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Scaling of metabolism in *Helix aspersa* snails: changes through ontogeny and response to selection for increased size

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SUMMARY

Though many are convinced otherwise, variability of the size-scaling of metabolism is widespread in nature, and the factors driving that remain unknown. Here we test a hypothesis that the increased expenditure associated with faster growth increases metabolic scaling. We compare metabolic scaling in the fast- and slow-growth phases of ontogeny of *Helix aspersa* snails artificially selected or not selected for increased adult size. The selected line evolved larger egg and adult sizes and a faster size-specific growth rate, without a change in the developmental rate. Both lines had comparable food consumption but the selected snails grew more efficiently and had lower metabolism early in ontogeny. Attainment of lower metabolism was accompanied by decreased shell production, indicating that the increased growth was fuelled partly at the expense of shell production. As predicted, the scaling of oxygen consumption with body mass was isometric or nearly isometric in the fast-growing (early) ontogenetic stage, and it became negatively allometric in the slow-growing (late) stage; metabolic scaling tended to be steeper in selected (fast-growing) than in control (slow-growing) snails; this difference disappeared later in ontogeny. Differences in metabolic scaling were not related to shifts in the scaling of metabolically inert shell. Our results support the view that changes in metabolic scaling through ontogeny and the variability of metabolic scaling between organisms can be affected by differential growth rates. We stress that future approaches to this phenomenon should consider the metabolic effects of cell size changes which underlie shifts in the growth pattern.

Key words: metabolism, growth rate, 3/4 power law, life history, allometry, cost of growth, body size, experimental evolution, growth efficiency, bioenergetics, food consumption, cell size, Bertalanffy's theory, metabolic theory.

INTRODUCTION

The energy transition within cells, called metabolism, is a basic characteristic of living things. Its rate is linked, as cause or effect, to most fundamental features of biological systems, such as cell and body sizes, rates of reproduction, growth and senescence, immunocompetence, mating success and genome evolution (von Bertalanffy, 1957; Sibly and Calow, 1986; Konarzewski, 1995; Kooijman, 2000; Lochmiller and Deerenberg, 2000; Furness, 2003; Kozłowski et al., 2003; Martin et al., 2003; Radwan et al., 2006). It is not surprising, then, that Rubner's (Rubner, 1883) discovery of the non-isometric increase of the metabolic rate with body size in dogs spurred a debate on the origin of size-scaling of metabolism (von Bertalanffy, 1951; McMahon, 1973; Schmidt-Nielsen, 1984; Sibly and Calow, 1986; West et al., 1997; Kooijman, 2000; Kozłowski et al., 2003; Glazier, 2006). The dispute has centered around two questions: what are the universal patterns in the size-scaling of metabolism, and how are the scalings to be explained (Glazier, 2005). Metabolic rate ($\dot{M}_{O_2}$) is usually assumed to be a power function of body mass (M_b),

$$\dot{M}_{O_2} = aM_b^b . \quad (1)$$

Most proponents of the idea of universal laws of metabolic scaling have argued that the size-scaling exponent b attains a constant value of 2/3 or 3/4 (Rubner, 1883; von Bertalanffy, 1951; von Bertalanffy, 1957; Heusner, 1982; Peters, 1983; Brown et al., 1997); models and hypotheses considering various biophysical constraints have been formulated to validate such exponent values theoretically (Glazier, 2005). However, the universality of 2/3 and 3/4 metabolic scaling has been questioned recently (e.g. Kozłowski et al., 2003; Glazier, 2005; Chown et al., 2007). Glazier's analysis of published data shows that variability of metabolic scaling and deviations from the 2/3 and 3/4 scaling modes are widespread in nature: the value of exponent b ranges from 0.38 to 1.11 in mammals, from 0.27 to 1.26 in squamate reptiles, and from –1.2 to 2.05 in invertebrates; among 642 intraspecific exponent values analyzed by Glazier, 45.8% differ significantly from b=2/3 and 50.2% deviate from b=3/4. It is important to identify the factors diversifying exponent b, not only to answer whether there are universal modes of metabolic scaling, but also to achieve a fuller understanding of phenotypic variability in nature. Metabolic scaling is expected to affect optimal resource allocation to growth and reproduction, so it should substantially influence the life history of organisms (Kozłowski and Teriokhin, 1999; Czarnołęski et al., 2003). Emerging evidence suggests that adaptive allocation responses to shifts in metabolic scaling can explain different ecological and evolutionary phenomena, such as the so-called 'temperature-size rule' in ectotherms (slower growth and larger final body size in colder environments) (Angilletta and Dunham,

2003; Kozłowski et al., 2004), or the interspecific patterns of body size distributions and life history allometries (Kozłowski and Weiner, 1997; Kindlemann et al., 1999; Kozłowski and Gawelczyk, 2002).

In this work we examine the link between size-scaling of metabolism and growth rate in *Helix aspersa* snails. We analyze shifts of metabolic scaling across snail ontogeny and compare scaling of metabolism in normal snails and snails artificially selected for increased adult size. First we provide a physiological and life history background for metabolic analysis by analyzing correlated responses of growth traits, food consumption and snail viability to selection. Then we test the hypothesis that metabolic scaling is steeper in fast-growing than in slow-growing organisms (Riisgård, 1998; Glazier, 2005) by comparing metabolic exponent b in fast- *versus* slow-growth phases of snail ontogeny, and in slow- *versus* fast-growing genetic lines of snails. Finally, we examine whether the metabolic scalings conform to the 2/3 and 3/4 power laws.

MATERIALS AND METHODS

Control and selection lines

Wild *Helix aspersa* (Müller) snails were used to create random-bred stock maintained on an experimental snail farm [Le Magneraud, INRA, France; details are published elsewhere (Dupont-Nivet et al., 1997)]. After three generations of random breeding, control and selection lines were derived (Dupont-Nivet et al., 2000). The control was maintained by breeding individuals randomly; each generation was produced from at least 30 families to maintain genetic variability. The selected line was produced by applying individual selection for increased adult mass; the proportion of selected animals was ~13% for the first five generations and ~30% after that. The individuals used in this study came from the seventh generation of both lines (tenth generation after the establishment of the snail stock). In the spring of 2002, 20 egg-layings per line were randomly taken from different parents (families). After hatching, 60 newborns were randomly chosen from each clutch and reared for 5 months. Each family group was kept in a wooden box (120×300×480 mm) lined with moist moss and covered with a plastic-mesh lid (Bonnet et al., 1990). The boxes (20 per line) were randomly located in an air-conditioned room (20°C, 90% relative humidity, 16 h:8 h L:D photoperiod). The snails were fed *ad libitum* with a compound food (Ets Arrive, France). Every week the boxes were cleaned, checked for dead animals, and supplied with fresh food.

Growth pattern

The egg-layings were weighed and scored for eggs. Immediately after hatching and every 3 weeks thereafter, the snails were weighed in family groups and counted, and average snail mass per family box at the time was calculated. Mass was measured to the nearest 0.01 g on an electronic balance. Typically, *H. aspersa* snails cease growth after development of a thickened lip at the shell aperture, the so-called peristome, and start reproducing (Baker, 2001). Such individuals were systematically removed from the boxes and weighed individually to the nearest 0.001 g. This nonrandom removal of larger individuals caused underestimation of average snail mass calculated in the periods following such removals. To reduce the bias, average mass at a given time was calculated from the mass of snails found in a box at that time and from the mass of snails removed from the box before.

The shape of the growth trajectory of an average snail in a family box was described by the logistic equation:

$$M_b = M_0 M_A / [M_0 + (M_A - M_0)e^{-kt}] , \quad (2)$$

as used for *Biomphalaria glabrata* snails (Plorin and Gilbertson, 1984), where M_b (g) is body mass at age t (days), M_0 (g) is the hypothetical body size at age equal to 0, M_A (g) is the asymptotic size and k (day^{-1}) is the growth rate coefficient. The equation was fitted to datasets on age and average snail mass in each box, using the least squares method with the Simplex-quasi Newton procedure (Statistica 6.1, StatSoft); average mass was weighted with the number of snails contributing to its calculation. Data on egg mass were not used for curve fitting; snail age was assumed to be zero days at the date of egg laying.

Logistic growth is characterized by an inflection (at body size M_b=0.5M_A), which demarcates ontogeny between two growth phases: growth accelerates with age before reaching the inflection; afterwards growth slows with age. Given this criterion, snails younger than the family-specific age at which the inflection was attained (hereafter $AGE_{inflect}$) were defined as fast growing, and older snails as slow growing. The rate of growth at the inflection point was calculated from a derivative of Eqn 2, $dM_b/dt = kM_b(M_A - M_b)/M_A$, and used as a family-specific index of maximum growth rate, GR_{max} (g day^{-1}).

Average egg number per clutch, average egg and hatchling mass, growth curve parameters M_0, M_A, k and maximum growth rate parameters GR_{max} and $AGE_{inflect}$ were compared with ANOVA (Statistica 6.1, StatSoft) in the control and size-selected lines. To normalize the distributions, the values of metric traits were transformed with decimal logarithms.

Consumption and growth

Weekly data on the amount of provided and uneaten food in each rearing box were combined in three-week sets to match the time intervals over which snail growth was monitored. Consumption over the intervals was calculated by subtracting the dry mass of uneaten food from the dry mass of food provided to the boxes; the dry mass of added food was estimated from a dry-to-wet mass regression derived from preliminary data. Samples of food and refuse were dried for 24 h at 103°C in a ventilated oven, and weighed dry to the nearest 0.01 g. Consumption was converted to daily ration per snail (C, g day^{-1}). Snail growth was measured over 3-week intervals as the gain of average snail mass in a box and converted to daily growth rate (GR, g day^{-1}). The average growth efficiency of a snail in a box (GE, g g^{-1}) was expressed by the ratio GR:C.

General linear models (GLMs) were used to compare consumption rate C, growth rate GR and growth efficiency GE between the control and size-selected lines (Statistica 6.1, StatSoft). The models included three grouping factors: snail line (fixed), family box nested within line (random), and the 3-week interval over which the data were recorded (random); average snail body mass at the beginning of each interval was covariate. The relationship of GR and GE with the covariate was linear within time intervals, but became nonlinear for pooled data, indicating that the slope of the within-interval relation was changing across intervals. To account for this phenomenon, the GLMs for growth rate and growth efficiency included the body size×time interval interaction. The analysis was performed only on data from the fast-growth period because the estimates of biomass increase and food consumption calculated from this period were least affected by snail removal and mortality. Prior to the analysis, the data were

transformed with decimal logarithms to normalize the distributions and linearize the relationships between variables.

Rates of development and mortality

Snail survivorship in rearing boxes (families) was expressed by the median life expectancy of snails at the beginning of life, calculated using the life table method (Statistica 6.1, StatSoft). Snails alive at the end of the experiment, snails removed from boxes after production of the peristome, and individuals used for metabolic measurements were treated as censored observations. The calculated median life expectancies were compared between the control and size-selected lines with the Kruskal–Wallis nonparametric test. A similar procedure was used to compare the age at which control and size-selected snails produced the peristome. Dead individuals, snails removed for metabolic measurements, and snails that did not develop the peristome by the end of experiment were treated as censored observations.

Table 1. Results of ANOVA comparing growth traits of control and size-selected lines of snails *Helix aspersa*

Trait	Mean trait values: Control line	Mean trait values: Size-selected line	*F*	*P*
Number of eggs in clutch	171.7	182.2	0.54	0.47
Egg size (g)	0.038	0.049	62.13	0.00000001
Hatchling size (g)	0.029	0.035	18.33	0.0001
M_0 (g)	0.041	0.065	4.41	0.04
M_A (g)	6.939	12.411	19.52	0.0001
k (day^{-1})	0.059	0.058	0.001	0.97
GR_{max} (g day^{-1})	0.101	0.181	18.37	0.0001
$AGE_{inflect}$ (days)	86.90	89.17	0.59	0.45

Family-specific trait values were used as data (20 families per line). M_0, M_A, and k are logistic growth curve parameters: M_0 is hypothetical initial body size at age equal to 0; M_A is asymptotic size; k is the growth rate coefficient; GR_{max} measures maximum growth rate (derivative of logistic growth curve at body size equal to $0.5M_A$); $AGE_{inflect}$ is the age at which GR_{max} is attained. Data on metric traits were transformed with decimal logarithms prior to analysis; displayed mean values were back-transformed.

Scaling of metabolism and shell mass

To measure metabolic rate, snails were sampled from the family boxes, placed individually in plastic containers with holes in the lids, and shipped *via* courier service in an isolation box to the Institute of Environmental Sciences (IES), Jagiellonian University in Kraków, Poland. To obtain a wide range of body sizes and to trace changes of metabolic scaling across ontogeny, seven samples were taken, at approximately 3-week intervals; the snails were between 11 and 33 days old at the first sampling. One snail per family was chosen during the first three samplings, and two snails per family in the following four samplings; only individuals weighing close to the mean for the box were chosen. Snails were received at IES after 4–5 days; they were sprayed with dechlorinated tapwater and kept for 48 h in a 20°C chamber under a 16 h:8 h L:D photoperiod. Fifteen snails per line, representing different families, were randomly chosen for measurement of metabolic rate; occasionally two snails of the same family were used. Snails were placed individually in 5×5 mm net curtain sacks to reduce their activity, and enclosed in flasks (volume 50, 600, 1600 ml, depending on snail size) connected to individual channels of a computer-controlled, closed-circuit respirometer (Micro-Oxymax, Columbus Instrument, USA). An Eppendorf tube with distilled water and a hole in the cap was placed in each flask to maintain humidity. Flasks with control line and size-selected line snails were placed alternately in 20°C chambers, kept illuminated during the measurements to reduce snail activity. Oxygen intake was measured for 6 h, and its consumption per hour was taken as a measure of metabolic rate (hereafter $\dot{M}_{O_2}$; $\mu l\ h^{-1}$). After the measurements the snails were weighed on an electronic balance to the nearest 0.001 g, then killed by freezing at –20°C for 24 h; soft parts were removed and weighed. Whole mass M and flesh mass M_F were used as measures of snail body size. Shell mass M_S was calculated by subtracting M_F from M.

To compare metabolic size-scaling between fast- and slow-growth ontogenetic phases, snails younger than the family-specific $AGE_{inflect}$ were defined as fast-growing, and older snails as slow-growing. Slopes of $\log_{10}$-$\log_{10}$ regressions of oxygen consumption $\dot{M}_{O_2}$ *versus* whole snail mass M and flesh mass M_F (exponent b in Eqn 1) and slopes of $\log_{10}$–$\log_{10}$ regressions of shell mass M_S *versus* flesh mass M_F were calculated for control line and size-selected line snails, separately for their fast- and slow-growth phases. Confidence intervals of regression slopes were used to compare metabolic exponents with theoretical b values 1, 0.75 and 0.67. The GLM method was used to compare body size-scaling of metabolic rate $\dot{M}_{O_2}$ in control *versus* size-selected lines, and in fast- *versus* slow-growth phases. The model included a fixed factor of snail line or growth phase, whole body mass M as covariate, and factor x covariate interaction. A significant interaction was taken to indicate a difference in size-scaling between snail lines or growth phases. To further investigate how metabolic scaling was affected by shell scaling, a similarly structured GLM analysis was performed on data on oxygen consumption and shell mass M_S in relation to snail flesh mass M_F as covariate. All data were $\log_{10}$-transformed prior to the analyses to normalize the distributions and to linearize the relationships between variables.

RESULTS

Growth pattern

Size-selected snails produced larger eggs and hatchlings than the control line; they attained larger initial and asymptotic sizes M_0 and M_A of the logistic curve; the two lines had similar logistic growth coefficients k (Table 1, Fig. 1). Maximum growth rate GR_{max} was

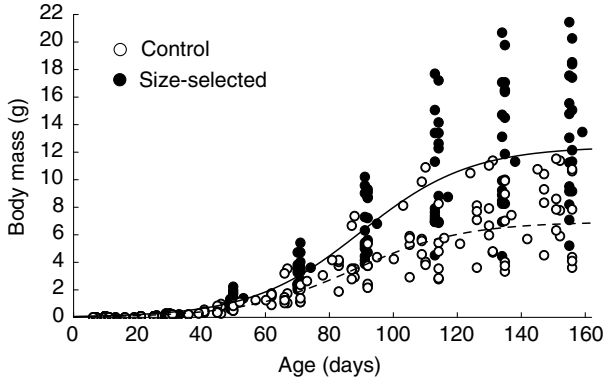

Fig. 1. Logistic growth curves of *Helix aspersa* snails from control and size-selected lines, based on values of averaged family-specific growth curve parameters (see Table 1). Symbols denote family-specific mean size of snails at a given age.

Table 2. Results of three general linear models testing differences in consumption, growth rate and growth efficiency of control and size-selected lines of *Helix aspersa* snails

Factor	Effect	*F*	*P*
Consumption rate *C* (g dry food day^{-1} snail^{-1})			
Snail line	Fixed	$1.09_{(1,122)}$	0.30
Box (snail line)	Random	$1.16_{(38,122)}$	0.27
Time interval	Random	$7.27_{(4,122)}$	0.00003
Body size	Fixed	$107.69_{(1,122)}$	0.000001
Growth rate *GR* (g mass gained day^{-1} snail^{-1})			
Snail line	Fixed	$33.24_{(1,118)}$	0.000001
Box (snail line)	Random	$1.63_{(38,118)}$	0.025
Time interval	Random	$5.02_{(4,118)}$	0.001
Body size	Fixed	$3.09_{(1,118)}$	0.096
Body size×time interval	Random	$4.89_{(4,118)}$	0.0011
Growth efficiency *GE* (*GR*×*C*$^{-1}$)			
Snail line	Fixed	$39.08_{(1,118)}$	0.000001
Box (snail line)	Random	$1.55_{(38,118)}$	0.0396
Time interval	Random	$10.08_{(4,118)}$	0.000001
Body size	Fixed	$0.68_{(1,118)}$	0.43
Body size×time interval	Random	$9.35_{(4,118)}$	0.000001

Degrees of freedom for *F* statistics are given in brackets. Snails were reared in 20 family boxes per line; average daily consumption of dry food (*C*) was calculated over 3-week intervals for an individual snail in a box; growth rate (*GR*) is average daily mass gain of a snail in a box, calculated from 3-week data on snail growth; growth efficiency is the *GR*:*C* ratio. The analyzed data are from the fast-growth phase of snail ontogeny, and were transformed with decimal logarithms prior to analysis.

higher in the size-selected snails, but the two lines attained it at a similar age (Table 1).

Consumption and growth

Food consumption rate *C* increased with snail size; it differed between three-week measurement intervals (Table 2, Fig. 2A), but not between snail lines or rearing boxes. GLM analysis showed that growth rate *GR* and growth efficiency *GE* were higher in the selected than in the control line (Table 2, Fig. 2B,C); *GR* and *GE* significantly differed between rearing boxes and between 3-week intervals. Growth rate and growth efficiency were related to body size, but this link was altered by the time interval, as indicated by the significant body size×time interval interaction for *GR* and *GE*.

Rates of development and mortality

The Kruskal–Wallis test showed that median life expectancy tended to be higher in the size-selected than in the control line (median values: 155.00 *versus* 130.55 days, H=3.55741, P=0.059). The median expected age at which snails reached the adult stage (presence of peristome) did not differ between the lines (Kruskal–Wallis test, H=1.551941, P=0.21).

Scaling of metabolism and shell mass

Fig. 3 shows the size-scaling of oxygen consumption and shell mass in the control and size-selected snails, and changes of these scalings through ontogeny. Table 3 reports the results of GLM analysis of scaling of metabolism and shell mass, and Table 4 gives estimates of the size-scaling exponents for the two traits.

The metabolic rate tended to increase isometrically with body size (b=1) in the fast-growth phase of the selected line (scaling with whole mass M and with flesh mass M_F; Table 4, Fig. 3C); the scaling exponents were higher than 0.75. In the fast-growth phase of the control line, oxygen consumption scaled isometrically with size (and with a slope steeper than 0.75) when metabolism was regressed against whole mass M, and almost isometrically (with a

Table 3. Results of general linear models testing the size-scaling of oxygen consumption and shell mass of *Helix aspersa* snails from control *versus* size-selected lines, and in fast-growth *versus* slow-growth phases

	Metabolism*×body mass		Metabolism*×flesh mass		Shell mass†×flesh mass	
Factor	*F*	*P*	*F*	*P*	*F*	*P*
Control line						
Growth phase	$4.78_{(1,92)}$	0.0313	$5.28_{(1,94)}$	0.024	$2.19_{(1,93)}$	0.14
Body size	$134.82_{(1,92)}$	0.000001	$112.38_{(1,94)}$	0.000001	$244.83_{(1,93)}$	0.000001
Growth phase×body size	$1.42_{(1,92)}$	0.24	$0.52_{(1,94)}$	0.47	$7.44_{(1,93)}$	0.0076
Size-selected line						
Growth phase	$2.14_{(1,89)}$	0.15	$1.58_{(1,89)}$	0.21	$1.10_{(1,89)}$	0.30
Body size	$146.86_{(1,89)}$	0.000001	$125.55_{(1,89)}$	0.000001	$303.02_{(1,89)}$	0.000001
Growth phase×body size	$4.11_{(1,89)}$	0.0457	$3.00_{(1,89)}$	0.0867	$3.48_{(1,89)}$	0.0653
Fast-growth phase						
Line	$0.003_{(1,97)}$	0.96	$0.62_{(1,98)}$	0.43	$0.43_{(1,97)}$	0.51
Body size	$852.46_{(1,97)}$	0.000001	$730.91_{(1,98)}$	0.000001	$1208.56_{(1,97)}$	0.000001
Line×body size	$3.00_{(1,97)}$	0.0867	$4.11_{(1,98)}$	0.0453	$3.08_{(1,97)}$	0.0826
Slow-growth phase						
Line	$0.005_{(1,84)}$	0.94	$0.001_{(1,85)}$	0.98	$0.08_{(1,85)}$	0.78
Body size	$75.86_{(1,84)}$	0.000001	$71.47_{(1,85)}$	0.000001	$187.00_{(1,85)}$	0.000001
Line×body size	$0.003_{(1,84)}$	0.95	$0.01_{(1,85)}$	0.92	$0.06_{(1,85)}$	0.80

*Metabolism measured as oxygen consumption (μl h^{-1}); †shell mass (g).

Growth rate accelerates with age in the fast-growth phase; it decelerates with age in the slow-growth phase. Oxygen consumption scaled with whole body mass of snails (body size including shell mass) or against snail flesh mass (body size without shell mass); shell mass scaled against flesh mass. Snail line and growth phase were treated as fixed factors; degrees of freedom for *F* statistics are given in brackets. Data were transformed with decimal logarithms prior to analysis.

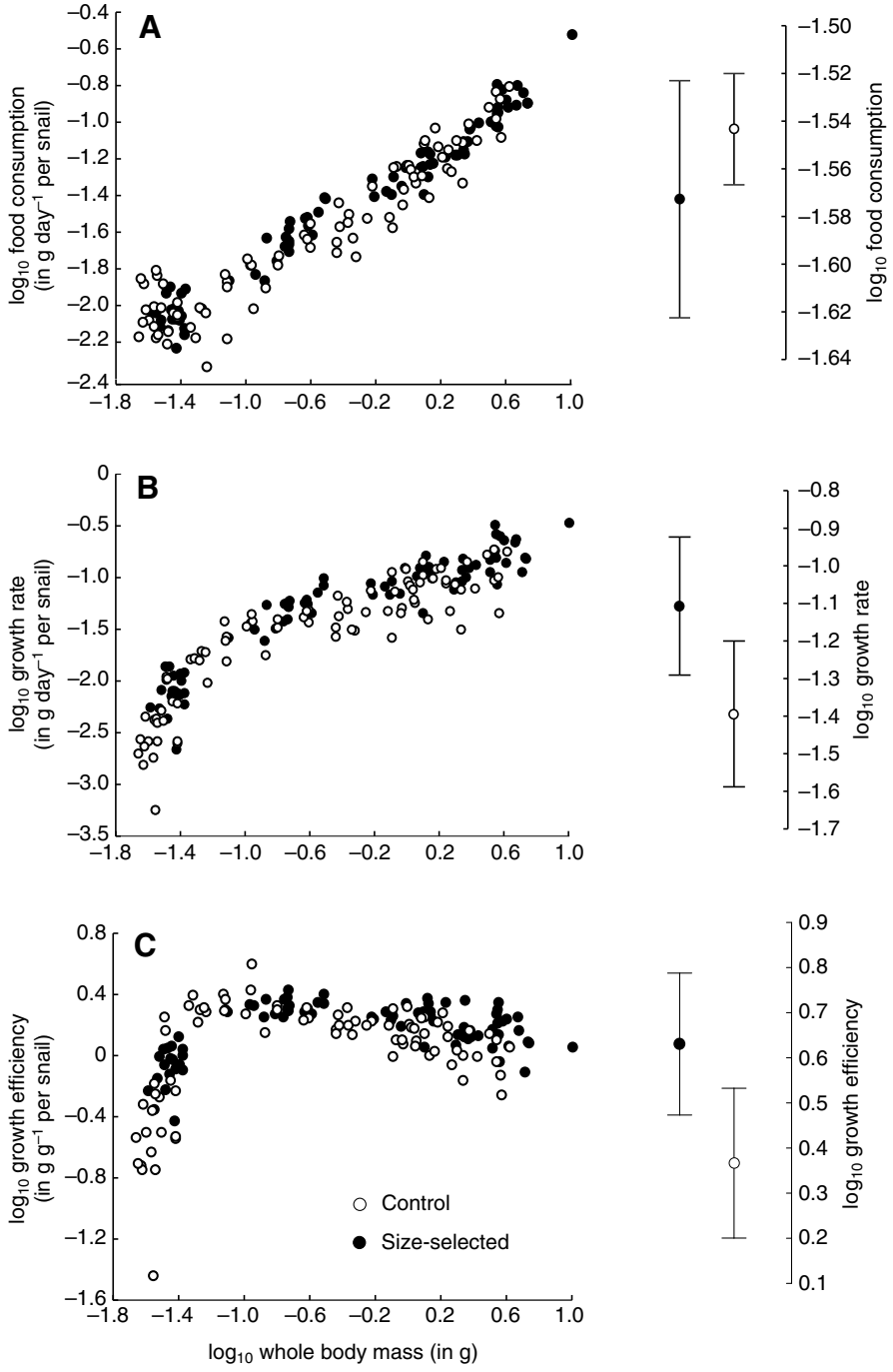

Fig. 2. Food consumption (A), growth rates (B) and growth efficiency (C) of control and size-selected *Helix aspersa* snails in the fast-growth ontogenetic phase (exponential-like part of growth curve; see Fig. 1). Growth efficiency is the ratio of the average increase of snail whole mass to food consumption. Symbols denote family-specific mean values calculated from data for 3-week intervals. Whisker charts show least-square means with 95% confidence intervals estimated at mean value of body mass, with the aid of GLM from Table 2.

slope steeper than 0.67 but not diverging from 0.75) when it was regressed against flesh mass M_F (Table 4). In the slow-growth phase, the scaling was significantly lower than 1 but not diverging from 0.67 and 0.75 in both lines.

The size-scaling of metabolism was shallower in the slow- than in the fast-growth phase (Tables 3, 4, Fig. 3A,B). This tendency was observed in both lines, whether metabolism was scaled with whole body mass M or with flesh mass M_F. GLM analysis showed that the shallowing of the slope of metabolic scaling through ontogeny was significant in the size-selected line when whole body mass was the size measure (Table 3: P=0.046 for interaction term) or marginally significant when the size measure was flesh mass (Table 3: P=0.087 for interaction term). In the control line, the ontogenetic shift in metabolic scaling was nonsignificant for both size measures (Table 3: P=0.24 and 0.47 for interaction terms).

In the fast-growth phase, the mass exponent for metabolism was larger for selected than for control snails (Tables 3, 4, Fig. 3C). The difference was significant when snail flesh mass was taken as the size measure (Table 3: P=0.045 for an interaction term); it was marginally significant when whole body mass was used (Table 3: P=0.087 for an interaction term). In the slow-growth phase, metabolism scaled at the same rate with size in both lines, no matter which measure of body size, M or M_F, was considered (Table 3: P=0.95 and 0.92 for interaction terms).

Shell mass M_S increased faster with flesh body mass M_F in the slow- than in the fast-growth phase (Table 4); the change in scaling was marginally significant in the size-selected line (Table 3: P=0.065 for interaction term, Fig. 3E) and significant in the control (Table 3: P=0.008 for interaction term, Fig. 3D). In the fast-growth phase, shell mass tended to increase faster with body size in the selected than in the control line (Table 3: P=0.0826 for interaction term, Fig. 3F). Shell scaling did not differ between lines in the slow-growth phase (Table 3: P=0.80 for interaction term).

DISCUSSION

Life history response to size selection

The growth pattern of *Helix aspersa* snails resembled a biphasic logistic-like trajectory, with a phase of accelerating growth early in ontogeny and a phase with decelerating growth later in life (Fig. 1). Selection for increased adult size substantially changed the characteristics of this growth: the size-selected snails attained larger initial size M_0 and higher maximum growth rate GR_{max}, and their growth curve asymptotic size M_A was almost double that of the control snails (Table 1). Development of larger adult size can proceed through three mechanisms, which are not mutually exclusive: by starting growth from larger initial size, by speeding-up size-specific growth, and by extending the growth period. Our data indicate that size-selection of *H. aspersa* snails produced larger adults *via* an increase of egg size and of the size-specific growth rate (Tables 1, 2, Fig. 2B). The evolution of adult size was not achieved through alteration of developmental rates because the two

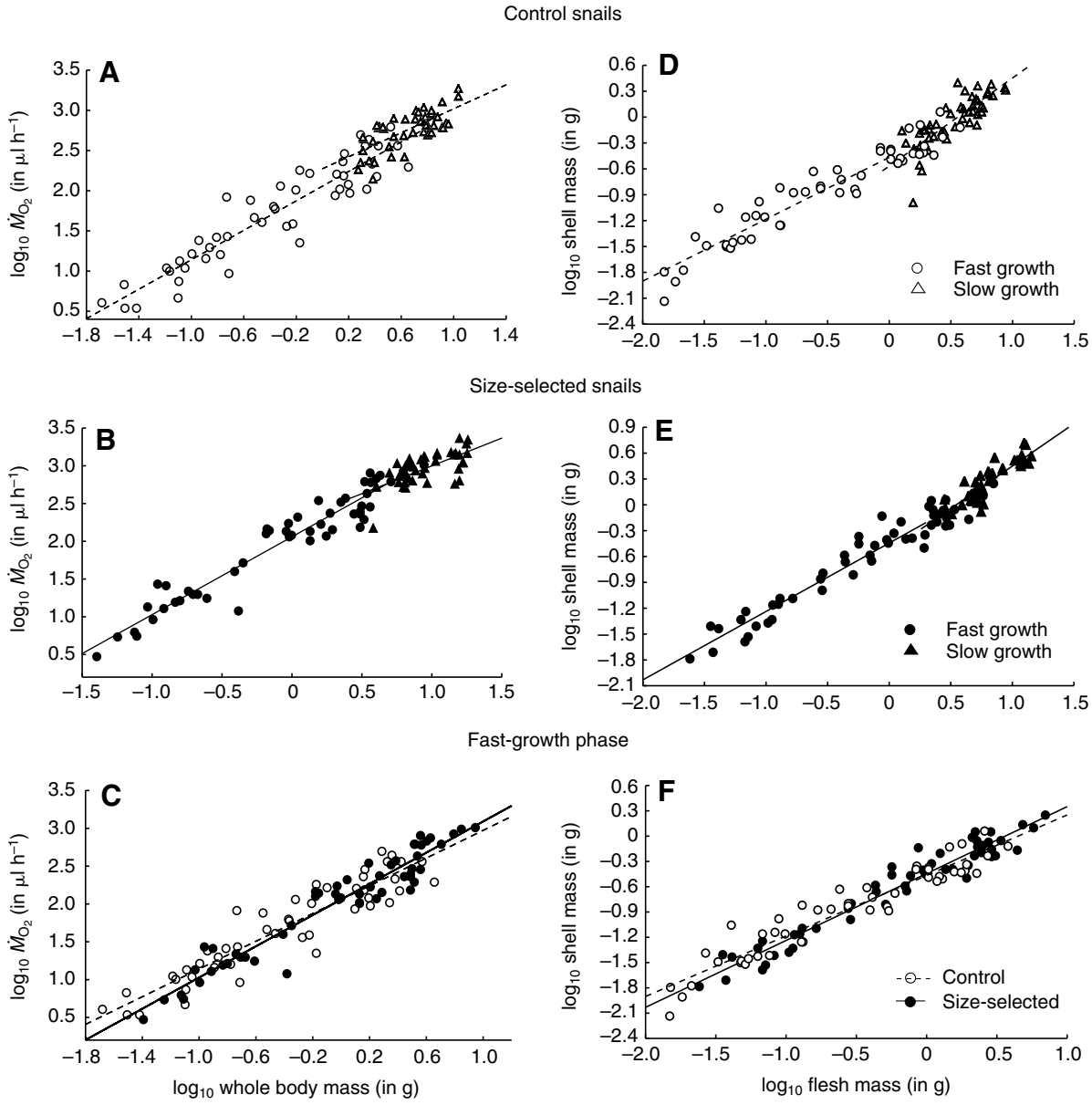

Fig. 3. Comparison of size-scaling of oxygen consumption (A–C) and shell mass (D–F) of control and size-selected *Helix aspersa* snails in the fast- and slow-growth phases of ontogeny. Growth rate accelerates with age in the fast-growth phase; it decelerates with age in the slow-growth phase. Symbols denote measures of individual snails.

lines attained the maximum growth rate at similar ages and entered the adult stage at comparable rates. To further probe the relative role of initial size and growth rate in size evolution, egg size would need to be manipulated in order to break the potential covariance between the early physiological state determined by egg size and growth performance later in life (Sinervo and Huey, 1990).

Increased growth rates are achieved through either (1) an increase of energy acquisition, (2) an increase of resource allocation

Table 4. Size-scaling exponents of metabolism and shell mass in slow-growing control and fast-growing size-selected lines of *Helix aspersa* snails

	Metabolism×whole body mass		Metabolism×flesh mass		Shell mass×flesh mass	
Growth phase:	Fast	Slow	Fast	Slow	Fast	Slow
Control line	0.9162±0.1072	0.7459±0.2222	0.8337±0.1086	0.7373±0.2142	0.7176±0.0675	1.0207±0.2163
Size-selected line	1.0316±0.0823	0.7360±0.2604	0.9688±0.0798	0.7094±0.2687	0.7939±0.0560	0.9845±0.1737

Exponent values are shown with ±confidence intervals and were calculated separately for the fast-growth phase of ontogeny (growth rate accelerates with age) and for the slow-growth phase (growth rate decelerates with age). The exponents are slopes of $\log_{10}$–$\log_{10}$ regressions of metabolism or shell mass *versus* body size measure.

to growth at the expense of other energy-demanding processes (e.g. reproduction, maintenance), or (3) lowering of the metabolic costs of growth (Glazier, 1990; Konarzewski, 1995; Czarnołęski and Kozłowski, 1998; Bayne, 1999; Konarzewski et al., 2000). In general, accelerated growth is expected to increase total metabolism as a result of elevated expenditures for biosynthesis and tissue deposition (Jörgensen, 1988), but the interdependence of mechanisms 1–3 can lead to different responses of total metabolism (Konarzewski, 1995). For example, fast-growing forms of the lake whitefish *Coregonus clupeaformis* had lower food consumption and lower metabolism than slow-growing dwarfs (Trudel et al., 2001); artificial selection for increased body size in oysters produced fast-growing individuals which consumed more food but used less oxygen due to lower costs of growth (joules respired per joule of growth) and decreased expenditure for maintenance (Bayne, 1999). Interestingly, MacLaury and Johnson (MacLaury and Johnson, 1972) demonstrated that selection for increased oxygen uptake can produce slow-growing organisms. In our study, fast- and slow-growing lines of *H. aspersa* had similar food consumption. Compared to the control line, the fast-growing selected line had higher growth efficiency (Table 2, Fig. 2C), and a lower (at smaller body sizes) or equal (at larger sizes) metabolic rate (Fig. 3C). These characteristics point to the role of alteration of resource allocation (2) and costliness of growth (3) in differentiating growth rates between the two lines. Evolution of growth rates through resource allocation must involve alterations in the energy provisioning of many functions which are interconnected in complex ways, thus generating a wide array of different tradeoffs (Metcalfe and Monaghan, 2001; Pigliucci and Preston, 2004; Czarnołęski et al., 2005). This study was not aimed at identifying such tradeoffs, but our data allow us to look at whether size-selected snails enhanced their growth at the expense of survivorship and shell production. We found a concerted response of shell production and oxygen consumption to size selection: in the phase of accelerating growth, the fast-growing selected line had a lower metabolic rate and produced lighter shells than the slow-growing control (Fig. 3C,F). Interestingly, the difference in metabolic rate persisted as long as the size-selected snails had lower shell mass than control snails: both lines became similar with respect to metabolic rates after attainment of body size M_F equal to 0.498 g, which almost exactly coincided with equalization of shell masses in the two lines at M_F=0.490 g. Our results suggest that the increased expenditure for tissue growth in the size-selected snails was at least in part covered at the expense of shell production. Costs of shell production are often considered an important part of the energy budget, and they are responsible for tradeoffs between shell elongation and thickening (Palmer, 1992) (but see Czarnołęski et al., 2006). Our analysis of snail mortality suggests that increased growth rate was not realized at the expense of processes that determined survivorship (e.g. maintenance). On the contrary, the mortality rate tended to be lower in the fast-growing selected line than in the slow-growing control line. We admit, however, that this finding might not be conclusive because, for logistical reasons, we only measured juvenile mortality, in laboratory conditions, under high food levels and in the absence of natural enemies. Adverse effects of increased growth early in life are often not evident until much later (Metcalfe and Monaghan, 2001; Monaghan and Haussmann, 2006); a fuller understanding of the costs of growth in snails would require analyses of lifespan and mortality under unfavorable conditions (infections, starvation, dehydration, hypothermia, estivation). For example, the impairment of shell production in the selected snails suggests their higher susceptibility to water loss through the shell, and less ability to withstand predatory attacks.

Growth rate and metabolic scaling

Expenditure for growth processes can constitute a significant part of total metabolism: in the toad *Bufo bufo* it reaches 60% of total metabolism (Jörgensen, 1988). Given that in fast-growing organisms the expenditure for biosynthesis and tissue deposition increases, and that the growth rate changes proportionally to body size, total metabolism is predicted to scale isometrically or almost isometrically with body mass in fast growers, and negatively allometrically in slow growers (Wieser, 1994; Riisgård, 1998). Reviving Bertalanffy's idea (von Bertalanffy, 1957), Glazier (Glazier, 2005) used this concept to distinguish four major types of intraspecific metabolic scaling, and he argued that much of the variability of metabolic scaling between organisms (individuals, species and higher taxa) can be explained by the effects of evolution of differential growth rates. For example, according to Glazier (Glazier, 2006), isometric metabolic scaling prevails in pelagic species because they evolved fast growth, whereas negative allometry dominates in benthic species growing relatively slowly. The results of our analysis of metabolic scaling in *H. aspersa* snails are generally consistent with the concept of coupling between growth rates and metabolic scaling. The size-dependence of metabolism was isometric or almost isometric in the fast-growing, early ontogenetic stages of snails (b=1.03 in the size-selected line, b=0.92 in the control line), and it was negatively allometric in the slow-growing late stages (b=0.74 in the size-selected line, b=0.75 in the control line) (Table 4, Fig. 3A,B); the ontogenetic shallowing of metabolic scaling was statistically significant in the selected but not in the control line (Table 3). The biphasic metabolic scaling detected in *H. aspersa* resembles Glazier's Type III, and it has been reported in a wide range of different organisms including copepods, marine invertebrates, insects, fish and mammals (Brody, 1945; Epp and Lewis, 1980; Muthukrishan and Pandian, 1987; Post and Lee, 1996). For example, the metabolism of fast-growing *Mytilus edulis* larvae and juveniles increased almost isometrically with size (b=~0.9) and negatively allometrically (b=~0.7) in slow-growing adult mussels (Riisgård, 1998). Our data also point to the importance of the growth rate in explaining the between-organism variability of the mass exponent for metabolism: scaling of metabolism was steeper in the fast-growing selected line than in the slow-growing control (Table 3, Fig. 3C; note that this difference persists only early in ontogeny).

Size-scaling of metabolism can be obscured by the allometry of metabolically inert biomass such as reserve and skeletal material (Glazier, 1991). For example, in the amphipod *Gammarus fossarum*, whose proportion of metabolically active protoplasm decreases while the proportion of metabolically inert chitin increases with body size, metabolism scaled to the power of 0.65 with whole body mass, but to the power of 0.95 in relation to protoplasm (Simčič and Brancelj, 2003). This demonstrates that an examination of coupling between metabolic scaling and growth rates in molluscs should incorporate changes in shell mass. Our data showed that the shell mass of *H. aspersa* snails constituted up to 68% of whole body mass; its size-scaling differed between the early and late phases of snail development (Table 3, Fig. 3D,E). Shell mass scaled to the power of 0.79 (selected line) and 0.72 (control line) with flesh mass in the fast-growth phase, and to the power of 0.98 and 1.02 in the slow-growth phase (Table 4), which means that the proportion of metabolically inert shell decreased with body size during early growth and remained approximately constant throughout the remainder of life. Although this suggests that shell size-scaling may have affected our assessment of scaling of

metabolism in the early (but not in the late) ontogenetic stages, the exponents for *H. aspersa* metabolism derived from the regressions of metabolism *versus* shell-free mass resembled the estimates calculated from the regressions of metabolism *versus* whole mass (Table 4). This result is similar to earlier findings (Simčič and Brancelj, 2003) that the metabolism of the amphipod *G. fossarum* scaled at similar rates with whole and with non-chitinous mass, despite the increase in the proportion of metabolically inert exoskeleton with body size. Our comparison of the size-scaling of shell in selected *versus* control lines of *H. aspersa* (Tables 3, 4, Fig. 3F) revealed that scaling in the two lines was similar when analyzed in late ontogenetic stages, but early in ontogeny the proportion of shell mass increased faster with body size in the selected than in the control line. Note that this difference cannot account for our finding that the metabolism of selected snails scaled faster with whole body mass than in the control. Just the opposite: such a difference should sharpen the between-line difference in metabolic scaling when data on shell-free mass are considered instead of data on whole mass, and we found such a tendency (Table 3). Overall, removing the effects of metabolically inert shell mass did not change the general picture of size-scaling of metabolism derived from the analysis based on the whole mass of snails (Table 3). This strengthens the primary evidence on the role of growth rates in explaining variability of metabolic scaling in *H. aspersa* snails.

Linking metabolic scaling, growth and cell size – future prospects

Altogether, our data showed that the size-scaling of metabolism in *H. aspersa* snails was isometric or nearly isometric, and significantly steeper than 2/3 and 3/4 size-scalings early in ontogeny, and became shallower and not different from the 2/3 and 3/4 scaling modes later in life (Table 4). These findings complement emerging evidence on the variability of the mass exponent for metabolism in nature (e.g. Kozłowski et al., 2003; Glazier, 2005; Glazier, 2006). The new research challenges the traditional conviction that 2/3 or 3/4 size-scalings are the norm, a view still dominating currently developed theories in ecology (Brown et al., 2004), and raises the question of the mechanisms explaining this diversity (Glazier, 2005; Chown et al., 2007). Our results favor the view that some part of this variability can be linked to variable growth rates, but we stress that this concept in its original form (*sensu* Wieser, 1994; Riisgård, 1998) overlooks the potential metabolic consequences of cellular processes associated with growth rate changes. Most organisms increase body size mainly through cell proliferation (hyperplasia) during early postembryonic development and thus with relatively little change in average cell size, but later in life mainly by cell growth and/or hypertrophy (Falconer et al., 1978; Atchley et al., 2000; Glazier, 2005). Given that larger cells require less energy per protoplasm volume than smaller cells for maintenance of ion gradients across cell membranes (Davison, 1955; Kozłowski et al., 2003), cellular changes through ontogeny alone should lead to nearly isometric scaling of metabolism early in life and to negative allometry of metabolism later in life, a phenomenon already postulated (Kayser and Heusner, 1964) and quoted (Medrano and Gall, 1976b). Thus, it is reasonable to suggest that biphasic size-scaling of metabolism is produced by the joined effects of ontogenetic shifts in energy expenditure for growth and changes in the relative roles of hyperplasia and hypertrophy in ontogenetic growth. Changes in the size and number of cells are also known to underlie responses to selection for growth traits (Medrano and Gall, 1976a; Falconer et al., 1978; Stevenson et al., 1995; Atchley et al., 1997; Atchley et al., 2000; Calboli et al., 2003). Partridge et al. (Partridge et al., 1999), for example, demonstrated that lab evolution of larger *Drosophila melanogaster* proceeded mainly through increasing cell number, and an evolutionary decrease in size was achieved mostly by reduction of cell size. Such cellular changes should contribute to coupling between growth rates and metabolic scaling on evolutionary scales, but this issue remains largely unexplored. In line with that view, Glazier (Glazier, 2005) suggested that dissimilar cellular mechanisms of ontogenetic growth could be a proximate explanation of shallower metabolic scaling in nematodes (0.677) than in squid species (~1): hypertrophy characterizes nematodes and hyperplasia prevails in squids. Similarly, Chown et al. (Chown et al., 2007) demonstrated that in ant species where changes in cell size are a main proximate mechanism explaining intraspecific variation in body size, metabolic rate scales isometrically with body size, whereas in the species where cell size does not contribute to body size variation, the scaling becomes negatively allometric. On a higher taxonomic level, Kozłowski et al. (Kozłowski et al., 2003) showed that differences in interspecific scaling of the basal metabolic rate between orders within mammals and birds are linked to differential size-scaling of genomes. Thus, on a macroevolutionary scale, cell size appears to change, at least in part, through alterations in the amount of DNA packed in nuclei, and the cellular outcome of this evolution can influence interspecific size-scaling of metabolism. We stress that future studies should reconcile the cellular and physiological (1–3) mechanisms associated with growth rate evolution, and should investigate their role in the origin of metabolic scaling variability on different levels of biological organization. Successful integration of these phenomena promises evolutionary explanations of different large-scale phenomena such as Bergmann's rule in ectotherms or patterns in interspecific body size distributions and life histories (Kozłowski and Weiner, 1997; Kindlemann et al., 1999; Kozłowski and Gawelczyk, 2002; Angilletta and Dunham, 2003; Kozłowski et al., 2003; Kozłowski et al., 2004).

LIST OF SYMBOLS AND ABBREVIATIONS

a	metabolic rate coefficient in Eqn 1
$AGE_{inflect}$	family-specific age at which growth rate attains maximum value (GR_{max}); inflection point in the logistic growth equation (Eqn 2) at M=0.5M_A
b	mass-scaling exponent for metabolism in Eqn 1
C	family-specific daily food consumption of an average snail
GE	family-specific growth efficiency; GR:C ratio
GR	family-specific daily growth rate of an average snail
GR_{max}	family-specific measure of maximum individual growth rate; derivative of logistic growth equation at M=0.5M_A
k	growth rate coefficient in logistic growth equation (Eqn 2)
M_A	asymptotic body mass in logistic growth equation (Eqn 2)
M_b	body mass in Eqn 1 and Eqn 2; M, whole body mass of an individual snail
M_F	flesh mass of an individual snail
$\dot{M}_{O_2}$	metabolic rate in Eqn 1; average hourly oxygen consumption of a snail
M_S	shell mass of an individual snail
M_0	initial body mass in logistic growth equation (Eqn 2)
t	snail age

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