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RNA expression and disease tolerance are associated with a “keystone mutation” in the ochre sea star *Pisaster ochraceus*

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An overdominant mutation in the elongation factor 1-alpha (EF1A) gene in the sea star *Pisaster ochraceus* has shown itself to mediate tolerance to "sea star wasting disease", a pandemic that has significantly reduced sea star populations on the Pacific coast of North America. Here we use RNA sequencing of healthy individuals to identify differences in constitutive expression of gene regions that may help explain this tolerance phenotype. Our results show that individuals carrying this single mutation have lower expression at a large contingent of gene regions, and it appears likely that the EF1A locus itself is similarly affected, with a 2-fold reduction in expression of some EF1A transcripts. Individuals without this mutation also appear to have a greater cellular response to temperature stress, which has been implicated in the outbreak of sea star wasting disease. Given the ecological significance of *P. ochraceus* and the key role of EF1A in cellular composition and maintenance, these results may be useful in predicting the evolutionary and demographic future for Pacific intertidal communities.

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Abstract

An overdominant mutation in the elongation factor 1-α (EF1A) gene in the sea star *Pisaster ochraceus* has shown itself to mediate tolerance to "sea star wasting disease", a pandemic that has significantly reduced sea star populations on the Pacific coast of North America. Here we use RNA sequencing of healthy individuals to identify differences in constitutive expression of gene regions that may help explain this tolerance phenotype. Our results show that individuals carrying this single mutation have lower expression at a large contingent of gene regions, and it appears likely that the EF1A locus itself is similarly affected, with a 2-fold reduction in expression of some EF1A transcripts. Individuals without this mutation also appear to have a greater cellular response to temperature stress, which has been implicated in the outbreak of sea star wasting disease. Given the ecological significance of *P. ochraceus* and the key role of EF1A in cellular composition and maintenance, these results may be useful in predicting the evolutionary and demographic future for Pacific intertidal communities.

Introduction

The sea star *Pisaster ochraceus* --- best known as a "keystone predator" that modifies the diversity of its intertidal community (Paine, 1969) --- harbors a mutation in the elongation factor 1-α (EF1A, hereafter) gene that is characterized as 'overdominant' (Pankey & Wares, 2009); that is, where heterozygous individuals (carrying one copy of this mutation) have dramatically higher fitness than either homozygote. At the time, with no apparent mechanism for this heterozygote advantage, Pankey and Wares (2009) noted that overdominance has often been associated with disease tolerance. However, our understanding of disease in marine organisms remains quite limited, with a few notable cases that have guided much of the research being done today (Jolles et al., 2002; Mydlarz, Jones & Harvell, 2006; Sutherland et al., 2011). A recent and dramatic pandemic known as "sea star wasting disease" (SSWD) has led to very high mortality in a large number of sea star species on the Pacific coast of North America (Hewson et al., 2014; Eisenlord et al., 2016; Menge et al., 2016). Field surveys of apparently healthy and diseased individuals of *P. ochraceus* suggested that individuals carrying the insertion mutation (*ins*) described by Pankey and Wares (2009) have lower prevalence of (or mortality from) SSWD than individuals homozygous for the wild-type sequence (*wild*; Wares & Schiebelhut, 2016).

The EF1A gene produces a “housekeeping” protein that is involved in translational elongation – forming peptide bonds between amino acids. However, EF1A appears to be involved in diverse cellular functions (Ejiri, 2002), and diversity at this gene has been implicated in variation in fitness in other metazoans (Stearns, 1993; Stearns & Kaiser, 1993). Though some of these other functions include interactions with environmental stress or pathogen responses (Bukovnic et al., 2009; Li et al., 2013; Schulz et al., 2014; Wei

et al., 2014), the mechanism by which the *ins* mutation, or something closely linked to it, affects the function of the EF1A gene or the cellular functions associated with SSWD (Hewson et al., 2014) remains unknown. Continued evaluation of this system has supported the results of Wares and Schiebelhut (2016), with indications that *ins* individuals are not resistant to the disease, but are more tolerant (M. Gravem, pers. comm.).

We can now query distinct genotypes for variation in RNA transcription to identify components of cellular and molecular networks that are associated with specific trait variation (Cohen et al., 2010). Here, a series of hypotheses are tested using RNA sequencing of a set of individuals of each genotype in *P. ochraceus* (the mutation is homozygous lethal, so there are only 2 genotypes (Pankey & Wares, 2009)). First, the *ins* mutation – which is within an intron between two coding subunits of the EF1A gene (Pankey & Wares, 2009) – could affect mRNA splicing and thus generate subfunctional or functionally distinct transcripts. If so, we may expect greater expression of EF1A in homozygotes or expression of distinct isoforms of EF1A in heterozygotes. Second, the mutation could influence the regulation of other genes, in which case we may detect significantly different expression of a set of loci between heterozygotes and homozygotes.

In addition, recent work has suggested that elevated sea surface temperatures could cross environmental thresholds that influence the appearance of SSWD (Bates, Hilton & Harley, 2009a; Eisenlord et al., 2016). In recent years, some regions in which SSWD has been prevalent have experienced temperature anomalies greater than 3°C (Eisenlord et al., 2016). Increased temperatures are also associated with changes in feeding (Sanford, 1999) and metabolism (Fly et al., 2012). Thus we coupled a temperature challenge trial with

behavioral observations and repeated RNA sequencing to understand how individuals respond to periods of elevated temperature or stress. In this case, we hypothesized that an interaction between environmental stress and cellular physiology could be indicated by distinct patterns of behavior or activity levels between the two EF1A genotypes (Dahlhoff, Buckley & Menge, 2001).

Our goal is to illuminate mechanisms by which EF1A *ins* heterozygotes in *P. ochraceus* may be protected from SSWD, as this information may guide exploration of why some sea stars are more susceptible than others to this disease. Additionally, this system provides an opportunity to explore how variation in expression of a gene or gene network that is of fundamental importance to organismal development, growth, and acclimation can affect the tolerance of an organism to disease.

Methods

Field and Lab

Individual *P. ochraceus* were collected from ~0m tidal depth within the Friday Harbor Laboratories marine reserve (Friday Harbor, WA, 48.54°N 123.01°W). Collections were made following written permission from the Associate Director of the Friday Harbor Laboratories. Individuals were placed in sea tables with ambient temperature, unfiltered, running sea water within 1 hour of collection and fed available bivalves *ad libitum*. After the experiment, all surviving individuals were returned to the field.

84

85 At the beginning of the experiment two samples (~25mg) of tube feet were removed from
86 each individual; one sample was placed in 95% undenatured ethanol (for genotyping as in
87 (Wares & Schiebelhut, 2016)), the other sample into RNALater (Thermo Fisher). Tissue
88 sampling was repeated following the heat trial described below. Distal tube feet were used
89 in part to minimize damage to individual *P. ochraceus*, and to standardize contrasts of
90 regulatory change (Montgomery & Mank, 2016). Individuals were kept in flow-through sea
91 tables in Vexar enclosures to ensure consistent individual identification. DNA samples were
92 tested for presence of SSaDV (the putative pathogen causing SSWD) using qPCR as in
93 Hewson et al. (2014).

94 Righting responses (Figure 1) were used to explore the physiological status of individuals
95 subjected to periods of elevated temperature. Increasing the temperature by ~3° is known
96 to influence the physiology of *P. ochraceus*. Flow-through temperature treatments were
97 performed as in Eisenlord et al (2016); individuals were maintained at +3°C for 8 days. Sea
98 table temperature was monitored 4x daily with digital thermometers and with Hobo Tidbit
99 data loggers. Righting response trials were performed as in Held and Harley (2009). We
100 recorded the time each individual required to flip from the aboral side to the point that the
101 majority of arms contacted the surface on their oral side. Trials were performed three
102 times in each condition: in ambient seawater, at the end of the temperature trial, and again
103 when individuals returned to ambient temperature. Individuals that did not right
104 themselves within 1 hour were considered unresponsive and were excluded from
105 subsequent analyses. Minimum and mean righting response times were recorded; these

values are examined across EF1A genotypes using a linear mixed-effects model using the *lmerTest* package (Kuznetsova, Bruun Brockhoff & Haubo Bojesen Christensen, 2016) in R version 3.3.2 (R Core Team, 2016).

RNA Sequencing and Comparison

Samples of tube feet stored in RNALater were thawed on ice and 25mg were removed for RNA isolation using a Qiagen RNEasy Mini-prep kit. A Qiagen TissueRuptor with sterile disposable pestles was used for homogenization of each sample. RNA samples were submitted to the Georgia Genomics Facility (GGF; dna.uga.edu) for stranded RNA library preparation (Illumina TruSeq LT) and subsequent quality checks using an Agilent 2100 BioAnalyzer. Libraries were sequenced in parallel (high output PE75) on an Illumina NextSeq 500 at GGF and then informatically demultiplexed.

Our pipeline followed Kelly et al. (2017), minus the utilization of *cd-hit* to reduce the sequence complexity in the data. Our goal was to identify potential differential expression of isoforms at EF1A and other loci, so all fragments were retained in the final assembly. Illumina adapter sequences were removed during the demultiplex step. FASTQ data were cleaned using Trimmomatic (Bolger, Lohse & Usadel, 2014) (default settings), and 2 whole transcriptome assemblies were generated using *in silico* read normalization in Trinity (Grabherr et al., 2011). The first assembly utilized data from all 20 RNA libraries; the second utilized only the data from 4 individuals, 2 of each genotype, chosen for high RIN values and read numbers, as we had been advised that this could lead to a higher-quality assembly. Trinity *de novo* assembly was performed on a Georgia Advanced Computing Resource Center 512GB node with 8 processors. Individual RNA libraries were then aligned

to the assemblies using Bowtie2 (Langmead & Salzberg, 2012) and the RSEM method (Li & Dewey, 2011) as in Haas et al. (2013).

All assembled Trinity clusters were used as *blastn* queries against the NCBI nr database, with the best hit for each (e-value < 10^{-6}) retained. A custom R script was used to collapse the expression count files by inferred gene and by BLAST homologies except where otherwise noted. Differential expression was quantified using edgeR (McCarthy, Chen & Smyth, 2012), filtering reads for a counts-per-million (CPM) >1 in at least 2 of the libraries. Other filtering combinations were attempted with similar results (V. K. Chandler, results not shown). Both negative binomial and empirical Bayes dispersion measures were estimated before testing for differences. To evaluate specific expression of EF1A, we considered all fragments that successfully BLAST to NCBI accession AB070232, a ~5kb sequence of the EF1A gene region from the confamilial *Asterias amurensis* (Wada et al., 2002), and also used sequence data (NCBI KY489762-KY489768) generated from cloning of *P. ochraceus* EF1A (Pankey & Wares, 2009) to evaluate expression of the focal intron region that harbors the *ins* mutation. These latter assemblies were performed using Geneious R10 (Biomatters).

Results

A total of 24 individuals were collected from the Friday Harbor Laboratories marine reserve, and 21 survived our lab trials (1 died of apparent SSWD; 2 from distinct external infections) and were returned to their original location. As in previous studies (Pankey & Wares, 2009), the ratio of heterozygotes (+/*ins*, or *ins* hereafter) to homozygotes (+/+ or *wild* hereafter) at the EF1A locus was ~1:1. In order of initial labeling, the first 5

individuals of each genotype that had complete behavioral data were selected for RNA sequencing (Supplemental Table 1). Each individual was genotyped 3 times from 3 separate tissue samples with no errors. These 10 individuals exhibited no visible signs of SSWD and tested negative for SSaDV.

Behavior

For the 10 individuals analyzed in full, righting response trials (Figure 1) suggested that *ins* heterozygotes righted themselves approximately 1.8 times faster than *wild* homozygotes (Supplemental Table 1; $p = 0.02$) at both temperatures. However, including all data on righting response (from all 17 individuals assayed for behavior) introduces higher variation in response by genotype; the effect is in the same direction but not significant. Results are consistent for analysis of minimum and mean times. Unresponsive individuals (n=6) in the full sample were evenly distributed across genotypes.

Sequenced RNA Diversity

Supplemental Table S1 provides information for each library used in transcriptome assemblies. Of the two *de novo* assemblies, the reduced-input transcriptome had greater length and quality of contigs (N_{50} of 1799 bp, median contig length 513, total assembled bases 179,034,265) and is the focus of subsequent analyses. Fragments that were differentially expressed (FDR < 0.01) between the two genotypes from the two Trinity assemblies were themselves *de novo* aligned in Geneious R10; 80.76% of contigs from one of the two assemblies aligned with one from the other.

To identify the statistical signal associated with EF1A genotype in these samples, we developed a permutational misassignment test to see what differential expression could be

identified if one individual from either group is misassigned; if one individual in each group is misassigned; or if two individuals from each group are misassigned to the other group. This is distinct from random assignment in that "random" could include misassigning all individuals from each genotype to the other, and the assignment problem is symmetric. For each iteration, the number of Trinity assemblies that are significantly different (FDR < 0.01) was identified and contrasted with the true classification. The results suggest that differentiation of the two genotypes is robust relative to the most extreme misassignments (Figure 2), and greater than 0.96 of all permutations. All permutations with higher counts of differentially expressed transcripts involve re-assignment of individual Po5 (*wild*); though genotype was confirmed for this individual, it is similar to the *ins* heterozygotes for many expression traits (see below).

Comparison of EF1A expression across genotypes (Hypothesis 1)

Following BLAST analysis, a total of 28 fragments sufficiently matched NCBI accession AB070232 (Wada et al., 2002), a ~5kb sequence of EF1A from *Asterias amurensis*. Individually, none of these fragments appear to be differentially expressed (FDR < 0.01) between *wild* and *ins* EF1A genotypes. Summing expression counts from these fragments suggests negligible difference in expression patterns (logFC 0). As noted above, individual Po5 (*wild*) exhibits an inconsistent expression pattern (Fig. 2, 3); if excluded, 6 fragments matching EF1A are differentially expressed (FDR < 0.01; Fig. 4C). These 6 fragments together have an average $\log_2$ fold change in expression of 0.99 suggesting that homozygotes have expression approximately double that of heterozygotes when Po5 is excluded. Summing across all putative EF1A homologs (excluding Po5) indicates no

195 significant expression differences. Assembly of RNA sequence fragments from libraries of
196 the two genotypes to *A. amurensis* EF1A sequence showed no obvious distinctions in
197 coverage of coding regions.

198 **Comparison of differential expression across genotypes (Hypothesis 2)**

199 There are strong differences in the constitutive expression patterns of the 5 *wild* and 5 *ins*
200 individuals assayed. There are 200 fragments exhibiting differential expression with FDR <
201 0.01, and 19 with FDR < 0.0001 (Figure 3). Only 5 of the FDR < 0.01 fragments are
202 identifiable via BLAST (Supplemental Table 2); 2 are of unclear function, but the remaining
203 3 include a Na/K/Ca exchange protein, an ATP-binding protein, and an amino acid
204 transporter identified in the sea star *Diplasterias*. As before, if Po5 is excluded, a greater
205 number (n = 419) of fragments exhibit differential expression (FDR < 0.01), suggesting that
206 this individual represents an inconsistent expression phenotype for its EF1A genotype.

207

208

209 The effect of the *ins* genotype appears to be inhibitory; Figure 4A shows only those
210 fragments that are differentially expressed between the two genotypes, and only 30 of 200
211 fragments with FDR < 0.01 exhibit higher expression in heterozygotes. Many of the
212 significantly elevated transcripts in heterozygotes are modestly expressed compared to the
213 significantly elevated transcripts from wild homozygotes. The average log CPM for
214 fragments with FDR < 0.01 that are more highly expressed in *ins* heterozygotes is 0.876
215 (maximum 6.154), while the same average for fragments that are more highly expressed in

wild homozygotes is 3.639 (maximum 11.217). A similar result is obtained when Po5 is excluded (Figure 4B).

Comparison of differential expression following heat exposure

Following exposure (8 days) to water warmed by +3°C, *wild* homozygotes exhibited a larger number of potential loci (n=38) that changed in expression (FDR < 0.01) than *ins* heterozygotes (n=6). If individual Po5 is excluded, the remaining *wild* homozygotes then exhibit 52 fragments that change in expression (FDR < 0.01), suggesting that the expression phenotype of this individual adds considerable variance to the expression patterns of homozygotes. Of all fragments identified as responding to the temperature treatment, 3 of 6 identified in the heterozygotes are also found among those that are differentially expressed in the homozygotes (whether or not Po5 is included). These results are suggestive that homozygous individuals experienced a greater net change in expression phenotype following exposure to heat than *ins* heterozygotes. However, a multidimensional scaling plot of all libraries (using logFC values) showed low support of differentiation of temperature-treated RNA samples from the ambient treatments of the same individuals (results not shown).

Discussion

A difficult aspect of RNA sequencing in non-model organisms is the inference of true transcripts from *de novo* assembly, with a potential for alignment error and resultant chimeric sequences. As such, we consider these results provisional with respect to whether EF1A itself exhibits change in expression between the two EF1A genotypes in *P. ochraceus*.

Two uncertainties remain: whether there is a single copy of EF1A in the *P. ochraceus* genome (the PCR marker itself has one primer in an intron region, so is likely specific to only one copy (Pankey & Wares, 2009)), and whether the *ins* mutation itself is causal of the expression and tolerance changes, or if it is a linked site. Of the 10 individuals sequenced in this study, one (Po5) appears to have expression characteristics that are inconsistent with other samples of either genotype. Genetic analysis of individual crosses and sequence diversity have suggested that the *ins* marker is a single locus but that linked diversity may also be important in this system (Pankey & Wares, 2009).

Exclusion of this single RNA library (Po5) from analysis of fragments that positively BLAST to a confamilial sequence of EF1A suggests that a group of homologous transcripts are differentially expressed between genotypes (Figure 3C). Whether these are differentially expressed isoforms of a single locus, or a differentially expressed copy of EF1A among multiple copies in the genome, requires further evaluation. Nevertheless, our results show that the EF1A *ins* mutation has a likely effect on the generation of mature mRNA transcripts of EF1A, as well as apparent regulatory effects on a large number of other loci. Homozygous *wild* individuals express some EF1A-like elements approximately twice as much as *ins* heterozygotes, suggesting that *ins*/*ins* homozygotes may not express this isoform or copy at all, perhaps the cause of early mortality (Pankey & Wares, 2009).

The intron that contains the *ins* mutation does not appear to contain coding sequence (Pankey & Wares, 2009). Nevertheless, there is evidence in other systems for intronic promoters and/or noncoding transcripts of biological effect (Leh et al., 1998; Relle et al., 2014; Gaidatzis et al., 2015), which could generate a distinct isoform of the locus in

question (Galante et al., 2004). Intron sequence is typically rare in transcriptomic data, and has been interpreted as a sign of DNA contamination or the presence of pre-mRNA from the nucleus. We have no evidence for the *ins* allele being transcribed; some *wild* intron transcript is found at very low levels in 2 of the RNA libraries. The original work characterizing intron-exon boundaries in EF1A, including the sea star *A. amurensis*, noted the evolutionary lability of introns in this gene (Wada et al., 2002). Overall, the high expression and critical role of EF1A for metazoan development and function makes this evolutionary and functional lability of particular interest.

Moonlighting roles of EF1a

If true, lower expression of EF1A in *ins* heterozygotes of *P. ochraceus* is intriguing given the lower mortality of these individuals to SSWD. Viruses rely on host protein synthesis machinery for their own replication (Walsh & Mohr, 2011; Walsh, Mathews & Mohr, 2013; Abbas, Kumar & Herbein, 2015). Though there has been a focus of the role of EF1A in replication of RNA viruses (Li et al., 2013; Wei et al., 2014), this is likely also true for the SSaDV virion that has been associated with SSWD (Hewson et al., 2014). SSaDV is a densovirus with a single-stranded DNA genome, and requires host polymerase and other components of the replication machinery to multiply. To the extent we understand replication in densoviruses, *ins* heterozygotes appear to maintain cellular conditions that could limit viral replication.

Additionally, expression of EF1A is linked to interactions with cytoskeletal proteins like tubulin and actin (Lamberti et al., 2004), with altered expression leading to different cellular phenotypes. Similarly, there are associations between EF1A expression and

apoptosis during times of cellular stress (Lamberti et al., 2004). This is likely an indirect interaction, and some studies show that cell death is limited when EF1A is overexpressed (Blanch et al., 2013; Abbas, Kumar & Herbein, 2015); organismal lifespan is similarly affected in one manipulative experiment of EF1A levels (Stearns & Kaiser, 1993). As apoptosis is implicated in some of the degenerative effects of SSWD in the sea star *Pycnopodia helianthoides* (Fuess et al., 2015; Gudenkauf & Hewson, 2015), and actin and other cytoskeletal proteins have an important role in maintaining cellular morphology, this network of coexpression will likely be of use in understanding the cascade leading to SSWD and its symptoms.

Other implicated gene regions

There are a large number of differentially expressed loci (as many as 419 in the instance of excluding individual Po5) between EF1A marker genotypes, but little information on the identity or function of these loci. Though a small number of fragments have sufficient BLAST homology to identified proteins (Supplementary Information S1), we are currently limited by the tremendous evolutionary divergence between *Pisaster* and other characterized genomes (the only assembled Asteroidea genome to date is *Patiria miniata* (echinobase.org), a taxon with a Jurassic divergence from *Pisaster*; C. Mah, personal comm.). Generating a more extensive list of loci that are coregulated with EF1A (or the *ins* marker) is perhaps of modest utility without better experimental data in this non-model organism (Hudson, Dalrymple & Reverter, 2012). We do not know if the differentially expressed loci are relatively rapidly evolving, or if these transcripts represent noncoding RNA; currently, these hypotheses are difficult to test with available resources (Dinger et al., 2008). Nevertheless it is intriguing that a large number of genic regions do appear to have a

regulated response that is distinct between the two EF1A genotypes. It is also notable that the regulatory effect of the *ins* mutation (or a linked polymorphism) has a consistent response - there is a clear asymmetry (Figures 4A-C) in expression of transcripts suggesting that the *ins* mutation affects a promoter region.

Our ability to understand the effects of differential genotype in *P. ochraceus* may also require an understanding of tissue specificity. Here, tube feet were used as simple non-invasive tissues for sampling because the health of the local population is of concern. Future efforts could target tissues more specific to immune response function. For example, EF1A is also thought to regulate interleukins (Schulz et al., 2014), one of the basic components of the echinoderm immune response (Mydlarz, Jones & Harvell, 2006; Leclerc & Otten, 2013); these are produced in the axial organ, stimulate coelomocytes and are associated with antiviral activity (Ghiasi et al., 2002).

Temperature treatment and behavior

Though the effects of our temperature treatment were modest, it is tempting to note that *wild* homozygotes differentially expressed nearly 10 times as many transcripts as *ins* heterozygotes following treatment. As elevated temperatures may accelerate SSWD (Bates, Hilton & Harley, 2009b; Eisenlord et al., 2016; but see Menge et al., 2016), the likelihood that the *ins* mutation ameliorates multiple forms of stressors on the health of an individual is worth further investigation. In comparison with previous studies on *P. ochraceus*, we note the distinct time scales of stress: from daily tidal fluctuations, to the 96-hour exposure in Bates et al. (2009b), to the months-long duration of temperature anomalies reported by Eisenlord et al. (2016). The association of transcriptional regulation and temperature

stress important for interpreting our results, but there is little fine-scale temporal understanding of how stress influences physiological and regulatory acclimation responses in many organisms. Some studies have shown elevated expression of EF1A in heat trials (Bukovnic et al., 2009); others show that increased availability of EF1A was associated with greater heat tolerance and longevity (Stearns & Kaiser, 1993). We lack understanding of the rate of transcriptional acclimation in many species, with few studies that bridge physiological responses and longer-term acclimation. Bay and Palumbi (2015) sampled coral (*Acropora*) tissue following a range of short-duration experiments (2, 7, and 11 days) that suggested that early stages of acclimation to stress involved few changes in baseline expression, but could dampen later responses to heat shock following exposure.

Here, behavioral righting responses were used to understand the response to heat as an influence on activity levels (Held & Harley, 2009). Heterozygous individuals tend to right themselves more quickly in a limited sample. However, individual-level variation was high and the biological effect of genotype on this response may be low or absent. Individuals appeared to be consistent in their response, *i.e.* individuals with long response times tended to do so at all treatments; whether this is associated in any way with effects of this genotype requires further consideration. Overall, we conclude that righting response is a noisy response variable and perhaps ineffective for assaying physiological contrasts. We are not the first to recognize this difficulty:

from Jennings, 1907 (1907):

"It could probably be said, in a word, that the starfish may, and does, in different cases, right itself in any conceivable way, - and indeed, in many ways that would not readily be conceived before they were observed."

Thus, other approaches such as respirometry (Fly et al., 2012) are needed to more directly understand stress response in *P. ochraceus*.

Evolutionary Implications

A polymorphism like this should not be stable unless there is some balance of benefits to both genotypes (Subramaniam & Rausher, 2000). With typical genotype frequencies in the wild (Pankey & Wares, 2009,Wares & Schiebelhut (2016)), approximately 1/16th of all offspring (1/4 of the offspring from 1/4 of the random mating events) are lost each generation to this polymorphism. Similar levels of reduced fitness are involved in explorations of Dobzhansky-Muller interactions associated with outbreeding depression (Sweigart, Fishman & Willis, 2006). This is a considerable mutational load attributed to a single polymorphism yet the sudden appearance, or incidence in recent decades, of high mortality events like SSWD is unlikely to be a sufficient mechanism for maintaining this polymorphism. The two allelic classes each harbor considerable levels of flanking diversity and appear to be relatively divergent and ancient (Pankey & Wares, 2009), and the high frequency of the *ins* allele throughout the range of *P. ochraceus* (Pankey & Wares, 2009; Wares & Schiebelhut, 2016) suggests its origin is not recent (Slatkin & Rannala, 2000).

The question remains, what has maintained this polymorphism, and what can we learn from EF1A about disease in other echinoderms - or more broadly, other animals? One consideration is whether EF1A is simply mediating a very general stress response. In other major epidemics, it has been noted that mortality has been highest in individuals that are weak *or* that have the strongest inflammatory/immune response to a pathogen (Lai, Ng & Cheng, 2015). If this is true, perhaps individuals with higher levels of constitutive EF1A

371 expression are more prone to extreme stress responses. However, there is little support for
372 this hypothesis; Wang et al (2011) note elevated expression of EF1A in response to pH and
373 heavy metal stressors in the shrimp *Litopenaeus*, but many other studies identify no
374 response to stress at this locus (Nicot et al., 2005), and in fact EF1A is often used as an
375 endogenous control in RT-PCR studies. The reality is that stress tolerance is thought to be
376 highly context-dependent (Berry et al., 2011; Bay & Palumbi, 2015) and may be difficult to
377 assess in a wild population such as the *Pisaster* surveyed here.

378 Whether environmental stress is a component or not, there is nevertheless a net
379 transcriptional difference between these EF1A genotypes that is associated with ins
380 heterozygotes being more tolerant of SSWD (Wares & Schiebelhut, 2016), and this
381 potential for increased survival against a huge diversity of likely marine pathogens merits
382 further exploration. If P. ochraceus individuals with higher expression of EF1A - or some
383 copy or isoform of it - have higher adult mortality during SSWD outbreaks, the only
384 mechanism we currently know that makes sense is an influence on viral replication (Li et
385 al., 2015). It remains to be seen whether the balance of fitness between wild individuals
386 and ins heterozygotes involves other life history trade-offs.

387 Conclusions

388 We have explored the differential expression of all transcripts, and EF1A in particular, in
389 samples of *P. ochraceus* from two EF1A genotypes in a natural population. Each individual
390 bears high levels of additional variation that mediates their responses to environment,
391 pathogens, and so on. The fact that this single 6-bp insertion mutation generates such
392 strong biological effect amidst the noise of other natural genomic diversity is

393 extraordinary. Certainly there are other examples of single mutations that confer
394 significant health and life history consequences on carriers (Aidoo et al., 2002; Drnevich et
395 al., 2004; Gemmell & Slate, 2006). Additionally, distinct phenotype classes within a species
396 often have distinct expression profiles (McDonald et al., 1977; Garg et al., 2016), including
397 instances of disease or tolerance phenotypes (Emilsson et al., 2008; O'Connor et al., 2017).
398 If we are correct about changes in expression of a common housekeeping gene between
399 these two phenotype classes, this is a novel report of how levels of EF1A expression
400 throughout an organism - with no currently-known tissue specificity - can mediate health
401 and survival. The fortuitous result of inconsistent expression changes in one individual
402 additionally suggests a recombination event that further defines the influence of this
403 genomic region. Our hopes are that further consideration of this system, in an ecologically
404 important sea star (Paine, 1969; Menge et al., 2016), will be of relevance for a more general
405 understanding of health and pathogen tolerance.

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418

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Figure 1. Aboral view of *Pisaster* in righting response trial. Photo by JPW.

Figure 2. Permutational misassignment of individuals and comparison with actual genotypic groups. Misassignments were directed to maintain (nearly) equal sample sizes in the two groups. The true grouping of individuals by EF1A genotype suggests a stronger signal (vertical dotted line) than almost all permutations; 'misassignments' with more extreme results involve reassignment of individual Po5.

Figure 3. Differential expression heatmap for transcripts with FDR < 10^{-4}. Color scores indicate *ln* counts for each transcript by library. Results are only shown for individuals at ambient sea water temperature; similar results are obtained with elevated water temperature.

Figure 4. Volcano plot of fragments that are differentially expressed (FDR < 0.01); other fragments not plotted. Contrast indicated with positive logFC values on the right for genes that have higher expression in *wild* homozygotes. Size of circle scaled by log CPM. Panel (A) includes all individuals in study; center panel (B) excludes *wild* individual Po5; right panel (C) includes only fragments with BLAST homology to EF1A, excluding individual Po5. Red dotted lines indicate FDR of 0.01; blue dotted line indicates two-fold change in expression in panel (C).

Figure 1

Image of Pisaster ochraceus

Figure 1. Aboral view of *Pisaster* in righting response trial. Photo by JPW.

Figure 2(on next page)

Randomized expression differences among libraries

Figure 2. Permutational misassignment of individuals and comparison with actual genotypic groups. Misassignments were directed to maintain (nearly) equal sample sizes in the two groups. The true grouping of individuals by EF1A genotype suggests a stronger signal (vertical dotted line) than almost all permutations; 'misassignments' with more extreme results involve reassignment of individual Po5.

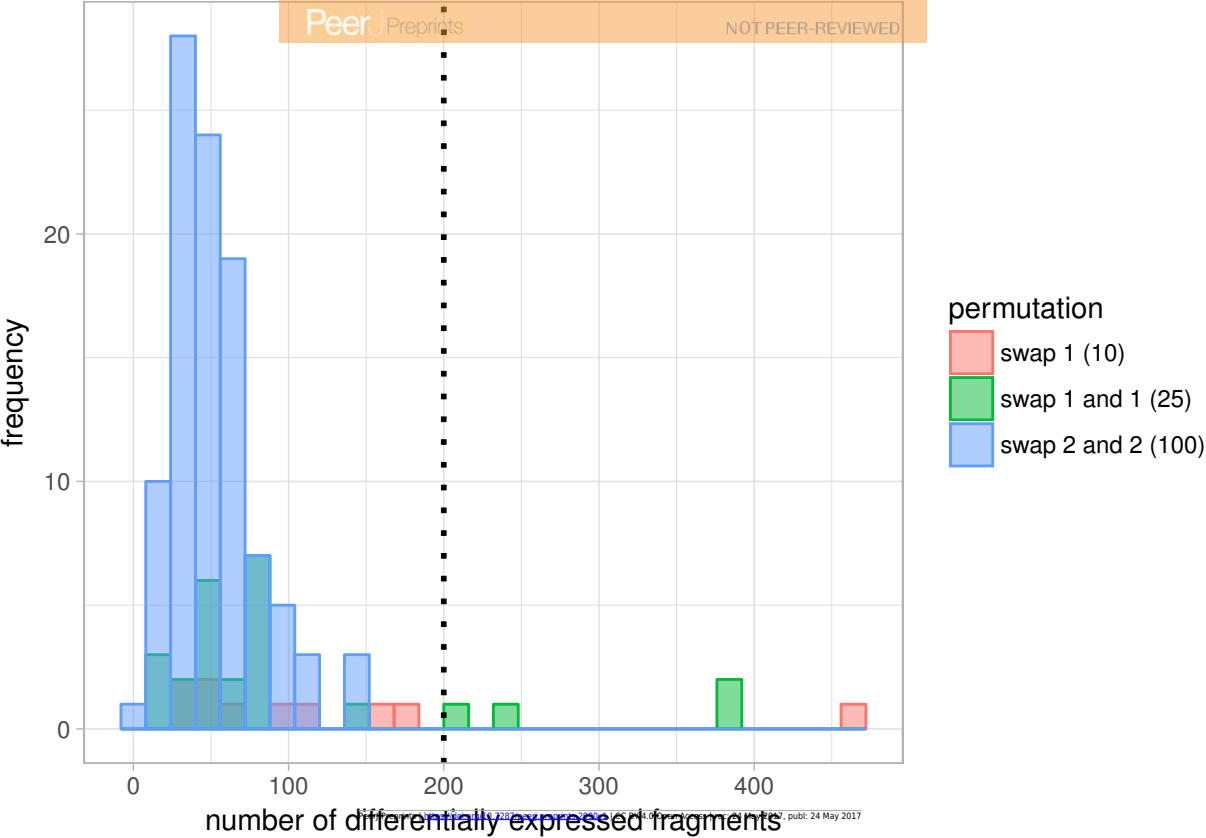

Figure 3(on next page)

Heatmap of differential expression

Figure 3. Differential expression heatmap for transcripts with FDR < 10^{-4}. Color scores indicate *ln* counts for each transcript by library. Results are only shown for individuals at ambient sea water temperature; similar results are obtained with elevated water temperature.

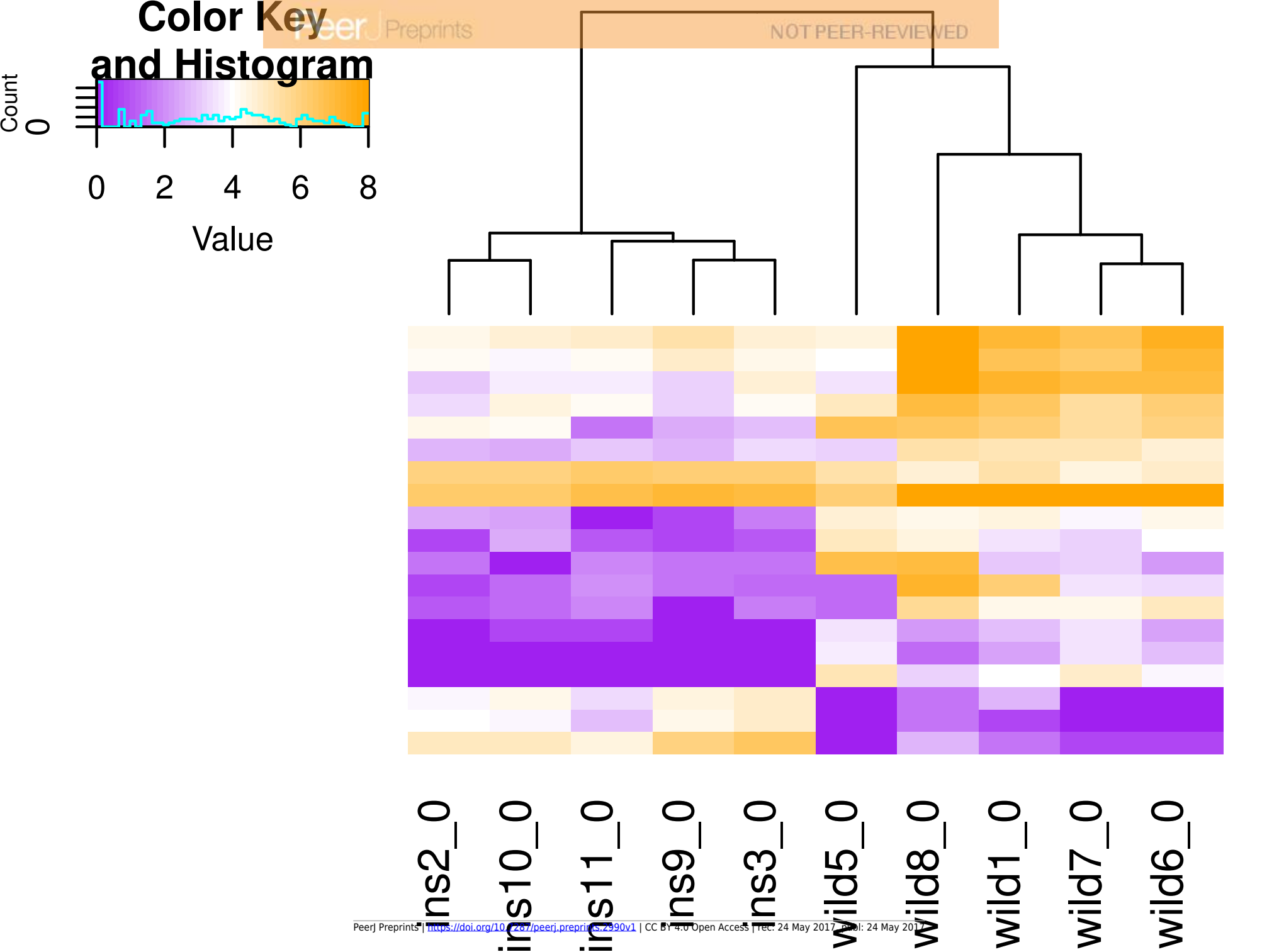

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Figure 4(on next page)

Volcano plots of differential expression

Figure 4. Volcano plot of fragments that are differentially expressed (FDR < 0.01); other fragments not plotted. Contrast indicated with positive logFC values on the right for genes that have higher expression in *wild* homozygotes. Size of circle scaled by log CPM. Panel (A) includes all individuals in study; center panel (B) excludes *wild* individual Po5; right panel (C) includes only fragments with BLAST homology to EF1A, excluding individual Po5. Red dotted lines indicate FDR of 0.01; blue dotted line indicates two-fold change in expression in panel (C).