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SHORT COMMUNICATION

Low cost of pulmonary ventilation in American alligators (*Alligator mississippiensis*) stimulated with doxapram

Nini Skovgaard[1,*], Dane A. Crossley[2] and Tobias Wang[1]

ABSTRACT

To determine the costs of pulmonary ventilation without imposing severe oxygen limitations or acidosis that normally accompany exposures to hypoxia or hypercapnia, we opted to pharmacologically stimulate ventilation with doxapram (5 and 10 mg kg^{-1}) in alligators. Doxapram is used clinically to alleviate ventilatory depression in response to anaesthesia and acts primarily on the peripheral oxygen-sensitive chemoreceptors. Using this approach, we investigated the hypothesis that pulmonary ventilation is relatively modest in comparison to resting metabolic rate in crocodilians and equipped seven juvenile alligators with masks for concurrent determination of ventilation and oxygen uptake. Doxapram elicited a dose-dependent and up to fourfold rise in ventilation, primarily by increasing ventilatory frequency. The accompanying rise in oxygen uptake was very small; ventilation in resting animals constitutes no more than 5% of resting metabolic rate. The conclusion that pulmonary ventilation is energetically cheap is consistent with earlier studies on alligators where ventilation was stimulated by hypoxia or hypercapnia.

KEY WORDS: Ventilatory response, Oxygen consumption, Cost of ventilation, Reptile, Ventilator stimulant, Chemoreceptor

INTRODUCTION

Pulmonary ventilation requires energy to overcome elastic and non-elastic forces when the lungs and, in particular, the chest wall are expanded during inhalation. Although both the structure of the gas exchange organs and the respiratory medium vary considerably amongst vertebrates, the cost of ventilation (COV) is considered to be quite low, of the order of 1–7% of resting metabolic rate, in fish, birds and mammals (Otis, 1950; Steffensen and Lomholt, 1983; Markley and Carrier, 2010). COV in reptiles, nevertheless, remains controversial, with estimates of 30% in unidirectional ventilated turtles, 52% in dormant tegu lizards and an incredible value of more than 60% in pregnant lizards (Kinney and White, 1977; Andrade and Abe, 1999; Munns, 2013), whilst other studies on turtles, alligators and awake tegu lizards provide considerably lower values (Jackson et al., 1991; Wang and Warburton, 1995; Skovgaard and Wang, 2004, 2007; Lee and Milsom, 2015).

The studies yielding low estimates of COV in reptiles are derived from simultaneous measurement of the rate of both oxygen uptake ($\dot{V}_{O_2}$) and ventilation ($\dot{V}_e$) where pulmonary ventilation typically has been stimulated by hypoxia or hypercapnia (Jackson et al., 1991; Wang and Warburton, 1995; Skovgaard and Wang, 2004). However, Munns (2013) also used hypercapnia to assess COV in the lizard *Tiliqua rugosa* and obtained estimates ranging from 20% in non-pregnant females to 63% 1 week prepartum and postpartum. The assessment of COV from simultaneous measurement of $\dot{V}_{O_2}$ and $\dot{V}_e$ assumes that the rise in $\dot{V}_{O_2}$ during exposure to the altered inspired gas composition can be ascribed solely to increased ventilation. However, the implicit constancy of all other energy-consuming processes during either hypoxia or hypercapnia is rather tenuous (Hicks and Wang, 2004), and this could therefore be argued to either underestimate or overestimate COV. It is nevertheless interesting that cervical vagotomy and hence elimination of pulmonary stretch receptor feedback and the accompanying rise in tidal volume, a response considered particularly costly, did not increase $\dot{V}_{O_2}$ in alligators (Skovgaard and Wang, 2007).

Given that both hypoxia and hypercapnia may affect $\dot{V}_{O_2}$, we opted to stimulate ventilation pharmacologically in alligators using the ventilatory stimulant doxapram. Doxapram was synthesised in the 1960s (Ward and Franko, 1962) and quickly gained wide usage in human as well as veterinary medicine to stimulate ventilation post-operatively in patients with ventilatory depression (Yost, 2006; Golder et al., 2013). In mammals, doxapram predominantly stimulates tidal volume, and while it was originally described to act on the central nervous system, most of the ventilatory response is now believed to stem from direct stimulation of peripheral chemoreceptors in the aortic and carotid bodies (Yost, 2006; Golder et al., 2013). Regardless of the exact mechanism, doxapram causes hyperventilation without changes in the inspired gases. It follows, therefore, that a rise in $\dot{V}_e$ upon doxapram administration should cause $\dot{V}_{O_2}$ to increase if COV is high. We tested this hypothesis in American alligators (*Alligator mississippiensis* Daudin 1802).

MATERIALS AND METHODS

Experimental animals

Experiments were undertaken on seven juvenile American alligators (*A. mississippiensis*) of undetermined sex weighing between 0.52 and 1.05 kg (0.72±0.09 kg, mean±s.e.m.). The animals were imported from Rockefeller Wildlife Refuge and shipped to Aarhus University, where they were kept in aquaria containing water at 27°C with access to dry platforms and basking lamps, allowing for behavioural thermoregulation. Food was withheld 3 days prior to experiments. All animals appeared healthy and grew considerably in captivity. Experiments were performed according to Danish Federal Regulations.

Anaesthesia and cannulation of the femoral artery

Anaesthesia was induced by inhalation of approximately 5% isoflurane (Isofluran, Baxter, Denmark) until all reflexes disappeared, allowing for intubation and mechanical ventilation with 1–2% isoflurane during the remaining surgery (~30 min). The femoral artery was accessed through a 3 cm incision on the dorsal

[1]Zoophysiology, Department of Bioscience, Aarhus University, 8000 Aarhus C, Denmark. [2]Developmental Integrative Biology Cluster, Department of Biological Sciences, University of North Texas, Denton, TX 76203, USA.

*Author for correspondence (nini.jensen@bios.au.dk)

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side of the leg. The muscles were separated along the connection between major muscle groups, and lidocaine was applied on the artery to prevent vasoconstriction. The vessel was occlusively cannulated with a PE50 catheter filled with heparinised (50 IU ml^{-1}) saline for measurement of systemic blood pressure. The catheter was secured to the skin, and the mask for measurements of ventilation was glued over the nostrils when the alligators resumed spontaneous ventilation and appeared to have recovered from anaesthesia. Then, the animals were left to recover overnight.

Measurements of blood pressure and derivation of heart rate

The catheter was connected to Baxter Edward (model PX600, Irvine, CA, USA) disposable pressure transducers and the signals were amplified using an in-house-built preamplifier. The pressure transducers were positioned at the level of the animal's heart and calibrated daily using a static water column. Signals from the pressure transducers were recorded with a Biopac MP100 data acquisition system (Biopac Systems, Inc., Goleta, CA, USA) at 100 Hz, and heart rate was derived from the pulsatile pressure signal.

Measurements of ventilation and oxygen uptake

$\dot{V}_e$ and $\dot{V}_{O_2}$ were measured simultaneously using pneumotachography (Glass et al., 1978, modified by Wang and Warburton, 1995). A mask constructed from Bioplast (Scheu-Dental, Iserlohn, Germany) was glued over the snout of the alligator and connected to a resistor (Fleisch tube) upstream from the animal, so a differential pressure transducer (Valendyne MP-45-1-871, Northridge, CA, USA) could monitor the pressure changes across the resistor. Downstream from the alligator, an AMETEK (Applied Electrochemistry Technologies, Pittsburgh, PA, USA) R1 flow control pump pulled air through the mask and a drying tube (Drierite, Xenia, OH, USA) before entering an AMETEK O_2 analyser. The flow rate (875 ml min^{-1}) was sufficient for breath-to-breath analysis of oxygen uptake. The signals from the differential pressure transducer and the gas analyser were recorded with a Biopac MP100 data acquisition system (Biopac Systems, Inc.) at 100 Hz. Masks were calibrated by simulating breaths of known volume and gas composition. The relationship between the electrical signal generated and the known tidal volumes and gas compositions could be accurately described by linear regression (R^2>0.99).

Experimental protocol

The animals were placed in a box maintained within a climatic chamber during measurements where they were shielded from visual and auditory disturbances, and maintained at 25°C. Resting values of ventilation, $\dot{V}_{O_2}$ and blood pressure were recorded for 1 h before two doses (5 and 10 mg kg^{-1}) of the ventilatory stimulant doxapram was administered. Initial experiments showed that intravascular injections of doxapram elicited an immediate and dose-dependent rise in breathing frequency that persisted for hours. Injections were separated by 1 h and were given in aliquots of 1 ml kg^{-1} through the catheter.

Data analysis and statistics

At each concentration of doxapram (0, 5 and 10 mg kg^{-1}), 15 representative and consecutive breaths were analysed to obtain breathing frequency, tidal volume and $\dot{V}_{O_2}$ using AcqKnowledge data analysis software (v 3.7.2, Biopac). The effects of doxapram on cardiorespiratory parameters were assessed using a repeated one-way ANOVA followed by Dunnett's *post hoc* test. Differences were considered statistically significant at a 95% confidence level (P<0.05). All data are presented as means±s.e.m.

RESULTS AND DISCUSSION

As in other studies on undisturbed resting juvenile alligators (Wang and Warburton, 1995; Farmer and Carrier, 2000a; Munns et al., 2005; Skovgaard and Wang, 2007), the ventilatory pattern consisted of single breaths commencing with an exhalation followed by an inhalation (Fig. 1). Also, the resting values of both $\dot{V}_{O_2}$ and $\dot{V}_e$ (Fig. 2) compare favourably with previous studies on fully recovered and undisturbed alligators using similar techniques (Wang and Warburton, 1995; Farmer and Carrier, 2000a; Munns et al., 2005; Skovgaard and Wang, 2007), but are higher than those reported by Branco and Wood (1993).

The intravascular injections of doxapram elicited a prompt and dose-dependent rise in pulmonary ventilation that persisted for hours. The approximately fourfold rise in $\dot{V}_e$ at the highest dose was almost entirely due to increased breathing frequency, whilst tidal volume remained unaffected (Fig. 2). The rise in frequency was accompanied by a significant, but modest, shortening of the inspiratory time from 4.9±0.6 to 3.8±0.2 s^{-1}, such that the ventilatory response was primarily mediated by shortening the non-ventilatory period between each breath (data not shown). This means that the energy expenditure to overcome elastic and non-elastic forces probably remained the same for each individual breath. Because each breath occurred alone, there is no confounding influence of elastic recoil having made subsequent breaths within an episode energetically cheaper than the first. Also, the small rise in $\dot{V}_{O_2}$ was entirely linear over the fourfold elevation of $\dot{V}_e$ (Fig. 3).

Because the ventilatory response was elicited without hypoxia or hypercapnia, the graphical expression of $\dot{V}_{O_2}$ as a function of $\dot{V}_e$ (Fig. 3) is likely to be devoid of any reductions in resting metabolic rate due to acidosis (as a result of hypercapnia) or hypoxia, although the hyperventilation must have incurred a respiratory alkalosis. The analysis in Fig. 3 yields a rather benign COV of 1.9 ml O_2 l^{-1} air, which is of the order of 5% of resting metabolic rate. This is consistent with other direct measurements in other vertebrates (Otis, 1950; Steffensen and Lomholt, 1983; Jackson et al., 1991; Wang and Warburton, 1995; Skovgaard and Wang, 2004, 2007; Markley and Carrier, 2010; Lee and Milsom, 2015), but lower than more indirect estimates in lizards (*Tupinambis* and *Tiliqua*) and several species of turtles (Kinney and White, 1977; Andrade and Abe, 1999; Skovgaard and Wang, 2004; Munns, 2013; Cordeiro et al., 2014; see also Lee and Milsom, 2015). As recently emphasised by Cordeiro et al. (2014)

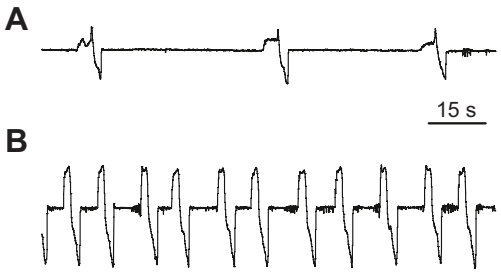

Fig. 1. Ventilatory pattern in *Alligator mississippiensis*. Original traces from one alligator showing breathing patterns at rest (A) and after injection of the ventilatory stimulant doxapram (10 mg kg^{-1}; B), demonstrating that doxapram primarily stimulates ventilation by increasing respiratory frequency.

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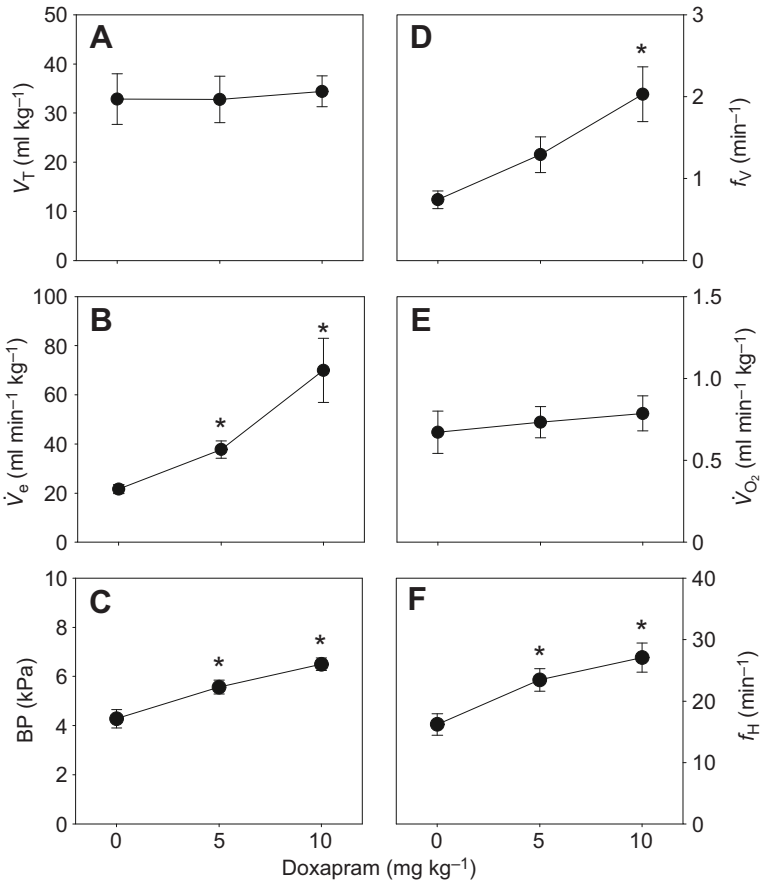

Fig. 2. Cardiorespiratory effects of doxapram in *A. mississippiensis*. The dose dependency of the effect of doxapram (5 and 10 mg kg^{-1}) on tidal volume (V_T, A), total ventilation ($\dot{V}_e$, B), blood pressure (BP, C), breathing frequency (f_V, D), oxygen uptake ($\dot{V}_{O_2}$, E) and heart rate (f_H, F). Values are means±s.e.m., N=7. An asterisk denotes values significantly different from resting values (i.e. prior to doxapram injection; P<0.05).

as well as Lee and Milsom (2015), it may be more relevant to compare the actual COV between species. Here, our estimation of 1.9 ml O_2 l^{-1} air is also lower than many of the previous studies on reptiles [see tables in Cordeiro et al. (2014) and Lee and Milsom (2015)], where particularly studies involving inhalation of hypoxic gas mixtures have resulted in values as high as 8–20 ml O_2 l^{-1} air, which may reflect that hypoxia causes stress and agitation leading to increased metabolism. This explanation could also explain why Wang and Warburton (1995) provided an absolute COV of around 5 ml O_2 l^{-1} air in hypoxia-exposed alligators, whereas exposure to hypercapnia resulted in a small reduction in $\dot{V}_{O_2}$ despite a marked ventilatory response (i.e. a paradoxical negative COV).

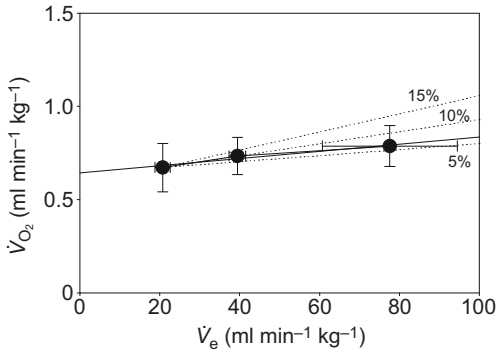

Fig. 3. Analysis of the oxidative cost of ventilation in *A. mississippiensis* at 25°C showing $\dot{V}_{O_2}$ at different levels of $\dot{V}_e$. Points represent mean values at each dose of doxapram and the solid line is a linear regression through these points with a slope of 1.9 ml O_2 l^{-1} air (y=0.0019x+0.642, r^2=0.945). Dotted lines depict calculated oxygen uptake–ventilation relationships for three different cost of ventilation values relative to resting metabolic rate. The calculations are based on average values for $\dot{V}_{O_2}$ and $\dot{V}_e$ at rest ($\dot{V}_{O_2}$=0.67 ml min^{-1} kg^{-1} and $\dot{V}_e$=20.73 ml min^{-1} kg^{-1}).

There is no reason to believe that the structurally rather complex lungs of alligators should support particularly low energetic costs (Perry, 1988), although possible energetic savings of the hepatic piston (Gans and Clark, 1976; Farmer and Carrier, 2000b) have not been taken into account. Likewise, the consequences of the newly discovered unidirectional flow in the crocodilian lung (Farmer and Sanders, 2010; Farmer, 2015) for flow resistive forces may also be worthwhile considering for future analyses of the elastic versus non-elastic contributions to COV in crocodilians.

Consistent with the original description of the physiological effects of doxapram in mammals (Ward and Franko, 1962), we observed a significant pressor effect, and the rise in mean arterial blood pressure of the alligators was accompanied by a dose-dependent tachycardia (Fig. 2). In the context of the present study, this cardiovascular effect means that cardiac work must also have increased, which, if anything, would have contributed to an overestimation of COV. In humans and other mammals, doxapram is also known for increasing anxiety and reducing sleep. If similar effects were elicited in the alligators, it is likely to have caused an elevation in $\dot{V}_{O_2}$, and would therefore lead to an overestimation of COV in the analysis presented in Fig. 3. We conclude that crocodilians indeed have a low COV and probably resemble other vertebrates in this context.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
All authors contributed to the conception and the experimental design. N.S. performed most of the experiments and the data analysis, and all authors contributed to writing the manuscript.

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