Investigating the complex association between viral ecology, environment, and Northeast Pacific sea star wasting

Ian Hewson[1]*, Kalia S.I. Bistolas[1], Eva Marie Quijano Cardé[2], Jason B. Button[1,4] Parker J. Foster[1,5], Jacob M. Flanzenbaum[1], Jan Kocian[3] and Chaunte K. Lewis[1,6]

SUPPLEMENTAL MATERIAL

i. Genomic study of densoviruses and historical detection:

Biogeographical and historical analyses of SSaDV prevalence by qPCR suggested the presence of the virus across multiple samples in time (Hewson et al., 2014). We expanded our study of historical specimens by obtaining a further 81 specimens of asteroid from museum collections (Supplemental Table 2). These new specimens focused on species heavily affected by SSWD from 2013 – 2015 (*Pycnopodia helianthoides*, *Pisaster ochraceus* and *Evasterias troscheli*) and increased both the annual range (1905 – 2012) and geographic range of samples. SSaDV was detected in a further 35 samples from 1905 to 2012 by qPCR using Hewson et al. (2014) primers targeting VP4 and NS1. These new data suggested that SSaDV had been present for longer than previously reported (1942) (Hewson et al., 2014). In addition to historical specimens, we obtained 134 asteroid samples collected in 2014 and 2015 to determine the geographic range of SSaDV (Supplemental Table 1). Amongst these, 12 samples were positive for SSaDV using qPCR targeting VP4 and NS1 (Hewson et al., 2014). The lack of reported SSWD in many of these samples, including from 1905 to 2012 (museum specimens), and positive detections in asymptomatic asteroids from False Bay (South Africa; n = 4 of 24 examined), the Oresund (n = 1 of 4 examined), the Waddenzee (n=2 of 10 examined), Okinawa (n = 1 of 9 examined) and Truk Lagoon (n = 3 of 15 examined), all suggested largely asymptomatic infections in species found in those locations (Supplemental Table 1). SSaDV detection was associated with wasting *Asterias amurensis* from Shandong Province, China, and *Asterias forbesii* from the Gulf of Maine (Hewson et al., 2014). We speculated that SSaDV may have strains which are more virulent in some species, and

that the SSaDV strain currently associated with mass mortality in the northeast pacific may have evolved from strains detected since 1905.

To understand the diversity of viruses associated with asymptomatic asteroids globally, metaviromic analyses of 15 asteroid samples were performed (Supplemental Table 12). All metaviromes yielded very few viral sequences (Supplemental Table 12). However, 5 densovirus-like contigs were found in 3 libraries (*Echinaster luzonicus* and *Astropecten polyacanthus* from Hong Kong and *Parvulastra exigua* from South Africa; Supplemental Table 13). While these densoviruses had best matches to marine invertebrate-associated densoviruses, they were only 43-64 % similar to SSaDV across their sequence.

In an attempt to further understand the genome sequence of SSaDV present in samples globally and historically worldwide, PCR primers and probes were designed around 5 loci on the SSaDV genome including NS1, VP4, VP1 and intergenic amplicons between NS1-VP4 and VP4-VP1 (Supplemental Table 14). Amplicons were then sequenced. PCR was successful for samples of *P. helianthoides* in which SSaDV was detected by qPCR from the Seattle Aquarium in 2013. PCR amplicons were successfully generated from a total of 13 historical specimens (of 36 attempted), but never of all 5 loci in a single individual. Sequencing revealed that amplicons shared no homology or identity with SSaDV. Similarly, while PCR amplicons were obtained from 7 individuals worldwide (of 36 attempted), only a handful (one VP4 from *A. amurensis* sharing 87% homology, and both VP4 and NS1 from *A. forbesii* from the Gulf of Maine, sharing 84% homology) shared any homology with SSaDV (i.e. were spurious amplicons). Taken together, these data suggest that densoviruses may be a common constituent of the asteroid nanobiome, yet are only in some cases found in SSWD-affected populations. Our data also do not support the presence of SSaDV in asteroid samples from before the current disease event, contrasting with previous work (Hewson et al., 2014).

The lack of association between SSaDV and SSWD signs in many individuals from 2013-2014 (Hewson et al., 2014) may have been due to targeting of tissues not affected by SSWD by qPCR. In an attempt to understand the tissue specificity (tropism) of

SSaDV, we obtained 1 asymptomatic and 2 SSWD-affected individuals of *P. ochraceus* from Langley Harbor, Whidbey Island, in February 2016. Individuals were frozen at -20^{o}C whole immediately after collection and shipped to the lab at Cornell. The presence of SSaDV in tissue samples of body wall, gonad, pyloric caeca, radial nerve, coelomic epithelium, papillae, pedicellaria, madroporite, and pyloric stomach was determined by qPCR. These data indicated the presence of SSaDV in all tissue types tested. Subsequently, we performed viral metagenomics on individual tissues of the individual used for tissue-level detection (body wall, gonad, pyloric caeca, ventral papillae and tube feet). Assembly of 14,141,126 reads resulted in 61,605 contigs > 500 nt long. Direct comparison of this contig spectrum to SSaDV yielded only one 481 nt long contig (POBW_31527) which bore similarity to SSaDV (83% nucleotide ID across 91% of the query).

The lack of SSaDV in metaviromes and our inability to detect SSaDV by general PCR in samples that provided positive detections by qPCR targeting VP4 and NS1 suggested that amplification of other densoviruses or spurious amplicons bearing homology to TaqMan probe developed in 2014 (Hewson et al., 2014) may have occurred. We therefore performed an alignment on SSaDV and its closest relatives (VP4 amplicons from *A. forbesii* and *A. amurensis*) along with other related densovirus sequences from *Cherax quadricarinatus* (Bochow et al., 2015) and the VP4 region of contigs V41_94, V42_5549 and POBW_31527 from metagenomes prepared from asymptomatic *P. ochraceus*, *E. luzonicus* and *A. polyacanthus* (Supplemental Fig. 10). New PCR primers were designed around a 249 bp segment of the VP4 region which would likely exclude amplification of the latter three sequences and other densoviruses that are found at NCBI. We named this new primer/probe “Wasting Asteroid associated Densoviruses” WAaDs to reflect their detection of densoviruses in asteriid populations experiencing wasting, but excluding densoviruses that were present in clearly asymptomatic asteriids and other species.

ii. Bacterial investigations

Full-length 16S rRNA sequencing of asymptomatic *Evasterias troscheli* from the Salish Sea and *Asterias forbesii* and *Asterias rubrens* from the Gulf of Maine demonstrate the

presence of Alphaproteobacteria (36% of sequences) and Mollicutes (26%), with fewer Gammaproteobacteria, Betaproteobacteria and Deltaproteobacteria (Supplemental Table 15). The Spirochaetes, Mollicutes and most Proteobacteria that were observed in Nakagawa et al. (Nakagawa et al., 2017) and in the survey reported here are not primary pathogens of SSWD, since they represent either well known copiotrophs that potentially consume decaying asteroid organic matter (Ivars-Martinez et al., 2008), or those which are normally associated with asymptomatic animals. To directly investigate the diversity of bacteria capable of consuming asteroid-derived organic matter, we cultivated bacteria on media containing solely filtered (< 0.7 µm) homogenized asteroid tissues as a nutritional source. We also cultivated bacteria from the surfaces of SSWD-affected and asymptomatic *P. ochraceus* on the *Vibrio*-selective Tris-Citrate-Bile Salts (TCBS) media. Bacterial isolates cultivated on media containing filtered tissue homogenates were overwhelmingly (94%) Gammaproteobacteria (most closely related to *Pseudoalteromonas* spp.), while bacteria cultivated from the surface of SSWD-affected *P. ochraceus* on TCBS included *Vibrio* spp., *Aliivibrio* sp. (Supplemental Table 16). Our detection of *Vibrio* sp. stimulated interest in bacterial etiology, since the genus includes several known pathogens of aquatic metazoan including *Vibrio anguillarum* and *Vibrio coralilyticus*. We performed bacterial challenge by inoculating asymptomatic *P. ochraceus* with overnight cultures of *Vibrio* sp. (BW_16-1). Bacterial challenge resulted in increased weight compared to controls, however animals remained asymptomatic throughout the experiment (120h) (Supplemental Fig. 8, Supplemental Fig. 9). Hence, the *Vibrio* spp. cultivated from SSWD-affected asteroid tissues in this survey are also unlikely to represent pathogens. Our data serve to demonstrate that genera of bacteria which include pathogenic species may not represent pathogens. However, this non-exhaustive investigation of the asteroid cellular microbiome does not exclude the possibility that SSWD is related to bacteria and archaea in other ways. For example, the microbiome of invertebrates may influence their health and disruptions may lead to compromised individuals and co-infection by bacteria and other pathogens may lead to disease but as solitary agents do not induce disease (reviewed in (Lee and Hase, 2014)). The association between SSWD and the cellular microbiome represents a promising area of future research.

SUPPLEMENTAL METHODS

a. Sample and museum specimen collection

Samples of common asteroids were collected from 13 locations worldwide (Supplemental Table 1). The asteroid samples were collected by hand or SCUBA diver. A ray from each individual was dissected using a clean scalpel or razor blade, and preserved immediately in >95% ethanol (or by freezing at -80^{o}C for samples from the Gulf of Maine and the Yellow Sea). Samples were shipped to the laboratory at Cornell for metagenomic analysis and detection of SSaDV by quantitative PCR. Museum specimens were collected as part of a previous study (Hewson et al., 2014), and additional tube feet specimens obtained from collections at the Museum of Natural History (Los Angeles) and California Academy of Sciences (San Francisco) to expand the date range and provide a greater suite of specimens for historical analysis of SSaDV presence (Supplemental Table 2). Tube feet from EtOH preserved specimens were pulled by sterilized forceps and placed into sterile 2 mL cryovials before transport at room temperature to the lab at Cornell University.

b. Preparation of metaviromes from asteroid tissues

Viral metagenomes were prepared from samples following the approaches of Vega Thurber (Thurber et al., 2009) and Hewson (Hewson et al., 2014). Briefly, 1 g of preserved or frozen tissue was homogenized in 25 mL of 0.02um –filtered PBS in a NutriBullet blender. The homogenate was centrifuged briefly at 3,000 x *g* to remove large tissue debris and ossicles, and the supernatant filtered through 0.2 µm Durapore filters to remove cellular debris. The filtrate was precipitated with PEG-8000 (1 g/ml) overnight. The filtrate was then centrifuged at 22,000 x g for 30 min, supernatant decanted, and the pellet resuspended in 1 ml 0.02 um filtered PBS. The resuspension was filtered through a 0.2 µm Acrodisc PES filter. Samples were then treated with TurboDNAse (5 U; Thermo Fisher Scientific), RNAse One (50 U; Promega) and Benzonase (250 U; Sigma-Aldrich) for 3 h at 37^{o}C. Nuclease activity was stopped by the addition of 0.2 vol ETDA (25 mM). Viral DNA in the purified suspension was extracted

using the Zymo Viral DNA kit. The DNA was amplified prior to sequencing using the WGA2 kit V2 (GE Biosciences) before submission to the Cornell Biotechnology Resource Center (BRC) for library preparation and sequencing. 24 samples were run on a single MiSeq 2 x 150bp lane. Sequence data is available at the NCBI under BioSample accessions SAMN08012637 - SAMN08012651.

c. Bioinformatic analyses of asteroid metaviromes

MiSeq libraries were imported into the CLC Genomics Workbench 4.0 and assembled individually with parameters of 0.5 overlap and 0.95 similarity. Resulting contigs were subject to three analyses. First, libraries were annotated by BLASTx against the non-redundant database using an e-value cut-off of 0.001. Contigs bearing matches at $e < 0.001$ to the non-redundant database were then vetted for viral matches, and matches to viruses were compared by BLASTn against the non-redundant and by tBLASTx against the RefSeq databases to ensure that they represented valid viral hits. Finally, the entire contig spectrum was compared by tBLASTx against a boutique database comprising the genomes of all known densoviruses (as of January 2016), and hits matching at $e < 0.001$ were compared to hits from comparisons against the nr and RefSeq databases to identify bona fide densoviral hits. The densovirus hits were further compared by BLASTx against the nr database in October 2017 to identify best match.

d. Quantification of SSaDV by VP4/NS1

For both biogeographic and historical samples, DNA was extracted from 200 mg of tissue (comprising tube feet for historical samples, and a ca. 2mm cross-section of a ray for biogeographic samples) using the Zymo Tissue & Insect Kit. The resulting DNA was subject to quantitative PCR using primer+probe sets VP4_F/VP4_R+VP4_Pr and NS1_F/NS1_R+NS1_Pr (Hewson et al., 2014).The conditions for qPCR were identical to those reported previously. qPCR was run in duplicate for each sample against duplicate oligonucleotide standards spanning 10^{10} to 10^{1} copies per reaction.

e. PCR amplification of genome fragments of SSaDV

Samples that returned positive detection for both VP4 and NS1 were further investigated to determine genetic variation within the two gene encoding regions of VP4 and NS1. PCR primers were designed to encompass five loci within the SSaDV genome (Supplemental Table 14). PCR amplification was performed for each primer pair in 50 µl reactions containing 1X PCR Buffer, 0.13 mM MgCl, 0.1 mM dNTPs, 200 pmol of each of the forwards and reverse primers, 2 ng uL^{-1} BSA and 2U Taq DNA Polymerase. Thermal cycling was preceded by a heating step at 94ºC for 5 min, followed by 35 cycles of denature at 94ºC for 30s, anneal at 55ºC for 30s and extend at 72ºC for 60s. At the conclusion of thermal cycling, reactions were subject to a 7 min final extension at 72ºC. Ten microliters of each PCR reaction were electrophoresed on an agarose gel, stained and visualized on a transilluminator. PCR amplicons of the predicted size were excised using a clean razor blade. DNA in gel slices was extracted using the Zymo Gel Extraction kit, cloned into pGEM-T vectors and then Sanger sequenced at the Cornell BRC.

f. Tissue-level SSaDV prevalence

SSaDV presence and load in *Pisaster ochraceus* was determined by qPCR targeting VP4 and NS1. Two asymptomatic individuals were collected from Langley, Whidbey Island, Salish Sea on 9 February 2016 by SCUBA diver, frozen at -20ºC and transported frozen to the laboratory at Cornell University. Once there, the individual was thawed and dissected into tissue types including body wall, coelomic epithelium, dorsal and ventral papillae, nerve ring and water vascular system. Tissues were placed into sterile bead beater tubes (Zymo Research) and processed using the Zymo Tissue and Insect Kit. Extracted DNA was subject to qPCR using SSaDV primers VP4 and NS1 as described above.

Metagenomes were prepared from several tissues (body wall, gonad, pyloric caeca, tube feet, and ventral papillae) in which large SSaDV loads were detected by qPCR targeting VP4 and NS1 (Hewson et al., 2014). Metavirome processing and sequencing was performed as described above for geographic samples. Sequence data is available at the NCBI under BioSample accession SAMN08012652. All 5 libraries were assembled

together into contiguous sequences using a 0.5 overlap and 0.95 similarity. Contigs were directly compared to the SSaDV genome by BLASTx using an E-value cutoff of 0.001.

g. Development of degenerate SSDV primer/probe set

A degenerate primer/probe set targeting three wasting asteroid associated densoviruses (SSaDV, VP4 sequences from *Asterias forbesii* and *Asterias amurensis* (GenBank accession MG550035)). An alignment of these densovirus genome fragments, together with contigs V41_94, V42_5549 and POBW_31527 (assembled from *Echinaster luzonicus, Astropecten polyacanthus* and *Pisaster ochraceus* metagenomes, respectively), and the closely related *Cherax quadricarinatus* densovirus (Bochow et al., 2015) was performed by ClustalW (Thompson et al., 1994). Degenerate primers were then developed manually by identifying a 249 nt region sharing the most similarity amongst the target genomes and having the most difference to non-target genome fragments (Supplemental Fig. 10). This resulted in the "Wasting Asteroid associated Densoviruses" (WAaDs) primer probe set (WAaDs_F 5'- ATCCYACTGGTGGTACAACWT-3'; WAaDs_R 5'- ARTARGGAATYTTRTGAGCACAKC-3'; and probe WAaDs_Pr 5'- CTTGAACGWAAAATGAGAGGWGGA-3'). WAaDs_F has 5 – 12 mismatches to other asteroid-associated densoviruses; WAaDs_R has 16 mismatches to V41_94; and WAaDs_Pr has 13 mismatches to V41_94. Outer qPCR primers were tested against tissue extracts of *P. helianthoides*, the *A. amurensis* sample from Shandong, and samples of *A. forbesii* from the Gulf of Maine. Amplicons were successfully obtained from these samples and Sanger sequenced to confirm their identity. The new VP4 primer set was also tested against samples for which the previous NS1/VP4 primer set were negative to ensure they did not amplify negative samples. Furthermore, the amplification efficiency and slope of replicate standards from all 3 stars was tested and fell within 1 order of magnitude of each other with equal slope. The amplicon from *P. helianthoides* was cloned into a pGEM-T plasmid and used as standards for all further qPCR using the WAaDs primer/probe set.

h. Bacterial 16S rRNA survey

Samples of *Evasterias troscheli* were collected in North Pacific shallow, coastal waters

on 8 August 2014. The samples were collected from two locations near Coupeville, WA: A pier (48.2376^{o}N, 122.7090^{o}W; n=6) and a beach (48.2150^{o}N, 122.7147^{o}W; n=4). Pier samples were removed directly from pier foundation support beams at low tide. Beach samples were removed from water at low tide (< 1m). Samples of *Asterias rubens* (n=6) and *Asterias forbesii* (n=2) were collected together at Shoals Marine laboratory, Appledore Island, ME (42.9850^{o}N, 70.6135^{o}W) within a subtidal zone by SCUBA divers on 20 July 2014. Each sample was placed in a cooler on ice and transferred back to the lab for dissection.

Bacterial community DNA was extracted from each sample using the ZR Fungal/Bacterial DNA MiniPrep (ZYMO Research Inc) kit, following manufacturer's protocol. Near full-length 16S rRNAs were amplified using the polymerase chain reaction (PCR) in 50 µL reactions containing 1X PCR buffer, 2.5 mM $MgCl_2$, 300 µM dNTPs, 200 pmol each of primers Bact-16S-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') (*15*) and Bact-16S-1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') (*17*), 0.2 ng mL^{-1} BSA, 5 U *Taq* DNA polymerase (Life Technologies) and 5 µL extracted DNA. Thermal cycling comprised 30 cycles of denature (94^{o}C) for 30s, anneal (54^{o}C) for 30s, and extend (71^{o}C) for 45s; followed by a 7 min final extension (71^{o}C). PCR products were electrophoresed on a 1% agarose gel in TBE buffer at 90V for 1 h, followed by staining with SYBR Gold (1 µL mL^{-1}) and visualized on a UV transilluminator. Bands containing amplicons were gels using a clean razor blade and purified using the ZR Gel Recovery Kit (ZYMO Research).

Purified amplicons were pooled with samples of matching location and species of asteroid. The pooled amplicons were then ligated into the pGEM-T vector and transformed into competent *E. coli* (JM109). The transformants were subject to blue/white screening on IPTG/X-Gal/Ampicillin plates per the pGEM-T kit (Promega) protocols. From each sample, 20 randomly selected colonies were grown overnight in 5 mL LB broth with ampicillin and with shaking at 37 ^{o}C. Liquid cultures were pelleted by centrifugation at 3,000 x *g* for 5 min. Plasmid DNA was extracted from isolates using Zyppy Plasmid Preparation Kit (ZYMO Research), following manufacturer's protocol,

followed by Sanger sequencing in forward and reverse directions at the Cornell Biotechnology Resource Center. Sequence data is available at NCBI under accessions MG518570 - MG518611.

i. Cultivation of bacteria from asteroid surfaces

Cultivable bacteria from seawater at a site where SSWD was present (Coupeville, WA, taken January 2014) and directly from asteroid (*Pisaster ochraceus*) surfaces were isolated on media containing only filtered sea star homogenate (SSM) and on the Vibrio-specific TCBS media, respectively. SSM was prepared by homogenizing a ~1cm ray section of *E. troscheli* in 20 mL of 0.02 µm –filtered seawater (collected from Langley, WA). Tissues were disrupted using a NutriBullet blender (NutriBullet LLC) for 1 min and centrifuged at 3,000 x *g* for 5 min to remove large particles. The homogenates were then filtered over 0.2 µm Durapore filters (Millipore). SSM comprised 450 mL of GF/F filtered seawater and 50 mL of filtered asteroid homogenate. SSM solid media (1.5% agar) was prepared in sterile petri dishes. Seawater from the Puget Sound (Bellingham, WA) was streaked onto SSM plates and incubated at 8ºC for 7 d. Randomly selected colonies were sub-streaked on fresh SSM to ensure isolation. Isolates were then grown overnight in 5 mL liquid SSM (i.e. without agar) with shaking at 20ºC. 16S rRNA was sequenced for each isolate (see above). Seawater from the surface of a wasting *P. ochraceus* was streaked onto TCBS media (Difco) using a sterile plastic inoculating loop. Cultures of bacteria were grown at 11ºC in the dark for 2 d. Colonies of bacteria (n=11) were randomly selected for 16S rRNA sequencing following the approach outlined above. Sequence data from cultures is available at NCBI under accessions MG496260-MG496270 and MG521825-MG521849.

j. Bacterial challenge experiment

A bacterial challenge experiment was performed to assess whether a cultivated strain of bacteria could induce SSWD. Individuals were acclimated to duplicate 37 L glass aquaria which contained artificial seawater (Instant Ocean). *Pisaster ochraceus* were equilibrated at 11ºC for ~ 1 year prior to the start of the experiment. A dense overnight culture of *Vibrio* spp. strain B16_1 was obtained by growing 50 mL at 25ºC with shaking in

moebus sea water (MSW) (Moebus, 1980). Individuals were amended with either sterile culture media (controls) or with the bacterial culture by direct injection into coelomic cavity. The wet weight of individuals along with visual inspection for SSWD signs was performed daily for 120h.

REFERENCES

Bochow, S., Condon, K., Elliman, J., and Owens, L. (2015). First complete genome of an Ambidensovirus; Cherax quadricarinatus densovirus, from freshwater crayfish *Cherax quadricarinatus*. *Mar Genomics* 24 Pt 3**,** 305-312.

Hewson, I., Button, J.B., Gudenkauf, B.M., Miner, B., Newton, A.L., Gaydos, J.K., Wynne, J., Groves, C.J., Hendler, G., Murray, M., Fradkin, S., Breitbart, M., Fahsbender, E., Lafferty, K.D., Kilpatrick, A.M., Miner, C.M., Raimondi, P., Lahner, L., Friedman, C.S., Daniels, S., Haulena, M., Marliave, J., Burge, C.A., Eisenlord, M.E., and Harvell, C.D. (2014). Densovirus associated with sea-star wasting disease and mass mortality. *Proceedings of the National Academy of Sciences of the United States of America* 111**,** 17276-17283.

Ivars-Martinez, E., Martin-Cuadrado, A.-B., D'auria, G., Mira, A., Ferriera, S., Johnson, J., Friedman, R., and Rodriguez-Valera, F. (2008). Comparative genomics of two ecotypes of the marine planktonic copiotroph *Alteromonas macleodii* suggests alternative lifestyles associated with different kinds of particulate organic matter. *ISME Journal* 2**,** 1194-1212.

Lee, W.J., and Hase, K. (2014). Gut microbiota-generated metabolites in animal health and disease. *Nature Chemical Biology* 10**,** 416-424.

Moebus, K. (1980). A method for the detection of bacteriophages from ocean water. *Helgolander Meeresuntersuchungen* 34**,** 1-14.

Nakagawa, S., Saito, H., Tame, A., Hirai, M., Yamaguchi, H., Sunata, T., Aida, M., Muto, H., Sawayama, S., and Takaki, Y. (2017). Microbiota in the coelomic fluid of two common coastal starfish species and characterization of an abundant Helicobacter-related taxon. *Scientific Reports* 7**,** 8764.

Thompson, J., Higgins, D., and Gibson, T. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22**,** 4673-4680.

Thurber, R.V., Haynes, M., Breitbart, M., Wegley, L., and Rohwer, F. (2009). Laboratory procedures to generate viral metagenomes. *Nature Protocols* 4**,** 470-483.