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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
Keywords: ventilation, breathing pattern, oxygen consumption, blood gases,
exercise, hypercapnia, crocodilian.

21 **Abstract**

22

23 Crocodylians 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 crocodylians, 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 30°C and 20°C; (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 30°C and 20°C, 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

47 **Introduction**

48

49 Crocodilians generate subatmospheric pulmonary pressures to inflate their lungs.

50 Unlike mammals, in which the diaphragm plays a central role, crocodilians lack a

51 muscular structure homologous or analogous to the mammalian diaphragm and a

52 combination of three other muscular mechanisms power ventilation; namely, the

53 intercostal, abdominal and diaphragmaticus muscles.

54 Intercostal muscles are active during both inspiration and expiration (Gans and

55 Clark, 1976). Inspiration is driven by cranial rotation of tripartite ribs which

56 increases thoracic volume, whereas caudal and medial rotation of the ribs decreases

57 thoracic volume during expiration (Claessens, 2009). The abdominal muscles act

58 to alter abdominal volume either by displacing the liver cranially during expiration

59 or by providing room for the caudal displacement of the liver during inspiration.

60 The rectus abdominis and transversus abdominis muscles are active during

61 inspiration and expiration (Gans and Clark, 1976; Naifeh et al., 1970), particularly

62 during exercise (Farmer and Carrier, 2000a). The rectus abdominis muscle (and

63 possibly the transversus abdominis) also rotate the pubic bones in the craniodorsal

64 direction and contribute to decreasing abdominal volume during expiration (Farmer

65 and Carrier, 2000a). The ischiopubis and ischiotruncus muscles act to increase

66 abdominal volume during inspiration by rotating the pubic bones ventrally (Farmer

67 and Carrier, 2000a).

68

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 crocodylians 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 Animals

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 (27-33°C), 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 \pm s.e.m., $0.98 \pm$
127 0.19 kg).

128

129 Surgical procedure

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 (Buprenorphine, 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 Lung Ventilation and gas exchange

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 through 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 a decrease in airflow and inspiration caused an 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 O₂ (F_{O₂}) and CO₂ (F_{CO₂}) (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,

$$189 \quad \dot{V}_{O_2} = \text{flow}^{\prime} \times (F^{\prime}IO_2 - F^{\prime}EO_2) / (1 - F^{\prime}IO_2)$$

190 where the subscripts I and E represent incurrent and excurrent gas, respectively, and
191 the superscript \prime (prime) represents dry CO₂-free gas. CO₂ was mathematically
192 scrubbed using $F^{\prime}O_2 = F_{O_2} / (1 - F_{CO_2})$.

$$193 \quad \dot{V}_{CO_2} = \text{flow}^{\prime} \times (F^{\prime}ECO_2 - F^{\prime}ICO_2) / (1 - F^{\prime}ICO_2)$$

194 where prime \prime represents dry O₂-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 ($\dot{V}_E = V_T \times f$), 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 O₂ ($ACR_{O_2} = \dot{V}_E/\dot{V}_{O_2}$) and CO₂

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

206 Blood gases

207 The arterial blood partial pressures of O₂ (PaO₂) and CO₂ (PaCO₂) and pH were measured
208 with BMS 3 Mk 2 and PHM 73 (Radiometer, Denmark), respectively, at the appropriate
209 test temperature (20°C or 30°C) via small blood samples (250-300µL) taken from the
210 arterial cannula and stored anaerobically on ice. The electrodes were calibrated before
211 and after each measurement. PaO₂ and PaCO₂ were measured every 30 s over 3 min and
212 regressed back to time zero to account for drift and/or O₂ consumption by the electrode;
213 pH was measured in incremental volumes of blood until the variation between successive
214 measurements was less than 0.005 units. The arterial oxygen content, CaO₂, of each
215 blood sample was determined on a 10µL subsample of blood using a galvanic cell
216 (Oxygen Content Analyser, OxyCon, University of Tasmania, Australia). Lactate
217 concentration was determined by an Accusport analyser (Boehringer Mannheim,
218 Mannheim, Germany) and haemoglobin concentration by the HemoCue analyser
219 (HemoCue AB, Ängelholm, Sweden). Note that neither analyser had been validated for
220 use with reptile blood.

221

222 Electromyography

223 Electromyographic signals were amplified and recorded using a Powerlab data
224 acquisition system (Model 8/30, AD Instruments, Bella Vista, NSW, Australia) and
225 analyzed using Powerlab Chart Pro software (AD Instruments, Bella Vista, NSW,
226 Australia).

227

228 Experimental protocol

229 Crocodiles were fasted for 7 days prior to surgery (to ensure a post absorptive state)
230 and were held at the 30°C 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 30°C (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 20°C. Ventilation, metabolic
241 rate and blood gases were measured again, once the crocodile's body temperature
242 had stabilized at 20°C for a minimum of 60 mins. The room temperature was then
243 slowly returned to 30°C and the crocodile's body temperature restabilized at 30°C
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% CO₂). After a minimum period of one hour at 30°C, 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% CO₂ for 10 minutes. The above experimental
253 protocol was then repeated no less than 48hrs after the diaphragmaticus muscle was
254 inactivated.

255

256 Data collection, analysis and statistics

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 Rest at 30°C

271

272 Crocodiles resting at 30°C displayed a typical crocodylian breathing pattern which
273 consisted of one or two consecutive breaths interspersed with long pauses (Fig 1A),

274 \dot{V}_E ($27.61 \pm 4.03 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), V_T ($15.56 \pm 3.27 \text{ mL} \cdot \text{kg}^{-1}$), f ($1.98 \pm 0.48 \text{ min}^{-1}$), \dot{V}_{O_2}
275 ($0.83 \pm 0.24 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), \dot{V}_{CO_2} ($0.70 \pm 0.19 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), 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 30°C (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 T_{np}
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 $0.77 \pm 0.43 \text{ mmol.L}^{-1}$ to $4.27 \pm 0.95 \text{ mmol.L}^{-1}$ (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 Hypercapnia at 30°C

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 30°C,
325 20°C or following inhalation of 5% CO₂ (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

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 crocodylians. 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 V_{T_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 V_{T_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 V_{T_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. V_{T_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 crocodylians 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.

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420 In conclusion, the contribution of the hepatic piston pump and costal ventilation, the two
421 primary ventilatory mechanisms in crocodylians, 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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486 **Figure 1:** Ventilatory airflow (arbitrary units) and associated diaphragmaticus muscle
487 EMG activity in one representative crocodile (mass 0.72 kg) at 30°C (A), 20°C (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 30°C 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_{T_I}/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 30°C 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 30°C 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 30°C 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 30°C 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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Figure 6: The effect of transection of the diaphragmaticus muscle on the partial pressure of arterial O₂ (PaO₂), the partial pressure of arterial CO₂ (PaCO₂) and the arterial O₂ content (CaO₂). Closed bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. † indicates a significant difference compared to 30°C in crocodiles with the same status of the diaphragmaticus muscle. There were no significant differences when comparing crocodiles with and without a functional diaphragmaticus muscle under the same experimental conditions. Data are mean±s.e.m., n=3.

Figure 7: The effect of transected of the diaphragmaticus muscle on the arterial pH (pHa), the arterial haemoglobin concentration ([Hb]a) and the arterial lactate concentration ([La]a). Closed bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. † indicates a significant difference compared to 30°C in crocodiles with the same status of the diaphragmaticus muscle. There were no significant differences when comparing crocodiles with and without a functional diaphragmaticus muscle under the same experimental conditions. Data are mean±s.e.m., n=3.