

Measuring oxygen uptake in fishes with bimodal respiration

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Respirometry is a robust method for measurement of oxygen uptake as a proxy for metabolic rate in fishes, and how species with bimodal respiration might meet their demands from water *v.* air has interested researchers for over a century. The challenges of measuring oxygen uptake from both water and air, preferably simultaneously, have been addressed in a variety of ways, which are briefly reviewed. These methods are not well-suited for the long-term measurements necessary to be certain of obtaining undisturbed patterns of respiratory partitioning, for example, to estimate traits such as standard metabolic rate. Such measurements require automated intermittent-closed respirometry that, for bimodal fishes, has only recently been developed. This paper describes two approaches in enough detail to be replicated by the interested researcher. These methods are for static respirometry. Measuring oxygen uptake by bimodal fishes during exercise poses specific challenges, which are described to aid the reader in designing experiments. The respiratory physiology and behaviour of air-breathing fishes is very complex and can easily be influenced by experimental conditions, and some general considerations are listed to facilitate the design of experiments. Air breathing is believed to have evolved in response to aquatic hypoxia and, probably, associated hypercapnia. The review ends by considering what realistic hypercapnia is, how hypercapnic tropical waters can become and how this might influence bimodal animals' gas exchange.

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Key words: respirometry; respiratory partitioning; intermittent-closed; oxygen consumption.

INTRODUCTION

Respiration of air-breathing fishes has interested researchers for at least a century; the earliest attempts to measure oxygen uptake ($\dot{M}O_2$) and respiratory partitioning date to the 19th century (Baumert, 1853; Jolyet & Regnard, 1877*a, b*). With technological advances in the 1960s, there was a massive renewed interest that endured up to 1980s, after which the literature has accumulated at a slower pace. The available general respirometry methods have been described and reviewed by several authors

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(Cech, 1990; Lighton, 2008), and studies of the respiration of air-breathing fishes were reviewed in detail in the iconic book by Graham (1997).

Bimodal respirometry requires a respirometer with both aquatic and aerial phases, in which to quantify changes in respiratory gas concentrations, and there have been many variations on their design. These have ranged from simple closed funnels placed over the fish (Todd & Ebeling, 1966; Graham, 1983; Juca-Chagas, 2004) to more complex custom-designed plexiglas set-ups allowing manipulation of flows of air and water through the relevant sections (Hughes & Singh, 1970; McMahon, 1970; Tamura *et al.*, 1976; Itazawa & Ishimatsu, 1981; Johnston *et al.*, 1983; Graham & Baird, 1984; Yu & Woo, 1985; McMahon & Burggren, 1987; Takeda *et al.*, 1999). Table I shows $\dot{M}O_2$ measurements for air-breathing fishes categorized according to the type of respirometry used. There are three dominant methods, all of which can and have been used in water and air, separately or in various combinations, to obtain simultaneous measurements of aquatic and aerial $\dot{M}O_2$ ($\dot{M}O_{2w}$ and $\dot{M}O_{2a}$, respectively). These are volumetric, closed and flow-through respirometry. This review will begin by providing a brief overview of these systems, their strengths and weaknesses.

This overview is followed by a detailed discussion of systems that have been developed to use the principles of intermittent-closed respirometry for both air and water phases, which is the major focus of the review. Intermittent-closed respirometry has become recognized as the gold standard for measurements of dynamic temporal variability in $\dot{M}O_2$ and derivation of standard metabolic rate (SMR) in water-breathing species (Steffensen, 1989; Svendsen *et al.*, 2016). Systems have recently been developed for bimodal respirometry allowing reliable estimates of, for example, diurnal patterns of oxygen uptake and respiratory partitioning, effects of specific dynamic action and estimates of SMR (Lefevre *et al.*, 2011a, b, 2012, 2014a; D. J. McKenzie, T. C. Belão, S. S. Killen & F. T. Rantin, unpubl. data).

The review is concluded with a discussion of some of the challenges associated with swimming respirometry, and some perspectives on future directions in respirometry of bimodally breathing fishes.

GENERAL RESPIROMETRY METHODS IN AIR-BREATHING FISHES: PAST AND PRESENT

The earliest attempts to measure $\dot{M}O_2$ in bimodal breathers involved volumetric respirometry of the air phase (Winterstein, 1912), which was later modified for measurements of aquatic respiration (Scholander & Edwards, 1942; Scholander, 1949). This approach was used up to the 1980s. Overall, in these simple systems, fish breathe from a constant pressure compartment of air and water, and CO_2 released by the fish is removed from the air using an appropriate absorbent such as KOH (Scholander & Edwards, 1942). The resulting decrease in volume in the air phase will reflect the sum of all CO_2 released by the fish, *i.e.* CO_2 released directly into the air and CO_2 released to the water and subsequently released to the air *via* diffusion, assuming that the two phases are in equilibrium. The volume of the air phase is then restored by manual addition of a precisely measured volume of air. This amount corresponds to the amount of CO_2 removed, which in turn corresponds to the released amount of CO_2 . This latter corresponds in some ratio to the amount of O_2 taken up by the fish, depending upon the substrata they are using for aerobic respiration. The strength of the volumetric

TABLE I. Overview of different methods used to measure oxygen uptake in air-breathing fishes

Phases measured	Method in water phase	Method in air phase	Species	References
Only water	Closed	None	<i>Amia calva</i>	Porteus <i>et al.</i> (2014)
			<i>Anguilla vulgaris</i>	Berg & Steen (1965)*
			<i>Hoplosternum littorale</i>	Brauner <i>et al.</i> (1995)
			<i>Periophthalmus sobrinus</i>	Teal & Carey (1967)*
			<i>Piabucina festae</i>	Graham <i>et al.</i> (1978)
	Flow-through	None	<i>Umbra limi</i>	Currie <i>et al.</i> (2010)
			<i>Amia calva</i>	McKenzie & Randall (1990)
			<i>Clarias batrachus</i>	Munshi <i>et al.</i> (1976)
				Tripathi <i>et al.</i> (2013)
			<i>Clarias gariepinus</i>	Belão <i>et al.</i> (2011)
			<i>Heteropneustes fossilis</i>	Munshi <i>et al.</i> (1978)
			<i>Hoplerhrynus unitaeniatus</i>	Oliveira <i>et al.</i> (2004)
			<i>Hoplosternum littorale</i>	Affonso & Rantin (2005)
			<i>Hypostomus regani</i>	Mattias <i>et al.</i> (1998)
Only air	None	Closed respirometry		Nelson <i>et al.</i> (2007)
			<i>Pterygoplichthys anisitsi</i>	da Cruz <i>et al.</i> (2012)
			<i>Rhinelepis strigose</i>	Takasusuki <i>et al.</i> (1998)
			<i>Amia calva</i>	McKenzie & Randall (1990)
			<i>Channa argus</i>	Glass <i>et al.</i> (1986)†
	Flow-through Sampling from ABO		<i>Gillichthys mirabilis</i>	Todd & Ebeling (1966)‡
			<i>Hoplerhrynus unitaeniatus</i>	Juca-Chagas (2004)
			<i>Hoplosternum littorale</i>	
			<i>Lepidosiren paradoxa</i>	
			<i>Periophthalmus sobrinus</i>	
			<i>Alticus kirki</i>	Teal & Carey (1967)*
			<i>Channa argus</i>	Martin & Lighton (1989)*
			<i>Megalops cyprinoides</i>	Itazawa & Ishimatsu (1981)
				Seymour <i>et al.</i> (2007)

TABLE I. Continued

Phases measured	Method in water phase	Method in air phase	Species	References
Not simultaneously	Closed	Volumetric Closed	<i>Calamoichthys calabaricus</i>	Pettit & Beitinger (1981)
			<i>Alticus kirki</i>	Brown <i>et al.</i> (1992)*
			<i>Anoplarchus purpurens</i>	Yoshiyama & Cech (1994)*
			<i>Ascelichthys rhodorus</i>	Yoshiyama & Cech (1994)*
			<i>Blennius pholis</i>	Pelster <i>et al.</i> (1988)*
			<i>Boleophthalmus chinensis</i>	Tamura <i>et al.</i> (1976)*
			<i>Caracanthus unipinna</i>	Nilsson <i>et al.</i> (2007)*
			<i>Cebidichthys violaceus</i>	Edwards & Cech (1990)*
			<i>Clinocottus globiceps</i>	Yoshiyama & Cech (1994)*
			<i>Electrophorus electricus</i>	Farber & Rahn (1970)§
			<i>Fundulus heteroclitus</i>	Halpin & Martin (1999)*
			<i>Galaxias maculatus</i>	Urbina <i>et al.</i> (2014)*
			<i>Gobiodon axillaris</i>	Nilsson <i>et al.</i> (2007)*
			<i>Gobiodon erythrosphilus</i>	
			<i>Gobiodon histrio</i>	
			<i>Gobiodon unicolor</i>	
			<i>Gobiodon acicularis</i>	
			<i>Gobiodon ceramensis</i>	
			<i>Gobiodon okinawae</i>	
			<i>Mnierpes macrocephalus</i>	Graham (1973)*
			<i>Neochanna burrowsius</i>	Meredith <i>et al.</i> (1982)*
				Urbina <i>et al.</i> (2014)*
				Yoshiyama & Cech (1994)*
			<i>Oligocottus maculosus</i>	
			<i>Oligocottus snyderi</i>	
			<i>Periophthalmus cantonensis</i>	Tamura <i>et al.</i> (1976)*
				Gordon <i>et al.</i> (1978)*
			<i>Sicyases sanguineus</i>	Gordon <i>et al.</i> (1970)*

TABLE I. Continued

Phases measured	Method in water phase	Method in air phase	Species	References
Simultaneously	Closed	Flow-through	<i>Betta splendens</i>	Alton <i>et al.</i> (2013)
			<i>Clinocottus analis</i>	Martin (1991)*
			<i>Lepidosiren paradoxa</i>	Amin-Naves <i>et al.</i> (2004)
	Closed	Volumetric	<i>Trichogaster leeri</i>	Alton <i>et al.</i> (2007)
			<i>Dallia pectoralis</i>	Crawford (1971)‡
			<i>Sicyopterus sanguineus</i>	Gordon <i>et al.</i> (1970)*
	Flow-through	Closed	<i>Periophthalmodon schlosseri</i>	Takeda <i>et al.</i> (1999)*
		Flow-through	<i>Chlamydogobius eremius</i>	Thompson & Withers (2002)*
			<i>Lipophrys pholis</i>	Steeger & Bridges (1995)*
			<i>Periophthalmus barbarus</i>	
		Volumetric	<i>Trichogaster pectoralis</i>	Natarajan & Rajulu (1982)*
	Intermittent-closed	Volumetric	<i>Helcogramma medium</i>	Innes & Wells (1985)*
	Volumetric	Volumetric	<i>Periophthalmus sobrinus</i>	Gordon <i>et al.</i> (1969)*
			<i>Tomocodon humeralis</i>	Eger (1971)*
	Closed	Closed	<i>Amia calva</i>	Johansen <i>et al.</i> (1970)
				McKenzie & Randall (1990)
			<i>Anabas testudineus</i>	Hughes & Singh (1970)
			<i>Ancistrus chagresi</i>	Graham (1983)
			<i>Arapaima gigas</i>	Stevens & Holeyton (1978a)
			<i>Brochis splendens</i>	Gee & Graham (1978)
			<i>Channa argus</i>	Itazawa & Ishimatsu (1981)
			<i>Channa maculata</i>	Yu & Woo (1985)
			<i>Clarias mossambicus</i>	Johnston <i>et al.</i> (1983)
			<i>Erythrinus erythrinus</i>	Stevens & Holeyton (1978b)
			<i>Hoplerethrinus unitaeniatus</i>	Stevens & Holeyton (1978b)
			<i>Hoplosternum thoracatum</i>	Gee & Graham (1978)
			<i>Lepisosteus osseus</i>	Rahn <i>et al.</i> (1971)

TABLE I. Continued

Phases measured	Method in water phase	Method in air phase	Species	References
			<i>Piabucina festae</i>	Graham <i>et al.</i> (1977)
			<i>Protopterus aethiopicus</i>	Seifert & Chapman (2006)
			<i>Protopterus annectens</i>	Ifitkar <i>et al.</i> (2008)
			<i>Protopterus dolloi</i>	Perry <i>et al.</i> (2005a, b)
			<i>Synbranchus marmoratus</i>	Graham & Baird (1984)
			<i>Trichogaster trichopterus</i>	Burggren (1979)
				Burggren & Haswell (1979)
				Seymour <i>et al.</i> (2004)
				Babiker (1979)
		Flow-through	<i>Megalops cyprinoides</i>	
		Sampling from ABO	<i>Clarias lazera</i>	Biswas <i>et al.</i> (1979)
			<i>Protopterus annectens</i>	Ojha <i>et al.</i> (1979)
			<i>Boleophthalmus boddarti</i>	Jordan (1976)
			<i>Channa marulius</i>	Patra <i>et al.</i> (1983)
		Volumetric	<i>Clarias batrachus</i>	Munshi <i>et al.</i> (1982)
			<i>Heteropneustes fossilis</i>	Lomholt & Johansen (1976)
			<i>Monopterusuchia</i>	Ghosh <i>et al.</i> (1986)
			<i>Notopterus chitala</i>	Eduardo <i>et al.</i> (1979)
			<i>Synbranchus marmoratus</i>	Natarajan & Rajulu (1982)*
			<i>Trichogaster pectoralis</i>	Johansen & Lenfant (1967)
			<i>Lepidosiren paradoxa</i>	Lenfant & Johansen (1968)
			<i>Protopterus aethiopicus</i>	Randall <i>et al.</i> (1981)
			<i>Amia calva</i>	McKenzie & Randall (1990)
				McKenzie <i>et al.</i> (1991)
				Farmer & Jackson (1998)
				Singh & Hughes (1971)
				Sacca & Burggren (1982)
		Exhaled air	<i>Clarias batrachus</i>	
		Sampling from ABO	<i>Calamoichthys calabaricus</i>	
		Closed		
	Exhaled water			
	Flow-through			

TABLE I. Continued

Phases measured	Method in water phase	Method in air phase	Species	References
			<i>Gymnotus carapo</i>	Liem <i>et al.</i> (1984)
			<i>Heteropneustes fossilis</i>	Hughes & Singh (1971)
			<i>Lepisosteus oculatus</i>	Farmer & Jackson (1998)
			<i>Misgurnus anguillicaudatus</i>	McMahon & Burggren (1987)
			<i>Polypterus senegalus</i>	Babiker (1984)
			<i>Protopterus aethiopicus</i>	McMahon (1970)
		Flow-through	<i>Clarias lazera</i>	Babiker (1979)
		Sampling from ABO	<i>Lepisosteus oculatus</i>	Smatresk & Cameron (1982 <i>a, b</i>)
			<i>Protopterus annectens</i>	Babiker (1979)
			<i>Hoplerhrynus unitaeniatus</i>	McKenzie <i>et al.</i> (2007 <i>a</i>)
		Closed	<i>Channa striata</i>	Lefevre <i>et al.</i> (2012)
		Intermittent-closed	<i>Clarias gariepinus</i>	D. J. McKenzie, T. C. Belão, S. S. Killen & F. T. Rantin (unpubl. data)
			<i>Dallia pectoralis</i>	Lefevre <i>et al.</i> (2014 <i>a</i>)
			<i>Pangasianodon hypophthalmus</i>	Lefevre <i>et al.</i> (2011 <i>a, b</i> , 2013)

ABO, air-breathing organ.

*Amphibious air breather.

†Air sampled and analysed from Douglas bag.

‡Aerial respiration measured in very hypoxic water to exclude aquatic $\dot{M}O_2$.

§Extraction and re-filling with air after each breath.

||Respirometer filled completely with water in-between breaths.

method is that it is rather intuitive, and does not require complex calculations to obtain $\dot{M}O_2$. One weakness of the method is that it requires constant involvement by the experimenter, with resulting unavoidable disturbance of the fish and potential effects on $\dot{M}O_2$ and respiratory partitioning. These systems are therefore not suitable for long-term measurements or for precise estimates of SMR. Also, for measurement of $\dot{M}O_{2w}$, it is a crucial assumption that the air and water phase are in equilibrium. For a fish breathing bimodally, the volumetric method will give the total CO_2 released, and therefore total $\dot{M}O_2$, but it is not possible to assess the respiratory partitioning between air and water, unless $\dot{M}O_{2w}$ is measured using a different method and then subtracted from the total. Furthermore, conversion of CO_2 produced to O_2 consumed and thus metabolic rate requires an understanding of the prevailing respiratory quotient (RQ). This can be measured (Ultsch, 1996) but not, of course, using a volumetric system! An RQ of 1 assumes that metabolism is fuelled 100% by carbohydrates and is often used simply for convenience. Relatively, little is known about patterns of substrate utilization in fishes and how this might be influenced by their nutritional state; whether they are feeding, post-absorptive, fasting or starving. The available evidence indicates that many fishes, especially carnivorous species, rely mostly on lipids and proteins as fuels, and that proteins may dominate during a feeding state but lipids have a greater role when fish are post-absorptive and fasting (Lauff & Wood, 1996a, b; McKenzie *et al.*, 2007b, 2014; Bar & Volkoff, 2012). If this is the case, applying an arbitrary RQ of 1 to convert $\dot{M}CO_2$ to $\dot{M}O_2$ will have underestimated actual metabolic rate because RQ is *c.* 0.9 for proteins and *c.* 0.7 for lipids in fishes (van den Thillart & Kesbeke, 1978; Kleiber, 1992; Wood, 2001). Measurements of substrate utilization have not been performed in air-breathing fishes. Despite these shortcomings, the volumetric method has been used frequently, and has been an important tool for measurement of aerial respiration before gas analysers and oxygen probes became available.

Closed respirometry is another method that has been used in many studies, for measurements in both air and water, separately or simultaneously. One of its major strengths is that it can be even simpler than volumetric respirometry, basically requiring some kind of container that fits the fish, and a way to directly measure O_2 concentration or partial pressure (pO_2), either through titration by the Winkler method, by use of gas analysers (mostly used for air samples), galvanic oxygen electrodes, or fibre-optic oxygen sensors (optodes). These measurements are direct and do not require an estimate of RQ to calculate $\dot{M}O_2$. The method relies on the fact that when a fish is breathing from a confined volume of air or water, the oxygen content will decrease, and from this decrease $\dot{M}O_2$ can be calculated (Lenfant, 1961; Cech, 1990; Lighton, 2008). Depending on the length of time, the system is held closed, problems with a reduction in pO_2 and accumulation of CO_2 and other waste products may arise, and this is one of the major weaknesses of closed respirometry (Steffensen, 1989). In most studies, this problem is circumvented to some degree by having several successive closed periods interspersed by flushing of the system. In one study, this procedure was repeated manually once per hour for 25 h (McMahon, 1970) but this again introduces the problem of fish disturbance and hence possible influences on, for example, surfacing behaviours.

Flow-through respirometry relies on the fact that oxygen uptake by the fish, in a confined but unsealed chamber, will elicit a small decline in oxygen levels between inflow and outflow, whether the medium is air or water. The strength of this system is that contrary to volumetric and closed respirometry, the method can be used to measure $\dot{M}O_2$ for extended periods of time because there is a constant flow of air or water

through the relevant chamber, keeping oxygen levels close to ambient all the time. In addition, it requires only a few more pumps and valves than closed respirometry, plus the equipment for direct measurement of oxygen. One of the weaknesses of flow-through respirometry, particularly in water, was pinpointed early by Steffensen (1989). This concerns (1) the risk of incorrect $\dot{M}O_2$ measurements if the flow rates are not constant, (2) the animal is not in a metabolic steady state and (3) washout characteristics are not taken into account. Particular care must be taken to return to a steady state if pO_2 in the water are changed up and down during the experiment, which is a common manipulation in studies on bimodal fishes. Even under steady-state conditions, flow-through systems will only provide a measure of $\dot{M}O_2$ averaged over time; they cannot accurately reveal the discontinuous patterns of respiration from air and water that are characteristic of many fishes with bimodal respiration (Graham, 1997). Because air breathing is arrhythmic and the volume of air breaths can be relatively small, such systems need to have a very low flow of air and sensitive oxygen analysers if they are to reliably measure $\dot{M}O_2$ from air, when a fish is not surfacing very frequently. A common variation on these systems, that is frequently used, is to have a flow-through aquatic phase combined with a closed air phase at one end, allowing spot samples to be taken to measure oxygen consumption from the air. This requires manipulation, but has the advantage that it allows accurate measures of low rates of aerial respiration, for example, by facultative air breathers in aquatic normoxia (Randall *et al.*, 1978, 1981; McKenzie & Randall, 1990).

In addition to the above approaches, some more specialized and less common ways of estimating $\dot{M}O_2$ in air-breathing fishes also deserve mention. Some researchers have, for example, placed a catheter behind the operculum to sample O_2 in the out-flowing water, to calculate aquatic $\dot{M}O_2$ by subtraction from the inhaled water content using Fick's principle (Johansen & Lenfant, 1967). While this approach is intuitive and is assumed to measure the amount extracted by the gills, it does require substantial handling and the catheter places constraints on the free movement of the fish. Also, it is unclear whether water flow can be accurately measured and whether the catheter samples a truly mixed exhalant water stream, or even whether it samples a mixture of exhaled water and the surrounding water volume. Catheterization has also been used to investigate changes in pO_2 and pCO_2 inside air-breathing organs (ABO; Lenfant & Johansen, 1968; Babiker, 1979; Ishimatsu & Itazawa, 1981; Smatresk & Cameron, 1982a, b; Seymour *et al.*, 2007). This approach obviously also requires fish handling and again the uncertainty as to whether the presence of a catheter affects behaviour and the spontaneity of visits to the surface. While the above methods generally may not be entirely suitable for estimation of whole animal SMR, they can be powerful approaches for detailed investigation of respiratory function of gills and ABOs, rather than to measure metabolic rate *per se*.

As described, all of the above can be used for both air and water, separately and in combination (Table I). Some of the earlier studies measured only aerial respiration (Munshi *et al.*, 1976, 1978) while others measured only aquatic respiration (Berg & Steen, 1965; Teal & Carey, 1967), and this approach is still in use (Tripathi *et al.*, 2013; Porteus *et al.*, 2014). Yet, others have confined fishes to either medium during the measurements and several studies have performed both successively on the same species. It may be appropriate to measure $\dot{M}O_{2w}$ and $\dot{M}O_{2a}$ separately and under confined conditions, for example, in amphibious skin breathers that cannot take up oxygen from air unless they are emerged (Gordon *et al.*, 1978), or specifically to assess the capacity

for $\dot{M}O_2$ through gills and skin alone, for example, to maintain SMR (Lefevre *et al.*, 2011a, 2014a). For questions that require measurement of total $\dot{M}O_2$ and its partitioning, however, it is necessary to measure $\dot{M}O_2$ from both air and water simultaneously. This has been carried out since the 1960s but, for some reason, the non-simultaneous approach appears to have dominated in recent decades. Graham (1997) points out the most obvious challenge in measuring aquatic and aerial respiration simultaneously, namely that O_2 (and CO_2) can diffuse from one phase to the other. This is particularly true when working with a hypoxic water phase, something that is frequently the case in studies on air-breathing fishes. The rates of diffusion can, however, be corrected for by running background measurements without a fish (Lefevre *et al.*, 2011a; McKenzie *et al.*, 2012) or, as suggested by Graham (1983), by measuring changes in the air phase with a unimodal water-breathing fish (that tolerates the particular hypoxia level used) in the respirometer.

SYSTEMS FOR AUTOMATED BIMODAL RESPIROMETRY USING INTERMITTENT-CLOSED TECHNIQUES

Historically, one of the major limitations of techniques for bimodal respirometry has been the need to be present to collect and process samples of water or gas phases; or to ensure that, for example, sensitive gas analysers were calibrated and functioning well. As a result, few studies comprised measurements for periods longer than about 8 h (Hedrick & Jones, 1993; Graham, 1997). It is well known that the behaviour of air-breathing fishes can be profoundly affected if they sense any risks associated with surfacing, such as might be created by experimenters manipulating their respirometer or just by entering the room (Lefevre *et al.*, 2011a). They may also have diurnal patterns of activity, nocturnally active species such as the marbled swamp eel *Synbranchus marmoratus* Bloch 1795, the African sharptooth catfish *Clarias gariepinus* (Burchill 1822) or the northern snakehead *Channa argus* (Cantor 1842) may show very different respiratory partitioning at night, when they forage and are active, compared with during the daytime, when they reduce activity and seek cover in the wild (Babiker, 1979; Munshi *et al.*, 1979; Ojha *et al.*, 1979; Boujard *et al.*, 1990; Ghosh *et al.*, 1990).

Following the development of intermittent-closed techniques for purely water-breathing fishes (Steffensen *et al.*, 1984; Svendsen *et al.*, 2016), the small community of researchers that study respiratory physiology of air-breathing fishes have given some thought as to how automated systems might be developed to allow measurements of $\dot{M}O_2$ from water and air on undisturbed animals over relatively extended periods. The first report of such a system was by Abe & Steffensen (1996a, b) who studied respiratory partitioning by the South American lungfish *Lepidosiren paradoxa* Fitzinger 1837 in aquatic normoxia and hypoxia, and by comparison to aestivating individuals. Although a diagram of the system is provided (Abe & Steffensen, 1996a), the methods unfortunately do not report how flushing of water and air phases was ensured, which is a critical issue in the design of such systems. It turns out that flushing and sealing of the compartments was ensured by a system of electronic valves (J. F. Steffensen, pers. comm.) similar to the system described in method 1 below.

Two systems are described in detail here, that both take their inspiration from the intermittent-closed technique of Steffensen (1989), applying it to both water and air phase. Method 1, first in use and published by Lefevre *et al.* (2011a), requires some

know-how of electronics, data acquisition, digital control systems and their associated programming, but offers greater control of the phases and flexibility of the alternating cycles so they can be fitted for a variety of experimental conditions. Method 2, only in use recently (D. J. McKenzie, T. C. Belão, S. S. Killen & F. T. Rantin, unpubl. data), is more simple to set up but ultimately provides less control of the system as it is collecting data, and therefore unexpected errors are more likely to occur.

METHOD 1

The basic layout of the system is shown in schematic form in Fig. 1. The triangular-shaped respirometer, made of, for example, plexiglas [acrylic glass or poly(methyl methacrylate)], is partly submerged in an outer water bath, the level of submergence determining the size of the air space located at the top of the respirometer. If running experiments at high temperature in a room with lower temperature and low humidity, water will evaporate and lower the water level, thereby changing the air space volume. To prevent this, the holding tank can be equipped with an overflow (positioned at the desired water level) to a second water supply, from which water is continually pumped back in the holding tank, maintaining the water level. Under humid conditions, however, this is not necessary. The triangular shape, originally developed for experiments on *S. marmoratus* but later used successfully on both striped catfish *Pangasianodon hypophthalmus* (Sauvage 1878) (Lefevre *et al.*, 2011a, b), striped snakehead *Channa striata* (Bloch 1793) (Lefevre *et al.*, 2012) and Alaska blackfish *Dallia pectoralis* Bean 1880 (Lefevre *et al.*, 2014a), has the advantage that it guides the fish towards the air space and minimizes the surface area of the air–water interface, which allows detection of small changes in air phase pO_2 . Furthermore, the absence of large horizontal surfaces ensures that any air bubbles released by the fish will flow upwards and be released back to the air space, not get trapped elsewhere in the system to cause errors in the measurements of $\dot{M}O_{2a}$ and, eventually, also reduction in the air space volume. The elongated bottom also makes it possible for the fish to rest in a natural horizontal position.

The air space is sealed by valves (*e.g.* 3/2-way direct acting solenoid brass or stainless steel valves from Christian Bürkert GmbH & Co. KG; www.burkert.com), one for inflow and one for outflow. It is important to use valves that seal completely even without a pressure drop across the closed valve. An air pump is connected either directly to the inflow valve, or through a water trap, to completely saturate the inflowing air with water. This can be necessary if experiments are performed with dry air, as the partial pressure of oxygen will change gradually as a result of the gradual increase in water vapour pressure as the air phase equilibrates with the water phase, the change being directly dependent on the temperature at which the experiment is performed. The pO_2 within the air chamber is measured by an O_2 sensor, where optodes are preferred in settings with unstable electrical supply (*e.g.* tropical field stations). The optodes and data acquisition system can be chosen independently, *e.g.* Fibox 3 or OXY-4 (Loligo Systems ApS; www.loligosystems.com) with optodes from PreSens (PreSens Precision Sensing GmbH; www.presens.de) or FireSting O_2 (PyroScience GmbH; www.pyro-science.com). The water phase contains a closed loop, with a submersible circulation pump (*e.g.* AquaBee UP 300, Aquabee Aquarientechnik; www.aquabee-aquarientechnik.de or Eiheim Universal 300, Eiheim GmbH; www.eiheim.com) ensuring continuous and gentle mixing of the water and

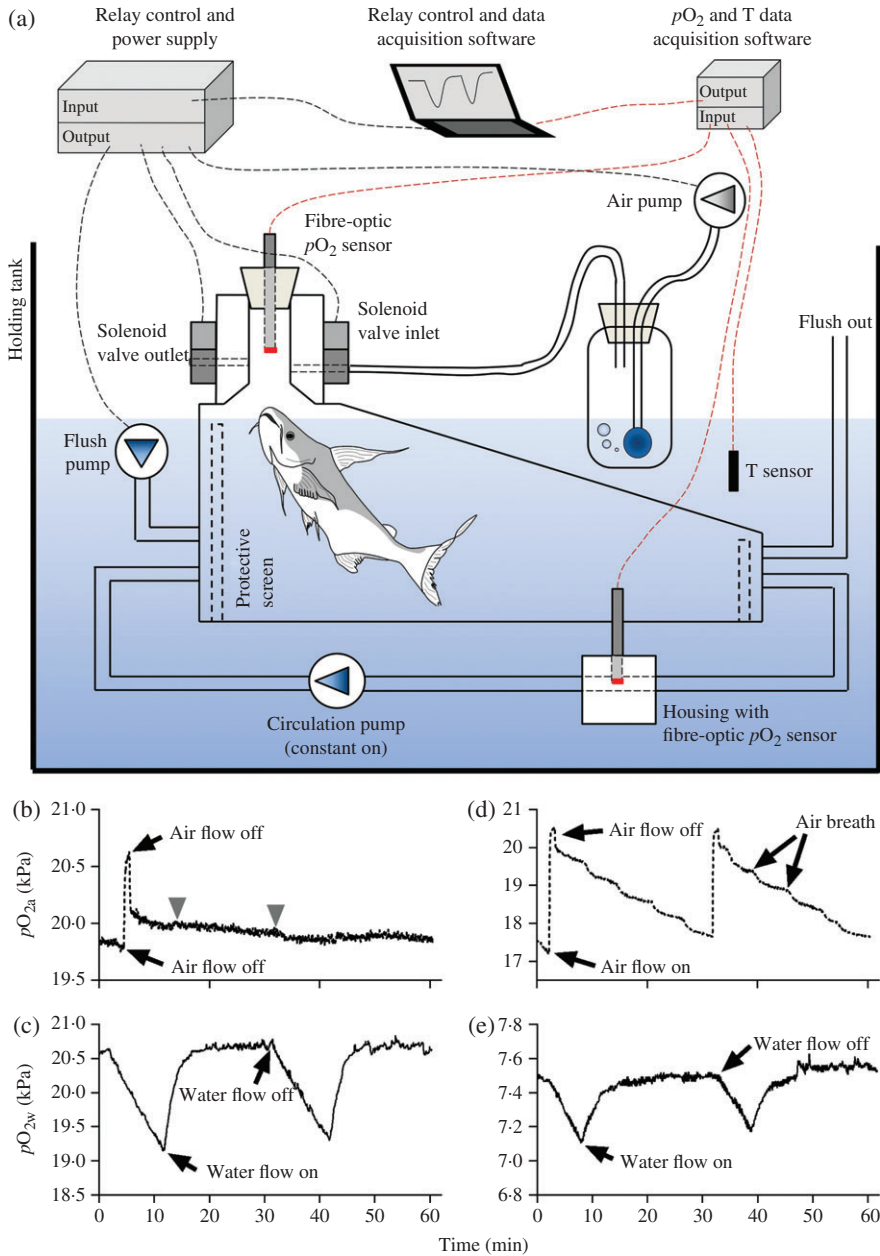


FIG. 1. (a) Diagram of a set-up that can be used for bimodal intermittent-closed respirometry. The solenoid valves, air pump and submersible pump for water phase flushing needs to be controlled either by a computer or programmable relays. A closed circulation loop passes water continuously over the pO_2 sensor and ensures gentle mixing within the chamber. A surfacing *Pangasianodon hypophthalmus* is depicted for illustration, as this was the first species on which this system was used. The water phase is flushed alternately with the air phase to avoid protrusion of water into the air phase. Representative pO_2 traces from an experiment with *P. hypophthalmus* are shown for air with (b) normoxic water, (c) normoxic water, (d) air with hypoxic water and (e) hypoxic water. Flushing of the water phase causes a slight increase in the pO_2 of the air phase, as indicated (▼) in (b). Illustration modified from Lefevre *et al.*, 2011a.

minimizing boundary layers at the sensor. As most O_2 sensor and data acquisition systems are multi-channelled, the meter used in the air phase can also be used for the water phase. The inflow to the water phase is a tube connected to a submersible pump (e.g. Eiheim Compact 300), whereas the outflow is a long tube connected under water, but protruding above the water surface to prevent inflow of oxygenated water. The standing water in the outflow tube slows the diffusion of O_2 from the air into the water phase to a minimum. It is important that the flow of this pump is large enough to exchange all water in the respirometer within reasonable time, while small enough not to create a current that disturbs the fish and forces it to actively maintain position. Both the inflow from circulation and flush pumps are located in front of the respirometer, to aid unidirectional flow. To reduce the pressure of the inflowing water, which may cause the fish to spend energy on maintaining position or even swim forward, a baffle consisting of a plastic plate with evenly spaced small holes can be placed in front of the inflows. Also, to prevent the fish from getting sucked into and stuck to the intake from the circulation pump, a small baffle should be placed in front of it.

The closing and opening of valves is controlled by relays programmed in appropriate software. This can be achieved using, for example, programmable national instrument relays (e.g. NI 9481) or dedicated hardware controllers such as the type used by Lefevre *et al.* (2011a). The advantage of this kind of system is that it offers great flexibility in the adjustment of flushing and closed periods as well as control of temperature and water pO_2 . A diagram of the overall alternating cycles in this bimodal intermittent-closed system is depicted in Fig. 2. The length of phase 1, the closed phase, depends on the relative size of the fish to the respirometer and its $\dot{M}O_2$, and should be adjusted accordingly as to avoid too great depletion of either phase's oxygen content. During phase 2, flushing of the water phase, it is important that the air space remains sealed, to prevent water from flooding the air space or changing the air volume. Once the flush pump has been turned off (phase 3), the air in the air space can very quickly be renewed by opening both valves (phase 4) and gently blowing air through the air space. Even if the outflow valve is open, the pressure inside the air space may increase slightly, causing the air volume to increase as well as the pO_2 [Fig. 1(b)]. Therefore, it is important to have a small interval after the inflow and air pump has been turned off, where the

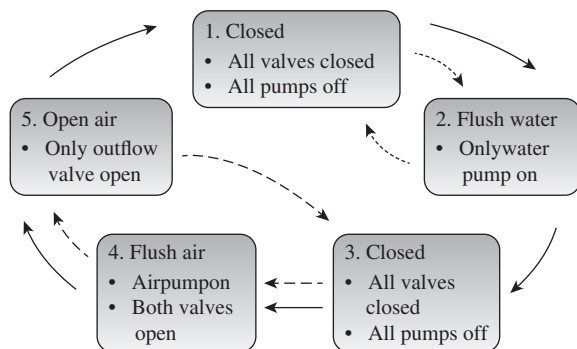


FIG. 2. Diagram of the cycle of events in method 1 bimodal intermittent-closed respirometry. Note that the cycle can be modified to have a repetition of phases 1 and 2 (.....), if, for example, the water is normoxic and dependence on air breathing is low, or a repetition of phases 3–5 (---), if dependence on air breathing is high.

outflow valve remains open (phase 5), so the water level and thereby air space volume and pressure can be restored to normal. Because of the effect flushing of a phase has on the other phase, it is preferable only to use the period when both phases are closed (either phase 1 or phase 3) for calculation of $\dot{M}O_{2w}$ and $\dot{M}O_{2a}$.

Both $\dot{M}O_{2w}$ and $\dot{M}O_{2a}$ are calculated from the rate of decline in pO_2 in each phase during the closed period [Fig. 1(b)–(e)]. $\dot{M}O_{2w}$ is calculated as described by Lefevre *et al.* (2011a) and Svendsen *et al.* (2016). If frequency is low enough and the size of air breaths large enough, it may be possible to discern single breaths as a stepwise pattern in pO_{2a} trace [Fig. 1(d)], but it is still the average slope that is used for calculation of $\dot{M}O_{2a}$, as it allows for easy correction for diffusion, simply by subtracting the background slope from the total slope. $\dot{M}O_{2a}$ can be calculated from the air ΔpO_2 using a standard curve made by removing a known volume from the air chamber and replacing this volume with N_2 gas, then correlating the volume of N_2 gas with the corresponding ΔpO_2 . The volume of O_2 displaced then corresponds to 20.95% of the injected N_2 volume. In this calculation, it is not necessary to calculate the exact volume of air in the air space, but obviously, the standard curve changes if the volume changes, once again underpinning the importance of a constant air volume. The total $\dot{M}O_2$ at each time interval can simply be calculated as the sum of $\dot{M}O_{2w}$ and $\dot{M}O_{2a}$, and the respiratory partitioning can, for example, be expressed as the percentage of the total $\dot{M}O_2$ that is derived from air.

METHOD 2

This method is shown in schematic form in Fig. 3. Exactly as for method 1, the bimodal respirometers are partially immersed in an outer bath of well-aerated water with a submerged water phase and an air phase that projects above the water surface. The cross-sectional design of the respirometer is also the same. As for method 1, the water level in the outer bath has to be maintained constant with an overflow, such that the level is above the outflow from the respirometer (Fig. 3). The volume of the air space was measured as described for method 1.

The $\dot{M}O_2$ from both phases is measured with an intermittent-closed technique modified for bimodal respirometry. In this example, using *C. gariepinus* with a mass of c. 350 g, the method alternated two periods within a 15 min cycle, controlled by a custom-designed electronic timer-relay (Tempus 1). In the first period, water and air chambers are closed to the exterior for 10 min and $\dot{M}O_2$ from both phases is recorded by optodes (OXY-10 Micro, PreSens Precision Sensing GmbH), which sampled either air or water. As for method 1, the aquatic phase is continuously gently mixed by a submerged pump (in this case, an Eheim 1048) within a closed tubing circuit, drawing water from a submerged outlet and delivering it to a submerged inlet. The alternate period is when the two phases, aquatic and aerial, are flushed simultaneously for 5 min to replenish oxygen levels, by activation of the common timer:relay. Exactly as for method 1, the water phase is flushed from a second inlet below the water surface, where a submersible pump (Eheim 1048) sitting in the outer bath delivers aerated water to the respirometer. The air phase is flushed by a membrane air pump (Tetratec APS 400, Tetra United Per Group; www.tetra-fish.com) that delivers air, humidified through a water trap, into an inlet in the roof of the air space. The flushing water and air both exit from the respirometer *via* a common outflow, a 50 mm long and 10 mm inner diameter polyvinyl chloride (PVC) tube lying just below, and horizontal to, the water surface

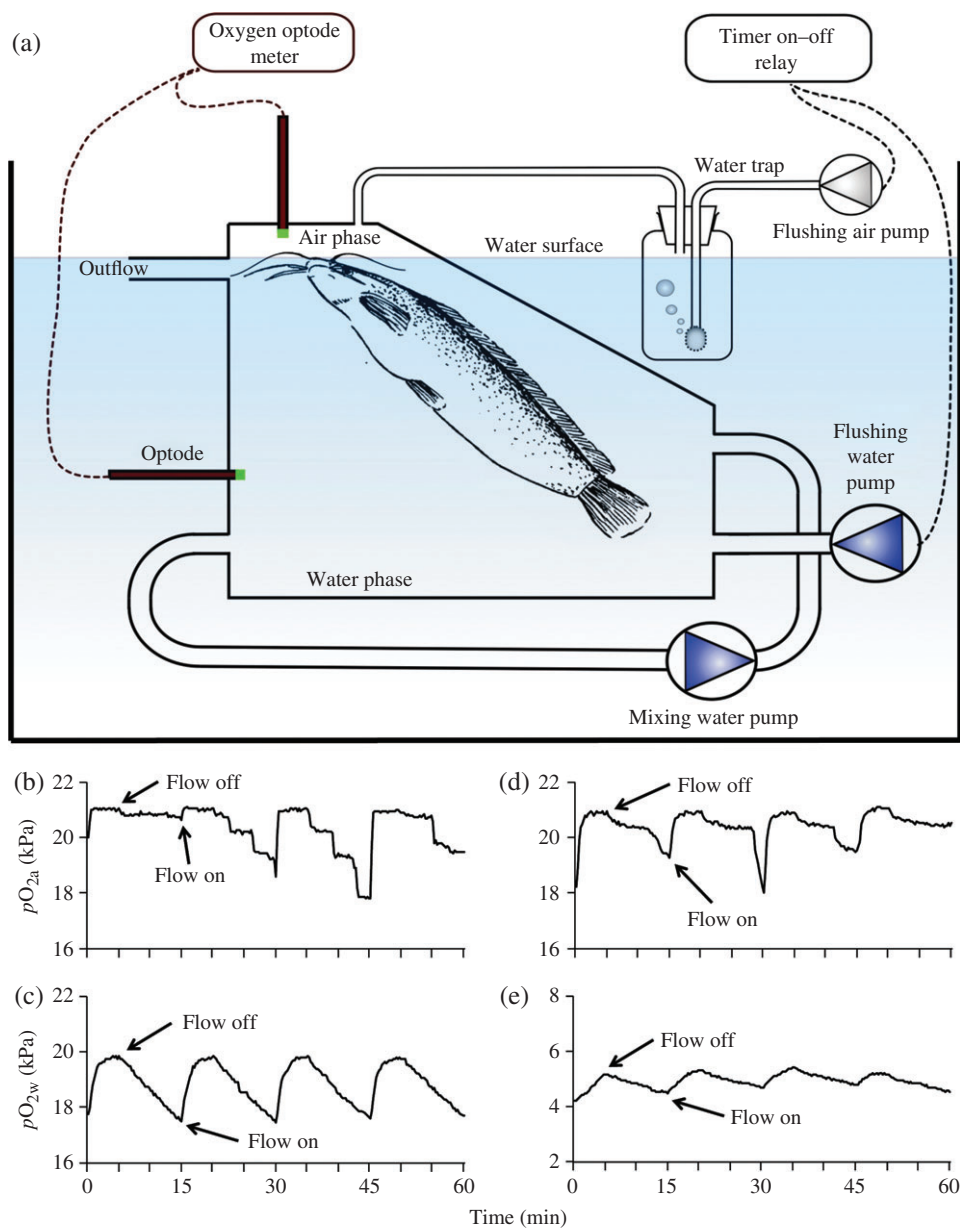


FIG. 3. (a) Diagram of a relatively simple set-up that can be used for bimodal intermittent-closed respirometry. This system uses a timer and relay to flush water and air phases simultaneously, with both flows exiting from a common outflow that lies parallel to, and just below, the water surface. A closed circulation loop gently mixes the water phase. A surfacing *Clarias gariepinus* is depicted for illustration, as this was the first species on which this system was used. Representative pO_2 traces from an experiment with *C. gariepinus* are shown for (b) air with normoxic water, (c) normoxic water, (d) air with hypoxic water and (e) hypoxic water. Flushing pressurizes the system slightly, which is visible as a slight increase in the pO_2 of the air phase when flow turns on, which then disappears when flow turns off. Aerial respiration can be seen as distinct declines in pO_2 of the air phase, it is not known whether each drop in pO_2 represents a single breath. Illustration modified from D. J. McKenzie, T. C. Belão, S. S. Killen & F. T. Rantin (unpubl. data).

(Fig. 3). This raises total pressure in the system by *c.* 2 kPa (0.02 atmospheres), an effect that is visible as a slight step upwards on the trace from the two optodes. When flushing stops, the system is no longer pressurized and water level in the respirometer equilibrates with the water level in the outer bath. At this point, the air phase is sealed by the water level in outer tank and respirometer, which equilibrates at the surface above the outflow, and by the water trap at the air pump inlet. The water phase is effectively sealed during the closed recirculation phase because the only means for gas exchange with the outside is by diffusion along the narrow outflow tube. This can be confirmed by making water inside the respirometers hypoxic and leaving the system in closed recirculation with the water surface to the aerial phase sealed with bubble wrap. In the current example, water pO_2 was reduced to 2 kPa and it then took over 24 h for the water phase to return to 70% atmospheric O_2 saturation (D. J. McKenzie, T. C. Belão, S. S. Killen & F. T. Rantin, unpubl. data). Under these conditions, there was little to be gained by making corrections for any measurements during a 10 min recirculation phase.

Although not shown on Fig. 3, as for method 1, baffles should be placed as with all such respirometers to avoid strong air or water currents at inflows and to avoid the fish interfering with outflows by, for example, resting against them. The same is true for optodes or electrodes, the tips should be set behind protection such as a small dome fashioned from plastic fencing, which allows free movement of fluids in front of the sensor tips but does not allow the fish to touch them. In aquatic hypoxia, corrections have to be made for the rate of transfer of oxygen from air to water during the 10 min recirculation phase, based upon measurements without fish in each respirometer.

In this case, oxygen levels in the water and air were measured with an optode system (OXY-10 Micro, Pre-Sens Precision Sensing GmbH), using eight channels such that four chambers were set up at the same time. Optodes were positioned to sample water and air for each respirometer (Fig. 3), with all data stored on a PC using the manufacturer's software. Absolute rates of O_2 uptake from air and water were calculated as described for method 1.

This method is simple and therefore multiple respirometers can easily be set up, so that many fishes can be run at one time. A disadvantage is that water levels in the whole system must remain stable, so that one can set them to be just above the common upper outflow (Fig. 3). Small variations in water level can cause problems such as changing the volume of the air space. Because this system has no valve at the outflow, a reduction in the water level in the outer tank can also cause the air space to be open to the outside air, rendering measurements of $\dot{M}O_{2a}$ completely impossible. Thus, care must be taken to have a constant water level, and to ensure that there is enough replacement into the system to compensate any evaporation. Thus, this system is simpler than method 1, but care must be taken to ensure constant water levels.

BIMODAL SWIMMING RESPIROMETRY

There have been relatively few studies of swimming respirometry on air-breathing fishes (Lefevre *et al.*, 2014b) and these have used one of the two methods. Seymour *et al.* (2007) catheterized the gas bladder of the Pacific tarpon *Megalops cyprinoides* (Broussonet 1782) and were able to derive $\dot{M}O_{2a}$ from the decline in pO_2 in the ABO between breaths, as the fish swam against a current of different speeds. This approach

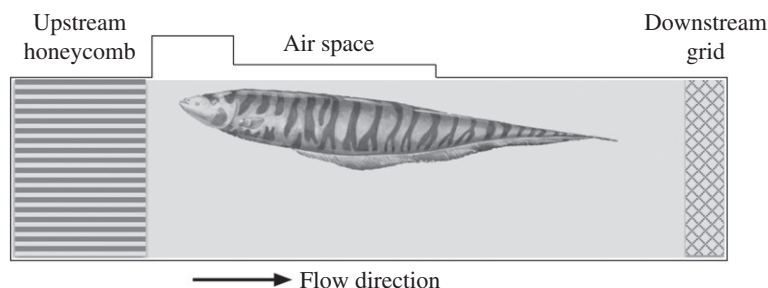


FIG. 4. Illustration of the sideways appearance of a swim tunnel modified for bimodal respirometry. The honeycomb is used to create an even and laminar flow. The air space is kept relatively small, and includes a longer but more flattened part, the purpose of which is to capture bubbles expelled by the fish, as these are carried backwards with the flowing water.

requires quite intensive surgical manipulation of the animal and an accurate estimate of the total volume of the ABO. As such, it is only a suitable method for large animals and for ABOs that are easy to cannulate, such as swimbladders. Given these caveats, such methods do have the advantage that the measurements of $\dot{M}O_2$ from air are absolute; there is no need to correct for complex rates of exchange of oxygen between aerial and aquatic phases which, as described below, can constitute a technical problem for systems where changes in pO_2 in air and water compartments are measured. In fact, the study by Seymour *et al.* (2007) did not measure $\dot{M}O_2$ from the water, as the *M. cyprinoides* swam in an open-topped flume.

The other studies have simply placed an air space at the upstream end of the swim chamber on a standard swim tunnel respirometer (Fig. 4), in particular a Steffensen-type (Seymour *et al.*, 2004; McKenzie *et al.*, 2012; Lefevre *et al.*, 2013). These have the advantage that the fish does not need to be manipulated surgically, and can be left to swim freely in the tunnel. When considering the design of this air space, it should be kept in mind, however, that the fish must be able to access it easily, without varying its natural swimming behaviour any more than it might in the wild. At the same time, the chamber should be small enough for pO_2 to change to a reliably measurable extent with air breathing by the fish. The mechanics of ventilation of the ABO must also be considered. All air-breathing fishes exhale first and then use a buccal force pump to push fresh air into their ABO so, as they descend back underwater after gulping at the surface, excess air usually exits as bubbles from their opercula (Graham, 1997). In the moving current of the swim tunnel, these bubbles will be dragged towards the back; to measure $\dot{M}O_2$ from the air, they must surface back into the air space and not be entrained underwater into the propeller. Indeed, the presence of a water current flowing below the air space poses a number of problems. These may be self-evident, but considering them here should help anyone that embarks on such studies to be aware of the pitfalls beforehand and so design their system and perform control experiments accordingly.

First among these is the fact that the swim tunnel current mixes the surface of the aquatic phase and so promotes exchange of gases with the aerial phase. The extent to which this exchange occurs will be proportional to the speed of the water current and to the gradient of pO_2 between water and air. This makes correction rather difficult, especially if a fish is consuming O_2 from both phases such that their pO_2 is not constant.

Measurements of aerobic swimming performance in fishes typically set the current at a known speed for a time long enough to ensure that the fish is exercising in a relatively steady state (Beamish, 1978; McKenzie, 2011). It is therefore necessary to perform control experiments at the fixed speeds that will be used in the experiment, without a fish, to measure rates of exchange of gases between air and water as a function of the pO_2 gradient between the phases. Bubbling with nitrogen can be used to modify the pO_2 of the water, and then the pO_2 of both phases recorded over, for example, the same time period as the increment in speed used in the swimming protocol. The air space should be flushed at regular intervals, to ensure that air pO_2 never declines below 95% saturation, and this should also then be performed throughout the actual swimming experiments. The reader will also realize that errors in estimating $\dot{M}O_2$ from air and water are virtually impossible to avoid if the fish is consuming from both compartments simultaneously, so that the pO_2 gradient is also changing continuously, something that is difficult to account for with control measurements. If experiments are performed in normoxia, and water and air phases are not allowed to decline below, for example, 90% saturation, then O_2 consumption by the fish should be significantly greater than the rate of passive exchange between phases, so errors should be relatively small. The exchange of gases between water and air will, however, be magnified if experiments are also performed in aquatic hypoxia, so careful tests of rates of exchange of oxygen as a function of pO_2 gradient and swim speed will be required. Although these necessary control measurements of exchange between the phases require considerable time, it is absolutely necessary when experiments are performed in hypoxia.

The other problem is the nature of the water flow through and below the air space. If the air space is set exactly to coincide with the top of the swimming channel, the water current may entrain bubbles at the downstream end of the air space. Entraining bubbles makes it impossible to measure $\dot{M}O_2$ accurately by the fish from either phase. The faster the current, the greater the pull on the air at the end of the airspace so, in general, the tendency to entrain bubbles will increase with current speed. This will not, however, necessarily be a linear effect with speed because the open water surface will develop a standing wave, the height and length of which will vary with the current speed and the design of the respirometer. The length of the air space will then determine how this wave meets the end of the space, and the tendency to entrain bubbles at different speeds. At certain speeds, the wave may dip below the end of the space and cause a serious problem of entrainment of bubbles; at other speeds, it may hit against the back of the space and have unknown effects on the flow profile below. If an attempt is made to try and reduce these problems by raising the water surface slightly higher than the upper boundary of the swim channel, one does not know the flow profile in the upper few millimetres of water, and how the current hitting against the end of the airspace affects this. Fishes are extremely efficient at exploiting any areas of lower flow in a swim tunnel (D. J. McKenzie & S. Lefevre, pers. obs.), making it difficult to know their actual swimming speed.

Overall, the outcome of these two sets of problems is that performing valid bimodal respirometry becomes increasingly difficult with swimming speed. Thus, it is easier to perform on fishes that do not swim to particularly high maximum aerobic speeds, such as the banded knifefish *Gymnotus carapo* L. 1758 (McKenzie *et al.*, 2012) rather than on athletic migratory air breather such as *P. hypophthalmus* (Lefevre *et al.*, 2013). It also follows that flushing and water phases can cause a variety of problems with bubbles and changes in levels of the two phases. As such, the application of automated

intermittent-closed techniques to swimming respirometry poses particular technical challenges and all studies to date have used manual methods to flush the air space (Burlerson *et al.*, 1998; Farmer & Jackson, 1998; Seymour *et al.*, 2004, 2007; McKenzie *et al.*, 2012; Lefevre *et al.*, 2013).

FUTURE PERSPECTIVES AND CONCLUSIONS

In the measurement of metabolism in air-breathing fishes, there has been an overwhelming focus on the effects of aquatic hypoxia on the partitioning of gas exchange. The excretion of CO₂ from these species is usually argued to be branchial, based on a combination of theoretical arguments concerning the very high CO₂ solubility in water and evidence from experiments with normocapnic or slightly hypercapnic water (Ultsch, 1996). Hypoxia in natural waters, however, is invariably accompanied by an associated hypercapnia, which in tropical waters with organic loading, can frequently reach 8.0 kPa (Shartau & Brauner, 2014) and thus significantly in excess of levels in most of the metabolic literature. Indeed, Willmer (1934) found in several Amazonian fish species that in waters with *p*CO₂ levels up to 3.3 kPa, branchial excretion dominated, whereas above that level ABO excretion became increasingly important. It is quite possible that ABO excretion is the dominant route of CO₂ excretion in a variety of air-breathing fishes inhabiting tropical waters susceptible to profound hypercapnia. Recent advances in aquatic *p*CO₂ sensors (Oxyguard; www.oxyguard.dk) are making it more straightforward to control aquatic *p*CO₂ levels independent of bicarbonate levels, controlling against both pH and *p*CO₂ by addition of CO₂ gas, and the newest probes have response times that may even be sufficient for respirometry measurements. This area is then worth revisiting in the near future, both with respect to clarifying routes of CO₂ excretion, and also allowing RQ estimation in fishes with bimodal breathing.

Although bimodal intermittent-closed respirometry, in principle, is relatively straightforward, it is important to remember that the air-breathing fishes are very diverse when it comes to behaviour, morphology and physiology (Johansen, 1970; Graham, 1997), which is important to take into account when designing the shape of respirometers and protocol of the experiment. Most obviously, the fish needs to be able to find and access the air space, and be able to move up and down to surface, in a way that closely resembles its natural behaviour. The development and use of systems that apply the intermittent-closed principle is a crucial step in the movement of the field to more complex studies, where partitioning and metabolic rate are studied in a broader context, such as ecology and behaviour, and the effects of environmental conditions such as hypercapnia as described above.

S.L. was financially supported by the Carlsberg Foundation and the Research Council of Norway. M.B. was supported by the Danish Ministry of Foreign Affairs (DANIDA), DFC no: 12-014AU and D.J.M. was supported by a CNPq Visiting Researcher Grant to the Department of Physiological Sciences at UFSCar São Carlos (CNPq processo n. 313621/2013). This review was invited following discussions at the COST Action FA1004 2nd Annual Conference on Conservation Physiology of Marine Fishes.

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