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The accessory role of the diaphragmaticus muscle in lung

ventilation in the estuarine crocodile *Crocodylus porosus*

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Short Title: Diaphragmaticus as accessory muscle of inspiration in crocodiles

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exercise, hypercapnia, crocodilian.

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21 **Abstract**

22

23 Crocodilians use a combination of three muscular mechanisms to effect lung

24 ventilation: the intercostal muscles producing thoracic movement, the abdominal

25 muscles producing pelvic rotation and gastralial translation, and the

26 diaphragmaticus muscle producing visceral displacement. Earlier studies suggested

27 that the diaphragmaticus is a primary muscle of inspiration in crocodilians, but

28 direct measurements of the diaphragmatic contribution to lung ventilation and gas

29 exchange have not been made to date. In this study, ventilation, metabolic rate and

30 arterial blood gases were measured from juvenile estuarine crocodiles under three

31 conditions: (i) while resting at 30ºC and 20ºC; (ii) while breathing hypercapnic

32 gases; and (iii) during immediate recovery from treadmill exercise. The relative

33 contribution of the diaphragmaticus was then determined by obtaining

34 measurements before and after transection of the muscle. The diaphragmaticus was

35 found to make only a limited contribution to lung ventilation while crocodiles were

36 resting at 30ºC and 20ºC, and during increased respiratory drive induced by

37 hypercapnic gas. However, the diaphragmaticus muscle was found to play a

38 significant role in facilitating a higher rate of inspiratory airflow in response to

39 exercise. Transection of the diaphragmaticus decreased the exercise-induced

40 increase in the rate of inspiration (with no compensatory increases in the duration

41 of inspiration), thus compromising the exercise induced increases in tidal volume

42 and minute ventilation. These results suggest that, in *C. porosus*, costal ventilation

43   alone is able to support metabolic demands at rest, and the diaphragmaticus is

44   largely an accessory muscle used at times of elevated metabolic demand.

45

46

Introduction

Crocodilians generate subatmospheric pulmonary pressures to inflate their lungs. Unlike mammals, in which the diaphragm plays a central role, crocodilians lack a muscular structure homologous or analogous to the mammalian diaphragm and a combination of three other muscular mechanisms power ventilation; namely, the intercostal, abdominal and diaphragmaticus muscles. Intercostal muscles are active during both inspiration and expiration (Gans and Clark, 1976). Inspiration is driven by cranial rotation of tripartite ribs which increases thoracic volume, whereas caudal and medial rotation of the ribs decreases thoracic volume during expiration (Claessens, 2009). The abdominal muscles act to alter abdominal volume either by displacing the liver cranially during expiration or by providing room for the caudal displacement of the liver during inspiration. The rectus abdominis and transversus abdominis muscles are active during inspiration and expiration (Gans and Clark, 1976; Naifeh et al., 1970), particularly during exercise (Farmer and Carrier, 2000a). The rectus abdominis muscle (and possibly the transversus abdominis) also rotate the pubic bones in the craniodorsal direction and contribute to decreasing abdominal volume during expiration (Farmer and Carrier, 2000a). The ischiopubis and ischiotruncus muscles act to increase abdominal volume during inspiration by rotating the pubic bones ventrally (Farmer and Carrier, 2000a).

69 The diaphragmaticus muscle of crocodilians is not homologous to the mammalian

70 diaphragm (Gans, 1970, Klein and Owerkowicz, 2006) and its main function may

71 have been non-respiratory (Uriona and Farmer, 2008) as crocodilian ancestors

72 became secondarily adapted to life in water (Seymour et al., 2004).   The

73 diaphragmaticus has been well described in caiman and alligator (Boelaert, 1942;

74 Claessens, 2009; Farmer and Carrier, 2000a; Gans and Clark, 1976; Naifeh et al.,

75 1970; Uriona and Farmer, 2006). In alligators, the two paired strap-like muscles

76 originate on the ischia and on the last gastralia and insert onto a connective tissue

77 sheath that surrounds the liver (Farmer and Carrier, 2000a). In caiman (Gans and

78 Clark, 1976) and crocodiles (Munns, pers. obs.), the origin of the diaphragmaticus

79 muscle differs slightly from that in alligators and encompasses the ischia and the

80 pubis. Contraction of the diaphragmaticus muscle pulls the liver caudally,

81 increasing thoracic volume and facilitating inspiration (Farmer and Carrier, 2000a;

82 Gans, 1971; Gans and Clark, 1976; Naifeh et al., 1970). The caudocranial

83 translation of the liver during the ventilatory cycle has been likened to a piston, and

84 hence the term 'hepatic piston pump' has been coined to describe the mechanism

85 powered by the diaphragmaticus muscle (Gans and Clark, 1976). The hepatic

86 piston pumping has been shown to effectively decouple terrestrial locomotor

87 mechanics from breathing mechanics in the American alligator (Farmer and

88 Carrier, 2000b), and thus may provide an functional advantage during exercise

89 compared to costal ventilation alone.

90 Previous studies have shown that lung ventilation in crocodilians can be effected by

91 various combinations of muscular mechanisms. In submerged caiman, lung

92   ventilation was achieved solely by use of the hepatic piston pump (Gans and Clark,

93   1976) with costal muscle activity being neither regular or obligatory (Gans, 1971).

94   In juvenile alligators on land, lung ventilation was achieved by a combination of

95   both costal and hepatic piston mechanisms (Farmer and Carrier, 2000a). These

96   studies suggest that the diaphragmaticus muscle plays a primary role in inspiration.

97   This argument is further supported by recent videoradiographic measurements of

98   lung volume in resting alligators (Claessens, 2009), where the diaphragmatic

99   contribution to lung inflation has been determined to range from 36-61% of

100   inspired tidal volume.

101

102   That the diaphragmaticus muscle is not absolutely necessary for effective lung

103   ventilation at rest has been demonstrated in hatchling and juvenile alligators with a

104   surgically transected diaphragmaticus (Hartzler et al., 2004; Uriona and Farmer,

105   2006). The loss of diaphragmatic function was found to result in significant

106   reductions in maximal inspiratory flow rate, but whether this adversely affected

107   respiratory gas exchange was not quantified.

108

109   The goal of our study is to determine the inspiratory importance of the

110   diaphragmatic muscle in juveniles of the estuarine crocodile (*Crocodylus porosus*

111   Schneider 1801). Extant crocodilians genera show differences in their habitat and

112   activity preferences (Webb et al., 1993), thus the relative contribution of the

113   diaphragmaticus muscle to lung ventilation may vary between groups. So far,

114   however, only *Alligator* and *Caiman* have been studied from this perspective. In

115   contrast to previous studies at a single temperature and at rest, we measured the

116   contribution of the diaphragmaticus muscle to lung ventilation, and its effect on gas

117   exchange, in crocodiles under altered respiratory demand associated with decreased

118   body temperature, recovery from forced exercise, and hypercapnia.

119

120   **Materials and Methods**

121   <u>Animals</u>

122   Five estuarine crocodiles (*Crocodylus porosus* Schneider 1801) of indeterminate

123   sex were obtained from the Koorana Crocodile Farm, Rockhampton, Australia, and

124   kept in aquaria with a thermal gradient (27-33ºC), full spectrum lighting

125   (14L:10D), free access to water and were fed a diet of whole rodents, fish, and

126   chicken pieces. Body weight ranged from 0.60 to 1.42 kg (mean ± s.e.m., 0.98 ±

127   0.19 kg).

128

129   <u>Surgical procedure</u>

130   Crocodiles were anaesthetised with halothane (Veterinary Companies of Australia,

131   Artarmon, NSW, Australia), intubated and artificially ventilated (Model 661,

132   Harvard Apparatus, Millis, Massachusetts, USA) with room air that had been

133   passed through a vapourizer (Fluotec 3, Cyprane Limited, Keighley, Yorkshire,

134   England). The vapourizer was initially set at 4-5% for induction of anaesthesia,

135   and was then reduced to 1-2% for surgical maintenance. Incision was made in the

136   skin and cervical muscles were carefully blunt-dissected to expose the underlying

137   carotid artery. The carotid artery was cannulated with heparinised polyethylene

138   tubing (I.D. 0.023, O.D. 0.038mm Microtube Extrusions, North Rocks, NSW,

139   Australia) and the tubing looped once prior to exiting the wound where it was

140   secured to the skin using two sutures. Incision site was closed with silk sutures.

141   EMG electrodes (0.05mm diameter copper wire) were inserted bilaterally (and

142   perpendicular to muscle fiber orientation) into the diaphragmaticus muscle via a 3-

143   4cm midline abdominal incision. A copper ground electrode (with frayed ends)

144   was also placed in the abdominal cavity. Leads from the electrodes were

145   subcutaneously tunneled to a dorsal exit just caudal to the hind limb. All incisions

146   were closed with interrupted sutures and treated with cyanoacrylate tissue adhesive

147   (Vetbond, 3M, St Paul, MN, USA). The cannula and lead wires were coiled and

148   taped to the back of the animal. Artificial ventilation with room air was continued

149   until the crocodile regained consciousness and initiated spontaneous breathing.

150   Intramuscular injections of the antibiotic Duplocillin (Intervet Australia, Bendigo

151   East, Victoria, Australia), and the analgesic Temgesic (Buprenophine, Reckitt

152   Benckiser, West Ryde, NSW, Australia) were given at the conclusion of surgery.

153   Duplocillin injections were repeated every second day after surgery. A minimum

154   recovery period of two days was allowed before experiments commenced.

155

156   Transection of the diaphragmaticus muscle

157   After the first set of experiments, crocodiles were anaesthetised for a second time

158   as described above. The diaphragmaticus muscle was exposed via the previous

159   incision site, and transected by surgically severing the muscle bellies from their

160   origin on the pubis and the ischia. After the incision was closed and animals

161   recovered as described above. Complete transection of the diaphragmaticus muscle

162   was confirmed for each animal by post mortem examination at the end of the study.

163

164   <u>Lung Ventilation and gas exchange</u>

165   Ventilation was measured using a mask constructed from the base of a 20ml

166   centrifuge tube, fitted with a plastic Y connector to which flexible tubing was

167   attached. The mask was placed over the snout of the crocodile, covering the

168   nostrils and the mouth and sealed to the body with a dental polyether impression

169   material (Impregum F, Henry Schein Halas, Brisbane, QLD, Australia). A pump

170   (Reciprotor AB, Sweden) pushed fresh room air through the mask at a constant

171   flow rate of 0.8-1.2 $L.min^{-1}$, depending on the size of the crocodile, controlled with

172   a mass flowmeter (Sierra Instruments, Monterey, CA, USA). Care was taken to

173   ensure that the flow rate though the mask exceeded the rate of inspiration, in order

174   to prevent rebreathing. Alterations in airflow due to ventilation were measured

175   using a pneumotachograph (MLT10L Respiratory Flow Head, AD Instruments,

176   Bella Vista, NSW, Australia) placed downstream of the mask, such that expirations

177   caused an decrease in airflow and inspiration caused a increase in airflow. Pressure

178   gradients induced by alterations in airflow across the pneumotachograph were

179   monitored using a differential pressure transducer connected to a carrier

180   demodulator (MP-45-1 and CD15, respectively; Validyne, Northridge, CA, USA).

181   The signal from the differential pressure transducer was calibrated by injecting and

182   withdrawing known volumes of gas from the sealed mask and was integrated to

183   obtain tidal volumes. Gas exiting the mask was sub-sampled, passed through the

184   desiccant anhydrous calcium sulfate (Drierite, Hammond, Xenia, OH, USA) and

185   analysed for fractional concentrations of O_2 (F_{O_2}) and CO_2 (F_{CO_2}) (ML206 gas

186   analyser, AD Instruments, Bella Vista, NSW, Australia). The rates of oxygen

187   consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were determined as

188   previously described by (Frappell et al., 1992). Briefly,

189    $\dot{V}_{O_2} = $ flow` x $(F`_{IO_2} - F`_{EO_2}) / (1 - F`_{IO_2})$

190   where the subscripts I and E represent incurrent and excurrent gas, respectively, and

191   the superscript ` (prime) represents dry CO_2-free gas. CO_2 was mathematically

192   scrubbed using $F`_{O_2} = F_{O_2} / (1 - F_{CO_2})$.

193    $\dot{V}_{CO_2} = $ flow` x $(F`_{ECO_2} - F`_{ICO_2}) / (1 - F`_{ICO_2})$

194   where prime ` represents dry O_2-free gas. Metabolic gas values are reported at

195   STPD (standard temperature and pressure, dry).

196

197   Breathing patterns were analysed in terms of tidal volume (V_T), breathing

198   frequency (*f*), minute ventilation ($\dot{V}_E = V_T$ x *f*), inspiratory and expiratory durations

199   (T_I and T_E), the duration of the non ventilatory period (T_{NVP}), rate of inspiratory

200   airflow (V_{TI}/T_I), air convection requirements for O_2 (ACR $O_2 = \dot{V}_E / \dot{V}_{O_2}$) and CO_2

201   (ACR $CO_2 = \dot{V}_E / \dot{V}_{CO_2}$) and respiratory exchange ratio (RER $= \dot{V}_{CO_2} / \dot{V}_{O_2}$). For

202   each test condition, an average of 40 consecutive breaths were analysed and

203   ventilatory volumes are reported at BTPS (body temperature and barometric

204   pressure, saturated).

205

<u>Blood gases</u>

The arterial blood partial pressures of O_2 (PaO_2) and CO_2 ($PaCO_2$) and pH were measured

with BMS 3 Mk 2 and PHM 73 (Radiometer, Denmark), respectively, at the appropriate

test temperature (20ºC or 30ºC) via small blood samples (250-300μL) taken from the

arterial cannula and stored anaerobically on ice. The electrodes were calibrated before

and after each measurement. PaO_2 and $PaCO_2$ were measured every 30 s over 3 min and

regressed back to time zero to account for drift and/or O_2 consumption by the electrode;

pH was measured in incremental volumes of blood until the variation between successive

measurements was less than 0.005 units. The arterial oxygen content, CaO_2, of each

blood sample was determined on a 10μL subsample of blood using a galvanic cell

(Oxygen Content Analyser, OxyCon, University of Tasmania, Australia). Lactate

concentration was determined by an Accusport analyser (Boehringer Mannheim,

Mannheim, Germany) and haemoglobin concentration by the HemoCue analyser

(HemoCue AB, Ängelholm, Sweden). Note that neither analyser had been validated for

use with reptile blood.

<u>Electromyography</u>

Electromyographic signals were amplified and recorded using a Powerlab data

acquisition system (Model 8/30, AD Instruments, Bella Vista, NSW, Australia) and

analyzed using Powerlab Chart Pro software (AD Instruments, Bella Vista, NSW,

Australia).

228   <u>Experimental protocol</u>

229   Crocodiles were fasted for 7 days prior to surgery (to ensure a post absorptive state)

230   and were held at the 30ºC for 2-3 days prior to experimentation (to ensure stable

231   respiratory and metabolic parameters). At the time of the experiment the body

232   temperature of the crocodiles was monitored via a thermocouple inserted ~ 5cm

233   into the cloaca (temperature pod, AD Instruments, Bella Vista, NSW, Australia). A

234   mask was fitted, the cannula and lead wires connected and the crocodile was placed

235   on a treadmill belt. The crocodile was left on the stationary treadmill belt for at

236   least one hour to obtain resting measurements for all variables at 30ºC (the effects

237   of handling and instrumentation have previously been shown to be non significant

238   after 60 mins) (Munns, 2000) . Reductions in respiratory drive were induced by

239   lowering body temperature. The room temperature was slowly reduced over 2-3

240   hours until the crocodile's body temperature reached 20ºC. Ventilation, metabolic

241   rate and blood gases were measured again, once the crocodile's body temperature

242   had stabilized at 20ºC for a minimum of 60 mins. The room temperature was then

243   slowly returned to 30ºC and the crocodile's body temperature restabilized at 30ºC

244   for at least 60 mins. Increases in centrally mediated respiratory drive were induced

245   by short bouts of moderate intensity exercise or administration of hypercapnic gas

246   (5% CO_2). After a minimum period of one hour at 30ºC, the crocodile was

247   exercised on the treadmill. The exercise period consisted of a two-minute exercise

248   bout at 1.0 km.hr^{-1}. Locomotion was initiated by gently tapping the treadmill belt

249   behind the crocodile or by lightly touching the crocodile's tail. Following exercise,

250   crocodiles were allowed to rest on the treadmill for a minimum of one hour (until

251 ventilation, blood gases and lactate concentrations had returned to pre-exercise

252 values) and then exposed to 5% CO_2 for 10 minutes. The above experimental

253 protocol was then repeated no less than 48hrs after the diaphragmaticus muscle was

254 inactivated.

255

256 <u>Data collection, analysis and statistics</u>

257 All signals were collected on a computer at 1 kHz using Chart data acquisition

258 software (AD Instruments, Bella Vista, NSW, Australia). Due to the intermittent

259 and variable nature of reptilian ventilation and the low breathing frequencies

260 employed at rest, ventilatory variables were calculated from the last 10 min of the

261 rest periods. To avoid locomotor interference on recorded signals (e.g., ventilation,

262 EMG signals), calculations were made from the first 25 breaths immediately

263 following exercise.

264

265 The effect of severing the diaphragmaticus muscle on all parameters was

266 determined using paired Dunnett's test (30°C resting as the control, $P<0.05$) and

267 paired t-tests ($P<0.05$). All data presented are mean ± s.e.m.

268

269 **Results**

270 <u>Rest at 30°C</u>

271

272 Crocodiles resting at 30ºC displayed a typical crocodilian breathing pattern which

273 consisted of one or two consecutive breaths interspersed with long pauses (Fig 1A),

274 $\dot{V}_E$ (27.61±4.03mL.kg^{-1}min^{-1}), V_T (15.56±3.27mL.kg^{-1}), f (1.98±0.48 min^{-1}), $\dot{V}_{O_2}$

275 (0.83±0.24 mL.kg^{-1}min^{-1}) , $\dot{V}_{CO_2}$ (0.70±0.19 mL.kg^{-1}min^{-1}), ACR O_2 (47.00±21.06),

276 ACR CO_2 (52.99±22.17) and RER (0.87±0.04) (Figs 2-5). EMG activity from the

277 diaphragmaticus muscle was typically associated with ventilation when crocodiles

278 were quietly resting at 30ºC (Fig 1).

279

280 At this temperature, transection of the diaphragmaticus muscle did not induce any

281 significant alterations in the ventilatory, respiratory or blood gas variables (Figs 6-

282 7, Table 1).

283

284 Rest at 20°C

285

286 A lower body temperature (T_B) altered the breathing pattern by increasing Tnvp

287 and T_I (Fig 2). Decreases in $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ (Fig 4) and V_{T_I}/T_I also accompanied a

288 decrease in T_B. Diaphragmatic EMG activity was not always evident during

289 inspiration, but when EMG activity was present, it was associated with inspiratory

290 flow (Fig 1B). At 20°C, transection of the diaphragmaticus muscle induced a

291 significant increase in V_T, with no change in any other ventilatory, respiratory or

292 blood gas parameter (Table 1).

293

294 Post-exercise recovery at 30°C

295   During the immediate recovery from treadmill exercise, minute ventilation ($\dot{V}_E$)

296   increased 9 fold (Fig 4), tidal volume (V_T) 2.7-fold (Fig 2), breathing frequency (*f*)

297   3.3-fold (Fig 3), rate of oxygen consumption ($\dot{V}_{O_2}$) 2.5-fold (Fig 4) and rate of

298   carbon dioxide production ($\dot{V}_{CO_2}$) 5.8-fold (Fig. 4), while blood lactate

299   concentration rose 5.6-fold from 0.77 ± 0.43mmol.L^{-1} to 4.27 ± 0.95mmol.L^{-1} (Fig 7).

300   The increase in V_T was achieved via both a 1.9-fold increase in the rate of

301   inspiratory flow (V_{T_I}/T_I) and a 1.6-fold increase in inspiratory time (T_I, Fig 2).

302   While PaO_2 remained unaltered by exercise, $PaCO_2$ significantly decreased (Fig 6).

303

304   All animals completed the exercise period both before and after inactivation of the

305   diaphragmaticus muscle. Exercise in crocodiles with an inactivated

306   diaphragmaticus muscle resulted in a reduction in the exercise induced elevation in

307   V_{T_I}/T_I , resulting in lower V_T (Fig 2) and $\dot{V}_E$ (Fig 4) compared to the same

308   crocodiles with intact diaphragmaticus muscles. $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were not

309   significantly elevated in crocodiles with inactivated diaphragmaticus muscles (Fig

310   4), and no significant alterations in blood gases were measured (Fig 6-7).

311

312   <u>Hypercapnia at 30°C</u>

313

314   At rest, inhalation of normoxic air with 5% CO_2 increased $\dot{V}_E$ 1.5-fold (Fig 4) via

315   1.5-fold increase in T_I and a 2.2-fold increase in V_T (Fig.2). There were no

316   significant alterations in T_{NVP} or f (Fig 3) or any other ventilatory parameter (Fig

317   5). EMG activity from the diaphragmaticus muscle was present during hypercapnic

318   exposure, however not all ventilations were associated with diaphragmaticus

319   activity (Fig 1). Transection of the diaphragmaticus muscle did not significantly

320   alter any ventilatory parameter during hypercapnic exposure (Table 1).

321

322   **Discussion**

323   Inactivation of the diaphragmaticus muscle in juvenile *Crocodylus porosus* did not induce

324   any significant alterations in ventilation, gas exchange or arterial blood gases at 30ºC,

325   20ºC or following inhalation of 5% CO_2 (Table 1). Loss of diaphragmatic function

326   disabled the hepatic piston pump, thus aspiration could only be achieved via alterations in

327   intercostal or abdominal muscle activities. The resting breathing patterns of crocodiles in

328   this study at both 20°C and 30°C, and in response to hypercapnia, were similar, both

329   before and after surgery, to those previously measured on juvenile alligators and

330   crocodiles under similar conditions (Farmer and Carrier, 2000c; Hartzler et al., 2006a;

331   Munns et al., 1998; Munns et al., 2005). This suggests that the surgical intervention did

332   not adversely alter the animals' breathing patterns and the consistency of ventilatory and

333   metabolic data both before and after surgery precluded the need for sham operated

334   controls.

335

336   Our results suggest that activity of the inspiratory muscles (such as the intercostals,

337   trapezius, anterior serratus and derived hypobranchial muscles of the neck) is able to

338   maintain ventilation, thus maintaining arterial oxygenation to support metabolic rate in

16

339 the absence of a functional hepatic piston pump. As such, they support the argument that

340 the diaphragmaticus muscle is an accessory, not primary, muscle of inspiration in

341 crocodiles.

342 Variation in respiratory muscle activity of the diaphragmaticus appears to exist based on

343 the physical environment and physiological condition of the crocodilians. It may vary in

344 animals on land versus in water, at rest versus undergoing exercise. Earlier studies

345 reported that intercostal muscle activity was not regular or obligatory during ventilation

346 in submerged caiman (Gans, 1971; Gans and Clark, 1976), whereas others reported that

347 lung ventilation can be effected solely by the use of the intercostal musculature in

348 juvenile alligators on land (Hartzler et al., 2004; Uriona and Farmer, 2006). Uriona and

349 Farmer (2006) also demonstrated that transection of the diaphragmaticus muscle did not

350 alter the maximum inspiratory volume, expired volume, inspiratory or expiratory times.

351 The same authors also propose that the diaphragmaticus muscle may have a limited

352 contribution to ventilation in fasted, standing alligators.   The differential role of the

353 diaphragmatic activity in an aquatic versus terrestrial environment has been highlighted

354 by Uriona and Farmer's (2008) findings that the diaphragmaticus is recruited in alligators

355 to control buoyancy and pitch during diving (Uriona and Farmer, 2008).

356

357 Some of the variation reported in activity of the diaphragmaticus and intercostal muscles

358 may be due to the use of different sized animals in the various studies. Relatively large

359 (up to 7.5kg) submerged caimans were used in studies that reported low EMG activity of

360 the intercostals and a high reliance on the diaphragmaticus muscle for inspiration (Gans,

361 1971; Gans and Clark, 1976). Videoradiographic studies in juvenile alligators (mass 0.72-

362 2.09kg) estimated that 36-61% of tidal volume was attributable to diaphragmaticus

363 activity and approximately 40% attributable to costosternal activity (Claessens, 2009),

364 though it should be noted that these estimates were calculated for tidal volumes 2-4 fold

365 larger than those measured at rest in this study. While the diaphragmaticus muscle is

366 well developed in adults, it is thin and translucent in juvenile crocodilians (pers. obs.).

367 Future investigations are needed to examine if the contribution of the diaphragmaticus

368 muscle to ventilation increases with age in crocodilians and whether any age related

369 increase in diaphragmaticus muscle recruitment is related to hypertrophy of the muscle or

370 to alterations in chest wall compliance.

371

372 Post exercise recovery caused significant alterations in ventilatory and respiratory

373 parameters ($\dot{V}_E$, V_T, f, V_{T_I}/T_I, T_I, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, Figs 2-5) and arterial lactate (Fig 7) in

374 crocodiles with an intact diaphragmaticus muscle. The changes in ventilation and

375 metabolic rates were not as extensive as those previously reported in exercising juvenile

376 alligators (Farmer and Carrier, 2000b; Munns et al., 2005). The discrepancy of our results

377 with those of earlier reports, however, is not surprising given differences in species used

378 (*Crocodylus* versus *Alligator*), experimental protocol (2-min period versus exhaustive

379 exercise), and acclimation to treadmill (none versus extensive). The aim of this

380 experiment was not to achieve maximum treadmill performance but rather to test if

381 adequate ventilation was maintained during elevated respiratory drive in the absence of a

382 functional hepatic piston pump.

383

384   Transection of the diaphragmaticus resulted in a reduced capacity for exercise recovery to

385   elevate VT_I/T_I (Fig 2), thus limiting the elevations in V_T (-19%) and $\dot{V}_E$ (-39%, Fig 4),

386   compared to the same crocodiles with an intact diaphragmaticus. Interestingly, post

387   exercise-induced elevations in V_T were achieved via increases in both VT_I/T_I and T_I,

388   whereas hypercapnia-induced increases in V_T of similar magnitude were supported solely

389   by increases in T_I. Increases in T_I reflect a delay in the centrally integrated inspiratory

390   "off-switch" (Munns et al., 1998) and as such are unlikely to be altered by the transection

391   of the diaphragmaticus muscle. However, increases in VT_I/T_I likely reflect an increase in

392   respiratory muscle recruitment, thus increasing the rate of inspiratory airflow. Effective

393   recruitment of the diaphragmaticus muscle to increase inspiratory airflow rates was

394   prevented in crocodiles with inactivated hepatic piston pumps, and thus V_T and $\dot{V}_E$ were

395   compromised during the recovery from exercise. VT_I/T_I was also impaired following

396   transection of the diaphragmaticus muscle in juvenile postprandial alligators (Uriona and

397   Farmer, 2008), thus the proposed role of the diaphragmaticus muscle in increasing

398   inspiratory airflow rates appears to include not only exercising but also digesting

399   crocodilians.

400

401   Under laboratory conditions, exercise in crocodilians is predominantly anaerobic; arterial

402   lactate concentrations increased by 5.6-fold after moderate activity in this study (Fig 7)

403   and by 16-fold following exhaustive exercise in alligators (Hartzler et al., 2006b). While

404   respiratory parameters tend to increase with treadmill speed, cardiovascular responses

405   appear to be "all or nothing" with maximal increases in heart rate, central venous

406   pressure, arterial blood pressure and venous return reached early in the exercise period,

407   and no further elevations triggered by increasing treadmill speed (Munns et al., 2005).

408   Exercise in crocodilians is also associated with a marked relative hyperventilation

409   (Farmer and Carrier, 2000b; Farmer and Carrier, 2000c; Hartzler et al., 2006b) which was

410   evident in this study by the increased ACR O_2 (Fig 5) and the decrease in $PaCO_2$ (Fig 6).

411   Exercising crocodiles rely on anaerobic metabolism which results in a low demand for

412   O_2. At the same time, a relative hyperventilation occurs during exercise and results in a

413   high O_2 supply. The combination anaerobic metabolism (thus low O_2 demand) and

414   relative hyperventilation (thus high O_2 supply) may limit the impact of the V_T and $\dot{V}_E$

415   constraints induced by transection of the diaphragmaticus muscle during exercise. Future

416   studies involving a greater range of treadmill speeds and exercise durations would be

417   required to more completely assess the contribution of the diaphragmaticus muscle (and

418   hence the hepatic piston pump) to exercise endurance.

419

420   In conclusion, the contribution of the hepatic piston pump and costal ventilation, the two

421   primary ventilatory mechanisms in crocodilians, appears to be highly plastic. In *C.*

422   *porosus*, the diaphragmaticus muscle appears to make only limited contributions to

423   maintaining ventilation, metabolic rate and arterial oxygenation at rest (both at preferred

424   and lowered body temperatures) and during increased respiratory drive induced by

425   hypercapnia. Tidal volume elevations produced by increasing the duration of inspiration

426   (as induced by hypercapnia) are not affected by inactivation of the diaphragmaticus

427   muscle. However, the diaphragmaticus muscle makes a significant contribution to

428   ventilation during the recovery from exercise, facilitating increases in inspiratory airflow

429   rates, and thus improving the increases in tidal volume and minute ventilation that would

430   otherwise be obtained.

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435

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486   **Figure 1:** Ventilatory airflow (arbitrary units) and associated diaphragmaticus muscle
487   EMG activity in one representative crocodile (mass 0.72 kg) at 30ºC (A), 20ºC (B) and
488   after hypercapnic gas exposure (C). Inspiration occurs when the airflow trace is above
489   zero and expiration when the trace is below zero. Periods of gular flutter (*) were present
490   in crocodiles at 30ºC and during hypercapnic exposure. Bar = 2 minutes.
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493   **Figure 2:** The effect of transection of the diaphragmaticus muscle on the duration of
494   inspiration (T_I), the rate of inspiration (V_{TI}/T_I) and tidal volume (V_T). Closed bars
495   indicate the control (intact diaphragmaticus) and the open bars indicate surgically-altered
496   (transected diaphragmaticus) animals. † indicates a significant difference compared to
497   30ºC in crocodiles with the same status of the diaphragmaticus muscle. * indicates a
498   significant difference compared to crocodiles with an intact diaphragmaticus muscle
499   under the same experimental conditions. Data are mean±s.e.m., n=5.
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502   **Figure 3:** The effect of transection of the diaphragmaticus muscle on the duration of
503   expiration (T_E), the duration of the non ventilatory period (T_{NVP}) and breathing frequency
504   (*f*). Closed bars indicate the control (intact diaphragmaticus) and the open bars indicate
505   transected diaphragmaticus muscles. † indicates a significant difference compared to
506   30ºC in crocodiles with the same status of the diaphragmaticus muscle. There were no
507   significant differences when comparing crocodiles with and without a functional
508   diaphragmaticus muscle under the same experimental conditions. Data are mean±s.e.m.,
509   n=5.
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512   **Figure 4:** The effect of transection of the diaphragmaticus muscle on the minute
513   ventilation ($\dot{V}_E$), the rate of oxygen consumption ($\dot{V}_{O_2}$) and the rate of carbon dioxide
514   production ($\dot{V}_{CO_2}$). Closed bars indicate the control (intact diaphragmaticus) and the open
515   bars indicate transected diaphragmaticus muscles. † indicates a significant difference
516   compared to 30ºC in crocodiles with the same status of the diaphragmaticus muscle. *
517   indicates a significant difference compared to crocodiles with a functional
518   diaphragmaticus muscle under the same experimental conditions. Data are mean±s.e.m.,
519   n=5.
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522   **Figure 5:** The effect of transection of the diaphragmaticus muscle on the respiratory
523   exchange ratio (R), the air convention requirement for oxygen (ACR O_2) and the air
524   convention requirement for carbon dioxide (ACR CO_2). Closed bars indicate the control
525   (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles.
526   † indicates a significant difference compared to 30ºC in crocodiles with the same status of
527   the diaphragmaticus muscle. * indicates a significant difference compared to crocodiles
528   with a functional diaphragmaticus muscle under the same experimental conditions. Data
529   are mean±s.e.m., n=5.

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532 **Figure 6:** The effect of transection of the diaphragmaticus muscle on the partial pressure
533 of arterial O_2 (PaO$_2$), the partial pressure of arterial CO_2 (PaCO$_2$) and the arterial O_2
534 content (CaO$_2$). Closed bars indicate the control (intact diaphragmaticus) and the open
535 bars indicate transected diaphragmaticus muscles. † indicates a significant difference
536 compared to 30ºC in crocodiles with the same status of the diaphragmaticus muscle.
537 There were no significant differences when comparing crocodiles with and without a
538 functional diaphragmaticus muscle under the same experimental conditions. Data are
539 mean±s.e.m., n=3.
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542 **Figure 7:** The effect of transected of the diaphragmaticus muscle on the arterial pH
543 (pHa), the arterial haemoglobin concentration ([Hb]a) and the arterial lactate
544 concentration ([La]a). Closed bars indicate the control (intact diaphragmaticus) and the
545 open bars indicate transected diaphragmaticus muscles. † indicates a significant
546 difference compared to 30ºC in crocodiles with the same status of the diaphragmaticus
547 muscle. There were no significant differences when comparing crocodiles with and
548 without a functional diaphragmaticus muscle under the same experimental conditions.
549 Data are mean±s.e.m., n=3.
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