose arbitrary positions for measurement on the dorsolateral side of the trunk, an orientator clock was applied (Howard and Reed, 2005). As a result, 10 selected skin sections of each specimen were captured with a magnification of 200 \times on a light microscope (Zeiss Axiokop; Carl Zeiss Microscopy GmbH, Germany) equipped with a digital camera (Leica DFC490; Leica Microsystems, Switzerland Ltd.), connected to a computer (software LAS V4.2; Leica Microsystems, Switzerland Ltd.). Assessment of total skin thickness and of its different components (dermis, epidermis, stratum corneum, periderm) as well as the distance between the external surface and most superficial capillaries were performed at five different points on the image (Fractionator principle), yielding 50 measurements per specimen. Measurements were made directly on the computer screen using a digital ruler (Image J software; National Institutes of Health, USA). The results of the measured slides have been averaged for each animal and the mean and SE were presented. If more than one specimen per age stage were measured, additional group means for the age stage (bold) are given (Table 2).

Using LM morphometry, the volume densities of capillaries (V_{vc}) in the 10 selected skin sections were obtained by point-counting methods (Howard and Reed, 2005), following the equation:

$$V_{vc} = P_c / P_{tot}$$

where P_c and P_{tot} are point counts on capillaries and total number of points on the skin tissue. The sample size per specimen was 150–200 point counts on each of the 10 selected skin images. The resulting 10 volume densities were averaged for each animal, the mean and SE were presented and again group means for age stages presented (Table 2).

3 | RESULTS

3.1 | General morphological development

Body size, body weight, and external morphology of the developing young *D. viverrinus* provide important information for evaluating properties for cutaneous gas exchange. The morphological development from neonate to day 60 is presented in Figure 2 and Table 1.

The crown-rump length (CRL) of the newborn *D. viverrinus* was 5.5 mm, and body weight was about 0.01 g. The external morphology of the newborn *D. viverrinus* (Figure 2a) was influenced

TABLE 1 List of Eastern quoll (*Dasyurus viverrinus*) specimens from the Hubrecht & Hill collection examined in this study

Age (remark)	No.	CRL (mm)	Stage	Section	Staining
(a) Sectioned specimens					
Neonate (newborn)	MS 134a	5.50	A	10 μ m, transversal	H&E
	MS 134b	5.50	A	10 μ m, transversal	H&E
(4–12 hours old)	MS 138a	5.45	B	10 μ m, longitudinal	H & E
(4–12 hours old)	MS 138b	5.45	B	10 μ m, transversal	H & E
2 dpn (26–30 hours old)	MS 142a	6.00	C	10 μ m, transversal	H & E
(26–30 hours old)	MS 142b	6.00	C	10 μ m, longitudinal	H & E
(26–30 hours old)	MS 142c	6.00	C	10 μ m, transversal	H & E
3 dpn	MS 146	6.50–7.25	between C + D	10 μ m, transversal	H & E
	MS 147	7.00	D	10 μ m, longitudinal	H & E
(3 days old)	MS148a	7.00	D	10 μ m, transversal	H & E
(3 days old)	MS 148e	7.00	D	10 μ m, transversal	H & E
4 dpn	MS 149a	8.00	E	10 μ m, transversal	H & E
	MS 150a	8.00	E	10 μ m, transversal	H & E
	MS151b	8.00	E	10 μ m, transversal	H & E
5 dpn	MS152a	9.00	between E + F	10 μ m, transversal	H & E
	MS152b	9.00	between E + F	10 μ m, transversal	H & E
	MS154b	8.50–9.00	F	longitudinal	H & E
6 dpn	MS156	9.50	F	transversal	H & E
	MS157	9.50–10.00	F	transversal	H & E
	MS161	10.00	F	6 μ m, transversal	H & E
7 dpn	MS158a	10.00	G	10 μ m, longitudinal	H & E
	MS158b	10.00	G	10 μ m, transversal	H & E
	MS159	10–10.50	G	10 μ m, transversal	H & E
	MS160	11.00	G	10 μ m, transversal	H & E
12 dpn	MS164a	13.50	H	longitudinal	H & E
	MS164b	13.50	H	transversal	H & E
	MS164c	13.50	H	longitudinal	H & E
	MS165	13.00	H	12 μ m, transversal	Zenker
16 dpn	MS169	15.00	I	15 μ m, transversal	H & E
	MS173	15.50	I	longitudinal	H & E
19 dpn	MS172a	17.00	I	transversal	H & E
(19 days old)	MS172b	16.50	I	transversal	H & E
25 dpn	MS174	19.00	between I + J	16 μ m, transversal	H & E
	MS175		about J	12 μ m, transversal	H & E
(25 days old)	MS176	20.00	J	transversal	H & E
29 dpn	MS177	23.00		transversal	H & E
35 dpn	MS180a	27.00	K	10 μ m, transversal	H & E
	MS180b	27.00	K	10 μ m, transversal	H & E
41 dpn (41 days old)	MS179		K	10 μ m, transversal	H & E
52 dpn	MS181	45.00–48.00	just over L	transversal	H & E
55 dpn (2 months old)	MS182		M	transversal	H & E

(Continues)

TABLE 1 (Continued)

Age (remark)	No.	CRL (mm)	Head length (mm)	Body weight (g)	Stage
(b) Specimens in alcohol					
Neonate	MA746d	5.50		0.01	A
2 dpn (36 hours old)	MA724	6.0	2.75	0.02	C
	MA746a	6.0		0.02	
3 dpn (48 hours old)	MA747a	6.75		0.05	
	MA744a	7.00	3.00	0.05	D
5 dpn (5–6 days old)	MA733	8.00	4.50	0.07	E
	MA744	8.00	4.50	0.07	E
7 dpn (7 days old)	MA721	9.50	5.50	0.12	F
	MA754	10–10.50	6.50	0.13	G
10 dpn	MA741	11.00	7.00	0.19	G
14 dpn (14 days old)	MA738	13.00	8.00	0.32	H
	MA731	13.75	8.00	0.35	H
19 dpn (19 days old)	MA745	16.50	10.00	0.60	I
	MA753	15.50	10.00	0.58	I
25 dpn (25 days old)	MA739	20.00	12.50	1.05	J
	MA750	24.00	13.50	1.45	J
35 dpn (35 days old)	MA734	26–28.00	16.50	2.50	
41 dpn (41 days old)	MA732	27–28	18.00	2.92	K
46 dpn (46 days old)	MA751	42.00	21.00	5.29	L
60 dpn (60 days old)	MA726	48.00	23.00	5.60	M
	MA727	48.00	22.50	5.42	

dpn, days post natum.

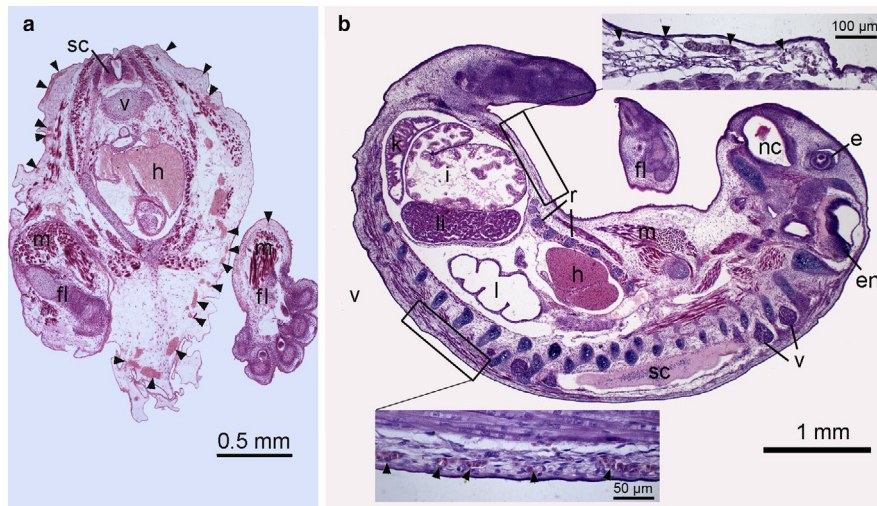


FIGURE 1 Light micrographs of a transversal and a longitudinal histological section through a newborn (a) and 2-day-old (b) *Dasyurus viverrinus*. Numerous superficial capillaries (arrowheads) were closely associated with the epidermis, providing an extremely thin diffusion barrier. Cutaneous capillaries were present in the dorsal, lateral, and ventral sides; however, capillary density was highest in the skin of the dorsolateral side of the trunk (b, insets). The general developmental degree of *D. viverrinus* was highly immature at birth, showing cartilaginous skeletal elements, lungs with only few partitions of the spherical sacs, and poor development of the gut and associated organs (i.e., liver, pancreas, mesonephros). Remarkable was the pronounced para-axial musculature, which enables crawling movement toward the maternal teat immediately after birth. e, eye primordium; en, encephalon; fl, fore limbs; h, heart; i, intestine; k, kidney; l, lung; m, musculature; nc, nasal cavity; r, rib; sc, spinal cord; v, vertebrae. Magnification is indicated by the scale bar

TABLE 2 Cutaneous morphometric data of *Dasyurus viverrinus* during the postnatal period. All values are means ± SE. For species with more than one specimen investigated, additional group means are presented

	Specimen no.	Entire Skin thickness, μm	Dermal thickness, μm	Epidermal thickness, μm	Stratum corneum thickness, μm	Periderm thickness, μm	Diffusion distance, μm	Volume density of capillaries in the skin, Vvc
Neonate	MS134a	44 ± 1	39 ± 1	5 ± 0	2 ± 0	1 ± 0	7 ± 0	0.44 ± 0.04
	MS134b	36 ± 1	30 ± 1	6 ± 0	2 ± 0	1 ± 0	10 ± 0	0.29 ± 0.02
	MS 138a	33 ± 1	27 ± 1	6 ± 0	2 ± 0	1 ± 0	9 ± 0	0.26 ± 0.03
	MS 138b	31 ± 1	24 ± 1	7 ± 0	2 ± 0	1 ± 0	10 ± 1	0.33 ± 0.05
	Mean	36 ± 3	30 ± 3	6 ± 0	2 ± 0	1 ± 0	9 ± 1	0.33 ± 0.04
2 dpn	MS142a	52 ± 2	42 ± 2	10 ± 0	3 ± 0	2 ± 0	19 ± 1	0.25 ± 0.03
	MS142b	45 ± 1	37 ± 1	8 ± 0	2 ± 0	2 ± 0	14 ± 1	0.15 ± 0.01
	MS142c	47 ± 1	31 ± 1	16 ± 0	5 ± 0	2 ± 0	31 ± 1	0.12 ± 0.01
	Mean	48 ± 2	37 ± 4	11 ± 2	3 ± 1	2 ± 0	21 ± 5	0.17 ± 0.04
	MS146	38 ± 2	26 ± 2	12 ± 0	4 ± 0	2 ± 0	19 ± 2	0.17 ± 0.02
3 dpn	MS147	46 ± 1	29 ± 1	17 ± 1	6 ± 0	2 ± 0	21 ± 1	0.11 ± 0.02
	MS148a	72 ± 1	49 ± 1	23 ± 0	7 ± 0	2 ± 0	57 ± 1	0.09 ± 0.01
	MS148e	59 ± 1	44 ± 1	15 ± 0	3 ± 0	1 ± 0	43 ± 1	0.09 ± 0.02
	Mean	54 ± 8	37 ± 6	17 ± 2	5 ± 1	2 ± 0	35 ± 9	0.12 ± 0.02
	MS149a	85 ± 2	59 ± 2	26 ± 1	5 ± 0	2 ± 0	54 ± 2	0.05 ± 0.02
4 dpn	MS150a	64 ± 1	45 ± 1	19 ± 0	7 ± 0	1 ± 0	33 ± 1	0.06 ± 0.01
	MS151b	85 ± 2	66 ± 2	19 ± 0	5 ± 0	1 ± 0	45 ± 2	0.07 ± 0.01
	Mean	78 ± 7	57 ± 6	21 ± 2	5 ± 1	1 ± 0	44 ± 6	0.06 ± 0.01
	MS152a	90 ± 1	64 ± 1	26 ± 0	5 ± 0	1 ± 0	51 ± 2	0.08 ± 0.01
	MS152b	119 ± 2	91 ± 2	28 ± 1	7 ± 0	-	79 ± 3	0.07 ± 0.01
5 dpn	MS154b	82 ± 1	58 ± 1	24 ± 0	5 ± 0	-	47 ± 2	0.05 ± 0.01
	Mean	97 ± 11	71 ± 10	26 ± 1	6 ± 1	-	59 ± 10	0.06 ± 0.01
	MS156	97 ± 2	76 ± 1	21 ± 1	5 ± 0	-	52 ± 1	0.03 ± 0.00
	MS157	100 ± 1	61 ± 1	39 ± 1	6 ± 0	-	74 ± 2	0.05 ± 0.01
	MS161	112 ± 2	88 ± 2	24 ± 1	5 ± 0	-	87 ± 3	0.04 ± 0.01
6 dpn	Mean	103 ± 4	75 ± 8	28 ± 5	6 ± 0	-	71 ± 10	0.04 ± 0.01
	MS158a	129 ± 4	106 ± 4	23 ± 1	6 ± 0	-	91 ± 3	0.06 ± 0.01
	MS158b	121 ± 3	88 ± 2	33 ± 1	5 ± 0	-	87 ± 3	0.03 ± 0.00
	MS159	112 ± 4	69 ± 3	43 ± 2	9 ± 0	-	87 ± 3	0.02 ± 0.00
	MS160	110 ± 3	84 ± 3	26 ± 0	7 ± 0	-	79 ± 3	0.06 ± 0.01
7 dpn	Mean	118 ± 5	87 ± 7	31 ± 4	7 ± 1	-	86 ± 3	0.05 ± 0.01

(Continues)

TABLE 2 (Continued)

	Specimen no.	Entire Skin thickness, μm	Dermal thickness, μm	Epidermal thickness, μm	Stratum corneum thickness, μm	Periderm thickness, μm	Diffusion distance, μm	Volume density of capillaries in the skin, V_{vc}
12 dpn	MS164a	165 \pm 4	131 \pm 4	34 \pm 1	8 \pm 0	-	89 \pm 2	0.02 \pm 0.00
	MS164b	157 \pm 2	118 \pm 2	39 \pm 1	9 \pm 0	-	127 \pm 3	0.01 \pm 0.00
	MS164c	118 \pm 3	91 \pm 2	27 \pm 1	6 \pm 0	-	73 \pm 3	0.03 \pm 0.00
	MS165	118 \pm 3	90 \pm 3	28 \pm 1	7 \pm 0	-	84 \pm 3	0.04 \pm 0.01
	Mean	140 \pm 12	108 \pm 9	32 \pm 3	7 \pm 1	-	93 \pm 12	0.02 \pm 0.01
16 dpn	MS169	119 \pm 3	89 \pm 3	30 \pm 1	6 \pm 0	-	81 \pm 2	0.02 \pm 0.00
	MS173	139 \pm 2	101 \pm 1	38 \pm 1	8 \pm 0	-	85 \pm 2	0.02 \pm 0.00
	Mean	129 \pm 10	95 \pm 7	34 \pm 4	7 \pm 1	-	83 \pm 2	0.02 \pm 0.00
19 dpn	MS172a	134 \pm 2	103 \pm 2	31 \pm 1	7 \pm 0	-	94 \pm 2	0.02 \pm 0.00
	MS172b	155 \pm 4	128 \pm 4	27 \pm 1	7 \pm 0	-	105 \pm 3	0.02 \pm 0.00
	Mean	145 \pm 11	116 \pm 11	29 \pm 2	7 \pm 0	-	99 \pm 6	0.02 \pm 0.00
25 dpn	MS174	151 \pm 1	121 \pm 1	30 \pm 0	8 \pm 0	-	99 \pm 2	0.02 \pm 0.00
	MS175	120 \pm 2	99 \pm 2	21 \pm 0	9 \pm 0	-	97 \pm 3	0.02 \pm 0.00
	MS176	157 \pm 3	128 \pm 3	29 \pm 1	8 \pm 0	-	122 \pm 3	0.02 \pm 0.00
	Mean	143 \pm 11	116 \pm 9	27 \pm 3	8 \pm 0	-	106 \pm 8	0.02 \pm 0.00
29 dpn	MS177	156 \pm 3	120 \pm 3	36 \pm 1	7 \pm 0	-	-	-
35 dpn	MS180a	176 \pm 4	145 \pm 5	31 \pm 1	5 \pm 0	-	105 \pm 5	0.02 \pm 0.00
	MS180b	170 \pm 3	125 \pm 3	45 \pm 1	6 \pm 0	-	124 \pm 4	0.02 \pm 0.00
	Mean	173 \pm 3	135 \pm 10	38 \pm 3	5 \pm 1	-	114 \pm 9	0.02 \pm 0.00
	MS179	178 \pm 3	124 \pm 3	54 \pm 2	6 \pm 0	-	139 \pm 3	0.02 \pm 0.00
52 dpn	MS181	260 \pm 2	212 \pm 3	48 \pm 1	12 \pm 0	-	166 \pm 5	0.02 \pm 0.00
55 dpn	MS182	284 \pm 9	234 \pm 9	50 \pm 1	10 \pm 1	-	172 \pm 7	0.02 \pm 0.00

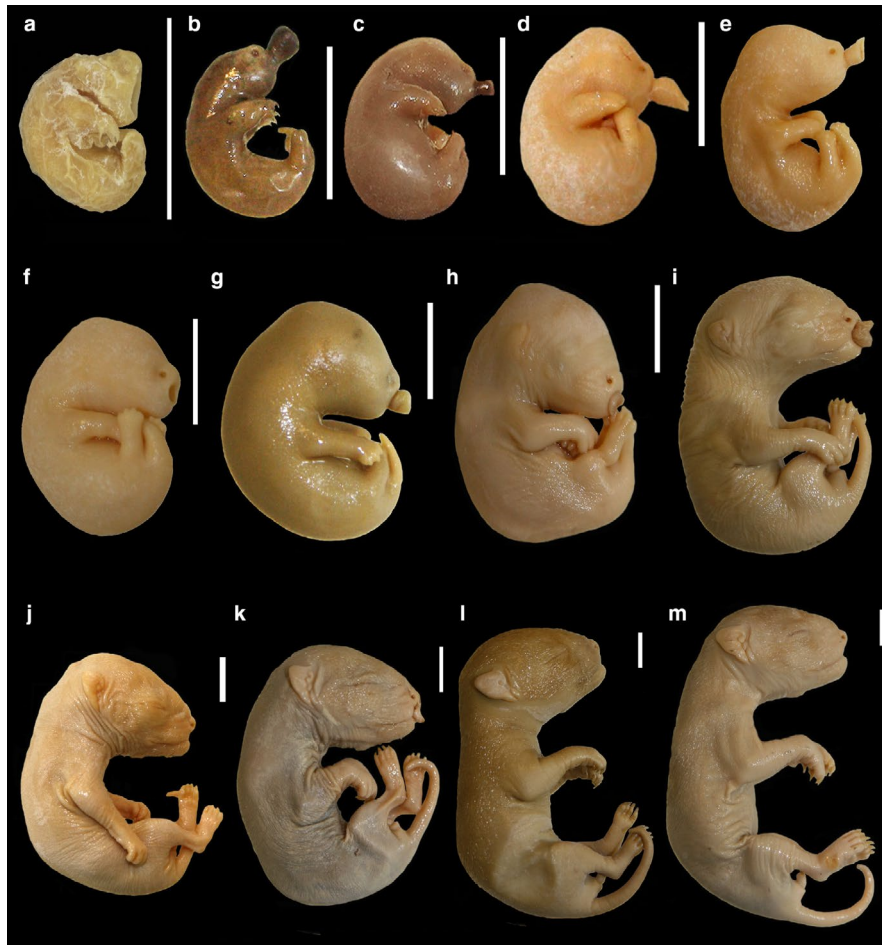


FIGURE 2 Postnatal development of *Dasyurus viverrinus*. Macrographs reflect the morphological transformation from neonate to day 60: neonate 0- to 12-hour-old (a), 2 dpn 26- to 30-hour-old (b), 3 dpn 48-hour-old (c), 5 dpn (d), 7 dpn (e), 10 dpn (f), 14 dpn (g), 19 dpn (h), 25 dpn (i), 35 dpn (j), 41 dpn (k), 46 dpn (l) and 60 dpn (m). The highly immature neonate (a) had strongly developed forelimbs and rudimentary hindlimbs, a massive oral shield, large prominent nostrils, eye and ear primordia were barely visible and a definitive neck was missing. During the first week the head was forming and by day 7 a definitive neck was visible; the nasal swelling was moderate and a simple oral shield was present. Around day 10 the hind limbs started to differentiate and digits became recognizable. A clear developmental progress was observed between days 14 and 19, where ear and eye primordia and the area of the later mystacial vibrissae became visible. The morphological transformation of fore- and hindlimbs started about this time. By day 25 a lateral lip line, eyelids, and auricle became visible, but were still fused. Pinnae became free from the head around day 35. First hairs erupted on the head around day 35, reaching the middle back by day 46. With day 60 the jaws started separating. Scale bar = 0.5 cm. Details to the specimens are provided in Table 1b

by the development of internal structures. The lungs were disproportionately small and the marked caudal taper of the body was associated with poor development of the gut and associated organs (i.e., liver, mesonephros) (Figure 1). The volume of the viscera of the torso was small in relation to overall skeleton-muscular components. The highly immature neonate of *D. viverrinus* had strong developed forelimbs and undifferentiated hindlimb paddles (Figure 2a). A definitive neck was missing and the mouth was lying deep within the massive oral shield. The medial nasal swelling contributed to a large prominent nostril. The eye primordium lacked retinal pigmentation and the ear primordium was barely discernable. During the first week, the head was forming and by day 7 a definitive neck was visible; the nasal swelling was moderate and a simple oral shield was present (Figure 2e). By day

7, the CRL measured 10 mm, and body weight was about 0.12 g. The differentiation of the hind limbs started around day 10 and digits became recognizable (Figure 2f). The facial development progressed rapidly between day 14 and 19. Ear and eye primordia and the area of the later mystacial vibrissae became visible (Figure 2h). The morphological transformation of fore- and hindlimbs started by this time as well. By day 25, a lateral lip line, eyelids, and auricle were discernable (Figure 2i). By this time the CRL measured 20–24 mm, and body weight was about 1–1.5 g. Pinnae were free from the head around day 35 (Figure 2j). First hairs erupted on the head around day 35, reaching the middle back by day 46 (Figure 2l). With day 60, the jaws started separating. By this time the CRL measured 48 mm and body weight was ~5.5 g. (Figure 2m).

3.2 | Postnatal development of the skin

The skin at the dorsolateral side of the newborn *D. viverrinus* measured $36 \pm 3 \mu\text{m}$. It was composed of a thin layer of epidermis ($6 \pm 0 \mu\text{m}$) in close contact with the capillary bed located on a layer of dermis ($30 \pm 3 \mu\text{m}$) (Figure 3a). The epidermis consisted of one to two layers of squamous cells followed by two layers of flattened partially keratinized cells, the stratum corneum ($2 \pm 0 \mu\text{m}$, darker staining), and the outermost surface layer, the periderm ($1 \pm 0 \mu\text{m}$, lighter staining; Figure 3a, right side with higher magnification), that formed together a double membrane. The dermis accounted for the largest part of the skin and consisted mainly of connective tissue and several capillaries. Hairs and hair follicles were completely absent in the skin of the newborn *D. viverrinus* and sebaceous and perspiratory glands were missing as well. Numerous superficial capillaries were encountered close to the epidermis and the capillary volume density (0.33 ± 0.04) was high (Figures 1a and 3a). A survey of the whole trunk of newborn *D. viverrinus* revealed that subcutaneous capillaries were not evenly distributed over the entire body (for detail see Section 2.3). Although cutaneous capillaries were present at the ventral side of the trunk as well (Figure 1a),

the capillary density was highest on the dorsolateral side of the trunk. The distance between the external surface of the skin and the most superficial capillaries (skin diffusion barrier) in the dorsolateral skin of the newborn *D. viverrinus* was very low and measured only $9 \pm 1 \mu\text{m}$.

In the consecutive 5 days (Figure 3b-e), the skin thickness increased from $48 \pm 2 \mu\text{m}$ to $97 \pm 11 \mu\text{m}$, due to an increase in dermal (37 ± 4 to $71 \pm 10 \mu\text{m}$) and epidermal (11 ± 2 to $26 \pm 1 \mu\text{m}$) layers. The periderm was discernible up to day 4, after that time the stratum corneum formed the outermost layer of the epidermis. At day 2, cutaneous capillaries were still in close proximity of the epidermis (Figure 3b). However, the diffusion distance increased to $21 \pm 5 \mu\text{m}$ and the capillary density decreased to 0.17 ± 0.04 . By day 3 (Figure 3c), the diffusion distance increased furthermore to $35 \pm 9 \mu\text{m}$. This resulted mainly from newly formed connective tissue layers in the dermis between the capillaries and the thickening epidermal layer. The capillary density decreased further to 0.12 ± 0.02 . From day 4 (Figure 3d), the capillary density was reduced to 0.06 ± 0.01 and remained on a low level until the end of the first week (0.05 ± 0.01 at 7 dpn). By day 5 postpartum, the layers of cells forming the epidermis were increased to approximately 7, a stratum basale with one layer of amorphous cells, a stratum spinosum composed

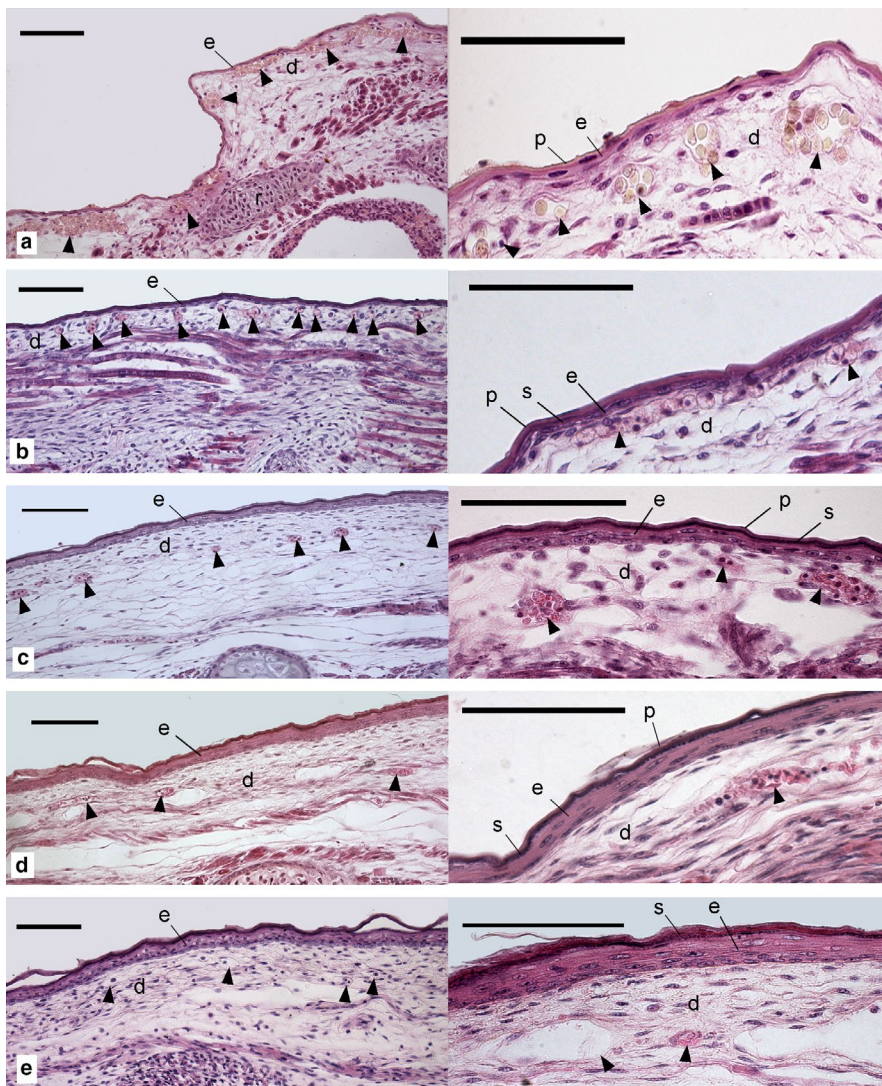


FIGURE 3 Histological sections comparing the structural skin development of *Dasyurus viverrinus* in the early postnatal period: neonate (a), 2 dpn (b), 3 dpn (c), 4 dpn (d), and 5 dpn (e). In the newborn and 2-day-old *D. viverrinus* capillaries (arrowheads) were numerous and closely associated with the epidermis. A lower capillary density and increasing diffusion distance became noticeable with progressive age. Up to the age of 4 days, the outer layer of the epidermis was formed by the periderm. An additional thin layer of keratin (stratum corneum) was visible between the periderm and the basal region of the epidermis. Hair follicles were completely absent. d, dermis; e, epidermis; r, rib; s, stratum corneum; p, periderm. Scale bar = $100 \mu\text{m}$. Magnification: $\times 200$ (left photograph), $\times 400$ (right photograph)

of approximately four layers of flattened cells, and a stratum corneum formed by keratinized cells were distinguishable (Figure 3e). The epidermis started to show the various layers present in the mature eastern quoll. With increasing thickness of the dermal and epidermal layers also the diffusion distance between the external surface and most superficial capillaries increased to $59 \pm 10 \mu\text{m}$. By days 6 and 7 postpartum (Figure 4a,b), the dermal layer showed a further increase in thickness by formation of additional connective tissue. The increase in thickness of the epidermis is primarily due to an increase in the thickness of the spinous layer. As a result, the diffusion distance between capillaries and skin surface increased and cutaneous capillaries were located mostly in the proximal part of the dermis. By day 12, follicle primordia (epithelial placodes), the first signs of hair formation were observed (Figure 4c). Cutaneous capillaries became rare and were found only in the proximal part of the dermis. From this time the value of capillary density stabilized at a value of 0.02 ± 0.00 (see Table 2). The later postnatal skin development in *D. viverrinus* from day 16 to day 55 postpartum (Figure 4d-j) was characterized by a further increase in total skin thickness (from 129 ± 10 to $284 \pm 9 \mu\text{m}$) resulting from the thickening of the dermal (from 95 ± 7 to $234 \pm 9 \mu\text{m}$) and epithelial (from 34 ± 4 to

$50 \pm 1 \mu\text{m}$) layers. By day 19, a subcutaneous layer of lipid cells (adipose tissue) was observed (Figure 4e). First hair follicles were present by day 25 (Figure 4f). The formation of hair follicles progressed and mature hair follicles with cells of sebaceous glands were seen by day 55 (Figure 4j). The measurements and morphometric results for the skin development of *D. viverrinus* in the postnatal period are summarized in Table 2.

3.3 | Structural development of the cardiac and respiratory system

In the neonate *D. viverrinus*, the numerous, partly elongated, cutaneous capillaries were located close to the epidermis (see Figures 1 and 5), forming a large area for gas exchange. Deeper lying capillaries were running through the dermis, indicating a transport of blood to and from the cutaneous capillaries (Figure 5c). These capillaries merged into larger blood vessels which were drawing from peripheral regions toward the venous system (Figure 5e). Several blood vessels merged into systemic veins, such as jugal veins and superior and inferior vena cava (Figure 5d,f), indicating the pathway of

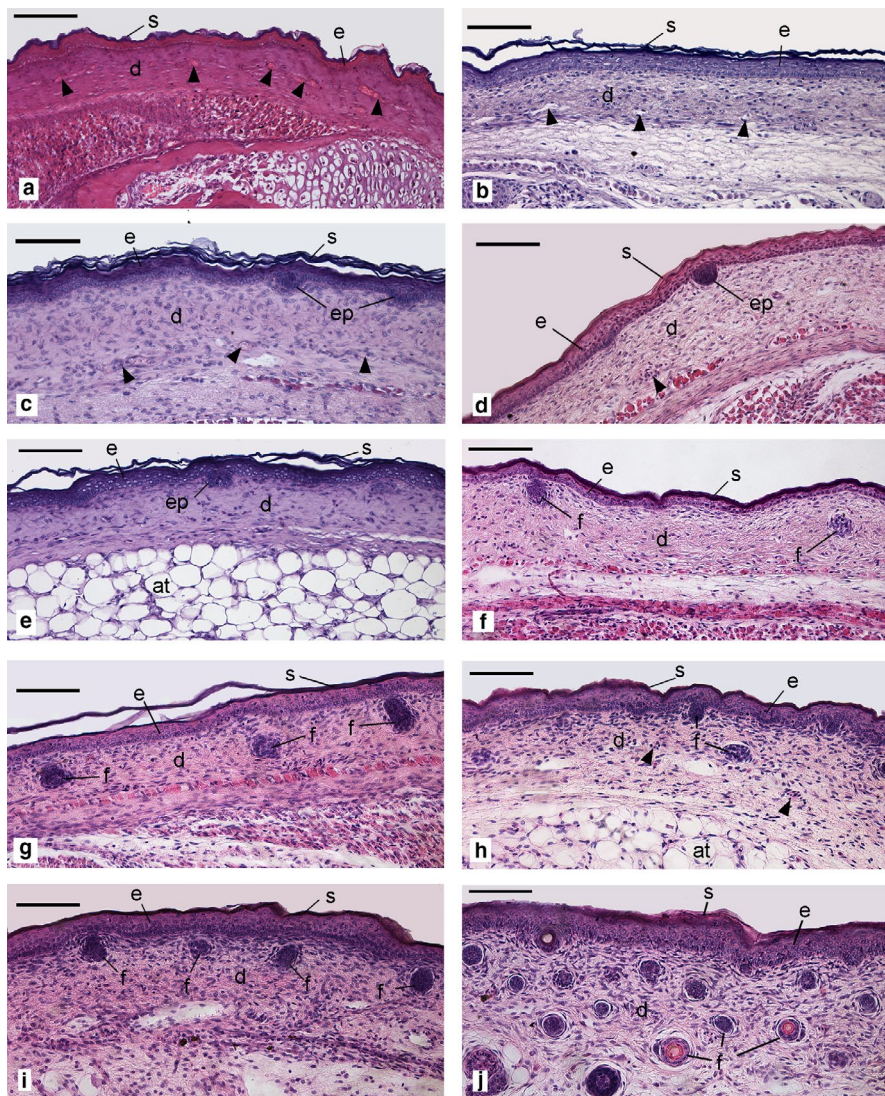


FIGURE 4 Histological sections comparing the structural skin development of *Dasyurus viverrinus* in the later postnatal period: 6 dpn (a), 7 dpn (b), 12 dpn (c), 16 dpn (d), 19 dpn (e), 25 dpn (f), 29 dpn (g), 35 dpn (h), 41 dpn (i), and 55 dpn (j). Capillaries (arrowheads) became less numerous and the diffusion distance between cutaneous capillaries and the skin increased further. A layer of keratin (stratum corneum, arrow) formed the outer rim of the epidermis, providing cover and protection of the skin. First signs of hair formation (epithelial placodes) were present by day 12. The formation of hair follicles progressed and mature hair follicles with cells of sebaceous glands (arrows) were visible by day 55. d, dermis; e, epidermis; ep, epithelia placodes; f, hair follicle; s, stratum corneum. Scale bar = 100 μm , Magnification: $\times 200$

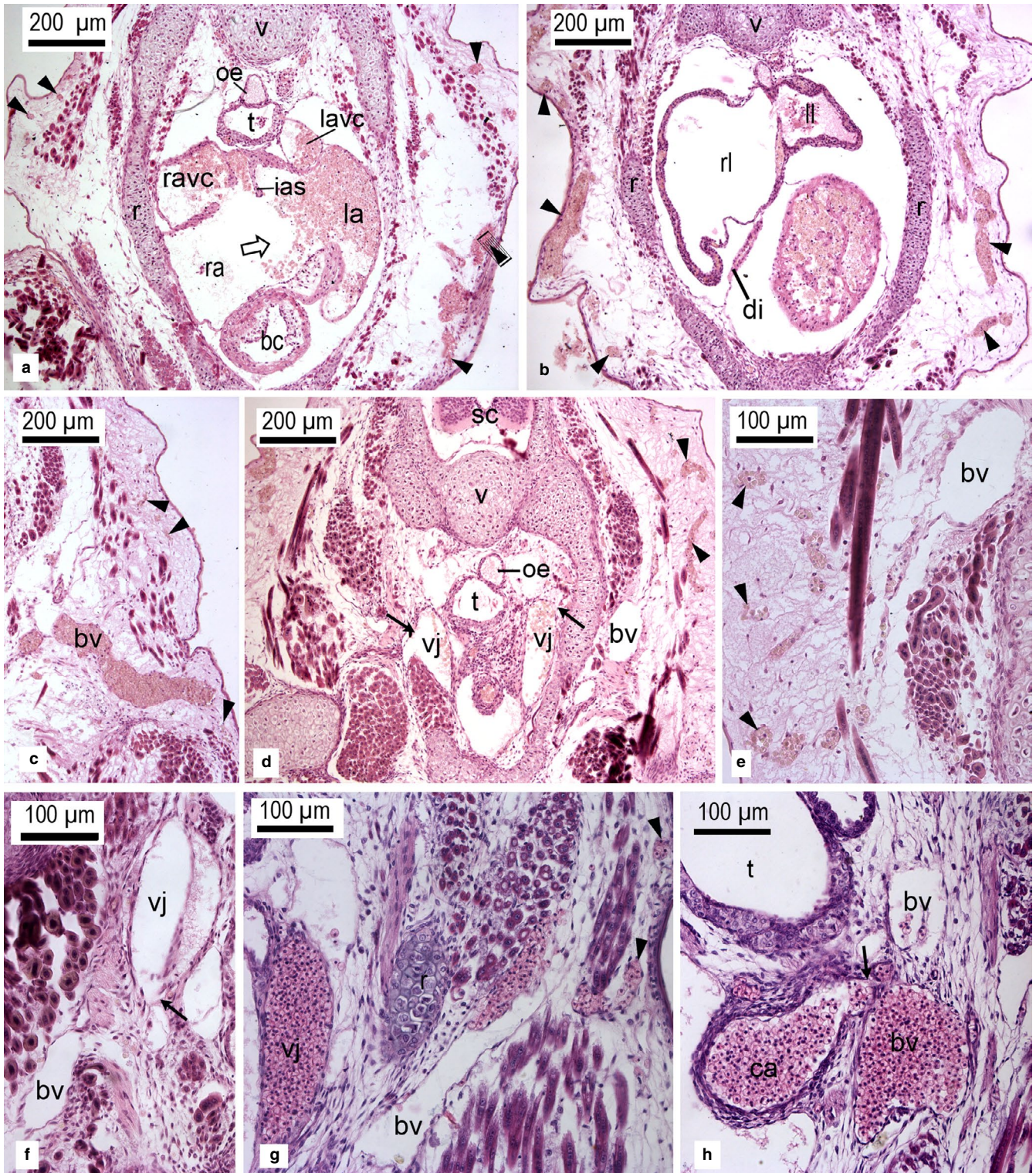
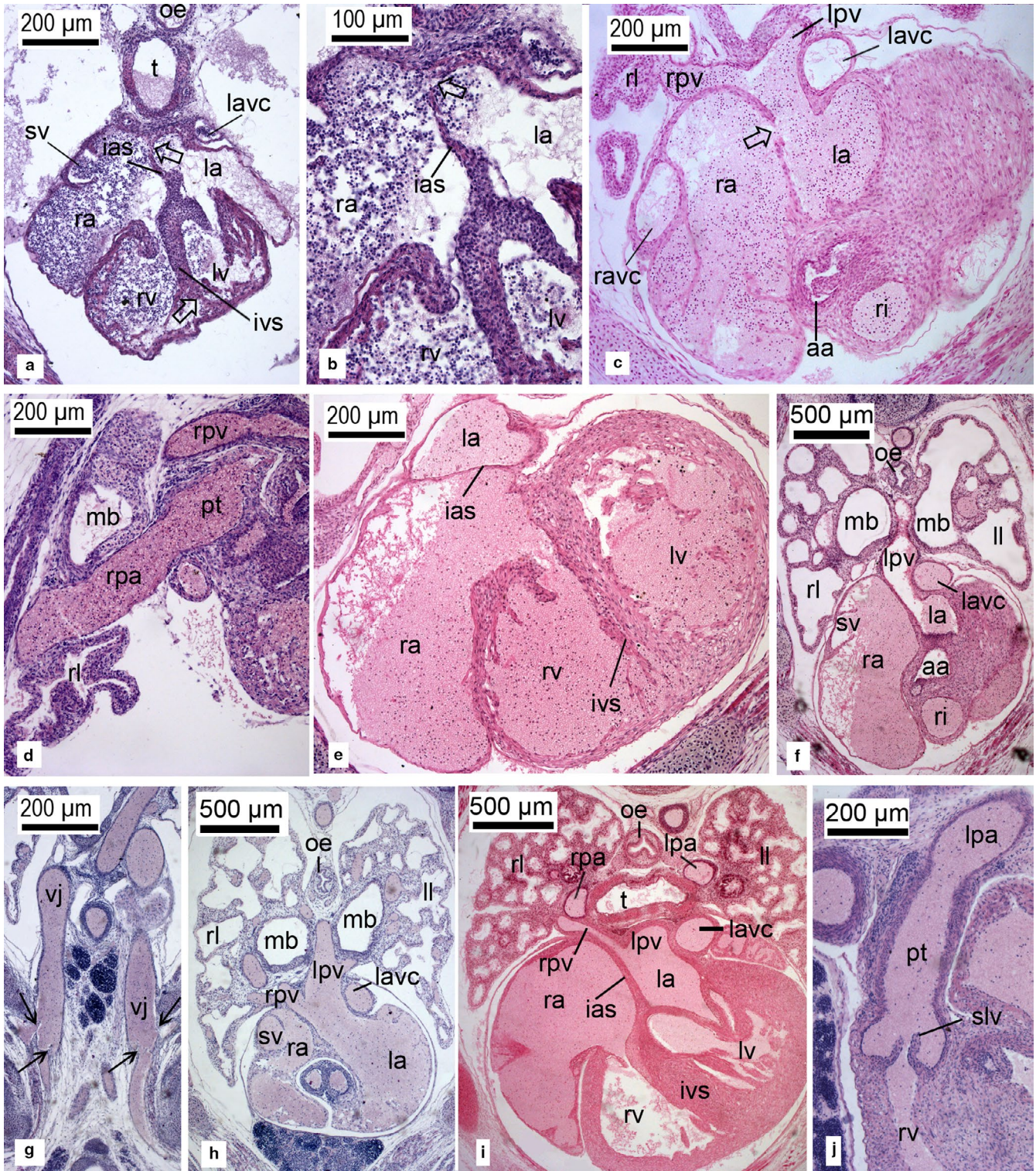


FIGURE 5 Light micrographs of the cardiorespiratory system of the neonate (a–f) and 2-day-old (g–h) *Dasyurus viverrinus*. The heart was highly immature with incomplete inter-atrial and inter-ventricular septa, permitting communication between the left and right side of the heart (interatrial opening indicated by open arrow). Numerous superficial cutaneous capillaries (indicated by arrowheads) were located close to the epidermis (a–e), allowing for transcutaneous gas exchange. The capillaries were communicating with larger capillaries and blood vessels running through the dermis (c, e), indicating a transport of blood to and from the cutaneous capillaries. Various large blood vessels were drawing from peripheral regions toward the major venous system (jugular veins and vena cava) and merged into them (indicated by black arrows c, f). The lung in the neonate *D. viverrinus* (b, only the right lung was ventilated in this specimen) was at the canalicular stage. Note the poorly developed diaphragm. bc, bulbus cordis; bv, blood vessel; ca, common carotid artery; di, diaphragm; ias, interatrial septum; la, left atrium; lavc, left anterior vena cava; ll, left lung; lv, left ventricle; oe, oesophagus; r, rib; ra, right atrium; ravc, right anterior vena cava; rl, right lung; rv, right ventricle; sc, spinal cord; t, trachea; v, vertebral body; vj, jugal vein. Magnification is indicated by the scale bar



communication between cutaneous and systemic vascular system. The situation persisted in the 2-day-old *D. viverrinus* (Figure 5g,h).

An unusual feature of the cardiac system in newborn *D. viverrinus* was the presence of a large opening connecting the ventricles of the heart. A wide opening connecting the atria, as found in many newborn marsupials, was present as well (Figure 5a). The ventricles and the atria were completely open, allowing for a mixture of oxygenated and deoxygenated blood. Division of the distal truncus arteriosus into

the aortic and pulmonary outflow tracts was complete in the neonate *D. viverrinus*. However, aortic and pulmonary valves were not evident yet and septation of the bulbus cordis was incomplete. Fusion of the right and left bulbar ridges had not yet occurred and the bulbar ridges were protruding into the lumen of the bulbus cordis (Figure 5a).

In the 3-day-old pouch young, division of the bulbus cordis was complete but there were still small interatrial and interventricular fenestrated openings (Figure 6a,b). In the 4-day-old young,

FIGURE 6 Light micrographs of the cardiorespiratory system of the developing *Dasyurus viverrinus* at the age of 3 (a, b), 4 (c, d), 5 (e, f), 6 (g, h), and 14 (i, j) days. The heart of the 3-day-old *D. viverrinus* had nearly complete interatrial and interventricular septa. The open arrows indicated the presence of fenestrations in the septa. By day 4 a fenestrated interatrial septum was still visible (c, open arrow), but the pulmonary circulation was greatly increased (d), indicating the final switch from cutaneous to solely pulmonary respiration. By day 5 the interatrial and interventricular septa were complete and the heart was fully subdivided into left and right side (e). At this age, the bronchial tree started developing and the pulmonary circulation was establishing (f). Remnants of junctions of blood vessels with jugal veins were still visible at day 6 (g, arrows indicate the inflow), but the respiratory function was restricted to the developing lung (h). By day 14, the heart was increasingly muscular and interatrial and interventricular increased in thickness (i), cardiac valves developed and became more pronounced (j). aa, ascending aorta; ias, interatrial septum; ivs, interventricular septum; la, left atrium; lavc, left anterior vena cava; ll, left lung; lpa, left pulmonary artery; lpv, left pulmonary vein; lv, left ventricle; mb, main bronchus; oe, oesophagus; pt, pulmonary trunk; ra, right atrium; ravc, right anterior vena cava; ri, infundibulum of the right ventricle rl, right lung; rpa, right pulmonary artery; rpv, right pulmonary vein; rv, right ventricle; slv, semilunar valves; sv, sinus venarum; t, trachea; vj, jugal vein. Magnification is indicated by the scale bar

a fenestrated interatrial septum was still visible (Figure 6c), allowing for a small communication between right and left atrium (Figure 6c). At this age the pulmonary circulation was greatly increased (Figure 6d). At 5 days the interatrial and interventricular septa were complete and the heart was fully subdivided in left and right atria and ventricles. The pulmonary circulation was establishing (Figure 6e,f). Remnants of junctions of blood vessels with jugal veins were still visible at day 6, however, the development of the pulmonary vascular system indicated a transition from cutaneous to pulmonary respiration (Figure 6g,h). At the age of 14 days the heart was increasingly muscular and interatrial and interventricular septa

increased in thickness (Figure 6i). The heart developed structurally, cardiac valves, such as semilunar valves in the right ventricle, developed and became more pronounced (Figure 6j).

At birth the respiratory system of *D. viverrinus* was highly immature. The lungs of the neonate were characterized by a few large tubular-like structures, primitive airways that one would expect to be present in the early canalicular stage (Figures 5b and 7a). The respiratory cavities were lined mainly by cuboidal epithelium interspersed with some respiratory capillaries (Figure 7a inset), providing only limited surface area for gas exchange. The large terminal air chambers opened from short main bronchi or

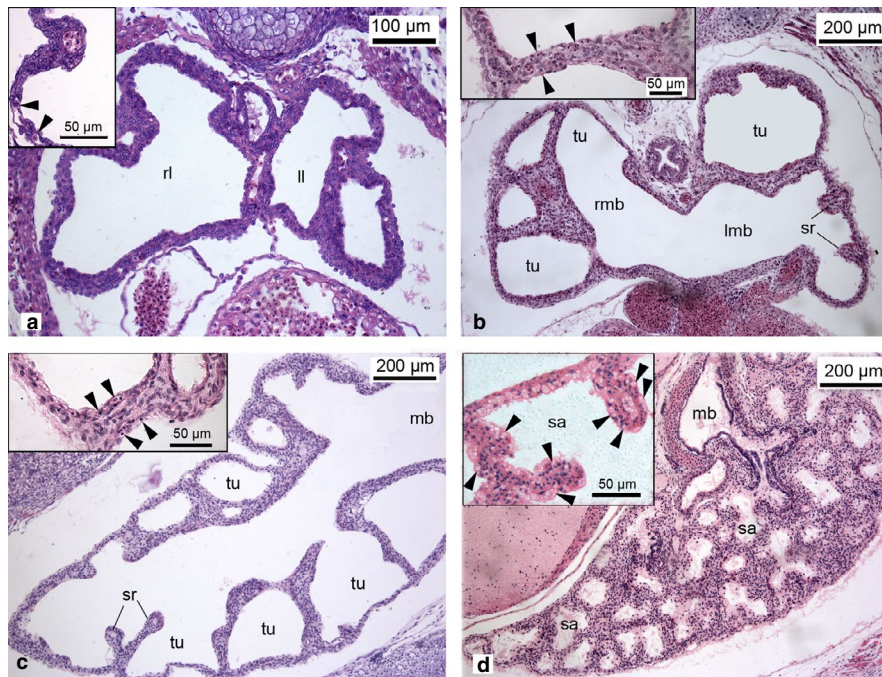


FIGURE 7 Light micrographs showing the coarse structural changes in the lung parenchyma during the early postnatal lung development of *Dasyurus viverrinus*. The lung of the neonate (a) was characterized by a few large tubular-like structures that appeared undivided. The epithelial lining of these airways was mostly cuboidal with a few interspersed respiratory capillaries (a, inset). By day 3, the lung became more compartmentalized by thick septal ridges (b). Thick intertubular septa were lined by parenchymal epithelium alternating with respiratory capillaries (b, inset). In the 5-day-old young (c), the subseptation of the lung continued and numerous smaller tubules resulted, a double capillary system was developing (c, inset), indicating the transition from the canalicular to the saccular stage. By day 14 (d), the lung was at the saccular period of lung development with numerous saccules and a double capillary system (d, inset). ll, left lung; lmb, left main bronchus; mb, main bronchus; rl, right lung; rmb, right main bronchus; sa, sacculus; sr, septal ridge; tu, tubules. Arrowheads indicate respiratory capillaries. Magnification is indicated by the scale bar

as it appeared directly from the trachea. By days 2 and 3 the lung became eventually more compartmentalized by thick septal ridges (Figure 7b). By this time the parenchyma comprised few large tubules, separated by thick septa. The intertubular septa were characterized by parenchymal epithelium alternating with capillaries lining the tubular walls (Figure 7b inset). The process of septation of the lung progressed during the following days and the pulmonary vascular system developed (Figure 6d). By day 5, the lung appeared well subdivided with several tubular structures and the formation of the bronchial tree started (Figures 6f and 7c). The septa between the tubules resembled a double capillary network, however, not forming a consistent double-capillary network yet (Figure 7c inset). During the next days, the septation of the lung parenchyma progressed rapidly, leading to a reduction in the size of the tubules (Figures 6h,i and 7d). Finally, the transition from the canalicular to the saccular stage took place. The tubules converted into sacculi and a true double capillary system, characteristic for the saccular stage, was established (Figure 7d inset). A diaphragm was present in neonate *D. viverrinus*, although only a few cells thick (Figure 5b).

4 | DISCUSSION

Hatching monotremes and newborn marsupials are distinguished from all other mammals by their relative immaturity and small size at birth, much of their development occurs postnatally in a nest or in the pouch attached to one of the maternal teats (Tyndale-Biscoe and Renfree, 1987; Grant, 1995; Renfree, 2006; Simpson *et al.*, 2011; Ferner *et al.*, 2017). The short gestation favored by marsupials delivers a newborn with a compromised respiratory system that must rely to varying degrees on transcutaneous gas exchange (Frappell and MacFarlane, 2006; Simpson *et al.*, 2011; Ferner, 2018). A graduation of lung development ranging from canalicular to saccular stage can be observed among newborn marsupials (Mess and Ferner, 2010). It follows the size variation in the sequence G1 to G3 as proposed by Hughes and Hall (1988).

Generally, marsupial species with very low birth weight have lungs at birth consisting of a few tubular air spaces (G1; e.g., *D. viverrinus*), neonates with intermediate birth weights have lungs consisting of large terminal sacculi separated by thick primary septa (G2; e.g., *Monodelphis domestica*, *Trichosurus vulpecula*, *Perameles nasuta*), and the ones with the highest birth weights have further subdivided lungs (G3; e.g., *Phascolarctos cinereus*, *Petrogale penicillata*, *M. eugenii*) (Krause and Leeson, 1975; Gemmell, 1986; Gemmell and Selwood, 1994; Runciman *et al.*, 1996; Ferner, 2018).

Increasing evidence from physiological studies suggests that the functional immaturity of the respiratory system at birth in marsupials necessitates recruitment of an alternative organ system such as the skin for gas exchange (Mortola *et al.*, 1999; Frappell and Mortola, 2000; MacFarlane and Frappell, 2001; MacFarlane *et al.*, 2002; Frappell and MacFarlane, 2006). In the newborn Julia creek dunnart (*S. douglasi*) and fat-tailed dunnart (*S. crassicaudata*),

gas exchange through the skin accounts for almost 100% of total gas exchange and is obviously a requirement because of the inefficient pulmonary ventilation (no breathing movements and volume changes) attributable to the immature neuromuscular development (Frappell and Mortola, 2000). The structural immaturity of the lung, poor muscle coordination, chest wall distortion, and lack of respiratory drive may impede efficient breathing during the first days in these smallest of newborn mammals (Simpson *et al.*, 2011). Although transcutaneous gas exchange might be most pronounced in dasyurid neonates, even in the relatively large newborn tamar wallaby, *M. eugenii* (~350 mg) gas exchange across the skin accounts for 33% of total gas exchange (MacFarlane and Frappell, 2001; MacFarlane *et al.*, 2002). This is made possible only by a low metabolic rate and a favorable body surface area. However, without an appropriate diffusion barrier, the gas exchange via the skin would not be possible.

The extremely thin skin of the newborn *D. viverrinus*, with short diffusion distances (blood-gas barrier) as well as a high capillary volume density, provides excellent preconditions for effective cutaneous gas exchange. The skin diffusion barrier of the newborn *D. viverrinus* measured only 9 μm . This is thinner than the skin diffusion barrier reported from other marsupial neonates (23–65 μm) and other species with cutaneous gas exchange as well (amphibians: 31–42 μm ; folded wing of the fruit bat *Epomorphus wahlbergi*: 27 μm) (Makanya *et al.*, 2007; Ferner, 2018). However, the value is close to the estimation of the epidermal thickness (9.8 μm) in the stretched wing web membrane of the fruit bat (Makanya and Mortola, 2007). It needs to be discussed that the diffusion distances obtained in this study have been estimated on histological sections only. Due to processing and possibly shrinking of the material, the values might be underestimated (see Section 2). Furthermore, the resolution of the histological material might be inadequate for the description of details of the blood-gas barrier. Transmission electron microscopy (TEM) would give more insight in cell composition and actual thickness of the blood gas barrier, but was not available for investigation. There is only one ultrastructural study that investigated postnatal skin development in a dasyurid marsupial, the northern native cat (*Dasyurus hallucatus*) (Pralomkarn *et al.*, 1990). The TEM pictures of the neonate *D. hallucatus* showed a similar structural development as observed in *D. viverrinus*. The dorsal skin was composed of a thin layer of epidermis in close contact with the capillary bed located on an equally thin layer of dermis (Pralomkarn *et al.*, 1990). Unfortunately, no values were reported for the diffusion barrier of *D. hallucatus*, so no comparison was possible. Another ultrastructural study reported a diffusion barrier of 30–35 μm in the skin of the more mature born quokka wallaby (*S. brachyurus*) (Makanya *et al.*, 2007).

Nevertheless, even diffusion distances ranging from 23 to 65 μm should be sufficient for gas exchange, since Stücker *et al.* (2002) have shown that cutaneous oxygen diffusion for the supply of underlying tissue can reach depths of 250–400 μm .

The capillary density decreases rapidly during the first four postnatal days in *D. viverrinus*. At the same time the diffusion

distance between cutaneous capillaries and the air increases due to new formed dermal and epidermal cell layers. By day 4, the diffusion distance via the skin is 5 times the value of the newborn *D. viverrinus*, but with 44 μm it is still in the range of other marsupial neonates. Parallel to the increase in diffusion distance, the capillary density decreased markedly from 0.33 in neonates to 0.06 by day 4. After the first postnatal week, the capillary density stabilized at a value of 0.02. From this time on cutaneous capillaries probably serve only the supply of the skin, but play no major role in cutaneous respiration. The skin of most vertebrates, like any other living tissue, receives blood only from the major systemic arteries, which typically deliver oxygenated blood (Feder and Burggren, 1985).

An explanation for the decrease in capillary density in the skin during the postnatal period remains hypothetical and the elucidation of the fate of the superficial capillaries would require ultrastructural investigation. What can be observed is an increasing distance of the capillaries toward the skin surface due to new-formed tissue in the outwards growing epidermal and dermal layers. The disappearance of capillaries, leading to a lower capillary density, might possibly follow mechanisms of vessel regression as reviewed by Korn and Augustin (2015). Vessel remodeling leading to pruning and regression is a common phenomenon during embryonic and postnatal development of mouse (Udan *et al.*, 2013; Franco *et al.*, 2015) and rat (Hughes and Chang-Ling, 2000). Cellular mechanisms, such as apoptosis of endothelial cells, and several molecular mediators might be involved in the regression process of the superficial capillaries. Furthermore, a reduction in blood flow toward the skin following cardiac remodeling might play a role. Changes in blood flow are known to act as regulators of vessel regression and non-perfused vessels are predisposed to vascular pruning (Ando and Yamamoto, 2009).

The rapid structural development of the skin and the lung in *D. viverrinus* suggests that the transition from cutaneous to pulmonary respiration occurs during the first 4 days. That seems not unlikely, since in another dasyurid species, the fat-tailed dunnart, no breathing movements at all were recorded in neonates. At day 3 very small deflections indicated the commencement of a coordinated breathing pattern and a number of augmented breaths were observed. Finally, by day 4, a rhythmic breathing pattern with frequent augmented breaths was apparent (Frappell and MacFarlane, 2006).

Our results confirm the high immaturity of the lung in newborn dasyurids reported in previous studies. The lungs of the neonate *D. viverrinus* consisted only of two respiratory cavities, superficially vascularized by respiratory capillaries which emerged directly from the trachea. In one specimen the left lung was not even ventilated (Figure 5b). It seems to be most probable that the respiratory system of the newborn might be very limited, if functioning at all. Contrary to the slow postnatal development generally assumed for marsupial offspring, the respiratory system of *D. viverrinus* developed very rapidly. By day 5 the lung parenchyma had structurally matured, the lung appeared well subdivided in saccular-like structures, and a simple bronchial tree was recognizable. In parallel, the pulmonary

circulation developed and became functional. That indicates that the respiratory function of the lung might be established at this time.

The pathways of cutaneous gas exchange must include not only the superficial subepidermal capillary network but also the blood transport to and from the skin as well as communication with the systemic circulation (Figure 8).

During the early postnatal period, the skin of *D. viverrinus* possesses a well-developed capillary network for cutaneous gas exchange. These superficial "gas exchange" dermal capillaries communicate with deeper lying "transport" capillaries that were running through the dermis, indicating a transport of blood to and from the subepidermal capillaries. These capillaries join into larger blood vessels that communicate with the arterial and venous system. Histological investigation indicated a blood flow from peripheral regions toward the heart and vice versa (Figure 8a).

This circulatory arrangement seems to be favorable, since cutaneous respiration is only a temporary solution in the early postnatal period in marsupials. Adult marsupials have a pulmonary and systemic circulation similar to eutherians. In marsupials, the cardiovascular system must develop to a functional stage within a gestation period as short as 19 days in the case of *D. viverrinus*. The newborn *D. viverrinus*, with a very low birth weight of 12.5 mg, shows the least degree of cardiac septation from all marsupial species investigated so far (Runciman *et al.*, 1995). It seems that the extent of cardiac maturity, similarly to the respiratory system, is related to birth weight rather than to length of the gestation (Gemmell and Nelson, 1988). The heart of the newborn *D. viverrinus* allows for a communication between the left and right side of the heart, provided by incomplete interatrial, inter-ventricular, and aortico-pulmonary septa (Hill and Hill, 1955; Baudinette *et al.*, 1988). A ductus arteriosus and an interatrial communication is a common phenomenon during the immediate postnatal period in marsupials (Baudinette *et al.*, 1988; Runciman *et al.*, 1995). However, in addition to the two common shunts, *D. viverrinus* has a large interventricular communication for up to 3 days after birth and the septation of the outflow tract is incomplete. The communication between the ventricles may allow blood flow in either direction and there may be mixing of right and left ventricular output in the outflow tract. Development of the outflow tracts and separation of the ventricles in marsupials is structurally similar to that seen in eutherians, but completion of the process of septation occurs in marsupials in the early postnatal period rather than before birth (Figure 8b) (Runciman *et al.*, 1995).

The presence of the right-to-left shunt prevents discreet pulmonary and systemic circuits. It permits blood to bypass the lungs and allows venous admixture of blood low in oxygen content (Frappell and MacFarlane, 2006). With closing of the shunts in the early postnatal period, the pulmonary and the systemic circulations are separated, all right ventricular output is directed to the lungs, and right and left ventricular volumes are balanced (Figure 8c). From this time on, around 3 days after birth, a transition from cutaneous to pulmonary respiration must occur. The rapid maturation of the lung, in parallel, allows for the pulmonary system to be functional in order to meet the metabolic needs of the organism.

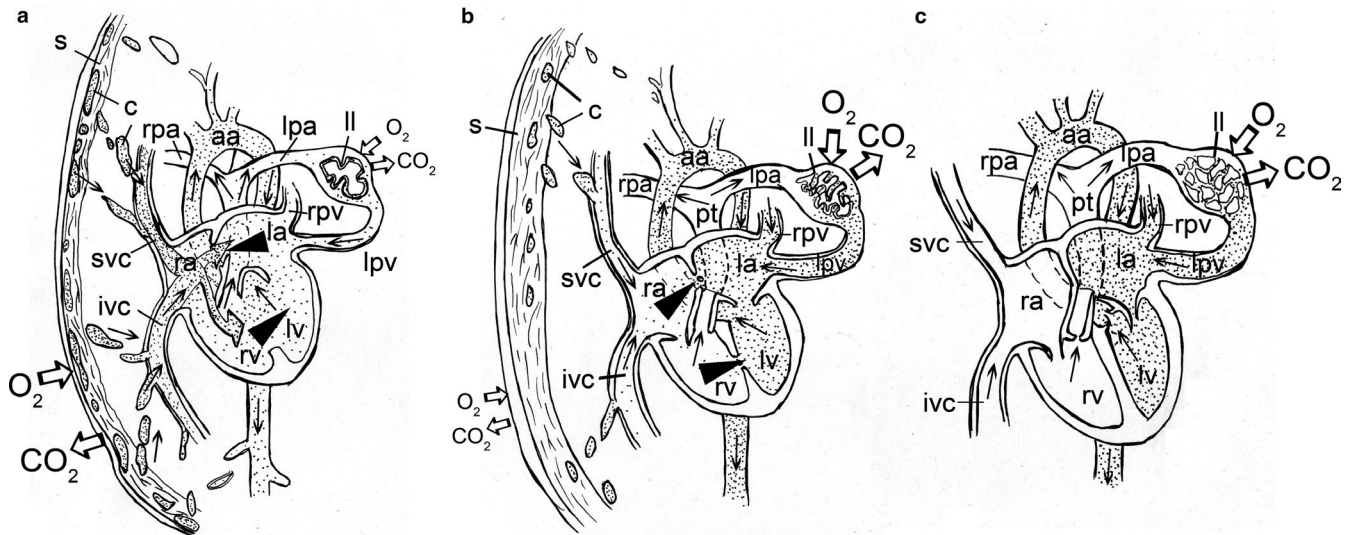


FIGURE 8 A diagrammatic representation of the circulation in the neonate (a), 3-day-old (b), and 6-day-old (c) *Dasyurus viverrinus*. In the neonate the major site for gas exchange seems to be the very thin skin. Cutaneous respiration exceeds the pulmonary gas exchange at this time of development. The oxygenated blood, coming from the skin, is transported via the venous system to the immature heart. The wide intra-atrial and intra-ventral openings allow for a mixture of oxygenated and deoxygenated blood. In the 3-day-old young the diffusion distance via the skin increases, the capillary density decreases, and the heart and the lungs are developing. Only small fenestrations in the intra-atrial and inter-ventral septa allow for a lower mixture of blood. A switch from cutaneous to pulmonary respiration takes place. By day 6 the intra-atrial and inter-ventral septa are closed and the heart is completely separated. The lung is the only gas exchanging organ and the oxygenated blood is coming from the lungs. The stippling indicates relatively well-oxygenated blood and the direction of blood flow is indicated by the arrows (modified from Runciman *et al.*, 1995). aa, ascending aorta; c, capillary; ivc, inferior vena cava; la, left atrium; ll, left lung; lpa, left pulmonary artery; lpv, left pulmonary vein; pt, pulmonary trunk; ra, right atrium; rpa, right pulmonary artery; rpv, right pulmonary vein; rv, right ventricle; s, skin; svc, superior vena cava

To discuss the cutaneous gas exchange of newborn marsupials, a comparison with obligate and facultative skin breathers might be useful. Cutaneous gas exchange is widespread among vertebrates. Although it is most prominent in amphibians (e.g., 100% in the Lungless salamander), this mode of respiration is also important in several other vertebrate groups, such as fish (e.g., 30% in the Mudskipper) and non-avian sauropsids (e.g., 20% in the Southern musk turtle) (Feder and Burggren, 1985). Aside from the physiological studies on pouch young marsupials, the only case of percutaneous gas exchange in mammals has been reported from bats. The structural modifications of the wing web membrane of the epauletted fruit bat (*Epomophorus wahlbergi*) permit a substantial contribution (6–10%) to the total gas exchange (Makanya and Mortola, 2007).

A prerequisite for cutaneous gas exchange in vertebrates seems to be a heart with an undivided ventricle. This cardiac situation can be found (with the exception of bats) in all skin-breathing species. In amphibians, the best-known skin breathers, the oxygenated blood from the lung reaches the left atrium, while deoxygenated blood from the systemic circulation enters the right atrium. The blood reaches the undivided ventricle and is pumped via the Conus arteriosus into the pulmonary and systemic circulations. Trabeculae in the ventricle allow for a separation of oxygenated and deoxygenated blood. A spirally fold in the Conus arteriosus conducts deoxygenated blood to the Arteria pulmocutanea, whose ramifications reach the skin and the lung (Remane *et al.*, 1994). Oxygenated blood coming from the skin enters, in contrast to that coming from the lung, the

right atrium. Since amphibians have a heart that consists of a single ventricle and two atria, it is possible that oxygenated blood from the skin mixes with that of the systemic circulation (Stephenson *et al.*, 2017). Dependent from the oxygen content in the environment amphibians can use shunts of the pulmocutaneous arteries to favor cutaneous or pulmonary circulation and bypass the systemic or pulmonary circulations, respectively (Feder and Burggren, 1985). Compared to newborn marsupials, the skin of adult anurans shows structural differences in the superficial subepidermal capillary network. Ramifications of the cutaneous arteries and veins join the subepidermal capillaries through segments running vertical to the skin surface, penetrating stratum compactum and stratum spongiosum of the dermis without branching in these layers (de Saint-Aubain, 1982). Amphibians, which perform cutaneous gas exchange lifelong, have developed permanent solutions, such as pulmocutaneous arteries and a well-structured subepidermal capillary network. These permanent adaptations related to skin breathing are not present in newborn marsupials. In consideration of the temporary role of cutaneous respiration in marsupial pouch young (see Mortola *et al.*, 1999; MacFarlane and Frappell, 2001) and the rapid structural changes in the pulmonary and systemic circulations (closing all shunts) in the early postnatal period, a temporary solution for the cardiovascular-cutaneous circulation is necessary.

The utility of the skin as a gas exchange organ depends on both morphological and physiological variables. Cutaneous gas exchange is diffusion limited (Piiper, 1988). The process of diffusion is defined

as the equilibrating flow of matter, through the random movement of molecules in a fluid or gas, from a high concentration to one of low concentration (Mortola, 2015). Diffusion via the skin is proportional to the skin surface area, the skin permeability to gases and the difference in gas concentration (gas partial pressure) on either side of the skin, and is inversely proportional to skin thickness (Feder and Burggren, 1985).

The skin diffusion distance of the newborn *D. viverrinus* (9 μm) offers an enormous diffusion barrier when compared to the blood-gas barrier of the lung in neonatal marsupials, which can be as thin as 0.3–0.6 μm (Runciman, 1994; Szdzyu *et al.*, 2008). However, percutaneous gas exchange is advantageous in that the animal is exposed to an infinite pool of air in the environment with high partial pressures of oxygen (PO_2 of about 150 mmHg). In contrast, the lung values of partial oxygen pressure reach 100 mmHg in the sacculi and 46 mmHg in the venous blood. Thus, the O_2 pressure gradient for the skin capillaries of newborn marsupials would be almost twice that in the lung. That would be equivalent to halving the diffusion distance (Makanya and Mortola, 2007). A high P_{50} value (PO_2 at which the blood is 50% saturated) in newborn marsupials, accomplished by a right-shifted P_{50} curve, ensures that O_2 can be unloaded at high PO_2 and diffusion over a greater distance becomes possible. This might be a special adaptation in marsupials toward the early postnatal period, when pulmonary circulation is limited and the immaturity of the lung function favors reliance on cutaneous gas exchange (Frappell and MacFarlane, 2006).

The physiological role of the cutaneous gas exchange area needs to be considered in relation to the surface area to mass ratio and the metabolic needs of the organism. Skin gas exchange is favored in the newborn marsupial because of a relatively large surface area to mass ratio and a low metabolic rate (Frappell and MacFarlane, 2006). Thus, it is not surprising to find the highest rates of cutaneous gas exchange (95–100%) among the dasyurids, which have the smallest and most immature of all marsupial neonates (10–18 mg). Taken into consideration that even the relatively large newborn tammar wallaby (~350 mg) reaches values of 30–40% cutaneous gas exchange, transcutaneous respiration should be possible in dasyurids for an extended period of time until body dimensions of newborn macropodids are attained. However, the early postnatal maturation of the cardiac system (closing of shunts) and possibly a rapid increase in the metabolic rate in the developing marsupial young force an early transition from cutaneous to pulmonary respiration.

Cutaneous respiration is often significant and crucial in invertebrates and lower vertebrates, even in organisms with small surface to mass ratio, as long as oxygen needs are low (Feder and Burggren, 1985; Mortola, 2015). However, among higher vertebrates, such as birds and mammals, the skin is seldom an important route for gas exchange. Cutaneous gas exchange in higher vertebrates is limited by the poor gas diffusion properties of the skin tissues (often furred or feathered), the low surface area to mass ratio resulting from large body size and the high metabolic demands (Makanya *et al.*, 2007; Mortola, 2015). However, the situation in young mammals and birds might be different. They are often born or hatched with thin

skin, richly supplied with blood and lacking fur or feathers, at least in altricial neonates or hatchlings (Szdzyu and Zeller, 2009; Ferner *et al.*, 2009, 2010, 2017). Cutaneous respiration may therefore be more important in the developing forms of such higher vertebrates than it is in the adults. There is probably significant cutaneous gas exchange in the mammalian embryo and fetus (Harpin and Rutter, 1983; Cartlidge and Rutter, 1987) and gas exchange through the shell membrane is the major avenue of respiration available for the eggs of birds and indeed of all egg-laying vertebrates (Rahn *et al.*, 1979).

However, even if the role of the skin in respiratory gas exchange is minimal, cutaneous excretion of CO_2 exchange might be more important. Due to a higher permeation coefficient, CO_2 diffuses through tissue 20 times more readily than O_2 (Feder and Burggren, 1985).

Increasing the number of blood vessels per unit area (i.e., skin capillary density) can also enhance the functional surface area of the skin (Feder and Burggren, 1985). The skin of amphibians is heavily vascularized and up to 95% of the total respiratory capillarization may be located in the skin (Czopek, 1965). The high value of capillary volume density in skin of the newborn *D. viverrinus*, reported here, was three- to fourfold higher than that of other marsupial neonates (Ferner, 2018). This suggests that skin gas exchange in the dasyurid neonate might be most pronounced. In all neonate marsupials, investigated so far, the epidermis of the skin was poorly developed with a thin stratum corneum and no hair follicles (Lyne *et al.*, 1970; Krause *et al.*, 1978; Pralomkarn *et al.*, 1990; Makanya *et al.*, 2007; Ferner, 2018). It provides only a weak barrier to transcutaneous gas exchange. The presence of numerous capillaries in the dermal layer of all marsupial neonates and the monotreme hatchling supports the notion that cutaneous exchange in newborn marsupials and probably also newly hatched monotremes might be commonplace (Ferner, 2018).

The poor developed respiratory system at one side and the pronounced subepidermal capillary network in the skin at the other side strongly suggest that the neonate *D. viverrinus* is almost totally dependent on skin gas exchange, as it has been reported for other newborn dasyurids (see Frappell and MacFarlane, 2006).

Generally, terrestrial organisms that use the skin as blood-gas barrier face a high risk of excessive water loss via the skin. Therefore, animals with percutaneous gas exchange often live in a moist environment (Mortola, 2015). The humidity of the pouch in marsupials helps the neonate to circumvent this problem and even under pouch conditions, cutaneous blood would be exposed to a relatively high PO_2 (Frappell and MacFarlane, 2006). However, there are 'pouch-less' and 'pseudo-pouched' marsupial species, especially among the dasyurids (e.g., *S. douglasi*, *Antechinus stuartii*, *Dasyuroides byrnie*, *D. viverrinus*) and didelphids (*M. domestica*), that lack the true, permanent pouches seen in other species (Woolley, 1974; Tyndale-Biscoe and Renfree, 1987). These species form temporary skin folds in the mammary region when reproducing, but the young will be still exposed to the air (Woolley *et al.*, 2002). Interestingly, there seems to be a correlation between being 'pouch-less' and high rates of

cutaneous gas exchange. The advantage of being exposed to an infinite pool of oxygen obviously outweighs the greater risk of desiccation.

There are some studies of skin development in marsupials (Henrikson, 1969; Lyne, 1970; Lyne *et al.*, 1970; Krause *et al.*, 1978; Pralomkarn *et al.*, 1990; Armstrong and Ferguson, 1995; Makanya *et al.*, 2007; Ferner, 2018). Some of these studies described the presence of a periderm (also called "epitrichium") in marsupials during the early postnatal period, which has been discussed as a structure preventing desiccation in the developing marsupial. The periderm is defined as "the superficial transient layer of epithelial cells of the embryonic epidermis" (Pralomkarn *et al.*, 1990). In eutherians, the periderm is observed prior to the formation of hair follicles and disappears when the hair shafts emerge through the epidermis (Hanson, 1947; Lyne and Hollis, 1972; Holbrook and Smith, 1981). Thus, in eutherians, the periderm is only present in utero and disappears with the eruption of hair. Hill and Hill (1955) described this structure in the newborn *D. viverrinus* as keratinized membrane, responsible for the protection of the young from desiccation. In *Dasyurus viverrinus* the periderm, the outer layer of the developing epidermis was discernable up to day 4, after that time the outermost layer of the epidermis was the stratum corneum. This is similar to the observation in other marsupial species, where the periderm disappears soon after birth (Krause *et al.*, 1978; Gemmell *et al.*, 1988). This disappearance occurs well before the appearance of hair follicles, which emerge by day 55 in *D. viverrinus*. Thus, the correlation between hair shafts and the disappearance of the periderm, observed in eutherians, is not present in marsupials.

All marsupial young, including those of *D. viverrinus*, are born without hair and are incapable of maintaining a steady body temperature (Szdzyu and Zeller, 2009; Wacker *et al.*, 2017). Because of their relatively large surface area, small marsupial young lose heat very rapidly when exposed to low ambient temperatures. However, the postnatal development of *D. viverrinus* (Figure 2, Table 1) indicates a rapid increase in body size. The rates of heat and water loss decrease with growth because the surface area does not increase as rapidly as does the size (for 100% increase in body weight, the surface increases by only 59%) (Hulbert, 1988). The thyroid gland, which influences skin structure and development of thermoregulation, is not present in newborn marsupials (Setchell, 1974; Pralomkarn *et al.*, 1990). Thyroid function, furring, and thermogenic capacity in the marsupial do not occur until approximately two-thirds of pouch occupancy (Geiser *et al.*, 1986). The development of thermoregulation in the fat-tailed dunnart (*S. crassicaudata*), a species with a similar birth weight (~10 mg) as *D. viverrinus*, was described as a continuum (Wacker *et al.*, 2017). It was characterized by three major thermoregulatory stages: (1) at 40 days, animals were unable to maintain a constant high body temperature during short-term cold exposure; (2) at 60 days, animals could maintain a high body temperature for some time; but then entered an apparent torpor bout and could only re-warm passively when basking under a heat lamp; (3) from ~90 days, they expressed prolonged torpor bouts and were able to re-warm endogenously. Also, *Dasyuroides byrnei*, another dasyurid species, is

not properly furred and capable of thermogenesis until day 70–80 (Geiser *et al.*, 1986). The appearance of hair and sebaceous glands at day 55 postpartum in *D. viverrinus* supports the view that thermogenic capacity develops late in the postnatal development and is correlated with furring and insulation. The formation of hair follicles is exactly at the same time as the detachment from the maternal teat (49–56 days) occurs. Hair and sebaceous glands are observed in the skin just prior to the mother leaving the young in a nest. At this time the skin has a similar structure to that of an altricial eutherian neonate (for comparison, see Ferner *et al.*, 2017; Ferner, 2018).

During a long period of postnatal skin development, the structural differentiation of the skin results in a functional shift from transcutaneous gas exchange in the beginning to insulation and thermoregulation in later life.

5 | CONCLUSION

The extremely small and immature born *D. viverrinus* possesses an extensive subepidermal capillary network, characterized by low diffusion distances and high capillary volume density, a well-developed vascular system for communication between cutaneous capillaries and cardiac system and an undivided ventricle. This provides optimal structural prerequisites for transcutaneous gas exchange. The duration of cutaneous gas exchange in the postnatal period seems to be determined by the maturation of the cardio-respiratory system. In particular, the closing of the shunts, resulting in the separation of the left and right ventricles necessitates a transition from cutaneous to pulmonary gas exchange. During postnatal development, the skin of *D. viverrinus* undergoes structural changes, resulting in a functional shift from cutaneous respiration toward insulation and protection. Although cutaneous gas exchange may be performed for only a short time period in marsupials, it plays a vital role for the survivability of these most immature of all mammalian neonates. Substantial cutaneous gas exchange in *D. viverrinus* and in other marsupial species during the neonatal and early postnatal period seems to be common and a required compensation for incomplete pulmonary architecture and impaired ventilation. The diversity of species across vertebrate groups that resort at least in part to cutaneous gas exchange suggests strongly that skin breathing is commonplace rather than exceptional in vertebrates and probably a phylogenetically old phenomenon.

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CONFLICTS OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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