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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 uncinate 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 abdominus 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 uncinate processes in birds varies with the degree of specialization to different

65 forms of locomotion [13]. The presence of uncinate processes is frequently overlooked in

66 crocodilians as these processes are cartilaginous, which may explain their absence in museum

67 and fossil specimens. Crocodilian uncinates 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 uncinate 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 uncinate

74 processes. The aims of our current study were (1) to provide the first detailed anatomical

75 description of muscle connections to the uncinate 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 uncinate 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 uncinate processes. The uncinate 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 uncinate 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 uncinate

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 uncinate 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 crocodilians are directly correlated with body

162   temperatures and an increase in ventilatory frequency [16]. Furthermore, in crocodilians

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 crocodilians 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 [18]. 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   crocodilians 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 [18]. 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 [19]. More common, however, is recruitment of hypaxial muscles to
180   assist breathing in other reptiles [15,20] or mammals [21]. 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 [22]. Some quadrupedal
189   dinosaurs also possessed analogous intercostal plates thought to play a role in respiration or
190   locomotion [23]. The trends for bipedality and enhanced cursoriality seen in dinosaurs [24] has
191   also evolved with the crocodilian lineage [25] indicating the necessary decoupling of ventilation
192   and locomotion was in place in the earliest archosaurs. Outside the archosaur lineage, broad,
193   plate-like uncinate processes are also reported in the early amphibians *Eyrops* and *Dissorophus*
194   [26]. Interestingly, given our findings for the alligator, the iliocostalis extended onto the ribs in
195   *Eryops* [27]. Uncinate processes are also reported in one species of extinct frog, *Prosalirus biti*
196   [28]. Any potential role of the uncinates 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 uncinate processes
199   during rib movements and reducing the work of breathing [29].

200

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

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218

219

Figure Legends

222 **Figure 1** (A) Diagram of the axial musculature of *A. mississippiensis* illustrating the iliocostalis (ic),

223 deep external oblique (eop), rectus abdominus (ra) and truncocaudalis (tc). Star indicates EMG

224 electrode placement in the ic. (B) muscles have been removed to highlight the uncinate processes,

225 (arrows, ucp). Cranial is to the left.

226

227 A   B   C   D
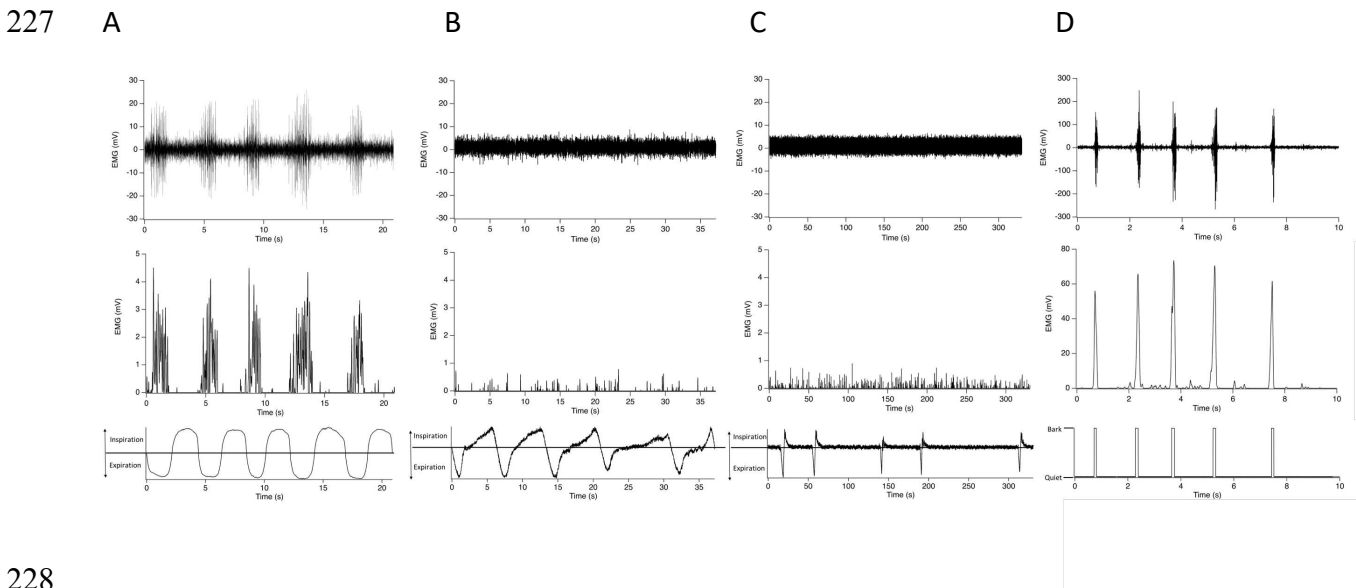
228

229 **Figure 2** Electromyography (EMG) of the iliocostalis muscle. The top trace in each panel is a

230 representative raw unprocessed EMG signal (mV), the middle trace is processed, rectified EMG

231 activity for all alligators (mV, band-pass filtered 60-250Hz) and the bottom trace indicates;

232 inspiration and expiration for panels A, B & C and denotes alarm distress vocalisation events for

233 panel D. All traces are from one alligator. The iliocostalis is active during expiration when alligators

234 were at rest and breathing at 28°C (A), demonstrates no activity during either expiration or

235 inspiration when at rest and breathing either rapidly (B) or more slowly (C) with the body

236 submerged in water at 18°C and demonstrated a marked increase in activity during expiration

237 associated with alarm vocalisations (D). Alarm vocalisation events (bottom trace, D) were

238 indicated by manually depressing a 5V trigger upon each vocalisation shown in the bottom trace,

239 hence the slight offset caused by the delay in reaction time to the alarm vocalisation event.

240

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