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

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

38

39 **1. Background**

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

51 The kinematics of the ribcage during ventilation in crocodilians are now well understood
52 [8]. During exhalation, the external and parasternal internal intercostals swing the ribs inwards
53 and backwards [9], contraction of the transversus abdominis moves the liver anteriorly and the
54 rectus abdominis pulls the hips forward and draws the belly in. During inhalation, intercostal
55 muscles swing the ribs outwards and forwards [9] and pelvic muscles draw back the hips. The
56 diaphragmaticus muscle, which runs from the hip to the caudal aspect of the liver, pulls the liver

57 back causing the lungs to inflate [9,10]. Crocodilian lungs are attached to the parietal pleura and
58 have deep costal impressions meaning that rather than sliding forwards and backwards they will
59 expand and contract with changes in thoracic volume [11].

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

77

78 **2. Materials and methods**

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

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

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

112 Breathing was monitored via a Validyne® pressure transducer (Model DP-45-16, Validyne
113 Engineering Corp, Northridge, CA, USA) connected to a pneumotach in line with either a face mask
114 made from a 50ml plastic beaker modified to add inlet and outlet tubes and sealed around the

115 alligator head with Bisico® (ISO4823, Typ 3, Bielefelder Dental Silicone GmbH, Germany) when
116 they were at rest and breathing or the outflow pipe of the breathing chamber above the water
117 tank for at rest breathing when the body was submerged (figure S2). In both cases air was drawn
118 through the system at 500 ml min⁻¹. Alarm distress vocalisations were elicited by gently tapping
119 on the head of the alligator with two fingers and were synced to EMG traces via manually
120 depressing a purpose built 5V trigger connected to the PowerLab®. Raw EMG data were band-
121 pass filtered (60 – 250 Hz), rectified and smoothed (Triangular Bartlett smoothing) in LabChart®.
122 In each respiratory cycle the processed signal was partitioned into 50 bins each for inspiration
123 and expiration, adjusted for cycle phase duration. In each bin, the mean intensity of EMG activity
124 (mV) and integrated signal were recorded allowing for quantification of the onset of muscle
125 activity. ANOVA on the sum of integrated EMG across 10 breaths in each animal was used to test
126 if muscle activity occurred in phase with either inspiration or expiration (tables S1, S2).

127 **3. Results**

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

138 We implanted electrodes into the body of the iliocostalis directly above the uncinat
139 processes on the vertebral ribs (figure 1a,b). When the alligators were at rest and breathing at,
140 13.21±0.77 breaths per minute (bpm) at an ambient temperature of 28°C phasic activity of the
141 iliocostalis was correlated with expiration (figure 2a, electronic supplementary material (esm)
142 figure S1, table S1). The activity of the iliocostalis muscles is not a result of the alligators wearing
143 the mask (figure S2). Interestingly, however, there was no phasic respiratory activity of the

144 iliocostalis muscle when the alligators were at rest and breathing but their body submerged in a
145 water tank at 18°C; both when the breathing frequency was fast (4.6 ± 0.92 bpm, figure 2b) or
146 when approximately 10-fold lower (1.57 ± 0.46 bpm, figure 2c, esm figure S3). The iliocostalis also
147 demonstrated significantly greater phasic activity (≈ 20 -fold) during expirations associated with
148 alarm distress vocalisation when compared to the animal breathing at rest (figure 2d, esm figure
149 S1, table S2).

150

151 **4. Discussion**

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

158 Our findings demonstrate that the iliocostalis, into which the uncinata processes are
159 embedded, is an accessory breathing muscle contributing to expiration during periods of
160 increased ventilatory frequency by moving the rib cage inwards and backwards. It is well
161 established that, as found here, breathing rates in crocodylians are directly correlated with body
162 temperatures and an increase in ventilatory frequency [16]. Furthermore, in crocodylians
163 alterations in the intensity of muscle action and recruitment of different muscles is known to
164 relate to differences in the rate of breathing. For example, the intensity of exercise influences the
165 mechanics of breathing [17]. When crocodylians are resting or walking the diaphragmaticus makes
166 a limited contribution. However, as the level and intensity of effort increase (during swimming
167 and high-speed walking) the diaphragmaticus becomes the principal contributor [17]. The
168 iliocostalis also functions during postural support and trunk bending during locomotion in some
169 reptiles [19]. The role described here, as an accessory breathing muscle, does not preclude the
170 iliocostalis from a dual role in support or sagittal bending of the body during locomotion in the
171 alligator, however this remains to be determined. In birds, for example, the appendicocostalis
172 muscle has a dual role during respiration and locomotion [12]. Future experiments examining if

173 there were any changes in muscle activity along the rostrocaudal axis of this muscle in
174 crocodylians may also shed new light on this potential dual role. The importance of recruitment
175 of the iliocostalis to assist expiration was confirmed by the relative increase in muscle activity
176 during vocalisation. Alligators have a large vocal repertoire that depends on active control of
177 laryngeal movements and expiration [19]. Recruitment of the iliocostalis to assist respiration is
178 not unique to alligators; in the garter snake a slip of the iliocostalis, the M. retractor costae biceps,
179 contributes to inhalation [20]. More common, however, is recruitment of hypaxial muscles to
180 assist breathing in other reptiles [15,21] or mammals [22]. The absence of expiratory activity of
181 the iliocostalis when the alligators were at rest and breathing either relatively slowly or quickly
182 with their body submerged is likely due to the decrease in metabolic rate at the lower body
183 temperature in the water. Under conditions of reduced aerobic demand hydrostatic pressure
184 acting on the abdomen which shifts the liver further into the ribcage, may facilitate passive
185 expiration [9].

186 Uncinate processes may be key accessory breathing structures across the tetrapod
187 lineage. They also occur in maniraptoran dinosaurs including velociraptor and the oviraptorids
188 and may have a role in avian-like breathing mechanics in these animals [23]. Some quadrupedal
189 dinosaurs also possessed analogous intercostal plates thought to play a role in respiration or
190 locomotion [24]. The trends for bipedality and enhanced cursoriality seen in dinosaurs [25] has
191 also evolved with the crocodylian lineage [26] indicating the necessary decoupling of ventilation
192 and locomotion was in place in the earliest archosaurs. Outside the archosaur lineage, broad,
193 plate-like uncinata processes are also reported in the early amphibians *Eryops* and *Dissorophus*
194 [27]. Interestingly, given our findings for the alligator, the iliocostalis extended onto the ribs in
195 *Eryops* [28]. Uncinate processes are also reported in one species of extinct frog, *Prosalirus biti*
196 [29]. Any potential role of the uncinate processes in amphibian breathing is unknown although expiration
197 is passive in extant anurans and passive or active in urodeles [2]. Pterosaurs have analogous
198 structures, sternocostapophyses, thought to have a similar function to the uncinata processes
199 during rib movements and reducing the work of breathing [30].

200

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

204

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206 with alligator husbandry and training.

207

208

209 **Figure Legends**

210
211 **Figure 1** (A) Diagram of the axial musculature of *A. mississippiensis* illustrating the iliocostalis (ic),
212 deep external oblique (eop), rectus abdominus (ra) and truncocaudalis (tc). Star indicates EMG
213 electrode placement in the ic. (B) muscles have been removed to highlight the uncinata processes,
214 (arrows, ucp). Cranial is to the left.

215
216 **Figure 2** Electromyography (EMG) of the iliocostalis muscle. The top trace in each panel is a
217 representative raw unprocessed EMG signal (mV), the middle trace is processed, rectified EMG
218 activity for all alligators (mV, band-pass filtered 60-250Hz) and the bottom trace indicates;
219 inspiration and expiration for panels A, B & C and denotes alarm distress vocalisation events for
220 panel D. All traces are from one alligator. The iliocostalis is active during expiration when alligators
221 were at rest and breathing at 28°C (A), demonstrates no activity during either expiration or
222 inspiration when at rest and breathing either rapidly (B) or more slowly (C) with the body
223 submerged in water at 18°C and demonstrated a marked increase in activity during expiration
224 associated with alarm vocalisations (D). Alarm vocalisation events (bottom trace, D) were
225 indicated by manually depressing a 5V trigger upon each vocalisation shown in the bottom trace,
226 hence the slight offset caused by the delay in reaction time to the alarm vocalisation event.

227
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