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Effect of closed *v*. intermittent-flow respirometry on hypoxia tolerance in the shiner perch *Cymatogaster aggregata*

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This study compares the critical oxygen saturation (O_{2crit}) levels of the shiner perch *Cymatogaster aggregata* obtained using two different methods wherein hypoxia is induced either by the fish's respiration (closed respirometry) or by degassing oxygen with nitrogen (intermittent-flow respirometry). Fish exhibited loss of equilibrium at a higher O_2 saturation in the closed respirometry method when compared with the intermittent-flow method. Utilization of closed respirometry yielded O_{2crit} measurements that were almost twice as high as those obtained with intermittent-flow respirometry. The lower hypoxia tolerance in closed respirometry is consistent with additional stress, caused by a build-up of ammonia and carbon dioxide and a faster rate in dissolved oxygen decline. The results indicate that these two methods of determining hypoxia tolerance in aquatic organisms are not comparable, and that much care should be given to method choice.

Key words: aquatic breathers; critical oxygen saturation level; hypoxic; physiology; respiration; stress.

INTRODUCTION

Low oxygen, or hypoxic, conditions are becoming more common in marine habitats. Run-off of agricultural fertilizers has led to prevalent and severe regions of hypoxia, known as coastal dead zones (Diaz & Rosenberg, 2008; Diaz & Breitburg, 2009; Zhang *et al.*, 2010) and low oxygen regions in the open ocean are expanding (Stramma *et al.*, 2011). These regional decreases in dissolved oxygen (DO) could lead to changes in habitat availability in a wide range of fishes (Diaz & Rosenberg, 2008; Stramma *et al.*, 2011).

To quantify the effects of hypoxia on fish ecology, researchers rely on accurate calculations of hypoxia tolerance. While fishes employ compensatory mechanisms, such as increased ventilation rate and depth, gill surface area and whole blood haemoglobin to maximize oxygen extraction under hypoxic conditions (Marvin & Heath, 1968; Mandic

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et al., 2009; Perry *et al.*, 2009), fishes become unable to regulate oxygen uptake at a species-specific critical oxygen saturation level (O_{2crit}, Schurmann & Steffensen, 1997; Claireaux *et al.*, 2000). At oxygen saturations below O_{2crit}, fishes become oxygen conformers [*i.e.* their metabolic rate becomes dependent on the ambient oxygen saturation and falls below standard metabolic rate (SMR); Davis, 1975; Cech *et al.*, 1979; Chapman *et al.*, 2002; Portner & Langenbuch, 2005; Perry *et al.*, 2009; Cech & Brauner, 2011]. O_{2crit} therefore denotes the saturation of DO when an individual must switch to anaerobic metabolism, with repercussions for metabolic equilibrium, acid–base balance and survival (Ultsch *et al.*, 1980; Portner & Langenbuch, 2005; Perry *et al.*, 2009; Mandic *et al.*, 2013). Although not a comprehensive measure (Speers-Roesch *et al.*, 2013), O_{2crit} remains a useful benchmark for understanding a fish species' hypoxia tolerance.

Optimal determination of O_{2crit} involves three steps: (1) measuring SMR of a calm, acclimated fish under normoxic conditions (over 80% oxygen saturation), (2) subjecting the fish to decreasing DO while measuring oxygen uptake rates ($\dot{M}O_2$, a proxy for metabolism; Nelson, 2016) and (3) calculating the level of oxygen saturation where the fish's metabolic rate falls below SMR and it becomes an O_2 conformer. This method relies on respirometry, placing an animal in a chamber and calculating the change in DO in the chamber over time (Steffensen, 1989; Cech, 1990).

Methods used to measure O_{2crit} vary among studies. The resting period (*i.e.* the time period over which the fish is left in the chamber to calm to SMR) ranges from 1 to 48 h in duration (Steffensen *et al.*, 1994; Nilsson & Ostlund-Nilsson, 2004; Nilsson *et al.*, 2010; Killen *et al.*, 2012; Aboagye & Allen, 2014; Eliason & Farrell, 2014). The resting period must (1) provide enough time for the fish to adjust to the respirometer and repay any oxygen debt resulting from the stress of the handling and transfer and (2) take into account any circadian cycle in the fish's metabolism (Chabot *et al.*, 2016). Very short resting time or an overestimation of SMR would, by definition, result in an overestimation of O_{2crit} (Claireaux & Chabot, 2016).

Another potential confounding factor is the way in which hypoxic conditions are created. The two most commonly employed methods are closed and intermittent-flow respirometry (*e.g.* closed: Steffensen *et al.*, 1994; Nilsson & Ostlund-Nilsson, 2004, and intermittent flow: Steffensen, 1989; Killen *et al.*, 2012; Eliason & Farrell, 2014). These methods usually differ in the following ways: (1) the time spent at different oxygen saturation levels, (2) the rate at which hypoxic conditions are reached and (3) the chemical composition of the water in the respirometry chamber. Any of these three factors could affect the fish's stress level, thus potentially influencing the response to hypoxia.

In the closed respirometry method, hypoxic conditions are created by the fish's own respiration over time (Kaufmann *et al.*, 1989; Steffensen, 1989; Cech, 1990). The fish's oxygen uptake rate $\left(\dot{M}O_2\right)$ controls the rate at which hypoxia is induced (*i.e.* the rate of oxygen depletion cannot be controlled and varies among fish in the experiment). Also, because there is no water exchange in the chamber, the closed method may induce confounding effects of hypercapnia and accumulation of nitrogenous wastes (Kaufmann *et al.*, 1989; Steffensen, 1989; Cech, 1990). While these effects may not be separable from the effects of hypoxia, they may replicate natural conditions under particular circumstances (*e.g.* a fish in a small tide pool, estuaries and coastal zones) and hence have some ecological relevance (Truchot, 1988; Burnett, 1997; Brix *et al.*, 1999; Mandic *et al.*, 2009; Mucci *et al.*, 2011).

Alternatively, in the intermittent-flow respirometry method, oxygen is degassed using nitrogen. Each fish is exposed to specific oxygen saturation levels for the same amount of time and the rate at which hypoxic conditions are reached is controlled among all replicate fish. The respirometry chamber is flushed after each measurement cycle, which prevents the build-up of wastes (Schurmann & Steffensen, 1997; Eriksen, 2002; Sollid *et al.*, 2003). Lastly, there is a lower risk of oxygen diffusion in the intermittent-flow method *v*. the closed due to a lower gradient in oxygen saturation along the walls of the respirometry chamber and tubing (M. Svendsen, unpublished data).

Despite long-standing concerns about the closed respirometry method (Keys, 1930; Tang, 1933), no study has yet examined how the method of simulating hypoxic conditions influences measures of hypoxia tolerance. This study tested whether the two most commonly used respirometry methods (closed and intermittent flow) produce comparable determinations of O_{2crit} in the shiner perch *Cymatogaster aggregata* Gibbons 1854.

MATERIALS AND METHODS

COLLECTION AND HUSBANDRY

Adult *C. aggregata* (standard length, $L_S = 8{\cdot}69 \pm 0{\cdot}08$ cm, wet mass, $16{\cdot}88 \pm 0{\cdot}40$ g, mean ± S.E.) were collected using beach seines at Jackson Beach, San Juan Island, Washington, U.S.A., between July and August 2013. Following capture, fish were transported to Friday Harbor Laboratories (FHL) and maintained in flow-through tanks (measuring 60 cm × 130 cm × 25 cm, length × width × height) supplied with filtered seawater under a natural light regime (14L:10D). Water temperature corresponded to ambient conditions in Puget Sound (13–15° C) during the experimental period.

EXPERIMENTAL SET-UP

The experiment was conducted in a closed system consisting of the experimental aquarium (70 l aquarium) and a 140 l sump. Water in this closed system was circulated from the sump to the experimental aquarium using an Eheim Universal 600 Pump (Eheim; www.eheim.com), before overflowing through a drain back into the sump. To minimize bacterial respiration, water was continuously circulated through a UV sterilisation system (Blagdon Pro UVC 36 W; www.blagdonforcehybrid.co.uk) (Johansen & Jones, 2011; Clark *et al.*, 2013). Water temperature was maintained at $14 \pm 0{\cdot}1$° C using an Ametek Pt100 temperature sensor (Ametek; www.jofra.com) and PR 5714 programmable relay (PR Electronics; www.prelectronics.dk) connected to and controlling the addition of water from a LAUDA-Brinkmann chiller (LAUDA-Brinkmann; www.lauda-brinkmann.com) to the sump.

The experimental aquarium was divided in half using 5 mm black netting sewn onto a metal frame. Two identical 0·5 l respirometry set-ups were placed in the aquarium, with one on each side of the divider. Fish were starved for a minimum of 48 h prior to experimentation so that they were in a post-absorptive state (Niimi & Beamish, 1974). As *C. aggregata* is a gregarious species (Eschmeyer *et al.*, 1983), four individuals collected on the same day as the focal individual were placed in the experimental aquarium around each of the respirometry chambers to avoid isolation stress (Pursche *et al.*, 2009). This was done for all trials.

The respirometry set-up consisted of a sealed 0·5 l chamber connected to a recirculating pump (which mixed water inside the respirometer) and a flushing pump (which pumps water from the aquarium in and out of the chamber) (Steffensen, 1989; Clark *et al.*, 2013; Svendsen *et al.*, 2016). DO concentration in the chamber was measured and logged using a FireSting fibre-optic oxygen meter (Pyro-science; www.pyro-science.com). The sensor was mounted in the recirculation loop, to ensure that flow was sufficient for the fast response time of the sensor, according to the manufacturer's recommendations.

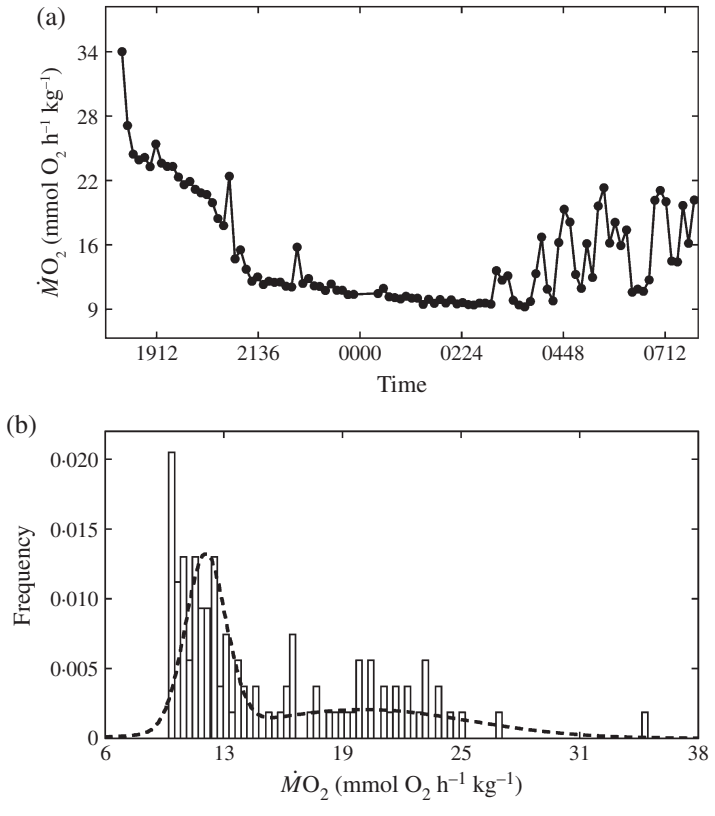

FIG. 1. (a) Plot of oxygen uptake rate $(\dot{M}O_2)$ during resting period of *Cymatogaster aggregata*. (b) Histogram demonstrating the double Gaussian method of determining standard metabolic rate (SMR).

HYPOXIA METHODS

To test whether the method of administering hypoxia affects measures of hypoxia tolerance, multiple trials were conducted using two different methods: (1) intermittent-flow respirometry and (2) closed respirometry. Two identical respirometers were run during each trial. In order to account for any pseudoreplication that may have occurred due to running two fish simultaneously, a mixed-effects model was employed with trial as a random effect.

Resting period

The fish were placed in the respirometry chamber 12–15 h prior to the experimental period in order to allow the fish to calm to SMR. During this period, intermittent-flow respirometry was utilized to measure oxygen uptake rate $(\dot{M}O_2)$. Each $\dot{M}O_2$ measurement was executed using a 180 s flush, 60 s wait and 240 s measurement period, which was monitored using the free AquaResp programme (www.aquaresp.com). During the 240 s measurement period, the respirometry chamber was sealed to prevent water exchange between the chamber and surrounding aquarium. The fish's $\dot{M}O_2$ reduced the oxygen saturation in the chamber by 10–15% during each measurement period. In general, fish showed a steep decrease in $\dot{M}O_2$ within 2–4 h following introduction into the chamber, leaving a minimum of 8–11 h for SMR determination [Fig. 1(a)]. The resting period was conducted at night as *C. aggregata* are known to be more active during the daytime (Brett & Zala, 1975; Thetmeyer, 1997; Porter, 2001).

Treatment 1: intermittent-flow respirometry

A fish ($L_S = 8{\cdot}56 \pm 0{\cdot}12$ cm, wet mass $= 16{\cdot}22 \pm 0{\cdot}52$ g, mean ± S.E.; $n = 9$) was randomly selected and placed into one of the respirometry chambers. Following the overnight resting period, the oxygen saturation of the water was systematically reduced by bubbling nitrogen through air stones in the experimental sump. The rate of nitrogen bubbling was controlled by an Oxyguard mini-oxygen probe (Oxyguard International; www.oxyguard.dk) connected to a PR 5714 programmable relay (Pr Electronics). The relay would open a solenoid valve allowing the injection of compressed nitrogen into the sump when the oxygen levels in the tank were above the pre-set oxygen level. This allowed $\dot{M}O_2$ to be measured at 100, 70, 50, 30, 25, 20 and 17% oxygen saturation for each fish (Domenici *et al.*, 2000). Three $\dot{M}O_2$ determinations were carried out at each oxygen saturation level. At the start and end of each measurement period, the respirometry chamber was flushed with waters of greater or equal DO concentration to ensure no oxygen leakage from the plastic (Stevens, 1992). Time between the oxygen saturation levels ranged between 15 and 20 min, for a total experimental duration of *c*. 5 h.

Treatment 2: closed respirometry

In this method, fish ($L_S = 8{\cdot}74 \pm 0{\cdot}21$ cm, wet mass $= 17{\cdot}40 \pm 1{\cdot}07$ g, mean ± S.E.; $n = 8$) were randomly selected and placed in the respirometry chamber. After the resting period, the chamber was sealed allowing the fish's respiration to reduce the oxygen saturation of the water. Each fish was kept in the chamber until either oxygen saturation reached 17% or the fish exhibited loss of equilibrium (LOE, *i.e*. was unable to remain upright), whichever occurred first. If the fish exhibited LOE, the oxygen saturation at first detection was recorded. At that point, the experiment was terminated and the chamber flushed. The length of the hypoxia experiment varied among trials due to differences in fish respiration rates. Trials lasted 24–54 min ($41 \pm 3{\cdot}5$ min, mean ± S.E., $n = 8$).

Bacterial respiration

For all trials, bacterial respiration was measured for 24 min (three cycles) both before and after trials. The average of the bacterial respiration was subtracted from measurements of $\dot{M}O_2$.

OXYGEN CONSUMPTION DATA ANALYSIS

To calculate $\dot{M}O_2$, a linear fit was applied to the oxygen time series for each 5 min period that the chamber was closed for intermittent-flow respirometry and every 5 min period for closed respirometry. The derived slope s could then be converted into $\dot{M}O_2$ (mmol O_2 h^{-1} kg^{-1}) using the following equation: $\dot{M}O_2 = sV_{resp}\alpha m^{-1}$, where V_{resp} is the volume of the respirometer minus the volume of the fish (l^{-1}), α is the solubility of oxygen in water (mmol O_2 l^{-1}) and m is the mass of the fish (kg). Oxygen saturation level corresponding to each measurement was estimated as the average of the oxygen levels at the beginning and end of the 5 min period.

For all methods, SMR was defined using a double Gaussian fit of the $\dot{M}O_2$ rates measured during normoxia [Fig. 1(b); Steffensen *et al.*, 1994; Chabot *et al.*, 2016], *i.e*. when oxygen saturation levels exceeded 80%. O_{2crit} was defined as the oxygen saturation level where the $\dot{M}O_2$ fell below SMR. To calculate O_{2crit}, a line was fitted to a sub-set of $\dot{M}O_2$ (mmol O_2 h^{-1} kg^{-1}) values v. the oxygen saturation level (O_{sat}%):

$$\dot{M}O_2 = sO_{sat} + b \tag{1}$$

where s and b are the derived slope and y-intercept. The sub-set included $\dot{M}O_2$ values, during which the fish's $\dot{M}O_2$ fell below and remained below SMR.

O_{2crit} was then calculated as the oxygen saturation level where $\dot{M}O_2$ (mmol O_2 h^{-1} kg^{-1}) equalled SMR $\left(\dot{M}O_{2\_SMR}\right)$: $O_{2crit} = \left(\dot{M}O_{2SMR} - b\right) s^{-1}$. In cases where $\dot{M}O_2$ increased rapidly, or spiked, prior to the steep decrease below SMR, the elevated $\dot{M}O_2$ was considered part of the physiological response to hypoxia (Herbert & Steffensen, 2005, 2006; Johansen *et al.*, 2006; Perry *et al.*, 2009). Because the $\dot{M}O_2$ was now dependent on the DO concentration, these points were included in the linear fit (equation 1; Fig. 2). O_{2crit} was calculated for all fish in both treatments, regardless of whether the fish underwent LOE.

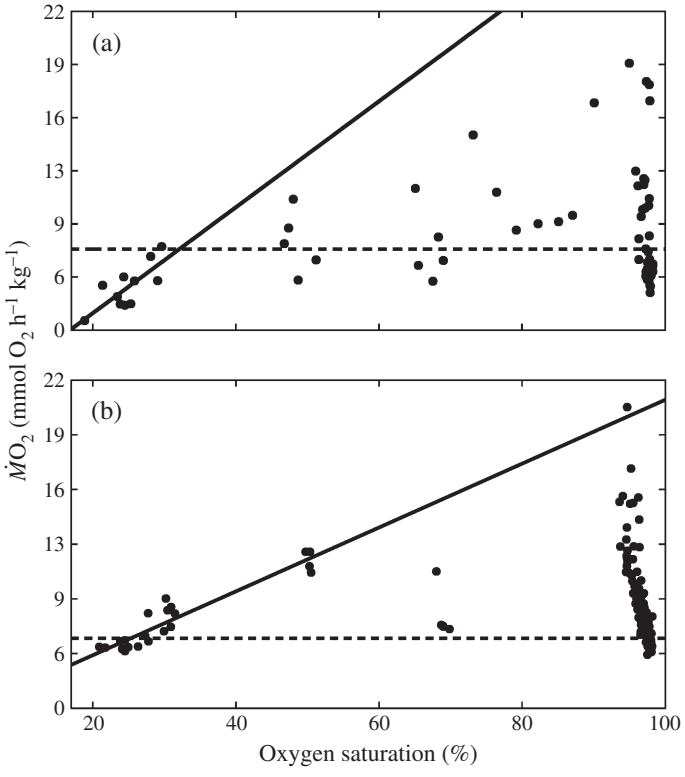

FIG. 2. Shown here are two shiner perch, *Cymatogaster aggregata*, from the intermittent treatment illustrating the calculation of critical oxygen saturation (O_{2crit}) in (a) a fish that did not exhibit an elevation in oxygen uptake rate ($\dot{M}O_2$) prior to the precipitous drop in $\dot{M}O_2$ below standard metabolic rate (SMR) and (b) a fish that did exhibit a peak in $\dot{M}O_2$. - - - -, SMR; ——— represents the line used to calculate O_{2crit}.

STATISTICAL ANALYSIS

Statistical analysis was conducted using the R statistical environment 3.1.1 (www.r-project.org). The effect of hypoxia administration method on O_{2crit}, the relationship between SMR and O_{2crit} and the difference in SMR between the methods were assessed using a mixed effects model (function lme in R package nlme; $P < 0{\cdot}05$; Lindstrom & Bates, 1988; Pinheiro *et al.*, 2015), with trial as a random effect.

All animal care and experimental protocols followed guidelines of Institutional Animal Care and Use Committee at the University of Washington (IACUC permit number 4208-3).

RESULTS

The two respirometry methods produced significantly different measures of O_{2crit} [Fig. 3; linear mixed effects (LME) model: $F_{1,6} = 25{\cdot}11$, $P < 0{\cdot}01$]. The intermittent-flow respirometry method produced values of O_{2crit} ($28{\cdot}1 \pm 1{\cdot}8$% oxygen saturation, mean ± S.E.) that were on average 1·7 fold lower than estimates made using the closed respirometry method ($46{\cdot}9 \pm 3{\cdot}4$% oxygen saturation, mean ± S.E.). Variability in SMR did not drive the results related to O_{2crit}, as no significant relationship was found between SMR and O_{2crit} (Fig. 4; LME: $F_{1,6} = 0{\cdot}002$, $P > 0{\cdot}05$) and SMR was not significantly different between methods (LME: $F_{1,6} = 0{\cdot}634$, $P > 0{\cdot}05$).

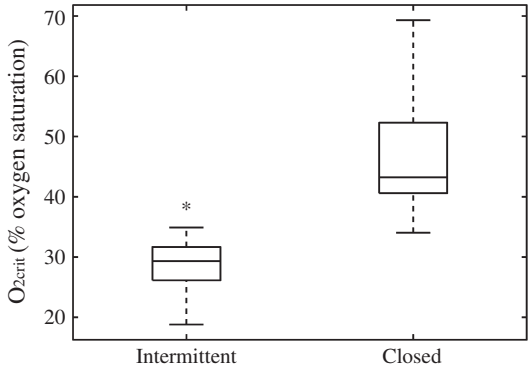

FIG. 3. Calculated per cent critical oxygen saturation (O_{2crit}) of *Cymatogaster aggregata* for intermittent-flow respirometry method and closed respirometry method. ∗, significant difference between the two methods ($P < 0{\cdot}05$). The whiskers on the box-plots represent the minimum and maximum values observed.

The methods also produced different percentages of fish exhibiting LOE. None of the individuals in the intermittent-flow respirometry method experienced LOE. This contrasted with the closed respirometry method, in which three of the eight fish experienced LOE prior to reaching the 17% oxygen saturation. For the three fish that exhibited LOE, the oxygen saturation at LOE (22, 30 and 55%) was directly related to the calculated O_{2crit} (55·7, 54·3 and 69·3%, respectively). The O_{2crit} values for the fish that exhibited LOE (56·1 ± 7·2% oxygen saturation, mean ± S.E.) were higher and more variable than the O_{2crit} values for fish in the closed respirometry method that did not exhibit LOE (42 ± 3·4% oxygen saturation, mean ± S.E.).

DISCUSSION

A variety of methods have been used in the literature to measure hypoxia tolerance in aquatic species (*e.g.* closed: Steffensen *et al.*, 1994; Nilsson & Ostlund-Nilsson, 2004,

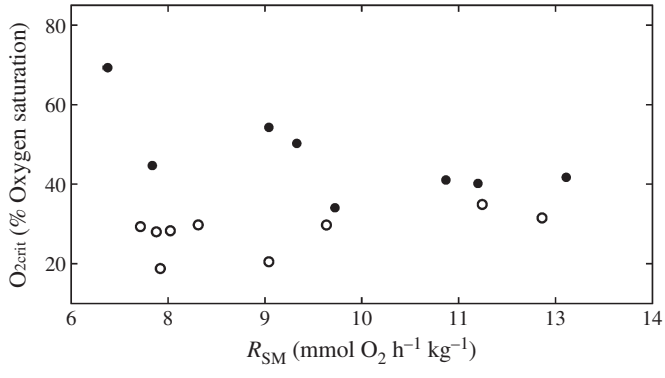

FIG. 4. Scatterplot of per cent critical oxygen saturation (O_{2crit}) as a function of standard metabolic rate (SMR; R_{SM}) for the intermittent-flow respirometry method (○) and the closed respirometry method (●) for *Cymatogaster aggregata.*

intermittent flow: Steffensen, 1989; Killen *et al.*, 2012; Eliason & Farrell, 2014). Differences in hypoxia administration technique have significant effects on O_{2crit}. This study suggests that caution is warranted when comparing results generated using closed *v*. intermittent-flow respirometry. Although issues of comparability between these techniques have been suggested in review articles (Keys, 1930; Kaufmann *et al.*, 1989; Steffensen, 1989; Cech, 1990; Clark *et al.*, 2013), this study is the first experimental comparison to confirm this issue.

There are a number of potential reasons that the closed respirometry method yielded higher measured O_{2crit} values than the intermittent-flow method. First, as there is no water exchange in the closed respirometry method, there may be uncontrolled build-up of carbon dioxide (CO_2) and toxic nitrogenous wastes in the chamber (Keys, 1930; Tang, 1933; Kaufmann *et al.*, 1989; Steffensen, 1989; Cech, 1990). Ammonia and CO_2 have been found to affect a variety of compensatory physiological mechanisms essential for maintaining $\dot{M}O_2$ under hypoxic conditions (Meade, 1985; Crocker & Cech, 2002). Elevated CO_2 (which leads to hypercapnia and reduced pH) can lead to reduced oxygen-binding capacity of haemoglobin (Riggs, 1970; Cech & Crocker, 2002), lowered arterial blood total oxygen content and arterial plasma pH (McKenzie *et al.*, 2002), increased levels of CO_2 in the blood (Cameron, 1976) and higher $\dot{M}O_2$ and ventilation frequency (Cech & Crocker, 2002; Crocker & Cech, 2002; McKenzie *et al.*, 2002), all of which can have profound effects on hypoxia tolerance in fishes (Mandic *et al.*, 2009; Perry *et al.*, 2009). Nitrogenous wastes, such as ammonia, can have detrimental effects on the fish's oxygen uptake mechanisms and physiology, including damage to the gill structure (Smart, 1976), disruptions to plasma ion concentrations (Remen *et al.*, 2008), reduced blood glucose (Remen *et al.*, 2008) and increased mortality (Ball, 1967; Magaud *et al.*, 1997; Vedel *et al.*, 1998). While the build-up of waste products was not quantified in this experiment, the concentration of CO_2 and ammonia in closed respirometry will inevitably be higher than those in intermittent flow due to the lack of water exchange. To fully understand the build-up of wastes in closed respirometry, a future study could examine ammonia and CO_2 concentration as a function of the ratio of fish to water volume, the fish's activity level and the residence time of the water within the respirometry chamber.

In addition, fish in the closed respirometry method exhibited increased incidence of LOE, which is known to be exacerbated by elevated levels of stress (Magaud *et al.*, 1997; Aboagye & Allen, 2014). Previous work suggests that there is a correlation between oxygen saturation at LOE and O_{2crit} (Mandic *et al.*, 2013), a finding supported by the closed respirometry results of this study. By comparison, no fish underwent LOE in the intermittent-flow respirometry method. The correlation between LOE and O_{2crit} indicates that the additional stressors linked to LOE will also result in overestimated O_{2crit}.

The negative effects of waste build-up on hypoxia tolerance could, however, be overestimated. For example, the literature contains conflicting reports on the effect of hypercapnia on hypoxia tolerance; while some fish species exhibit reduced hypoxia tolerance (Ultsch *et al.*, 1980; Cruz-Neto & Steffensen, 1997) under hypercapnic conditions, others are unaffected by this change in water quality (Cochran & Burnett, 1996; McKenzie *et al.*, 2003), indicating that sensitivity to hypercapnia under hypoxic conditions is probably species-specific. In addition, the excretion of nitrogenous wastes was probably reduced as these fish were starved prior to experimentation (Carter *et al.*, 2001) and

may have been less detrimental as ammonia toxicity is reduced in low pH conditions (Meade, 1985; Randall & Tsui, 2002).

Trials that employed closed respirometry were far shorter than those conducted using intermittent-flow respirometry (*c*. 1 *v*. 5 h). These shorter trials result in a more acute exposure to hypoxia, a greater rate of decrease in oxygen saturation and a lower temporal resolution of measurements (Kaufmann *et al.*, 1989; Steffensen, 1989; Cech, 1990; Clark *et al.*, 2013), which could affect the measurement and calculation of O_{2crit}. Uncontrolled decline rates in DO is a common problem in closed respirometry studies, as the rate of decrease will depend on an individual fish's activity, ventilation frequency and stress level as well as the fish's size relative to the respirometer. To examine the influence of the rate of oxygen decline on O_{2crit}, future experiments could compare the two methods while attempting to standardize the rates of oxygen depletion. This could be accomplished either by increasing the rate of oxygen depletion in the intermittent-flow respirometry method to match the rate of decline in the closed method or, by decreasing the rate of oxygen depletion in the closed method, by increasing the size of the respirometry chamber to match the rate of decline in the intermittent-flow method. While these studies would be useful, controlling the rate of oxygen depletion in the closed respirometry method is challenging because of variability in $\dot{M}O_2$ between individuals.

Careful measurements of SMR are also essential to attain accurate estimates of O_{2crit}. Researchers must account for both the stress of the transfer to the respirometer as well as any circadian rhythms of the species. The length of the resting period must be sufficient to repay any oxygen debt resulting from the stress of the transfer to the respirometer and to allow the individual to recover to SMR (Claireaux & Chabot, 2016). The resting period should also correspond to time periods of naturally low activity for the species (Brett & Zala, 1975; Thetmeyer, 1997; Claireaux & Chabot, 2016).

In conclusion, the current results suggest that different methods of hypoxia administration do not produce comparable measures of hypoxia tolerance in *C. aggregata*. While the closed method may be appropriate in understanding the hypoxic response under specific natural conditions (*e.g.* fish residing in low flow regimes or tide pools), the overall recommendation is to use intermittent-flow respirometry when measuring O_{2crit} if the aim is to strictly measure the hypoxia tolerance of a species. These data suggest that the intermittent-flow respirometry method provides a more repeatable and precise (less variable) measurement in comparison with the closed respirometry method. Unlike closed respirometry, intermittent-flow respirometry reduces the potential for confounding factors (*e.g.* hypercapnia and build-up of nitrogenous wastes), controls for the duration of hypoxia treatment (*e.g.* the duration of hypoxia exposure and the progression rate to hypoxic conditions) and allows for precise measures of O_{2crit}.

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