

## RESEARCH ARTICLE

# The accessory role of the diaphragmaticus muscle in lung ventilation in the estuarine crocodile *Crocodylus porosus*

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### SUMMARY

**Crocodylians use a combination of three muscular mechanisms to effect lung ventilation: the intercostal muscles producing thoracic movement, the abdominal muscles producing pelvic rotation and gastralial translation, and the diaphragmaticus muscle producing visceral displacement. Earlier studies suggested that the diaphragmaticus is a primary muscle of inspiration in crocodylians, 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.**

Key words: ventilation, breathing pattern, oxygen consumption, blood gases, exercise, hypercapnia, crocodylian.

### INTRODUCTION

Crocodylians generate subatmospheric pulmonary pressures to inflate their lungs. Unlike mammals, in which the diaphragm plays a central role, crocodylians 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 crocodylians is not homologous to the mammalian diaphragm (Gans, 1971; Klein and Owerkowicz, 2006) and its main function may have been non-respiratory (Uriona and Farmer, 2008) as crocodylian 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 gastralial and insert onto a connective tissue sheath that surrounds the liver (Farmer and Carrier, 2000a). In caiman (Gans and Clark, 1976) and crocodiles (S.L.M., personal observation), 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 a functional advantage during exercise compared with costal ventilation alone.

Previous studies have shown that lung ventilation in crocodylians 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 nor 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 diaphragmatic 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% to 61% of inspired tidal volume.

Evidence that the diaphragmatic 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; however, the effect on 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*). Extant crocodylians 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 (14 h:10 h light:dark), free access to water and were fed a diet of whole rodents, fish and chicken pieces. Body mass ranged from 0.60 kg to 1.42 kg (mean  $\pm$  s.e.m., 0.98 $\pm$ 0.19 kg). Experiments were conducted under institutional animal ethics permit [LTU 04/37(L)].

### Surgical procedure

Crocodiles were anaesthetised with halothane (Veterinary Companies of Australia, Artarmon, NSW, Australia), intubated and artificially ventilated (Model 661, Harvard Apparatus, Millis, MA, USA) with room air that had been passed through a vapourizer (Fluotec 3, Cyprane Limited, Keighley, Yorkshire, UK). The vapourizer was initially set at 4–5% for the induction of anaesthesia, and was then reduced to 1–2% for surgical maintenance. An 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 mm, o.d. 0.038 mm; Microtube Extrusions, North Rocks, NSW, Australia) and the tubing was looped once prior to exiting the wound where it was secured to the skin using two sutures. The incision site was closed with silk sutures.

Electromyography (EMG) electrodes (0.05 mm diameter copper wire) were inserted bilaterally (and perpendicular to muscle fibre orientation) into the diaphragmaticus muscle *via* a 3–4 cm midline

abdominal incision. A copper ground electrode (with frayed ends) was also placed in the abdominal cavity. Leads from the electrodes were subcutaneously tunnelled to a dorsal exit just caudal to the hindlimb. 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, VIC, 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 2 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 20 ml 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, Skara, Sweden) pushed fresh room air through the mask at a constant flow rate of 0.8–1.2 l min<sup>-1</sup>, 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 through 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 sulphate (Drierite, Hammond, Xenia, OH, USA) and analysed for fractional concentrations of O<sub>2</sub> ( $F_{O_2}$ ) and CO<sub>2</sub> ( $F_{CO_2}$ ) (ML206 gas analyser, AD Instruments). 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. (Frappell et al., 1992). Briefly:

$$\dot{V}_{O_2} = \text{flow}' \times (F'I_{O_2} - F'E_{O_2}) / (1 - F'I_{O_2}), \quad (1)$$

where I and E represent incurrent and excurrent gas, respectively, and the superscript ' (prime) represents dry CO<sub>2</sub>-free gas. CO<sub>2</sub> was mathematically scrubbed using  $F'I_{O_2} = F_{O_2} / (1 - F_{CO_2})$ .

$$\dot{V}_{CO_2} = \text{flow}' \times (F'E_{CO_2} - F'I_{CO_2}) / (1 - F'I_{CO_2}), \quad (2)$$

where prime (') represents dry O<sub>2</sub>-free gas. Metabolic gas values are reported at standard temperature and pressure, dry (STPD).

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 ( $V_{T_I}/T_I$ ), air convection requirements for O<sub>2</sub> ( $ACR_{O_2} = \dot{V}_E/\dot{V}_{O_2}$ ) and CO<sub>2</sub> ( $ACR_{CO_2} = \dot{V}_E/\dot{V}_{CO_2}$ ) and respiratory exchange ratio ( $RER = \dot{V}_{CO_2}/\dot{V}_{O_2}$ ). For each test condition, a mean of 40 consecutive breaths were analysed, and ventilatory volumes are reported at body temperature and barometric pressure, saturated (BTPS).

#### Blood gases

The arterial blood partial pressures of O<sub>2</sub> ( $P_{aO_2}$ ) and CO<sub>2</sub> ( $P_{aCO_2}$ ) and pH were measured with BMS 3 Mk 2 and PHM 73 (Radiometer, Brønshøj, Denmark), respectively, at the appropriate test temperature (20°C or 30°C) *via* small blood samples (250–300 µl) taken from the arterial cannula and stored anaerobically on ice. The electrodes were calibrated before and after each measurement.  $P_{aO_2}$  and  $P_{aCO_2}$  were measured every 30 s for 3 min and regressed back to time zero to account for drift and/or O<sub>2</sub> 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 ( $Ca_{O_2}$ ) of each blood sample was determined from a 10 µl subsample of blood using a galvanic cell (Oxygen Content Analyser, OxyCon, University of Tasmania, TAS, 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) and analysed using Powerlab Chart Pro software (AD Instruments).

#### Experimental protocol

Crocodiles were fasted for 7 days prior to surgery (to ensure a post-absorptive state) and were held at 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 ( $T_B$ ) of the crocodiles was monitored *via* a thermocouple inserted ~5 cm into the cloaca (temperature pod, AD Instruments). A mask was fitted, the cannula and lead wires were connected, and the crocodile was placed on a treadmill belt. The crocodile was left on the stationary treadmill belt for at least 1 h 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 min) (Munns, 2000). Reductions in respiratory drive were induced by lowering  $T_B$ . The room temperature was slowly reduced over 2–3 h until the crocodile's  $T_B$  reached 20°C. Ventilation, metabolic rate and blood gases were measured again, once the crocodile's  $T_B$  had stabilised at 20°C for a minimum of 1 h. The room temperature was then slowly returned to 30°C and the crocodile's  $T_B$  restabilised at 30°C for at least 1 h. Increases in centrally mediated respiratory drive were induced by short bouts of moderate-intensity exercise or by the administration of hypercapnic gas (5% CO<sub>2</sub>). After a minimum period of 1 h at 30°C, the crocodile was exercised on the treadmill. The exercise period consisted of a two-minute exercise bout at 1.0 km h<sup>-1</sup>. Locomotion was initiated by gently tapping the treadmill belt behind the crocodile or by lightly

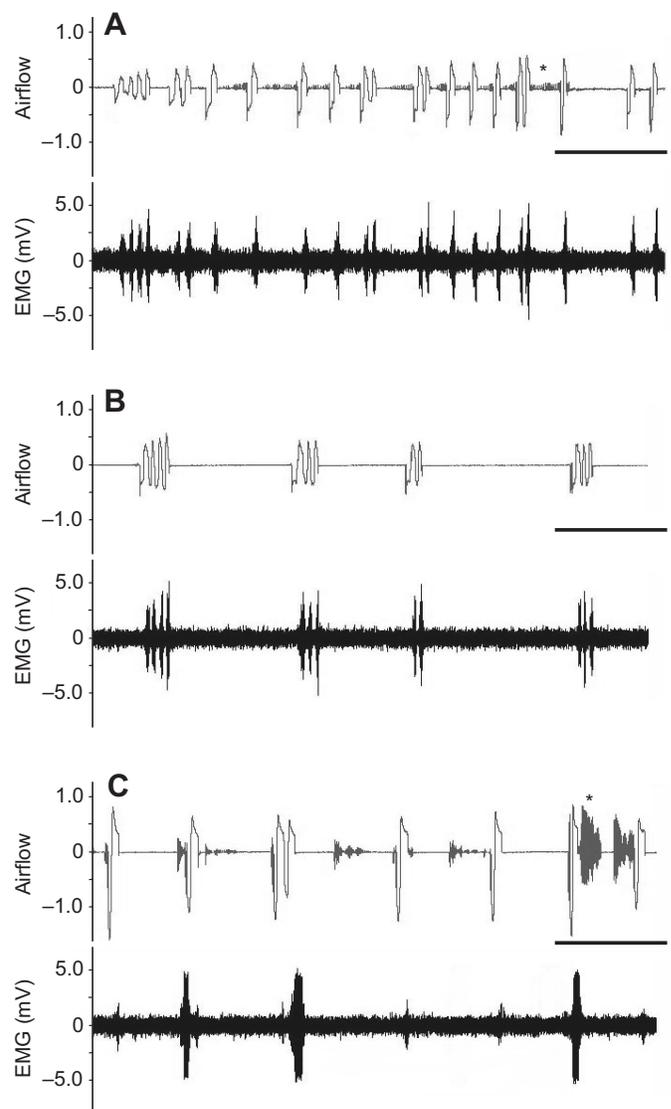


Fig. 1. Ventilatory airflow (arbitrary units) and associated diaphragmaticus muscle electromyography (EMG) activity in one representative crocodile (mass 0.72 kg) at 30°C (A), 20°C (B) and after hypercapnic gas exposure (C). Inspiration occurs when the airflow trace is above zero and expiration occurs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

touching the crocodile's tail. Following exercise, crocodiles were allowed to rest on the treadmill for a minimum of 1 h (until ventilation, blood gases and lactate concentrations had returned to pre-exercise values) and then exposed to 5% CO<sub>2</sub> for 10 min. The above experimental protocol was then repeated no less than 48 h 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). Because of 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.

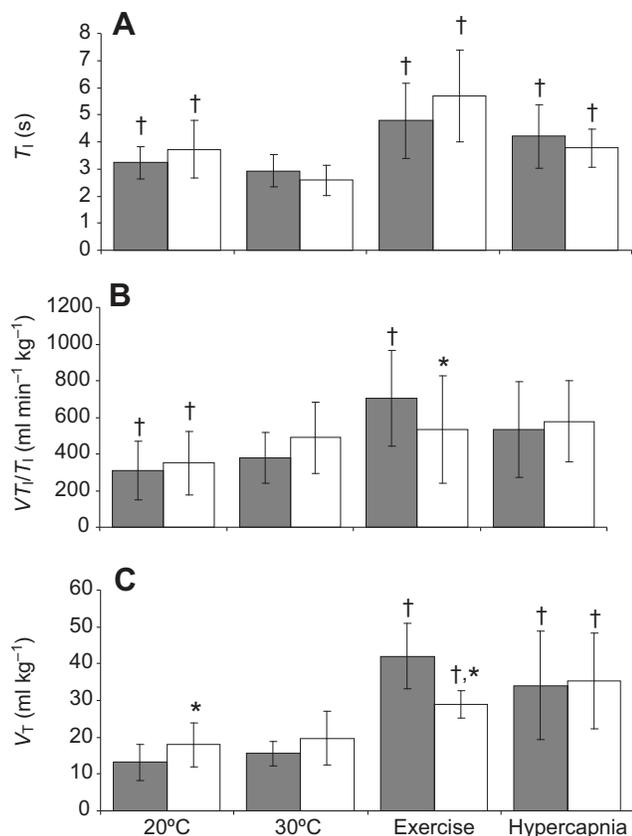


Fig. 2. The effect of transection of the diaphragmatic muscle on (A) the duration of inspiration ( $T_i$ ), (B) the rate of inspiration ( $V_T/T_i$ ) and (C) tidal volume ( $V_T$ ). Filled bars indicate the control (intact diaphragmaticus) and the open bars indicate surgically altered (transected diaphragmaticus) animals. †Significant difference compared with 30°C in crocodiles with the same status of the diaphragmaticus muscle. \*Significant difference compared with crocodiles with an intact diaphragmaticus muscle under the same experimental conditions. Data are means  $\pm$  s.e.m.,  $N=5$ .

The effect of severing the diaphragmaticus muscle on all parameters was determined using a paired Dunnett's test (30°C resting as the control,  $P<0.05$ ) and paired  $t$ -tests ( $P<0.05$ ). All data presented are means  $\pm$  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),  $\dot{V}_E$  ( $27.61 \pm 4.03$  ml kg<sup>-1</sup> min<sup>-1</sup>),  $V_T$  ( $15.56 \pm 3.27$  ml kg<sup>-1</sup>),  $f$  ( $1.98 \pm 0.48$  min<sup>-1</sup>),  $\dot{V}_{O_2}$  ( $0.83 \pm 0.24$  ml kg<sup>-1</sup> min<sup>-1</sup>),  $\dot{V}_{CO_2}$  ( $0.70 \pm 0.19$  ml kg<sup>-1</sup> min<sup>-1</sup>), ACR O<sub>2</sub> ( $47.00 \pm 21.06$ ), ACR CO<sub>2</sub> ( $52.99 \pm 22.17$ ) 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 and 7, Table 1).

### Rest at 20°C

A lower  $T_B$  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  $V_T/T_i$  also accompanied

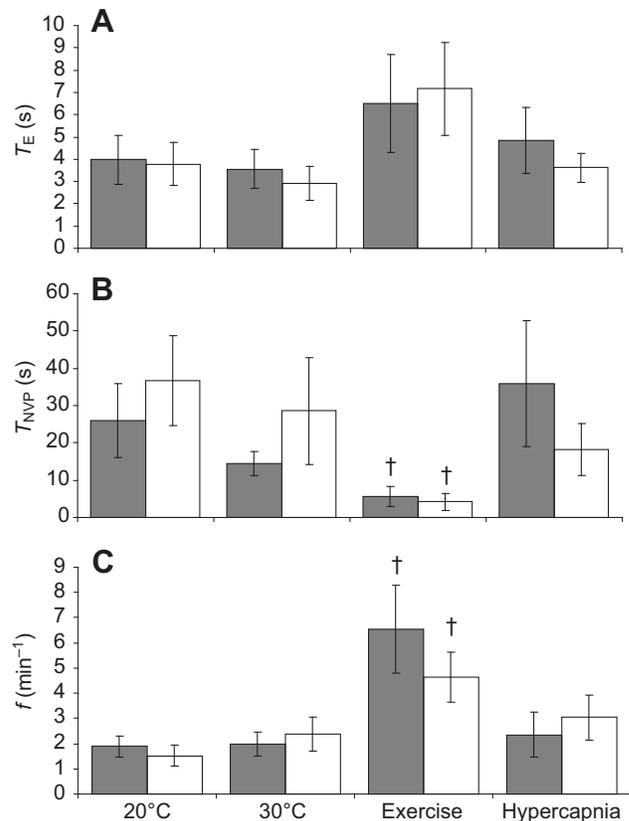


Fig. 3. The effect of transection of the diaphragmatic muscle on (A) the duration of expiration ( $T_E$ ), (B) the duration of the non-ventilatory period ( $T_{NVP}$ ) and (C) breathing frequency ( $f$ ). Filled bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. †Significant difference compared with 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 means  $\pm$  s.e.m.,  $N=5$ .

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,  $\dot{V}_E$  increased 9-fold (Fig. 4),  $V_T$  increased 2.7-fold (Fig. 2),  $f$  increased 3.3-fold (Fig. 3),  $\dot{V}_{O_2}$  increased 2.5-fold (Fig. 4) and  $\dot{V}_{CO_2}$  increased 5.8-fold (Fig. 4), while blood lactate concentration rose 5.6-fold from  $0.77 \pm 0.43$  mmol l<sup>-1</sup> to  $4.27 \pm 0.95$  mmol l<sup>-1</sup> (Fig. 7). The increase in  $V_T$  was achieved *via* both a 1.9-fold increase in  $V_T/T_i$  and a 1.6-fold increase in  $T_i$  (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  $V_T/T_i$ , resulting in lower  $V_T$  (Fig. 2) and  $\dot{V}_E$  (Fig. 4) compared with the same crocodiles with intact diaphragmaticus muscles.  $\dot{V}_{O_2}$  and  $\dot{V}_{CO_2}$  were not significantly elevated in crocodiles with inactivated

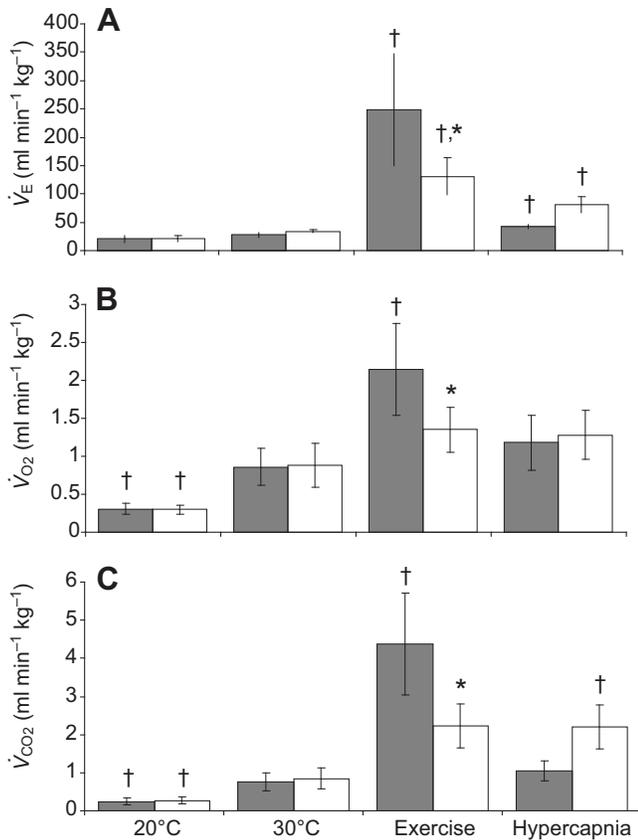


Fig. 4. The effect of transection of the diaphragmaticus muscle on (A) the minute ventilation ( $\dot{V}_E$ ), (B) the rate of oxygen consumption ( $\dot{V}_{O_2}$ ) and (C) the rate of carbon dioxide production ( $\dot{V}_{CO_2}$ ). Filled bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. †Significant difference compared with 30°C in crocodiles with the same status of the diaphragmaticus muscle. \*Significant difference compared with crocodiles with a functional diaphragmaticus muscle under the same experimental conditions. Data are means  $\pm$  s.e.m.,  $N=5$ .

diaphragmaticus muscles (Fig. 4), and no significant alterations in blood gases were measured (Figs 6, 7).

#### Hypercapnia at 30°C

At rest, inhalation of normoxic air with 5%  $CO_2$  increased  $\dot{V}_E$  1.5-fold (Fig. 4) *via* a 1.5-fold increase in  $T_I$  and a 2.2-fold increase in  $V_T$  (Fig. 2). There were no significant alterations in  $T_{NVP}$  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 *C. porosus* did not induce any significant alterations in ventilation, gas exchange or arterial blood gases at 30°C, 20°C 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 20°C and 30°C, and in

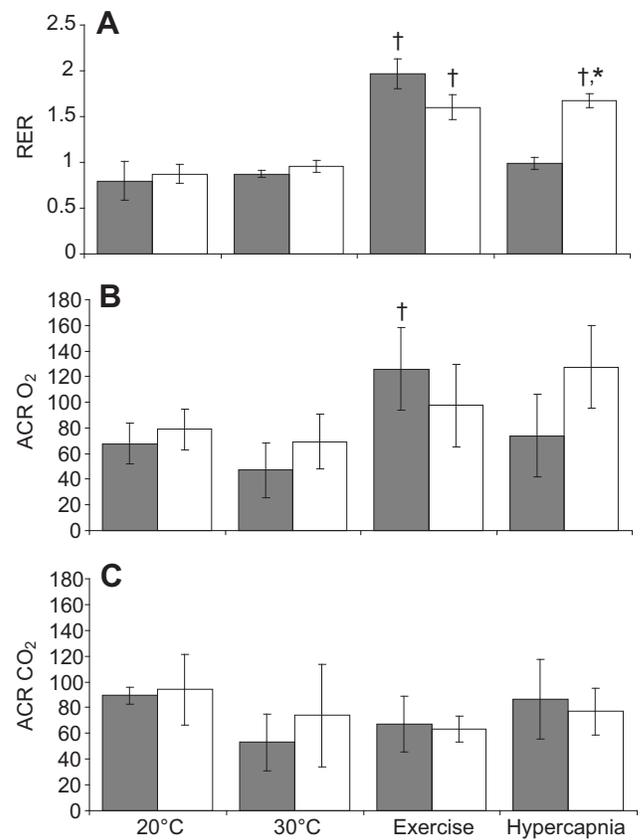


Fig. 5. The effect of transection of the diaphragmaticus muscle on (A) the respiratory exchange ratio (RER), (B) the air convection requirement for oxygen (ACR  $O_2$ ) and (C) the air convection requirement for carbon dioxide (ACR  $CO_2$ ). Filled bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. †Significant difference compared to 30°C in crocodiles with the same status of the diaphragmaticus muscle. \*Significant difference compared with crocodiles with a functional diaphragmaticus muscle under the same experimental conditions. Data are means  $\pm$  s.e.m.,  $N=5$ .

response to hypercapnia, were similar, both before and after surgery, to those previously measured in 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 a 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

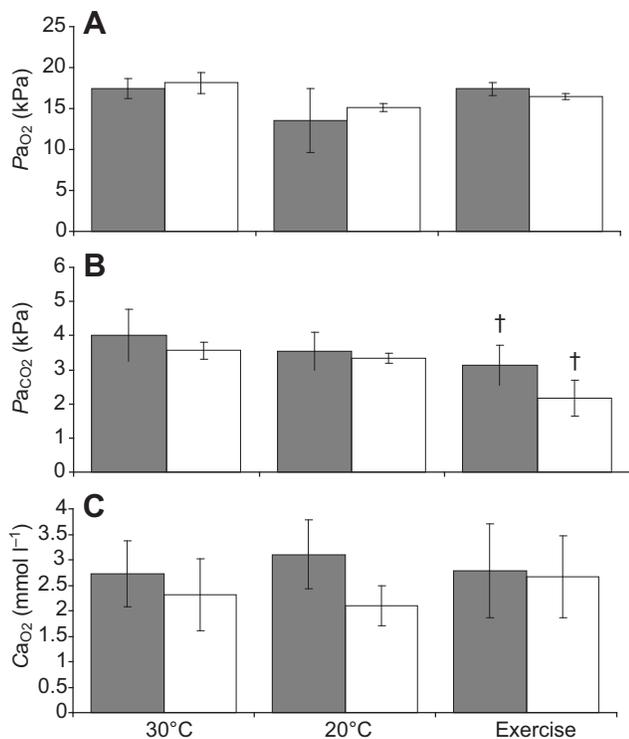


Fig. 6. The effect of transection of the diaphragmaticus muscle on (A) the partial pressure of arterial  $O_2$  ( $P_{aO_2}$ ), (B) the partial pressure of arterial  $CO_2$  ( $P_{aCO_2}$ ) and (C) the arterial  $O_2$  content ( $Ca_{O_2}$ ). Filled bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. <sup>†</sup>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 means  $\pm$  s.e.m.,  $N=3$ .

by the use of the intercostal musculature in juvenile alligators on land (Hartzler et al., 2004; Uriona and Farmer, 2006). Uriona and Farmer also demonstrated that transection of the diaphragmaticus muscle did not alter the maximum inspiratory volume, expired volume, inspiratory or expiratory times (Uriona and Farmer, 2006). The same authors also propose that the diaphragmaticus muscle may have a limited contribution 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 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 7.5 kg) 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 0.72–2.09 kg) estimated that 36–61% of  $V_T$  was attributable to diaphragmatic activity and ~40% attributable to costosternal activity (Claessens, 2009), although it should be noted that these estimates were calculated for  $V_T$  values 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 (S.L.M.,

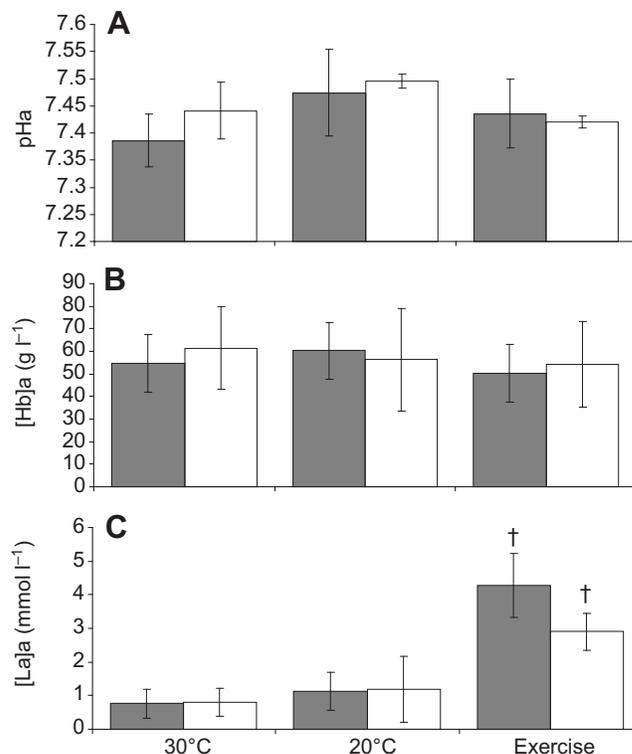


Fig. 7. The effect of transection of the diaphragmaticus muscle on (A) the arterial pH (pHa), (B) the arterial haemoglobin concentration ([Hb]a) and (C) the arterial lactate concentration ([La]a). Filled bars indicate the control (intact diaphragmaticus) and the open bars indicate transected diaphragmaticus muscles. <sup>†</sup>Significant difference compared with 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 means  $\pm$  s.e.m.,  $N=3$ .

personal observations). 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 ( $\dot{V}_E$ ,  $V_T$ ,  $f$ ,  $V_T/T_I$ ,  $T_I$ ,  $\dot{V}_{O_2}$  and  $\dot{V}_{CO_2}$ ; 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 the differences in the species used (*Crocodylus* *versus* *Alligator*), experimental protocol (2 min period 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  $V_T/T_I$  (Fig. 2), thus limiting the elevations in  $V_T$  (–19%) and  $\dot{V}_E$  (–39%, Fig. 4), compared with the same crocodiles with an intact diaphragmaticus. Interestingly, post-exercise-induced elevations in  $V_T$  were achieved *via* increases in both  $V_T/T_I$  and  $T_I$  whereas hypercapnia-induced increases in  $V_T$  of

Table 1. The effect of inactivation of the diaphragmaticus muscle on ventilation, metabolic rate and arterial blood gases

	30°C	P-value	20°C	P-value	Exercise recovery	P-value	Hypercapnia	P-value
$T_I$	94.54±18.48	0.597	118.61±24.05	0.639	142.04±62.41	0.639	105.45±25.57	0.620
$T_E$	94.68±24.52	0.623	112.89±31.41	0.911	147.51±69.25	0.810	102.12±32.19	0.565
$T_{NVP}$	192.08±86.20	0.375	171.95±76.70	0.351	110.11±81.32	0.710	88.92±46.13	0.181
$V_T$	119.15±23.27	0.473	143.75±7.82	<b>0.026</b>	81.54±24.29	<b>0.045</b>	139.87±37.88	0.866
$f$	117.76±23.12	0.366	82.05±13.76	0.397	90.21±30.29	0.431	152.62±21.87	0.138
$\dot{V}_E$	128.19±16.56	0.093	116.98±20.26	0.721	61.12±11.54	<b>0.044</b>	191.43±27.19	0.051
$V_T/T_I$	135.81±25.22	0.237	150.25±47.89	0.228	64.66±12.45	<b>0.028</b>	139.29±36.48	0.550
$V_{O_2}$	95.39±10.75	0.630	103.28±22.837	0.883	80.50±24.09	<b>0.046</b>	152.16±27.14	0.300
$V_{CO_2}$	106.08±16.76	0.361	136.44±46.18	0.526	73.04±28.67	<b>0.041</b>	224.58±69.58	0.055
RER	110.47±10.69	0.408	136.03±36.10	0.714	82.97±10.27	0.153	177.15±14.13	<b>0.023</b>
ACR O <sub>2</sub>	137.09±15.44	0.252	132.35±32.36	0.688	96.67±29.01	0.436	228.71±152.85	0.624
ACR CO <sub>2</sub>	128.59±20.38	0.348	104.19±26.78	0.869	130.78±49.65	0.891	147.10±77.96	0.817
[La]a	111.94±26.42	0.910	120.59±16.81	0.500	71.28±10.24	0.186		
[Hb]a	112.64±18.72	0.648	84.88±23.96	0.781	100.72±16.79	0.658		
CaO <sub>2</sub>	82.09±12.50	0.358	80.79±9.80	0.432	101.80±10.23	0.643		
PaO <sub>2</sub>	105.72±13.53	0.798	128.56±43.55	0.928	95.09±6.01	0.489		
PaCO <sub>2</sub>	106.99±18.57	0.990	108.82±28.52	0.987	77.31±28.38	0.374		
pHa	100.75±0.15	0.056	101.36±0.15	0.086	99.81±0.96	0.155		

Data presented are % change in animals with a transected diaphragmaticus relative to animals with intact diaphragmaticus muscle, means ± s.e.m.,  $N=5$  (blood gases  $N=3$ ).  $P$ -values from paired  $t$ -tests. Significant differences are indicated in bold. See text for definitions of abbreviations used.

similar magnitude were supported solely by increases in  $T_I$ . Increases in  $T_I$  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  $V_T/T_I$  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  $V_T$  and  $\dot{V}_E$  were compromised during the recovery from exercise.  $V_T/T_I$  was also impaired following transection of the diaphragmaticus muscle in juvenile post-prandial 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<sub>2</sub> (Fig. 5) and the decrease in PaCO<sub>2</sub> (Fig. 6). Exercising crocodiles rely on anaerobic metabolism, which results in a low demand for O<sub>2</sub>. At the same time, a relative hyperventilation occurs during exercise and results in a high O<sub>2</sub> supply. The combination anaerobic metabolism (thus, low O<sub>2</sub> demand) and relative hyperventilation (thus, high O<sub>2</sub> supply) may limit the impact of the  $V_T$  and  $\dot{V}_E$  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.  $V_T$  elevations produced by increasing the duration of inspiration (as induced by hypercapnia) are not affected by the 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  $V_T$  and  $\dot{V}_E$  that would otherwise be obtained.

#### LIST OF ABBREVIATIONS

ACR CO <sub>2</sub>	air convection requirement for CO <sub>2</sub>
ACR O <sub>2</sub>	air convection requirement for O <sub>2</sub>
CaO <sub>2</sub>	arterial O <sub>2</sub> content
$f$	breathing frequency
$F_{CO_2}$	fractional concentration of CO <sub>2</sub>
$F_{O_2}$	fractional concentration of O <sub>2</sub>
[Hb]a	arterial haemoglobin concentration
[La]a	arterial lactate concentration
PaCO <sub>2</sub>	arterial blood partial pressure of CO <sub>2</sub>
PaO <sub>2</sub>	arterial blood partial pressure of O <sub>2</sub>
pHa	arterial pH
RER	respiratory exchange ratio
$T_B$	body temperature
$T_E$	expiratory duration
$T_I$	inspiratory duration
$T_{NVP}$	duration of non-ventilatory period
$V_T$	tidal volume
$V_T/T_I$	rate of inspiratory airflow
$\dot{V}_{CO_2}$	rate of CO <sub>2</sub> consumption
$\dot{V}_E$	minute ventilation
$\dot{V}_{O_2}$	rate of O <sub>2</sub> consumption

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