Thermal stress and predation risk trigger distinct transcriptomic responses in the intertidal snail *Nucella lapillus*

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Abstract

Thermal stress and predation risk have profound effects on rocky shore organisms, triggering changes in their feeding behaviour, morphology and metabolism. Studies of thermal stress have shown that underpinning such changes in several intertidal species are specific shifts in gene and protein expression (e.g. upregulation of heat-shock proteins). But relatively few studies have examined genetic responses to predation risk. Here, we use next-generation RNA sequencing (RNA-seq) to examine the transcriptomic (mRNA) response of the snail *Nucella lapillus* to thermal stress and predation risk. We found that like other intertidal species, *N. lapillus* displays a pronounced genetic response to thermal stress by upregulating many heat-shock proteins and other molecular chaperones. In contrast, the presence of a crab predator (*Carcinus maenas*) triggered few significant changes in gene expression in our experiment, and this response showed no significant overlap with the snail’s response to thermal stress. These different gene expression profiles suggest that thermal stress and predation risk could pose distinct and potentially additive challenges for *N. lapillus* and that genetic responses to biotic stresses such as predation risk might be more complex and less uniform across species than genetic responses to abiotic stresses such as thermal stress.

Keywords: nonconsumptive effects, *Nucella lapillus*, predation risk, RNA-seq, thermal stress, transcriptomics

Received 15 October 2014; accepted 28 October 2014

Introduction

Rocky shore organisms must regularly cope with intense abiotic and biotic stresses. Chief among these is thermal stress, which often determines intertidal organisms’ biogeographic ranges and clinal zonation (Somero 2002; Helmuth et al. 2006). Thermal stress can also alter foraging behaviour (Jones & Boulding 1999; Pincebourde et al. 2008), select for heat-resistant phenotypes (Etter 1988; Dong et al. 2008) and induce physiological and cellular changes (Lockwood et al. 2010; Connor & Gracey 2011). Cellular responses (e.g. changes in protein and gene expression) to thermal stress have been studied in a variety of intertidal organisms, including algae (Collén et al. 2007; Pearson et al. 2010; Liu et al. 2013a), mussels (Gracey et al. 2008; Lockwood et al. 2010; Connor & Gracey 2011; Place et al. 2012) and oysters (Lang et al. 2009). A common pattern in these studies was upregulation of genes encoding heat-shock proteins, which act as molecular chaperones during cellular stress (Feder & Hofmann 1999), as well as upregulation of genes encoding apoptosis regulators and other protein chaperones.

Recently, a growing body of work has demonstrated that predation risk can also be a major source of stress for rocky shore organisms (Long & Hay 2012; Benedetti-Cecchi & Trussell 2014). In addition to exerting well-known consumptive effects on their prey – whereby a
predator alters prey population density by actively consuming individuals (Paine 1966) – predators also exert nonconsumptive effects, where the risk of predation alone alters prey behaviour and morphology (Trussell et al. 2006a). Rocky shore organisms often detect predation risk via waterborne cues (Trussell et al. 2003) and respond by reducing foraging activity, which can produce cascading indirect effects throughout the community. The strength of these effects can rival or exceed those caused by predators actively consuming prey (Trussell et al. 2006a). Moreover, as with thermal stress, emerging evidence indicates that stress imposed by predation risk can trigger elevated respiration (Rovero et al. 1999) and increased production of heat-shock proteins (Pijanowska & Kloc 2004; Pauwels et al. 2005; Slos & Stoks 2008), antioxidant enzymes (Slos & Stoks 2008) and stress hormones (Cree et al. 2009; Sheriff et al. 2009). These physiological shifts probably exert additional energetic costs on prey species and may explain why some prey grow less efficiently under predation risk (Trussell et al. 2006b, 2008).

To date, only a few studies have examined the genetic responses of organisms to predation risk. These studies were carried out on planktonic crustaceans (Pauwels et al. 2005; Schwarzenberger et al. 2009; Miyakawa et al. 2010; Spanier et al. 2010), tadpoles (Mori et al. 2009), guppies (Fraser et al. 2011), threespined sticklebacks (Sanogo et al. 2011) and goldfish (Kagawa & Mugiya 2002). Many earlier studies examining molecular responses to predation risk used methods similar to those used to study abiotic stresses such as thermal and oxidative stress, often focusing on expression of heat-shock proteins or antioxidant enzymes (Kagawa & Mugiya 2002; Pijanowska & Kloc 2004; Pauwels et al. 2005; Slos & Stoks 2008). But later studies, employing high-throughput tools such as microarrays or transcriptomics, have shown few similarities in how species change gene expression in response to predation risk, which contrasts with well-established gene expression patterns during thermal stress (Mori et al. 2009; Fraser et al. 2011). Consequently, it is difficult to conclude from research to date whether predation stress triggers similar transcriptomic responses across many species – as heat stress does – or whether each species mounts a unique type of genetic response to its predators.

Here, we examine the genetic responses of the intertidal snail _Nucella lapillus_ to both thermal stress and predation risk. _Nucella lapillus_ sits in the middle of a three-level food chain, between its predator (the crab _Carcinus maenas_) and prey (the mussel _Mytilus edulis_ and the barnacle _Semibalanus balanoides_). In this system, waterborne cues from _C. maenas_ induce decreased feeding, lower growth rates and thicker shells in _N. lapillus_ (Stickle et al. 1985; Palmer 1990; Trussell et al. 2003, 2006b); in turn, reductions in feeding rates can produce strong cascading indirect effects in the resident community (Trussell et al. 2003). Past work has also documented the effects of thermal stress on the snail’s color polymorphisms (Etter 1988), population structure (Chu et al. 2014a) and protein expression (Gardestrom et al. 2007). Using RNA-seq, we sought to further understand the genetic responses to a well-characterized abiotic stress (thermal stress) and a less-understood biotic stress (predation risk) within this well-studied intertidal food chain. In addition, we sought to uncover the potential overlap between these genetic responses to better understand how the interactive effects of thermal stress and predation risk may influence _N. lapillus_. Although thermal stress and predation risk often have similar organismal and ecological effects – for example, decreased foraging and lower metabolic efficiency—we found that _N. lapillus_’s genetic responses to these factors were markedly different. The snail responded to thermal stress in a manner similar to other species, but we found few significant changes in gene expression in response to predation risk. In the context of past research, our findings suggest that genetic responses to biotic stresses, such as predation risk, may be more complex and less uniform across species than genetic responses to abiotic stresses, such as thermal stress.

**Methods**

To examine the genetic responses of _N. lapillus_ to thermal stress and predation risk, we exposed snails to three treatments: control conditions (no stress), risk cues from the predatory green crab _C. maenas_ (predation risk) and elevated temperature (thermal stress). In June 2010, we collected snails from Nahant, MA, near Northeastern University’s Marine Science Center, where we performed our experiments. First, snails were acclimated for almost two months in aquaria with an ambient supply of seawater from the Marine Science Center’s flow-through system and a steady supply of mussel prey (_M. edulis_). During the acclimation period, the snails were not directly exposed to crab risk cues beyond any trace cues that may have come in from the sea water system. Second, snails were placed in experimental mesocosms for a total of 17 days (during August and September 2010). Third, all experimental snails were flash-frozen in liquid nitrogen for genetic processing.

Each mesocosm had an independent water supply and a population of 30 mussels as prey, which was replenished every 6 days. Three control-treatment snails were kept at ambient temperature [15.75 ± 1.1 °C (mean ± 1
standard supply of seawater from the Marine Science Center’s flow-through system. For our predation risk treatment, four snails were kept at ambient temperature, but throughout the 17-day experimental period, their water supply first passed through a chamber containing a green crab (C. maenas) feeding on N. lapillus to simulate predation risk, as in Trussell et al. (2006b). For our thermal stress treatment, two snails were kept in mesocosms at ambient temperature without crabs and with an ambient supply of seawater. These snails were removed from the mesocosms, brought to an internal body temperature of 33 °C under a heat lamp over a period of 4.5 h at a rate of 1.8 °C/min and finally placed back in ambient-temperature mesocosms for a 2-h recovery period before we flash-froze them for RNA-seq.

We began RNA-seq library preparation by homogenizing a total of 25 mg of tissue from each snail’s head, foot and mantle in TRI Reagent. We extracted total RNA following the manufacturer’s protocol and quantified it using an Agilent Bioanalyzer and an RNA 6000 Pico Chip. We used only samples that had an RNA integrity number (RIN) higher than 7. We then extracted mRNA by poly A+ selection using Dynabeads Oligo(dT) from Life Technologies. We prepared unstranded Illumina mRNA-seq libraries as in Craig et al. (2008), with the following modifications: we used fragmentase (NEB) to fragment the cDNA libraries, we size-selected for cDNA fragments ~250 base pairs in length, and we used custom 4-base-pair barcodes for each individual. We also used random primers during first-strand synthesis to avoid 3’ bias from Oligo(dT) primers. We quantified our final libraries using a Bioanalyzer DNA 1000 chip and sequenced them on an Illumina GAIIx using single-end, 50-base-pair reads.

To identify differentially expressed genes from our Illumina sequence data, we first used custom Perl scripts to filter sequence output to remove sequence reads having a Phred quality score <30, more than one ambiguous nucleotide or Illumina adapter sequences. We also filtered these sequence reads for rDNA contamination using the National Center for Biotechnology Information’s (NCBI) metazoan data set and bowtie v 0.12.7 (Langmead et al. 2009), applying default parameters. To assemble our RNA sequences into contigs – each representing a putative mRNA transcript – we used Trinity with default parameters, except that we set the path-reinforcement distance to 85 to account for potential polyploidy (Grabherr et al. 2011). We annotated the assembled transcriptome with BLASTX v 2.2.27+ against the UniProtKB Swiss-Prot database, using the BLOSUM50 substitution matrix and an e-value cut-off of 10^{-5}. We used the relatively permissive BLOSUM50 matrix because of the scarcity of genetic data for mol-
family regulator 5, myeloperoxidase, hemocyte protein-glutamine gamma-glutamyltransferase), oxidation stress (peroxidase) and metabolism (acyloxyacyl hydrolase, acidic phospholipase A2 PA4) (Table 1, Fig. 2). The most strongly upregulated genes were for heat-shock 70-kDa protein cognate 1 and DNAJ homologue subfamily B member 4 (also known as heat-shock protein 40), both of which operate as molecular chaperones during stress (Feder & Hofmann 1999; Borges et al. 2005). None of the downregulated transcripts mapped to a protein-coding gene. In sum, the most significantly upregulated genes were associated with stress response, protein folding, metabolism and inflammatory response (Gene Ontology Consortium, http://www.geneontology.org; Fig. 2).

In contrast, our DESEQ2 analyses identified only three differentially expressed transcripts in snails exposed to predation risk (Fig. 1), and none of these three were differentially expressed in snails exposed to elevated temperature. Of the three differentially expressed transcripts induced by predation risk, one was upregulated and two were downregulated. The upregulated transcript did not annotate to a known gene and, from raw read counts, appears to have been differentially expressed in only one snail (Grubbs’ test, $M = 17.25$, $SD = 23.23$, $P < 0.05$; Chu et al. 2014c: read count table). The two downregulated transcripts both annotated to saxiphilin, a toxin-binding protein involved in iron transport (Morabito & Moczydlowski 1994; Negri & Llewellyn 1998; Fig. 2; Table 1).

Discussion

New, next-generation sequencing technologies are enabling transcriptomic analysis with much greater depth, detail and precision than before (Mardis 2008). Compared with a previously published transcriptome used to study N. lapillus’s response to tributyltin, a pollutant that acts as an endocrine disrupter (Pascoal et al. 2013), our transcriptome assembly was both larger and more annotated. The previous Roche 454-based transcriptome assembled using Roche 454’s Newbler assembly software was approximately one-third the size of our assembly, with a lower annotation rate of $∼2.5\%$ (Pascoal et al. 2013), compared with our annotation rate of 23.1%. These differences reflect both the increasing throughput of next-generation sequencing platforms and the growing power of short-read assembly algorithms like those used in Trinity (Grabherr et al. 2011).

Our RNA-seq analyses revealed distinct gene expression responses to thermal stress and predation risk among the N. lapillus in our experiments. Despite similar potential effects exerted by both these stresses – such as decreased feeding and growth efficiency – the snails evidently compensated for each stress via independent processes. The difference between the genetic responses to thermal stress and predation risk suggests that these stressors could be additive in their impact on N. lapillus, as each stress appeared to trigger separate genetic pathways.

We found that N. lapillus had a genetic response to thermal stress similar to those of other intertidal organisms (Lang et al. 2009; Place et al. 2012), involving the upregulation of genes associated with multiple heat-shock proteins, apoptosis and other molecular chaperones. Heat-shock proteins are a well-described set of proteins mediating cellular damage, which are often upregulated in response to elevated temperature and other environmental stresses, such as oxidation, and these proteins occur in all three phylogenetic domains (Feder & Hofmann 1999; Kregel 2002). The magnitude of N. lapillus’s response, including upregulating heat-shock 70 protein by more than 23 times, probably reflects the extreme temperature variation that the species experiences in the intertidal zone (Somero 2002). We also observed a strong upregulation (by 8.69 times) of acyloxyacyl hydrolase, which is associated with inflammation responses (Hagen et al. 1991; Cody et al. 1997) and lipid metabolism (Munford & Hunter 1992). These differentially expressed genes show that when exposed to
high temperatures, *N. lapillus* mounts a significant response, stabilizing cellular and molecular machinery and potentially altering metabolic pathways to meet additional energetic needs. These results align with thermal-stress experiments in other species, which often show similar shifts in gene expression (Lang et al. 2009; Connor & Gracey 2011; Place et al. 2012).

In contrast to the response we observed to thermal stress, we found a less pronounced response to predation risk. The single upregulated transcript appears to

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**Table 1** Gene annotations of differentially expressed transcripts in snails exposed to elevated temperature (thermal stress) and predation risk (predation risk). *n* values in parentheses indicate that multiple differentially expressed transcripts annotated to this gene, and in these cases, we report the range of *P*-values found.

<table>
<thead>
<tr>
<th>Gene transcript annotation</th>
<th>Uniprot ID</th>
<th>log2-fold change</th>
<th>Adjusted <em>P</em>-value</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thermal stress</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-shock 70-kDa protein 1</td>
<td>P02826</td>
<td>4.53</td>
<td>3.82e-09</td>
<td>Heat-shock proteins act as molecular chaperones during heat and other stresses (Feder &amp; Hofmann 1999).</td>
</tr>
<tr>
<td>Heat-shock 70-kDa protein 16</td>
<td>Q9SAB1</td>
<td>2.73</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Heat-shock cognate 70-kDa protein 1*</td>
<td>P36415</td>
<td>2.59</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Heat-shock protein 83</td>
<td>O16087</td>
<td>2.34</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>78-kDa glucose-regulated protein (n = 3)</td>
<td>P34935</td>
<td>2.69–2.70</td>
<td>0.005</td>
<td>Belongs to the heat-shock protein 70 family; involved in protein folding in the endoplasmic reticulum (Chang et al. 1987).</td>
</tr>
<tr>
<td><strong>Predation risk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saxiphilin (n = 2)</td>
<td>P31226</td>
<td>2.78–2.74</td>
<td>0.093</td>
<td>Binds saxotoxin, a toxin found in shellfish (Negri &amp; Llewellyn 1998).</td>
</tr>
</tbody>
</table>

*Heat-shock cognate 70-kDa protein 1 was the second most significant blast hit (e-value = 6e-15). The most significant blast hit was a heat-shock 70-kDa protein from chloroplast membranes.*
risk triggers changes in gene expression: perhaps the sampling missed the temporal scale at which predation risk has been shown to induce strong changes in the expressed transcripts, as defined by the Gene Ontology project.

These genes are unannotated and merit further investigation because of their potential role in responses to thermal stress and predation risk. In addition, it appears that six related genes that were downregulated in response to thermal stress appear to be similarly downregulated in response to predation risk [Chu et al. 2014c: read count table, transcripts comp34986_c0_seq(1, 4–8)], although DESEQ2 did not identify these genes as statistically significant. These genes are unannotated and merit further investigation because of their potential role in responses to both thermal stress and predation risk.

We were surprised that few genes were differentially expressed in response to predation risk, given that such risk has been shown to induce strong changes in the snail’s behaviour [e.g. predator avoidance (Matassa & Trussell 2011)], morphology [e.g. shell thickening (Palmer 1990)] and metabolism [e.g. reduced growth efficiency (Trussell et al. 2006a,b)]. It may be that our sampling missed the temporal scale at which predation risk triggers changes in gene expression: perhaps the snails had not yet responded to predator cues or they had become acclimated or desensitized to them during the experiment. Such a sampling effect would appear unlikely, however, given that (i) the ecological effects of predation risk in N. lapillus persist at high levels for weeks or longer (Matassa & Trussell 2011); (ii) snails were acclimated in crabfree mesocosms before all experiments, thus minimizing potential pre-experimental exposure to predator cues; and (iii) we sampled during a peak period of predation risk effects documented in this system (Trussell et al. 2006a,b). Moreover, other studies have detected significant physiological effects after as little as 12 days of incubation (Matassa & Trussell 2011), and snails that were maintained in parallel with this experiment (from the same collection and acclimation period, used in a different study) showed significant decreased growth efficiency and foraging behaviour in responses to crab cues during our 17-day incubation period (Miller et al. 2014). It is also possible that N. lapillus’s genetic response to predation risk is tissue specific, so that, by pooling tissue types, we might have missed a response.

Finally, our depth of sequencing may not have been sufficient to capture a response to predation risk, particularly if changes in gene expression were modest. This possibility could have been exacerbated by DESEQ2’s analysis pipeline, which filters out genes if the mean of normalized counts is below a given threshold (Anders & Huber 2010). By filtering out such low-expression genes, DESEQ2 reduces the probability of false positives and increases statistical power (Bourgon et al. 2010), but it also increases the chance of false negatives for genes expressed at very low levels. Our sequencing depth may also not have been sufficient to cover N. lapillus’s genome, which is estimated to be approximately 2.6 gigabases (Pascoe et al. 2004). This species’ genome has not been sequenced, and mapping to a de novo transcriptome assembly is inherently less efficient than mapping to a sequenced genome.

Our results suggest that when applying transcriptomics to study complex ecological phenomena, one must keep in mind the limitations of RNA-seq and take into account the experimental design and sequencing depth used. The Encyclopedia of DNA Elements (ENCODE) Consortium’s guidelines for RNA-seq studies suggest a minimum of two biological replicates and 30 million paired-end reads per sample in human genomic studies (https://www.encodeproject.org/), although some research has reported diminishing returns after 10 million reads (Liu et al. 2013b). Indeed, quite a few recent studies do not meet ENCODE’s read-depth standards, in part because of sequencing cost. Such variations highlight the importance of experimental context when interpreting RNA-seq results, which are comparative. In
our study, the detection of few differentially expressed genes in response to predation risk probably suggests that responding to predation involves more subtle changes in gene expression than responding to thermal stress. More detailed sampling and sequencing may be needed to uncover these processes. As sequencing technology and RNA-seq methodologies continue to mature, the sensitivity of these tools will no doubt grow and costs decrease, which should enable us to find and clarify phenomena we might have missed at first.

Even so, if more frequent sampling, tissue separation or greater sequencing depth were necessary to identify differentially expressed genes in response to predation risk, these factors alone would not likely explain the comparatively less extensive genetic response we found in response to predation risk. These results suggest that the genetic response to predation risk might also involve more moderate changes to gene expression and complex shifts in behaviour and physiology than its response to thermal stress. Although mitigating thermal stress requires upregulating molecular chaperones to maintain crucial cellular processes, avoiding predation involves hiding (Matassa & Trussell 2011), increased shell production (Palmer 1990) and changes in metabolism (Trussell et al. 2006b) in *N. lapillus*, and these plastic responses probably involve a diverse complement of genes. Future work with deeper sequencing, more sampling over multiple time points and sampling of individual tissue types may improve our ability to detect changes in gene expression associated with behavioural responses to predation risk in *N. lapillus* and other species.

In addition, when considered within the context of previous research on predator-induced gene expression, our data suggest that the genetic response to predation risk could be less uniform across species than the response to thermal stress. Thermal stress is often considered one of the most fundamental stresses an organism can confront, and studies of genetic responses to thermal stress from species from all three phylogenetic domains show a similar pattern of upregulating heat-shock proteins and other chaperone proteins (Feder & Hofmann 1999). Although past studies using single- or multiple-gene techniques found that some prey species upregulate heat-shock proteins in response to predators (Kagawa & Mugiya 2002; Pijanowska & Kloc 2004; Slos & Stoks 2008), more recent studies using microarrays and transcriptomics in other species found that the presence of predators does not always trigger the upregulation of stress-mediating genes associated with thermal or oxidation stress. These studies found different responses to predation risk for each organism (Mori et al. 2009; Miyakawa et al. 2010; Fraser et al. 2011). Nishimura and colleagues, for example, have studied predator-induced phenotypes in the tadpole of *Rana pipica*, which can produce a ‘bulgy’ morphology that prevents predation by larval salamanders (Kishida et al. 2007). Using microarrays, the researchers found that bulgy-morph tadpoles differentially expressed a number of genes mediating cell adhesion and structure, including the upregulation of NADH dehydrogenase, aldehyde dehydrogenase, and uromodulin-like genes and downregulation of keratin-related genes (Mori et al. 2009). Their results suggest that changes in tadpole gene expression were specifically linked to the pronounced phenotypic changes preventing predation. Besides our study, only one other has used high-throughput transcriptomic techniques to assess genetic responses to predation risk. This study examined a species of guppy (*Poecilia reticulata*) and found that two genes encoding cerebellin proteins were the most upregulated in response to cues from a fish predator (Fraser et al. 2011).

These studies indicate that not all species upregulate typical stress proteins in response to predation risk, and particular responses for each species may reflect the diversity of behavioural and physiological strategies taken by different organisms to reduce their vulnerability to predation. A better understanding of the genetic underpinnings of complex biotic stresses will require high-throughput transcriptomic approaches to identify specialized responses, because such responses are likely to be missed by lower-throughput analyses.

**Conclusions**

Using RNA-seq analyses, we found notably different genetic responses to thermal stress and predation risk in *N. lapillus*. Thermal stress invoked vigorous upregulation of many stress-related genes, similar to responses in other intertidal organisms. In contrast, predation risk triggered a much less marked response. These results suggest that thermal stress and predation risk may present fundamentally different challenges to *N. lapillus*. 
In the context of previous studies, our findings suggest that genetic responses to biotic stresses, such as the risk of predation, may be less uniform than genetic responses to abiotic stresses, such as thermal stress, and that further studies using transcriptomic tools may uncover unique and subtle genetic responses mounted by different species to avoid predation.

Acknowledgements

Funding was generously provided by National Science Foundation grants OCE-0727628 to GCT, OCE-0848345 to SVV and OCE-0963010 to the Marine Science Center as part of the Academic Research Infrastructure Recovery and Reinvestment Program. This is contribution number 321 from the Marine Science Center. We thank Ellen W. Chu for comments on the manuscript. For scripts and more information, please visit http://nathanieldavidchu.wordpress.com.

References


L.P.M., G.C.T. and S.V.V. conceived and designed the research. L.P.M. prepared sequencing libraries. N.D.C. and S.T.K. analysed data. N.D.C. wrote the first draft, and L.P.M., G.C.T. and S.V.V. contributed significantly to revisions.

**Data accessibility**


**Supporting information**

Additional supporting information may be found in the online version of this article.

**Table S1** Illumina read representation of individual snails.

**Table S2** *Nucella lapillus* transcriptome assembly metrics.