

RESEARCH ARTICLE

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

Yiping Luo*, Dingcong He, Ge Li, Hang Xie, Yurong Zhang and Qingda Huang

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 differences 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 does not exclude another mechanism based on the recent contextual multimodal theory.

KEY WORDS: Body mass, Metabolic rate, Erythrocyte

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 a 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 interspecifically 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 et al., 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 determines the relative importance of the constraints (Glazier, 2005, 2008, 2009, 2010). According to the MLB hypothesis, a species with a higher RMR has a lower intraspecific b -value (Glazier, 2005, 2010). In contrast, 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*) from 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 MR 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 M is attributed entirely to changes in cell size, or 1.0 if cell size remains unchanged and variation in M 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 MR scaling is more

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Received 2 December 2014; Accepted 9 March 2015

List of symbols and abbreviations

b_R	intraspecific scaling exponent of resting metabolic rate
CM	cell metabolism
CMT	contextual multimodal theory
FAS	factorial aerobic scope
M	body mass
MLB	metabolic-level boundaries
MR	metabolic rate
MTE	metabolic theory of ecology
RBC	red blood cell
RMR	resting metabolic rate

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 to cell size in grass carp (Zhang et al., 2014) and among several species of eyelid geckos (Starostová et al., 2009), when correcting for M . 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 hypothesise 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

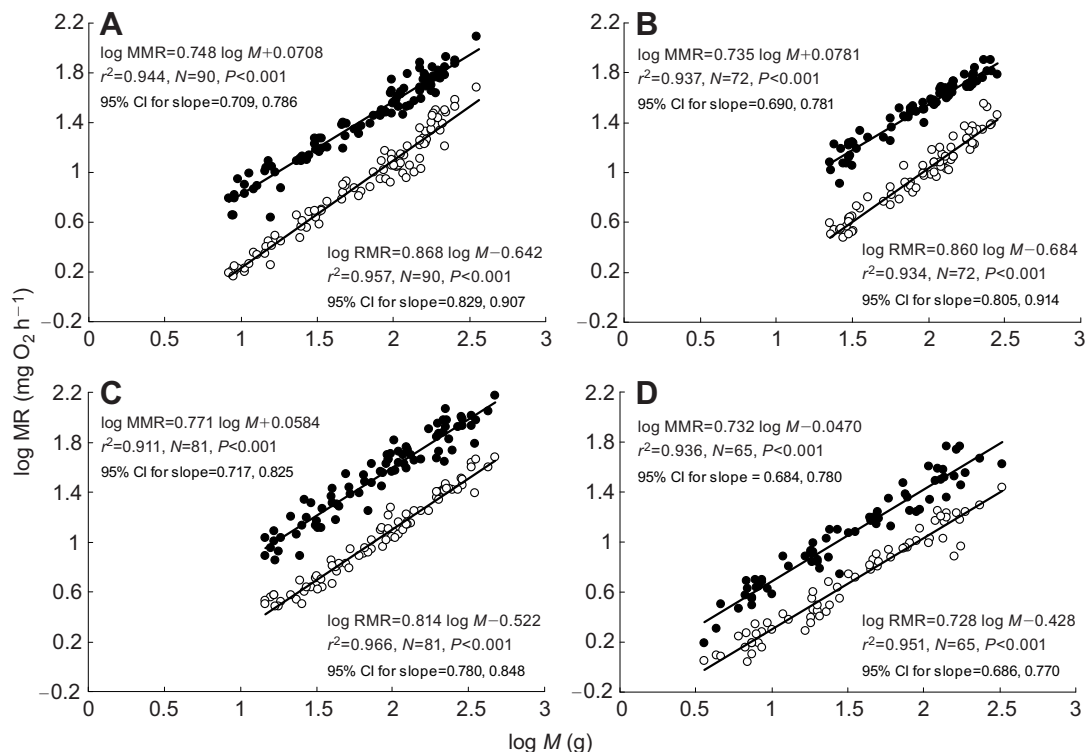


Fig. 1. Metabolic rate (MR) of individual fish versus body mass (M) in each of the four species of cyprinids. The filled circles represent the maximum metabolic rate (MMR) and the open circles represent the resting metabolic rate (RMR). (A) *Hypophthalmichthys molitrix*; (B) *Aristichthys nobilis*; (C) *Cyprinus carpio*; (D) *Coreius guichenoti*. CI, confidence interval.

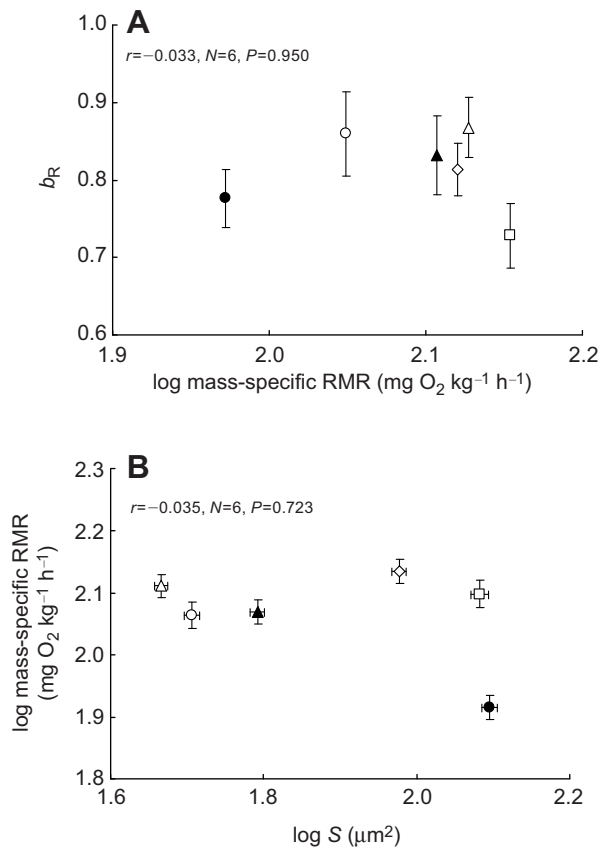


Fig. 2. Correlation between mass-specific RMR and the scaling exponent of RMR (b_R) and red blood cell areas (S) among six species of fish. (A) b_R versus RMR; (B) RMR versus S . Data are presented as mean values with 95% CI. Filled circles: *Carassius auratus* (data from Huang et al., 2013); open circles: *Aristichthys nobilis*; filled triangles: *Ctenopharyngodon idellus* (data from Zhang et al., 2014); open triangles: *Hypophthalmichthys molitrix*; open diamonds: *Cyprinus carpio*; open squares: *Coreius guichenoti*.

Hypophthalmichthys molitrix (Valenciennes 1844), bighead carp *Aristichthys nobilis* (J. Richardson 1845), common carp *Cyprinus carpio* Linnaeus 1758, and largemouth bronze gudgeon *Coreius guichenoti* (Sauvage and Dabry de Thiersant 1874)] 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 the 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 MR; (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 of each species increased to peak values after exhaustive exercise and then recovered to pre-exercise values (see supplementary material Fig. S1). 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 from 2.05 to 2.15 $\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$. Mass-specific RMR at the midpoint of the regression was not significantly correlated with 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 the 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 for *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 MMR when controlling for M (see supplementary material Fig. S2). 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 previously reported values for other teleosts (Killen et al., 2007, 2010; White et al., 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 this value may just be an empirical average value (Glazier, 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 four species increased with M in the present study. Our results show that predictions based on the MTE hypothesis are not applicable in the context of intraspecific metabolic scaling.

Our results support the CM hypothesis that the allometric scaling of RMR with M may be partly attributed to the increase in cell size. However, the slopes (range 0.0164–0.0407) of the relationship between S and M suggest that cell size has only minor effects on intraspecific metabolic scaling. The CM hypothesis has been supported by reports that MR is negatively correlated with RBC size in 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), the grass carp (Zhang et al., 2014) and all species in our study when controlled for M . For *H. molitrix* and *C. carpio*, in contrast, the RMR 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 MR may not be general across species with very similar MRs. Significant correlations between cell size and MR may exist in more diverse species with a broader range of MRs, as shown by Glazier (2014b). RBC size increased with M 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 M in some other species (Huang et al., 2013; Starostová et al., 2013). Among the species in the present study, the species with

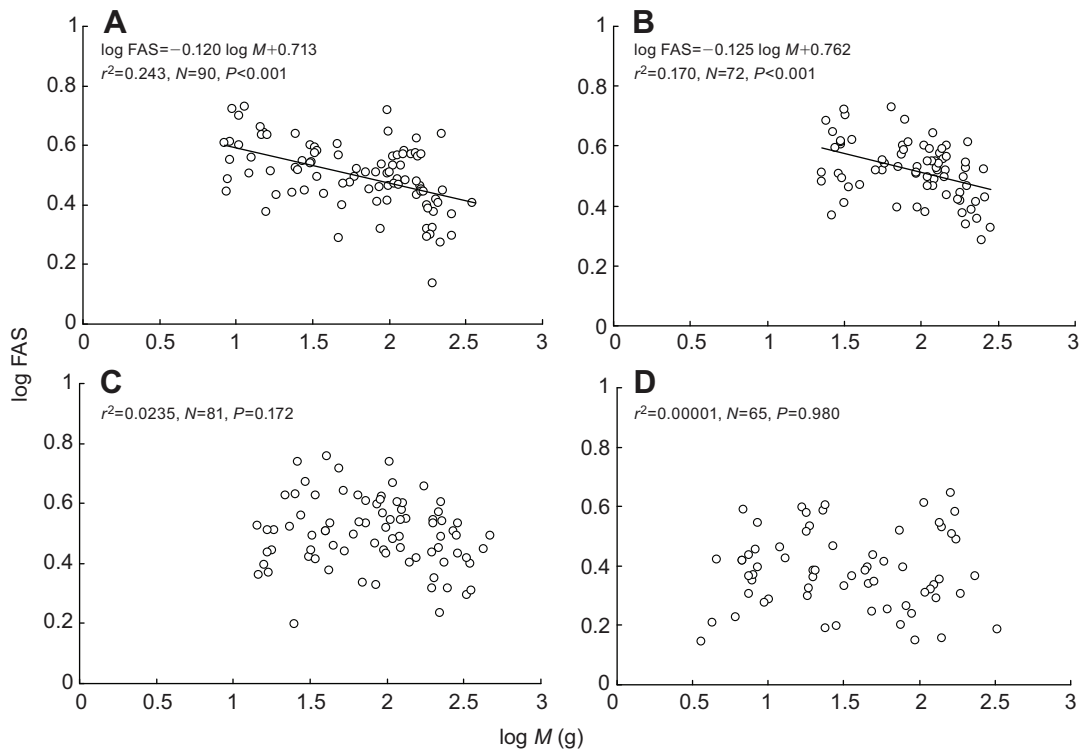


Fig. 3. Relationship between factorial aerobic scope (FAS) and M of each species. (A) *Hypophthalmichthys molitrix*; (B) *Aristichthys nobilis*; (C) *Cyprinus carpio*; (D) *Coreius guichenoti*.

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 M (Porter and Brand, 1995; Savage et al., 2007; Burpee et al., 2010).

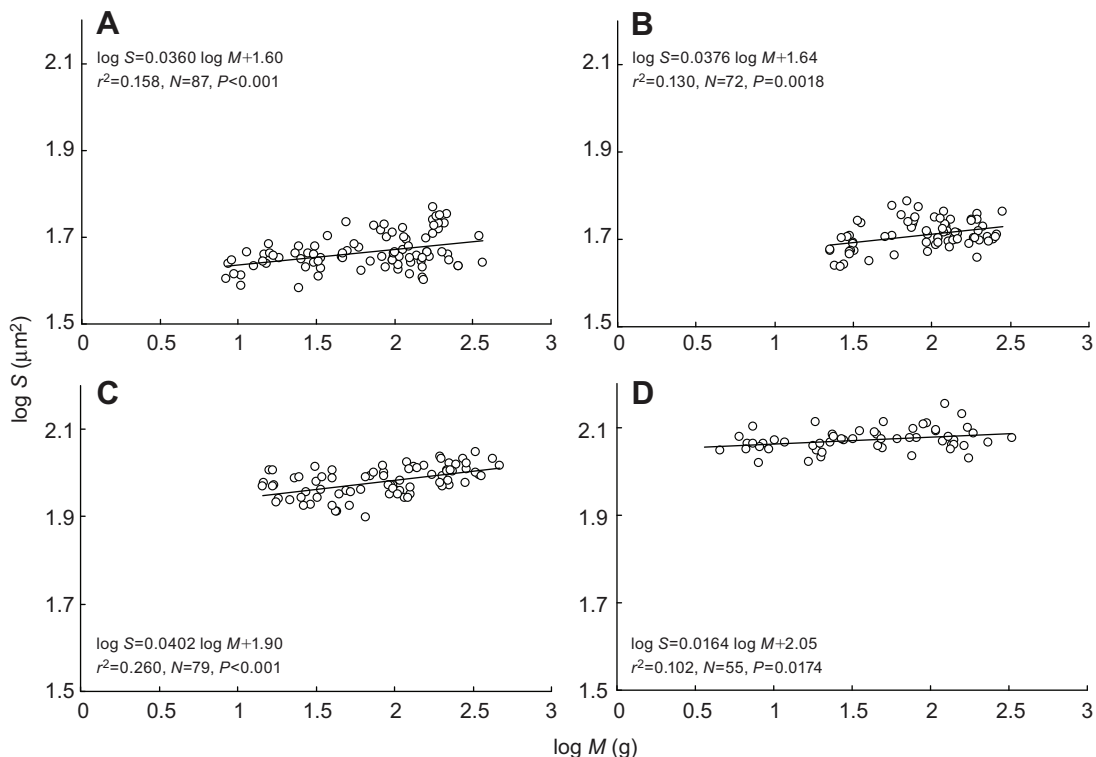


Fig. 4. Relationship between S and M in each species. (A) *Hypophthalmichthys molitrix*; (B) *Aristichthys nobilis*; (C) *Cyprinus carpio*; (D) *Coreius guichenoti*.

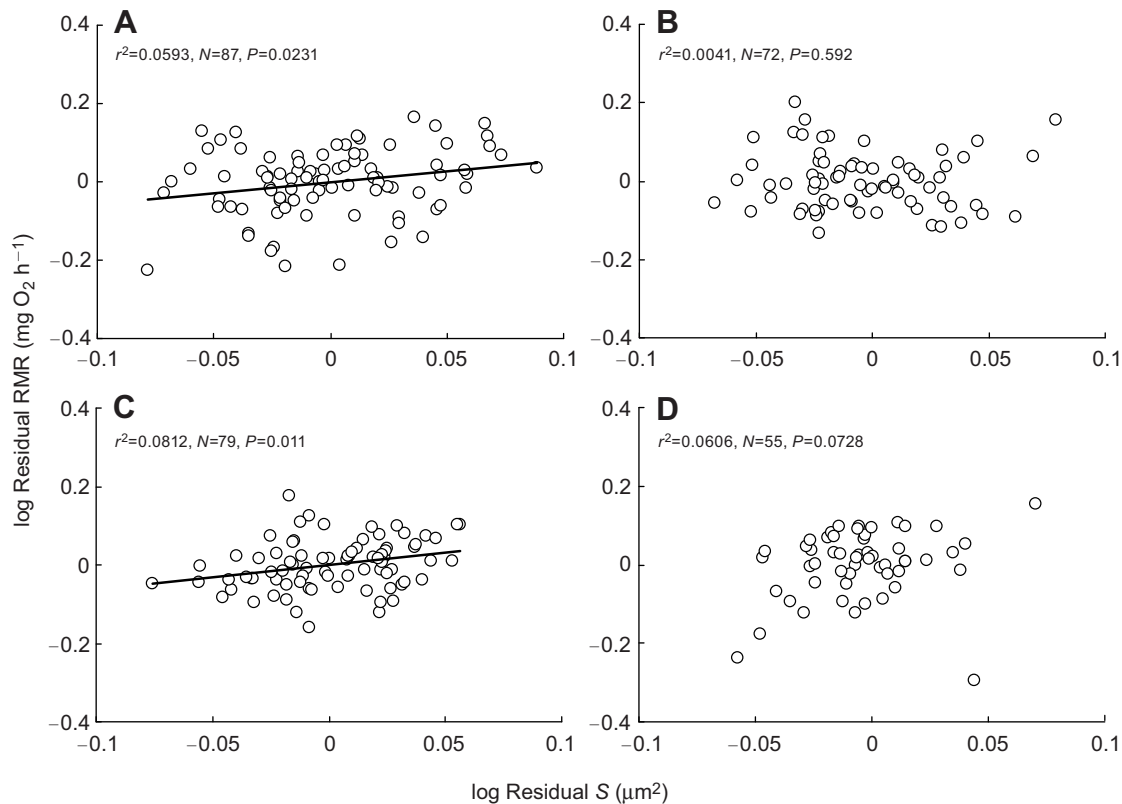


Fig. 5. Correlation between the residual S and the residual RMR of each species. (A) *Hypophthalmichthys molitrix*; (B) *Aristichthys nobilis*; (C) *Cyprinus carpio*; (D) *Coreius guichenoti*.

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 level. 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 lower 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 size was negatively correlated with metabolic level, which in turn was negatively correlated with b (Glazier, 2014b). In our results, by contrast, b was negatively correlated with RBC size when metabolic level was invariant. Following the MLB hypothesis (Glazier, 2005, 2010), metabolic level, b , and cell size can be integrated into one model (Fig. 7), by which the findings of either Glazier (2014b) or the present study follow the predictions of the MLB hypothesis.

This suggests that the mechanisms 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 M 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 M (Glazier, 2005, 2009). The low b_M in the present study suggests that muscular energy expenditure has a limited contribution 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 a minor (~1%) part of M .

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 M increases. FAS of fish generally increases or remains unchanged as M increases, implying that aerobic capacity is important as the body grows (Brett, 1965; Beamish, 1978; Armstrong et al., 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 a gill-rake net on their gill arches. The size of the gill-rakes gradually increases as the body grows such that they cover the gill filament, which may reduce the respiratory gas exchange capacity (Jirásek et al., 1981; Hampl et al., 1983;

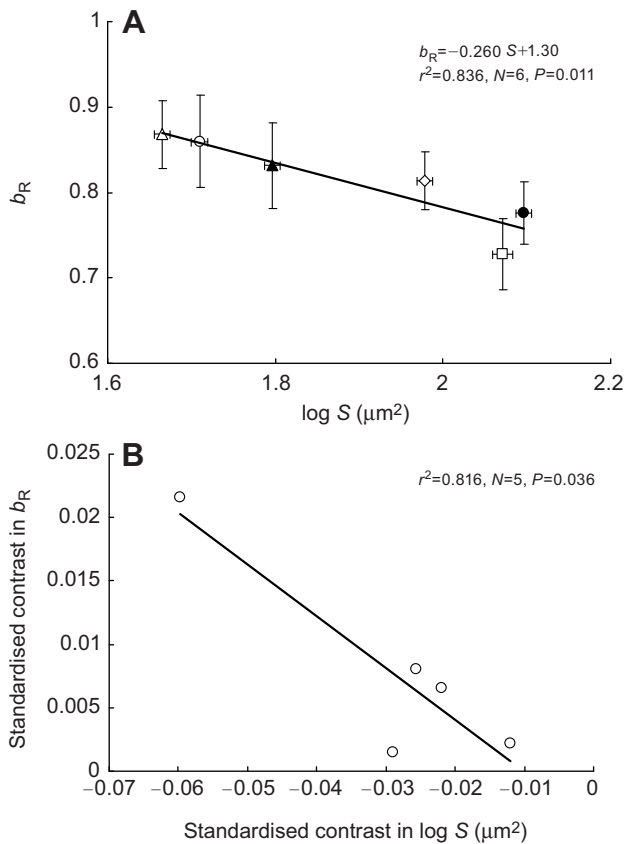


Fig. 6. Correlation between S and b_R of RMR among the six fish species.

(A) Correlation of the raw data among species and the 95% 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 et al. (2005), with phylogenetic relatedness and branch lengths (supplementary material Fig. S3) assigned from the results of Wang et al. (2012a).

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 a 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, such that our mechanism and that proposed by Glazier (2014b) are not mutually exclusive.

MATERIALS AND METHODS

Experimental animals, *H. molitrix*, *A. nobilis*, *C. carpio* and *C. guichenoti*, with different body sizes (range 3–460 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 2 weeks prior to study. During acclimation, the water temperature was maintained at $25 \pm 1^\circ\text{C}$, the oxygen concentration was maintained above 90% saturation, the ammonia concentration was kept below 0.015 mg l^{-1} and the photoperiod was 14 h:10 h light:dark, as in our previous studies (Huang et al., 2013; Zhang et al., 2014). Fish were fed with a commercial diet (1% of M) once at 18:00 h 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 accordance with the ethical requirements of the Animal Care Committee of the Fisheries Science Institution of Southwest University, China,

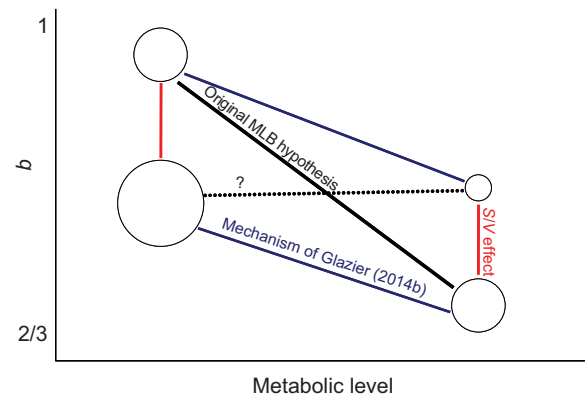


Fig. 7. Illustration of the hypothetical relationships among metabolic level, the intraspecific scaling exponent b and cell size. Different circle sizes represent differences in cell size among species. The black solid line shows that b is negatively correlated with metabolic level according to the original mechanism of the metabolic-level boundaries (MLB) hypothesis (Glazier, 2005; Killen 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. The red solid lines show 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. The blue solid lines show 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). The black dotted line shows a potential extreme case, in which species with a 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.

and requirements of environment and housing facilities for laboratory animals in China (Gb/T14925-2001).

The respirometer used for MR 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 the 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 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 min) as previously described (Wang et al., 2012b). The water flow rate was adjusted to ensure that 95% of the chamber water was replaced within 1 min (Steffensen, 1989) and to ensure a $>7 \text{ mg l}^{-1}$ 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 \dot{M}_{O_2} ($\text{mg O}_2 \text{ h}^{-1}$):

$$\dot{M}_{\text{O}_2} = \Delta[\text{O}_2] \times v, \quad (1)$$

where $\Delta[\text{O}_2]$ is the difference in oxygen concentration ($\text{mg O}_2 \text{ l}^{-1}$) 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 the acclimation period, the fish were fasted for 24 h and M was measured to the nearest 0.1 g. The ranges in M 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 1 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 h, 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 min post-exercise, 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 the oxygen consumption rate during this period was used as the MMR. FAS was also calculated as the ratio of the MMR to the RMR.

When the MR had recovered after the chasing exercise, the fish were anaesthetised for blood sampling with 0.15 g l⁻¹ tricaine methanesulphonate (MS-222). Blood was taken from the caudal vessels with a syringe treated with anticoagulant (1 g sodium fluoride:3 g potassium oxalate) and transferred to an anticoagulant-treated centrifuge tube on ice. The length (L_{RBC}) and width (W_{RBC}) 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 $S=L_{RBC}\times W_{RBC}\times\pi/4$ (Zhu et al., 2012). The mean values of L_{RBC} , W_{RBC} 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 MR 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 the 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 supplementary material Fig. S3) 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 \pm s.e.m.

Acknowledgements

We thank Mr Bo Zhang for his help in fish collection. We sincerely thank the anonymous reviewers for their suggestions and comments.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.L. designed the study; Y.L., G.L., Y.Z., D.H. and Q.H. performed the research; Y.L., D.H. and H.X. analysed the data; Y.L. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

Funding

This study was supported by the National Natural Science Foundation of China (31000958); the Natural Science Foundation Project of CQ (CSTC2013cyjA80023); and the Fundamental Research Funds for the Central Universities (XDJK2014C157).

Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.117739/-DC1>

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