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Interspecific Differences in Hypoxia-Induced Gill Remodeling in Carp

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ABSTRACT

The gills of many fish, but in particular those of crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*), are capable of extensive remodeling in response to changes in oxygen (O_2), temperature, and exercise. In this study, we investigated the interspecific variation in hypoxia-induced gill modeling and hypoxia tolerance in 10 closely related groups of cyprinids (nine species, with two strains of *Cyprinus carpio*). There was significant variation in hypoxia tolerance, measured as the O_2 tension (Po_2) at which fish lost equilibrium (LOE_{crit}), among the 10 groups of carp. In normoxia, there was a significant, phylogenetically independent relationship between mass-specific gill surface area and LOE_{crit}, with the more hypoxia-tolerant carp having smaller gills than their less hypoxia-tolerant relatives. All groups of carp, except the Chinese bream (*Megalobrama pellegrini*), increased mass-specific gill surface area in response to 48 h of exposure to hypoxia (0.7 kPa) through reductions in the interlamellar cell mass (ILCM) volume. The magnitude of the hypoxia-induced reduction in the ILCM was negatively correlated with LOE_{crit} (and thus positively correlated with hypoxia tolerance), independent of phylogeny. The hypoxia-induced changes in gill morphology resulted in reduced variation in mass-specific gill surface area among species and eliminated the relationship between LOE_{crit} and mass-specific gill surface area. While behavioral responses to hypoxia differed among the carp groups, there were no significant relationships between hypoxia tolerance and the Po_2 at which aquatic surface respiration (ASR) was initiated or the total number of ASR events observed during progressive hypoxia. Our results are the first to show that the extent of gill remodeling in cyprinids is associated with hypoxia tolerance in a phylogenetically independent fashion.

Introduction

Environmental hypoxia can be common in many aquatic habitats (Val et al. 2006; Diaz and Breitburg 2009), and the fish that inhabit these waters have evolved a suite of strategies to enhance hypoxic survival. These strategies serve to either enhance oxygen (O_2) uptake from the O_2-depleted water or prolong survival when environmental O_2 tensions (Po_2) are below levels where routine metabolic rate can be maintained. Crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*), both members of the family Cyprinidae, are considered the champions of hypoxia (and even anoxia) tolerance, surviving months of severe hypoxia at cool temperatures (Van den Thillart et al. 1976; Walker and Johansen 1977). This impressive hypoxia tolerance is associated with their high hemoglobin-O_2 binding affinity (Weber and Lykkeboe 1978; Lomholt and Johansen 1979; Sollid et al. 2005*b*), large hepatic glycogen stores, alternative anaerobic pathways (including ethanol production (Shoubridge and Hochachka 1980; Johnston and Bernard 1983), and an ability to undergo reversible depression of metabolic rate (Van Waversveld et al. 1989; Muusze et al. 1998; Nilsson and Ostlund-Nilsson 2008). More recently, Sollid et al. (2003) also showed that crucian carp and goldfish are capable of a dramatic remodeling of their gill morphology in response to hypoxia, greatly increasing gill surface area and O_2 diffusing capacity (Nilsson et al. 2012; Sollid et al. 2003; Tzaneva et al. 2011*a*, 2011*b*).

Since the first description of hypoxia-induced gill remodeling in crucian carp (Sollid et al. 2003), similar temperature- and exercise-dependent responses in crucian carp and/or goldfish have been described (Sollid et al. 2005*b*; Sollid and Nilsson 2006; Fu et al. 2011; Brauner et al. 2011; Nilsson et al. 2012; Perry et al. 2012). In all these studies, it is clearly shown that crucian carp and goldfish under resting, normoxic conditions

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Table 1: List of groups, mean weight, and GenBank accession numbers

Species	Common name	Mean weight ± SEM (g)	GenBank accession number
Spinibarbus sinensis	Qingbo	2.35 ± .37	KC808485
Cyprinus carpio	Common carp	17.48 ± 1.46	KC808480
C. carpio	Koi	21.88 ± 1.63	KC808479
Carassius auratus	Goldfish	4.26 ± .19	KC808478
Carassius carassius	Crucian carp	10.80 ± .70	KC808477
Megalobrama pellegrini	Chinese bream	8.80 ± .29	KC808486
Hypophthalmichthys molitrix	Silver carp	14.71 ± .79	KC808484
Aristichthys nobilis	Bighead carp	7.46 ± 1.36	KC808481
Ctenopharyngodon idellus	Grass carp	10.90 ± 1.44	KC808482
Mylopharyngodon piceus	Black carp	6.57 ± .64	KC808483

at low temperature possess a large interlamellar cell mass (ILCM; Sollid et al. 2003, 2005*b*), which substantially reduces total gill surface area. When O_2 is plentiful or metabolic demands are low, the smaller gill surface area is thought to minimize ionoregulatory challenges (Sollid et al. 2003; Nilsson et al. 2012) or provide a barrier to toxic substances or pathogens that target the gill (Nilsson et al. 2012). However, upon exposure to hypoxia or conditions that increase metabolic demands, a low gill surface area may impede O_2 uptake, and both crucian carp and goldfish respond by reducing the size of the ILCM, exposing the lamellae and increasing the functional surface area of the gill. In general, this response requires days to complete at low temperatures, because it involves increasing the rate of apoptosis and decreasing the rate of mitosis in the ILCM, although faster responses have been noted at higher temperatures or during sustained exercise (Sollid et al. 2005*a*; Brauner et al. 2011; Tzaneva et al. 2011*a*; Perry et al. 2012).

The list of species that possess an ILCM and subsequently undergo gill remodeling in response to changes in O_2 requirements has increased, and beyond crucian carp and goldfish, hypoxia-induced gill remodeling has also been shown to occur in the scaleless carp (*Gymnocypris przewalskii*; Matey et al. 2008) and the mangrove killifish (*Kryptolebias marmoratus*; Ong et al. 2007), both hypoxia-tolerant species, albeit not to the same extent as crucian carp and goldfish. Although it is tempting to assign adaptive value to hypoxia-induced gill remodeling as a response to enhance hypoxia survival, assigning adaptive value to a physiological trait requires evidence that the trait has been selected for in taxa that experience the relevant environmental challenge (Garland et al. 1992). Multispecies comparative analysis offers one way to collect such evidence, whereby an investigator can look for a correlation among measures of, in this case, hypoxia tolerance and the extent of gill remodeling observed during hypoxia exposure across multiple species. Multispecies comparative analysis must take into account the phylogenetic relationship between the study species (Felsenstein 1985; Garland and Adolph 1994; Garland et al. 2005), using methods such as phylogenetically independent contrasts (PICs), in order to reduce the inherent statistical errors associated with comparing species that have a shared ancestry described by a phylogeny. This approach has been used in other groups of fish to identify putatively adaptive traits involved in hypoxia tolerance (Mandic et al. 2009*a*, 2009*b*, 2013), but to date, no phylogenetically corrected comparative analysis has been performed on cyprinids, and the relationship between the capacity and extent of gill remodeling and hypoxia tolerance is largely unknown.

In this study, we used a phylogenetically corrected comparative approach to determine whether hypoxia-induced gill remodeling is a general feature of cyprinids or related to their ability to survive hypoxia. To accomplish this goal, we selected 10 groups from the family Cyprinidae (including two strains of the common carp *Cyprinus carpio*) from central China that are found in environments ranging from stagnant ponds that can be prone to eutrophication and winter ice cover (in the case of crucian carp, goldfish, and common carp) to faster-moving rivers (e.g., qingbo *Spinibarbus sinensis*, silver carp *Hypophthalmichthys molitrix*, black carp *Mylopharyngodon piceus*, and Chinese bream *Megalobrama pellegrini*; Kong et al. 2007; Yi et al. 2010; Lou 1990). Other species (e.g., black carp and grass carp *Ctenopharyngodon idellus*) are known to occupy the full spectrum of freshwater habitats. We propose a hypothesis for the phylogenetic relationship among the study groups based on the cytochrome *b* sequence, and we use the phylogenetic information in our comparative analysis of hypoxia tolerance, assessed as the Po_2 at which individuals showed a loss of equilibrium, and our measures of the extent of gill remodeling observed during a 48-h exposure to a Po_2 of 0.7 kPa at 12°C. Variation in routine metabolic rate ($\dot{M}O_2$), the Po_2 at which fish transition from being oxyregulators to being oxyconformers (P_{crit}), and characteristics of aquatic surface respiration (ASR) were also examined in the 10 groups of cyprinids. This work presents the first comparative analysis of the relationship between hypoxia tolerance and gill remodeling in closely related species of carp.

Material and Methods

Experimental Fish and Holding Conditions

We used 10 groups of carp, which consisted of nine different species and two strains of one species (*Cyprinus carpio* include both common carp and koi; see table 1). All fish were obtained from a local market in Chongqinq, China. We treated common carp and koi separately in our comparative analysis because

they are genetically distinct strains (see phylogeny in fig. 1*b*). Fish were transported to Chongqing Normal University, Chongqing, and held in a 4,000-L concrete tank in well-aerated freshwater for 2 wk before experimentation. Water was changed twice daily. Each species was held in a separate, flow-through container within the same system. During the holding period, temperature was maintained at 12°C, and Po_2 was always at or above 20 kPa. Fish were fed to satiation daily with commercial feed. All experiments were carried out in accordance with the guidelines on the humane treatment of laboratory animals as established by the Ministry of Science and Technology of the People's Republic of China.

Loss of Equilibrium and Aquatic Surface Respiration

In order to quantify hypoxia tolerance, we determined the Po_2 at which individuals showed loss of equilibrium (LOE). Briefly, for a given species, 10 individuals were transferred from the holding tank to one of four chambers in a partitioned 120-L tank and held under flow-through conditions at 12°C for 12 h before the experiment. At the start of the experiment, a mesh screen was placed below the surface of the water to prevent the fish from accessing the air-water interface. Inflowing water was shut off, and nitrogen gas was introduced to the tank to rapidly decrease Po_2 from normoxic levels (~20 kPa) to 11.4 kPa over the course of 1 h. After this point, Po_2 was decreased in a stepwise fashion as follows: the Po_2 was held at 11.4 kPa for 1 h, then decreased to 5.7 kPa over 30 min, held at 5.7 kPa for 30 min, decreased to 2.7 kPa over 30 min, and held at this new Po_2 for an additional 30 min. From this point on, Po_2 was decreased in a similar stepwise manner (decreased over ~30 min and then held at the new level for 30 min), but in smaller increments of 0.2 kPa, to a final level of 0 kPa. When a fish showed LOE, defined as failure to maintain dorsoventral orientation, the Po_2 and time of exposure to that Po_2 were recorded. The critical oxygen tension for LOE, or LOE_{crit}, was determined in a fashion analogous to Brett's (1964) equation for calculating critical swimming speeds:

$$LOE_{crit} = Po_{2_i} - \left(\frac{t_i}{t_{ii}}\right)Po_{2_{ii}},$$

where Po_{2_i} is the penultimate oxygen tension, $Po_{2_{ii}}$ is the decrease in oxygen tension at each increment (0.2 kPa), t_i is the time required for the fish to lose equilibrium at the final Po_2, and t_{ii} is the time held at each Po_2. The protocol was repeated three times for each species, and each replicate consisted of 10 fish per species. Throughout the protocol, we also monitored attempted ASR behaviors in all 10 groups. Briefly, the number of ASR attempts was monitored in 3-min intervals over the 30 min required to change the Po_2 and the 30 min at each Po_2 (total 60 min of monitoring time). During each 3-min interval, an individual was defined as performing ASR if it made contact with the mesh suspended below the water-to-air interface at least once.

Whole-Animal Respirometry

Routine oxygen consumption rate ($\dot{M}O_2$) and critical oxygen tension of $\dot{M}O_2$ (P_{crit}) were determined on individual fish by means of closed respirometry based on protocols described in Zhang et al. (2010). The respirometry chambers were cylindrical and made of poly(methyl methacrylate), with an adjustable volume (190–740 mL) that was scaled to the size of the fish to achieve a ratio of volume to biomass of approximately 40–80 mL g^{-1}. At the beginning of a trial, fish were placed into individual respirometry chambers and held under flow-through conditions for 24 h to recover from handling stress. Water Po_2 was maintained at 20 kPa during the recovery period, and temperature was maintained at 12° ± 0.5°C (mean ± SEM; $N = 9$–12) throughout the acclimation period and the determination of $\dot{M}O_2$ and P_{crit}. At the beginning of a trial, the respirometer was closed, and water was recirculated by connecting the inflow and outflow tubes of the respirometer via a submersible pump. Water Po_2 was measured continuously with an oxygen electrode (HQ20, Hach) and recorded every 3 min until the fish showed signs of distress or water Po_2 reached 0.7 kPa, whichever came first. If the fish showed signs of distress, the trial was terminated immediately, and the data were not included in the analysis. At the end of each trial, fish were removed from the respirometer and euthanized with an overdose of tricaine methanesulfonate (MS-222: 2 g L^{-1}), and the weight and body length were measured. The volume of water in the respirometer was also measured. Routine metabolic rate ($\dot{M}O_2$) was calculated as described in Fu et al. (2011) and is given as the mean $\dot{M}O_2$ above P_{crit}. Critical oxygen tensions were determined with a virtual BASIC program developed by Yeager and Ultsch (1989) and based on a two-segment linear model above and below an inflection point, where the intersection is defined as P_{crit}.

Hypoxia Exposure

In order to assess gill remodeling, fish were exposed to 0.7 kPa for up to 48 h at 12.2° ± 0.9°C. Briefly, 30 fish of each species were transferred from the holding tank to a 40-L perforated plastic bucket and held in a 4,000-L concrete tank. Fish were allowed to recover from the transfer under well-aerated conditions for a period of 48 h, during which they were not fed. At the start of the experiment, a normoxic control "0-h" sample was taken from six to eight individuals of each species. To sample the fish, individuals were quickly netted from the perforated bucket and transferred to a separate bucket (maintained at 12°C) containing $NaHCO_3$-buffered (2 g L^{-1}) MS-222 (1 g L^{-1}). Once the fish ceased ventilating, fork length and weight were measured. Muscle, liver, red blood cells, plasma, and gills were sampled and frozen in liquid N_2 for biochemical analysis for a subsequent study. The gill basket from the right-hand side of the fish was removed, rinsed in freshwater, and placed in cold Karnovsky's fixative (Karnovsky 1965) for morphological analysis (see below). Once the normoxic sampling was complete, water Po_2 was reduced to 0.7 kPa over 30 min by bubbling N_2 gas into the tank,

and the water Po_2 was maintained at this level for 48 h after N_2 was first introduced into the tank. A plastic mesh was suspended 5 cm below the water surface to prevent the fish from accessing the air-water interface. Furthermore, the surface was covered with plastic bubble wrap to help minimize oxygen ingress, oxygen tensions in the water were monitored continuously, and adjustments to inflowing N_2 were made as needed to keep oxygen tension constant. Fish were sampled as described above at 8 and 48 h of hypoxia exposure.

Gill Morphology

The fixed gills were transferred to San Diego State University for morphometric analysis using scanning electron microscopy (SEM) and light microscopy (LM). Briefly, a 4–5-mm section from the middle section of each gill arch was cut into two equal pieces, each with 10–12 filaments in both anterior and posterior rows (see Brauner et al. 2011 for details). One piece of each gill arch was used for SEM, and these sections were rinsed in phosphate-buffered saline, postfixed in 1% osmium tetroxide for 1 h, and dehydrated in ascending concentrations of ethanol from 30% to 100%. The specimens were critical-point dried with liquid CO_2, mounted, sputter-coated with gold-palladium, and examined with a Hitachi S 2700 scanning electron microscope at the accelerating voltage of 20 kV. The other half of each gill arch, to be used for LM, was refixed in Serra fixative for 4 h, dehydrated in ethanol, cleared in xylene, and then embedded in paraffin wax. Thick serial paraffin sections of 4–5 mm were cut on a microtome (Microm HM-355), mounted on glass slides, dewaxed in xylene, rehydrated in a descending ethanol series to distilled water, and stained with hematoxylin and eosin. Slides were examined in a Nikon Eclipse E200 microscope (Melville, NY).

Both SEM and LM data were used to determine the degree of gill remodeling at 0, 8, and 48 h of hypoxia exposure on the basis of 80 measurements per fish (10 lamellae in each of 10 filaments per fish). The following parameters were measured to estimate changes in the protruding lamellar surface and the ILCM: (1) protruding lamella height (determined by LM), (2) protruding lamella thickness (determined by LM), (3) basal length of protruding lamella (determined by SEM), (4) ILCM height (determined by LM), and (5) distance between lamellae (determined SEM), according to the methods described in Matey et al. (2008). Protruding lamella surface area was calculated from parameters 1–3, and the volume of the ILCM was calculated from parameters 3–5 according to Sollid et al. (2003) and Matey et al. (2008). To determine whether any reduction of the ILCM during gill remodeling was associated with epithelial cell loss, the number of cell nuclei in the interlamellar spaces were counted via LM.

To calculate the surface area for the protruding lamellae (i.e., the functional respiratory surface), an estimation of the area based on half an ellipse was made as follows:

$$a = pl.$$

Here, a is the respiratory surface area of the lamellae, l is the basal length of the protruding lamellae, p is the ellipse perimeter formula divided by 2,

$$p = \frac{2\pi\sqrt{(1/2)(r^2 + h^2)}}{2},$$

and

$$r = t/2,$$

where h is the height of the protruding lamellae and t is the thickness of the lamellae.

Phylogenetic Analysis

Genomic DNA was extracted from the white muscle of three individuals of each species with the DNeasy Tissue Kit (Qiagen), and the cytochrome *b* (cyt *b*) gene sequence was then amplified via PCR with the following primer pair: 5′-GAC TTG AAA AAC CAC CGT TG-3′ (forward primer) and 5′-CTC CGA TCT CCG GAT TAC AAG AC-3′ (reverse primer). PCR products were sequenced directly with BIGDYE Terminator chemistry and high-throughput sequence analysis (ver. 3.1; Applied Biosystems 3730S 48-capillary sequencer). For each sample, the resulting PCR product was sequenced in both directions, and from all three individuals a consensus sequence for each species was established. Sequences have been submitted to GenBank, and the cyt *b* accession numbers are given in table 1.

In order to construct a phylogentic tree for the carp groups used in this study, sequences were aligned with ClustalW and formatted as a nexus file in Mega 3.1 (Kumar et al. 2004). Sequences were then imported into PAUP to construct a maximum likelihood gene tree, and Modeltest (Posada and Crandall 1998) was used to determine the likelihood ratio test that best fitted the sequence data. A heuristics search was used to create the tree with bootstrap analysis of 1,000 pseudoreplicates. *Danio rerio* was included as an outgroup; the cyt *b* sequence available in GenBank (accession no. EU241427.1) was used.

Statistical Analyses

All direct comparisons between groups were analyzed for significance with one-way ANOVA in Graphpad Prism (http://graphpad.com) followed by a Tukey's post hoc test to compare between groups. The effects of body mass on our traits was examined with correlations comparing the trait of interest to body mass for each taxa. There were no significant relationships between any body mass and any trait examined in this study. All percent-change comparisons were analyzed with one-way ANOVAs on data that were arcsine transformed. Pearson correlations (non-PIC) were analyzed in Graphpad Prism. PICs and PIC correlations were performed with the PDAP module (Midford et al. 2003) in Mesquite (Maddison and Maddison 2004) on the basis of the maximum likelihood tree described above and the procedures outlined in (Mandic et al. 2009*b*). For all statistical analyses, significance was assumed at $P < 0.05$.

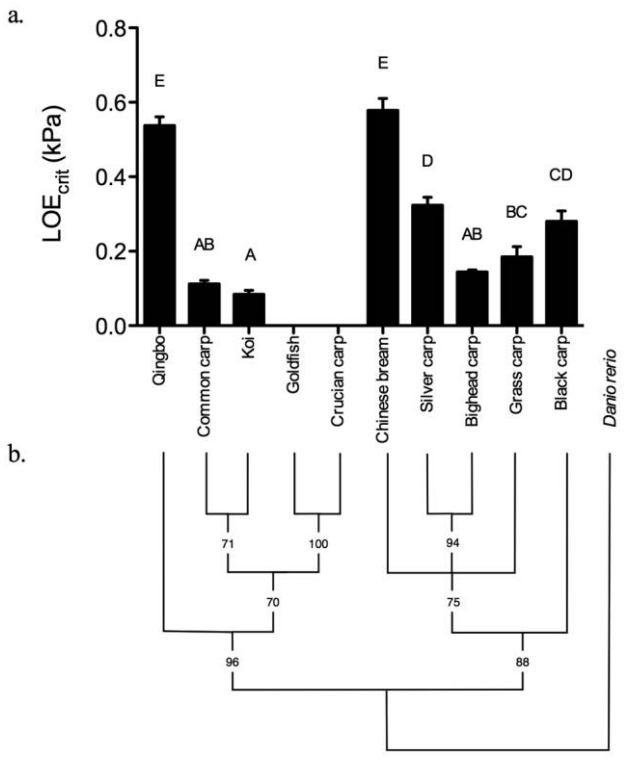

Figure 1. Loss of equilibrium (LOE, in kPa; *a*) and maximum likelihood tree (*b*) for partial cytochrome *b* sequences, with *Danio rerio* as an outgroup. Node bootstrap support values from 1,000 replicates are shown for groups with greater than 50% support. Data are represented as mean + SEM. Letters that differ indicate statistically significant differences between groups.

Results

Phylogeny

Our comparative analysis was performed on 10 groups of carp, including two strains of *Cyprinus carpio*. Phylogenetic analysis based on 900 bp of the cyt *b* sequence from the 10 groups of carp yielded a well-resolved maximum likelihood phylogeny, with zebrafish (*Danio rerio*) as an out-group (fig. 1). Two distinct clades were formed, one of which included koi and common carp (*C. carpio*), goldfish (*Carassius auratus*), and crucian carp (*Carassius carassius*), as well as qingbo (*Spinibarbus sinensis*; fig. 1). The second grouping was composed of Chinese bream (*Megalobrama pellegrini*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), grass carp (*Ctenopharyngodon idellus*), and black carp (*Mylopharyngodon piceus*). There was some variation in body mass among the groups of carp used in our comparative analysis, with the two strains of *C. carpio* being significantly larger than the other groups (table 1); however, there were no significant relationships between body mass and any physiological parameter, and therefore the responses described below are unlikely to be influenced by size over the range used in this study.

Hypoxia Tolerance

There was substantial variation in LOE_{crit} among the 10 groups of carp studied (fig. 1). Crucian carp and goldfish were the most hypoxia tolerant, and they did not show LOE, even after 1 h at 0 kPa at 12°C (these two species were subsequently removed from the statistical analyses shown in fig. 1*a*). After the two anoxia-tolerant species, the koi and common carp were the most hypoxia tolerant, and these, plus the anoxia-tolerant species, were phylogenetically most related to each other. Of the remaining six species, there was significant variation in hypoxia tolerance. Chinese bream and qingbo were the most hypoxia-intolerant species examined, and interestingly, they were in separate phylogenetic clades (fig. 1*b*). The remaining four species showed intermediate tolerance to hypoxia.

Routine $\dot{M}O_2$ and P_{crit}

There was significant variation in P_{crit} among the species examined, with Chinese bream and grass carp having higher P_{crit} values than the other eight groups (fig. 2). However, there was

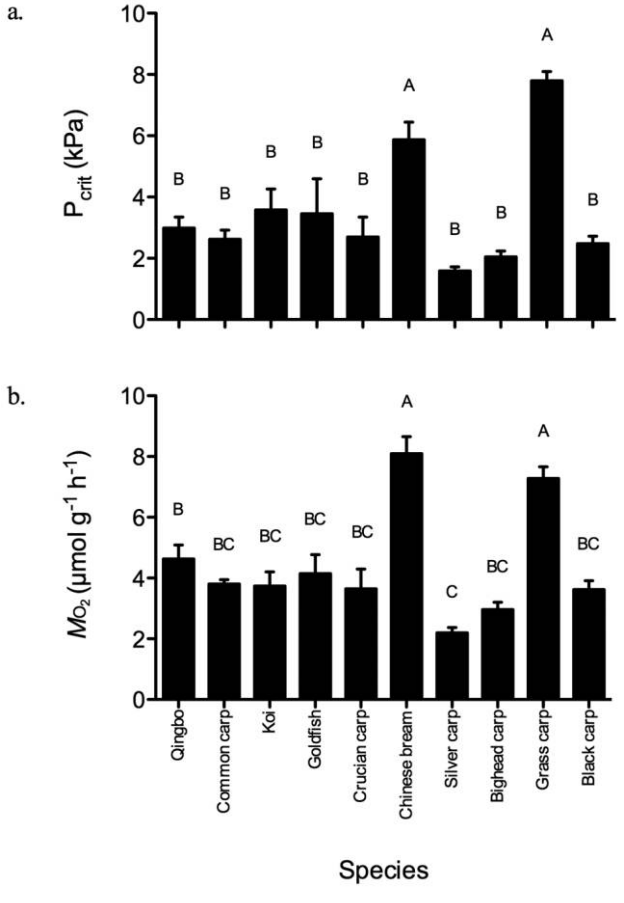

Figure 2. Critical oxygen tension (P_{crit}; *a*) and oxygen consumption rate ($\dot{M}O_2$; *b*) determined for carp groups. Data are represented as mean + SEM. Letters that differ indicate statistically significant differences between groups ($P < 0.05$).

no significant correlation between P_{crit} and LOE_{crit} in both Pearson and PIC correlations (Pearson: $R^2 = 0.004$; $P = 0.60$; PIC: $R^2 = 0.0002$; $P = 0.97$). There were significant differences in $\dot{M}O_2$ between groups, with Chinese bream and grass carp having higher $\dot{M}O_2$ than the other eight groups (fig. 2). Silver carp had the lowest $\dot{M}O_2$, but this was significant only relative to Chinese bream, grass carp, and qingbo. There was no significant correlation between $\dot{M}O_2$ and LOE_{crit} in both Pearson correlations and PIC correlations (Pearson: $R^2 = 0.13$; $P = 0.30$; PIC: $R^2 = 0.15$; $P = 0.29$).

Gill Morphology

In normoxia-acclimated carp, there was large variation in mass-specific lamellae area and the percentage of interlamellar space filled by the ILCM (figs. 3–5). Mass-specific lamellae area was largest in the qingbo, Chinese bream, and black carp, whereas goldfish, koi, and silver carp had the smallest mass-specific lamellae areas (fig. 4*a*, 4*b*). The variation in mass-specific lamellae area of normoxia-acclimated carp was positively correlated with LOE_{crit} ($R^2 = 0.53$, $P = 0.02$; fig. 4*b*), whereby species with low mass-specific lamellae area were more hypoxia tolerant (fig. 4*b*). This correlation remained significant even after phylogeny was taken into account ($R^2 = 0.44$, $P = 0.03$; fig. 4*b*). For most species, there was a progressive increase in mass-specific lamellae area after 48 h of exposure to hypoxia when compared to normoxia, except for Chinese bream (fig. 4*a*). The largest changes in lamella area were observed in common carp, in which it almost doubled after 48 h of exposure to hypoxia. Mass-specific lamellae area after 48 h of hypoxia exposure was not significantly correlated with LOE_{crit} (Pearson correlation: $R^2 = 0.30$; $P = 0.10$; PIC: $R^2 = 0.34$; $P = 0.07$; fig. 4*c*).

The hypoxia-induced changes in mass-specific gill surface area (fig. 4) were associated with significant changes in several morphological parameters. The ILCM height decreased significantly over the 48-h hypoxia exposure in all species, with the exception of Chinese bream (fig. 5*a*; two-way ANOVA; $P < 0.001$). The largest and most rapid (within 8 h) changes in the ILCM occurred in crucian carp, goldfish, and common carp, with 30%, 27%, and 19% decreases in lamellar coverage by the ILCM, respectively, over 8 h of hypoxia exposure (fig. 5). After 48 h of hypoxia exposure, goldfish and crucian carp had reduced lamellar coverage by the ILCM by about half compared with the normoxic controls (fig. 5*a*). Only after 48 h of exposure did bighead, silver, and black carp also show large changes in ILCM height. The change in the ILCM was less than 5% in Chinese bream and qingbo over 48 h of hypoxic exposure, significantly less than that of all other species in this study (fig. 5*b*).

In addition to a significant phylogenetically independent correlation between the change in the ILCM and LOE_{crit} over 48 h ($R^2 = 0.67$; $P = 0.005$; fig. 5*b*), there was also a significant correlation between these parameters over 8 h ($R^2 = 0.75$; $P = 0.04$; data not shown), but the relationship was not phylogenetically independent ($R^2 = 0.32$; $P = 0.15$). By 48 h into exposure to hypoxia, all carp species had less than 50% of their lamellar area covered by the ILCM (fig. 5*a*). Interestingly, ILCM

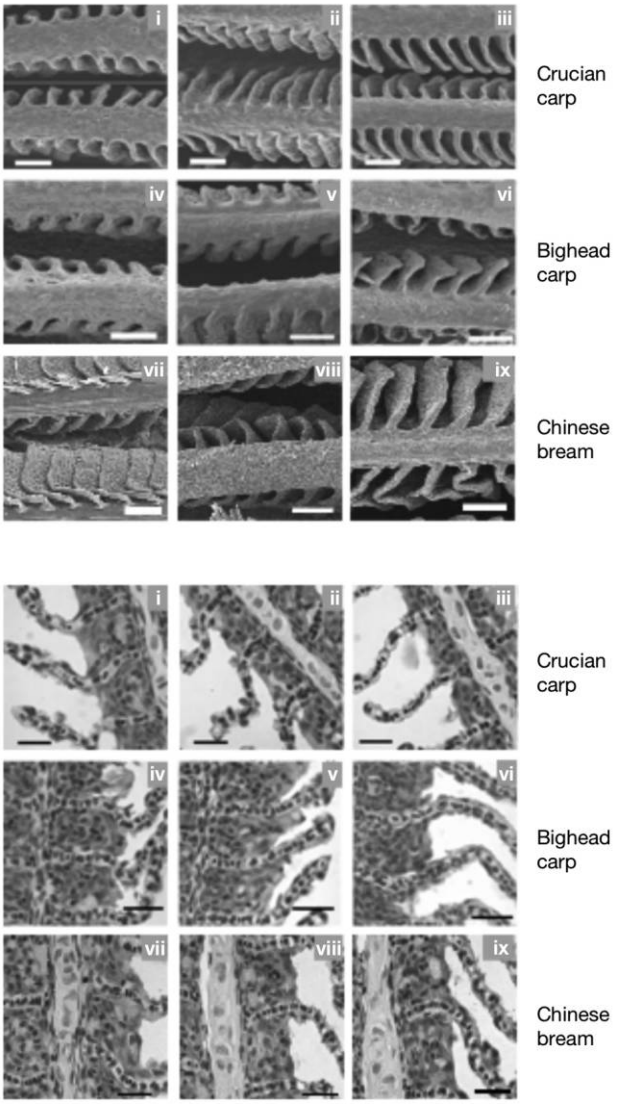

Figure 3. Scanning electron micrographs (*top*) and light microscope micrographs (*bottom*) of the gill lamella for crucian carp, bighead carp, and Chinese bream over a 48-h hypoxia exposure. Scale bars = 50 μm.

thickness never dropped below 20% of the total lamellae height in all 10 groups after 48 h of hypoxia exposure. Also of note were the number of nuclei in the ILCM, which decreased significantly over the 48-h period in crucian carp, goldfish, common carp, and silver carp (table 2). Overall, the percent change in the ILCM over 48 h can be explained by the variation in LOE_{crit}, using PIC ($R^2 = 0.67$; $P = 0.005$; fig. 5*b*).

Aquatic Surface Respiration

Goldfish and crucian carp started ASR at a higher Po_2 than the other groups of carp (table 3). Bighead carp began ASR at the lowest Po_2 of all the carp examined (table 3). The O_2 tension where the highest total number of ASR events was observed (referred to as "Peak ASR" in table 3) was highest for qingbo

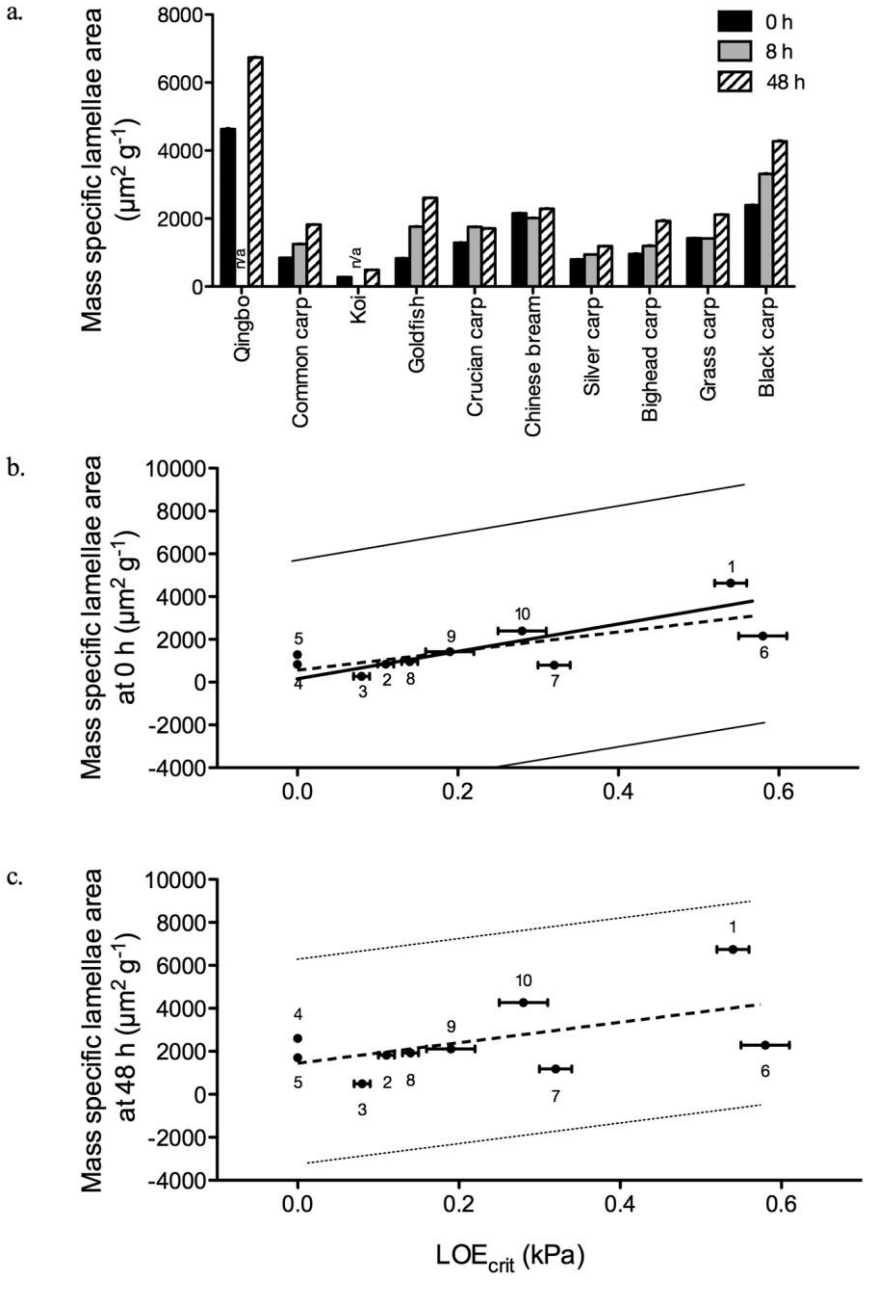

Figure 4. Mass-specific lamellae area (*a*), mass-specific lamellae area at 0 h (*b*), and mass-specific lamellae area at 48 h (*c*) from gills of carp exposed to hypoxia for 48 h. Data are represented as mean + SEM. However, vertical error bars are not visible for most gill measurements. In *b*, the Pearson correlation is displayed as a dashed line, and the independent contrasts are shown as a solid line with 95% confidence intervals. In *c*, the independent contrast was not significant, and so only the Pearson correlation is displayed, with 95% confidence intervals. The numbers 1–10 represent the different groups: 1 = qingbo, 2 = common carp, 3 = koi, 4 = goldfish, 5 = crucian carp, 6 = Chinese bream, 7 = silver carp, 8 = bighead carp, 9 = grass carp, and 10 = black carp. LOE_{crit} = O_2 tension at which fish lost equilibrium.

and Chinese bream, 0.91 kPa for both, significantly higher than that seen in the other eight groups of carp (table 3). The total number of ASR events displayed at peak ASR was highest in goldfish, crucian carp, koi, and common carp (one-way ANOVA; $P < 0.001$). The total number of ASR events counted at $P_{O_2} \geq 0.2$ kPa was highest in the goldfish and crucian carp and lowest in the silver carp (table 3).

At peak ASR, LOE_{crit} was correlated with P_{O_2}; less-tolerant carp reached peak ASR at higher environmental P_{O_2} than more-tolerant carp ($R^2 = 0.47$, $P = 0.03$; correlation not shown). However, this relationship was not significant when phylogeny was taken into account in the correlation. Despite the large variation in total number of ASR events displayed by different species, there was no significant correlation between the total

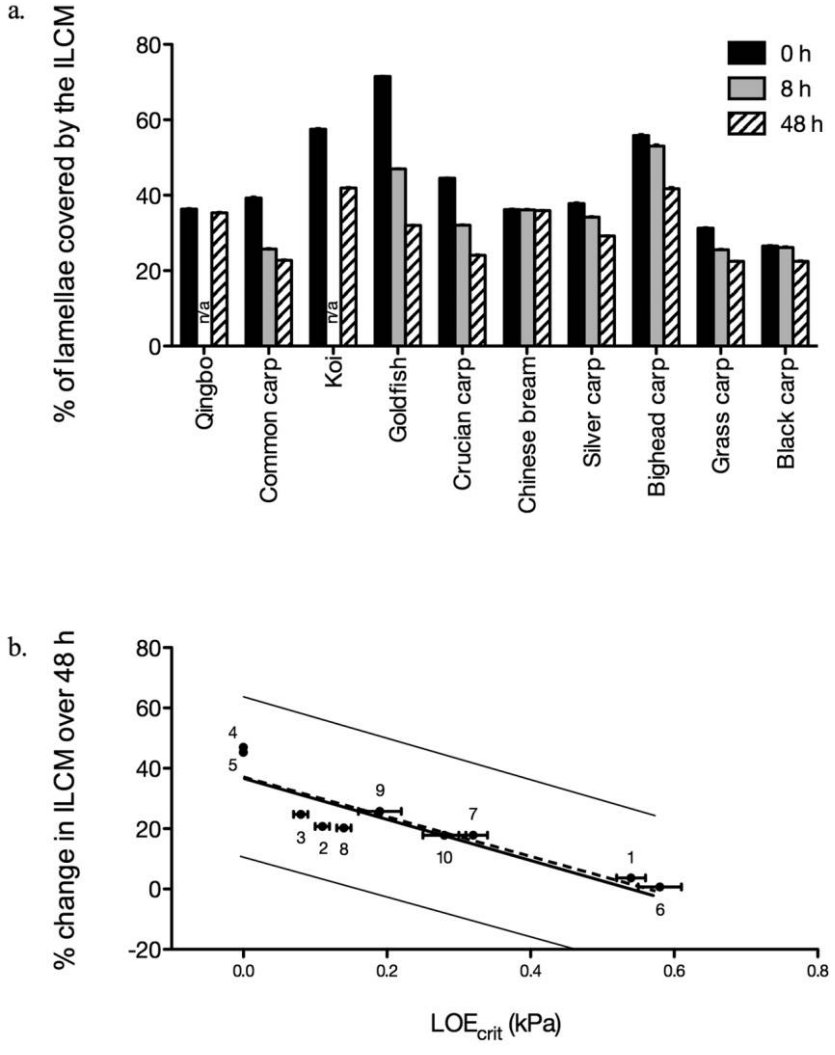

Figure 5. Effects of hypoxia exposure (0.7 kPa) over 48 h on percent coverage of the lamellae by the interlamellar cell mass (ILCM; *a*) and the percent change in the ILCM over the 48-h experiment (*b*). Data are represented as mean + SEM; however, vertical error bars are not visible. In *b*, the Pearson correlation is displayed as a dashed line, and the independent contrast is shown as a solid line; 95% confidence intervals are shown. See figure 4 legend for more details. LOE_{crit} = O_2 tension at which fish lost equilibrium.

number of ASR events and LOE_{crit}, under both a standard linear model and PIC.

Discussion

Since the discovery that crucian carp and goldfish are capable of extensive gill remodeling in response to hypoxia exposure (Sollid et al. 2003), many studies have shown similar responses in a growing number of teleost fish in response to a variety of environmental perturbations (see Brauner et al. 2004; Ong et al. 2007; Nilsson et al. 2012; Perry et al. 2012 for reviews). Our study is the first to explicitly examine whether there is variation in the extent of gill remodeling among species of carp and whether this variation in gill remodeling is phylogenetically independently correlated with hypoxia tolerance. The results of this study point to three novel findings: (1) all but one group of carp studied here are capable of remodeling their gills in response to environmental hypoxia exposure; (2) the extent of gill remodeling is related to hypoxia tolerance in a phylogenetically independent manner; and (3) the extensive gill remodeling seen in the more hypoxia-tolerant groups in response to hypoxia exposure serves to increase their gill surface area to values that are similar to those seen in the less hypoxia-tolerant groups held under normoxia.

Modern comparative methods aim to identify the repeated evolution of a trait across multiple species while attempting to take into account the confounding effects of shared ancestry (i.e., phylogeny; Felsenstein 1985). We used 10 groups of carp, including two strains of *Cyprinus carpio*, plus an outgroup (*Danio rerio*) and constructed a well-resolved maximum likelihood phylogeny based on the cyt *b* gene sequence (fig. 1). Our phy-

Table 2: Quantification of changes in gill morphology in 10 groups of carp exposed to hypoxia (0.7 kPa) for 0, 8, and 48 h

	ILCM volume (μm^3)			Number of nuclei		
Species	0 h	8 h	48 h	0 h	8 h	48 h
Qingbo	$5.4 \times 10^{4\,A}$	NA	$5.4 \times 10^{4\,A}$	8 ± 2^{A}	NA	7 ± 2^{A}
Common carp	$9.7 \times 10^{4\,A}$	$8.8 \times 10^{4\,B}$	$8.8 \times 10^{4\,B}$	20 ± 2^{A}	17 ± 1^{B}	14 ± 2^{C}
Koi	$10.3 \times 10^{4\,A}$	NA	$8.7 \times 10^{4\,B}$	15 ± 2^{A}	NA	10 ± 1^{A}
Goldfish	$9.6 \times 10^{4\,A}$	$7.7 \times 10^{4\,B}$	$6.1 \times 10^{4\,C}$	15 ± 1^{A}	11 ± 2^{B}	10 ± 2^{B}
Crucian carp	$14.1 \times 10^{4\,A}$	$10.4 \times 10^{4\,B}$	$8.4 \times 10^{4\,C}$	18 ± 2^{A}	14 ± 2^{B}	10 ± 2^{C}
Chinese bream	$13.8 \times 10^{4\,A}$	$13.8 \times 10^{4\,A}$	$13.8 \times 10^{4\,A}$	17 ± 2^{A}	16 ± 3^{A}	16 ± 2^{A}
Silver carp	$6.8 \times 10^{4\,A}$	$6.8 \times 10^{4\,A}$	$6.5 \times 10^{4\,B}$	16 ± 2^{A}	13 ± 1^{B}	11 ± 1^{C}
Bighead carp	$10.8 \times 10^{4\,A}$	$10.6 \times 10^{4\,A}$	$10.4 \times 10^{4\,A}$	20 ± 2^{A}	19 ± 2^{A}	15 ± 1^{B}
Grass carp	$8.1 \times 10^{4\,A}$	$7.2 \times 10^{4\,B}$	$6.8 \times 10^{4\,C}$	14 ± 1^{A}	13 ± 2^{A}	10 ± 1^{B}
Black carp	$7.2 \times 10^{4\,A}$	$7.2 \times 10^{4\,A}$	$7.2 \times 10^{4\,A}$	12 ± 2^{A}	12 ± 1^{A}	11 ± 2^{A}

Note. Letters that differ indicate statistically significant differences between sampling times. ILCM = interlamellar cell mass; NA = not available.

logeny is in agreement with other published phylogenies (Wang et al. 2007, 2008, 2012). On the basis of mitochondrial genome sequences, Wang et al. (2012) showed that grass carp and silver carp were more closely related to each other than to black carp, which is also shown in our phylogeny, where black carp diverged from the other two species earlier. The close relationship between bighead carp and silver carp seen in our phylogeny is also supported by other published phylogenies (Wang et al. 2008, 2012). Furthermore, Wang et al. (2008) suggested that grass carp be placed in the subfamily Leuciscinae, which includes black carp, silver carp, and bighead carp, as opposed to the subfamily Cyprininae, which contain crucian carp, goldfish, and koi/common carp. This general division of our species into two distinct groups is also supported by our maximum likelihood analysis (fig. 1*b*).

Among the 10 groups of carp used in this study, we observed significant variation in hypoxia tolerance, as assessed by LOE_{crit}, and it is clear that phylogeny explains some of the observed variation. Briefly, LOE_{crit} should represent the lowest oxygen tension that a fish can survive without losing equilibrium. However, it is important to note that this experiment was not run indefinitely, and thus species that did not lose equilibrium over the course of the experiment have an LOE_{crit} value of 0 kPa. In general, crucian carp and goldfish, the only members of the *Carassius* genus used in this study, are closely related and they also had the lowest (undetectable) LOE_{crit} values. This impressive anoxia tolerance is consistent with published results, which show that even at room temperature, these two species are capable of surviving complete anoxia for more than 16 and 48 h, respectively (e.g., Blazka 1958; Van den Thillart et al. 1976; Shoubridge and Hochachka 1980; Johnston and Bernard 1983; Nilsson and Renshaw 2004; Bickler and Buck 2007), and that anoxia survival time increases as temperature decreases (Van den Thillart et al. 1983). Despite the fact that the two most hypoxia-/anoxia-tolerant species are closely related to each other, it is notable that among the other eight groups of carp examined, variation in hypoxia tolerance is spread across the phylogeny. Common carp, koi (both *C. carpio*), and bighead carp were the next–most hypoxia-tolerant groups, and they had representatives in each of the two major clades shown in figure 1. Furthermore, the least hypoxia-tolerant species, qingbo and Chinese bream, are in separate phylogenetic clades. Overall, the variation in hypoxia tolerance seen across the phylogeny is conducive to the application of PIC for examining the correlated evolution of hypoxia tolerance.

Critical oxygen tensions of $\dot{M}_{O_2}$ (P_{crit}) are often used as a proxy measure of hypoxia tolerance, with the assumption that an animal with a lower P_{crit} will be able to sustain a routine metabolic rate to lower oxygen tension. Indeed, among 12 species of fish from the superfamily Cottoidea (commonly called sculpins), Mandic et al. (2009*b*) described significant variation in P_{crit} that was directly related to the frequency and duration of hypoxia exposure in their natural environment (the marine nearshore environment). Furthermore, there was a significant correlation between P_{crit} and the time required for 50% of a group of fish to lose equilibrium (LOE_{50}) when exposed to 0.85 kPa, but this relationship was influenced by phylogeny, and thus the PIC correlation was not significant. Among our 10 groups of carp, we also observed significant variation in P_{crit} (fig. 2*a*), but the variation in P_{crit} was not correlated with LOE_{crit}. Similar results have also been observed among nine groups (10 species, including three subspecies of *D. rerio*) of fish from the genera *Danio* and *Devario*, where we observed very little variation in P_{crit} among the groups of fish despite significant variation in time to LOE at 12 torr (L. Yao and J. G. Richards, unpublished results). As a result, there is accumulating evidence to suggest that P_{crit} is not a direct measure of hypoxia tolerance but rather that it should be considered a proxy for whole-animal oxygen uptake capacity. In the carp, there was a strong association between P_{crit} and $\dot{M}_{O_2}$ ($R^2 = 0.85$; $P = 0.0002$), which further suggests that P_{crit} is a measure of oxygen uptake and not of hypoxia tolerance per se.

The most striking outcomes of this study are that all the carp studied, except the Chinese bream, are capable of remodeling

Table 3: Characteristics of aquatic surface respiration (ASR) in 10 groups of cyprinids in response to progressive decreases in Po_2

	First ASR		Peak ASR		
Species	Mean Po_2 (kPa)	No. events	Mean Po_2 (kPa)	No. events	Total no. events
Qingbo	1.59^{B}	2 ± 2	$.91^{A}$	171 ± 25	476 ± 35^{BCD}
Common carp	$.91^{BC}$	28 ± 19	$.23^{AB}$	184 ± 16	470 ± 134^{BCD}
Koi	1.82^{B}	13 ± 7	$.23^{AB}$	198 ± 2	716 ± 235^{BC}
Goldfish	2.27^{A}	15 ± 9	$.45^{AB}$	200	1,399 ± 50^{A}
Crucian carp	2.27^{A}	1 ± 1	$.00^{B}$	200	834 ± 144^{B}
Chinese bream	1.82^{B}	2 ± 2	$.91^{A}$	141 ± 23	390 ± 34^{BCD}
Silver carp	$.68^{BC}$	1 ± 1	$.23^{B}$	43 ± 14	49 ± 15^{D}
Bighead carp	$.45^{C}$	10 ± 5	$.23^{B}$	126 ± 6	288 ± 28^{CD}
Grass carp	$.68^{BC}$	43 ± 18	$.45^{AB}$	127 ± 20	255 ± 54^{CD}
Black carp	1.13^{BC}	1 ± 1	$.45^{AB}$	140 ± 40	286 ± 74^{CD}

Note. Letters that differ indicate statistically significant differences between groups.

their gills in response to 48 h of exposure to 0.7 kPa at 12°C and that the extent of gill remodeling in all groups is phylogenetically independent and correlated with LOE_{crit}. The most hypoxia-tolerant species (e.g., crucian carp and goldfish) undergo much greater gill remodeling than the less hypoxia-tolerant groups (fig. 5*b*), which results in increases in mass-specific gill surface area (fig. 4*a*). This phylogenetically independent relationship points to gill remodeling as an adaptation to hypoxia that contributes to increasing hypoxia tolerance by increasing gill surface area. While the ability to change gill structure in response to hypoxia is clearly related to hypoxia tolerance, the resulting mass-specific lamellae area shows no significant relationship with LOE_{crit} (fig. 4*c*). That is, hypoxia-induced gill remodeling serves to increase the lamellar area in hypoxia-tolerant carp to values similar to those seen in the less hypoxia-tolerant carp (fig. 4*c*). Therefore, it seems that the more hypoxia-tolerant species are better able to tolerate low environmental oxygen tensions despite the lack of a real advantage in gill size, which may, in part, be due to their high hemoglobin-O_2 binding affinities and other metabolic adjustments that are known to occur in the hypoxia-tolerant carp used in this study (Burggren 1982; Sollid et al. 2005*a*; Sollid and Nilsson 2006; Nilsson et al. 2012).

Another striking outcome of our study is the observation that the mass-specific lamellae area measured in normoxia-acclimated fish was correlated with hypoxia tolerance in a phylogenetically independent fashion (fig. 4*b*). Specifically, hypoxia-tolerant carp have much smaller gills, when held under normoxic conditions, than their hypoxia-sensitive relatives. The questions of why hypoxia-tolerant carp have small gills in normoxia and what the advantage of gill remodeling is have been asked before (see Nilsson et al. 2012), but they remain unanswered. The results of our analysis clearly indicate that gill size in normoxia is related to hypoxia tolerance and, by extension, perhaps to life history. Our most hypoxia-tolerant species, crucian carp and goldfish, are well known to inhabit slow-moving ponds and lakes that can freeze over in the winter (Van den Thillart et al. 1976; Hyvärinen et al. 1985; Holopainen et al. 1997). Some of our more hypoxia-sensitive species, on the other hand, including qingbo (Kong et al. 2007), silver carp, and black carp (Yi et al. 2010), are known to inhabit faster-flowing rivers and have streamlined body form. As a result, it is intriguing to speculate that there is an inverse relationship between hypoxia tolerance and swimming performance in the carp used in this study, which may partially explain how life history is related to gill surface area and gill remodeling. Fish that inhabit faster-flowing water require larger gills to support the metabolic demands of swimming (high aerobic scope), while fish inhabiting slower-moving waters do not require large gills, since their aerobic scope is generally lower (Fu et al. 2012). Indeed, compared to active salmonids like rainbow trout (*Oncorhynchus mykiss*), the common carp has lower aerobic scope (Ultsch et al. 1980). In hypoxia-tolerant carp, reducing gill surface area through proliferation of an ILCM may alleviate the possible consequences of a high gill surface area in normoxia (Nilsson et al. 2012).

The gill is a multifunctional organ that is involved in both gas exchange and ion regulation. In freshwater environments, fish with greater gill surface areas for increased oxygen uptake must also face the physiological trade-off of an associated increase in ion loss, known as the osmorespiratory compromise (see Gonzalez 2011 for a review). In our study, goldfish had the highest lamellar coverage with ILCM in normoxia and, along with crucian carp and *C. carpio*, showed the largest capacity for change in gill surface area. This loss of gill surface area in normoxia in these species may be permissible because of the high hemoglobin-O_2 affinity of *Carassius* sp., which may facilitate continued oxygen uptake in normoxia despite reduced respiratory surface area. The smaller gill will consequently reduce ion loss and water influx in normoxia and thus limit the energetic costs of the active uptake of ions. Other explanations for why hypoxia-tolerant carp have small gills in normoxia have been suggested, including the proliferation of the ILCM as a response to exposure to toxic substances or pathogens (Nilsson et al. 2012). Nilsson et al. (2012) demonstrated that in the case of crucian carp infected with gill flukes, there are no reductions in the ILCM even when fish are exposed to hypoxia. Such issues

with water quality or risk of infection were likely not the case in our study and are unlikely to be related to variation in hypoxia tolerance as reported here.

Recent published literature has shown great variation in the time course over which gill remodeling occurs in response to metabolic challenges. The original work of Sollid et al. (2003) in crucian carp showed that it took up to 3 d to see a decrease in the number of cells in the ILCM and that the reductions in ILCM volume were the result of increased apoptosis and decreased mitosis. More recent evidence indicates that this process can occur much faster (within roughly 6 h; Sollid et al. 2005*b*) in crucian carp exposed to decreasing oxygen tensions, and in goldfish, Tzaneva et al. (2011*b*) observed a ~18% decrease in ILCM volume in goldfish exposed to hypoxia for only 30 min. Collectively, these data suggest that more than one mechanism may be responsible for changing ILCM volume in response to hypoxia or other metabolic challenges, where slow changes may be the result of changes in apoptosis and mitosis and faster responses may be due to possible shedding of ILCM cells, although the latter mechanism has yet to be elucidated (see Nilsson et al. 2012 for more detail). It is possible that the rate of change in the ILCM may be species specific and also under selection in hypoxia-tolerant species, unlike that in more sensitive species. However, it is difficult to resolve how the rate of change is associated with hypoxia tolerance in the time points selected in this study.

Compared with that in other studies that have quantified gill remodeling in crucian carp, the extent of gill remodeling noted in crucian carp in our study is low, which is likely the result of relatively low coverage of the lamellae by the ILCM under normoxic conditions. Our experiments were conducted at 12°C, which is an intermediate temperature from the range used in previous studies. Much of the research examining gill remodeling was performed at ~8°C (Sollid et al. 2003; Sollid and Nilsson 2006; Nilsson et al. 2007; Tzaneva et al. 2011*a*), and under those conditions both crucian carp and goldfish have almost no protruding lamellae in normoxia. As temperature increases, there is a decrease in the ILCM and an increase in protruding lamellar area, such that in goldfish exposed to 15° or 25°C there is almost no ILCM present in normoxia (Sollid et al. 2005*b*; Nilsson et al. 2007). As a result, it is likely that the larger protruding lamellar area seen in our normoxic carp, compared with that in previous work, is due to temperature differences. Another possible explanation for why our more hypoxia-tolerant carp species, specifically crucian carp and goldfish, have more protruding lamellae in normoxia than those in other studies may also be related to the time of year, and as far as we are aware, no study has assessed whether time of year has an effect on gill morphology. Variation in other morphological and biochemical traits occurs seasonally, and it is well known that crucian carp increase liver size and glycogen content in late fall and early November in preparation for winter (Hyvärinen et al. 1985). It is possible that gill morphology also changes in response to season.

Hypoxia survival necessitates changes at many levels of biological organization, including behavior; however, few studies have shown a strong relationship between hypoxia-induced behaviors and hypoxia tolerance (Mandic et al. 2009*a*). ASR is a behavioral response to hypoxia that involves a fish selectively ventilating at the better-oxygenated air-water interface. In our study, ASR was observed in all groups, and it was initiated at oxygen tensions much lower than their P_{crit} (fig. 2; table 3). This delay in surface breathing may suggest a predator avoidance strategy (Sloman et al. 2006), which may be particularly important, given the small carp used in our study. In general, hypoxia-tolerant fishes are thought to delay initiating ASR in favor of reducing metabolic demand (Yoshiyama et al. 1995); however, in our groups of cyprinids, we see that the most hypoxia-tolerant carp initiate their first ASR events at Po_2 values higher than those for less hypoxia-tolerant carp. At first glance, this seems counterintuitive, but a similar relationship was observed in sculpins (Mandic et al. 2009*a*), where the more hypoxia-tolerant sculpins initiated ASR at higher Po_2 values than less hypoxia-tolerant sculpins. These data suggest that the most hypoxia-tolerant species are more sensitive to changes in Po_2 and initiate ASR behavior at higher Po_2 values in an attempt to sustain aerobic metabolism at the lowest possible environmental Po_2, thus conserving precious "anaerobic" substrates. This may be of particular importance to crucian carp and goldfish, which may endure severe hypoxia/anoxia for long periods of time. The Po_2 at peak ASR was correlated with hypoxia tolerance, such that the more hypoxia-tolerant carp showed their maximum number of attempts at ASR at environmental Po_2 values lower than those for less hypoxia-tolerant species. Since ASR behavior is associated with increased risk of predation and increased energetic costs (Kramer 1987), it seems reasonable that fish that inhabit environments prone to hypoxia have physiological and biochemical strategies that allow the hypoxia-tolerant carp to withstand lower environmental Po_2 without increasing risky behavior.

Overall, there were significant differences in hypoxia tolerance among the 10 groups of carp examined in this study. These differences were primarily driven by more-tolerant species displaying a greater propensity for gill remodeling. Furthermore, there were significant standard linear correlations, as well as phylogenetically independent correlations, between LOE_{crit} and both the percent change in the ILCM over 48 h of hypoxia exposure and the mass-specific lamellae area in normoxia. Thus, the capacity for gill remodeling is likely an adaptive trait to increase hypoxia tolerance. Behavior was also important in mitigating the effects of low environmental oxygen, as the most hypoxia-tolerant carp species exhibited ASR attempts more often and at a higher Po_2 than the other species examined in this study.

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