A peer-reviewed version of this preprint was published in PeerJ on 29 March 2016.

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Wares JP, Schiebelhut LM. 2016. 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". PeerJ 4:e1876 https://doi.org/10.7717/peerj.1876

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*.

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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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Abstract

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 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*.

Introduction

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

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

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

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

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

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

Methods

Pisaster and Disease Status

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

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

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

Temporal Comparisons

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

Molecular Methods

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

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

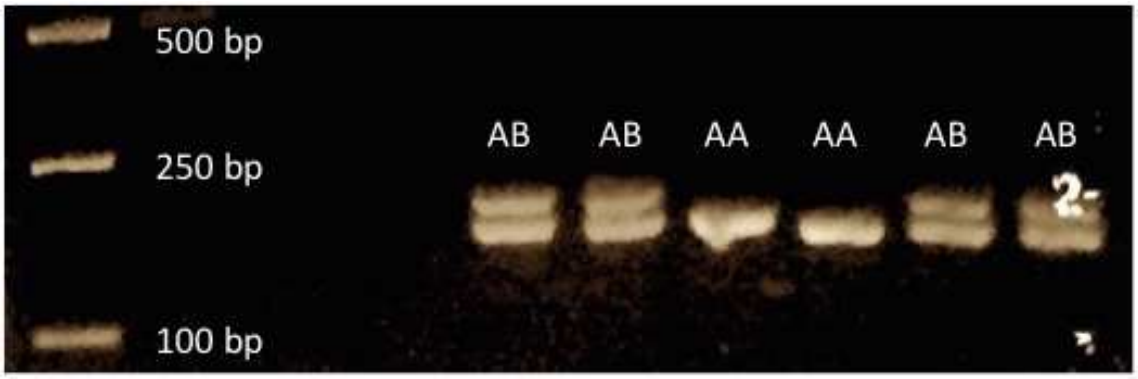

Figure 1. *Results from analysis of 6 individuals (right side of image) on 2% agarose gel following PCR amplification as noted in Methods. Fragments only vary by 6bp in length so gel must be run for ~60 minutes under typical conditions. Heterozygotes are denoted 'AB' and homozygotes denoted 'AA' on gel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

Statistical Analyses

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

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

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

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

Results

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

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

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

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

Discussion

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

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

Evolutionary Response

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

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

Disease in Sea Stars

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

Parallels with Malaria

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

Conclusions

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

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

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

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

Acknowledgments

The authors would like to thank Collin Closek (Wares Lab alumnus) and Mo Turner for coordinating tissue samples from FHL, Mike Hart and John Drake for discussion of the idea and early drafts of the manuscript, California Sea Grant College Program grant #2012-R/ENV-223PD and National Science Foundation grant OCE-1243958 and OCE-1243970 to Michael Dawson, Rick Grosberg and Brian Gaylord, and NSF Ecology of Infectious Diseases funding (OCE-1015342) to Wares and UGA colleagues, members of the Wares Lab at UGA and colleagues at the Odum School of Ecology. Tim Makinde generated much of the genotypic data at UGA. We thank Sarah Abboud, Charlsie Berg, Anny Calderon, Lorely Chavez, Brendan Cornwell, Michael N Dawson, Madlen Friedrich, Brian Gaylord, Alehandra Guzman, Brittany Jellison, Laura Jurgens, Shawn Knapp, Kelly McClintock, Holly Mondo, Mira Parekh, Emily Ramirez, Mariana Rocha de Souza, Adam Rosso, Stephen Sanchez, Holly Swift, Sabah Ul Hussan, and Jesse Wilson for assisting with California collections in 2012–2014. Thanks to Mike Hart, Vanessa Guerra, and David Breault for specimen collection and handling of Nanaimo tissues in 2015, Peter Raimondi and PISCO for other coastal samples collected in 2014–2015, and Sarah Gravem for organizing the SSWD-themed session at the Western Society of Naturalists meeting in 2015 from which we gained perspective and useful comments on this project.

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