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

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Running headline: Transcriptomic responses to intertidal stress

23 **ABSTRACT**

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

39 **INTRODUCTION**

40 Rocky shore organisms must regularly cope with intense abiotic and biotic stresses. Chief among
41 these is thermal stress, which often determines intertidal organisms' biogeographic ranges and
42 clinal zonation (Somero 2002; Helmuth *et al.* 2006). Thermal stress can also alter foraging
43 behavior (Jones and Boulding 1999; Pincebourde *et al.* 2008), select for heat-resistant phenotypes
44 (Etter 1988; Dong *et al.* 2008), and induce physiological and cellular changes (Lockwood *et al.*
45 2010; Connor and Gracey 2011). Cellular responses (e.g., changes in protein and gene
46 expression) to thermal stress have been studied in a variety of intertidal organisms, including
47 algae (Collén *et al.* 2007; Pearson *et al.* 2010; Liu *et al.* 2013), mussels (Gracey *et al.* 2008;
48 Lockwood *et al.* 2010; Connor and Gracey 2011; Place *et al.* 2012), and oysters (Lang *et al.*
49 2009). A common pattern in these studies was upregulation of genes encoding heat-shock
50 proteins, which act as molecular chaperones during cellular stress (Feder and Hofmann 1999), as
51 well as upregulation of genes encoding apoptosis regulators and other protein chaperones.
52 Recently, a growing body of work has demonstrated that predation risk can also be a
53 major source of stress for rocky shore organisms (Long and Hay 2012; Benedetti-Cecchi and
54 Trussell 2014). In addition to exerting well-known consumptive effects on their prey—whereby a
55 predator alters prey population density by actively consuming individuals (Paine 1966)—
56 predators also exert nonconsumptive effects, where the risk of predation alone alters prey
57 behavior and morphology (Trussell *et al.* 2006a). Rocky shore organisms often detect predation
58 risk via waterborne cues (Trussell *et al.* 2003) and respond by reducing foraging activity, which
59 can produce cascading indirect effects throughout the community. The strength of these effects
60 can rival or exceed those caused by predators actively consuming prey (Trussell *et al.* 2006a).
61 Moreover, as with thermal stress, emerging evidence indicates that stress imposed by predation

62 risk can trigger elevated respiration (Rovero *et al.* 1999) and increased production of heat-shock

63 proteins (Pijanowska and Kloc 2004; Pauwels *et al.* 2005; Slos and Stoks 2008), antioxidant

64 enzymes (Slos and Stoks 2008), and stress hormones (Creel *et al.* 2009; Sheriff *et al.* 2009).

65 These physiological shifts likely exert additional energetic costs on prey species and may explain

66 why some prey grow less efficiently under predation risk (Trussell *et al.* 2006b, 2008).

67 To date, only a few studies have examined the genetic responses of organisms to

68 predation risk. These studies were done on planktonic crustaceans (Pauwels *et al.* 2005;

69 Schwarzenberger *et al.* 2009; Miyakawa *et al.* 2010; Spanier *et al.* 2010), tadpoles (Mori *et al.*

70 2009), guppies (Fraser *et al.* 2011), three-spined sticklebacks (Sanogo *et al.* 2011), and goldfish

71 (Kagawa and Mugiya 2002). Many earlier studies examining molecular responses to predation

72 risk used methods similar to those used to study abiotic stresses such as thermal and oxidative

73 stress, often focusing on expression of heat-shock proteins or antioxidant enzymes (Kagawa and

74 Mugiya 2002; Pijanowska and Kloc 2004; Pauwels *et al.* 2005; Slos and Stoks 2008). But later

75 studies, employing high-throughput tools such as microarrays or transcriptomics, have shown few

76 similarities in how species change gene expression in response to predation risk, which contrasts

77 with well-established gene expression patterns during thermal stress (Mori *et al.* 2009; Fraser *et*

78 *al.* 2011). Consequently, it is difficult to conclude from research to date whether predation stress

79 triggers similar transcriptomic responses across many species—as heat stress does—or whether

80 each species mounts a unique type of genetic response to its predators.

81 Here, we examine the genetic responses of the intertidal snail *Nucella lapillus* to both

82 thermal stress and predation risk. *Nucella lapillus* sits in the middle of a three-level food chain,

83 between its predator (the crab *Carcinus maenas*) and prey (the mussel *Mytilus edulis* and the

84 barnacle *Semibalanus balanoides*). In this system, waterborne cues from *C. maenas* induce

85 decreased feeding, lower growth rates, and thicker shells in *N. lapillus* (Stickle *et al.* 1985;
86 Palmer 1990; Trussell *et al.* 2003, 2006b); in turn, reductions in feeding rates can produce strong
87 cascading indirect effects in the resident community (Trussell *et al.* 2003). Past work has also
88 documented the effects of thermal stress on the snail's color polymorphisms (Etter 1988),
89 population structure (Chu *et al.* 2014), and protein expression (Gardeström *et al.* 2007). Using
90 RNA-seq, we sought to further understand the genetic responses to a well-characterized abiotic
91 stress (thermal stress) and a less-understood biotic stress (predation risk) within this well-studied
92 intertidal food chain. In addition, we sought to uncover the potential overlap between these
93 genetic responses to better understand how the interactive effects of thermal stress and predation
94 risk may influence *N. lapillus*. Although thermal stress and predation risk often have similar
95 organismal and ecological effects—e.g., decreased foraging and lower metabolic efficiency—we
96 found that *N. lapillus*'s genetic responses to these factors were markedly different. The snail
97 responded to thermal stress in a manner similar to other species, but we found few significant
98 changes in gene expression in response to predation risk. In the context of past research, our
99 findings suggest that genetic responses to biotic stresses, such as predation risk, may be more
100 complex and less uniform across species than genetic responses to abiotic stresses, such as
101 thermal stress.
102

103 **METHODS**

104 To examine the genetic responses of *N. lapillus* to thermal stress and predation risk, we exposed
105 snails to three treatments: control conditions (no stress), risk cues from the predatory green crab
106 *C. maenas* (predation risk), and elevated temperature (thermal stress). In June 2010, we collected
107 snails from Nahant, MA, near Northeastern University's Marine Science Center, where we

108 performed our experiments. First, snails were acclimated for almost two months in aquaria with
109 an ambient supply of seawater from the Marine Science Center's flow-through system and a
110 steady supply of mussel prey (*M. edulis*). During the acclimation period, the snails were not
111 directly exposed to crab risk cues beyond any trace cues that may have come in from the sea
112 water system. Second, snails were placed in experimental mesocosms for a total of 17 days
113 (during August and September 2010). Third, all experimental snails were flash-frozen in liquid
114 nitrogen for genetic processing.

115 Each mesocosm had an independent water supply and a population of 30 mussels as prey,
116 which was replenished every 6 days. Three control-treatment snails were kept at ambient
117 temperature (15.75 ± 1.1 °C [mean ± 1 standard deviation], total range 12.1 – 20.3 °C) with an
118 ambient supply of seawater from the Marine Science Center's flow-through system. For our
119 predation risk treatment, four snails were kept at ambient temperature, but throughout the 17-day
120 experimental period, their water supply first passed through a chamber containing a green crab
121 (*C. maenas*) feeding on *N. lapillus* to simulate predation risk, as in Trussell *et al.* (2006b). For our
122 thermal stress treatment, two snails were kept in mesocosms at ambient temperature without
123 crabs and with an ambient supply of seawater. These snails were removed from the mesocosms,
124 brought to an internal body temperature of 33 °C under a heat lamp over a period of 4.5 hours at a
125 rate of 1.8 °C min^{-1}, and finally placed back in ambient-temperature mesocosms for a 2-hour
126 recovery period before we flash-froze them for RNA-seq.

127 We began RNA-seq library preparation by homogenizing at total of 25 mg of tissue from
128 each snail's head, foot, and mantle tissue in TRI Reagent. We extracted total RNA following the
129 manufacturer's protocol and quantified total RNA using an Agilent Bioanalyzer and an RNA
130 6000 Pico Chip (see Figure S1). We used only samples that had an RNA integrity number (RIN)

131 higher than 7. We then extracted mRNA by poly A+ selection using Dynabeads Oligo(dT) from
132 Life Technologies. We prepared unstranded Illumina mRNA-seq libraries as in Craig *et al.*
133 (2008), with the following modifications: we used fragmentase (NEB) to fragment the cDNA
134 libraries, we size-selected for cDNA fragments ~250 base pairs in length, and we used custom 4-
135 base-pair barcodes for each individual. We also used random primers during first-strand synthesis
136 to avoid 3' bias from Oligo(dT) primers. We quantified our final libraries using a Bioanalyzer
137 DNA 1000 chip and sequenced them on an Illumina GAIIx using single-end, 50-base-pair reads.
138 To identify differentially expressed genes from our Illumina sequence data, we first used
139 custom Perl scripts to filter sequence output to remove sequence reads having a Phred Quality
140 score less than 30, more than one ambiguous nucleotide, or Illumina adapter sequences. We also
141 filtered these sequence reads for rRNA contamination using the National Center for
142 Biotechnology Information's (NCBI) metazoan dataset and Bowtie v 0.12.7 (Langmead *et al.*
143 2009), applying default parameters. To assemble our RNA sequences into contigs—each
144 representing a putative mRNA transcript—we used Trinity with default parameters, except that
145 we set the path-reinforcement distance to 85 to account for potential polyploidy (Grabherr *et al.*
146 2011). We annotated the assembled transcriptome with Blastx v 2.2.27+ against the UniProtKB
147 Swiss-Prot database, using the BLOSUM50 substitution matrix and an e-value cutoff of 10^{-5}. We
148 used the relatively permissive BLOSUM50 matrix because of the scarcity of genetic data for
149 molluscs. We used Bowtie v 0.12.7 to map RNA-seq reads from each individual snail to the
150 annotated transcriptome and used Express to quantify for each snail the number of reads mapping
151 to each individual assembled transcript (Langmead *et al.* 2009; Roberts and Pachter 2013). The
152 software package eXpress did not detect any significant 3' bias because of poly A+ selection
153 (Dryad, express files). We analyzed differential expression using the DESeq2 package in R v

154 3.0.2, using the default adjusted *P*-value cutoff of 0.1 (Anders and Huber 2010). We also tested

155 *P*-value cutoffs of 0.05, 0.2, and 0.3. At a cutoff of 0.05, there were no differentially expressed

156 transcripts in the snails exposed to predation risk, and at 0.2 and 0.3 there were no additional

157 differentially expressed transcripts beyond those identified by the default *P*-value cutoff of 0.1.

158 Default independent filtering during DESeq2 analysis filtered out transcripts unlikely to be

159 differentially expressed using the mean of normalized counts as a filer statistic (Anders and

160 Huber 2010). We plotted biological processes associated with differentially expressed genes

161 according to their functional similarity using Sim_{Rel} (Schlicker *et al.* 2006) and REVIGO (Supek

162 *et al.* 2011), setting the allowed similarity to 0.7 and querying the UniProt database.

163

164 **RESULTS**

165 Our RNA-seq analysis produced a total of 42,079,889 single-end, 50-base-pair reads after quality

166 filtering. Each individual was represented by 1,324,128 to 7,037,731 reads (Table S1). Using

167 pooled reads from all individuals, we assembled a 44.2-megabase transcriptome of 90,674

168 transcripts with an *N50* statistic of 413, meaning that over 50 percent of transcripts were longer

169 than 413 nucleotides (Table S2); 20,922 of the transcripts (23%) annotated to a known protein-

170 coding gene.

171 Our DESeq2 analyses identified 141 transcripts that were differentially expressed in snails

172 exposed to elevated temperature versus snails kept at ambient temperature (Figure 1), and 29 of

173 those differentially expressed transcripts annotated to 26 known protein-coding genes (Table 1,

174 Figure 2). Of the 141 transcripts differentially expressed in snails exposed to elevated

175 temperature, 135 of them were upregulated and 6 were downregulated. The upregulated genes

176 included six heat-shock proteins; an additional protein chaperone (BAG family regulator 5, which

177 activates heat-shock protein 70); as well as genes mediating immune defenses (myeloperoxidase),
178 inflammation (acyloxyacyl hydrolase), apoptosis (baculovial IAP repeat-containing protein 2,
179 BAG family regulator 5, myeloperoxidase, hemocyte protein-glutamine gamma-
180 glutamyltransferase), oxidation stress (peroxidase), and metabolism (acyloxyacyl hydrolase,
181 acidic phospholipase A2 PA4) (Table 1, Figure 2). The most strongly upregulated genes were for
182 heat-shock 70 kDa protein cognate 1 and DNAJ homolog subfamily B member 4 (also known as
183 heat-shock protein 40), both of which operate as molecular chaperones during stress (Feder and
184 Hofmann 1999; Borges *et al.* 2005). None of the downregulated transcripts mapped to a protein-
185 coding gene. In sum, the most significantly upregulated genes were associated with stress
186 response, protein folding, metabolism, and inflammatory response (Gene Ontology Consortium,
187 http://www.geneontology.org; Figure 2).
188 In contrast, our DESeq analyses identified only three differentially expressed transcripts
189 in snails exposed to predation risk (Figure 1), and none of these three were differentially
190 expressed in snails exposed to elevated temperature. Of the three differentially expressed
191 transcripts induced by predation risk, one was upregulated and two were downregulated. The
192 upregulated transcript did not annotate to a known gene and, from raw read counts, appears to
193 have been differentially expressed in only one snail (Grubbs' test, $M = 17.25$, $SD = 23.23$, $P <$
194 0.05; Supplementary File 2). The two downregulated transcripts both annotated to saxiphilin, a
195 toxin-binding protein involved in iron transport (Morabito and Moczydlowski 1994; Negri and
196 Llewellyn 1998; Figure 2; Table 1).
197
198 **DISCUSSION**
199 New, next-generation sequencing technologies are enabling transcriptomic analysis with much

200 greater depth, detail, and precision than before (Mardis 2008). Compared with a previously

201 published transcriptome used to study *N. lapillus*'s response to tributyltin, a pollutant that acts as

202 an endocrine disrupter (Pascoal *et al.* 2013), our transcriptome assembly was both larger and

203 more annotated. The previous Roche 454-based transcriptome assembled using Roche 454's

204 Newbler assembly software was approximately one-third the size of our assembly, with a lower

205 annotation rate of approximately 2.5% (Pascoal *et al.* 2013), compared with our annotation rate of

206 23.1%. These differences reflect both the increasing throughput of next-generation sequencing

207 platforms and the growing power of short-read assembly algorithms like those used in Trinity

208 (Grabherr *et al.* 2011).

209 Our RNA-seq analyses revealed distinct gene expression responses to thermal stress and

210 predation risk among the *N. lapillus* in our experiments. Despite similar potential effects exerted

211 by both these stresses—such as decreased feeding and growth efficiency—the snails evidently

212 compensated for each stress via independent processes. The difference between the genetic

213 responses to thermal stress and predation risk suggests that these stressors could be additive in

214 their impact on *N. lapillus*, as each stress appeared to trigger separate genetic pathways.

215 We found that *N. lapillus* had a genetic response to thermal stress similar to those of other

216 intertidal organisms (Lang *et al.* 2009; Place *et al.* 2012), involving the upregulation of genes

217 associated with multiple heat-shock proteins, apoptosis, and other molecular chaperones. Heat-

218 shock proteins are a well-described set of proteins mediating cellular damage, which are often

219 upregulated in response to elevated temperature and other environmental stresses, such as

220 oxidation, and these proteins occur in all three phylogenetic domains (Feder and Hofmann 1999;

221 Kregel 2002). The magnitude of *N. lapillus*'s response, including upregulating heat-shock 70

222 protein by more than 23 times, likely reflects the extreme temperature variation that the species

223 experiences in the intertidal zone (Somero 2002). We also observed a strong upregulation (by
224 8.69 times) of acyloxyacyl hydrolase, which is associated with inflammation responses (Hagen *et*
225 *al.* 1991; Cody *et al.* 1997) and lipid metabolism (Munford and Hunter 1992). These
226 differentially expressed genes show that when exposed to high temperatures, *N. lapillus* mounts a
227 significant response, stabilizing cellular and molecular machinery and potentially altering
228 metabolic pathways to meet additional energetic needs. These results align with thermal-stress
229 experiments in other species, which often show similar shifts in gene expression (Lang *et al.*
230 2009; Connor and Gracey 2011; Place *et al.* 2012).

231 In contrast to the response we observed to thermal stress, we found a less pronounced
232 response to predation risk. The single upregulated transcript appears to have been upregulated in
233 only one of the snails exposed to predation risk (Supplementary File 2). The two downregulated
234 transcripts annotated to saxiphilin, a protein that binds saxotoxin, a well-known paralytic shellfish
235 toxin (Negri and Llewellyn 1998). It is unclear what role this protein might play in a response to
236 predation risk. In addition, it appears that six related genes that were downregulated in response
237 to thermal stress appear to be similarly downregulated in response to predation risk (Dryad, read
238 count table: transcripts comp34986_c0_seq[1, 4-8]), although DESeq2 did not identify these
239 genes as statistically significant. These genes are unannotated and merit further investigation
240 because of their potential role in responses to both thermal stress and predation risk.

241 We were surprised that few genes were differentially expressed in response to predation
242 risk, given that such risk has been shown to induce strong changes in the snail's behavior [e.g.,
243 predator avoidance (Matassa and Trussell 2011)], morphology [e.g., shell thickening(Palmer
244 1990)], and metabolism [e.g., reduced growth efficiency (Trussell *et al.* 2006b)]. It may be that
245 our sampling missed the temporal scale at which predation risk triggers changes in gene

246 expression: perhaps the snails had not yet responded to predator cues or they had become
247 acclimated or desensitized to them during the experiment. Such a sampling effect would appear
248 unlikely, however, given that (1) the ecological effects of predation risk in *N. lapillus* persist at
249 high levels for weeks or longer (Matassa and Trussell 2011); (2) snails were acclimated in crab-
250 free mesocosms before all experiments, thus minimizing potential preexperimental exposure to
251 predator cues; and we sampled during a peak period of predation risk effects documented in this
252 system (Trussell *et al.* 2006a, 2006b). Furthermore, other studies have detected significant
253 physiological effects after as little as 12 days of incubation (Matassa and Trussell 2011), and
254 snails that were maintained in parallel with this experiment (from the same collection and
255 acclimation period, used in a different study) showed significant decreased growth efficiency and
256 foraging behavior in responses to crab cues during our 17-day incubation period (Miller *et al.*
257 2014). It is also possible that *N. lapillus*'s genetic response to predation risk is tissue specific, so
258 by pooling tissue types, we might have missed a response.
259 Finally, our depth of sequencing may not have been sufficient to capture a response to predation
260 risk, particularly if changes in gene expression were modest. This possibility could have been
261 exacerbated by DESeq2's analysis pipeline, which filters out genes if the mean of normalized
262 counts is below a given threshold (Anders and Huber 2010). By filtering out such low-expression
263 genes, DESeq2 reduces the probability of false positives and increases statistical power (Bourgon
264 *et al.* 2010), but it also increase the chance of false negatives for genes expressed at very low
265 levels. Our sequencing depth may also not have been sufficient to cover *N. lapillus*'s genome,
266 which is estimated to be approximately 2.6 gigabases (Pascoe *et al*. 2004). This species' genome
267 has not been sequenced, and mapping to a *de novo* transcriptome assembly is inherently less
268 efficient than mapping to a sequenced genome.

269 Our results suggest that when applying transcriptomics to study complex ecological
270 phenomena, one must keep in mind the limitations of RNA-seq and take into account the
271 experimental design and sequencing depth used. The Encyclopedia of DNA Elements
272 (ENCODE) Consortium's guidelines for RNA-seq studies suggest a minimum of two biological
273 replicates and 30 million paired-end reads per sample in human studies
274 (https://www.encodeproject.org/), although some research has reported diminishing returns after
275 10 million reads (Liu *et al*. 2013). Indeed, quite a few recent studies do not meet ENCODE's read
276 depth standards, in part, because of the sequencing costs. Such variations highlight the
277 importance of experimental context when interpreting RNA-seq results, which are comparative.
278 In our study, the detection of few differentially expressed genes in response to predation risk
279 likely suggests that responding to predation involves more subtle changes in gene expression than
280 responding to thermal stress. More detailed sampling and sequencing may be needed to uncover
281 these processes. As sequencing technology and RNA-seq methodologies continue to mature, the
282 sensitivity of these tools will no doubt grow and costs decrease, which should enable us to find
283 and clarify phenomena we might have missed at first.

284 Even so, if more frequent sampling, tissue separation, or greater sequencing depth were
285 necessary to identify differentially expressed genes in response to predation risk, these factors
286 alone would not likely explain the comparatively less extensive genetic response we found to
287 predation risk than to thermal stress. This notable difference is consistent with the idea that
288 elevated temperature poses a more critical threat to *N. lapillus* than predation and that the extreme
289 daily and seasonal thermal fluctuations of the intertidal zone are the primary drivers of stress on
290 rocky shores (Helmuth and Hofmann 2001; Somero 2002). Even though the ecological impacts of
291 moderate thermal stress and predation risk can be similar in magnitude (Miller *et al.* 2014), the

292 intensity of thermal stress in intertidal habitats may require a more extensive genetic response to
293 survive periods of high temperature. In contrast, the spatial distribution and temporal scales of
294 predator risk vary greatly (Turner and Montgomery 2003) and may require fewer genetic
295 changes.

296 *N. lapillus*'s relatively modest genetic response to predation risk might also involve more
297 moderate changes to gene expression and complex shifts in behavior and physiology than its
298 response to thermal stress. Although mitigating thermal stress requires upregulating molecular
299 chaperones to maintain crucial cellular processes, avoiding predation involves hiding (Matassa
300 and Trussell 2011), increased shell production (Palmer 1990), and changes in metabolism
301 (Trussell *et al.* 2006b) in *N. lapillus*, and these plastic responses likely involve a diverse
302 complement of genes. Future work with deeper sequencing, more sampling over multiple time
303 points, and sampling of individual tissue types may improve our ability to detect changes in gene
304 expression associated with behavioral responses to predation risk in *N. lapillus* and other species.

305 In addition, when considered within the context of previous research on predator-induced
306 gene expression, our data suggest that the genetic response to predation risk might be less
307 uniform across species than the response to thermal stress. Thermal stress is often considered one
308 of the most fundamental stresses an organism can confront, and studies of genetic responses to
309 thermal stress from species from all three phylogenetic domains show a similar pattern of
310 upregulating heat-shock proteins and other chaperone proteins (Feder and Hofmann 1999).
311 Although past studies using single- or multiple-gene techniques found that some prey species
312 upregulate heat-shock proteins in response to predators (Kagawa and Mugiya 2002; Pijanowska
313 and Kloc 2004; Slos and Stoks 2008), more recent studies using microarrays and transcriptomics
314 in other species found that the presence of predators does not always trigger the upregulation of

315 stress-mediating genes associated with thermal or oxidation stress. These studies found different
316 responses to predation risk for each organism (Mori *et al.* 2009; Miyakawa *et al.* 2010; Fraser *et*
317 *al.* 2011). Nishimura and colleagues, for example, have studied predator-induced phenotypes in
318 the tadpole of *Rana pirica*, which can produce a "bulgy" morphology that prevents predation by
319 larval salamanders (Kishida *et al.* 2007). Using microarrays, the researchers found that bulgy-
320 morph tadpoles differentially expressed a number of genes mediating cell adhesion and structure,
321 including the upregulation of NADH dehydrogenase, aldehyde dehydrogenase, and uromodulin-
322 like genes and downregulation of keratin-related genes (Mori *et al.* 2009). Their results suggest
323 that changes in tadpole gene expression were specifically linked to the pronounced phenotypic
324 changes preventing predation. Besides our study, only one other has used high-throughput
325 transcriptomic techniques to assess genetic responses to predation risk. This study examined a
326 species of guppy (*Poecilia reticulata*) and found that two genes encoding cerebellin proteins were
327 the most upregulated in response to cues from a fish predator (Fraser *et al.* 2011). These studies
328 indicate that not all species upregulate typical stress proteins in response to predation risk, and
329 particular responses for each species may reflect the diversity of behavioral and physiological
330 strategies taken by different organisms to reduce their vulnerability to predation. A better
331 understanding of the genetic underpinnings of complex biotic stresses will require high-
332 throughput transcriptomic approaches to identify specialized responses, because such responses
333 are likely to be missed by lower-throughput analyses.
334
335 **CONCLUSIONS**
336 Using RNA-seq analyses, we found notably different genetic responses to thermal stress and
337 predation risk in *N. lapillus*. Thermal stress invoked vigorous upregulation of many stress-related

338 genes, similar to responses in other intertidal organisms. In contrast, predation risk triggered a
339 much less marked response. These results suggest that thermal stress and predation risk may
340 present fundamentally different challenges to *N. lapillus*. In the context of previous studies, our
341 findings suggest that genetic responses to biotic stresses, such as the risk of predation, may be
342 less uniform than genetic responses to abiotic stresses, such as thermal stress, and that further
343 studies using transcriptomic tools may further uncover unique and subtle genetic responses
344 mounted by different species to avoid predation.
345

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351 http://nathanieldavidchu.wordpress.com.
352

353 **DATA ACCESSIBILITY**
354 -RNA-seq raw sequence reads: NCBI SRA-SRX357400
355 -Transcriptome assembly: Dryad repository-doi: 10.5061/dryad.610dd
356 -Express files will be deposited in the Dryad repository
357

358 **AUTHOR CONTRIBUTIONS**
359 LPM, GCT, and SVV conceived and designed the research. LPM prepared sequencing libraries.
360 NDC and STK analyzed data. NDC wrote the first draft, and LPM, GCT, and SVV contributed

361 significantly to revisions.

362

363 **TABLES**

364 Table 1. Gene annotations of differentially expressed transcripts in snails exposed to elevated

365 temperature (thermal stress) and predation risk (predation risk). *n* values in parentheses indicate

366 that multiple differentially expressed transcripts annotated to this gene, and in these cases, we

367 report the range of *P*-values found.

Gene transcript annotation	**Uniprot ID**	**$\log_2$ fold change**	**Adjusted *P*-value**	**Activity**
		Thermal stress		
Heat-shock 70 kDa protein cognate 1	P02826	4.53	3.82e-09	Heat-shock proteins act as molecular chaperones during heat and other stresses (Feder and Hoffmann 1999).
Heat-shock 70 kDa protein 16	Q9SAB1	2.73	0.005	
Heat-shock cognate 70 kDa protein 1*	P36415	2.59	0.008	
Heat-shock protein 83	O16087	2.34	0.039	
78 kDa glucose-regulated protein (*n* = 3)	P34935	2.69–2.70	0.005	Belongs to the heat-shock protein 70 family; involved in

				protein folding in the endoplasmic reticulum (Chang *et al.* 1987).
BAG family molecular chaperone regulator 5 (nucleotide exchanger for HSP70)	Q5QJC9	2.82	0.004	Acts as a protein chaperone and activates heat-shock proteins (Kalia *et al.* 2004).
DnaJ homolog subfamily B member 4 (probable chaperone) (n = 2)	Q9D832	4.40–4.44	3.82e-09	Acts as a protein chaperone, and is also known as heat-shock protein 40 (Borges *et al.* 2005).
Baculoviral IAP repeat-containing protein 2 (n = 2)	Q62210	2.56–2.61	0.015–0.018	Mediates cellular apoptosis (Rothe et al. 1995).
Myeloperoxidase	P05164	2.54	0.020	Involved in immune defense (Nauseef *et al.* 1996), apoptosis regulation (Wagner *et al.* 2000), and inflammation responses (Hashinka *et al.* 1988).
Acyloxyacyl hydrolase	P28039	3.12	0.001	Involved in inflammation responses and lipid

				metabolism (Hagen et al. 1991).
Peroxidase (*n* = 2)	Q01603	2.47–2.49	0.027–0.029	Breaks down free-radical products of inefficient cellular metabolism (Ng *et al.* 1992).
Hemocyte protein-glutamine gamma-glutamyltransferase (protein synthesis catalyst)	Q05187	2.13	0.098	Catalyzes peptide cross-linking and potentially plays a role in programmed cell death (Tokunaga *et al.* 1993).
Prestin	Q9JKQ2	2.27	0.085	Acts as a motor protein and helps regulate cell shape (Zheng *et al.* 2000).
Acidic phospholipase A2 PA4	P80003	2.36	0.055	Involved in lipid metabolism and host defense (Dennis et al. 1991).
Patched domain-containing protein 3 (sperm development)	Q0EEE2	2.66	0.011	Potentially regulates hedgehog signaling in male germ cells (Fan et al. 2007).

IgE-binding protein (*n* = 2)	P03975	2.42–2.50	0.021–0.032	Mediates immunoglobulin (antibody) responses (Ishizaka 1984).
Retrovirus-related Pol polyprotein from transposon 412 (*n* = 3)	P10394	2.30–2.73	0.007–0.065	Acts as a transposable element (Yuki *et al.* 1986).
RNA-directed DNA polymerase from mobile element jockey	P21329	2.44	0.035	Acts as a mobile DNA element (Mizrokhi and Mazo 1990).
Predation risk				
Saxiphilin (*n* = 2)	P31226	-2.78–2.74	0.093	Binds saxotoxin, a toxin found in shellfish (Negri and Llewellyn 1998).

368 *Heat-shock cognate 70 kDa protein 1 was the second most significant blast hit (e-value = 6e-
369 15). The most significant Blast hit was a heat-shock 70 protein from chloroplast membranes.
370

371 **FIGURE LEGENDS**

372 Figure 1. Differential gene expression in (a) snails exposed to elevated temperature versus control

373 snails and (b) snails exposed to predation risk versus control snails. Each point in these volcano
374 plots represents a gene transcript; red dots are differentially expressed transcripts that had an
375 adjusted *P*-value of < 0.1.
376
377 Figure 2. Biological processes associated with differentially expressed transcripts, as defined by
378 the Gene Ontology project. Red circles represent genes differentially expressed in snails exposed
379 to elevated temperatures, and blue circles represent those from snails exposed to predation risk.
380 Circle size is proportional to the absolute value of the $\log_{10}$ of the reported DESeq2 *P*-value, with
381 larger circles indicating a more statistically significant differentially expressed transcript.
382 Biological processes are plotted according to their functional similarity using the Sim_{Rel}
383 measurement (Schlicker *et al.* 2006) and REVIGO (Supek *et al.* 2011).

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581

a.

Log_2(fold change)

4
2
0
-2
-4

1e-01 1e+01 1e+03 1e+05

Mean of normalized counts

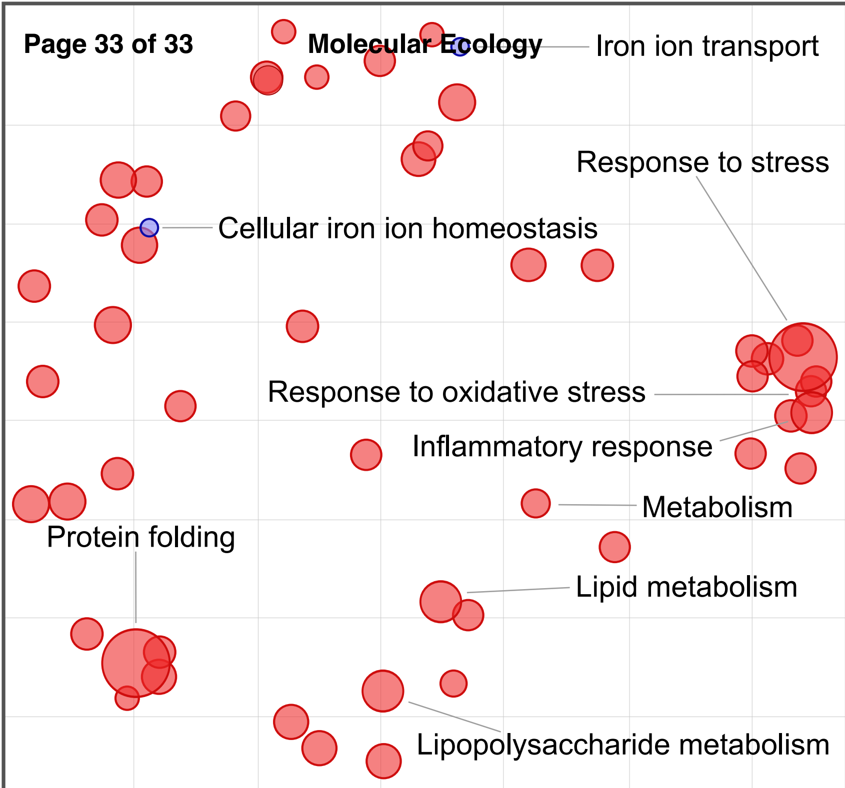
b.

Log_2(fold change)

4
2
0
-2
-4

1e-01 1e+01 1e+03 1e+05

Mean of normalized counts


Page 33 of 33
Molecular Ecology
Iron ion transport
Response to stress
Cellular iron ion homeostasis
Response to oxidative stress
Inflammatory response
Metabolism
Protein folding
Lipid metabolism
Lipopolysaccharide metabolism

Supporting Information

Table S1. Illumina read representation of individual snails.

Sample	**Filtered Reads**	**Mapping Reads**	**Annotated, Mapping Reads**
AC_006	6,020,717	3,432,767	718,576
AC_008	7,037,731	4,289,831	893,364
AC_43	1,324,128	702,278	210,358
AC_42	1,857,540	883,589	273,892
ANC_003	5,782,131	3,568,060	715,620
ANC_34	5,577,022	2,594,866	927,301
ANC_41	4,575,798	2,293,349	911,726
HNC_31	3,677,763	1,891,007	657,755
HNC_37	6,227,059	3,002,492	941,372

Table S2. *Nucella lapillus* transciprtome assembly metrics.

Total base length of assembly	**Contigs**	**Annotated contigs**
44,210,724	90,674	20,922