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RESEARCH ARTICLE

Interspecific variation in hypoxia tolerance and hypoxia acclimation responses in killifish from the family Fundulidae

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ABSTRACT

Hypoxia is a pervasive stressor in aquatic environments, and both phenotypic plasticity and evolutionary adaptation could shape the ability to cope with hypoxia. We investigated evolved variation in hypoxia tolerance and the hypoxia acclimation response across fundulid killifishes that naturally experience different patterns of hypoxia exposure. We compared resting O_2 consumption rate ($\dot{M}_{O_2}$), and various indices of hypoxia tolerance [critical O_2 tension (P_{crit}), regulation index (RI), O_2 tension (P_{O_2}) at loss of equilibrium (P_{LOE}) and time to LOE (t_{LOE}) at 0.6 kPa O_2] in *Fundulus confluentus*, *Fundulus diaphanus*, *Fundulus heteroclitus*, *Fundulus rathbuni*, *Lucania goodei* and *Lucania parva*. We examined the effects of chronic (28 days) exposure to constant hypoxia (2 kPa) or nocturnal intermittent hypoxia (12 h normoxia:12 h hypoxia) in a subset of species. Some species exhibited a two-breakpoint model in $\dot{M}_{O_2}$ caused by early, modest declines in $\dot{M}_{O_2}$ in moderate hypoxia. We found that hypoxia tolerance varied appreciably across species: *F. confluentus* was the most tolerant (lowest P_{LOE} and P_{crit}, longest t_{LOE}), whereas *F. rathbuni* and *F. diaphanus* were the least tolerant. However, there was not a consistent pattern of interspecific variation for different indices of hypoxia tolerance, with or without taking phylogenetic relatedness into account, probably because these different indices are underlain by partially distinct mechanisms. Hypoxia acclimation generally improved hypoxia tolerance, but the magnitude of plasticity and responsiveness to different hypoxia patterns varied interspecifically. Our results therefore suggest that hypoxia tolerance is a complex trait that is best appreciated by considering multiple indices of tolerance.

KEY WORDS: Hypoxia resistance, Evolutionary physiology, Phylogenetically independent contrasts, Respirometry, Diurnal hypoxia

INTRODUCTION

Hypoxia is a pervasive stressor in aquatic environments that can originate from natural and anthropogenic events (Breitburg et al., 2009; Diaz and Breitburg, 2009; Diaz and Rosenberg, 2008). Aquatic hypoxia is most simply defined as a reduction in water O_2 levels that compromises some aspect of physiological function (Farrell and Richards, 2009). Severe bouts of hypoxia are often implicated in fish kills, and hypoxic episodes can have broad ecological implications by reducing available habitat, altering species distributions, and changing trophic relationships (Breitburg et al., 2009; Mallin et al., 2006). Even brief exposure to less severe reductions in O_2 availability can also affect fish physiology. Hypoxia-prone zones like tidal pools and estuaries are typically occupied by relatively hypoxia-tolerant organisms (Bickler and Buck, 2007; Chapman et al., 2002, 1995; Mandic et al., 2009b; Pollock et al., 2007; Richards, 2011; Wu, 2002).

Several metrics are used as indices of hypoxia tolerance in aquatic organisms. Many species maintain resting rates of oxygen consumption ($\dot{M}_{O_2}$) across a range of high O_2 tensions (P_{O_2}) but will exhibit progressive declines in $\dot{M}_{O_2}$ at low P_{O_2}. The transition from oxyregulation to oxyconformation of $\dot{M}_{O_2}$, which occurs at a P_{O_2} that is termed the critical O_2 tension (P_{crit}), is one common measure of hypoxia tolerance (Regan et al., 2019; Richards, 2009; Rogers et al., 2016). P_{crit} is often calculated as the breakpoint in a two-segmented linear regression (Yeager and Ultsch, 1989). However, studies have found that some species do not exhibit true oxyregulation; some species appear to oxyconform, in which $\dot{M}_{O_2}$ declines progressively as P_{O_2} falls from normoxic levels (Urbina et al., 2012; Wood, 2018), and the patterns of $\dot{M}_{O_2}$ variation cannot be adequately fitted using two-segmented regression. For this and other reasons, the value of P_{crit} as an index of hypoxia tolerance has been debated recently (Regan et al., 2019; Wood, 2018). Alternative metrics that summarize the P_{O_2} dependence of $\dot{M}_{O_2}$, such as the regulation index (a measure of the relative degree of oxyregulation), have been proposed to overcome these criticisms (Mueller and Seymour, 2011; Wood, 2018), but these metrics may represent different physiological information (Regan et al., 2019). Hypoxia tolerance is also reflected by the ability to resist the loss of equilibrium (LOE) in severe hypoxia, as either the time to LOE at a constant level of severe hypoxia (t_{LOE}) or the P_{O_2} at LOE (P_{LOE}) during progressive hypoxia (Borowiec et al., 2016; Crans et al., 2015; Dhillon et al., 2013; Mandic et al., 2013; McBryan et al., 2016). These various indices of hypoxia tolerance have sometimes (Dan et al., 2014; Mandic et al., 2013; Yang et al., 2013), but often not (Crans et al., 2015; Dhillon et al., 2013; Fu et al., 2014; Mathers et al., 2014; Speers-Roesch et al., 2013), been found to co-vary across species.

Fish can respond to and cope with hypoxia by enacting cardiorespiratory adjustments that help maintain cellular O_2 supply, and thus maintain aerobic metabolism, or by reducing cellular O_2 demands via anaerobic metabolism or metabolic depression (Farrell and Richards, 2009; Richards, 2009, 2010). The ability to maintain cellular O_2 supply can obviate the need to reduce cellular O_2 demands, and the relative ability of fish to safeguard cellular O_2 levels should affect their ability to survive in hypoxia. For this reason, indices of hypoxia tolerance that reflect the ability to maintain cellular O_2 supply, such as P_{crit}, are expected to correlate with those that reflect imminent death, such as t_{LOE} or P_{LOE} (McBryan et al., 2016; Speers-Roesch et al., 2013). However, survival in hypoxia could also vary between species depending on

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Received 27 June 2019; Accepted 20 January 2020

Journal of Experimental Biology (2020) 223, jeb209692. doi:10.1242/jeb.209692

List of abbreviations

ASR	aquatic surface respiration
LOE	loss of equilibrium
$\dot{M}_{O_2}$	rate of O_2 consumption
P_{crit}	critical O_2 tension
P_{LOE}	P_{O_2} at loss of equilibrium
P_{O_2}	partial pressure of O_2
RI	regulation index
t_{LOE}	time to loss of equilibrium

their relative ability to reduce cellular O_2 demands, even between species encountering the same reduction in cellular O_2 levels, and in such cases there might be a poor correlation between P_{crit} and t_{LOE} or P_{LOE} (Barnes et al., 2011; Crans et al., 2015; Dhillon et al., 2013; Speers-Roesch et al., 2013). By this rationale, a poor correlation between P_{crit} and t_{LOE} or P_{LOE} could suggest that a reduction in cellular O_2 demands, or the ability to cope with the secondary consequences of such a reduction (e.g. metabolic acidosis), plays a greater role in determining variation in the ability to survive hypoxia exposure.

Variation in hypoxia tolerance results from developmental plasticity, adult phenotypic plasticity and/or evolutionary innovation, in association with variation in many underlying physiological traits. Adult fish can show substantial plasticity that improves hypoxia tolerance in response to chronic hypoxia exposure, in association with morphological and physiological changes in a number of underlying traits involved in oxygen uptake, transport and utilization (Borowiec et al., 2015; Burnett et al., 2007; Du et al., 2016; Fu et al., 2011; Greaney et al., 1980; Martinez et al., 2006; Søllid et al., 2003; Sollid and Nilsson, 2006). Exposure to hypoxia during early development can also can elicit changes in hypoxia tolerance and in other phenotypic traits that persist into adulthood (Blank and Burggren, 2014; Heinrich et al., 2011; Robertson et al., 2014). Enhanced hypoxia tolerance can also evolve over generations, such as when species evolve to become more specialized for life in hypoxia-prone environments, and can be associated with evolved changes in the underlying determinants of O_2 transport and utilization (Hopkins and Powell, 2001; Mandic et al., 2009b; Regan et al., 2017b; Richards, 2011). However, we know much less about the interactions between these processes – namely, the extent to which the plastic responses to chronic hypoxia might differ between species. Some evidence from African cichlids suggests that adaptation to hypoxia-prone swamps can attenuate the changes in brain size in response to developmental hypoxia (Chapman et al., 2008; Crispo and Chapman, 2010), but we know relatively little about the magnitude of interspecific variation in the plasticity of hypoxia tolerance across closely related species.

Killifish from the family Fundulidae (Fig. 1) are well suited for evaluating the roles of phenotypic plasticity and evolutionary innovation in the tolerance of challenging environments. Species of this family are widely distributed and occupy habitats spanning a range of dissolved oxygen, salinity, pH, temperature and other environmental factors (Burnett et al., 2007; Whitehead, 2010; Whitehead et al., 2013). Considerable variation in physiological tolerance of environmental stressors occurs across this family, and even closely related sister taxa can have very different tolerances (Griffith, 1974; Nordlie, 2006; Whitehead, 2010; Whitehead et al., 2013), making Fundulidae a particularly useful model for evolutionary physiology (Burnett et al., 2007). *Fundulus heteroclitus* exhibits significant plasticity to chronic hypoxia (Borowiec et al., 2015, 2018; Du et al., 2016; Martinez et al., 2006) as well as intraspecific variation in hypoxia tolerance (McBryan et al., 2016), but it is unclear how hypoxia tolerance and its plasticity vary across the family. Therefore, our first two objectives were to characterize (i) the interspecific variation in hypoxia tolerance across Fundulidae and (ii) whether there is interspecific variation in the plasticity of hypoxia tolerance in response to chronic hypoxia. Finally, given recent debate surrounding P_{crit} and the most appropriate indices of hypoxia tolerance in fishes (Regan et al., 2019; Wood, 2018), our third objective was (iii) to examine whether there is a strong correlation or an uncoupling of different indices of hypoxia tolerance across a variety of taxa and patterns of hypoxia exposure.

MATERIALS AND METHODS

Study animals and husbandry

Wild populations of killifish were collected with minnow traps, dip nets or seine nets. *Fundulus confluentus* Goode and Bean 1879 and *Fundulus heteroclitus* (Linnaeus 1766) were collected from Jekyll Island, GA, USA (31.1039°N, 81.4061°W) with minnow traps. *Lucania parva* (Baird and Girard 1855) and *Lucania goodei* Jordan 1880 were collected from the Wakulla River, FL, USA (30.1761°N, 84.245°W) using dip nets. *Fundulus diaphanus* (Lesueur 1817) and *Fundulus rathbuni* Jordan and Meek 1889 were collected from Lake Opinicon, ON, Canada (44.559°N, −76.328°W) and Chapel Hill, NC, USA (35.9266474N, −79.0318428W), respectively, with a beach seine. Fish were treated with a 24 h cupramine soak (to treat ectoparasites) followed by 2 weeks of praziquantel treatment (to treat internal worm infections) upon arrival at Louisiana State University, during which they were held at a salinity comparable to that at the collection site (i.e. <0.2 ppt or 12 ppt). Fish were then slowly acclimated to common 0.1 ppt conditions over the course of the next 1–2 weeks and were then held at that salinity until any experimentation began (see below). Experiments were carried out at Louisiana State University for all taxa except *F. diaphanus*, for which experiments were carried out at McMaster University, and care was taken to provide consistent housing conditions (similar densities, aquarium sizes, etc.) and equivalent experimental treatments across all species at both sites.

At Louisiana State University, fish were initially housed in a filtered recirculating rack system in aerated (normoxic) water (~20 kPa, 8 mg O_2 l^{-1}) with a salinity of 0.1 ppt, at room temperature (22–26°C) on a 12 h light:12 h dark photoperiod, and were then transferred to 35 l aquaria for 28 day experimental acclimation. At McMaster University, fish were initially housed in 35 l glass aquaria under the conditions described above and were kept in these aquaria for experimental acclimation. Fish at both sites were fed daily with commercial fish pellets (Cargill, Minneapolis, MN, USA). Water chemistry was monitored at least once per week (measurements were similar across tanks and ranged as follows: ammonia <1.0 ppm, nitrates <10 ppm, nitrites <1.0 ppm; pH 8.0–8.3), and water changes were performed as necessary to maintain good water quality. Acclimations to normoxia and hypoxia (see below), respirometry and hypoxia tolerance measurements were conducted in aquaria at least 1 month after arrival at McMaster University or Louisiana State University. All fish were held at room temperature during all acclimations and hypoxia tolerance experiments. Daily temperatures ranged from 22.0 to 26.0°C over the course of the study, but average temperatures for acclimations and hypoxia tolerance experiments did not vary between species or acclimation P_{O_2}. For normoxia and hypoxia acclimations, fish were prevented from accessing the water surface using a plastic grid

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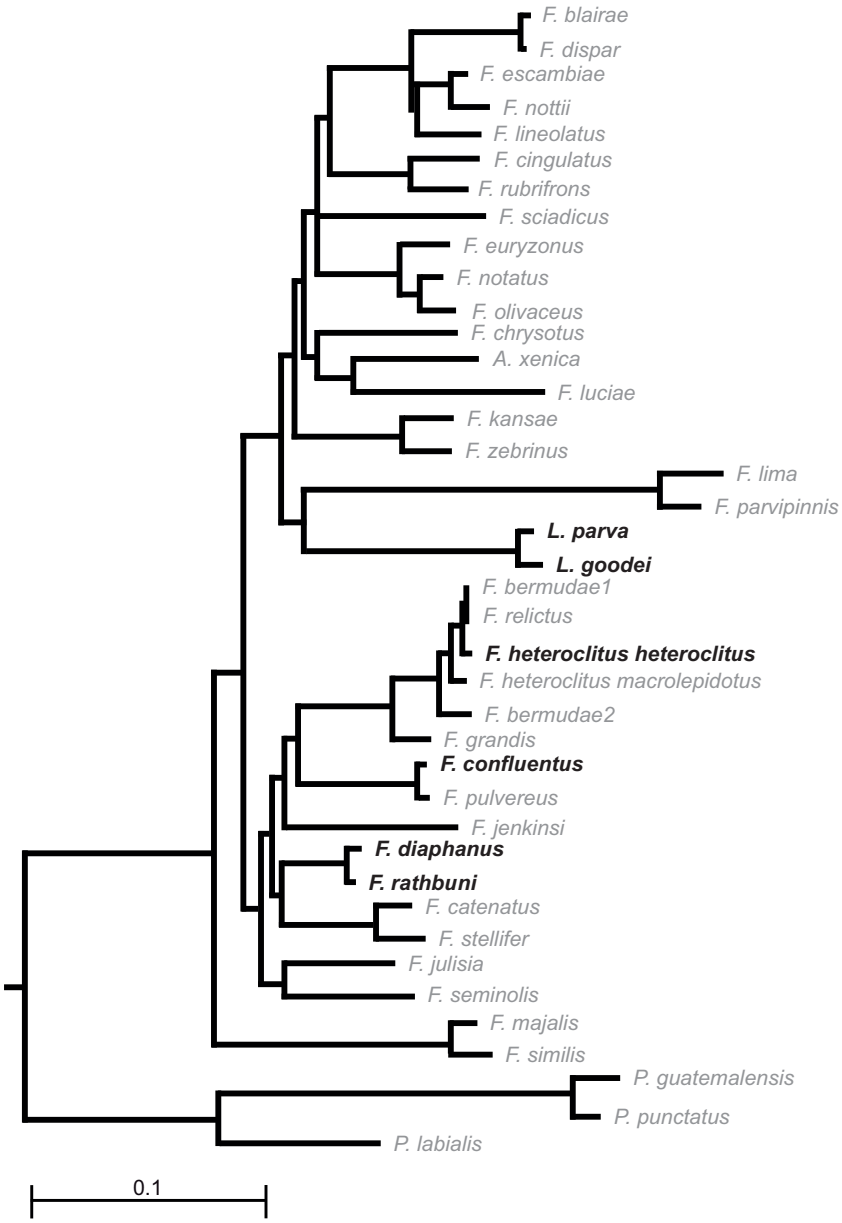

Fig. 1. Phylogeny of Fundulidae killifishes. Phylogeny was determined by maximum likelihood using the consensus sequence of the cytochrome *b* gene for each species, generated previously (Whitehead, 2010) and adapted for this investigation. The species used in this study are indicated by bold text. Branch lengths are representative of the relative evolutionary distance between taxa. Two capture locations for *F. bermudae*: 1, Evan's Pond; 2, Blue Hole.

barrier over which we laid bubble wrap to minimize the diffusion of O_2 from the atmosphere into the water in the aquarium. Because of space limitations, we were restricted to a single tank per treatment, and so did not include tank effects in our analysis. Nevertheless, there were no measurable differences between tanks, with the exception of the desired variation in acclimation P_{O_2} (hysteresis reported below). All procedures for collecting wild fish and for subsequent experimental treatments were approved by the institutional animal ethics boards of each institution.

Chronic hypoxia exposure

A subset of species (*F. rathbuni*, *L. parva*, *L. goodei*) were also exposed for 28 days to constant hypoxia or to nocturnal intermittent hypoxia (12 h hypoxia:12 h normoxia, matched to the photoperiod), as previously described (Borowiec et al., 2015, 2018). Constant hypoxia (P_{O_2} set point of 2 kPa O_2 with a 0.1 kPa hysteresis) was maintained by bubbling the water with nitrogen gas, mediated by a feedback loop using a fibre-optic oxygen sensor (Loligo Systems, Tjele, Denmark) to regulate the action of a solenoid valve controlling the flow of nitrogen. Intermittent hypoxia was maintained using the same feedback loop as for constant hypoxia, except that hypoxia (2 kPa) was only maintained at night (19:00 h to 07:00 h local time), and the water was bubbled with air to reoxygenate and maintain normoxia during the day. Respirometry and hypoxia tolerance measurements (see below) were conducted after the completion of chronic exposure. Average body mass for fish acclimated to intermittent hypoxia was as follows: *F. rathbuni*, 1.44±0.22 g and 1.08±0.24 g (t_{LOE} only); *L. parva*, 0.41±0.04 g and 0.55±0.08 g (t_{LOE} only); *L. goodei*, 0.32±0.02 g and 0.41±0.04 g (t_{LOE} only). Average body mass for fish acclimated to constant hypoxia was as follows: *F. rathbuni*, 1.00±0.20 g and 1.26±0.24 g (t_{LOE} only); *L. parva*, 0.33±0.05 g and 0.45±0.08 g (t_{LOE} only); *L. goodei*, 0.31±0.03 g and 0.34±0.04 g (t_{LOE} only). See Fig. 4 for the body mass of normoxia-acclimated fish.

Respirometry and hypoxia tolerance measurements

Stop-flow respirometry was used to measure resting $\dot{M}_{O_2}$, P_{crit}, regulation index (RI) and P_{LOE} (Fig. 2A), using methods consistent

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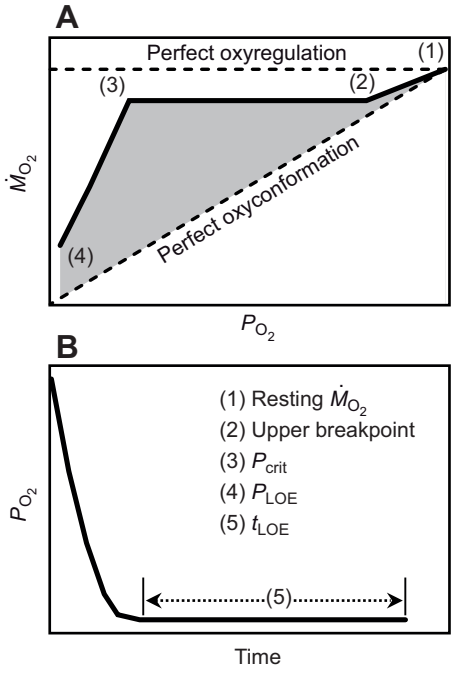

Fig. 2. Hypothetical illustration of the relationship between O_2 consumption rate ($\dot{M}_{O_2}$) and O_2 pressure (P_{O_2}), as well as the hypoxia tolerance metrics measured in this study. (A) Resting $\dot{M}_{O_2}$ was determined for each fish in normoxia (1), and a progressive hypoxia protocol was used to determine the critical O_2 tension (P_{crit}, the lower breakpoint in the $\dot{M}_{O_2}$–P_{O_2} relationship) (3), and the P_{O_2} at which the fish displays loss of equilibrium (P_{LOE}) (4). A minority of individuals also showed an upper breakpoint (2) in the $\dot{M}_{O_2}$–P_{O_2} relationship (see Materials and Methods for further details). The overall response of $\dot{M}_{O_2}$ to progressive hypoxia was used to calculate the regulation index (RI), the ratio of the area bound between the measured $\dot{M}_{O_2}$ and the line of perfect oxyconformation (indicated by the shaded region) and the area bound between the lines of perfect oxyregulation and perfect oxyconformation. (B) In a separate experiment, time to LOE (t_{LOE}) (5) was calculated as the time elapsed between when the water P_{O_2} first reached 0.6 kPa and when the fish lost equilibrium.

with those we have described previously for *F. heteroclitus* (Borowiec et al., 2015). Fish were habituated overnight in normoxia to a 70 ml cylindrical respirometry chamber that was situated in a large darkened buffer tank. The chamber was connected to a flush pump that circulated water from the buffer tank through the chamber in a 'flushing circuit'. A second pump circulated water from the chamber in a closed loop (a 'recirculating circuit') across a flow-through fibre-optic O_2 sensor (PreSens, Regensburg, Germany). Water flow through both circuits was driven by pumps controlled by AutoResp software (Loligo Systems). Pumps in both the flushing and recirculating circuit were active during the overnight habituation to the respirometry chamber.

$\dot{M}_{O_2}$ measurements began the following morning, and sequential activation and deactivation of the pumps allowed measurement of the change in O_2 concentration due to fish respiration. During flush periods, both pumps were active, the chamber received a steady flow of water from the buffer tank, and no measurements of $\dot{M}_{O_2}$ were conducted. During measurement periods, the flushing pump was deactivated, but continued pumping through the recirculating circuit allowed for measurement of the decline in water oxygen content due to fish respiration. First, resting $\dot{M}_{O_2}$ (indicated by '1' in Fig. 2A) was measured in normoxia. We then measured $\dot{M}_{O_2}$ throughout a progressive stepwise hypoxia protocol, in which the P_{O_2} of the buffer tank was reduced from ∼20 kPa to 2 kPa in ∼2 kPa steps (∼15 min per step) using the O_2 control system described above, allowing calculation of $\dot{M}_{O_2}$ at 20, 18, 16, 14, 12, 10, 8, 6, 4 and 2 kPa. After $\dot{M}_{O_2}$ was measured at 2 kPa, the chamber was isolated from the buffer tank (by deactivating the flushing circuit) and the fish consumed the remaining O_2 until LOE, and the P_{LOE} was recorded (indicated by '4' in Fig. 2A). During this period, we recorded $\dot{M}_{O_2}$ at roughly 1.5, 1.0 and 0.5 kPa, but measurements were not possible at each of these nominal P_{O_2} in every individual (e.g. if the fish had earlier lost equilibrium). A modest number of small fish could not consume O_2 below 0.5 kPa, so we had to open the chamber to the buffer tank and bubble the tank with nitrogen until the fish reached LOE.

$\dot{M}_{O_2}$ was calculated from the change in chamber O_2 concentration over time, as previously recommended (Clark et al., 2013), and is expressed relative to body mass. P_{crit} was calculated from the $\dot{M}_{O_2}$ and P_{O_2} data using the R package 'segmented' (Muggeo, 2008), which allows the identification and calculation of multiple breakpoints within a single $\dot{M}_{O_2}$–P_{O_2} curve. To calculate the P_{crit} of an individual fish, we used the average $\dot{M}_{O_2}$ measurement from 2–3 replicates for the set point P_{O_2} at each step. The segmented regression model was fitted to a general linear model of the data (Muggeo, 2008). Most individuals exhibited the expected two-segment association between $\dot{M}_{O_2}$ and P_{O_2}, such that P_{crit} could be calculated as the single breakpoint using the $\dot{M}_{O_2}$ data across all P_{O_2} (i.e. in the manner described by Yeager and Ultsch, 1989). However, for some individuals from a subset of species, there were two P_{O_2} breakpoints in the $\dot{M}_{O_2}$–P_{O_2} relationship (indicated by '2' and '3' in Fig. 2A) rather than a single breakpoint. This pattern resulted from a decline in $\dot{M}_{O_2}$ across a narrow range of P_{O_2} just below normoxia, followed by a stabilization of $\dot{M}_{O_2}$ across a broader range of intermediate P_{O_2} (i.e. an absence or appreciable reduction in the slope of decline), and then another phase of more steeply declining $\dot{M}_{O_2}$ (Fig. 2A). There has recently been criticism of the lack of standardized approaches and a tendency for data pruning in calculations of P_{crit}, possibly in an effort to force a two-segment association on $\dot{M}_{O_2}$–P_{O_2} data that do not exhibit this pattern of variation (Wood, 2018). With this criticism in mind, we calculated the P_{O_2} at which each of the two apparent breakpoints occurred, and designated the breakpoint that occurred at the lower P_{O_2} as the P_{crit}. For only a very small number of individuals across all treatment groups (N=1 each of *F. rathbuni* and *L. parva*), the model could not converge upon a P_{crit} value as either the single breakpoint or the lower of two breakpoints in the $\dot{M}_{O_2}$–P_{O_2} relationship, so we do not report P_{crit} values for these individuals.

We also used the $\dot{M}_{O_2}$ and P_{O_2} data to calculate RI for each individual, which provides a relative measure of the degree of oxyregulation by comparing the $\dot{M}_{O_2}$ measured across P_{O_2} with the $\dot{M}_{O_2}$ expected from perfect oxyconformation (Mueller and Seymour, 2011). P_{crit} and RI are both indices that describe how $\dot{M}_{O_2}$ reacts to changes in P_{O_2}, but they are calculated in different ways and they represent different aspects of this relationship. Whereas P_{crit} determines the major breakpoint P_{O_2} in the $\dot{M}_{O_2}$–P_{O_2} relationship, RI is a metric of the relative degree of oxyconformity across the entirety of the $\dot{M}_{O_2}$–P_{O_2} relationship from perfect oxyconformity (0) to perfect oxyregulation (1). RI was calculated by first determining the lines of perfect oxyconformation and perfect oxyregulation, which were the lines from the $\dot{M}_{O_2}$ recorded at a P_{O_2} of ∼20 kPa and the origin (0, 0) or a horizontal line at that $\dot{M}_{O_2}$ to a P_{O_2} of zero, respectively. RI was then calculated as the area bound by the individual's measured $\dot{M}_{O_2}$–P_{O_2} relationship and the oxyconformity line (indicated by the shaded region in Fig. 2A), divided by the triangular area bound by the oxyregulation and oxyconformity

lines. Therefore, a RI of 1 describes perfect maintenance of the $\dot{M}_{O_2}$ recorded at 20 kPa during hypoxia exposure, whereas a RI of 0 describes perfect oxyconformation.

t_{LOE} at a sustained level of severe hypoxia was measured in all species (Fig. 2B). Fish were held individually in small chambers (which did not allow access to the surface) within an aquarium of aerated (normoxic) water for an overnight period. During this period, water was continuously circulated through the chambers with aquarium pumps. The following morning, buffer tank P_{O_2} was rapidly decreased to 0.6 kPa (0.3 mg O_2 l^{-1}), which typically took less than 5–15 min, by the rapid bubbling of nitrogen gas. This P_{O_2} was held steady until the fish lost equilibrium, and t_{LOE} was calculated beginning from the time when the P_{O_2} in the aquaria first reached 0.6 kPa ('5' in Fig. 2B). We chose 0.6 kPa because it probably represents a considerable acute hypoxia stressor for even hypoxia-acclimated fish, being well below the P_{crit} of *F. heteroclitus* acclimated for 7 days to 2 kPa hypoxia (Borowiec et al., 2015). Accordingly, animals that lost equilibrium before the P_{O_2} reached 0.6 kPa were assigned a negative t_{LOE}, which was reflective of the difference in time between when LOE occurred and when the aquarium reached 0.6 kPa.

Statistics and phylogenetic analyses

Data were first checked for normality using a Shapiro–Wilk test (data not shown). For data across normoxia-acclimated fish, we used one-way ANOVA (on ranks in cases when normality was not confirmed) followed by Dunn's or Dunnett's *post hoc* tests (as appropriate to the type of ANOVA used), to examine the change in $\dot{M}_{O_2}$ as a function of P_{O_2} within a species or to compare hypoxia tolerance metrics between taxa. We similarly used two-way ANOVA to test for effects of hypoxia acclimation, species and their interaction. We used least squares linear regression to test for relationships between body mass and $\dot{M}_{O_2}$ or indices of hypoxia tolerance, and for relationships between $\dot{M}_{O_2}$ and indices of hypoxia tolerance. Data from the same set of normoxia-acclimated *F. rathbuni*, *L. parva* and *L. goodei* were used for both the interspecific comparisons (Figs 3–7) and the hypoxia acclimation comparisons (Figs 8 and 9). These statistical analyses were performed using R Studio or GraphPad Prism software (La Jolla, CA, USA).

Phylogenetically independent contrasts were calculated using Mesquite (http://www.mesquiteproject.org) using the PDAP module (http://mesquiteproject.org/pdap_mesquite). We used a previously published, robust maximum likelihood phylogeny of the Fundulidae based on the consensus sequence of the cytochrome *b* gene for each species (Whitehead, 2010) (Fig. 1). We pruned the tree to only include species for which we measured all character data (species names in bold in Fig. 1), and we used this tree for phylogenetically independent contrasts analysis. Positivized unstandardized contrasts were calculated from absolute data and then standardized by dividing them by the standard deviation of that contrast (e.g. square root of the sum of corrected branch lengths), as previously recommended (Garland et al., 1992; http://www.mesquiteproject.org). We used least squares linear regressions on standardized contrasts to test in a phylogenetically independent manner for the same relationships between body mass, $\dot{M}_{O_2}$ and indices of hypoxia tolerance that are described above.

RESULTS

Variation in hypoxia tolerance across Fundulidae

We quantified the changes in resting $\dot{M}_{O_2}$ during progressive hypoxia (Fig. 3) in 6 species from multiple lineages of fundulid killifish (Fig. 1), in fish that were well acclimated to normoxia in freshwater (0.1 ppt). The response of $\dot{M}_{O_2}$ to declining P_{O_2} varied between species (Fig. 3), but all species had a significant main effect of P_{O_2} on $\dot{M}_{O_2}$ (Fig. 3A, *F. rathbuni*, $F_{12,171}$=4.30, P<0.0001; Fig. 3B, *F. diaphanus*, H_{13}=37.29, P=0.0002; Fig. 3C, *L. parva*, H_{13}=46.26, P<0.0001; Fig. 3D, *L. goodei*, H_{13}=33.74, P=0.0007; Fig. 3E, *F. heteroclitus*, H_{13}=21.61, P=0.0421; Fig. 3F, *F. confluentus*, H_{13}=34.77, P=0.0005). There was variation in the P_{O_2} at which $\dot{M}_{O_2}$ first exhibited statistically significant declines relative to $\dot{M}_{O_2}$ in normoxia, with the extremes represented by *L. parva*, the species that reduced $\dot{M}_{O_2}$ at the highest P_{O_2} (occurring at ∼10 kPa), and *F. heteroclitus* and *F. confluentus*, species that maintained $\dot{M}_{O_2}$ statistically similar to resting $\dot{M}_{O_2}$ until very low (∼0.5 kPa) P_{O_2}.

Along with the variation in the P_{O_2} at which $\dot{M}_{O_2}$ first decreased from normoxic $\dot{M}_{O_2}$, the general pattern of the $\dot{M}_{O_2}$–P_{O_2} curve also differed between taxa. For example, in *F. diaphanus* and *L. goodei*, $\dot{M}_{O_2}$ changed very little until a low P_{O_2} was reached, at which point it declined (Fig. 3B,D). Other species, such as *F. confluentus* and *F. heteroclitus*, seemed to show a weaker pattern of oxyregulation, with slight declines in $\dot{M}_{O_2}$ at high levels of declining P_{O_2} (Fig. 3E,F), but nevertheless showed the typical two-segment $\dot{M}_{O_2}$–P_{O_2} relationship with a single breakpoint P_{O_2} that we considered to be P_{crit}. However, some species (e.g. *F. rathbuni*, *L. parva*) exhibited an early decline in $\dot{M}_{O_2}$ during mild hypoxia (P_{O_2} just below normoxia) followed by stabilization of $\dot{M}_{O_2}$ in moderate hypoxia (with the delineation between the two occurring at an upper breakpoint P_{O_2}), and then a second sharper decline in $\dot{M}_{O_2}$ at low P_{O_2} below P_{crit} (Fig. 3A,C). In such cases, in which there were three clear segments in the $\dot{M}_{O_2}$–P_{O_2} curve, we considered the lower breakpoint to represent P_{crit}.

There was a significant main effect of species on resting $\dot{M}_{O_2}$ in normoxia (H_6=49.64, P<0.0001) and this appeared to be partially driven by the relatively high $\dot{M}_{O_2}$ of *L. parva* and *L. goodei*, as well as the low $\dot{M}_{O_2}$ of *F. diaphanus* compared with those of other taxa (Fig. 3). This variation appeared to result from the relationship between $\dot{M}_{O_2}$ and body mass. As expected, resting $\dot{M}_{O_2}$ in normoxia was negatively correlated with body mass, such that larger animals had lower mass-specific O_2 demands (Fig. 4A), and this relationship remained significant after accounting for evolutionary relatedness between taxa using phylogenetically independent contrasts (Fig. 4B). These relationships remained true even after removing *F. diaphanus* from the regression in Fig. 4A, or after removing the strong *F. diaphanous–F. rathbuni* contrast from the regression in Fig. 4B (data not shown).

Indices of hypoxia tolerance varied appreciably between species (Fig. 5). There were highly significant main effects of species on t_{LOE} (H_6=71.23, P<0.0001; Fig. 5A), P_{LOE} (H_6=31.34, P<0.0001; Fig. 5B) and P_{crit} (H_6=26.56, P<0.0001; Fig. 5C), all of which suggested that *F. confluentus* was the most hypoxia-tolerant species (longest t_{LOE}, lowest P_{LOE}, and tied for lowest P_{crit}), *F. rathbuni* and *F. diaphanus* were the least hypoxia-tolerant species (shortest t_{LOE}, highest P_{LOE}, highest P_{crit}), and the other species had intermediate tolerance. RI also varied across species (Fig. 5D), as reflected by a significant main effect of species (H_6=16.98, P=0.0045), but did not follow a similar pattern to P_{crit} or to the other indices of hypoxia tolerance. RI was generally greater than zero, suggesting that all species exhibited some degree of oxyregulation in hypoxia. Although our different hypoxia tolerance metrics generally agreed with each other (except RI) in species representing the extremes of hypoxia tolerance (i.e. *F. confluentus* and *F. rathbuni*), the rank order of species with intermediate levels of tolerance was inconsistent.

Differences in body mass did not appear to make a major contribution to the interspecific variation in hypoxia tolerance. The

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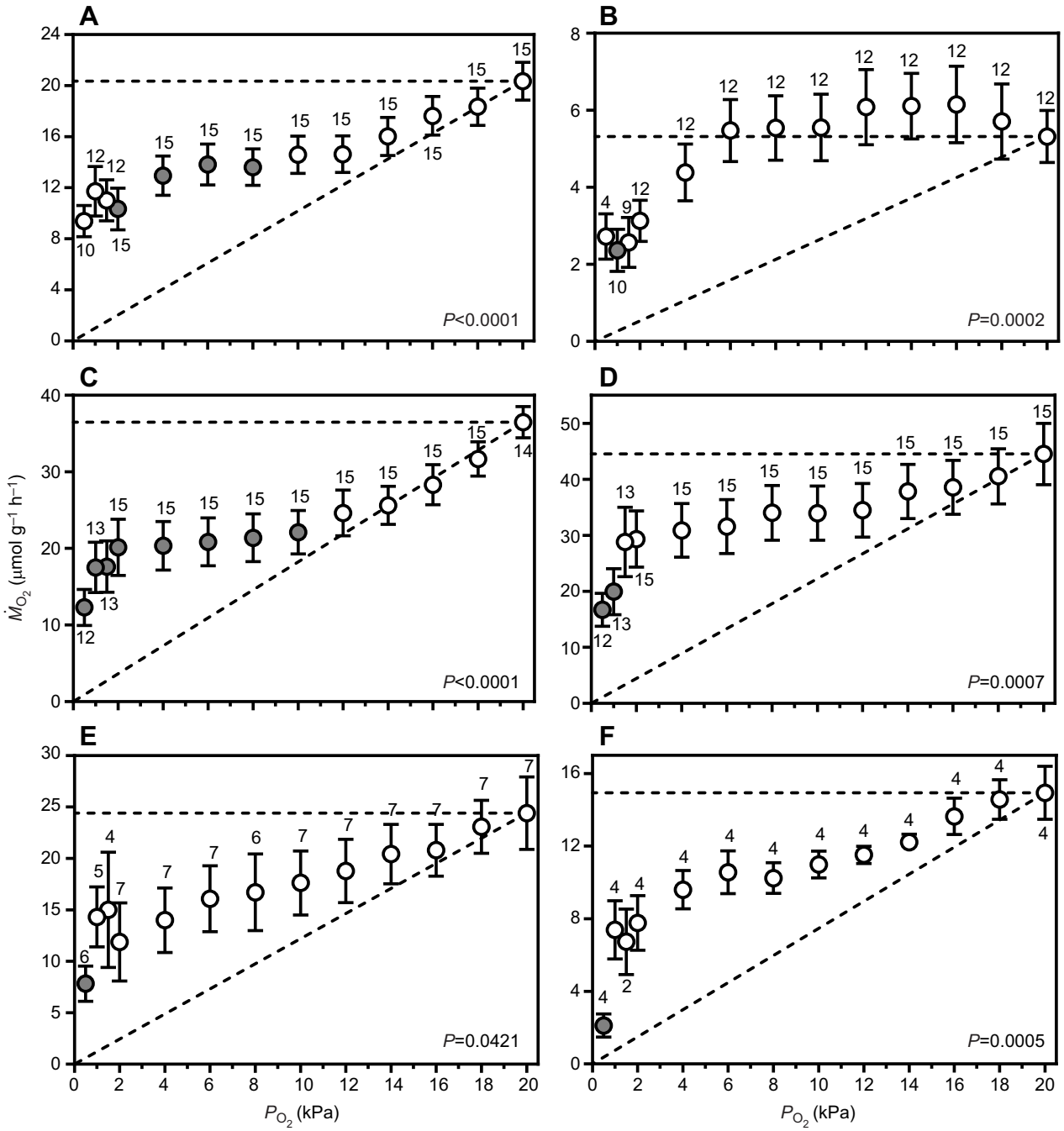

Fig. 3. Response of $\dot{M}_{O_2}$ to declines in P_{O_2} in 6 killifish species acclimated to normoxia. (A) *Fundulus rathbuni*; (B) *Fundulus diaphanus*; (C) *Lucania parva*; (D) *Lucania goodei*; (E) *Fundulus heteroclitus*; and (F) *Fundulus confluentus*. Dashed lines represent the $\dot{M}_{O_2}$ expected with perfect oxyregulation (maintenance of resting $\dot{M}_{O_2}$) and perfect oxyconformation (linear decline of $\dot{M}_{O_2}$ in hypoxia, to $\dot{M}_{O_2}$=0 at P_{O_2}=0). Data are presented as means±s.e.m. with sample sizes reported above each data point (see Materials and Methods). The *P*-values for the main effects of P_{O_2} from one-way ANOVA are indicated within the panels. $\dot{M}_{O_2}$ measurements that are statistically different from resting $\dot{M}_{O_2}$ in normoxia (20 kPa) via *post hoc* tests are indicated by grey symbols, and measurements that are not statistically different from resting $\dot{M}_{O_2}$ are indicated by white symbols.

correlations of t_{LOE}, P_{LOE} or P_{crit} with body mass were not significant for the allometric regressions using either absolute values or phylogenetically independent contrasts (Table 1). The allometric regressions of RI to body mass were significant before (Fig. 6A) and after (Fig. 6C) phylogenetic correction, suggesting that larger species have a higher RI (Table 1). Fish with a lower mass-specific $\dot{M}_{O_2}$ also had a higher RI (Fig. 6B), but this result was only significant after phylogenetic correction (Fig. 6D, Table 1). However, these significant correlations were entirely driven by the single contrast between the sister taxa pair of *F. rathbuni* and *F. diaphanus*, which differ appreciably in body mass (Fig. 4A), $\dot{M}_{O_2}$ (Fig. 3A,B) and RI (Fig. 5D).

Relationship between indices of hypoxia tolerance

Given the substantial variation in metrics associated with hypoxia tolerance across Fundulidae (Fig. 5), we examined how different indices of hypoxia tolerance correlated with each other (Table 1). P_{LOE} and t_{LOE} exhibited very similar patterns of variation across

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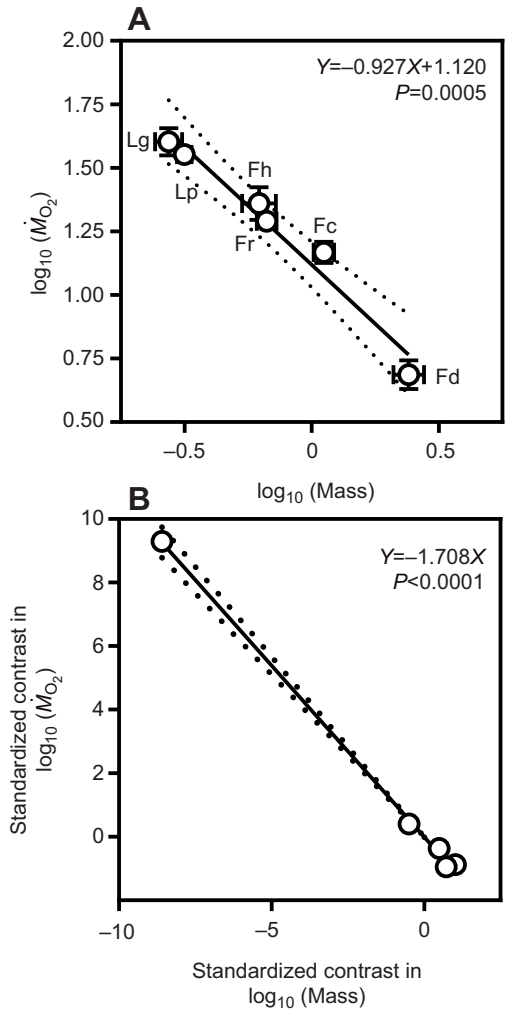

Fig. 4. Relationship between $\dot{M}_{O_2}$ and body mass in normoxia. (A) Absolute values (means±s.e.m.). (B) Phylogenetically independent contrasts. Least squares linear regressions (solid line) and 95% confidence bands (indicated by dotted lines) are shown, with equations and *P*-values reported in each panel. Species abbreviations and sample sizes for absolute values were as follows: Fc, *F. confluentus* (*N*=4); Fd, *F. diaphanus* (*N*=12); Fh, *F. heteroclitus* (*N*=7); Fr, *F. rathbuni* (*N*=15); Lg, *L. goodei* (*N*=15); Lp, *L. parva* (*N*=15). Other statistical information is reported in Table 1.

species (Fig. 5A,B), and the negative correlation between the absolute values of these traits neared significance (P=0.076). There was also a significant positive correlation between the absolute values of P_{LOE} and P_{crit} (Fig. 7A), but this correlation was not significant after phylogenetic correction (Fig. 7B, Table 1). RI showed a significant negative correlation with P_{crit} after accounting for evolutionary relatedness between taxa (Fig. 7D) using phylogenetically independent contrasts, but the correlation of absolute data was not significant (Fig. 7C).

Effects of hypoxia acclimation on hypoxia tolerance

We examined the plasticity of hypoxia tolerance in response to chronic hypoxia exposure in a subset of species – *F. rathbuni*, *L. goodei* and *L. parva* – that appeared to differ in hypoxia tolerance in normoxia (Fig. 5). This was done using two distinct patterns of chronic hypoxia: constant sustained hypoxia and diel cycles of nocturnal hypoxia (‘intermittent hypoxia’, 12 h hypoxia:12 h normoxia, matched closely to the photoperiod). Hypoxia acclimation appeared to affect the qualitative pattern of the response of $\dot{M}_{O_2}$ to declining P_{O_2}, particularly in *F. rathbuni* and *L. parva*, such that animals generally reduced $\dot{M}_{O_2}$ less at higher P_{O_2} (i.e. a less pronounced drop in $\dot{M}_{O_2}$ above the upper P_{O_2} breakpoint) after acclimation to constant and/or intermittent hypoxia (Fig. 8). The change in the $\dot{M}_{O_2}$–P_{O_2} curve was most dramatic in *F. rathbuni*, where the P_{O_2} at which $\dot{M}_{O_2}$ was first statistically different from $\dot{M}_{O_2}$ at 20 kPa decreased from 8 kPa in normoxia-acclimated fish to ∼0.5 kPa in hypoxia-acclimated fish (Fig. 8A,D,G).

Hypoxia acclimation led to significant improvements in some (though not all) indices of hypoxia tolerance, as reflected by significant main effects of hypoxia acclimation on t_{LOE} ($F_{2,117}$=22.32, P<0.0001), P_{LOE} ($F_{2,80}$=17.73, P<0.0001) and RI ($F_{2,91}$=10.10, P=0.0001), but not on P_{crit} ($F_{2,89}$=1.21, P=0.30) (Fig. 9). Although there was some qualitative variation between the responses to constant hypoxia and intermittent hypoxia, both patterns of hypoxia exposure generally appeared to improve most metrics of hypoxia tolerance. These changes were not associated with any significant changes in resting $\dot{M}_{O_2}$, which was unaffected by hypoxia acclimation ($F_{2,91}$=1.52, P=0.23), and there was no species×environment interaction for this trait ($F_{4,91}$=0.47, P=0.76) (Fig. 9E). As a result, the species differences in resting $\dot{M}_{O_2}$ that were observed in normoxia (Fig. 3) were found to persist across acclimation environments (significant main effect of species, $F_{2,91}$=27.52, P<0.0001).

There were species differences in the effects of chronic hypoxia on some indices of tolerance (Fig. 9). The strongest example of these differences was the highly significant species×environment interaction for t_{LOE} ($F_{4,117}$=8.57, P<0.0001), for which there was also a significant main effect of species ($F_{2,117}$=95.81, P<0.0001). This significant interaction was driven by the large increase in t_{LOE} in *L. goodei* in response to both patterns of chronic hypoxia and the increase in *L. parva* in response to intermittent hypoxia, with no effects of hypoxia acclimation in *F. rathbuni* (Fig. 9A). P_{crit} did not show a significant main effect of species ($F_{2,89}$=0.48, P=0.62), but there was a significant species×environment interaction ($F_{4,89}$=2.90, P=0.026) driven by the strong reduction in P_{crit} in *F. rathbuni* after hypoxia acclimation (Fig. 9C). Neither P_{LOE} ($F_{4,80}$=1.90, P=0.12) nor RI ($F_{4,91}$=1.84, P=0.13) had a significant species×hypoxia acclimation interaction, but in both cases, the significant main effects of hypoxia acclimation on hypoxia tolerance appeared to be driven by responses in only one or two of the three species. The effects of hypoxia acclimation on P_{LOE} were driven by the much stronger reductions in *F. rathbuni* than in either *Lucania* species (Fig. 9B). The effects of hypoxia acclimation on RI were driven by increases in both *F. rathbuni* and *L. parva* after constant hypoxia, and in only *F. rathbuni* after intermittent hypoxia (Fig. 9D). These patterns of variation suggest that the responses of each index of hypoxia tolerance to hypoxia acclimation are uncoupled, and there is interspecific variation in the magnitude of these responses.

DISCUSSION

We compared data across closely related taxa and in response to hypoxia acclimation to investigate the interspecific variation and plasticity of hypoxia tolerance in fundulid killifishes. $\dot{M}_{O_2}$ and various metrics of hypoxia tolerance (t_{LOE}, P_{LOE}, P_{crit} and RI) varied substantially across species (Figs 3 and 5). No single metric could fully describe the variation across species (Fig. 5), and the interspecific variation in different indices of hypoxia tolerance often did not correlate (Fig. 7, Table 1). Hypoxia acclimation generally improved hypoxia tolerance based on some indices (t_{LOE}, P_{LOE} and

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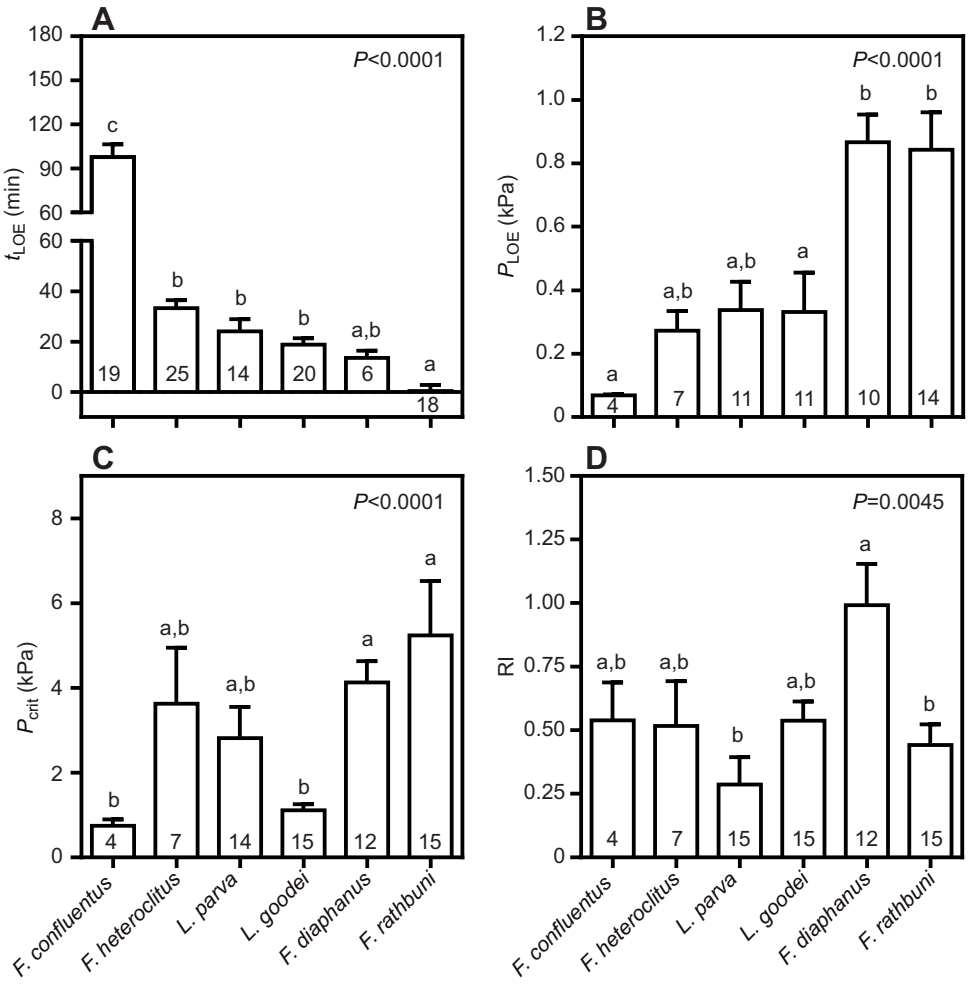

Fig. 5. Variation in hypoxia tolerance indices across 6 killifish species. (A) Time to loss of equilibrium (t_{LOE}), (B) P_{O_2} at loss of equilibrium (P_{LOE}), (C) critical O_2 tension (P_{crit}) and (D) regulation index (RI). Data are presented as means±s.e.m., and sample sizes are reported within each bar. The *P*-values for the main effects of species in one-way ANOVA are reported in each panel (see Materials and Methods), and dissimilar letters indicate a significant pairwise difference between species according to *post hoc* tests.

RI), but there was interspecific variation in the magnitude of this plasticity and in the indices of tolerance that were altered by chronic hypoxia (Figs 8 and 9). Our results suggest that hypoxia tolerance is a complex trait that is best appreciated by fully characterizing the $\dot{M}_{O_2}$–P_{O_2} relationship and by considering multiple indices of hypoxia tolerance.

Evolution of hypoxia tolerance across Fundulidae killifishes

There was appreciable interspecific variation in hypoxia tolerance (Fig. 5). Relative to that of other teleost fish, the P_{crit} of fundulid species ranged from comparable to (*F. heteroclitus*, *F. rathbuni*, *F. diaphanus*, *L. parva*) to far below (*F. confluentus*, *L. goodei*) the typical P_{crit} across various species of fish held at a similar

Table 1. Relationships between body mass, O_2 consumption rate and hypoxia tolerance metrics in normoxia-acclimated fish

		Absolute values				Phylogenetically independent		
X	*Y*	Slope	Intercept	r^2	*P*	Slope	r^2	*P*
$\log_{10}(M_b)$	$\log_{10}(\dot{M}_{O_2})$	−0.927	1.120	0.9640	0.0005	−1.708	0.9984	<0.0001
M_b	P_{crit}	0.316	3.597	0.0184	0.80	−0.287	0.0215	0.60
M_b	RI	0.246	0.315	0.8541	0.0084	0.279	0.9368	0.0013
M_b	P_{LOE}	0.189	0.273	0.2654	0.30	0.016	0.0337	0.54
M_b	t_{LOE}	1.101	28.86	0.0029	0.92	8.884	0.2882	0.20
$\dot{M}_{O_2}$	P_{crit}	−0.019	4.377	0.0181	0.80	0.002	−0.0591	0.98
$\dot{M}_{O_2}$	RI	−0.011	0.822	0.4506	0.14	−0.030	0.7135	0.029
$\dot{M}_{O_2}$	P_{LOE}	−0.009	0.686	0.1732	0.41	−0.002	0.0417	0.53
$\dot{M}_{O_2}$	t_{LOE}	−0.492	43.07	0.0417	0.70	−0.831	0.5352	0.076
P_{crit}	RI	0.022	0.489	0.0026	0.76	−0.310	0.6585	0.042
P_{crit}	P_{LOE}	0.153	0.004	0.6830	0.043	0.008	−0.0588	0.83
P_{crit}	t_{LOE}	−13.01	81.91	0.6136	0.065	−2.067	−0.0585	0.64
RI	P_{LOE}	0.655	0.094	0.2256	0.34	0.053	0.0183	0.57
RI	t_{LOE}	−11.39	37.40	0.0061	0.89	20.48	0.3759	0.15
P_{LOE}	t_{LOE}	−81.06	68.00	0.5874	0.076	−51.52	−0.0216	0.56

M_b, body mass (g); $\dot{M}_{O_2}$, O_2 consumption rate (μmol g^{-1} h^{-1}); P_{crit}, critical O_2 tension (kPa); P_{LOE}, O_2 tension at loss of equilibrium (kPa); RI, regulation index; t_{LOE}, time to loss of equilibrium at 0.6 kPa (min).

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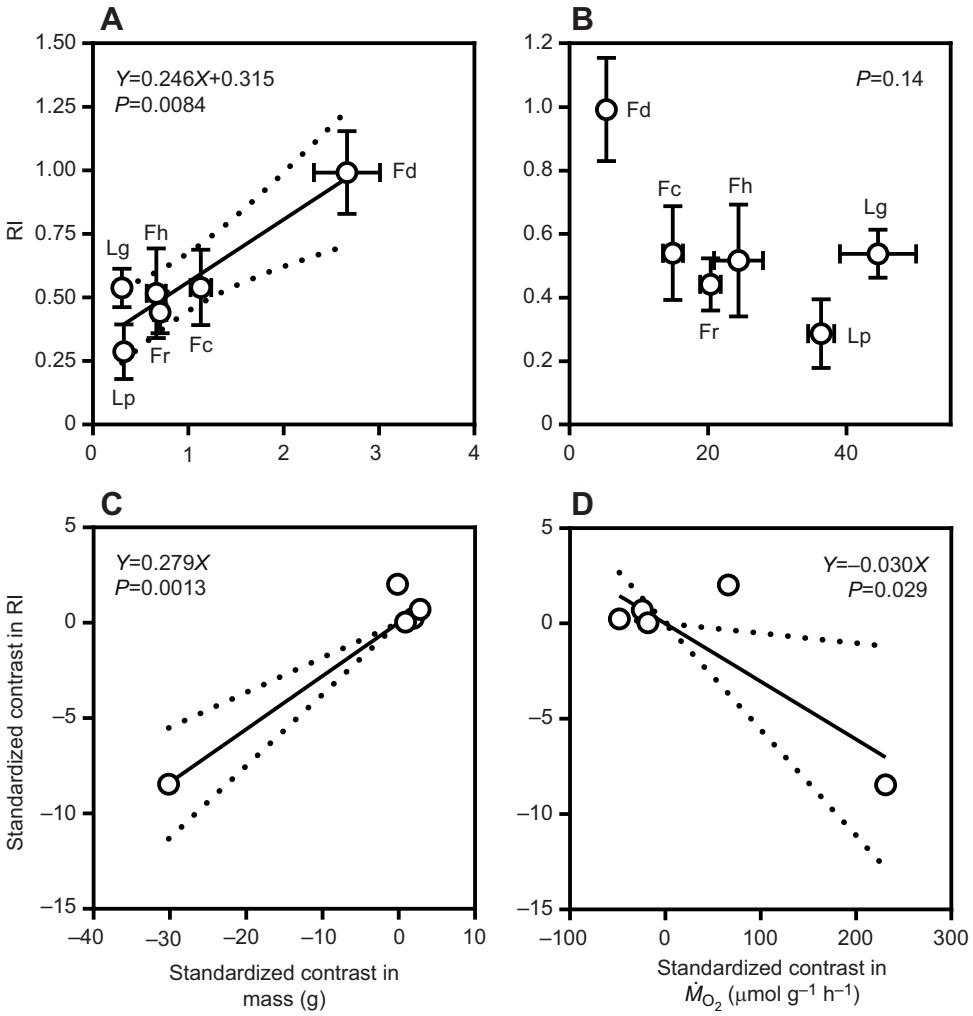

Fig. 6. Correlation between RI and body mass (left) and between RI and $\dot{M}_{O_2}$ (right). (A,B) Absolute values (means± s.e.m.). (C,D) Phylogenetically independent contrasts. Least squares linear regressions (solid line) and 95% confidence bands (indicated by dotted lines) are shown, with equations and *P*-values reported in each panel. Species abbreviations and sample sizes for absolute values were as follows: Fc, *F. confluentus* (*N*=4); Fd, *F. diaphanus* (*N*=12); Fh, *F. heteroclitus* (*N*=7); Fr, *F. rathbuni* (*N*=15); Lg, *L. goodei* (*N*=15); Lp, *L. parva* (*N*=15). Other statistical information is reported in Table 1.

temperature, based on a recent meta-analysis (Rogers et al., 2016). The P_{LOE} and t_{LOE} reported here in *Fundulus* and *Lucania* are lower than previous measurements in centrarchids (Borowiec et al., 2016; Crans et al., 2015), and are comparable to some previous measurements across common carp and other cyprinids (Dhillon et al., 2013; Fu et al., 2014), suggesting that Fundulidae killifish are relatively tolerant of extreme hypoxia. Among the species examined in this study, *F. confluentus* was the most hypoxia tolerant overall and the closely related pair of *F. rathbuni* and *F. diaphanus* had the weakest tolerance (according to the pattern of variation in t_{LOE}, P_{LOE} and P_{crit}). This may reflect differences in the native habitat between these species: *F. rathbuni* and *F. diaphanus* are typically constrained to freshwater streams or lakes (which are often well oxygenated but can become hypoxic, such as during seasonal ice-cover for *F. diaphanus*), whereas *F. confluentus* are widely distributed across the highly variable, and frequently hypoxic (Diaz, 2001), estuaries of the southern Atlantic and the Gulf of Mexico (Griffith, 1974; Nordlie, 2006; Whitehead, 2010). Some of the interspecific variation in P_{crit} could also arise from differences in O_2 supply capacity as a result of interspecific variation in active metabolism and aerobic scope (Farrell and Richards, 2009; Zhang et al., 2018). Better characterization of the habitat occupied by different fundulid species is needed to more clearly understand the relationship between species distribution and hypoxia tolerance in this family, but similar associations have been observed in other taxa. For example, variation in traits associated with hypoxia tolerance are related to differences in distribution between North American *Lepomis* sunfish, based on the exclusion of the less tolerant bluegill but not the more tolerant pumpkinseed from northern lakes that experience winterkill events (Farwell et al., 2007).

In general, the different indices of hypoxia tolerance often did not correlate with each other (Table 1), as previously observed in other taxa (Crans et al., 2015; Dhillon et al., 2013; Speers-Roesch et al., 2013), probably because these different metrics are partially underlain by distinct physiological mechanisms (Borowiec et al., 2016; Nilsson and Renshaw, 2004). Fish that maintain resting $\dot{M}_{O_2}$ into deeper levels of hypoxia (perhaps from having a higher O_2 transport capacity) would be expected to have lower P_{crit} and higher RI (Perry et al., 2009; Richards, 2009). If the mechanisms supporting this ability are associated with increases in cellular O_2 levels, they may also contribute to helping fish resist losing equilibrium in hypoxia, which could help explain the correlation between P_{crit} and P_{LOE} observed here (Fig. 7A). However, the correlation between the phylogenetically independent contrasts of these traits was not significant, nor were many other correlations between P_{LOE} or t_{LOE} and traits expected to have a respiratory component (P_{crit} and RI) (Table 1). Therefore, the interspecific variation in P_{LOE} and t_{LOE} reported here (Fig. 5) is probably explained at least partly by physiological factors independent of the ability to maintain aerobic metabolism in hypoxia.

Variation in the ability to maintain equilibrium in hypoxia could have instead resulted from variation in the relative use of metabolic depression or anaerobic metabolism to help match cellular ATP

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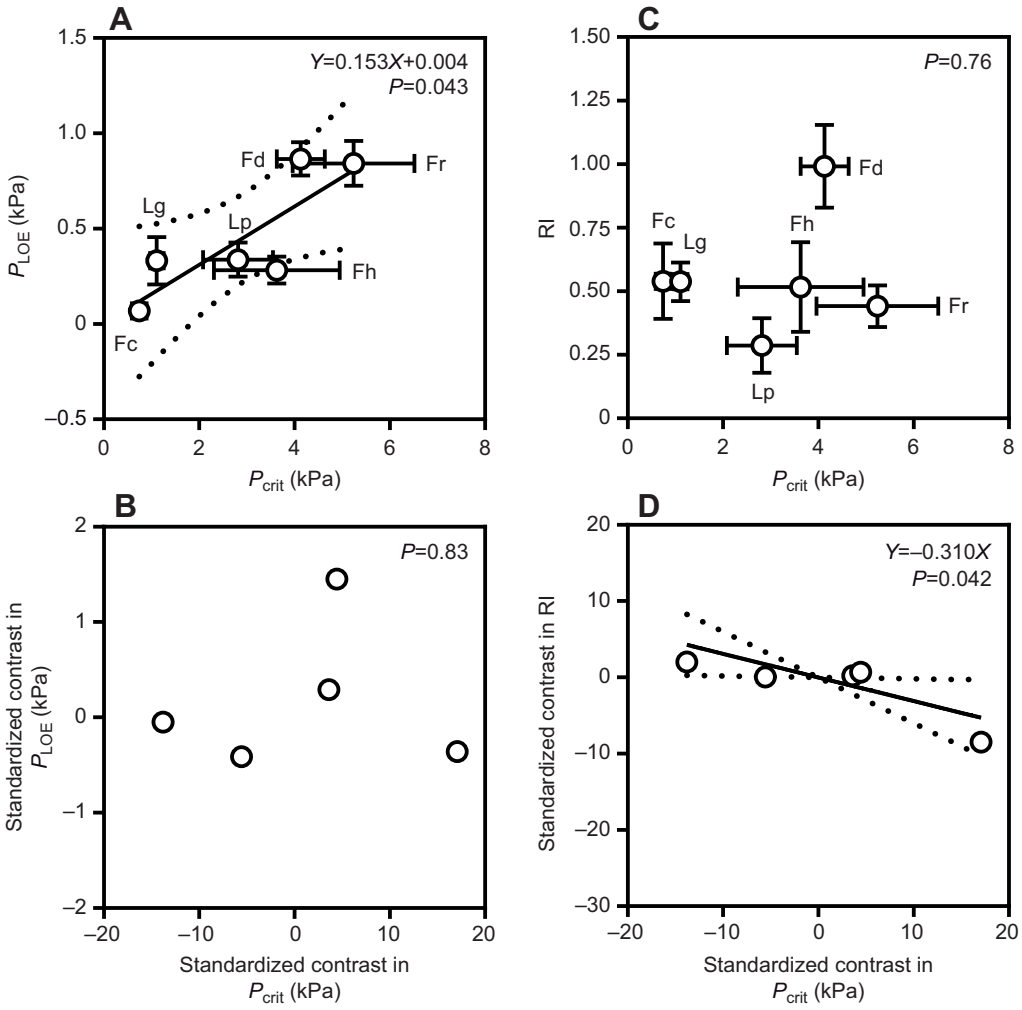

Fig. 7. Correlation between P_{crit} and P_{LOE} (left) and between P_{crit} and RI (right). (A,C) Absolute values (means±s.e.m.). (B,D) Phylogenetically independent contrasts. Least squares linear regressions (solid line) and 95% confidence band (indicated by dotted lines) are shown, with equations and *P*-values reported in each panel. Species abbreviations and sample sizes for absolute values (A,B) were as follows: Fc, *F. confluentus* (*N*=4, 4); Fd, *F. diaphanus* (*N*=10, 12); Fh, *F. heteroclitus* (*N*=7, 7); Fr, *F. rathbuni* (*N*=14, 15); Lg, *L. goodei* (*N*=11, 15); Lp, *L. parva* (*N*=11, 15). Other statistical information is reported in Table 1.

supply and demand, or in the relative development and sensitivity to metabolic acidosis, rather than variation in the ability to maintain cellular O_2 supply. Metabolic depression can reduce O_2/ATP demands and stretch limited fuel stores through a general reduction in energetically expensive processes in the cell (Guppy and Withers, 1999; Hochachka et al., 1996; Nilsson and Renshaw, 2004). Interspecific variation in the use of metabolic depression could have contributed to some of the observed variation in hypoxia tolerance, although resting $\dot{M}_{O_2}$ in normoxia was not generally observed to be associated with variation in hypoxia tolerance (e.g. the hypoxia-intolerant *F. diaphanus* had the lowest resting $\dot{M}_{O_2}$ of all species, and *L. goodei* and *L. parva*, which had by far the highest resting $\dot{M}_{O_2}$, were intermediate in their hypoxia tolerance). Variation in the capacity and availability of fuel reserves to support anaerobic metabolism could also have been important; in sculpins, for example, interspecific variation in t_{LOE} is associated with variation in glycogen reserves and lactate dehydrogenase activity in the brain (Mandic et al., 2013; Speers-Roesch et al., 2013). The detrimental effects of metabolic acidosis may have differed between species as well; among triplefin fish, for example, hypoxia-tolerant species are less susceptible to acidosis-induced mitochondrial dysfunction (Devaux et al., 2019).

There was not always a consistent association between P_{LOE} and t_{LOE} (Table 1), largely because there appeared to be discordance between these traits in some species (e.g. *F. diaphanus* had high P_{LOE} but intermediate t_{LOE}). This may be reflective of differences in the underlying physiological mechanism of what causes LOE in each situation, at least in some species. For example, t_{LOE} has been associated with resistance of brain ATP depletion and anaerobic capacity, whereas the mechanisms underlying P_{LOE} are less well understood (Mandic et al., 2013; Speers-Roesch et al., 2013). A potential cause of such differences may lie in disparities in the rate of hypoxia induction between protocols, which was ∼2 h during the stepwise reductions in P_{O_2} that led to our measurement of P_{LOE} but was <15 min for measurement of t_{LOE}. Variation in the rate of hypoxia induction over the same order of magnitude has been shown to affect P_{crit} in goldfish, such that slower rates of induction (8 h) were beneficial because they provided more time to reduce P_{crit}, via increases in gill surface area and haemoglobin–O_2 binding affinity, whereas faster rates of induction (∼0.5–1.5 h) did not (Regan and Richards, 2017). Alternatively, some species may have suffered detrimental effects of the prolonged exposure to moderate hypoxia that occurred in the stepwise hypoxia protocol that was used to determine P_{LOE}, such that the slower rate of hypoxia induction was a disadvantage for some species compared with others. This last possibility may explain one of the findings reported here, that *F. diaphanus* were able to maintain equilibrium for nearly 20 min on average in the t_{LOE} experiment, when P_{O_2} was rapidly reduced to 0.6 kPa (Fig. 5A), but they exhibited a P_{LOE} of ∼0.8 kPa in the stepwise hypoxia experiment when hypoxia was induced more slowly. This distinction could have arisen if anaerobic by-products had accumulated over the long period of exposure to

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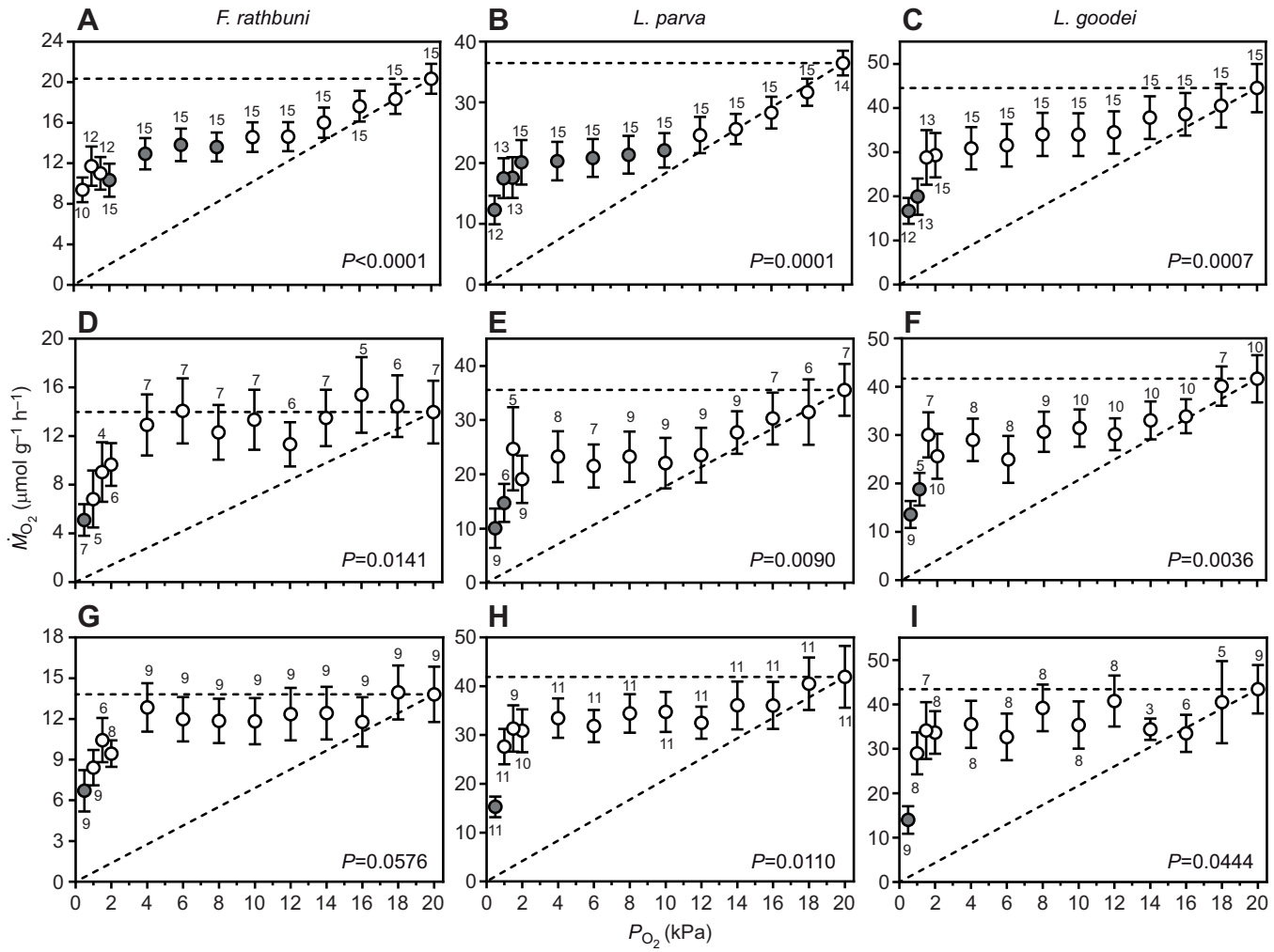

Fig. 8. Response of $\dot{M}_{O_2}$ to progressive hypoxia in 3 killifish species. (A–C) $\dot{M}_{O_2}$–P_{O_2} relationships in fish acclimated to normoxia (statistical results in text). (D–F) $\dot{M}_{O_2}$–P_{O_2} relationships after acclimation to nocturnal intermittent hypoxia (12 h hypoxia:12 h normoxia) (D: *F. rathbuni*, H_{13}=25.15, P=0.0141; E: *L. parva*, H_{13}=26.55, P=0.0090; and F: *L. goodie*, H_{13}=29.24, P=0.0036). (G–I) $\dot{M}_{O_2}$–P_{O_2} relationships after acclimation to constant hypoxia (G: *F. rathbuni*, H_{13}=20.54, P=0.0576; H: *L. parva*, $F_{12,127}$=2.30, P=0.011; and I: *L. goodie*, H_{13}=21.43, P=0.0444). Dashed lines represent the $\dot{M}_{O_2}$ expected with perfect oxyregulation (maintenance of resting $\dot{M}_{O_2}$) and perfect oxyconformation (linear decline of $\dot{M}_{O_2}$ in hypoxia, to $\dot{M}_{O_2}$=0 at P_{O_2}=0). Data are presented as means±s.e.m. with samples sizes reported above each data point (see Materials and Methods). The P-values for the main effects of P_{O_2} in one-way ANOVA are indicated in the panels. $\dot{M}_{O_2}$ measurements that are statistically different from resting $\dot{M}_{O_2}$ in normoxia (20 kPa) via *post hoc* tests are indicated by grey symbols, and measurements that are not statistically different from resting $\dot{M}_{O_2}$ are indicated by white symbols.

stepwise hypoxia, rising to levels that elicited LOE before the fish reached 0.6 kPa O_2.

There was a negative correlation between P_{crit} and RI, but only after phylogenetic correction (Fig. 7D). The RI has been proposed by some to be a preferable alternative to P_{crit} (Mueller and Seymour, 2011; Wood, 2018), and we had expected a stronger association between P_{crit} and RI, given that both of these metrics aim to describe aspects of how hypoxia affects $\dot{M}_{O_2}$. However, while P_{crit} describes the breakpoint between oxyconformation and oxyregulation, it is possible for $\dot{M}_{O_2}$ to vary above P_{crit}, which could affect RI with little effect on P_{crit}. RI and P_{crit} could be well correlated among species that exhibit only a single breakpoint in the $\dot{M}_{O_2}$–P_{O_2} relationship. However, the effects of modest hypoxia on $\dot{M}_{O_2}$ that led to the two-breakpoint pattern in the $\dot{M}_{O_2}$–P_{O_2} relationship in some species may have disrupted the correlation between these metrics. These issues emphasize the value in reporting the full $\dot{M}_{O_2}$–P_{O_2} relationship to consider how its shape may help explain the relationship (or lack thereof) between RI and P_{crit}.

RI was the only metric that did not distinguish the most tolerant killifish species (*F. confluentus*) from the least tolerant (*F. rathbuni*) (Fig. 5). Our data also suggest that the RI does not adequately describe the $\dot{M}_{O_2}$ responses of some species to hypoxia. For example, the very low RI (~0.25) of *L. parva* could be mistakenly interpreted as evidence for oxyconformity of this species, but the $\dot{M}_{O_2}$–P_{O_2} relationship clearly shows that this species maintains a stable $\dot{M}_{O_2}$ across a broad range of P_{O_2} from 10 kPa down to its P_{crit} of ~3 kPa (Fig. 3). *Fundulus rathbuni* and *F. diaphanus*, two closely related species that have similar P_{crit} and similarly poor hypoxia tolerance (as also reflected by P_{LOE} and t_{LOE}), have very different RI values as a result of the 'upper breakpoint' pattern seen in *F. rathbuni* but not in *F. diaphanus*. Our data suggest that neither P_{crit} nor RI can adequately represent the nuances of the $\dot{M}_{O_2}$–P_{O_2} relationship that we observed in some species of killifish, and that the pattern of interspecific variation in RI is largely inconsistent with multiple other indices of hypoxia tolerance. As such, RI may have limited value as a metric of hypoxia tolerance for *Fundulus* species.

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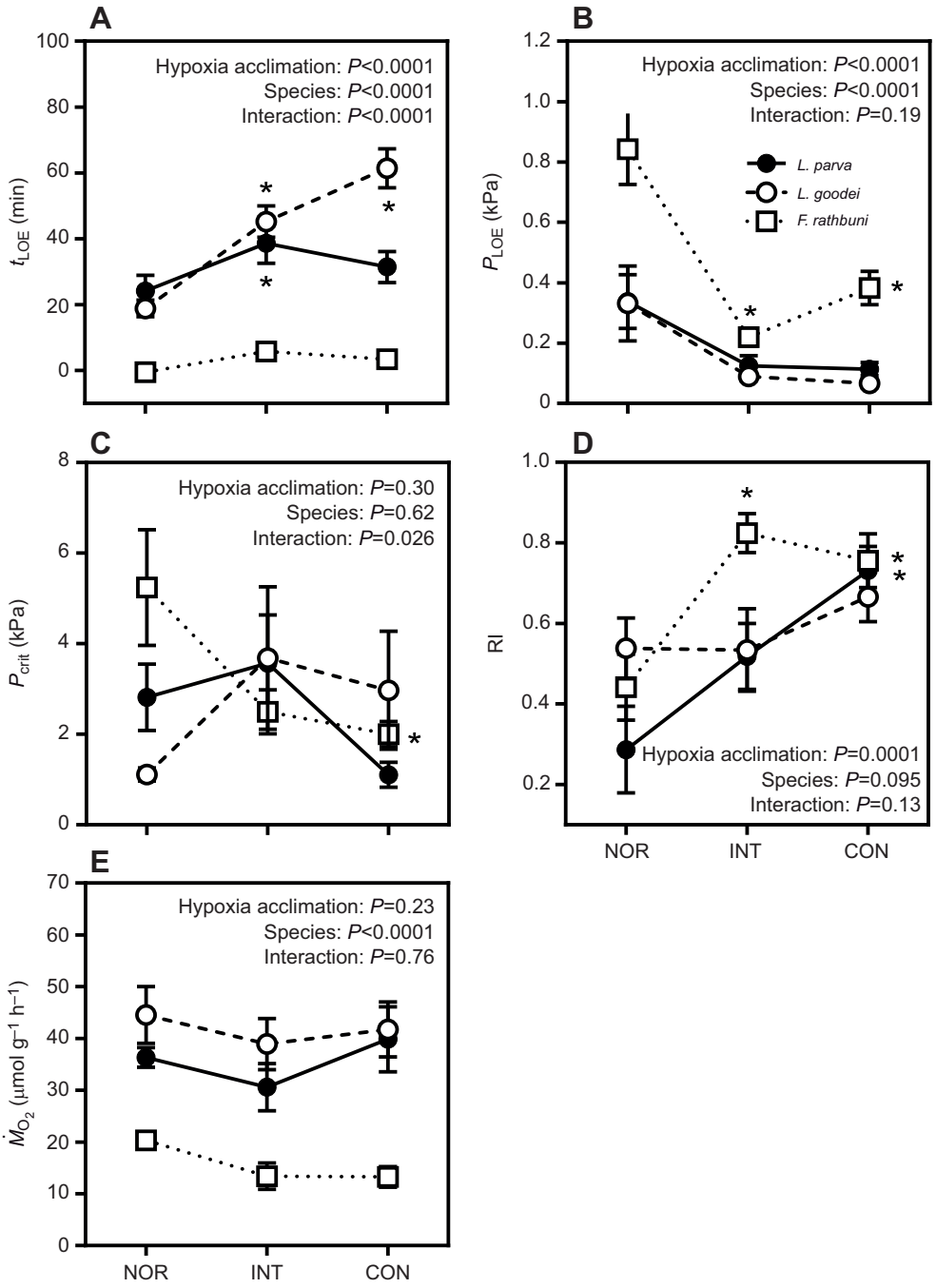

Fig. 9. Effects of acclimation for 28 days to nocturnal intermittent hypoxia or constant hypoxia at 2 kPa O_2 on hypoxia tolerance indices in 3 killifish species. (A) t_{LOE}, (B) P_{LOE}, (C) P_{crit}, (D) RI and (E) resting $\dot{M}_{O_2}$. NOR, normoxia; INT, nocturnal intermittent hypoxia (12 h hypoxia at 2 kPa O_2:12 h normoxia); CON, constant hypoxia. The *P*-values for the main effects of species, hypoxia acclimation and their interaction in two-way ANOVA are reported in each panel. *Significant ($P<0.05$) within-species pairwise differences from the normoxia-acclimated animals via *post hoc* tests. Samples sizes for normoxia, intermittent hypoxia and constant hypoxia, respectively, are as follows: *F. rathbuni* (A) N=18, 15, 14; (B) N=14, 7, 9; (C) N=15, 7, 8; (D) N=15, 7, 9; (E) N=15, 7, 9; *L. goodei* (A) N=20, 15, 10; (B) N=11, 10, 8; (C) N=15, 10, 9; (D) N=15, 10, 9; (E) N=15, 10, 9; *L. parva* (A) N=14, 11, 8; (B) N=11, 8, 11; (C) N=14, 9, 11; (D) N=15, 9, 11; (E) N=15, 9, 11.

Although some of the distinctions between P_{crit} and RI probably arise from the upper breakpoint pattern seen in the $\dot{M}_{O_2}$–P_{O_2} relationship for some species, the cause of this pattern is not entirely clear. One possibility is that some species are especially sensitive to the very subtle effects of disturbance at the beginning of an experiment (e.g. inactivation of the flush pump, etc.), such that resting normoxic $\dot{M}_{O_2}$ was elevated and fish only returned to a stable standard metabolic rate after they became accustomed to these minor disturbances after a few flush-measurement cycles. The species that exhibited this upper breakpoint pattern (*L. parva*, *L. goodei* and *F. rathbuni*) also appeared to be relatively skittish in laboratory conditions and may be more sensitive to minor disturbance, providing some anecdotal support for this possibility. Another possibility for the upper breakpoint pattern in some species is that animals were sensing and responding to minor changes in P_{O_2} with modest facultative reductions in resting $\dot{M}_{O_2}$ above P_{crit}. Regardless of the underlying cause of the upper breakpoint pattern, it tends to reduce RI and thus exaggerates the apparent level of oxyconformity, probably without affecting P_{crit} (if calculated using three-segment regression as done here), P_{LOE} or t_{LOE}. Regardless, the R-script used in this study was suitable for modelling both patterns of respirometry data even though the cause of each pattern is unclear.

While this investigation focused on physiological indices of hypoxia tolerance, fish also make important behavioural responses as well, such as the threshold P_{O_2} and/or rate at which aquatic surface respiration (ASR) occurs. ASR increases as P_{O_2} declines and greatly enhances survival in hypoxic water (Kramer and McClure, 1982). Interestingly, hypoxia-tolerant sculpins perform ASR at a

higher P_{O_2} than do hypoxia-intolerant sculpins, suggesting ASR may be an important behavioural strategy to survive hypoxia in fishes (Mandic et al., 2009a). While we did not investigate the use of ASR in this study (our fish were prevented from accessing the surface throughout hypoxia acclimation and respirometry experiments), the use of ASR could modulate the P_{O_2} experienced by fish and thus allow for persistence in hypoxic waters, particularly in species like *F. rathbuni* that have relatively low hypoxia tolerance. As both behavioural and physiological responses are important for coping with environmental stress in wild fishes, correlating behavioural responses to hypoxia with physiological indices of hypoxia tolerance across fundulids would be a useful area of future study.

Overall, our findings suggest that multiple indices of hypoxia tolerance are needed to appreciate interspecific variation in how fish cope with hypoxia, even when comparing very closely related species, as was done in this study. We agree with recent suggestions (Regan et al., 2019; Wood, 2018) that full characterization of the $\dot{M}_{O_2}$–P_{O_2} relationship can provide valuable insight that is not represented by the metrics that are calculated from this relationship (i.e. P_{crit} and RI). P_{LOE} and t_{LOE}, indices that are measured directly and reflect the ability to survive hypoxia, are also critical indices of hypoxia tolerance that are often not correlated with P_{crit} or RI (Table 1) (Speers-Roesch et al., 2013). The critical need to consider multiple metrics comes from evidence that evolutionary variation in the ability to live in hypoxic environments can result from variation in some but not all metrics of hypoxia tolerance. For example, in the example of North American *Lepomis* sunfish that is discussed above, evolutionary variation in hypoxia tolerance that underlies differences in species distribution in the wild can be explained by interspecific differences in t_{LOE} but not P_{crit} (Borowiec et al., 2016; Farwell et al., 2007; Mathers et al., 2014). Among some other species, species differences in distribution can be explained by differences in P_{crit} (Chapman et al., 2002; Richards, 2011). Furthermore, considering multiple metrics of hypoxia tolerance may provide a more nuanced understanding of the ecological implications of hypoxia. For example, heavy use of anaerobic metabolism below P_{crit} may prolong survival in hypoxia, but deplete energy reserves for the performance of ecologically relevant traits (e.g. foraging, reproduction, etc.). Given that no single metric can fully explain how fish respond to and cope with hypoxia, and that no single metric can reliably predict interspecific variation in hypoxic niche, we believe that metrics for describing both aerobic respiration and survival in hypoxia should be included in future studies aiming to characterize hypoxia tolerance.

Hypoxia acclimation improves hypoxia tolerance

Hypoxia acclimation generally improved hypoxia tolerance, as reflected by the various indices of tolerance measured here, including reduced P_{LOE}, increased t_{LOE} and increased RI. Broadening of the P_{O_2} range for sustaining resting metabolism and/or body posture are common responses to hypoxia acclimation in fishes (Borowiec et al., 2015, 2018; Fu et al., 2011; Regan et al., 2017b; Richards, 2009), and could reflect the combined impact of physiological changes that increase branchial O_2 uptake or circulatory O_2 transport (Matey et al., 2008; Perry et al., 2009; Wells, 2009), adjust the use of anaerobic metabolism (Richards, 2009; Richards et al., 2008; Vornanen et al., 2009) and actively reduce cellular ATP demands (Boutilier, 2001; Hochachka et al., 1996; Richards, 2010). In *F. heteroclitus*, the mechanisms used to improve hypoxia tolerance after acclimation appear to differ between patterns of hypoxia exposure. Acclimation to constant hypoxia induces a pronounced ∼50% reduction in whole-animal $\dot{M}_{O_2}$, well beyond the reduction observed in naive fish exposed to acute hypoxia, in association with reductions in gill-filament length and in the oxidative capacity of the muscle (Borowiec et al., 2015, 2018). Whether similar mechanisms underlie the improvements in hypoxia tolerance observed here is unclear, insofar as resting $\dot{M}_{O_2}$ was unaffected by acclimation across species (Fig. 9). If these species are capable of metabolic depression, its use may be reserved for more severe levels of hypoxia, as observed in goldfish (Regan et al., 2017a). Acclimation of *F. heteroclitus* to intermittent hypoxia, by contrast, leads to increases in $\dot{M}_{O_2}$ in hypoxia, and to increases in the oxidative and gluconeogenic capacities of the liver that could hasten the speed of recovery from anaerobic metabolism (Borowiec et al., 2015, 2018). These distinct coping mechanisms enacted by different patterns of hypoxia exposure are both effective at maintaining cellular ATP content and avoiding metabolic acidosis during hypoxia (Borowiec et al., 2018), and improving hypoxia tolerance (Borowiec et al., 2015).

There were interspecific differences in the magnitude of the response to chronic hypoxia, suggesting that there is evolutionary variation in the phenotypic plasticity associated with hypoxia acclimation across fundulid killifishes (Figs 8 and 9). *Fundulus rathbuni* exhibited greater plasticity than *L. parva* and *L. goodei* for several traits (P_{LOE}, P_{crit}, RI). One explanation for this may be differences in the signal for plasticity across taxa (e.g. *F. rathbuni* may experience a greater decrease in tissue P_{O_2} during hypoxia acclimation). Related to this possibility, the severity of hypoxia relative P_{crit} could affect the plastic response, as fish were chronically exposed to a P_{O_2} that was below the normoxic P_{crit} for two species (*F. rathbuni* and *L. parva*) but above it for the other species (*L. goodei*). This may help explain why P_{crit} was reduced by exposure to constant hypoxia in *F. rathbuni* and *L. parva* but not in *L. goodei* (Fig. 9C). Another possible explanation is that each species differs in its inherent capacity for plasticity, potentially as a result of interspecific variation in the activation and capacity of different coping mechanisms during exposure to chronic hypoxia. This may explain why *L. goodei*, which had the lowest P_{crit} of the three species, showed such a strong improvement in t_{LOE} in response to constant hypoxia (Fig. 9A). Relatively few detailed investigations into interspecific variation in phenotypic plasticity in response to chronic hypoxia have been done in other fishes, but some previous studies have revealed interspecific variation in how metabolic function and gene expression respond to days of hypoxia exposure (Fu et al., 2014; Mandic et al., 2014). Such interspecific variation in plastic responses to hypoxia probably has important implications for the ecology and distribution of different fish species.

Phenotypic plasticity can facilitate (or sometimes impede) colonization of novel environments, and the magnitude of plasticity can evolve (Fordyce, 2006; Ghalambor et al., 2007; Storz et al., 2010). The observed improvements in hypoxia tolerance associated with hypoxia acclimation probably help fundulid killifish colonize hypoxic environments in the wild. Most of the killifish species studied here were relatively tolerant of hypoxia by standard measures, and many inhabit environments that can become quite hypoxic, including estuaries (*F. heteroclitus*, *F. confluentus*, *L. parva*) and freshwater lakes that experience seasonal ice-cover and hypoxia (*F. diaphanus*) (Hasler et al., 2009; Nordlie, 2006; Whitehead, 2010). Our findings suggest that hypoxia tolerance is plastic across fundulids, but the manifestation of plasticity can differ between species, which could contribute to the broad distribution of this family across North America (Nordlie, 2006; Whitehead, 2010).

Acknowledgements
We thank Tiffany Simms Lindsay, Kat Munley, Charlie Brown and other members of the Galvez lab for their assistance. We also thank Catie Ivy for her help with troubleshooting our R script. Matt D. Regan and Jeff G. Richards provided excellent comments on a pre-submission version of the manuscript. We also thank the anonymous reviewers for their comments on a previous version of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.G.B., F.G., G.R.S.; Methodology: B.G.B., R.D.H., C.D.H., G.R.S.; Formal analysis: B.G.B., C.D.H., F.G., G.R.S.; Investigation: B.G.B., R.D.H.; Resources: F.G., G.R.S.; Data curation: B.G.B.; Writing - original draft: B.G.B.; Writing - review & editing: B.G.B., R.D.H., C.D.H., F.G., G.R.S.; Visualization: B.G.B., G.R.S.; Supervision: B.G.B., F.G., G.R.S.; Project administration: B.G.B., F.G., G.R.S.; Funding acquisition: B.G.B., R.D.H., F.G., G.R.S.

Funding
The equipment and operational costs of this research were supported by funds from McMaster University, the Canadian Foundation for Innovation, the Ontario Ministry of Research and Innovation, a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to G.R.S., and a National Science Foundation grant and a National Institutes of Environmental Health Sciences grant (R01 ES021934-01) to F.G. B.G.B. was supported by an Ontario Graduate Scholarship and a National Institutes of Environmental Health Sciences postgraduate scholarship, as well as travel funding from McMaster University and the Society for Experimental Biology. R.D.H. was supported by the Louisiana State University President's Future Leaders in Research scholarship, an American Physiological Society undergraduate research fellowship, a Louisiana Sea Grant Award, a Youth Activity Fund from the Explorer Club, and travel awards through Louisiana State University Discover and Louisiana State University Office of Research. G.R.S. is supported by the Canada Research Chairs program. Deposited in PMC for release after 12 months.

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