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A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (P_{crit})

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Hypoxia is a common occurrence in aquatic habitats, and it is becoming an increasingly frequent and widespread environmental perturbation, primarily as the result of anthropogenic nutrient enrichment and climate change. An in-depth understanding of the hypoxia tolerance of fishes, and how this varies among individuals and species, is required to make accurate predictions of future ecological impacts and to provide better information for conservation and fisheries management. The critical oxygen level (P_{crit}) has been widely used as a quantifiable trait of hypoxia tolerance. It is defined as the oxygen level below which the animal can no longer maintain a stable rate of oxygen uptake (oxyregulate) and uptake becomes dependent on ambient oxygen availability (the animal transitions to oxyconforming). A comprehensive database of P_{crit} values, comprising 331 measurements from 96 published studies, covering 151 fish species from 58 families, provides the most extensive and up-to-date analysis of hypoxia tolerance in teleosts. Methodologies for determining P_{crit} are critically examined to evaluate its usefulness as an indicator of hypoxia tolerance in fishes. Various abiotic and biotic factors that interact with hypoxia are analysed for their effect on P_{crit}, including temperature, CO_2, acidification, toxic metals and feeding. Salinity, temperature, body mass and routine metabolic rate were strongly correlated with P_{crit}; 20% of variation in the P_{crit} data set was explained by these four variables. An important methodological issue not previously considered is the inconsistent increase in partial pressure of CO_2 within a closed respirometer during the measurement of P_{crit}. Modelling suggests that the final partial pressure of CO_2 reached can vary from 650 to 3500 μatm depending on the ambient pH and salinity, with potentially major effects on blood acid–base balance and P_{crit} itself. This database will form part of a widely accessible repository of physiological trait data that will serve as a resource to facilitate future studies of fish ecology, conservation and management.

Key words: Carbon dioxide, critical oxygen tension, metabolic rate, oxygen and capacity limitation of thermal tolerance, physiological trait

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Introduction

In recent decades, there has been growing concern regarding the increasingly widespread and frequent occurrence of hypoxia in aquatic environments, associated with the increased discovery of hypoxic zones globally (Diaz, 2001; Diaz and Breitburg, 2009; Zhang *et al*., 2010). Although periods of hypoxia can develop naturally in many aquatic systems, anthropogenic influences have been shown to be a major driver of hypoxic events in both freshwater and marine habitats (Friedrich *et al*., 2014). In particular, eutrophication associated with increased anthropogenic nutrient loading of lakes, rivers and coastal waters leads to blooms of algae and phytoplankton, the death of which subsequently fuels microbial respiration and the depletion of dissolved oxygen (Smith, 2003). Hypoxia has been shown to result in losses of biodiversity and to trigger widespread mortality events (Vaquer-Sunyer and Duarte, 2008). In the marine environment, more than 400 coastal systems have been reported as eutrophication-associated 'dead zones' (Diaz and Rosenberg, 2008). Global warming is likely to exacerbate hypoxia in aquatic systems owing to increased microbial respiration rates and reduced oxygen solubility with increasing water temperatures (McBryan *et al*., 2013). In addition, potential modifications to oceanic circulation linked to future climate change are predicted to result in greater stratification and 'deoxygenation' of the oceans (Keeling and Garcia, 2002; Keeling *et al*., 2009). In summary, in the future, reduced oxygen concentrations are predicted to occur more extensively, more frequently and for longer periods of time (IPCC, 2014). Fish are among the more hypoxia sensitive of aquatic taxa and, as such, the sequential loss of fauna from aquatic ecosystems during hypoxic events is commonly initiated by the loss or relocation of fish populations (Vaquer-Sunyer and Duarte, 2008). Understanding the physiological responses of individual organisms to environmental stressors, such as hypoxia, provides a mechanistic link between environmental change and population-level effects, which may be key to predicting future ecological impacts (Chown, 2012; Seebacher and Franklin, 2012; Cooke *et al*., 2013).

Fishes can show various behavioural responses to hypoxia, such as rising to the surface to breathe the uppermost layer of water in contact with air, increasing activity to escape the hypoxic area or decreasing activity to reduce oxygen demand (Chapman and McKenzie, 2009; Urbina *et al*., 2011; Domenici *et al*., 2012). Beyond these behavioural responses, fishes can engage numerous profound physiological responses, such as changes in ventilation, cardiac activity and haemoglobin–O_2 binding (Richards, 2009). These physiological responses work primarily to sustain oxygen extraction from the environment in order to maintain aerobic ATP production. This allows the majority of fishes to maintain stable oxygen uptake rates across a wide range of ambient partial pressures of oxygen (P_{O_2}), a response known as 'oxyregulation' (reviewed by Perry *et al*., 2009). When, however, oxygen reduces to a threshold below which oxygen uptake rate cannot be maintained, oxygen uptake declines linearly with a decrease in ambient P_{O_2}, a response known as 'oxyconforming' (Pörtner and Grieshaber, 1993; Claireaux and Chabot, 2016). This threshold, when oxygen uptake transitions from regulation to conforming, is referred to as the critical P_{O_2} (P_{crit}; Beamish, 1964; Ultsch *et al*., 1978). As a measure of whole-animal oxygen extraction capacity, which varies extensively across species and among populations, P_{crit} is widely used to describe the degree of hypoxia tolerance in fishes (Ultsch *et al*., 1978; Chapman *et al*., 2002; Nilsson *et al*., 2007a,b; Mandic *et al*., 2009; reviewed by Chapman and McKenzie, 2009; Speers-Roesch *et al*., 2012).

Oxygen, the key variable in P_{crit} measurements, is used by aerobic organisms as an electron acceptor in order to drive the production of ATP. As such, the rate of oxygen uptake is widely considered as a proxy for the rate of aerobic metabolism, at least when in a steady state (Brown *et al*., 2004; Nelson, 2016). Standard metabolic rate (SMR) is the oxygen uptake rate of an entirely inactive, post-absorptive fish and reflects its minimal cost of living at a given temperature (Beamish and Mookherjii, 1964; Chabot *et al*., 2016). Routine metabolic rate (RMR) provides a similar estimate of the cost of living but takes into account energy expended on maintaining posture and making the small movements that are typical of most fishes even when in a quiescent state (McBryan *et al*., 2013). In contrast, maximal metabolic rate (MMR) is the highest rate of oxygen uptake that can be attained in defined environmental conditions (Clark *et al*., 2013; Norin and Clark, 2016). The difference between SMR and MMR is referred to as aerobic scope and provides for the oxygen demands of higher functions, such as locomotion, growth, behaviour and reproduction (Farrell and Richards, 2009; Claireaux and Chabot, 2016). In the context of this aerobic hierarchy, several levels of critical P_{O_2} are represented in Figure 1. As this conceptual diagram illustrates, MMR is the

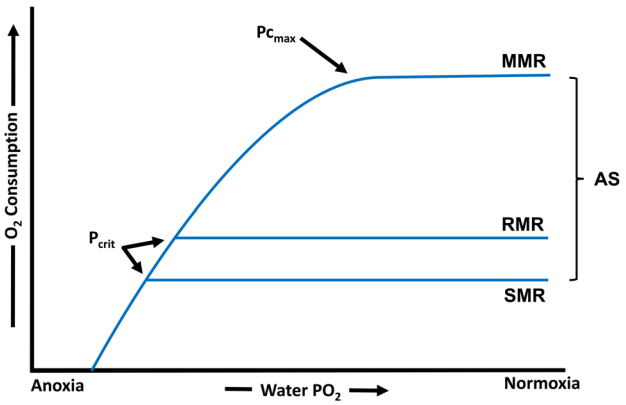

Figure 1: Diagram illustrating the conceptual idea of the effects of hypoxia on the standard metabolic rate (SMR), routine metabolic rate (RMR), maximal metabolic rate (MMR) and aerobic scope (AS) of an oxyregulator. This and may not apply to species with facultative metabolic depression below the critical oxygen level (P_{crit}). Pc_{max} is defined as the critical exeternal oxygen partial pressure at which oxygen supply no longer meets the maximum demand for oxygen (Portner, 2010).

first rate to become limited as ambient oxygen decreases (Pc_{max}), from which point a decline in MMR leads to a reduction in aerobic scope. Secondly, the P_{crit} for RMR is reached, whereby oxygen supply cannot sustain even minimal levels of aerobic activity. Finally, the P_{crit} for SMR indicates that oxygen supply cannot meet even basic oxygen demands (Pörtner and Lannig, 2009; Claireaux and Chabot, 2016). Below this threshold, anaerobiosis or suppression of metabolic rate are required to sustain life (Richards, 2009). Each of the three levels of P_{crit} may indicate the difference between mortality and survival. If so, P_{crit} may have major implications for the fitness of fishes living in environments prone to hypoxia and, as such, each of these levels can be considered as functional traits (McGill *et al*., 2006; Claireaux and Chabot, 2016).

The examination of trait variation across populations and communities, and its ecological implications, are increasingly becoming the basis for predicting and potentially mitigating the effects on biodiversity of environmental change (Chown, 2012). Such trait-based approaches are facilitated by the collection and dissemination of trait data. Large-scale multi-trait databases have been compiled for various taxa, including plants (Kattge *et al*., 2011), mammals (Jones *et al*., 2009), marine polychaetes (Faulwetter *et al*., 2014) and North American freshwater fishes (Frimpong and Angermeier, 2009). As a quantifiable measure of hypoxia tolerance that is measured on individuals and is applicable at population level, P_{crit} is useful for incorporation into trait-based approaches to the conservation physiology of fishes (Frimpong and Angermeier, 2009).

The field of fish physiology has generated a large body of literature on P_{crit}, across a wide range of species and in highly variable abiotic and biotic conditions (Perry *et al*., 2009). Owing to the discrete and nuanced nature of each study, it is challenging to make broad generalizations. The aims of the present work were as follows: (i) to assemble a database of the P_{crit} values reported for fishes, from published literature, in a format suitable for future incorporation into multi-trait-based analyses; (ii) to analyse the data to identify how biotic and abiotic factors (particularly temperature) interact with hypoxia and affect P_{crit}; and (iii) to appraise methodologies for measuring P_{crit} critically, and thereby evaluate its usefulness for quantifying hypoxia tolerance in fishes. This new analysis not only provides an opportunity for further quantitative considerations but also serves as a tangible link between the physiology and the conservation of fishes.

Methods

Literature search

The citation and abstract indexes, Scopus® and Web of Science®, were used to collect relevant peer-reviewed literature. The literature search was conducted in December 2014 using the following terms: 'critical oxygen', 'critical PO_2', 'oxygen threshold', 'P_{crit}', 'oxyregulate', 'oxyconform' or 'hypoxia tolerance'. Approximately 400 papers from relevant subject areas were identified. Each of these articles was individually assessed for relevance based on their title and abstract. Finally, 144 papers were downloaded for a full read of the manuscript. Of these, only 96 papers reported P_{crit} measurements in at least one fish species.

Database construction

In order to maximize the future usefulness of the database and to ensure that it fully reflects the variation in abiotic/biotic conditions in which P_{crit} has previously been measured in fishes, it was necessary to extract multiple parameters from each study. For each P_{crit} entry, 66 columns summarize information on the species and origin, acclimation parameters, animal characteristics, experimental method, results, statistical analyses, general comments and bibliographic information (Table 1). The database was constructed as a single Microsoft Excel file, with individual columns for each parameter and rows for each P_{crit} determination in a particular species or treatment group. As such, a single study may occupy several rows depending on the number of treatment groups and/or species for which P_{crit} is reported. Values for P_{crit} were reported in a variety of different oxygen units across the literature (millimetres of mercury, torr, percentage air saturation, milligrams of oxygen per litre and micromolar), but were converted here to a partial pressure of oxygen (in kilopascals) based on oxygen solubility values reported by Green and Carrit (1967) and assuming standard atmospheric pressure at sea level (760 mmHg), if not otherwise reported. Likewise, all values of oxygen uptake rate were converted to milligrams of oxygen per kilogram per hour. To enable unbiased inter-species comparison, a subset of the full database was produced, which included only those P_{crit} measurements made in fishes meeting the following conditions: (i) in an unfed or post-absorptive state; (ii) undergoing no additional (to hypoxia) abiotic stressor; and (iii) where temperature acclimation lasted for >2 days.

Database analysis

The frequency of P_{crit} measurements across families and climate zones was calculated based on the full database. However, comparisons of P_{crit} values were made using the subset 'control' database described above. Based on the latitude of where the studies were conducted, each entry was labelled as tropical, sub-tropical, temperature or polar. Analysis of variance was used to test for an effect of climate zone on P_{crit} using the Sidak *post hoc* test.

Potential influences of varying respirometry methodologies and hypoxia exposure methods on P_{crit} were explored using the subset 'control' database, in which there are 297 data points. Similar to the full database, the majority of studies measured P_{crit} using closed static respirometry on individual fish, where oxygen is reduced via the oxygen consumption of the fish ($n = 202$). Where there were sufficient data to compare methods between respirometry methods within a species, a Student's unpaired *t*-test was used to compare between groups. It was not possible to test for differences in hypoxia exposure methods within species because there were insufficient data from at least two methods.

Table 1: List of the parameters incorporated into the database alongside each reported critical oxygen level value

Species and origin	Stock acclimation	Sample characteristics	Experimental method	Results	Statistical analysis	Comments and reference
Family	Holding time	Sample size	Respirometry type	Oxy regulating or conforming	Statistical method	Comments
Genus	Acclimation temperature	Mean mass	BMR/RMR/SMR/MMR	M_{O_2}	P_{crit} calculation method	Reference
Species	Acclimation salinity	Mass SD	Determination method	Critical P_{O_2}	SMR determination	Year
Origin	P_{O_2} units	Mass SEM	Swimming speed	Critical P_{O_2} range		Corresponding Author
Latitude and longitude	Acclimation P_{O_2}	Mass range upper	Hypoxia method	Critical P_{O_2} SD		DOI
	Acclimation pH	Mass range lower	Rate of hypoxia onset	Critical P_{O_2} SEM		Full citation
	Acclimation time	Mean length	P_{O_2} set-point time	Critical P_{O_2} units		
	Diet	Length SD	Minimal P_{O_2}	Air breathing threshold		
	Energy content	Length SEM	P_{O_2} unit	Common P_{O_2} units		
	Ration unit	Length range upper	Salinity			
	Ration size	Length range lower	Temperature			
	Photoperiod (light:dark)	Life stage	pH			
	Feeding regimen	Sex	P_{CO_2}			
		Last feed	Photoperiod (light:dark)			
			Access to air			

Abbreviations: BMR, basal metabolic rate; DOI, digital object identifier; MMR, maximal metabolic rate; M_{O_2}, oxygen uptake rate; P_{CO_2}, partial pressure of carbon dioxide; P_{crit}, critical oxygen level; P_{O_2}, partial pressure of oxygen; RMR, routine metabolic rate; SMR, standard metabolic rate.

Stepwise multiple linear regression analysis was used to develop a model for predicting P_{crit} based on biotic (body mass, RMR) and abiotic (temperature, salinity) variables. Earlier analysis detected no significant within-species effect of respirometry method (closed or flow through) on P_{crit}, and it was therefore not included in the linear regression model. Acclimation variables such as temperature, P_{O_2} and salinity were not included in this analysis because they were very highly correlated with the equivalent variables reported during the trials. Minimal P_{O_2} was not included in the model because it is driven by P_{crit}.

As the multivariate model identified salinity as a relevant factor, the potential effect of salinity on P_{crit} was explored further by comparing P_{crit} values measured in seawater (150 entries from 82 species) with P_{crit} values measured in freshwater (116 entries from 50 species). This approach was taken because most of the studies were conducted either in freshwater [~0.1 practical salinity units (PSU)] or seawater (~30–38 PSU). Values of P_{crit} were calculated as the partial pressure of oxygen (in kilopascals) and as the concentration of oxygen (in milligrams per litre), using the solubility coefficient based on experimental temperature and salinity (Green and Carrit, 1967). Potential differences between groups were then tested by a Mann–Whitney *U*-test, because normality assumptions were violated.

Results and discussion

Database coverage

Of the 96 studies reviewed, 331 measurements of P_{crit} across 151 species were incorporated into the database. Across the global database, 58 families are represented, with Cyprinidae ($n = 44$), Pomacentridae ($n = 41$), Gobiidae ($n = 24$), Cichildae ($n = 23$), Salmonidae ($n = 19$), Cottidae ($n = 18$), Apogonidae ($n = 17$), Percidae ($n = 13$) and Sparidae ($n = 12$) the most frequently represented. Freshwater and marine (including euryhaline) species account for 40 and 60% of P_{crit} entries, respectively. Water temperatures at which P_{crit} values were determined ranged between −1.5 and 36°C, with a mean (±SD) of 21.7 ± 7.6°C. Values for P_{crit} over the entire data set ranged between 1.02 kPa (*Pseudocrenilabrus multicolor victoriae*; Reardon and Chapman, 2010) and 16.2 kPa (*Solea*

solea larvae; McKenzie *et al.*, 2008) with a mean (±SD) P_{crit} in the 'control' data set of 5.15 ± 2.21 kPa. Plots of species and their reported P_{crit} values from the subset data set are provided in the Supplementary Data (Supplementary Fig. 1).

The geographical coverage of the database includes at least one entry from every continent, although North America, Europe and Australasia are by far the most heavily represented and, when combined, account for 87% of P_{crit} entries. Perhaps unsurprisingly, most studies of P_{crit} in fishes have been concentrated around the major fish physiology research groups in Europe, North America and Australia. Arguably, this introduces an element of bias into the database, given the incomplete representation of all habitats and species at a global scale. Based on the full database, tropical studies are the most frequently represented ($n = 125$ P_{crit} measurements, dominated by Lizard Island Research Station, Australia, $n = 98$), followed by subtropical ($n = 104$) and temperate regions ($n = 100$), dominated by Canada and Europe. The polar regions are the most under-represented ($n = 2$). Within the subset 'control' database, there was a significant difference in mean P_{crit} across climatic regions (ANOVA, $F_{2,297} = 4.054$, $P = 0.018$), where tropical fishes had the lowest P_{crit} (mean ± SEM: 4.92 ± 0.190 kPa) < sub-tropical fishes (5.0 ± 0.24 kPa) < temperate fishes (5.74 ± 0.24 kPa). However, the Sidak *post hoc* test suggested that P_{crit} values for tropical fishes were significantly lower only than temperate fishes ($P = 0.021$). There was no difference in mean P_{crit} between subtropical and either tropical ($P = 0.991$) or temperate P_{crit} ($P = 0.085$). Owing to low sample size, the polar P_{crit} values were not included in the ANOVA across temperatures but, interestingly, had a higher mean P_{crit} than the other three climatic zones (7.9 ± 1.6 kPa).

Additionally, the species studied tend to be those conducive to respirometry trials. In particular, large, active or highly sensitive species, such as those of the Scombridae family (tuna, mackerels and bonitos) are generally under-represented in the literature (Blank *et al.*, 2007). For example, the majority of P_{crit} values reported in the database were measured on fish <1 kg body mass.

Methodology used to determine critical oxygen level

The relationship between ambient P_{O_2} and oxygen uptake in fishes has been investigated since the study of Keys (1930). Even at that early stage, there was considerable discussion among physiologists regarding the validity of different methodologies. Technological developments, particularly methods for measuring dissolved oxygen content such as galvanic oxygen electrodes and, more recently, fibre-optic sensors, have made the performance of high-resolution measurements of oxygen uptake in fishes increasingly common (Clark *et al.*, 2013; Nelson, 2016). Nevertheless, the literature examined for the purpose of building this database is characterized by considerable variation in terms of methods used to determine P_{crit}. For example, the majority of studies (56%) used closed respirometry for P_{crit} estimates, 21% used flow-through respirometry, 20% used intermittent respirometry, and 3% used other approaches, such as indirect estimation of gill oxygen uptake (Table 2). Most studies (70%) depleted ambient oxygen through the fish's own respiration, whereas 30% of studies bubbled nitrogen gas into the water to reduce ambient oxygen levels. The majority of studies (80%) measured RMR for P_{crit} estimates; the remaining 20% measured SMR. These methodological differences and their implications are important to consider when interpreting collated P_{crit} data.

Closed respirometry, whereby the fish is placed within a sealed chamber from which water is intermittently sampled for measurement of dissolved oxygen content, provides the simplest method of measuring oxygen uptake rate (Steffensen, 1989), as follows:

$$M_{O_2} = [(V_r - V_f) \times \Delta O_2] \div (\Delta t \times bw),$$

where M_{O_2} represents oxygen uptake rate, V_r is respirometer volume, V_f is fish volume, ΔO_2 is change in ambient oxygen content, t is time, and bw is fish mass ('body weight'). Importantly, water needs to be recirculated within the chamber to ensure adequate mixing, thus preventing the stratification of dissolved

Table 2: The breakdown of the number of data points representing each respirometry type and oxygen removal method in the subset database

	Oxygen depletion method					
Respirometry type	Fish respiration	N_2 equilibration	N_2 and O_2 equilibration	N_2 and CO_2 equilibration	N_2, O_2 and air equilibration	Total
Closed static (individual)	202	1	0	0	0	203
Closed static (grouped)	13	0	0	0	0	13
Closed flow-through (individual)	13	14	0	0	3	30
Intermittent flow (individual)	13	26	2	1	0	42
Mesocosm (grouped, large tuna)	0	1	0	0	0	1
Open flow-through (grouped)	7	0	0	0	0	7
Opercular mask	1	0	0	0	0	1

oxygen within the chamber (Keys, 1930). Whether spontaneous movements and ventilation are sufficient to provide mixing depends on the species and achieving the correct fish-to-respirometer volume ratio. For closed determinations of P_{crit}, hypoxia is generated by allowing the fish to deplete available oxygen through its own respiration, therefore negating the need to strip dissolved oxygen from the water artificially through equilibration with nitrogen. For this reason, closed respirometry is particularly useful for conducting measurements of P_{crit} in the field or at remote locations where facilities such as a supply of N_2 may not be readily available (Rosenberger and Chapman, 2000; Nilsson *et al*., 2007b).

However, there are several important considerations regarding the use of closed respirometry for determination of P_{crit}. For instance, the rate of oxygen depletion during closed respirometry is determined by the ratio of fish size (or oxygen uptake rate) to respirometer volume. A lack of control over the development of hypoxia can be problematic in comparative studies that use the same respirometer to measure P_{crit} in fish of different size and/or metabolic rate. As an illustrative example, the depletion of oxygen levels from 20 to 1 kPa by Australian barramundi (*Lates calcarifer*) took between 1.5 and 4 h depending on the temperature (26 or 36°C; Collins *et al*., 2013). From our database, it is evident that there is very little, if any, standardization in terms of the rate of oxygen depletion between P_{crit} studies, irrespective of which respirometry method is employed. This is in contrast to measurements of other physiological threshold traits, such as the determination of critical temperature, which tends to be made at consistent warming or cooling rates among studies (0.2–0.3°C min^{-1}; Beitinger *et al*., 2000; Mora and Maya, 2006; Murchie *et al*., 2011). It is unclear whether the rate of decline in ambient oxygen will significantly affect P_{crit}, but it is likely that a longer time scale would allow for greater respiratory adjustments, and hence, reveal lower P_{crit} values than more acute hypoxic exposures. Indeed, our own anecdotal observations in European flounder (*Platichthys flesus*) suggest that these fish tend to oxyconform across the entire range of ambient P_{O_2} when exposed to a very rapid reduction of oxygen (from 21 to 2 kPa in <2 h).

A further issue associated with closed respirometry is the build-up of the waste products of metabolism, in particular CO_2 (Keys, 1930; Steffensen 1989; Urbina *et al*., 2012). It has been argued that the level of CO_2 accumulation within a closed respirometer is unlikely to impact on CO_2 excretion by fishes significantly, given that they normally exhibit a blood partial pressure of CO_2 (P_{CO_2}) of around 2–4 mmHg, much higher than normal ambient levels (Ishimatsu *et al*., 2005; Nilsson *et al*., 2007a). However, a precedent has been set, albeit at more severe levels of hypercarbia (2.25–20 mmHg), to show that elevated P_{CO_2} can increase P_{crit} in European eels (*Anguilla anguilla*; Cruz-Neto and Steffensen, 1997), although no effect on P_{crit} was observed when eels were given enough time to acclimate fully in terms of acid–base regulation (McKenzie *et al*., 2003), or in spot fish (*Leiostomus xanthurus*) and mummichog (*Fundulus heteroclitus*; Cochran and Burnett, 1996). Given the potential influence of hypercarbia, it would be prudent to report any change in water P_{CO_2} alongside values for P_{crit} that have been determined through closed respirometry, but this has rarely been the case throughout the existing literature. A single study so far has evaluated this potential confounding factor in determining P_{crit}, but in this unusual oxyconforming species (inanga, *Galaxias maculatus*) elevated P_{CO_2} had no effect on oxygen uptake rate at any level of ambient oxygen (Urbina *et al*., 2012). Furthermore, the authors pointed out that the effect of CO_2 on M_{O_2} in fishes appears to be species specific (Gilmour, 2001; Ishimatsu *et al*., 2008).

An important issue that does not appear to have been considered previously is that the extent to which P_{CO_2} increases within a closed respirometer will be highly dependent on the starting water chemistry, in particular pH and salinity (Fig. 2). A higher seawater pH indicates a greater total alkalinity (TA). In turn, this gives increased capacity for buffering added CO_2 and limiting the increase in P_{CO_2} for a given increase in total CO_2 attributable to net excretion by the fish in a respirometer. Therefore, the lower the starting water pH, the larger the

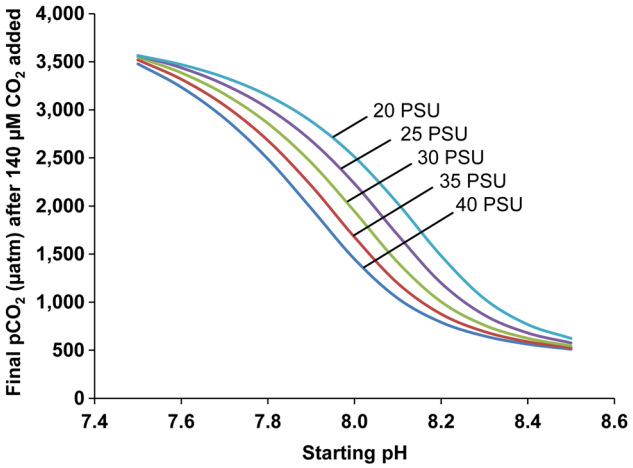

Figure 2: Model of the estimated partial pressure of carbon dioxide (P_{CO_2}) reached, in water of different salinities and starting pH values, after the addition of 140 µM CO_2. The value of 140 µM approximates the increase in total CO_2 attributable to excretion by a fish at 15°C during a closed respirometry experiment. In this theoretical example, the oxygen level is allowed to decline as a result of respiration from a normoxic partial pressure of >20 kPa (~245 µM) to a common P_{crit} value of ~6 kPa (~74 µM), and we have assumed a respiratory quotient (CO_2 excreted ÷ O_2 consumed) of 0.85 for fish (Kieffer *et al*., 1998). At each starting pH, the total alkalinity (TA) and total CO_2 were calculated from the pH and assuming equilibration with atmospheric P_{CO_2} (395 µatm). When excreted CO_2 is dissolved in water, the total CO_2 increases accordingly (in this case, by 140 µM) but TA remains unchanged (Riebesell *et al*., 2010). For each starting pH, we therefore used the CO2sys program (for the national bureau of standards pH scale) to calculate the final P_{CO_2} that would result from increasing total CO_2 by 140 µM while TA remained constant. This was repeated for salinities of 20, 25, 30, 35 and 40 practical salinity units (PSU) and starting pH values of 7.5–8.5 to cover ranges experienced in many marine laboratories.

overall change in P_{CO_2} over the course of the P_{crit} measurement. From the models shown in Figure 2, it is clear that pH has a massive influence on the ambient P_{CO_2} reached within such a closed respirometry scenario, with final P_{CO_2} values ranging by 5-fold, from ~650 µatm (0.49 mmHg) to ~3500 µatm (2.66 mmHg) at the highest (8.5) and lowest (7.5) starting pH values shown, respectively. Note that even the lowest of these final P_{CO_2} values has been shown (in experiments designed to mimic future 'ocean acidification' scenarios) to have significant detrimental effects in fishes (Munday *et al.*, 2009). When the starting pH is low, the highest P_{CO_2} values of ~3500 µatm occur, which are more than 3.5 times higher than the 'business as usual' for end-of-century global CO_2 projections (representative concentration pathway scenario 8.5; Meinshausen *et al.*, 2011). It is also relevant to note that salinity has a major modulating effect, in particular within the middle of the range of starting pH values. For example, at a starting pH of 8.0, the final P_{CO_2} will vary from slightly <1500 µatm (1.14 mmHg) at the highest salinity (40 PSU) to >2500 µatm (1.90 mmHg) at the lowest salinity (20 PSU).

The larger ambient P_{CO_2} values indicated above would certainly be expected to cause significant blood acid–base disturbance during the time scale of a typical closed respirometry experiment (minutes to hours) and thus have the potential to influence P_{crit} via alterations in the oxygen binding affinity of haemoglobin. It is therefore important to recognize this variability in P_{CO_2} when conducting closed respirometry experiments to determine hypoxia tolerance, and particularly, when interpreting P_{crit} measurements.

Flow-through respirometry is a technique whereby oxygen content of the inflowing (O_2,in) and outflowing (O_2,out) water is continuously measured at a fixed water flow rate through the respirometer (F_w). By application of the Fick principle, oxygen uptake (M_{O_2}) is determined by:

$$M_{O_2} = F_w(O_2,\text{in} - O_2,\text{out}) \div \text{bw}.$$

Although flow-through respirometry avoids the accumulation of metabolites in the chamber, it suffers from problems primarily related to the 'wash-out' effect, whereby a significant lag can develop between changes in the fish's real M_{O_2} and changes in observed O_2,out. The degree of wash-out depends on the dilution factor, which is a function of water mixing, volume and flow rate (Steffensen, 1989).

Intermittent flow-through respirometry is generally considered the ideal method of M_{O_2} determination in fishes because it involves none of the problems associated with closed or flow-through techniques (Steffensen, 1989; Clark *et al.*, 2013). The term 'intermittent' or 'semi-closed' in this context refers to the transitioning between a closed phase for determination of M_{O_2} and a flush phase for restoring O_2 to a set level and removing metabolites from the respirometer. As the equipment and software for automating flush–recirculation cycles and simultaneous data acquisition from multiple chambers have become more sophisticated and widely available, intermittent flow-through respirometry has been increasingly used (Svendsen *et al.*, 2016). However, P_{crit} measurements via this preferred technique account for only 20% of values incorporated into the present database.

Flow-through techniques allow for the supply of hypoxic water to the respirometry chamber. This hypoxic water can be produced by bubbling with N_2 via a solenoid valve linked to an O_2 probe (Schurmann and Steffensen, 1997) or by bubbling with set gas mixtures of variable O_2 and N_2 content. Both methods allow for finer control of the hypoxic exposure compared with allowing the fish to deplete ambient oxygen levels dependent on its own M_{O_2}. Progressive hypoxia can be generated in a stepwise fashion such that multiple M_{O_2} measurements can be made at a specific P_{O_2}, thereby increasing the likelihood of determining an M_{O_2} that is representative of true SMR or RMR (Rantin *et al.*, 1993).

Using the present database, we were able to explore differences in respirometry methods within three species, Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*), for which the sample size for at least two methods was greater than $n > 2$. Between closed static or closed flow-through respirometers, there was no difference in P_{crit} of common carp (Student's unpaired *t*-test, $t = 1.429$, d.f. = 6, $P = 0.203$). Likewise, between closed, static respirometers (individual fish) and open flow respirometry (with grouped fish), there was no difference in P_{crit} in Atlantic salmon (Student's unpaired *t*-test, $t = -0.678$, d.f. = 8, $P = 0.517$). There was no difference in P_{crit} between closed, flow-through or intermittent flow-through respirometry within Nile tilapia (Student's unpaired *t*-test, $t = -0.644$, d.f. = 6, $P = 0.543$). In both Atlantic salmon and common carp, oxygen levels were reduced by the respiration of the fish, whereas in Nile tilapia the oxygen was reduced by nitrogen equilibration. A direct comparison in the shiner perch (*Cymatogaster aggregata*) found, however, that P_{crit} measured by intermittent flow-through respirometry was significantly lower than that measured by closed respirometry (Snyder *et al.*, 2016). Thus, more direct comparisons are needed to investigate whether the two most common methodologies might provide different estimates of P_{crit}.

To determine P_{crit}, M_{O_2} is plotted against ambient P_{O_2} in order to identify the inflection point at which M_{O_2} transitions from being independent of ambient oxygen to dependent on ambient oxygen. Within this procedure, a great deal of subtle variation exists among studies. Most obvious is the differential use of SMR or RMR, with the majority (84%) of studies reporting a P_{crit} for RMR. Arguably, the P_{crit} exhibited for RMR is more ecologically relevant, given that this level of M_{O_2} is likely to be exhibited most of the time in the field (Ultsch *et al.*, 1978; Pörtner, 2010). Indeed, for some highly active species, such as salmonids, P_{crit} determined during active swimming may be most useful in considering the ecological implications of hypoxia (Fry, 1957). Activity level may affect P_{crit} in unexpected ways, such as in the Adriatic sturgeon (*Acipenser naccarii*), which exhibits a well-developed ability to

oxyregulate (P_{crit} = 4.9 ± 0.5 kPa) when permitted to swim at a low sustained speed but oxyconforms across the entire range of declining ambient oxygen when its activity is restricted in a static respirometer (McKenzie *et al.*, 2007). Some species exhibit a relatively high P_{crit} for RMR at a P_{O_2} that is well above the P_{50} (half of the hemoglobin oxygen binding sites are saturated with oxygen) of their haemoglobin. In these instances, P_{crit} may indicate a behavioural change and not simply a physical limitation of oxygen supply (McBryan *et al.*, 2013).

Of the studies that determine the P_{crit} for SMR, the methods used for quantifying SMR vary considerably. Some studies use the single lowest M_{O_2} value recorded at normoxia, whereas others take the average of a set number of the lowest M_{O_2} values (Iversen et al., 2010). More sophisticated and robust methods involve extrapolating the average M_{O_2} measured at specified swimming speeds back to zero activity (Wilson *et al.*, 1994; Cook *et al.*, 2014) or the use of percentiles and frequency distributions to assess all normoxic M_{O_2} data (Dupont-Prinet *et al.*, 2013). As the critical level for basal metabolism, P_{crit} determinations based on SMR should theoretically reflect a true physiological limitation of oxygen extraction capacity (McBryan *et al.*, 2013), although this may not be true in species for which metabolic depression below P_{crit} has a facultative component. Given that the P_{crit} for RMR is likely to be encountered at higher P_{O_2} than that for SMR (Fig. 1), intra- or inter-species comparisons among studies reporting different levels of P_{crit} may not be entirely valid. Whether SMR or RMR measurements are used to reflect normoxic M_{O_2}, it is essential that sufficient time is allowed for the fish to acclimate to the respirometry chamber; otherwise, apparent reductions in M_{O_2} as hypoxia develops may be an artefact of increasing habituation rather than true oxyconforming (Nilsson *et al.*, 2004).

The method used to establish the point of intersection between continuous oxyregulation and oxyconforming M_{O_2} data is also inconsistent among studies. The slope of these lines will determine the P_{crit} and vice versa; therefore, determining which data points should be included within each line is critical to establishing an accurate estimate of P_{crit} (Yeager and Ultsch, 1989). This can be achieved graphically by fitting a least-squares linear regression through data points that show a progressive decline in M_{O_2} such that it intersects with a regression line fitted through normoxic M_{O_2} data (Monteiro *et al.*, 2013). A number of mathematical methods for performing so-called piece-wise or segmented linear regression analyses are available, which provide greater robustness to estimates of P_{crit} and are used in the majority of studies incorporated into the present database (Nickerson *et al.*, 1989; Yeager and Ultsch, 1989; Leiva *et al.*, 2015). These approaches assume that the response of M_{O_2} to declining P_{O_2} is biphasic and consists of two entirely linear elements, with an abrupt transition between the two. Such assumptions are not necessarily met by real-world data, and indeed, concentration-dependent reaction kinetics make truly linear relationships between M_{O_2} and P_{O_2} unlikely (Marshall *et al.*, 2013). Recent developments in non-linear regression techniques are now being promoted as a more accurate approach to determining biological thresholds such as P_{crit} (Stinchcombe and Kirkpatrick, 2012; Marshall *et al.*, 2013).

Critical oxygen level as a hypoxia tolerance trait

A low P_{crit} is generally associated with greater hypoxia tolerance because it indicates a higher capacity for oxygen extraction and tissue delivery at low P_{O_2} (Mandic *et al.*, 2009). Maintaining aerobic metabolism during hypoxia is advantageous because it is up to 30-fold more efficient than anaerobic ATP production (per unit substrate consumed) and avoids accumulation of the deleterious by-products (e.g. H^+) of anaerobic metabolism (Richards, 2009). Hypoxia-induced physiological modifications that increase oxygen extraction capacity, such as increased gill surface area (Nilsson, 2007) and haemoglobin–O_2 binding (Brix *et al.*, 1999), are observed in fishes that frequently encounter hypoxia, suggesting that maintaining aerobic metabolism is a primary hypoxia survival strategy (Mandic *et al.*, 2009). However, when ambient P_{O_2} declines below P_{crit}, survival depends on the availability of substrate for O_2-independent ATP production (primarily glycolysis) and the ability to reduce metabolic demand (Richards, 2009).

How long a fish can maintain a balance between ATP demand and supply below its P_{crit}, and thus delay the onset of cellular dysfunction, necrosis and subsequent death, is a key component of hypoxia tolerance (Nilsson and Östlund-Nilsson, 2008; Urbina and Glover, 2012; Speers-Roesch *et al.*, 2013). Speers-Roesch *et al.* (2013) showed that P_{crit} does not entirely predict hypoxia tolerance at lower oxygen levels. The authors used three species of sculpin (*Blepsias cirrhosis*, *Leptocottus armatushave* and *Oligocottus maculosus*), which exhibit different P_{crit} values (1.76, 1.48 and 1.03 kPa, respectively), and exposed them to hypoxia levels that were 30% below each of their respective P_{crit} values while recording the time to loss of equilibrium. The loss of equilibrium was consistent between only two of the three species (*L. armatushave* and *O. maculosus*). Similar relative hypoxia exposures in the epaulette shark (*Hemiscyllium ocellatum*) and shovelnose ray (*Aptychotrema rostrata*) revealed lower lactate accumulation in epaulette sharks, indicating enhanced metabolic depression in this species (Speers-Roesch *et al.*, 2012). Furthermore, Nilsson and Östlund-Nilsson (2008) showed that P_{crit} did not correlate with body mass in juvenile and adult damselfish (Pomacentridae) ranging between 10 mg and 40 g but that smaller fish were much less tolerant to hypoxia below P_{crit}, owing to their limited capacity for meeting ATP demand through anaerobic metabolism. These findings were further supported in *G. maculatus* (Urbina and Glover, 2013). These results illustrate the benefit of considering P_{crit} alongside other methods of determining hypoxia tolerance, such as measurements of tissue-specific lactate accumulation and determinations of the loss of equilibrium of 50% of the fish, in order to assess overall hypoxia tolerance (Urbina and Glover, 2013; Speers-Roesch *et al.*, 2013; Claireaux and Chabot, 2016).

A recent review by Salin *et al.* (2015) argues that whole-animal oxygen consumption measurements may provide only a partial proxy for energy metabolism because of variation, within and between individuals, in the amount of ATP produced per molecule of oxygen consumed by mitochondria (P/O ratio). Environmental factors such as ambient temperature, food intake and diet composition have been shown both to increase and to decrease P/O ratios in the mitochondria of a variety of organisms (Salin *et al.*, 2015). Hence, conclusions based on oxygen consumption rate alone could lead to misleading conclusions regarding respiratory performance during environmental changes. To our knowledge, the effect of hypoxia on P/O ratios in fish has yet to be investigated, and as such, provides an interesting avenue for further research.

As a hypoxia-tolerance trait, low P_{crit} can often, but not always, indicate an ability to survive in hypoxic water. It does not consider the use of hypoxia-avoidance strategies, such as adaptations for emersion, aquatic surface respiration and air breathing (Chapman and McKenzie, 2009). The inanga (*G. maculatus*), which inhabits lowland streams prone to severe hypoxia, is a rare example of a fish species that appears to be an entirely obligate oxyconformer and thus demonstrates no discernible P_{crit} (Urbina *et al.*, 2012). Likewise, several species of Gymnotiform electric fishes from South America, which inhabit naturally hypoxic floodplain pools, also appear to be obligate oxyconformers with no P_{crit} (Reardon E. E., personal communication), an observation that is also anecdotally supported in *Brachyhypopmus brevirostris* (Crampton, 1998). In some of these species, such as the inanga, a lack of scales and a large surface area-to-volume ratio indicate a high capacity for cutaneous O_2 uptake whilst emersed, and hence, provide a short-term means to escape aquatic hypoxia (Urbina *et al.*, 2011). The oxygen thresholds for aquatic surface respiration, air breathing and emergence were incorporated into the database, but only where they have been reported alongside P_{crit} measurements. Such examples demonstrate the limitation of P_{crit} as a universal and comparative measure of hypoxia tolerance between species and emphasize the benefit of multi-trait-based approaches.

Biotic and abiotic interactions

Environmental stressors, such as hypoxia, rarely occur in isolation, and the interaction between stressors is of key concern in the context of predicting the ecological impacts of future environmental change (Crain *et al.*, 2008). As a typical threshold effect, the response of fish to hypoxia is likely to result in 'ecological surprises', whereby seemingly resilient populations suddenly collapse once a critical threshold is crossed (McBryan *et al.*, 2013). Additive or synergistic interactions with hypoxia could hasten the arrival of such thresholds, meaning that small environmental shifts could result in large effects on the performance of a population. Theoretically, any abiotic or biotic factor that affects either oxygen supply (cardiorespiratory capacity) or oxygen demand (metabolic rate) of an individual, and the balance therein, will have implications for its hypoxia tolerance. As an indicator of hypoxia tolerance, the effects of a wide range of abiotic and biotic interactions on P_{crit} in fish have been published (Table 3).

The stepwise multiple linear regression found that biotic (body mass, RMR) and abiotic (temperature, salinity) variables were highly correlated with P_{crit} (see Table 4). A significant regression ($F_{4,1154} = 10.565$, $P < 0.001$) predicted 19.5% of the variation in the data, based on an adjusted r^2 (multiple linear regression). Predicted P_{crit} is equal to $5.689 + 0.047$ (salinity) $- 0.083$(temperature) $+ 1.931$(body mass) $+ 0.001$ (RMR), where salinity is measured in practical salinity units, temperature in degrees Celsius, body mass in kilograms, and RMR in milligrams of oxygen per litre. All four variables were significant predictors of P_{crit} in the full model (Table 4).

Temperature is by far the most widely studied abiotic factor potentially interacting with hypoxia (reported in 30 species) and is particularly relevant, given ongoing global climate change (Ficke *et al.*, 2007; Pörtner, 2010). As ectotherms, oxygen demand in fishes increases in a roughly exponential manner with temperature (inter-species mean Q_{10} of 1.83; Clarke and Johnston, 1999), and the intrinsic link between temperature and environmental hypoxia has become the basis of an overarching concept termed 'oxygen and capacity limitation of thermal tolerance' (Pörtner, 2001, 2010). Essentially, this concept suggests that the thermal tolerance of ectotherms is dictated by their capacity to meet the oxygen demands of aerobic metabolism. Increased temperature both elevates basal oxygen demand (SMR) and reduces oxygen supply (via its effect on oxygen solubility), whereas hypoxia reduces the oxygen supply. Hence, temperature and hypoxia are likely to act synergistically in fishes. Within species, increasing temperature generally results in a higher P_{crit}, but among species, the slope of the relationship between temperature and P_{crit} is highly variable (Fig. 3). For example, the Atlantic salmon (*S. salar*) exhibits a steep linear increase of P_{crit} in comparison to the shallower slope seen in the common carp (*C. carpio*) across a similar temperature range (Ott *et al.*, 1980; Remen *et al.*, 2013). A surprising exception to the generally positive intra-species correlation between temperature and P_{crit} was observed in four out of six species of darter (*Etheostoma*), for which P_{crit} was lower at 20 than 10°C (Ultsch *et al.*, 1978). Variation in the sensitivity of species to temperature in terms of hypoxia tolerance may arise because of differences in their potential for thermal acclimation. Explanations for this variation may include reducing the metabolic impact of increased temperature or enhancing oxygen extraction capacity (Ott *et al.*, 1980; Pörtner, 2010). Species exhibit highly contrasting capacities for plastic acclimation responses. At opposite ends of this spectrum, crucian carp (*Carassius carassius*) can dramatically increase respiratory surface area through gill remodelling in response to temperature and hypoxia (Sollid *et al.*, 2005), whereas certain tropical reef fish species (*Ostorhinchus doederleini* and *Pomacentrus moluccensis*) demonstrate no thermal acclimation ability even over a relatively modest temperature range (29–32°C; Nilsson *et al.*, 2010).

Table 3: Summary of biotic and abiotic factors and their interactions with the intra-species critical oxygen level as reported by studies included in the database

Variable	Species	Effect on P_{crit}	Reference
Increasing temperature			
	Gadus morhua	Increase	Schurmann and Steffensen (1997)
	Lates calcarifer	Increase	Collins *et al.* (2013)
	Scyliorhinus canicula	Increase	Butler and Taylor (1975)
	Salmo salar	Increase	Barnes *et al.* (2011)
	S. salar	Increase	Remen *et al.* (2013)
	Dentex dentex	Increase	Cerezo Valverde et al. (2006)
	Tautogolabrus adspersus	Increase	Corkum and Gamperl (2009)
	Gadus ogac	Increase	Corkum and Gamperl (2009)
	Bellapiscis medius	Increase	Hilton *et al.* (2008)
	Bellapiscis lesleyae	Increase	Hilton *et al.* (2008)
	Morone saxatilis	Increase	Lapointe *et al.* (2014)
	Carassius carassius	Increase	Sollid *et al.* (2005)
	Gobiodon histrio	Increase	Sørensen et al. (2014)
	Gobiodon erythrospilus	Increase	Sørensen et al. (2014)
	Oreochromis niloticus	Increase	Fernandes and Rantin (1989)
	Cyprinus carpio	Increase	Ott *et al.* (1980)
	Oncorhynchus mykiss	Increase	Ott *et al.* (1980)
	Pomacentrus moluccensis	Increase	Nilsson *et al.* (2010)
	Ostorhinchus doederleini	Increase	Nilsson *et al.* (2010)
	Carassius auratus grandoculis	No effect	Yamanaka *et al.* (2013)
	Etheostoma boschungi	Decrease	Ultsch *et al.* (1978)
	Etheostoma fusiforme	Decrease	Ultsch *et al.* (1978)
	Etheostoma flabellare	Decrease	Ultsch *et al.* (1978)
	Etheostoma rufilineatum	Decrease	Ultsch *et al.* (1978)
Increasing salinity			
	Cottus asper	Decrease	Henriksson *et al.* (2008)
	Leptocottus armatus	No effect	Henriksson *et al.* (2008)
	Cyprinus carpio	Increase	De Boeck et al. (2000)
	Cyprinodon ariegatus	Increase	Haney and Nordlie (1997)
Increased P_{CO_2}			
	Fundulus heteroclitus	No effect	Cochran and Burnett (1996)
	Leiostomus xanthurus	No effect	Cochran and Burnett (1996)
	Anguilla anguilla	Increase	Cruz-Neto and Steffensen (1997)
	Platichthys flesus	Increase	Rogers (2015)
Hypoxic acclimation			
	Pagrus auratus	No effect	Cook *et al.* (2013)
	S. salar	No effect	Remen *et al.* (2013)
	Hemiscyllium ocellatum	Decrease	Routley *et al.* (2002)
	Spinibarbus sinensis	Decrease	Dan *et al.* (2014)
	C. auratus	Decrease	Fu *et al.* (2011)
	Poecilia latipinna	Decrease	Timmerman and Chapman (2004 a,b)

(*Continued*)

Table 3: continued

Variable	Species	Effect on P_{crit}	Reference
Reared in hypoxic environment			
	Pseudocrenilabrus multicolor	Decrease	Reardon and Chapman (2010)
Exercise pre-conditioning			
	C. auratus	Decrease	Fu *et al.* (2011)
Fed			
	Astronotus ocellatus	Increase	De Boeck et al. (2013)
	Oreochromis niloticus	Increase	Mamun *et al.* (2013)
	Perca fluviatilis	Increase	Thuy *et al.* (2010)
Fatty acid-enriched diet			
	Solea solea (larvae)	Decrease	McKenzie *et al.* (2008)
	S. solea (juveniles)	Decrease	McKenzie *et al.* (2008)
Increasing body mass			
	Hypostomus plecostomus	Decrease	Perna and Fernandes (1996)
	Astronotus ocellatus	Decrease	Sloman *et al.* (2006)
	Pomacentridae	No effect	Nilsson and Östlund-Nilsson (2008)
Pre- to post-settlement (larvae)			
	Chromis atripectoralis	Decrease	Nilsson *et al.* (2007a,b)
	Pomacentrus amboinensis	Decrease	Nilsson *et al.* (2007a,b)
Larvae to juveniles			
	C. auratus grandoculis	Decrease	Yamanaka *et al.* (2013)
Juveniles to adults			
	Reinhardtius hippoglossoides	Decrease	Dupont-Prinet *et al.* (2013)
Increasing brood size (mouthbrooders)			
	Zoramia fragilis	Increase	Östlun-Nilsson and Nilsson (2004)
	Zoramia leptacantha	Increase	Östlun-Nilsson and Nilsson (2004)
Mycobacteriosis infection			
	Morone saxatilis	Increase	Lapointe *et al.* (2014)
Acidified water			
	Salmo gairdneri	Increase	Ultsch *et al.* (1980)
	Cyprinus carpio	Increase	Ultsch *et al.* (1980)
Metal exposure			
	Brycon amazonicus	Increase	Monteiro *et al.* (2013) (Hg^{2+})
	C. carassius	Increase	Schjolden *et al.* (2007) (Cu^{2+})
	Perca fluviatilis	Increase	Bilberg *et al.* (2010) ($AgNO_3$)
	P. fluviatilis	Increase	Bilberg *et al.* (2010) (nano-Ag)
Organophosphate exposure			
	Oreochromis niloticus	Increase	Thomaz *et al.* (2009)
Anaemia			
	Pagrus auratus	Increase	Cook *et al.* (2011)

Abbreviations: P_{CO_2}, partial pressure of carbon dioxide; P_{crit}, critical oxygen level.

Table 4: Results of the stepwise linear regression analysis where salinity, body mass, routine metabolic rate (RMR) and temperature had zero-order *r* correlations with P_{crit} ($P < 0.05$) and with each other, where values were reported

	Zero-order *r* ($n = 159$)							
Variable	Salinity (psu)	Temperature (°C)	Body mass (kg)	RMR (mg O_2 l^{-1})	P_{crit} (kPa)	β	sr^2	b
Salinity		0.317	–0.165	0.354	0.279	0.346	0.099	0.047
Temperature				0.366	–0.141	–0.314	0.081	–0.083
Body mass				–0.166	0.166	0.242	0.056	1.931
RMR					0.17	0.202	0.032	0.001
Mean	23.54	23.1	0.1	323.84	5.4	Intercept = 4.027		
SD	15.36	7.9	0.3	434.04	2.1	Adjusted $r^2 = 0.195$	$P < 0.001$	

Abbreviations: P_{crit}, critical oxygen level; RMR, routine metabolic rate. In the full model, all four variables were significant predictors of P_{crit}.

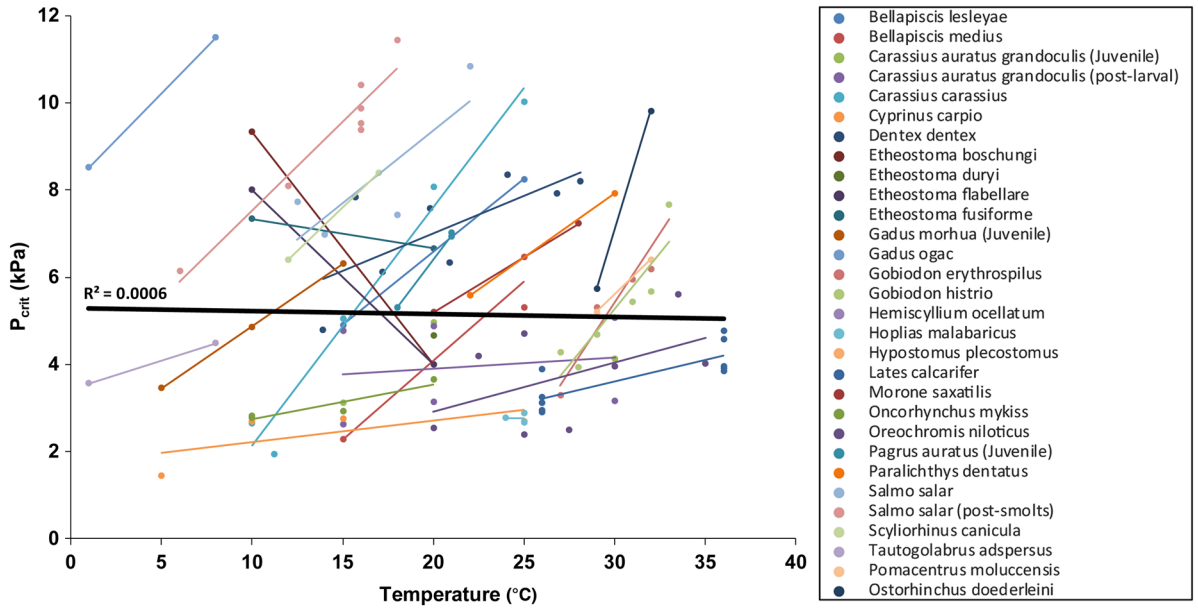

Figure 3: The effect of temperature on inter-species critical oxygen level (P_{crit}; black dashed line) and intra-species P_{crit} (continuous lines).

Unlike intra-species P_{crit}, there is no apparent relationship between temperature and inter-species P_{crit} (Fig. 3), suggesting that evolution may have nullified the thermal sensitivity of hypoxia tolerance across species. It has been shown that the difference in RMR between a typical cold-water and warm-water fish is less than expected, given the thermal sensitivity of RMR within individual species (intra-species median $Q_{10} = 2.4$; Clarke and Johnston, 1999). In addition, gill surface area appears to scale in a linear manner with metabolic rate, implying that natural selection equips fishes with the oxygen extraction capacity required to match demand at higher temperatures (Nilsson and Östlund-Nilsson, 2008). Selective pressures for small gills, such as the osmorespiratory compromise (Nilsson, 1986; Gonzalez and McDonald, 1992; Urbina and Glover, 2015), gill parasites and risks associated with gill injury, are likely to limit respiratory surface area so that oxygen extraction capacity does not exceed that required by a particular species for survival in its natural range (Nilsson, 2007). Thus, generalizations regarding hypoxia tolerance across temperatures cannot be established firmly at the inter-species level.

Although salinity has long been recognized as a key environmental factor, studies evaluating the effects of salinity on P_{crit} are scarce. A previous study in the euryhaline sheephead minnow (*Ciprinodon variegatus*), acclimated to salinities from freshwater (0 PSU) to hypersaline waters (100 PSU), showed a marked effect on P_{crit} (Haney and Nordlie 1997) as environmental salinity rose. Inter-specific comparisons in the database agree with this previous intra-specific finding; that is, salinity had a significant influence on P_{crit}, whereby freshwater

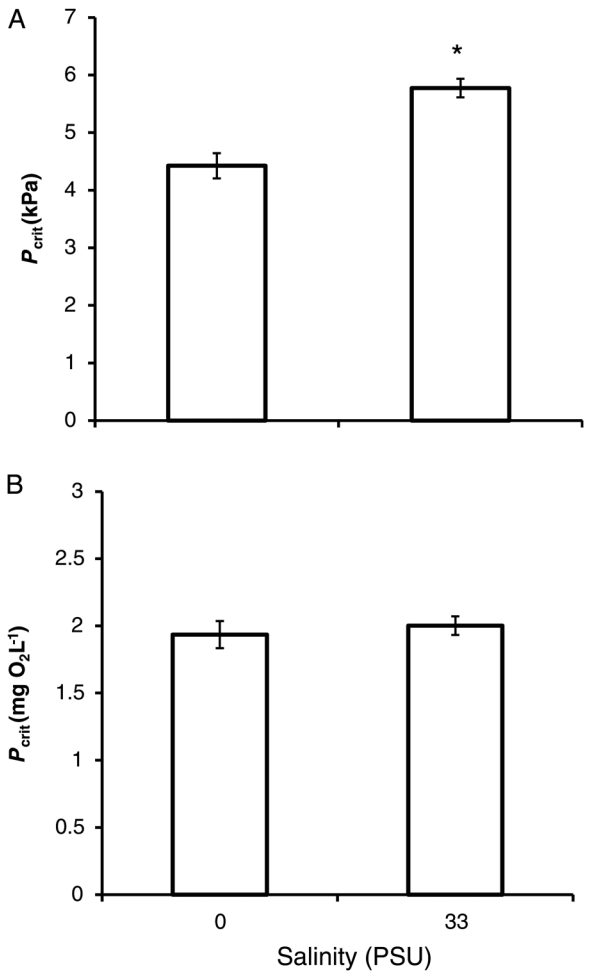

Figure 4: The effect of environmental salinity on inter-species critical oxygen level (P_{crit}), expressed as partial pressure of oxygen (in kilopascals; **A**) and concentration of oxygen (in milligrams per litre; **B**). Data are shown as means + SEM, including data from 82 species in seawater and 50 species in freshwater. *Unpaired *t*-test, significant when $P < 0.050$.

species (including a few euryhaline species) presented a 23% lower P_{crit} than seawater species (also including a few euryhaline species; Fig. 4A; $P \leq 0.001$).

As explained in earlier sections, any factor influencing the oxygen demand (metabolic rate) of an individual will be likely to have implications for its hypoxia tolerance. Given that teleost fishes must maintain a tight regulation of their internal salts and water composition (osmolality), as external salinity changes or becomes extreme, fishes must expend increased efforts to maintain internal homeostasis (Urbina and Glover, 2015). As many of the mechanisms of osmoregulation involve the action of ATP-driven pumps (i.e. Na^+,K^+-ATPase) in order to pump ions against a concentration gradient, increased costs of osmoregulation may explain, in part, some of these differences in P_{crit}, at least for intra-specific comparisons. However, from our database (inter-specific), where more freshwater vs. seawater species comparison are presented, it is likely that other mechanisms are explaining differences in P_{crit}. Given that seawater species separated million years ago from a freshwater ancestor (actinoptyergians, 300–180 million years ago; Vega and Wiens, 2012), both fresh- and seawater species have adapted to their respective environments, and therefore, have also optimized their energy allocated to osmoregulation. Thus, the differences in P_{crit} found in the present study, rather than being explained by energy-related/oxygen demand issues, could be associated with intrinsic characteristics of both media (freshwater vs. seawater). Owing to differences in size, organic matter load and stability, hypoxia is much more prevalent and common in freshwater than in seawater environments. As such, the driver for an enhanced hypoxia tolerance (lower P_{crit}) could potentially explain the lower P_{crit} found in freshwater species. A future phylogenetic analysis might contribute to test this hypothesis.

It is also worth noting that the difference found in P_{crit} when presented as the partial pressure of oxygen (in kilopascals) was no longer found when P_{crit} was calculated as the concentration (in milligrams per litre; (Fig. 4B; $P > 0.05$). This could potentially highlight the importance of working with partial pressure, because this is what drives diffusion when considering gases. Alternatively, it could indicate that the oxygen concentration is more relevant when considering P_{crit} values, because it determines the total amount of oxygen that is potentially available for diffusion as water flows over the gills, i.e. for the same oxygen uptake, salinity (through its effect on solubility) will have a big effect on the difference between inspired and expired P_{O_2}.

The biological processes that consume O_2 also produce CO_2; therefore, hypoxia and hypercarbia can often co-occur in aquatic environments (Ultsch, 1996; Cruz-Neto and Steffensen, 1997; Gilmour, 2001). Despite this, the interactive effect of environmental hypercarbia on hypoxia tolerance has been relatively understudied. As previously discussed (Table 3), there are conflicting reports within the available literature regarding to the effect of hypercarbia on the P_{crit} of fishes (Cochran and Burnett, 1996; Cruz-Neto and Steffensen, 1997; McKenzie *et al.*, 2003). The most likely mechanism by which hypercarbia could negatively impact hypoxia tolerance is through respiratory acidosis, leading to Bohr/Root effects on haemoglobin and reduced oxygen transport capacity (Jensen *et al.*, 1993; Cruz-Neto and Steffensen, 1997). In this respect, hypercarbia is partly akin to the far more extreme acidosis that can occur in poorly buffered freshwater environments subjected to acid precipitation or drainage. Acidification of the surrounding water by addition of sulphuric acid (water pH range 7.4–4.0, at constant atmospheric P_{CO_2}) increases P_{crit} in both rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*; Ultsch *et al.*, 1980). The time required to compensate for acid–base disturbance is highly variable among species (10–24 h during moderate hypercarbia; Melzner *et al.*, 2009), and as such, the effect of hypercarbia and acidification on hypoxia tolerance is likely to be dependent

largely on the species in question as well as the severity and duration of the hypercarbic or acid exposure (Jensen *et al*., 1993).

Exposure to toxicants, such as trace metal contamination, appears to reduce hypoxia tolerance in fishes. Specifically, exposure to elevated concentrations of copper (300 μg l^{-1}), mercury (150 μg l^{-1}) and silver (63 μg l^{-1}) have been demonstrated to increase P_{crit} in various species (Table 3). The accumulation of toxic metals on the gills can stimulate the hypersecretion of mucus, which acts as a barrier to diffusion of external toxicants into the blood (McDonald and Wood, 1993; Wilson *et al*., 1994). In addition, some trace metals cause hyperplasia and hypertrophy of gill epithelia cells that results in the fusing and thickening of gill lamellae (Schjolden *et al*., 2007; Bilberg *et al*., 2010). As a consequence, respiratory function is compromised as a result of reduced diffusion area and increased diffusion distance (McDonald and Wood, 1993). The organophosphate insecticide trichlorfon has been shown to increase P_{crit} by inducing similar changes in gill morphology as well as by promoting vasoconstriction that reduces lamellar blood flow in Nile tilapia (*Oreochromis niloticus*; Thomaz *et al*., 2009). These potential interactions between toxic contaminants and hypoxia in fishes are clearly of concern, particularly given that both stressors predominantly threaten freshwater and coastal marine systems and are therefore likely to coincide (McDonald and Wood, 1993; Diaz and Rosenberg, 2008).

Determinations of P_{crit} in fishes have almost universally been made in unfed, post-absorptive individuals which, although providing a useful basis for comparing absolute hypoxia tolerance among species and individuals, does not fully account for the digestive state typical of fishes in their natural setting. An increase in oxygen uptake following ingestion of food, termed specific dynamic action (SDA), is required in order to meet the energetic costs associated with mechanical and biochemical digestion and assimilation (Jobling, 1993). Shortly after a meal, oxygen uptake in fish typically rises rapidly, reaching a peak two to three times higher than pre-fed levels within a few hours. The shape and duration of the SDA is highly dependent on the species in question as well as the meal size and composition (Secor, 2009). Measurements of P_{crit} in fishes undergoing SDA have revealed significant increases in P_{crit} compared with unfed control fishes, showing that increased aerobic demand during digestion has negative consequences for hypoxia tolerance (Table 3). In common perch (*Perca fluviatilis*) force-fed a 5% body mass ration, P_{crit} at 20 h post-feeding was increased by 1.44-fold compared with sham-fed individuals (Thuy *et al*., 2010). Likewise, oscars (*Astronotus ocellatus*) fasted for 14 days showed a 1.6-fold lower P_{crit} than individuals fed a daily 1% body mass ration up to 24 h prior to P_{crit} determination (De Boeck et al., 2013). In such experiments, the requirement for a stable M_{O_2} on which to base a determination of P_{crit} means that measurements at peak SDA are not feasible, and thus, are likely to underestimate the effect of digestion on hypoxia tolerance (Thuy *et al*., 2010).

Several studies have investigated the effect of hypoxia acclimation on P_{crit} (Table3). Broadly, short-term physiological acclimation to hypoxia appears to be achieved through either enhanced O_2 extraction capacity or metabolic depression. In goldfish (*Carassius auratus*), 48 h of severe (0.63 kPa) hypoxia induced dramatic increases in both lamellar surface area and blood haemoglobin content, leading to a 49% reduction in P_{crit} compared with individuals held at normoxia (Fu *et al*., 2011). Likewise, sailfin molly (*Poecilia latipinna*) demonstrated increased haemoglobin and red blood cell concentrations and a reduced P_{crit} following a 6 week exposure to severe hypoxia (Timmerman and Chapman, 2004a). Depression of RMR at normoxia and a subsequent reduction in P_{crit} following chronic hypoxic exposure has been observed in the epaulette shark (*H. ocellatum*; Routley *et al*., 2002) and qingbo (*Spinibarbus sinensis*; Dan *et al*., 2014). However, some less hypoxia-tolerant species appear to demonstrate no physiological acclimation potential through hypoxic pre-conditioning. Daily exposure to 6 h of moderate hypoxia (10.5 kPa) for 33 days had no effect on P_{crit} in post-smolt Atlantic salmon (*S. salar*; Remen *et al*., 2013). Additionally, chronic (6 week) moderate hypoxia produced no change in the P_{crit} of juvenile snapper (*Pagrus auratus*; Cook *et al*., 2013).

As hypoxia is likely to become an increasingly predominant aquatic perturbation in the future (Vaquer-Sunyer and Duarte, 2008; Keeling *et al*., 2009), the degree of physiological plasticity for hypoxia tolerance will be a key determinant of species performance. The potential for long-term and transgenerational hypoxia acclimation with respect to P_{crit} has been largely unstudied. A transgenerational transfer of hypoxia tolerance has been demonstrated in zebrafish (*Danio rerio*) larvae after 2–4 weeks of parental hypoxia exposure, but this was based on determinations of time to loss of equilibrium (4 kPa O_2) rather than through measurement of P_{crit} (Ho and Burggren, 2012). Reardon and Chapman (2010) demonstrated a strong element of developmental plasticity in the P_{crit} of the Egyptian mouthbrooder (*Pseudocrenilabrus multicolor*) when reared in hypoxic conditions. In addition, intraspecies population effects on P_{crit} across habitats of differing O_2 regimens have been observed in several species, indicating that a high degree of phenotypic plasticity for P_{crit} exists within these populations (Timmerman and Chapman, 2004b; Reardon and Chapman 2010; Fu *et al*., 2011).

Future applications

The comprehensive P_{crit} database presented here provides the opportunity for a variety of further analyses with potential to offer fundamental physiological, as well as wider ecological, insights. For example, further analyses could involve comparing species P_{crit} values within a phylogenetic context as a means to investigate the evolutionary relationships of hypoxia tolerance among species (Mandic *et al*., 2009). Likewise, combining species P_{crit} data with information on the spatial distribution of populations would help to refine our understanding of the ecological relevance of P_{crit} as a physiological trait. Such an analysis would be particularly relevant to predicting the

impacts on fish populations likely to arise from the increasingly widespread occurrence of hypoxic zones in aquatic environments around the globe (Friedrich *et al*., 2014). Given the variability found in the reported P_{crit} for different fish species, it is likely that hypoxic events will have consequences that are very dependent on individual species. This highlights the complexity of predicting the effects that hypoxia will have at community and ecosystem levels, and the potential for hypoxia to have differential effects on predator-prey interactions, migrations, and ultimately, global fisheries.

The integration of the present database with similar databases of other widely measured physiological parameters in fishes should offer useful insights into interactions among traits. Such physiological data are of great value for improving the predictive capacity of models as an aid to the management and conservation of aquatic systems (Jørgensen et al., 2012; Cooke *et al*., 2013). Traits for which databases are currently under construction include the metabolic response to feeding (SDA), aerobic scope, growth rate and critical temperature. On completion, the combined data set will be made widely accessible via an online data repository facility, such as that provided by Dryad (http://datadryad.org/). Thus, it is envisaged that these data will prove to be a tangible link between the field of fish physiology and future studies of ecology, conservation and management.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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