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Origin of the unique ventilatory apparatus of turtles

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; Bever, G S ; Rubidge, Bruce S ; de Queiroz, Kevin

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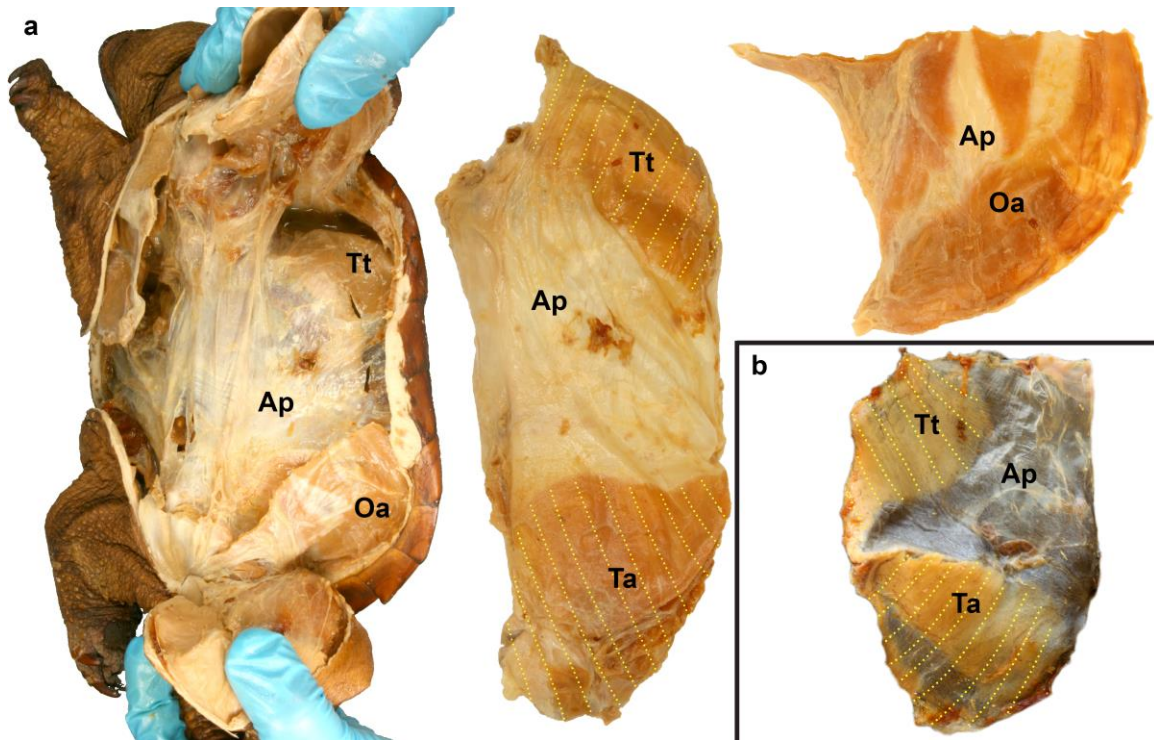
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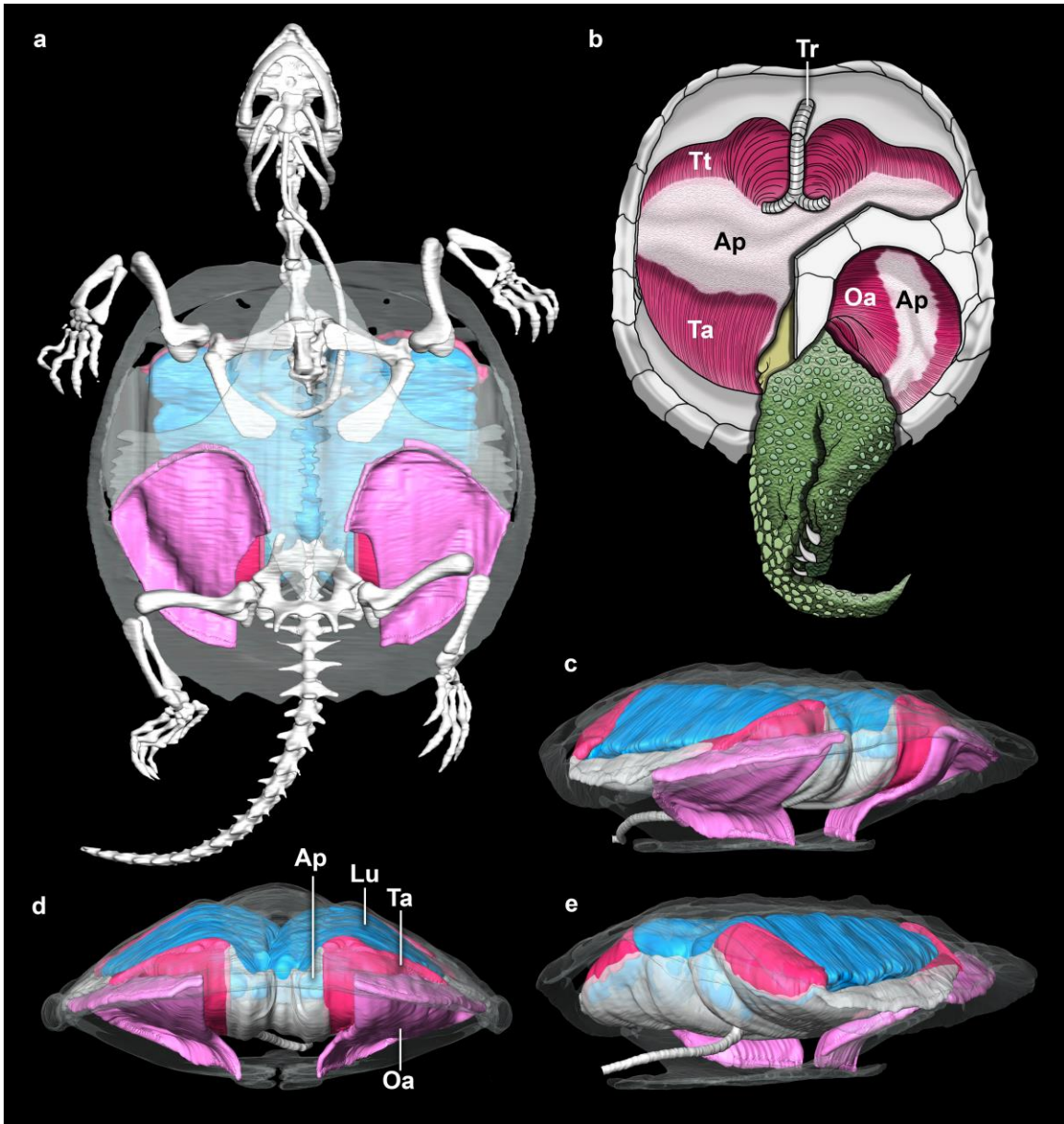
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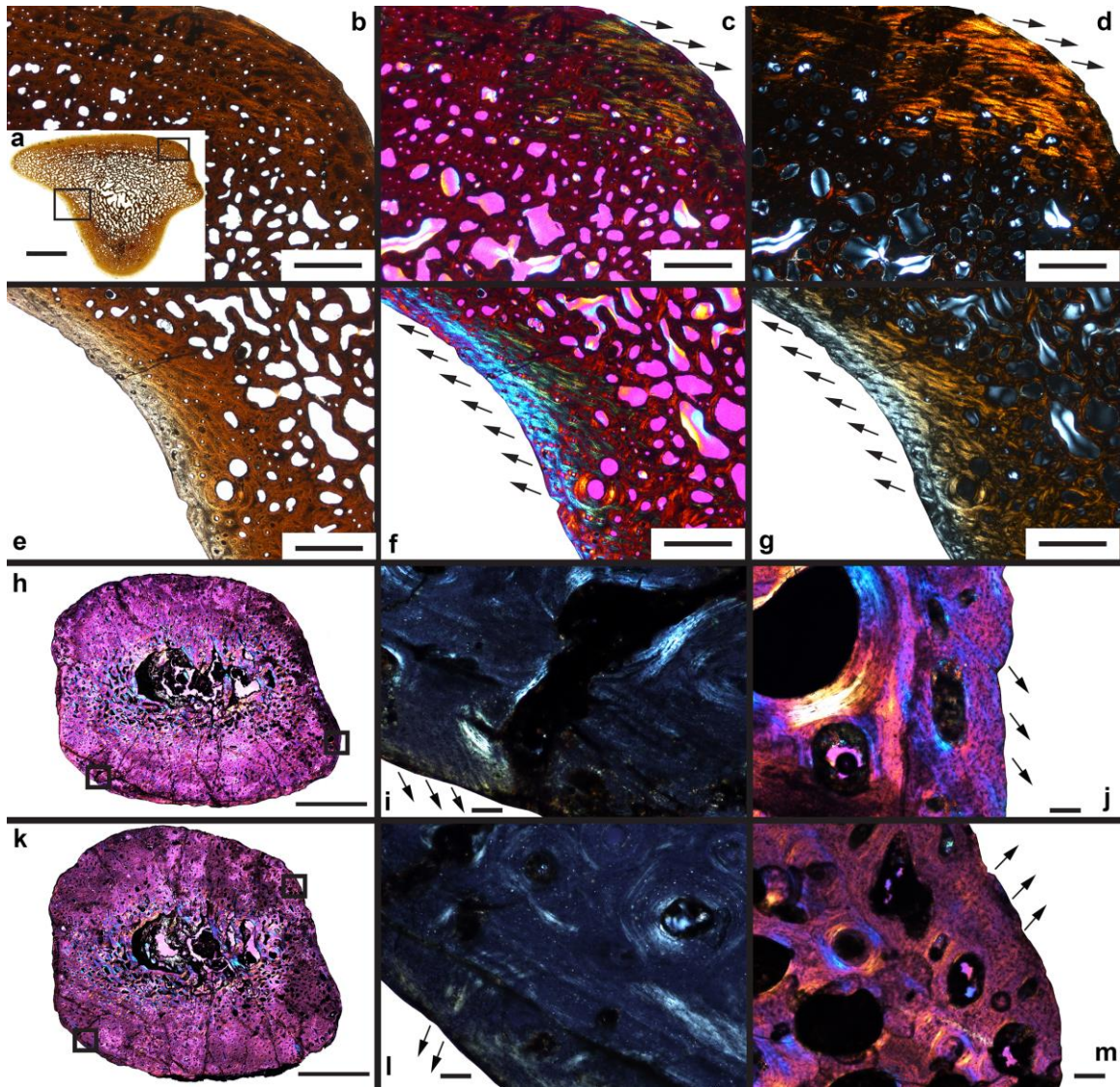
Supplementary Figure 1: Respiratory muscles of cryptodire turtles. a, Ventral view of a snapping turtle, *Chelydra serpentina* (Chelydridae) (ZMB uncataloged), with the plastron removed (**a** left), showing the major respiratory muscles *in situ*. The *M. transversus thoracis* (Tt) originates on thoracic ribs two and three and the *M. transversus abdominis* (Ta) originates on thoracic ribs five and six; both insert into a tendinous aponeurosis (Ap) that lies superficial to the peritoneum. The excised left ventral portion of the peritoneum (**a** middle) with the associated *M. transversus thoracis* and *M. transversus abdominis* overlain with dotted lines which indicate the orientation of the muscle fibers. The excised left *M. obliquus abdominis* (**a** right) is composed of distinct muscle bellies, each of which inserts into a tendinous aponeurosis (Ap). **b**, Ventral view of a Chinese softshelled turtle, *Pelodiscus sinensis* (Trionychidae) (PIMUZ lab#2009.72IW; Carapace length = 82 mm), with the excised ventral portion of the peritoneum with the orientation of muscle fibers indicated by dotted lines showing the

major expiratory muscles *in situ*. The *M. transversus thoracis* and *M. transversus abdominis* are well developed in this turtle and, at least laterally, nearly completely enclose the peritoneum. The *M. transversus thoracis* originates on thoracic ribs 2-4 and the *M. transversus abdominis* originates on thoracic ribs 5-8.



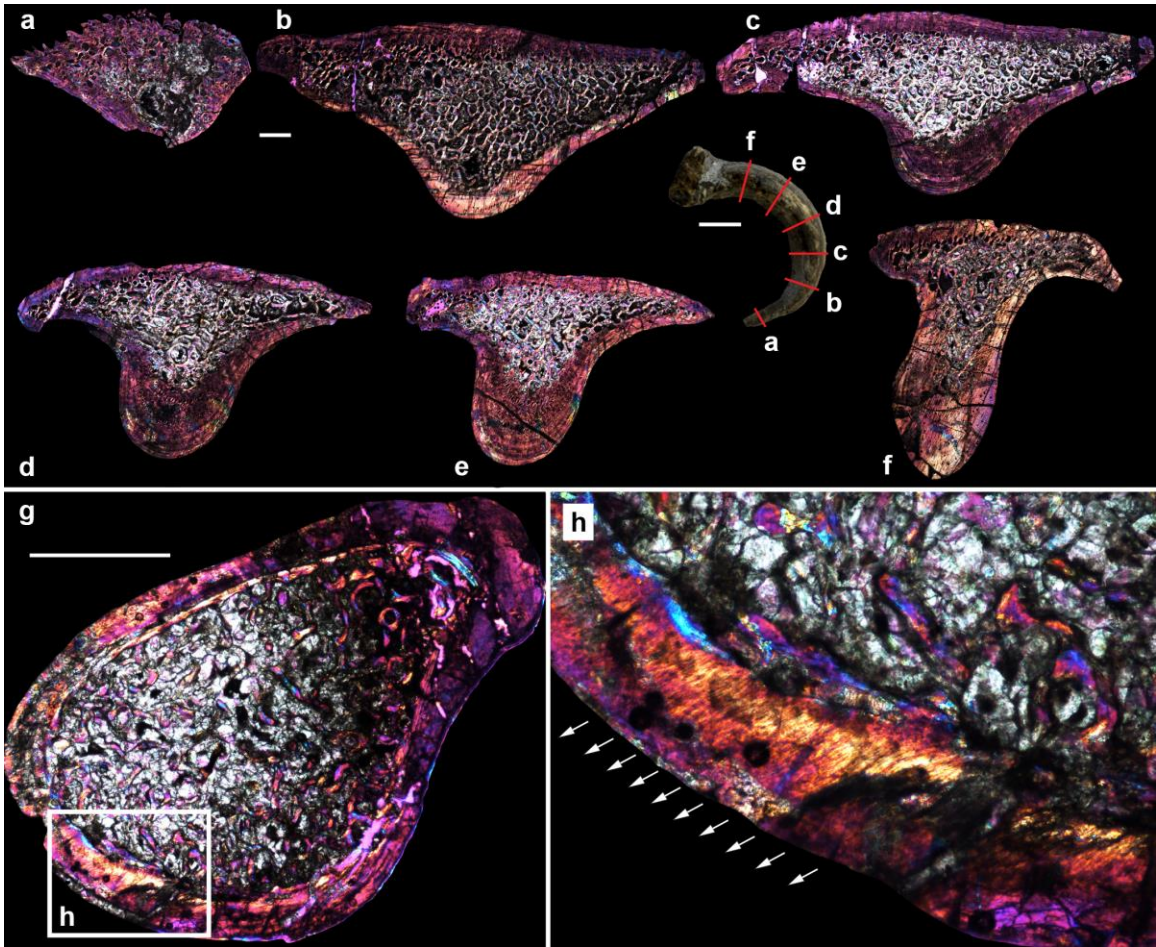
Supplementary Figure 2: Respiratory muscles of a snapping turtle, *Chelydra serpentina* (Cryptodira: Chelydridae). **a**, Computed tomography rendering of a specimen (Uncatalogued University of Utah) in ventral view with different regions segmented: skeleton (white and gray), lung (blue), *M. obliquus abdominis* (pink) and *M. transversus* (red). **b**, Diagram of the muscular ventilatory apparatus in ventral view with the plastron removed. Redrawn from Mitchell and Morehouse⁸. **c-e**, Digital surface models of the shell, lungs, trachea and ventilatory muscles in left caudolateral (**c**), caudal (**d**) and caudal (**e**) views.

(**d**), and left craniolateral (**e**) views with different anatomical components segmented as in **a**. Lu = lung; Oa = *M. oblique abdominis*; Ta = *M. transversus abdominis*; Tr = trachea; Ap = tendinous aponeurosis; Tt = *M. transversus thoracis*.



Supplementary Figure 3. Histological preparations from extinct amniotes showing the presence of Sharpey's fibers (ShF) on both the cranial and caudal edges of the dorsal ribs that have *Mm. intercostales* attachment sites. a-g, Histological cross-section of a dorsal rib of a specimen (MRF 254; Scale bar = 5 mm) of *Thescelosaurus neglectus* (Archosauria) in normal light with close-up images of the cranial (b-d) and caudal (e-g) edges of the rib. Scale bar = 1 mm. h-m, Histological cross-section of a dorsal rib of a specimen (BP/1/7280; Scale bar = 5 mm in h and k) of *Pareiasaurus* sp.

(Parareptilia) under polarized light with close-up images of the cranial (**i, j**) and caudal edges (**l, m**) under cross-polarized (**i, l**) and polarized (**j, m**) light. Scale bar = 200 μm .



Supplementary Figure 4: Histological preparations of the right fifth dorsal rib and a limb fragment of a specimen (BP/1/7024) of *Eunotosaurus africanus* in cross section under polarized light. a-f, The right fifth dorsal rib shows no signs of Sharpey's fibers (ShF) along the entire length of the rib. Scale bar = 1 cm. g, h, A limb fragment from the same specimen preserves ShF (white arrows in close-up view in h) and indicates the lack of ShF in the dorsal rib is not the result of diagenetic alterations. Scale bar = 1 cm.

Supplementary Discussion : Homology and terminology of the respiratory muscles in turtles

The body wall of amniotes is composed of a complicated set of muscles that is subdivided into an epaxial and a hypaxial group¹³. The hypaxial group plays a crucial role in breathing, but is also involved in locomotion, which results in a mechanical conflict between respiration and locomotion^{8,9}. Of these hypaxial muscles, which are divided into layers with different fiber orientations¹³, the abdominal *M. obliquus* and abdominal *M. transversus* are responsible for lung ventilation in extant turtles. Other hypaxial muscles, such as the *Mm. intercostales*, are reduced and eventually lost in embryonic ontogeny (Fig. 1b to c, 2, and Supplementary Figs. 1-2)³. The *M. obliquus* and *M. transversus* are found in all turtles¹⁴ and are active during lung ventilation⁶. See Table 1 for a summary of the main muscles and their synonymy.

M. obliquus

In most extant amniotes, the abdominal *M. obliquus* occurs as a *M. obliquus externus* lateral to the ribs and as a *M. obliquus internus* medial to them. Both *M. obliquus* groups can be further subdivided¹³. The *M. obliquus*-complex extends between the shoulder and pelvic girdles and originates from tendinous sheaths attached to each dorsal rib (Fig. 2d) in tuataras (*Sphenodon punctatus*)³². A nearly identical condition is found in squamates^{32,56} and is inferred to represent the basal amniote condition². The *M. obliquus* is innervated by the caudal intercostal nerves^{S1}.

In turtles a single element from the abdominal *M. obliquus* group, usually designated the *M. obliquus abdominis*⁵ (but see the *M. abdominis lateralis* of Ogushi¹⁶),

is present (Fig. 1b to c, 2a to c, and Supplementary Figs. 1-2). Although Rathke⁴⁵ homologized the muscle in question to the *M. obliquus internus* (as *M. obliquus internus abdominis*), its derivation, in part, from the *M. obliquus externus* cannot be excluded. Indeed, the fragmentary appearance of the chelonian *M. obliquus abdominis* (Supplementary Fig. 1a) suggests it may derive from multiple sources, although additional developmental data are needed. The chelonian *M. obliquus abdominis* is a highly modified, inverted cup-shaped muscle located cranial to each hindlimb that originates on the caudolateral portion of the carapace and plastron and inserts on the aponeurosis of the *M. transversus*⁶ (Fig. 1b to c, 2a to c, and Supplementary Figs. 1-2) with a dorsal attachment to the ventral surface of the caudal aspect of the *M. transversus*. As in other amniotes, it is innervated by the caudal intercostal nerves⁵.

M. transversus

In tetrapods, the abdominal *M. transversus* is the deepest layer of the hypaxial body wall muscles. In *Sphenodon punctatus* the *M. transversus* (Fig. 2e to f) lies just superficial to the peritoneum, separated by the transversalis fascia, and extends continuously between the first dorsal rib that connects to the sternum through the last pre-pelvic rib³². The *M. transversus* originates via tendinous sheaths from the ventral surface of each bony rib. The fibers of the three cranial-most bundles insert on the dorsolateral margin of the sternum, whereas those of the remaining bundles insert into a tendinous aponeurosis that stretches over the ventral body midline, covering the peritoneum⁵² (Fig. 2e to f) A nearly identical condition is found in squamates^{32,56} and was inferred to represent the ancestral amniote condition². The *M. transversus* can be further subdivided

into the *M. transversus thoracis* and *M. transversus abdominis*^{32,56,S2} both of which are innervated by the ventral rami of the spinal nerves³².

In most species of turtles, a tendinous aponeurosis separates the thoracic and abdominal elements of the *M. transversus* (Fig. 1b to c, 2a to c, and Supplementary Figs. 1-2). However, in some species (e.g. *Pelodiscus sinensis*; Supplementary Fig. 1b), the two elements nearly converge medially to form a complete muscular sheath, resembling the plesiomorphic condition (e.g. *Sphenodon punctatus*, Fig. 2e).

Like most previous authors, we identify the caudal portion of the *M. transversus* as the *M. transversus abdominis*^{5,7,11,12,14,48} (but see Ogushi¹⁶ who termed the entire sheath in *P. sinensis* the *M. tensor pleuro-peritonei*). The *M. transversus abdominis* originates from the posterior portion of the carapace (generally dorsal ribs five, six, or seven) and inserts into a tendinous aponeurosis ventrally (Fig. 1b to c, 2a to c, and Supplementary Figs. 1-2). The tendinous aponeurosis lies just superficial to the peritoneum. The *M. transversus abdominis* is innervated by the posterior (= ventral, see below) rami of the spinal nerves in turtles^{5,16,34}.

The turtle *M. transversus thoracis* originates on the ventral side of the cranial portion of the carapace (generally dorsal ribs two and three), and encapsulates the anterior portion of the coelomic cavity and thereby also the cranial portion of the lungs^{36,37} (Fig. 1b to c, 2a to c, and Supplementary Figs. 1-2). The *M. transversus thoracis* inserts ventrally into a tendinous aponeurosis that stretches over the entire width of the ventral peritoneum, similarly to the *M. transversus abdominis*. The *M. transversus thoracis* is innervated by the posterior (= ventral, see below) rami of the spinal nerves (Bojanus⁵ termed the muscle “*M. diaphragmaticus*”). Ogushi¹⁶ described a corresponding

innervation for the cranial portion of his “*M. tensor pleuro-peritonei*” (of which our *M. transversus thoracis* represents the anterior portion) and further stated that *Pelodiscus sinensis* has an anterior and a posterior ramus of the associated spinal nerve, the latter innervating the “*M. tensor pleuro-peritonei*.” He furthermore convincingly demonstrated the homology of these rami with the dorsal (= turtle anterior) and ventral (= turtle posterior) rami of other vertebrates, thus revealing another source for confusion regarding the homology of this muscle. Due to the upright posture of humans, human anatomists refer to the dorsal rami of the spinal nerves as the “posterior rami” and the ventral rami of the spinal nerves as the “anterior rami”^{S3}; the spatial terminology is the other way around in quadrupeds, including turtles.

A muscle named “*M. diaphragmaticus*” was recognized by Bojanus⁵ in *Emys orbicularis* (Fig. 2a). This muscle originates from the same position as the *M. transversus thoracis* (see above) and at least the dorsal portion also runs superficial to the peritoneum^{5,15,16} (Fig. 2a). However, it does not extend as far ventrally along the peritoneum as does the *M. transversus thoracis* of *Pelodiscus sinensis*. Instead it only dorsally and cranially (and partly laterally) envelops the cranial part of the lung. It is also found in the intracoelomic *Septum postpulmonale* directly ventral to the lungs and thus lies within the coelomic cavity^{15,36,37}. Unfortunately, muscles with identical names but doubtful homology have been described in turtles, mammals and crocodiles². The *M. diaphragmaticus* of mammals and crocodilians is caudal to the lungs, whereas the “*M. diaphragmaticus*” of turtles is cranial and ventral to the lungs (Fig. 1b to c, 2a to c, and Supplementary Figs. 1-2) and there is widespread agreement that these muscles are not homologous among the three groups². Rather the “*M. diaphragmaticus*” of turtles has a

similar origination on the ventral portion of the dorsal ribs, a similar position superficial to the peritoneum, and the same innervation pattern^{5,15,16} (Fig. 1b to c, 2a to c, and Supplementary Figs. 1-2) as the *M. transversus thoracis* of other amniotes, and we therefore consider it homologous to that muscle.

Other muscles used in turtle respiration:

One muscle associated with the shoulder girdle, the *M. serratus* (Fig. 2a), is also directly involved in turtle lung ventilation (Supplementary Discussion 1). This muscle was named *M. serratus magnus* by Bojanus⁵ and *M. carapaco-scapulo-coracoideus* by Ogushi¹⁶. We recommend maintaining use of the name *M. serratus* until embryological data disprove its homology to the *M. serratus* of other tetrapods, which not only exhibits a corresponding topography but is also known to serve an inspiratory function in humans^{S3}. Also the *M. pectoralis* (Fig. 2a), through its ability to retract the anterior extremities, can be recruited for ventilation. The *M. pectoralis* is only one example of a diverse suite of girdle-associated, mainly locomotory muscles, that can contribute to chelonian breathing¹⁰.

Finally, some turtles have a very thin and enigmatic muscle that is directly associated with the lungs. The *M. pulmonalis* of Hansemann⁴⁶ = *M. striatum pulmonale* of George & Shah⁴⁷ lies beneath the “*M. diaphragmaticus*” (= *M. transversus thoracis*). Its homology is unknown and its function has never been studied.

Supplementary Methods : List of material analyzed by the authors

Institutional Abbreviations: AM = Albany Museum, Grahamstown, South Africa; BP/1/ = Evolutionary Studies Institute (formerly the Bernard Price Institute), University of the Witwatersrand, Johannesburg, South Africa; BMNH = The Natural History Museum, London, UK; CM = Council for Geosciences, Pretoria, South Africa; GPIT = Paläontologische Lehr- und Schausammlung, University of Tübingen, Germany; IVPP = Institute for Vertebrate Paleontology and Paleoanthropology, Beijing, China; MRF = Marmarth Research Foundation, Marmarth ND, USA; NMQR = National Museum, Bloemfontein, South Africa; PIMUZ = Paläontologisches Institut und Museum der Universität Zürich, Zurich, Switzerland; SAM-PK = Iziko Museums of South Africa, Cape Town, South Africa; SMNS = Staatliches Museum für Naturkunde Stuttgart, Stuttgart, Germany; YPM = Yale Peabody Museum of Natural History, Yale University, New Haven CT, USA; USNM = National Museum of Natural History, Washington DC, USA; ZFMK = Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany; ZMB = Museum für Naturkunde, Berlin, Germany; ZSM = Zoologische Staatssammlung München, Munich, Germany

EXTANT TAXA

Dissections:

TESTUDINES: CRYPTODIRA

Carettochelyidae: <i>Carettochelys insculpta</i>	ZFMK 94265, fluid specimen. ZFMK 94266, fluid specimen. ZSM 374/2001, fluid specimen. Uncatalogued fresh specimen, donated by M. Baur (Reptilienauffangstation München, Munich, Germany) to M. Lambertz.
Chelydridae: <i>Chelydra serpentina</i>	USNM 324328, fluid specimen. USNM 334981, fluid specimen. ZMB uncatalogued, five fluid specimens (Fig. 6a and Supplementary Fig. 1a). University of Utah uncatalogued, fresh specimen (Fig. 1, b to c and Supplementary Fig. 2).
<i>Macrochelys temminckii</i>	ZMB uncatalogued, fluid specimen.
Emydidae: <i>Clemmys guttata</i>	ZFMK uncatalogued, fluid specimen.
<i>Trachemys scripta</i>	Rheinische Friedrich-Wilhelms-Universität Bonn uncatalogued, four fluid specimens (Fig. 6b).

Geoemydidae	<i>Sacalia quadriocellata</i>	ZFMK uncatalogued, fluid specimen.
Kinosternoidea	<i>Claudius angustatus</i>	ZMB uncatalogued, five fluid specimens.
	<i>Kinosternon acutum</i>	SMNS 3742, fluid specimen.
	<i>Kinosternon baurii</i>	SMNS 3744, fluid specimen.
		ZFMK uncatalogued, two fluid specimens.
	<i>Kinosternon flavescens</i>	ZSM 335/1978, fluid specimen.
	<i>Kinosternon hirtipes</i>	ZSM uncatalogued, two fluid specimens.
	<i>Kinosternon leucostomum</i>	SMNS 7166, fluid specimen.
	<i>Kinosternon scorpioides</i>	Uncatalogued fresh specimen, donated by Rheinische Friedrich-Wilhelms-Universität Bonn to M. Lambertz. SMNS 4658, fluid specimen.
	<i>Staurotypus triporcatus</i>	ZMB uncatalogued, fluid specimen.
	<i>Sternotherus carinatus</i>	ZSM 438/2001, fluid specimen.
		ZSM 439/2001, fluid specimen.
	<i>Sternotherus minor</i>	ZSM 440/2001, fluid specimen.
	<i>Sternotherus odoratus</i>	ZMB uncatalogued, two fluid specimens.
Platysternidae	<i>Platysternon megacephalum</i>	ZSM 380/2001, fluid specimen.
		ZMB uncatalogued, four fluid specimens.

		Uncatalogued fresh specimen, donated by M. Baur (Reptilienauffangstation München, Munich, Germany) to M. Lambertz.
Testudinidae	<i>Geochelone elegans</i>	ZFMK uncatalogued, fluid specimen.
	<i>Testudo hermanni</i>	Rheinische Friedrich-Wilhelms-Universität Bonn uncatalogued, four fresh specimens.
Trionychidae	<i>Pelodiscus sinensis</i>	PIMUZ lab#2009.72IW, fluid specimen (Supplementary Fig. 1b).
TESTUDINES: PLEURODIRA		
Chelidae	<i>Chelus fimbriatus</i>	ZFMK uncatalogued, fluid specimen
	<i>Emydura subglobosa</i>	USNM 574459, fluid specimen (Fig. 2, b to c).
Podocnemidae	<i>Podocnemis unifilis</i>	Uncatalogued fresh specimen, donated by A. S. Abe to M. Lambertz.
LEPIDOSAURIA: RHYNCHOCEPHALIA		
	<i>Sphenodon punctatus</i>	ZSM 1318/2006, fluid specimen (Fig. 2f).
FOSSIL MATERIAL		
<i>Eunotosaurus africanus</i> :		
	AM 5999:	impression of a nearly complete articulated specimen with an articulated shoulder girdle.
	BP/1/7198:	disarticulated dorsal ribs and vertebrae.
	BP/1/7027:	fragmentary dorsal ribs and vertebrae.
	BP/1/7024:	posterior two thirds of an axial skeleton (Fig. 3c and Supplementary Fig. 4).
	BP/1/6218:	posterior two thirds of an axial skeleton and portions of the pelvis.
	BP/1/5677:	fragmentary dorsal ribs and vertebrae.
	BP/1/3514:	disarticulated dorsal vertebrae and ribs.
	BP/1/3515:	disarticulated dorsal vertebrae and ribs.
	BMNH R 1968 (Holotype):	disarticulated dorsal vertebrae and associated dorsal ribs and associated limb elements.
	BMNH R 4949:	disarticulated dorsal vertebrae and associated dorsal ribs.

BMNH R 4054: nodule containing articulated dorsal vertebrae and ribs.
BMNH R 49424: nodule containing articulated dorsal vertebrae and ribs.
BMNH R 49423: highly eroded nodule containing partial dorsal vertebrae and ribs.
CM 71: articulated dorsal vertebrae and ribs.
CM 86-341: beautifully preserved partial skull, completely articulated neck with a few cervical ribs, and complete carapace (nine dorsal vertebrae and nine pairs of dorsal ribs) (Figs. 3, a to b and Fig. 4, e to j).
CM 775: articulated posterior dorsal vertebrae and ribs.
CM 777: articulated skull, neck, elongate cervical ribs, shoulder girdle, limb elements, and cranial half of carapace including dorsal vertebrae and ribs (Fig. 3d).
NMQR 3299: mostly complete skeleton including an articulated shoulder girdle.
NMQR 3466: isolated dorsal rib.
NMQR 3474: impression of a mostly articulated skeleton.
NMQR 3486: isolated dorsal rib.
NMQR 3490: isolated dorsal rib.
NMQR 3500: isolated dorsal rib.
Fransie Pienaar Museum, Prince Albert, 2014/269: mostly complete skeleton including an articulated shoulder girdle and pelvis.
SAM-PK-K207: plastically deformed, articulated dorsal ribs and vertebrae.
SAM-PK-K1132: mostly complete series of dorsal ribs and vertebrae with partial shoulder girdle.
SAM-PK-K1133: complete shell with articulated shoulder girdle.
SAM-PK-K7611: plastically deformed, articulated dorsal ribs and vertebrae.
SAM-PK-K7670: highly weathered nodule with mostly complete skeleton including cranial two-thirds dorsal ribs and vertebrae, impressions of the cervical vertebrae, and an impression of the skull.
SAM-PK-K7909: weathered nodule complete shell with articulated neck, impression of the skull, and complete shoulder girdle.
SAM-PK-K7910: plastically deformed, articulated series of dorsal ribs and vertebrae.
SAM-PK-K7911: mid-series section of articulated dorsal vertebrae and ribs.
SAM-PK-K4328: impression of back two-thirds of an articulated specimen, including dorsal ribs, vertebrae, partial pelvis, and first few caudal vertebrae.
SAM-PK-K11954: highly weathered nodule containing mid-section with dorsal vertebrae and ribs.
SAM-PK-K1509: mid-series section of articulated dorsal vertebrae and ribs.
SAM-PK-K1673: isolated dorsal rib.
USNM 23099: disarticulated dorsal ribs, vertebrae, limb elements, pelvic girdle, and carpal and tarsal elements.

Odontochelys semitestacea:

IVPP V15639: holotype, complete articulated skeleton.
IVPP V13240: paratype, complete articulated skeleton (Fig. 3, e to f).
IVPP V15653: referred specimen, incomplete disarticulated skeleton.

Proganochelys quenstedti

GPIT (uncataloged holotype): internal mold of shell showing distinct T-shaped
(cross-section) ribs.

SMNS 10012: partial carapace with internal surface and external surface preserved in
separate pieces.

SMNS 15759: crushed skull, mandibles, and right half of shell.

SMNS 16980: nearly complete skeleton.

SMNS 17203: carapace and plastron.

SMNS 17204: carapace and plastron.

Supplementary References:

1. Putz, R., in *Benninghoff Anatomie - Makroskopische Anatomie, Embryologie und Histologie des Menschen, Band 1: Zellen- und Gewebelehre, Entwicklungsbiologie, Bewegungsapparat, Herz-Kreislauf-System, Immunsystem, Atem- und Verdauungsapparat*, (eds Drenckhahn, D. & Zenker, W.) 245–324 (Urban & Schwarzenberg, München-Wien-Baltimore, 1994)
2. Bhullar, B.-A. S. A reevaluation of the unusual abdominal musculature of squamate reptiles (Reptilia: Squamata). *Anat. Rec.* **292**, 1154–1161
3. Drenckhahn, D. in *Benninghoff Anatomie - Makroskopische Anatomie, Embryologie und Histologie des Menschen, Band 1: Zellen- und Gewebelehre, Entwicklungsbiologie, Bewegungsapparat, Herz-Kreislauf-System, Immunsystem, Atem- und Verdauungsapparat*, (eds Drenckhahn, D. & Zenker, W.) 405–470 (Urban & Schwarzenberg, München-Wien-Baltimore, 1994)