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Two-tiered defense strategy may compensate for predator avoidance costs of an ecosystem engineer

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Abstract

Inducing defenses to deter predators is a necessary process theorized to incur costs. Although studies have investigated defense trade-offs, quantifying trade-offs is challenging and costs are often inferred. Additionally, prey employ strategies to reduce costs, making costs difficult to predict. Our purpose was to investigate induced defense costs by characterizing the defense mechanisms and costs in eastern oysters (*Crassostrea virginica*). In the field, newly-settled oysters exposed to blue crab (*Callinectes sapidus*) exudates grew stronger shells containing less percent organic material than oysters in controls. In natural populations, shell density was negatively correlated with shell thickness, further suggesting oysters thicken their shells by increasing low-density calcium carbonate. Reproductive investment showed an increasingly negative relationship with thickness as density decreased (and induction increased). In a laboratory experiment, oysters exposed to a temporal gradient in risk grew heavier shells in all crab treatments, but only grew stronger shells under constant exposure. Collectively, these results suggest oysters initially react to predators by adding inexpensive calcium carbonate to their shells to quickly outgrow risk. However, in high-risk environments, oysters may increase the production of costly organic material to increase the shell strength. Thus, oysters demonstrate a two-tier mechanism allowing them to cheaply escape predation at lower risk but to build stronger shells at greater expense when warranted. These results illuminate the complex strategies prey deploy to balance predation risk and defense costs as well as the importance of understanding these strategies to accurately predict predator effects.

Introduction

To reduce their chances of being eaten, prey organisms often use defenses including morphological structures like spines or hard shells, distasteful chemicals, or altered behavior to reduce their risk of consumption (Cronin 2001; Hay 2009). Many organisms use plastic or induced defenses that are

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employed only when injury or death are imminent (Tollrian and Harvell 1999; Ferrari et al. 2010). Induced defenses are predicated on the idea that defenses are costly to produce because they divert energy away from growth and reproduction. As a result, plastic defenses allow prey to limit these costs to circumstances where risk and defense value are high (Harvell 1990; Cronin 2001). Defense costs have been effectively demonstrated for a few species (Lively 1986; Baldwin 1998; Van Buskirk 2000; Relyea 2002). For example, acorn barnacles (*Chthamalus anisopoma*) developing a bent morphology in response to gastropod predators experience lower rates of predation but also slower growth and reduced egg production compared to individuals of the conic morph (Lively 1986). Tadpoles of several anuran species which alter body and tail size ratios in response to dragonfly predators experience increased survivorship but reduced growth rates compared to undefended conspecifics (Van Buskirk 2000). Wild tobacco plants (*Nicotiana attenuata*) produce more nicotine when grazers abound, but incur reduced seed production (Baldwin 1998).

However, costs associated with producing predator-resistant morphologies are often difficult to discern. Many studies infer costs or estimate them using proxy variables such as changes in growth or foraging, and the previously described studies measuring defense fecundity trade-offs are both rare and valuable (Langerhans and Dewitt 2002). Where costs are inferred, inferences are often based on a limited understanding of the mechanisms prey use to produce defenses, which can lead to an incorrect understanding of the costs and benefits of induced defenses. For example, the snail *Nucella lamellosa* develops a thicker, stronger shell in response to crab predators (Bourdeau 2010). However, *N. lamellosa* also behaviorally defends against predators through reduced foraging and fortified morphology is produced under conditions of limited food availability (Bourdeau 2010). Therefore, under conditions of predation risk, *N. lamellosa* reduces feeding to reduce apparency to predators, mimicking conditions of limited food availability. They continue to produce energetically inexpensive inorganic shell material, altering their growth patterns and passively producing a predator-resistant morphology. Thus, the costs to *N. lamellosa* stem from their behavioral response (feeding reduction) and not from diverting energy resources to shell production. This example clearly demonstrates the risks of assuming defenses are inherently costly.

However, induced defenses are highly diverse, even within taxa; for example, predator-resistant snail shell morphologies can be costly. *Nucella lapillus*, unlike *N. lamellosa*, produce different shell types with and without crab predator exudates, even when consuming equal amounts of food in laboratory assays (Large et al. 2012; Large and Smee 2013). Additionally, freshwater snails *Physella virgata* delay reproduction and grow larger to reach a size refuge in response to crayfish predators (Covich and Crowl 1990), which may lower their lifetime reproductive potential (Langerhans and Dewitt 2002). Unlike the morphological changes in *N. lamellosa*, *P. virgata* life history changes result from resource reallocation toward growth in response to predators (Covich and Crowl 1990).

To investigate mechanisms and costs associated with plastic prey defenses, we selected the eastern oyster (*Crassostrea virginica*) as a model species. Oysters decrease their susceptibility to predators by growing heavier, stronger shells in response to predator exudates (Newell et al. 2007; Lord and Whitlatch 2012; Johnson and Smee 2012; Robinson et al. 2014; Scherer et al. 2016). Creating more predator-resistant oyster shells may divert resources away from soft tissue growth and/or fecundity (Lord and Whitlatch 2012; Johnson and Smee 2012) and, thus, incur long-term costs in terms of growth and reproduction. However, the mechanism by which oysters alter their shells to deter predators and the associated long-term costs remain uninvestigated. This process may be complex in oysters and other bivalves,

whose shells consist of both organic and inorganic components. The organic protein component is energetically more expensive to produce than the inorganic calcium carbonate (Frieder et al. 2016; Lee et al. 2016), which comprises the bulk of oyster shells (Currey and Taylor 1974). Despite its quantity, the organic component confers much of the shell's strength (Taylor and Kennedy 1969; Zuschin et al. 2001; Avery and Etter 2006; Lee et al. 2011), although both components contribute to shell durability (Avery and Etter 2006; Lombardi et al. 2013). Therefore, to strengthen their shells, oysters may change the type and/or amount of either of these shell constituents and, given the differences in energy requirements, costs of oyster defenses will depend strongly on the mechanism of induction.

Oysters are a foundation species, creating reefs that provide habitat for numerous organisms (Grabowski and Peterson 2007). They are a commercial fishery and provide numerous services, such as shoreline protection and water filtration (Grabowski and Peterson 2007). Thus, they are both ecologically and economically important. However, > 85% of oyster reef habitat worldwide has been lost (Beck et al. 2011). Further, calcification is expected to become increasingly expensive under future coastal ocean acidification conditions, especially in higher latitude regions (Waldbusser et al. 2015), potentially altering the ability of oysters to respond to their predators. Thus, it is important to understand how long-term relationships between shell and soft tissue production influence oyster fecundity to contribute to our understanding of oyster reef ecology under changing environmental conditions. To improve our understanding of their morphological defenses, we exposed oysters to predator exudates in the field and measured changes in shell weight, strength, and chemical composition. We then investigated the relationship of shell density and thickness with oyster investment in somatic and gonad tissue. Finally, these results were compared with shell morphology of oysters reared under a gradient of predation risk in laboratory conditions.

Materials and methods

Field induction and amino acid analysis

Dead, adult oyster shells containing naturally settled oyster spat (*Crassostrea virginica* Gmelin, 1791, < 1 cm) were obtained from the Texas Parks and Wildlife Department (TPWD) following their 2014 trawl surveys in Copano Bay, Texas, USA. These spat-containing shells were deployed in cages in the field from mid-April to late October and reared either with or without exudates from predatory blue crabs (*Callinectes sapidus* Rathbun, 1896). Cages (0.5 × 0.5 × 0.25 m) were constructed from lumber and

covered with plastic mesh (1.0 cm^2 openings). In the center of each cage, a small compartment was constructed from the same plastic mesh (10 cm diameter × 50 cm tall). Shells containing oyster spat were placed along the outer edges of each cage to a depth of approximately half the cage. The inner compartments either contained a single adult blue crab (10–13 cm carapace width; predator treatment, $n = 8$) or were left empty (control, $n = 9$). Control and predator cages were placed haphazardly along the leeward edge of an oyster reef in Saint Charles Bay, Texas, USA and separated from each other by at least 2 m. Cages were checked weekly and were cleaned of drift algae and repaired if necessary. Blue crabs were fed one market-sized oyster (~ 5 cm in length, collected from adjacent natural reefs) per week and dead crabs were replaced as needed.

In October, all cages were retrieved and 15 oyster spat (mean shell diameter = 16.0 ± 0.36 mm, range = 8.3–26.4 mm) were obtained from each cage for data collection. Shell metrics were measured using destructive techniques; i.e., drying prior to measuring shell weight may alter shell strength and crushing shells to measure strength results in the loss of small amounts of shell material necessary for weight and composition data. Therefore, the same oysters were not measured for weight, strength, and shell composition. Instead, five spat from each cage were measured for each of the three shell metrics (weight, strength, composition).

Oysters grow heavier, stronger shells in response to predator exudates (Robinson et al. 2014; Scherer et al. 2016). Therefore, we measured shell weight and strength as metrics of oyster responses to caged crabs. Shell weight was measured on the right valve, cleaned of soft tissue and fouling organisms. Shells were dried at 38 °C for 48 h and weighed using a high precision electronic balance to the nearest 0.001 g. Shell crushing force is a measure of shell strength (Zuschin et al. 2001) and a proxy for the force required by predators to crush the shell. Crushing force was measured using a Kistler 5995 charge amplifier and Kistler 9222 force sensor (sensu Robinson et al. 2014). The settings for this force sensor are sensitivity 19 and range 2000. The force sensor was used to crush the shell by applying constant pressure to the center of the articulated right valve until structural failure. The maximum force exerted is then measured and output by the charge amplifier.

The final five oysters from each cage underwent amino acid analysis, conducted by the Liu lab at the University of Texas at Austin Marine Science Institute, to identify and quantify amino acids present and assess shell chemical composition (detailed protocol in Liu et al. 2008). Dried shell samples were ground with a mortar and pestle and stored in a desiccator until analysis. Ground samples were hydrolyzed in 10 mL centrifuge tubes with 1 mL 6 M HCl while nitrogen gas was blown gently into tubes to prevent

oxidation. The sealed tubes filled with nitrogen gas were placed in a Pierce Heating Module at 110 °C for 20 h. Large, unhydrolyzable particles were filtered out with a 0.2 mm filter. The acid solution was heated and evaporated under nitrogen gas. Residual material was dissolved in 1.0 mL distilled water. Large particles were again filtered through a 0.2-mm filter before high-performance liquid chromatography (HPLC) analysis. Total hydrolysable amino acids were measured using HPLC with fluorescence detection after precolumn o-phthaldialdehyde (OPA) derivatization (Liu et al. 2008). Briefly, amino acid derivatives are separated on a 25 cm × 5-μm Alltech Alltima C$_{18}$ column with a mobile phase flow rate of 1 mL min^{-1}. A binary gradient of 0.05 M sodium acetate (pH 7.5) and 5% THF (eluant A) and methanol (eluant B) was used, ramping from 20% B to 50% B in 40 min, then to 100% B in 20 min. OPA-derivatized amino acids are detected by fluorescence and identified by retention time as compared with authentic Pierce™ standards. Duplicate analyses generally agreed within 10%.

All statistical data analyses were performed in R 3.0.1 (R Core Team 2013). Shell weight and crushing force were compared between treatments using one-way ANOVAs. The significance of cage as a random factor was tested to ensure no effect and removed from the final models. The total proportion amino acid content was compared using a t test. Assumptions for analysis of variance were assessed using diagnostic plots as well as the Shapiro–Wilks test for normality and the Browne–Forsythe–Levene test for homoscedasticity. Data met all assumptions. Data for this and all other portions of the study are available from the data library Pangaea (Scherer et al. 2018).

Relationship between shell and soft tissue

Adult oysters (> 5 cm shell length) were acquired from TPWD following spring trawl surveys in 2014 and 2017 in Copano Bay, Texas. We held oysters without food for 3 weeks prior to data collection to allow oysters to expel digestive waste because digestive tissue cannot be effectively separated from gonad tissue, and gut material could influence gonad tissue weight. Prior to data collection, oysters were shucked without penetrating the soft tissue mass and the gonad tissue was classified as either intact (Fig. 1a) or partially released (i.e., gamete release previously initiated; Fig. 1b). Oysters with less than 75% of intact gonad mass were rejected as neither gonad weight nor soft tissue weight could be reliably determined. Soft tissue was then separated from the shell and transferred to a weigh tin. Gonad tissue was carefully cut away from somatic tissue and placed in a separate weigh tin. Tissue samples were dried at 38 °C for 48 h and weighed using a high precision electronic balance to the nearest 0.001 g. Then, we calculated an index
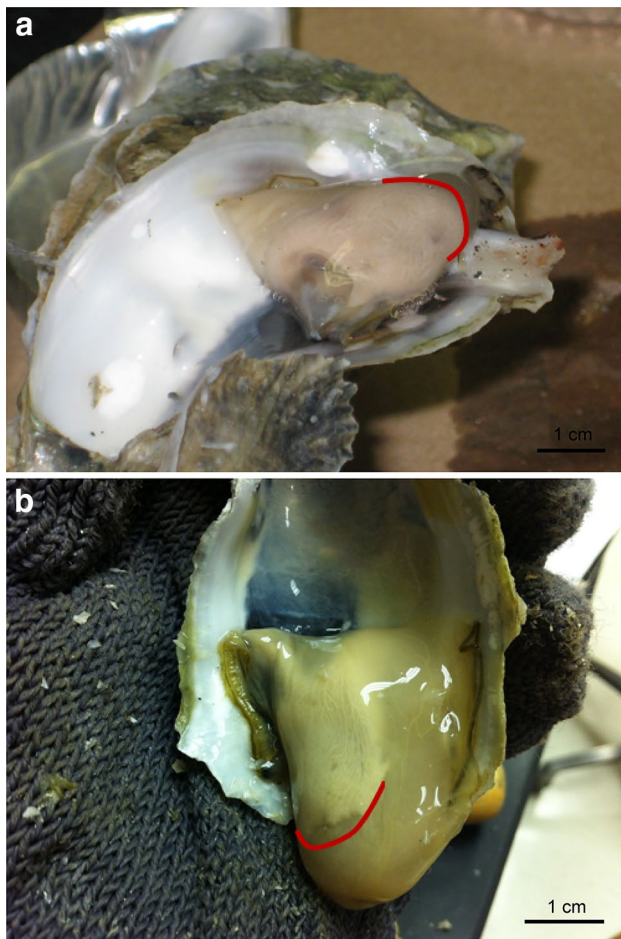
Fig. 1 a Intact gonad tissue, indicated by smooth, rounded lower edge of opaque gonad tissue reaching red line indicating edge of gonad mass. **b** Partially released gonad, indicated by ragged lower edge of opaque gonad tissue which does not form a smooth, rounded edge reaching red line. Photo credit to A. E. Scherer

for gonad tissue, hereafter termed as gonad index (GI), by dividing gonad tissue weight by somatic tissue weight.

Shell metrics were measured on the right valve of the oyster shell as left valves are cemented to the substrate and cannot be effectively cleaned of associated material. Shells were first cleaned of such fouling material and measured for volume of the shell mass using water displacement following shell submersion. Photos of the inner and outer shell surfaces were taken (two photos per oyster) and assessed for cross-sectional area using the freehand tool in ImageJ (Schneider et al. 2012; Lord and Whitlatch 2012). Two researchers analyzed each photo (four measurements per oysters) and an average was calculated from these analyses. Shells were dried and weighed as described for the soft tissue masses. Shell thickness was then calculated as shell volume over cross-sectional area and shell density as shell weight over volume.

Analyses were completed using JMP Pro 13. Results were considered significant at $\alpha = 0.05$ but those below $\alpha = 0.1$ are also discussed because they may indicate biologically meaningful defense costs. We calculated the correlation between shell thickness and shell density using data from all oysters measured ($n = 126$) to assess the relationship between shell characteristics. We then performed regression analyses predicting absolute somatic tissue weight and gonad index using shell thickness and density. The analysis for somatic weight included data from all oysters measured ($n = 126$). To test for size-independent relationships, somatic weight data were standardized to the mean surface area using the linear relationship and residuals for somatic tissue predicted by the cross-sectional area. Somatic tissue values were first transformed using a 0.25 power transformation to improve conformity to assumptions of linearity.

The same regression analysis was performed for gonad index using the subset of oysters for which the gonad mass was completely intact ($n = 72$). For gonad index and shell thickness, size independence was again achieved using linear relationships with cross-sectional area following transformation (0.75 power transformation for gonad index data and 0.5 power transformation for thickness data) to conform to assumptions of linearity.

Where significant interaction effects were detected, the relationship of shell and soft tissue metrics was explored using the interaction and prediction profile features in JMP Pro 13. This feature allows the user to dynamically explore the effects of multiple factors on the dependent variable. Therefore, it is useful for qualitatively assessing the effect of continuous factors on variables of interest when factor interactions are significant.

Laboratory study and variable predation risk

In the summer of 2013, oyster larvae were purchased from the Auburn University Shellfish Laboratory at the Dauphin Island Sea Lab, Alabama, USA and settled onto clean, sun-bleached oyster shell (hereafter spat). At ~1 mm, spat were thinned to ~10 oysters per shell and transferred to 32 opaque tanks at densities of 3 shells per tank. Tanks were randomly assigned to four treatments ($n_{treatment} = 8$) along a gradient of predation intensity, created through weekly predator residency times: 0 h (control), 2, 24, or 168 h (constant predator). Blue crab predators (carapace width 10–13 cm, average width 10.7 cm) were collected locally and fed refrigerated tissue from one market-sized oyster (5.5–7.5 cm) in the experimental tanks once a week. Oyster spat were held in plastic mesh pouches (1 cm^2) to protect them from consumption. Tanks were provided with 20 L of aerated artificial seawater (Instant Ocean™) maintained at 25–27 °C and salinity 25. Water changes were conducted weekly following blue

crab feeding to remove waste and prevent build-up of cues. Oysters were fed 5 mL of Phytoplex phytoplankton daily.

Following the eight-week exposure period, three spat each were measured for shell dry weight and crushing force. Shell weight was measured by removing spat from their settlement shells, drying them at 38 °C for 48 h, and weighing them using a high precision electronic balance to the nearest 0.001 g. Although soft tissue cannot be effectively removed at this size, it comprises < 1% of total weight at this stage in development (Robinson et al. 2014). Shell crushing force was measured on the right value following the same procedures as described for the field study oysters. For this size class of oysters, we used the Kistler 5995 charge amplifier and a Kistler 9203 force sensor with sensitivity 19 and range 50 (sensu Robinson et al. 2014).

All statistical analyses were completed using R 3.0.1 (R Core Team 2013). Two-way nested mixed ANOVA's using Type III Sums of Squares were used, with predator treatment as a fixed factor and tank as a nested random factor. Assumptions for analysis of variance were assessed using diagnostic plots as well as the Shapiro–Wilks test for normality and the Browne–Forsythe–Levene test for homoscedasticity. Shell weight was transformed using a square root transformation and crushing force using a natural log transformation to improve conformity to the assumption of normality. The var-Power function in the nlme package was applied to the fitted data to improve conformity to the assumption of homogeneity of variance (Pinheiro et al. 2016). For significant models, pairwise comparisons were made using Tukey's test with a Westfall correction to control for familywise type I error.

Results

Field induction and amino acid analysis

Shell crushing force was significantly higher for oysters in cages with predators ($X \pm SD = 129.05 \pm 5.14$ N, $n = 8$) than in control cages [$X \pm SD = 135.61 \pm 8.02$ N, $n = 9$; ANOVA, $F(1,1) = 8.54$, $P = 0.005$; Fig. 2a]. Oysters in predator cages ($X \pm SD = 0.23 \pm 0.03$ g, $n = 8$) tended to have heavier shells than those in control cages ($X \pm SD = 0.18 \pm 0.01$ g, $n = 9$), although this result was significant only at $\alpha = 0.1$ [AVOVA, $F(1,1) = 3.17$, $P = 0.079$; Fig. 2b].

Oysters reared in predator treatments ($X \pm SD = 0.07 \pm 0.003$ µg mg^{-1}, $n = 8$) had significantly lower amino acid content (µg mg^{-1}) than oysters reared in control treatments ($X \pm SD = 0.09 \pm 0.007$ µg mg^{-1}, $n = 9$; T test, $t11.6 = 2.74$, $P = 0.002$; Fig. 2c). Additionally, 15 individual amino acids were detected in tested oyster shells, and all but one (*gamma*-aminobutyric acid) had a greater mean concentration in control treatments (Table 1).
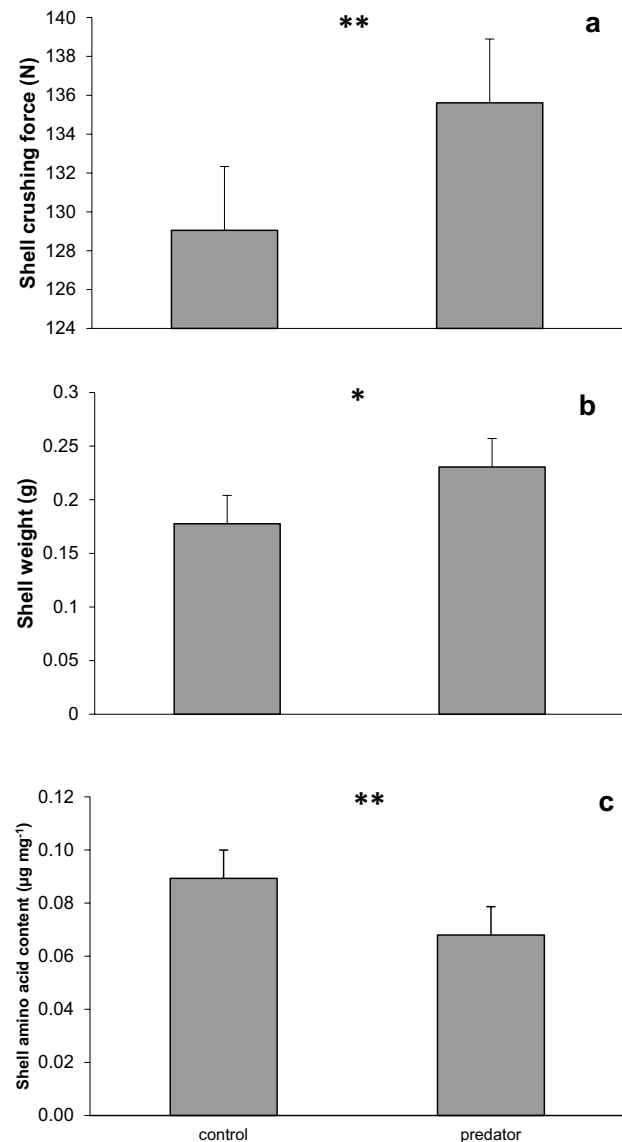
Fig. 2 Mean + standard error, $n_{control} = 9$, $n_{predator} = 8$ for **a** shell compression force, **b** shell weight, **c** shell amino acid content for oyster spat reared under field conditions in either predator present or control treatments. Significance at $\alpha = 0.05$ denoted with **, $\alpha = 0.1$ with *

Relationship between shell and soft tissue

Shell characteristics (density and thickness) were significantly negatively correlated (Pearson correlation, $r = -0.49$, $P < 0.001$; Fig. 3). For somatic tissue weight, a positive linear relationship was detected with shell cross-sectional area (a proxy for oyster size; Linear regression, $r^2 = 0.42$, $F1,124 = 90.10$, $P < 0.001$). The interaction of shell thickness and density was not significant and was removed from the model. The final model was significant (multiple regression, $F2,123 = 14.22$, $P < 0.0001$; Table 2), and indicated positive effects of both thickness (estimate = 0.024,

Table 1 Amino acids identified in shells of oysters reared under field conditions and mean ± standard deviation relative amounts in control and predator cages

Amino acid	Control cages (µg mg^{-1})	Predator cages (µg mg^{-1})
Aspartic acid	0.0893 ± 0.020	0.0680 ± 0.0083
Glutamic acid	0.0893 ± 0.020	0.0680 ± 0.0083
Histidine	0.0893 ± 0.020	0.0680 ± 0.0083
Serine	0.0893 + 0.020	0.0680 ± 0.0083
Arginine	0.0893 ± 0.020	0.0680 ± 0.0083
Glycine	0.0226 ± 0.0046	0.0148 ± 0.0012
Threonine	0.0028 ± 0.0011	0.0021 ± 0.00039
Alanine	0.0091 ± 0.0036	0.0680 ± 0.000013
Tyrosine	0.0045 ± 0.0013	0.0032 ± 0.00015
gamma-**Aminobutyric acid**	**0.0000 ± 0.000036**	**0.0000 ± 0.00046**
Methionine	0.0005 ± 0.00029	0.0003 ± 0.00015
Valine	0.0036 ± 0.0011	0.0029 ± 0.00046
Phenylalanine	0.0025 ± 0.00068	0.0021 ± 0.00041
Isoleucine	0.0022 ± 0.00069	0.0019 ± 0.00038
Leucine	0.0039 ± 0.0012	0.0032 ± 0.00056

The single amino acid not more abundant in predator cages in bold
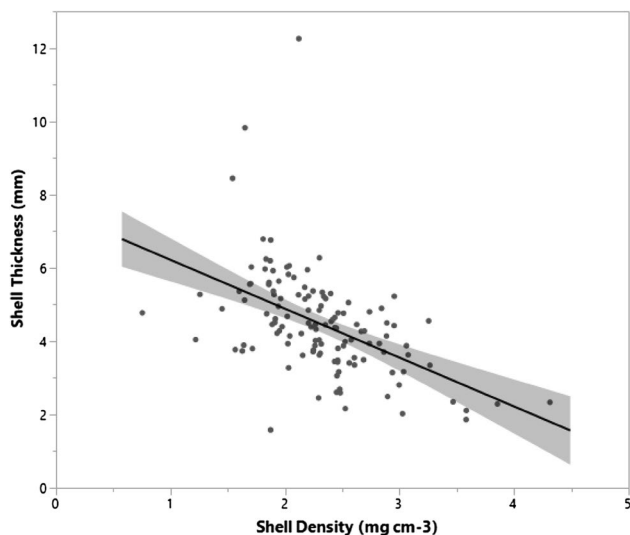
Fig. 3 Correlation between shell density and thickness. Black line represents line of best fit, shaded region represents 95% confidence interval calculated by Graph Builder in JMP Pro 13

$P < 0.001$) and density (estimate = 0.033, $P = 0.008$) on somatic tissue weight.

For gonad index, relationships with size (cross sectional area) were detected for both gonad index (Linear regression, $r^2 = 0.31$, $F1,70 = 30.85$, $P < 0.0001$) and thickness (Linear regression, $r^2 = 0.08$, $F1,70 = 5.86$, $P = 0.02$). The final model for gonad index included shell thickness, density, and their interaction. Although the overall model was

not significant (multiple regression, $F3,68 = 2.05$, $P = 0.12$; Table 3), the interaction between shell thickness and density was significant at $\alpha = 0.1$ ($P = 0.060$). This indicated the relationship between shell thickness and soft tissue metrics varied according to shell density. As a result, significant relationships in some portion of this plane may have been hidden in the overall model by non-significant relationships in other portions of this plane. The interaction profile demonstrated that, when density was high (and thickness low), variation in thickness had a positive effect on gonad index, but when density was low (and thickness high), the effect of thickness was negative (Fig. 4; this interaction can be interactively explored using the data in Online Resource 1 and the code for the prediction profiler in Online Resource 2).

Laboratory study and variable predation risk

Significant differences were found for both metrics: weight [$n_{treatment} = 8$; ANOVA, $F(3,85) = 9.21$, $P < 0.0001$; Table 4, Fig. 5a] and crushing force [$n_{treatment} = 8$; ANOVA, $F(3,28) = 6.40$, $P = 0.002$; Table 4, Fig. 5b]. Westfall comparisons showed that constant predator treatments (168 h) were greater than control treatments (0 h) for both shell metrics. For shell weight, intermediate treatments (2, 24 h) were not statistically different from constant predator treatments but, for crushing force, these treatments were not significantly different from controls.

Discussion

Oysters reared under field conditions responded to predators by producing shells that were heavier and stronger, consistent with earlier laboratory studies (Newell et al. 2007; Lord and Whitlatch 2012; Robinson et al. 2014; Scherer et al. 2016). Even though it composes ≤ 5% of shell weight (Currey and Taylor 1974), we expected oyster shells in predation treatments to also contain relatively more organic material, as the organic component of mollusc shells confers much of the shell strength (Taylor and Kennedy 1969; Zuschin et al. 2001; Avery and Etter 2006; Lee et al. 2011) through increased shell pliability (Newell et al. 2007), shearing, energy absorption, and prevention of crack propagation (Currey and Taylor 1974). However, oyster shells in predator treatments contained relatively less organic amino acid material, perhaps because this organic component is generated slowly and is expensive to produce (2.1 versus 1.1 J mg^{-1} CaCO$_3$; Lee et al. 2016 and Frieder et al. 2016 respectively).

This reduction in organic shell content suggests that oysters increased the production of inorganic calcium carbonate in response to predation risk. Although calcium carbonate shell material is weaker than organic shell material (Currey

Table 2 Summary table for somatic tissue weight model

Factor	Estimate	Standard error	t value	P value
Intercept	0.57	0.043	13.43	<0.0001**
Thickness	0.024	0.0046	5.33	<0.0001**
Density	0.033	0.012	2.69	0.0080**
Residual standard error	R^2	F-statistic	df	P value
0.063	0.19	14.22	2/123	<0.0001**

Statistics for full model listed below statistics on individual factors within model. Significance at $\alpha = 0.05$ denoted with **, $\alpha = 0.1$ with *

Table 3 Summary table for gonad index model

| Factor	Estimate	Standard error	t value	P value
Intercept	1.31	0.43	3.05	0.0032**
Thickness	−0.16	0.15	−1.04	0.30
Density	0.042	0.077	0.55	0.59
Interaction	0.38	0.20	1.92	0.060*
Residual standard error	R^2	F-statistic	df	P value
0.28	0.083	2.05	3/68	0.16

Statistics for full model listed below statistics on individual factors within model. Significance at $\alpha = 0.05$ denoted with **, $\alpha = 0.1$ with *

and Taylor 1974), this material is less energetically expensive to produce (1.1 J mg^{-1}; Frieder et al. 2016), contributes somewhat to shell strength (Avery and Etter 2006; Lombardi et al. 2013) and, under conditions favorable for calcification, can be laid quickly to increase shell volume (Carter 1980; Lee et al. 2011). Avery and Etter (2006) found the same increases in shell strength and inorganic content in *Nucella lapillus* and suggested that it was more adaptive for *N. lapillus* to escape predators by quickly reaching a size refuge than to deter them by maximizing shell strength. This theory is also supported by previous data (Scherer 2017), which found that oyster shells have greater overall shell strength, but are weaker per unit of shell material (i.e., shells are composed of weaker material), when reared with predators.

Further, it is unknown whether oysters alter the microstructure of inorganic shell material under conditions of predation risk. Oyster shells are composed primarily of calcite (Lee et al. 2011) and preliminary data suggest that oysters do not alter their proportion of calcite versus aragonite under conditions of predation risk (Scherer 2017). However, inorganic material of oyster shells consists of two components, the folia and the chalky layer. Both components provide unique mechanical properties which may influence the way shells respond to mechanical stress. Folia is the harder component and provides support to the shell, similar to a skeleton in the human body (Lee et al. 2011). The chalky layer can quickly fill volume in the shell, increases force absorption, and is very brittle, allowing layers of shell to

be sacrificed during predator encounters without harm to internal tissues (Lee et al. 2011). It was outside the scope of this study to investigate changes in these microstructures in response to predation risk, but this provides a valuable avenue for future research of this defense mechanism.

Our results contrast with those of Newell et al. (2007), who found oysters (*Crassostrea virginica*) exposed to mud crabs (*Rhithropanopeus harrisii*) significantly increased organic shell content while those exposed to blue crabs did not alter the relative make-up of organic to inorganic material in their shells. Unrecognized differences in methodology, such as the effect of food availability (Bourdeau 2010) or the influence of possible differences in water chemistry on the construction of calcium carbonate (Barton et al. 2012, 2015), may contribute to these differences. Further, different findings here which indicate oysters increase inorganic shell material in response to blue crabs may be explained by the use of the less accurate weight-loss-upon-ignition method by Newell et al. (2007), which is known to overestimate organic content (Goulletquer and Wolowicz 1989). Additionally, different predator species may trigger different responses in prey (Bourdeau 2009). Newell et al. (2007) found that oysters increase organic shell content in response to mud crabs, a smaller, slower species of intermediate predator, which we did not test in this study, although small individuals were present near the cages. However, Robinson et al. (2014) found that oysters exposed to mud crab exudates produced shells that were stronger without increases in shell weight,
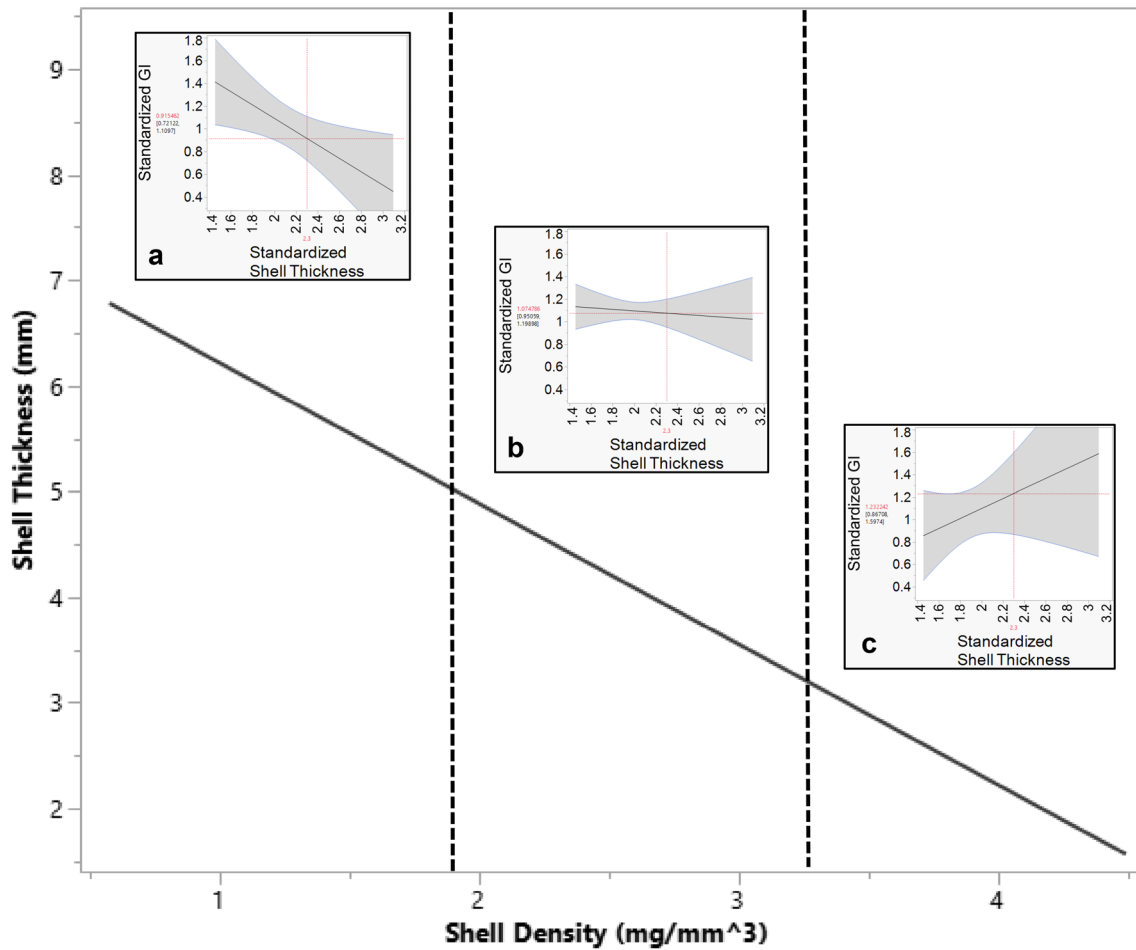
Fig. 4 Graphical display of interaction between shell thickness and density on gonad index. Background graph is inverse linear relationship between shell density and thickness (Fig. 2). Insert graphs taken from interactive profiler in JMP (data and code available in Online resources) demonstrate the influence of variation in shell thickness on gonad index for shells with **a** low, **b** intermediate, **c** high density

Table 4 $X_{\text{treatment}} \pm$ SD of shell metrics

Shell metric	0 h	2 h	24 h	168 h
Weight (mg)	0.029 ± 0.029	0.29 ± 0.054	0.30 ± 0.060	0.23 ± 0.067
Crushing force (N)	0.89 ± 0.078	0.95 ± 0.065	0.93 ± 0.081	1.82 ± 0.20

suggesting that oysters may employ a different defense mechanism in response to mud crab predators.

Increased shell thickness is a common defense in molluscs generally (Appleton and Palmer 1988; Covich and Crowl 1990; Bourdeau 2010; Johnson and Smee 2012) and oysters specifically (Newell et al. 2007; Lord and Whitlatch 2012; Johnson and Smee 2012; Robinson et al. 2014; Scherer et al. 2016) but, as both thickness and density are known to contribute positively to shell strength (Zuschin et al. 2001; Newell et al. 2007; Lombardi et al. 2013), we hypothesized both would increase under conditions of risk. In contrast, we found a strong inverse relationship between shell thickness and density. This may be attributable to the nature of shell inorganic material. The microstructure of this material influences its density; although the chalky layer has a higher density than the folia, pore space within this layer reduces the density of the chalky layer below that of the folia (Lee et al. 2011). It is unknown whether inorganic shell microstructure changes under conditions of predation risk, but it is possible that oysters increase the production of the chalky layer, either to more quickly increase shell volume or for the mechanical benefits it provides. Thus, shell density may decrease when oysters quickly increase shell inorganic material under conditions of risk if oysters increase the proportion of chalky microstructures.
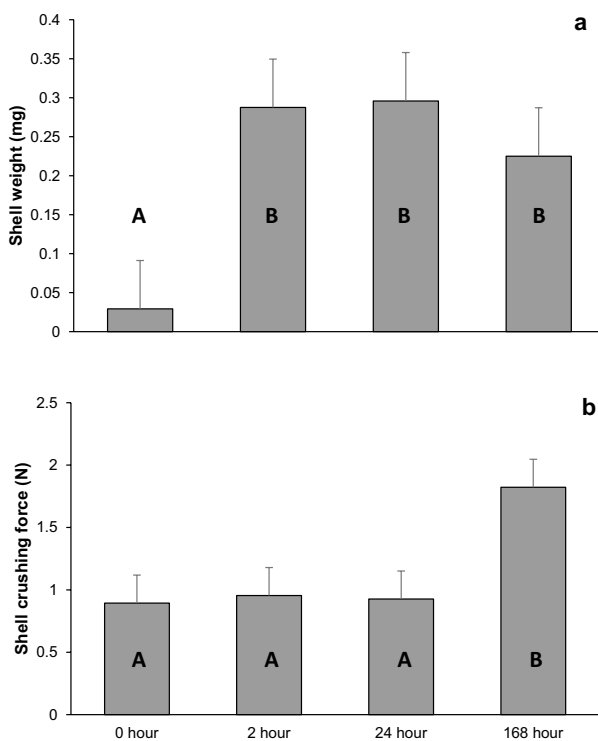
Fig. 5 Mean + standard error, $n_{treatment} = 8$ for **a** shell weight, **b** shell compression force of oyster spat reared under laboratory conditions with variable predator presence. Letter designations represent results of pairwise comparisons; treatments sharing a letter not statistically different from one another

Based on the phenotypic plasticity theory, our initial hypothesis assumed that increasing shell fortification was costly and would reduce oyster investment in growth (somatic tissue) and reproduction (gonad index). Due to the low energy costs of calcium carbonate production under current conditions (Frieder et al. 2016), it is no longer possible to assume the existence of such costs; in contrast, we found that shell metrics have a significant positive effect on somatic tissue weight. However, we also assumed that oysters altered shell morphology to increase shell strength. If, rather than strength, oysters increase calcium carbonate to increase shell growth rates, associated changes in soft tissue may be necessary to accommodate size increases. Our method of assessing soft tissue via weight provides no method to assess the quality of this soft tissue and further research is necessary to investigate this theory and possible effects of increased growth on the quality of soft tissue.

Although we were not able to detect significant negative effects of shell thickness or density on soft tissue or gonad index, we are hesitant to assume these defenses incur no costs to oysters for several reasons. First, our model for gonad index, although not significant overall, did contain an interaction which was significant at $\alpha = 0.1$. Calcified

structures serve many functions and are a compromise of diverse pressures (Gazeau et al. 2007; Bourdeau 2010; Amaral et al. 2012; Barton et al. 2012, 2015; Lord and Whitlatch 2014), making patterns difficult to discern. Yet, we found that a significant interaction between the effects of shell density and thickness on investment in gonad tissue. This interaction indicates the relationship between shell thickness and gonad index changes for different values of shell density, perhaps due to changes in the mechanism by which oysters alter their morphology. Given the strong inverse relationship between thickness and density, we demonstrated that increases in shell thickness had more negative impacts on gonad index at lower density values (Fig. 4), when we assume predation risk is highest. We hypothesize that, as risk increases, the benefits of defending grow to outweigh additional costs and it becomes beneficial for oysters to increase energy-expensive organic shell material (in addition to calcium carbonate). This shift in strategy would explain the shift in the relationship between shell characteristics and gonad index which results in a more negative relationship and higher cost at these more intense levels of risk.

Although we were unable to test this defense strategy directly, the results of our laboratory study provide an indirect test of our hypothesis. Under this mechanism, oysters would begin increasing calcium carbonate at low levels of predation risk. This would result in the increases in shell weight we observed for all levels of predation risk. However, organic material, which maximizes shell strength, would only be increased at higher levels of risk and only become detectable when risk reached a certain threshold. This would explain increases in oyster shell strength only at the highest tested level of predation risk. These results support the presence of a novel and complex mechanism of defense induction in oysters, which seeks to minimize defense costs by escaping predators at intermediate levels of risk, but switches to deterring predators at high levels of risk where defense benefits are maximized. This potential mechanism warrants additional studies assessing shell and tissue growth relationships among oysters exposed to various levels of predation risk to fully address this relationship. Further, these results demonstrate the value of carefully quantifying defense mechanisms and costs, as they contrast with previous assumptions and provide very different predictions regarding predator effects on oyster reef ecosystems.

Our experiment likely represents a conservative estimate of defense costs in this species for several reasons. First, our sample size was small and few oysters were found with density values near the minimum or maximum, which limited our statistical power when testing stronger relationships with gonad tissue in this region. Second, the small energy demand to produce calcium carbonate may make the costs of defenses small and difficult to detect, especially if the cost is shared between multiple

processes. Other potential costs, such as limitations on body volume (Lively 1986; Trussell and Nicklin 2002; Avery and Etter 2006) or immune responses (Kroeker et al. 2014; Duong and McCauley 2016), may share or suffer disproportionately greater costs if oysters prioritize and preserve growth and reproduction. Further, calcified shells are influenced by numerous pressures, including predation as well as temperature (Lord and Whitlatch 2014), pH (Gazeau et al. 2007; Amaral et al. 2012; Barton et al. 2012) and carbonate saturation state (Waldbusser et al. 2015), and food availability (Bourdeau 2010; Scherer and Smee 2017). It will require additional research to tease apart ecologically important patterns in shell costs due to predation amongst all the natural forces influencing shell structure. Third, adult oysters do not respond to predation risk (market size > 5 cm; Johnson and Smee 2012) and there is uncertainty regarding how strongly induction is maintained as oysters grow. Thus, costs may not be as apparent in the adult oysters (65.5 ± 0.89 mm) we used to ascertain relationships between shell thickness, density, and fecundity and may be more apparent in younger oysters, which are known to produce less soft tissue under conditions of risk (Johnson and Smee 2012). Studies that address these factors will provide clearer measurements of any defense costs and may strengthen evidence for a two-tiered system of defense induction.

Finally, costs which are small or insignificant under current conditions may not remain so. Baseline studies that establish an understanding of relationships between shell characteristics and predation risk will become increasingly important in the face of ocean acidification, which will likely alter the costs of shell production and maintenance. Decreasing ocean pH and carbonate saturation state will provide great challenges for calcifying organisms by making the formation and maintenance of shells more expensive. Several species are known to reduce calcification under conditions of ocean acidification (Gazeau et al. 2007; Bibby et al. 2007; Gaylord et al. 2011; Melatunan et al. 2013; Coleman et al. 2014; Mackenzie et al. 2014; Waldbusser et al. 2015) in ways which can alter prey defenses and species interactions (Bibby et al. 2007; Kroeker et al. 2014). Conditions of ocean acidification have been shown to reduce calcification (Gazeau et al. 2007; Amaral et al. 2012; Barton et al. 2012), growth, and larval production (Barton et al. 2012, 2015) in oysters in ways which can increase oyster consumption by predators (Amaral et al. 2012). Thus, costs which are small or nonexistent under current conditions may become larger and more difficult to pay as conditions change. However, not all organisms will be influenced equally by these changes (Coleman et al. 2014), and predator prey interactions will become increasingly difficult to predict if the factors of importance are poorly understood under current conditions (Kroeker et al. 2014).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Data availability Data for all portions of the study are available from the data library Pangaea (Scherer et al. 2018).

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Amaral V, Cabral HN, Bishop MJ (2012) Effects of estuarine acidification on predator-prey interactions. Mar Ecol Prog Ser 445:117–127. https://doi.org/10.3354/meps09487

Appleton RD, Palmer AR (1988) Water-borne stimuli released by predatory crabs and damaged prey induce more predator-resistant shells in a marine gastropod. Proc Natl Acad Sci USA 85:4387–4391

Avery R, Etter RJ (2006) Microstructural differences in the reinforcement of a gastropod shell against predation. Mar Ecol Prog Ser 323:159–170

Baldwin IT (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proc Natl Acad Sci USA 95:8113–8118

Barton A, Hales B, Waldbusser GG, Langdon C, Feely RA (2012) The Pacific oyster, *Crassostrea gigas*, shows negative correlation to naturally elevated carbon dioxide levels: implications for near-term ocean acidification effects. Limnol Oceanogr 57:698–710. https://doi.org/10.4319/lo.2012.57.3.0698

Barton A, Waldbusser GG, Feely RA, Weisberg SB, Newton JA, Hales B, Cudd S, Eudeline B, Langdon CJ, Jefferds I, King T, Suhrbier A, McLaughlin K (2015) Impacts of coastal acidification on the Pacific Northwest shellfish industry and adaptation startegies implemented in response. Oceanography 28:146–159. https://doi.org/10.5670/oceanog.2015.38

Beck MW, Brumbaugh RD, Airoldi L, Carranza A, Coen LD, Crawford C, Defeo O, Edgar GJ, Hancock B, Kay MC, Lenihan HS, Luckenbach MW, Toropova CL, Zhang G, Guo X (2011) Oyster reefs at risk and recommendations for conservation, restoration, and management. Bioscience 61:107–116. https://doi.org/10.1525/bio.2011.61.2.5

Bibby R, Cleall-Harding P, Rundle S, Widdicombe S, Spicer J (2007) Ocean acidification disrupts induced defences in the intertidal gastropod *Littorina littorea*. Biol Lett 3:699–701. https://doi.org/10.1098/rsbl.2007.0457

Bourdeau PE (2009) Prioritized phenotypic responses to combined predators in a marine snail. Ecology 90:1659–1669

Bourdeau PE (2010) An inducible morphological defence is a passive by-product of behaviour in a marine snail. Proc R Soc B Biol Sci 277:455–462. https://doi.org/10.1098/rspb.2009.1295

Carter JG (1980) Environmental and biological controls of bivalve shell mineralogy and microstructure. In: Rhoads DC, Lutz RA (eds) Skeletal growth of aquatic organisms. Plenum, New York, pp 69–113

Coleman DW, Byrne M, Davis AR (2014) Molluscs on acid: gastropod shell repair and strength in acidifying oceans. Mar Ecol Prog Ser 509:203–211. https://doi.org/10.3354/meps10887

Covich AP, Crowl TA (1990) Predator-induced life-history shifts in a freshwater snail. Science 247(4945):949. https://doi.org/10.1126/science.247.4945.949

Cronin G (2001) Resource allocation in seaweeds and marine invertebrates: chemical defense patterns in relation to defense theories. In: McClintock JB, Baker BJ (eds) Chemical ecology. CRC, Boca Raton, pp 325–353

Currey JD, Taylor JD (1974) The mechanical behavior of some molluscan hard tissues. J Zool 173:395–406. https://doi.org/10.1111/j.1469-7998.1974.tb04122.x

Duong TM, McCauley SJ (2016) The effects of non-consumptive predation stress on immune response in a larval libellulid dragonfly. Ecology 27:1605–1610

Ferrari MCO, Wisenden BD, Chivers DP (2010) Chemical ecology of predator–prey interactions in aquatic ecosystems: a review and prospectus. Can J Zool 88:698–724. https://doi.org/10.1139/Z10-029

Frieder CA, Applebaum SL, Pan T-CF, Hedgecock D, Manahan DT (2016) Metabolic cost of calcification in bivalve larvae under experimental ocean acidification. J Mar Sci. https://doi.org/10.1093/nar/gkw1002

Gaylord B, Hill TM, Sanford E, Lenz EA, Jacobs LA, Sato KN, Russell AD, Hettinger A (2011) Functional impacts of ocean acidification in an ecologically critical foundation species. J Exp Biol 214:2586–2594. https://doi.org/10.1242/jeb.055939

Gazeau F, Quiblier C, Jansen JM, Gattuso J-P, Middelburg JJ, Heip CHR (2007) Impact of elevated CO_2 on shellfish calcification. Geophys Res Lett 34:L07603. https://doi.org/10.1029/2006GL028554

Goulletquer P, Wolowicz M (1989) The shell of *Cardium edule*, *Cardium glaucum* and *Ruditapes philippinarum*: organic content, composition and energy value, as determined by different methods. J Mar Biol Assoc U K 69:563–572. https://doi.org/10.1017/S0025315400030976

Grabowski JH, Peterson CH (2007) Restoring oyster reefs to recover ecosystem services. Theor Ecol Ser 4:281–298. https://doi.org/10.1016/S1875-306X(07)80017-7

Harvell CD (1990) The ecology and evolution of inducible defenses. Q Rev Biol 65:323–340

Hay ME (2009) Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. Ann Rev Mar Sci 1:193–212. https://doi.org/10.1146/annurev.marine.010908.163708

Johnson KD, Smee DL (2012) Size matters for risk assessment and resource allocation in bivalves. Mar Ecol Prog Ser 462:103–110. https://doi.org/10.3354/meps09804

Kroeker KJ, Sanford E, Jellison BM, Gaylord B (2014) Predicting the effects of ocean acidification on predator-prey interactions: a conceptual framework based on coastal molluscs. Biol Bull 226:211–222. https://doi.org/10.1016/j.biocon.2013.11.034

Langerhans RB, Dewitt TJ (2002) Plasticity constrained: over-generalized induction cues cause maladaptive phenotypes. Evol Ecol Res 4:857–870

Large SI, Smee DL (2013) Biogeographic variation in behavioral and morphological responses to predation risk. Oecologia 171:961–969. https://doi.org/10.1007/s00442-012-2450-5

Large SI, Torres P, Smee DL (2012) Behavior and morphology of *Nucella lapillus* influenced by predator type and predator diet. Aquat Biol 16:189–196. https://doi.org/10.3354/ab00452

Lee S-W, Jang Y-N, Ryu K-W, Chae S-C, Lee Y-H, Jeon C-W (2011) Mechanical characteristics and morphological effect of complex crossed structure in biomaterials: fracture mechanics and microstructure of chalky layer in oyster shell. Micron 42:60–70. https://doi.org/10.1016/j.micron.2010.08.001

Lee JW, Applebaum SL, Manahan DT (2016) Metabolic cost of protein synthesis in larvae of the Pacific oyster (*Crassostrea gigas*) is fixed across genotype, phenotype, and environmental temperature. Biol Bull 230:175–187

Liu Z, Lee C, Aller RC (2008) Drying effects on decomposition of salt marsh sediment and on lysine sorption. J Mar Res 66:665–689

Lively CM (1986) Competition, comparative life histories, and maintenance of shell dimorphism in a barnacle. Ecology 67:858–864

Lombardi SA, Chon GD, Lee JJ, Lane HA, Paynter KT (2013) Shell hardness and compressive strength of the eastern oyster, *Crassostrea virginica*, and the Asian oyster, *Crassostrea ariakensis*. Biol Bull 225:175–183

Lord JP, Whitlatch RB (2012) Inducible defenses in the eastern oyster *Crassostrea virginica* Gmelin in response to the presence of the predatory oyster drill *Urosalpinx cinerea* Say in Long Island Sound. Mar Biol 159:1177–1182. https://doi.org/10.1007/s00227-012-1896-7

Lord J, Whitlatch R (2014) Latitudinal patterns of shell thickness and metabolism in the eastern oyster *Crassostrea virginica* along the east coast of North America. Mar Biol 161:1487–1497. https://doi.org/10.1007/s00227-014-2434-6

Mackenzie CL, Ormondroyd GA, Curling SF, Ball RJ, Whiteley NM, Malham SK (2014) Ocean warming, more than acidification, reduces shell strength in a commercial shellfish species during food limitation. PLoS One 9:e86764. https://doi.org/10.1371/journal.pone.0086764

Melatunan S, Calosi P, Rundle SD, Widdicombe S, Moody AJ (2013) Effects of ocean acidification and elevated temperature on shell plasticity and its energetic basis in an intertidal gastropod. Mar Ecol Prog Ser 472:155–168. https://doi.org/10.3354/meps10046

Newell RIE, Kennedy VS, Shaw KS (2007) Comparative vulnerability to predators, and induced defense responses, of eastern oysters *Crassostrea virginica* and non-native *Crassostrea ariakensis* oysters in Chesapeake Bay. Mar Biol 152:449–460. https://doi.org/10.1007/s00227-007-0706-0

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2016) nlme: Linear and nonlinear mixed effects models. R package version 3.1-137. https://CRAN.R-project.org/package=nlme. Accessed Feb 2017

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Relyea RA (2002) Costs of phenotypic plasticity. Am Nat 159:272–282. https://doi.org/10.1086/338540

Robinson EM, Lunt J, Marshall CD, Smee DL (2014) Eastern oysters *Crassostrea virginica* deter crab predators by altering their morphology in response to crab cues. Aquat Biol 20:111–118. https://doi.org/10.3354/ab00549

Scherer AE (2017) Characterization of an induced morphological defense in the eastern oyster *Crassostrea virginica*. Dissertation, Texas A&M Univeristy-Corpus Christi, Corpus Christi

Scherer AE, Smee DL (2017) Eastern oysters *Crassostrea virginica* produce plastic morphological defenses in response to crab predators despite resource limitation. Biol Bull 233:144–150. https://doi.org/10.1086/695470

Scherer AE, Lunt J, Draper AM, Smee DL (2016) Phenotypic plasticity in oysters (*Crassostrea virginica*) mediated by chemical signals

from predators and injured prey. Invertebr Biol 135:97–107. https://doi.org/10.1111/ivb.12120

Scherer AE, Bird CE, McCutcheon MR, Hu X, Smee DL (2018) Shell (weight, density, thickness, crushing force, amino acid content) and soft tissue metrics (somatic tissue weight, gonad index) for eastern oysters *Crassostrea virginica* reared under variable predation conditions (S. Texas, 2013–14). PANGAEA. https://doi.pangaea.de/10.1594/PANGAEA.892092

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. https://doi.org/10.1038/nmeth.2089

Taylor JD, Kennedy WJ (1969) The influence of the periostracum on the shell structure of bivalve molluscs. Calcif Tissue Res 3:274–283

Tollrian R, Harvell CD (eds) (1999) the ecology and evolution of inducible defenses. Princeton University Press, Princeton

Trussell GC, Nicklin MO (2002) Cue sensitivity, inducible defense, and trade-offs in a marine snail. Ecology 83:1635–1647

Van Buskirk J (2000) The costs of an inducible defense in anuran larvae. Ecology 81:2813–2821

Waldbusser GG, Hales B, Langdon CJ, Haley BA, Schrader P, Brunner EL, Gray MW, Miller CA, Gimenez I (2015) Saturation-state sensitivity of marine bivalve larvae to ocean acidification. Nat Clim Chang 5:273–280. https://doi.org/10.1038/NCLIMATE2479

Zuschin M, Stanton RJ, Stanton RJ Jr (2001) Experimental measurement of shell strength and its taphonomic interpretation. Palaios 16:161–170