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What doesn't kill them makes them stronger: An association between elongation factor 1- α overdominance in the sea star *Pisaster ochraceus* and "sea star wasting disease"

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In recent years, a massive plague has killed millions of sea stars, of many different species, along the Pacific coast of North America. This disease, known as 'sea star wasting disease' (SSWD), is thought to be caused by viral infection. In the affected sea star *Pisaster ochraceus*, previous work had identified that the elongation factor 1- α (EF1A) locus harbored an intronic insertion allele that is lethal when homozygous yet appears to be maintained at moderate frequency in populations through increased fitness for heterozygotes. The environmental conditions supporting this increased fitness are unknown, but overdominance is often associated with disease. Here, we evaluate populations of *P. ochraceus* to identify the relationship between SSWD and EF1A genotype. Our data suggest that there may be significantly decreased infection or mortality rates in individuals that are heterozygous at this locus. These results suggest further studies to understand the functional relationship between diversity at EF1A and survival in *P. ochraceus*.

1 **What doesn't kill them makes them stronger: An association between**
2 **elongation factor 1- α overdominance in the sea star *Pisaster***
3 ***ochraceus* and "sea star wasting disease"**

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9 **Abstract**

10 In recent years, a massive plague has killed millions of sea stars, of many different species,
11 along the Pacific coast of North America. This disease, known as 'sea star wasting disease'
12 (SSWD), is thought to be caused by viral infection. In the affected sea star *Pisaster*
13 *ochraceus*, previous work had identified that the elongation factor 1- α locus harbored an
14 intronic insertion allele that is lethal when homozygous yet appears to be maintained at
15 moderate frequency in populations through increased fitness for heterozygotes. The
16 environmental conditions supporting this increased fitness are unknown, but
17 overdominance is often associated with disease. Here, we evaluate populations of *P.*
18 *ochraceus* to identify the relationship between SSWD and EF1A genotype. Our data suggest
19 that there may be significantly decreased infection or mortality rates in individuals that are
20 heterozygous at this locus. These results suggest further studies to understand the
21 functional relationship between diversity at EF1A and survival in *P. ochraceus*.

22 **Introduction**

23 One of the more stunning recent news stories pertaining to ocean health was the massive
24 die-off of sea stars on both coasts of North America via a necrotic syndrome now known as
25 sea star wasting disease (SSWD) (Hewson et al. 2014). Similar die-offs have happened in
26 earlier decades (Eckert, Engle, and Kushner 1999; Becker 2006), though none as extensive
27 as in 2013–2014. Hewson et al. (2014) identified a candidate densovirus that is in greater
28 abundance in diseased sea stars, and may be a causal agent; however, there is much yet to
29 be learned. As sea stars are key predators in marine benthic ecosystems, the impacts of
30 disease on these organisms could dramatically restructure coastal communities (Paine
31 1966). Thus, we address here the potential for one species to respond to disease via natural
32 genetic variation.

33 During disease outbreaks, biologists are keen to know whether populations will exhibit any
34 resistance to a pathogen. Thus, management studies may include surveys of genetic

35 diversity to identify the potential for evolving resistance, or genetic rescue from other
36 regions (Whiteley et al. 2015); such studies may also provide insight into the extent of
37 population structure and gene flow among regions. Following a routine analysis of genetic
38 variation in the sea star *Pisaster ochraceus* (Harley et al. 2006), Pankey and Wares (2009)
39 identified an insertion mutation in an intron of the elongation factor 1- α gene (hereafter
40 EF1A) that appeared to exhibit overdominance. In this case, the insertion is lethal when
41 homozygous (Pankey and Wares 2009), yet the average frequency of the insertion allele
42 was ~ 0.24 along the Pacific coast of North America. These observations suggest that the
43 heterozygote has a significant fitness advantage in an unknown environmental setting.
44 Overdominance is often associated with resistance to disease or toxins, however, and
45 Pankey and Wares (2009), referring to what is now called SSWD, speculated that:

46 *“widespread die-offs on the west coast of North America... could exert a substantial selective*
47 *force on Pisaster. Given the prevalence of pathogen resistance in earlier studies of*
48 *overdominance, we believe this to be a probable explanation for the maintenance of the*
49 *described ... polymorphism.”*

50 There is concern that elevated sea temperature is a component of the SSWD outbreak
51 (Bates, Hilton, and Harley 2009; Hewson et al. 2014). The relationship between expression
52 of EF1A and thermal tolerance has been identified in other metazoans (Stearns, Kaiser, and
53 Hillesheim 1993; Buckley, Gracey, and Somero 2006), and functions in part through rapid
54 co-production of proteins associated with the heat shock response. Though Pankey and
55 Wares (2009) were not able to detect EF1A expression differences among individuals of
56 differing genotype, we were not able at the time to control for a number of environmental
57 factors nor the possible action of splice variants. Here, we posit an indirect mechanistic
58 relationship between temperature, the effect of expression of EF1A, and SSWD.

59 With as little as is known about this disease and marine disease in general (Mydlarz, Jones,
60 and Harvell 2006), this is at best an educated guess. However, it is useful to know what
61 potential *P. ochraceus* and other sea stars have for surviving this outbreak and natural
62 patterns of genetic variation, and whether subsequent generations will be more resistant
63 or tolerant of similar pathogens. Here we evaluate this simple polymorphism from
64 populations of *P. ochraceus* collected prior to and following the SSWD outbreak, as well as
65 focus on extant individuals and their disease status. We ask whether there are frequency
66 shifts of the two genotypes at this locus that may be associated with resistance to infection,
67 and evaluate efforts to explore similar genomic variation in other species.

68 **Methods**

69 ***Pisaster* and Disease Status**

70 Collections were made in 2014–2015 from locations in central California, Oregon, coastal
71 Washington and the San Juan Islands (WA), and Nanaimo (Vancouver), and categorized by
72 health status using the Pacific Rocky Intertidal Monitoring Network classification
73 (<http://www.eeb.ucsc.edu/pacificrockyintertidal/index.html>; Table 1). Complete
74 information on collection location, individual sizes, permitting, and other metadata are in

75 Supplemental Data S1. All permits are listed in Supplement S1. Specimens were collected
 76 under California Department of Fish and Wildlife permits #11794 to L. Schiebelhut and
 77 #603 to M. N. Dawson, California State Parks permit to M. N. Dawson, and National Parks
 78 permits PORE-2012-SCI-0038, PORE-2013-SCI-0033, and REDW-2014-SCI-0018.
 79 Collections at Nanaimo were under Dept. of Fisheries and Oceans site permit at Pacific
 80 Biological Station; tissues from the San Juan Islands were re-sampled from those collected
 81 for Hewson et al. (2014).

Site/Region	Lat	Long	SSWD + / +	SSWD + / ins	OK + / +	OK + / ins	Effect
Nanaimo, Vancouver, BC	49.2	124	8	4	7	5	0.089
Olympic Peninsula, WA	48.5	125	5	0	11	4	0.31
San Juan Island, WA	48.5	123	25	17	15	18	0.14
Cape Meares, OR	45.5	124	1	0	4	5	0.2
Seal Rock, OR	44.5	124.1	1	2	1	6	0.25
Coquille Point, OR	43.1	124.4	7	0	1	2	0.88
Damnation Creek, CA	41.7	124.1	1	1	4	4	0
Sonoma County, CA	38.7	123.4	8	1	22	18	0.21
San Francisco Bay, CA	38.0	122.8	0	0	7	13	0
Overall			56	25	72	75	0.19

82 **Table 1.** Sample sizes from each regional collection of individuals (see Supplemental Table 1
 83 for additional sampling information); samples are listed by health status as well as genotype
 84 (+/+ wild type, +/ins for the heterozygote genotype). "Effect" refers to the difference in
 85 proportion of EF1A homozygotes that exhibit SSWD and the proportion of heterozygotes with
 86 SSWD; positive numbers suggest a higher proportion of homozygotes with SSWD.

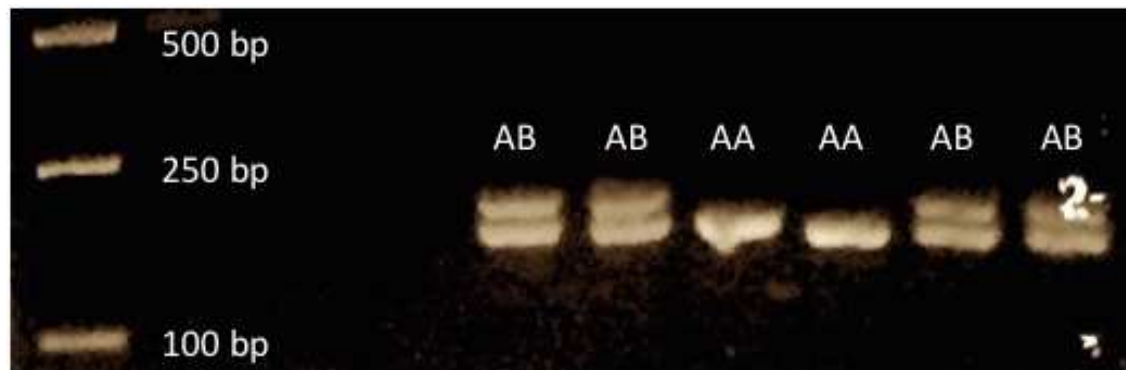
87 Temporal Comparisons

88 In addition to individuals explicitly assessed for health status, we also considered the
 89 potential for genotype (and allele) frequency change following a related disease outbreak.
 90 Previous EF1A genotype/allele frequency information from specimens collected in 2003–
 91 2004 are available for many locations along the Pacific coast (Pankey and Wares 2009). In
 92 central California, tissues from 4 locations (Sonoma County) were obtained in both 2012
 93 (pre-outbreak) as well as 2014 (the tissues noted in previous section from these sites).
 94 Thus genetic frequencies from 3 time points can be assessed. Along the Oregon,
 95 Washington, and San Juan Island coastal regions, the tissues noted in previous section
 96 (from 2014–2015) can be compared to the genetic frequency information from 2003–2004
 97 tissues.

98 Molecular Methods

99 As in Pankey and Wares (2009), primers PisEF1-F (5'-aggctgccgataccttcaa-3') and PisEF1-
 100 R (5'-gctagtatctgttctgtgtgactgc-3') were used to determine individual EF1A genotypes by

101 scoring length-polymorphic PCR products on 2% (or greater) agarose gels. About 10% of
102 individuals were multiply genotyped so that genotype error rate (Pompanon et al. 2005)
103 could be assessed. An example of this polymorphism is shown in Figure 1.



104

105 **Figure 1.** Results from analysis of 6 individuals (right side of image) on 2% agarose gel
106 following PCR amplification as noted in Methods. Fragments only vary by 6bp in length so gel
107 must be run for ~60 minutes under typical conditions. Heterozygotes are denoted 'AB' and
108 homozygotes denoted 'AA' on gel image (white "2" at far right is a marking on gel tray). Size
109 ladder is shown at left side of gel.

110 Statistical Analyses

111 Our first approach is to ask whether the frequency distribution of the two EF1A genotypes
112 differs between diseased and healthy individuals of *P. ochraceus*. Specimens from distinct
113 locations are grouped when sites are within 50km of each other and each regional sample
114 is evaluated separately as well as combined. Separate analyses recognize the potential for
115 heterogeneity at related quantitative traits despite apparent phylogeographic homogeneity
116 (Harley et al. 2006), as well as distinct environmental influences, while pooled analysis
117 augments statistical power.

118 Regional and combined data are analyzed first with a Fisher's exact test. Additionally,
119 following Gerrodette (2011), we estimate the effect size of genotype on SSWD mortality.
120 Here we assume that the genotype frequencies are binomially distributed (with associated
121 sampling error) and estimate the difference in proportion of disease incidence (also here
122 assumed binomial) between homozygotes and heterozygotes. Again, these probability
123 distributions are estimated for each individual regional/temporal sample. These same
124 statistical methods were applied to evaluate genotype frequency changes between
125 population samples from before and after the 2013–14 SSWD outbreak, as described
126 above.

127 To evaluate the probability that heterozygous individuals have a higher probability of
128 avoiding or surviving SSWD, logistic regression of the complete dataset is performed with
129 models that incorporate individual size (when available, measured from center of disk to
130 tip of an arm), genotype at EF1A, and region of collection. Models of single factors were
131 evaluated, and for factors that exhibit significant variation additive and interaction models
132 were also evaluated and compared using AIC values. The model with greatest AIC weight is

133 considered to explain most of the variation in the system. All statistical analyses were
134 performed using R (R Core Team 2015).

135 Results

136 The genotype error rate was 0 for 21 individuals (out of 228 in this study) that were
137 repeatedly genotyped. Two individuals initially presented a very faint second band on gel,
138 but subsequent repeat amplification of one of these confirmed it as homozygous (note: this
139 polymorphism has been assessed as an overdominant Mendelian locus (Pankey and Wares
140 2009), so we do not think this represents variation in paralog amplification success).

141 Data from each location (Table 1) were analyzed with a basic Fisher's exact test. Each
142 regional sample was in the predicted direction with a higher likelihood of SSWD among
143 homozygotes than heterozygotes; no single location or regional grouping presented
144 contrasts that were statistically significant (results not shown). Certainly a component of
145 individual regional/temporal samples is that with modest sample sizes, statistical power is
146 low. Combining all samples leads to a statistically significant result (p-value 0.0035).

147 When considering all available information related to disease risk in our samples, models
148 representing single factors (size, sample location, and genotype) and combinations of
149 factors were compared using AIC against a null (no factor) model. Size (radius) was not
150 significant and was dropped from subsequent analyses, while location ($p < 0.001$) and
151 genotype were both significant factors ($p = 0.00615$). Given the remaining main effects and
152 interactions, we found the AIC weight was strongest for models with both factors included
153 (AIC weight 0.226) and with both factors included with interaction between (AIC weight
154 0.756). When both factors are included, both are still significant ($p < 0.01$); when the model
155 includes interactions, genotype is important but the additional parameterization reduces
156 power ($p = 0.0526$), location is not significant, and the interaction term is not significant
157 ($p = 0.0694$). A small sample of individuals ($n = 14$) had no size measurement available
158 (Supplement S1) but exhibited no significant effect (effect=0.05, $p = 1$) of genotype.

159 Despite the hypothesis of increased fitness for EF1 heterozygotes under these conditions,
160 the frequency of the insertion (*ins*) allele in central California only appears to decrease
161 through time, from approximately 0.27 ($n = 33$) in 2003–2004 (Pankey and Wares 2009) to
162 0.24 ($n = 40$) in 2012 and 0.25 ($n = 40$) in 2014–2015. However, with sampling error these
163 frequencies are statistically unchanged and a larger sample comparison may be necessary
164 to explore this component of our evaluation. In the San Juan Islands, the frequency of the
165 *ins* allele decreased slightly from 0.27 in 2003 ($n = 62$) to 0.2533 in 2014–2015 ($n = 75$),
166 again not supporting a hypothesis of selection increasing or maintaining the *ins* frequency
167 (but also not a statistically significant difference in frequency). The same can be said for
168 contrasts along the Oregon and Washington coasts, where the overall frequency is
169 effectively unchanged.

170 Discussion

171 Our data show that our hypothesis for a relationship between disease status (SSWD) and
172 an apparent overdominant polymorphism in *P. ochraceus* is strongly supported—results
173 from each sample are in the predicted direction, and overall there is clear evidence that
174 sick individuals are more likely to be EF1A homozygotes than heterozygotes. The net effect
175 size of 0.19 is very similar to the fitness differential between genotypes (~0.2) proposed by
176 Pankey and Wares (2009) given simulations of overdominance. The variation in effect seen
177 among regional samples—and the apparent statistical interaction between sample location
178 and genotypic effect on disease status—is likely influenced both by modest sample sizes as
179 well as distinct exposure histories. It is likely that each of our regional samples has been
180 exposed to distinct temperature profile histories (Bates, Hilton, and Harley 2009), and it is
181 possible that despite apparent population genetic homogeneity (Harley et al. 2006) that
182 undetected evolutionary changes have led to distinct reaction norms among regional
183 samples. Additionally, the timing of arrival and effect of the densovirus that causes SSWD
184 may lead to distinct evolutionary dynamics across regions.

185 In previous work (Bates, Hilton, and Harley 2009; Hewson et al. 2014) it has been
186 evaluated whether size was a predictive factor in disease status; both research groups
187 found no clear statistical association (though it appeared there is a negative relationship
188 between densovirus abundance and size in *Pycnopodia helianthoides*; Hewson et al. 2014).
189 Our overall sample also finds no association between sea star radius and disease status nor
190 an interaction with genotype. Analysis of the large sample from the San Juan Islands does
191 suggest a statistically significant ($p < 0.01$) pattern in which small individuals are more
192 likely to exhibit SSWD (results not shown); however, our sample in this case was selected
193 non-randomly with respect to size and disease status and so we discount this result in the
194 face of the larger analysis and previous analyses.

195 Evolutionary Response

196 Despite the apparent and predicted effect in our samples, we do not see the hypothesized
197 evolutionary response—the frequency of the *ins* mutation has apparently not increased in
198 recent years. If EF1A in *P. ochraceus* truly evolves via overdominance, where the
199 heterozygote is significantly more fit under certain environmental conditions, then we
200 would expect this allele to increase in frequency when exposed to a relevant mass
201 mortality event. However, it is also not entirely clear what proportion of individuals have
202 died in recent years as a result of SSWD (though estimates from intertidal surveys are high
203 enough that some frequency response is warranted). Thus, detection of this change could
204 be masked in part by simple stochastic changes (e.g. genetic drift) in local populations of *P.*
205 *ochraceus*. Of course, there are other forms of mortality in sea stars like *P. ochraceus*
206 (Jurgens et al. 2015), and so it is still likely that we are seeing an indirect interaction
207 between recent die-offs and individual-level responses that appear to be genotype
208 dependent. Preliminary evidence (L.S., unpublished observations) suggest higher
209 frequency of the *ins* allele at some California sites, and some of our sampled locations had
210 higher frequencies of SSWD after tissues were harvested. Thus further study, tracking

211 frequency of the EF1A *ins* polymorphism through time with detailed information on
212 disease and other environmental factors at local sites, is warranted.

213 Disease in Sea Stars

214 With limited understanding of immune response in most echinoderms (Mydlarz, Jones, and
215 Harvell 2006), the problem of SSWD is difficult enough to explore in *P. ochraceus*, let alone
216 the many other species affected in the recent outbreak. In another species of sea star (*P.*
217 *helianthoides*), Fuess et al. (2015) have identified some of the genomic components that are
218 upregulated in response to viral exposure; however, we know of no similar (apparent)
219 overdominant system in these other asteroids, and as yet still know very little about how
220 this polymorphism in *P. ochraceus* influences EF1A expression, alternate splicing events, or
221 what genes may be linked to this region and thus affected. Our interest in exploring this
222 particular case has little to do with solving the problem of disease, and more about the
223 question of what demographics will be like for *P. ochraceus* in an increasingly warmer—
224 and disease-affected—environment (Harvell et al. 2002). If disease like SSWD interacts
225 with the EF1A polymorphism as noted here, and the frequency of the deleterious *ins* allele
226 increases, this could also indicate increased reproductive loss through homozygous
227 lethality, which could decrease the potential for populations to rebound from crashes.

228 Parallels with Malaria

229 Aidoo et al. (2002) note that "sickle cell trait" (carrying a single *S* allele of hemoglobin)
230 provides ~60% protection against overall mortality, mostly in the first 16 months of life;
231 being a carrier is not a guarantee against infection. Other studies have focused on specific
232 malarial parasites, and note that children heterozygous for the *S* hemoglobin allele have
233 approximately one-tenth the mortality risk from *Plasmodium falciparum* as those
234 homozygous for normal alleles (Cholera et al. 2008). In the absence of cohort data, it is
235 difficult to estimate the level of disease or mortality protection that any single
236 polymorphism can provide (Aidoo et al. 2002). Until such recent studies, the claim of
237 overdominant selection on hemoglobin genotypes, based on the relationship between the
238 frequency of the *S* allele and the prevalence of malaria (Allison 1954), was only correlative.
239 This is still a clear case of overdominance, but is illustrative that increased heterozygote
240 fitness does not require absolute protection against the associated risk factor—*e.g.*, that all
241 individuals of *P. ochraceus* with SSWD would be homozygous for the wild-type EF1 allele
242 identified in Pankey and Wares (2009), nor that healthy individuals would all be
243 heterozygotes—as there are many components to disease avoidance, tolerance, or
244 resistance.

245 Conclusions

246 At this time, with limited sampling (and recognizing that our samples themselves may not
247 be random from populations of *P. ochraceus*), our results suggest an intriguing (but
248 probably indirect) relationship between SSWD susceptibility and the EF1A polymorphism
249 described. The direction of effect is consistent in *all* subsamples, and the magnitude of
250 effect overall is comparable to predictions based on simulations of overdominance in this

251 system given the observed frequency of the *ins* allele and lethality of *ins* homozygotes
252 (Pankey and Wares 2009). Nevertheless, we do not see an increase in the frequency of the
253 *ins* allele over time in our samples and so we remain curious about the dynamics of
254 overdominance in this system.

255 It is possible that regulation and expression of EF1A is influenced by this polymorphism in
256 a way that alters an individuals' tolerance or capacity for heat stress, and in a warming
257 climate and ocean it is known that disease and mortality are higher in large part because of
258 physiological stress modifying an organisms response to pathogens (Harvell et al. 2002).
259 Further work is needed not only to examine the association shown here, but also to identify
260 (i) whether size or maturity is truly important in this relationship, (ii) whether individuals
261 of different genotype do have distinct constitutive or regulated patterns of expression of
262 EF1A or related/linked genes, and (iii) whether there are genotype-driven differences in
263 mortality of individuals under thermal stress (which can affect feeding rates as well as
264 physiological factors in *P. ochraceus*; (Sanford 2002)).

265 In the meantime, we emphasize that this system is so easy to explore as a low-budget
266 research project or teaching tool that there are opportunities to work as a community to
267 greatly expand our understanding of the maintenance of the overdominant EF1A diversity
268 in *P. ochraceus*, perhaps for other pertinent variables of interest. We would encourage any
269 interested colleagues to ensure that sufficient metadata are associated with any such
270 comparative study so that data can continue to be updated, and we will facilitate this
271 crowd-sourcing of analysis by maintaining a dynamic analytical database through JPW.

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