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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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Abstract

Crocodilians use a combination of three muscular mechanisms to effect lung ventilation: the intercostal muscles producing thoracic movement, the abdominal muscles producing pelvic rotation and gastralia translation, and the diaphragmaticus muscle producing visceral displacement. Earlier studies suggested that the diaphragmaticus is a primary muscle of inspiration in crocodilians, but direct measurements of the diaphragmatic contribution to lung ventilation and gas exchange have not been made to date. In this study, ventilation, metabolic rate and arterial blood gases were measured from juvenile estuarine crocodiles under three conditions: (i) while resting at 30°C and 20°C; (ii) while breathing hypercapnic gases; and (iii) during immediate recovery from treadmill exercise. The relative contribution of the diaphragmaticus was then determined by obtaining measurements before and after transection of the muscle. The diaphragmaticus was found to make only a limited contribution to lung ventilation while crocodiles were resting at 30°C and 20°C, and during increased respiratory drive induced by hypercapnic gas. However, the diaphragmaticus muscle was found to play a significant role in facilitating a higher rate of inspiratory airflow in response to exercise. Transection of the diaphragmaticus decreased the exercise-induced increase in the rate of inspiration (with no compensatory increases in the duration of inspiration), thus compromising the exercise induced increases in tidal volume and minute ventilation. These results suggest that, in *C. porosus*, costal ventilation
alone is able to support metabolic demands at rest, and the diaphragmaticus is
largely an accessory muscle used at times of elevated metabolic demand.
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).
The diaphragmaticus muscle of crocodilians is not homologous to the mammalian diaphragm (Gans, 1970, Klein and Owerkowicz, 2006) and its main function may have been non-respiratory (Uriona and Farmer, 2008) as crocodilian ancestors became secondarily adapted to life in water (Seymour et al., 2004). The diaphragmaticus has been well described in caiman and alligator (Boelaert, 1942; Claessens, 2009; Farmer and Carrier, 2000a; Gans and Clark, 1976; Naifeh et al., 1970; Uriona and Farmer, 2006). In alligators, the two paired strap-like muscles originate on the ischia and on the last gastralia and insert onto a connective tissue sheath that surrounds the liver (Farmer and Carrier, 2000a). In caiman (Gans and Clark, 1976) and crocodiles (Munns, pers. obs.), the origin of the diaphragmaticus muscle differs slightly from that in alligators and encompasses the ischia and the pubis. Contraction of the diaphragmaticus muscle pulls the liver caudally, increasing thoracic volume and facilitating inspiration (Farmer and Carrier, 2000a; Gans, 1971; Gans and Clark, 1976; Naifeh et al., 1970). The caudocranial translation of the liver during the ventilatory cycle has been likened to a piston, and hence the term ‘hepatic piston pump’ has been coined to describe the mechanism powered by the diaphragmaticus muscle (Gans and Clark, 1976). The hepatic piston pumping has been shown to effectively decouple terrestrial locomotor mechanics from breathing mechanics in the American alligator (Farmer and Carrier, 2000b), and thus may provide an functional advantage during exercise compared to costal ventilation alone.

Previous studies have shown that lung ventilation in crocodilians can be effected by various combinations of muscular mechanisms. In submerged caiman, lung
ventilation was achieved solely by use of the hepatic piston pump (Gans and Clark, 1976) with costal muscle activity being neither regular or obligatory (Gans, 1971). In juvenile alligators on land, lung ventilation was achieved by a combination of both costal and hepatic piston mechanisms (Farmer and Carrier, 2000a). These studies suggest that the diaphragmaticus muscle plays a primary role in inspiration. This argument is further supported by recent videoradiographic measurements of lung volume in resting alligators (Claessens, 2009), where the diaphragmatic contribution to lung inflation has been determined to range from 36-61% of inspired tidal volume.

That the diaphragmaticus muscle is not absolutely necessary for effective lung ventilation at rest has been demonstrated in hatchling and juvenile alligators with a surgically transected diaphragmaticus (Hartzler et al., 2004; Uriona and Farmer, 2006). The loss of diaphragmatic function was found to result in significant reductions in maximal inspiratory flow rate, but whether this adversely affected respiratory gas exchange was not quantified.

The goal of our study is to determine the inspiratory importance of the diaphragmatic muscle in juveniles of the estuarine crocodile (Crocodylus porosus Schneider 1801). Extant crocodilians genera show differences in their habitat and activity preferences (Webb et al., 1993), thus the relative contribution of the diaphragmaticus muscle to lung ventilation may vary between groups. So far, however, only Alligator and Caiman have been studied from this perspective. In
contrast to previous studies at a single temperature and at rest, we measured the
contribution of the diaphragmaticus muscle to lung ventilation, and its effect on gas
exchange, in crocodiles under altered respiratory demand associated with decreased
body temperature, recovery from forced exercise, and hypercapnia.

Materials and Methods

Animals

Five estuarine crocodiles (*Crocodylus porosus* Schneider 1801) of indeterminate
sex were obtained from the Koorana Crocodile Farm, Rockhampton, Australia, and
kept in aquaria with a thermal gradient (27-33°C), full spectrum lighting
(14L:10D), free access to water and were fed a diet of whole rodents, fish, and
chicken pieces. Body weight ranged from 0.60 to 1.42 kg (mean ± s.e.m., 0.98 ±
0.19 kg).

Surgical procedure

Crocodiles were anaesthetised with halothane (Veterinary Companies of Australia,
Artarmon, NSW, Australia), intubated and artificially ventilated (Model 661,
Harvard Apparatus, Millis, Massachusetts, USA) with room air that had been
passed through a vapourizer (Fluotec 3, Cyprane Limited, Keighley, Yorkshire,
England). The vapourizer was initially set at 4-5% for induction of anaesthesia,
and was then reduced to 1-2% for surgical maintenance. Incision was made in the
skin and cervical muscles were carefully blunt-dissected to expose the underlying
carotid artery. The carotid artery was cannulated with heparinised polyethylene
tubing (I.D. 0.023, O.D. 0.038mm Microtube Extrusions, North Rocks, NSW, Australia) and the tubing looped once prior to exiting the wound where it was secured to the skin using two sutures. Incision site was closed with silk sutures. EMG electrodes (0.05mm diameter copper wire) were inserted bilaterally (and perpendicular to muscle fiber orientation) into the diaphragmaticus muscle via a 3-4cm midline abdominal incision. A copper ground electrode (with frayed ends) was also placed in the abdominal cavity. Leads from the electrodes were subcutaneously tunneled to a dorsal exit just caudal to the hind limb. All incisions were closed with interrupted sutures and treated with cyanoacrylate tissue adhesive (Vetbond, 3M, St Paul, MN, USA). The cannula and lead wires were coiled and taped to the back of the animal. Artificial ventilation with room air was continued until the crocodile regained consciousness and initiated spontaneous breathing. Intramuscular injections of the antibiotic Duplocillin (Intervet Australia, Bendigo East, Victoria, Australia), and the analgesic Temgesic (Buprenorphine, Reckitt Benckiser, West Ryde, NSW, Australia) were given at the conclusion of surgery. Duplocillin injections were repeated every second day after surgery. A minimum recovery period of two days was allowed before experiments commenced.

Transection of the diaphragmaticus muscle

After the first set of experiments, crocodiles were anaesthetised for a second time as described above. The diaphragmaticus muscle was exposed via the previous incision site, and transected by surgically severing the muscle bellies from their origin on the pubis and the ischia. After the incision was closed and animals
recovered as described above. Complete transection of the diaphragmaticus muscle was confirmed for each animal by post mortem examination at the end of the study.

**Lung Ventilation and gas exchange**

Ventilation was measured using a mask constructed from the base of a 20ml centrifuge tube, fitted with a plastic Y connector to which flexible tubing was attached. The mask was placed over the snout of the crocodile, covering the nostrils and the mouth and sealed to the body with a dental polyether impression material (Impregum F, Henry Schein Halas, Brisbane, QLD, Australia). A pump (Reciprotor AB, Sweden) pushed fresh room air through the mask at a constant flow rate of 0.8-1.2 L.min\(^{-1}\), depending on the size of the crocodile, controlled with a mass flowmeter (Sierra Instruments, Monterey, CA, USA). Care was taken to ensure that the flow rate though the mask exceeded the rate of inspiration, in order to prevent rebreathing. Alterations in airflow due to ventilation were measured using a pneumotachograph (MLT10L Respiratory Flow Head, AD Instruments, Bella Vista, NSW, Australia) placed downstream of the mask, such that expirations caused a decrease in airflow and inspiration caused an increase in airflow. Pressure gradients induced by alterations in airflow across the pneumotachograph were monitored using a differential pressure transducer connected to a carrier demodulator (MP-45-1 and CD15, respectively; Validyne, Northridge, CA, USA). The signal from the differential pressure transducer was calibrated by injecting and withdrawing known volumes of gas from the sealed mask and was integrated to obtain tidal volumes. Gas exiting the mask was sub-sampled, passed through the
desiccant anhydrous calcium sulfate (Drierite, Hammond, Xenia, OH, USA) and
analysed for fractional concentrations of O2 (F_O2) and CO2 (F_CO2) (ML206 gas
analyser, AD Instruments, Bella Vista, NSW, Australia). The rates of oxygen
consumption (\(\dot{V}_{O_2}\)) and carbon dioxide production (\(\dot{V}_{CO_2}\)) were determined as
previously described by (Frappell et al., 1992). Briefly,
\[
\dot{V}_{O_2} = \text{flow} \times (F'_{I O_2} - F'_{E O_2}) / (1 - F'_{I O_2})
\]
where the subscripts I and E represent incurrent and excurrent gas, respectively, and
the superscript ' (prime) represents dry CO2-free gas. CO2 was mathematically
scrubbed using \(F'_{O_2} = F_{O_2} / (1 - F_{CO2})\).
\[
\dot{V}_{CO_2} = \text{flow} \times (F'_{ECO_2} - F'_{ICO_2}) / (1 - F'_{ICO_2})
\]
where prime ' represents dry O2-free gas. Metabolic gas values are reported at
STPD (standard temperature and pressure, dry).

Breathing patterns were analysed in terms of tidal volume (\(V_T\)), breathing
frequency (f), minute ventilation (\(\dot{V}_E = V_T \times f\)), inspiratory and expiratory durations
\(T_I\) and \(T_E\), the duration of the non ventilatory period (\(T_{NVP}\)), rate of inspiratory
airflow (\(V_T/T_I\)), air convection requirements for O2 (ACR O2 = \(\dot{V}_E/\dot{V}_{O_2}\)) and CO2
(ACR CO2 = \(\dot{V}_E/\dot{V}_{CO_2}\)) and respiratory exchange ratio (RER = \(\dot{V}_{CO_2}/\dot{V}_{O_2}\)). For
each test condition, an average of 40 consecutive breaths were analysed and
ventilatory volumes are reported at BTPS (body temperature and barometric
pressure, saturated).
Blood gases

The arterial blood partial pressures of O₂ (PaO₂) and CO₂ (PaCO₂) and pH were measured with BMS 3 Mk 2 and PHM 73 (Radiometer, Denmark), respectively, at the appropriate test temperature (20°C or 30°C) 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. PaO₂ and PaCO₂ were measured every 30 s over 3 min and regressed back to time zero to account for drift and/or O₂ 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, CaO₂, 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.

Electromyography

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).
Experimental protocol

Crocodiles were fasted for 7 days prior to surgery (to ensure a post absorptive state) and were held at the 30°C for 2-3 days prior to experimentation (to ensure stable respiratory and metabolic parameters). At the time of the experiment the body temperature of the crocodiles was monitored via a thermocouple inserted ~ 5cm into the cloaca (temperature pod, AD Instruments, Bella Vista, NSW, Australia). A mask was fitted, the cannula and lead wires connected and the crocodile was placed on a treadmill belt. The crocodile was left on the stationary treadmill belt for at least one hour to obtain resting measurements for all variables at 30°C (the effects of handling and instrumentation have previously been shown to be non significant after 60 mins) (Munns, 2000). Reductions in respiratory drive were induced by lowering body temperature. The room temperature was slowly reduced over 2-3 hours until the crocodile’s body temperature reached 20°C. Ventilation, metabolic rate and blood gases were measured again, once the crocodile’s body temperature had stabilized at 20°C for a minimum of 60 mins. The room temperature was then slowly returned to 30°C and the crocodile’s body temperature restabilized at 30°C for at least 60 mins. Increases in centrally mediated respiratory drive were induced by short bouts of moderate intensity exercise or administration of hypercapnic gas (5% CO₂). After a minimum period of one hour at 30°C, the crocodile was exercised on the treadmill. The exercise period consisted of a two-minute exercise bout at 1.0 km.hr⁻¹. Locomotion was initiated by gently tapping the treadmill belt behind the crocodile or by lightly touching the crocodile’s tail. Following exercise, crocodiles were allowed to rest on the treadmill for a minimum of one hour (until
ventilation, blood gases and lactate concentrations had returned to pre-exercise values) and then exposed to 5% CO₂ for 10 minutes. The above experimental protocol was then repeated no less than 48hrs after the diaphragmaticus muscle was inactivated.

Data collection, analysis and statistics

All signals were collected on a computer at 1 kHz using Chart data acquisition software (AD Instruments, Bella Vista, NSW, Australia). Due to the intermittent and variable nature of reptilian ventilation and the low breathing frequencies employed at rest, ventilatory variables were calculated from the last 10 min of the rest periods. To avoid locomotor interference on recorded signals (e.g., ventilation, EMG signals), calculations were made from the first 25 breaths immediately following exercise.

The effect of severing the diaphragmaticus muscle on all parameters was determined using paired Dunnett’s test (30°C resting as the control, P<0.05) and paired t-tests (P<0.05). All data presented are mean ± s.e.m.

Results

Rest at 30°C

Crocodiles resting at 30°C displayed a typical crocodilian breathing pattern which consisted of one or two consecutive breaths interspersed with long pauses (Fig 1A),
\( \dot{V}_E (27.61 \pm 4.03 \text{mL.kg}^{-1}\text{min}^{-1}) \), \( \dot{V}_T (15.56 \pm 3.27 \text{mL.kg}^{-1}) \), \( f (1.98 \pm 0.48 \text{ min}^{-1}) \), \( \dot{V}_{O_2} \\
(0.83 \pm 0.24 \text{ mL.kg}^{-1}\text{min}^{-1}) \), \( \dot{V}_{CO_2} (0.70 \pm 0.19 \text{ mL.kg}^{-1}\text{min}^{-1}) \), ACR \( O_2 \) (47.00 \pm 21.06), ACR \( CO_2 \) (52.99 \pm 22.17) and RER (0.87 \pm 0.04) (Figs 2-5). EMG activity from the diaphragmaticus muscle was typically associated with ventilation when crocodiles were quietly resting at 30°C (Fig 1).

At this temperature, transection of the diaphragmaticus muscle did not induce any significant alterations in the ventilatory, respiratory or blood gas variables (Figs 6-7, Table 1).

Rest at 20°C

A lower body temperature (\( T_B \)) altered the breathing pattern by increasing \( T_{nvp} \) and \( T_I \) (Fig 2). Decreases in \( \dot{V}_{O_2} \), \( \dot{V}_{CO_2} \) (Fig 4) and \( VT/T_I \) also accompanied a decrease in \( T_B \). Diaphragmatic EMG activity was not always evident during inspiration, but when EMG activity was present, it was associated with inspiratory flow (Fig 1B). At 20°C, transection of the diaphragmaticus muscle induced a significant increase in \( V_T \), with no change in any other ventilatory, respiratory or blood gas parameter (Table 1).

Post-exercise recovery at 30°C
During the immediate recovery from treadmill exercise, minute ventilation ($V_E$) increased 9-fold (Fig 4), tidal volume ($V_T$) 2.7-fold (Fig 2), breathing frequency ($f$) 3.3-fold (Fig 3), rate of oxygen consumption ($V_{O_2}$) 2.5-fold (Fig 4) and rate of carbon dioxide production ($V_{CO_2}$) 5.8-fold (Fig. 4), while blood lactate concentration rose 5.6-fold from 0.77±0.43mmol.L$^{-1}$ to 4.27±0.95mmol.L$^{-1}$ (Fig 7).

The increase in $V_T$ was achieved via both a 1.9-fold increase in the rate of inspiratory flow ($V_{TI}/T_I$) and a 1.6-fold increase in inspiratory time ($T_I$, Fig 2). While $P_{aO_2}$ remained unaltered by exercise, $P_{aCO_2}$ significantly decreased (Fig 6).

All animals completed the exercise period both before and after inactivation of the diaphragmaticus muscle. Exercise in crocodiles with an inactivated diaphragmaticus muscle resulted in a reduction in the exercise induced elevation in $V_{TI}/T_I$, resulting in lower $V_T$ (Fig 2) and $V_E$ (Fig 4) compared to the same crocodiles with intact diaphragmaticus muscles. $V_{O_2}$ and $V_{CO_2}$ were not significantly elevated in crocodiles with inactivated diaphragmaticus muscles (Fig 4), and no significant alterations in blood gases were measured (Fig 6-7).

Hypercapnia at 30°C

At rest, inhalation of normoxic air with 5% CO$_2$ increased $V_E$ 1.5-fold (Fig 4) via 1.5-fold increase in $T_I$ and a 2.2-fold increase in $V_T$ (Fig 2). There were no
significant alterations in \( T_{\text{NVP}} \) or \( f \) (Fig 3) or any other ventilatory parameter (Fig 5). EMG activity from the diaphragmaticus muscle was present during hypercapnic exposure, however not all ventilations were associated with diaphragmatic activity (Fig 1). Transection of the diaphragmaticus muscle did not significantly alter any ventilatory parameter during hypercapnic exposure (Table 1).

Discussion

Inactivation of the diaphragmaticus muscle in juvenile *Crocodylus porosus* did not induce any significant alterations in ventilation, gas exchange or arterial blood gases at 30ºC, 20ºC or following inhalation of 5% CO\(_2\) (Table 1). Loss of diaphragmatic function disabled the hepatic piston pump, thus aspiration could only be achieved via alterations in intercostal or abdominal muscle activities. The resting breathing patterns of crocodiles in this study at both 20ºC and 30ºC, and in response to hypercapnia, were similar, both before and after surgery, to those previously measured on juvenile alligators and crocodiles under similar conditions (Farmer and Carrier, 2000c; Hartzler et al., 2006a; Munns et al., 1998; Munns et al., 2005). This suggests that the surgical intervention did not adversely alter the animals’ breathing patterns and the consistency of ventilatory and metabolic data both before and after surgery precluded the need for sham operated controls.

Our results suggest that activity of the inspiratory muscles (such as the intercostals, trapezius, anterior serratus and derived hypobranchial muscles of the neck) is able to maintain ventilation, thus maintaining arterial oxygenation to support metabolic rate in
the absence of a functional hepatic piston pump. As such, they support the argument that
the diaphragmaticus muscle is an accessory, not primary, muscle of inspiration in
crocodiles.

Variation in respiratory muscle activity of the diaphragmaticus appears to exist based on
the physical environment and physiological condition of the crocodilians. It may vary in
animals on land versus in water, at rest versus undergoing exercise. Earlier studies
reported that intercostal muscle activity was not regular or obligatory during ventilation
in submerged caiman (Gans, 1971; Gans and Clark, 1976), whereas others reported that
lung ventilation can be effected solely by the use of the intercostal musculature in
juvenile alligators on land (Hartzler et al., 2004; Uriona and Farmer, 2006). Uriona and
Farmer (2006) also demonstrated that transection of the diaphragmaticus muscle did not
alter the maximum inspiratory volume, expired volume, inspiratory or expiratory times.
The same authors also propose that the diaphragmaticus muscle may have a limited
collection to ventilation in fasted, standing alligators. The differential role of the
diaphragmatic activity in an aquatic versus terrestrial environment has been highlighted
by Uriona and Farmer’s (2008) findings that the diaphragmaticus is recruited in alligators
to control buoyancy and pitch during diving (Uriona and Farmer, 2008).

Some of the variation reported in activity of the diaphragmaticus and intercostal muscles
may be due to the use of different sized animals in the various studies. Relatively large
(up to 7.5kg) submerged caimans were used in studies that reported low EMG activity of
the intercostals and a high reliance on the diaphragmaticus muscle for inspiration (Gans,
1971; Gans and Clark, 1976). Videoradiographic studies in juvenile alligators (mass 0.72-
2.09kg) estimated that 36-61% of tidal volume was attributable to diaphragmaticus activity and approximately 40% attributable to costosternal activity (Claessens, 2009), though it should be noted that these estimates were calculated for tidal volumes 2-4 fold larger than those measured at rest in this study. While the diaphragmaticus muscle is well developed in adults, it is thin and translucent in juvenile crocodilians (pers. obs.). Future investigations are needed to examine if the contribution of the diaphragmaticus muscle to ventilation increases with age in crocodilians and whether any age related increase in diaphragmaticus muscle recruitment is related to hypertrophy of the muscle or to alterations in chest wall compliance.

Post exercise recovery caused significant alterations in ventilatory and respiratory parameters ($\dot{V}_E$, $V_T$, $f$, $V_{TI}/T_I$, $T_I$, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, Figs 2-5) and arterial lactate (Fig 7) in crocodiles with an intact diaphragmaticus muscle. The changes in ventilation and metabolic rates were not as extensive as those previously reported in exercising juvenile alligators (Farmer and Carrier, 2000b; Munns et al., 2005). The discrepancy of our results with those of earlier reports, however, is not surprising given differences in species used (Crocodylus versus Alligator), experimental protocol (2-min period versus exhaustive exercise), and acclimation to treadmill (none versus extensive). The aim of this experiment was not to achieve maximum treadmill performance but rather to test if adequate ventilation was maintained during elevated respiratory drive in the absence of a functional hepatic piston pump.
Transection of the diaphragmaticus resulted in a reduced capacity for exercise recovery to elevate VT/Ti (Fig 2), thus limiting the elevations in VT (-19%) and VE (-39%, Fig 4), compared to the same crocodiles with an intact diaphragmaticus. Interestingly, post exercise-induced elevations in VT were achieved via increases in both VTi/Ti and Ti, whereas hypercapnia-induced increases in VT of similar magnitude were supported solely by increases in Ti. Increases in Ti reflect a delay in the centrally integrated inspiratory “off-switch” (Munns et al., 1998) and as such are unlikely to be altered by the transection of the diaphragmaticus muscle. However, increases in VTi/Ti likely reflect an increase in respiratory muscle recruitment, thus increasing the rate of inspiratory airflow. Effective recruitment of the diaphragmaticus muscle to increase inspiratory airflow rates was prevented in crocodiles with inactivated hepatic piston pumps, and thus VT and VE were compromised during the recovery from exercise. VTi/Ti was also impaired following transection of the diaphragmaticus muscle in juvenile postprandial alligators (Uriona and Farmer, 2008), thus the proposed role of the diaphragmaticus muscle in increasing inspiratory airflow rates appears to include not only exercising but also digesting crocodilians.

Under laboratory conditions, exercise in crocodilians is predominantly anaerobic; arterial lactate concentrations increased by 5.6-fold after moderate activity in this study (Fig 7) and by 16-fold following exhaustive exercise in alligators (Hartzler et al., 2006b). While respiratory parameters tend to increase with treadmill speed, cardiovascular responses appear to be “all or nothing” with maximal increases in heart rate, central venous pressure, arterial blood pressure and venous return reached early in the exercise period,
and no further elevations triggered by increasing treadmill speed (Munns et al., 2005).

Exercise in crocodilians is also associated with a marked relative hyperventilation (Farmer and Carrier, 2000b; Farmer and Carrier, 2000c; Hartzler et al., 2006b) which was evident in this study by the increased ACR O$_2$ (Fig 5) and the decrease in PaCO$_2$ (Fig 6).

Exercising crocodiles rely on anaerobic metabolism which results in a low demand for O$_2$. At the same time, a relative hyperventilation occurs during exercise and results in a high O$_2$ supply. The combination anaerobic metabolism (thus low O$_2$ demand) and relative hyperventilation (thus high O$_2$ supply) may limit the impact of the V$_T$ and V$_E$ constraints induced by transection of the diaphragmaticus muscle during exercise. Future studies involving a greater range of treadmill speeds and exercise durations would be required to more completely assess the contribution of the diaphragmaticus muscle (and hence the hepatic piston pump) to exercise endurance.

In conclusion, the contribution of the hepatic piston pump and costal ventilation, the two primary ventilatory mechanisms in crocodilians, appears to be highly plastic. In *C. porosus*, the diaphragmaticus muscle appears to make only limited contributions to maintaining ventilation, metabolic rate and arterial oxygenation at rest (both at preferred and lowered body temperatures) and during increased respiratory drive induced by hypercapnia. Tidal volume elevations produced by increasing the duration of inspiration (as induced by hypercapnia) are not affected by inactivation of the diaphragmaticus muscle. However, the diaphragmaticus muscle makes a significant contribution to ventilation during the recovery from exercise, facilitating increases in inspiratory airflow.
rates, and thus improving the increases in tidal volume and minute ventilation that would otherwise be obtained.

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Figure 1: Ventilatory airflow (arbitrary units) and associated diaphragmaticus muscle EMG activity in one representative crocodile (mass 0.72 kg) at 30°C (A), 20°C (B) and after hypercapnic gas exposure (C). Inspiration occurs when the airflow trace is above zero and expiration when the trace is below zero. Periods of gular flutter (*) were present in crocodiles at 30°C and during hypercapnic exposure. Bar = 2 minutes.

Figure 2: The effect of transection of the diaphragmaticus muscle on the duration of inspiration (T₁), the rate of inspiration (VT₁/T₁) and tidal volume (V₁). Closed bars indicate the control (intact diaphragmaticus) and the open bars indicate surgically-altered (transected diaphragmaticus) animals. † indicates a significant difference compared to 30°C in crocodiles with the same status of the diaphragmaticus muscle. * indicates a significant difference compared to crocodiles with an intact diaphragmaticus muscle under the same experimental conditions. Data are mean±s.e.m., n=5.

Figure 3: The effect of transection of the diaphragmaticus muscle on the duration of expiration (Tₑ), the duration of the non ventilatory period (T_NVP) and breathing frequency (f). 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=5.

Figure 4: The effect of transection of the diaphragmaticus muscle on the minute ventilation (Vₑ), the rate of oxygen consumption (V_O₂) and the rate of carbon dioxide production (V_{CO₂}). 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. * indicates a significant difference compared to crocodiles with a functional diaphragmaticus muscle under the same experimental conditions. Data are mean±s.e.m., n=5.

Figure 5: The effect of transection of the diaphragmaticus muscle on the respiratory exchange ratio (R), the air convention requirement for oxygen (ACR O₂) and the air convention requirement for carbon dioxide (ACR CO₂). 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. * indicates a significant difference compared to crocodiles with a functional diaphragmaticus muscle under the same experimental conditions. Data are mean±s.e.m., n=5.
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 30ºC 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 diaphragmaticus muscle on the arterial pH (pHa), the arterial haemoglobin concentration ([Hb]a) and the arterial lactate concentration ([Lac]a). Closed bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. † indicates a significant difference compared to 30ºC 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.