Intraspecific metabolic scaling exponent depends on red blood cell size in fishes

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Abstract

The metabolic-level boundaries (MLB) hypothesis and the cell metabolism (CM) hypothesis have been proposed to explain the body mass scaling of metabolic rate. The MLB hypothesis focuses mainly on the influence of the metabolic level on the relative importance of volume and surface area constraints. The CM hypothesis focuses on the variation of cell size as the body grows. The surface area to volume ratio of individual cells may vary among species with different cell sizes, by which surface area constraints on metabolic scaling may change according to the MLB hypothesis. The present study aimed to extend the MLB and the CM hypotheses by proposing that, in addition to metabolic level, the varying cell surface area constraints among species also influence the intraspecific scaling exponents. The red blood cell area (S), and intraspecific scaling exponents for resting ($b_R$) and maximum metabolic rates of four species of cyprinids were assessed. The scaling exponents varied among species, but mass-specific resting metabolic rates (RMR) of each species were similar. No significant correlation was found between $S$ and mass-specific RMR among species. As predicted, a significantly negative relationship exists between $S$ and $b_R$ among species. The results suggest that the varying $b_R$ could be attributed to cell size difference among species, as those with larger cells may face stronger surface boundary limits, as predicted by the MLB hypothesis. This mechanism represents an additional way of relating the MLB and the CM hypotheses and is not mutually exclusive to another mechanism based on the recent contextual multimodal theory.

Key-words: body mass, metabolic rate, red blood cell
Introduction

The relationship between metabolic rate \((MR)\) and body mass \((M)\) is an important issue in many areas of biology. \(MR\) typically scales with \(M\) according to the equation: \(MR = aM^b\), where \(a\) is the constant and \(b\) is the scaling exponent. This relationship has been addressed by many studies over the past century. Several important hypotheses regarding the scaling of \(MR\) have been proposed in recent years. Of these, the metabolic theory of ecology (MTE) proposes a universal value of 0.75 inter- or intraspecifically, as a result of the assumed geometry of optimised resource distribution fractal networks (West et al., 1997; Brown et al., 2004). However, many studies have found that the intraspecific \(b\)-value \((b_R)\) for resting metabolic rate \((RMR)\) varies among species and depends on a species’ lifestyle, ontogenetic phases, and ecological factors (Post and Lee, 1996; Glazier, 2005; White and Seymour, 2006; Killen et al., 2007, 2010; Yagi et al., 2010). Especially in fish, the \(b_R\) ranges widely from 0.38 to 1.29 (mostly between 0.66 and 1), deviating from the 0.75 scaling law (Clarke and Johnston, 1999; Bokma, 2004; Killen et al., 2007, 2010; Zhang et al., 2014). It has been proposed that this variation in metabolic scaling may be better explained by a meta-mechanistic theory composed of multiple mechanisms, each of which acts contingently based on various modulating contextual factors (internal or external), rather than by a deterministically mechanistic theory based on a single deterministic mechanism (Glazier, 2014a,b).

The metabolic-level boundaries (MLB) hypothesis proposed that volume and surface area constraints (scaling as \(M^1\) and \(M^{2/3}\), respectively) act as boundary limits on \(b\), while the metabolic level \((L)\) determines the relative importance of the constraints (Glazier, 2005, 2008, 2009, 2010). According to the MLB hypothesis, a species with higher \(RMR\) has a lower intraspecific \(b\)-value (Glazier, 2005, 2010). On the other hand, a value of 1 has been proposed for the \(b\)-value \((b_M)\) for the maximum metabolic rate \((MMR)\), as \(MMR\) mostly represents energy expenditure by muscle, and the scaling of muscle mass is proportional to \(M^1\) (Glazier, 2005, 2010). The inverse relationship between metabolic level and \(b\) for \(RMR\) has been modelled in 89 species of fish (Killen et al., 2010) and could effectively predict the intraspecific scaling exponent of some fish species, e.g., the crucian carp \((Carassius auratus)\) by our previous study (Huang et al., 2013). However, the low coefficient (0.18) of determination for this model (Killen et al., 2010) implies that species with similar metabolic levels may vary greatly in their scaling exponent as a consequence of other variables. More work is needed to determine whether the intraspecific \(b\)-value varies among species with similar \(RMR\). The MLB hypothesis assumes that both the volume and surface area constraints,
along with metabolic level, contribute to the intraspecific $b$, but focuses mainly on the influence of the metabolic level on $b$. Factors that directly affect the relative contribution of a surface or volume-related process to metabolic scaling should also affect $b$ (Glazier, 2014b). Therefore, in addition to the metabolic level, the varying volume and surface area constraints among species could be hypothesised to influence the intraspecific $b$-value.

The cell metabolism (CM) hypothesis proposed that larger cells have a relatively lower metabolic rate because of their smaller surface area/volume ratio (Davison, 1955, 1956; Kozłowski, 2003). Therefore, the scaling exponent $b$ should be 0.67 if the variation in body mass is attributed entirely to changes in cell size, or 1.0 if cell size remains unchanged and body mass variation is entirely due to differences in cell number (Davison, 1955; Kozłowski et al., 2003). The CM hypothesis has been supported by some studies (Chown et al., 2007; Pis, 2008; Starostová et al., 2009). Most of these studies adopted the red blood cell (RBC) size as a proxy for the general cell size of an organism, because of its importance in oxygen transport (Starostová et al., 2009, 2013; Maciak et al., 2011). Recently, a study based on arithmetic data suggested that metabolic rate scaling is more linear in species with an invariant RBC size during ontogeny, while species whose RBC size increases with body size during ontogeny should show an allometric relationship between $MR$ and body size (Starostová et al., 2013). However, when the data of Starostová et al. (2013) were log-transformed, the scaling exponents did not appear to agree with the CM hypothesis (Glazier, 2013). Our previous studies found an invariant RBC size along with a $b$-value of 0.776 in crucian carp, but an increasing RBC size along with a larger $b$-value of 0.831 in grass carp ($Ctenopharyngodon idellus$) (Huang et al., 2013; Zhang et al., 2014). This suggested that the CM hypothesis could only partly account for metabolic scaling, as it focused on the variation of cell size as the body grows, but ignored the interspecific differences of cell size and the surface area/volume ratio. The variation in RBC area among species is obvious, especially in fish (range approximately 30-fold) (Gregory, 2013). The large variation of cell size among species may result in differences in their intraspecific scaling exponents. Recently, based on a contextual multimodal theory (CMT) (Glazier, 2014a), an interesting review proposed to reconcile the MLB hypothesis with the CM hypothesis by hypothesising that organisms with small cells should have higher metabolic level values than those of related organisms with large cells, and that small-celled organisms should also have lower $b$ values than related large-celled organisms (Glazier, 2014b). This prediction was confirmed by a meta-analysis of 22 species of teleost fishes: RBC size was significantly negatively correlated with metabolic level, but positively correlated with $b$ (Glazier, 2014b). However, metabolic level was not negatively
related with cell size in grass carp (Zhang et al., 2014) and among several species of eyelid geckos (Starostová et al., 2009), when correcting for body mass. More work is needed to confirm a negative correlation between metabolic levels and cell size. As the varying volume and surface area constraints among species may influence the intraspecific $b$-value, we propose an alternative mechanism to unite the CM hypothesis with the MLB hypothesis, predicting that a species (within those species with similar metabolic levels) with larger cell sizes has more pronounced surface area constraints on metabolic scaling, resulting in $b$ decreasing toward 2/3, as predicted by the MLB hypothesis. Therefore, we hypothesize that an inverse relationship exists between cell size and intraspecific $b$-value among species with similar metabolic levels.

We previously reported on RBC size and metabolic scaling in two closely related species of cyprinids, crucian carp and grass carp, and found significant differences in their RBC size and $b_R$, but similarity in their metabolic levels (Huang et al., 2013; Zhang et al., 2014). However, it has been proposed that using two-species comparisons is inadequate for studying adaptation for both logical and statistical problems (Garland and Adolph, 1994). Thus, the limited data from only two species is insufficient to test the hypothesis we propose above. In the present study, the RBC size and intraspecific scaling for resting and maximum metabolic rates of four more closely related species of cyprinids (silver carp *Hypophthalmichthys molitrix*, bighead carp *Aristichthys nobilis*, common carp *Cyprinus carpio*, and largemouth bronze gudgeon *Coreius guichenoti*) were assessed under the same controlled experimental conditions. The RBC size and scaling exponent results of crucian carp and grass carp (Huang et al., 2013; Zhang et al., 2014) are also included for comparison. These species are widely distributed in the basin of Yangtze River and their phylogenetic relationship has been clearly constructed by molecular analyses (Wang et al., 2012a), allowing us to control for the effects of phylogeny on metabolic scaling, though the species are closely related. We aimed to determine: 1. whether these closely related species differ in their RBC size, metabolic level, and scaling exponent of metabolic rate; 2. whether RBC size negatively correlates with metabolic level among species; and 3. whether an inverse relationship exists between RBC size and the scaling exponent of $RMR$.

**Results**

The $MR$ values of each species increased to their peak values after exhaustive exercise and then recovered to their pre-exercise values (see Fig. S1 in supplementary material). The $b_R$ varied significantly from 0.728 to 0.868 among the four experimental species and was higher
than the expected 0.75, except in *C. guichenoti* (Fig. 1). The log-transformed values of mass-specific *RMR* at the midpoint of the regression of the four species were close to each other, ranging 2.05 to 2.15 mgO$_2$ kg$^{-1}$ h$^{-1}$. Mass-specific *RMR* at the midpoint of the regression were not significantly correlated with their $b_R$ ($r = -0.033$, $n = 6$, $p = 0.950$) among species (Fig. 2A). The $b_M$ was lower than 1 in each species and was even lower than $b_R$ in *H. molitrix* and *A. nobilis* (Fig. 1). As a result, the factorial aerobic scope (FAS) of *C. carpio* and *C. guichenoti* remained unchanged, whereas the FAS of *H. molitrix* and *A. nobilis* decreased with increasing $M$ (Fig. 3).

*RBC* area ($S$) increased with $M$ in all four species (Fig. 4) with slopes not significantly different among species ($F_{3, 289} = 1.56$, $p = 0.198$). $S$ was significantly different among species, and their mass corrected mean values ranged from 46.4 to 121.0 μm$^2$. *H. molitrix* had the smallest $S$, followed by *A. nobilis* and *C. carpio*, and *C. guichenoti* had the largest $S$. Positive intraspecific correlations between values of $S$ and *RMR* were found in *H. molitrix* and *C. carpio* when controlling for $M$, but no significant correlations were found for *A. nobilis* or *C. guichenoti* (Fig. 5). $S$ in any of the four species was not correlated with their *MMR* when controlling for $M$ (see Fig. S2 in supplementary material). No significant correlation was found between mass-corrected mean values of $S$ and mass-specific *RMR* among species (Fig. 2B). Interestingly, significantly negative relationships were found between the mass-corrected mean values of $S$ of each species and their $b_R$ (Fig. 6A). After correction for the phylogenetic effects, the negative relationship was still significant (Fig. 6B).

**Discussion**

Values for $b_R$ vary significantly among the four experimental species of cyprinids, but lie within the range (mostly 0.66-1) of previous reported values for other teleosts (Killen et al., 2007, 2010; White and Seymour, 2006). The $b_R$ in three of the four experimental species did not correspond with the value of 0.75 suggested by the MTE hypothesis (West et al., 1997; Brown et al., 2004). This is not surprising, as the value of 0.75 by the MTE hypothesis may just be an empirical average value (Glaizer, 2005). Extrapolating from MTE, Savage et al. (2007) proposed that the cell size of quickly dividing cells, e.g., RBC, should be body size invariant. However, the $S$ of all the four species increased with body mass in the present study. Our results show that predictions based on the MTE hypothesis are inapplicable in the context of intraspecific metabolic scaling.

Our results support the CM hypothesis that the allometric scaling of *RMR* with body mass may be partly attributed to the increase in cell size. However, the slopes (range 0.0164-0.0407)
of the relationship between $S$ and body mass suggest that cell size has only minor effects on intraspecific metabolic scaling. The CM hypothesis has been supported by reports that metabolic rate negatively correlated with RBC size of the spined loach (Cobitis taenia) (Maciak et al., 2011) and C. auratus (Huang et al., 2013), but not by observations on several species of eyelid geckos (Starostová et al., 2009), in the grass carp (Zhang et al., 2014), and in any species in our study when controlled for body mass. For H. molitrix and C. carpio, in contrast, the $RMR$ even correlated positively with $S$. This suggests that variation of cell size may only partly explain the difference of $RMR$, and that the negative relationship between cell size and metabolic rate may not be general across species with very similar metabolic rates. Significant correlations between cell size and metabolic rate may exist in more diverse species with a broader range of metabolic rates, as shown by Glazier (2014b). RBC size increased with body mass in spined loach (Maciak et al., 2011), grass carp (Zhang et al., 2014), and several species of geckos (Starostová et al., 2013), but did not vary with body mass in some other species (Huang et al., 2013; Starostová et al., 2013). Compared among the species in the present study, the species with relative higher scaling slopes for RBC size did not show lower $b_R$. This indicates that the CM hypothesis may not be generally applicable to intraspecific metabolic scaling of different species, and needs to be revised. Possible explanations proposed by some previous studies include variations in cell metabolic activities between cell types or along ontogenesis (Kozłowski et al., 2010), and the possibility that cell membrane permeability and the volume and activity of mitochondria are dependent on body mass (Porter and Brand, 1995; Savage et al., 2007; Burpee et al., 2010).

The MLB hypothesis attributes scaling of $RMR$ to increases in metabolic level, intensifying the surface area constraints and possibly explaining the negative relationship observed between $b_R$ and metabolic levels among many fish species (Glazier, 2010; Killen et al., 2010). The experimental fish in the present study are closely related species and their mass corrected average $RMR$ varies within only a narrow range. A consequence of the small variation in metabolic level between species is the absence of a significant negative correlation between $b_R$ and metabolic levels. However, an interesting observation in the present study is that the intraspecific $b_R$ of the closely related species correlates negatively with their $S$. $S$ varied approximately 2.6-fold among the species in the present study. Species with a larger cell size may have a smaller surface to volume ratio, which may intensify the surface boundary limits on $b_R$ predicted by the MLB hypothesis (Glazier, 2005, 2010), thus resulting in a smaller $b_R$. In contrast, species with smaller cell sizes may face less surface boundary limits and consequently have a larger $b_R$. This suggests that not only the variation of metabolic level, but
also the variation of cell surface area, may contribute to metabolic scaling. Based on the CMT (Glazier, 2014a), a recent review relating the MLB hypothesis to the CM hypothesis was recently published by Glazier (2014b). RBC size was positively correlated with \( b \) among 22 species of teleost fishes, which was explained using the MLB hypothesis: RBC was negatively correlated with metabolic level, which in turn was negatively correlated with \( b \) (Glazier 2014b). In our results, by contrast, \( b \) is negatively correlated with RBC when metabolic level is invariant. Following the MLB hypothesis (Glazier, 2005, 2010), metabolic level, \( b \), and cell size can be integrated into one model (Fig. 7), by which either the findings of Glazier (2014b) or the present study follow the predictions of the MLB hypothesis. It suggests that the mechanism proposed by Glazier (2014b) and our present study are not mutually exclusive. Our results supply additional data for species with similar metabolic levels but different cell sizes relevant to the relationship of the MLB hypothesis to the CM hypothesis.

The \( b_M \) values of all species we studied were less than 1, suggesting that MMR may not scale isometrically with body mass in these fish. MMR scales approximately isometrically in many athletic fish, e.g., salmonids (Brett, 1965; Brett and Glass, 1973; Wieser, 1985), which was attributed to the increasing importance of volume-related muscular energy expenditure on metabolism during exercise, and the linear increase of muscle mass in proportion to body mass (Glazier, 2005, 2009). The low \( b_M \) in the present study suggests that muscular energy expenditure contributes limitedly to whole-body metabolism in these fish species. The majority of fish muscle consists of white muscle with low metabolic activity. In this study, the red muscle of these cyprinids contributed only minor (~1%) part of their body mass.

The low \( b_M \) of these cyprinids causes FAS to remain unchanged in \( C. \) carpio and \( C. \) guichenoti, or even decrease in \( H. \) molitrix, and \( A. \) nobilis, as body mass increases. FAS of fish generally increases or remains unchanged as body mass increases, implying that aerobic capacity is important as the body grows (Brett, 1965; Beamish, 1978; Armstrong, 1992; Post and Lee, 1996; Killen et al., 2007; Huang et al., 2013; Zhang et al., 2014). The unusual decrease of FAS in \( H. \) molitrix, and \( A. \) nobilis, may be explained by special characteristics of their gill morphology. As filter-feeding species, \( H. \) molitrix and \( A. \) nobilis have gill-rakes net on their gill arches. The sizes of gill-rakes gradually increase as body grows and cover gill filament, which may reduce the respiratory gas exchange capacity (Jirásek, 1981; Hampl, 1983; Dong et al., 1992; Sun and Meng, 1992) and restrict them to inhabiting only the upper layers of water, with high oxygen availability.

In conclusion, we found that the closely related species of cyprinids have similar \( RMR \), but varying \( b_R \) and RBC size. The varying \( b_R \) could be attributed to cell size differences among
species, as those with larger cells may face stronger surface boundary limits, as predicted by the MLB hypothesis (Glazier, 2005, 2010). Our results may provide new mechanism to explain metabolic scaling by uniting the MLB hypothesis and the CM hypothesis. This mechanism represents an additional way of relating the MLB and CM hypotheses and is not mutually exclusive to the mechanism proposed by (Glazier, 2014b).

**Materials and Methods**

Experimental animals, *H. molitrix, A. nobilis, C. carpio*, and *C. guichenoti* with different body sizes (range 3 to 460 g) were collected from local fisheries in Chongqing, China, in October 2013. Fish were acclimated in a rearing system at the Fisheries Science Institute of Southwest University for two weeks prior to study. During acclimation, the temperature of water was maintained at 25±1 °C, the oxygen concentration was maintained above 90 % saturation, the ammonia concentration was kept below 0.015 mg l⁻¹, the photoperiod was 14 L: 10D, as in our previous studies (Huang et al., 2013; Zhang et al., 2014). Fish were fed with commercial diets (1 % of body mass) once at 18:00 every day. The chemical composition of the diet was 6.3 % moisture, 30.3 % protein, 2.9 % fat, and 10.0 % digestible carbohydrate. Animal handling and experiments were conducted in according with the ethical requirements of the Animal Care of the Fisheries Science Institution of Southwest University, China and requirements of environment and housing facilities for laboratory animals of China (Gb/T14925-2001).

The respirometer used for metabolic rate determination was a continuous flow design as described by Wang et al. (2012b). Chambers with different volume (0.13, 0.52, 0.86, and 1.20 L) were used depending on the size of fish. Up to 14 fish were subjected to measurements at the same time. Each fish was put into a chamber individually and one empty chamber was used as a control for the background oxygen consumption. The dissolved oxygen concentration was measured at the outlet of the chamber using an oxygen meter (HQ30, Hach Company, Loveland CO, USA). The water flow rate through the respirometer chamber was determined by collecting the outflow from each tube into a beaker over different time intervals (in minutes) as previously described by Wang et al. (2012b). The water flow rate was adjusted to ensure that 95 % of the chamber water was replaced within 1 minute (Steffensen, 1989) and to ensure a >7 mg L⁻¹ oxygen concentration in the outlet water, to avoid physiological stress as described by Zhang et al. (2014). The following formula was used to calculate the individual oxygen consumption rate $MO_2$ (mg O₂ h⁻¹):

$$MO_2 = \Delta DO_2 \times v \quad (1)$$
where $\Delta DO_2$ is the difference in oxygen concentration (mg O$_2$ L$^{-1}$) between an experimental chamber and the control chamber and $v$ is the water flow rate in the experimental chamber (L h$^{-1}$).

At the end of acclimation, the fish were fasted for 24 h and their body masses were weighed to nearest 0.1 g. The body mass ranges for *H. molitrix*, *A. nobilis*, *C. carpio*, and *C. guichenoti* were 8.4-366 g, 22.6-282.3 g, 14.5-471.7 g, and 3.6-330.3 g, respectively. Each fish was transferred individually to the respirometer chamber and allowed to adapt for one day prior to oxygen consumption measurement under the same conditions as the acclimation period. Then, oxygen consumption rate was measured at every hour for 4 hours, and the mean value of the last three measurements was used as the *RMR* for that individual (Huang et al., 2013; Zhang et al., 2014). After measuring *RMR*, the fish were transferred individually to a chasing tank and were chased for 5 min to exhaustion as previously described (Huang et al., 2013; Zhang et al., 2014). After chasing, the fish were immediately returned to the respirometer chambers and the oxygen consumption rates were measured at 1-min intervals for the first 10 post-exercise min, and then at 15, 20, 30, 40, 60, 80, 100, 120, 140, and 160 min, until the oxygen consumption rate returned to within 120% of the *RMR*. The maximal value of oxygen consumption rate during this period was used as *MMR*. The following metabolic parameters were also calculated: *FAS*, calculated as the ratio of the *MMR* to the *RMR*.

When the *MR* recovered from the chasing exercise, the fish were anesthetised for blood sampling by 0.15 g L$^{-1}$ tricaine methanesulfonate (MS-222). Blood was taken from the caudal vessels with an anticoagulated (sodium fluoride: potassium oxalate = 1:3 g g$^{-1}$) syringe and transferred to an anticoagulated centrifuge tube on ice. The length (*LC*) and width (*WC*) of 50 randomly selected RBCs were measured for each fish using a digital light microscope with a video camera linked to computer image analysis software (EV5680B, Aigo Digital Technology Co., Ltd, Beijing, China). The *S* of RBC was estimated using the equation $S = LC \times WC \times \pi / 4$ (Zhu et al., 2012). The mean values of *LC*, *WC*, and *S* for the 50 RBCs were determined for each fish. Because some individuals were too small for blood sampling, the final numbers of blood samples were 87, 72, 79, and 55 for *H. molitrix*, *A. nobilis*, *C. carpio*, and *C. guichenoti*, respectively.

**Data analysis**

The data were calculated by Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA) and were transformed to base-10 logarithms prior to statistical analysis. The statistical tests were completed by using STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, USA). Pearson’s
correlation and ordinary least square regression were used to analyse the relationship between $M$ and each of the other parameters of each species, and ANCOVA were used to compare the slopes of the regressions using $M$ as a covariate. T-tests were used to compare the slopes of MRs with 1 or 0.75. Pearson’s product moment correlation analyses were also used to test the mass-independent correlations between residual values of $S$, $RMR$ ($rRMR$), and $MMR$ ($rMMR$) of each species. To analyse the relationship between $S$ and $b_R$, our previous results for two closely related species (Huang et al., 2013; Zhang et al., 2014) were included. The interspecific correlations between the values of mass-specific $RMR$ at the midpoint of regression and $b_R$ or $S$ were analysed by Pearson’s correlation. Reduced Major Axis (RMA) regression was used for the relationship between $S$ and $b_R$ by using RMA software version 1.21 (Bohonak and van der Linde, 2004). To correct for the influence of phylogenetic relatedness, phylogenetically independent contrasts (PIC) of $S$ and $b_R$ of each species were calculated as described in Garland et al. (2005), with phylogenetic relatedness and branch lengths (see Fig. S3 in supplementary material) assigned from the results by Wang et al. (2012a). Then, the PICs were used for Pearson’s correlation. The 95% confidence interval (CI) was used for the slopes of $RMR$ and $MMR$. P-values less than 0.05 were considered statistically significant. Data are presented as means ± s.e.m.

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Competing interests
The authors declare no competing financial interests.

Author contributions
YL designed the study, YL, GL, YZ, DH, and QH performed the research, YL, DH, and HX analysed the data, YL wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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References


Figures

**Figure 1.** Metabolic rate (mg O$_2$ h$^{-1}$) of individual fish versus body mass ($M$; g) in each of the four species of cyprinids. The filled circles represent the maximum metabolic rate (MMR; mg O$_2$ h$^{-1}$) and the open circles represent the resting metabolic rate (RMR; mg O$_2$ h$^{-1}$). A: *Hypophthalmichthys molitrix*; B: *Aristichthys nobilis*; C: *Cyprinus carpio*; D: *Coreius guichenoti.*
Figure 2. Correlations between mass-specific resting metabolic rate (RMR; mg O$_2$ kg$^{-1}$ h$^{-1}$) and scaling exponent of RMR ($b_R$) (A), and red blood cell areas ($S$; $\mu$m$^2$) and mass-specific RMR (mg O$_2$ kg$^{-1}$ h$^{-1}$) (B) among six species of fish. Data are presented as mean values with 95% confidence interval (CI). Filled circles: *Carassius auratus* by Huang et al. (2013); open circles: *Aristichthys nobilis*; filled triangles: *Ctenopharyngodon idellus* by Zhang et al. (2014); open triangles: *Hypophthalmichthys molitrix*; open diamonds: *Cyprinus carpio*; open squares: *Coreius guichenoti*. 
Figure 3. Relationships between factorial aerobic scope (FAS) and body mass (M; g) of each species. A: *Hypophthalmichthys molitrix*; B: *Aristichthys nobilis*; C: *Cyprinus carpio*; D: *Coreius guichenoti*.
Figure 4. Relationships between red blood cell areas ($S; \mu m^2$) and body mass ($M; g$) in each species. A: *Hypophthalmichthys molitrix*; B: *Aristichthys nobilis*; C: *Cyprinus carpio*; D: *Coreius guichenoti*.
Figure 5. Correlations between the residual red blood cell areas ($S; \mu m^2$) and the residual resting metabolic rate ($RMR; mg O_2 h^{-1}$) of each species. A: Hypophthalmichthys molitrix; B: Aristichthys nobilis; C: Cyprinus carpio; D: Coreius guichenoti.
Figure 6. Correlations between red blood cell areas ($S; \mu m^2$) and scaling exponents ($b_R$) of resting metabolic rates among the six fish species. A: correlation of the raw data among species, and the 95% confidence interval (CI) are given for each data point; B: correlation of the phylogenetically independent contrasts (PIC) of $S$ and $b_R$ of each species using the methods described in Garland, Bennett and Rezende (2005), with phylogenetical relatedness and branch lengths (Figure S1) assigned from the results by Wang et al. (2012a).
Figure 7. Illustration of hypothetical relationships among metabolic level, the intraspecific scaling exponent, $b$, and cell size. Circles: Different circle sizes represent difference in cell sizes among species. Black solid line: It shows that $b$ is negatively correlated with metabolic level according to the original mechanism of the MLB hypothesis (Glazier 2005; Kilien et al. 2010), and in this case the variation of $b$ is fully attributed to changes in the metabolic level, when cell size is invariant. Red solid line: It shows that the variation of $b$ is fully attributed to changes in cell size: species with smaller cell sizes have less surface area ($S$) /volume ($V$) limits, and thus larger $b$, when metabolic level is invariant, as observed in the present study. Blue solid line: It shows that cell size is negatively correlated with metabolic level, which in turn is negatively correlated with $b$, as predicted by the MLB hypothesis. Thus, $b$ is positively correlated with cell size as reported by Glazier (2014b). Black dashed line: It shows a potential extreme case, in which species with different metabolic level may have similar $b$ because $S/V$ is not limiting for any metabolic level. However, the possibility of this case is currently unknown.