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RESEARCH ARTICLE

Physiological tradeoffs may underlie the evolution of hypoxia tolerance and exercise performance in sunfish (Centrarchidae)

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ABSTRACT

Tradeoffs between hypoxia tolerance and aerobic exercise performance appear to exist in some fish taxa, even though both of these traits are often associated with a high O_2 transport capacity. We examined the physiological basis for this potential tradeoff in four species of sunfish from the family Centrarchidae. Hypoxia tolerance was greatest in rock bass, intermediate in pumpkinseed and bluegill and lowest in largemouth bass, based on measurements of critical O_2 tension (P_{crit}) and O_2 tension at loss of equilibrium (P_{O_2} at LOE). Consistent with there being a tradeoff between hypoxia tolerance and aerobic exercise capacity, the least hypoxia-tolerant species had the highest critical swimming speed (U_{crit}) during normoxia and suffered the greatest decrease in U_{crit} in hypoxia. There was also a positive correlation between U_{crit} in normoxia and P_{O_2} at LOE, which remained significant after accounting for phylogeny using phylogenetically independent contrasts. Several sub-organismal traits appeared to contribute to both hypoxia tolerance and aerobic exercise capacity (reflected by traits that were highest in both rock bass and largemouth bass), such as the gas-exchange surface area of the gills, the pH sensitivity of haemoglobin–O_2 affinity, and the activities of lactate dehydrogenase and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver. Some other sub-organismal traits were uniquely associated with either hypoxia tolerance (low sensitivity of haemoglobin–O_2 affinity to organic phosphates, high pyruvate kinase and lactate dehydrogenase activities in the heart) or aerobic exercise capacity (capillarity and fibre size of the axial swimming muscle). Therefore, the cumulative influence of a variety of respiratory and metabolic traits can result in physiological tradeoffs associated with the evolution of hypoxia tolerance and aerobic exercise performance in fish.

KEY WORDS: Respiration, Oxygen cascade, Critical O_2 tension, Energy metabolism, Centrarchids, Muscle histology

INTRODUCTION

Hypoxia is a regular occurrence in many freshwater ecosystems. It can result from both natural and anthropogenic events, including eutrophication, prolonged ice cover, stratification of the water column, and when respiration exceeds the rate of diffusion from the atmosphere (Diaz, 2001; Diaz and Rosenberg, 2008; Breitburg et al., 2009). When severe, hypoxia can be the cause of fish kills, changes in trophic patterns and loss of habitat (Mallin et al., 2006), and it is believed that the incidence and severity of aquatic hypoxia will increase in the future as global temperatures rise and as urbanization and pollution continue (Diaz, 2001).

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Hypoxia tolerance in fish is associated with a variety of physiological traits, arising via plasticity (e.g. acclimatization or developmental plasticity) or evolutionary specialization (e.g. genetic adaptation). These can include adjustments in traits that improve oxygen uptake from the water and oxygen transport to tissues, increase anaerobic ATP production, or depress ATP demands in hypoxia (Boutilier, 2001; Bickler and Buck, 2007; Richards et al., 2009; Borowiec et al., 2015). For example, many tolerant species have an increased capacity for O_2 transport in hypoxia compared with intolerant species, the underlying mechanisms of which can include a high functional area for oxygen uptake at the gills and/or a high haemoglobin (Hb)–O_2 affinity (Nilsson, 2007; Chapman et al., 2008; Mandic et al., 2009), and they tend to live a relatively sedentary lifestyle (Chapman and McKenzie, 2009). In contrast to the growing appreciation of the mechanisms fish use to cope with hypoxia, the potential tradeoffs associated with the evolution of hypoxia tolerance are not well understood.

Several observations suggest that there may be physiological tradeoffs between hypoxia tolerance and aerobic exercise capacity in some fish taxa. For example, it has been found that cyprinid fish species from habitats with high water flows (wild-caught but acclimated to laboratory conditions) tend to have a higher capacity for sustained swimming but a lesser hypoxia tolerance than species from slow flowing habitats, suggesting that there is a mechanistic tradeoff between hypoxia tolerance and performance among closely related species, even after phylogenetic relationships are taken into account (Fu et al., 2014). An increase in hypoxia tolerance coincident with a decrease in sustained swimming performance has been observed during development in coral reef damselfish (Nilsson et al., 2007). It is also well known that many active species (e.g. tuna, rainbow trout) are very sensitive to hypoxia, even though they possess extremely high O_2 transport capacities to support high rates of aerobic metabolism (Davis, 1975; Gooding et al., 1981; Bushnell et al., 1990; Matey et al., 2011). However, tradeoffs between hypoxia tolerance and exercise performance are not always observed. In carp, for example, hypoxia acclimation increases prolonged swimming capacity, and swim training improves hypoxia tolerance (Fu et al., 2011). It is therefore possible that tradeoffs between hypoxia tolerance and aerobic exercise capacity are an idiosyncratic feature of only some fish taxa.

Centrarchidae is a family of four genera and 32 species of North American freshwater sunfish (Near et al., 2004) that exhibit appreciable variation in hypoxia tolerance and aerobic exercise capacity. For example, the pumpkinseed sunfish [*Lepomis gibbosus* (Linnaeus 1758)] can tolerate deep hypoxia for longer periods than its congener, the bluegill sunfish [*Lepomis macrochirus* (Rafinesque 1810)] (Farwell et al., 2007; Mathers et al., 2014). Largemouth bass [*Micropterus salmoides* (Lacépède 1802)] also appear to be more tolerant of hypoxia than smallmouth bass

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List of symbols and abbreviations

BL	body length
C:F	capillary to fibre ratio
CD	capillary density
COX	cytochrome *c* oxidase
CS	citrate synthase
Hb	haemoglobin
HOAD	3-hydroxyacyl-CoA dehydrogenase
LDH	lactate dehydrogenase
LOE	loss of equilibrium
M_b	body mass
Mb	myoglobin
MCHC	mean cell Hb concentration
$\dot{M}_{O_2}$	rate of oxygen consumption
$\dot{M}_{O_2,max}$	maximal $\dot{M}_{O_2}$
P_{50}	P_{O_2} at which Hb is 50% saturated
P_{crit}	critical oxygen tension
P_{O_2}	partial pressure of oxygen
PEPCK	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
SDH	succinate dehydrogenase
TGSA	total gill surface area
U_{crit}	critical swimming speed

(*Micropterus dolomieu*), as largemouth bass maintain higher blood O_2 content in hypoxia and can maintain blood pH at levels of hypoxia that induce a metabolic acidosis in smallmouth bass (Furimsky et al., 2003). Largemouth bass and smallmouth bass are generally good swimmers, with higher sustained swimming performance than both bluegill and pumpkinseed (Brett and Sutherland, 1965; Dahlberg et al., 1968; Kelsch, 1996).

The objectives of this study were to (i) determine whether there is an apparent tradeoff between hypoxia tolerance and aerobic exercise capacity among a group of centrarchid species [largemouth bass, bluegill, pumpkinseed, and rock bass, *Ambloplites rupestri* (Rafinesque 1817)] inhabiting a similar environment (Lake Opinicon, Canada), and (ii) examine the underlying physiological determinants of hypoxia tolerance, aerobic exercise capacity and the potential tradeoff between them from across the oxygen transport pathway. We studied fish that were well acclimated to common conditions in the lab to eliminate variation arising from reversible physiological plasticity (though not the persistent irreversible effects of developmental plasticity or parental environment) and to help determine the inherent evolved differences between species.

RESULTS

Hypoxia tolerance and resting O_2 consumption rate

There was distinct variation in hypoxia tolerance across species, as evidenced by differences in critical oxygen tension (P_{crit}) – the oxygen tension (P_{O_2}) at which fish transition from oxyregulating to oxyconforming – and the P_{O_2} at loss of equilibrium (LOE) (Fig. 1A). Rock bass had the lowest P_{crit} (~2.3 kPa) and P_{O_2} at LOE (~0.7 kPa) of any of the species. P_{crit} was similar in the remaining species, but largemouth bass had a higher P_{O_2} at LOE (~1.6 kPa) than all other species. The pattern of variation in resting O_2 consumption rate ($\dot{M}_{O_2}$) was similar to the variation in P_{crit} (in mg O_2 kg^{-1} body mass h^{-1}: rock bass, 59.7±6.2; pumpkinseed, 81.6±5.2; bluegill, 79.4±4.4; largemouth bass, 86.1±12.2), but the differences were only marginally significant (P=0.053). These results suggest that rock bass are the most hypoxia tolerant of the species examined, followed by pumpkinseed and bluegill, and that largemouth bass are the least tolerant.

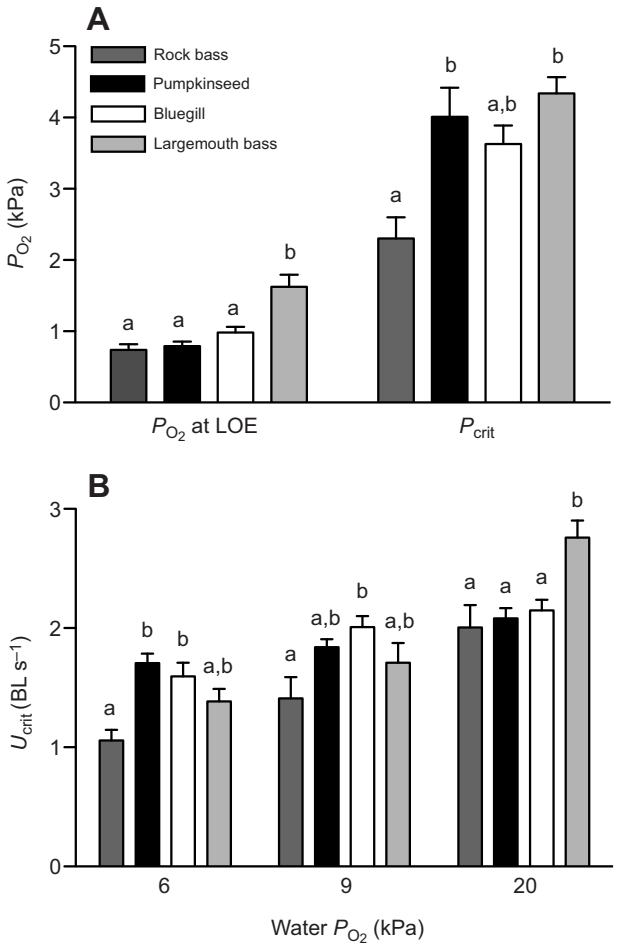

Fig. 1. Hypoxia tolerance and aerobic swimming capacity varied across species. (A) Critical oxygen tension (P_{crit}; P=0.009) and oxygen tension (P_{O_2}) at loss of equilibrium (LOE; P<0.001) both varied across rock bass (N=8), pumpkinseed (N=17), bluegill (N=13) and largemouth bass (N=7) as assessed by one-factor ANOVA. (B) There were significant main effects of species and water P_{O_2}, as well as a significant species×P_{O_2} interaction, on critical swimming speed (U_{crit}) (all P<0.001 in two-factor ANOVA). Within each measurement, different letters indicate a significant pairwise difference between species (P<0.05).

Aerobic exercise capacity

Aerobic swimming performance in normoxia (P_{O_2}≈20 kPa) varied across centrarchid species in a similar manner to the variation in P_{O_2} at LOE (Fig. 1B). Largemouth bass had a 28–38% higher critical swimming speed (U_{crit}) than rock bass, pumpkinseed and bluegill. Maximal O_2 consumption rate ($\dot{M}_{O_2,max}$) in normoxia was also highest on average in largemouth bass, and there was significant overall variation in $\dot{M}_{O_2,max}$ between species (Fig. 2), but the variation in $\dot{M}_{O_2,max}$ between species was generally lower in magnitude than the variation in U_{crit}. This may have arisen because of variation in body shape between species – largemouth bass are more streamlined with a higher fineness ratio (body length: body depth of 3.47±0.10, compared with 2.33±0.02 in rock bass, 1.98±0.02 in pumpkinseed and 2.00±0.01 in bluegill; P<0.001) – which could alter the relationship between U_{crit} and $\dot{M}_{O_2,max}$. Nevertheless, our data suggest that largemouth bass have the greatest capacity for performing sustainable exercise in normoxia.

Hypoxia had a substantial effect on aerobic swimming performance, particularly in largemouth bass and rock bass

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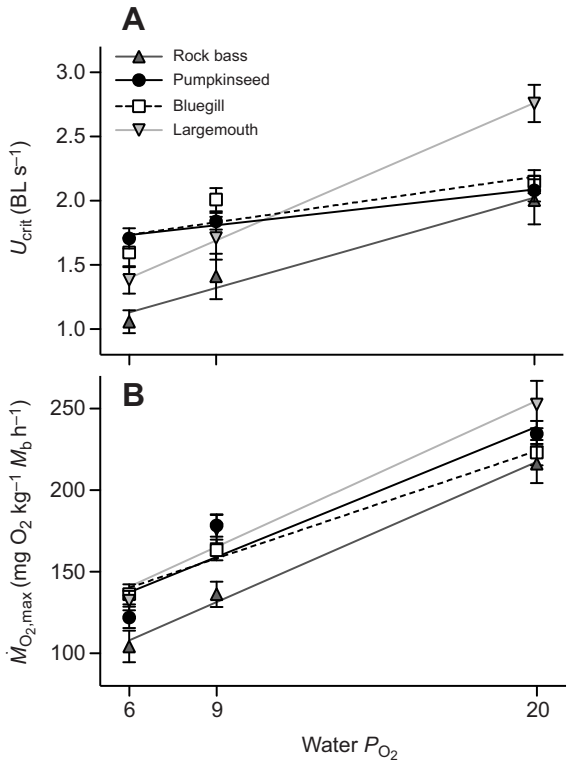

Fig. 2. The effect of hypoxia on aerobic swimming capacity varied across species. (A) The effect of reductions in water P_{O_2} on U_{crit} varied between species, as reflected by differences in the slopes of regressions of U_{crit} to water P_{O_2} (rock bass, 0.064±0.015; pumpkinseed, 0.025±0.007; bluegill, 0.032±0.010; largemouth bass, 0.097±0.013; P<0.0001 in ANCOVA). (B) There were no significant differences between species in the slopes of regressions of maximal O_2 consumption rate ($\dot{M}_{O_2,max}$) to water P_{O_2} (rock bass, 7.81±0.94; pumpkinseed, 7.26±0.75; bluegill, 5.90±0.65; largemouth bass, 8.14±1.02; P=0.306) but there were significant overall differences in $\dot{M}_{O_2,max}$ (P=0.0005 for intercepts in ANCOVA). M_b, body mass.

(Fig. 1B). In these species, U_{crit} in mild hypoxia (water P_{O_2} of 9 kPa) was only ~60% and ~67% of what it was in normoxia, respectively, and U_{crit} in moderate hypoxia (water P_{O_2} of 6 kPa) was only ~50% of normoxic values (6 kPa). Hypoxia had a lesser effect on U_{crit} in pumpkinseed and bluegill, as U_{crit} in mild hypoxia (≥90% of normoxic values) and moderate hypoxia (~80% of normoxic values) was a much higher proportion of U_{crit} in normoxia. Interspecific variation in the effects of hypoxia on swimming performance was reflected by a significant species×P_{O_2} interaction on U_{crit} (Fig. 1B). There were also significant differences between species in the slopes of regressions of U_{crit} to water P_{O_2}, with the largest slope and thus the greatest decline in U_{crit} with hypoxia exhibited by largemouth bass (Fig. 2A). This pattern of variation was less evident in the slopes of regressions of $\dot{M}_{O_2,max}$ to water P_{O_2} (Fig. 2B).

Correlation between hypoxia tolerance and aerobic exercise capacity

The pattern of variation in P_{O_2} at LOE and aerobic swimming performance suggested that better swimming fish (i.e. largemouth bass) are less hypoxia tolerant. There was a corresponding positive correlation between the species means of U_{crit} in normoxia and of P_{O_2} at LOE [U_{crit}=0.85±0.07 (P_{O_2} at LOE)+1.37±0.07, R^2=0.988, P=0.006]. This correlation remained significant after accounting for phylogeny using phylogenetically independent contrasts (Fig. 3).

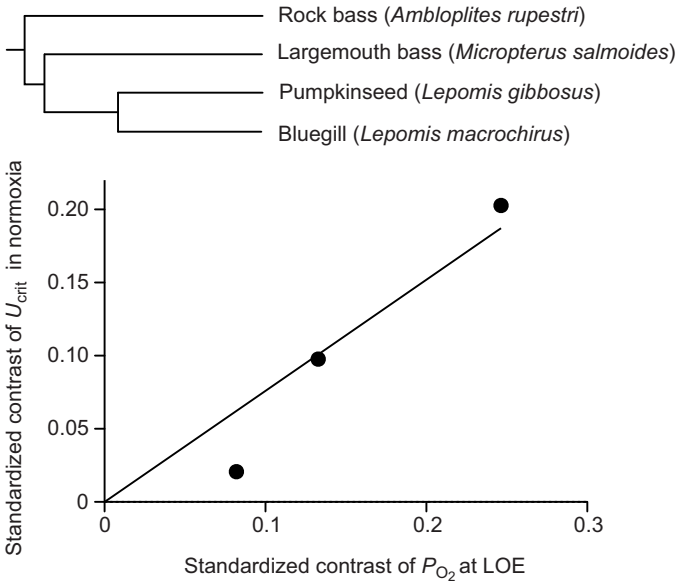

Fig. 3. There was a phylogenetically independent relationship between hypoxia tolerance and aerobic swimming capacity in normoxia. The phylogenetic tree shown was used for phylogenetically independent contrast analysis using an exponential transformation of branch lengths. There was a significant positive correlation between the standardized independent contrasts of U_{crit} and P_{O_2} at LOE (Pearson product-moment correlation coefficient, 0.980; reduced major axis slope, 0.775; P=0.010).

However, the correlation between U_{crit} and P_{crit} was not significant (data not shown).

Gill morphology

There were substantial differences in gill morphology between species, with the most hypoxia-tolerant species and the best-swimming species having the greatest surface areas for branchial gas exchange (Fig. 4). Rock bass and largemouth bass had 20–40% larger total gill surface area (TGSA) on average than both pumpkinseed and bluegill, a difference that was significant in

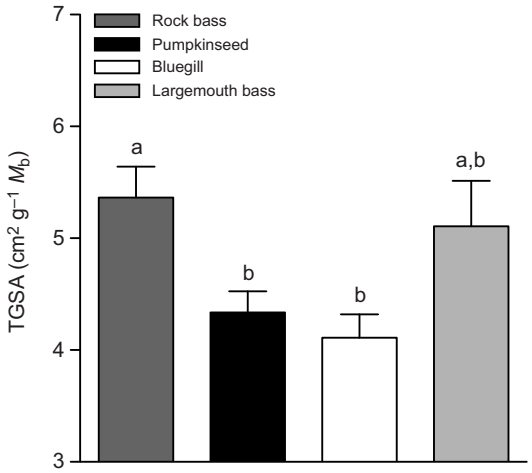

Fig. 4. Gill surface area was greatest in rock bass and largemouth bass. There was significant variation in total gill surface area (TGSA, expressed relative to M_b) across rock bass (N=8), pumpkinseed (N=16), bluegill (N=11) and largemouth bass (N=5) as assessed by one-factor ANOVA (P=0.003). Different letters indicate a significant pairwise difference between species (P<0.05).

Table 1. Gill morphometrics

	Rock bass	Pumpkinseed	Bluegill	Largemouth bass
Total filament number	1649±36[a]	1255±27[b]	1277±19[b]	2194±201[c]
Average filament length (mm)	3.47±0.11[a]	3.57±0.07[a]	3.67±0.15[a]	2.73±0.19[b]
Lamellar density (mm^{-1})	23.9±0.3	25.2±0.7	27.2±1.3	27.5±0.5
Lamellar bilateral surface area (mm^2)	0.242±0.020[a,b]	0.339±0.008[c]	0.247±0.011[a]	0.183±0.004[b]

Data were compared using one-factor ANOVA (P<0.001, P<0.001, P=0.063 and P<0.001, respectively) and are shown as means±s.e.m. (N as in Fig. 4). Within each morphometric measurement, different letters indicate a significant pairwise difference between species (P<0.05).

rock bass and neared significance in largemouth bass (P=0.092). Most of this variation in TGSA could be accounted for by variation in the number of gill filaments, which more than compensated for the shorter average filament length and smaller lamellae of largemouth bass (Table 1). Although pumpkinseed possessed lamellae with a relatively large surface area, TGSA was comparable to that of bluegill because of the offsetting effects of the other morphometric traits on TGSA (Table 1).

Hb–O_2 binding and haematology

There was appreciable variation in the effects of allosteric modifiers on Hb–O_2 affinity between species. P_{50} (the P_{O_2} at which Hb is 50% saturated) of stripped Hb was invariant across species at pH 7.8 and 7.4, suggesting that differences in inherent O_2 affinity do not underlie hypoxia tolerance or aerobic exercise capacity (Table 2). However, heightened pH sensitivity of Hb may underlie both hypoxia tolerance and aerobic exercise capacity, based on the greater increase in P_{50} from pH 7.4 to 7.0 in rock bass and largemouth bass in the presence of ATP and GTP (Fig. 5A). P_{50} was more sensitive to ATP and GTP at pH 7.0 in largemouth bass than in the other species (Fig. 5B). The P_{50} we measured in largemouth bass at pH 7.0 in the presence of ATP and GTP is very similar to a previous P_{50} measurement at 20°C on whole-blood from the same species (Furimsky et al., 2003). In contrast, there were no differences between species in blood Hb content, haematocrit or mean cell Hb concentration (MCHC) (Table 3).

Phenotype of the swimming muscle

Capillarity of the oxidative (red) fibre region of the axial swimming muscle was highest in the best swimming species, largemouth bass (Fig. 6A). Largemouth bass had both the highest density of capillaries (1.5- to 2.3-fold higher than other species) and the highest capillary to fibre ratio (1.3- to 2.1-fold higher than other species) in the oxidative muscle. Largemouth bass also had higher capillary density in the glycolytic (white) muscle, but capillary to fibre ratio was relatively low in largemouth bass compared with the other species (Fig. 6B). This distinction between indices of capillarity may have arisen because largemouth bass also had the smallest glycolytic muscle fibres (Table 4).

Although there were large differences in capillarity between species, there was no variation in the abundance of oxidative fibres in the axial musculature, or in the maximal activity of oxidative enzymes. The transverse area of oxidative fibres was similar between species, assessed as both total absolute area scaled to body mass or areal density (Fig. 6C). There were also no differences in average oxidative fibre size (Table 4) or in the total number of oxidative fibres in the axial musculature (Fig. 6D). Consistent with these findings, there were no differences in the activities of citrate synthase (CS; which catalyses the first step of the citric acid cycle) or cytochrome *c* oxidase (COX; complex IV and the terminal O_2 acceptor in the electron transport chain) assayed in samples that contained the entirety of the red and white muscle in one lateral hemisphere (Fig. 7A).

There was variation between species in the activity of enzymes involved in carbohydrate and lipid catabolism in the swimming muscle (Fig. 7A). The activity of the glycolytic enzyme pyruvate kinase (PK) was highest in rock bass and lowest in largemouth bass, in possible association with the variation in hypoxia tolerance. However, the substantial variation in both lactate dehydrogenase (LDH) and the β-oxidation enzyme 3-hydroxyacyl-CoA dehydrogenase (HOAD) did not occur in parallel to the variation in hypoxia tolerance or exercise performance.

Characteristics of the heart

There was substantial variation in heart mass across species, with largemouth bass – the best swimming species examined – having the largest heart (Table 3). Curiously, bluegill had very small hearts compared with the other species, such that largemouth bass had hearts that were almost twice as large as those of bluegill.

Variation in the biochemical capacity for glycolysis and anaerobic metabolism in the heart appeared to parallel the variation in hypoxia tolerance (Fig. 7B). Rock bass had 1.5- to 1.9-fold higher maximal activity of PK in the heart than all other species, and had 1.5- to 1.7-fold higher LDH activity than bluegill and largemouth bass. A similar pattern of variation in PK and LDH was observed when the aggregate activity across the whole heart was calculated (i.e. product of heart mass and the activities reported in Fig. 7B; data not shown). Neither oxidative capacity nor the

Table 2. Haemoglobin–O_2 binding affinity

	Rock bass	Pumpkinseed	Bluegill	Largemouth bass
Stripped Hb				
pH 7.8	0.59±0.04	0.52±0.02	0.44±0.01	0.50±0.04
pH 7.4	0.64±0.04	0.61±0.04	0.58±0.02	0.66±0.08
pH 7.0	1.66±0.12[a]	1.08±0.07[b]	0.94±0.04[b]	1.20±0.15[b]
Hb+2ATP+1GTP				
pH 7.4	0.69±0.05	0.58±0.02	0.53±0.02	0.73±0.02
pH 7.0	2.49±0.11[a]	1.56±0.07[b]	1.43±0.10[b]	2.95±0.30[c]

Data are the O_2 tension (kPa) at which haemoglobin (Hb) is 50% saturated (P_{50}), shown as means±s.e.m. (N as in Fig. 5), and were compared using two-factor ANOVA (P<0.001 for main effects of species and treatment, as well as for the species×treatment interaction).
Within each treatment, different letters indicate a significant pairwise difference between species (P<0.05).

Journal of Experimental Biology (2015) 218, 3264-3275 doi:10.1242/jeb.124602

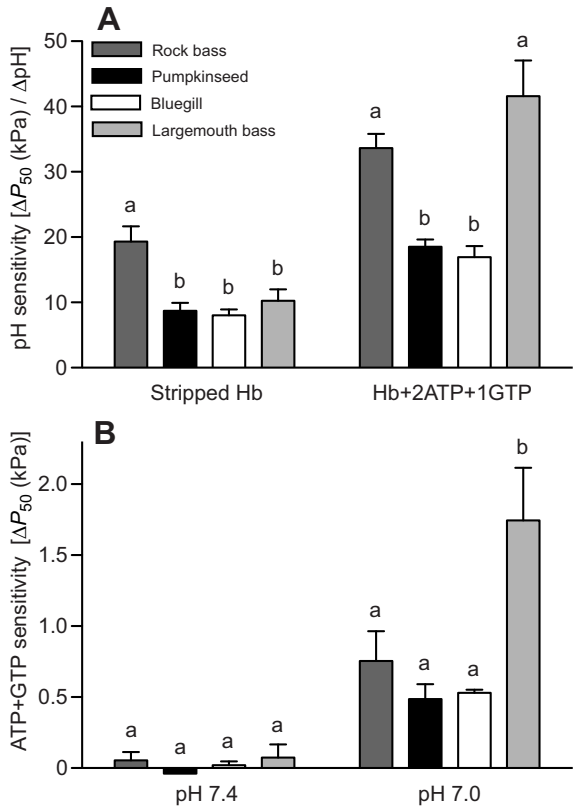

Fig. 5. The sensitivity of haemoglobin (Hb)–O_2 binding to pH and organic phosphates varied across species. (A) There were significant main effects of species and the presence of organic phosphates (ATP and GTP), as well as a significant species×organic phosphate interaction, on pH sensitivity (all P<0.001 in two-factor ANOVA). pH sensitivity was measured as the increase in P_{O_2} at which Hb is 50% saturated (P_{50}) between pH 7.4 and pH 7.0, normalized to a 1.0 pH unit change. (B) There were significant main effects of species and pH, as well as a significant species×pH interaction, on the increase in P_{50} caused by ATP and GTP (in molar ratios of 2 and 1 to tetrameric Hb) (all P<0.001). All measurements were made at 25°C in the presence of 50 mmol l^{-1} NaCl. Within each treatment, different letters indicate a significant pairwise difference between species (P<0.05) (N as follows: rock bass, 8; pumpkinseed, 15; bluegill, 10; largemouth bass, 5).

capacity for β-oxidation in the heart appeared to be clearly related to the variation in hypoxia tolerance or aerobic exercise capacity, because COX activity and myoglobin content ([Mb]) were invariant across all species, CS activity was highest in pumpkinseed and bluegill, and HOAD only differed in pumpkinseed (Fig. 7B, Table 3).

Enzyme activity in the liver and brain

There was appreciable variation in enzyme activity in the liver that was associated with hypoxia tolerance and/or aerobic exercise capacity (Fig. 7C). The activity of both LDH and phosphoenolpyruvate carboxykinase (PEPCK; an enzyme involved in gluconeogenesis) was elevated in both rock bass (the most hypoxia-tolerant species) and largemouth bass (the best swimmer) relative to pumpkinseed and bluegill. Liver PK activity was also uniquely elevated by 2- to 3-fold in largemouth bass, and COX activity appeared to be reduced in this species as well (only significant compared with pumpkinseed). Pumpkinseed had the highest activity of HOAD in the liver, but this was not related to the variation in hypoxia tolerance or aerobic exercise capacity. Enzyme activities in the brain were either invariant across species (CS, COX and LDH) or exhibited variation that was not associated with hypoxia tolerance or aerobic exercise capacity (Fig. 7D).

DISCUSSION

The potential tradeoff between hypoxia tolerance and aerobic exercise capacity in fish is paradoxical when considering that both traits rely on a high capacity for O_2 transport. Our current findings in fish from the family Centrarchidae, along with recent observations in other taxa (Fu et al., 2014), suggest that this tradeoff may exist among closely related species. We also identified a variety of respiratory and metabolic traits from across the O_2 cascade that vary in association with hypoxia tolerance and/or aerobic exercise capacity. Our data suggest that several sub-organismal traits underlie both hypoxia tolerance and aerobic exercise capacity, including a large surface area for gas exchange at the gills, a high pH sensitivity of Hb–O_2 binding, and a high gluconeogenic capacity in the liver. A variety of other sub-organismal traits distinguish hypoxia tolerance and aerobic exercise capacity – such as the sensitivity of Hb to organic phosphates, muscle capillarity and the capacity for anaerobic energy production in the heart – and may therefore contribute to a potential tradeoff.

Physiological basis for hypoxia tolerance

Rock bass appeared to have the greatest hypoxia tolerance among the species examined, with a lower P_{crit} than all the others and a P_{O_2} at LOE that was less than that of largemouth bass and similar to (or slightly less than on average) that of pumpkinseed and bluegill. The P_{crit} of rock bass is low compared with that of most other fish (Nilsson and Östlund-Nilsson, 2008; Scott et al., 2008; Mandic et al., 2009; Borowiec et al., 2015), suggesting that this species possesses an enhanced capacity to extract and transport oxygen and to sustain routine metabolism across a wide range of water P_{O_2}. P_{crit} is often, but not always, related to the ability to resist losing equilibrium in hypoxia (Speers-Roesch et al., 2013), probably because it is underlain by different sub-organismal physiological traits, which perhaps accounts for the discrepancy in the interspecific variation exhibited for P_{crit} and P_{O_2} at LOE observed here. In fact, the determinants of P_{O_2} at LOE may differ from those

Table 3. Haematology, heart size and heart myoglobin

	Rock bass	Pumpkinseed	Bluegill	Largemouth bass
Blood [Hb] (g dl^{-1})	6.47±0.56	5.81±0.69	6.01±0.79	5.1±0.7
Haematocrit (%)	31.0±1.9	30.3±0.9	28.0±1.3	28.3±1.7
MCHC (g dl^{-1})	20.7±1.2	19.4±2.2	20.7±1.2	17.8±1.5
Heart mass (% M_b)	0.147±0.006^{a,b}	0.148±0.008^{b}	0.104±0.010^{a}	0.187±0.024^{b}
Heart [Mb]	1.80±0.34	3.17±0.64	2.65±0.43	2.91±0.46

MCHC, mean cell Hb concentration; M_b, body mass; [Mb], myoglobin content (mg g^{-1} heart tissue).
Data are means±s.e.m. and were compared using one-factor ANOVA (P=0.815, P=0.330, P=0.830, P<0.001 and P=0.256, respectively), with different letters indicating a significant pairwise difference between species for each measurement (P<0.05).
N as in Fig. 1 except for [Mb] (rock bass, N=5; pumpkinseed, N=12; bluegill, N=7; largemouth bass, N=4).

Journal of Experimental Biology (2015) 218, 3264-3275 doi:10.1242/jeb.124602

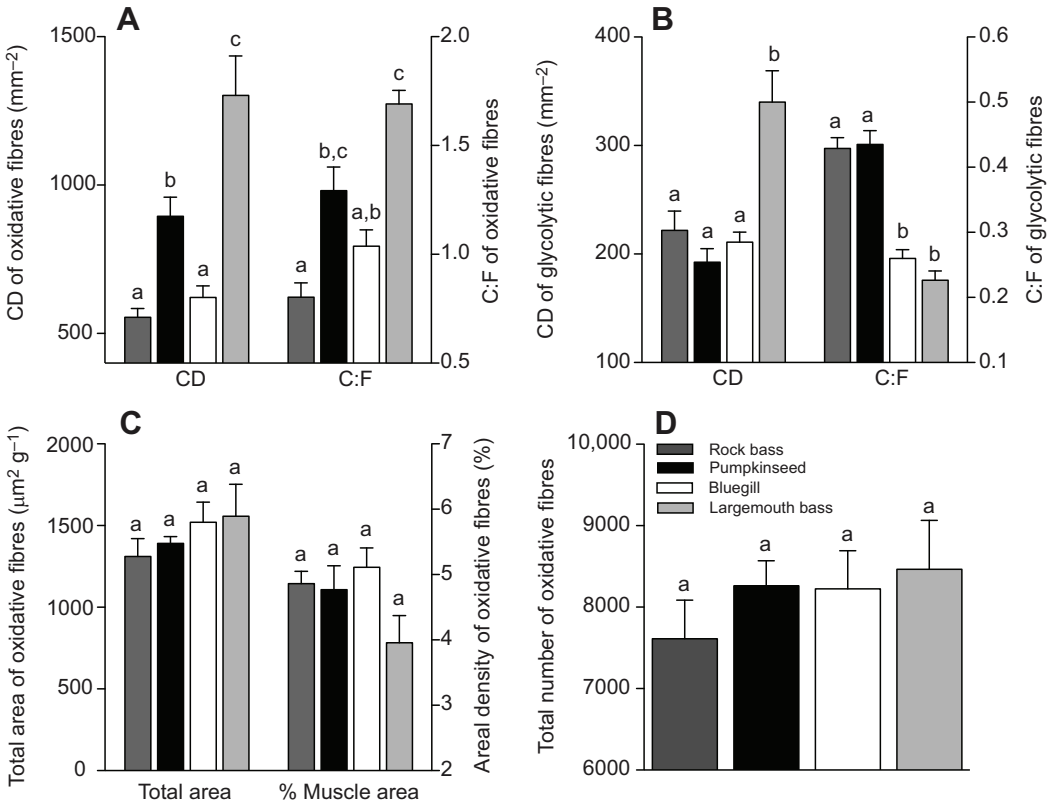

Fig. 6. Capillarity of the swimming muscle was higher in largemouth bass than in other centrarchids. Capillarity, expressed as capillary density (CD) and capillary to fibre ratio (C:F), varied across species in both the oxidative (A) and glycolytic (B) regions of the axial swimming muscle (all $P<0.001$ in one-factor ANOVA). In contrast, there were no significant differences between species in the area of oxidative fibres in the muscle (C), expressed in either absolute units ($P=0.437$) or as a percentage of the transverse area of the entire musculature ($P=0.204$), or in the total number of oxidative fibres ($P=0.667$) (D). Within each histological measurement, different letters indicate a significant pairwise difference between species ($P<0.05$) (N as follows: rock bass, 7; pumpkinseed, 12; bluegill, 9; largemouth bass, 5).

that dictate the time to LOE at a constant P_{O_2}. For example, pumpkinseed are able to avoid LOE at ∼2 kPa for a much longer duration than bluegill (Mathers et al., 2014), even though P_{crit} and the P_{O_2} at LOE in our progressive hypoxia protocol were similar between species (∼0.8–1.0 kPa; Fig. 1). Interestingly, it has been suggested that the northern range of centrarchid species is limited by hypoxia tolerance, due to there being more frequent potential for winterkill events from ice cover in the north, and among the species studied, rock bass have the northernmost range limit and largemouth bass have the southernmost (Bailey and Smith, 1981; Near et al., 2003).

The sensitivity of Hb–O_2 binding to organic phosphates was much lower in rock bass, pumpkinseed and bluegill than in largemouth bass (Fig. 5). The molecular mechanism for these differences are unclear, but could result from Hb sequence variation at or around ATP/GTP binding sites, or from species differences in the relative expression of Hb isoforms (e.g. anodic and cathodic isoforms differ in their sensitivity to H^+ and organic phosphates) (Weber and Fago, 2004). The lower ATP/GTP sensitivity we observed in the more hypoxia-tolerant fish is consistent with the frequent reductions in sensitivity to allosteric modifiers that have been observed in many hypoxia-tolerant vertebrates as a mechanism to increase Hb–O_2 affinity and enhance O_2 loading (Weber, 2007). Furthermore, the concentration of organic phosphates in red cells tends to be low in vertebrate species for which Hb P_{50} is insensitive to organic phosphates (Nikinmaa, 1990), and acclimation of fish to hypoxia can reduce the concentration of organic phosphates in red cells (Wood and Johansen, 1972; Jensen and Weber, 1985; Weber and Jensen, 1988). Therefore, *in vivo* P_{50} may have been higher in largemouth bass (due to interspecific differences in the sensitivity to and possibly concentrations of organic phosphates), which could have impaired branchial O_2 loading in hypoxia and contributed to the lesser hypoxia tolerance of this species compared with rock bass, pumpkinseed and bluegill.

The biochemical capacity of the heart for glycolysis and anaerobic metabolism, as reflected by the activities of PK and LDH, appeared to vary in parallel to the variation in hypoxia tolerance (Fig. 7B). The spongy myocardial tissue may be especially O_2 limited during hypoxia, because it is supplied with O_2 from the venous blood that has already perfused other upstream tissues in the peripheral circulation (Steffensen and Farrell, 1998;

Table 4. Muscle fibre size

	Oxidative fibres (μm^2)	Glycolytic fibres (μm^2)
Rock bass	1455±103	4696±408^a
Pumpkinseed	1473±91	5419±358^a
Bluegill	1674±65	4805±200^a
Largemouth bass	1357±150	3035±277^b

The transverse area of oxidative (red) and glycolytic (white) muscle fibres is shown as means±s.e.m. (N as in Fig. 6), and was compared using one-factor ANOVA ($P=0.196$ and $P=0.001$, respectively).
Within each fibre type, different letters indicate a significant pairwise difference between species ($P<0.05$).

Journal of Experimental Biology (2015) 218, 3264-3275 doi:10.1242/jeb.124602

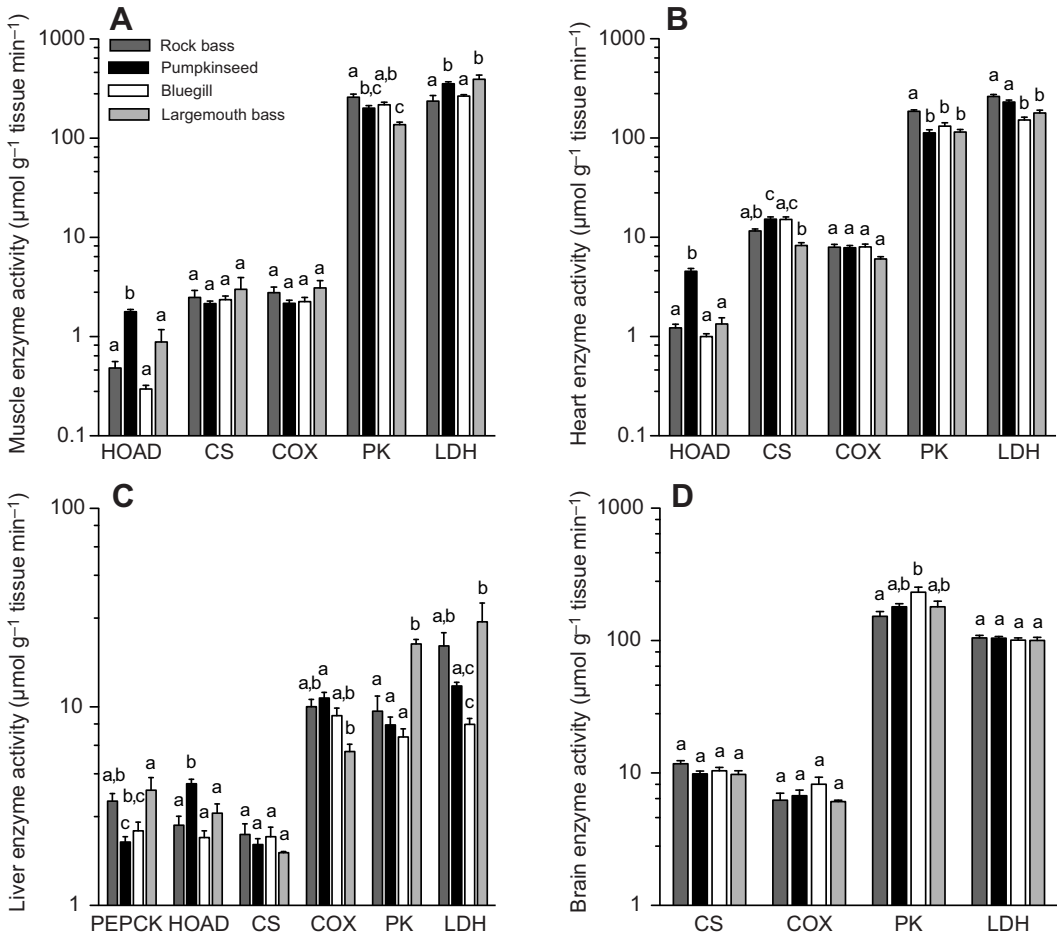

Fig. 7. Maximal activities of metabolic enzymes varied across species. (A) In the muscle, 3-hydroxyacyl-CoA dehydrogenase (HOAD), pyruvate kinase (PK) and lactate dehydrogenase (LDH) varied across species (all $P<0.001$ in one-factor ANOVA), but citrate synthase (CS) and cytochrome *c* oxidase (COX) did not vary ($P=0.498$ and $P=0.136$, respectively). (B) HOAD, CS, PK and LDH varied across species in heart (all $P<0.001$), but COX did not vary ($P=0.144$). (C) Phosphoenolpyruvate carboxykinase (PEPCK), HOAD, COX, PK and LDH varied across species in the liver (all $P<0.001$ except COX, $P=0.006$), but CS did not vary ($P=0.629$). (D) CS ($P=0.152$), COX ($P=0.326$) and LDH ($P=0.834$) were invariant in the brain, but there was modest variation in PK ($P=0.015$). Within each tissue enzyme measurement, different letters indicate a significant pairwise difference between species ($P<0.05$) (*N* as follows: rock bass, 8; pumpkinseed, 16; bluegill, 12; largemouth bass, 5).

Scott et al., 2008), so the spongy myocardium may require the use of anaerobic metabolism to maintain ATP supply. Consistent with this possibility, hypoxia acclimation increases the activity of glycolytic and anaerobic enzymes in the heart of some hypoxia-tolerant species (Chippari-Gomes et al., 2005; Martínez et al., 2006). The increase in LDH activity in particular may also increase the capacity of the heart to oxidize the lactate produced by other tissues in hypoxia. The activity of PK in the muscle also varied between species in relation to the observed differences in hypoxia tolerance, even though there were no differences in fibre-type composition. However, there were no interspecific differences in enzyme activity in the brain underlying the variation in hypoxia tolerance, consistent with recent observations in some fish taxa (Crocker et al., 2013) but inconsistent with some others (Mandic et al., 2013).

Physiological basis of aerobic exercise capacity

Largemouth bass had the greatest aerobic swimming performance in normoxia among all species examined, consistent with previous findings (Brett and Sutherland, 1965; Dahlberg et al., 1968; Kelsch, 1996). However, this difference was extremely dependent on water P_{O_2}, because U_{crit} was much more sensitive to hypoxia in largemouth bass than in pumpkinseed and bluegill (Fig. 1B, Fig. 2A). The limiting effect of hypoxia on aerobic exercise capacity has been documented in many other species (Lefrançois et al., 2005; Dutil et al., 2007), so the relative insensitivity of pumpkinseed and bluegill to hypoxia is somewhat surprising and may help these species to remain active if their environment becomes mildly hypoxic.

Largemouth bass were clearly distinguished from other species by having the highest capillarity in the swimming muscle, a feature that should have a strong influence on muscle performance and aerobic capacity (Wagner, 1996; Scott and Milsom, 2006). High performing species, such as lamnid sharks and tunas, also have high capillarity in the red muscle (Bernal et al., 2001), and swim training often increases the capillarity of red muscle (Sänger, 1992). However, capillarity and oxidative capacity are usually tightly linked (Hepple, 2000), and our observation that these traits did not co-vary between species (Figs 6, 7) is uncommon. The importance of the high capillarity in the glycolytic muscle of largemouth bass is less clear, but it could contribute to swimming performance at very high speeds when the white muscle is first recruited (Rome et al., 1984), or facilitate the clearance of lactate and metabolic acid during recovery from exhaustive exercise (Egginton, 2011).

Journal of Experimental Biology (2015) 218, 3264-3275 doi:10.1242/jeb.124602

Traits underlying both hypoxia tolerance and exercise performance

Some sub-organismal traits appeared to be related to both hypoxia tolerance and aerobic exercise capacity, as reflected by those traits that were highest in both rock bass and largemouth bass. TGSA was one such trait (Fig. 4), consistent with the crucial role of the gills in oxygen uptake, as well as previous observations that the capacity of the gills for gas exchange increases in response to hypoxia exposure (Chapman et al., 1999; Sollid et al., 2003) and is greater in fish with greater hypoxia tolerance (Mandic et al., 2009). Larger gills are also characteristic of the high performance tunas and lamnid sharks (Bernal et al., 2001).

A heightened pH sensitivity of Hb–O_2 binding also appears to be associated with both hypoxia tolerance and aerobic exercise capacity (Fig. 5). This high pH sensitivity could be beneficial by enhancing the unloading of O_2 from Hb as the red cells acidify while travelling through systemic capillaries, but it could make blood O_2 transport more susceptible to metabolic acidosis. However, largemouth bass only had a high pH sensitivity in the presence of organic phosphates, whereas rock bass had a high pH sensitivity in both their presence and their absence. Organic phosphates are known to amplify the Bohr effect (Burggren et al., 1991), so the observed species differences in how H^+ and organic phosphates interact to affect O_2 binding was somewhat unexpected, and may relate to the relative insensitivity of rock bass Hb to ATP and GTP.

Both aerobic exercise capacity and hypoxia tolerance also appeared to be associated with an elevated capacity for gluconeogenesis in the liver, reflected by a higher PEPCK activity in both rock and largemouth bass (Fig. 7C). The concurrent elevation of LDH activity in these species could further suggest that there is a high capacity for using lactate as a fuel source for gluconeogenesis in these species. If so, a high gluconeogenic capacity in the liver could facilitate lactate clearance from the blood during or in recovery from exercise or hypoxic exposure. Consistent with an important role of gluconeogenesis in hypoxia, acclimation to hypoxia increases the activities of enzymes involved in this process (PEPCK, malate dehydrogenase and fructose 1,6-bisphosphatase) in the liver of killifish (Martínez et al., 2006; Borowiec et al., 2015).

Physiological basis for tradeoffs between hypoxia tolerance and aerobic performance

The potential tradeoff between hypoxia tolerance and aerobic exercise capacity across centrarchid species is consistent with previous findings in other fish taxa (Nilsson et al., 2007; Fu et al., 2014). The best swimming performer in normoxia – largemouth bass – also had the lowest hypoxia tolerance and the sharpest decline in performance when swimming in hypoxia, and the most hypoxia-tolerant species – rock bass – was the poorest performer in normoxia (Figs 1, 2). Furthermore, there was a positive relationship between P_{O_2} at LOE and U_{crit}, both with and without accounting for phylogeny (Fig. 3). However, the existence of a tradeoff was not well supported when P_{crit} was used as an index of hypoxia tolerance, as there was an apparent disconnect between the interspecific variation in P_{crit} and that in U_{crit} (Fig. 1). Nevertheless, the apparent tradeoff that may exist between hypoxia tolerance (at least as reflected by P_{O_2} at LOE) and aerobic exercise capacity is probably underpinned by the cumulative influence of a variety of respiratory and metabolic traits.

Tradeoffs between hypoxia tolerance and aerobic exercise performance may arise from the conflicting influence of Hb–O_2 affinity on branchial O_2 uptake and peripheral O_2 unloading (Powers et al., 1979; Burggren et al., 1991). The evolution of hypoxia tolerance is often associated with a high Hb–O_2 affinity because it improves branchial O_2 uptake in hypoxia (Weber and Fago, 2004; Mandic et al., 2009). However, a low P_{50} may reduce O_2 unloading at respiring tissues because it reduces mean capillary P_{O_2} and thus the driving force for diffusion. Aerobic exercise capacity should therefore be increased by a lower Hb–O_2 affinity as long as the arterial blood can still be fully saturated with oxygen in normoxia. As a result, the evolution of Hb–O_2 affinity to facilitate either hypoxia tolerance or aerobic exercise capacity may come at the expense of having Hb that is less well suited to the other trait. We did not observe any interspecific variation in inherent P_{50} that was associated with the variation in hypoxia tolerance or aerobic exercise capacity, but there was associated variation in Hb sensitivity to organic phosphates. If this variation was paralleled by similar interspecific variation in *in vivo* P_{50}, then it is likely that Hb–O_2 affinity does indeed contribute to the apparent tradeoff between hypoxia tolerance and aerobic exercise capacity.

Tradeoffs between hypoxia tolerance and aerobic exercise performance could also arise from the conflicting influence of resting metabolic rate on each of these traits. Numerous studies have shown that low resting $\dot{M}_{O_2}$ are associated with hypoxia tolerance (Nilsson and Östlund-Nilsson, 2008; Mandic et al., 2009). Our data were consistent with this expectation insofar as the marginally significant variation in resting $\dot{M}_{O_2}$ mirrored the variation in P_{crit} (see Results). However, there is also an association between resting $\dot{M}_{O_2}$ and $\dot{M}_{O_2,max}$ across vertebrates, such that species that have high $\dot{M}_{O_2,max}$ also have high resting metabolic rates (Bennett and Ruben, 1979). Therefore, changes in resting metabolic rate associated with the evolution of either hypoxia tolerance or aerobic exercise capacity could come at the expense of impairments in the other trait.

The nature of oxygen supply to the heart could also underlie tradeoffs between hypoxia tolerance and aerobic exercise capacity. The spongy myocardium of the heart receives its oxygen supply from the venous blood in the heart lumen (Steffensen and Farrell, 1998), so cardiac performance can become impaired if the heart becomes deprived of oxygen during exercise (when muscle extracts appreciable O_2 from the venous blood) or exposure to hypoxia (which can lead to hypoxaemia). Cardiac impairment could be especially severe in hypoxia if (i) venous O_2 is depleted by the combined influence of arterial hypoxaemia and high O_2 extraction from the blood, and (ii) the heart does not possess a high anaerobic capacity to help maintain ATP supply during O_2 deprivation. This could be the case in largemouth bass, whose hearts have a lower anaerobic potential than those of other centrarchids (Fig. 7B) and whose swimming muscle (the most abundant tissue in the body) has high capillarity and is probably capable of high O_2 extraction in hypoxia. Therefore, this unique combination of traits associated with aerobic exercise performance in largemouth bass could potentiate O_2 limitation to the heart during hypoxia, and thereby reduce hypoxia tolerance.

Evolution, developmental plasticity or parental effects?

It is possible that the intraspecific variation we observed resulted partly from developmental plasticity or parental effects, in addition to inherent evolved differences between species. We studied wild-caught fish that were well acclimated to common conditions in the lab, which eliminated the influence of reversible plasticity on our results, but this approach cannot eliminate the differences between species that are caused by irreversible developmental plasticity. Developmental plasticity can have a strong influence on adult physiology (West-Eberhard, 2003; Scott and Johnston, 2012;

Journal of Experimental Biology (2015) 218, 3264-3275 doi:10.1242/jeb.124602

Schnurr et al., 2014), and developmental hypoxia in particular can have persistent effects on hypoxia tolerance, the gas-exchange organs, and the activities of metabolic enzymes in several tissues (Crocker et al., 2013; Blank and Burggren, 2014; Robertson et al., 2014). Exposure of parent zebrafish to hypoxia has also been shown to improve the hypoxia tolerance of their offspring (Ho and Burggren, 2012), suggesting that trans-generational effects could have also influenced some of our observations. Nevertheless, all of the fish we studied were caught from a single and relatively shallow lake to help minimize the variation in parental and developmental environment between species. It is also possible that some of the variation we observed was caused by differences in the developmental stage of the species studied, which has been shown to influence hypoxia tolerance in some species (Sloman et al., 2006), because the rock bass, pumpkinseed and bluegill studied were much closer to their fully mature size than were the largemouth bass. Future work is needed to disentangle these possibilities and to understand how hypoxia tolerance and aerobic exercise capacity interact with other aspects of each species' physiology, morphology and behaviour.

MATERIALS AND METHODS

Study animals

Fish were collected from Lake Opinicon, ON, Canada (44.559°N, −76.328°W) by angling or seining in August and October 2012. We caught an overlapping size range of largemouth bass (M_b ranged from 36.8 to 85.1 g, mean±s.e.m. of 54.5±7.5 g), rock bass (36.6 to 93.8 g, 62.7±6.2 g), bluegill (49.1 to 117.3 g, 80.0±5.3 g) and pumpkinseed (53.1 to 128.9 g, 87.8±4.3 g). Fish were then transported to McMaster University and housed in 500 l flow-through tanks on a 12 h:12 h light:dark photoperiod. Tanks were supplied with dechlorinated, aerated City of Hamilton tap water at 12–15°C. Fish were fed a mix of commercially purchased squid or beef organs (heart, liver, kidney) four to five times weekly (~3% M_b daily). Fish were housed for at least a month under these conditions before any experimentation took place. Passive integrated transponder tags (PIT) (Biomark, Boise, ID, USA) were implanted under anaesthesia into the body cavity of each fish to allow ongoing identification of individuals. All animal procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

Genotyping was conducted to clearly distinguish purebred pumpkinseed from bluegill–pumpkinseed hybrids, which have a very similar outward appearance, using a previously described PCR approach (Near et al., 2004; Mathers et al., 2014). DNA was extracted from small clips of the caudal fin using a REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, Oakville, ON, Canada). The nuclear S7 fragment was amplified in Ready Mix Taq PCR Reaction Mix (Sigma-Aldrich) following the manufacturer's suggested reaction conditions, using the forward primer TGTAACGGGGAGCAGTTAGC and the reverse primer ACAGCCGATGTAGGAAACAG. The PCR consisted of 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 59°C) and elongation (30 s at 72°C). Amplified products were electrophoresed on a 3% agarose gel. Purebred pumpkinseeds exhibit a single band at 385 bp but hybrids exhibit two bands at 363 and 385 bp.

Swimming respirometry

Swimming performance and $\dot{M}_{O_2}$ were measured at 15°C in fish that were fasted for ~24 h, using a 5 l swim-tunnel respirometer submerged in a ~60 l buffer tank (Loligo Systems, Tjele, Denmark). Each fish was swum at a water P_{O_2} of 20, 9 and 6 kPa. The order of each swim trial was randomized, and fish were allowed to recover for at least 2 weeks between trials. P_{O_2} in the buffer tank was maintained as previously described (Borowiec et al., 2015), and P_{O_2} in the swim tunnel was measured continuously using a fibre-optic O_2 sensor (PreSens, Regensburg, Germany). Fish were weighed and measured for standard body length (BL) before each swim trial and were allowed 30–60 min to habituate to the swim tunnel. The swimming trials then started at a velocity of 0.33 BL s^{-1}. Swimming velocity was increased by 0.33 BL s^{-1} every 10 min until the fish was exhausted (tunnel water speeds were calibrated with an inline flow meter; HFA, Höntzsch GmbH, Waiblingen, Germany). Two successive cycles of flush for 90 s (when the tunnel was open to the buffer tank) and measurement for 210 s (when the tunnel was closed) were used to measure $\dot{M}_{O_2}$ at each speed. Exhaustion was defined as when the fish could no longer maintain its position in the water flow and was forced into the screen at the back of the chamber for >30 s, despite repeated attempts to motivate it to resume swimming (e.g. tapping the chamber, shining a light, etc.). U_{crit} was calculated as previously described (Brett and Sutherland, 1965), and $\dot{M}_{O_2,max}$ was recorded as the maximum $\dot{M}_{O_2}$ value that was measured during the course of the trial.

Resting respirometry and hypoxia tolerance measurements

Resting $\dot{M}_{O_2}$ was measured by stop-flow respirometry during stepwise reductions in P_{O_2} in fish that were fasted for ~24 h, at least 2 weeks after completing the last swim trial. Fish were held in respirometry chambers (volume of 2 l) that were continuously flushed with normoxic water for at least 10 h before measurements began. Fish were then subject to a progressive step-wise hypoxia protocol using commercially available equipment for aquatic respirometry (Loligo Systems) as previously described (Borowiec et al., 2015). Briefly, $\dot{M}_{O_2}$ was measured in normoxia (100% air saturation) and at each P_{O_2} from 100% to 20% air saturation in steps of 10% every 20 min. Each 20 min step contained two successive flush and measurement periods of 5 min each. The 20 min step at 20% air saturation was followed by another step at 15% saturation, and then the chamber was sealed and the fish was allowed to consume the O_2 in the chamber until it lost equilibrium (at which point the P_{O_2} at LOE was noted). The P_{crit} – the inflection point of the relationship between $\dot{M}_{O_2}$ and P_{O_2} when fish transition from oxyregulating to oxyconforming – was determined using Regress software (Yeager and Ultsch, 1989).

Sampling

Fish were killed with an overdose of buffered MS-222 (1 g l^{-1}) and sampled within 30 min of reaching LOE. A transverse cut was made through the trunk at the anterior base of the anal fin. Blood was collected into capillary tubes and centrifuged for 5 min at 14,000 rpm to measure haematocrit, and the packed red blood cells were frozen in liquid N_2 and stored at −80°C for later measurements of Hb–O_2 binding. An additional 6 µl blood sample was taken to measure Hb content (using Drabkin's Reagent, following the manufacturer's instructions; Sigma-Aldrich). A second transverse cut was then made ~2–3 mm anterior to the first section, and this resulting block of muscle was frozen in liquid N_2 and stored at −80°C for later use in enzyme assays (see below). A third transverse cut was made ~2–3 mm posterior to the first cut, an image was taken to measure the total transverse area of the musculature, and one lateral hemisphere of the section was cut into dorsal, midline and ventral blocks of tissue for histology. These three blocks were then covered in embedding medium (Shandon Cryomatrix, Fisher Scientific, Ottawa, ON, Canada), frozen on cork in liquid N_2-cooled isopentane, and stored at −80°C until sectioning was performed. The entire heart was weighed, and the heart, brain and liver were frozen in liquid N_2 for later use in enzyme assays. The entire gill basket was removed from each fish, fixed for ~24 h at 4°C (in 2% paraformaldehyde and 2% glutaraldehyde in phosphate-buffered saline, PBS, at pH 7.8), and then stored in PBS at 4°C until morphometric analyses were performed.

Gill morphometrics

Total gill surface area was measured on the left arches using standard methods (Hughes, 1984). The four arches were dissected and digital pictures of all filaments were taken using a stereomicroscope to count filament number and measure the length of all filaments. The density of lamellae on the filaments (i.e. the average number of lamellae per filament length) was measured along the length of every tenth filament on the first gill arch. Five transverse sections through each tenth filament were used to quantify lamellar area. All measurements were made using ImageJ software (Rasband, 2014). TGSA was calculated by the equation TGSA=2LnB, where L is the total filament length (product of filament number and average

Journal of Experimental Biology

filament length), n is lamellar density and B is the average bilateral surface area of the lamellae (mm^2), all of which was doubled to account for there being two sides to a fish (Hughes, 1984). TGSA was expressed relative to M_b.

Hb–O_2 binding affinity

Oxygen equilibrium curves were determined for Hb isolated from the packed red cells. Cells were first lysed in 15 volumes of 10 mmol l^{-1} Tris (pH 7.4) and then centrifuged at 15,000 ***g*** for 10 min at 4°C. The supernatant containing Hb was then stripped free of the allosteric modifiers ATP and GTP using nucleic acid cleanup columns (Bio-Spin 30 Tris Columns, Bio-Rad Laboratories, Mississauga, ON, Canada), following instructions from the manufacturer. Hb content of the stripped Hb solution was measured with Drabkin's Reagent, and the solution was stored at −80°C until assayed. Oxygen dissociation curves were generated at 25°C using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA), following the manufacturer's recommendations, for 75 mg l^{-1} Hb in 5 ml of buffer containing 50 mmol l^{-1} Tris, 50 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA, 0.1% bovine serum albumin and 0.2% antifoaming agent (TCS Scientific). Hb–O_2 affinity (P_{50}, the P_{O_2} at which Hb is 50% saturated) was calculated using Hemox Analytical Software (TCS Scientific). P_{50} was measured for stripped Hb in the absence and presence of ATP and GTP (in molar ratios of 2 and 1 to tetrameric Hb, respectively) at pH 7.4 and 7.0, and for stripped Hb in the absence of ATP and GTP at pH 7.8. The chloride and allosteric modifier conditions were selected to approximate those in fish red blood cells (Nikinmaa, 1990). pH sensitivity (the combined potential effects of both Bohr and Root effects, which cannot be clearly distinguished using the Hemox Analyzer) was measured as the difference in P_{50} measured at 7.0 and 7.4, normalized to a 1.0 pH unit change.

Muscle histology

Muscle fibre-type composition and capillarity were determined from transverse sections of the entire swimming muscle, taken where the rostral base of the anal fin joins the body surface. Muscle blocks were sectioned 10 µm thick in a cryostat (CM1850, Leica Microsystems, Wetzlar, Germany) at −20°C, mounted on Superfrost Plus slides (Fisher Scientific), and air-dried. We stained freshly sectioned tissue for succinate dehydrogenase (SDH) activity to identify oxidative fibres, and the remaining slides were stored at −80°C until stained to identify capillaries. SDH activity was stained by incubating slides for 1 h at room temperature in a working buffer of 41.7 mmol l^{-1} Na_2HPO_4, 8.3 mmol l^{-1} NaH_2PO_4, 80 mmol l^{-1} sodium succinate, 0.1% NBT at pH 7.6. Capillaries were identified by staining for ATPase activity in a neutral Pb^{2+}-containing medium (Rosenblatt et al., 1987). Slides were first fixed for 5 min at 4°C in a PBS solution containing 4% paraformaldehyde and 68 mmol l^{-1} $CaCl_2$ (pH 7.6). Slides were then stained in a solution of 1 mmol l^{-1} ATP, 3.8 mmol l^{-1} $Pb(NO_3)_2$, 6.5 mmol l^{-1} $CaCl_2$, 300 mg gelatin and 100 mmol l^{-1} Tris (pH 7.2) for 1 h at 37°C, and were mounted with Aquamount (Fisher Scientific). Stained tissue was imaged with a Nikon Eclipse E800 light microscope (Melville, NY, USA).

The total transverse area and total number of oxidative fibres was measured from SDH-positive staining. The total area was calculated as twice (to account for there being two sides of the fish) the sum of all transverse areas of oxidative muscle in the three blocks taken for each fish. The areal density of oxidative fibres (%) was determined by dividing the total transverse area of oxidative fibres by the total transverse area of the musculature that was determined from the image taken during sampling. Capillary density and the number of capillaries per muscle fibre were quantified in areas that were clearly oxidative (red) muscle and glycolytic (white) muscle. The average transverse area of each fibre type was calculated as the total area imaged for each type divided by the number of fibres within that area. All measurements were made in ImageJ.

Enzyme and Mb assays

The maximal activities of several enzymes were assayed at 25°C using standard methods that have been previously described (Schnurr et al., 2014; Borowiec et al., 2015) with a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Tissue was homogenized on ice in 20 volumes of homogenization buffer (20 mmol l^{-1} Hepes, 1 mmol l^{-1} EDTA and 0.1% Triton X-100) at pH 7.0. Preliminary assays were carried out to determine the substrate concentrations needed to elicit maximal enzyme activity. COX activity was measured on freshly homogenized samples in 50 mmol l^{-1} Tris containing 100 µmol l^{-1} of fully reduced cytochrome *c* and 0.5% Tween-20 (pH 8.0). The remaining assays were performed after one or more freeze–thaw cycles, the number of which was consistent for each enzyme. PEPCK activity was assayed in the liver under the following conditions (concentrations in mmol l^{-1}): 1.1 phosphoenolpyruvate (PEP), 0.15 NADH, 0.5 dGDP, 20 $NaHCO_3$, 1 $MnCl_2$, 50 imidazole, and excess coupling enzyme (20 U ml^{-1} malate dehydrogenase). PEPCK activity was not detected in the muscle, brain or heart tissue. The remaining enzymes were assayed in all tissues (except that HOAD was not assayed in brain) under the following conditions (concentrations in mmol l^{-1}): CS, 0.5 oxaloacetate, 0.3 acetyl-CoA, 0.1 DTNB, 50 Tris, pH 8.0; HOAD, 0.05 acetoacetyl-CoA, 0.3 NADH, 50 imidazole, pH 7.2; LDH, 1 pyruvate, 0.15 NADH, 50 Hepes, pH 7.4; PK, 5 PEP, 0.15 NADH, 5 ADP, 100 KCl, 10 $MgCl_2$, 0.010 fructose 1,6-bisphosphate, 50 Mops, and excess coupling enzyme (10 U ml^{-1} LDH), pH 7.4. All enzyme assays were run in triplicate by measuring the rate of change in absorbance at 550 nm (COX), 412 nm (CS) or 340 nm (HOAD, LDH, PEPCK and PK). Activities were determined by subtracting the background reaction rate without a key substrate from the rates measured in the presence of all substrates. We used extinction coefficients (ε) of 28.5 and 13.6 optical density (mmol l^{-1})$^{-1}$ cm^{-1} for COX and CS assays, respectively, and determined ε empirically for HOAD, LDH, PEPCK and PK by constructing standard curves of absorbance versus NADH in the buffers appropriate for each assay. Mb content was also measured in heart homogenates as previously described (Borowiec et al., 2015).

Molecular phylogeny

We used concatenated DNA sequences for ND2, S7 and Tmo from GenBank to reconstruct a Centrarchidae phylogeny from an earlier publication (Near et al., 2004), where sequence accession numbers can be found. Sequences were aligned in Mesquite (Maddison and Maddison, 2014), and MrModeltest version 2.3 (Nylander, 2004) was used to determine the model of DNA evolution that best fitted the data based on the Akaike information criterion (GTR+I+G). BEAST version 1.8.1 (Drummond et al., 2012) was then used to generate the phylogenetic tree using Bayesian phylogenetics with 10^7 generations, and the posterior distribution was assessed to determine the appropriate burnin (10^6 generations) using Tracer version 1.6 (Rambaut et al., 2014). The resulting tree with branch lengths was pruned to contain the four species of interest and then used for phylogenetically independent contrast analysis (see below).

Statistics

Phylogenetically independent contrast analysis was performed using the PDAP module of Mesquite (Midford et al., 2005; Maddison and Maddison, 2014) to assess whether the relationship between hypoxia tolerance and exercise performance persisted after taking into account the effects of phylogeny. Raw contrasts were calculated for absolute data, which were then plotted against their standard deviations (square roots of sums of branch lengths). Exponential transformation of branch lengths eliminated all significant relationships between raw contrasts and their standard deviations, so standardized independent contrasts for all data were calculated using these transformed branch lengths (Garland et al., 1992).

Data are reported as means+s.e.m. or ±s.e.m., with the exception of contrast data for which only species means were analysed. One- or two-factor ANOVA followed by Bonferroni multiple comparisons post-tests were used to compare between species. ANCOVA was used to compare the effects of water P_{O_2} on U_{crit} and $\dot{M}_{O_2,max}$ between species. A significance level of $P<0.05$ was used throughout.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
K.D.C. led the majority of the experimentation, data collection, analysis and drafting of the manuscript. N.A.P. and G.R.S. contributed to data collection and analysis. G.R.S. designed and supervised the experiments. All authors contributed to the interpretation of data and to writing the manuscript. All authors approved the manuscript.

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