

# Mechanism of a plastic phenotypic response: predator-induced shell thickening in the intertidal gastropod *Littorina obtusata*

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evolutionary potential;  
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green crab;  
*Littorina obtusata*;  
*Carcinus maenas*.

## Abstract

Phenotypic plasticity has been the object of considerable interest over the past several decades, but in few cases are mechanisms underlying plastic responses well understood. For example, it is unclear whether predator-induced changes in gastropod shell morphology represent an active physiological response or a by-product of reduced feeding. We address this question by manipulating feeding and growth of intertidal snails, *Littorina obtusata*, using two approaches: (i) exposure to predation cues from green crabs *Carcinus maenas* and (ii) reduced food availability, and quantifying growth in shell length, shell mass, and body mass, as well as production of faecal material and shell microstructural characteristics (mineralogy and organic fraction) after 96 days. We demonstrate that *L. obtusata* actively increases calcification rate in response to predation threat, and that this response entails energetic and developmental costs. That this induced response is not strictly tied to the animal's behaviour should enhance its evolutionary potential.

## Introduction

Phenotypic plasticity, i.e. the ability of a particular genotype to produce different phenotypes in response to environmental variation (West-Eberhard, 1989; Thompson, 1991; Via *et al.*, 1995; Zhivotovsky *et al.*, 1996; DeWitt *et al.*, 1998; Pigliucci, 2005), has been the object of considerable interest and debate over the past two decades; recent reviews can be found in DeWitt & Scheiner (2004a), Pigliucci & Preston (2004), and Pigliucci (2005). However, plasticity was not part of the neo-Darwinian synthesis, and it has only recently been incorporated into theoretical models of evolutionary potentials and trajectories (see Sarkar, 2004 for an insightful historical overview). Furthermore, rigorous empirical tests concerning the adaptive value of plasticity remain rare (DeWitt & Scheiner, 2004b), and in very few cases are the mechanisms underlying plastic responses well understood (Windig *et al.*, 2004).

Inducible defences are amongst the best-documented and most taxonomically widespread examples of phenotypic plasticity; they are behavioural, morphological, or

physiological modifications generally induced by 'predator cues', and which increase resistance to predatory attacks (see review by Harvell, 1990). Examples of induced defences among animals in response to chemical cues released by a predator, or predatory event, include the production of larger spines in the colonial marine bryozoan *Membranipora membranacea* (Harvell, 1992), bending of the lateral plates of the barnacle *Chthamalus anisopoma* (Lively *et al.*, 2000), production of neckteeth by water fleas, *Daphnia* spp. (Tollrian, 1995), production of thicker and/or more ornamental shells by different species of mollusc (Appleton & Palmer, 1988; Palmer, 1990; Trussell, 1996; Cheung *et al.*, 2004), changes in tail and body colour and shape of amphibian tadpoles (McCollum & Leimberger, 1997; Van Buskirk & McCollum, 2000; Teplitsky *et al.*, 2003; LaFiandra & Babbitt, 2004; Morre *et al.*, 2004), and development of deeper bodies in crucian carp, *Carassius carassius* (Vøllestad *et al.*, 2004).

Predator-induced morphological defences have been documented in several species of intertidal gastropods (*Nucella lamellose*: Appleton & Palmer, 1988; *Nucella lapillus*: Palmer, 1990; *Littorina obtusata*: Trussell, 1996; *Littorina subrotundata*: Dalziel & Boulding, 2005). The specific cues responsible for these phenotypic changes are not known, but they are chemical in nature and related to the foraging activity of predators. In particular,

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experiments have shown that gastropods exposed to effluents from predatory crabs fed conspecific snails develop thicker, or more ornamented shells than those exposed to crabs that are starved or fed alternative types of food, which in turn develop better-defended shells than snails not exposed to any type of predation effluent (Appleton & Palmer, 1988; Palmer, 1990; Trussell & Nicklin, 2002). Similar phenotypically plastic responses have been documented in numerous species of invertebrates and vertebrates (Kats & Dill, 1998).

Very little empirical work has been done to elucidate the proximate control of induced defences in gastropod molluscs, or any other animal for that matter. One outstanding question, which has significant implications for the costs and evolutionary potential of this plastic response, is whether predator-enhanced shell thickness and ornamentation is an active physiological response to predation risk, or a developmental by-product of a behavioural change induced by predation cues (Palmer, 1990; Trussell, 1996; Trussell & Etter, 2001; Trussell & Nicklin, 2002). More specifically, perhaps predation cues cause an active increase in the rate at which calcium carbonate is deposited by the mantle into the gastropod's shell; if the rate of linear shell translation (i.e. increase of shell length along the axis of coiling) is unchanged, then this increased calcification rate will result in the production of a relatively thicker shell. Alternatively, predation cues may cause snails to reduce feeding activity, which in turn could cause a reduction in the rate of body growth and linear shell translation (e.g. Behrens Yamada *et al.*, 1998; Trussell *et al.*, 2003); if calcification rate remains unchanged, then this reduced shell elongation will cause more calcium carbonate to be deposited in any area of the shell parallel to the axis of translation. In support of this latter hypothesis, studies have shown that predation cues can reduce both snail grazing activity and linear shell translation (e.g. Behrens Yamada *et al.*, 1998; Trussell *et al.*, 2003), and that growth rate is intrinsically related to the shape (Kemp & Bertness, 1984; Boulding & Hay, 1993; DeWitt, 1998; Yeap *et al.*, 2001) and mass (Kemp & Bertness, 1984; Boulding & Hay, 1993) of gastropod shells. A third possibility is that both these mechanisms contribute to predation-induced increases in gastropod shell thickness.

The main objective of this study was to determine which of these mechanisms was responsible for the production of thicker and heavier shells by *L. obtusata* snails raised in the presence of effluents from green crabs, *C. maenas*, feeding on conspecific snails (Trussell, 1996; Trussell & Nicklin, 2002). There is evidence that this induced defence response is adaptive (see Discussion), and that it may be partly responsible for spatio-temporal patterns of variation in snail phenotype associated with the introduction of the European green crab *C. maenas* to the eastern coast of North America (Trussell, 1996, 2000; Trussell & Smith, 2000). We conducted a laboratory experiment in which snail growth was manipulated by

two different approaches: (i) exposure to predation cues (effluents from *C. maenas* feeding on *L. obtusata* snails) and (ii) varying food availability, and we compared patterns of linear shell growth and shell mass growth with patterns predicted (see Materials and methods) by the three different mechanisms of predator-enhanced shell thickening outlined above. We also quantified snail body mass growth, production of faecal material, as well as shell aperture area and micro-structural characteristics (mineralogy and organic fraction) to further investigate the benefits, costs and evolutionary significance of this plastic response. We demonstrate that *L. obtusata* actively increases its rate of shell material deposition in response to predation threat, and that this response entails both energetic and developmental costs. That this induced response is not strictly tied to, and hence constrained by, the animal's behaviour should enhance its potential to evolve by natural selection.

## Materials and methods

### Collection and initial morphological measurements

Small *L. obtusata* were randomly collected at mid-tide level (3.86 m above mean low water) from St Andrews (45°04'08.45"N, 67°02'14.59"W), NB, until ~400 snails 4.5–6.0 mm in shell length (see Trussell, 1996) were obtained. In the lab, we measured shell length of all experimental animals using digital callipers ( $\pm 0.01$  mm), and then shell mass and body tissue mass using a nondestructive weighing technique developed by Palmer (1982). This technique, which is based on the fact that the specific gravity of a snail's body tissue is similar to that of sea water, involves weighing each snail submerged in seawater and also in air, and then using regression equations, developed separately with sacrificed animals, to estimate their dry shell and body tissue masses, respectively, from these whole-snail mass measurements (Palmer, 1982). To obtain submerged masses, a Mettler AE240 balance ( $\pm 0.00001$  g) was placed above an aquarium filled with seawater maintained at approximately 18 °C, and a small weighting boat was suspended by a fine copper wire from the underside of the balance. Before being transferred to the weighing boat, each snail was chased into its shell while still underwater to remove any air bubbles that might be present within the shell, which would have affected the submerged mass estimate; we ensured the aperture remained full of water during transfer to the weighing boat, so no new air bubbles entered the shell. After the submerged mass was recorded, whole snail mass in air was ascertained by first air drying each snail for 3 h, then chasing the animal into its shell with tissue and removing any excess water, and finally weighing the whole snail in air.

In order to establish standard curves, an additional 20 snails 4.26–7.71 mm (size range slightly greater than that of experimental snails) were weighted using the same

procedure, then sacrificed and dried at 60 °C for 48 h to obtain actual dry shell mass and dry body mass. Dry shell mass was then regressed against submerged mass, and the resulting equation (dry shell mass =  $1.59 \times$  submerged mass +  $6.88 \times 10^{-5}$ ,  $n = 20$ ,  $r^2 = 0.999$ ,  $P < 0.001$ ) was used to estimate dry shell mass of all experimental snails, based on their submerged mass. Similarly, dry tissue mass of sacrificed snails was regressed against their wet tissue mass (whole mass in air – estimated dry shell mass) and the resulting equation (dry tissue mass =  $0.243 \times$  wet tissue mass –  $4.0 \times 10^{-5}$ ,  $n = 20$ ,  $r^2 = 0.969$ ,  $P < 0.001$ ) used to estimate dry body mass of experimental snails.

Following these measurements, snails were individually marked by gluing small coded tags (2 mm diameter) to their shells using epoxy glue and clear nail polish as a sealant; this allowed us to assess growth of individual snails.

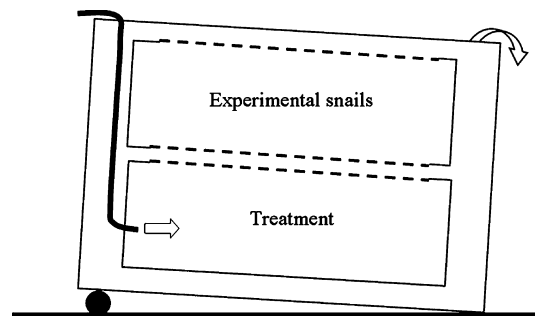
### Experimental design

Snails were randomly distributed among the following four treatments: a 'lab-control', in which snails had unlimited access to food and were not exposed to predation cues; two 'food-deprivation treatments', in which snails were not exposed to predation cues but had limited and varied access to food (i.e. 1 or 2 days of every 3-day feeding cycle); and a 'predation-risk treatment', in which snails had unlimited access to food, but were exposed to predatory effluents. Our design was not fully factorial because we were only interested in particular contrasts. There were four replicates of each treatment, and the experiment lasted 96 days.

The two food-deprivation treatments were used to cause reduced and variable growth rates in snails not exposed to predation cues, and hence enable particular contrasts with individuals from the predation-risk treatment. More specifically, under the 'growth rate hypothesis' snails that display reduced shell length growth are predicted to have a heavier shell at any given length than individuals that attained the same length more rapidly. Furthermore, and more importantly to the test of our hypothesis, shell mass of snails that displayed a reduced rate of shell elongation should be similar whether this reduction is caused by predator-avoidance behaviour (predation-risk treatment) or limited food availability (food-deprivation treatment), as long as the rate of shell elongation is similar between these treatments. However, and somewhat surprisingly (see Discussion), snails exposed to predation cues did not exhibit a significant reduction in linear shell translation relative to control individuals, and hence the food-deprived snails were not useful to discriminate between the two hypothesized mechanisms of predator-induced shell thickening. For this reason, and for the sake of brevity, we do not describe the methods and results pertaining to the food-deprivation treatments.

Trials were run in 3.8 L tanks ( $22 \times 14 \times 18$  cm), which housed two smaller containers ( $13 \times 6 \times 13$  cm) stacked on top of one another (Fig. 1). The top container held 15 experimental snails and their food (a handful of *Ascophyllum nodosum*), and had one  $10 \times 10$  cm hole cut in its lid and one in its bottom; both holes were covered with a fibreglass mesh screen ( $1.5 \times 1.3$  mm). The bottom container, which also had a mesh-covered hole in its lid, housed the different treatments (see below). All tanks were held on a seatable with flow-through seawater. Incoming water flowed through a silicon tube directly into the bottom container, at approximately  $0.1 \text{ L min}^{-1}$ , which ensured effluent propagation through both screen layers and into the upper container (Fig. 1). Every three days, all containers were opened and dead snails were removed (19 of 120 snails over course of experiment). At this time, all tanks were also rotated by one position on the seatable, in order to minimize potential lab-positional biases (e.g. varying light intensity, flow rate). Water temperature varied between 11 and 13.5 °C over the course of the experiment.

For the predation-risk treatment, one male crab (carapace width 35–40 mm) was housed in the bottom container and provided with five *L. obtusata* snails per day (same size range as the experimental snails); on average, crabs killed and consumed 4 snails per day. The experimental snails were not in physical contact with the crab, but were exposed to chemicals exuded by the predator and its foraging activity (see tank description above). Crabs were replaced with newly collected field crabs approximately every 15 days; individuals that moulted were replaced within 1 day.



**Fig. 1** Design of each 3.8 L treatment tank ( $22 \times 14 \times 18$  cm). Seawater entered from the tube on the left of the holding tank ( $\sim 0.1 \text{ L min}^{-1}$ ), flowed directly into the lower container ( $13 \times 6 \times 13$  cm), which housed the green crab fed *L. obtusata* snails in the predation treatment, then passed through mesh screening (hatched lines) to the upper container ( $13 \times 6 \times 13$  cm), which contained 15 experimental snails and their food, and finally drains off the right side of the tank. All tanks were tilted slightly so that water exited from the side opposite to where it entered, enhancing water circulation.

### Assessment of snail grazing activity

In order to determine whether predation cues affected the feeding activity of snails, we quantified production of faecal material by a sub-group of predation-exposed and control snails every sixth 3-day cycle over the course of the experiment (i.e. every 18 days, and a total of five times). For each day of a 3-day assessment cycle, we quantified faecal production of four randomly-selected snails (using individual ID codes) from each replicate container. The 32 snails (8 containers  $\times$  4 snails per container) that were assessed for faecal production on a particular day were placed individually in the wells of tissue culture trays containing fresh seawater and no food. After 48 h in the culture trays, all snails were returned to their respective tank and the number of faecal pellets each individual produced was counted. The faecal count values used for statistical analyses were obtained by averaging the number of faecal pellets produced by all 12 snails taken from the same replicate container over a given 3-day cycle (4 snails  $\times$  3 days). Therefore, each replicate container generated a single faecal count datum for each of the five faecal assessment periods.

### Final measurements

At the end of the 96-day experimental period, we quantified shell length, dry shell mass, dry body mass and inner aperture area of each experimental snail. In half of these we also quantified organic content of new shell material deposited during the experiment, and in the other half we quantified thickness of different mineral layers of the new shell.

#### *Body mass, shell mass and shell inner aperture area*

We quantified dry shell mass and dry body mass by dissecting each snail and drying shell material and soft tissues at 60 °C for 48 h. We measured inner aperture area by placing each shell under a dissection microscope so that the plane of the aperture was level with the microscope lens, and then taking a digital picture with Qcapture™ (version 2.66). Using OpenLab™ 3.0.4 software, we then traced the inner circumference of the aperture and calculated its total area (mm<sup>2</sup>). We quantified the inner, rather than the outer, area of the aperture because it is more likely to affect predation by shell-entry.

#### *Shell microstructure characteristics*

After dissections, snails were randomly divided in two groups for quantification of shell microstructure characteristics; half the snails from each of the 4 replicate cages of each treatment were used to determine the percentage of organic material in newly deposited shell material (control: 26 snails; predation-risk treatment: 25 snails) and the other half was used to determine the thickness of

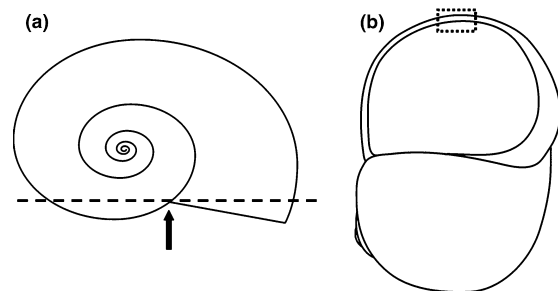
the irregular-prismatic and first cross-lamellar shell layers (control: 24 snails; predation-cue: 24 snails). The 21 snails that either died ( $n = 19$ ) during the experiment or had their shell damaged during final measurements ( $n = 2$ ) were not used in these analyses.

#### *Organic content of shell*

To determine whether predation risk affected the amount of organic material snails deposited in their shell, we first carefully clipped off, using fine needle-nosed pliers, the edge of the shell (~2.0–3.5 mm) up to a visible 'line of disturbance' on the snails' shell, which corresponded to the time they were brought in the lab. We then used a mortar and pestle to crush the shell samples into a fine powder, which we then wrapped in pre-weighed pieces of aluminium foil and dried at 60 °C for at least 18 h to obtain an estimate of dry shell mass. Samples were then ashed in a muffle furnace at 500 °C for 3 h, and re-weighed. The difference between the dry shell mass and the ashed mass, which reflects organic material being lost during ashing, was divided by dry shell mass to yield the percent organic content of each shell sample.

#### *Thickness of mineral shell layers*

To measure the thickness of the irregular-prismatic and first crossed-lamellar layers of shell material deposited during the experiment, we obtained a transverse section of the body whorl close to the shell aperture (Fig. 2a); note that our section does not contain the second cross-lamellar layer, which is deposited close to the shell's apex (see Taylor & Reid, 1990). Sectioning was done by first applying clear nail polish to the edge of the aperture, both inside and outside, to help reduce chipping. Then, shell edges were sanded using two weights of sand paper, first 600 grade, followed by 1500 grade. Shells were sanded by manually grinding the entire aperture against the sand paper until reaching the inner aperture in the vicinity of the suture between the new shell whorl and



**Fig. 2** Two views of a *Littorina obtusata* shell showing (a) the plane along which the cross-section was prepared (dash line) and (b) the approximate location where photos were taken to measure the thickness of different shell layers. The cross section was obtained by sanding the shell with sand paper until reaching the inner aperture in the vicinity of the suture between the new shell whorl and the old shell whorl (indicated by arrow).

the old whorl (Fig. 2a). The sanded surface was polished by gently rubbing it with Brasso®, a multipurpose metal polish, and then shells were rinsed in distilled water (Barroso *et al.*, 2005). Sectioned surfaces were soaked in Feigl's Solution for 7 min before being rinsed again with distilled water (adapted from Kido, 1996); Feigl's solution stains aragonite a dark metallic colour, but it does not stain calcite (Schneidermann & Sandberg, 1971), which enabled us to visualize the crossed-lamellar and irregular-prismatic shell layers, because in *L. obtusata* the former is made of aragonite and the latter of calcite (Taylor & Reid, 1990).

Stained shell preparations were positioned under a dissecting scope, and then a portion of the surface (see Fig. 2b) was photographed at 10× magnification using QCapture™. Photos were taken in the vicinity of the mid point of the arch of the aperture (Fig. 2b), ensuring that the fine crossed-lamellar layer had not been chipped in the area photographed. Each shell was re-positioned and re-photographed five times, to reduce biases related to shell orientation, as it was virtually impossible to position the section perfectly parallel to the plane of the camera. The thickness of both the cross-lamellar and irregular-prismatic layers were then measured using OpenLab™. For each of the five photographs, thickness of each shell layer was estimated in five areas evenly distributed across the photograph. For the analyses, we used the average of the 25 measurements made for each mineral layer and shell (5 images × 5 measurements per image).

## Statistical analyses

### *Mechanism of shell thickening: shell length and mass growth*

We first investigated the mechanism underlying the snail's induced defence by comparing shell length growth and shell mass growth between predation-risk and control snails (see Table 1 for predictions), where growth

**Table 1** Predicted variation in shell length growth and shell mass growth between snails grown in the presence (P) and absence (NP) of predation cues (see Materials and methods) under three hypothesized mechanisms of predator-induced shell thickening.

Shell trait	Calcification rate hypothesis	Growth rate hypothesis	Both hypothesized mechanisms
Shell length growth	P = NP	P < NP	P < NP
Shell mass growth	P > NP	P = NP	P > NP

Under the 'calcification rate hypothesis', predation-exposed snails are predicted to increase in shell length at a same rate, but in shell mass at a faster rate, than snails in control group. Under the 'growth rate hypothesis', predation-exposed snails are predicted to display reduced shell length growth, but similar increments in shell mass, as snails in control group. A third possibility is that both mechanisms contribute to enhanced shell thickening in snails exposed to predation cues.

was estimated as trait value at the end of the experiment minus its value at the beginning of the experiment. We analysed variation in shell length and shell mass growth separately using nested ANOVAs, in which treatment was a fixed-effect factor and replicate cages were nested within treatment. In these and all subsequent nested ANOVAs, the MS for the nested term was used, instead of the model error term, to compute the *F*-ratio of the treatment factor to reflect true level of replication (i.e. the cage, and not individual snails) for this treatment (Zar, 1999). Assumptions of normality and homoscedasticity for these, and all other analyses, were tested on model residuals using the Shapiro-Wilk *W* and Levene tests, respectively.

To further investigate the mechanism underlying the snail's induced defence, we performed a nested ANCOVA in which shell mass growth was the dependent variable, treatment was a fixed-effect factor, shell length growth was the covariate and replicate cages were nested within treatment. The data were log transformed in order to meet model assumptions. The model was first run with the interaction term between the covariate and treatment; however, as  $P > 0.25$  for this interaction, it was removed in order to simplify the model (Hendrix *et al.*, 1982).

### *Body mass growth*

We analysed variation in body mass growth (body mass at end of experiment minus mass at beginning) using a nested ANOVA, in which treatment was a fixed-effect factor and replicate cages were nested within treatment. The residuals of this analysis violated model assumptions ( $W = 0.966$ ,  $P = 0.053$ ;  $F_{1,105} = 6.826$ ,  $P = 0.010$ ), and no transformation remedied this situation. We nevertheless interpret results of this analysis, because ANOVAs are robust to such small assumption violations (Zar, 1999), and the observed effect (i.e. differences in body mass growth between predation-risk and control snails) was large and highly significant.

### *Faecal production*

The effect of predation risk on faecal production was analysed using a mixed-model ANOVA, in which the dependent variable was the number of faecal pellets produced per snail in 48 h, treatment was a fixed-effect factor, and assessment cycle (i.e. time block) was a random-effect blocking variable. The MS for the interaction term (Treatment × Time Block) was used as the error term to assess the effect of treatment (Newman *et al.*, 1997).

### *Aperture area*

A nested ANCOVA was used to analyse variation in inner aperture area at the end of the experiment, with treatment as a fixed-effect factor, replicate cages nested within treatment and final shell length as a covariate. As a significant interaction between treatment and the

covariate was found, the Johnson–Neyman Technique (Huitema, 1980) was used to determine at what point(s) along the covariate ( $x$ ) axis the treatments significantly diverged from one another.

#### Organic content

We used a nested ANCOVA to analyse percent organics data, with treatment as a fixed-effect factor, replicate cages nested within treatment and shell length growth as a covariate. Shell length growth explained a significant portion of variance in the data, and it indicated a potential energetic constraint related to the rapid deposition of shell material (see Results and Discussion). The data was log transformed to meet model assumptions. As the interaction term was not significant ( $P > 0.25$ ), it was removed to simplify the model (Hendrix *et al.*, 1982).

When we began analysing the percent organics data, the presence of an outlying datum was identified among the predation-exposed snails (see Fig. 6). Considering the minute size of shell samples being processed ( $7.36 \text{ mg} \pm 5.47 \text{ mg}$ ), this outlier may well be the result of procedural error, possibly as a result of the presence of dust or moisture on the sample prior to ashing, which would have been lost upon ashing. We therefore analysed the percent organics data twice, once with and once without the outlier, but conclusions were the same for both analyses; statistical results presented here do not include the outlying datum.

#### Thickness of different shell layers

The thickness of the two mineral shell layers, the first crossed-lamellar and the irregular-prismatic, was analysed separately using nested ANOVAs, in which treatment was a fixed-effect factor and replicate cages were nested within treatment. Shell length (or length growth) was not used as covariate in these analyses, because it did not explain a significant amount of variation in the data. The irregular-prismatic layer data were log-transformed to meet model assumptions.

All statistical analyses were done using JMP™ version 5, and a significance level of 0.05 was used for all hypothesis tests.

## Results

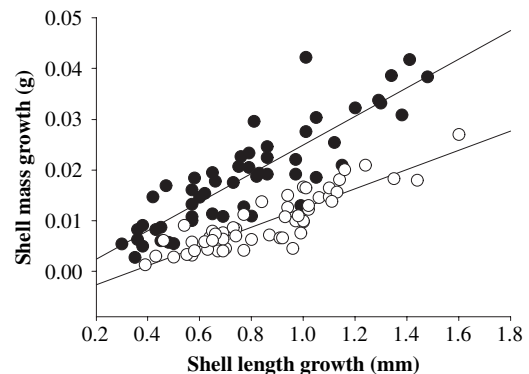
### Mechanism of shell thickening: shell length and mass growth

The nested ANOVAs performed to determine the mechanism of shell thickening indicated that there was no significant difference in shell length growth ( $F_{1,6} = 0.515$ ,  $P = 0.500$ ) between snails exposed ( $0.778 \pm 0.096 \text{ mm}$ ) and not exposed ( $0.855 \pm 0.056 \text{ mm}$ ) to predation cues. However, these snails showed a significant difference in shell mass growth ( $F_{1,6} = 9.905$ ,  $P = 0.020$ ), with shell mass increment of predation-risk snails ( $1.86 \times 10^{-2} \pm 2.17 \times 10^{-3} \text{ g}$ ) being 91.1% greater than

**Table 2** Results of the nested ANCOVA of (log) shell mass growth (increase in shell mass from start of experiment to the end) of snails grown for 96 days in the presence and absence of predation cues (a crab eating conspecific snails).

Source	DF	Sum of squares	Error term	F ratio	Prob > F
Treatment	1	3.478	Replicate [Treat.]	43.098	< 0.001
Replicate [Treat.]	6	0.484	Model	4.769	< 0.001
Log shell length growth	1	4.871	Model	287.804	< 0.001
Model error	98	1.659			

The covariate is (log) shell length growth. In addition to the usual model parameters, the table also shows the error term used to assess significance of different factors in the model.



**Fig. 3** Scatter plot of shell mass growth (increase in shell mass from start of experiment to the end) relative to shell length growth of snails raised for 96 days in the presence of effluent from a crab eating conspecific snails (predation treatment: closed circles) and in the absence of predation cues (control: open circles).

that of control individuals ( $9.75 \times 10^{-3} \pm 1.40 \times 10^{-3} \text{ g}$ ). Accordingly, when compared for a same amount of shell length growth, snails exposed to crabs eating conspecific snails deposited significantly more shell material than snails maintained in the absence of predation cues (Table 2, Fig. 3).

### Body mass growth and faecal production

There was a highly significant effect of predation treatment on body mass growth and production of faecal pellets by snails during the experiment (Table 3, Fig. 4). Snails exposed to effluent of predators eating conspecific snails produced fewer faecal pellets and had smaller increases in body mass than control individuals (Fig. 4).

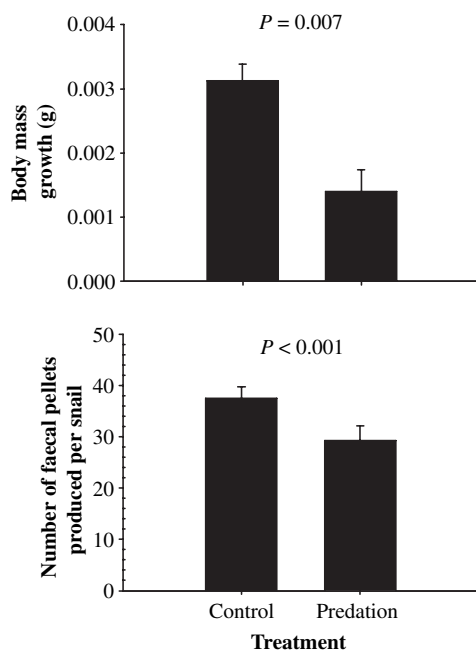
### Inner aperture

The ANCOVA of inner aperture area revealed a significant interaction ( $F_{1,93} = 12.052$ ,  $P < 0.05$ ) between treatment

**Table 3** Results of the nested ANOVA of body mass growth (increase from the start of experiment to the end) and number of faecal pellets produced by snails grown for 96 days in the presence and absence of predation cues (a crab eating conspecific snails).

Source	DF	Sum of squares	Error term	F ratio	Prob > F
Body mass growth					
Treatment	1	$8.404 \times 10^{-5}$	Replicate [Pred. treat.]	16.495	0.007
Replicate [Pred. treat.]	6	$3.057 \times 10^{-5}$	Model	3.602	0.003
Model error	99	$1.400 \times 10^{-4}$			
Number of faecal pellets					
Treatment (T)	1	171.541	T × B	138.307	< 0.001
Time block (B)	4	266.651	Model	15.702	< 0.001
T × B	4	4.961	Model	0.292	0.881
Model error	30	127.364			

In addition to the usual model parameters, the table also shows the error term used to assess significance of different factors in the model.

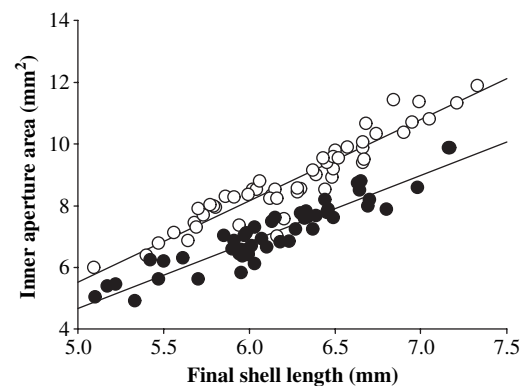


**Fig. 4** Mean ( $\pm$ SE, calculated using mean values for each replicate cage) body mass growth (top) and number ( $\pm$ SE, calculated using mean values for each time block) of faecal pellets produced in 48 h (bottom) by snails grown for 96 days in the presence and absence of predation cues (a crab eating conspecific snails).

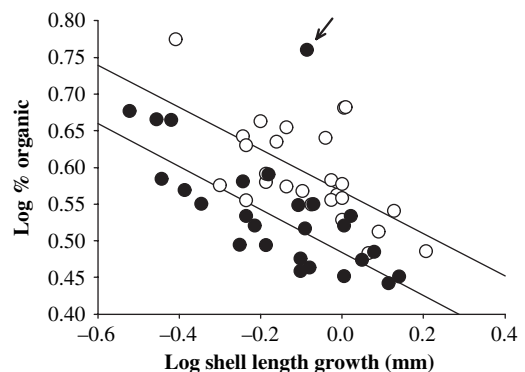
and the covariate, final shell length. When this interaction was investigated with the Johnson–Neyman technique, it was found that predation-exposed snails were predicted to have a significantly smaller aperture area than control individuals when the covariate, final length, was greater than 4.44 mm, which was the case of all snails at the end of the experiment (Fig. 5).

### Organic content

There was a negative relationship between a snail's shell length growth and the percent organic material present



**Fig. 5** Relation between inner aperture area and final shell length of snails grown for 96 days in the presence of cues from a crab eating conspecific snails (predation treatment: closed circles) and absence of such cues (control: open circles).



**Fig. 6** Scatter plot showing the (log) percentage of organic material present in new shell material deposited by snails grown for 96 days in the presence of predation cues (predation exposed; closed circles) and in the absence of such cues (control; open circles), relative to their (log) shell length growth during the experiment. The outlying datum (see Materials and methods for analysis details) present in the predation treatment is highlighted.

in new shell material it deposited during the experiment (Fig. 6). Furthermore, for a similar growth in shell length, predation-exposed snails were found to have significantly smaller fractions of organic material in their shell than individuals not exposed to predation cues (Fig. 6, Table 4).

### Shell layer thickness

There was a significant effect of treatment on the thickness of the irregular-prismatic layer (Table 5, Fig. 7), with it being 94.5% thicker in snails exposed to predation cues ( $0.300 \pm 6.24 \times 10^{-3}$  mm) than in control individuals ( $0.154 \pm 5.99 \times 10^{-3}$  mm). In contrast, the first cross-lamellar layer was 50% thicker in the shell of snails not exposed to predation cues ( $0.021 \pm 1.72 \times 10^{-3}$  mm) than in that of snails exposed to predation cues ( $0.014 \pm 3.09 \times 10^{-3}$  mm), but this difference was not statistically significant (Table 5, Fig. 7). It should be noted that the cross-lamellar layer was very thin and difficult to measure, and for three of the 48 shells prepared for these analyses such a layer was not detected, all within the predation-risk treatment; as there was no indication that the first cross-lamellar layer of these three shells had been chipped during preparation, it may be that the layer did not stain on these particular shells or perhaps it was too thin to detect. When we re-ran this analysis without these three shells, conclusions remain unchanged; the difference in thickness of the first cross-lamellar layer dropped to 29.8%,

and again the effect of treatment was not significant ( $F_{1,6} = 3.129$ ,  $P = 0.127$ ).

### Discussion

During our study, *L. obtusata* exposed to effluents from green crabs, *C. maenas*, feeding on conspecific snails (hereafter 'predation-exposed snails') produced on average 91% more shell material than snails not exposed to predation cues (hereafter 'control snails'). This induced response was reflected by dramatic differences in thickness of the irregular-prismatic calcite layer of new shell material deposited during the experiment, which was on average 95% greater in predation-exposed snails than in control individuals. Note that the absolute thickness of the much thinner crossed-lamellar aragonite layer, which represented only approximately 4% and 12% (based on thickness differences between the two shell layers) of new shell material deposited during the experiment by predation-exposed and control snails, respectively, did not differ significantly between the two groups of snails. This lack of a significant effect of predation treatment on the size of the crossed-lamellar layer may be questioned on procedural (see Results) and statistical grounds (i.e. low power; effect size was 48%), but it is important to stress that the results for this layer are opposite those obtained for the irregular-prismatic layer; the crossed-lamellar layer tended to be thinner, not thicker, in predation-exposed snails than in control individuals. The significance of this apparent selective

Source	DF	Sum of squares	Error term	F ratio	Prob > F
Treatment	1	$8.2918 \times 10^{-2}$	Replicate [Treat.]	131.075	< 0.0001
Replicate [Treat.]	6	$3.7956 \times 10^{-3}$	Model	0.259	0.953
Log shell length growth	1	$9.5671 \times 10^{-2}$	Model	39.214	< 0.0001
Model error	42	0.1025			

This analysis was done without the outlying datum (see Materials and methods and Fig. 6 for details). In addition to the usual model parameters, the table also shows the error terms used to assess significance of different factors in model.

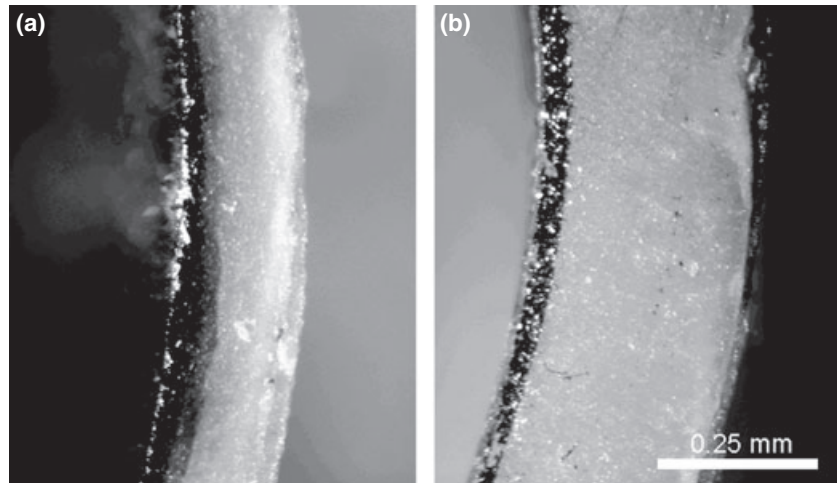
**Table 4** Results of the nested ANCOVA of (log) percentage of organic material in the shells of snails grown for 96 days in the presence and absence of predation cues (a crab eating conspecific snails), with (log) shell length growth as a covariate.

Source	DF	Sum of squares	Error term	F ratio	Prob > F
Log irregular-prismatic thickness					
Treatment	1	0.8682	Replicate [Treat.]	190.117	< 0.0001
Replicate [Treat.]	6	0.0274	Model	1.067	0.398
Model error	40	0.1713			
First cross-lamellar thickness					
Treatment	1	$5.5891 \times 10^{-4}$	Replicate [Treat.]	3.579	0.107
Replicate [Treat.]	6	$9.3711 \times 10^{-4}$	Model	1.543	0.189
Model error	40	$4.0490 \times 10^{-3}$			

In addition to the usual model parameters, the table also shows the error terms used to assess significance of different factors in model.

**Table 5** Results of the nested ANOVAs on the thickness of the (log) irregular-prismatic and first cross-lamellar shell layers of snails grown for 96 days in the presence and absence of predation cues (a crab eating conspecific snails).

**Fig. 7** Photographs showing the two main shell layers of a snail from the control (a) and predation (b) treatments (see Fig. 2b for photo location) that displayed a similar increase in shell length during the experiment. The shiny black strip on the left side of each shell section is the first cross-lamellar layer stained with Feigl's stain, and the much-thicker, light-coloured strip to the right of the cross-lamellar layer is the irregular-prismatic layer. Notice how much thicker the irregular-prismatic layer is in the shell of the predation-exposed versus control snail. Both photographs were taken at 10× magnification and are shown at same scale.



deposition of different shell layers in response to predation cues is difficult to ascertain. Prismatic layers generally have higher tensile and bending strength, but slightly lower compressive strength, than cross-lamellar layers. However, the properties of these different layers vary greatly among species of molluscs (Currey, 1988), and no tests have yet been done on the mechanical strength of different shell layers of littorinid snails.

Similar effects of predation cues on the overall thickness of *L. obtusata* shells have been reported by others (Trussell, 1996; Trussell & Nicklin, 2002), and results of recent experiments indicate that this response enhances the snail's resistance to shell-crushing predation. In particular, Trussell & Nicklin (2002) found that more mechanical force is needed to crack shells of *L. obtusata* that have been thickened because of prolonged exposure to effluents from *C. maenas* feeding on conspecific snails, and recent experiments have shown that inter-population variation in shell thickness comparable to variation reported by Trussell and Nicklin's (2002) greatly affects *L. obtusata*'s susceptibility to predation in staged encounters with *C. maenas* in the lab (Smith, 2004; Rochette *et al.*, 2007).

### Mechanism of predation-risk induced shell thickening

Two different mechanisms could account for the increased length-standardized shell thickness of snails exposed to predation cues, i.e. constant linear shell translation with increased deposition rate of shell material, or reduced linear translation of the shell in conjunction with constant deposition of shell material (Palmer, 1990; Trussell, 1996; Trussell & Etter, 2001). Our results strongly support the 'increased calcification rate' hypothesis, and are not consistent with the 'reduced growth rate' hypothesis. In particular, linear shell growth of predation-exposed and control snails was similar, and for a same amount of linear shell growth, the former

deposited more shell material than the latter. For example, a predation-exposed snail that displayed 0.82 mm in linear shell translation, which is the mean linear growth observed during the experiment, is predicted to have deposited 118% more shell material than a control individual that displayed the same linear shell growth. To our knowledge our study is the first to discriminate between these two potential mechanisms of predation-enhanced calcification in gastropod molluscs. However, comparing patterns of shell calcification of starved and well-fed *N. lapillus*, Palmer (1990) similarly concluded that changes in shell morphology induced by effluents from the predatory crab *Cancer pagurus* were unlikely the result of changes in snail growth rate alone.

That this induced response was not accompanied by a reduction in linear shell translation, as predicted by the 'reduced growth rate' hypothesis, probably enhances resistance to crab predation. Because the amount of force a crab claw can exert on an object decreases towards the tips of the fingers, less force can be applied on larger objects, which can not be inserted as far towards the base of the claw's fingers. Previous studies have indeed shown that larger *L. obtusata* snails are less susceptible to predation by *C. maenas* than smaller individuals (Smith, 2004; Rochette *et al.*, 2007). Although it is unclear how much of this difference is owed to shell thickness versus overall size of the snail, both factors are likely important. Therefore, maximizing both linear shell translation and shell thickening in order to more rapidly reach a larger overall size with a thicker shell may better protect *L. obtusata* against shell-crushing predation than either one of these responses would by itself. Furthermore, maintaining a high rate of linear shell translation may partly mitigate the negative effect shell thickening has on internal shell volume (Palmer, 1981), and hence snail fecundity (see below).

Interestingly, the lack of support for the 'reduced growth rate' hypothesis did not appear to be due to

predation cues not affecting snail behaviour, but rather to this behavioural response not causing reduced linear shell translation, as was expected. More specifically, predation cues seem to have negatively affected the grazing activity of predation-exposed snails, as these produced 22% fewer faecal pellets throughout the experiment than control individuals. Although our experimental set up did not allow easy observation and quantification of snail behaviour, we believe that variation in our admittedly crude estimate of grazing activity reflects true differences in feeding rates among groups of snails subjected to different treatments. The size and shape of faecal pellets varied relatively little, and showed no obvious differences between groups of snails. Furthermore, snails that were offered algae on only one of every three days of a feeding cycle produced a similar number of faecal pellets throughout the experiment as did predation-exposed snails (data not shown). Therefore, snails appear to have reduced their feeding activity in response to predation risk, as has been reported in numerous other studies on gastropods (e.g. Palmer, 1990; Richardson & Brown, 1992; Rochette & Himmelman, 1996; Serra *et al.*, 1997; Behrens Yamada *et al.*, 1998; Rochette *et al.*, 1999), but they maintained a similar rate of linear shell translation as control individuals.

Although our study demonstrates unequivocally that *L. obtusata* snails can actively increase the physiological machinery of shell calcification in response to predation risk, conditions in nature (e.g. availability of food or water-borne minerals) may not always enable this to be done at no cost to linear shell translation, as was observed in our study, because there is likely an upper limit to the rate at which gastropods can deposit shell material (Palmer, 1981; Kemp & Bertness, 1984). Nevertheless, that this induced defence is actively modulated and not strictly tied to the snail's feeding behaviour should increase its evolutionary potential, because there are clearly contexts in which genetic and/or developmental co-variance between reduced grazing and increased calcification would be mal-adaptive. More specifically, whereas snail grazing activity and growth are undoubtedly compromised by numerous biotic (e.g. low food quality or availability) and abiotic (e.g. high desiccation risk) factors, indiscriminate production of a thicker and heavier shell under all such conditions would often offer no advantage, while entailing important costs (see below). Whereas it is well recognized that genetic correlations can profoundly affect the evolution of 'fixed traits' (Lande & Arnold, 1983), far lesser consideration has been given to the effect of environmentally-mediated phenotypic correlations on the evolution of trait plasticity.

### Cost of predation-risk induced shell thickening

The production of thicker and heavier shells can entail both developmental and energetic costs to gastropods

(Palmer, 1981), which helps explain why all gastropods do not produce maximally armoured shells all the time. Although our study was not designed to quantify and qualify these costs, it did clearly indicate they exist. In particular, growth of body tissue was on average 55% less in predation-exposed snails than in control individuals (see also Appleton & Palmer, 1988; Palmer, 1990; Trussell & Nicklin, 2002; Trussell *et al.*, 2003), which likely represents a significant fitness cost, because intra-specific variation in fecundity is linked to growth and body size in gastropods (Spight & Emlen, 1976; Hughes & Answer, 1982; Palmer, 1983).

We can think of at least four, non exclusive, explanations for the smaller body of predation-exposed snails, two based on energetic, one on developmental, and one on adaptationist considerations. Firstly, the reduced grazing activity of predation-exposed snails relative to control individuals, as evidenced by faecal pellet counts, should have resulted in the former having less energy available for somatic and gonadic growth than the latter, assuming similar assimilation efficiency of food by these two groups of snails.

Secondly, increased investment of energy in production of shell material, including calorific content of the organic matrix as well as metabolic cost of synthesizing and depositing this matrix along with mineral crystals, may have resulted in less energy available for the growth of soft body tissue in predation-exposed snails relative to control individuals. Patterns of variation in the organic matrix are particularly informative in this regard. Whereas the organic matrix constitutes only 1–5% of the mass of a gastropod shell (Marin & Luquet, 2004), it plays a major role in defining its micro-structural characteristics (Falini *et al.*, 1996; Marin & Luquet, 2004) and structural integrity (Currey & Taylor, 1974; Currey, 1988; Zuschin *et al.*, 2003). Therefore, other things being equal, we would have expected the organic fraction of newly deposited shell material to be greater in predation-exposed than control snails, but we observed the exact opposite; e.g. when comparing snails that grew 0.81 mm in shell length (the mean observed linear growth), the organic fraction of shells from control snails was 21% greater than that of predation-exposed snails, suggesting a significant energetic cost of calcification. This cost is also suggested by the negative relation we observed, for both predation-exposed and control snails, between linear shell translation and % organics of new shell material; snails that displayed greater elongation along the coiling axis appeared unable to build as extensive an organic matrix in their shell as individuals that grew less. Both of these patterns are consistent with the substantial cost of the organic matrix of the shell relative to its calcium carbonate component on a per weight basis (Palmer, 1992).

Thirdly, because carbonate exoskeletons such as gastropod shells are thickened from the inside (Wilbur & Saleuddin, 1983), the reduced body size of predation-

exposed snails may reflect a developmental by-product of enhanced shell thickening. Assuming that the axis of coiling is unchanged, individuals that produce a thicker shell (such as those exposed to predation cues) will have decreased internal shell volume, and consequently reduced space available for somatic and/or reproductive tissue, relative to individuals that produce a thinner shell.

Fourthly, the smaller body of predation-exposed snails might reflect an adaptive response to shell-probing predation by *C. maenas* (e.g. Johannesson, 1986; Rochette *et al.*, 2007), as it should enable snails to retract further inside their shell and away from the grasp of the crab's fingers (see also Palmer, 1990). The decreased size of the inner shell aperture of snails exposed to predation cues may similarly help reduce predation by shell-entry (Rochette *et al.*, 2007). However, it is unclear whether these morphological changes are specific responses to predation risk, or a by-product of any factor or process that would cause snails to produce a thicker shell. Clearly, further manipulative experiments should be done to elucidate the adaptive value and developmental inter-dependence of shell thickness, body mass and aperture area in this and other gastropod species. For example, experiments could be done that involve different factors (e.g. predation cues and water temperature) affecting shell calcification. If reduced body size and aperture area are not specific responses to predation risk, then 'predation-exposed' and 'temperature-treated' snails that show similar changes in shell thickness should also have similar shell aperture areas and body masses. In contrast, if these traits are direct and adaptive responses to predation risk, then one would predict a greater decrease in body size and aperture area for predation-exposed than temperature-treated snails that show similar increases in shell thickness.

## Summary and conclusions

To further our understanding of trait plasticity evolution, empirical work is needed that addresses explicit hypotheses concerning the adaptive value, costs, constraints and mechanisms of trait plasticity and plastic expressions (DeWitt & Scheiner, 2004b). With respect to mechanism, which is the domain of this study, correlated characters present a particular challenge; plastic traits thought to be direct responses to particular environmental cue(s) may instead be by-products of developmentally- or genetically-correlated responses. Few studies have tested this possibility (but see DeWitt, 1998), which is consequential because genetic and developmental correlations are frequent and likely have profound implications for trait plasticity evolution.

The increased shell thickness of *L. obtusata* snails exposed to predation cues, as documented in this and other recent studies, appears to be the result of an active increase in the rate of calcification, rather than an

indirect consequence of reduced feeding given threatening stimuli. Therefore, the evolution of this plastic physiological response is not strictly tied to, and hence constrained by, the animal's behaviour. This is not to say, obviously, that grazing potential and growth rate have no bearing on the animal's physiological response and morphology (see above). One timely avenue for future research into the mechanism of this plastic response involves assessing its modulation by the separate and interacting effects of feeding opportunity, mineral availability (used for calcification) and predation risk; whereas experimental studies conducted to date have typically involved 'optimum' conditions for trait induction (e.g. *ad libitum* food supply and continual exposure to water-borne minerals and predation cues), in reality these all vary in a marked and predictable manner over small spatial scales throughout the snail's vertical distribution in the intertidal zone, and they likely constrain the trait values the species is able to display. Furthermore, these selective gradients may have contributed to genetic variation in trait plasticity and developmental reaction norms, a hypothesis that could be addressed using a quantitative genetic approach and common-gardening experiments that better simulate the range of conditions snails experience in nature.

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