Ontogenetic phase shifts in metabolism: links to development and anti-predator adaptation

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The allometric relationships between resting metabolism (VO_2) and body mass (M), $VO_2 = a_i M^b$, are considered a fundamental law of nature. A distinction though needs to be made between the ontogeny (within a species) and phylogeny (among species) of metabolism. However, the nature and significance of the intraspecific allometry (ontogeny of metabolism) have not been established in fishes. In this study, we present experimental evidence that a puffer fish ranging 0.0008–3 g in wet body mass has four distinct allometric phases in which three stepwise increases in scaling constants (a_i , $i = 1-4$), i.e. ontogenetic phase shifts in metabolism, occur with growth during its early life stages at around 0.002, 0.01 and 0.1 g, keeping each scaling exponent constant in each phase ($b = 0.795$). Three stepwise increases in a_i accompanied behavioural and morphological changes and three peaks of severe cannibalism, in which the majority of predation occurred on smaller fish that had a lower value of a_i . Though fishes are generally highly fecund, producing a large number of small eggs, their survivability is very low. These results suggest that individuals with the ability to rapidly grow and step up ' a_i ' develop more anti-predator adaptation as a result of the decreased predatory risk.

Keywords: allometry; cannibalism; growth; scaling; metabolism; mortality

1. INTRODUCTION

The relationship between resting metabolic rate (VO_2) and body mass (M) in animals is expressed by the allometric formula

$$VO_2 = aM^b, \quad (1.1)$$

where ' a ' is a scaling constant and ' b ' is the scaling exponent. The VO_2 of animals generally scales negatively allometrically to body mass, because the scaling exponent (b) is smaller than unity. This phenomenon is well known both interspecifically (among species) and intraspecifically (within a species) (Kleiber 1932; Brody 1945; Zeuthen 1953; Winberg 1956; Hemmingsen 1960; Peters 1983; Schmidt-Nielsen 1984; Glazier 2005, 2006; White *et al.* 2006; Moses *et al.* 2008). In mammals, the scaling exponent is reported to be steeper interspecifically than intraspecifically (Heusner 1982; Feldman & McMahon 1983; Makarieva *et al.* 2009).

The ontogeny of metabolism (intraspecific changes in VO_2 with growth) is a complex process in which several phases can be distinguished throughout the whole life cycle (Wieser 1984). Metabolic scaling is expected to

affect optimal resource allocation to growth and maintenance, so it should substantially influence the life history of organisms (Kozłowski & Teriokhin 1999; Czarnołęski *et al.* 2003; Hou *et al.* 2008). It is important to identify the factors diversifying metabolic processes, not only to answer how metabolic rate changes with size and growth stage, but also to achieve a fuller understanding of its ecological implications in nature. However, because of the small size and fragility of teleost larvae, the significance of ontogenetic effects on metabolism has not been established.

In early ontogenetic stages, it has been often reported that VO_2 scales intraspecifically isometrically to body mass, i.e. b is equal or near to 1, in aquatic invertebrates for larval and young stages (Zeuthen 1953; Glazier 2005, 2006), in fishes for larval and juvenile stages (Giguère *et al.* 1988; Oikawa *et al.* 1991; Post & Lee 1996; Finn *et al.* 2002; Moran & Wells 2007), in a terrestrial snail for early stage (Czarnołęski *et al.* 2008), and in a bird for chick stages (Seymour *et al.* 2008). Isometric metabolic scaling of ontogeny, interposed between two negative allometries (middle segment of the tri-phasic allometries), has been reported in marine invertebrates such as *Mytilus* and *Asterias* over 1000-fold difference in body mass (Zeuthen 1953). Isometric metabolic scaling has also been reported, both inter- and intraspecifically, in terrestrial plants for seedling and sapling (Reich *et al.* 2006).

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Although such an isometric scaling should follow if the metabolic rate is simply dictated by the energy demand of maintaining body tissue with growth, assuming that this cost is a constant per unit mass (Glazier 2005), the reason for such an isometric hypothesis was not immediately obvious and challenged research workers to come up with an explanation.

However, to date, there has not been a thorough evaluation of allometric and isometric scaling in teleosts. This study was undertaken to establish the relationship between metabolic rate and body mass during early life of the tiger puffer, *Takifugu rubripes* (Temminck and Schlegel). In the tiger puffer, similar phenomena reported in mammals (Heusner 1982; Feldman & McMahon 1983) are expected to occur.

The tiger puffer spawn demersal adhesive eggs, and hatch at the mouth of bays such as Hakata Bay and Ariake Sound, Kyushu Island, Japan. Hatched larvae flow and grow in the Bay and the Sound. Juveniles grown at approximately 0.1 g move to estuary and mudflats in the Bay and the Sound, and grow there until approximately 10 g. After that, they move offshore in the Bay and the Sound or around the bay mouth (Hidaka *et al.* 1988; Takita & Intong 1991). Tiger puffer larvae and juveniles display a high degree of cannibalism during their early life history under rearing conditions (Suzuki *et al.* 1995), although we have little information about cannibalism under natural conditions. They are three times larger in body mass at hatching than fishes spawning isolated epipelagic eggs such as a sea bream *Pagrus major* and a tuna *Thunnus albacares*, which are less fragile, and easily grow under culture. Therefore, the tiger puffer can be a model fish for quantitative studies on respirometry and cannibalism under culture conditions to provide a better understanding of ecological implication on them under nature.

In this paper, we show in the tiger puffer, oxygen consumption (VO_2) varies or scales with body mass (M) as $\text{VO}_2 = a_i M^{0.795}$, and that three stepwise increases in scaling constants ' a_i ', i.e. ontogenetic phase shifts in metabolism, occur with growth at three determined developmental stages, accompanied by three peaks of severe cannibalism. Our results of predator–prey interaction owing to cannibalism suggest that individuals with earlier stepped-up a_i are in a position to eat those of lower a_i to develop more anti-predator adaptation.

2. MATERIAL AND METHODS

(a) Fish used

Tiger puffer larvae were hatched from artificially inseminated eggs obtained from wild parents that were captured by fishermen. The larvae and juveniles were fed a sufficient supply of live rotifers, *Brachionus rotundiformis*, daily two times between 2 and 24 days after hatching, live brine shrimp, *Artemia* sp., larvae daily two times between 14 and 37 days after hatching and artificial diets daily five times thereafter. Live diets were fortified with essential fatty acids, EPA and DHA, before feeding. The water temperature ranged between 19 and 22°C, and the salinity 32–33 ppt during rearing. Fish used in the respirometry study were not fed for 3–24 h before experiments, depending on the developmental stage, and were held at 20°C.

Wet body mass during the larval stage was determined as follows: the weight of a histological cover glass was first measured, followed by placing several larvae (sufficient to amount to a few milligrams) on the cover glass with an excess of sea water. The sea water was carefully blotted using a small piece of filter paper under a binocular microscope. The weight of the cover glass (including the larvae) was measured, and the initial weight of the cover glass was subtracted.

Prior to the determination of body mass in the respirometry study or fixation in the morphological study, tiger puffer larvae and juveniles were chilled to 2–4°C in sea water, and immobilized.

(b) Respirometry

We measured oxygen consumption, as a proxy for metabolism, in larval and juvenile puffer fish ranging in size between 0.00068 (wet body mass, just after hatch) and 3.0 g (57 days old). Resting routine rates of oxygen consumption (i.e. intermediate between the resting and routine activity states; Cech 1990) were determined in fasted larvae and juveniles at 20°C using one of four methods depending on the developmental stage of the fish—a closed method (electronic supplementary material, figure S1), a semi-closed method equipped with an oxygen electrode (electronic supplementary material, figure S2), a semi-closed method without an oxygen electrode and a continuous flow method (electronic supplementary material, figure S3), based on our previous study (Oikawa *et al.* 1991). The closed method was based on depletion of oxygen in water in a sealed oxygen bottle, and the continuous flow method was based on loss of oxygen and the rate of water flow through a respiration chamber. The semi-closed method was essentially a closed method in which the chamber was very slowly flushed with air-saturated water before determination and closed during determination. Detailed data of the four types of respirometry employed in this study are summarized in electronic supplementary material, table S1.

The larvae were carefully selected for the measurement of oxygen consumption according to our previous study (Oikawa *et al.* 1991). The selected larvae were placed in the respiration chamber 1–2 h later after selection. Fish placed in the respiration chamber were allowed to settle for a certain period before measurement to remove stress caused by handling (electronic supplementary material, table S1). In the closed method, larvae just after hatching to 6 days were settled in standing water instead of running water because of their poor swimming ability (electronic supplementary material, figure S1). The initial concentration of oxygen in water was therefore lower than in the later stages (electronic supplementary material, table S1).

A blank chamber without fish was used to eliminate background respiration. In the two types of semi-closed method, the bottle that received water flowing out of the respiration chamber was used as the blank chamber (BC in electronic supplementary material, figure S2). This bottle was sealed at the beginning of determination of oxygen consumption by fish, placed in the water-bath for the respiration chamber during determination, and the oxygen concentration in the bottle was determined at the end of the measurement. By using this value as the initial oxygen concentration in the respiration chamber, background respiration was cancelled.

Oxygen consumption by the oxygen electrode was imperceptible. In the continuous flow method, a respiration chamber of the same size was used without fish as a blank chamber (electronic supplementary material, figure S3). Oxygen concentration in water flowing out of the blank chamber was used as the value in water flowing into the respiration chamber.

Wet body mass of experimental fish was directly determined immediately after respirometry, excepting the closed method. In the closed method, body mass was indirectly estimated from other individuals similar in body size to them, because they were fixed when dissolved oxygen concentration of water in the respiration chamber was determined by Winkler's titration method.

(c) Morphological study

Fish were fixed in 2.5 per cent glutaraldehyde fixative (Oikawa *et al.* 1999), for 24 h in a refrigerator, and preserved in 70 per cent ethanol. Following fixation, the fish were stained with alizarin red-S using the clearing and staining technique (Sire *et al.* 1997), and observed under a binocular microscope.

(d) Daily mortality owing to cannibalism

Tiger puffer larvae and juveniles display a high degree of cannibalism during their early life history (Suzuki *et al.* 1995). They have a relatively small gape size. Therefore, they seldom swallow a whole body, instead biting small portions of the body surface during an attack on conspecifics. A quantitative evaluation of daily mortality owing to cannibalism can be made by collecting the bodies of cannibalized fish from the bottom of the rearing tank, though some fish were missed as mentioned below.

We measured daily mortality owing to cannibalism in larval and juvenile puffer fish between 8 and 45 days after hatching. Experiments were conducted in 2001 and 2005. We used two 1000 l tanks stocked with 3000 juveniles (27 days old) in 2001, and two 100 l tanks stocked with 1500 larvae (7 days old) in 2005. In 2005, the fish were moved from 100 l tanks to 200 l tanks 23 days after hatching, and to 500 l tanks 29 days after hatching. They were fed sufficient food, as mentioned above, to avoid starvation. Daily mortality was calculated as the number of dead individuals collected at 9.00 and 18.00 h each day. The dead individuals were preserved in 5 per cent neutral formalin fixative for determination of body mass. Some survivors were occasionally sampled randomly from the tanks, and preserved in formalin fixative to estimate their body masses. At the end of the cannibalism experiment, the number of survivors was counted. The recovered number of individuals at the end of the experiment (45 days old) was 5661 (initial number: 6000) in the experiment 2001 and 2746 (initial number: 3000) in the experiment 2005. Therefore, there were some missed fish. Daily mortality was calculated using initial number of individuals, because we were not able to know when they were missed.

Observation of cannibalistic behaviour was visually carried out on 8–45-day-old larvae and juveniles. Some fish with predator–prey interactions were caught, introduced in a beaker to record cannibalistic behaviour with a video camera and fixed in glutaraldehyde fixative to determine their body mass. Two 500 l tanks, which accommodated about 5000 larvae just after hatching, were prepared for the study.

Body mass was indirectly estimated in dead specimens that had been cannibalized and preserved in formalin fixative or glutaraldehyde fixative. We estimated body mass based on total length (L'_t , mm), for fish with a complete caudal fin, or from the body length (L'_b , mm), for fish with an incomplete caudal fin, or from the eye diameter (D_e , mm), for fish in which neither the total length nor the body length was available. Body length was estimated using $L'_b = 6.2D_e^{1.096}$. Total length (L_t) or body length (L_b) prior to fixation was estimated using $L_t = 1.053L'_t$ or $L_b = 1.053L'_b$ for specimens fixed in formalin fixative, and $L_t = 1.029L'_t$ or $L_b = 1.029L'_b$ for specimens fixed in glutaraldehyde fixative. The constant values, 1.053 and 1.029, were derived from coefficients to correct for shrinkage with fixation (Oikawa *et al.* 1999). Wet body mass M (mg) was estimated using $M = cL_t^d$ (electronic supplementary material, figure S4) or $M = cL_b^d$ (electronic supplementary material, figure S5), where 'c' and 'd' are constants. Constants c and d are given in electronic supplementary material, tables S2 and S3.

3. RESULTS

There was no substantial difference among the results of the four different methods of respirometry (figure 1 and electronic supplementary material, figure S6). Therefore, the values obtained were used without any distinction for the relationship between oxygen consumption and body mass.

Rates of oxygen consumption (VO_2 in $\mu\text{l O}_2 \text{ fish}^{-1} \text{ min}^{-1}$) in relation to body mass (M in g) are plotted in figure 1. Mass-specific rates of oxygen consumption (VO_2/M in $\mu\text{l O}_2 \text{ g}^{-1} \text{ min}^{-1}$) are also presented. VO_2 increased daily from just after hatching to 3 days after hatching, with virtually no increase in body mass. Following this, body mass increased. We fitted four negative allometric relationships between VO_2 and M between 0.0008 (4 days old) and 3 g (57 days old), interposing three transitional phases of VO_2 at approximately 0.002, 0.01 and 0.1 g of body mass (figure 1).

We applied two models to compare the four negative allometric relationships, excepting the transitional phases:

$$VO_2 = a_i M^b \quad (3.1)$$

for each incidence of negative allometry, and

$$VO_2 = \alpha M^{\bar{b}} \quad (3.2)$$

for the overall line constituting these four negative allometries (Brownlee 1965; Feldman & McMahon 1983). a_i represents an intragroup scaling constant of the i 'th group, and α the intergroup one of the four groups.

Equations (3.1) and (3.2) were rewritten as

$$y_{ij} = \log a_i + \bar{b}x_{ij} + \varepsilon_{ij} \quad (3.3)$$

and

$$y_{ij} = \log \alpha + \bar{b}x_{ij} + E_{ij}, \quad (3.4)$$

where y_{ij} is $\log VO_2$, x_{ij} is $\log M$ and ε_{ij} and E_{ij} represent the random intra- and intergroup variation in metabolism (electronic supplementary material, figure S12). To estimate $\bar{b}$ and $\log \alpha$, we used the ordinary least-squares regression to minimize the sum of squares of μ_i , which is the vertical distance of the group mean ($\bar{x}_i, \bar{y}_i$) from

Figure 1. Ontogenetic changes in the relationship between the rate of respiration (VO_2 , diamonds) and body mass (M) in the tiger puffer. The relationship between the mass-specific rate of respiration (VO_2/M , circles) and body mass is also given. The coloured symbols on VO_2/M signify the four methods of respirometry (blue, closed method; white, semi-closed method equipped with an oxygen electrode; green, semi-closed method without an oxygen electrode; yellow, continuous flow method). Points in the closed method are the mean values of five measurements with different groups with similar body mass of the same age. Points in the semi-closed method equipped with an oxygen electrode and in the continuous flow method are the mean values of two to four and three repeated measurements of the same individuals, respectively. Points in the semi-closed method without an oxygen electrode are usually the values for a single measurement. The coefficients of variation (s.d./mean) were generally less than 0.20, and the standard deviations were too small to be shown in the figure. The coloured symbols on VO_2/M signify the year in which data were collected (red, 1997; blue, 2001; yellow, 2005; green, 2006; white, 2007). Results for each year are presented in electronic supplementary material, figures S7–S11. The vertical blue lines at around 0.0007 g represent VO_2 and VO_2/M , which increased daily from just after hatching to 3 days after hatching, with virtually no increase in body mass. Ranges covered by the four solid black lines each on VO_2 and VO_2/M indicate intragroup phases of negative allometry. Small symbols (ranges not covered by the solid lines) represent values during the transitional phases. The broken lines both on VO_2 and VO_2/M represent the intergroup lines excluding the transitional phases. Regression analysis of each line on VO_2 is presented in table 1, and ANCOVA in table 2. I: $\text{VO}_2 = 2.75M^{0.795}$; II: $\text{VO}_2 = 3.29M^{0.795}$; III: $\text{VO}_2 = 4.68M^{0.795}$; IV: $\text{VO}_2 = 7.25M^{0.795}$. I': $\text{VO}_2/M = 2.75M^{-0.205}$; II': $\text{VO}_2/M = 3.29M^{-0.205}$; III': $\text{VO}_2/M = 4.68M^{-0.205}$; IV': $\text{VO}_2/M = 7.25M^{-0.205}$.

the overall line (intergroup line) (electronic supplementary material, figure S12) (Brownlee 1965).

Regression analysis of each group is given in rows 1–4 of table 1. The scaling exponent was significantly smaller than unity in any regression line ($p < 0.05$). The slopes of the individual lines ($\hat{b}$) in each of the four groups were not significantly different ($F_{3,111} = 2.42$, $p = 0.07$; one-way analysis of covariance (ANCOVA)). The intragroup scaling exponent was estimated to be $\hat{b} = 0.795 \pm 0.019$ (estimate $\pm$ s.e.m.), and logarithm of the scaling

constants in each group at $\hat{b} = 0.795$ was estimated as follows: $\log a_1 = 0.440 \pm 0.058$, $\log a_2 = 0.517 \pm 0.049$, $\log a_3 = 0.671 \pm 0.033$ and $\log a_4 = 0.861 \pm 0.010$. The scaling constants were calculated as follows: $a_1 = 2.75$, $a_2 = 3.29$, $a_3 = 4.68$ and $a_4 = 7.25$. Regression analysis of intergroup is given in rows 5–7 of table 1. The intergroup scaling exponent was estimated to be $\bar{b} = 0.948 \pm 0.002$.

Because $\log a_i$ is geometrically equal to $(\log \alpha + \mu_i + (\bar{b} - \hat{b})\bar{x}_i)$ (electronic supplementary material, figure S12),

Table 1. Intragroup (rows 1–4) and intergroup (rows 5–7) regression analysis of the relationship between $\log \text{VO}_2$ (VO_2 , oxygen consumption in $\mu\text{l O}_2 \text{ fish}^{-1} \text{ min}^{-1}$) and $\log M$ (M , body mass in g) in the tiger puffer. N , number of determinations; p , the difference of the scaling exponent from unity, examined by Student's t -test, two-tailed. R^2 , squared correlation coefficient between $\log M$ and $\log \text{VO}_2$.

group	N	range of body mass (g)	scaling constant	scaling exponent (mean $\pm$ s.e.m.)	p	R^2
1 ^a	26	0.00076–0.0017	0.80	0.610 ± 0.061	1.36×10^{-6}	0.806
2 ^a	29	0.0020–0.0058	4.88	0.864 ± 0.062	3.53×10^{-2}	0.879
3 ^a	38	0.010–0.055	4.88	0.805 ± 0.029	6.01×10^{-8}	0.957
4 ^a	26	0.20–3.0	7.26	0.798 ± 0.035	6.64×10^{-6}	0.955
1–4 ^b	119	0.00076–3.0	$\alpha = 7.97$	$\bar{b} = 0.948 \pm 0.002$	3.77×10^{-61}	0.9997
1–4 ^c	119	0.00076–3.0	7.73	0.940 ± 0.005	6.16×10^{-20}	0.996
total ^{c,d}	160	0.00076–3.0	7.83	0.943 ± 0.005	3.68×10^{-22}	0.996

^aParameters were estimated to minimize the sum of squares of ε_{ij} in each group.

^bParameters were estimated to minimize the sum of squares of μ_i .

^cParameters were estimated to minimize the sum of squares of E_{ij} .

^dIncluding the transitional phases.

Table 2. ANCOVA table for respirometry in the tiger puffer based on the model (equation (3.5)).

term	sum of squares	degrees of freedom	mean square	mean-square ratio	p
$\log \alpha$	79.548947	1	79.548947	36 283	1.37×10^{-144}
μ_i	0.034108	2	0.017054	7.78	6.81×10^{-4}
$(\bar{b} - \hat{b})\bar{x}_i$	0.128467	1	0.128467	58.59	6.81×10^{-12}
$\hat{b}x_{ij}$	107.984365	1	107.984365	49 252	3.90×10^{-152}
ε_{ij}	0.249942	114	0.002192		
total (about mean)	108.396881	118			
total (about zero)	187.945828	119			

equation (3.3) was rewritten as follows (Feldman & McMahon 1983):

$$y_{ij} = \log \alpha + \mu_i + (\bar{b} - \hat{b})\bar{x}_i + \hat{b}x_{ij} + \varepsilon_{ij}. \quad (3.5)$$

A one-way ANCOVA was carried out to clarify the validity of equation (3.5), and the results are provided in table 2. The μ_i 's were not zero ($F_{2,114} = 7.78$, $p = 0.00068$; one-way ANCOVA), meaning that the group means did not lie on the overall line. The intragroup scaling exponent $\hat{b} = 0.795$ was significantly different from the intergroup exponent $\bar{b} = 0.948$ ($F_{1,114} = 58.6$, $p = 6.8 \times 10^{-12}$; one-way ANCOVA). This implies that the intragroup scaling constant (a_i) increased significantly from $a_1 = 2.75$ to $a_4 = 7.25$ with increase in body mass (figure 1). Thus, the metabolic rate is expressed by $\text{VO}_2 = a_i M^{0.795}$, in which a_i increased three times during the transitional phases.

The increases in a_i were accompanied by morphological and behavioural changes during the transitional phases. Individuals that were heavier than 0.002, 0.01 or 0.1 g began to attack the smaller individuals. At approximately 0.002 g, the pectoral fin rays and the formation of plate-like larval teeth were completed. We also observed primordia of the dorsal, anal and caudal fin rays. Larvae heavier than 0.002 g were able to swim backwards using their pectoral fins, and therefore were able to repeatedly attack during a series of attacks on smaller individuals. During the second transitional phase, at approximately 0.01 g, we observed flexion

of the notochordal urostyle, post-flexion larvae metamorphosed into juveniles, completion of the formation of the gills and pseudobranch and alternation of juveniles' swimming mode from the larval style, using the pectoral fins, to the puffer-type style, using pectoral, caudal, dorsal and anal fins. During the third transitional phase, at approximately 0.1 g, squamation of the body proceeded and larval teeth were replaced with the adult form. Morphological and behavioural changes are summarized in table 3.

We observed three peaks in cannibalism corresponding to the three increases in a_i . Daily mortality, between 8 and 45 days after hatching, is given in figure 2a and electronic supplementary material, figure S13a. Mortality peaked following the first appearance of rapid growing fish that were larger than 0.002, 0.01 or 0.1 g, at which time a_i also increased, and ceased following the disappearance of slow-growing fish smaller than these threshold body sizes (figure 2b and electronic supplementary material, figure S13b). In general, the majority of predation occurred on smaller fish that had a lower a_i (less than 0.002, 0.002–0.01 or 0.01–0.1 g) (figure 3a–f), although a small amount of cannibalism occurred among fish within a phase (figure 3a,d).

4. DISCUSSION

Ontogenetic phase shifts in metabolism occurred, because the scaling constant a_i increased three times during the transitional phases at approximately 0.002, approximately

Table 3. Morphological and behavioural changes during the transitional phases in relation to increase of the scaling constant a_i .

transitional phase between (wet body mass in g)	morphological change	behavioural change
a_1 and a_2 (approx. 0.002)	pectoral fin rays completed plate-like larval teeth formed primordia of the dorsal, anal and caudal fin rays appeared digestive system fully formed ^a	larvae > approx. 0.002 g were able to swim backwards larvae > approx. 0.002 g began to attack smaller individuals independent mid-water swimming ^a
a_2 and a_3 (approx. 0.01)	flexion of the notocordal urostyle, post-flexion larvae metamorphosed into juveniles gills and pseudobranch formed visual system fully formed ^a	swimming mode changed from the larval style to the puffer-type style juveniles > approx. 0.01 g attacked smaller individuals active benethic swimming ^a negative phototaxis ^a
a_3 and a_4 (approx. 0.1)	squamation of the body proceeded larval teeth were replaced with the adult form anterior and posterior nostrils formed ^a	juveniles > approx. 0.1 g attacked smaller individuals benthic dwelling after sunset ^a juveniles at approximately 0.1 g move to estuary and neighbouring mudflats ^{b,c}

^aSuzuki *et al.* 1995.^bHidaka *et al.* 1988.^cTakita & Intong 1991.

0.01 and approximately 0.1 g, keeping each scaling exponent constant in each phase ($b = 0.795$). Three peaks in cannibalism corresponding to the three increases in a_i were observed. The increases in a_i were also accompanied by morphological and behavioural changes (table 3). These results suggest that rapid growth enables individuals to enter the next growth phase, associated with higher oxygen consumption, earlier than slow-growing fish. Individuals that enter the next growth phase with higher oxygen consumption are considered to become stronger, with higher motility accompanying morphological and behavioural changes than extrapolated from the previous growth phase with lower oxygen consumption. Fish at approximately 0.01 g have the visual system fully formed through the pseudobranch formation (table 3), because the arterial blood oxygenated in the first gill arch is supplied to the choroid of eyes through the pseudobranch (Harder 1975). As a consequence, fast-growing fish are able to cannibalize their slower growing conspecifics.

However, mortality in nature is more likely to occur by interspecific predation. Therefore, the present results of cannibalism may be an artefact of the condition of culture. But, intracohort cannibalism under natural conditions has been reported to occur in fishes such as the Atlantic mackerel *Scomber scombrus* (Hillgruber *et al.* 1997; Hillgruber & Kloppmann 2001) and a yellowtail *Seriola quinqueradiata* (Sakakura & Tsukamoto 1996) during early life stages. We consider that cannibalism in nature occurs in the tiger puffer to a less extent than interspecific predation, although we have little information about cannibalism under natural conditions in the tiger puffer.

Our results confirm abrupt or gradual increases, within a relatively narrow size range, in VO_2 or a_i during the ontogeny of metabolism. Previously, VO_2 was thought

to scale isometrically or near-isometrically to body mass ($b \approx 1$) during the ontogeny of metabolism in fishes (Giguère *et al.* 1988; Oikawa *et al.* 1991; Post & Lee 1996; Finn *et al.* 2002). Our results in relation to metabolic scaling suggest that isometric or near-isometric metabolic scalings early in the ontogeny reported so far can be produced by a combined effect of stepwise increases in a_i to make an isometric or a near-isometric metabolic scaling. Therefore, ontogenetic phase shifts would not be phenomena specific to the tiger puffer. However, further respirometric studies should be carefully carried out on various fish species to solve this matter.

The models we used to describe resting routine metabolism (equations (3.1) and (3.2)) are essentially the same as those describing the phylogeny and ontogeny of metabolism in seven species of mammals (*Peromyscus m.*, mice, rats, cats, dogs, sheep and cattle) in which the scaling exponent of the overall line ($\bar{b}$) equals 0.75 (interspecific comparison), and that of each species ($\hat{b}$) equals 0.67 (intraspecific comparison) (Feldman & McMahon 1983). (Note that $\bar{b} = 0.75$ is estimated to make the sum of μ_i zero, and that $\bar{b}$ is estimated to be 0.78 to minimize the sum of squares of μ_i .) In these studies, the scaling constant (a_i in watts at 1 kg body mass) increased threefold from 1.9 for *Peromyscus m.* to 6.1 for cattle with the size of the animal (Heusner 1982).

During the evolution of ectothermic and endothermic animals, there have been conspicuous increases in the scaling constants (Hemmingsen 1960; Zotin & Lamprecht 1996; Makarieva *et al.* 2008). Such increases imply that increases in body size during evolution are accompanied by an increase in the complexity of body organization (Zotin & Lamprecht 1996). Without an increase in the scaling constant, VO_2 would probably constrain the development of larger organisms.

Figure 2. Changes in daily mortality, owing to cannibalism, with an increase in growth. Diamonds show results in 2005, and circles in 2001. (a) Changes in daily mortality following hatching. The open and solid symbols signify the two experimental tanks. There were three peaks in daily mortality between 8 and 45 days after hatching (2005, diamonds; peak 1: 10–17, peak 2: 18–24 and peak 3: 35–41 days old) (2001, circles; peak 3: 28–45 days old). (b) Changes in the body mass, expressed as the geometric mean $\pm$ 1 s.d. (number of fish), of cannibalized individuals (grey) in the two experimental tanks together and survivors (blue) randomly sampled from either of the two experimental tanks following hatching in the experiment 2005. These body masses and the number of fish in peak 3 in the experiment 2001 are given in electronic supplementary material, figure S13. Note that the number of dead fish (grey) does not represent the actual number of cannibalized individuals, but the number for which we were able to estimate body mass from the total or body length, or the eye diameter. Percentage ratios of the number of fish, for which body mass was estimated, to the number of fish cannibalized are given in electronic supplementary material, figure S14. The estimated body masses of maximally grown individuals cultured in other tanks are also shown in the red diamonds. Those in peak 3 in the experiment 2001 are given in electronic supplementary material, figure S13. The ontogenetic phase shifts in metabolism corresponding to figure 1 are shown as the coloured zones (pink, phase I; yellow, phase II; light green, phase III; light blue, phase IV). The transitional phases are shown as the white zones between phases I and II, II and III and III and IV. Each peak in daily mortality was associated with the appearance of fish larger than 0.002, 0.01 or 0.1 g (red arrows), and ceased at approximately the time slow-growing fish in survivors had grown larger than these threshold body masses (blue arrows).

During the transitional phase at approximately 0.01 g, metamorphosis from the larval to the juvenile stage is thought to occur, in part owing to an increase in thyroxine release, which may explain the increase in a_i at this time. However, the mechanism(s) for the increase in a_i at approximately 0.002 and 0.1 g are currently unknown. Behaviourally, they move to estuary and neighbouring mudflats at approximately 0.1 g, and grow there for a while in Hakata Bay and Ariake Sound (Hidaka *et al.* 1988; Takita & Intong 1991).

Like most fishes, the tiger puffer produces a large number of small eggs. Our results suggest that rapidly growing individuals are able to progress more rapidly to the next developmental phase having a higher metabolic activity. As a consequence, individuals with a higher metabolism–body mass relationship are more successful during predator–prey interactions than slow-growing

individuals that have a lower metabolic rate, developing more anti-predator adaptation. Under natural conditions, although cannibalism is not going to have the same impact as it has in an aquarium as the larvae are likely to be dispersed, an increase in the scaling constant (a_i) with growth during early development implies that the survival of rapidly growing individuals will be greater and that slow-growing individuals are more likely to become prey as a result of predation, whether it is inter-specific predation or intraspecific cannibalism. This explanation supports the ‘growth–mortality’ hypothesis, which postulates that larger, faster growing and faster developing individuals have a higher probability of survival (Hare & Cowen 1997).

Experimental procedures followed the guidance for Animal Experiments in Faculty of Agriculture and in the Graduate

Figure 3. Size distribution of dead individuals cannibalized during each peak in daily mortality ((a) peak 1, $n = 793$; (b) peak 2, $n = 353$; (c) peak 3 in 2005, $n = 83$; (d) peak 3 in 2001, $n = 3084$). ‘ n ’ shows the number of cannibalized fish within each peak. Size classes are made to be evenly divided on the logarithmic scale in each peak. The prey size within each peak in cannibalism was almost within 0.002 in peak 1, 0.01 in peak 2 and 0.1 g in peak 3. The gross mean of body mass of prey (solid diamonds) and survivors (open diamonds) within each peak is shown as the geometric mean $\pm$ 1 s.d. (number of fish) ((e) peak 1; (f) peak 2; (g) peak 3 in 2005; (h) peak 3 in 2001). Geometric mean and s.d. of body mass of prey within each peak are weighted with the daily number of cannibalized fish.

Course of Kyushu University, with registrations of M.Y. and S.O. to Pursuers for Animal Experiments.

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