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Associated costs and benefits of a defended phenotype across multiple environments

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Summary

1. Many organisms respond to threats such as stress and predation by expressing a defended phenotype (phenotypic plasticity) or inducing the expression of a defended phenotype in offspring (transgenerational phenotypic plasticity). While defended phenotypes can increase resistance to a predator or stress, in the absence of the inducing agent defended phenotypes often have poorer performance. Producing a defended phenotype unnecessarily has been termed a phenotype-environment mismatch.

2. Most studies have focused on the benefits of a defended phenotype along a single environmental gradient (i.e. the presence/absence of the inducing agent) but in nature, organisms must face conditions that vary across a number of environmental gradients simultaneously. By focusing on the costs and benefits of a defended phenotype in a single dimension alone we risk underestimating the strength and likelihood of phenotype-environment mismatches.

3. For the marine bryozoan *Bugula neritina*, we examined the performance of individuals with an induced, defended phenotype (pollution resistance) relative to individuals with an undefended phenotype across a number of different environments. We found that individuals with the defended phenotype were more sensitive to osmotic stress, but surprisingly, were less susceptible to predation than individuals with the undefended phenotype.

4. Our findings suggest that the costs and benefits associated with expressing a defended phenotype are more complex than previously realized because the full consequences of induced phenotypes are only unmasked when performance in multiple environments is examined.

Key-words: inducible defences, maternal effect, phenotypic plasticity

Introduction

Phenotypic plasticity is the expression of different phenotypes by a single genotype in response to different environmental conditions (Schlichting 1986; Stearns 1992). In variable environments, phenotypic plasticity can increase the match between the phenotype and the environment, thereby increasing average fitness (Bradshaw 1965; Levins 1968; Dewitt, Sih & Wilson 1998). One of the most well-studied aspects of phenotypic plasticity is the expression of a 'defended' phenotype in response to a predator or other stress. For example, the water flea *Daphnia cucullata* develops a defended phenotype in response to chemical cues from predators and individuals expressing the defended phenotype suffer lower rates of predation than undefended individuals (Agrawal, Laforsch & Tollrian 1999). Defended phenotypes carry a fitness benefit in the presence of an inducing agent, but they are not produced constantly, suggesting that there is a downside or trade-off associated with their expression.

While defensive or induced phenotypes can increase resistance to a predator or stress, these benefits usually come with associated costs (Tollrian & Dodson 1999). For example, the acorn barnacle *Chthamalus anisopoma* expresses a predator-resistant morphology in the presence of predators, but individuals with the defended phenotype grow more slowly and are less fecund than individuals with the undefended phenotype – a major fitness cost in the absence of predators (Lively 1986). The risk of suffering fitness costs associated with unnecessarily expressing a defended phenotype may explain why defended phenotypes are not continually expressed: there is a net fitness benefit to producing a defended phenotype only when the threat of predation or stress is very high. The reduction in performance associated with producing a defended phenotype unnecessarily has been termed a 'phenotype-environment mismatch' (Dewitt, Sih & Wilson 1998). From an ecological standpoint, phenotype-environment mismatches can strongly affect population connectivity

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(Marshall *et al.* 2010), so it is important to determine the likelihood of such mismatches occurring. From an evolutionary standpoint, if we are to understand the selection pressures acting on phenotypic plasticity, then we must characterize the associated costs and benefits of different phenotypes (Dewitt, Sih & Wilson 1998). Typically, the costs and benefits of a defended phenotype have been examined in a single dimension (i.e. performance in the presence and absence of the inducing agent: Relyea 2001; Hoverman & Relyea 2009), but additional costs and benefits may occur in multiple dimensions simultaneously.

In nature, the environment that an organism faces is more complex than the simple presence or absence of a single predator or stress (Hoverman & Relyea 2009). Similarly, the induction of a defended phenotype may affect the performance of the focal organism in ways that are unrelated to its susceptibility to the inducing agent. By focusing on the costs and benefits of a defended phenotype in a single dimension alone we risk underestimating the strength and likelihood of phenotype-environment mismatches. Examining the costs and benefits of an induced phenotype across multiple environments should result in a more complete understanding of the evolutionary causes and ecological consequences of phenotypic plasticity. Multi-dimensional phenotypic plasticity studies were rare until relatively recently (Bourdeau 2009; Freeman, Meszaros & Byers 2009; Hoverman & Relyea 2009; Mirza & Pyle 2009). The majority of recent studies on this issue have been restricted to the examination of the associated costs and benefits of predation-resistance in the presence of multiple predators and typically focus on plasticity within a single generation. Whether sources of stress other than predation that induce defended phenotypes have similar associated costs and benefits remains largely unknown and we know of no studies that have examined the associated costs and benefits of a defended phenotype that is induced across generations.

Maternal effects can be viewed as a common form of transgenerational phenotypic plasticity where the offspring phenotype is affected by the maternal phenotype/environment so the environmental experiences in one generation affect the expression of phenotypes in the following generation (Mousseau & Dingle 1991; Fox, Thakar & Mousseau 1997; Agrawal, Laforsch & Tollrian 1999; Marshall & Uller 2007). Transgenerational plasticity, just like phenotypic plasticity within a single generation, can buffer offspring against changes in the environment by increasing the likelihood of a good offspring phenotype-environment match. Because offspring frequently disperse away from the maternal environment however (Pechenik 1999; Fowler 2005), the environmental cue and phenotypic response can be decoupled in both space and time. This decoupling creates a much greater potential for phenotype-environment mismatching, but there have been limited examinations of the phenotype-environment mismatches generated by maternal effects and none has examined the potential for phenotype-environment mismatches across multiple environmental dimensions. Here, we examine the potential for phenotype-environment mismatches as a result of transgenerational plasticity across multiple offspring environments.

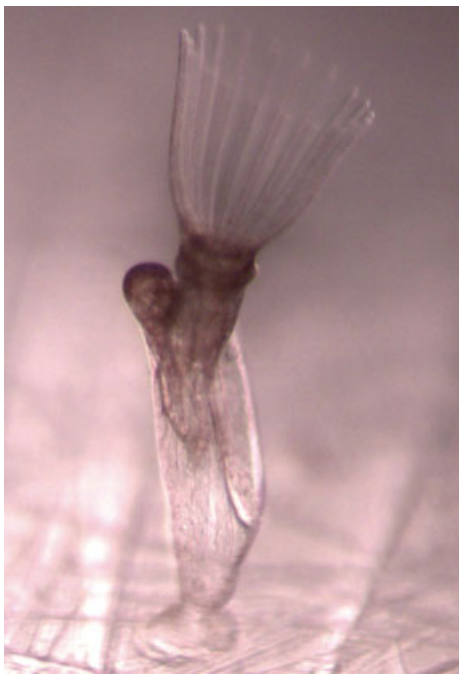

Fig. 1. Image of *Bugula neritina* shortly after settlement.

In the marine bryozoan *Bugula neritina* (Fig. 1), copper-exposed mothers produce offspring that are more resistant to copper exposure relative to the offspring of copper-naïve mothers (Marshall 2008). However, offspring from copper-exposed mothers grow poorly in the absence of copper (Marshall 2008) suggesting that there is a physiological cost associated with copper resistance. In this study, we examine the effect that maternally derived copper resistance has on offspring performance in two alternative environments. Specifically, we examined early post-metamorphic survival of copper-resistant offspring after their exposure to low salinity or predators. We chose to examine performance in environments of osmotic stress and predation because variation in salinity is a very common physiological stress in *B. neritina's* habitat and predation is major source of mortality in this species (D. J. Marshall, unpublished data). By examining the associated costs and benefits of the defended phenotype (copper resistance) under different environmental conditions, we hope to more fully understand the associated repercussions of producing defended phenotypes.

Materials and methods

Bugula neritina is an arborescent bryozoan that is a common member of the fouling community worldwide. *B. neritina* grows by asexual budding of the zooids and broods its larvae for approximately 1 week in specialized zooids called ovicells (Marshall 2008). Collection and field components of our experiments were conducted at Moreton Bay Boat Harbour at Redcliffe, Brisbane and Mooloolaba Marina at Mooloolaba on the Sunshine Coast. Experimental subjects were transported to and from the field sites in insulated containers filled with seawater.

Copper solutions were mixed from 1000 μg Cu L^{-1} stock solutions that were prepared using analytical-reagent grade $CuSO_4$ (copper II sulphate anhydrous; Sigma Chemicals, Balcatta, Perth, WA, Australia) and filtered seawater (FSW). FSW was prepared by collecting seawater from the field and filtering it with 0·45-μm filter paper using a vacuum filter; it was also re-aerated prior to use. Experimental containers were acid washed by soaking in 5% nitric acid for at least 12 h and were then rinsed with Reverse Osmosis (RO) freshwater prior to use. Copper storage containers and experimental containers used in the treatment of *B. neritina* individuals with copper were also pre-soaked overnight in the appropriate concentrations of copper solution in order to minimize the reduction in copper via chelation to these containers during the treatment process (Batley, Apte & Stauber 1999). Larvae were settled in Petri dishes that were pre-roughened using sand paper and conditioned for at least 1 day in aquaria containing seawater from the appropriate field site in order to allow for the development of biofilms which encourage larval settlement. Any larvae that did not settle after 3 h were discarded along with the treatment solutions and the number of settled individuals was recorded in each instance.

To obtain offspring from copper-exposed mothers and copper-naïve mothers, we used the same methods as described in detail by Marshall (2008). Here, we will only describe our methods briefly. Thirty colonies that were not brooding larvae (see Marshall 2008 for details) were individually exposed to either a 300 μg Cu L^{-1} solution (15 replicate colonies) or copper-free FSW (15 replicate colonies) in the laboratory for 6 h, placed back into the field to brood larvae for 1 week and then returned to the laboratory where they were spawned using standard methods (Marshall, Bolton & Keough 2003). This experimental protocol induces mothers that have been exposed to copper to produce larvae that are larger and more resistant to copper than larvae from mothers that have not been exposed to copper (Marshall 2008).

Once we had generated offspring from copper-exposed mothers (i.e. copper-resistant offspring) and offspring from copper-naïve mothers, we were interested in whether these offspring differed in their susceptibility to lower levels of salinity. Salinity can frequently drop to 26 ppt following rain at our study site and is therefore an important and common stress for *B. neritina* (D. J. Marshall, unpublished data). From each of the 15 copper-exposed and 15 copper-naïve maternal colonies, we collected 20 larvae and placed 10 larvae into each of two 30 mm Petri dishes and allowed them 3 h to settle. We then manipulated the salinity of the offspring environment by diluting the FSW with reverse osmosis freshwater and used an orthogonal design where offspring from each mother were exposed to either 26‰ or 35‰ for 6 h. After 6 h, each dish was rinsed and refilled with 35‰ FSW and then placed into a 22 °C constant temperature cabinet to allow the settlers to complete metamorphosis. We then recorded the proportion of settled individuals in each treatment that were still alive after 5 days. Individuals that showed normal development with no deformation of the feeding structure were classed as 'alive' and individuals that disappeared or showed deformation or absence of the feeding structure were classed as 'dead' (Marshall 2008).

We repeated this experiment using colonies from the Mooloolaba marina. This second experimental run was conducted during late spring when water temperatures are warmer so the maternal generation were exposed to their respective treatments and then redeployed to the field for 5 days instead of seven as the brooding period tends to be shorter at this time of the year (D.T. Moran, personal observation). Once again we used 30 maternal colonies and larvae were obtained from 15 copper-exposed mothers and 15 copper-naïve mothers. Data from both of these experiments were combined and analysed together with run as a blocking factor, giving a combined total of 30 replicates within each maternal treatment.

We were also interested in whether offspring from copper-exposed mothers were more or less susceptible to predation than offspring from copper-naïve mothers. To compare the resistance to predation between offspring from copper-exposed mothers and offspring from copper-naïve mothers, we collected an additional 10 larvae from each of the maternal colonies that were used in the previous experiment (15 copper-exposed mothers and 15 copper-naïve mothers). We then placed the larvae from each maternal colony into a single 30 mm Petri dish, giving a total of 30 dishes, and allowed them 3 h to settle. We obtained 6·93 ± 0·63 (mean ± SE) settlers per dish from copper-naïve mothers and 6·67 ± 0·76 settlers per dish from copper-exposed mothers. Following settlement, we introduced an unknown species of predatory flatworm into the dishes at a ratio of one flatworm to every two *B. neritina* settlers. The flatworms we used are voracious predator of both fully grown *B. neritina* colonies and new settlers and can obliterate entire populations within a few weeks (D.T. Moran, personal observation). Flatworms used in this experiment (and later experiments) were starved for 4 h prior to their introduction to the dishes. We counted the number of survivors in each dish after 3 h of exposure to predation.

We found that offspring from copper-exposed mothers suffered less predation than offspring from copper-naïve mothers (see Results) and so in another set of experiments we attempted to disentangle some of the potentially competing mechanisms that may have driven our results: either large individuals could be sheltered from predation by a size refuge, or predators might be avoiding contaminated prey.

Exposure to copper is known to affect offspring size in *Bugula neritina* (Marshall 2008) and so we were interested in whether larger offspring were more resistant to predation regardless of whether they came from copper-exposed or copper-naïve mothers. If larger offspring are more resistant to predation, we can then attribute the increased predation resistance of offspring from copper-exposed mothers to the associated change in larval size. We induced 10 copper-naïve colonies to spawn using the previously outlined methods, collected the larvae and classified them into two groups, one containing 'large' larvae and one containing 'small' larvae. In Run one of this experiment, larvae in the large group ranged in size from 0·54 to 0·66 mm^2 and larvae in the small group ranged from 0·40 to 0·54 mm^2. In Run two, larvae in the large group ranged from 0·51 to 0·64 mm^2 and from 0·32 to 0·49 mm^2 in the small group. For each treatment (large and small) we evenly distributed 100 larvae into 10 pre-conditioned Petri dishes and allowed them 3 h to settle. After 3 h we counted the number of settlers, discarded any larvae that had not settled and introduced the predatory flatworms at a density of one flatworm for every two settlers. Average settlement ± SE was fairly uniform across runs and treatments (run one: small 8·60 ± 0·31 large 9·10 ± 0·35; run two: small 9·30 ± 0·26 large 9·50 ± 0·31). We measured survivorship of the settlers after 3 h of exposure to the predators.

An alternative hypothesis for why offspring from copper-exposed mothers were less susceptible to predation is that offspring from toxicant-exposed parents can contain higher levels of toxicant within their tissues (Wu, Lin & Yang 2008) and therefore may be avoided by predators (Filser, Wittmann & Lang 2000). To test this hypothesis, we examined whether predators avoided consuming settlers that had been exposed to copper very recently and therefore presumably had high concentrations of copper in their tissues relative to settlers that had not been exposed to copper (Evans *et al.* 2006). We induced 10 copper-naïve colonies to spawn as outlined before and larvae were

then allocated to pre-conditioned petri-dishes randomly so that each petri-dish held 10 larvae. There were 26 pre-conditioned Petri dishes in run one and 32 pre-conditioned petri dishes in run two. We allowed the larvae to settle for 3 h during which time half of the Petri dishes were filled with FSW while the other half were filled with a solution of 100 μg Cu L^{-1}. We chose a lower copper concentration to expose the settling larvae to because 300 μg Cu L^{-1} is lethal to new settlers (D. J. Marshall, unpublished data). After 3 h, the seawater solutions in both treatments were discarded and the Petri dishes were rinsed in seawater and filled with FSW. We then counted the number of settlers and introduced the predatory flatworms into the Petri dishes at the same ratio as used in our previous experiments. Average settlement ± SE was again fairly uniform across runs and treatments (run one: FSW 9·46 ± 0·24, Cu 9·92 ± 0·21; run two: FSW 9·38 ± 0·20, Cu 9·87 ± 0·19).We measured survivorship of the settlers after 3 h of exposure to predators.

For our analyses of the salinity experiment and the initial predation experiment, we used a partly nested ANOVA where both fixed and random factors were included in the analysis. Maternal environment and offspring environment are both fixed factors while the identity of individual mothers is a random factor that is nested within maternal environment. For our later predation experiments we used a mixed-effect ANOVA where larval size (big or small) and larval environment (Cu or FSW) are both fixed factors. Where more than one experimental run was used, we included run as a random factor. We utilized the recommendations of Quinn & Keough (2002) and used $P > 0{\cdot}25$ as a criterion for model reduction as well as visual inspection and changes in R^2 values to help inform our decisions on what terms were retained in the final models.

Results

There was a significant interaction between the maternal environment (300 μg Cu L^{-1} or FSW) and the salinity (26‰ or 35‰) of the offspring environment on the survival of offspring following metamorphosis (Fig. 2, Table 1). This interaction was due to the presence of a survival differential between offspring from mothers with different exposure histories when offspring experienced low salinity. At 26‰, offspring from copper-naïve mothers had significantly higher survival than offspring from copper-exposed mothers, but at 35‰, there was no difference in survival between offspring from either maternal environment.

The survival of offspring after 3 h of exposure to predation was ~15% higher among the offspring of copper-exposed mothers than among the offspring of copper-naïve mothers (ANOVA, $F_{1,28} = 4{\cdot}28$, $P = 0{\cdot}048$) (Fig. 3).

Neither offspring size alone (Table 2) nor direct exposure of larvae to copper (Table 2) affected the survival of settlers after exposure to predation for 3 h.

Discussion

The transgenerational induction of copper resistance had complex effects on offspring performance under different conditions. We found that copper-resistant offspring performed poorly relative to copper-susceptible offspring in low salinity but copper-resistant offspring were more resistant to a common predator. Our findings suggest that the costs and benefits associated with expressing a defended phenotype can be multifarious and complex. Previous studies have shown that producing a defended phenotype in the absence of the inducing agent results in a phenotype-environment mismatch, and ultimately, a fitness cost (Dewitt, Sih & Wilson 1998). We have uncovered additional complexities associated with producing defended phenotypes: the fitness cost and benefits associated with the defended phenotype of copper resistance change dramatically depending on the broader environmental context in which this defended phenotype is expressed. Previous studies on the induction of predation-resistant phenotypes have also found what they term 'survival trade-offs' across predator environments – induced phenotypes that are resistant to one predator can increase susceptibility to other predators that employ functionally different foraging strategies (Tollrian & Dodson 1999; Kishida & Nishimura 2005; Bourdeau 2009; Hoverman & Relyea 2009). It seems that similar survival trade-offs can also occur across physiological environments: copper-susceptible offspring have a survival

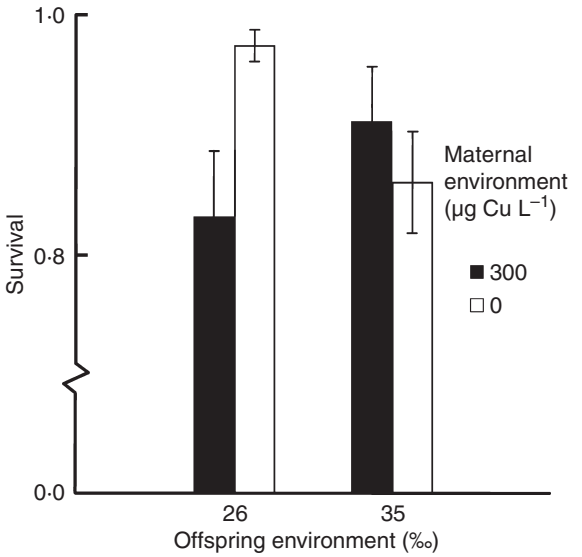

Fig. 2. Effect of offspring environment and maternal environment on survival of *Bugula neritina* offspring after 5 days in the laboratory. Bars represent survival (mean ± SE) of offspring at low and normal salinity. Open bars indicate survival of offspring from toxicant-naïve mothers, and shaded bars indicate survival of offspring from mothers that were exposed to 300 μg Cu L^{-1} a week before spawning.

Table 1. Effect of maternal environment (Cu or FSW) and offspring environment (26‰ or 35‰) on the survival of *Bugula neritina* settlers after 5 days (Non-significant interactions ($P \geq 0{\cdot}25$) have been excluded from the final model)

Source	d.f.	MS	*F*	*P*
Maternal environment	1	0·007	0·176	0·676
Offspring environment	1	0·001	0·026	0·872
Run	1	0·026	0·618	0·435
Maternal env. × Offspring env.	1	0·214	5·149	0·027
Maternal environment × Run	1	0·308	7·391	0·009
Maternal ID (Maternal environment)	29	0·043	1·033	0·445
Error	58	0·042		

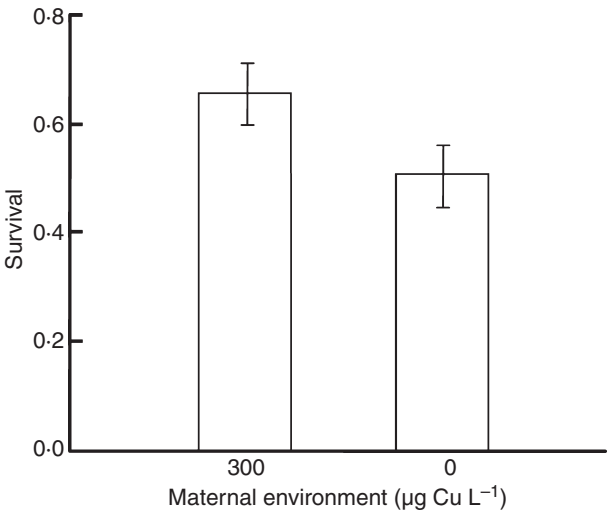

Fig. 3. Effect of maternal environment on the survival of *Bugula neritina* offspring after 3 h of exposure to flatworm predators. Bars represent survival (mean ± SE) of offspring.

Table 2. Effect of (a) offspring size and (b) larval exposure history (copper or FSW) on the survival of *Bugula neritina* after 3 h of exposure to predators

Source	d.f.	MS	*F*	*P*
(a) Offspring size				
Larval size	1	0·001	0·019	0·913
Run	1	2·075	94·333	< 0·001
Larval size × Run	1	0·075	3·421	0·073
Error	36	0·022		
(b) Larval exposure history				
Larval environment	1	0·096	2·657	0·350
Run	1	0·837	21·493	< 0·001
Larval env. × Run	1	0·036	0·933	0·338
Error	53	0·039		

advantage in low salinity environments that copper-resistant offspring are unable to achieve.

There are a number of possible mechanisms for the differences in performance between copper-susceptible and copper-resistant offspring that are observed in the two salinities. Copper-resistant offspring are poorer competitors (Marshall 2008), suggesting that copper resistance is energetically costly to maintain. The physiological requirements of maintaining copper resistance may thus interact with the metabolic needs of coping with osmotic stress, allowing copper-susceptible offspring to increase survival in low salinities while copper-resistant offspring are unable to do so. There is some support for this suggestion: Legras *et al*. (2000) found a physiological link between coping with low salinities and coping with high levels of heavy metals: low salinity environments can have detrimental effects on the production of proteins that increase resistance to heavy metals (metallothionein) and this increased heavy metal accumulation in two species of crabs. Alternatively, the exposure of mothers to copper could cause epigenetic damage to offspring such that they are less effective at coping with the physiological stress associated with reduced salinity environments. While the precise mechanism is unclear, it seems that copper-susceptible offspring perform better than copper-resistant offspring when they are both exposed to osmotic stress (26‰) even though their performances were similar at regular salinity (35‰). This variation in performance illustrates that the fitness consequences of defended phenotypes depend on the broader environmental context in which the phenotype is expressed.

A surprising result from our study is that offspring from copper-exposed mothers were more resistant to predation than the offspring from copper-naïve mothers. In contrast to our study, previous studies tend to find an opposite effect: for example, Relyea & Mills (2001) found that toxicants exacerbated the impacts of predators. We assessed two potential mechanisms for the increase in predation resistance but none of our experiments provided a clear explanation. Copper-exposed *B. neritina* mothers produce larger offspring (Marshall 2008) and previous studies have shown that some flatworm predators prefer smaller prey (Sih & Moore 1993). We hypothesized that offspring from copper-exposed mothers suffered less predation because they were within a size refuge but when we tested this idea with large and small larvae from copper-naïve mothers, we found no size advantage. It is possible that offspring size still drove our observations in the initial predation experiment if we generated a very large size difference that was not adequately represented in this subsequent study. Such a possibility is unlikely however given that the size differences we used in our experiments here exceed the differences previously observed in this system (Marshall 2008). In some systems, predators avoid contaminated prey (Filser, Wittmann & Lang 2000). If offspring from copper-exposed mothers have trace amounts of copper in them, flatworms may avoid consuming them. When we tested this hypothesis we found that flatworms consumed copper-exposed settlers in equal proportions to copper-free settlers. Finally, some studies have found that toxicant exposed prey can be of poorer quality and so are avoided by predators (Romeis, Dutton & Bigler 2004), but given that copper-resistant offspring are actually larger in this species (Marshall 2008) and are not sheltered by a size refuge (as found in this study), such an effect seems unlikely. Regardless of the mechanism, *B. neritina* offspring from copper-exposed mothers are less likely to be consumed by flatworms, and therefore gain a selective advantage over the offspring of copper-naïve mothers when predators are abundant. This finding is unusual given that many studies in this field have shown that the expression of a defended phenotype generally only carries fitness benefits when the inducing stress is present (see Hoverman & Relyea 2009). To our knowledge, this is the first study to find that there are benefits associated with the expression of a defended phenotype in the absence of the inducing agent. As far as we are aware, the influence of these associated benefits on the evolution of induced phenotypes has not been explored theoretically and we eagerly await studies that determine whether such associated benefits are common and whether they affect the evolution of phenotypic plasticity more generally.

Our results suggest that the links between maternal experience and offspring phenotype are more complex than previously recognized. Mothers face the formidable challenge of manipulating offspring phenotype so as to maximize performance for a combination of environments – for instance, under osmotic stress copper-resistant offspring perform poorly compared to copper-susceptible offspring but there are additional benefits to copper-resistance if encounters with predators are likely. We have no data on the co-occurrence of different environmental variables in our system, but mothers do change the phenotype of their offspring in response to copper in this system (Marshall 2008) and this suggests that the overall benefits gained by copper-resistant individuals outweigh the potential costs. However, if decreased salinity regularly co-occurs with high levels of copper pollution, as it does in some regions (see Sinex & Wright 1988; Ramos, Inoue & Ohde 2004), then our results suggest that copper pollution might have negative impacts on populations even when organisms have evolved responses to the pollutant itself – copper-resistant individuals could suffer higher mortality than copper-susceptible individuals during periods of lower salinity. Alternatively, if copper pollution and osmotic stress do not co-occur regularly or, if there is a regular co-occurrence of copper pollution and predation pressure than the opposite is true and copper-resistant individuals are likely to gain a selective advantage, it is therefore important that phenotypic plasticity and induced defences be examined with environmental context in mind.

Via & Lande (1985) highlighted the importance of recognizing the costs of phenotypic plasticity for understanding its evolution and maintenance. More than 20 years on, the costs associated with phenotypic plasticity are still the subject of intense debate. Recently, Callahan, Maughan & Steiner (2008) categorized the costs of phenotypic plasticity into two groups: the 'phenotypic' costs and the 'plasticity' costs – the former focuses on the costs associated with expressing a defended phenotype, while the later focuses on the costs of having a phenotypically flexible genotype. Our findings suggest that the consequences of expressing certain phenotypes are more complicated than previously realized because the full negative or positive effects of the induced phenotype are only unmasked under multiple environmental conditions. Subsequently, examining the fitness costs and benefits of a defended phenotype along only one environmental gradient may lead to a misestimation of the repercussions of expressing that phenotype. We therefore suggest that future studies should examine the costs and benefits of expressing defended phenotypes across more than the single environmental dimension of the presence and absence of the inducing agent, and rather should examine performance across several environmental dimensions.

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