

NATURAL SELECTION REDUCES ENERGY METABOLISM IN THE GARDEN SNAIL, *HELIX ASPERSA* (*CORNU ASPERSUM*)

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Phenotypic selection is widely recognized as the primary cause of adaptive evolution in natural populations, a fact that has been documented frequently over the last few decades, mainly in morphological and life-history traits. The energetic definition of fitness predicts that natural selection will maximize the residual energy available for growth and reproduction, suggesting that energy metabolism could be a target of selection. To address this problem, we chose the garden snail, *Helix aspersa* (*Cornu aspersum*). We performed a seminatural experiment for measuring phenotypic selection on standard metabolic rate (SMR), the minimum cost of maintenance in ectotherm organisms. To discount selection on correlated traits, we included two additional whole-organism performance traits (mean speed and maximum force of dislodgement). We found a combination of linear (negative directional selection, $\beta = -0.106 \pm 0.06$; $P = 0.001$) and quadratic (stabilizing selection, $\gamma = -0.012 \pm 0.033$; $P = 0.061$) selection on SMR. Correlational selection was not significant for any possible pair of traits. This suggests that individuals with average-to-reduced SMRs were promoted by selection. To the best of our knowledge, this is the first study showing significant directional selection on the obligatory cost of maintenance in an animal, providing support for the energetic definition of fitness.

KEY WORDS: Directional selection, energy budget, fitness component, performance, standard metabolic rate, survival.

Historically, natural selection has been recognized as the main mechanism explaining adaptive evolution (Endler 1986). This claim is supported by extensive empirical studies on morphological (e.g., Janzen 1993; Blanckenhorn et al. 1999; Barbraud 2000; Jann et al. 2000; Preziosi and Fairbairn 2000; Merila et al. 2001; Haerty et al. 2003; Garant et al. 2004; Young et al. 2004); and life-history traits (Reznick et al. 1990; Sinervo and Huey 1990; Lindén et al. 1992; Sinervo et al. 1992; McGregor 1996; Reznick et al. 1996; Fox 2000; Sinervo et al. 2000; Mauck et al. 2004; Brommer et al. 2005; Reznick and Ghalambor 2005) in plants and animals. However, a somewhat unexplored topic in evolutionary biology is the origin of physiological adaptations, for which the demonstrations of phenotypic selection are scarce (Endler 1986; Conner 2001; Hoekstra et al. 2001; Kingsolver et al. 2001; Kingsolver and Pfennig 2007), with some exceptions such as starvation and ethanol resistance in *Drosophila* (Rion and Kawecki 2007; Fry

et al. 2008) or the effects of the corticosterone on survival in lizards (Comendant et al. 2003; Meylan and Clobert 2005).

One of the most ecologically relevant physiological traits is maintenance metabolism (i.e., basal metabolic rate for endotherms and standard metabolic rate (SMR), for ectotherms), the minimum energy requirements to be alive, and also an important part of the overall energy budget (Hammond and Diamond 1997; McNab 2002). In fact, in some ectotherms, SMR can account for up to 50% of an individual's total daily energy expenditure (Congdon et al. 1999). Not surprisingly, physiological ecologists have expended considerable effort in studying energy metabolism in a number of animal species and situations (Hinds et al. 1995; Dohm et al. 2001; Mueller and Diamond 2001; Addo-Bediako et al. 2002; McNab 2002; Speakman et al. 2003; White et al. 2007). However, despite the apparent importance of energy metabolism and its likely effect on fitness (Konarzewski

et al. 2005; Cano and Nicieza 2006), few studies have attempted to establish this link directly, yielding mixed results (Hayes and O'Connor 1999; Jackson et al. 2001).

Certainly, the many logistical obstacles that the measurement of physiological and performance traits entail, combined with the requirements of phenotypic selection experiments (i.e., large sample size, permanent individual identification, good recapture success, low emigration probability) may well explain the few phenotypic selection studies on physiological traits in the literature. For this reason, in this study we performed a semi-natural experiment using an especially amenable model organism for phenotypic selection studies, the common garden snail (*Helix aspersa*). We tested two contrary arguments that could be invoked to infer the relationship between SMR and fitness. First, SMR could be considered a cost affecting survival, which is a consequence of the energetic definition of fitness (i.e., individuals with higher maintenance costs have less energy surplus to allocate to other functions) (Sibly and Calow 1986; Brown et al. 1993; Bochdansky et al. 2005). Thus, it is predictable that directional selection will act to reduce the magnitude of SMR. Second, SMR could be considered as a byproduct of other enhanced whole-organism physiological traits that increase fitness (i.e., individuals with higher whole-organism capacities also exhibit higher maintenance costs because of the larger metabolic machinery that these capacities require) (Alexander 1999; Le Galliard et al. 2004). In this case, correlational selection augmenting the combination of SMR and one or more performance traits would be expected. With the goal of testing these hypotheses, we captured, marked, and measured juvenile individuals of the land snail, *H. aspersa*. We recorded SMR, and whole-organism performance traits such as mean speed (MS) and maximum force of dislodgment (MFD). We quantified selection on these traits by evaluating survival of the snails in semi-natural enclosures, during their active season (spring-summer). We also determined selection on a proxy of body size.

Material and Methods

SAMPLING AND MAINTENANCE OF ANIMALS

Two hundred eighty-five juvenile individuals of the land snail *H. aspersa*, of approximately the same body size (mass: 4.26 ± 0.77 g, approximate age: less than one year) (Madec et al. 2000) were collected from under plants and rocks in public parks in two localities: Valdivia (39°48'S, 73°14'W; 9 m) and Viña del Mar (33°4'S, 71°31'W; 5 m) (central-Southern Chile). Juveniles of this species can be easily identified by the absence of a thickened lip at the shell aperture, called the peristome, which indicates adulthood (Czarnoleski et al. 2008). Prior to trait measurement, individuals were acclimatized for one month to standard condi-

tions (20°C, 12:12 light:dark) and fed with maize flour and rabbit pellets. Relative humidity was maintained at high levels by sprinkling the interior of the boxes with water, daily. Each individual was marked with a small plastic numbered tag and no individual became reproductive during the study period.

TRAIT MEASUREMENTS

The main trait that we measured was SMR, which is defined as the obligatory energy cost required to support basic life functions (McNab 2002), and must be measured in animals at rest and in a post absorptive state (without digestion, see below) (Willmer et al. 2000). In this study SMR was measured as the rate of CO₂ production within an open system as described in Nespolo et al. (2007). Each animal was measured over a 45-min period, but SMR was estimated as the total average of the last 30 min of each recording. Respirometric trials were performed during the day, which corresponds to the resting phase in this species (Bailey 1975; Attia et al. 1997). In addition, animals were deprived of food for 18 h prior to measurement, which was enough time to attain a postabsorptive state based on preliminary measurements of mean retention time in this species (P. Artacho, T. Catalán, and R.F. Nespolo, unpubl. ms.). A postabsorptive state is necessary because of the heat increase caused by feeding due to the specific dynamic action of digestion (Lighton 1989; Bradley et al. 2003; Nespolo et al. 2005). Also, animal activity was visually monitored at intervals of ca. 10 min. The few recordings of animals that were active during the metabolic measurements were discarded.

In brief, CO₂ production was measured continuously with an infra-red CO₂ analyzer (LI-COR LI6262, Lincoln, NE) capable of resolving differences of 1 part per million of CO₂ in air (Nespolo et al. 2007). The analyzer was calibrated periodically against two kinds of gas (CO₂-free air, and a commercial mix of 291 PPM of CO₂). Although there was almost no drift between calibrations, we performed baseline measurements before and after each recording. The arrangement of the respirometry system was as follows: air at 120 mL min⁻¹ was pumped sequentially through a drierite-soda lime-drierite column, a flow meter that maintained flow rate within ± 1% of the desired rate, and a transparent respirometry chamber with a volume of 60 mL. Each animal was located in a different, isolated metabolic chamber. We had eight simultaneous metabolic chambers (three of which were empty, as blanks). All metabolic trials were performed at 20°C, the mean daily temperature in the field.

Given that the analyzer provides parts-per-thousands, and SMR should be reported as a rate, we transformed the recordings as follows. From the respirometric recordings and based on the configuration of the system (i.e., flowmeter was upstream from

the chamber and both, CO₂ and water are scrubbed), we computed the following variables (see Withers 1977):

$$\text{VCO}_2 (\text{ml CO}_2 \text{ min}^{-1}) = (\text{FeCO}_2 \times \text{FR}) / [(1 - \text{FeCO}_2) \times (1 - 1/\text{RQ})]$$

Where VCO₂ = rate of CO₂ production; FeCO₂ = excurrent fractional concentration of CO₂; FR = flow rate (mL min⁻¹); RQ = respiratory quotient, assumed equal to 0.85 in herbivorous animals (Rogowitz and Chappell 2000).

In addition to energy metabolism, we measured traits that putatively represent performance in an organism such as mean speed (MS) and maximum force of dislodgement (MDF). MS was estimated by measuring the mucus track left by each individual on a dark surface over a 10-min period. Before each measurement the surface was cleaned to avoid the probable inhibition of locomotion provoked by the mucus left by the previous individual (Baur 1988). Mucus tracks were measured immediately after each trial with a metric tape (± 10 mm). MDF was evaluated, by means of a 400 g dynamometer, as the force necessary to detach the individual from the substrate. One hour before the measurements a small length of nylon thread was attached to the top of the shell with a drop of glue. Individuals were then placed on a glass surface to induce maximum adhesion (Trussell 1997). A dynamometer was quickly connected to the thread on the snail's shell and slowly pulled up until the individual detached from the substrate. All measurements were performed at 20°C and with a prior fasting period of 18 h, and were assessed at the beginning of the active cycle (i.e., night) (Bailey 1975; Attia et al. 1997).

Finally, two proxies of body size (body mass, Mb, and shell size, SS), plus foot length (FL) and width (FW) were estimated using digital images. Each individual was measured for SMR, performance, and morphological traits during the same period (SMR during day, performance and morphology in the beginning at night). Because the shell measures and body mass were correlated (body mass–shell height: $r = 0.17$, $P = 0.03$; body mass–shell length: $r = 0.18$, $P = 0.02$; body mass–shell aperture: $r = 0.24$, $P = 0.002$; shell height–shell length: $r = 0.97$, $P < 0.001$; shell height–shell aperture: $r = 0.79$, $P < 0.001$; shell length–shell aperture: $r = 0.87$, $P < 0.001$), we summarized the variation of these variables into principal components, and for the selection analysis we used only the first component (variance explained by the first component = 70.47%), hereafter referred to as Body size. Because individuals were preselected to be of approximately similar body masses, this variable had a low (non-significant) correlation with all traits. Analyses were performed on both, residuals from linear regressions with body mass and on trait values, and results were identical.

FIELD WORK

At the beginning of the spring, we released all the measured snails in three replicated field enclosures of 64 m² (100 snails per enclosure) with a density of 1.6 snails per m², closely mimicking the density and habitat of the original populations (i.e., enclosures contained the same plants found at the collection sites). We determined the survival in the field enclosures at the end of the summer of 2007 (seven months later). Thus, the period in which we performed the selection experiment corresponds to the end of hibernation and the beginning of the active season. One experimental enclosure was eliminated from the study because it was attacked by rats in the first month of the experiment.

After release, we exhaustively sampled each experimental enclosure monthly to collect dead snails (empty shells). The experiment ended in March (end of the austral summer) when all snails were captured (dead or alive) using specific snail bait to increase the probability of capturing living snails. Finally, the complete cover of plants and grass was removed and all shell remains were collected. Estimates of survival were based on recapture data under the assumption that nonrecaptured individuals actually died. In other species, such as birds (Sheldon et al. 2003), mammals (Hayes and O'Connor 1999) and reptiles (Sinervo et al. 2000) this is a critical issue because animals migrate, reducing the statistical power of the selection analysis. This fact obligates the marking, measuring, and releasing of great numbers of individuals (thousands, to detect nonlinear selection). In our case, we could collect and identify most of the dead individuals ($N = 74$) due to the tagged empty shells. Still, a small number of shells were found destroyed ($N = 32$) and it was impossible to identify their numbers. However, the home range of terrestrial snails is less than 100 m² (Aubri et al. 2006) and we did not find a single individual (dead or alive) outside the enclosures during the seven months of sampling.

STATISTICS

Absolute fitness was measured as a binary variable: 1 surviving snails and 0 dead snails (Brodie and Janzen 1996). The statistical analysis has two parts, parameter estimation and significance testing (see also Fairbain and Reeve 2001). First, linear, nonlinear, and correlational selection gradients were estimated using linear regression models (Lande and Arnold 1983; Brodie and Janzen 1996) for each enclosure separately, and for pooled data with survival as the dependent variable; SMR, MS, MDF, and body size as independent variables; and locality (two levels) and enclosure (two levels) as categorical factors. Multivariate gradients estimate the strength of selection acting directly on each trait when the indirect effects of selection acting on the other traits have been removed. Linear and nonlinear selection gradients estimate the direction and magnitude of directional selection, and stabilizing and/or disruptive selection, respectively. Correlational

selection, however, describes the selection on increasing positive or negative covariance between two traits. Second, because our fitness estimator is a binomial variable (alive or dead); the statistical significance of the association between survival and phenotypic traits was tested with a logistic regression (Brodie and Janzen 1996), with a logit link function (Statistica, version 6.1, StatSoft, Tulsa, OK). All phenotypic traits were standardized to mean 0 and variance 1 prior to analysis. We present the statistical probabilities derived from the χ^2 statistic of the logistic analysis. The surface of selection (i.e., shape) was estimated by univariate cubic splines also on standardized traits, using the program glms ver.4.0 (Schluter 1988). Finally, we compared the SMR between live ($N = 79$) and dead ($N = 106$, including destroyed and nonidentified shells) snails using t -tests.

Results

The logistic regressions for the two experimental enclosures were similar (the linear selection gradient was significant) for SMR. However, for the other performance traits (i.e., MFD and MS) both enclosures exhibited similar trends, but with nonsignificant

Table 1. Results from the analysis of selection using a logistic regression on pooled data from two field enclosures. Analyses were performed on standardized traits (mean 0, standard deviation 1). The linear term (β_i) evaluates the magnitude of directional selection; quadratic term (γ_i) estimates the impact of stabilizing/disruptive selection; whereas the cross-product (γ_{ij}) indicates correlational selection between pairs of traits. The statistical probabilities derived from χ^2 of the logistic analysis are presented. The significant gradients are indicated in bold.

Variables	Estimates (\pm SE)	χ^2	P
Locality	–	3.838	0.147
Field enclosure	–	0.564	0.453
Locality \times Field enclosure		2.230	0.328
SMR (β_i)	-0.106 ± 0.059	10.203	0.001
MS (β_i)	-0.089 ± 0.051	2.120	0.145
MFD (β_i)	0.086 ± 0.059	2.351	0.125
Body size (β_i)	0.060 ± 0.045	1.973	0.160
SMR (γ_i)	-0.012 ± 0.033	3.515	0.061
MS (γ_i)	0.07 ± 0.045	3.052	0.081
MFD (γ_i)	0.037 ± 0.051	0.964	0.326
Body size (γ_i)	-0.008 ± 0.034	0.026	0.872
SMR–MS (γ_{ij})	-0.054 ± 0.059	0.006	0.937
SMR–MDF (γ_{ij})	-0.003 ± 0.048	0.539	0.463
SMR–Body size (γ_{ij})	0.041 ± 0.052	1.439	0.230
MS–MDF (γ_{ij})	0.048 ± 0.062	1.342	0.247
MS–Body size (γ_{ij})	-0.038 ± 0.046	0.561	0.454
MDF–Body size (γ_{ij})	0.002 ± 0.047	0.051	0.821

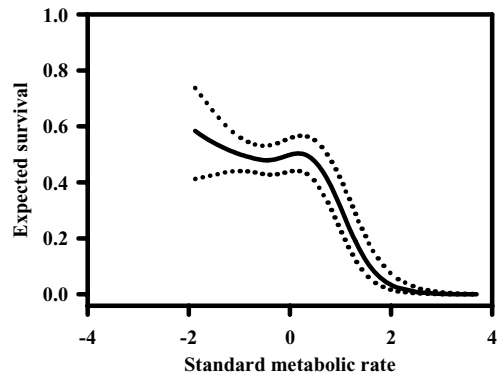


Figure 1. Selection surface showing the relationships between survival probability and standard metabolic rate. Dotted lines represents 95% confidence intervals estimated from 1000 bootstrap replications.

regressions. In other words, the selection gradient over SMR was strong enough to be detected in each enclosure separately. To improve the statistical power we pooled the data from both enclosures, and this logistic regression showed that neither experimental enclosure nor locality had a significant effect on the variable survival (Table 1). Our principal finding indicates that SMR experienced a combination of negative directional and, stabilizing selection (Fig. 1, Table 1). The average of SMR of live snails was 20% smaller ($x \pm$ SE: 0.16 ± 0.008 mLCO₂ h⁻¹) than that of dead snails (0.20 ± 0.006 mLCO₂ h⁻¹; $t_{183} = 3.39$; $P < 0.0001$). On the other hand, neither MS, MDF nor body size were significantly associated with the survival (Table 1). Correlational selection was not significant for any possible pair of traits (Table 1). Because body mass variation was controlled experimentally, by choosing land snails of approximately same body size, there was no change in the results after removing this variable from the analysis, or applying statistical control to it (i.e., using residuals).

Discussion

A basic tenet in evolutionary physiology is the energetic definition of fitness, which predicts that natural selection maximizes the residual energy available for growth and reproduction (Sibly and Calow 1986; Brown et al. 1993; Bochdansky et al. 2005). Hence, in the absence of compensations in other capacities, high maintenance costs would be synonymous with low fitness. This assumption has become an untested dogma in evolutionary physiology (see Bochdansky et al. 2005), but recently Czarnoleski et al. (2008) approached it elegantly by studying *H. aspersa* snails using artificial selection. These authors found that the main correlated response to artificial selection for large body size was a reduction in energy metabolism in juvenile stages, suggesting that terrestrial snails are severely constrained by energy, especially

during growth. Actually, the energy budget of land snails is seriously constrained by at least two expensive functions. First, the mode of locomotion of slugs and snails is one of the most costly in nature (Denny 1980). Second, and specifically in the immature stages of *H. aspersa*, the cost of shell production has a considerable impact on the overall energy budget (Czarnoleski et al. 2008). Also, Bochdansky et al. (2005) provided indirect evidence suggesting selection against high metabolic rates in juvenile fish maintained in laboratory conditions, by measuring otolith hatch check area as a proxy of metabolic rate. Here we provide direct evidence of natural selection toward reducing SMR in juvenile terrestrial snails, which would ultimately support the energetic definition of fitness. Actually, our results suggest that individuals with average-to-reduced SMRs are promoted by selection. We found a linear selection coefficient of -0.106 , which means that a decrease of one standard deviation in metabolism is associated with an increase of 11% in relative fitness (Conner 2001). Even assuming a conservative value for heritability for an ectotherm's energy metabolism (e.g., 0.35) (Rantala and Roff 2006), and no indirect selection, this magnitude of selection would cause the trait to be reduced by one standard deviation in only 26 generations (Conner 2001), and would support the expectation of adaptive shifts toward lower standard metabolism in *H. aspersa*.

To test our second hypothesis: maintenance metabolism is a byproduct of other enhanced whole-organism physiological traits, we included additional traits related to whole-organism performance (MS and maximum force of dislodgement, MFD) in the selection analysis. However, we did not find any evidence of correlational selection on any combination of these traits with SMR. In addition, body size exhibited neither a significant gradient nor correlation with SMR, which discounts the influence of this variable on our results.

Why would snails with high metabolic rates die? We believe that the reasons stem from the fact that *H. aspersa* is highly constrained by energy allocation patterns. As noted before, locomotion and shell production are both very expensive processes in land snails (Czarnoleski et al. 2008). Therefore, individuals with high SMR could have less surplus energy to assign to other processes, compromising functions such as growth (Steyermark 2002) or immunocompetence (Freitak et al. 2003; Ksiazek et al. 2007). Also, a high SMR would represent a higher energetic budget that necessitates more time to be spent foraging, elevating predation risk. However, there should be a limit in the decrease of SMR because animals need a minimum level of energy for maintenance. This is supported by the fact that stabilizing selection on SMR was also detected.

We are aware that we evaluated only one component of fitness (survival), which does not indicate anything with respect to fecundity. However, we selected pre-reproductive individuals to partially unify both survival and fecundity. In other words, indi-

viduals that died in this phase also have zero fecundity because they did not attain maturity. Therefore, the differential survival of pre-reproductive individuals may be an important source of variation in lifetime reproductive success. Thus, it is predictable that characters that influence the survival of juveniles in natural populations could be under strong selection (Civantos and Forsman 2000).

The fact that we found a combination of negative directional and stabilizing selection on SMR is another interesting point emerging from this study. The only two studies that have attempted to test if there is a link between metabolic rate and fitness (measured as survival) have shown a positive relationship between these two variables (Hayes and O'Connor 1999; Jackson et al. 2001). This is in agreement with other studies showing that selection would favor high levels of performance (or positive directional selection), and in a few cases would favor disruptive or stabilizing selection (Bennett and Huey 1990; Husak et al. 2006; Irschick et al. 2008). However, few if any studies have explored the direction of selection in traits that represent costs. In this case, negative directional selection would be expected, as we found in land snails.

In terms of the magnitude of selection, our linear selection gradient for SMR was $\beta = -0.106$, which is lower than the overall median of $\beta = 0.16$ reported in Kingsolver et al. (2001) for morphological and life-history/phenological traits. On the other hand, quadratic selection gradients seem to be typically lower in magnitude (overall median $\gamma = 0.10$, Kingsolver et al. 2001), and our data are in agreement with this because our quadratic coefficient was only $\gamma = -0.012$.

In summary, our principal result suggests that physiological traits in animals could be the target of current natural selection, in this case for maintenance costs, optimizing the energy budget in animals (Alexander 1999). One question still to be answered however is, to what extent are these metabolic costs and organismal capacities, translated across the life table, ultimately reflected in the net reproductive rate (i.e., absolute fitness)?

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