Differential Expression in *Hematodinium sp.*

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```
library("kableExtra")
library("tidyverse")
```

```
## -- Attaching packages --------------------------------------- tidyverse 1.3.0 --

## v ggplot2 3.3.2     v purrr   0.3.4
## v tibble  3.0.4     v dplyr   1.0.2
## v tidyr   1.1.2     v stringr 1.4.0
## v readr   1.4.0     v forcats 0.5.0

## -- Conflicts ------------------------------------------ tidyverse_conflicts() --
## x dplyr::filter()     masks stats::filter()
## x dplyr::group_rows() masks kableExtra::group_rows()
## x dplyr::lag()        masks stats::lag()
```

IMPORTANT: Much of my methods in this draft are based on Grace's wording for her paper's methods (since I'm using her samples, much of my method is hers). Check if this is OK or if it needs to be rewritten from scratch (or heavily rephrased).

Methods

Collection

400 male *C. bairdi* were collected using crab pots from Stephen's Passage in southeastern Alaska in late October 2017. Collections were made by the Alaska Department of Fish and Game (ADF&G). Stephen's Passage was selected due a consistently high prevalence of *Hematodinium*, approximately 50% (ADF&G, unpub. data), and due to its proximity to Juneau, AK. Crab were transported to Ted Stevens Marine Research Institute (TSMRI, NOAA facility, Juneau, AK) within TK hours of capture. Upon arrival, they were placed in TK liter flow-through seawater tanks at 7.5°C - the benthic water temperature within Stephen's Passage at time of capture.

Verification of Infection with *Hematodinium*

200 µl of hemolymph was withdrawn from each individual and preserved in 800 µl 95% ethanol. 200 µl of the ethanol-preserved hemolymph was centrifuged, and the supernatant was discarded. The pelleted material was then air-dried. DNA was extracted using invertebrate lysis buffer and following Ivanova et al.(2006). Extraction proctocol was modified by performing two washes with Wash Buffer and adjusting eluted DNA (50 µl) to 10 mM Tris-CI, pH 8.0, and 0.1 mM EDTA. The extracted DNA was subjected to two rounds of

Table 1: Individual libraries of infected crab

Crab ID	Treatment group	Day 0 sample ID	Day 2 sample ID	Day 17 sample ID
A	Ambient	178	359	463
B	Ambient	118	349	481
C	Ambient	132	334	485
G	Elevated	173	272	NA
H	Elevated	72	294	NA
I	Elevated	127	280	NA

PCR with two primer pairs designed for the small subunit (SSU) rRNA gene of *Hematodinium* spp. - Univ-F-15 / Hemat-R-1654 (Gruebl et al. 2002), and Hemat 18Sf / Hemat 18Sr (Bower et al. 2004). Post-PCR, reaction aliquots were pooled and visualized on ethidium bromide-stained 2% agarose gels. Samples were scored as positive if both *Hematodinium* spp. bands were visible on the gel, and scored as negative when neither fragment amplified. Samples where one band amplified were scored as TK.

Varying Temperature

All crabs acclimated at 7.5°C for nine days. 120 crabs were then [TK randomly?] selected for temperature treatments. Crabs that did not appear to have recovered from capture stress were not selected. A 0.2 ml sample of hemolymph was taken from each selected crab and preserved in 1200 µl RNAlater.10 infected crabs were placed in each of six replicate tanks, along with 10 uninfected crabs. Over the course of two days (day 0 to day 2), the water temperature in three tanks was gradually elevated to 10°C. In the remaining three tanks, water temperature was maintained at 7.5°C. After two days (day 2), and at the conclusion of the temperature trial (day 17), another 0.2 ml [TK: verify amt] of hemolymph was sampled and preserved in RNAlater for each surviving crab.

RNA Extraction and Sequencing

Hemolymph samples (n = TK) were centrifuged at 14000 g for 10 minutes. RNA was extracted with Quick DNA/RNA Microprep Plus kit (Zymo Research) using the manufacturer's protocol. 2 µl samples were run on Qubit 3.0 using the Qubit RNA HS Kit (Invitrogen) to determine RNA quantification. Six infected crabs were selected [TK: based on RNA yield?] - three from the elevated-temperature treatment group and three from the ambient-temperature treatment group.RNA was sent to the Northwest Genomics Center at Foege Hall at the University of Washington for library construction and sequencing. Libraries were created from all available samples of the selected crabs (Table 1). Due to a mass mortality event within those tanks, no libraries from day 17 are available for the elevated-temperature treatment group.

Transcriptome Assembly and Annotation

TK. Believe Grace's chapter describes Transcriptome 3.0, need to check if Transcriptome 2.0 construction method matches.

Differential Expression Analysis

An index of TK transcriptome was created with kallisto (TK citation), and each library was pseudoaligned to obtain counts. An abundance matrix for each pairwise comparison (Table 2) was then created using Trinity (v2.TK, TK citation?). Differential contig expression was calculated using a negative binomial GLM [TK: check if correct] using the R package DESeq2. Read counts were normalized using size factors and fit to a negative binomial distribution. The Wald test for significance of GLM terms for each comparison was used

Table 2: PairwiseComparisons

Pair 1			Pair 2			
Crab IDs	Temp. when sampled	Sample day	Crab IDs	Temp. when sampled	Sample day	DEGs analyzed individually?
A,B,C	Ambient	0	A,B,C	Ambient	2	No
A,B,C	Ambient	0	A,B,C	Ambient	17	No
A,B,C	Ambient	2	A,B,C	Ambient	17	No
A,B,C	Ambient	2	G,H,I	Elevated	2	Yes
G,H,I	Ambient	0	G,H,I	Elevated	2	Yes

to obtain unadjusted p-values. For comparisons of crabs at different temperatures, a table of significantly differentially-expressed transcripts (Benjamini-Hochberg adjusted $p < 0.05$) was also obtained (Table 2).

Enrichment Analysis

For each comparison (Table 2), the output from DESeq2 was cross-referenced with the annotated transcriptome and the UniProt database (citation TK) to produce a table of UniProt Accession IDs and GO terms, along with a table of UniProt Accession IDs and unadjusted p-values. GO categories were then tested for significant enrichment with the R package GO-MWU (citation TK), which utilizes the Mann-Whitney U test.

Individual DEG Examination

Process TK, haven't completed this yet