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Interspecies variation in hypoxia tolerance, swimming performance and plasticity in cyprinids that prefer different habitats

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Running title: Hypoxia tolerance and swimming in carps

Abstract This study quantified and compared hypoxia tolerance and swim performance among cyprinid fish species from rapid-flow, slow-flow and intermediate-flow habitats (4 species per habitat) in China. In addition, we explored effects of short-term acclimation on swim performance, maximum metabolic rate ($\dot{M}_{O_2\ max}$) and gill remodelling to detect habitat-associated patterns of plastic response to hypoxia. Indices of hypoxia tolerance included oxygen threshold for loss of equilibrium (LOE_{50}) and aquatic surface respiration (ASR_{50}) and critical oxygen tension for routine metabolic rate (P_{crit}). Critical swimming speed (U_{crit}) and $\dot{M}_{O_2\ max}$ were measured under normoxic and hypoxic conditions after 48 hours acclimation to normoxia and hypoxia, and gill remodelling was estimated after 48 hours of hypoxia exposure. Both traditional ANCOVA and phylogenetically independent contrast (PDANOVA) analyses showed that fish species from rapid-flow habitat exhibited lower LOE_{50} compared to fish from intermediate and slow-flow habitats. Habitat-specific difference in P_{crit} and U_{crit} were detected using PDANOVA but not traditional ANCOVA analyses, with fish species from rapid-flow habitat exhibited lower P_{crit} but higher U_{crit} compared to fish from intermediate and slow-flow habitats. Fish species from rapid-flow habitats were also characterized by less plasticity in swim performance and gill morphology in response to hypoxia acclimation compared to species from slow-flow habitats, but a greater drop in swim performance in response to acute hypoxia exposure. The study detected a habitat-specific difference in hypoxia tolerance, swimming performance and its plasticity among fish from habitats with different flow conditions possibly due to the long-term adaptation to the habitat caused by selection stress. The phylogenetically independent contrasts were more powerful than traditional statistical analyses according to the habitat effects in both hypoxia tolerance and swimming performance in this study.

Keyword: hypoxia tolerance, preferred habitat, swimming performance, phenotypic plasticity,

gill morphology

Introduction

Oxygen availability in aquatic habitats is a major environmental factor that influences the ecology, behaviour and physiology of fish (Martínez et al., 2011). Hypoxia (defined as any 'level of DO low enough to negatively impact the behaviour and (or) physiology of an organism') occurs naturally in many aquatic systems (Pollock et al., 2007). However, the frequency and extent of hypoxia is increasing associated with anthropogenic activities such as eutrophication and pollution of water bodies (Rabalais et al., 2010). Therefore, it has become increasingly important to understand the mechanisms that fish use to persist and survive under hypoxic conditions and to identify indicators of hypoxia tolerance that can be compared across species and systems. In this study, we use a closely related group of cyprinid fishes from China to explore habitat-associated hypoxia tolerance, relationships among tolerance indices and mechanisms that may contribute to the interspecific patterns observed.

Critical O_2 tension for routine oxygen consumption rate (P_{crit}) is the minimum O_2 level required to sustain routine oxygen consumption rate ($\dot{M}_{O_2\ rout}$), and is considered to be an indicator of an animal's hypoxia tolerance (Ultsch et al., 1978; Mandic et al., 2009). In addition to P_{crit}, the O_2 threshold for the loss of equilibrium (LOE), which represents the partial pressure of oxygen at which the fish can maintain balance, is also a frequently used indicator of hypoxia tolerance (Barnes et al., 2011; Mandic et al., 2013). Aquatic surface respiration (ASR) whereby fish breathe water from surface film is a common response of water-breathing fish to extreme hypoxia (Shingles et al., 2005; Sloman et al., 2006). ASR is hypothesised to be triggered by environmental oxygen tensions at which respiratory mechanisms fail to compensate for environmental hypoxia (Takasusuki et al., 1998). Therefore, the O_2 threshold for ASR is another potentially useful hypoxia tolerance indictor in addition to P_{crit} and LOE.

In many fish species, swimming performance is postulated to be a central determinant of Darwinian fitness (Brett, 1964; Plaut, 2001; Blake, 2004). In fish, the determination of maximum sustainable swimming speed or critical swimming speed (U_{crit}) is widely used to evaluate aerobic swimming performance (Gregory and Wood, 1998; Plaut, 2001; Lee et al., 2003; MacNutt et al., 2004). Aerobic swimming performance may be limited by either O_2 uptake and delivery or the aerobic metabolic capacity of the muscle. Ecologically, decreased swimming performance, which includes both swimming speed and swimming efficiency, in hypoxic water may render animals more vulnerable to predation and may also affect the foraging efficiency of predators (Abrahams et al., 2007). Therefore, the mechanism by which fish can maintain their swimming ability under hypoxic conditions may be closely related to their survival in such an environment.

The ability of fish to maintain their swimming ability under hypoxic condition may be affected by acclimation to hypoxia, i.e. acclimation may induce a phenotypically plastic response that improves tolerance to hypoxic stress. For example, the $\dot{M}_{O_2\,max}$ and swimming performance of goldfish (*Carassius auratus*) improved significantly after acclimation to hypoxia for 48 h (Fu et al., 2011). And, in several species of cyprinids, including the crucian carp (*Carassius carassius*) (Sollid et al., 2003), goldfish (Sollid et al., 2005; Mitrovic et al., 2009) and scaleless carp (*Gymnocypris przewalskii*) (Matey et al., 2008), hypoxia exposure has been found to result in dramatic changes in gill morphology (Sollid et al., 2003) that reduces the water-blood diffusion distance (Matey et al., 2008).

Hypoxia tolerance and physiological plasticity may differ between fish that live in rapid-flow habitats and fish that live in slow-water habitats. Rapid-flow rivers often exhibit little DO change and generally have high DO levels, whereas some small, isolated bodyies of water,

such as ponds, exhibit large daily DO fluctuations. The Cyprinidae is one of the largest families of vertebrates in the world. This family has a wide geographic distribution, including mainland Eurasia, Japan, the East Indian Islands, Africa and North America. There are approximately 532 species of cyprinid within approximately 132 genera in China, and the phylogenetic relationships among these fish are well-documented (Wang, 2005). Because of considerable morphological and physiological diversity, cyprinids exist in a wide variety of habitats (Howes, 1991). Therefore, we investigated the hypoxia tolerance, swimming performance and plastic response to hypoxia in 12 cyprinid species that live in slow-flow, intermediate-flow or rapid-flow habitats (Table 1). The objective of this study was to test whether hypoxia tolerance, gill remodelling ability, swimming performance and plasticity are related to habitat. To test our hypotheses, we used both traditional statistical analyses and phylogenetically independent contrasts.

Materials and methods

Experimental fish and holding conditions

Juveniles of the cyprinid species, *Schizothorax prenanti* (SP), *Ohychostoma sima* (OS), *Spinibarbus sinensis* (SS), *Carassius auratus* (AA), *Carassius carassius* (CA), *Cyprinus carpio* (CC), *Aristichthys nobilis* (AN), *Hypophthalmichthys molitrix* (HM), *Parabramis pekinensis* (PP), *Ctenopharyngodon idellus* (CI), *Ctenopharyngodon piceus* (CP) and *Zacco platypus* (ZP), were either collected by local fisherman or caught by hook-and-line angling from a local river or lake, except goldfish that were bought from local market of Chongqing City in southwest China. Based on our investigation on local fishery catchment and water velocity where fish were collected, we classified the 12 species into 3 groups: rapid-flow, intermediate-flow, and slow-flow (Table 1). The fish were maintained in a re-circulating-water rearing system at

Chongqing Normal University for at least 2 weeks prior to experimentation. During this time, the temperature of the de-chlorinated freshwater was maintained at 15.0 ± 0.5 °C and the oxygen content was maintained above 10 mg L^{-1}. The photoperiod was 12L:12D. One tenth of the water was replaced daily with freshwater to maintain good water quality. Throughout the experimental period, the fish were fed daily to satiation with commercial forage until 48 h before the experimental trials. Fish were used only once in any experimental trial. All procedures were conducted in accordance with the national animal regulations.

Measurement of hypoxia indicators

ASR_{50} and LOE_{50}

To quantify hypoxia tolerance, we determined the oxygen tension at which individual fish ASR_{50} thresholds and LOE_{50} thresholds during the same experiment. Briefly, for a given species, 3 groups of 10 individual fish were transferred from the holding tank to a 30 L tank and held under flow conditions for 4 h prior to the experiment. At the start of the experiment, a mesh screen was placed below the waterline to prevent the fish from accessing the water-air interface. Inflowing water was shut off and nitrogen gas was introduced into the tank to rapidly decrease the DO from normoxic levels of approximately 10 mg L^{-1} to 5 mg L^{-1}, then 2.5 mg L^{-1}, then 1.2 mg L^{-1}. Thereafter, the oxygen tension was decreased in the same step-wise manner but in smaller steps of 0.1 mg L^{-1} to a final DO of 0 mg L^{-1}. Fish were held for 1 h intervals at each DO level and the change of the DO between steps required less than 1 min. At each DO, the total number of attempts of ASR and LOE were counted over 20 successive 3 min intervals. An attempt to perform ASR of individual fish was defined as the point where the fish made contact with the mesh surface suspended below the water-to-air interface and the ASR_{50} value of any fish group was defined as the point at which 5 of 10 individual fish made contact with the mesh

surface suspended below the water-air interface for 3 consecutive observations. The elapsed time between ASR of the 1st and the 5th fish was 40 to 80 min in all species except goldfish and crucian carp that showed relatively large variation (1 to 4 h). LOE of individual fish was defined as the failure of the fish to maintain dorsoventral orientation and the LOE_{50} value of any fish group was defined as the DO point at which 5 of 10 individual fish failed to maintain a dorsoventral orientation for 3 consecutive observations. The elapsed time between LOE of the 1st and the 5th fish was 20 to 60 min in all experiment fish species except goldfish and crucian carp that showed no LOE.

Determination of $\dot{M}_{O_2\,rout}$ *and* P_{crit}

After 48 h fasting, 15 fish were randomly selected from each experimental group and placed in 160 mL respirometers for measurement of $\dot{M}_{O_2}$ and P_{crit} (Zhang et al., 2010). The fish were allowed to recover from transfer to the respirometer for 4 h. During this time, continuously aerated water flowed (3 cm s^{-1}) through the respirometer. Subsequently, the respirometer was closed and $\dot{M}_{O_2}$ was measured over a range of water DO values as the fish depleted the oxygen within the closed respirometer, beginning at approximately 95% saturation and decreasing to 1% saturation with a duration of 90 to 120 min. If the fish showed movements such as struggling and moving back and forth during the experiment, the data from the trial were discarded. To measure DO, the circulating water from the respirometer was drawn from the respirometer by a peristaltic pump, forced past a DO probe (HQ20, Hach Company, Loveland, CO, USA) housed in a sealed thermostated chamber, and then returned to the respirometer. The temperature of the system was maintained at 15 ± 0.2 °C.

The following formula was used to calculate the $\dot{M}_{O_2}$ (mg kg^{-1} h^{-1}) of individual fish:

$$\dot{M}_{O_2} = ([O_2]_k - [O_2]_{k+1})\ VOL / (t \times m) \quad (1)$$

where $[O_2]_k$ is the oxygen concentration (mg L^{-1}) at time point k and $[O_2]_{k+1}$ is the oxygen concentration (mg L^{-1}) at the next time point. These values were calculated according to the O_2 solubility coefficient in water at the corresponding temperature and pressure. VOL (L) is the total volume of the respirometer minus the volume of the fish, *t* (h) is the interval between time points k and k+1 and *m* (kg) is the body mass of the fish. To account for effects of body size, the $\dot{M}_{O_2}$ was adjusted to a standard body mass of 1 kg using a mass exponent of 0.75 (Reidy et al., 2000).

The P_{crit} is the point at which $\dot{M}_{O_2\ rout}$ could no longer be maintained with a further reduction in the water O_2 tension and was estimated for the individual fish with the two-segment linear model described by Yeager and Ultsch (1989).

Acclimation to hypoxia

After the 2-week habituation period, the fish were fasted for 48 h, and 60 fish of similar size were randomly selected and divided into the hypoxia acclimation group and control acclimation group. The water temperature was maintained at 15 °C. Thirty fish were transferred to a 120 L exposure chamber in which hypoxia was achieved by covering the surface of the water with translucent plastic and bubbling nitrogen into the water (Matey et al., 2008). The DO was reduced from aerated values of 10 mg L^{-1} to 0.3 mg L^{-1} over 1 h and was then maintained at 0.3 mg L^{-1} for 48 h. During this time, the water DO was continuously monitored using a DO probe.

Measurement of U_{crit}

After 48 h acclimation to hypoxic or normoxic (control group) conditions, 20 fish were selected from each group and subjected to a critical swimming speed (U_{crit}) test in either normoxic water (10 mg L^{-1}, n = 10) or hypoxic water (1 mg L^{-1}, n = 10). The water DO ranged

from 10.2 to 10.4 mg L^{-1} for normoxic swimming conditions and from 1 to 1.2 mg L^{-1} for hypoxic swimming conditions.

A Brett-type swimming tunnel respirometer with a swim chamber with 19.87 cm^2 cross-sectional area was used to measure the U_{crit} (total volume 3.5 L, see detail in Li et al., 2010 and Pang et al., 2010) of the fish. The fish were individually transferred into the swim tunnel and allowed to recover at either normoxic or hypoxic water for 1 h (Fu et al., 2011). The water temperature in the swim chamber was controlled at 15 ± 0.2 °C The water velocity was 3 cm s^{-1} during habituation period. The water velocity was increased in 5 cm s^{-1} increments every 30 min until the fish became fatigued. Fatigue was defined as the time at which the fish failed to move off the rear honeycomb screen of the swim chamber for 20 s (Lee et al., 2003). The U_{crit} was calculated for the individual fish using Brett's equation (Brett, 1964) as follows:

$$U_{crit} = V + (t / T)\,\Delta V \qquad (2)$$

where V is the fastest speed at which the fish swam for the entire time period (cm s^{-1}), ΔV is the velocity increment (5 cm s^{-1}), T is the prescribed period of swimming per speed (30 min) and t is the length of time that the fish swam at the final speed (min). The swim tunnel was designed to switch between a closed mode and an open mode. The closed mode was for respirometry, and the open mode was to replenish the oxygen levels. In the open mode, the respirometer was supplied with 15 °C water supplied from a 350 L reservoir tank at a flow rate of 500 mL min^{-1}. For the normoxic conditions, the water in the reservoir tank was fully aerated (10 mg L^{-1}), whereas for the hypoxic conditions, the surface of the reservoir tank was covered with translucent plastic and the water bubbled with nitrogen to achieve a nominal water DO of 1 mg L^{-1}.

In the closed mode, the tunnel was isolated from the reservoir tank and water was recirculated

within the system. A small volume of water was drawn from the sealed respirometer by a peristaltic pump, forced past a DO probe housed in a sealed temperature-controlled chamber, and then returned to the respirometer. The oxygen concentration (mg L^{-1}) was recorded once every 2 min. The $\dot{M}_{O_2}$ (mg kg^{-1} h^{-1}) of the individual fish while swimming was calculated from the depletion of oxygen according to the following equation:

$$\dot{M}_{O_2} = 60 \text{ slope VOL} / m \quad (3)$$

where slope (mg L^{-1} min^{-1}) is the decrease in the water DO per min, VOL is the total volume of the respirometer (3.5 L) minus the volume of the fish and *m* is the body mass (kg) of the fish. The slope was obtained through linear regression between time (min) and water DO (mg L^{-1}). Only slopes with an $r^2 > 0.95$ were considered for the analyses. During $\dot{M}_{O_2}$ measurements DO was never allowed to drop by more than 0.25 mg L^{-1} in either the normoxic or hypoxic U_{crit} determinations. The maximal $\dot{M}_{O_2}$ during the U_{crit} test was defined as the maximum $\dot{M}_{O_2}$ ($\dot{M}_{O_2\,max}$). The metabolic rate was adjusted to a standard body mass of 1 kg using a mass exponent of 0.75 (Reidy et al., 2000).

Gill morphology

The gill morphology of only 4 fish species was evaluated, *S. prenanti*, *O. sima*, *S. sinensis* and *Z. platypus*, because the gill morphology change that was elicited by hypoxia acclimation of the other 8 species has previously been performed by our laboratory (Dhillon et al., 2013, Table 1).

After 48 h acclimation to hypoxic or control conditions, 4 fish from each group were immediately euthanised using neutralised tricaine methanesulphonate (MS-222, 50 mg L^{-1}) and terminally sampled. The second gill arch from the right side of each fish was removed, rinsed, and immediately fixed in cold Karnovsky's fixative for scanning electron microscopy (SEM) (at

the Third Military Medical University, Chongqing, China).

The middle part of each fixed gill arch (5 mm) with up to 20 filaments in both the anterior and posterior rows was used for scanning electron microscopy. All fixed gill tissues were rinsed in phosphate-buffered saline (PBS) and post-fixed in 1% osmium tetroxide for 1 h. The gill tissues were dehydrated in ascending concentrations of ethanol from 30% to 100%, critical-point dried with liquid CO_2, mounted on stubs, sputter-coated with gold–palladium, and examined with a Hitachi S 3400 scanning electron microscope (SEM) at an accelerating voltage of 15 kV. The protruding lamella height was measured to estimate changes in the gill morphology.

Data analysis

Statistics17 was used for data analysis. P values < 0.05 were considered statistically significant, and all data are presented as the mean ± SEM.

The effects of the habitats and species (nested within habitat) on hypoxia tolerance and swimming performance (we only tested the swimming performance of control group measured under normoxic condition) were determined by a nested analysis of covariance (ANCOVA, body size used as the covariate). One-way ANCOVA which followed by a Duncan multiple-comparison posthoc test was used to detect differences among habitats. The effect of species and DO (oxygen concentration in which the trait was measured), and their interaction on U_{crit} and $\dot{M}_{O_2\ max}$ was performed by two-way ANCOVA. The effect of hypoxia acclimation on U_{crit} and $\dot{M}_{O_2\ max}$ was detected using t-tests. For the 12 fish species, Pearson correlation was used to examine the relationship between ASR_{50} and LOE_{50}, and P_{crit} and $\dot{M}_{O_2\ rest.}$

We also conducted phylogenetically independent ANOVAs, which tested for differences in hypoxia tolerance and swimming performance (for the control group measured under normoxia) among species inhabiting environments under different flow conditions. We used the PDSIMUL

and PDANOVA programs (Garland et al., 1993) to perform phylogenetically independent ANOVAs. Using these programs, we simulated trait evolution as Brownian motion with the means and variances of the simulations set to the means and variances of the original data. We performed 1000 simulations, producing a null distribution of F-statistics against which the F-value of one way ANOVA from the actual data could be compared to assess the statistical significance (i.e., we determined how different the observed patterns were from those expected via genetic drift alone). We constructed a best-estimate phylogenetic hypothesis for this group of species based on previous morphological and molecular studies (Fig. 1). All branch lengths were set as equal to one.

Results

Hypoxia tolerance differences based on habitat

ASR_{50}

Effects of species on ASR_{50} were significant while neither habitat nor covariate (fish length) was significant (nested ANCOVA, Fig. 2A). The habitat effect on ASR_{50} was also not significant when controlling for phylogeny (PDANOVA, $P = 0.429$).

LOE_{50}

Both habitat and species showed significant effects, while body length showed no effect on LOE_{50} (nested ANCOVA, Fig. 2A). The habitat's effect on LOE_{50} was also significant when controlling for phylogeny (PDANOVA, $P = 0.004$). The LOE_{50} of fish species from rapid-flow water exhibited significantly higher LOE_{50} values than the intermediate group while the latter exhibited significantly higher LOE_{50} values than the fish species from slow-flow habitats (one-way ANCOVA, $P < 0.001$, Fig. 2B). Neither AA nor CA showed any sign of LOE_{50} after exposure to DO-free water for 1 h.

The ASR_{50} value was positively related to LOE_{50} among 12 species (R^2 = 0.580, P = 0.004, Fig. 2C).

P_{crit} and $\dot{M}_{O_2\ rest}$

The P_{crit} varied from 0.78 ± 0.05 in AN to 3.00 ± 0.38 mg O_2 L^{-1} in ZP (Fig. 3A), whereas the $\dot{M}_{O_2\ rest}$ varied from 29.5 ± 2.1 in PP to 63.6 ± 4.7 mg O_2 kg^{-1} h^{-1} in SP (Fig. 3B). There was no significant difference in neither P_{crit} nor $\dot{M}_{O_2\ rest}$ among habitats (nested ANCOVA, P > 0.05). However, the habitat effect was significant for P_{crit} when controlling for phylogeny (Fig. 3A, B). One-way ANCOVA indicated a significant difference in P_{crit} between the rapid-flow group and the other 2 groups. The mean P_{crit} value was positively related to mean $\dot{M}_{O_2\ rest}$ among 12 species (R^2 = 0.341, P = 0.038, Fig. 3C).

U_{crit} differences based on habitat

U_{crit}

In the control group (normoxia), the fish species showed great variation in U_{crit} ranging from 7.05 BL s^{-1} in OS to 3.62 BL s^{-1} in CI (Fig. 3A). Both species and body length showed significant effects (P < 0.001) while habitat showed no significant effect on U_{crit} (nested ANCOVA , Fig. 2A). However, the habitat's effect on U_{crit} was showed using phylogenetic analysis (PDANOVA, P = 0.046). The U_{crit} of the rapid-flow fish species was significantly higher than the other fish groups (one-way ANCOVA , P < 0.001).

Sensitivity to hypoxia

Under hypoxic conditions, all fish species showed significantly lower U_{crit} values (P < 0.001). The U_{crit} value of control fish decreased by 21 to 60%, with the percentage reduction being greater in fish from rapid-flow environments (significant species by condition interaction, P < 0.001, Fig. 4A, B).

Acclimation effect

After 48 h hypoxia acclimation, only CA and AA showed significantly higher U_{crit} values under normoxic conditions, whereas both SP and PP showed significantly lower U_{crit} values compared to the control group (Fig. 4A, B, $P < 0.05$). However, when measured under hypoxic conditions, half the fish species from the intermediate group, all 4 fish species from the slow-flow group showed significantly higher U_{crit} values compared to the control fish under hypoxic conditions. Therefore, the hypoxia acclimation had no effect on the U_{crit} measured under hypoxia for the rapid-flow species, but significantly improved the U_{crit} measured under hypoxia for the slow-flow species.

$\dot{M}_{O_2\ max}$ differences based on habitat

$\dot{M}_{O_2\ max}$

The $\dot{M}_{O_2\ max}$ also showed great variation among the different species. SP showed the highest $\dot{M}_{O_2\ max}$ value with 267 mg O_2 kg^{-1} h^{-1}, whereas CA showed the lowest value with 117 mg O_2 kg^{-1} h^{-1}(Fig. 3C). However, neither habitat nor body mass showed significant effects on $\dot{M}_{O_2\ max}$ (nested ANCOVA, Fig. 5A). The habitat effect on $\dot{M}_{O_2\ max}$ was also not significant when controlling for phylogeny (PDANOVA, $P = 0.142$).

Sensitivity to hypoxia

Under hypoxic conditions, all fish species showed significantly lower $\dot{M}_{O_2\ max}$ values ($P < 0.001$). The $\dot{M}_{O_2\ max}$ value of control fish decreased by 53 to 81%, with the percentage reduction being greater in fish from rapid-flow environments (significant species by condition interaction, $P < 0.001$, Fig. 5A, B).

Acclimation effect

After 48 h hypoxia acclimation, only CA showed significantly higher $\dot{M}_{O_2\,max}$ values under normoxic conditions, whereas AN showed significantly lower $\dot{M}_{O_2\,max}$ values compared to the control group (Fig. 5A, B) ($P < 0.05$). However, under hypoxic conditions, half the fish species from the intermediate group and 3 of 4 fish species from the slow-flow group showed significantly higher $\dot{M}_{O_2\,max}$ values compared to the control fish ($P < 0.05$). Hypoxia acclimation had no effect on the $\dot{M}_{O_2\,max}$ for all 4 rapid-flow fish species.

Gill morphology

All 4 fish species that were evaluated exhibited no gill morphology changes after hypoxia acclimation as indicated by the protruding lamella height. The protruding lamella heights were 62.1 ± 4.2 (N= 4 for all 4 species both before and after hypoxia acclimation) *vs.* 54.5 ± 2.9, 47.0 ± 3.1 *vs.* 37.6 ± 3.3, 51.8 ± 2.0 *vs.* 53.8 ± 1.3 and 75.2 ± 2.1 *vs.* 62.0 ± 3.8 μm in the hypoxia-acclimated and non-acclimated SP, OS, SS and ZP, respectively.

Discussion

The primary objective of this study was to answer the question of whether hypoxia tolerance and swimming performance differed in fish species that live in different habitats. Habitat-specific differences in hypoxia tolerance and swimming performance were detected by phylogenetically independent contrast while only hypoxia tolerance (as suggested by LOE_{50}) was detected by traditional contrast in this study, suggesting phylogenetically independent contrast proves to be a more powerful test of habitat that the traditional analyses. The fish species from rapid-flow habitats showed lower hypoxia tolerance but better swimming performance and greater sensitivity to DO change compared to slow-flow fish, as expected. Furthermore, the fish from the slow-flow habitats showed improved swimming and respiratory capacities when measured under hypoxic conditions after hypoxia acclimation, possibly related

to changes in the gill morphology, whereas none of rapid-flow fish showed any improvement in aerobic swimming performance after hypoxia acclimation.

Hypoxia tolerance and preferred habitat

Habitat-specific differences in hypoxia tolerance as suggested by LOE_{50} and P_{crit} were detected by phylogenetically independent contrast in this study. However, the habita-specific difference in P_{crit} was not showed by traditional analyses. The difference between these two methods is the latter take phylogenetic relationship into account. It suggested the phylogenetically independent contrast may be more powerful than traditional nested ANOVA. It has been demonstrated that P_{crit} is quite changeable and is affected by the routine metabolic rate and, therefore, nutritional status (Hochachka, 1986; Guppy and Withers, 1999), hypoxia acclimation (Fu et al., 2011) and temperature (Barnes et al., 2011). A positive relationship between P_{crit} and $\dot{M}_{O_2\ rest}$ also showed in the present study (Fig. 3C). Another important reason that P_{crit} may not be an appropriate indicator for hypoxia tolerance is that it neglects the role of anaerobic metabolic capacity in hypoxia tolerance (this is particularly true for both AA and CA in the present study).

An animal's ability to tolerate environmental fluctuations requires the integration and coordination of behavioural, physiological and biochemical processes (Sloman et al., 2008). Behavioural response of hypoxia using ASR may be beneficial for surviving hypoxia; however, the threshold for initiating ASR may be affected by perceived predation risk (Sloman et al., 2008). Although it may be predicted that ASR is triggered by environmental DO levels at which the respiratory mechanisms fail to compensate for environmental hypoxia (Takasusuki et al., 1998), other studies have also supported the hypothesis that there is an element of flexibility in the performance of ASR (Sloman et al., 2008). In this study, there were no significant

differences in the ASR among fish with different preferred habitats. Furthermore, both AA and CA, the champions of anoxia-tolerance, showed a relatively higher ASR threshold, suggesting that ASR may not be an appropriate hypoxia tolerance indicator for these 2 fish species (Fig. 2). This may be because some bold fish species, such as AA and CA in this study, performed ASR early whereas other fish did not. We can also speculate that ASR may change as a result of predator stress variation.

Nevertheless, this study suggested that the LOE might be a better indicator of hypoxia tolerance, whereas P_{crit} and ASR may not be appropriate for some species because of physiological and behavioural factors.

Swimming performance, gill morphology, plasticity and preferred habitat

Cyprinids living in rapid-flow water showed higher U_{crit} values compared to slow-flow fish species which is independent of phylogenetic relationship, which is consistent with our hypothesis. Similar to P_{crit}, traditional contrast showed no significant difference among fish species from different habitats, which again suggesting that phylogenetically independent contrast was more powerful to detect the habitat difference than traditional analyses. Furthermore, the swimming performance of the rapid–flow fish was more sensitive to the DO change. This may reflect more stable and high DO conditions in rapid-flow habitats. By contrast, it may be critical for slow-flow fish species to maintain the majority of swimming performance under hypoxic conditions to survive in such an environment.

We determined whether swimming performance showed adaptive plasticity after hypoxia acclimation and whether such plasticity differed among groups of fish species from different preferred habitats. All 4 fish species showed no improvement in U_{crit} after hypoxia acclimation either under normoxic or hypoxic conditions. However, in the slow-flow fish species, all 4 fish

391 species showed improved swimming performance under hypoxic conditions, whereas AA and
392 CA also showed improved swimming performance under normoxic conditions. Similar to
393 swimming performance, the fish species from slow-flow habitats also showed significant gill
394 morphology changes whereas fish species from rapid-flow habitats showed less or no gill
395 plasticity after 48 h hypoxia acclimation. It suggested that the improved swimming and
396 respiratory capacities after hypoxia acclimation might partially due to the gill morphological
397 change. Interestingly, we provide evidence here that gill remodelling may not be
398 phylogenetically dependent because closely related fish, such as and PP and CI, showed
399 alternative morphological responses after hypoxia acclimation. We further suggest that
400 swimming performance and gill flexibility are habitat-specific as long-term adaption to the
401 habitat DO condition. Besides morphological change, the physiological mechanism might also
402 contribute to the improved swimming performance of hypoxia-acclimated fish. It has been
403 found that hypoxia acclimation results in an increase in [hemoglobin] (Hb) and blood oxygen
404 carrying capacity (Wells et al., 1989; Silkin and Silkina, 2005), an increase in the number of
405 muscle mitochondria and muscle myoglobin concentration, and a higher capillarization of
406 muscle, which improves the extraction and utilization of circulating oxygen stores at low DO
407 (Sänger, 1993).

408 The acclimation effect on U_{crit} was more profound when fish that were tested under low DO.
409 Increased O_2 uptake capacity, which may have been elicited by hypoxia acclimation, may not
410 result in increased swimming performance under normoxic conditions because the limiting
411 factor is not likely to be the availability of O_2. However, when measured under hypoxia,
412 slow-flow fish showed significant swimming plasticity whereas rapid-flow fish did not.

413 In conclusion, this study clearly demonstrated that there was a phylogenetically-independent

habitat-specific difference in both hypoxia tolerance and swimming performance among fish from habitats with different flow conditions. This difference may reflect response to flow regime and associated DO differences among habitats. Rapid-flow fish showed poor hypoxia tolerance but stronger swimming performance than the slow-flow fish, as expected. The slow-flow fish also displayed significant gill morphology changes and swimming performance improvement after hypoxia acclimation, whereas rapid-flow fish showed no such ability. Furthermore, the swimming performance of the slow-flow fish was less sensitive to DO change compared to the rapid-flow fish. The differences in sensitivity and plasticity of swimming performance in the fish from habitats with different flow conditions may be due to differences in O_2 fluctuation among the different habitats. The study also suggested that the phylogenetically independent contrast might be more powerful than traditional statistical approach when detected the habitat difference in both swimming performance and hypoxia tolerance.

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Table 1. Biological information and body size of the 12 fish species used in this study.

Common name	Latin name	Preferred habitat	Distribution *	Collected cites	Body mass (g)	Body length (cm)	Gill remodelling ability
Mountain carp (SP)	*Schizothorax prenanti*	Rapid-flow	M(1.5-4)	M (2-4)	8.72±0.35	8.49±0.13	NO (Present Study)
Sharp-jaw barbell (OS)	*Onychostoma sima*	Rapid-flow	R(1.5-4)	R (1.5-4)	3.92±0.23	6.34±0.16	NO (Present Study)
Qingbo (SS)	*Spinibarbus sinensis*	Rapid-flow	R(1.5-4)	R (1.5-4)	6.61±0.24	7.10±0.08	NO (Present Study)
Chinese hook snout carp (ZP)	*Zacco platypus*	Rapid-flow	R, M(1.5-4)	R (1.5-4)	6.79±0.10	4.69±0.20	NO (Present Study)
Silver carp (HM)	*Hypophthalmichthys molitrix*	Intermediate	L, R(0-2)	L (0)	7.30±0.08	6.48±0.25	YES (Dhillon et al., 2013)
Chinese bream (PP)	*Parabramis pekinensis*	Intermediate	L, R(0-2)	L (0)	9.05±0.32	7.96±0.12	NO (Dhillon et al., 2013)
Grass carp (CI)	*Ctenopharyngodon idellus*	Intermediate	L, R(0-3)	L (0)	9.22±0.23	9.24±0.12	YES (Dhillon et al., 2013)
Black carp (CP)	*Ctenopharyngodon piceus*	Intermediate	L, R(0-2)	L (0)	6.27±0.09	4.29±0.15	NO (Dhillon et al., 2013)
Goldfish (AA)	*Carassius auratus*	Slow-flow	/	/	6.45±0.36	5.85±0.14	YES (Dhillon et al., 2013)
Crucian carp (CA)	*Carassius carassius*	Slow- flow	P, L, R(0-1)	L (0)	8.60±0.40	6.96±0.14	YES (Dhillon et al., 2013)
Common carp (CC)	*Cyprinus carpio*	Slow- flow	P, L, R(0-3)	L (0)	7.05±0.23	6.40±0.08	YES (Dhillon et al., 2013)
Bighead carp (AN)	*Aristichthys nobilis*	Slow- flow	L(0)	L (0)	11.27±0.44	8.73±0.14	YES (Dhillon et al., 2013)

* L: lake, P: pond, R: River, M: mountain steam. Distribution is based on our investigation, local fish catchment and information from local fishermen. The number in brackets is the water velocity (m s^{-1}). Common carp and crucian carp are classified as slow–flow type because they live well in pond.

Captions

Fig. 1. The phylogenetic relationship among the 12 experimental cyprinid species (the hierarchical topology tree was built based on the data of Wang 2005). See Table 1 for more information regarding each of the selected fish species.

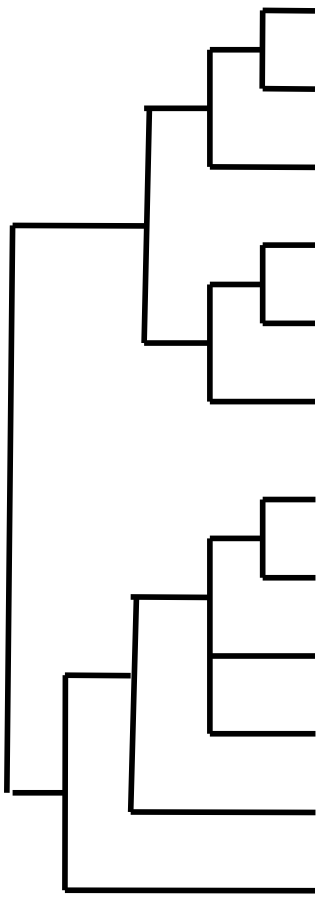
Fig. 2. The oxygen threshold for aquatic surface respiration (ASR_{50}, $mgO_2\ L^{-1}$) (Fig. 2A) and loss of equilibrium (LOE_{50}, $mgO_2\ L^{-1}$) (Fig. 2B) of 12 different cyprinid fish species and the relationship between ASR_{50} and LOE_{50} among 12 fish species from three different habitats (rapid-flow, blank circle; intermediate, grey circle; slow-flow, dark circle) (Fig. 2C). Shared letters A, B, C indicated no difference in the trait measured (ANCOVA with habitat as a factor (n = 3) and Duncan post-hoc tests) among groups of fish species from different habitat types (rapid-flow, blank column; intermediate, grained column; slow-flow, filled column). The results of nested ANCOVA according to different preferred habitats and species (nested within habitat) and PDANOVA were listed above the figure. $, the LOE_{50} of AA and CA were zero.

Fig. 3. The critical oxygen tension for routine metabolic rate (P_{crit}, $mgO_2\ L^{-1}$) (Fig. 3A) and routine metabolic rate ($\dot{M}_{O_2\ rest}$, $mgO_2\ kg^{-1}\ h^{-1}$) (Fig. 3B) of 12 different cyprinid fish species and the relationship between P_{crit} and $\dot{M}_{O_2\ rest}$ among 12 fish species from three different habitats (rapid-flow, blank circle; intermediate, grey circle; slow-flow, dark circle) (Fig. 3C). Shared letters A, B, C indicated no difference in the trait measured (ANCOVA with habitat as a factor (n = 3) and Duncan post-hoc tests) among groups of fish species from different habitat types (rapid-flow, blank column; intermediate, grained column; slow-flow, filled column). The results

of nested ANCOVA according to different preferred habitats and species (nested within habitat) and PDANOVA were listed above the figure.

Fig. 4. The U_{crit} (BL s^{-1}) of the control (blank column) and 48 h hypoxia-acclimated (filled column) cyprinid fish species measured under normoxic (Fig. 4A) and hypoxic (Fig. 4B) conditions. Shared letters A, B, C indicated no difference in the trait measured (ANCOVA with habitat as a factor (n = 3) and Duncan post-hoc tests) among groups of fish species from different habitat types. * indicated significant difference in U_{crit} between the hypoxia-acclimated and non-acclimated fish (t-test). The results of nested ANCOVA according to different preferred habitats and species (nested within habitat) and PDANOVA (we only compared control fish measured under normoxic condition) were listed above the figure.

Fig. 5. The $\dot{M}_{O_2\ max}$ ($mgO_2\ kg^{-1}\ h^{-1}$) of of the control (blank column) and 48 h hypoxia-acclimated (filled column) cyprinid fish species measured under normoxic (Fig. 5A) and hypoxic (Fig. 5B) conditions. * indicated significant difference in $\dot{M}_{O_2\ max}$ between the hypoxia-acclimated and non-acclimated fish (t-test). The results of nested ANCOVA according to different preferred habitats and species (nested within habitat) and PDANOVA (we only compared control fish measured under normoxic condition) were listed above the figure.



Latin name	Species abbreviation	Habitat type
Carassius auratus	AA	Slow-flow
Carassius carassius	CA	Slow-flow
Cyprinus carpio	CC	Slow-flow
Schizothorax prenanti	SP	Rapid-flow
Onychostoma sima	OS	Rapid-flow
Spinibarbus sinensis	SS	Rapid-flow
Aristichthys nobilis	AN	Slow-flow
Hypophthalmichthys molitrix	HM	Intermediate
Parabramis pekinensis	PP	Intermediate
Ctenopharyngodon idellus	CI	Intermediate
Ctenopharyngodon piceus	CP	Intermediate
Zacco platypus	ZP	Rapid-flow

Fig. 1

A) ASR_{50}

Nested ANCOVA: Habitat effect $F_{2, 9} = 4.08$, $P = 0.055$
Species effect $F_{3, 3} = 8.47$, $P < 0.001$
Body length effect $F_1 = 0.671$, P = 0.421
PDANOVA (Habitat effect): CV = 32.15, $P = 0.429$

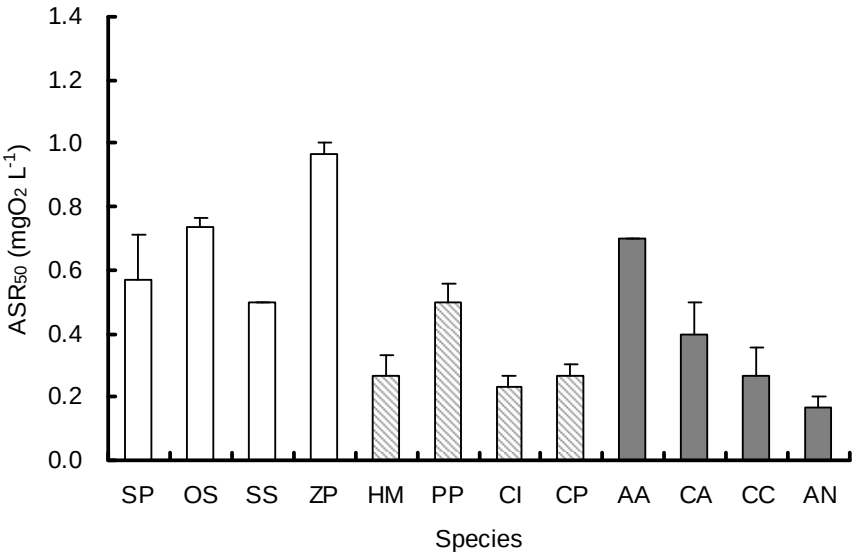


Fig. 2A

B) LOE_{50}

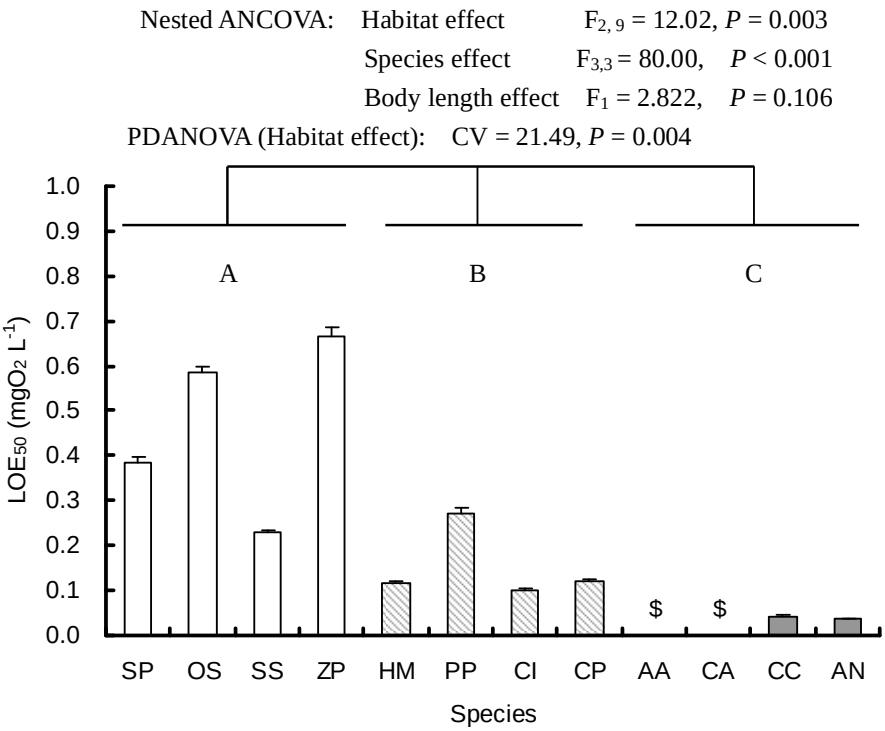


Fig. 2B

C) Correlation between ASR_{50} and LOE_{50}

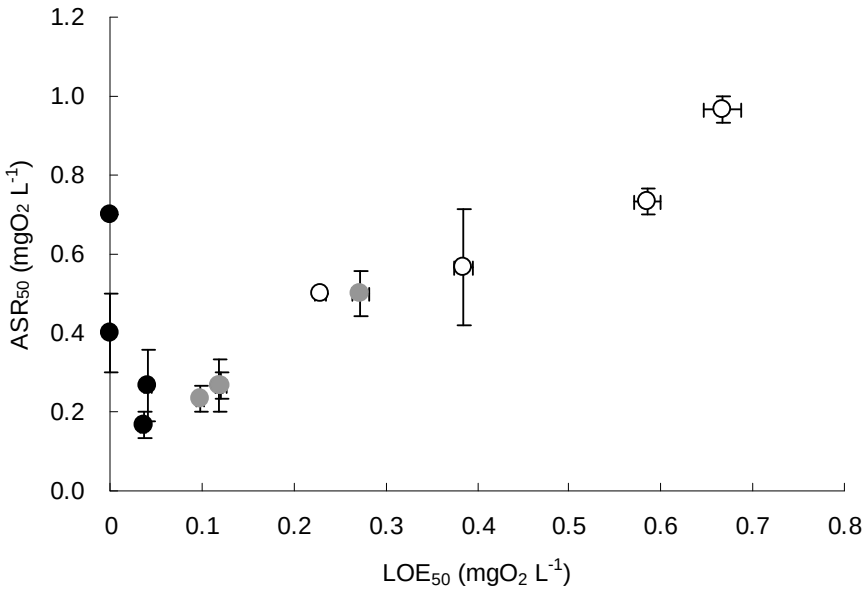


Fig. 2C

A) P_{crit}

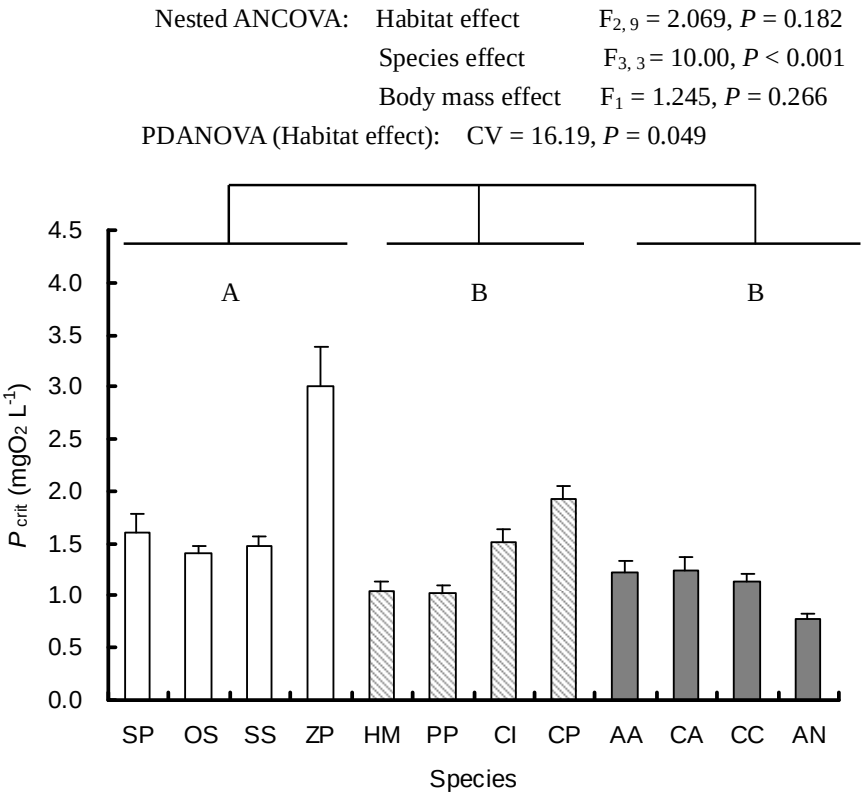


Fig. 3A

B) $\dot{M}_{O_2\ rest}$

Nested ANCOVA: Habitat effect $F_{2, 9} = 1.006$, $P = 0.403$
Species effect $F_{3, 3} = 9.44$, $P < 0.001$
Body mass effect $F_1 = 2.92$, $P = 0.089$
PDANOVA (Habitat effect): CV = 11.09, $P = 0.143$

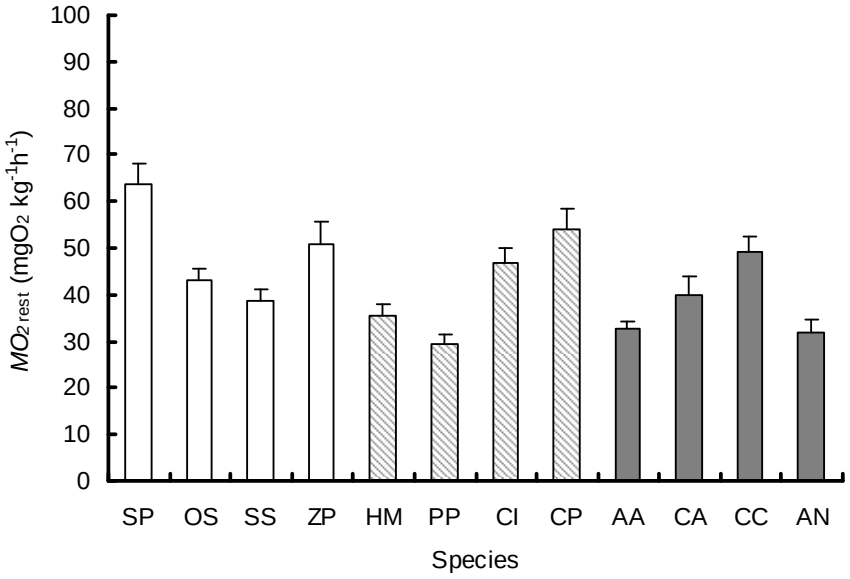


Fig. 3B

C) Correlation between P_{crit} and $\dot{M}_{O_2\ rest}$

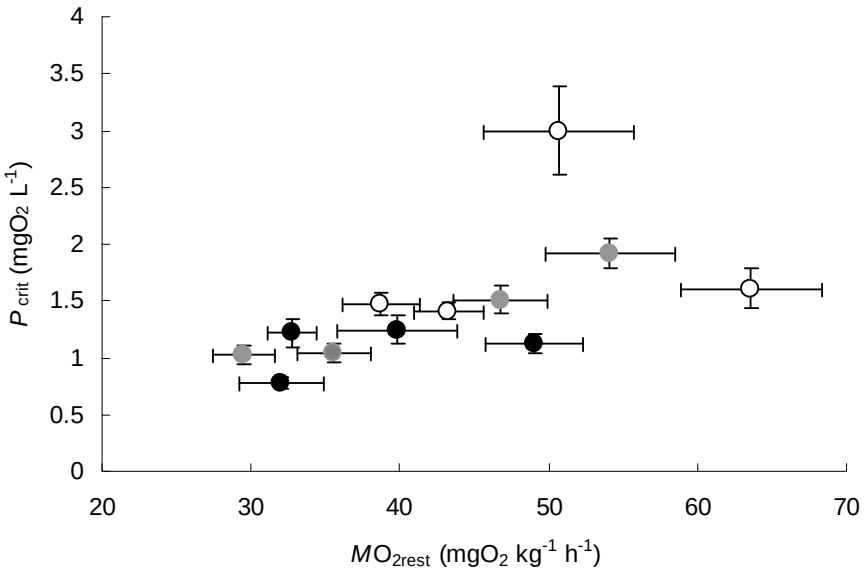
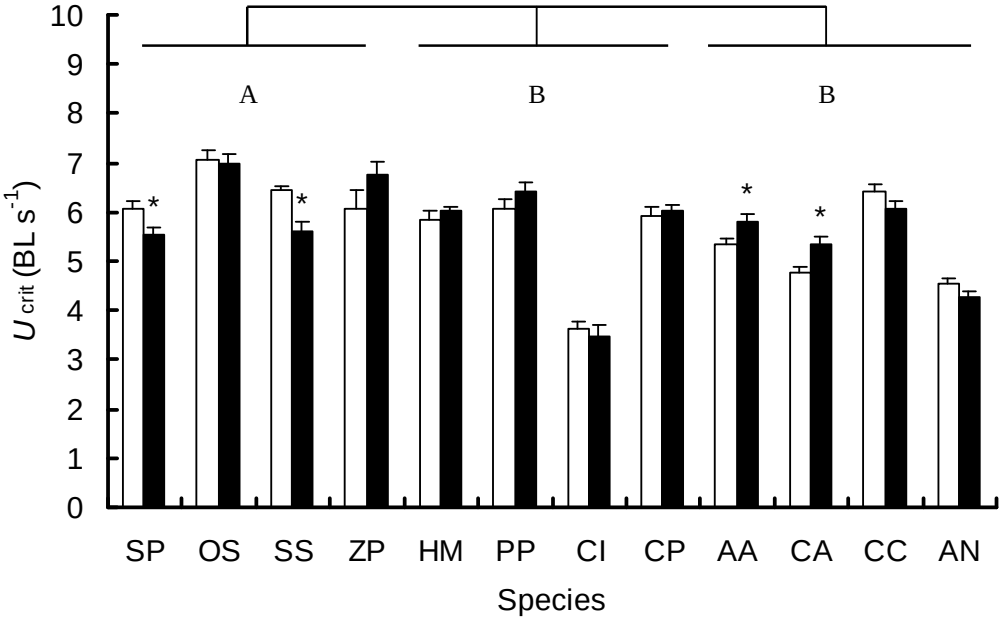


Fig. 3C

A) Normoxic condition

Nested ANCOVA: Habitat effect $F_{2, 9} = 4.21$, $P = 0.051$
Species effect $F_{3, 3} = 15.42$, $P < 0.001$
Body length effect $F_1 = 31.99$, $P < 0.001$
PDANOVA (Habitat effect): CV = 19.60, $P = 0.046$



B) Hypoxic condition

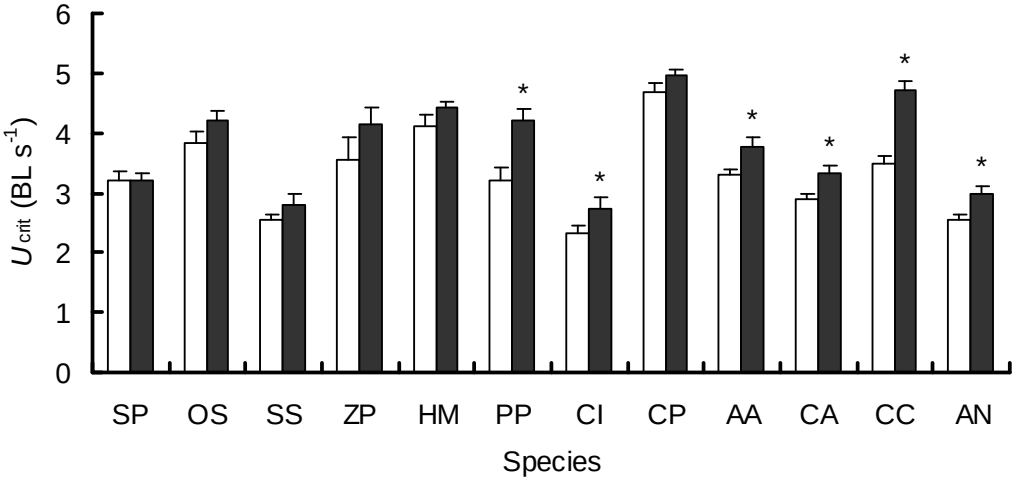
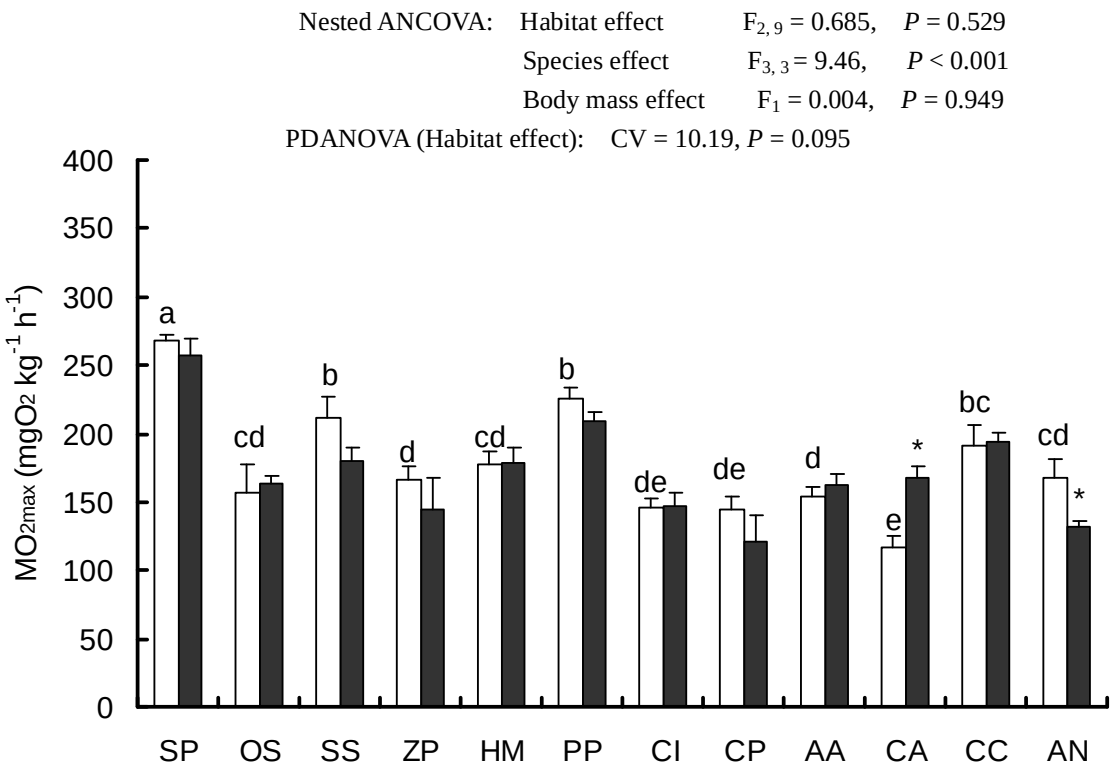


Fig. 4

A) Normoxic condition



B) Hypoxic condition

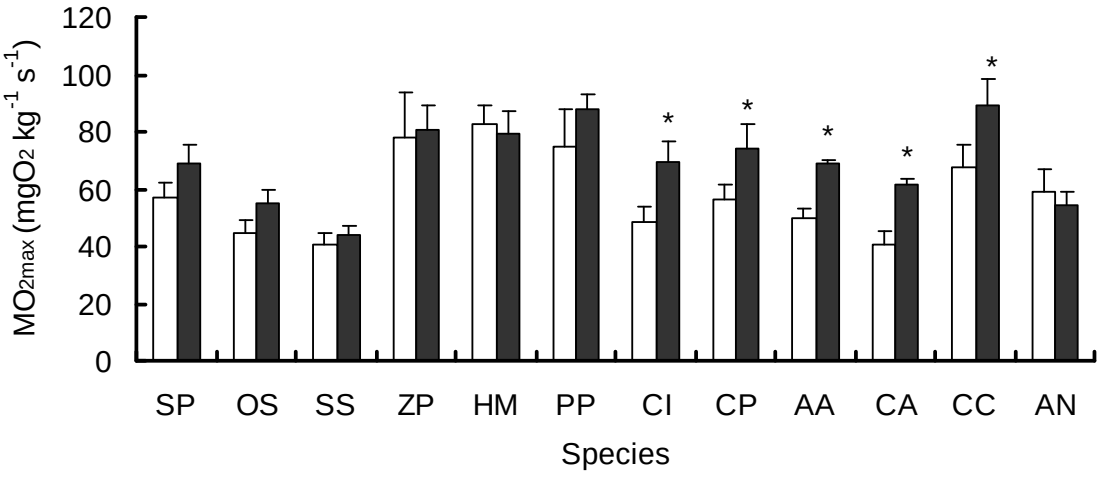


Fig. 5