



SYMPOSIUM

Atmospheric Oxygen Levels Affect Mudskipper Terrestrial Performance: Implications for Early Tetrapods

Corey J. Jew,^{1,*} Nicholas C. Wegner,^{2,*†} Yuzo Yanagitsuru,* Martin Tresguerres* and Jeffrey B. Graham*

*Center for Marine Biotechnology and Biomedicine and Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093, USA; †Fisheries Resource Division, Southwest Fisheries Science Center, NOAA Fisheries, La Jolla, CA 92037, USA

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¹E-mail: cjew@ucsd.edu

²E-mail: nwegner@ucsd.edu

Synopsis The Japanese mudskipper (*Periophthalmus modestus*), an amphibious fish that possesses many respiratory and locomotive specializations for sojourns onto land, was used as a model to study how changing atmospheric oxygen concentrations during the middle and late Paleozoic Era (400–250 million years ago) may have influenced the emergence and subsequent radiation of the first tetrapods. The effects of different atmospheric oxygen concentrations (hyperoxia = 35%, normoxia = 21%, and hypoxia = 7% O₂) on terrestrial performance were tested during exercise on a terrestrial treadmill and during recovery from exhaustive exercise. Endurance and elevated post-exercise oxygen consumption (EPOC; the immediate O₂ debt repaid post-exercise) correlated with atmospheric oxygen concentration indicating that when additional oxygen is available *P. modestus* can increase oxygen utilization both during and following exercise. The time required post-exercise for mudskippers to return to a resting metabolic rate did not differ between treatments. However, in normoxia, oxygen consumption increased above hyperoxic values 13–20 h post-exercise suggesting a delayed repayment of the incurred oxygen debt. Finally, following exercise, ventilatory movements associated with buccopharyngeal aerial respiration returned to their rest-like pattern more quickly at higher concentrations of oxygen. Taken together, the results of this study show that *P. modestus* can exercise longer and recover quicker under higher oxygen concentrations. Similarities between *P. modestus* and early tetrapods suggest that increasing atmospheric oxygen levels during the middle and late Paleozoic allowed for elevated aerobic capacity and improved terrestrial performance, and likely led to an accelerated diversification and expansion of vertebrate life into the terrestrial biosphere.

Introduction

The composition of the Earth's atmosphere has fluctuated throughout history, being molded by the forces of climate, tectonics, asteroid impacts, and the appearance and abundance of life (Knoll 2003). The timing and magnitude of fluctuations in atmospheric oxygen have not only been heavily shaped by biological processes but have also likely impacted the evolutionary history of life on this planet. The appearance of cyanobacteria and algae in the fossil record correlates with rises in atmospheric oxygen 2.7 and 1 billion years ago, respectively

(reviewed by Xiong and Bauer 2002), while relatively sharp drops in oxygen have been suggested as a factor in large losses of biodiversity (extinction events) (Bernier et al. 2007). Although many researchers have correlated a number of biological phenomena with changes in the Earth's atmosphere throughout the Phanerozoic Eon (from 550 million years ago [mya] to the present), modeled oxygen levels as well as paleontological data generally lack the temporal resolution required to provide direct association (i.e., cause and effect) between atmospheric changes and a biological response

(Powell 2010; Graham et al. 2013). Nevertheless, there is general consensus among historic models of the Earth's atmospheric composition that oxygen concentration increase during the middle and late Paleozoic Era (400–250 mya), reaching 30–35% during the late Paleozoic oxygen pulse (~320–260 mya), a period linked to several transformative evolutionary events.

Graham et al. (1995) hypothesized that the late Paleozoic oxygen pulse (likely caused by the appearance of vascular land plants) may have released the constraints of diffusion for some organisms and allowed for the evolution of insect gigantism and the emergence of costly aerobic processes, such as insect flight. These proposed implications are supported by the fossil record showing gigantism in a diverse lineage of insects, with prehistoric dragonflies having wingspans extending up to 70 cm during the late Carboniferous and Permian (320–250 mya) (May 1982; Shear and Kukalová-Peck 1990; Carpenter 1992). Graham et al. (1995) also proposed that increased availability of oxygen may have fueled the diversification and ecological radiation of early tetrapods by allowing for elevated aerobic capacity and the concomitant increase in terrestrial performance. This hypothesis is supported by paleontological data showing that the Tetrapodomorpha came onto land during the Middle Devonian (~350 mya) (although potential terrestrial trackways have been found as early as 395 mya) (Niedzwiedzki et al. 2010), when atmospheric oxygen was rising from a low (~7%) ~375 mya to above the present-day level (20.95%). Oxygen continued to rise into the Carboniferous, reaching above 30% as the tetrapods underwent an explosive diversification and achieved an almost global distribution by the Early Permian (~290 mya) (Clack 2012).

A number of studies have sought to validate the hypothesis of Graham et al. (1995) by examining the effects of oxygen concentration on extant organisms. These have focused mainly on insects and have shown increased performance for a few species reared in hyperoxia, while general reductions in flight-performance for those reared in hypoxia (reviewed by Harrison et al. 2010). Multigenerational studies have shown increased body size and a decreased investment in respiratory tracheae for several species housed in hyperoxia, while in hypoxia body size is reduced and tracheal investment is increased. Harrison et al. (2010) proposed that due to the elevated diffusive flux of oxygen, hyperoxia reduces the demand for tracheal investment and allows for gigantism by decreasing the energetic and spatial cost of the respiratory system, thus increasing limb strength and reducing

the displacement of vital tissues. In vertebrates, Owerkowicz et al. (2009) has shown $\dot{V}O_2$ (rate of oxygen consumption) during digestion, $\dot{V}O_2$ per breath, and growth rate all correlate with the availability of oxygen (12–30% O_2) for the American alligator (*Alligator mississippiensis*). However, little other comparable research has been conducted on vertebrate physiology in the context of Paleozoic oxygen levels. This is largely due to the long generation times of vertebrates and the lack of extant groups closely related to the Tetrapodomorpha.

This study is an attempt to further uncover the physiological implications of fluctuations in Paleozoic oxygen concentration on the tetrapod lineage using an extant vertebrate model, the Japanese mudskipper, *Periophthalmus modestus*. Mudskippers (Teleostei: Gobiidae), which appeared during the early to mid Tertiary (40–50 mya) (Flück et al. 2007), are amphibious intertidal fishes inhabiting mangroves and mudflats of the Indo-Pacific region and are the pinnacle of the actinopterygian (ray-finned fish) progression onto land (Murdy 1989). These fishes voluntarily emerge from water and possess numerous adaptations for terrestrial life including modified fins used for terrestrial locomotion, flattened lenses, and steeply shaped corneas adapted for aerial vision, the ability to detect air borne sounds, and a highly vascularized buccopharyngeal chamber, and in some species, high-density areas of subcutaneous capillaries used for air breathing (reviewed by Clayton 1993; Graham 1997; Schwab 2003; Sayer 2005; Graham et al. 2007). With these adaptations, mudskippers spend a large portion of their time and are highly active on land. Although mudskippers are not closely related to tetrapods and have independently acquired adaptations for terrestrial life, their recent land invasion uniquely separates them from other extant vertebrate models for studying the physiological changes of the vertebrate transition to land (Graham and Lee 2004). This study examines the effect of atmospheric oxygen concentration on *P. modestus* terrestrial performance (exercise and recovery) in hyperoxia (35% O_2 , the highest level estimated during the Paleozoic), normoxia (21%, present day O_2 level), and hypoxia (7%, the lowest estimate during the Devonian, ~375 mya) to better understand the influence of Paleozoic oxygen concentrations on the evolution and radiation of early tetrapods.

Materials and methods

Acquisition and animal care

Periophthalmus modestus were collected from mudflats in Ashikari, Saga Prefecture, Japan, and shipped

by air to San Diego, CA, USA, during the summers of 2010 through 2012. Upon arrival, fish were sorted according to size and held in partially filled aquaria with brackish water (15 ppt). Each aquarium contained filtration, water heaters set at 25°C, and submerged and floating structures so that mudskippers could seek refuge and emerge from the water at will. Mudskippers were fed bloodworms and minced shrimp twice a week but were not fed for at least 48 h prior to experimentation. All mudskipper husbandry and experimentation was approved by the University of California, San Diego, Institutional Animal Care and Use Committee (Protocol no. S05372).

Endurance on a treadmill

In order to assess the effects of atmospheric oxygen concentration on mudskipper terrestrial endurance, 17 *P. modestus* (5.08 ± 0.62 g, mean \pm SD, sex not differentiated) were exercised to exhaustion under three regimes (hyperoxia = 35%, normoxia = 21%, and hypoxia = 7% O₂) using a miniature, enclosed terrestrial treadmill ($75 \times 17.5 \times 20$ cm, $l \times w \times h$) (Fig. 1). Atmospheric oxygen level within the enclosure was controlled by throttling the valves connected to an O₂ and N₂ tank and was monitored using a Cameron Instrument Co (Port Aransas, TX) DO-051 Clark-type oxygen electrode. The enclosure was maintained at 25°C using a heat lamp and reservoir of warm water held below the treadmill platform. The treadmill was kept moist by a drip tube in the roof of the enclosure. All fish were allowed to acclimate to the treadmill environment for 1 min before exercise, following which the treadmill was set to a speed of 5.5 cm s^{-1}

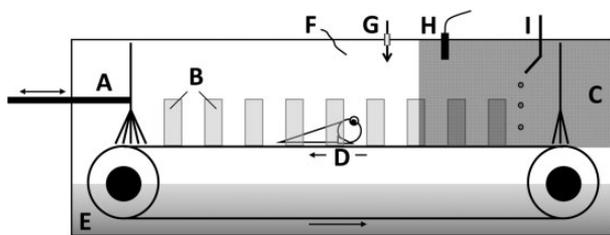


Fig. 1 Enclosed terrestrial treadmill used to exercise mudskippers. (A) Moveable plastic bristle brush used to prod mudskippers forward, (B) vertical bars painted on the side of the enclosure to help mudskippers visually maintain their position on the treadmill, (C) shaded “refuge” created by black plastic, (D) tread surface (arrows indicate direction of tread rotation), (E) warm water reservoir at 25°C, (F) thermocouple to monitor atmospheric temperature, (G) gas input valve to adjust atmospheric concentration of oxygen, (H) Clark-type electrode used to monitor atmospheric concentration of oxygen, (I) drip tube to keep tread moist.

(based on preliminary trials to elicit a natural velocity fast enough to force exhaustion). Fish were encouraged to “skip” and “crutch” forward (Harris 1960) by shading the front of the treadmill with black plastic (to simulate a refuge) and by prodding them with a plastic-bristled brush from behind. Mudskippers were exercised to exhaustion, defined by when a fish would fall against the brush at the back of the treadmill and no longer respond to prodding, or for a maximum of 15 min. Each fish was exercised three or more times at each oxygen concentration in random order to determine mean individual endurance under each condition (all fish in these and subsequently described experiments were allowed to recover for a minimum of 48 h between trials).

Recovery from exercise

To determine the effect of atmospheric oxygen concentration on terrestrial post-exercise recovery, nine mudskippers (4.40 ± 0.47 g) were exercised on the treadmill (5.5 cm s^{-1} under normoxia) until exhaustion and their recovery was monitored for up to 22 h in a flow-through respirometry system held at 35%, 21%, or 7% O₂. Resting metabolic rate (resting $\dot{V}O_2$), time to return to resting $\dot{V}O_2$, and excess post-exercise oxygen consumption (EPOC) were determined for each individual fish recovering under each oxygen concentration using averaged and subsampled data at 5 min intervals. Resting $\dot{V}O_2$ for each individual was determined by the mean of the 2-h segment in which $\dot{V}O_2$ was the lowest during the recovery record. Time to return to resting $\dot{V}O_2$ was defined as the time required post-exercise for a fish’s elevated metabolic rate to first return to its respective resting $\dot{V}O_2$. EPOC, the immediate repayment of the oxygen debt incurred during exercise, was calculated as the total amount of oxygen consumed above resting $\dot{V}O_2$ between time “0” (end of exercise and placement into the respirometer) and the time to return to resting $\dot{V}O_2$.

The flow-through respirometry system used to determine these variables consisted of a holding chamber (30 cm^3 glass syringe) connected to oxygen, carbon dioxide, and water vapor analyzers (Oxzilla II, CA-10, and RH-300, Sable Systems International, Las Vegas, NV). Compressed gas of the desired oxygen concentration (35%, 21%, or 7% O₂, balanced by N₂) was forced through the respirometer at 30 ml min^{-1} (regulated by a Sable Systems SS-4). Before entry into the holding chamber, gas was scrubbed of residual carbon dioxide with a column of Ascarite II (Thomas Scientific, Swedesboro, NJ) and passed through a bubbler for humidification. Leaving the chamber, carbon dioxide and water vapor pressure (WVP) were measured and then

moisture was scrubbed from the gas stream using a drierite (W.A. Hammond Drierite Company, Ltd, Xenia, OH) column to increase definition in measuring oxygen. The holding chamber was kept at 25°C by a surrounding coil of copper tubing connected to a Neslab (Portsmouth, NH) RTE-Series refrigerated bath/circulator. Disturbances from light and sound were minimized by covering the chamber in dark plastic and vacating the laboratory.

Partitioning respiration

In preliminary respirometry trials, it was noticed that following exercise, mudskipper aerial oxygen consumption and carbon dioxide production were generally smooth traces interspersed with peaks of elevated gas exchange (Fig. 2). Video recordings synchronized with respirometry records showed these peaks to be associated with mudskippers releasing gas held in the buccopharyngeal chamber. Assuming cutaneous respiration is therefore responsible for the generally smooth metabolic signal while the buccopharyngeal chamber contributes the peaks above this rate, the respiratory partitioning between the two sites can be determined. To examine the effects of atmospheric oxygen concentration on the partitioning of mudskipper respiration between the buccopharyngeal chamber and the skin during peak exercise recovery, 17 fish (4.21 ± 0.79 g) were exercised to exhaustion (in normoxia at 5.5 cm s^{-1}) and placed in the respirometer with an elevated gas flow-through rate of 200 ml min^{-1} to increase the resolution of the metabolic signal. Oxygen consumption

and carbon dioxide production were recorded in each of the three oxygen regimes for the first 90 min post-exercise. Gas exchange at the buccopharyngeal chamber (bucco $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$) were calculated by integrating the peaks above the skin rates (skin $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$) (Fig. 2).

Data analysis

Due to the censoring of the treadmill exercise data beyond 15 min, a survival analysis and log-rank test with a *post-hoc* pairwise comparison was used to determine statistical significance ($P < 0.05$) of mudskipper endurance between treatments.

Respirometry data were analyzed using Expedata (Sables Systems International). Data on $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ were corrected for drift using values obtained by bypassing the respirometry chamber every 2 h during recovery. Background respiration was calculated by subtracting blank-chamber measurements made before and after each trial. Carbon dioxide measurements were corrected for WVP using the equation:

$$\text{CO}_2 = \frac{\text{CO}_{2\text{wvp}} \cdot \text{BP}}{\text{BP} - \text{WVP}}, \quad (1)$$

where $\text{CO}_{2\text{wvp}}$ is the uncorrected fractional concentration of carbon dioxide and BP is the barometric pressure. Mudskipper oxygen consumption and carbon dioxide production were calculated using the formulas:

$$\dot{V}\text{O}_2 = \text{FR} \cdot (\text{F}_1\text{O}_2 - \text{F}_E\text{O}_2) \quad \text{and} \quad (2)$$

$$\dot{V}\text{CO}_2 = \text{FR} \cdot (\text{F}_E\text{CO}_2 - \text{F}_1\text{CO}_2), \quad (3)$$

where FR is the flow rate and F_1 and F_E are the initial and end fractional concentrations of the respective gases.

When comparing treatments in exercise recovery and partitioning data, statistical significance ($P < 0.05$) was determined for all parameters using repeated-measures ANOVA with a *post-hoc* Tukey test or, if only two groups were compared, a paired *t*-test in R (version 2.15.2).

Results

Endurance

Mudskipper endurance in hyperoxia (12.33 ± 2.49 min) was significantly greater than in normoxia (8.54 ± 2.43 min), which was significantly greater than in hypoxia (4.90 ± 0.94 min).

Recovery from exercise

Mean post-exercise oxygen consumption as a function of atmospheric oxygen concentration is shown

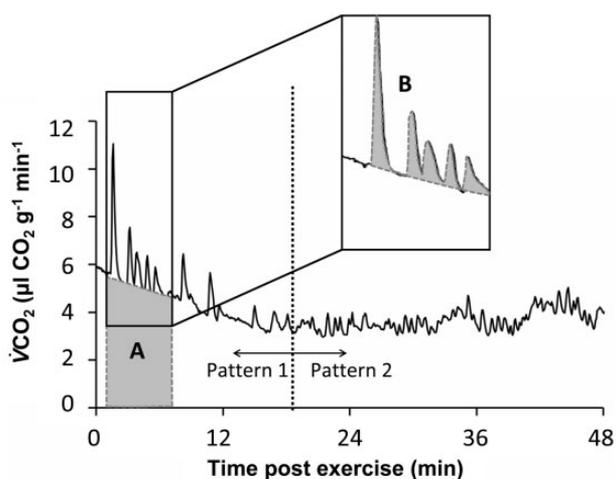


Fig. 2 Sample high-resolution trace of carbon dioxide production from *P. modestus* recovering in normoxia following exhaustive exercise. Shown is the relative contribution of the skin (A) and buccopharyngeal chamber (B) to gas exchange. Transition in ventilatory behavior from Pattern 1 to Pattern 2 is indicated by the dashed line.

in Fig. 3. Resting $\dot{V}O_2$ for *P. modestus* during post-exercise recovery did not significantly differ between hyperoxia ($1.94 \pm 0.40 \mu\text{l O}_2 \text{ min}^{-1} \text{ g}^{-1}$) and normoxia ($2.09 \pm 0.73 \mu\text{l O}_2 \text{ min}^{-1} \text{ g}^{-1}$), but both were significantly higher than hypoxia ($1.42 \pm 0.30 \mu\text{l O}_2 \text{ min}^{-1} \text{ g}^{-1}$). Time to return to resting $\dot{V}O_2$ did not differ significantly between oxygen conditions with a mean across treatments of 4.87 ± 2.19 h. EPOC differed significantly between all treatments, with hyperoxia ($580 \pm 290 \mu\text{l O}_2 \text{ g}^{-1}$) being the highest, followed by normoxia ($390 \pm 170 \mu\text{l O}_2 \text{ g}^{-1}$), and lastly hypoxia ($130 \pm 210 \mu\text{l O}_2 \text{ g}^{-1}$). Following the initial EPOC, oxygen

consumption was found to be significantly elevated above resting $\dot{V}O_2$ (paired *t*-test) between 13 and 20 h post-exercise with total oxygen consumption during this time significantly higher in normoxia ($202 \pm 158 \mu\text{l O}_2 \text{ g}^{-1}$) than in hyperoxia ($78 \pm 107 \mu\text{l O}_2 \text{ g}^{-1}$); however, neither differed from hypoxia ($158 \pm 87 \mu\text{l O}_2 \text{ g}^{-1}$).

Respiratory partitioning

Table 1 summarizes the partitioning of mudskipper post-exercise gas exchange between the buccopharyngeal chamber and skin under different atmospheric

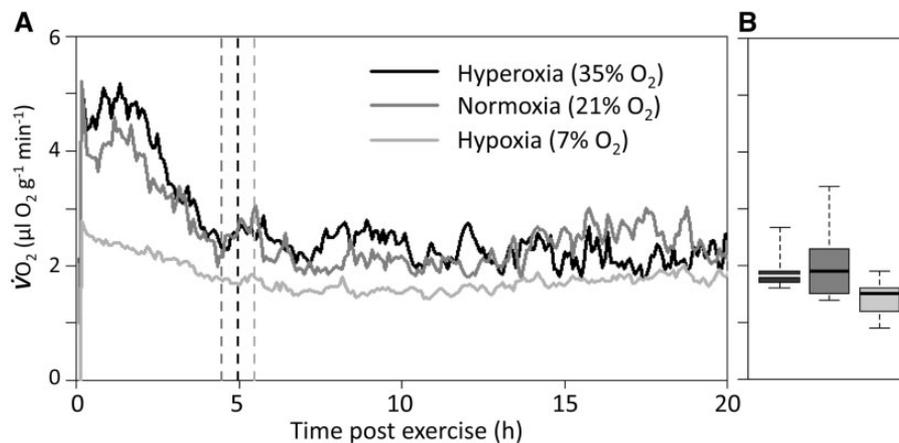


Fig. 3 (A) Mean post-exercise oxygen consumption for nine *P. modestus* recovering in three atmospheric oxygen concentrations: hyperoxia (35% O_2), normoxia (21% O_2), and hypoxia (7% O_2). Dashed vertical lines show the mean time to return to resting $\dot{V}O_2$ for each treatment. (B) Box plot of resting $\dot{V}O_2$ showing minimum, first quartile, median, third quartile, and maximum under each concentration of oxygen: hyperoxia (black), normoxia (gray), and hypoxia (light gray).

Table 1 Respiratory partitioning (mean \pm SD) between the skin and buccopharyngeal (bucco) chamber for 17 *P. modestus* during Pattern 1 of post-exercise recovery under different atmospheric concentrations of oxygen

	Hyperoxia (35% O_2)	Normoxia (21% O_2)	Hypoxia (7% O_2)
Total $\dot{V}O_2$ ($\mu\text{l O}_2 \text{ g}^{-1} \text{ min}^{-1}$)	5.16 ± 1.52^a	3.87 ± 1.12^b	2.36 ± 0.73^c
Bucco $\dot{V}O_2$ ($\mu\text{l O}_2 \text{ g}^{-1} \text{ min}^{-1}$)	0.81 ± 0.48^a	0.48 ± 0.28^b	0.40 ± 0.14^b
Skin $\dot{V}O_2$ ($\mu\text{l O}_2 \text{ g}^{-1} \text{ min}^{-1}$)	4.34 ± 1.47^a	3.39 ± 1.12^b	1.96 ± 0.67^c
% Bucco $\dot{V}O_2$	16.35 ± 8.27^a	13.01 ± 9.13^a	18.00 ± 5.84^a
$\dot{V}O_2/\text{ventilation}$ ($\mu\text{l O}_2 \text{ breath}^{-1}$)	7.72 ± 3.89^a	4.42 ± 2.15^b	2.90 ± 1.63^b
Total $\dot{V}CO_2$ ($\mu\text{l CO}_2 \text{ g}^{-1} \text{ min}^{-1}$)	4.95 ± 1.18^a	4.44 ± 1.07^a	2.90 ± 0.36^b
Bucco $\dot{V}CO_2$ ($\mu\text{l CO}_2 \text{ g}^{-1} \text{ min}^{-1}$)	0.42 ± 0.27^a	0.32 ± 0.18^a	0.42 ± 0.15^a
Skin $\dot{V}CO_2$ ($\mu\text{l CO}_2 \text{ g}^{-1} \text{ min}^{-1}$)	4.53 ± 1.10^a	4.13 ± 1.02^a	2.48 ± 0.36^b
% Bucco $\dot{V}CO_2$	8.42 ± 5.10^a	7.37 ± 4.96^a	14.40 ± 5.10^b
$\dot{V}CO_2/\text{ventilation}$ ($\mu\text{l CO}_2 \text{ breath}^{-1}$)	3.54 ± 1.36^a	2.77 ± 0.93^b	2.68 ± 1.02^b
Ventilation frequency (min^{-1})	0.51 ± 0.27^a	0.52 ± 0.30^a	0.68 ± 0.27^a
Time post-exercise of transition from Pattern 1 to Pattern 2 (min)	19.13 ± 11.45^a	29.02 ± 17.42^b	NA ^d

Statistical significance between treatments is indicated by different superscripts (a, b, and c). Values with the same superscript do not differ significantly. ^dVentilatory pattern did not transition from Pattern 1 to Pattern 2 under hypoxia.

concentrations of oxygen. Total $\dot{V}O_2$ was directly related to oxygen concentration, being the highest in hyperoxia, followed by normoxia, and hypoxia. Skin $\dot{V}O_2$ followed the same trend and was significantly elevated with each increase in oxygen concentration. Bucco $\dot{V}O_2$ and breath-specific $\dot{V}O_2$ were significantly elevated in hyperoxia but did not differ between normoxia and hypoxia. The relative contribution of the buccopharyngeal chamber to total $\dot{V}O_2$ did not significantly differ between oxygen conditions with a mean of $15.79 \pm 7.99\%$.

Total $\dot{V}CO_2$ and skin $\dot{V}CO_2$ were significantly lower in hypoxia than in either hyperoxia or normoxia. Bucco $\dot{V}CO_2$ was the same across all treatments with a mean of $0.38 \pm 0.21 \mu\text{l O}_2 \text{g}^{-1} \text{min}^{-1}$, while breath-specific $\dot{V}CO_2$ was significantly elevated in hyperoxia over values obtained in normoxia and hypoxia, which did not differ from each other. The contribution of bucco $\dot{V}CO_2$ was significantly increased in hypoxia ($14.40 \pm 5.10\%$) but did not differ between hyperoxia and normoxia ($8.08 \pm 4.93\%$).

The relative contribution of the skin and buccopharyngeal chamber to gas exchange was calculated using the large and clearly defined spikes in respirometry (CO_2 and O_2) traces associated with *P. modestus* holding and releasing large mouthfuls of gas (Fig. 2). In both hyperoxia and normoxia, however, these clearly defined spikes (Pattern 1 in Fig. 2) transitioned to a second pattern with more frequent, smaller, and less defined peaks. Pattern 2 was therefore defined by when the CO_2 record could no longer be used to determine individual breaths (Fig. 2), which precluded determination of respiratory partitioning. Video observation of *P. modestus* behavior during Pattern 2 revealed an increase in mouth and opercular movements, in which quick short breaths and only partial or slow filling and emptying of the buccal chamber occurred (Pattern 2 closely matches that observed for *P. modestus* at rest, albeit with more ventilatory movements following exercise). The transition to Pattern 2 occurred significantly sooner in hyperoxia ($19.13 \pm 11.45 \text{ min}$) than in normoxia ($29.02 \pm 17.42 \text{ min}$). In hypoxia, *P. modestus* did not transition to Pattern 2, and thus large defined breaths continued throughout the 90 min record following exercise.

In hyperoxia and normoxia, data on respiratory partitioning between the buccopharyngeal chamber and skin could only be determined during Pattern 1. Hence, all data in Table 1 refer to measurements made during Pattern 1. Ventilation frequency during Pattern 1 was not significantly different between oxygen treatments (mean for all conditions = $0.57 \pm 0.29 \text{ breaths min}^{-1}$).

Discussion

This study reveals that terrestrial performance in *P. modestus* is directly correlated with the atmospheric concentration of oxygen, with higher oxygen levels increasing endurance and hastening post-exercise recovery (as indicated by a higher EPOC and quicker return to resting-like buccopharyngeal air-breathing movements following exhaustive exercise). Assuming physiological similarities between mudskippers and primitive tetrapods, these results support the hypothesis that the rising atmospheric oxygen concentration during the middle and late Paleozoic supplied a physiological advantage that likely facilitated the tetrapod radiation on land.

The effects of atmospheric oxygen on mudskipper terrestrial performance

It has long been recognized that mudskippers of the genera *Periophthalmus* and *Periophthalmodon* can fully satisfy resting metabolic oxygen demand when emerged from water and breathing air (Teal and Carey 1967; Gordon et al. 1969; Tamura et al. 1976; Kok et al. 1998; Takeda et al. 1999). The resting metabolic rates of *P. modestus* determined in this study under both hyperoxia ($1.94 \pm 0.40 \mu\text{l O}_2 \text{min}^{-1} \text{g}^{-1}$) and normoxia ($2.09 \pm 0.73 \mu\text{l O}_2 \text{min}^{-1} \text{g}^{-1}$) are similar to those measured by Tamura et al. (1976) ($2.15 \mu\text{l O}_2 \text{min}^{-1} \text{g}^{-1}$) for *P. modestus* in air, indicating that this species, like other members of its genus, can fully satisfy routine metabolic oxygen demands on land. Although not statistically different, the slightly lower resting rate of *P. modestus* in hyperoxia could indicate an increased diffusive flux of oxygen along the step-wise progression from the respiratory epithelium to the tissues (i.e., oxygen cascade), resulting in a reduction in cardiac work and perhaps indicating a slight benefit of a hyperoxic atmosphere while at rest, similar to the lowered cost of breathing in alligators reared in hyperoxia (Owerkowicz et al. 2009). The depressed resting metabolic rate in *P. modestus* under hypoxia indicates that this oxygen concentration (7% $\text{O}_2 = 53 \text{ Torr}$) is below the minimum partial pressure required to sustain routine metabolic rate in air (i.e., critical PO_2). This is consistent with the aerial critical PO_2 observed for most snakes, alligators, and lizards ($\sim 10\% \text{ O}_2$; Boyer 1966), as well as other amphibious animals that rely heavily on cutaneous respiration, such as *Desmognathus fuscus*, a lungless salamander (8–10% O_2 ; Sheafor et al. 2000).

Although mudskipper resting metabolism can be fully met in normoxia, active metabolism is greatly enhanced under hyperoxic conditions. Although oxygen consumption could not be directly measured

for *P. modestus* during exercise (a closed chamber small enough would have interfered with mudskipper crutching and hopping), the increase in exercise endurance and post-exercise $\dot{V}O_2$ under hyperoxia (and decrease in hypoxia) suggests enhanced utilization of oxygen (aerobic scope) at higher oxygen concentrations. This increased aerobic scope during exercise in hyperoxia likely reduces mudskipper reliance on anaerobic metabolism and delays the onset of an exercise limit caused by the depletion of energy stores (glycogen, ATP, and phosphocreatine), the buildup of anaerobic byproducts (lactate and H^+) (Wood 1991; Kieffer 2000), or a deficiency in delivery of oxygen to the heart (Farrell 2002).

In addition to increased endurance, hyperoxia appears to hasten mudskipper recovery following exercise. Although the time required post-exercise for *P. modestus* to return to resting $\dot{V}O_2$ did not differ between treatments, a higher EPOC under hyperoxia indicates a more complete recovery from exercise compared with normoxia. This is supported in that ~ 13 h post-exercise mudskippers in normoxia showed a larger increase in $\dot{V}O_2$ (Fig. 3) that was similar in magnitude to the difference in the initial EPOC between hyperoxia and normoxia. This suggests a delayed recovery in normoxia. Because the processing of lactate and other anaerobic byproducts and the repayment of muscle glycogen to pre-exercise levels can take more than 12–24 h for a fish exercised to exhaustion (Piiper et al. 1972; Wood et al. 1977; Wood 1991; Richards et al. 2002), a prolonged recovery period is not unexpected. However, it remains unclear why mudskipper oxygen consumption after an initial EPOC remains close to resting until ~ 13 h post-exercise.

A faster recovery from exercise under elevated oxygen levels is also demonstrated by the quicker return to a rest-like ventilatory pattern (Pattern 1 to Pattern 2; Fig. 2). Ventilation Pattern 1 (large, distinct breath-hold-and-release events) in Fig. 2 has been observed in other mudskippers (Singh and Munshi 1968; Graham 1997; Aguilar et al. 2000) and appears to be a respiratory response in *P. modestus* to both functional (exercise-induced) and environmental hypoxia. This breath-hold behavior has been hypothesized to aid in oxygen absorption by using the buccopharyngeal muscles to compress the air breath, thereby increasing the pressure of the held volume above ambient (Graham 1997). This would increase the PO_2 and the diffusive driving force of oxygen into the blood. Although this hypothesis has never been substantiated by pressure measurements or by oxygen diffusion rates under different breathing regimes (i.e., Pattern 1 vs.

Pattern 2), the use of breath holding immediately following exercise supports its use for increasing oxygen uptake, and thus the transition from Pattern 1 to Pattern 2 likely indicates a partial release from oxygen deprivation.

Mudskipper terrestrial performance in comparison to other lower vertebrates

Similar to terrestrial performance in mudskippers, solely water-breathing fishes show depleted performance (usually measured in terms of a maximum sustainable swimming velocity, U_{crit}) in hypoxia (Davis et al. 1963; Dahlberg et al. 1968; Jones 1971; Kaufmann and Wieser 1992). However, these fishes generally do not appear to benefit from oxygen tensions above normoxia (Davis et al. 1963; Dahlberg et al. 1968; Duthie and Hughes 1987; Kaufmann and Wieser 1992; Jones et al. 2008). In small larval fishes (2–120 mg), environmental oxygen can readily diffuse throughout the body in normoxia (hence there is little reliance on the gills or on circulation as in larger fish), and thus hyperoxia appears to offer little advantage (Kaufmann and Wieser 1992). For larger fishes, which rely heavily on the gills, performance in hyperoxia is likely limited by the carrying capacity of the cardiovascular system (Farrell 2002). During exercise, venous blood- O_2 levels drop due to increased utilization of oxygen by the skeletal muscles, and because the myocardium in most fishes receives the majority of its oxygen from the venous return (Farrell 2002), cardiac output quickly peaks during exercise.

The ability of *P. modestus* to increase oxygen consumption in hyperoxia is likely associated with differences from most other fishes in the primary sites of oxygen uptake from the environment. Most water-breathing fishes primarily use the gills (Feder and Burggren 1985), while *P. modestus* meets up to 84% of its demand for oxygen via the skin post-exercise (this study) and 76% while at rest (Tamura et al. 1976). Although the gills of *P. modestus* appear somewhat modified for use in air (short and twisted filaments and thick lamellae may help reduce the collapse of gills out of water), their functional surface area is still significantly reduced, leaving the epithelial lining of the buccopharyngeal chamber likely responsible for a large portion of non-cutaneous oxygen absorption (Low et al. 1990; Graham et al. 2007). Both the skin and buccopharyngeal epithelium are perfused by blood originating from efferent brachial and systemic arteries and drained by systemic veins, thereby running in parallel and by-passing the systemic circulation that provides

oxygen to the tissues (Das 1934; Schöttle 1931; Niva et al. 1981; Ishimatsu 2012). Although oxygenated blood from the skin and buccopharyngeal epithelium mixes with deoxygenated blood returning from the rest of the body, the oxygen level in mixed venous blood is elevated. This increases oxygen availability to the heart, allowing for increased cardiac output during exercise and additional blood flow to the respiratory and exercising tissues.

The reliance on cutaneous and buccopharyngeal respiration by *P. modestus* thus appears to allow for increased exercise performance in comparison to fishes that use the gills as the primary source of oxygen and generally have a low cutaneous respiratory component. Other air-breathing fishes such as the Pacific Tarpon (*Megalops cyprinoides*), which possesses a respiratory gas bladder with a pulmonary circulation that also runs in-parallel to the systemic circulation, can similarly increase cardiac oxygenation during exercise by breathing air (Clark et al. 2007). The circulatory pattern and consequently exercise physiology of *P. modestus* (and some other air-breathing fishes) appears more similar to basal extant tetrapods such as the lungless salamander, *Batrachoseps attenuates*, which also has a high cutaneous respiratory component and shows elevated performance in hyperoxia (Feder and Olsen 1978), than to modern fishes that respire solely aquatically.

Implications for the radiation and diversification of early tetrapods

Our findings support the hypothesis (Graham et al. 1995) that the rise in atmospheric oxygen during the middle and late Paleozoic (400–250 mya) increased terrestrial performance in early tetrapods and likely facilitated their radiation from the water's edge.

Models suggest that the Earth's atmosphere was hypoxic during the early to mid Devonian, reaching a minimum as low as 7% ~375 mya (Bergman et al. 2004). As most extant air-breathing fishes are found in marginal aquatic habitats that are exposed to frequent or chronic hypoxia (Graham 1997), the early Devonian drop in atmospheric oxygen (that would have exacerbated aquatic hypoxia) likely selected for air breathing among fishes close to the surface and may have encouraged hypoxic emersion behavior (Graham and Lee 2004; Graham and Wegner 2010; Clack 2012). However, as seen in *P. modestus*, hypoxia would have likely limited the aerobic ability needed for terrestrial activity, hence constraining the radiation of the early fish-like tetrapods.

As the oxygen concentration in the atmosphere rose through the middle and into the late Paleozoic, reaching a peak of 30–35% by ~320

mya, primitive tetrapods likely benefited in a manner similar to *P. modestus* from an increased aerobic scope, enhanced endurance, and faster recovery following functional hypoxia (exercise), ultimately increasing their performance on land. In addition to lungs (likely a step up, but analogous in function to the mudskipper buccopharyngeal chamber), early tetrapods (such as modern amphibians and reptiles) probably relied on cutaneous respiration for breathing air (Clack 2012); this is supported in that early tetrapod scales were of dermal origin and show grooves for vascularization across and through to the epidermis (Frolich 1997; Clack 2012). With early tetrapods likely having a fish-like two-chambered heart (Farmer 1999; Hicks 2002), respiration at the lungs and skin would have likely augmented venous oxygen delivery to the heart, resulting in increased cardiac output and blood flow to the exercising muscle, and ultimately, a higher total aerobic capacity that could take advantage of a hyperoxic atmosphere. With time, increased reliance upon lungs, the complete loss of gills, and the development of a mechanism allowing for separation of oxygenated and deoxygenated blood (i.e., with the development of a three- or four-chambered heart), complete with a well-developed coronary circulation, would have allowed tetrapods to further exploit their new niche and ultimately withstand the hypoxic atmosphere that followed the Paleozoic.

Studying mudskippers and other extant amphibious fishes can give us a window into deep time and provide insight into how Paleozoic oxygen concentrations influenced both the physiology and evolution of early tetrapods. Although this and similar studies are inherently acute in nature and thus limit our ability to draw inference as to events that took place over geologic time scales, examination of closely related species at different stages along the water-to-land transition can provide insight at different stages of tetrapod evolution. Specifically, the four mudskipper genera provide a phylogenetic continuum in which to study the sequence of adaptations associated with the transition to life on land (Graham et al. 2007), and future research can ask how hyperoxia and other variables would affect fish models more or less specialized for terrestrial life (i.e., for *Periophthalmodon* the most terrestrial genus of mudskipper, vs. *Boleophthalmus* and *Scarteloas* which are less amphibious than *P. modestus*). How would our results differ in an air-breathing fish with a lower capacity for cutaneous respiration or with a different specialized air-breathing organ (i.e., lung or respiratory gas bladder with a pulmonary

circulation)? The use of other extant vertebrates with primitive air-breathing and cardiovascular morphologies, such as amphibians, or other air-breathing and amphibious fishes, as well as long-term acclimation, developmental, and multigenerational studies, can greatly add to the breadth of empirical data available to paint a clearer picture of how the timing of fluctuations in atmospheric oxygen played a role in the evolution of life on this planet during the middle and late Paleozoic Era.

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