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COMMENTARY

The fallacy of the P_{crit} – are there more useful alternatives?

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ABSTRACT

P_{crit} – generally defined as the P_{O_2} below which the animal can no longer maintain a stable rate of O_2 consumption ($\dot{M}_{O_2}$), such that $\dot{M}_{O_2}$ becomes dependent upon P_{O_2} – provides a single number into which a vast amount of experimental effort has been invested. Here, with specific reference to water-breathers, I argue that this focus on the P_{crit} is not useful for six reasons: (1) calculation of P_{crit} usually involves selective data editing; (2) the value of P_{crit} depends greatly on the way it is determined; (3) there is no good theoretical justification for the concept; (4) P_{crit} is not the transition point from aerobic to anaerobic metabolism, and it disguises what is really going on; (5) P_{crit} is not a reliable index of hypoxia tolerance; and (6) P_{crit} carries minimal information content. Preferable alternatives are loss of equilibrium (LOE) tests for hypoxia tolerance, and experimental description of full $\dot{M}_{O_2}$ versus P_{O_2} profiles accompanied by measurements of ventilation, lactate appearance and metabolic rate by calorimetry. If the goal is to assess the ability of the animal to regulate $\dot{M}_{O_2}$ from this profile in a mathematical fashion, promising, more informative alternatives to P_{crit} are the regulation index and Michaelis–Menten or sigmoidal allosteric analyses.

KEY WORDS: Critical oxygen tension, Standard metabolic rate, Routine metabolic rate, Active metabolic rate, Regulation index, Michaelis–Menten analysis, Oxyconformation, Oxyregulation, Water-breathers

Introduction

This critical Commentary focuses on the concept of the P_{crit} specifically in water-breathers, and particularly in fish, where the high density and viscosity of the respiratory medium makes the cost of breathing much higher than in air-breathers (Dejours, 1988). The concept originated with the theoretical work of Tang (1933), who examined the relationship between P_{O_2} and $\dot{M}_{O_2}$ in a variety of whole animals, plants, tissues and cells. He realized that, in most cases, these were best described by hyperbolic relationships with the dependent variable $\dot{M}_{O_2}$ (the rate of O_2 consumption) on the ordinate, and the independent variable P_{O_2} (the partial pressure of O_2) on the abscissa. He defined P_{crit} as 'the tension at which the curve approaches saturation'. This differs from the usage applied today, where P_{crit} is generally defined as 'the P_{O_2} below which the animal can no longer maintain a stable $\dot{M}_{O_2}$, such that $\dot{M}_{O_2}$ becomes dependent upon P_{O_2}' (Rogers et al., 2016). This usage can probably be traced back to the pioneering investigations of F. E. J. Fry on fish (e.g. Fry, 1947; Fry and Hart, 1948) and the idealized plots that he showed of two intersecting lines for $\dot{M}_{O_2}$ versus P_{O_2}, one with zero slope in a high P_{O_2} range (region of oxyregulation; see Glossary), and one with positive slope in a lower P_{O_2} range (region of oxyconformation; see Glossary) (Fig. 1). Fry actually called the P_{O_2} at the point of intersection 'the incipient limiting level' when determined for active (swimming) metabolism, and the 'level of no excess activity' (after Lindroth, 1942) when determined for standard metabolism. In various formats, these plots have been featured in many textbooks of comparative physiology (e.g. Prosser, 1973; Hoar, 1983; Schmidt-Nielsen, 1997; Hill et al., 2004), and have been remarkably effective in promoting the P_{crit} concept, though perhaps not an understanding of the limitations accompanying it.

As any researcher who has worked with real organisms will know, such relationships rarely occur in individual animals, and real data are not neatly ordered into two intersecting lines, one with zero slope and one with positive slope (see Box 1). Despite this, researchers have persevered using a variety of methodological and statistical techniques to determine this single number (P_{crit}), largely because it is widely believed to provide a reliable index of hypoxia tolerance – i.e. the lower the P_{crit}, the greater the hypoxia tolerance (but see below). Indeed, a recent excellent review (Rogers et al., 2016) found 331 P_{crit} measurements for fish alone, and there are probably a comparable number for aquatic invertebrates. I will argue that while the $\dot{M}_{O_2}$ versus P_{O_2} relationships in these studies are very valuable, the focus on the P_{crit} is misguided and counter-productive. I will provide six reasons why the P_{crit} is flawed, and finish by proposing more useful approaches for quantifying hypoxia tolerance and the relationship between $\dot{M}_{O_2}$ and P_{O_2}.

Calculation of P_{crit} usually involves selective data editing

Any scientific method that routinely involves the choice by the experimenter to use some data points and not others is worrisome. The basis of this choice is invariably that the real data do not fit the rigid paradigm of the P_{crit} – i.e. the animal did not do what it was 'expected' to do. In informal conversation, practitioners of P_{crit} determination will often comment that the data from certain animals had to be discarded, or individual $\dot{M}_{O_2}$ points had to be ignored, because they just didn't fit the model. Admirably, some researchers report this in the methods section of their papers and provide objective criteria or justification for how they edited the data.

For example, Richards et al. (2008), Henriksson et al. (2008) and McBryan et al. (2016) all noted that 'any fish that struggled was removed from the data set', while Dhillon et al. (2013) excluded data from fish which 'showed signs of distress'. McBryan et al. (2016) were also concerned about an increase that often occurred in $\dot{M}_{O_2}$ at declining P_{O_2} before P_{crit} was reached in killifish (see panel B in Box 1), which would cause a negative slope to the upper line. They therefore provided criteria for substituting a constant routine $\dot{M}_{O_2}$ value so the upper line would have a zero slope. Snyder et al. (2016), by contrast, studying shiner perch, kept a zero slope to the upper line based on the standard metabolic rate (SMR), but used some but not all of these elevated $\dot{M}_{O_2}$ points at declining P_{O_2} to define the slope of the lower line, 'as the elevated $\dot{M}_{O_2}$ was considered part of the physiological response to hypoxia'. As

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List of symbols and abbreviations

K_m	partial pressure of O_2 at which the rate of O_2 consumption is 50% of maximal under a defined condition, in a Michaelis–Menten relationship
LOE	loss of equilibrium
LOE_{crit}	critical oxygen tension at which an animal loses equilibrium
$\dot{M}_{O_2}$	rate of O_2 consumption
$\dot{M}_{O_2,max}$	maximum rate of O_2 consumption under a defined condition
MMR	maximum sustainable aerobic metabolic rate
P_{50}	partial pressure of O_2 at which the rate of O_2 consumption is 50% of maximal under a defined condition, in a sigmoidal allosteric relationship
P_{crit}	critical O_2 tension, the partial pressure of O_2 below which the animal can no longer maintain a stable $\dot{M}_{O_2}$ such that $\dot{M}_{O_2}$ becomes dependent upon partial pressure
P_{O_2}	partial pressure or tension of O_2
RI	regulation index
RMR	routine aerobic metabolic rate of a resting, non-feeding animal exhibiting only spontaneous activity
SMR	minimum aerobic metabolic rate needed to supply the needs of a fasting, resting animal at zero activity level

general guidance for P_{crit} determination, Claireaux and Chabot (2016) advocated 'removing outlying (low) $\dot{M}_{O_2}$ values, i.e. values that are so low that they are not expected until the animal is below P_{crit}'. In their example plots, they also excluded many values that may have been above SMR. Ultsch et al. (1981), studying toadfish, stated somewhat enigmatically that $\dot{M}_{O_2}$ values were accepted as estimates of SMR only 'if they did not differ by more than ±10% of the mean value of the bordering steady state SMRs'. Yeager and Ultsch (1989) noted that their method for calculating P_{crit} did not work for about 25% of the datasets in the literature. Barnes et al. (2011) excluded from their P_{crit} analysis the 44% of Atlantic salmon tested that did not meet their criteria for oxyregulation. Regan et al. (2017), studying goldfish 'excluded from our routine $\dot{M}_{O_2}$ estimation any $\dot{M}_{O_2}$ value that exceeded 1.5 times the standard deviation of an individual's average $\dot{M}_{O_2}$ between 13 and 21 kPa' for the line at high P_{O_2}, whereas for the line at low P_{O_2}, Regan and Richards (2017) used only '$\dot{M}_{O_2}$ values that were >15% below the mean routine $\dot{M}_{O_2}$ value'. McBryan et al. (2016) did the same, but used a threshold of '>12% of calculated routine $\dot{M}_{O_2}$'. This is just a small sample for illustration, but sufficient to demonstrate the heterogeneity of approaches used in data editing to calculate P_{crit}. I conclude that if a calculation protocol ignores a significant fraction of the real data, the resulting value is suspect, because it does not reflect how the animal performed, but rather how the experimenter wanted it to perform.

Glossary

Blood shunting
Redirection of blood flow to an alternative pathway.

Closed-system respirometry
The measurement of O_2 consumption by O_2 depletion in a sealed chamber.

Intermittent-flow respirometry
The measurement of O_2 consumption by sequentially flushing or regassing, then closing the respirometer, usually by automation, and then recording O_2 depletion during the short closed periods.

O_2 dissociation curve
The relationship between the partial pressure of O_2 and the percentage saturation of the blood with O_2.

Open-system respirometry
The measurement of O_2 consumption by the decrease in O_2 concentration between the inlet and outlet of a chamber receiving constant water flow.

Oxyconformation
The situation where O_2 consumption rate falls in direct proportion to the partial pressure of O_2 in the environment.

Oxyregulation
The situation where O_2 consumption rate is maintained constant, independent of the partial pressure of O_2 in the environment.

Regulation index (RI)
A relative measure of oxyregulation ability.

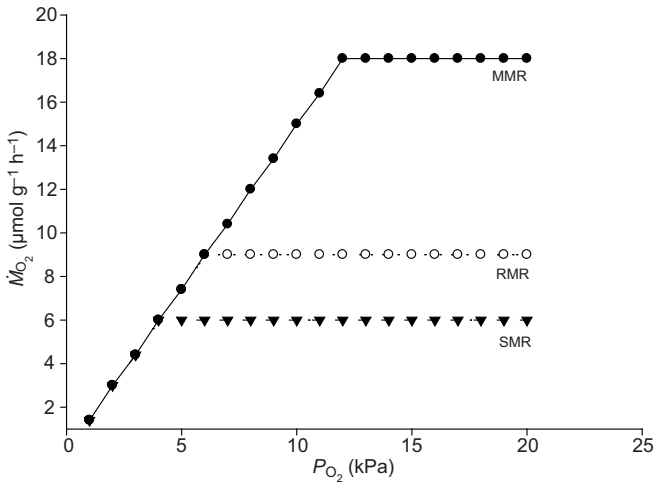

Fig. 1. The original framework of Fry (Fry, 1947; Fry and Hart, 1948) from which modern versions of the P_{crit} approach have evolved. The diagonal line is the line of respiratory dependence, where $\dot{M}_{O_2}$ (the rate of O_2 consumption) is directly proportional to ambient P_{O_2} (the O_2 partial pressure). The lowest horizontal line represents the standard metabolic rate (SMR), and the intersection of this line with the line of respiratory dependence gives the P_{O_2} for Fry's 'level of no excess activity' – in modern parlance, the critical O_2 tension under SMR conditions ($P_{crit,SMR}$). The middle horizontal line represents the routine metabolic rate (RMR), and the intersection of this line with the line of respiratory dependence gives the critical O_2 tension under RMR conditions ($P_{crit,RMR}$). The highest horizontal line represents the maximum metabolic rate (MMR), the $\dot{M}_{O_2}$ at the maximal activity level that is sustainable aerobically, and the intersection of this line with the line of respiratory dependence gives the P_{O_2} for Fry's 'incipient limiting level'.

The value of P_{crit} depends greatly on the way it is determined

Setting aside differences in the calculation method and the criteria for data editing, the value of P_{crit} obtained appears to depend very much on how the experiment has been done. How else can we explain the great variation in P_{crit} values for the same species reported by different investigators in Table 1, and illustrated for a single subspecies in Fig. 2? There has been much concern about possible differences associated with closed- versus open-system versus intermittent-flow respirometry (see Glossary), especially due to the potential for the build-up of waste (particularly CO_2) in the former. This concern may have been unwarranted, because there is surprisingly little evidence that this really matters. In their meta-analysis, Rogers et al. (2016) found no consistent differences for most species where parallel data were available, and this has been reinforced by nose-to-nose comparative studies (e.g. Cochran and Burnett, 1996; Regan and Richards, 2017), and studies where P_{crit} was determined at elevated P_{CO_2} levels (e.g. Heinrich et al., 2014), though exceptions exist (e.g. Snyder et al., 2016). In my opinion, closed-system respirometry, which has been used in the majority (56%) of fish studies to date (Rogers et al., 2016), is preferred simply because it is more ecologically realistic; natural hypoxia

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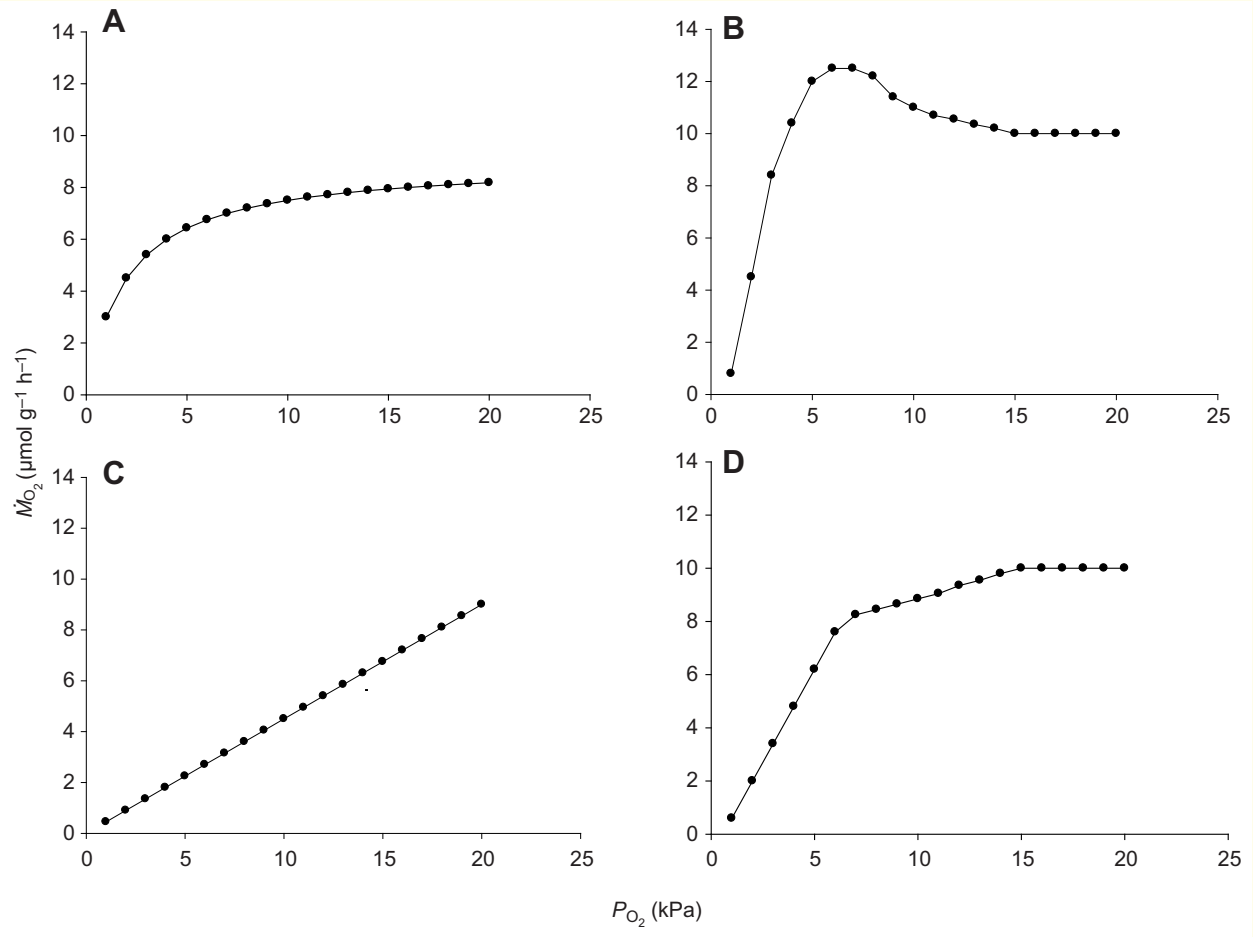
Box 1. How real data patterns challenge the P_{crit} paradigm

Although the original P_{crit} concept was that the slope of the relationship between the partial pressure of O_2 (P_{O_2}) and the rate of O_2 consumption ($\dot{M}_{O_2}$) at higher P_{O_2} values should be zero (i.e. $\dot{M}_{O_2}$ should be completely independent of P_{O_2} above the P_{crit}), this constraint has been abandoned by many investigators in the face of experimental data where the slope in the higher P_{O_2} range is clearly either positive or negative. These investigators have resorted to ignoring data that do not fit the paradigm or simply fitting the best two lines to the available data, or to more complex non-linear regression techniques to estimate P_{crit}. Indeed, there has been much debate about how best to 'fit square pegs into round holes' – i.e. how best to impose this rigid theoretical paradigm onto a messy biological reality with at least 13 different methods proposed to get the best possible estimate of P_{crit} (e.g. Yeager and Ultsch, 1989; Nickerson et al., 1989; Mueller and Seymour, 2011; Marshall et al., 2013; Claireaux and Chabot, 2016). The four panels of the figure show the relationships that are most commonly reported in actual experiments, illustrating how actual $\dot{M}_{O_2}$ data depart from the Fry ideal of Fig. 1. (A) Hyperbolic relationships – e.g. sturgeon (Nonnotte et al., 1993), epaulette shark (Routley et al., 2002; Speers-Roesch et al., 2012), grass shrimp (Cochran and Burnett, 1996), catfish (Zhang et al., 2010), tambaqui (Giacomin et al., 2018), goldfish (Regan et al., 2017). (B) As ambient P_{O_2} declines, $\dot{M}_{O_2}$ is initially close to stable, then increases before it eventually declines – e.g. brook trout, common carp and goldfish (Beamish, 1964), rainbow trout (Marvin and Heath, 1968; Ott et al., 1980). (C) $\dot{M}_{O_2}$ declines in almost direct proportion to the decrease in ambient P_{O_2} (i.e. oxyconformation)–e.g. toadfish (Hall, 1929), catfish (Marvin and Heath, 1968), sturgeon (Burggren and Randall, 1978), plaice (Steffensen et al., 1982), common carp (Lomholt and Johansen, 1979). (D) As ambient P_{O_2} declines, $\dot{M}_{O_2}$ first decreases with a more or less constant slope, and then transitions to a much sharper slope of decline – e.g. crucian carp (Nilsson, 1992), zebrafish (Feng et al., 2016), branquinha (Johannsson et al., 2018).



almost always involves concomitant CO_2 build-up, as originally noted by Fry and Hart (1948). An additional benefit is that it is much easier than other methods.

A more serious concern is whether the P_{crit} is determined under standard (SMR) or routine metabolic rate (RMR) conditions, a factor that is often overlooked when comparing P_{crit} values amongst species. Clearly, it is easier to do the experiment under RMR conditions, explaining why 84% of the fish studies in the literature used RMR (Rogers et al., 2016). Very probably, this involves less data editing, as it is so difficult to define SMR, and maintain SMR conditions. Like the P_{crit}, SMR is an artificial experimental construct which almost never occurs in nature. Based on the original paradigm of Fry and Hart (1948) and its conceptualization by Claireaux and Chabot (2016), we would expect P_{crit} to be higher under RMR than SMR conditions and indeed the higher RMR is above SMR, the higher the expected P_{crit} until Fry's 'incipient limiting level' is reached at maximum sustainable aerobic metabolic rate (MMR) (Fig. 1). While considerable evidence suggests that this general trend is true, I am aware of no rigorous comparison of P_{crit} values determined under normal resting laboratory criteria (i.e. well-acclimated, fasted, undisturbed animals) for RMR versus SMR on the same species. Regardless, in accord with Rogers et al. (2016),

Table 1. A selection of P_{crit} values reported in the literature for hypoxia-sensitive and hypoxia-tolerant fish

Species	Common name	P_{crit}	Conditions	Temperature (°C)	Data editing	Reference
Hypoxia-sensitive species						
Oncorhynchus mykiss	Rainbow trout	4.0–4.7	RMR	12	–	Scott et al. (2014)
		2.0–3.0	SMR	7–8	*	Zhang et al. (2018)
		2.8	SMR	10	*	Ott et al. (1980)
		2.9	SMR	15	*	Ott et al. (1980)
		3.7	SMR	20	*	Ott et al. (1980)
		12.6	RMR	12	–	Marvin and Heath (1968)
Salmo salar	Atlantic salmon	7.0–7.4	RMR	14–18	*	Barnes et al. (2011)
		10.8	RMR	22	*	Barnes et al. (2011)
Lepomis macrochirus	Bluegill sunfish	13.6	RMR	18	–	Robertson et al. (2015)
		15.4	RMR	25	–	Marvin and Heath (1968)
		13.6	RMR	13	–	Spitzer et al. (1969)
		3.6	RMR	15	–	Crans et al. (2015)
		4.6–6.3	RMR	25	–	Borowiec et al. (2015)
Aptychotrema rostrata	Shovelnose ray	7.2	RMR	28	–	Speers-Roesch et al. (2012)
Hypoxia-tolerant species						
Cyprinus carpio	Common carp	2.7	SMR	10	*	Ott et al. (1980)
		2.8	SMR	15	*	Ott et al. (1980)
		2.7	SMR	25	*	Ott et al. (1980)
		2.6	RMR	12	*	Dhillon et al. (2013)
		Conformer	RMR	–	–	Lomholt and Johansen (1979)
Carassius carassius	Crucian carp	2.7	RMR	12	*	Dhillon et al. (2013)
		3.1	RMR	18	–	Nilsson (1992)
		1.8	RMR	8	–	Sollid et al. (2003)
Carassius auratus	Goldfish	3.5	RMR	12	*	Dhillon et al. (2013)
		1.4	SMR	15	*	Fry and Hart (1948)
		3.3	SMR	35	*	Fry and Hart (1948)
		1.0–2.6	RMR	17	*	Regan and Richards (2017)
		0.7–3.0	RMR	17	*	Regan et al. (2017)
Fundulus heteroclitus	Killifish	8.5	RMR	19	*	Richards et al. (2008)
		4.4–5.3	RMR	20	*	McBryan et al. (2016)
		4.7	RMR	30	–	Cochran and Burnett (1996)
		3.7–5.3	RMR	21	–	Borowiec et al. (2015)
		Conformer	RMR	18	–	Blewett et al. (2013)
Astronotus ocellatus	Amazon oscar	4.5	RMR	28	–	De Boeck et al. (2013)
		6.7–9.3	RMR	28	–	Sloman et al. (2006)
		4.6–9.9	RMR	28	–	Scott et al. (2008)
Colossoma macropomum	Tambaqui	5.4	RMR	30	–	Saint-Paul (1984)
		5.5	RMR	28	–	Giacomin et al. (2018)
		3.9	RMR	28	–	Robertson et al. (2015)
Cyprinodon variegatus	Sheepshead minnow	7.6	RMR	20	–	Haney and Nordlie (1997)
Opsanus tau	Oyster toadfish	2.9–4.9	SMR	22	*	Ultsch et al. (1981)
		Conformer	RMR	20–21	–	Hall (1929)
Hemiscyllum ocellatum	Epaulette shark	6.5	RMR	25	–	Routley et al. (2002)
		8.1	RMR	28–29	*	Heinrich et al. (2014)
		5.1	RMR	28	–	Speers-Roesch et al. (2012)

SMR, standard metabolic rate; RMR, routine metabolic rate. *Criteria for selecting which data to use or not use were specified in the methods section of the respective article.

I favour the use of RMR for these types of studies as it is likely to be 'more ecologically relevant – in the field'.

The rate of P_{O_2} decline is another important but non-standardized factor highlighted by Rogers et al. (2016) that seems to greatly affect P_{crit}. Snyder et al. (2016) and Regan and Richards (2017) concluded that a slower rate of P_{O_2} decline resulted in a much lower P_{crit} in shiner perch and goldfish, respectively, presumably because a longer period allows more time for functional adjustments such as gill remodelling and improvement of the oxygen affinity of the haemoglobin. These can start to occur very rapidly (e.g. Tetens and Christensen, 1987; Matey et al., 2011), certainly within the time frame (minutes to a few hours) of a typical P_{crit} experiment. Alternatively, it may also allow more time for other adjustments such as the down-regulation of total metabolic rate or the up-regulation of anaerobic metabolism, which may explain the oxyconforming results of Blewett et al. (2013) on the killifish in Fig. 2.

The killifish is generally recognized as being very hypoxia tolerant (Burnett et al., 2007). Fig. 2 illustrates for a single subspecies, the northern race (*Fundulus heteroclitus macrolepidotus*), the heterogeneity of reported $\dot{M}_{O_2}$ versus P_{O_2} relationships. All tests were done at 18–21°C with well-acclimated, fasting, undisturbed fish of similar size under RMR conditions, though there were differences in salinity (4–20 ppt) and methodology (closed system versus intermittent flow) amongst studies. Routine $\dot{M}_{O_2}$ values at normoxic P_{O_2} varied by no more than 50%, yet the profiles at lower P_{O_2} differed greatly (Fig. 2). Indeed P_{crit} values varied from no P_{crit} (almost perfect oxyconformation; Blewett et al., 2013) to 8.5 kPa (Richards et al., 2008). The speed of hypoxia induction is undoubtedly an important issue. In the dataset where there was no P_{crit}, the P_{O_2} was gradually lowered over 7 h (Blewett et al., 2013), which may have allowed the animals to progressively suppress their metabolic rate and/or increase anaerobic metabolism so as to manifest as

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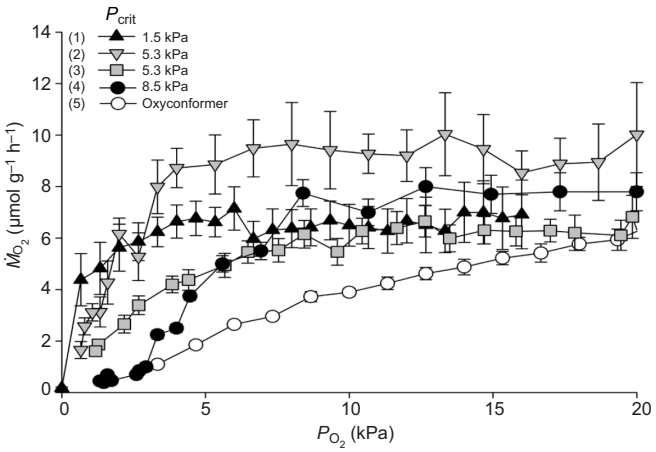

Fig. 2. Heterogeneity of $\dot{M}_{O_2}$ versus P_{O_2} relationships for a single fish species. A comparison of profiles of $\dot{M}_{O_2}$ (RMR conditions) versus ambient P_{O_2} in the northern subspecies of the killifish (*Fundulus heteroclitus macrolepidotus*), determined in different laboratories on well-acclimated, fasting, undisturbed fish of similar size (~5 g), at similar temperature (18–21°C) under RMR conditions, illustrating the heterogeneity of reported relationships and associated P_{crit} values (means±s.e.m.). The P_{crit} values are those reported by the authors. There were differences in salinity (4–20 ppt) and methodology (closed-system versus intermittent-flow respirometry) amongst studies. (1) M. Giacomin, P. Schulte and C.M.W., unpublished – 11 ppt, closed-system respirometry, P_{crit}=1.5 kPa; (2) McBryan et al. (2016) – 20 ppt, closed-system respirometry, P_{crit}=5.3 kPa; (3) Borowiec et al. (2015) – 4 ppt, intermittent-flow respirometry, P_{crit}=5.3 kPa; (4) Richards et al. (2008) – 10 ppt, closed-system respirometry, P_{crit}=8.5 kPa; (5) T. Blewett and C.M.W., unpublished; see also Blewett et al. (2013) – 16 ppt, closed-system respirometry, no P_{crit} (oxyconformer).

oxyconformers. In contrast, much more rapid lowering over 1–3 h resulted in P_{crit} values ranging from 4 to 8.5 kPa, although within this time frame, these values were not correlated to the measurement period. Interestingly, the two almost identical values (~5.3 kPa) were obtained using different methods (Borowiec et al., 2015: intermittent flow; McBryan et al., 2016: closed system), but as the salinities were different and only one of the studies used data editing, interpretation is confounded. Nevertheless, such wide unexplained variation among different laboratories in P_{crit} determination within a single subspecies is troublesome, and lessens trust in the index.

There is no good theoretical justification for P_{crit}

Setting aside the methodological criticisms above, the theoretical basis of the P_{crit} is questionable. The P_{crit} assumes a single, quantifiable point (P_{O_2}) at which the transition from oxyregulation to oxyconformation occurs. Yet, biological processes almost always reflect a smooth continuum of change, and it is difficult to see how or why such a complex multi-step process as O_2 consumption should exhibit a single sharp transition point. Certainly, the O_2 dissociation curve (see Glossary) of the blood does not have one (Dejours, 1988). Indeed, even the originators of the concept recognized that the real dependence of $\dot{M}_{O_2}$ on environmental P_{O_2} was closer to hyperbolic (Tang, 1933; Fry, 1947), and this is accepted by some modern workers in the field (e.g. Mueller and Seymour, 2011; Marshall et al., 2013; Claireaux and Chabot, 2016). However, even a hyperbolic relationship may not always be true, and there are a range of non-linear functions (e.g. Mueller and Seymour, 2011; Marshall et al., 2013) that may better fit the data in individual instances (Box 1; see also the next section and 'Concluding remarks', below).

Furthermore, Fry (1947) noted that the greatest respiratory dependence (i.e. the clearest transition from oxyregulation to oxyconformation) is seen not in resting animals but in animals respiring at their MMR. Indeed, Fry (1947) advocated measuring SMR separately, and then defining the relationship between MMR and P_{O_2}. The hypothetical intersection of a horizontal SMR line with the MMR versus P_{O_2} relationship would give his 'level of no excess activity' or P_{crit} (Fig. 1). This is close to the approach advocated by Claireaux and Chabot (2016), but differs greatly from most current approaches, which attempt to actually determine P_{crit} directly by progressively lowering P_{O_2} for animals respiring at their SMR or RMR, and then applying one of the many available calculation techniques (e.g. Marshall et al., 2013) to best estimate the transition point as P_{crit}. Regardless, none of these approaches provide a theoretical justification for a sharp transition point. I conclude that in the absence of such a theoretical framework, it is difficult to see any real benefit in trying to determine P_{crit}.

P_{crit} is not the transition point from aerobic to anaerobic metabolism, and it disguises what is really going on

The often-stated assumption is that, above the P_{crit}, the animal is able to meet all its needs for SMR or RMR aerobically (region of oxyregulation), whereas below P_{crit}, aerobic metabolism is reduced and an increasing amount of anaerobic metabolism is needed (region of oxyconformation). This is not true. Some contribution of anaerobic metabolism occurs even at high environmental P_{O_2} – otherwise, animals would not normally have lactate in their blood and tissues when respiring under normoxia (e.g. Nonnotte et al., 1993; Maxime et al., 2000; Routley et al., 2002). More importantly, when organisms at RMR or SMR are subjected to progressively declining P_{O_2}, the animal makes adjustments to improve the conditions for O_2 uptake long before the region of oxyconformation is reached. These include changes in the pattern of cardiac output (bradycardia, increased stroke volume), changes in effective gill permeability [lamellar recruitment, thinning of the diffusion barrier, blood shunting (see Glossary) in the gills], changes in arterial–venous O_2 content difference and, most importantly, increases in ventilation (discussed by Perry et al., 2009). This was first noted by van Dam (1938), and there are many reports in the literature of ventilation increasing long before the apparent P_{crit} is reached during declining P_{O_2} treatments. Particularly clear examples are summarized in review papers – see, for example, fig. 2 of McMahon (1988) (crabs) and fig. 5.3 of Perry et al. (2009) (teleost fish).

While all of these adjustments carry metabolic cost, the greatest is undoubtedly the expense of increased breathing. Estimates of the cost of breathing such a dense, viscous medium as water range greatly (0.2–70% of metabolic rate; McMahon, 1988), but 10–20% is probably a reasonable value (Schumann and Piiper, 1966; Cameron and Cech, 1970; Edwards, 1971; Jones and Schwarzfeld, 1974; Steffensen, 1985; Farrell and Steffensen, 1987), and this ventilatory cost will increase in absolute terms as ventilatory frequency and ventilatory stroke volume increase. Therefore, as P_{O_2} declines, an increasing percentage of total $\dot{M}_{O_2}$ is consumed by increased breathing and other physiological adjustments, so less and less is available for the maintenance needs of SMR or RMR, which must now be either fuelled by anaerobic metabolism or reduced by a depression of true metabolic rate. Both are likely to occur, long before apparent P_{crit} is reached. While absolute $\dot{M}_{O_2}$ may be maintained down to the apparent P_{crit}, the needs of SMR or RMR are not. Indeed, the apparent P_{crit} often occurs around the P_{O_2} at which the animal abandons hyperventilation (Perry et al., 2009), probably because it is so expensive. As McMahon (1988) notes: 'all of the additional O_2 acquired is used to fuel the pumps, with no net gain to the organism'.

The study of Maxime et al. (2000), who subjected turbot to a progressive decline of P_{O_2} down to 2.7 kPa over about 300 min, is particularly informative. The apparent P_{crit} under SMR conditions was about 4 kPa. However, ventilatory frequency and stroke volume, and plasma and muscle lactate concentrations had all increased significantly by the time a P_{O_2} of 8 kPa was reached, and upon restoration of normoxia, the O_2 debt repaid over the next 360 min was 16-fold greater than the O_2 deficit exhibited in the 100 min period during which P_{O_2} declined from 4 kPa to 2.7 kPa. Clearly, a massive anaerobic contribution had occurred prior to the point of apparent P_{crit}.

The earlier quotation from McMahon (1988) captures an important issue that P_{crit} practitioners wish would not happen: 'additional O_2' is often taken up as the animal hyperventilates, so even a hyperbolic relationship may not apply for the $\dot{M}_{O_2}$ versus P_{O_2} plot. Instead, $\dot{M}_{O_2}$ actually increases as P_{O_2} falls (see panel B in Box 1), due mainly to the increased costs of this hyperventilation (though excitement may also contribute) and the slope of the upper 'oxyregulation' line becomes negative and often bumpy. This was first reported by van Dam (1938). The phenomenon is especially evident when the experimenter is attempting to measure P_{crit} under SMR conditions. The classic work by Beamish (1964; one of Fry's students) is an excellent example, and indeed this pattern had been anticipated by Fry (1947) himself. Beamish (1964) used a warm-bulb flowmeter to measure spontaneous activity, and estimated the $\dot{M}_{O_2}$ at SMR by extrapolation to zero activity. Using brook trout, carp and goldfish, he found that this $\dot{M}_{O_2}$ increased by 20–80% as P_{O_2} declined, and only below a much lower P_{O_2} did $\dot{M}_{O_2}$ finally decline in the region of oxyconformation. He attributed the increase to the cost of hyperventilation. Ott et al. (1980) reported similar phenomena in rainbow trout. In summary, I conclude that a simple P_{crit} value disguises all of this physiological complexity, and therefore it is misleading.

The P_{crit} is not a reliable index of hypoxia tolerance

The reason why P_{crit} has been so often measured is because it is widely believed to provide a reliable indicator of hypoxia tolerance – the lower the P_{crit}, the greater the hypoxia tolerance (e.g. Mandic et al., 2009). It probably does not. Some of the most hypoxia-tolerant fish, capable of resisting severe hypoxia for prolonged periods, have unremarkable P_{crit} values, which often vary considerably among reports (Table 1). Examples of organisms where apparent P_{crit} values appear to overlap those of hypoxia-sensitive species include killifish, Amazon oscar, tambaqui, oyster toadfish and epaulette shark (Table 1). In many cases, the strategy for survival in hypoxia of these animals is not the ability to maintain $\dot{M}_{O_2}$ but rather the ability to suppress metabolic rate (Nilsson and Renshaw, 2004): in the words of Kjell Johansen: 'turning down the pilot light' (Hochachka and Somero, 2002).

The P_{crit} value would be expected to work best as a measure of hypoxia tolerance within a single species studied by the same investigators, but the evidence is problematical. Sometimes it works (e.g. McBryan et al., 2016), but often it does not. For example, in killifish, significant changes in P_{crit} caused by acclimation to various hypoxia regimes were generally not accompanied by significant changes in the critical oxygen tension at which the fish lost equilibrium (LOE_{crit}) (Borowiec et al., 2015). In the sheepshead minnow, acclimation to progressively higher salinities (40–100 ppt) was accompanied by progressive increases in P_{crit}, but the P_{O_2} causing lethality did not change (Haney and Nordlie, 1997). Differences in strain and ploidy of trout were accompanied by differences in LOE_{crit} but not in P_{crit} (Scott et al., 2014).

The P_{crit} would also be expected to predict hypoxia tolerance when studied in closely related species within the same lab by the same investigators. However, Dhillon et al. (2013) found no relationship between P_{crit} and LOE_{crit} in nine closely related carp species, whether the data were corrected for phylogeny or not. Fu et al. (2014) concluded that LOE_{crit} was a more reliable indicator than P_{crit} of hypoxia tolerance in 12 different cyprinids. Mandic et al. (2013) had slightly greater success, showing a significant correlation between P_{crit} and time to loss of equilibrium (LOE) in constant hypoxia (0.85 kPa) in 11 closely related sculpin species. However, the correlation lost significance when corrected for phylogeny. An additional study by Speers-Roesch et al. (2013) conducted at a fixed percentage (30%) of P_{crit} for three of these sculpin species also failed to show the correlation. Overall, I conclude that the P_{crit} value is not a useful indicator of hypoxia tolerance: there are more reliable and easier ways to measure hypoxia tolerance, which are explored below.

P_{crit} carries minimal information content

There is now an unfortunate tendency to report only P_{crit} values, and not the whole relationship between $\dot{M}_{O_2}$ and P_{O_2}. P_{crit} is simply the P_{O_2} approximating an inflection point, which means very little. Some traditional (e.g. Yeager and Ultsch, 1989) and modern methods (e.g. Claireaux and Chabot, 2016) for P_{crit} determination simply impose two lines on the data and do not attempt to describe the relationship between $\dot{M}_{O_2}$ and P_{O_2}. As pointed out by Mueller and Seymour (2011), a P_{crit} value says nothing about the relationship above or below the P_{crit}. For example, is the slope above the P_{crit} positive (Box 1, panel D) or negative (Box 1, panel B)? Is the whole relationship hyperbolic (Box 1, panel A), or is it closer to a straight line (Box 1, panel C), or to two straight lines (Fig. 1)? How close or distant from oxyconformation is the whole relationship? Does $\dot{M}_{O_2}$ continue right down to zero P_{O_2}? Indeed, a low apparent P_{crit} value does not necessarily even indicate a greater ability to regulate $\dot{M}_{O_2}$ in the face of hypoxia, as the $\dot{M}_{O_2}$ above the P_{crit} may have actually increased before it decreased (Box 1, panel B), or decreased more gradually before it decreased more steeply (Box 1, panel D).

Concluding remarks: the alternatives

If the goal of the experimenter is to simply measure hypoxia tolerance, a much easier and better way is to measure LOE_{crit} or time to LOE, because these are straightforward and relatively non-subjective determinations: when the animal can no longer maintain equilibrium, it is ecologically dead, a meaningful endpoint. For both, however, the conditions must be standardized in terms of the criteria for LOE (e.g. first overturn, or failure to right the body position after prodding), rate of P_{O_2} decline for LOEcrit and absolute P_{O_2} for time to LOE. To date, however, this has not been done, with each investigator tending to use their own species-specific protocols, so there is a need to develop standardized guidelines for LOE tests.

If the goal is to describe the relationships between $\dot{M}_{O_2}$ and P_{O_2}, then there is nothing the matter with just doing this for different species and treatments, and then comparing specific points and slopes on the $\dot{M}_{O_2}$ versus P_{O_2} profiles, rather than just focusing in on one number, the apparent P_{crit}. If the goal is to understand what is really going on, then these profile determinations should be accompanied by other physiological measures that provide mechanistic information. Two very important ones are the quantification of breathing (to assess the role of ventilatory costs) and the measurement of blood or tissue lactate (to assess the onset

and extent of increased anaerobic metabolism), both as a function of declining P_{O_2}. A third is to measure the total metabolic rate by direct calorimetry (van Ginneken et al., 1994; Regan et al., 2013), so as to evaluate the extent of metabolic depression, although the technology is not yet widely available. Finally, if the goal is to assess the ability of the animal to regulate $\dot{M}_{O_2}$ from these profiles and express this mathematically, there are two approaches that are much better than P_{crit}: the regulation index and Michaelis–Menten analysis.

The first was originally conceived by Alexander and McMahon (2004), who called it the 'regulation value' (R). It was then further developed on a different mathematical basis and popularized by Mueller and Seymour (2011), who termed it the 'regulation index' (RI; Fig. 3), the term which is more widely used today. The approach can be applied to $\dot{M}_{O_2}$ recorded under either standard or routine conditions. Briefly, the RI provides a relative measure of regulatory ability by calculating the area under the $\dot{M}_{O_2}$ versus P_{O_2} curve that is greater than a linear trend [i.e. the area above the diagonal line of oxyconformation joining the $\dot{M}_{O_2}$ at normoxia (e.g. X $\dot{M}_{O_2}$ at 20.9 kPa) with the origin (0 $\dot{M}_{O_2}$ at 0 kPa)] and then dividing it by the total available area calculated in the same way for perfect oxyregulation (i.e. the area whose upper bound is defined by the horizontal line of perfect regulation). As defined by Mueller and Seymour (2011), this fractional index can vary from 0.0 (perfect oxyconformation) to 1.0 (perfect oxyregulation), but my own interpretation is that there is no reason why the value could not be above 1.0 for organisms in which $\dot{M}_{O_2}$ increases greatly above normoxic values before declining, or less than 0.0 for organisms where $\dot{M}_{O_2}$ falls below the line of perfect oxyconformation; indeed, the original approach of Alexander and McMahon (2004) allowed for this possibility (see their fig. 1, where an R value less than 50% represents an RI value less than 0.0). One valuable feature of the RI is that it captures information embodied in the whole $\dot{M}_{O_2}$ versus P_{O_2} profile, not just in a single apparent P_{crit} value. Another benefit is that it is not restricted to any particular model, but can be used with the equation that best fits the data.

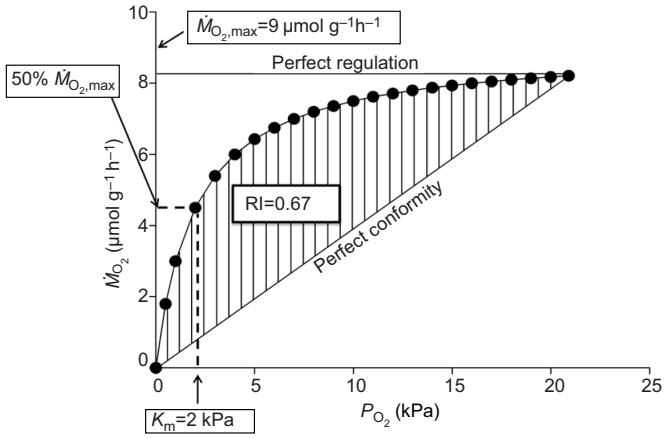

Fig. 3. Alternative analyses of $\dot{M}_{O_2}$ versus P_{O_2} profiles. Two alternative analyses of $\dot{M}_{O_2}$ versus P_{O_2} profiles that are more useful than the P_{crit} approach: the regulation index (RI) and Michaelis–Menten analysis (which can be extended to a sigmoidal allosteric analysis) (see text for details). A hypothetical hyperbolic relationship with a K_m of 2 kPa and an $\dot{M}_{O_2,max}$ of 9 µmol g^{-1} h^{-1} is shown, bracketed by the lines of perfect conformity and perfect regulation. The cross-hatched area divided by the total area available between these lines represents the RI, which is 0.67 in this example. Note that the $\dot{M}_{O_2,max}$ occurs at a higher P_{O_2} than 20.9 kPa (air saturation). As defined for the RI, the horizontal line is drawn for the $\dot{M}_{O_2}$ (just above 8.0) recorded at air saturation. The approaches can be applied to $\dot{M}_{O_2}$ recorded under either standard or routine conditions.

The second approach to assessing the ability of the animal to regulate $\dot{M}_{O_2}$ is based on enzyme kinetics. This approach uses a non-linear regression model to fit a curve to the entire dataset, and from this derive constants that describe the affinity of the whole animal for O_2, and the capacity of the animal for O_2 uptake. One such model that has proven useful is the Michaelis–Menten equation:

$$\dot{M}_{O_2} = \frac{\dot{M}_{O_2,max} \times P_{O_2}}{K_m + P_{O_2}}. \quad (1)$$

By analogy to enzyme kinetics, P_{O_2} is the substrate, the organism is the enzyme and $\dot{M}_{O_2}$ is the rate (Fig. 3). The $\dot{M}_{O_2,max}$ is the maximum $\dot{M}_{O_2}$ under the experimental conditions (which may be approximately at or above the $\dot{M}_{O_2}$ under normoxia) and K_m is the P_{O_2} at which $\dot{M}_{O_2}$ is 50% of $\dot{M}_{O_2,max}$. In essence, K_m represents the affinity of the organism for O_2: the lower the K_m, the greater the affinity. This works well when the $\dot{M}_{O_2}$ versus P_{O_2} profile approximates a hyperbolic relationship. In other situations, the profile may better approximate a sigmoidal relationship, and here an allosteric sigmoidal model would be more appropriate (Dr R. S. Seymour, personal communication):

$$\dot{M}_{O_2} = \frac{\dot{M}_{O_2,max} \times P_{O_2}^h}{P_{50}^h + P_{O_2}^h}.$$

where P_{50} (analogous to the K_m) is the P_{O_2} that produces half of $\dot{M}_{O_2,max}$ and h is the Hill's number (the slope of the relationship on double logged axes; if h=1.0, then the equation is the same as the Michaelis–Menten equation).

While the RI can still be determined from these non-linear regression approaches (e.g. Fig. 3), they provide much more information than the RI, because the derived constants (K_m or P_{50}, $\dot{M}_{O_2,max}$ and h) describe the whole relationship mathematically. I would argue that the affinity of the organism for O_2 is much more meaningful than the P_{crit}, as it integrates the net effect of all the gradually changing processes that occur as the organism encounters declining P_{O_2}. It would, for example, be very instructive to compare this K_m or P_{50} value with the whole-blood P_{50} for O_2 among species and experimental treatments. There is additional information content in $\dot{M}_{O_2,max}$, as it predicts what the $\dot{M}_{O_2}$ would be under these conditions if environmental P_{O_2} availability were not a limiting factor. To illustrate, as activity level increases, and RMR moves upwards towards MMR (Fig. 1), we would expect $\dot{M}_{O_2,max}$ to increase. And if at the same time, the O_2 diffusing capacity of the gills were to increase through changes in their effective permeability to O_2 (see the section on the transition from aerobic to anaerobic metabolism, above), we would expect K_m or P_{50} to decrease. And finally, if there really is ever a need to extract an estimate of the apparent P_{crit} value, this can be done mathematically using the 'greatest difference' method or other approaches if $\dot{M}_{O_2,max}$, K_m or P_{50}, and h are known, as explained by Mueller and Seymour (2011) and Marshall et al. (2013). Use of all of these alternative approaches to the P_{crit} summarized in this section will help improve our understanding and quantification of hypoxia tolerance in water-breathers.

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