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Control of breathing in African lungfish (*Protopterus dolloi*): A comparison of aquatic and cocooned (terrestrialized) animals

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Abstract

African lungfish, *Protopterus dolloi* exhibited constant rates of O_2 consumption before (0.95 ± 0.07 mmol kg^{-1} h^{-1}), during (1.21 ± 0.32 mmol kg^{-1} h^{-1}) and after (1.14 ± 0.14 mmol kg^{-1} h^{-1}) extended periods (1–2 months) of terrestrialization while cocooned. Although a breathing event in terrestrialized fish consisted of multiple bouts of inspiration and expiration in rapid succession, the mean frequency of pulmonary breathing events was unaltered in the terrestrialized fish (16.7 ± 1.4 h^{-1} *versus* 20.1 ± 4.9 h^{-1} in the aquatic and terrestrialized fish, respectively). Hypoxia (~20 mmHg) increased the frequency of breathing events by 16 and 23 h^{-1} in the aquatic and terrestrialized fish, respectively. Hyperoxia (~550 mmHg) decreased breathing event frequency by 10 and 15 h^{-1} in the aquatic and terrestrialized animals. Aquatic hypercapnia (~37.5 mmHg) increased pulmonary breathing frequency (from 15.3 ± 2.3 to 28.7 ± 5.4 h^{-1}) in free swimming lungfish, whereas aerial hypercapnia was without effect in aquatic or terrestrialized fish.
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1. Introduction

African lungfish (genus *Protopterus*) are bimodal breathers that use both gills and lungs for respiratory gas transfer (see Burggren and Johansen, 1986; Graham, 1997). Because the vast majority of O_2 uptake of adult lungfish occurs over the lung (Lenfant and Johansen, 1968; Lahiri et al., 1970; McMahon, 1970; Johansen et al., 1976), African lungfish are obligate air breathers that die if denied access to air. CO_2 excretion, on the other hand, normally occurs largely across the gills (Burggren and Johansen, 1986) although the lung is the predominant route of CO_2 transfer in the slender African lungfish (*P. dolloi*; Perry et al., 2005a). The mechanics of pulmonary ventilation have been well characterized in *Protopterus*, where inspiration is actively driven by a buccal force pump, while expiration involves passive elastic recoil (McMahon, 1969).

A fascinating feature of African lungfish is their ability to survive for years without access to water (Smith, 1931). During drought, some species of African lungfish descend into the mud to form a burrow that is connected to the external environment by a narrow opening that forms a breathing tube (Smith, 1930; Greenwood, 1986). While in the mud, the fish exude mucus that hardens to form a thin water-impermeable cocoon that envelops the fish with exception of the small opening at the mouth that allows for pulmonary ventilation (Dubois, 1892; Smith, 1931; Delaney et al., 1974; Lomholt et al., 1975; Greenwood, 1986; Lomholt, 1993). Aestivation is associated with a gradual reduction in metabolic rate (Smith, 1930; Delaney et al., 1974; Fishman et al., 1986) although it is uncertain whether fish ever enter true torpor. Indeed, although cocooned and motionless, aestivating *Protopterus aethiopicus* continue to respond to sensory stimuli with bradycardia and altered breathing (Delaney et al., 1974; Fishman et al., 1986; Sturla et al., 2002). The aestivating fish switch from ammonotelism to ureagenesis to prevent ammonia toxicity (Smith, 1930; Janssens, 1964), while excretion of urine ceases (Smith, 1930) to reduce dehydration.

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The ventilatory pattern of aestivating lungfish is characterized by periods of apnea interspersed with bouts of tachypnea (Lomholt et al., 1975; Lomholt, 1993; Delaney and Fishman, 1977). Interestingly, overall breathing frequency increases in the early stages of aestivation and never appears to fall below pre-aestivation rates even after prolonged submergence in mud (Delaney et al., 1974; Fishman et al., 1986). Although early studies reported that aestivating lungfish displayed aspiration breathing (Dubois, 1892; Lomholt et al., 1975), it is now largely accepted that the aestivating fish continue to exploit a buccal force pump even though the mouth remains open during all phases of ventilation (Delaney and Fishman, 1977).

The slender African lungfish (*P. dolloi*) apparently does not aestivate in its natural habitat (Greenwood, 1986) yet seems unique within the genus *Protopterus* because it can form a cocoon and "aestivate" (aestivation may be an inappropriate description of the physiological state of *P. dolloi* while cocooned because it is unclear as to whether metabolic rate is lowered. Hence the term "terrestrialization" was coined (Wood et al., 2006) and will be used throughout this paper) successfully under laboratory conditions while remaining on the surface (Chew et al., 2004). Thus, unlike lungfish naturally aestivating in mud or artificial burrows (Janssens, 1964; Delaney et al., 1974), which experience a marked increase in functional respiratory dead space, terrestrialized *P. dolloi* is able to freely breathe the ambient air. Consequently, *P. dolloi* breathing at similar minute volumes while terrestrialized is less likely than other species of *Protopterus* to exhibit changes in blood gases and therefore may not experience certain cues that may be involved in depressing metabolism. Thus, a first component of this study was designed to assess the consequences of terrestrialization on metabolic rate, blood gases and breathing in *P. dolloi* to compare with species that aestivate below ground.

Aside from a single report of aestivating African lungfish hyperventilating in response to hypercapnia (Smith, 1930), there are no data on the control of breathing in the aestivating state (e.g. see Fishman et al., 1986). *P. dolloi* is a convenient animal on which to compare ventilatory control in aquatic and terrestrialized animals because they will remain cocooned and motionless at the surface where the composition of the inspired air is easily manipulated. Therefore, a second component of this study was designed to compare the ventilatory responses of free swimming and terrestrialized *P. dolloi* to hypoxia, hyperoxia and hypercapnia.

2. Materials and methods

2.1. Experimental animals

Experiments were performed in Singapore (National University of Singapore), Ottawa (University of Ottawa) and Aarhus (Aarhus University) using lungfish from a common source. Adult slender African lungfish (*Protopterus dolloi*), weighing 146.4 ± 6.7 g [mean ± standard error of the mean (S.E.M.), $N = 71$], were imported from central Africa through a local fish farm in Singapore. In Singapore, specimens were maintained in plastic aquaria filled with dechlorinated water, containing 2.3 mmol l^{-1} Na^+, 0.54 mmol l^{-1} K^+, 0.95 mmol l^{-1} Ca^{2+}, 0.08 mmol l^{-1} Mg^{2+}, 3.4 mmol l^{-1} Cl^- and 0.6 mmol l^{-1} M HCO_3^-, at pH 7.0 at 25 °C; water was changed daily. No attempt was made to separate the sexes. The lungfish were acclimated to laboratory conditions for at least 1 month. During this period, they were fed frozen mosquito larvae (blood worms), but food was withheld 96 h prior to experiments, so the gut could be emptied of all food and waste.

Fish were shipped to Ottawa by air cargo in partially filled bags of oxygenated water contained within insulated containers. Upon arrival, fish were placed individually into covered plastic containers containing 2–3 l of dechlorinated city of Ottawa tap water at 25 °C. The water was changed every second day or sooner if there was obvious fouling. The fish were kept in a sealed room at a photoperiod of 10 h light:14 h darkness and were fed on alternate days with frozen blood worms or pieces of rainbow trout flesh. Fish were allowed to acclimate to these conditions for at least 1 month prior to beginning experiments.

2.2. Terrestrialization

Terrestrialization was induced according to Chew et al. (2004). Briefly, unfed fish (3–4 days without food) were transferred to plastic containers containing 10 ml of dechlorinated water. Typically, the water would evaporate within 3–4 days; during this time the secretion of mucus led to the formation of a thin cocoon. After 4–5 days, the motionless fish was encased in a hardened cocoon. To reduce dehydration, terrestrialized fish were sprayed with 1–2 ml of water at 6 day intervals. In Singapore, the terrestrialized fish were maintained in darkened compartments in an outdoor laboratory with an average ambient humidity of approximately 80%.

After at least 1 month of terrestrialization, fish were transported to the University of Ottawa and Aarhus University by air in carry-on luggage. In Ottawa and Aarhus, they were kept in plastic chambers at 25 °C in rooms humidified to approximately 75%. They were sprayed at 6-day intervals with dechlorinated tap water. To minimize disturbances, the terrestrialized fish were kept behind opaque curtains.

2.3. Surgical procedures

Non-terrestrialized fish were anaesthetized in a solution of MS-222 (ethyl-*p*-aminobenzoate; 1.0 g l^{-1}) adjusted to neutral pH with $NaHCO_3$ (2 g l^{-1}). After cessation of breathing movements, the fish were transferred to an operating table where they were draped with paper towels soaked with anaesthetic solution. In this way, they were kept moist and deeply anaesthetized for the duration of the surgery. To allow blood sampling, a cannula (Clay-Adams PE 50 polyethylene tubing) was inserted into the dorsal aorta according to standard surgical procedures (Axelsson and Fritsche, 1994). Briefly, a lateral incision (~2 cm in length) was made at the vent approximately 3 mm below the lateral line. The dorsal aorta was exposed and the cannula was inserted via a small incision and advanced at least

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