Impacts of temperature on the molecular response of shellfish

Grace Alan Crandall

A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2020

Committee:

Steven Roberts

Pamela Jensen

Chelsea Wood

Program Authorized to Offer Degree:

School of Aquatic and Fishery Sciences

University of Washington

Abstract

Impacts of temperature on the molecular response of shellfish

Grace Alan Crandall

Chair of the Supervisory Committee:
Dr. Steven Roberts
School of Aquatic and Fishery Sciences

Climate change is causing the average global ocean temperature to increase. In the face of ocean warming, the majority of marine organisms will be impacted by this shift in thermal regimes. There will likely be some species, and some life stages of species, that will fare better than others. In this thesis, this concept is explored in two organisms. The first organism is the Pacific oyster (*Crassostrea gigas*), which is a valuable aquaculture product that also provides important ecosystem benefits. Larval Pacific oysters were grown in two temperature treatments, 23 ˚C and 29 ˚C, and proteomics was used to determine how temperature impacted the molecular response in the oysters. Processes related to growth and development were more abundant in oysters grown in 29˚C, while processes related to immune response were less abundant. The second organism is the Alaska Tanner crab (*Chionoecetes bairdi*), which is an important species in a commercial fishery. Specifically, I focused on southeast Alaska Tanner crab populations, which have a high prevalence of infection with an endoparasite called *Hematodinium* sp., and

will have to face ocean warming. Tanner crab infected with *Hematodinium* sp. and uninfected crab were exposed to different temperature treatments - ambient (7.5˚C), elevated (10˚C), and decreased (4˚C). Transcriptomics was used to determine how temperature and infection with *Hematodinium* sp. influenced the molecular response in Tanner crab. Primary processes that were influenced by temperature were lipid storage, transcription, and morphogenesis. The genes associated with lipid storage and transcription have roles in immune function and clear expression pattern differences between temperature treatments. There were no distinct expression patterns of the genes associated with morphogenesis, and while it is a process that is less expected, it could be attributed to hemocyte morphology or type change in response to temperature or infection with *Hematodinium* sp. This thesis provides insight into how increasing ocean temperature will affect two important shellfish species, and the data associated with each project can be used for further research into shellfish physiology.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor, Steven Roberts. He has been my advisor through my different roles in his lab since 2014, and has helped me become the scientist that I am today.

I would like to thank Pam Jensen, co-PI on the crab project, and Chelsea Wood for being on my thesis committee and for sharing so much of their expertise with me, both in terms of the science and being a grad student in general.

I would like to thank Sam White for all of the help in the lab and seemingly unending patience as he answered my countless questions related to lab work.

I would like to thank Emma Timmens-Schiffman for all of the instruction and insight she provided during the challenging proteomic data analysis phase of the oyster project.

I would also like to thank my fellow grad students in the Roberts Lab – Yaamini and Laura – and post-doc Shelly, and all of the graduate students in the School of Aquatic and Fishery Sciences, who have made my time in graduate school better in countless ways.

Finally, I'd like to acknowledge the National Pacific Research Board (NPRB) for the funding provided to support the crab project (NPRB Project 1705) and supporting my position as a research assistant.

DEDICATION

To Mom, Dad, Jen, Caitlin, Julian, Emily, Bess, John, Riley, and Tom

Chapter 1. INTRODUCTION

Oysters, such as the Pacific oyster (*Crassostrea gigas*), are a valuable aquaculture product that also provides ecosystem benefits. Oysters are sessile as adults and form reefs that provide habitat for fish, invertebrates, and marine flora, and as filter feeders, oysters can have a positive influence on water quality (Coen et al. 2007; reviewed in Newell 2004). Pacific oysters are broadcast spawners, releasing eggs and sperm into the water column where fertilization occurs. Larval settlement generally occurs two to three weeks after fertilization and involves the metamorphosis from free-swimming pelagic larvae to sessile juvenile oysters. This metamorphosis is an energy-intensive process during which larvae undergo complex behavioral and morphological changes.

As with many coastal marine organisms, changes in temperature can have an impact on *C. gigas* physiology. In Ireland, mass mortality events of hatchery-produced adult Pacific oysters out-planted at two sites were linked to high-temperature stress in the summer months (Malham et al., 2009). Further investigation in laboratory experiments showed decreased immune function at 21˚C compared to 12˚C by way of decreased phagocytosis in the hemolymph (Malham et al. 2009). Often, temperature changes are accompanied by changes in other environmental factors that can also have negative impacts. Ko et al. (2014) showed that low pH and low salinity combined with high temperature delays Pacific oyster larval growth rate before settlement and metamorphosis. These impacts of temperature can affect both cultured and wild oysters and are likely to be realized on a more frequent basis with climate change-induced ocean warming.

While natural and uncontrollable temperature increases can be harmful, some commercial hatcheries have used increased temperatures in controlled conditions to achieve better outcomes. For many bivalves, increased temperature is often used to initiate spawning in the hatchery by

mimicking spring conditions, when spawning naturally occurs (FAO 2004). Increased temperature is also used to improve metabolism and growth in young oysters in hatchery settings. A comparison of larval physiology and early juvenile development of the Pacific oyster at five different temperatures (17, 22, 25, 27, and 32°C) concluded that optimal growth rates and greatest settlement occurred at 27°C (Rico-Villa et al. 2009). Higher temperatures accelerate biological processes, including respiration and metabolism, perhaps allowing young bivalves to "cruise" through the stressful metamorphosis period. However, there is a likely limit to any realized benefit with respect to temperature and duration of exposure.

Insight into molecular physiology at the protein level could help to explain the differences in larval success between different temperatures since the presence of certain proteins will show which genes are being translated as a physiological response to the given environment. An unbiased global proteomics survey can identify all proteins present in organisms at the time of sampling. This information can be leveraged to decipher the biological processes at play in various temperature treatments. Although it is still considered a novel approach, proteomics has been used in several studies of Pacific oyster response to a range of relevant environmental drivers and life stages (Venkataraman et al. 2019; Huan et al. 2012; Dineshram et al. 2012).

In this study, we used shotgun proteomics to understand how temperature impacts the physiology of recently settled oysters in a hatchery setting. We employed data-independent acquisition (DIA), which is a method of tandem mass spectrometry that essentially provides full proteome coverage by eliminating the need for mass spectrometry (MS) scans in data acquisition (Gillet et al. 2012; Venable et al. 2004). The goal of this experiment was to compare the proteomic responses of the oysters in the two temperature regimes and in the process develop a robust proteome that could build upon current *Crassostrea gigas* proteomic resources. These

results contribute to our understanding of how temperature conditions impact oysters in hatchery settings, and suggest that oysters in the wild may be impacted by increasing temperature during the post-settlement stage.

1.2 METHODS

1.2.1 *Oyster rearing and phenotype*

Adult oysters were strip spawned and eggs and sperm were combined for fertilization. Oyster larvae were reared for 19 days before competent larvae were split between two silos, each a different temperature regime — conventional commercial conditions (23°C) and elevated temperature (29°C) — with 1.1 million *Crassostrea gigas* larvae reared in one fiberglass silo (45.72 diameter) per treatment. All seawater was pumped from Dabob Bay, WA, filtered through sequentially decreasing filter bags of 25 µm, 10 µm, and 5 µm, and treated with sodium carbonate (Na_2CO_3) to reach a pH set point of 8.4. All oysters received the same mixed high-density microalgae diet (flagellates: *Isochrysis* spp., *Pavlova* spp., *Nannochloropsis* spp., *Rhodomonas* spp., and *Tetraselmis* spp.). Effluent algal densities were targeted at 100K cells/ml. Microcultch graded from 180-315 µm was used as substrate for settlement and added to the oyster silos at 20 days post-fertilization (dpf). At 24 dpf (5 days after initiation of temperature treatment), oysters were screened to determine size and settlement rate, and then were returned to their respective silos. Oyster seed from each temperature regime was sampled for proteomic analysis on 24 and 27 dpf, after 5 and 8 days of temperature exposure, respectively. Oyster seed (approximately 500 µl) was flash frozen in liquid nitrogen prior to storage at -80˚C. The remaining seed was reared until 33 dpf, then screened to determine size and mortality (**Figure 1**).

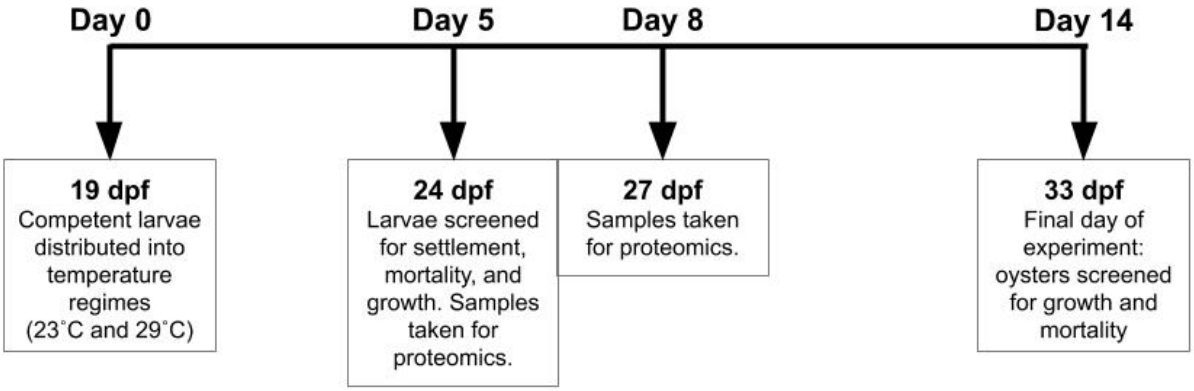

Figure 1. Experimental timeline. At 19 dpf, *Crassostrea gigas* larvae were exposed to either 23°C or 29°C for 14 days. Proteomic samples were taken at 24 and 27 dpf, or days 5 and 8 of temperature exposure respectively. Settlement, growth, and mortality were assessed at 24 dpf (5 days of temperature exposure) and 33 dpf (14 days temperature exposure).

1.2.2 *Proteomic sample preparation*

Prior to sample preparation, four larval samples (24 and 27 dpf, duplicates at each time point) were pooled for each treatment such that the resultant pool contained 250 µl of larvae. To each pooled sample, 50mM NH_4HCO_3 + 6M urea (500 µl) was added and larvae were homogenized using a pestle. Samples were centrifuged at 2000 x g for 5 minutes. Supernatant (150 µl) was pipetted from each sample and placed into new tubes. The supernatant was sonicated three times each for 5 seconds, cooling samples in between sonication rounds using an ethanol and dry ice bath for 5 seconds. Protein concentration was determined using a Pierce™ BCA Protein Assay Kit according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA USA).

Samples were digested and desalted for mass spectrometry as described in Timmins-Schiffman et al. (Timmins-Schiffman et al. 2017). The dried peptides were reconstituted in 100 µl 3% acetonitrile + 0.1% formic acid and stored at -80°C. Data Independent Acquisition (DIA) was performed to assess protein abundance patterns via liquid chromatography tandem mass

spectrometry (LC-MS/MS) with a Q-Exactive mass spectrometer (Thermo Fisher). Samples were analyzed in MS1 over 400–900 *m*/*z* range with 30k resolution in four separate injections with ranges of 400-525 *m/z*, 535-650 *m/z*, 650-775 *m/z,* and 775-900 *m/z*, and from 450 to 850 *m*/*z* in MS2 with 4*m*/*z* isolation windows 60 K resolution.

1.2.3 *Proteomic data analysis*

Raw mass spectrometry files were converted to .mzML format using MSConvert from the ProteoWizard Toolkit version 3.0 (Chambers et al. 2012). Resulting files and the *Crassostrea gigas* deduced proteome (Crandall 2020; data/Cg_Giga_cont_prtc_AA.fasta) were used to create a chromatogram library using EncyclopeDIA with Walnut version 0.6.14 (Searle et al. 2018). Specific protocol details are provided in supplementary material (Crandall 2020; protocols/01-EncyclopeDIA-protocol.md) The chromatogram library, *Crassostrea gigas* proteome, and .mzML files were imported into Skyline Daily version 4.1.9.18271 (MacLean et al. 2010), which provides a means of setting filters, viewing spectral data for quality inspection, and exporting the data for downstream analyses (Crandall 2020; protocols/02-SkylineDaily-protocol.md).

Spectral data and proteins detected were exported for use in MS Stats (version 3.12.3 (Choi et al. 2014)). Within MS Stats, the two sampling dates (5 and 8 days of temperature treatment) were combined and treated as replicates to compare protein abundances between temperatures (Crandall 2020; protocols/01-MSstats-Cgseed-DIA.R). Pooling the sampling dates provided a more robust analysis of the dominant trends in temperature response to compensate for the small number of samples. From the list of proteins, significantly differentially abundant proteins were identified from proteins detected by MS Stats using a threshold of >2.00 and <-2.00 log-2 fold change in RStudio (version 1.1.453 (R Core Team 2015)) (Crandall 2020; protocols/02-Cgseed-diff-exp.R). Specific protocol details are provided (Crandall 2020;

protocols/03-MSStats-protocol.md) DAVID, version 6.8 (Huang, Sherman, and Lempicki 2009a, [b] 2009), was used to identify the enriched Gene Ontology terms from the list of differentially abundant proteins in relation to all detected proteins. Additionally, enriched GO terms from the detected proteins were characterized in relation to the *Crassostrea gigas* proteome in order to capture the abundant biological processes present at this developmental stage, irrespective of temperature treatment, and enriched GO terms were identified using a < 0.05 FDR cutoff. The enriched GO terms from each protein list and their respective fold enrichment values were visualized separately using REViGO (Supek et al. 2011).

1.3 RESULTS

1.3.1 *Phenotype*

At 24 dpf (5 days into temperature treatment), oysters reared at 29˚C had a 22.6% settlement rate with a weighted average screen size of 560 µm. Approximately 25% of seed grown at 29˚C were 710 µm and larger. Oysters grown at 23˚C had a 9.2% settlement rate with a size of 363 µm at 24 dpf, with no seed exceeding 710 µm. At 29 dpf (10 days into temperature experiment), ciliates were visible in the 23˚C treatment. By 33 dpf, no oysters were alive at 23˚C, while survival of oysters grown at 29˚C was 86%.

1.3.2 *Proteomics*

There were 2,808 detected proteins (Crandall 2020; analyses/20190403-2015Cgseed-protcomp.csv**)** ~6.9% of the proteins described in the *Crassostrea gigas* proteome as determined by MS Stats. These detected proteins were enriched for 108 biological process GO terms compared to the full *Crassostrea gigas* proteome (Crandall 2020; analyses/revigo-detected-

proteome-108.csv). Enriched processes were primarily related to metabolism and growth (**Figure 2**).

Of the 2,808 detected proteins, 69 were differentially abundant between the 23˚C and 29℃ treatments. The differentially abundant proteins contributed to 18 enriched GO biological processes (**Figure 3).** Thirty-six proteins were more abundant in the 29˚C treatment, while 33 were more abundant in 23˚C treatment (Crandall 2020; analyses/proteins_comp_annot_threshold.csv). Further analysis of the differentially abundant proteins identified enriched biological processes in the samples when compared with the proteome. Proteins significantly more abundant in oysters grown in 29˚C were to do with biological processes primarily related to growth (**Table 1**), while those in oysters grown in 23˚C contributed to immune response (**Table 2**).

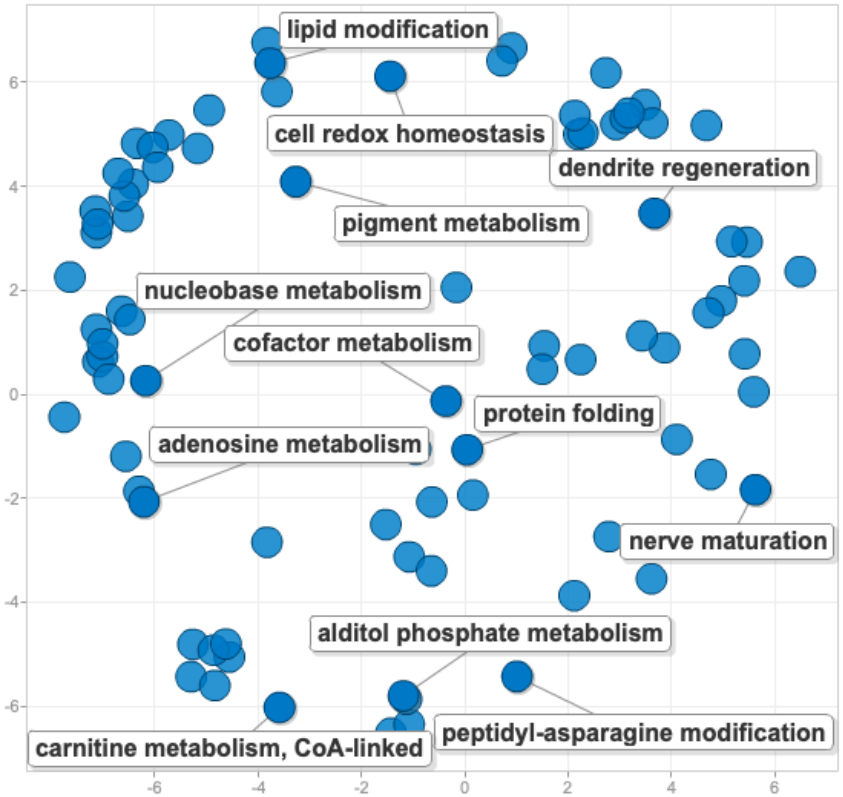

Figure 2. Relationship of the enriched gene ontology terms from the list of detected oyster proteins, showing the processes enriched, irrespective of temperature treatment. The color and size of the dots are the same for all processes enriched. The dots are grouped based on semantic

similarity, meaning that more similar terms - and processes - are grouped closer together. Processes enriched are related to basic functioning and growth.

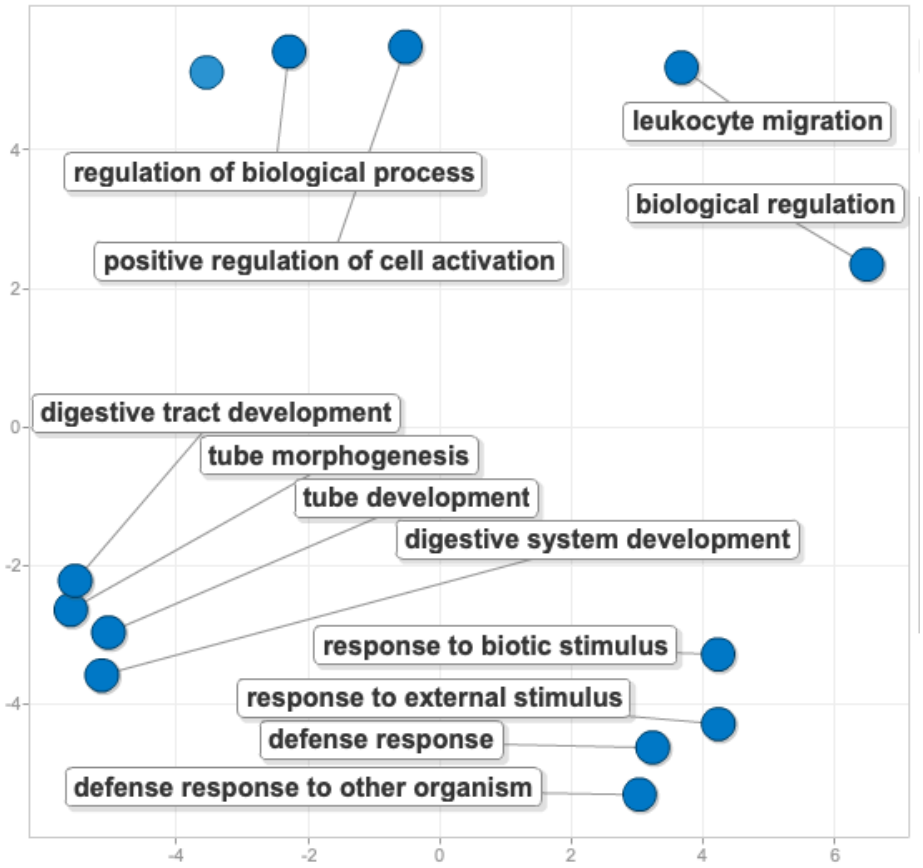

Figure 3. Relationship of the enriched gene ontology terms from the list of differentially abundant proteins. The color and size of the dots are the same for all processes enriched. The dots are grouped closer based on semantic similarity, meaning that more similar terms - and processes - are grouped closer together. Immune response functions are shown, as are metabolism and growth functions.

Table 1. Enriched gene ontology terms of proteins that were more abundant in the 29℃-exposed oyster seed than in the 23˚C seed.

Term	Fold Enrichment	Function
GO:0055123	14.37	digestive system development
GO:0035295	4.57	tube development
GO:0048568	7.66	embryonic organ development
GO:0007389	6.21	pattern specification process
GO:0045428	19.16	regulation of nitric oxide biosynthetic process
GO:0006809	19.16	nitric oxide biosynthetic process
GO:0009791	5.34	post-embryonic development

Table 2. Enriched gene ontology terms of proteins that were more abundant in the 23˚C-exposed oyster seed than in the 29˚C-exposed seed.

Term	Fold Enrichment	Function
GO:0044712	6.68	single-organism catabolic process
GO:0044419	5.60	interspecies interaction between organisms
GO:0044403	5.60	symbiosis, encompassing mutualism through parasitism
GO:1901136	20.50	carbohydrate derivative catabolic process

1.4 DISCUSSION

Using novel proteomics techniques, this study identified proteins that occur in different abundances when comparing larval Pacific oysters exposed to two different temperatures. Based on differential protein abundance, we found that the biological processes detected in oysters in the high-temperature treatment were related to metabolism and growth, while the biological processes detected in oysters exposed to the low-temperature treatment were involved in immune system response. Given the observance of ciliates at lower temperatures, the latter was expected.

Across the two temperature treatments, the whole oyster larval proteome primarily consisted of proteins related to metabolism, growth, and development. These findings are in agreement with the life history stage sampled, where growth rates are elevated and significant physiological changes are occurring related to somatic organization. In another study, researchers used *in silico* approaches to identify genes associated with larval settlement of *Crassostrea gigas* (Foulon et al. 2019). Approximately 27% of genes described by Foulon et al (2019) had protein complements expressed in the current study. This not only validates the relevance of the developmental role of these proteins but also provides valuable resources for future work focused on metamorphosis and larval adhesion. Additionally, this comparison

highlights the robust nature of the proteome developed as part of this study, along with the value of the DIA proteomic approach.

The growth and development of oyster larvae were impacted by exposure to 29°C. The higher temperature likely promoted elevated metabolic rates, which in turn supported elevated growth and development. Higher temperatures between 28-30˚C have been shown to promote higher rates of metabolism and growth in another oyster species, *Crassostrea corteziensis,* in the juvenile spat life stage (Cáceres-Puig et al. 2007). Another possibility for the observed proteomic trend is that in the absence of other stressors, such as the ciliates observed at the lower temperature, there was an increased relative allocation of energy towards growth and development. This is further supported by the phenotype data, where the oysters in the 29℃ treatment had higher settlement rates, greater size, no ciliates, and 86% survival, as compared to the 23℃ treatment group which had lower settlement, smaller size, and 100% mortality by the end of the experiment.

The oyster larvae samples from the 23℃ treatment had higher abundances of proteins associated with immune response when compared to the larvae at elevated temperature. At 29 dpf, ciliates were observed in the silo at 23℃ and by 33 dpf, all the oysters in the 23℃ treatment were dead. The predominant proteomic response was an immune response to parasites, supporting the idea that the oysters were initiating immune responses. Ciliate presence could have negatively impacted survival, either directly through parasitism or indirectly through increased energy allocation towards immune responses and away from critical maintenance processes. Ciliates have been a problem in hatcheries for decades, and are associated with significant mortality events in early development bivalves (Elston et al. 1999). Ciliates may prefer colder temperatures or may not be able to survive at higher temperatures, protecting larval

oysters at 29ºC against potential infections and the associated cost of launching an immune response. Alternatively, larvae may be physiologically compromised at lower temperatures, making them more susceptible to ciliates. Future research is certainly needed to attempt to disentangle these phenomena and continue to elucidate factors contributing to improved survival in oysters.

Our findings can help hatchery workers, managers, and conservationists predict how temperature is and will impact oysters at this developmental stage in hatchery settings. The findings support an improved practice of increasing the temperature during the early developmental stage after settlement to improve growth and survival. However further studies should investigate the optimal length of time and during which phase of development the larvae should be reared at elevated temperature. In addition, the annotated proteome developed as part of this work will be a valuable tool for future studies on bivalve development including providing specific targets for protein regulation studies in oysters as well as a reference for gene discovery in less studied bivalves.

Chapter 2. INTRODUCTION

Southern Tanner crab (*Chionoecetes bairdi*), hereafter referred to as Tanner crabs, are an important species for commercial, recreational, and subsistence fishing. They occur in relatively shallow waters along the continental shelf, from the Bering Sea in Alaska through coastal Oregon (Jadamec et al. 1999), and are found at temperatures ranging from -2˚C to 9˚C (Nielsen et al. 2007). In southeast Alaska, Tanner crab stocks supported a $21 million commercial fishery in 2014 (NMFS 2015). Both males and females undergo a 'terminal molt', which means that once they reach a certain size and age, they have one last molt, and are then in their final adult shell for the remainder of their life (Jadamec et al. 1999). This life-history strategy differs from that in many other Crustacea, which continually molt throughout life.

There are many threats to Tanner crab during all phases of their life, among them ocean warming and disease. One disease, bitter crab disease (BCD), has been termed the "principal threat" to Alaska Tanner crab stocks, as identified by the Alaska Department of Fish and Game (ADF&G) (ADF&G 2020). BCD is caused by a parasitic dinoflagellate of the genus *Hematodinium*, and Tanner crab in southeast Alaska experience high infection rates, approaching 100% in some areas (Meyers et al., 1987, 1990; Eaton et al. 1991; Love et al. 1993; Bednarski et al. 2011). *Hematodinium* spp. infects 40+ decapod crustacean species worldwide (Morado 2011), and its spread around the world has followed warming trends in the Atlantic and Pacific Oceans (Morado et al. 2011). This has implications for *Hematodinium* spp. to continue to spread to naive Tanner crab and other crustacean populations as the global average ocean temperatures continue to increase as a result of climate change. There are two described *Hematodinium* species (Chatton and Poisson 1931; Hudson and Shields 1994), and an additional recognized but undescribed species that infects Tanner crab (Jensen et al. 2010).

The mode of transmission is unknown, as well as whether *Hematodinium* always causes direct mortality, or if it can be a chronic condition (Gornik et al. 2010) and the extent to which death comes via secondary factors such as the increased risk of predation to lethargic crab (Butler et al. 2014). The disease alone is concerning, but with the mounting threat of increasing ocean temperatures, it is important to understand how these two threats will interact to affect Alaska's Tanner crab populations. In the Atlantic Ocean in the closely related species *Ch opilio*, infection with *Hematodinium* has been correlated with increased temperatures (Shields et al. 2007). In other species and marine ecosystems, it has been documented that increasing temperature decreases the hosts' ability to mount an immune defense against pathogens (Bruno et al. 2007), increases the prevalence of disease (Groner et al. 2018), and can increase a pathogen's ability to grow and reproduce, thus enhancing infectivity potential (Harvell et al. 2002).

If a pathogen can get through a crustacean's exoskeleton, the second line of defense is their innate immune system. Crustacean innate immunity centers around the activity of their hemocytes, of which there are three types: hyaline cells (phagocytosis), granular cells (melanization, antimicrobial peptides, cytotoxicity), and semi-granular cells (encapsulation, early non-self recognition, coagulation; Lin and Söderhäll 2011). Granular cells and semi-granular cells are critical in initiating and sustaining the cascade of responses that are involved in the major innate immune responses of the prophenoloxidase (proPO) system and melanization (Cerenius and Soderhall 2004). The immune response begins when host protein recognition proteins (PRPs) recognize non-self pathogen-associated molecular patterns (PAMPs) (e.g. bacterial peptidoglycans), which initiates an immune response that leads to the production of melanin, which binds to nucleophiles on pathogen surfaces and encapsulates pathogens

(Cerenius et al. 2008; Nappi and Ottaviani 2000). There are other innate immune response pathways, with some of the important processes described in Verbruggen et al. (2015).

Although *Hematodinium* spp. transmission remains a black box, research has uncovered some potential pathways. It is possible that the pathogen can enter the host during molting, as that is a physiologically stressful time for the crab, leaving them vulnerable to parasite infection through microtears in their soft cuticle (reviewed in Rowley et al. 2015). It is also possible that infection and transmission occur through the water column by way of moribund infected hosts shedding infective-stage dinospores into the water, and subsequently, the dinospores infecting other crustaceans (Meyers et al. 1990). It is also not well understood whether there is a host immune response to *Hematodinium* spp., as there is no evidence of parasite encapsulation, and it is thought that *Hematodinium* spp. may be able to conceal itself from the host immune system through molecular mimicry or evasion (reviewed in Rowley et al. 2015). The complex life cycle of *Hematodinium* spp. is not well understood and may vary between host or parasite species, but amoeboid trophonts, arachnoid trophonts, and sporonts have been observed in hosts, along with 2 forms of bi-flagellated dinospores observed in hosts, with the dinospores released into the sediment and/or water column (Frischer et al. 2006; Butler et al. 2014).

In crustacean hosts with BCD, there are often some outward signs that aid with diagnosis, as well as technical methods of detection. BCD causes milky, opaque hemolymph and pinkish discoloration and cooked-like appearance to carapace, and lethargic behavior (Eaton et al. 1991) in many decapods. A healthy crab has clear hemolymph, but in infected crab, *Hematodinium* proliferates throughout the hemal spaces, causing the hemolymph to appear milky. Eventually, all organ systems become impacted and ultimately, the parasite outcompetes the host for nutrients and oxygen, leading to host lethargy and subsequent suffocation. While BCD is not

harmful to humans, it renders their meat chalky and bitter, which makes them unmarketable. As such, the Tanner crab fishery has suffered direct economic loss (Meyers et al. 1987, 1990). Other infections can present similar gross signs, such as the milky hemolymph syndrome (MHS) described in *C. opilio*, which causes opaque hemolymph in response to infection with a bacilliform virus (Kon et al. 2011), so additional detection methods should be used for confirmation of *Hematodinium* spp. presence. Common methods are conventional PCR (Jensen et al. 2010) or quantitative PCR (Crosson 2011), to detect *Hematodinium* spp. DNA, and examining hemolymph smears or other host tissues to directly detect *Hematodinium* (Shields 2017).

We aimed to investigate how temperature and infection with *Hematodinium* impact Tanner crab physiology. We generated a *Ch. bairdi* transcriptome and used it for differential gene expression analysis. Our results demonstrate how environmental conditions are impacting crab physiology and provide insight as to how *Hematodinium* sp.-infected southeast Alaska Tanner crab may fare as ocean temperatures continue to rise.

2.2 METHODS

2.2.1 *Tanner crab collection and disease status determination*

In late October 2017, 400 male Tanner crab were collected using crab pots by the ADF&G in Stephen's Passage, near Juneau, Southeast Alaska. Stephen's Passage was selected because its long term *Hematodinium* sp. rate of infection in Tanner crab is ~50% (ADF&G unpub. data). The crab were transported to Ted Stevens Marine Research Institute (TSMRI, NOAA facility, Juneau, AK) and placed in flow-through seawater tanks at 7.5°C, the benthic water temperature in Stephen's Passage when captured.

Using a sterile needle and syringe, hemolymph was withdrawn for smears and visualization of *Hematodinium* sp. infection. From each crab, 200 ul of hemolymph was withdrawn and preserved in 800 µl 95% ethanol for conventional PCR (cPCR) detection of *Hematodinium* sp. infection. For extraction of total genomic DNA, 200 µl of ethanol-preserved hemolymph was centrifuged to pellet the solids, the supernatant discarded, and the pelleted material air-dried and processed as tissue. DNA was extracted following Ivanova et al. (2006) using invertebrate lysis buffer and modified by performing 2 washes with Wash Buffer, and adjusting eluted DNA (50 µl) to 10 mM Tris-Cl, pH 8.0, and 0.1 mM EDTA. Genomic DNA was subjected to 2 rounds of PCR (**Table 3**) with 2 different primer pairs designed for the small subunit (SSU) rRNA gene of *Hematodinium* spp.: Univ-F-15 and Hemat-R-1654 (Gruebl et al. 2002) and Hemat 18Sf and Hemat 18Sr (Bower et al. 2004). Reaction aliquots were pooled post-PCR and visualized on ethidium bromide-stained 2% agarose gels. Samples were scored as positive when both *Hematodinium* sp. bands of the expected size were visible on the gel, and negative when neither fragment amplified. DNA extraction and cPCR were conducted at TSMRI.

For a more sensitive assay of *Hematodinium* sp. presence, extracted DNA was subjected to qPCR following (Crosson 2011) using Hemat-F-214 and Hemat-R-370, and Hemat-P, a *Hematodinium*-specific hydrolysis probe, labeled with a 5' FAM fluorophore and a 3' Black Hole Quencher®-1 (LGC Biosearch Technologies, Inc., Novato, California). Thermal profile and reagents were as in Crosson 2011, except GoTaq Probe qPCR Master Mix (Promega, Madison, WI) was used; amplifications were performed in CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Richmond, CA). A *Hematodinium* standard curve of 1 copy to 3 million genes copies was provided by Lisa Crosson. All samples, standards and negative

controls were tested in duplicate. A preliminary subset of samples was amplified with an exogenous internal amplification control, to verify a lack of reaction inhibition (Nolan et al. 2006, in Crosson 2011). Samples were deemed positive when the mean copy number of the two replicates was equal to or exceeded the assay's lower limit of detection, as determined by comparison to standard curves. Total genomic DNA from 100 µl aliquots of the RNA-preserved hemolymph from Day 17 was extracted and subjected to qPCR.

Table 3. *Hematodinium* spp. rDNA primer pairs, sequence, amplicon size, PCR profile[1] and primer sources. Standard PCR temperature profile was: denaturation at 94°C for 1 min, number of cycles as designated at 94°C for 15 s, annealing temperature as designated for 15 s, extension as designated, followed by a final hold at extension temperature for 5 min.

Primer pair	Sequence (5' – 3')	Expected amplicon size (bp)	No. ampl. cycles	Annealing temp. (˚C)	Extension time (s) & temp. (°C)	Primer source
Univ-F-15	CTC CCA GTA GTC ATA TGC	1682	34	59°	120 s, 68°	Gruebl et al. 2002
Hemat-R-1654	GGC TGC CGT CCG AAT TAT TCA C					
Hemat 18Sf	GAA CCG AAC CAA GCT CTG CTT GGC C	450	32	72°	45 s, 72°	Bower et al. 2004
Hemat 18Sr						

CCA AAG GGT GCA CCG ATC GCT TCA A

[1] PCR reactions contained: 3 µl genomic DNA, 200 µM dNTP (Bioline USA Inc., Taunton, MA), 0.05 U/µl One *Taq*® DNA polymerase (New England BioLabs Inc., Ipswich, MA, USA), 1.0 µM each primer, 1× buffer, and molecular grade water to 25 µl.

2.2.2 *Experimental design*

The crab were allowed to acclimatize at 7.5˚C for 9 days. At the end of the acclimatization period, of the crab that appeared to have recovered from capture stress, 180 were selected for temperature treatments, such that half were infected and half were uninfected as determined by cPCR. Twenty crab (10 infected and 10 uninfected) were placed in each of nine replicate tanks at 7.5˚C. Over the course of day 0 through day 2, the temperature in 6 tanks was gradually adjusted to the final experiment temperatures of 10˚C (elevated) in 3 tanks and 4˚C (decreased) in 3 tanks; the remaining 3 tanks were kept at 7.5˚C. Prior to the initiation of the temperature adjustments (day 0), 0.2 ml hemolymph was sampled and preserved in 1200 ul RNAlater ® (Qiagen) for transcriptomic analyses. Hemolymph was sampled again after 2 days and on day 17 of the temperature trial; the experiment ended on day 17.

2.2.3 *RNA extraction and sequencing*

Hemolymph samples were centrifuged for 10 minutes at 14000 g with RNA extracted using Quick DNA/RNA Microprep Plus Kit (Zymo Research) according to the manufacturer's

protocol. Samples were run (2 ul) on Qubit 3.0 using the Qubit RNA HS Kit (Invitrogen) to determine RNA quantification. Samples (Crandall 2020; sample-list_pool-RNAseq.xlsx) were pooled (**Table 4**) and submitted to Northwest Genomics Center at Foege Hall at the University of Washington, where RNA-seq libraries were constructed and sequenced.

Table 4. RNA-seq libraries sequenced. For some of the pooled samples, temperature treatments were combined or infection statuses were combined.

Library ID	**Sample day**	**Infection status**	**Temperature treatment**
1	17	*combined*	*combined*
2	0	infected	ambient
3	0	uninfected	ambient
4	2	infected	*combined*
5	2	uninfected	*combined*
6	17	infected	*combined*
7	17	uninfected	*combined*
8	2	infected	decreased
9	2	uninfected	decreased
10	2	infected	elevated
11	2	uninfected	elevated

2.2.4 *Transcriptome assembly and annotation*

Raw sequence data were assessed using FastQC (v0.11.8; Andrews 2010) and MultiQC (v1.6; Ewels et al. 2016) pre- and post-trimming. Data were quality trimmed using fastp (v.0.20.0; (Chen et al. 2018)) with the "--detect_adapter_for_pe" setting. A transcriptome was *de novo* assembled from libraries 1-11 (**Table 4**) using Trinity (v2.9.0; Grabherr et al. 2011; Haas et al. 2013). Trimmed sequencing reads were functionally annotated with a combination of

DIAMOND BLASTx (0.9.26; Huson et al. 2016) and MEGAN6 (6.18.3; Huson et al. 2016). Annotation with DIAMOND BLASTx was run against NCBI nr database (downloaded 20190925). The resulting DAA files were converted to RMA6 files for importing into MEGAN6 with the daa2rma utility, using the following MEGAN6 mapping files: prot_acc2tax-Jul2019X1.abin, acc2interpro-Jul2019X.abin, acc2eggnog-Jul2019X.abin. This work was facilitated through the use of advanced computational, storage, and networking infrastructure provided by the Hyak supercomputer system at the University of Washington.

To perform taxonomic classification, RMA6 files were imported into MEGAN6 using the default Naive LCA settings and the following mapping files: prot_acc2tax-Jul2019X1.abin, acc2interpro-Jul2019X.abin, acc2eggnog-Jul2019X.abin, acc2seed-May2015xx.abin. All mapping files were downloaded from the MEGAN6 website (https://software-ab.informatik.uni-tuebingen.de/download/megan6/old.html). Transcriptome completeness was assessed using BUSCO (v3; Simão et al. 2015; Waterhouse et al. 2018) with the metazoa_odb9 in transcriptome mode and species set as fly. The transcriptome was annotated using DIAMOND BLASTx (v0.9.29; Buchfink, Xie, and Huson 2015) against the UniProt/SwissProt database (downloaded 20200123). Corresponding Gene Ontology information was obtained based on the UniProt GO databases.

2.2.5 *Differential gene expression analysis*

Gene expression differences were assessed by the comparison of RNA-seq data between infected and uninfected crab sampled during the trial. Specifically, this included libraries 8, 9, 10, and 11. Kallisto was used to obtain count data for each library and an abundance matrix was then produced using a perl script (abundance_estimates_to_matrix.pl) provided as part of Trinity (v2.8.6). Differential expression of contigs was calculated using a negative binomial GLM in the

R package DESeq2. The read counts were first normalized using the size factors method and fit to a negative binomial distribution. Significantly differential contig expression (Benjamini-Hochberg adjusted $p<0.05$) between infected and uninfected crab was determined using the Wald test for significance of GLM terms.

Further analyses were performed to address how temperature affects the contigs differentially expressed between infected and uninfected crab. Differential expression of contigs was calculated between libraries 8 and 10 combined and libraries 9 and 11 combined including temperature treatment (decreased or elevated) in a multifactor design formula. From the results of that differential expression of contigs, a contrast was performed between the two temperature treatments to extract the differentially expressed contigs related to infection with *Hematodinium* that are influenced by temperature.

2.2.6 *Enrichment analysis*

Gene enrichment analysis was performed using DAVID v. 6.8 (Huang et al. 2009a; Huang et al. 2009b). The Uniprot Accession IDs from the crab transcriptome were set as the background, and the gene lists were the Uniprot Accession IDs from the differentially expressed contig list related to infection status that were influenced by temperature. The enriched GO terms and their respective fold enrichment values were visualized separately using REViGO (Supek et al. 2011).

2.3 RESULTS

2.3.1 *Survival*

On day 4 of the temperature trial, a mortality event began in the elevated temperature tanks (**Figure 4**). By day 10, 95% of the crab at the elevated temperature had perished. Over the

course of the experiment, one crab died in the decreased temperature treatment and three mortalities occurred in the ambient temperature treatment.

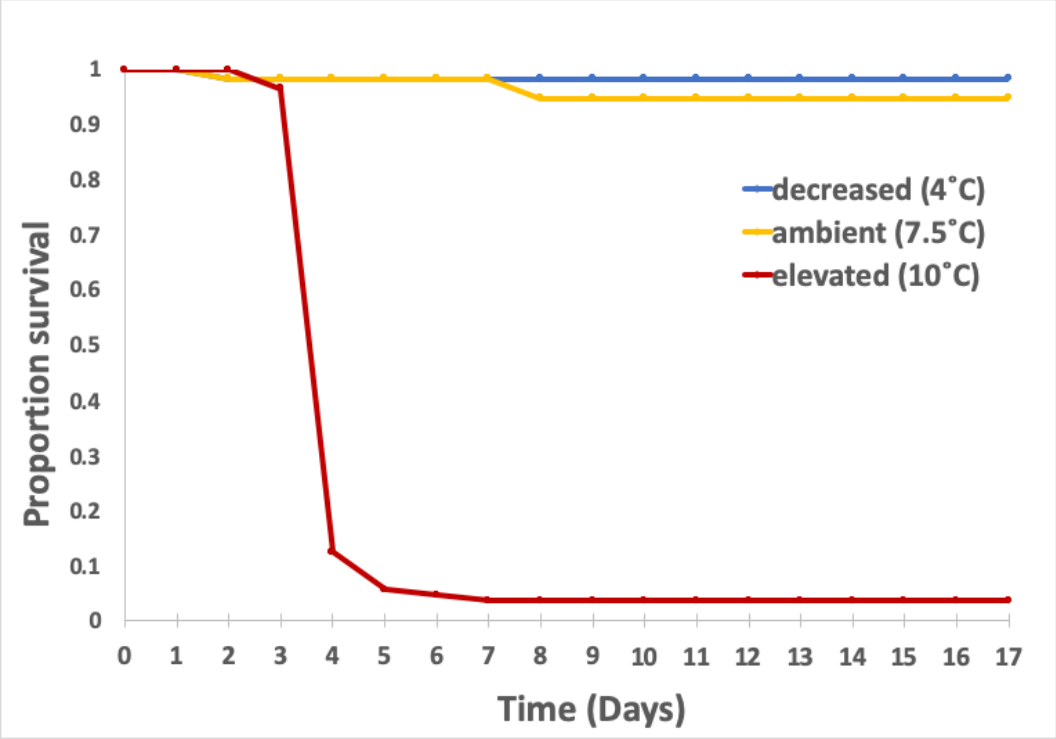

Figure 4. Survival associated with each treatment over time. The first hemolymph sampling for RNAseq occurred on Day 0. The second occurred on Day 2. The majority of crab in the elevated temperature tanks died between Day 3 and Day 9. The final hemolymph sampling day occurred on Day 17, with only 3 crabs remaining in the elevated temperature treatment.

2.3.2 *Transcriptome assembly and annotation*

Assembly of 143,543,003 base pairs (bp) resulted in 78,649 contigs. The median contig length was 1522bp, with an average contig length of 1825.11bp and an N50 of 2580bp. (Crandall 2020; cbai_transcriptome_v3.1.zip). Of the 78,649 contigs, 50,038 were able to be annotated using the Swiss-Prot databases, with 25,188 having corresponding Gene Ontology information (Crandall 2020; transcriptome3.1-blast-GO.txt). Gene ontology slim (GOslim) terms were extracted (**Figure 5**). Genes involved in stress response (11046) were identified and annotated

(Crandall 2020; stress-response-genes-tr3.1.txt). Comparison between this study's transcriptome assembly statistics and other crustacean transcriptome assembly statistics is listed in **Table 5**.

Table 5. Comparison of *de novo* transcriptome assembly statistics between the Tanner crab (this study), white leg shrimp (*Litopenaeus vannamei*) transcriptome (Ghaffari et al. 2014), and European shore crab (*Carcinus maenas*) transcriptome (Verbruggen et al. 2015).

	C. bairdi (this study)	*L. vannamei* (Ghaffari et al. 2014)	*C. maenas* (Verbruggen et al. 2015)
Contigs	78,649	87,307	212,427
Median contig length	1522 bp	429 bp	380 bp
Average contig length	1825.11 bp	1137.44 bp	992 bp
N50	2,580 bp	2,701 bp	2,102 bp

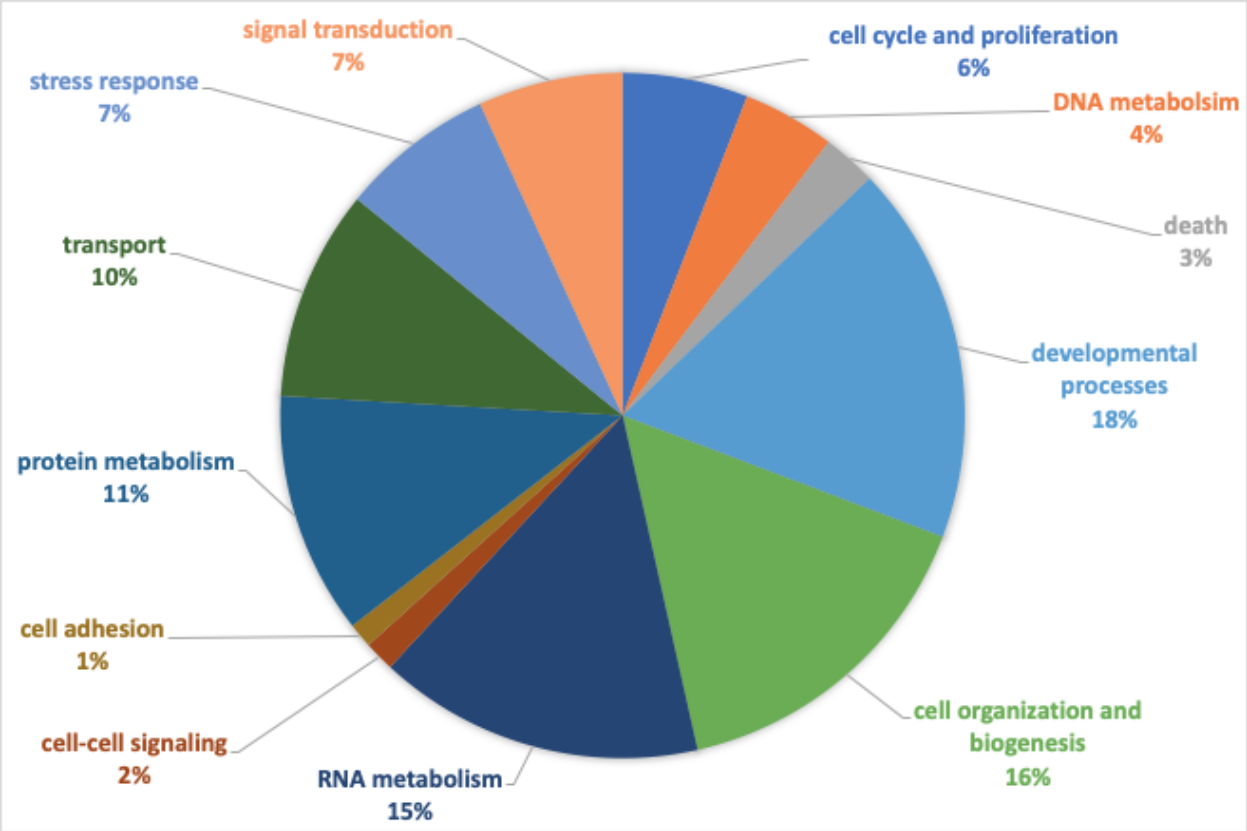

Figure 5. The proportion of GOslim terms for the biological processes identified in the crab transcriptome. Non-descriptive GOslim terms (e.g., "other biological processes") were omitted.

The percent composition of the pie represents the relative number of contigs from the crab transcriptome that contribute to that GOslim term.

2.3.3 *Differential gene expression and enrichment*

A total of 408 differentially expressed contigs were identified, 341 of which were annotated when comparing infection status. There were 357 expressed at an elevated level in the infected crabs and 51 expressed at a decreased level in infected individuals (**Figure 6**). (Crandall 2020; DEGlist-infection_temp-annot.txt). A total of 123 of the 408 differentially expressed contigs related to infection with *Hematodinium* were influenced by temperature treatment, 103 of which were annotated. There were 70 contigs expressed at an elevated level in the elevated temperature, and 53 expressed at a decreased level in the elevated temperature treatment (**Figure 7, Figure 8**). (Crandall 2020; DEGlist-contrasttemp-annot.txt).

In further analysis of the infection-related differentially expressed genes influenced by temperature, there were 15 enriched biological processes when compared to the transcriptome, (Crandall 2020; contast_temp-DAVID.txt). The primary biological processes were related to lipid storage, transcription, and morphogenesis.

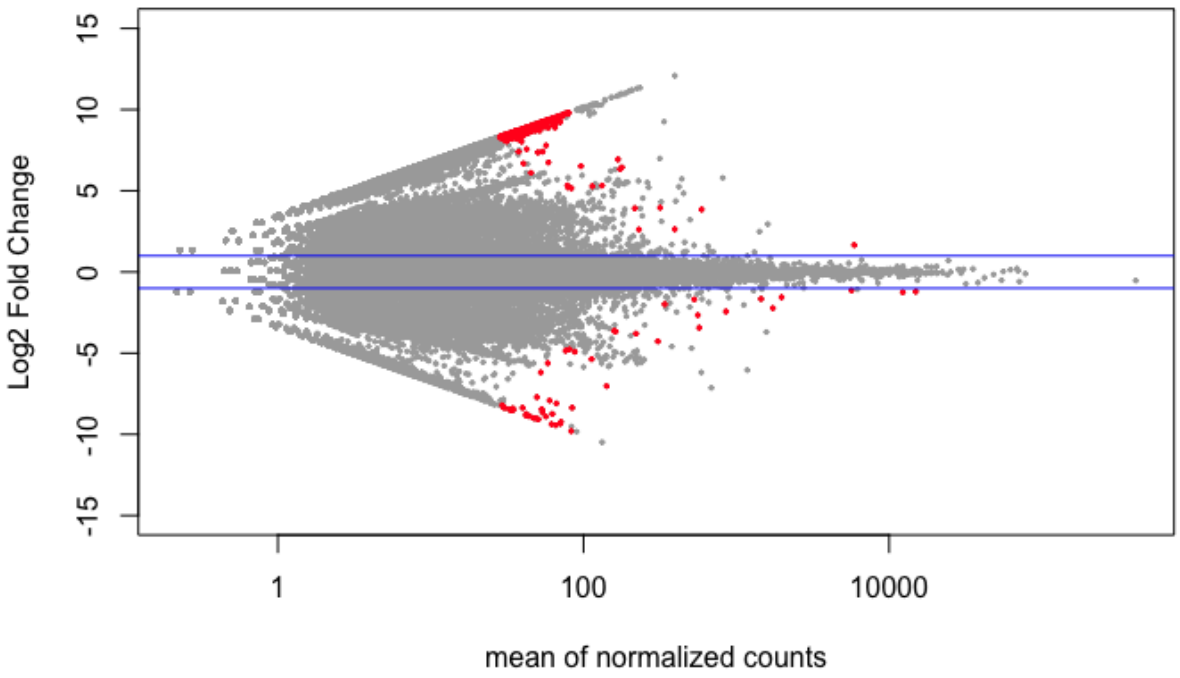

Figure 6. Gene expression levels in crab hemolymph in *Hematodinium*-infected and uninfected crabs, taking temperature treatment into account. Each dot represents a single contig with red indicating those contigs determined to be differentially expressed (>2 fold change and adjusted $p<0.05$) based on infection status. 357 contigs expressed at an elevated level in the infected individuals (>2 Log2 Fold Change) and 51 contigs expressed at a decreased level in infected individuals (<-2 Log2 Fold Change).

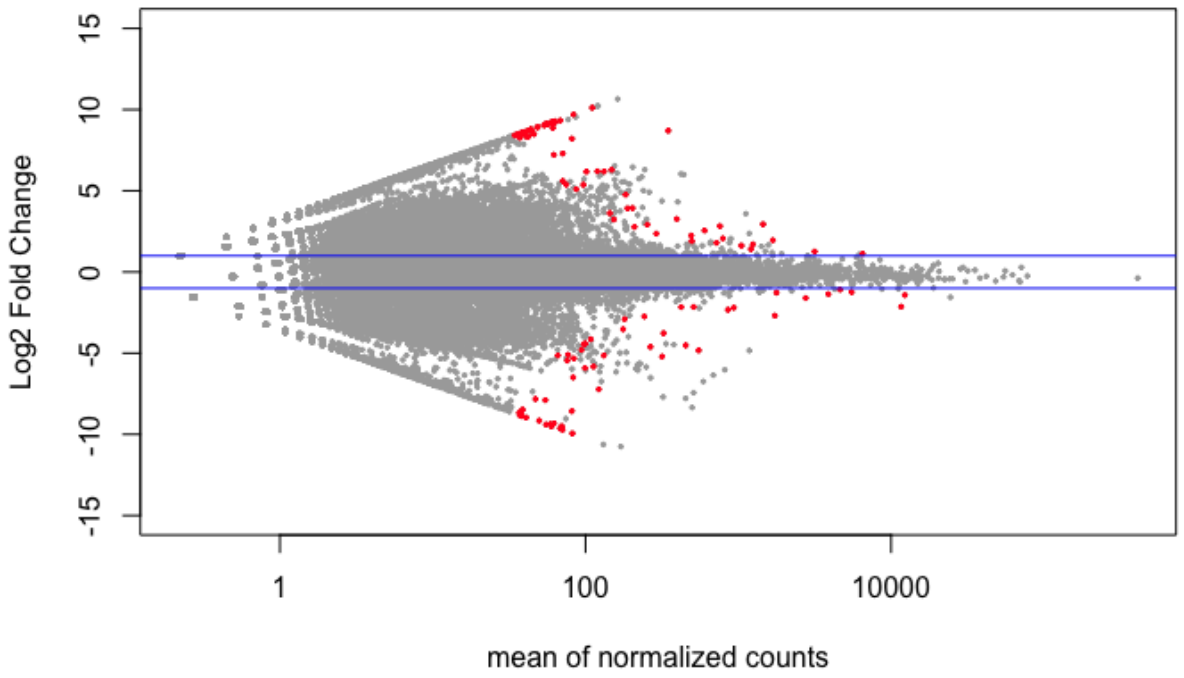

Figure 7. Gene expression levels in crab hemolymph in infected and uninfected crabs – genes related to infection status that are driven by temperature treatment. Each dot represents a single contig with red indicating those contigs determined to be associated with infection and significantly influenced by temperature (>2 fold change and adjusted $p<0.05$). 70 contigs expressed at an elevated level in the infected individuals in elevated temperature (>2 Log2 Fold Change) and 53 contigs expressed at a decreased level in infected individuals in elevated temperature treatment (<-2 Log2 Fold Change).

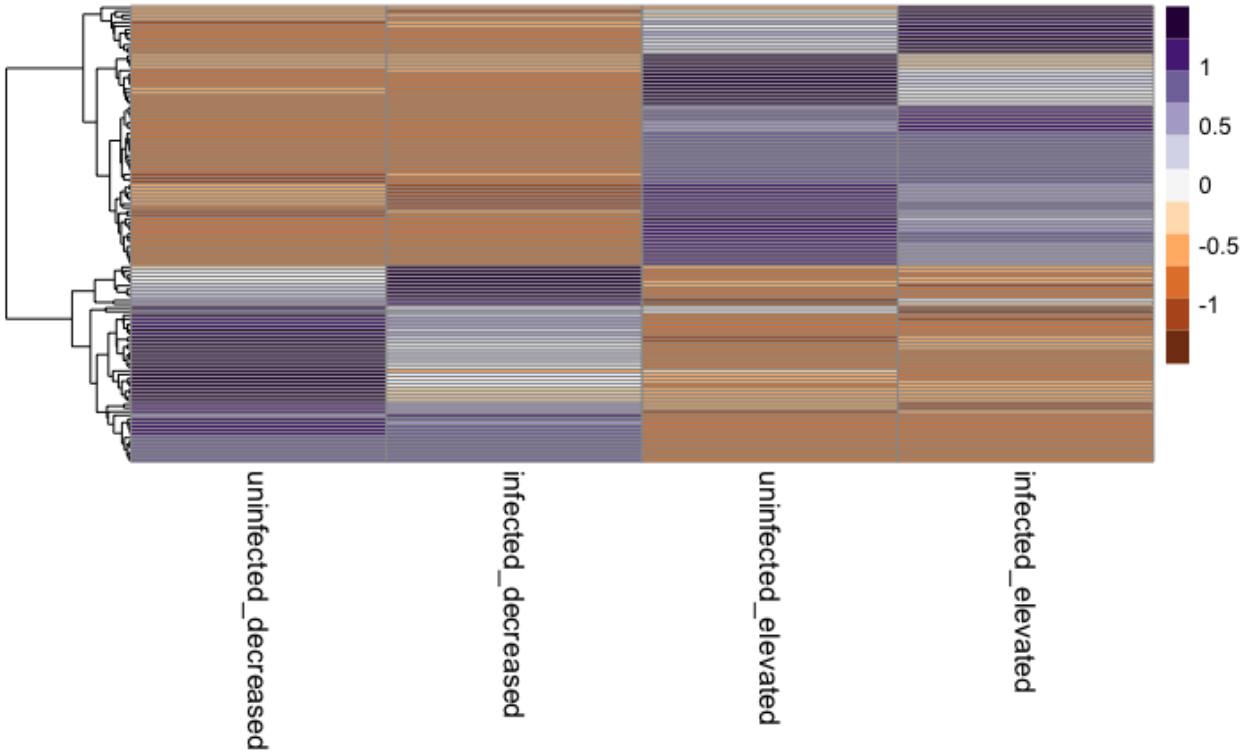

Figure 8. Heatmap of the differentially expressed contigs related to infection with *Hematodinium* that are influenced by temperature treatment. Each row is a differentially expressed gene, with the blue/purple coloration indicating higher expression, and orange/brown coloration indicating lower expression. The cladogram on the left groups genes that are more similar.

2.4 DISCUSSION

In this study, we have made progress towards understanding the mechanisms behind the influence of temperature on infection with the endoparasite *Hematodinium* on the immune response of one of its hosts, *Ch. bairdi*. We produced and annotated a *Ch. bairdi* hemolymph transcriptome from *Hematodinium* sp.-infected and uninfected crab exposed to different temperature treatments. This transcriptome, annotation, and RNAseq data from the libraries used to create the transcriptome are publicly available and can be used for further research on Tanner crab. Using the transcriptome and libraries of interest, we were able to identify genes that were

differentially expressed between *Hematodinium* sp.-infected crabs and uninfected crabs as well as identifying which of these genes were significantly influenced by temperature.

The Tanner crab hemolymph transcriptome is comparable to transcriptomes of the whiteleg shrimp (*Litopenaeus vannamei*) (Ghaffari et al. 2014) and the European shore crab (*Carcinus maenas*) (Verbruggen et al. 2015), with the shrimp transcriptome being more similar in size than the shore crab to the *Ch. bairdi* transcriptome. Ghaffari et al. (2014) assembled the shrimp transcriptome from RNA-seq data from the abdominal muscles, hepatopancreas, gills, and pleopods of one male shrimp. Verbruggen et al. (2015) assembled the European shore crab transcriptome from RNA-seq data from 12 pooled libraries of 12 tissues and organs from adult males and females. The larger number of individuals and tissues likely contributed to the higher number of contigs identified in the *C. maenas* transcriptome.

As part of the Tanner crab transcriptome, a number of stress response genes were identified and annotated. Several of the expressed genes are involved in immunity. Some are involved in the prophenoloxidase system and melanization pathway, such as proPO, phenoloxidase, lozenge, peroxinectin, and serine protease, among others. Lozenge regulates the expression of proPO (Tang 2009; Verbruggen et al. 2015), while peroxinectin contributes to the adhesion of hemocytes to pathogens (Liu et al. 2005; Verbruggen et al. 2015). There are also genes present that contribute to other innate immune responses, such as the toll-like receptors, which bind to PAMPs (Kingsolver et al. 2013; Verbruggen et al. 2015). Additionally, dicer-1 was identified, which is part of the RNAi pathway involved in antiviral innate immune response (Wang et al. 2014; Verbruggen et al. 2015). And as part of the endocytosis pathway, Rab GTPases and others were detected, which aid in phagocytosis and macropinocytosis, and the elimination of pathogens (Egami 2016).

Some other stress response genes were also found. Specifically, Hsp70-Hsp90 organizing protein 3 (AtHop3) was detected, which has a wide range of functions relating to the chaperone activity of cellular responses. While referred to as "heat shock" proteins, this group of molecular chaperones are involved in responding to stress, such as heavy metals, viral or bacterial infections, as well as thermal stress (Yang et al. 2013). Because the transcriptome combines temperature treatment and infection statuses, it cannot be determined if the presence of these proteins is part of an immune response against a pathogen, against thermal stress, or is in fact a pathogen gene because as there is no sequenced genome available for either *Ch. bairdi* or *Hematodinium*, it is possible that the transcriptome developed didn't filter out all *Hematodinium*-related transcripts. There has been evidence of increased expression of heat shock proteins 70 and 90 in response to bacterial and viral infections in prawns (Chaurasia et al. 2016), as well as evidence of increased Hsp70 expression in response to bacterial, osmotic, and temperature stress (Yang et al. 2013). Additionally, there is evidence that knocking out Hsp70 in the nauplii stage of *Artemia franciscana* decreases their tolerance to both bacterial infections and heat stress (Iryani et al. 2017). Histone-lysine N-methyltransferase (SETD2) was expressed, implying that crab gene expression, or pathogen gene expression, may be modified in response to stressors.

Among some of the genes that were differentially expressed between *Hematodinium* sp.-infected crabs and uninfected crabs there was unc-112-related protein (Fit1), signal peptide peptidase-like 2b (SPPL2B), and eukaryotic translation initiation factor 2-alpha kinase 4 (EIF2AK4) expressed, which each may have roles in immunity. In the whiteleg shrimp, *L. vannamei*, unc-112-related proteins were detected in the immune transcriptome, and this protein has the role of cell adhesion (Robalino et al. 2007). Signal peptide peptidase-like 2b may play a role in the regulation of innate immune response, as described in the annotated list of

differentially expressed genes in the supplemental material. The eukaryotic initiation factor 2-alpha contributes to the immune response of *L. vannamei* to the white spot syndrome virus (Xu et al. 2014). While these genes have not been studied in crabs, shrimp are crustaceans and these processes are likely conserved across crustacean species.

Enrichment analysis of the differentially expressed genes driven by temperature treatment with respect to infection status yielded three primary biological processes: lipid storage, transcription, and morphogenesis. Alteration in lipid storage and transcription provide insight into how temperature impacts energy allocation in *Hematodinium* sp.-infected crabs, with many of the genes associated with these processes having a role in immune function. A gene of interest that is associated with lipid storage process codes for the protein E3 ubiquitin protein ligase. This gene was expressed higher in crab in the decreased temperature treatment, with higher expression in uninfected crabs. This protein has been determined to be associated with immune response in the shrimp *Marsupenaeus japonicus* to white spot syndrome virus (Huang et al. 2012), and has been identified as contributing to the endocytosis immune pathway in the European shore crab, *C. maenas* (Verbruggen et al. 2015), implying that the *Hematodinium* sp.-infected crabs in the decreased temperature are launching immune responses to some pathogenic stressor, whether it be a virus or *Hematodinium* sp. One of the several genes associated with transcription is Histone deacetylase complex subunit Sin3a. This gene was expressed at higher levels in crab in the elevated temperature treatment, with higher expression in uninfected crabs. While this particular gene is best described in humans, some of its functions relate to activation of the innate immune response, indicating that infected crab in the elevated temperature are also launching immune responses. The enriched process of morphogenesis was less expected, but it could possibly suggest changes in hemocyte morphology or type. There were many genes that

are associated with this process with no distinct expression pattern between temperature treatments or infection statuses. When thinking about the high mortality that occurred in the elevated temperature treatment where nearly all crabs died, it could be that the elevated temperature changed hemocyte morphology or type in such a way that it increased the risk of mortality. It is also possible that *Hematodinium* sp. may be modulating hemocyte morphology, though more research is needed.

2.4.1 *Conclusions*

The high prevalence of BCD in Alaska is an ongoing threat, and with increasing ocean temperatures as a result of anthropogenic climate change, understanding the interaction of temperature and disease and its effect on crab is important. While there is still much to be learned from the differences between infected and uninfected crab, some important next steps would be to make comparisons between infection status influenced by temperature treatment over the time series of experimental days to see if there is a trackable change in crab stress response over the duration of infection and temperature exposure. Additionally, because RNAseq involves extracting RNA from all cells present in a sample, there is the possibility of assembling a partial transcriptome of *Hematodinium* and performing differential gene expression analyses on *Hematodinium* over time and between temperature treatments. This could be an important piece in understanding how ocean warming may impact the infectivity of *Hematodinium*. Finally, the publicly available assembled *Ch. bairdi* transcriptome provides a valuable resource for future research in transcriptomics and associated immune and stress responses of Tanner crab and other decapods.

REFERENCES

Alaska Department of Fish and Game (ADF&G). (last visit: 4/15/2020). http://www.adfg.alaska.gov/index.cfm?adfg=tannercrab.main

Andrews, S. 2010. "FASTQC: A Quality Control Tool for High Throughput Sequence Data". Available Online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bednarski, J., C. E. Siddon, G. H. Bishop, and J. F. Morado. 2011. "Overview of Bitter Crab Disease in Tanner Crabs, Chionoecetes Bairdi, in Southeast Alaska from 2001 to 2008." *Biology and Management of Exploited Crab Populations under Climate Change*. https://doi.org/10.4027/bmecpcc.2010.07.

Bower, Susan M., Ryan B. Carnegie, Benjamin Goh, Simon R. M. Jones, Geoffrey J. Lowe, and Michelle W. S. Mak. 2004. "Preferential PCR Amplification of Parasitic Protistan Small Subunit rDNA from Metazoan Tissues." *The Journal of Eukaryotic Microbiology*. https://doi.org/10.1111/j.1550-7408.2004.tb00574.x.

Bruno, John F., Elizabeth R. Selig, Kenneth S. Casey, Cathie A. Page, Bette L. Willis, C. Drew Harvell, Hugh Sweatman, and Amy M. Melendy. 2007. "Thermal Stress and Coral Cover as Drivers of Coral Disease Outbreaks." *PLoS Biology* 5 (6): e124.

Buchfink, Benjamin, Chao Xie, and Daniel H. Huson. 2015. "Fast and Sensitive Protein Alignment Using DIAMOND." *Nature Methods* 12 (1): 59–60.

Butler, Mark J., John M. Tiggelaar, Jeffrey D. Shields, and Mark J. Butler. 2014. "Effects of the Parasitic Dinoflagellate Hematodinium Perezi on Blue Crab (Callinectes Sapidus) Behavior and Predation." *Journal of Experimental Marine Biology and Ecology*. https://doi.org/10.1016/j.jembe.2014.09.008.

Cáceres-Puig, Jorge I., Fernando Abasolo-Pacheco, José M. Mazón-Suastegui, Alfonso N. Maeda-Martínez, and Pedro E. Saucedo. 2007. "Effect of Temperature on Growth and Survival of Crassostrea Corteziensis Spat during Late-Nursery Culturing at the Hatchery." *Aquaculture*. https://doi.org/10.1016/j.aquaculture.2007.06.030.

Cerenius, Lage, Bok Luel Lee, and Kenneth Söderhäll. 2008. "The proPO-System: Pros and Cons for Its Role in Invertebrate Immunity." *Trends in Immunology* 29 (6): 263–71.

Cerenius, Lage, and Kenneth Soderhall. 2004. "The Prophenoloxidase-Activating System in Invertebrates." *Immunological Reviews*. https://doi.org/10.1111/j.0105-2896.2004.00116.x.

Chambers, Matthew C., Brendan Maclean, Robert Burke, Dario Amodei, Daniel L. Ruderman, Steffen Neumann, Laurent Gatto, et al. 2012. "A Cross-Platform Toolkit for Mass Spectrometry and Proteomics." *Nature Biotechnology* 30 (10): 918–20.

Chatton, E., Poisson, R. 1931. "Sur l'existence, dans le sang des crabs, de peridiniens parasites *Hematodinium perezi* n. G., n sp. (Syndinidae). CR Sceances Soc. Biol Paris 105: 553-557.

Chaurasia, Mukesh Kumar, Faizal Nizam, Gayathri Ravichandran, Mariadhas Valan Arasu, Naif Abdullah Al-Dhabi, Aziz Arshad, Preetham Elumalai, and Jesu Arockiaraj. 2016. "Molecular Importance of Prawn Large Heat Shock Proteins 60, 70 and 90." *Fish & Shellfish Immunology* 48 (January): 228–38.

Chen, Shifu, Yanqing Zhou, Yaru Chen, and Jia Gu. 2018. "Fastp: An Ultra-Fast All-in-One FASTQ Preprocessor." *Bioinformatics* 34 (17): i884–90.

Crandall, G. 2020. Supplemental Materials for MS Thesis - Crab Chapter. https://zenodo.org/record/3906883#.XvOsNi2ZMcg

Choi, Meena, Ching-Yun Chang, Timothy Clough, Daniel Broudy, Trevor Killeen, Brendan MacLean, and Olga Vitek. 2014. "MSstats: An R Package for Statistical Analysis of Quantitative Mass Spectrometry-Based Proteomic Experiments." *Bioinformatics*.

https://doi.org/10.1093/bioinformatics/btu305.
Coen, Lauren D., Robert D. Brumbaugh, David Bushek, Ray Grizzle, Mark W. Luckenbach, Martin H. Posey, Sean P. Powers, and S. Gregory Tolley. 2007. "Ecosystem services related to oyster restoration". *Marine Ecology Progress Series* 341: 303-307.
Crandall, Grace, and Steven B. Roberts. 2020. grace-ac/paper-pacific.oyster-larvae: for reference in MS Thesis. GitHub repository archive: https://zenodo.org/record/3906832#.XvOeni2ZMcg
Crandall, Grace. 2020. Supplemental Materials for MS Thesis - Crab Chapter. https://zenodo.org/record/3906883#.XvOsNi2ZMcg
Crosson, Lisa M. 2011. "Development and Validation of a Quantitative Real-Time Polymerase Chain Reaction (qPCR) Assay to Assess the Impact of Hematodinium, a Parasitic Dinoflagellate, on Tanner Crab Populations in Alaska." University of Washington.
Dineshram, R., Kelvin K. W. Wong, Shu Xiao, Ziniu Yu, Pei Yuan Qian, and Vengatesen Thiyagarajan. 2012. "Analysis of Pacific Oyster Larval Proteome and Its Response to High-CO2." *Marine Pollution Bulletin* 64 (10): 2160–67.
Eaton, W. D., D. C. Love, C. Botelho, T. R. Meyers, K. Imamura, and T. Koeneman. 1991. "Preliminary Results on the Seasonality and Life Cycle of the Parasitic Dinoflagellate Causing Bitter Crab Disease in Alaskan Tanner Crabs (Chionoecetes Bairdi)." *Journal of Invertebrate Pathology* 57 (3): 426–34.
Elston, Ralph A., Dan Cheney, Paul Frelier, and Denis Lynn. 1999. "Invasive Orchitophryid Ciliate Infections in Juvenile Pacific and Kumomoto Oysters, Crassostrea Gigas and Crassostrea Sikamea." *Aquaculture*. https://doi.org/10.1016/s0044-8486(98)00512-2.
Egami, Youhei. 2016. "Molecular Imaging Analysis of Rab GTPases in the Regulation of Phagocytosis and Macropinocytosis." *Anatomical Science International* 91 (1): 35–42.
Ewels, Philip, Måns Magnusson, Sverker Lundin, and Max Käller. 2016. "MultiQC: Summarize Analysis Results for Multiple Tools and Samples in a Single Report." *Bioinformatics* 32 (19): 3047–48.
FAO. 2004. "Hatchery Culture of Bivalves." http://www.fao.org/3/y5720e/y5720e09.htm.
Foulon, Valentin, Pierre Boudry, Sébastien Artigaud, Fabienne Guérard, and Claire Hellio. 2019. "In Silico Analysis of Pacific Oyster (Crassostrea Gigas) Transcriptome over Developmental Stages Reveals Candidate Genes for Larval Settlement." *International Journal of Molecular Sciences* 20 (1). https://doi.org/10.3390/ijms20010197.
Frischer, M. E., R. F. Lee, M. A. Sheppard, A. Mauer, F. Rambow, M. Neumann, J. E. Brofft, T. Wizenmann, and J. M. Danforth. 2006. "Evidence for a Free-Living Life Stage of the Blue Crab Parasitic Dinoflagellate, Hematodinium Sp." *Harmful Algae*. https://doi.org/10.1016/j.hal.2005.11.002.
Ghaffari, Noushin, Alejandro Sanchez-Flores, Ryan Doan, Karina D. Garcia-Orozco, Patricia L. Chen, Adrian Ochoa-Leyva, Alonso A. Lopez-Zavala, et al. 2014. "Novel Transcriptome Assembly and Improved Annotation of the Whiteleg Shrimp (Litopenaeus Vannamei), a Dominant Crustacean in Global Seafood Mariculture." *Scientific Reports* 4 (November): 7081.
Gillet, Ludovic C., Pedro Navarro, Stephen Tate, Hannes Röst, Nathalie Selevsek, Lukas Reiter, Ron Bonner, and Ruedi Aebersold. 2012. "Targeted Data Extraction of the MS/MS Spectra Generated by Data-Independent Acquisition: A New Concept for Consistent and Accurate Proteome Analysis." *Molecular & Cellular Proteomics: MCP* 11 (6): O111.016717.
Gornik, Sebastian G., Amaya Albalat, Robert J. A. Atkinson, Graham H. Coombs, and Douglas M. Neil. 2010. "The Influence of Defined Ante-Mortem Stressors on the Early Post-Mortem Biochemical Processes in the Abdominal Muscle of the Norway lobster,Nephrops

norvegicus(Linnaeus, 1758).” *Marine Biology Research*. https://doi.org/10.1080/17451000903147468.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, et al. 2011. *Full-Length Transcriptome Assembly from RNA-Seq Data without a Reference Genome* (version 29). https://doi.org/10.1038/nbt.1883.

Groner, Maya L., Jeffrey D. Shields, Donald F. Landers Jr, John Swenarton, and John M. Hoenig. 2018. “Rising Temperatures, Molting Phenology, and Epizootic Shell Disease in the American Lobster.” *The American Naturalist* 192 (5): E163–77.

Gruebl, Tomas, Marc E. Frischer, Michael Sheppard, Monika Neumann, Andreas N. Maurer, and Richard F. Lee. 2002. “Development of an 18S rRNA Gene-Targeted PCR-Based Diagnostic for the Blue Crab Parasite Hematodinium Sp.” *Diseases of Aquatic Organisms* 49 (1): 61–70.

Haas, Brian J., Alexie Papanicolaou, Moran Yassour, Manfred Grabherr, Philip D. Blood, Joshua Bowden, Matthew Brian Couger, et al. 2013. “De Novo Transcript Sequence Reconstruction from RNA-Seq Using the Trinity Platform for Reference Generation and Analysis.” *Nature Protocols* 8 (8): 1494–1512.

Harvell, C. Drew, Charles E. Mitchell, Jessica R. Ward, Sonia Altizer, Andrew P. Dobson, Richard S. Ostfeld, and Michael D. Samuel. 2002. “Climate Warming and Disease Risks for Terrestrial and Marine Biota.” *Science* 296 (5576): 2158–62.

Huan, P., Wang, H., Dong, B., & Liu, B. 2012. “Identification of differentially expressed proteins involved in the early larval development of the Pacific oyster Crassostrea gigas”. *Journal of Proteomics* (Vol. 75, Issue 13, pp. 3855–3865).

Huang, D. W., Sherman, B.T., and Lempicki, R.A. 2009a. “Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, 4(1), 44-57.

Huang, D. W., Sherman, B.T., and Lempicki, R.A. 2009b. “Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists”. *Nucleic Acids Research*, 37(1), 1-13.

Hudson, D. A., and J. D. Shields. 1994. “Hematodinium Australis N. Sp., a Parasitic Dinoflagellate of the Sand Crab Portunus Pelagicus from Moreton Bay, Australia.” *Diseases of Aquatic Organisms*. https://doi.org/10.3354/dao019109.

Huson, Daniel H., Sina Beier, Isabell Flade, Anna Górska, Mohamed El-Hadidi, Suparna Mitra, Hans-Joachim Ruscheweyh, and Rewati Tappu. 2016. “MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data.” *PLoS Computational Biology* 12 (6): e1004957.

Iryani, Mat Taib Mimi, Thomas H. MacRae, Sheethal Panchakshari, Jiabo Tan, Peter Bossier, Mohd Effendy Abd Wahid, and Yeong Yik Sung. 2017. “Knockdown of Heat Shock Protein 70 (Hsp70) by RNAi Reduces the Tolerance of Artemia Franciscana Nauplii to Heat and Bacterial Infection.” *Journal of Experimental Marine Biology and Ecology*. https://doi.org/10.1016/j.jembe.2016.12.004.

Jadamec, L. S., W. E. Donaldson, and P. Cullenberg. 1999. “Biological Field Techniques for Chionoecetes Crabs.” https://doi.org/10.4027/bftcc.1999.

Jensen, Pamela C., Katy Califf, Vanessa Lowe, Lorenz Hauser, and J. Frank Morado. 2010. “Molecular Detection of Hematodinium Sp. in Northeast Pacific Chionoecetes Spp. and Evidence of Two Species in the Northern Hemisphere.” *Diseases of Aquatic Organisms* 89 (2): 155–66.

Kingsolver, Megan B., Zhijing Huang, and Richard W. Hardy. 2013. “Insect Antiviral Innate

Immunity: Pathways, Effectors, and Connections." *Journal of Molecular Biology*. https://doi.org/10.1016/j.jmb.2013.10.006.

Ko, Ginger W. K., R. Dineshram, Camilla Campanati, Vera B. S. Chan, Jon Havenhand, and Vengatesen Thiyagarajan. 2014. "Interactive Effects of Ocean Acidification, Elevated Temperature, and Reduced Salinity on Early-Life Stages of the Pacific Oyster." *Environmental Science & Technology*. https://doi.org/10.1021/es501611u.

Kon, Toshi, Tadashi Isshiki, Toshiaki Miyadai, and Yoshiharu Honma. 2011. "Milky Hemolymph Syndrome Associated with an Intranuclear Bacilliform Virus in Snow Crab Chionoecetes Opilio from the Sea of Japan." *Fisheries Science*. https://doi.org/10.1007/s12562-011-0405-0.

Lin, Xionghui, and Irene Söderhäll. 2011. "Crustacean Hematopoiesis and the Astakine Cytokines." *Blood*. https://doi.org/10.1182/blood-2010-11-320614.

Liu, Chun-Hung, Winton Cheng, and Jiann-Chu Chen. 2005. "The Peroxinectin of White Shrimp Litopenaeus Vannamei Is Synthesised in the Semi-Granular and Granular Cells, and Its Transcription Is up-Regulated with Vibrio Alginolyticus Infection." *Fish & Shellfish Immunology*. https://doi.org/10.1016/j.fsi.2004.10.005.

Love, D. C., S. D. Rice, D. A. Moles, and W. D. Eaton. 1993. "Seasonal Prevalence and Intensity of Bitter Crab Dinoflagellate Infection and Host Mortality in Alaskan Tanner Crabs Chionoecetes Bairdi from Auke Bay, Alaska, USA." *Diseases of Aquatic Organisms*. https://doi.org/10.3354/dao015001.

MacLean, Brendan, Daniela M. Tomazela, Nicholas Shulman, Matthew Chambers, Gregory L. Finney, Barbara Frewen, Randall Kern, David L. Tabb, Daniel C. Liebler, and Michael J. MacCoss. 2010. "Skyline: An Open Source Document Editor for Creating and Analyzing Targeted Proteomics Experiments." *Bioinformatics* 26 (7): 966–68.

Malham, Shelagh K., Elizabeth Cotter, Selena O'Keeffe, Sharon Lynch, Sarah C. Culloty, Jonathan W. King, John W. Latchford, and Andy R. Beaumont. 2009. "Summer Mortality of the Pacific Oyster, Crassostrea Gigas, in the Irish Sea: The Influence of Temperature and Nutrients on Health and Survival." *Aquaculture* 287 (1): 128–38.

Meyers, T. R., C. Botelho, T. M. Koeneman, S. Short, and K. Imamura. 1990. "Distribution of Bitter Crab Dinoflagellate Syndrome in Southeast Alaskan Tanner Crabs Chionoecetes Bairdi." *Diseases of Aquatic Organisms*. https://doi.org/10.3354/dao009037.

Meyers, T. R., T. M. Koeneman, C. Botelho, and S. Short. 1987. "Bitter Crab Disease: A Fatal Dinoflagellate Infection and Marketing Problem for Alaskan Tanner Crabs Chionoecetes Bairdi." *Diseases of Aquatic Organisms*. https://doi.org/10.3354/dao003195.

Morado, J. F. 2011. "Protistan Diseases of Commercially Important Crabs: A Review." *Journal of Invertebrate Pathology* 106 (1): 27–53.

Morado, J. F., E. G. Dawe, D. Mullowney, C. A. Shavey, V. C. Lowe, and R. J. Cawthorn. 2011. "Climate Change and the Worldwide Emergence of Hematodinium-Associated Disease: Is There Evidence for a Relationship?" *Biology and Management of Exploited Crab Populations under Climate Change*. https://doi.org/10.4027/bmecpcc.2010.08.

Nappi, Anthony J., and Enzo Ottaviani. 2000. "Cytotoxicity and Cytotoxic Molecules in Invertebrates." *BioEssays*. https://doi.org/3.0.co;2-4">10.1002/(sici)1521-1878(200005)22:5<469::aid-bies9>3.0.co;2-4

National Marine Fisheries Service. 2015. Fisheries of the United States, 2014. U.S. Department of Commerce, NOAA Current Fishery Statistics No.2014. Available at: https://www.st.nmfs.noaa.gov/commercialfisheries/fus/fus14/index

Newell, Roger I. E. 2004. "Ecosystem influences of natural and cultivated populations of

suspension-feeding bivalve molluscs: a review". *Journal of Shellfish Research* 23 (1).
Nielsen, Julie K., S. James Taggart, Thomas C. Shirley, and Jennifer Mondragon. 2007. "Spatial Distribution of Juvenile and Adult Female Tanner Crabs (Chionoecetes Bairdi) in a Glacial Fjord Ecosystem: Implications for Recruitment Processes." *ICES Journal of Marine Science*. https://doi.org/10.1093/icesjms/fsm158.
R Core Team. 2015. *R: A Language and Environment for Statistical Computing* (version 3.2.3). R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org.
Rico-Villa, B., S. Pouvreau, and R. Robert. 2009. "Influence of Food Density and Temperature on Ingestion, Growth and Settlement of Pacific Oyster Larvae, Crassostrea Gigas." *Aquaculture*. https://doi.org/10.1016/j.aquaculture.2008.10.054.
Robalino, Javier, Jonas S. Almeida, David McKillen, Joan Colglazier, Harold F. Trent 3rd, Yian Ann Chen, Megan E. T. Peck, et al. 2007. "Insights into the Immune Transcriptome of the Shrimp Litopenaeus Vannamei: Tissue-Specific Expression Profiles and Transcriptomic Responses to Immune Challenge." *Physiological Genomics* 29 (1): 44–56.
Rowley, Andrew F., Amanda L. Smith, and Charlotte E. Davies. 2015. "How Does the Dinoflagellate Parasite Hematodinium Outsmart the Immune System of Its Crustacean Hosts?" *PLoS Pathogens* 11 (5): e1004724.
Searle, Brian C., Lindsay K. Pino, Jarrett D. Egertson, Ying S. Ting, Robert T. Lawrence, Brendan X. MacLean, Judit Villén, and Michael J. MacCoss. 2018. "Chromatogram Libraries Improve Peptide Detection and Quantification by Data Independent Acquisition Mass Spectrometry." *Nature Communications*. https://doi.org/10.1038/s41467-018-07454-w.
Shields, Jeffrey D. 2017. "Collection Techniques for the Analyses of Pathogens in Crustaceans." *Journal of Crustacean Biology*. https://doi.org/10.1093/jcbiol/rux077.
Shields, Jeffrey D., David M. Taylor, Paul G. O'Keefe, Eugene Colbourne, and Elaine Hynick. 2007. "Epidemiological Determinants in Outbreaks of Bitter Crab Disease (Hematodinium Sp.) in Snow Crabs Chionoecetes Opilio from Conception Bay, Newfoundland, Canada." *Diseases of Aquatic Organisms* 77 (1): 61–72.
Simão, Felipe A., Robert M. Waterhouse, Panagiotis Ioannidis, Evgenia V. Kriventseva, and Evgeny M. Zdobnov. 2015. "BUSCO: Assessing Genome Assembly and Annotation Completeness with Single-Copy Orthologs." *Bioinformatics* 31 (19): 3210–12.
Supek, Fran, Matko Bošnjak, Nives Škunca, and Tomislav Šmuc. 2011. "REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms." *PloS One* 6 (7): e21800.
Tang, Huaping. 2009. "Regulation and Function of the Melanization Reaction in Drosophila." *Fly* 3 (1): 105–11.
Timmins-Schiffman, Emma B., Grace A. Crandall, Brent Vadopalas, Michael E. Riffle, Brook L. Nunn, and Steven B. Roberts. 2017. "Integrating Discovery-Driven Proteomics and Selected Reaction Monitoring To Develop a Noninvasive Assay for Geoduck Reproductive Maturation." *Journal of Proteome Research* 16 (9): 3298–3309.
Venable, John D., Meng-Qiu Dong, James Wohlschlegel, Andrew Dillin, and John R. Yates. 2004. "Automated Approach for Quantitative Analysis of Complex Peptide Mixtures from Tandem Mass Spectra." *Nature Methods* 1 (1): 39–45.
Venkataraman, Y. R., Timmins-Schiffman, E., Horwith, M. J., Lowe, A. T., Nunn, B., Vadopalas, B., Spencer, L. H., & Roberts, S. B. 2019. "Characterization of Pacific oyster (Crassostrea gigas) proteomic response to natural environmental differences". https://doi.org/10.1101/460204
Verbruggen, Bas, Lisa K. Bickley, Eduarda M. Santos, Charles R. Tyler, Grant D. Stentiford, Kelly S. Bateman, and Ronny van Aerle. 2015. "De Novo Assembly of the Carcinus Maenas Transcriptome and Characterization of Innate Immune System Pathways." *BMC Genomics*

16 (June): 458.
Wang, Pei-Hui, Tianzhi Huang, Xiaobo Zhang, and Jian-Guo He. 2014. “Antiviral Defense in Shrimp: From Innate Immunity to Viral Infection.” *Antiviral Research* 108 (August): 129–41.
Waterhouse, Robert M., Mathieu Seppey, Felipe A. Simão, Mosè Manni, Panagiotis Ioannidis, Guennadi Klioutchnikov, Evgenia V. Kriventseva, and Evgeny M. Zdobnov. 2018. “BUSCO Applications from Quality Assessments to Gene Prediction and Phylogenomics.” *Molecular Biology and Evolution* 35 (3): 543–48.
Xu, Jingxiang, Lingwei Ruan, and Hong Shi. 2014. “eIF2α of Litopenaeus Vannamei Involved in Shrimp Immune Response to WSSV Infection.” *Fish & Shellfish Immunology*. https://doi.org/10.1016/j.fsi.2014.08.016.
Yang, Ya ’nan, Haihui Ye, Huiyang Huang, Shaojing Li, Xueliang Liu, Xianglan Zeng, and Jie Gong. 2013. “Expression of Hsp70 in the Mud Crab, Scylla Paramamosain in Response to Bacterial, Osmotic, and Thermal Stress.” *Cell Stress & Chaperones* 18 (4): 475–82.
Zhang, Y., Fonslow, B. R., Shan, B., Baek, M.-C., & Yates, J. R., 3rd. (2013). Protein analysis shotgun/bottom-up proteomics. *Chemical Reviews*, *113*(4), 2343–2394.