Natural Transmission of *Hematodinium perezi* in Juvenile Blue Crabs (*Callinectes sapidus*) in the Laboratory

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Keywords: Dinoflagellate, Parasite, qPCR, Mortality, Sporulation, Crustacea

Abstract

Hematodinium perezi is a dinoflagellate endoparasitic in marine crustaceans, primarily decapods. It is known to occur in juvenile blue crabs, *Callinectes sapidus*, at high prevalence levels and has severe pathogenic effects in this host. The life history outside the host has not been experimentally investigated and, until now, transmission using dinospores has not been successful. We investigated the natural transmission dynamics of *H. perezi* in the laboratory using small juvenile crabs, which are highly susceptible to infection in the field, and elevated temperatures, which are known to stimulate dinospore production. Natural water-borne transmission to naïve crabs varied between 7-100% and was not correlated with dinospore densities measured from their aquaria water. Infections appeared to develop quickly in naïve hosts at 25 °C, suggesting that elevated temperatures as seen in the late summer and early autumn have a strong influence on the transmission of *H. perezi* in natural systems.

1. Introduction

Hematodinium species are parasitic dinoflagellates that infect more than 40 species of crustaceans, all of which are found in the hemolymph of the host (Stentiford and Shields, 2005; Small, 2012). The type species, *H. perezi* was first described from *Carcinus maenas* and *Liocarcinus depurator* in France (Chatton and Poisson, 1931). It was first reported in blue crabs *Callinectes sapidus* from North Carolina, Georgia, and Florida as *Hematodinium* sp. by Newman and Johnson (1975) and then reported in other states, ranging from Delaware to Texas (Messick, 1994; Messick and Shields, 2000). Molecular analysis of the ITS1 region of *H. perezi* shows that three genotypes of this species infect disparate crustacean hosts from different geographic regions. Genotype I is found in decapods from northern European waters, genotype II from portunids from China, and genotype III from blue crabs and other crustaceans from the eastern United States of America (Small, 2012).

Hematodinium perezi is an obligate parasite and obtains nutrition from the host through osmotrophy (Shields, 1994; Shields et al., 2003). It is highly pathogenic in adult blue crabs, with 100% mortality in naturally infected crabs held for 35 days (Messick and Shields, 2000) and 86% mortality in experimentally infected crabs held for 40 days (Shields and Squyars, 2000). The mortality is associated with glycogen depletion (Shields et al., 2003), organ malfunction, and respiratory dysfunction (Field et al., 1992; Shields and Squyars, 2000) resulting from proliferation of the parasite and its resulting burden on the host's metabolic processes. In juvenile blue crabs, progression of the parasite is strongly temperature dependent, leading to rapid and high mortality of infected crabs at 25 °C (Huchin-Mian et al., 2018).

Juvenile blue crabs are highly susceptible to *H. perezi* in high salinity areas (26-34 psu) and become infected shortly after settlement and recruitment (Messick and Shields, 2000; Small

et al., 2019). The parasite has a bimodal prevalence in blue crabs, with a small peak in summer and a much higher peak in autumn (Messick, 1994; Messick and Shields, 2000; Sheppard et al., 2003). In sentinel studies, naïve crabs held in off-bottom cages deployed in endemic areas become infected within 3 days of deployment and heavy infections develop within 7 to 14 days (Huchin-Mian et al., 2017; Shields et al., 2017).

Few studies have focused on natural transmission of *H. perezi*, but there is strong circumstantial evidence that the dinospores are the transmissive stage (Frischer et al., 2006; Huchin-Mian et al., 2017; Shields et al., 2017). *In vitro* culture has shown that species of *Hematodinium* produce two types of dinospores (Appleton and Vickerman, 1996, Li et al., 2011), the micro-dinospore and the macro-dinospore, but their function remains unknown. In blue crabs, these dinospores are released from their adult hosts into the water in large quantities (up to 10^8 dinospores/ml hemolymph) (Shields and Squyars, 2000; Huchin-Mian et al., 2018). Sporulation leads to damage of the gill lamellae as the spores exit through the less sclerotized tissues, resulting in perforation of the gills, which is fatal in many host species (Meyers et al., 1987; Appleton and Vickerman, 1998; Wheeler et al., 2007). After sporulation, motile dinospores can be observed in aquaria for 3-5 days with a microscope and are detectable with qPCR for up to 7 days (Li et al., 2010). The DNA of free-living stages of *H. perezi* in the environment was first detected in water samples using real-time PCR by Frischer et al. (2006). This observation has since been verified in both water and sediment samples at densities ranging from 1-100+ cells/l using additional molecular primers with higher sensitivity (Li et al., 2010; Hanif et al., 2013; Lycett and Pitula, 2017). A recent cohabitation trial using infected mudflat crabs, *Helice tientsinensis* and healthy swimming crabs, *Portunus trituberculatus*, showed that

naïve crabs can become infected with *H. perezi* (genotype II) when exposed to infected individuals, further supporting water-borne transmission (Huang et al., 2021).

Our goal is to understand the transmission dynamics of *H. perezi* to blue crabs. We undertook three objectives as part of this goal: 1) to determine if natural water-borne transmission can occur in a laboratory setting; 2) to assess spore densities in relation to infections using natural transmission; 3) to examine patterns in spore densities in water samples in relation to transmission.

2. Material and methods

2.1. Crab collection

Juvenile blue crabs < 30 mm carapace width (CW) were collected using dipnets from two locations in October 2020 and September 2021. Unexposed naïve crabs were collected from a non-endemic area, Mobjack Bay (37°18′N, 76°24′W), VA, a sub-estuary of Chesapeake Bay. Infected donor crabs were collected from a hyper-endemic area, Cobb Bay (37°19.234′N, 75°47.120′W), VA, a coastal bay on the Delmarva Peninsula. Crabs were transported back to the Virginia Institute of Marine Science (VIMS) in coolers. Naïve crabs were caught a week prior to donor crabs and held in individual cells in a shallow recirculating system at 20 psu as in Shields et al. (2017). During the holding period, salinity was gradually adjusted to 30 psu with two water changes and crabs were fed twice with pelleted food (pellet size 0.5 mm; Omega One™). Donor crabs were held in two aerated 76-liter aquaria at 30 psu overnight and transferred into experimental aquaria on the next day. As a preliminary assessment on the infection status of the donor crabs, 10 crabs from Cobb Bay were dissected and assessed for background infections using the neutral red diagnostic (see below). Crabs from Mobjack Bay have never been found

with *H. perezi* infections (Shields et al., 2017); however, to ensure they were uninfected, 10 crabs from Mobjack Bay were dissected and assessed for infections using the same method.

2.2. Experimental design

The experiment was conducted in 2020 and repeated in 2021 with the same design. Each crab was placed individually in a 90-ml urinalysis cup (Fisher Scientific®) that had ~20 drilled holes (~ 8 mm dia) for water flow and a layer of crushed coral to provide substrate and to prevent floating. In each 38 L aquarium, 15 donor crabs and 15 naïve crabs were put in these cups, which were then placed randomly in the aquarium. Replicates consisted of seven aquaria stocked in this manner. A control group consisting of 30 naïve crabs held in an aquarium as above was also run to assess crab mortality. Crabs were kept in 20 L of artificial sea water at 30 psu and 25 °C. For temperature control, aquaria were held in 5 cm of water in a fiberglass container (water bath) equipped with two aquarium heaters. Because the room temperature was 23-27 °C, no acclimation was needed. Mortality was monitored daily. Donor crabs were replaced when they died to maintain similar biomass and potential for dinospore release into the water. Each week, crabs were fed twice with pellets and 30% water changes were conducted to maintain water quality. All surviving crabs were dissected and diagnosed after 30 days, except for two aquaria, which were kept for 40 days.

2.3. Crab assessment

The carapace width (including epibranchial spines) of all crabs was measured in mm before dissection. For *H. perezi* diagnosis, crabs were individually assessed by cutting each in half width-wise with a sterile razor blade on a clean microscope slide. The dissection smear was

stained with 0.3% neutral red in saline buffer, and examined at 200× and 400× with a transmitted light microscope as in Shields et al. (2017). The life history stages were determined based on morphology of the parasite cells present in the smear. Life history stages were categorized as filamentous trophonts, amoeboid trophonts, clump colonies, and prespores. The relative intensity of infection was determined by the number of parasites per microscopic field at 200×. Light infections had 1-5 parasites, moderate infections had 5-20 parasites, and heavy infections had >20 parasites. Most dead crabs were not diagnosable due to their rapid degradation at room temperature; thus, they were only recorded for survival analysis.

2.4. Water sample processing

In the first experiment (2020), water samples were taken every three days to track densities of *H. perezi* stages in the tanks. Samples were taken at the surface of each aquarium, starting on the first day of the experiment. The sampling interval was based on previous work on the longevity of spores in tank water (Li et al., 2010). In the second experiment (2021) there were two differences in water sampling: 1) water samples were taken from the bottom of the aquarium instead of at the surface; and 2) water samples were taken daily from Day 12 to Day 21 to obtain a finer scale (daily) of observation over this time period.

To process water samples, aliquots of 100 ml of aquarium water were filtered through a 3 µm Nuclepore™ membrane (Whatman®), using a sterile or bleach-cleaned filter flask assembly and a vacuum pump. The filters were kept in a -80 °C freezer until processed for quantitative measurements using real-time PCR assays. DNA was extracted using the Qiagen QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) with modifications to the manufacturer's instructions as described in Wolny et al. (2020). All samples were eluted in 100µl ATE buffer

and passed over the column two times (Audemard et al., 2004). To check for contamination, all extractions completed within the same day included a blank column without actual sample as a negative control.

2.5. Real-time PCR

The Taqman assay derived from Hanif et al. (2013) was used for real-time PCR on all water samples. The assay targeted the ITS2 region with primer set, ITS2For (5'-AGGTCTAATGCTTGTTGGCC-3') and ITS2Rev (5'-CACTAGTCCGAAAACCTGTG-3') and a Taqman probe, HemITS2 probe (6-FAM-ACCGCTACTCTTCTTCCGCCCT-BHQ1). The reagents were used with primers at 0.9μM, probe at 0.1μM, and with Taqman Fast Advanced Mix (Life Technologies, Austin, TX) to a volume of 16μl. A standard curve was constructed with 10-fold serial dilutions using gBlocks Gene Fragments (5'-CTTATGACCCAGTTTAGGTCTAATGCTTGTTGGCCGAGAGTTACGCTGCATGGTTATACCGCTACTCTTCTTCCGCCCTTTATTGTGATAGTACACAGGTTTTCGGACTAGTGACCTCTATTGCATGTACG-3') (Integrated DNA Technologies, Coraville, IA) starting with a concentration at 2.8×10^7 gene copies/μl. Real-time PCR was conducted on duplicates of a serial dilution of the gBlocks, extraction samples, a negative control for extraction, and a no-template control with distilled water as template. Each reaction had 4μl of template added to reach a final volume of 20μl. For the 2021 water samples, the concentration of the probe was adjusted to 0.25μM and the final volume of each reaction was 10μl with 2μl of template.

Thermocycling was carried out on an Applied Biosystems Fast7500 thermocycler with initial heating to 95°C for 20 seconds, followed by 40 cycles of by 95°C for 3 seconds and then by 60°C for 30 seconds. The number of *Hematodinium* gene copies in the water samples was

plotted as a C_T curve versus copy number. The limit of quantification of the gBlock was 28 gene copies per qPCR reaction using 4µl of DNA, which translates to 7000 copies/L, or ~20 cells/L. Thus, values below this number are considered as below the quantification limit and not reported.

2.6. Data analysis

Transmission success in each replicate was calculated as the percentage of naïve crabs infected with *H. perezi*, diagnosed as positive by dissection smears, at the end of the experiment. The mean size of naïve crabs in each aquarium was compared using one-way ANOVA and post hoc Tukey's test. The mean size of infected and non-infected naïve crabs was compared using a Student's t-test. Correlation between average density of *H. perezi* gene copy and prevalence in naïve crabs was tested for the 2020 and 2021 experiments. To analyze the effect of mortality and year on maximum *H. perezi* gene copies, a three-way ANOVA was used with data binned into three groups based on experiment days: day 0-9, day 10-21, and day 22-30. Kaplan-Meier survival curves were plotted for donor and naïve crabs in each aquarium. The survival time of the replaced donor crabs were counted from the day of replacement. Pairwise comparisons were performed on survival curves between the control group and each treatment group using the log-rank test in the R package survminer. All statistical analyses were carried out in R 4.2.0 (R Core Team 2022).

3. Results

Prevalence levels in the pre-sample of crabs from Cobb Bay were 80% and 90% in 2020 and 2021, respectively; hence, crabs from this area were used as infected "donor" crabs. Pre-samples of crabs from Mobjack Bay were uninfected as expected and served as naïve crabs.

3.1. Transmission dynamics of donor and naïve crabs

Natural transmission of *H. perezi* in juvenile blue crabs occurred in five aquaria in the 2020 experiment and six aquaria in the 2021 experiment. In aquaria with successful transmission, prevalence in naïve crabs greatly varied in both experiments, ranging from 17-100% in 2020 and 7-69% in 2021 (Table 1). The mean size of donor crabs (CW 17.0 ± 5.1 mm) did not differ between aquaria (one-way ANOVA, $p = 0.48$). The mean size of naïve crabs (CW 17.8 ± 5.7 mm) did not differ between aquaria (one-way ANOVA, $p = 0.61$). There was no difference in the mean size of naïve crabs in relation to infection status ($t = -1.51$, $df = 199$, $p = 0.13$).

Of the infected crabs in the pre-sample from Cobb Bay, 75% had heavy infections. The filamentous trophont stage, the stage indicative of early infection, was observed in 17% of the pre-sampled donor crabs. At the end of the experiment, more than 75% of the surviving donor crabs were heavily infected. Only one donor crab had filamentous trophonts at the end of the experiment, an indication of either new infection in an uninfected donor or reinfection. More than 30% of donor crabs had the prespore stage, indicating late infection. At the end of the experiment, 50% of the naïve crabs that became infected had heavy infections (Figure 1A). None of the infected naïve crabs had filamentous trophonts, indicating that infections had progressed past the early stages of infection. All infected naïve crabs had mid to late-stage infections and

17% had the prespore stage (Figure 1B), indicating that sporulation, the release of spores, was imminent.

3.2. *Hematodinium* sporulation by qPCR

Quantitative assessment of *H. perezi* DNA in the aquaria exhibited sharp, ephemeral spikes in abundance. In 2020, peaks in the number of *H. perezi* ITS2 gene copies in the water were detected on days 9, 15, and 18 in six aquaria (Figure 2). A total of 14 signals were detected. In four aquaria, only one peak signal was detected during the duration of the experiment. The number of gene copies ranged from 8.1×10^3 copies/L to 9.4×10^5 copies/L. In 2021, *H. perezi* DNA was detected more frequently and in higher densities. A total of 74 signals were detected. The number of gene copies ranged from 7.2×10^3 copies/L to 2.1×10^7 copies/L. Over a three-day average, the signals appeared in peaks on days 6, 15, and 24 (Figure 3A). In addition, aquarium 3 had an earlier peak on day 3. From day 12 to day 21, the density of gene copies fluctuated markedly in each aquarium, but most remained high, above 10^5/L. High densities of gene copies, i.e., those over 5×10^6 copies/L, decreased 100-fold by the next day (Figure 3B). The average density of *H. perezi* gene copies was not correlated with prevalence levels in naïve crabs in either year (2020, t=0.23, df=5, p=0.83, r=0.10; 2021, t=-1.23, df=5, p=0.27, r=-0.48). This holds true when tanks without transmission were removed (2020, t=-0.45, df=3, p=0.68, r=-0.25; 2021, t=-0.54, df=4, p=0.62 r=-0.26).

3.3. Survival, mortality, and sporulation

Survival of the infected donor crabs showed sharp declines over the first 20 days in each experiment, with more than 50% mortality in all aquaria. In both years, two clustered mortality

events occurred over short time periods, with an average of a 25% decrease in survival over a three-day period in each aquarium. In 2020, mortality events occurred on or around day 11 and day 19. In 2021, mortality events occurred earlier, on or around day 6 and day 17. The survival of the uninfected control crabs was 93% in 2020 and 83% in 2021 experiment (Figure 4). The survival of the exposed naïve crabs was above 75% in all aquaria and was not significantly different from the crabs in the control treatment (log-rank test, $p > 0.05$ for all groups). A three-way ANOVA revealed a significant effect of mortality and year on maximum *H. perezi* gene copies and a significant interaction between mortality and year (Table 2). Thus, sporulation appeared linked with mortality of donor crabs because the maximum number of *H. perezi* gene copies over time peaked with donor mortality, which was highest in the period 10-21 days after the start of each experiment.

4. Discussion

This study is the first report of natural transmission of *Hematodinium perezi* to blue crabs in the laboratory. Our results are in general agreement with the cohabitation trial done by Huang et al. (2021), but we further support our findings with a quantitative assessment of *H. perezi* DNA demonstrating direct, waterborne transmission. Although transmission was natural in our system, the resulting prevalence in naïve crabs varied widely between aquaria. Prevalence levels from natural transmission experiments varied from 29.6% in a cohabitation trial (Huang et al., 2021) to 12.7%-72% in sentinel studies in the field (Huchin-Mian et al., 2017; Shields et al., 2017). These difference in transmission success may be due to variation in host susceptibility, dinospore densities, dinospore viability, the ephemeral nature of dinospore release, environmental conditions, or combinations of these variables. For example, crabs are known to

sporulate non-infectious prespores or effete dinospores (Meyers et al., 1987; Huchin-Mian et al., 2018), possibly in relation to handling or confinement stress. In addition, dinospore viability is highly ephemeral, lasting only a few days and is significantly affected by time since release and changes in salinity (Coffey et al., 2012). These factors may explain the non-significant correlation between the density of *H. perezi* gene copies and prevalence levels in naïve crabs in this study. In contrast, Lycett and Pitula (2017) showed a positive correlation between environmental DNA density of *H. perezi* and prevalence in juvenile crabs from trawl surveys. Additional work is needed to better understand this aspect of the transmission dynamics of this system.

The high proportion of heavy infections in naïve crabs indicates that the transmission and development of *H. perezi* occurs over a short time period with infections establishing and developing quickly at the elevated temperature of 25°C. Similarly, heavy infection developed rapidly in naïve juvenile crabs after an exposure period of 7 d in sentinel studies in the field (Huchin-mian et al., 2017; Shields et al., 2017). Although infected naïve crabs were found with heavy infections, as indicated by the presence of the prespore stage in this current study, little mortality occurred in the naïve crabs during the experiments or the mortality occurred early, prior to infection by *H. perezi*. One explanation for the lack of infection mortality in these crabs is that the development period of infection may not have been long enough to proceed to sporulation. Naïve crabs were likely infected within three days of a sporulation event because dinospores are short-lived (Li et al., 2010; Coffey et al., 2012; Huchin-Mian et al., 2018). Thus, the maximum time for the development of patent infections, i.e., those ready to release new dinospores, would be 10-20 days. In adult crabs, mortality occurred over 5 to 40 days in inoculation trials (Shields & Squyars, 2000; Coffey et al., 2012) and in 4 days from natural

infection (Frischer et al., 2006). However, most of those crabs died without sporulation, indicating that the life cycle of *H. perezi* in blue crabs could be longer than expected. Another possibility is that sporogony/sporulation requires a trigger, such as changes in temperature, declines in metabolic products such as glucose, or other signals. This is supported by Huchin-Mian et al. (2018) who observed naturally infected crabs surviving for extended periods with moderate and heavy infections at 20°C and below. In addition, only 31.6% of their infected crabs held at 25°C released dinospores (Huchin-mian et al., 2018). Our results suggest that once acclimated to 25°C, infected naïve crabs may also survive an extended period with heavy infections before the parasite undergoes sporogony.

In the present study, the timing of sporulation, shown by days with high densities in *H. perezi* gene copy, was roughly consistent between aquaria. That is, the elevated temperature of 25°C may have contributed to an apparent synchrony in the release of dinospores from crabs as seen by the somewhat consistent sporulation occurring around 10-20 d after confinement at this temperature. This is also reflected by significant interactions between mortality and binned time. When high *H. perezi* gene copies were detected in water, donor mortality likely followed due to damage caused by sporulation. Moreover, in 2021, *H. perezi* DNA was detected earlier, potentially due to the higher environmental temperature (27 °C) that was occurring when the experiment started, expediting, or initiating sporulation events. This may partly explain the variation of prevalence in different seasons seen in the field, indicating an optimal temperature range for transmission (Gandy et al., 2015; Lycett and Pitula, 2017; Sullivan and Neigel, 2017). In the Chesapeake Bay region and in the coastal bays of Georgia, peak prevalence levels typically occur in early autumn (Messick & Shields, 2000; Frischer et al., 2006). In mid-summer, transmission may be hampered at temperatures greater than 25°C with infected crabs dying prior

to sporulation due to metabolic stress. However, because heavily infected crabs may survive for prolonged periods below 25°C, sporulation may only occur when temperature fluctuations trigger the progression of *H. perezi* into next stage. Temperature increases can clearly stimulate production as overwintering crabs with natural infection show logarithmic increases in the density of *H. perezi* cells when held under elevated temperature (Shields et al., 2015).

There were differences in findings between the 2020 and 2021 experiments. These are most likely due to variation in infection intensities, experimental variation between years, and sampling differences between years. For example, in the 2021 experiment, *H. perezi* DNA was detected at higher frequencies than in the 2020 experiment, and the maximum densities of gene copies/L detected were 10-fold higher. Although reagent and template quantities were different for qPCR between 2020 and 2021, the efficiency and range of detection was not affected. The mean size of donor crabs was not significantly different between aquaria. Indeed, the main difference between replicates was sampling location in the aquaria. The higher gene copy densities detected in 2021 may have resulted from sampling the bottom of the aquaria vs. the top of the aquaria in 2020. Surface water has more current flow and mixing than bottom water and may be a less preferred environment for the dinospores. However, in 2021, daily samples between day 12 to day 21 typically had higher densities of *H. perezi*, above 10^5 gene copies/L in most aquaria. Although the dinospores of *H. perezi* are known to be short-lived, small-scale variation in spore densities has not been previously reported. It may be due to a number of factors. 1) Although there was evidence of more dinospores being released in 2021, mortality of infected crabs was not different between years and crab biomass was similar between years. 2) Dinospores may be longer lived than previously thought, but the spikes in abundance and patterns in detection support a short-lived dinospore, possibly as a gamete. 3) An unknown cyst

stage may be present in benthic samples, but there is at present no evidence for this. 4) There are two types of dinospores in the life cycle, the micro-dinospore and the macro-dinospore (Chatton and Poisson, 1931; Li et al., 2011; Huchin-Mian et al., 2018). The two dinospores likely have different functional behaviors, either swimming into the water column for rapid dispersal (e.g., rapidly swimming micro-dinospore), or sinking to the benthos (e.g., slowly swimming macro-dinospore). Their short viability and behavior are suggestive of gametes. Such differences in behavior could account for different distributions in the water column, a feature that we likely missed in our sampling and one that needs more experimental investigation to determine their importance in transmission of this parasite.

Dinospores have been proposed as the infectious stage in many studies (Shields, 1994; Frischer et al., 2006; Li et al., 2010; Lycett and Pitula, 2017; Shields et al., 2017), but the functions of the two dinospore types have not been considered. Although laboratory transmission with dinospores has not been reported, the short period of transmission in this study and in several field studies indicates that either the dinospores are the transmissive stage or they lead to an infectious stage. All syndinid dinoflagellate produce micro- and macro- dinospores. Meyers et al. (1987) conjectured that they may be gametes, but was later ruled out because each has similar DNA mass comparing to the vegetative stage and both are infectious by injection (Eaton et al., 1991). With successful transmission in this study and the ability to successfully generate spores using temperature changes (Huchin-Mian et al., 2018), the long-standing question regarding *H. perezi* having two spore types can now be investigated in more detail.

5. Conclusions

Our findings on transmission dynamics of *H. perezi* in juvenile blue crabs confirm the long-standing notion that the parasite is transmitted via water-borne transmission of dinospores without an intermediate host. The high mortality of donor crabs and subsequent peaks of *H. perezi* DNA in the water help to explain the natural seasonality of prevalence observed in the Delmarva Peninsula. With alterations in the experimental design, natural transmission in the laboratory may provide an opportunity to further understand the mode of transmission, aspects of viability of the dinospores, early development of *H. perezi* in the host, and its interaction with the host immune system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was partially supported by the National Science Foundation (EEID Program grant # 2207343), the Virginia Institute of Marine Science, and the Willard A. Van Engel Fellowship program. The authors thank Richard Snyder, Sean Fate, Edward Smith, Gail Scott, Victoria Simmons, Gabe Thompson, and Alexandra Schneider for their invaluable technical assistance.

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Tables

Table 1. Prevalence of *Hematodinium perezi* in naïve crabs sampled from experimental and control aquarium at the end of the experiment. Each tank started with 15 live donor crabs and 15 live naïve crabs. Dead donor crabs were replaced prior to day 20, with the total number dying indicated. Controls consisted of 30 naïve crabs at start of the experiment.

Aquarium	No. of dead donors	No. of naïve crabs	Prevalence (%)
2020 Experiment			
1	8	15	100
2	19	15	67
3	13	14	64
4	14	15	60
5	10	12	17
6	9	14	0
7	15	13	0
Control		27	0
2021 Experiment			
1	11	13	69
2	11	15	53
3	13	14	36
4	21	14	14
5	11	15	13
6	16	14	7
7	13	15	0
Control		25	0

Table 2. Three-way ANOVA on the maximum number of *H. perezi* gene copies in relation to donor crab mortality, time, and year. Data on time were binned into three groups based on experiment days: 0-9, 10-21, and 22-30 days. Year was included as a predictor due to the difference in water sampling in 2020 and 2021. The assumption of equal variances was not met.

Predictor	Sum of Squares	df	Mean Square	F value	Pr (>F)
Mortality	33.28	1	33.28	13.189	0.00087 ***
Year	92.13	1	92.13	36.516	6.09e-07 ***
Time	15.63	2	7.82	3.098	0.05734
Mortality*Year	14.71	1	14.71	5.832	0.02094 *
Residuals	90.83	36	2.52		

Note: * indicates $p < 0.05$, *** indicates $p < 0.001$

Figure Legends

Figure 1. Status of crabs infected with *Hematodinium perezi* during this study. (A) Proportional frequency of light, moderate and heavy intensity infection in crabs from the preliminary sample, and crabs held as donors or naïve crabs that became infected. A preliminary sample of crabs was dissected from the hyperendemic location where donors were obtained and were assessed before the start of the experiment. Donor and naïve crabs were assessed at the end of the experiment. Crabs from the two experiments were pooled for this analysis. (B) Proportional frequency of life history stages observed in crabs from the preliminary sample, donors, and naïve crabs that became infected. A preliminary sample of crabs was dissected from the hyperendemic location where donors were obtained and were assessed before the start of the experiment. Donor and naïve crabs were assessed at the end of the experiment. Lighter color bar indicates earlier stage of infection.

Figure 2. Densities of ITS2 gene copies of *Hematodinium perezi* estimated from real-time PCR in the 2020 experiment. Every three days, 100 ml of water samples were taken from the surface in each aquarium. Quantification limit is at 7000 copies/L.

Figure 3. Densities of ITS2 gene copies of *Hematodinium perezi* estimated from real-time PCR in the 2021 experiment. Every three days, 250 ml of water samples were taken from the bottom in each aquarium from day 0 to day 12, and day 21 to day 30. From day 12 to day 21, daily water samples were taken (inset). (A) ITS2 gene copies every three days with 3-day average from day 12 to day 21. (B) Daily ITS2 gene copies between day 12 and day 21. Quantification limit is at 7000 copies/L.

Figure 4. Survival curves of donor crabs in the treatment groups and naive crabs in the control group over 30 days in (A) 2020 and (B) 2021 experiments. Control crabs are naïve, uninfected crabs (black line). Replicates with donor and naive. Two aquaria in 2020 were kept for more than 30 days without significant mortality.

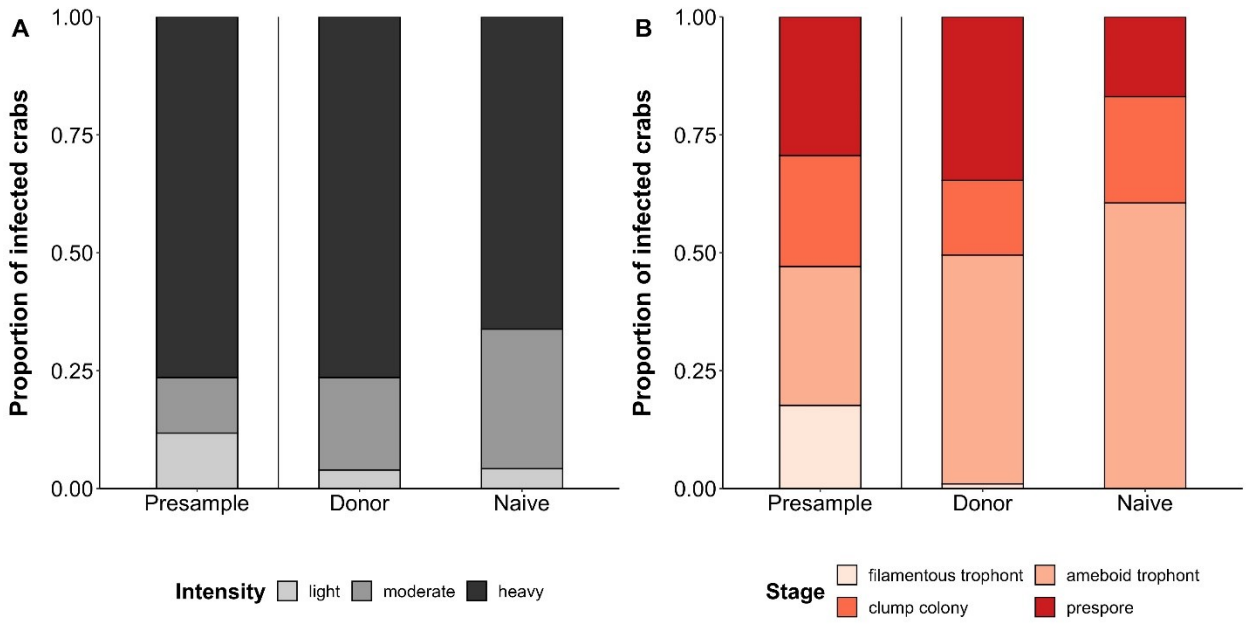

Figure 1. Status of crabs infected with *Hematodinium perezi* during this study. (A) Proportional frequency of light, moderate and heavy intensity infection in crabs from the preliminary sample (n=17), and crabs held as donors (n=102) or naïve crabs (n=71) that became infected. A preliminary sample of crabs was dissected from the hyperendemic location where donors were obtained and were assessed before the start of the experiment. Donor and naïve crabs were assessed at the end of each experiment. Crabs from the two experiments were pooled for this analysis. (B) Proportional frequency of life history stages observed in crabs from the preliminary sample, donors, and naïve crabs that became infected. Sample sizes as in (A). A preliminary sample of crabs was dissected from the hyperendemic location where donors were obtained and were assessed before the start of the experiment. Donor and naïve crabs were assessed at the end of the experiment. Lighter color bar indicates earlier stages of infection.

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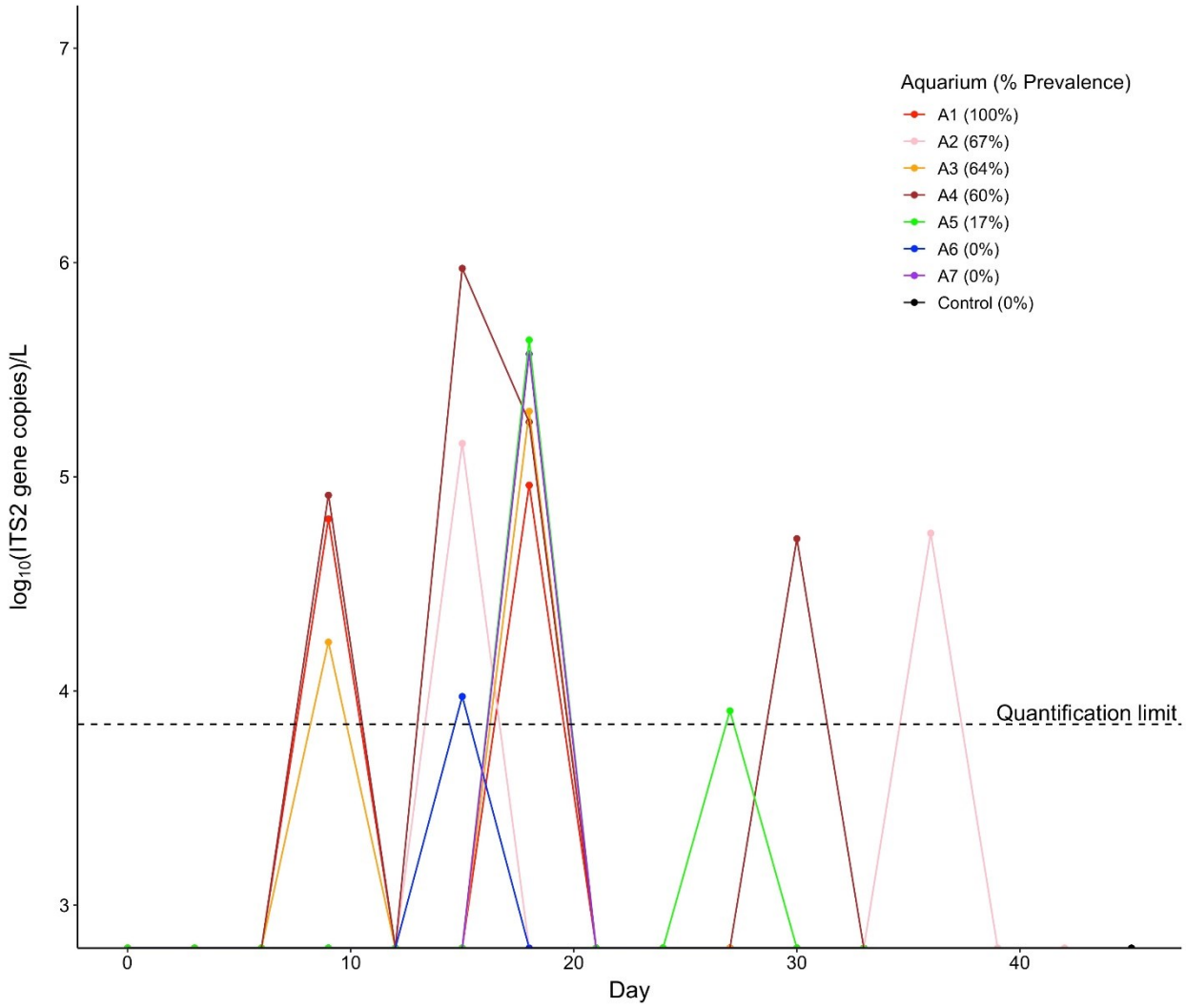

525 **Figure 2.** Densities of ITS2 gene copies of *Hematodinium perezi* estimated from real-time PCR
526 in the 2020 experiment. Every three days, 100 ml of water samples were taken from the surface
527 in each aquarium. Quantification limit (dotted line) is 7000 copies/L.

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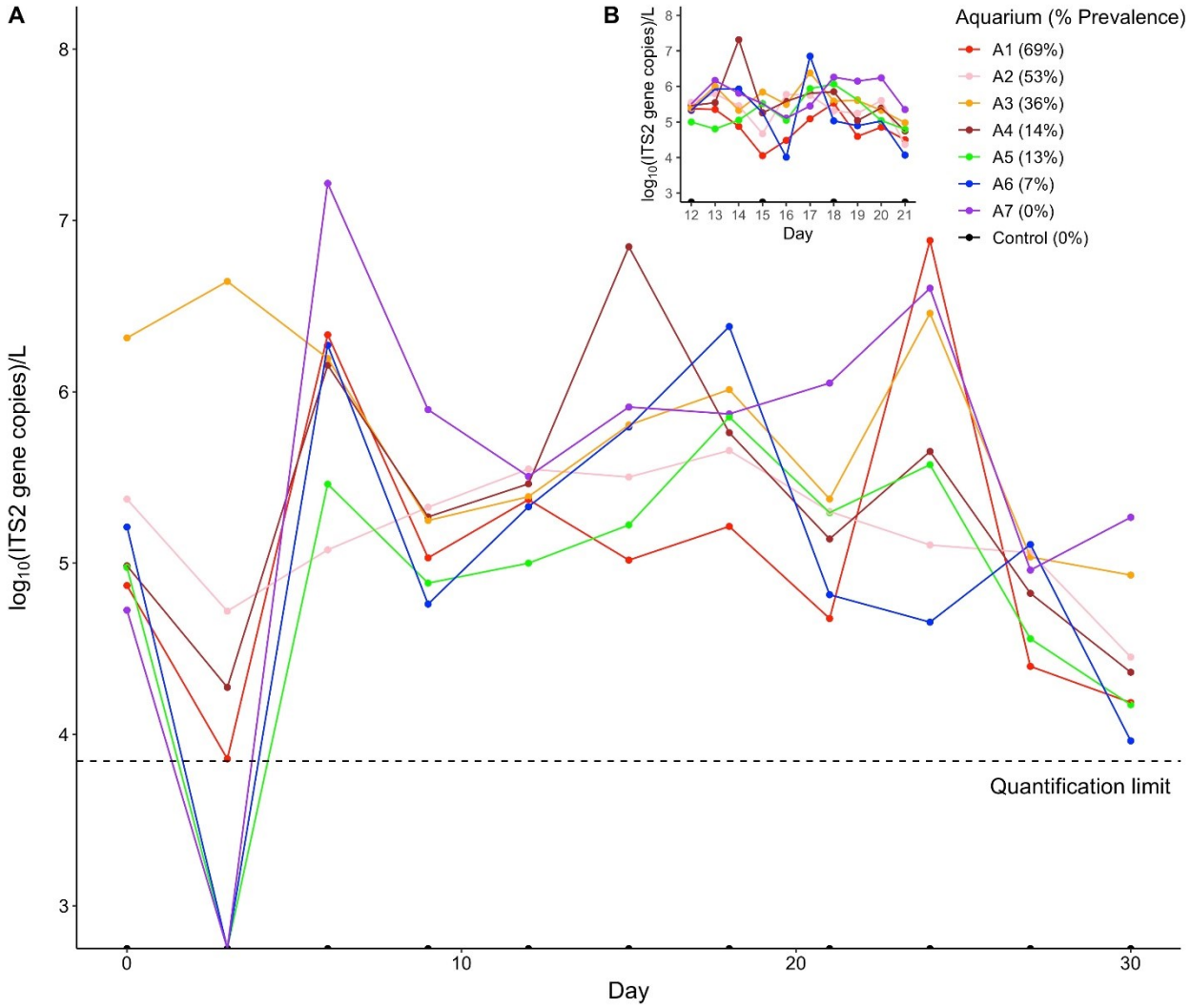

Figure 3. Densities of ITS2 gene copies of *Hematodinium perezi* estimated from real-time PCR in the 2021 experiment. Every three days, 250 ml of water samples were taken from the bottom in each aquarium from day 0 to day 12, and day 21 to day 30. From day 12 to day 21, daily water samples were taken (inset). (A) ITS2 gene copies every three days with 3-day average from day 12 to day 21. (B) Daily ITS2 gene copies between day 12 and day 21. Quantification limit (dotted line) is 7000 copies/L.

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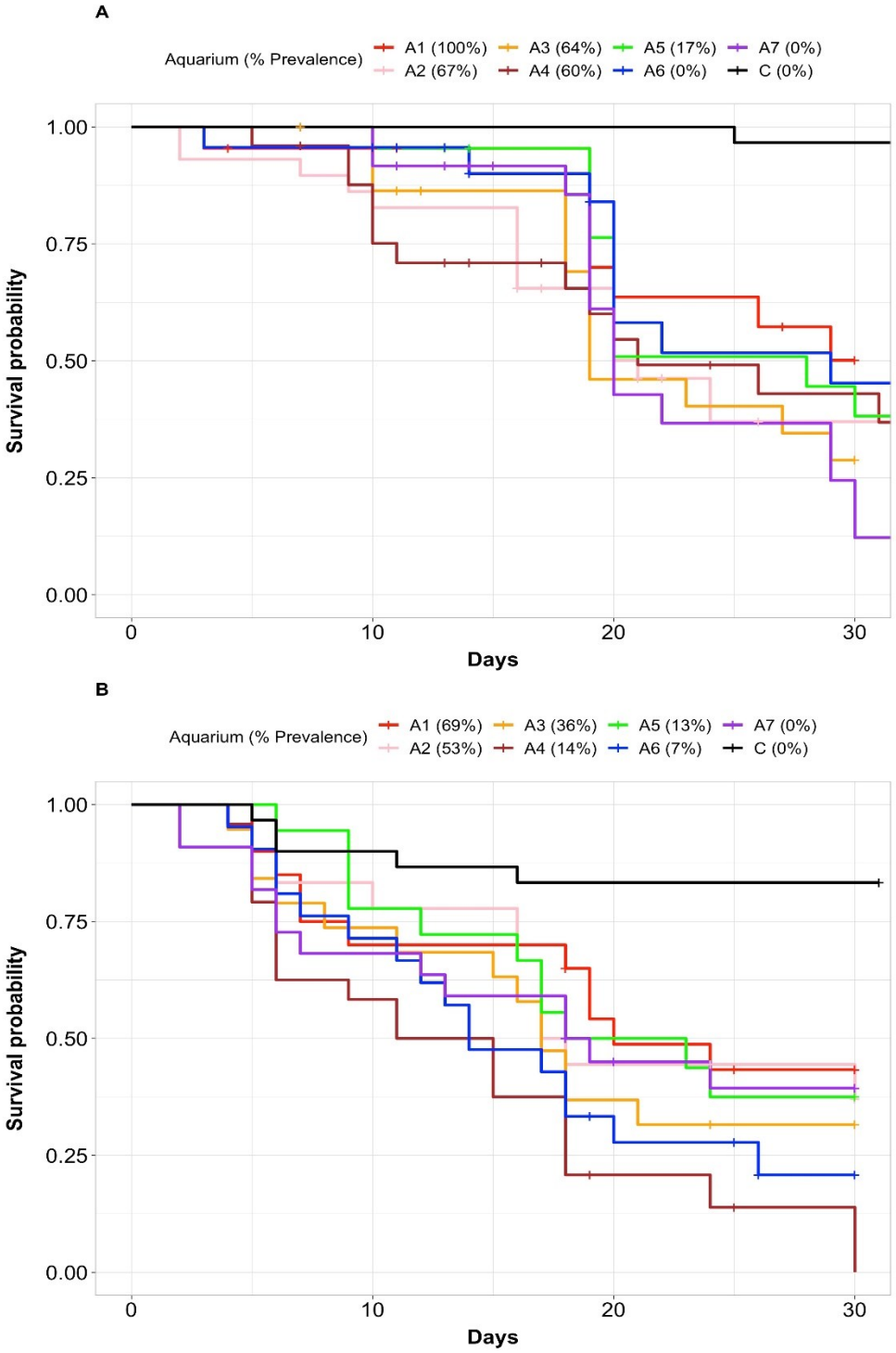

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Figure 4. Survival curves of donor crabs (colored lines) in the treatment groups and naive crabs in the control group over 30 days in the (A) 2020 and (B) 2021 experiments. Control crabs are naïve, uninfected crabs (black line). Experimental replicates consisted of 15 donor and 15 naïve crabs. Two aquaria in 2020 were kept for more than 30 days without significant mortality (last 10 day not shown to retain scale).