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# A novel accessory respiratory muscle in the American alligator (*Alligator mississippiensis*)

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The muscles that effect lung ventilation are key to understanding the evolutionary constraints on animal form and function. Here, through electromyography, we demonstrate a newly discovered respiratory function for the iliocostalis muscle in the American alligator (*Alligator mississippiensis*). The iliocostalis is active during expiration when breathing on land at 28°C and this activity is mediated through the uncinate processes on the vertebral ribs. There was also an increase in muscle activity during the forced expirations of alarm distress vocalisations. Interestingly we did not find any respiratory activity in the iliocostalis when the alligators were breathing with their body submerged in water at 18°C, which resulted in a reduced breathing frequency. The iliocostalis is an accessory breathing muscle that alligators are able to recruit in to assist expiration under certain conditions.

## 1. Background

Crocodilians are large, semiaquatic reptiles that first appeared in the Late Cretaceous and share a common ancestor with birds. Like all amniotes, crocodilians use an aspiration pump for ventilation [1,2]. In birds and crocodilians the pump generates a unidirectional airflow through the lungs [3,4]. Inspiration, expiration or both are active processes in vertebrates and can therefore only be facilitated through muscle action [5]. There are many similarities between the respiratory systems of birds and crocodilians; their lungs are multi-chambered; the internal chambers spiral in a clockwise manner and they have an asymmetrical branching pattern [6]. And there are also similarities in the topography of the intrapulmonary bronchus, bronchi [7] and aerodynamic valves [4]. Unidirectional airflow through the lungs of alligators, akin to that seen in birds, was most likely present in the basal archosaurs from the Triassic, predating the evolution of birds [4].

The kinematics of the ribcage during ventilation in crocodilians are now well understood [8]. During exhalation, the external and parasternal internal intercostals swing the ribs inwards and backwards [9], contraction of the transversus abdominis moves the liver anteriorly and the rectus abdominus pulls the hips forward and draws the belly in. During inhalation, intercostal muscles swing the ribs outwards and forwards [9] and pelvic muscles draw back the hips. The diaphragmaticus muscle, which runs from the hip to the caudal aspect of the liver, pulls the liver back causing the lungs to inflate [9,10]. Crocodilian lungs are attached to the parietal pleura and have deep costal impressions meaning that rather than sliding forwards and backwards they will expand and contract with changes in thoracic volume [11].

Uncinate processes are projections that extend posteriorly off the proximal surface of the vertebral ribs, and are another shared characteristic between crocodilians and birds [12]. In birds these processes function as levers and are involved in inspiration and expiration through the action of the appendicocostalis and the external oblique muscles, respectively [12]. The morphology of the uncinate processes in birds varies with the degree of specialization to different forms of locomotion [13]. The presence of uncinate processes is frequently overlooked in crocodilians as these processes are cartilaginous, which may explain their absence in museum and fossil specimens. Crocodilian uncinates are morphologically distinct from those of birds being semi-circular, flattened and broad. However, similar to birds they project from the vertebral ribs, extend caudally and are associated with thoracic musculature (figure 1). The proximity to the uncinate processes suggests that the iliocostalis is a likely candidate for potential respiratory function. Of the muscles currently described and documented as having a role during respiration in the alligator (Inspiration: diaphragmaticus, ischiotruncus, ischiopubis, intercostals. Expiration: rectus abdominis, transversus abdominis, intercostals), none has any attachment to the uncinate processes. The aims of our current study were (1) to provide the first detailed anatomical description of muscle connections to the uncinate processes and (2) to determine if any muscles connected to the processes were active during ventilation in *A. mississippiensis*.

## 2. Materials and methods

We examined electromyography (EMG) activity of the iliocostalis muscle under three conditions: (1) at rest and breathing at an ambient temperature of 28°C, (2) at rest and breathing at a 10-fold lower breathing frequency while the body was submerged in a water tank at 18°C with their front legs supported on a block, keeping the head in a breathing chamber and (3) during forced expirations when making alarm distress vocalisations at an ambient temperature of 28°C. Experiments were conducted on 5-8 female alligators hatched from eggs collected in Louisiana and raised in the animal unit at the University of North Texas (mean ± SE body mass 1395±150g;

age 2years) housed in fiberglass pens (0.7x2x0.7m) in freshwater and fed commercial alligator food twice weekly. Animals were maintained on a 12h:12h light:dark cycle at 28°C, the same temperature at which the mask and barking experiments were conducted; the temperature of the water in the tank was 18°C.

Anaesthesia was induced by placing the head of the alligator into a plastic tube containing cotton gauze soaked in isoflurane (Henry Schein Animal Health, Dublin, OH, USA). The trachea was then intubated with Tygon® tubing and alligators were ventilated (5 breaths min$^{-1}$, 30mL min$^{-1}$) using a mechanical ventilator (model 665, Harvard Apparatus, Holliston, MA, USA). Isoflurane was maintained at 1.5-3% during surgery using an isoflurane vaporizer (Highland Medical Equipment, Temecula, CA, USA). Body temperature was maintained at 28±0.5°C with a heating lamp connected to a Thermistemp® temperature controller (model 71A, YSI, Yellow Springs, OH, USA). Surgery was minimally invasive and animals were fully recovered after 24 h and prior to any data collection. Following completion of all experimentation animals were killed by an overdose of pentobarbitone (150mg kg$^{-1}$, Fatal-Plus®, Vortech Pharmaceutical, Dearborn, MI, USA) and used for anatomical investigation and confirmation of EMG electrode placement. All EMG data were collected on equipment from Telemetry Research Limited® (Auckland, New Zealand). On the left side of the animals, the intercostal spaces between the 3rd and 4th dorsal ribs were located by palpating the skin above the site. A 2-3cm incision was then made to expose the target area of the iliocostalis muscle. A Dual Biopotential Transmitter (TR40BB) was then sutured onto the back of each animal at approximately the midpoint along the vertebral column. The shielded EMG electrodes (diameter 1mm) were then tunnelled under the skin from the posterior margin of the thoracic region (2-3cm from EMG implantation site) to the incision above the iliocostalis muscle. EMG electrodes were then sewn directly into the muscle belly of the iliocostalis above the uncinate processes. Data from the transmitter were telemetered to a receiving unit (TR102, filtered above 1000 and below 100Hz). All data were collected on a PowerLab® 16/35 running Chart® v7-8 (ADInstruments, Colorado Springs, CO, USA).

Breathing was monitored via a Validyne® pressure transducer (Model DP-45-16, Validyne Engineering Corp, Northridge, CA, USA) connected to a pneumotach in line with either a face mask made from a 50ml plastic beaker modified to add inlet and outlet tubes and sealed around the alligator head with Bisico® (ISO4823, Typ 3, Bielefelder Dental Silicone GmbH, Germany) when they were at rest and breathing or the outflow pipe of the breathing chamber above the water tank for at rest breathing when the body was submerged (figure S2). In both cases air was drawn through the system at 500 ml min$^{-1}$. Alarm distress vocalisations were elicited by gently tapping on the head of the alligator with two fingers and were synced to EMG traces via manually depressing a purpose built 5V trigger connected to the PowerLab®. Raw EMG data were band-pass filtered (60 – 250 Hz), rectified and smoothed (Triangular Bartlett smoothing) in LabChart®. In each respiratory cycle the processed signal was partitioned into 50 bins each for inspiration and expiration, adjusted for cycle phase duration. In each bin, the mean intensity of EMG activity (mV) and integrated signal were recorded allowing for quantification of the onset of muscle activity. ANOVA on the sum of integrated EMG across 10 breaths in each animal was used to test if muscle activity occurred in phase with either inspiration or expiration (tables S1, S2).

## 3. Results

The iliocostalis (figure 1a) is a segmented muscle, which is connected to the vertebrae and extends superficially across the vertebral ribs. It also occupies the intercostal spaces and is attached to the uncinate processes. The uncinate processes sit within the myosepta, which segment the iliocostalis muscle, superficial to and dorsal to the ventral external intercostals (figure 1b). Iliocostalis muscle fibres originate from the posterior edge of the vertebral ribs and embed the uncinate processes, making it likely that they will be removed during any de-fleshing of the skeleton. The superficial (figure 1a) and deep external oblique, extends across the intermediate and sternal rib portions, the superficial part attaches to the fascia overlying the iliocostalis. The layers of external oblique have attachments to the rectus abdominis and truncocaudalis (figure 1a).

We implanted electrodes into the body of the iliocostalis directly above the uncinate processes on the vertebral ribs (figure 1a,b). When the alligators were at rest and breathing at, 13.21±0.77 breaths per minute (bpm) at an ambient temperature of 28°C phasic activity of the iliocostalis was correlated with expiration (figure 2a, electronic supplementary material (esm) figure S1, table S1). The activity of the iliocostalis muscles is not a result of the alligators wearing the mask (figure S2). Interestingly, however, there was no phasic respiratory activity of the iliocostalis muscle when the alligators were at rest and breathing but their body submerged in a water tank at 18°C; both when the breathing frequency was fast (4.6±0.92 bpm, figure 2b) or when approximately 10-fold lower (1.57± 0.46 bpm, figure 2c, esm figure S3). The iliocostalis also demonstrated significantly greater phasic activity (≈20-fold) during expirations associated with alarm distress vocalisation when compared to the animal breathing at rest (figure 2d, esm figure S1, table S2).

## 4. Discussion

Understanding the evolution of respiratory structures is complicated as soft tissues, such as the lung rarely fossilise. Furthermore, all tetrapods evolved from fish ancestors and the divergence of the hypaxial muscles from a locomotor to breathing role creates the possibility for antagonistic functions. Overcoming Carrier's constraint, as this conflict is known [14, 15], has led to the evolution of novel accessory breathing structures and muscle recruitment across the tetrapod lineage.

Our findings demonstrate that the iliocostalis, into which the uncinate processes are embedded, is an accessory breathing muscle contributing to expiration during periods of increased ventilatory frequency by moving the rib cage inwards and backwards. It is well established that, as found here, breathing rates in crocodilians are directly correlated with body temperatures and an increase in ventilatory frequency [16]. Furthermore, in crocodilians alterations in the intensity of muscle action and recruitment of different muscles is known to relate to differences in the rate of breathing. For example, the intensity of exercise influences the mechanics of breathing [17]. When crocodilians are resting or walking the diaphragmaticus makes a limited contribution. However, as the level and intensity of effort increase (during swimming and high-speed walking) the diaphragmaticus becomes the principal contributor [17]. The iliocostalis also functions during postural support and trunk bending during locomotion in some reptiles [18]. The role described here, as an accessory breathing muscle, does not preclude the iliocostalis from a dual role in support or sagittal bending of the body during locomotion in the alligator, however this remains to be determined. In birds, for example, the appendicocostalis muscle has a dual role during respiration and locomotion [12]. Future experiments examining if there were any changes in muscle activity along the rostrocaudal axis of this muscle in crocodilians may also shed new light on this potential dual role. The importance of recruitment of the iliocostalis to assist expiration was confirmed by the relative increase in muscle activity during vocalisation. Alligators have a large vocal repertoire that depends on active control of laryngeal movements and expiration [18]. Recruitment of the iliocostalis to assist respiration is not unique to alligators; in the garter snake a slip of the iliocostalis, the M. retractor costae biceps, contributes to inhalation [19]. More common, however, is recruitment of hypaxial muscles to assist breathing in other reptiles [15,20] or mammals [21]. The absence of expiratory activity of the iliocostalis when the alligators were at rest and breathing either relatively slowly or quickly with their body submerged is likely due to the decrease in metabolic rate at the lower body temperature in the water. Under conditions of reduced aerobic demand hydrostatic pressure acting on the abdomen which shifts the liver further into the ribcage, may facilitate passive expiration [9].

Uncinate processes may be key accessory breathing structures across the tetrapod lineage. They also occur in maniraptoran dinosaurs including velociraptor and the oviraptorids and may have a role in avian-like breathing mechanics in these animals [22]. Some quadrupedal dinosaurs also possessed analogous intercostal plates thought to play a role in respiration or locomotion [23]. The trends for bipedality and enhanced cursoriality seen in dinosaurs [24] has also evolved with the crocodilian lineage [25] indicating the necessary decoupling of ventilation and locomotion was in place in the earliest archosaurs. Outside the archosaur lineage, broad, plate-like uncinate processes are also reported in the early amphibians *Eyrops* and *Dissorophus* [26]. Interestingly, given our findings for the alligator, the iliocostalis extended onto the ribs in *Eryops* [27]. Uncinate processes are also reported in one species of extinct frog, *Prosalirus biti* [28]. Any potential role of the uncinates in amphibian breathing is unknown although expiration is passive in extant anurans and passive or active in urodeles [2]. Pterosaurs have analogous structures, sternocostapophyses, thought to have a similar function to the uncinate processes during rib movements and reducing the work of breathing [29].

Demonstrating an accessory respiratory function for the iliocostalis, mediated through the uncinate processes, suggests that flexibility in the mechanics of breathing in crocodilians linked to changes in respiratory demand likely evolved in basal Archosaurs.

**Authors' contributions.** J.R.C. conceived the idea and designed the study. J.R.C., K.A.R.R., P.G.T., W.I.S., R.J.B., R.M.E., and D.A.C.II collected, interpreted and analysed data. R.M.E. facilitated alligator egg collection. All authors drafted and revised the manuscript. All authors agree to be held accountable for the content therein and approve the final version of this manuscript.

**Competing interests.** The authors declare that they have no competing interests.

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## Figure Legends

**Figure 1** (A) Diagram of the axial musculature of *A. mississippiensis* illustrating the iliocostalis (ic), deep external oblique (eop), rectus abdominus (ra) and truncocaudalis (tc). Star indicates EMG electrode placement in the ic. (B) muscles have been removed to highlight the uncinate processes, (arrows, ucp). Cranial is to the left.

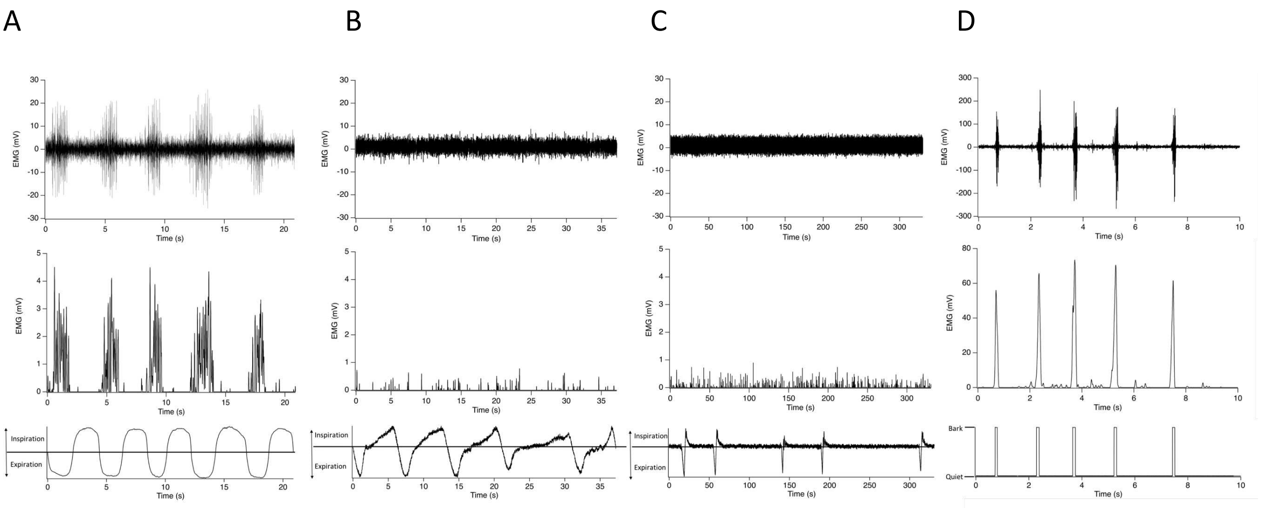

**Figure 2** Electromyography (EMG) of the iliocostalis muscle. The top trace in each panel is a representative raw unprocessed EMG signal (mV), the middle trace is processed, rectified EMG activity for all alligators (mV, band-pass filtered 60-250Hz) and the bottom trace indicates; inspiration and expiration for panels A, B & C and denotes alarm distress vocalisation events for panel D. All traces are from one alligator. The iliocostalis is active during expiration when alligators were at rest and breathing at 28°C (A), demonstrates no activity during either expiration or inspiration when at rest and breathing either rapidly (B) or more slowly (C) with the body submerged in water at 18°C and demonstrated a marked increase in activity during expiration associated with alarm vocalisations (D). Alarm vocalisation events (bottom trace, D) were indicated by manually depressing a 5V trigger upon each vocalisation shown in the bottom trace, hence the slight offset caused by the delay in reaction time to the alarm vocalisation event.

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