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ORIGINAL PAPER

Exhaustive exercise training enhances aerobic capacity in American alligator (*Alligator mississippiensis*)

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Abstract The oxygen transport system in mammals is extensively remodelled in response to repeated bouts of activity, but many reptiles appear to be 'metabolically inflexible' in response to exercise training. A recent report showed that estuarine crocodiles (*Crocodylus porosus*) increase their maximum metabolic rate in response to exhaustive treadmill training, and in the present study, we confirm this response in another crocodilian, American alligator (*Alligator mississippiensis*). We further specify the nature of the crocodilian training response by analysing effects of training on aerobic [citrate synthase (CS)] and anaerobic [lactate dehydrogenase (LDH)] enzyme activities in selected skeletal muscles, ventricular and skeletal muscle masses and haematocrit. Compared to sedentary control animals, alligators regularly trained for 15 months on a treadmill (run group) or in a flume (swim group) exhibited peak oxygen consumption rates higher by 27 and 16%, respectively. Run and swim exercise training significantly increased ventricular mass (~11%) and haematocrit (~11%), but not the mass of skeletal muscles. However, exercise training did not alter CS or LDH activities of skeletal muscles. Similar to mammals, alligators respond to exercise training by increasing convective oxygen transport mechanisms, specifically heart size (potentially greater stroke volume) and haematocrit (increased oxygen carrying-capacity of the blood). Unlike mammals, but similar to squamate reptiles, alligators do not also increase citrate synthase activity of the skeletal muscles in response to exercise.

Keywords Alligator · Citrate synthase · Exercise · Metabolic rate · Oxygen consumption

Abbreviations

CF	Caudofemoralis
CS	Citrate synthase
DP	Diaphragmaticus
GC	Gastrocnemius
IAP	Intra-abdominal pressure
KWRS	Kruskal–Wallis rank sums
LDH	Lactate dehydrogenase
Q_{tot}	Cardiac output
RER	Respiratory exchange ratio
SC	Splenius capitis
SNK	Student Newman–Keuls post hoc test

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$\dot{V}_A$	Alveolar ventilation
$\dot{V}_{CO_2}$	Mass-specific carbon dioxide production rate
$\dot{V}_{O_2}$	Mass-specific oxygen consumption rate
WRS	Wilcoxon rank sums

Introduction

The oxygen transport system in mammals is extensively remodeled in response to repeated bouts of activity. In mammals, chronic exercise training causes concomitant increases in both oxidative enzyme activities and convective oxygen transport and an overall increase in maximum oxygen consumption ($\dot{V}_{O_2}$ max) (e.g., human—Carter et al. 2000; horse—Evans and Rose 1988; rat—Gleeson et al. 1983; mouse—Kemi et al. 2002; dog—Musch et al. 1985). However, among non-mammalian vertebrates, the effects of chronic exercise training on oxygen transport are not well documented. Increases in catabolic enzyme activities have been measured for a chondrichthyan (Gruber and Dickson 1997), several osteichthyan fishes (e.g. Farrell et al. 1991; Davison 1997), two anuran amphibians (Cummings 1979; Miller and Camilliere 1981), an agamid lizard (Garland et al. 1987) and two birds (Butler and Turner 1988; Lundgren and Kiessling 1986). No consistent evidence exists for other non-mammalian vertebrates. At the whole-animal level, an increase in $\dot{V}_{O_2}$ max in response to exercise training has been demonstrated in relatively few non-mammalian vertebrates, with data for two teleosts (chinook salmon—Gallaugher et al. 2001; dark barbel catfish—Liu et al. 2009), one archosaurian reptile (estuarine crocodile—Owerkowicz and Baudinette 2008) and one bird (tufted duck—Butler and Turner 1988). Studies in phylogenetically diverse lizards have failed to find a significant exercise training effect for skeletal muscle aerobic enzymatic activity or whole-animal aerobic metabolism (Conley et al. 1995; Garland et al. 1987; Gleeson 1979; Thompson 1997; A. M. Szucsik et al., unpublished). Consequently, lizards have been considered 'metabolically inflexible' (Gleeson 1979).

In contrast to lizards, a recent study suggests that crocodilians may have a more plastic metabolic response to exercise training (Owerkowicz and Baudinette 2008). Daily exhaustive exercise on a treadmill resulted in a significant increase in aerobic capacity (+28%) and walking endurance (+82%) in juvenile male estuarine crocodiles (*Crocodylus porosus* Schneider 1801). The purpose of our study was to determine whether exercise can increase $\dot{V}_{O_2}$ in another crocodilian species, American alligator (*Alligator mississippiensis* Daudin 1801) and to ascertain if exercise training alters major aerobic or anaerobic enzyme activities of skeletal muscle. We subjected juvenile female alligators to either a swimming or running exhaustive exercise training protocol and compared them to a sedentary control group. After 15 months of training, we measured the rates of oxygen consumption and carbon dioxide production for alligators subjected to a modified graded exercise test on a treadmill. Following sacrifice and tissue harvest, we examined changes in animals' haematocrit and wet heart mass. We also measured the activity of citrate synthase and lactate dehydrogenase in selected skeletal muscles of exercise-trained and sedentary alligators.

Materials and methods

Animals

American alligator eggs ($N = 35$; *A. mississippiensis*) were obtained from the Rockefeller Wildlife Refuge (RWR) in Grand Chenier, LA, USA and transported by air freight to the University of California, Irvine (UCI). Eggs were potted in moist vermiculite and incubated at 30°C. This ensured that all alligator hatchlings were female (confirmed by cloacal examination). After hatching (August–September 2005), alligators were group housed in 1 × 2.5 × 1 m fiberglass tanks at 30°C with free access to water and basking sites. Animals were fed an ad libitum diet of live goldfish or ground whole chicken, 2–3 times per week. Approval for animal use in this study was given by the UCI Institutional Animal Care and Use Committee (protocol #1999-2123).

Sham surgery

From January–March 2006, alligators underwent a sham surgery as a control for other studies. At the time of surgery (5–7 month old animals), mean mass was 65 ± 2.1 g. Briefly, following anaesthesia induction with isoflurane (Isoflo®; Abbott laboratories, North Chicago, IL, USA), the animal was intubated and artificially ventilated using a SAR-830 Ventilator (CWE, Ardmore, PA, USA), with a vaporiser (Foregger Fluomatic, Smithtown, NY, USA) providing 1–2% isoflurane. The pericardium and great vessels were exposed. The pericardium was incised to expose the ventricles and atria and sewn shut with 6-0 silk suture. The musculature, sternum and skin were sewn shut in succession with 3-0 silk suture (Ethicon, Somerville, NJ, USA). Following surgery, animals were artificially ventilated on room air until voluntary breathing resumed. Intramuscular injections of the antibiotic enrofloxacin (10 mg kg^{-1}; Baytril; Bayer Corporation, Shawnee Mission, KS, USA) and the analgesic flunixin meglumine (5 mg kg^{-1}; Flunixamine; Fort Dodge, Madison, NJ, USA) were given at the conclusion of surgery, and enrofloxacin was given for two additional days post-operative. Food was withheld for 5–7 days following surgery. All animals were

fully recovered (2.5–5 months post-operative) by the beginning of the 15-month exercise training protocol.

Exercise training protocol

At approximately 9 months of age (June 2006), alligators were randomly assigned to three groups: run, swim, and sedentary ($N = 12$, 12 and 11, respectively). Animals in the run and swim groups were exercised to exhaustion on approximately each Monday, Wednesday and Friday and every other Sunday on a treadmill (1.0–2.0 km h^{-1}) or in a swim flume (0.5–1.0 km h^{-1}) until sacrifice in October–December 2007. Our exercise training protocol was much longer than those typically employed in mammalian studies (e.g. Carter et al. 2000; Evans and Rose 1988; Gleeson et al. 1983; Kemi et al. 2002; Musch et al. 1985), and the long duration was chosen because previous studies on exercise training in squamates have not demonstrated skeletal muscle plasticity in response to training (Conley et al. 1995; Garland et al. 1987; Gleeson 1979; Thompson 1997; A. M. Szucsik et al., unpublished). Animals were encouraged to run or swim by tapping them on the tail by hand or with a pair of long forceps. Exercise bouts were kept at ~5 min by gradually increasing flume and treadmill speeds over the training period, as animals grew. Five minutes is a typical endurance time for 1–2 kg crocodilians exercising at a ~0.75–2.0 km h^{-1} (Farmer and Carrier 2000; Munns et al. 2005; Owerkowicz and Baudinette 2008). Endurance was defined as time to exhaustion (to the nearest 15 s), at which point the animal failed to respond to repeated stimulation and was judged unable to maintain treadmill or flume speed. Animals in the sedentary group were not exercised and were only handled once every fortnight for the purpose of body size measurements. During transport to the treadmill room or the flume room for exercise, all animals were kept in darkened containers to minimise handling stress.

$\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ of exercise-trained and sedentary alligators

Following 15 months of exercise training (in September 2007), the run, swim and sedentary groups of alligators underwent a graded exercise test on a motorised treadmill in order to estimate $\dot{V}_{O_2}$ peak. We refer to the highest oxygen consumption measured for alligators in this study at any single treadmill speed (last 30–40 s at each speed) or recovery (first 30–40 s following cessation of exercise) as '$\dot{V}_{O_2}$ peak', rather than '$\dot{V}_{O_2}$ max'. $\dot{V}_{O_2}$ max is commonly used to indicate a steady state of sustained maximum oxygen consumption, measured during sustained locomotion at a steady speed. However, it is relatively difficult for crocodilians to reach a classic stable metabolic state on a treadmill, given their lack of cooperation to locomote on a treadmill and limited ability to sustain a rigorous exercise level (e.g. 3–7 min total, see Farmer and Carrier 2000; Munns et al. 2005; Owerkowicz and Baudinette 2008). In contrast to prior studies on crocodilians, we did not rest animals between successive speeds. Instead, we thoroughly exhausted alligators during a single bout of exercise by increasing treadmill speed every minute until exhaustion. Hence, given the short duration that animals spent at each treadmill speed, and that we define $\dot{V}_{O_2}$ peak as the highest oxygen-consumption value measured during any one treadmill speed or recovery, we believe that $\dot{V}_{O_2}$ peak is an appropriate distinction from $\dot{V}_{O_2}$ max.

Alligators were fasted for 3–4 days prior to experimentation, and measures were made at 30°C in a temperature-controlled environmental chamber. Experiments were performed between 1400-000 hours for six consecutive days, and the training schedule of exercise-trained animals was suspended for 3–6 days prior to experimentation to ensure animals were fully recovered before the graded exercise test (Hartzler et al. 2006). At the time of the graded exercise test (~24-month-old animals), mean mass was 1.1 ± 0.2 kg for the run group, 1.2 ± 0.1 kg for the swim group and 1.7 ± 0.1 kg for the sedentary group. To collect expired gases, animals were fitted with a mask constructed from acetate sheeting. The mask had two holes (inlet and outlet) connected to flexible tubing (Tygon; 5 mm ID, 2 mm wall). With the animal's mouth taped shut, the mask was affixed over its nostrils and sealed air-tight with Impregum (3 M ESPE, Seefeld, Germany). Room air was pulled through the mask by pumps at a rate of 1.0–4.4 L min^{-1}, depending on animal size, and expired air was sub-sampled 8 cm downstream of the nostrils (via 2 mm ID Tygon tubing) at a flow rate of 150 ml min^{-1}. Bias flow through the mask was similar to rates published previously (Farmer and Carrier 2000; Hartzler et al. 2006; Munns et al. 2005; Owerkowicz and Baudinette 2008). Prior to the treadmill test, flow rates were tested across the range of animal sizes by rolling alligators to exhaustion and monitoring exhaled gases during subsequent recovery when the animals were breathing vigorously and frequently. Flow rates were then increased until carbon dioxide could not be detected escaping into the inlet tubing near the mask, which ensured collection of all exhaled gases and prevented rebreathing. Animals that were rolled were not a part of an exercise test for at least one week. Proper flow rates were applied to appropriately sized animals and ensured that no CO_2 escaped into the inlet tubing. All flow rates were maintained using calibrated rotameters (Cole-Parmer, Vernon Hills, IL, USA); one rotameter maintained bias flow through the mask (downstream flow at 1.0–4.4 L min^{-1}) and the other maintained an appropriate sub-sampling flow rate (150 ml min^{-1}) for the gas analysers. Wet, sub-sampled air was pulled first through a carbon dioxide analyser (CD-3A, Applied

Electrochemistry Instruments, Pittsburgh, PA, USA), then through a Drierite (anhydrous calcium sulphate; W.A. Hammond Drierite Co., Xenia, OH, USA) column, and finally through an oxygen analyser (S-3A, Applied Electrochemistry Instruments, Pittsburgh, PA, USA). Signals from the carbon dioxide and oxygen analysers were collected on a computer at 20 Hz using an A/D MP100 board and Acq*Knowledge* data-acquisition software (v.3.8.1 Biopac, Goleta, CA, USA).

An alligator was placed on the treadmill and remained stationary for 1–2 min ('pre-exercise'). The animal was then stimulated to run as steadily as possible for 1 min at each of the following successively increasing speeds: 0.5, 1.0, 1.5 and 2.0 km h^{-1}. Expired gases were collected continuously, and $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were estimated from the respective mean values in expired air during the last 30–40 s of pre-exercise and each treadmill speed. Animals were run until exhaustion, with exhaustion defined as refusal to run or attempt to run for $\sim$10 s. Exhaustion occurred at either 1.5 or 2.0 km h^{-1}. Expired gases were collected after exercise ended ('recovery') and the mean values in expired air from the first 30-40 s were used to estimate recovery $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. Prior experiments have shown that some reptiles have a locomotor constraint on oxygen transport during exercise (Carrier 1987; Farmer and Hicks 2000; Frappell et al. 2002; Munns et al. 2004; Owerkowicz et al. 1999), but previous studies on alligators have not demonstrated a locomotor constraint on cardiopulmonary convection during exercise (Farmer and Carrier 2000; Munns et al. 2005). Since we did not rest alligators between successive increasing treadmill speeds (Farmer and Carrier 2000; Munns et al. 2005), it was necessary, therefore, to determine if gas exchange during immediate recovery from exercise was different from gas exchange during the highest treadmill speed. Equations derived from Withers (1977) were used to calculate $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ rates at STPD for pre-exercise, each treadmill speed and recovery:

$$\dot{V}_{O_2} = \frac{\dot{V}_E(F_{I_{O_2}} - F_{E_{O_2}}) - F_{I_{O_2}}(\dot{V}_{CO_2})}{1 - F_{I_{O_2}}} \quad (1)$$

$$\dot{V}_{CO_2} = \frac{\dot{V}_{out}(F_{E_{CO_2}} - F_{I_{CO_2}}) - F_{I_{CO_2}}(\dot{V}_{O_2} - \dot{V}_{EWL})}{1 - F_{I_{CO_2}}} \quad (2)$$

where $\dot{V}_{O_2}$ is oxygen consumption rate (ml O_2 min^{-1}), $\dot{V}_{CO_2}$ is carbon dioxide production rate (ml CO_2 min^{-1}), $\dot{V}_{out}$ is excurrent airflow rate across the mask (ml min^{-1}; STP), $\dot{V}_E$ is dried excurrent airflow rate (ml·min^{-1}; STPD), F_I is fraction of inspired gas, F_E is fraction of expired gas and $\dot{V}_{EWL}$ is evaporative water loss rate (ml H_2O ·min^{-1}). $\dot{V}_{EWL}$ was not measured directly, but calculated using published minute ventilation rates for alligators during exercise and recovery (Farmer and Carrier 2000), with the assumption of full relative humidity of exhaled air. At the time of the $\dot{V}_{O_2}$ peak test, sedentary animals were significantly heavier than animals in either exercise group (ANOVA: $F_{2,32} = 3.3$, $P = 0.025$). It is likely that the energy expended during exercise training limited the growth of exercise-trained alligators, compared to sedentary controls; however, exercise-trained alligators did not appear chronically stressed. We express $\dot{V}_{O_2}$ in mass-specific terms because regression of raw values against mass yielded a high correlation coefficient ($R^2 = 0.88$, Simple Linear Regression, $P < 0.0001$).

$\dot{V}_{O_2}$ peak and $\dot{V}_{CO_2}$ peak values were taken as the highest oxygen consumption and carbon dioxide production rates, respectively, measured during any one treadmill speed or during recovery. Following the graded exercise test, each animal in the run and swim groups resumed its regular exercise regimen until it was sacrificed (1–3 months later).

Euthanasia, blood sample collection and tissue harvest

All alligators were sacrificed at age 26–27 months (October–December 2007). Mean mass at the time of sacrifice was 1.3 ± 0.2 kg for the run group, 1.4 ± 0.1 kg for the swim group and 2.0 ± 0.1 kg for the sedentary group. In order to assure that animals had fully recovered from their most recent bout of exercise, exercise training was suspended 2–4 days before each animal's sacrifice (Hartzler et al. 2006). Animals were fasted for 2–6 days prior to sacrifice. Following induction of anaesthesia with isoflurane, the animal was intubated and artificially ventilated (2–5% isoflurane; see 'Sham surgery', above). Blood samples were taken from the supraspinal vein using pre-heparinised 3 ml syringes, and haematocrit determined in triplicate (±1%) using a haematocrit microcentrifuge (3 min at 9,500*g*, Model Z231M; Hermle Labortechnik, Wehingen, Germany) and Micro-Hematocrit Capillary Tube Reader (Lancer®; Brunswick Company, St. Louis, MO, USA). Skeletal muscles were harvested sequentially (gastrocnemius, caudofemoralis, diaphragmaticus and splenius capitis) while the animal continued to be artificially ventilated under anaesthesia. The gastrocnemius (knee and ankle flexor), caudofemoralis (femoral retractor and rotator) and diaphragmaticus (liver retractor) were all likely stimulated during exercise, whereas splenius capitis (neck extensor) was likely not involved in running and swimming exercise motions and was chosen as a 'control' skeletal muscle. While under anesthesia and following removal of the skeletal muscles, animals were euthanised by excision of the heart. The atria and great vessels were carefully dissected away from both ventricles. The ventricles and skeletal muscles were blotted lightly with gauze, and respective wet masses measured on an analytical

balance (±0.001 g; Mettler AE 163; Mettler-Toledo Inc., Columbus, OH, USA). Samples of each skeletal muscle tissue (taken from the middle portion of each muscle) were promptly freeze-clamped with copper tongs pre-cooled in liquid nitrogen and stored at −80°C until homogenisation for enzyme assays.

Enzyme assays

We determined citrate synthase activity (CS; units g^{-1} tissue) and lactate dehydrogenase activity (LDH; units g^{-1} tissue) in triplicate at 30°C for each skeletal muscle using methods modified from Seebacher et al. (2003). Only fresh homogenates, kept on ice, were used for assays. Frozen tissues (0.045–0.070 g) were homogenised on ice using nine volumes of extraction buffer (pH 7.5). Extraction buffer consisted of 50 mmol L^{-1} imidazole/HCl, 2 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} ethylene diamine tetra-acetic acid (EDTA), 1 mmol L^{-1} reduced glutathione and 0.1% Triton X-100. Using a microplate reader (PowerWave XS; Biotech Instruments, Winooski, Vermont, USA), CS activity was estimated as reduction of 5,5′ dithiobis-(2-nitrobenzoic) acid (DTNB) at 412 nm and LDH activity as absorbance of NADH at 340 nm. Aliquots of original homogenates were further diluted by a factor of 2 for LDH samples and by 300 for CS samples. The CS assay medium consisted of 100 mmol L^{-1} Tris/HCl (pH 8.0), 1.0 mmol L^{-1} DTNB, 3.0 mmol L^{-1} acetyl CoA and 5.0 mmol L^{-1} oxaloacetate. The millimolar extinction coefficient of DTNB is 14.1. The LDH assay medium consisted of 100 mmol L^{-1} potassium phosphate buffer (KH_2PO_4/K_2HPO_4, pH 7.0), 0.16 mmol L^{-1} NADH and 4.0 mmol L^{-1} pyruvate. The millimolar extinction coefficient of NADH is 6.22. Sedentary animals were significantly heavier than exercise-trained animals at the time of sacrifice and tissue harvest (ANOVA: $F_{2,32} = 3.3, P < 0.001$), but expressing enzyme activities in mass-specific terms does not change results of the statistical analyses. Therefore, we chose to present enzyme activities in units g^{-1} tissue for comparison with a previous report of American alligator enzyme plasticity (Seebacher et al. 2003).

Statistical analyses

Mass-specific $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (ml gas kg^{-1} min^{-1}) was compared using a Kruskal–Wallis rank sums (KWRS; $\alpha = 0.05$) test between exercise groups (run, swim, sedentary) within each treadmill speed (mean value calculated from individual average expired gas values taken from last 30–40 s at 0.5, 1.0, 1.5 and 2.0 km h^{-1}) and for recovery (mean value calculated from individual average expired gas values taken from last 30–40 s) and $\dot{V}_{O_2}$ peak. Respiratory exchange ratio (RER; $\dot{V}_{CO_2}/\dot{V}_{O_2}$) was compared between exercise groups within each treadmill speed and recovery using a KWRS test ($\alpha = 0.05$). A significant χ^2 from a KWRS test was followed by post hoc comparisons using Wilcoxon rank sums (WRS) tests with Bonferroni adjustment ($\alpha = 0.0167$). Non-parametric tests were used because of platykurtic data. For ventricular and skeletal muscle wet mass, a one-way analysis of variance (ANOVA) compared mass-specific values (gram of tissue kg $animal^{-1}$) between exercise groups, and enzyme activities were also compared between groups using a one-way ANOVA. Haematocrit was compared between exercise groups using a one-way ANOVA on arcsine square root transformed fractions. A significant ANOVA ($\alpha = 0.05$) was followed by a Student Newman–Keuls (SNK) post hoc test ($\alpha = 0.05$).

Mass-specific $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ and RER were also compared within each exercise group between sequential treadmill speeds using repeated-measures ANOVA, followed by post hoc comparisons using one-tailed paired *t* tests with Bonferroni adjustment ($\alpha = 0.025$). Between the highest treadmill speed and recovery, a two-tailed paired *t* test with Bonferroni adjustment was used ($\alpha = 0.025$).

Throughout the text, mean values are given ±SE.

Results

Exercise training

The mean number of separate exhaustive exercise bouts was 178 ± 14 and 196 ± 1 for the run and swim groups, respectively, from the age of ~9 to ~25 months (prior to graded exercise test). For an individual exercise bout, animals in the run group exercised for an average of 4.5 ± 0.06 min and in the swim group for 5.5 ± 0.06 min (grand mean of weekly mean values taken to the nearest 15 s). These averages yield an approximate total of 70 min per month of exercise (5 min × 14 days per month), a value similar to the one likely obtained by Farmer and Carrier (2000) and Munns et al. (2005), but less than the more rigorous training schedule employed by Owerkowicz and Baudinette (2008).

$\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and RER

Compared to animals in the sedentary group, American alligators regularly exercised for 15 months showed a significant elevation in maximal oxygen consumption ($\dot{V}_{O_2}$ peak; KWRS test: $\chi^2_{2,32} = 12.34, P < 0.01$, WRS test with $\alpha = 0.0167$ for run > sedentary: $\chi^2 = 9.82, P < 0.01$ and for swim > sedentary: $\chi^2 = 7.67, P < 0.01$; Figs. 1, 2). $\dot{V}_{O_2}$ peak occurred at the recovery stage for 11 of 12 animals in the run group, for 9 of 12 animals in the swim group, and for 6 of 11 animals in the sedentary group. Recovery $\dot{V}_{O_2}$ was

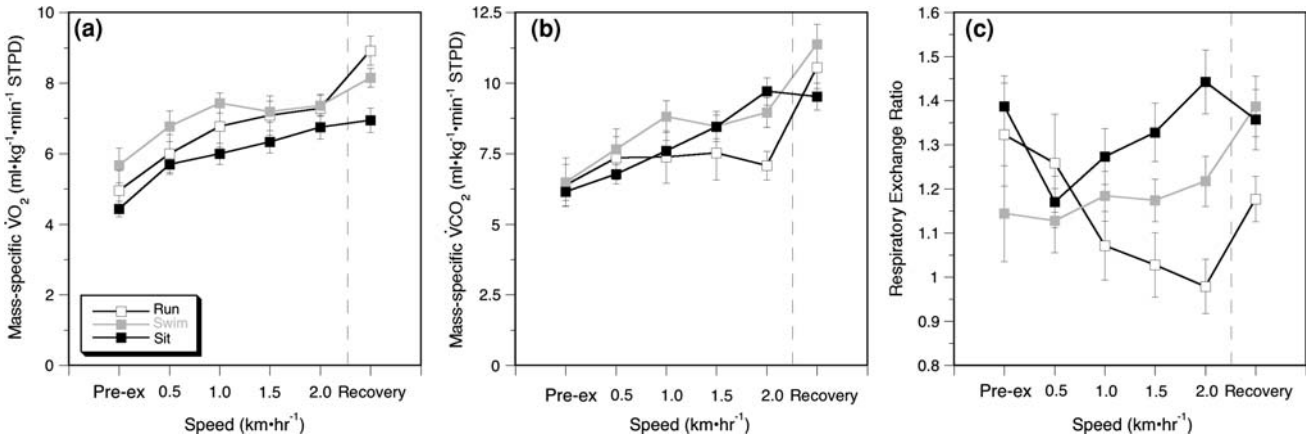

Fig. 1 Mean oxygen consumption rate (**a**), carbon dioxide production rate (**b**) and respiratory exchange ratio (**c**) before (Pre-ex), during (0.5–2.0 km h^{-1}) and after (recovery) a graded treadmill exercise test for alligators from different exercise groups, run, swim and sedentary (sit). All groups showed a significant increase in $\dot{V}_{O_2}$ with speed. The run group showed no trend towards increasing $\dot{V}_{CO_2}$ with increasing treadmill speed, and hence, displayed lower RERs than the swim and sedentary (sit) groups. *Error bars* are SE

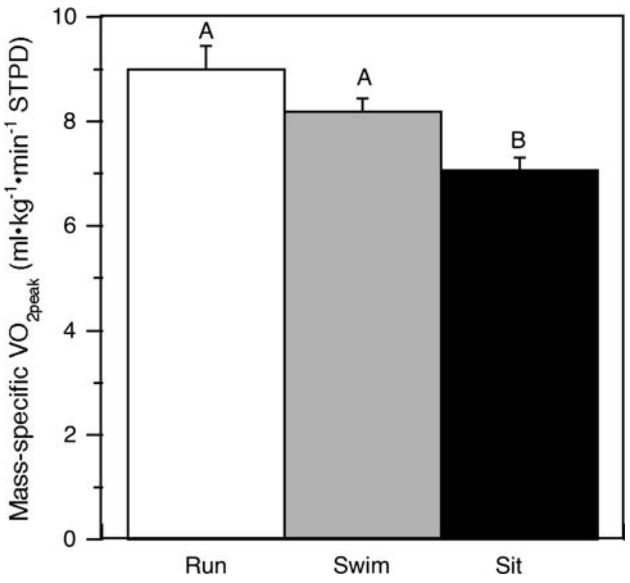

Fig. 2 Mean mass-specific peak oxygen consumption rate recorded during a graded treadmill exercise test for alligators from different exercise groups, run, swim and sedentary (sit). Run and swim groups had significantly higher $\dot{V}_{O_2}$ peak than the sedentary (sit) group. *Uppercase letters* above error bars indicate significant differences between groups derived from Kruskal–Wallis rank sums test ($\alpha = 0.05$) followed by Wilcoxon rank sums test with Bonferroni correction ($\alpha = 0.0167$). *Error bars* are SE

significantly higher for the run group, compared to sedentary (KWRS test: $\chi^2_{2,32} = 13.71$, $P < 0.01$, WRS test: $\chi^2 > 9.4$, $P < 0.01$). $\dot{V}_{O_2}$ at 1.0 km h^{-1} was significantly higher for the swim group, compared to sedentary (KWRS test: $\chi^2_{2,32} = 7.03$, $P = 0.03$, WRS test: $\chi^2 = 7.33$, $P < 0.01$). Compared to the sedentary group, $\dot{V}_{CO_2}$ trended lower at 2.0 km h^{-1} for the run group (KWRS test: $\chi^2_{2,32} = 6.94$, $P = 0.03$, WRS test: $\chi^2 = 5.44$, $P = 0.02$) but not for the swim group. Compared to sedentary animals, RER was significantly lower for the run group at 1.5 (KWRS test: $\chi^2_{2,32} = 8.38$, $P = 0.02$, WRS test: $\chi^2 = 7.33$, $P < 0.01$) and 2.0 km h^{-1} (KWRS test: $\chi^2_{2,32} = 10.38$, $P < 0.01$, WRS test: $\chi^2 = 6.76$, $P < 0.01$) and nearly significant during recovery (KWRS test: $\chi^2_{2,32} = 7.18$, $P = 0.03$, WRS test: $\chi^2 = 3.88$, $P = 0.05$). Compared to the swim group, RER was lower for the run group at 2.0 km h^{-1} (KWRS test: $\chi^2_{2,32} = 10.38$, $P < 0.01$, WRS test: $\chi^2 = 5.61$, $P = 0.018$) and recovery (KWRS test: $\chi^2_{2,32} = 7.18$, $P = 0.03$, WRS test: $\chi^2 = 5.88$, $P = 0.015$).

All three groups also showed a significant increase in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ with increasing treadmill speed (repeated-measures ANOVA: $F_{4,18} > 9.59$, $P < 0.001$). For all three groups, $\dot{V}_{O_2}$ at 0.5 km h^{-1} was significantly greater when compared to pre-exercise values (one-tailed paired *t* test with Bonferroni correction, $\alpha = 0.025$, $P < 0.01$), indicating the alligators were not overly agitated at the beginning of the exercise test. However, given considerable inter-individual variability in $\dot{V}_{O_2}$ at each speed, no significant difference was detected in $\dot{V}_{O_2}$ between successive speeds (one-tailed paired *t* test with Bonferroni correction, $\alpha = 0.025$, $P > 0.05$), except within the run group between speed 0.5 and 1.0 km h^{-1} ($P < 0.01$). At the end of the exercise, both the run and swim group showed a significant rise in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ during recovery (two-tailed paired *t* test with Bonferroni correction, $\alpha = 0.025$, $P < 0.02$). The sedentary group showed a significant rise in $\dot{V}_{O_2}$ during recovery (two-tailed paired *t* test with Bonferroni correction, $\alpha = 0.025$, $P = 0.015$), but not in $\dot{V}_{CO_2}$ ($P = 0.56$).

The RER steadily decreased with increasing treadmill speed, then spiked sharply during recovery for animals in the run group. In contrast, RER remained relatively constant during increasing treadmill speed for the swim group, then spiked during recovery. For the sedentary group, RER

increased with increasing treadmill speed, then dropped during recovery. The only significant differences were within the run and swim group, where RER spiked significantly during recovery (two-tailed paired t test with Bonferroni correction, $\alpha = 0.025$, $P < 0.02$; Fig. 1).

Ventricular and skeletal muscle mass and haematocrit

Mass-specific ventricular wet mass (combined ventricles; gram of tissue kg animal^{-1}) was significantly increased by an average of 11.5% in run and swim animals, compared to sedentary animals (one-way ANOVA: $F_{2,32} = 9.31$, $P < 0.001$, SNK $\alpha = 0.05$; Fig. 3a). For haematocrit, a similar average increase relative to sedentary animals was seen for both run (12.7%) and swim (9.9%) alligators (ANOVA on arcsine square root transformed fractions: $F_{2,32} = 4.62$, $P = 0.017$, SNK $\alpha = 0.05$; Fig. 3b). The skeletal muscles tested (gastrocnemius, caudofemoralis, diaphragmaticus and splenius capitis) did not show any significant change in mass-specific wet mass with exercise training (Table 1; one-way ANOVA: $F_{2,32} < 3.32$, $P > 0.05$); however, data for the caudofemoralis was suggestive of increasing muscle mass for both run and swim animals (one-way ANOVA: $F_{2,32} = 3.34$, $P = 0.053$).

Enzyme assays

No consistent trend for an increase or decrease in either aerobic (CS) or anaerobic (LDH) enzyme activities was seen across skeletal muscles tested (one-way ANOVA: $F_{2,11 \text{ or } 13} < 1.98$, $P > 0.18$; Fig. 4).

Discussion

Exhaustive exercise training increases maximum $\dot{V}_{O_2}$ in crocodilians

Exhaustive exercise training increased peak oxygen consumption ($\dot{V}_{O_2}$ peak) of a non-avian archosaur, American alligator. This result is in agreement with a recent report that exercise training increased $\dot{V}_{O_2}$ max in another crocodilian, estuarine crocodile (Owerkowicz and Baudinette 2008). Compared to sedentary control animals, alligators habitually trained on the treadmill (run) or in the flume (swim) exhibited $\dot{V}_{O_2}$ peak values 27 and 16% higher, respectively. The magnitude of this training effect is similar to the result obtained by Owerkowicz and Baudinette (2008) on juvenile estuarine crocodiles trained on a treadmill (post-training $\dot{V}_{O_2}$ max higher, on average, by 28% than pre-training values). The exercise sessions in each study followed similar protocols, with animals exercised to exhaustion during each training bout, although the frequency and duration of training differed. This approach was quite different from exercise training protocols employed in other vertebrate studies, which have found that training intensities corresponding to 75% $\dot{V}_{O_2}$ max can elicit significant increases in peak or maximum $\dot{V}_{O_2}$ (Adams et al. 1975; Bedford et al. 1979; Kieffer et al. 1998). Except when habituated to an exercise protocol (Farmer and Carrier 2000), crocodilians had previously shown an 'all-or-nothing' $\dot{V}_{O_2}$ response to exercise (Munns et al. 2005; Owerkowicz and Baudinette 2008), i.e., any treadmill speed elicits an anaerobic response which

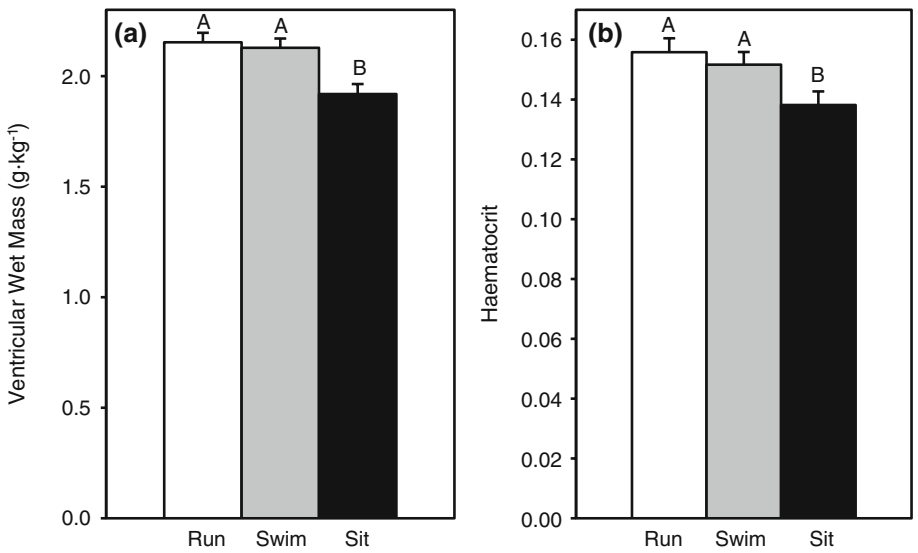

Fig. 3 Mean mass-specific ventricular wet mass (**a**) and mean haematocrit (**b**) for alligators from different exercise groups, run, swim and sedentary (sit). *Uppercase letters* above error bars indicate significant differences between groups within each metric derived from ANOVA followed by SNK post hoc test ($\alpha = 0.05$) (for haematocrit, fractional values were arcsine square root transformed). *Error bars* are SE

Table 1 Mass-specific skeletal muscle masses for selected skeletal muscles for alligators from different exercise groups: run, swim and sedentary

Group (*N*)	Gastrocnemius (g kg animal^{-1}) ± SE	Caudofemoralis (g kg animal^{-1}) ± SE	Diaphragmaticus (g kg animal^{-1}) ± SE	Splenius capitis (g kg animal^{-1}) ± SE
Run (12)	1.85 ± 0.04	16.03 ± 0.29	4.60 ± 0.09	2.83 ± 0.05
Swim (12)	1.83 ± 0.04	15.72 ± 0.26	4.18 ± 0.22	2.85 ± 0.09
Sedentary (11)	1.86 ± 0.05	14.94 ± 0.37	4.80 ± 0.13	2.91 ± 0.05

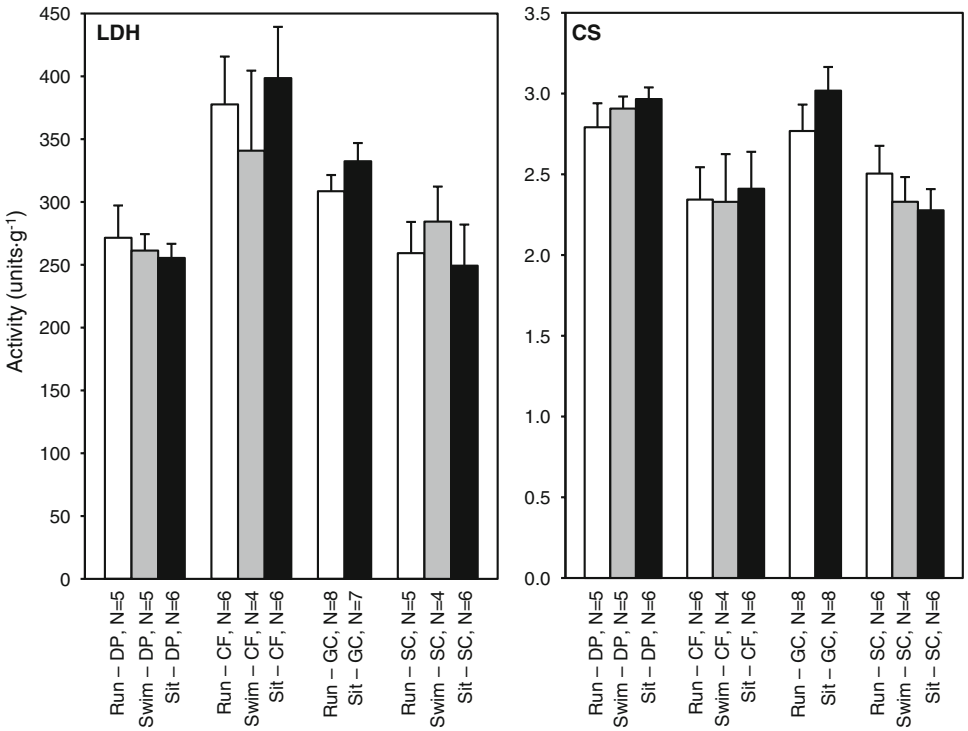

Fig. 4 Mean lactate dehydrogenase (LDH) and citrate synthase (CS) activities for skeletal muscles at 30°C for subsets of alligators from different exercise groups, run, swim and sedentary (sit). *DP* diaphragmaticus, *CF* caudofemoralis, *GC* gastrocnemius, *SC* splenius capitis. Aerobic (CS) and anaerobic (LDH) enzyme activity appeared unaffected by exercise training. *Error bars* are SE

suggests that energy demand exceeds $\dot{V}_{O_2}$ max. Therefore, flume and treadmill speeds were chosen to allow individual ~5 min training bouts over the course of the study.

In Owerkowicz and Baudinette (2008), juvenile male crocodiles were kept on a strict diet to limit their growth and maintain an approximately constant body mass (~1 kg) and were exercised more intensively (up to four times a day, 6 days each week for 16 weeks). In the present study, diet and growth of the juvenile female alligators were not limited, and the exercise frequency was lower (once a day, 7 times every 2 weeks) but continued over a much longer period (15 months). These data suggest that exhaustive exercise elicits a training effect in crocodilians of both sexes at variable periods of ontogenetic growth using different levels of training frequency.

Comparison with gas exchange data for other crocodilians

$\dot{V}_{O_2}$ peak of treadmill-trained alligators averaged 9.0 ml $O_2 \cdot kg^{-1}$ min^{-1}, that of flume-trained alligators—8.2 ml O_2 kg^{-1} min^{-1}, and that of sedentary alligators—7.1 ml O_2 kg^{-1} min^{-1} (Fig. 2). These values are very similar to the $\dot{V}_{O_2}$ max obtained in previous studies on American alligator (8.0–9.3 ml O_2 kg^{-1} min^{-1}) and estuarine crocodile (8.5 ml O_2 kg^{-1} min^{-1}) habituated to treadmill exercise (Farmer and Carrier 2000; Munns et al. 2005; Owerkowicz and Baudinette 2008). Results of our study are in agreement with Farmer and Carrier (2000), showing that alligators can demonstrate a graded $\dot{V}_{O_2}$ response with exercise intensity (i.e., significant increase in $\dot{V}_{O_2}$ with increasing speed), in contrast to previous reports

indicating an 'all or nothing' response for crocodilians to graded exercise (Munns et al. 2005; Owerkowicz and Baudinette 2008).

It is likely that the high RERs during 'pre-exercise' were a result of handling stress (metabolic acidosis) and hypoventilation (respiratory acidosis) that occurs when one picks up, holds and masks a crocodilian (i.e., they tend to struggle and hypoventilate). Following 'pre-exercise', animals were running on a treadmill, and CO_2 was quickly blown off during frequent breathing. With increasing treadmill speed, animals in the run and swim groups generally had lower RERs relative to sedentary animals. This observation reflects that chronic exercise training in alligators (regardless of the nature of the training regime—run or swim) can induce a more aerobic physiology capable of carrying (increased haematocrit) and delivering (increased heart size) more oxygen as exertion approaches maximal levels.

Plasticity of oxygen transport in crocodilians, squamates and mammals

Similar to mammals, American alligator demonstrated increased $\dot{V}_{O_2}$ (Carter et al. 2000; Evans and Rose 1988; Gleeson et al. 1983; Kemi et al. 2002; Musch et al. 1985), haematocrit (Brun et al. 2000; Ajmani et al. 2003) and heart size with exercise training (Richey and Brown 1998; Rowell 1974; Scheuer and Tipton 1977; Shephard and Balady 1999). Previous studies have failed to elicit an exercise training response in $\dot{V}_{O_2}$ for phylogenetically diverse squamates (Conley et al. 1995; Garland et al. 1987; Gleeson 1979; Thompson 1997; A. M. Szucsik et al., unpublished) and do not report consistent changes in cardiac morphology or haematocrit. For example, Garland et al. (1987) reported statistically higher haematocrit for exercise-trained *Ctenophorus* (*Amphibolurus*) *nuchalis*; however, haematocrit also tended to increase for sedentary, captive *C.* (*A.*) *nuchalis* compared to field-fresh lizards. Increased haematocrit can be a sign of over-training in human athletes (Brun et al. 2000; Gaudard et al. 2003) and may indicate that our exercise regime was stressful. However, crocodilian haematocrit is between a third and half that of humans, and the relatively moderate but significant increase in haematocrit demonstrated by exercise-trained alligators may represent an important and chronic mechanism to increase oxygen transport. Exercise training studies on lizards have not demonstrated an increase in ventricular size due to exercise training, with either no change in heart mass in response to exhaustive exercise training (Gleeson 1979) or a decrease in heart mass in response to exercise training (Garland et al. 1987). Importantly, increased heart mass can mediate an adaptive increase in stroke volume and $\dot{V}_{O_2}$ max in mammals (Gleeson 1979; Richey and Brown 1998; Rowell 1974; Scheuer and Tipton 1977), thereby aiding this convective step of the oxygen cascade.

In alligators, it has been demonstrated that venous return from the peripheral tissues to the heart is not limited by their locomotion (Munns et al. 2005), although in certain lizards the circulation limits the oxygen cascade during locomotion (Farmer and Hicks 2000; Frappell et al. 2002; Munns et al. 2004). Elevated intraabdominal pressure (IAP) limits venous return in *Varanus exanthematicus*, with peak venous return occurring after strenuous exercise has ceased (Munns et al. 2004). *V. mertensi* showed a decreased blood convection requirement ($Q_{tot}/\dot{V}_{O_2}$) with a large arterial-venous oxygen content difference and right-shifted Bohr curve indicating that circulation (Q_{tot}) limited the oxygen cascade (Frappell et al. 2002). Similarly, *Iguana iguana* showed a large increase in venous return following the end of strenuous treadmill exercise (Farmer and Hicks 2000). In contrast, venous return increased along with IAP in alligators during exercise and decreased during recovery, suggesting that alligators, unlike most squamates, are able to consistently return blood to their heart during strenuous exercise (Munns et al. 2005). It is possible that an increase in venous return during exercise stimulated the observed ventricular enlargement, as is thought to occur in mammals (McMullen et al. 2005; Pluim et al. 2000).

However, previous studies, which found no apparent constraints on convective oxygen transport during exercise in alligators (lung ventilation—Farmer and Carrier 2000; venous return—Munns et al. 2005), stand in contrast to our finding in this study: an increase in $\dot{V}_{O_2}$ following cessation of exercise (recovery) indicates possible locomotor constraint(s) on oxygen transport during exercise or $\dot{V}_A/\dot{Q}$ inhomogeneity. The multicameral lungs of alligators allow for relatively good $\dot{V}_A/\dot{Q}$ matching under resting conditions in anesthetised and conscious animals (Powell and Gray 1989; Powell and Hopkins 2004), but it is not known how $\dot{V}_A/\dot{Q}$ values change with exercise in crocodilians. It appears that exercise reduces the efficiency of O_2 exchange in many mammals and *V. exanthematicus*, but not in emu (*Dromaius novaehollandiae*; reviewed in Powell and Hopkins 2004). Our continuous, exhaustive exercise treadmill test may have limited optimal lung filling due to axial movements not observed when alligators run at a constant treadmill speed for multiple minutes (i.e. a 'steady state'; Farmer and Carrier 2000; Munns et al. 2005; Owerkowicz and Baudinette 2008), and the observed increased $\dot{V}_{O_2}$ during recovery from a continuous exhaustive exercise bout could have resulted from the recruitment of unventilated or unperfused regions of the lung.

Handling stress of alligators during the 15 months of exercise training could be a confounding factor in our study. Handling of exercise-trained animals before a bout

of exercise consisted of picking-up the animal, placing her into a dark container and transporting her to the treadmill or flume room (the treadmill room was often the vivarium room where the animals were housed). It is known that chronic stress can cause cardiomyopathy (e.g., Sharkey et al. 2005; Wittstein et al. 2005), however, no reported case, in either animal studies or human studies, correlates hypertrophic cardiomyopathy with increased aerobic performance. In humans, hypertrophic cardiomyopathy is a genetic disorder associated with a reduction in peak oxygen consumption, and lower peak oxygen consumption has been used to differentiate mild hypertrophic cardiomyopathy from an 'athlete's heart' (for a review see, Lauschke and Maisch 2009). It is, therefore, not likely that handling stress caused exercise-trained alligators to have increased heart mass and increased aerobic capacity. In addition, exercise-trained animals did not show signs of chronic stress (e.g., wasting, refusal to eat, aggression).

Seebacher et al. (2003) demonstrated that American alligators show thermal compensation for both CS and LDH activities. For example, when measured at 15°C, tail muscle extracts collected during the winter demonstrate higher enzyme activities than summer muscle extracts. Our enzymatic activity levels for LDH ($\sim$350 units g^{-1}) in caudofemoralis were very similar to values obtained for crocodilians acclimated to $\sim$30°C by Seebacher et al. (2003) and Seebacher and James (2008), whereas our values for CS ($\sim$2.3 units g^{-1}) in caudofemoralis are only slightly lower. It appears that temperature, but not exercise training, is a potent modulator of enzyme capacity in crocodilians.

Unlike the marked increase in aerobic enzyme capacity observed following long or short-term aerobic training in mammals (Baldwin et al. 1973; Holloszy and Booth 1976; Talanian et al. 2007), our data on American alligator are in agreement with previous molecular and biochemical research demonstrating a lack of plasticity in skeletal muscle at a molecular level for squamates when measured at a common temperature (Conley et al. 1995; Garland et al. 1987; Gleeson 1979; Szucsik et al. 2008; A. M. Szucsik et al., unpublished; Thompson 1997). CS activities in the ventricle and pyruvate kinase activity in the liver were elevated in *C.* (*A.*) *nuchalis* following an 8-week training schedule (Garland et al. 1987), however, Garland et al. (1987) also found that sedentary lizards had higher aerobic enzyme activity in thigh extensor muscles than exercise-trained lizards, contrary to what would be expected following an exercise training regime. Recently, it was demonstrated that chronic electrical stimulation for 8 weeks increases mitochondrial biogenesis in a hindlimb muscle of *V. exanthematicus* (Schaeffer et al. 2007), but in a separate study, 32 treadmill training bouts at 75% of $\dot{V}_{O_2}$ max over 8 weeks did not increase $\dot{V}_{O_2}$ max or alter myosin heavy chain expression in skeletal muscles of *V. exanthematicus* (A. M. Szucsik et al., unpublished). It remains to be tested whether a long-term and exhaustive training protocol, used in the present study and that on the estuarine crocodile (Owerkowicz and Baudinette 2008), can elicit an increase in heart mass, haematocrit or $\dot{V}_{O_2}$ max in a squamate.

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