

COMMENTARY

The utility and determination of P_{crit} in fishes

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ABSTRACT

The critical O₂ tension (P_{crit}) is the lowest P_{O_2} at which an animal can maintain some benchmark rate of O₂ uptake (\dot{M}_{O_2}). This P_{O_2} has long served as a comparator of hypoxia tolerance in fishes and aquatic invertebrates, but its usefulness in this role, particularly when applied to fishes, has recently been questioned. We believe that P_{crit} remains a useful comparator of hypoxia tolerance provided it is determined using the proper methods and hypoxia tolerance is clearly defined. Here, we review the available methods for each of the three steps of P_{crit} determination: (1) measuring the most appropriate benchmark \dot{M}_{O_2} state for P_{crit} determination ($\dot{M}_{\text{O}_2,\text{std}}$, the \dot{M}_{O_2} required to support standard metabolic rate); (2) reducing water P_{O_2} ; and (3) calculating P_{crit} from the \dot{M}_{O_2} versus P_{O_2} curve. We make suggestions on best practices for each step and for how to report P_{crit} results to maximize their comparative value. We also discuss the concept of hypoxia tolerance and how P_{crit} relates to a fish's overall hypoxia tolerance. When appropriate methods are used, P_{crit} provides useful comparative physiological and ecological information about the aerobic contributions to a fish's hypoxic survival. When paired with other hypoxia-related physiological measurements (e.g. lactate accumulation, calorimetry-based measurements of metabolic depression, loss-of-equilibrium experiments), P_{crit} contributes to a comprehensive understanding of how a fish combines aerobic metabolism, anaerobic metabolism and metabolic depression in an overall strategy for hypoxia tolerance.

KEY WORDS: Critical oxygen tension, Fish, Hypoxia, P_{crit} , Respirometry, Standard metabolic rate

Introduction

Innumerable studies of fishes have measured their metabolic rate – expressed as O₂ uptake rate (\dot{M}_{O_2} ; on the assumption that there is no significant anaerobic metabolism when O₂ is readily available) – and the effects of different variables upon it. One such variable is water P_{O_2} (P_{wO_2}); many studies have analyzed how \dot{M}_{O_2} changes as P_{wO_2} decreases. Typically \dot{M}_{O_2} changes only slightly, if at all, until P_{wO_2} reaches a level that is too low for the fish to extract sufficient quantities of O₂ to maintain a baseline maintenance \dot{M}_{O_2} ($\dot{M}_{\text{O}_2,\text{std}}$, which supports standard metabolic rate, SMR; see Glossary); \dot{M}_{O_2} starts to decline as P_{wO_2} decreases further. The P_{wO_2} at which this decrease starts – i.e. the lowest P_{wO_2} at which $\dot{M}_{\text{O}_2,\text{std}}$ can be sustained – has been designated the critical O₂ tension (P_c or P_{crit} ; see Glossary), and has been the subject of many investigations. It is generally assumed that a fish with a lower P_{crit} is more adapted to hypoxia than one with a higher P_{crit} , analogous to the assumption

that an animal with a low blood P_{50} (e.g. a llama) is more adapted to hypoxia than one with a higher P_{50} . Recently, the meaningfulness of P_{crit} has been criticized on both methodological and theoretical grounds, with the suggestion that it should be abandoned (Wood, 2018). We disagree (Regan et al., 2019), and present our reasoning here. It is not our intention to review all the physiological, biochemical and behavioral mechanisms surrounding P_{crit} (see Richards, 2009, 2011; Pörtner and Grieshaber, 1993; Wells, 2009). Rather, we will concentrate almost entirely on the standards for the determination of $\dot{M}_{\text{O}_2,\text{std}}$, and its use to calculate P_{crit} . Standardized methods would enable comparisons among studies and highlight the relevance of P_{crit} to ecology. The bulk of our discussion will deal with fishes, as a large number of such studies focus on this taxon.

Defining P_{crit}

Fig. 1 is a commonly used representation, often applied to fishes, of the various \dot{M}_{O_2} states and how they are affected by ambient P_{O_2} . These states include $\dot{M}_{\text{O}_2,\text{std}}$ (the \dot{M}_{O_2} required to aerobically support SMR), $\dot{M}_{\text{O}_2,\text{rtn}}$ (the \dot{M}_{O_2} required to aerobically support routine metabolic rate; RMR; see Glossary) and $\dot{M}_{\text{O}_2,\text{dep}}$ (the \dot{M}_{O_2} required to support a regulated state of metabolic depression). Several assumptions are made in Fig. 1. One is that the relationship is biphasic with two distinct linear portions. The slope of the curve in the zone of oxyregulation (i.e. at P_{wO_2} values between normoxia and P_{crit}) is assumed to be zero. However, this is not always the case, nor is it required to accurately calculate P_{crit} ; all that is required for this calculation is a sharp change in slope ($d\dot{M}_{\text{O}_2}/dP_{\text{O}_2}$) at the break point. Another assumption is that extending the relationship in the zone of oxyconformation will intersect the origin, which is usually not the case.

P_{crit} is the lowest P_{wO_2} at which the animal can maintain some benchmark \dot{M}_{O_2} state. Although the term P_{crit} has been applied to both $\dot{M}_{\text{O}_2,\text{rtn}}$ (Fig. 1, point A) and $\dot{M}_{\text{O}_2,\text{std}}$ (point B), in most cases this is a misnomer for $\dot{M}_{\text{O}_2,\text{rtn}}$. This point represents the P_{wO_2} at which the fish can no longer aerobically support RMR (i.e. the metabolic rate of a post-absorptive fish that includes the costs of minor activity within the respirometer). Therefore, the situation is not critical for the fish; if the animal lowers its activity level, then ambient P_{wO_2} will be sufficient to support its metabolic needs. Nevertheless, many studies have reported point A as the P_{crit} . More appropriate is $\dot{M}_{\text{O}_2,\text{std}}$ (point B), where the fish is at SMR (i.e. the metabolic rate of a post-absorptive fish that is completely inactive). While it is probably true that organisms in the wild are rarely at SMR, the P_{crit} calculated at point B is physiologically, ecologically and methodologically relevant for three reasons: (1) at the point B P_{crit} , the fish's aerobic scope (see Glossary), which has progressively decreased from normoxic P_{wO_2} , reaches zero, leaving only anaerobic glycolysis to fuel metabolic functions beyond baseline maintenance functions (Claireaux and Chabot, 2016); (2) at P_{wO_2} below P_{crit} , maintaining energy balance requires enhanced reliance on anaerobic glycolysis and/or the induction of a regulated metabolic depression (point C): life at or below the point B P_{crit} is therefore ultimately unsustainable; (3) $\dot{M}_{\text{O}_2,\text{std}}$ is a more consistent and reliable benchmark \dot{M}_{O_2} state compared with the inherently

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List of symbols and abbreviations

CF	continuous flow
CS	closed system
IF	intermittent flow
LOE	loss of equilibrium, especially the P_{O_2} at which it occurs
\dot{M}_{O_2}	rate of O_2 consumption
$\dot{M}_{O_2,dep}$	\dot{M}_{O_2} that supports a regulated state of metabolic depression
$\dot{M}_{O_2,max}$	\dot{M}_{O_2} that supports maximal metabolic rate
$\dot{M}_{O_2,rtn}$	\dot{M}_{O_2} that supports routine metabolic rate
$\dot{M}_{O_2,std}$	\dot{M}_{O_2} that supports standard metabolic rate
P_{50}	partial pressure of O_2 at which the blood or hemoglobin is 50% saturated with O_2
P_{crit}	critical O_2 tension
P_{wO_2}	partial pressure of O_2 in water
P_{wCO_2}	partial pressure of CO_2 in water
RI	regulation index
RMR	routine metabolic rate
SMR	standard metabolic rate

variable $\dot{M}_{O_2,rtn}$. Consequently, P_{crit} calculations based on $\dot{M}_{O_2,std}$ are more reproducible and comparable than those based on $\dot{M}_{O_2,rtn}$. For these reasons, point B is most relevant to P_{crit} and will be the focus of our discussion.

Two additional points about $\dot{M}_{O_2,std}$ and its relationship to P_{crit} are worth mentioning. First, while $\dot{M}_{O_2,std}$ is preferable to $\dot{M}_{O_2,rtn}$ as a P_{crit} calculation benchmark, there are conditions under which $\dot{M}_{O_2,rtn}$ is sufficient or even preferred. For example, within a single study, P_{crit} based on $\dot{M}_{O_2,rtn}$ can be an effective comparator so long as the investigator is consistent in their $\dot{M}_{O_2,rtn}$ measurement technique. And for species for which $\dot{M}_{O_2,std}$ values are virtually impossible to measure (e.g. those that use ram-jet ventilation; see Glossary), $\dot{M}_{O_2,rtn}$ is the only option. Second, as mentioned by Wood (2018), it is impossible to know from \dot{M}_{O_2} data alone what metabolic processes are supported by the O_2 taken up by the fish. Therefore,

Glossary**Aerobic scope**

The difference between $\dot{M}_{O_2,std}$ and $\dot{M}_{O_2,max}$ in resting, unfed animals.

Buccal pumping

A ventilation method whereby the respiratory medium (e.g. water for fishes) is drawn across the exchange surface (e.g. gills) via pressure changes generated by the mouth.

Oxyconformer

An animal in which \dot{M}_{O_2} declines in direct proportion to declining environmental P_{O_2} .

Oxyregulator

An animal that maintains \dot{M}_{O_2} independent of environmental P_{O_2} .

 P_{crit}

The partial pressure of O_2 below which the animal can no longer maintain a stable \dot{M}_{O_2} ; below P_{crit} , \dot{M}_{O_2} becomes dependent upon partial pressure (tension) of O_2 .

Ram-jet ventilation

A ventilation method used by some fishes whereby an open mouth and continuous swimming allow water to continually flow over the gills.

Regulation index (RI)

A dimensionless relative measure of hypoxia tolerance (more specifically, oxyregulatory ability) that ranges from 0 to 1.

Routine metabolic rate (RMR)

Metabolic rate of a fasting, resting animal exhibiting spontaneous activity, typically measured as \dot{M}_{O_2} .

Standard metabolic rate (SMR)

Metabolic rate of a fasting, resting animal exhibiting no spontaneous activity, typically measured as \dot{M}_{O_2} .

even if an \dot{M}_{O_2} value measured at a moderately hypoxic (i.e. supra- P_{crit}) P_{wO_2} is the same as the $\dot{M}_{O_2,std}$ value measured in normoxia, it is impossible to know whether it is supporting the same maintenance processes that comprise SMR. When P_{wO_2} is reduced, there may be (and probably is) a reallocation of resources, including O_2 , towards a different suite of processes (e.g. ventilation) that may nevertheless sum to a value of \dot{M}_{O_2} similar to normoxic $\dot{M}_{O_2,std}$. While these \dot{M}_{O_2} values at moderately hypoxic P_{wO_2} may not be SMR, they represent baseline \dot{M}_{O_2} values at each P_{wO_2} . We will therefore refer to them as $\dot{M}_{O_2,std}$ even if the unknown suite of processes that comprise them are different from those of baseline \dot{M}_{O_2} in normoxia.

Are there fishes with no P_{crit} between anoxia and normoxia, i.e. oxyconformers?

Most fishes studied to date have been found to be oxyregulators (see Glossary) over a wide range of P_{wO_2} , though some have been reported to be oxyconformers (see Glossary). Debate exists about whether truly oxyconforming species exist (e.g. Steffensen, 2006), and this debate is relevant to P_{crit} , because P_{crit} represents the lowest environmental P_{O_2} at which an animal can regulate \dot{M}_{O_2} . Therefore, a truly oxyconforming animal has no P_{crit} , and this may muddle the underlying theory of P_{crit} and reduce its comparative value.

The majority of studies of \dot{M}_{O_2} and P_{crit} have been done on small to moderate-sized teleosts, which pass water over their gills by buccal pumping (see Glossary). These studies have mostly been performed in respiration chambers with zero or moderate flow through the chamber. Under such conditions, the highly efficient gills should have no problem transporting sufficient quantities of O_2 into the blood to sustain $\dot{M}_{O_2,std}$ over a reasonable range of P_{wO_2} . As a result, the vast majority of teleosts have been found to be oxyregulators, but there are occasional reports of oxyconformation. One of the best known was Hall's (1929) study of toadfish – results that appeared in several textbooks for decades. Using a flow-through system, Hall's data clearly showed toadfish to be oxyconformers. But Ultsch et al. (1981) questioned why the toadfish should be an oxyconformer when the vast majority of fishes studied since 1929 were oxyregulators. Using a more sophisticated respirometry system, they demonstrated that toadfish from the same area as Hall's are clearly oxyregulators.

Other oxyconformers have been reported, but this small group is being whittled down by subsequent studies. Common carp (*Cyprinus carpio*) were suggested to be oxyconformers (Lomholt and Johansen, 1979), but they clearly are not (Dhillon et al., 2013; Ott et al., 1980; Ultsch et al., 1980; Yamanaka et al., 2007; He et al., 2015), which is not surprising considering their low hemoglobin P_{50} (Weber and Lykkeboe, 1978; Burggren, 1982). The Mayan cichlid (*Mayaheros urophthalmus*) was reported as an oxyconformer (Martinez-Palacios and Ross, 1986) but has recently been shown to be a strong oxyregulator at three different temperatures (Burggren et al., 2019). Subrahmanyam (1980) reported all four estuarine species he studied to be oxyconformers, one of which was *Fundulus grandis*. But in a study specifically designed to test this hypothesis, Virani and Rees (2000) found this species to be an oxyregulator. Another of the four species, *Leiostomus xanthurus*, has also subsequently been found to be an oxyregulator (Cochran and Burnett, 1996). *Fundulus heteroclitus* was reported as an oxyconformer (Blewett et al., 2013), but a number of other studies found it to be an oxyregulator (Borowiec et al., 2015; Cochran and Burnett, 1996; Richards et al., 2008; McBryan et al., 2016). Both male and female plainfin midshipman (*Porichthys notatus*) have been reported to be oxyconformers (Craig et al., 2014;

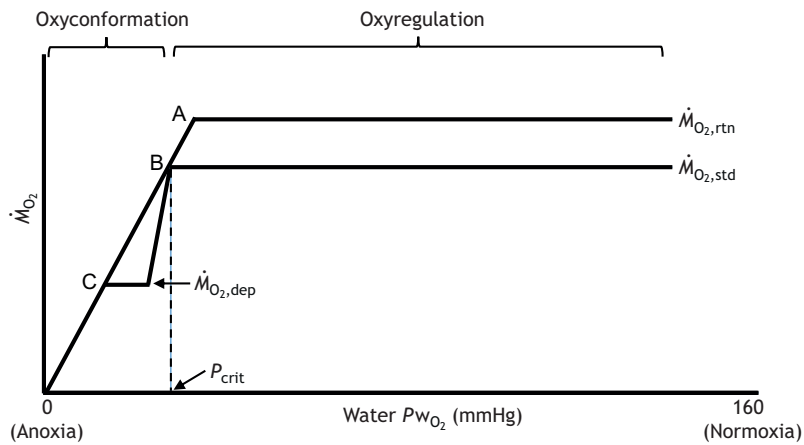


Fig. 1. Hypothetical relationships between rate of \dot{M}_{O_2} consumption (\dot{M}_{O_2}) and P_{O_2} . $\dot{M}_{O_2,rtn}$ represents the \dot{M}_{O_2} state for routine metabolic rate, $\dot{M}_{O_2,std}$ is the \dot{M}_{O_2} state for standard metabolic rate and $\dot{M}_{O_2,dep}$ is the \dot{M}_{O_2} for a regulated and depressed aerobic metabolic rate. Points A–C are the critical O_2 tension (P_{crit}) for each \dot{M}_{O_2} state level. Zones of oxyregulation and oxyconformation are shown for the $\dot{M}_{O_2,std}$ P_{crit} curve. Pw_{O_2} , partial pressure of O_2 in water.

Lemoine et al., 2014). However, there have been no additional studies to affirm the finding that this species is oxyconforming.

Sturgeons were reported to be oxyconformers by Burggren and Randall (1978), but subsequent studies found them to be oxyregulators (Ruer et al., 1987; Crocker and Cech, 2002; Randall et al., 1982; Nonette et al., 1993). Interestingly, McKenzie et al. (2007) found that when the sturgeon *Acipenser naccarii* was exposed to progressive hypoxia under static conditions, it was an oxyconformer; however, when allowed to swim at a low sustained speed, it could regulate \dot{M}_{O_2} down to a P_{O_2} of 37 mmHg (4.9 kPa). While this fish does not use ram-jet ventilation in the classic sense, the results do raise a point about the importance of swimming in some fishes if one is attempting to determine the $\dot{M}_{O_2,std}$ and/or P_{crit} .

The fishes discussed above use buccal pumps to irrigate their gills. Fishes that swim constantly and/or use ram-jet ventilation present some special problems in determining their $\dot{M}_{O_2,std}$ and P_{crit} (reviewed by Bushnell and Jones, 1994). Potentially, they could appear to be oxyconformers if constrained within a respiration chamber, when they might be oxyregulators if allowed to swim or provided with a high enough flow within the chamber to ventilate the gills through an open mouth. Several such species have been found to increase swimming speed and/or mouth gape as water becomes hypoxic (bonnethead shark, *Sphyrna tiburo*: Parsons and Carlson, 1998; several species of tuna: Bushnell and Brill, 1992; Gooding et al., 1981). Therefore, even if P_{crit} is determined under static conditions, it may have little relevance to their natural state, and the P_{crit} thus determined might well be reduced with moderate swimming that allows improved gill ventilation. The situation is further complicated with fishes that are not obligate ram-jet ventilators. For example, Steffensen (1985) found a 10.2% reduction in \dot{M}_{O_2} in rainbow trout when they switched from buccal pumping to ram-jet ventilation; however, that study did not attempt to determine a P_{crit} for each ventilation method. Thus, when comparing the P_{crit} of different species, one should consider the modes of ventilation and the lifestyles.

We are aware of no studies that deal with P_{crit} considerations for constantly swimming elasmobranchs. There have been studies on sharks, many of which swim constantly, at least during a significant portion of a 24 h day, but few of these studies were on swimming animals. *Scyliorhinus stellaris* was reported to be an oxyconformer by Piiper et al. (1970), but Hughes and Umezawa (1968) found that the related *Scyliorhinus canicula* could regulate down to at least 80 mmHg (10.7 kPa), and Butler and Taylor (1975) also found this species to be an oxyregulator at 12 and 17°C. Even the purportedly

hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*; Routley et al., 2002) is an oxyregulator down to 38 mmHg (5.1 kPa), as is the shovelnose ray (*Aptychotrema rostrata*), with a P_{crit} of 54 mmHg (7.2 kPa) (Speers-Roesch et al., 2012). An interesting question is whether such fishes, which normally swim constantly, would have a lower P_{crit} if allowed to swim at slow speeds, or would the extra activity raise their P_{crit} ? And from an ecological viewpoint, would the P_{crit} determined at normal swimming speeds be the most relevant? Clearly, interspecies comparisons of P_{crit} among elasmobranchs and other ram-ventilating fishes are more complicated than among the majority of teleosts, especially freshwater species.

One species deserves special mention. The inanga (*Galaxias maculatus*) has been reported to be an oxyconformer in studies that used both closed systems and intermittent-flow systems (Urbina et al., 2012; Urbina and Glover, 2013). The fish is especially interesting because it is scaleless and obtains about 1/3 of its O_2 in normoxic water cutaneously. When the water becomes severely hypoxic, it emerges (Urbina et al., 2011), and can increase its cutaneous O_2 uptake significantly. Nevertheless, the fish has functional gills that supply 2/3 of its O_2 requirements in normoxic water, and apparently can upregulate its cutaneous O_2 uptake, so it is not evident why it should not be an oxyregulator over at least a moderate range of Pw_{O_2} , with perhaps a comparatively high P_{crit} .

In summary, we believe that almost all, if not all, fishes are oxyconformers over some appreciable range of Pw_{O_2} , and that findings otherwise are likely to be due to methodology or chance.

Respirometry techniques for measuring $\dot{M}_{O_2,std}$ and reducing Pw_{O_2}

Properly determining P_{crit} involves three processes: (1) measuring $\dot{M}_{O_2,std}$; (2) reducing Pw_{O_2} ; and (3) calculating P_{crit} from the \dot{M}_{O_2} versus Pw_{O_2} curve. Respirometry is used to accomplish steps 1 and 2, and below we discuss the relevant advantages and disadvantages of three common respirometry techniques (see Table 1) – closed system (CS), intermittent flow (IF) and continuous flow (CF). We then discuss P_{crit} calculation methods, and end with our recommended best practices for each step.

CS respirometry

CS respirometry involves placing the fish in a sealed, gas-impermeable respirometry chamber with a P_{O_2} sensor and a well-mixed water volume. The fish's \dot{M}_{O_2} is determined from the rate at which its respiration reduces Pw_{O_2} from some starting Pw_{O_2} to some lower target Pw_{O_2} . For measuring $\dot{M}_{O_2,std}$, CS respirometry on its own (i.e. without the serial normoxic \dot{M}_{O_2} measurements made

Table 1. Advantages and disadvantages of different respirometry techniques for determining $\dot{M}_{O_2, \text{std}}$ and P_{crit}

Respirometry method	Advantages	Disadvantages
Closed system (CS)	Simplest, least expensive system	CO ₂ and ammonia accumulation Can reveal $\dot{M}_{O_2, \text{std}}$ only when paired with IF Uncertain $\dot{M}_{O_2, \text{std}}$ measurements jeopardize P_{crit} calculations No direct control of $P_{W_{O_2}}$ decline rate
Intermittent flow (IF)	Reduces CO ₂ and ammonia accumulation Serial \dot{M}_{O_2} measurements can reveal $\dot{M}_{O_2, \text{std}}$ Accurate $\dot{M}_{O_2, \text{std}}$ measurements enable accurate P_{crit} calculations Enables control of $P_{W_{O_2}}$ decline rate	Complex system requiring numerous components and (usually) automation Measuring \dot{M}_{O_2} at a particular $P_{W_{O_2}}$ requires exposing fish to a lower $P_{W_{O_2}}$ which, at hypoxic $P_{W_{O_2}}$, could influence P_{crit}
Continuous flow (CF)	Eliminates CO ₂ and ammonia accumulation Continuous \dot{M}_{O_2} measurements can reveal $\dot{M}_{O_2, \text{std}}$ Accurate $\dot{M}_{O_2, \text{std}}$ measurements enable accurate P_{crit} calculation Enables control of $P_{W_{O_2}}$ decline rate Does not pre-expose fish to lower $P_{W_{O_2}}$	Complex system requiring numerous components and (usually) automation Washout calculations are required for \dot{M}_{O_2} calculation during periods of P_{O_2} flux

\dot{M}_{O_2} , rate of O₂ consumption; $\dot{M}_{O_2, \text{std}}$, \dot{M}_{O_2} state for standard metabolic rate; P_{crit} , critical O₂ tension; $P_{W_{O_2}}$, partial pressure of O₂ in water.

during IF; see below) is problematic, because the investigator has no way of knowing whether the \dot{M}_{O_2} measured in normoxia is $\dot{M}_{O_2, \text{std}}$ or, more likely, some \dot{M}_{O_2} between $\dot{M}_{O_2, \text{std}}$ and the \dot{M}_{O_2} that supports maximal metabolic rate, $\dot{M}_{O_2, \text{max}}$ (i.e. $\dot{M}_{O_2, \text{rtn}}$). The uncertainty around this benchmark normoxic \dot{M}_{O_2} state consequently reduces the effectiveness of CS respirometry for determining P_{crit} . CS respirometry relies on the fish's respiration to reduce $P_{W_{O_2}}$. The rate of $P_{W_{O_2}}$ decline is therefore a function of the fish's metabolic rate and the water volume of the chamber, which leaves the investigator with only indirect control over this rate through control of water volume. Recommended water volume:fish mass ratios of 20:1 to 100:1 (Clark et al., 2013) typically result in $P_{W_{O_2}}$ being reduced from normoxia to terminal $P_{W_{O_2}}$ in 1–2 h. This rate may be higher than what the species experiences in its natural environment, but it may outpace the onset of P_{crit} -influencing hypoxic acclimations that are difficult to control for (e.g. Regan and Richards, 2017).

Furthermore, a closed system means that metabolic waste such as CO₂ and ammonia will accumulate in the water. Though often touted as a disadvantage of CS respirometry, CO₂ buildup is unlikely to affect $\dot{M}_{O_2, \text{std}}$ significantly – even if a fish consumes all the O₂ in the water, the water P_{CO_2} ($P_{W_{\text{CO}_2}}$) will not exceed 5 mmHg (0.7 kPa), and studies on a range of species have shown that much higher $P_{W_{\text{CO}_2}}$ values [up to 90 mmHg (12 kPa) in one case] have no effect on \dot{M}_{O_2} (Beamish, 1964; Cochran and Burnett, 1996; Randall et al., 1976; Sloman et al., 2008; Cruz-Neto and Steffensen, 1997; Crocker and Cech, 2002). However, it is possible that the elevated $P_{W_{\text{CO}_2}}$ may elevate P_{crit} through possible red blood cell acidification and subsequent reduction of hemoglobin–O₂ binding affinity. The effects of ammonia are not well studied, but most studies on P_{crit} use fasting fishes, so ammonia production should be minimal.

When using CS respirometry, it should be noted that the fish's attempts to escape the hypoxic conditions throughout the uncontrolled decline in $P_{W_{O_2}}$ often elevate \dot{M}_{O_2} far above $\dot{M}_{O_2, \text{std}}$. Some species (e.g. goldfish) tend not to display such escape responses. However, many species do, and the result is a scatter of \dot{M}_{O_2} values as a function of $P_{W_{O_2}}$ (Fig. 2). Calculating P_{crit} from such a data set requires the investigator to apply a set of criteria to isolate the \dot{M}_{O_2} values that best reflect $\dot{M}_{O_2, \text{std}}$.

IF respirometry

IF respirometry involves intermittently flushing the chamber with fresh water between \dot{M}_{O_2} measurements that are taken using CS

respirometry. Flushing replenishes the chamber with O₂, eliminates the buildup of waste products that might affect \dot{M}_{O_2} , and allows for serial \dot{M}_{O_2} measurements within a narrow $P_{W_{O_2}}$ range. For these reasons, IF respirometry has been widely touted in reviews (Clark et al., 2013; Snyder et al., 2016; Chabot et al., 2016; Eriksen, 2002; Svendsen et al., 2016; Steffensen, 1989). For measuring $\dot{M}_{O_2, \text{std}}$, IF respirometry enables serial normoxic \dot{M}_{O_2} measurements over a long habituation period (≥ 24 h), to which the investigator may then apply some criteria to determine which data points to use for the $\dot{M}_{O_2, \text{std}}$ estimation (e.g. Steffensen et al., 1994; Murchie et al., 2011; Snyder et al., 2016; Chabot et al., 2016). The result is an accurate estimation of $\dot{M}_{O_2, \text{std}}$. For reducing $P_{W_{O_2}}$, the investigator reduces the $P_{W_{O_2}}$ of sump (or incurrent) water in a stepwise fashion. This prevents the accumulation of end products in the fish chamber (though, as mentioned above, this may not be a problem) and allows hypoxic $P_{W_{O_2}}$ environments to be sustained long enough to outlast the fish's acute behavioral responses. Furthermore, as in normoxia, serial \dot{M}_{O_2} measurements can be made at each hypoxic $P_{W_{O_2}}$, meaning reasonable estimates of $\dot{M}_{O_2, \text{std}}$ at each moderately hypoxic (i.e. supra- P_{crit}) $P_{W_{O_2}}$ are possible. This reduces the scatter of \dot{M}_{O_2} values in the oxyregulation portion of the \dot{M}_{O_2} versus P_{crit}

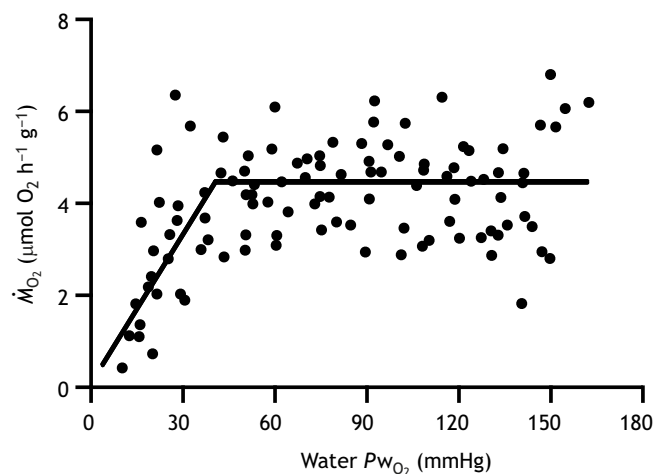


Fig. 2. Data scatter resulting from the use of closed-system respirometry, which does not account for activity. Example shown is for *Etheostoma squamiceps* (Ultsch et al., 1978).

curve, and ultimately makes for a more straightforward P_{crit} calculation. However, it also requires considerable time, which, when coupled with the time required to equilibrate the P_{wO_2} of the sump and chamber water volumes, results in a relatively long P_{crit} trial of ~ 5 h. This is sufficiently long for some species to induce hypoxia acclimation responses (e.g. Regan and Richards, 2017), which may jeopardize the comparative value of the resulting P_{crit} . Perhaps for this reason or the relative complexity of performing IF respirometry at progressively lower P_{wO_2} values, investigators often use IF respirometry to make normoxic $\dot{M}_{\text{O}_2, \text{std}}$ measurements, then abandon flushing when reducing P_{wO_2} in favor of straight CS respirometry. This approach presents the same drawbacks described above for CS respirometry. Some investigators mitigate these drawbacks by using a hybrid approach, where IF is used between normoxia and some moderately hypoxic P_{wO_2} that is predicted to be above the fish's P_{crit} (e.g. 8 kPa), and then CS is used from that P_{wO_2} down to the terminal P_{wO_2} (e.g. Borowiec et al., 2015; Crans et al., 2015).

CF respirometry

CF respirometry (sometimes called flow-through respirometry) involves supplying the fish with a continuous flow of fresh water; \dot{M}_{O_2} is measured as the difference between incurrent and excurrent values of P_{wO_2} multiplied by the water flow rate. For measuring $\dot{M}_{\text{O}_2, \text{std}}$, the continuous recording of excurrent P_{wO_2} enables a continuous calculation of \dot{M}_{O_2} . As with IF respirometry, when a sufficient habituation period (≥ 24 h) is used and some criteria are applied to these \dot{M}_{O_2} values, the result is an accurate estimate of $\dot{M}_{\text{O}_2, \text{std}}$ (Fig. 3). For reducing P_{wO_2} , the investigator reduces sump (or incurrent) P_{wO_2} in a stepwise fashion. This offers similar benefits to IF respirometry – no waste product accumulation and the ability to maintain any P_{wO_2} environment long enough for the fish to reach a stable \dot{M}_{O_2} approaching $\dot{M}_{\text{O}_2, \text{std}}$. In fact, CF enables the fish's P_{wO_2} environment to be held relatively stable. This is not possible with CS or IF because they require P_{wO_2} of the respirometer water volume to span the median P_{wO_2} for which \dot{M}_{O_2} is measured. This pre-exposes the fish to lower P_{wO_2} , which, particularly at P_{wO_2} around the P_{crit} , may be problematic. CF respirometry avoids this complication. However, reducing P_{wO_2} with CF respirometry results in a lag period during the washout phase as the next P_{wO_2} equilibrates across the chamber and both inflow and outflow P_{O_2} sensors. The typical flow-through equation cannot be used to calculate \dot{M}_{O_2} during this lag period, and this has been cited

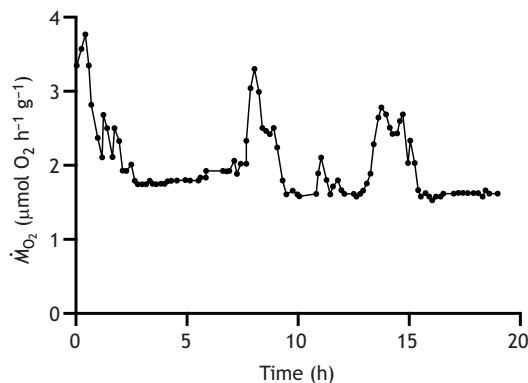


Fig. 3. \dot{M}_{O_2} of a carp (*Cyprinus carpio*) measured using a continuous flow system. Data are from Ultsch et al. (1980). $\dot{M}_{\text{O}_2, \text{std}}$ values are visually apparent as the lowest \dot{M}_{O_2} values (i.e. between 15 and 20 h) and may be quantitatively isolated using some applied criteria (e.g. lowest 10% of \dot{M}_{O_2} values).

as a problem with CF respirometry (Clark et al., 2013; Rosewarne et al. 2016; Svendsen et al., 2016). However, there are additional equations that allow \dot{M}_{O_2} to be accurately calculated during these lag periods (Niimi, 1978; Steffensen, 1989; Ultsch and Duke, 1990; Ultsch and Anderson, 1988; Ultsch et al., 1980, 1981), thereby shortening the time over which a P_{crit} trial using CF respirometry can be run.

Calculating P_{crit}

There are different methods to calculate P_{crit} from the \dot{M}_{O_2} versus P_{wO_2} curve, some of which use more data than others. According to a recent P_{crit} meta-analysis (Rogers et al., 2016), the most widely used method of P_{crit} calculation is a segmented linear regression technique such as that of Yeager and Ultsch (1989). This method uses all (or most) of the \dot{M}_{O_2} data and assumes the \dot{M}_{O_2} response of fishes to declining P_{wO_2} is approximately biphasic with two distinct linear portions, an oxyregulation line and an oxyconformation line (Fig. 1). The P_{wO_2} at which these lines intersect is the P_{crit} . The Yeager and Ultsch (1989) method puts no restrictions on the placement of data points. It looks at all sets of data points for two-line fits by starting with \dot{M}_{O_2} at the lowest P_{wO_2} in the zone of oxyconformation, plotting a regression line for the first three points, and assigning remaining points to the second line. It then continues the calculations for each line, adding one point at a time to the progressively higher P_{wO_2} . The process continues until all but the last three points are on the oxyconformation line. The best fit is then defined as the set of two regression lines that give the lowest summed sum of squares of error (SSE). This method is widely applicable because many fishes studied to date display a biphasic response of \dot{M}_{O_2} to declining P_{wO_2} , the result of their oxyregulatory abilities. However, not all species (or individuals) do, and applying this segmented linear regression technique to them may overestimate P_{crit} .

There are different ways to calculate P_{crit} from a non-biphasic \dot{M}_{O_2} versus P_{wO_2} curve. Non-linear regression (e.g. Marshall et al., 2013) and Akaike information criterion (Cobbs and Alexander, 2018) do so using all of the available data, but they are complex and perhaps for this reason are not widely used. More common techniques involve omitting certain \dot{M}_{O_2} data points from the P_{crit} calculation that are thought to be unrepresentative of $\dot{M}_{\text{O}_2, \text{std}}$, essentially transforming a non-biphasic curve into a biphasic one. The trick here of course is determining which \dot{M}_{O_2} values are kept and which are omitted, as critically assessed by Wood (2018); this is accomplished by applying some criteria to eliminate \dot{M}_{O_2} values that obviously exceed or underestimate $\dot{M}_{\text{O}_2, \text{std}}$. The remaining data may then be input into a program such as that of Yeager and Ultsch (1989) or Claireaux and Chabot (2016) to calculate P_{crit} . Perhaps the simplest method involves anchoring the oxyregulation line at the $\dot{M}_{\text{O}_2, \text{std}}$ value determined in normoxia (i.e. >17 kPa P_{wO_2}), and then extending this value leftward, effectively disregarding the \dot{M}_{O_2} values between normoxia and P_{crit} (e.g. Snyder et al., 2016). The oxyconformation portion of the curve is then determined by some criteria (e.g. any \dot{M}_{O_2} values $>15\%$ below the $\dot{M}_{\text{O}_2, \text{std}}$ line), regressed, and the intersection of this line with the extrapolated $\dot{M}_{\text{O}_2, \text{std}}$ line is the P_{crit} . This method is relatively easy to execute and eliminates the influence of $\dot{M}_{\text{O}_2, \text{rtm}}$ values at intermediate P_{wO_2} values such as activity-related \dot{M}_{O_2} elevations as P_{wO_2} approaches P_{crit} . However, the influence of such activity-related $\dot{M}_{\text{O}_2, \text{rtm}}$ values can be minimized by applying IF or CF respirometry as described above, which also avoids the assumption that the slope of the oxyregulation line is zero. Importantly, although the \dot{M}_{O_2} values at intermediate P_{wO_2} are not used in the P_{crit} calculation, the investigator must nevertheless choose a rate of P_{wO_2} decline that is appropriate for the question being addressed and

use this rate consistently to control for hypoxia acclimation responses that may affect P_{crit} (Regan and Richards, 2017).

This normoxia-anchored $\dot{M}_{\text{O}_2,\text{std}}$ method may be used in a different, more complex way to determine P_{crit} . First, $\dot{M}_{\text{O}_2,\text{max}}$ is measured at various $P_{\text{W}_{\text{O}_2}}$, generating a curve that has a reduced $\dot{M}_{\text{O}_2,\text{max}}$ as $P_{\text{W}_{\text{O}_2}}$ decreases. Next, $\dot{M}_{\text{O}_2,\text{std}}$ is determined at normoxia and extended leftward until it intersects the linear regression line of $\dot{M}_{\text{O}_2,\text{max}}$ and $P_{\text{W}_{\text{O}_2}}$. While this method effectively reveals the $P_{\text{W}_{\text{O}_2}}$ at which aerobic scope reaches zero (a property of P_{crit} as defined by Fry's concept of aerobic scope; Fry, 1971; Claireaux and Chabot, 2016) and has been used previously (e.g. Claireaux et al., 2000), it is much less practical than methods based on $\dot{M}_{\text{O}_2,\text{std}}$ alone and is virtually impossible to perform on a single individual without the confounding training effects of repeatedly determining $\dot{M}_{\text{O}_2,\text{max}}$.

Recommendations

When selecting from the array of available respirometry and calculation methods in order to determine P_{crit} , we feel that the most important issue to consider is how the P_{crit} data will be used. No single combination of respirometry and calculation is ideal for all situations and scientific questions. For example, if one intends to compare P_{crit} data with existing literature values, then it is advisable to duplicate the methods of those studies as closely as possible. In many cases, this will be difficult, especially if the previous studies based their calculations on $\dot{M}_{\text{O}_2,\text{rtm}}$ instead of $\dot{M}_{\text{O}_2,\text{std}}$. Alternatively, if the intended use is to duplicate the O_2 dynamics of a species' natural environment, then it is advisable to use IF or CF respirometry so as to control the rate of $P_{\text{W}_{\text{O}_2}}$ decline as precisely as possible. Or, if the experimental species is a ram ventilator, then it is advisable to conduct P_{crit} trials in a swim flume even if this precludes the use of $\dot{M}_{\text{O}_2,\text{std}}$ as the benchmark oxyregulatory \dot{M}_{O_2} state.

Aside from scenarios like these, we feel that there are certain best practices that should be followed when performing respirometry and calculating P_{crit} . Foremost, the respirometry experiments should be conducted as carefully as possible. A calculated P_{crit} is only as accurate as the \dot{M}_{O_2} data on which it is based, and it is probably true that variation in respirometry experiments explains far more of the inter-study P_{crit} variation highlighted by Wood (2018) than variation in P_{crit} calculation method. The reader is guided to reviews by Clark et al. (2013) and Chabot et al. (2016) for details on best respirometry practices, but, briefly, these experiments should involve a habituation period of ≥ 24 h and a well-circulated water volume, and should control for P_{O_2} sensor drift and background microbial respiration. Importantly, unless the research question requires $\dot{M}_{\text{O}_2,\text{rtm}}$, the respirometry method should generate data from which accurate estimates of normoxic $\dot{M}_{\text{O}_2,\text{std}}$ can be obtained. This requires either IF or CF respirometry. Depending on the research question, generating $\dot{M}_{\text{O}_2,\text{std}}$ values not just at normoxia but at $P_{\text{W}_{\text{O}_2}}$ values throughout the \dot{M}_{O_2} versus $P_{\text{W}_{\text{O}_2}}$ curve is ideal.

For $P_{\text{W}_{\text{O}_2}}$ reduction, the technique depends largely on the desired rate of hypoxia induction, as these rates may significantly impact P_{crit} (Regan and Richards, 2017). High rates are useful for questions involving cross-species comparisons, which would benefit from determining an 'innate' P_{crit} that is minimally influenced by hypoxia acclimation. For this, CS respirometry is ideal, particularly when using a chamber volume:fish mass of $\sim 30:1$ (with the caveat that the fish's activity at moderately hypoxic $P_{\text{W}_{\text{O}_2}}$ may hamper an accurate P_{crit} calculation). Low, controlled rates of hypoxia induction are useful when addressing questions regarding hypoxia acclimation and/or when duplicating the O_2 dynamics of a species' native hypoxic environment. For these, IF or CF respirometry is ideal, both of which have the added benefits over CS of preventing

metabolic end-product accumulation and enabling best estimates of $\dot{M}_{\text{O}_2,\text{std}}$ at each $P_{\text{W}_{\text{O}_2}}$.

For P_{crit} calculation, we feel that a method based on empirical data is better than one based on extrapolation. Therefore, methods that use all (or most) available \dot{M}_{O_2} versus $P_{\text{W}_{\text{O}_2}}$ data, such as Yeager and Ultsch (1989) and Claireaux and Chabot (2016), are advised over methods that, for example, ignore \dot{M}_{O_2} values at moderately hypoxic $P_{\text{W}_{\text{O}_2}}$ (i.e. supra- P_{crit}) and instead anchor the oxyregulation line at the normoxic $\dot{M}_{\text{O}_2,\text{std}}$ value. This is most effectively done with \dot{M}_{O_2} data that approximate $\dot{M}_{\text{O}_2,\text{std}}$ at moderately hypoxic $P_{\text{W}_{\text{O}_2}}$. In all cases, accurate measurements of $\dot{M}_{\text{O}_2,\text{std}}$ in normoxia (i.e. ≥ 17 kPa $P_{\text{W}_{\text{O}_2}}$) are required. These can be made using methods outlined in Chabot et al. (2016), and they serve as a benchmark for \dot{M}_{O_2} values at moderately hypoxic $P_{\text{W}_{\text{O}_2}}$. If values approximating $\dot{M}_{\text{O}_2,\text{std}}$ cannot be measured at moderately hypoxic $P_{\text{W}_{\text{O}_2}}$ for either biological or methodological reasons (i.e. the curve is non-biphasic), then the investigator must decide whether to calculate P_{crit} using either a non-linear approach or one that anchors the oxyregulation line at the normoxic $\dot{M}_{\text{O}_2,\text{std}}$ value. In any case, all \dot{M}_{O_2} versus $P_{\text{W}_{\text{O}_2}}$ data should be presented.

Finally, there are best practices for reporting P_{crit} results that maximize their value to the research community. It is imperative that the methods used for respirometry, reducing $P_{\text{W}_{\text{O}_2}}$, and calculating P_{crit} are clearly described. Furthermore, the raw respirometry data should be presented, either in the manuscript or as supplementary material. So long as the respirometry experiments are properly executed and reported, the \dot{M}_{O_2} versus $P_{\text{W}_{\text{O}_2}}$ data will be useable by readers, thus enhancing the comparative value of the P_{crit} results. Furthermore, when comparing literature P_{crit} values, investigators should analyze these values and the methods used to determine them carefully to ensure the comparison is appropriate.

Conclusions: does P_{crit} give useful comparative information on hypoxia tolerance?

Tolerance can be defined in different ways. Generally, tolerance is the capacity to endure continued subjection to something without adverse reaction. In the fish-hypoxia literature, tolerance is rarely defined, but investigators are often concerned only with the limits of endurance, not continued endurance. These are different definitions with different ecological implications; 'continued endurance' implies sustained survival, reproduction and the capacity to do work, whereas 'limits of endurance' implies merely surviving. Neither definition is more valid than the other, but investigators should clearly state which they mean (or how they define synonymous phrases such as hypoxic performance, sensitivity and resistance), because certain metrics of 'tolerance' may be more appropriate for some definitions than others. Nevertheless, P_{crit} and most other metrics of tolerance are tied most closely to the 'limits of endurance' definition, and so we will proceed using this definition.

As previously mentioned, at P_{crit} , a fish retains zero aerobic scope for activity. While maintenance functions may be fueled aerobically at this $P_{\text{W}_{\text{O}_2}}$, routine activities relevant to biological fitness need to be fueled anaerobically. This is unsustainable – it is limited by finite fuel stores and accumulation of deleterious waste products – and thus P_{crit} does not realistically represent the lowest $P_{\text{W}_{\text{O}_2}}$ at which a fish can survive indefinitely. Rather, P_{crit} defines the lowest $P_{\text{W}_{\text{O}_2}}$ at which maintenance functions are supported aerobically, capturing the suite of aerobic contributions to hypoxia tolerance along a fish's O_2 transport cascade in a single value. Because the sum of these maintenance functions can be accurately measured as $\dot{M}_{\text{O}_2,\text{std}}$, P_{crit} serves as an effective comparator of the aerobic contributions to overall hypoxia tolerance. And because aerobic metabolism is a

significant contributor to hypoxia tolerance (and P_{crit} strongly correlates with the nadir $P_{W_{O_2}}$ of a species' natural environment; Childress and Seibel, 1998; Mandic et al., 2009; Rogers et al., 2016), P_{crit} is closely associated with overall hypoxia tolerance. We see no reason to discard this view if appropriate methodology is used to determine P_{crit} .

Another often-used measurement of hypoxia tolerance is loss of equilibrium (LOE), which can be quantified in two ways: (1) the time at which the fish can no longer right itself (i.e. loses equilibrium) when held at some hypoxic $P_{W_{O_2}}$; or (2) the $P_{W_{O_2}}$ at which the fish loses equilibrium under conditions of continually decreasing $P_{W_{O_2}}$. The longer the time or the lower the $P_{W_{O_2}}$, the more hypoxia tolerant the fish. Survival at either LOE point is obviously unsustainable (at least under the experimental conditions), but by accounting for the contributions of anaerobic metabolism and metabolic depression to an animal's hypoxic survival, LOE serves as a comprehensive index of hypoxia tolerance. P_{crit} , being associated with aerobic metabolism, does not account for these contributions. However, this does not negate the value of P_{crit} ; it simply means that P_{crit} represents something different (i.e. the capacity for aerobic metabolism in hypoxia, something LOE does not reveal).

Another indicator of hypoxia tolerance is the regulation index (RI; see Glossary) (Mueller and Seymour, 2011), which quantifies an animal's oxyregulatory ability. The RI is a dimensionless number that ranges from zero (perfect oxyconformation) to one (perfect oxyregulation) over a range of P_{O_2} from high (e.g. normoxic) to zero (anoxic). A fish with a higher RI would be considered more adapted to hypoxia than one with a lower RI. The model is most useful when the relationship between \dot{M}_{O_2} and P_{O_2} is a gradual curve, but in most cases with fishes it is not – the broken-stick model is the most common result. In this situation, one can still calculate an RI, but the same conclusion will be drawn – the fish with the lower P_{crit} will have the higher RI. Moreover, P_{crit} contains some informational value, as it has units, while the RI has none.

In summary, we assert that P_{crit} is a useful comparator of hypoxia tolerance, so long as it is determined and reported using best practices that maximize its comparative value among studies. We also assert that CF and IF respirometry give the most reliable estimates of $\dot{M}_{O_2, std}$, which under most circumstances is prerequisite to calculating P_{crit} . Once one has reliable $\dot{M}_{O_2, std}$ data over a large range of $P_{W_{O_2}}$, the calculation of P_{crit} , at least for fishes, is straightforward.

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Competing interests

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