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A METHODOLOGY TO PRODUCE SPECIFIC-PATHOGEN-FREE PENAEID SHRIMP FOR USE IN EMPIRICAL INVESTIGATIONS OF PARASITE ECOLOGY

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ABSTRACT Emerging infectious diseases in marine systems threaten food security and ecosystem function. Experiments to determine drivers of transmission and mortality from emerging pathogens require a reliable supply of disease-free animals. Imperfect detection of subclinical, latent, and early stage infections, however, necessitates effective treatments to remove infection from animals with unknown infection status. In this study, a procedure utilizing elevated temperatures and over-the-counter parasiticides to remove *Hyalophysa lynni* was developed. The apostome ciliate *H. lynni* is responsible for shrimp black gill (sBG) disease in commercially important southeastern shrimp fishery species, including *Litopenaeus setiferus* (white shrimp). Following a series of pilot studies to determine parasiticides combination and dosage, *L. setiferus* of unknown infection status were exposed to either 20°C or 30°C artificial seawater, and a parasiticide cocktail of metronidazole (3.15 mg L−1) and Cu+2 (0.5 mg L−1) or a medication-free control for 14 days in a fully crossed experimental design. All treatments, except for the 20°C without medications treatment, significantly reduced pathogen prevalence. The 30°C artificial seawater with parasiticide treatment resulted in complete curing of the experimental population, but shrimp mortality in this treatment was high (50%). This high mortality may have contributed to the low parasite prevalence found in this treatment by differentially culling infected individuals. If this is the case, the parasiticides may lower the parasite prevalence both by curing infections and heightening stress and subsequent mortality of diseased individuals. Although this procedure was developed to produce *H. lynni*-free shrimp to facilitate experimental studies of sBG disease, it is likely that this methodology would effectively produce specific-pathogen-free individuals in other crustacean species.

KEY WORDS: *Litopenaeus setiferus*, *Crustacea*, *Hyalophysa lynni*, shrimp black gill, disease, parasiticides, gill parasite, apostome ciliate

INTRODUCTION

Many marine systems are experiencing emerging infectious diseases that cause significant mortality in commercially and ecologically important species, threatening food security, and ecosystem function (Lafferty et al. 2015, Byers 2020). Research into the drivers of disease in these systems is often hindered by challenges in disease detection, identification of major pathways of transmission, and factors affecting host mortality. Experimental laboratory studies where uninfected hosts are exposed to potential sources of infection are crucial for understanding how processes such as transmission and recovery from infection are influenced by both biotic (e.g., host density) and abiotic factors (e.g., environmental temperature). In wild systems where a source of uninfected stock is not available, developing methods to produce specific-pathogen-free (SPF) hosts to facilitate empirical studies is critical. These uninfected hosts can then be used as susceptible individuals to measure rates of disease transmission in laboratory or field settings. This is particularly applicable for crustacean hosts, which depend on an innate immune system (Vazquez et al. 2009, Hauton 2012), allowing recovered individuals to become reinfected with the pathogen of interest. One such system is shrimp black gill disease (sBG) that is currently of concern in the South Atlantic Bight and Gulf of Mexico (Frischer et al. 2017, 2022).

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The shrimping industry is one of the largest and most valuable commercial fisheries in the Southeastern US, including the state of Georgia (Gillett 2008, Georgia Commercial Seafood Landings 2019). All examined species of penaeid shrimp, including the two species of shrimp that comprise the majority of the Georgia fishery, white shrimp (*Litopenaeus setiferus* Linnaeus) and brown shrimp (*Farfantepenaeus aztecus* Ives), are susceptible to infection by the apostome ciliate *Hyalophysa lynni* (Landers et al. 2020). The life cycle of *H. lynni* has a simple life cycle with a latent stage (phoront), a parasitic stage (trophont), a reproductive stage (tomont), and a free-swimming, transmissible stage (tomite). The life cycle of *H. lynni* is similar to nonpathogenic apostome ciliates, including the closely related species *Hyalophysa chattoni*, except that the trophont stage feeds on living tissue and elicits the response of the shrimp innate immune system (Landers et al. 2020). During the phoront stage, the ciliate has yet to invade the host gill tissue and shrimp exhibit clean gill tissue. Once *H. lynni* has developed into the trophont stage, the generalized immune response of the shrimp forms the melanized nodules that are responsible for the characteristic darkened appearance of gill tissue. This response further leads to necrosis of the gill tissue and loss of gill lamellae (Landers et al. 2020). Mortality caused by sBG is thought to result from the inability of infected shrimp to absorb adequate oxygen from the water, leading to death in low oxygen environments or because of increased vulnerability to predation (Gooding et al. 2020). The evidence for a direct causal effect of the ciliate on shrimp populations is still under investigation. Although the decline

in commercial shrimp landings has coincided with the emergence of sBG (Frischer et al. 2017), fisheries-independent data have been unable to detect a relationship between white shrimp abundance estimated from Catch Per Unit Effort (CPUE) and sBG prevalence (Fowler et al. 2018).

Warm temperatures are thought to interact with both the presence and severity of sBG (Frischer et al. 2017, Fowler et al. 2018). In the South Atlantic Bight region, shrimp with black gills are only observed in the warmer months of the year (April–October) when water temperatures in the estuary are generally above 18°C (Power & Walker 2001, Verity & Borkman 2002). Once shrimp have mounted an immune defense against the ciliate, the darkened gill tissue is present until the shrimp host molts its exoskeleton. It is currently unknown whether molting eliminates the parasite from the host, and/or if shrimp hosts can shed the parasite without molting. Although the duration of the *Hyalophysa lynni* life cycle is unknown, increasing temperature likely accelerates the speed that *H. lynni* can complete its life cycle (Müller 2002, Weisse et al. 2002, Fels & Kaltz 2006). This may contribute to the high prevalence observed in the warm water months (Frischer et al. 2017), although across years the mean prevalence in summer is negatively correlated with the water temperature of the preceding winter (Kendrick et al. 2021). Additionally, increases in shrimp mortality are associated with warmer water temperatures (Frischer et al. 2017); this is likely because of the interaction among temperature, ciliate infection intensity, gill structure degradation, and low levels of dissolved oxygen in warm water (Frischer et al. 2018).

Although the seasonal pattern of sBG prevalence is well-documented, the factors influencing this pattern are not yet understood. Although the Georgia Department of Natural Resources Coastal Resources Division (GA CRD) has conducted monthly trawling surveys to monitor shrimp stocks since 1976 (Georgia Ecological Monitoring Trawl Survey; Page 2012) and has recorded the prevalence of sBG beginning in 1996 (C. Belcher, GA CRD, personal communication), there is little understanding of the factors that drive transmission of the ciliate. Previous experiments attempting to quantify transmission and mortality have been hindered by the lack of known uninfected animals (Frischer et al. 2018). Early or mild *Hyalophysa lynni* infections often do not result in the visible appearance of dark gill tissue, thus, these first experiments were unable to determine *a priori* if the animals in their experiments with white gills were uninfected or in the nascent stages of infection (Frischer et al. 2018).

Specific-pathogen-free (SPF) animals (i.e., laboratory animals that are guaranteed to be free of particular pathogens) are commonly used in studies of human and animal health (e.g., clinical trials) as well as commercial production industries. These animals are frequently used to measure the transmission and pathology of diseases of concern (Povey & Hale 1974, Kerr et al. 2017, Tomamichel et al. 2021). In crustacea, SPF animals are most commonly used as "seed" stock to prevent losses from disease in aquaculture (Lotz 1997, Lightner et al. 2009, Lightner & Redman 2012). Research conducted on infectious diseases, including white spot syndrome in shrimp, has extensively used SPF *Litopenaeus vannamei* in studies measuring pathogenicity, transmission, and host genetic resistance (Khadijah et al. 2003, Escobedo-Bonilla et al. 2005, 2007). Captive breeding by isolating young animals into populations that are never exposed to the pathogen are typically used to produce SPF animals (Barry & Strelow 2008), although they are also sourced from geographic areas or facilities that have a known absence of an infectious agent (Dahl et al. 2011, Tomamichel et al. 2021). There is concern that SPF animals procured in this way will respond differently to experimental manipulation than normal animals due to differences in their microbiomes and local animals acquiring a genetic predisposition toward tolerance of an endemic pathogen and/or local environmental conditions (Schade et al. 2014, Letson et al. 2019, Dobson et al. 2020). Therefore, it is preferable to source animals from the local environment to use in experiments measuring factors influencing transmission and pathology of an endemic disease.

The purpose of this study was to develop a method of removing *Hyalophysa lynni* from white shrimp sourced from the local environment to enable their use in other experimental investigations. A mixture of two over-the-counter aquarium drugs metronidazole (3.15 mg L^{-1}) and Cu^{+2} (0.5 mg L^{-1}) (Metroplex and Cupramine; Seachem, Madison, GA) and a no-drug control were experimentally administered over two temperature treatments (20°C and 30°C) in a fully crossed design. Temperature was important to manipulate because it plays a key role in many aquatic host and parasite processes, including metabolism (Gehman et al. 2018, Byers 2021). Cupramine and Metroplex are commonly used to treat a variety of parasitic protozoans and anaerobic bacterial infections in finfish, including the parasitic ciliate *Cryptocaryon irritans*, which is a common pest in the ornamental fish trade. These medications are administered directly to the aquarium water, making them a convenient and effective way to treat the external stages of parasitic infections. Dosages, experimental temperatures, and shrimp density were chosen based on a series of range-finding pilot studies described in the following section.

MATERIALS AND METHODS

Temperature, Density, and Dose Range-Finding Pilot Studies

A series of range-finding experiments performed between 2017 and 2019 identified the appropriate temperature conditions, shrimp density, length of treatment, and parasiticide dosage and combination. Because of the small sample sizes, limited replication, and substantial inter-experiment variability, results from the pilot studies were used to guide the main experiment and not formally analyzed. In 2017, three studies were performed that experimented with the dose and combination of commercially available parasiticides: Metroplex, Paraguard, and Cupramine, and their effect on shrimp survival. In these studies, five shrimp (*Litopenaeus setiferus*) per treatment were maintained in either 5-gallon (19 L) or 10-gallon (38 L) aquaria with a range and combination of the commercially available parasiticides. Dosages that ranged between 0.5 and 2 times, and up to 5 times, the dosages for aquarium fish recommended by the manufacturer (Seachem), were tested. Mortality, visual disease status, and molting frequency were monitored. Typically, disease status was monitored by assessing the visible condition of the shrimp each day, and at the beginning and end of each study using a polymerase chain reaction (PCR)-based diagnostic assay (Frischer et al. 2017). Shrimp were caught locally from the Wassaw Sound estuary, GA. Temperature was maintained

at approximately 25°C but varied by up to 5°C over the course of these studies. Aquaria were fully aerated and each shrimp was fed either ~1 g of squid or commercial shrimp pellets per day. Treatments lasted for 2 wk in each study as recommended by Seachem for the treatment of *Cryptocaryon irritans*. Water changes (50%) were conducted every other day with fresh drug cocktails added to maintain target drug dosages. These experiments found that notable mortality (≥30%) occurred in the 3× (1.5 mg L^{-1}) Cupramine and 1× (4.2 mg L^{-1}) and 2× (8.4 mg L^{-1}) Metroplex treatments.

Further preliminary studies documented the reduction in *Hyalophysa lynni* prevalence under various experimental conditions. An additional experiment in 2017, that held *Litopenaeus setiferus* of unknown infection status in static filtered seawater at approximately 25°C, found that dosing shrimp with a 2× dose of Cupramine (Cu^{+2}; 1 mg L^{-1}) or a 0.5× dose of Metroplex (2.1 mg L^{-1}) reduced the prevalence of *H. lynni* infections from 50% (as measured from an initial subsample) to 0%, although this was not statistically different from the unmedicated control (25% prevalence). This experiment was conducted with shrimp husbandry protocols similar to the abovementioned studies and suggested that 14 days was sufficient to allow all shrimp to complete a molt cycle during the experimental treatment. Additionally, this study noted high mortality in the Cupramine treatment (33%). In 2018, an experiment housed *L. setiferus* individually in 15 L artificial seawater without medications at 31°C and found that only a single shrimp tested positive for *H. lynni* with PCR (4%), whereas 69% of shrimp held communally during the same time period tested positive for the ciliate. This experiment suggested that low host density, artificial seawater, and elevated temperatures may induce host recovery without the use of parasiticides. In 2019, an attempt to reduce prevalence in large batches of shrimp while mitigating losses due to mortality housed 20 shrimp in 44 L at approximately 20°C and dosed with 3.15 mg L^{-1} (0.75×) dose of Metroplex. Although few shrimp died over this 14-day study, this treatment was unable to reduce the prevalence of sBG infection. Together, these studies suggest that reducing the prevalence of sBG is possible, but it remained unclear if the use of parasiticides was necessary or if temperature played a significant role in host recovery. Thus, the main experiment utilizes the less lethal, but most effective, combination of drugs and dosages [Metroplex (metronidazole; 3.15 mg L^{-1}, 0.75× dose) and Cupramine (Cu^{+2}; 0.5 mg L^{-1}, 1× dose)] and tests the effect of two temperatures on the efficacy of this drug cocktail. The density of shrimp in tanks for the main experiment was limited to a relatively low density of 10 shrimp per 44-L tank because low density had been successful in the 2018 trials.

Shrimp Collection and Experimental Facilities

Approximately 200 white shrimp were purchased from a local bait shop in July 2019. These shrimp had been collected from the Vernon River in the Ogeechee Estuary (31.9° N, 81.1° W), were all approximately the same length (82 mm, SE = 3.9 mm) and 79% were female. Shrimp were transported from the bait shop (20 min) to the University of Georgia, Skidaway Institute of Oceanography (SkIO) in Savannah, GA, in 60-L coolers with 100 shrimp per cooler; coolers were filled with water from the estuary and aerated vigorously. At the laboratory, shrimp were held for 4 days in two 125-L tanks that received flowing natural seawater with vigorous aeration. This holding period allowed the animals to acclimate to laboratory conditions. Twenty shrimp were euthanized and processed for sBG detection (as described in the following) immediately upon arrival at SkIO (initial sample) and again after the 4-day holding period (postacclimation sample) to determine whether this holding procedure altered the prevalence of *Hyalophysa lynni* (Fig. 1). Shrimp were fed a commercial pellet feed daily (Hyper-Intensive Shrimp 35; Zeigler Inc.), approximately 1 g of feed per animal. Shrimp were then placed into experimental conditions.

Experimental Setup

The effect of temperature and the efficacy of the aquarium medications to rid shrimp hosts of the *Hyalophysa lynni* parasite were tested. This experiment took place between July 17 and 31, 2019. Artificial sea salt (Instant Ocean; Aquarium Systems, Inc.) was mixed with ambient temperature fresh water to a salinity of 28–30 to create artificial sea water that was free of the *H. lynni* parasite. The artificial seawater was then pumped into eight 44-L tanks that were held in a climate-controlled laboratory at 20°C, and into four 44-L tanks housed in a separate, seawater laboratory (Fig. 1). The four tanks in the seawater laboratory were partially submerged in a water bath of flowing estuary water to maintain the temperature similar to that in the estuary. Over the course of the study temperature in the estuary and experimental treatments ranged from 29°C to 32°C, henceforth referred to as the 30°C treatment. Ten shrimp were then added to each tank (1 shrimp/4.4 L). Metroplex (metronidazole 3.15 mg L^{-1}) and Cupramine (Cu^{+2} 0.5 mg L^{-1}) were mixed with 408 mL of warm freshwater, and 35 mL of this drug cocktail was administered to four tanks in the 20°C treatment and two tanks in the 30°C treatment. The remaining four tanks in the 20°C treatment and two tanks in the 30°C treatment were left unmedicated.

Shrimp molts and dead shrimp were removed daily to prevent the water from fouling. Every other day, a 50% water change was performed with water that was held at the same temperature as the experimental tanks, and the drug cocktail was readministered. After 14 days, all remaining shrimp were euthanized and processed for *Hyalophysa lynni* detection. A 14-day period was chosen based on the recommended treatment duration for *Cryptocaryon irritans* infection from the manufacturer and to correspond with a typical *Penaeid* shrimp molt cycle (Corteel et al. 2012).

Shrimp Black Gill Detection

The length of each sampled shrimp was measured to the nearest millimeter as measured from the tip of the rostrum to the end of the tail, and sex and visual presence of sBG was recorded and scored based on the severity of melanization of gill tissue (clean, light brown, brown, and black). Visual sBG was used to evaluate the presence of symptomatic infection. Gill tissue was removed from euthanized shrimp and preserved in 1.5 mL of 70% non-denatured ethanol for molecular analysis. Total Genomic DNA from ethanol preserved tissue was purified using the DNeasy blood and tissue kit (QIAGEN Inc., Valencia, CA) and *Hyalophysa lynni* infection detected by PCR analysis as previously described (Frischer et al. 2017).

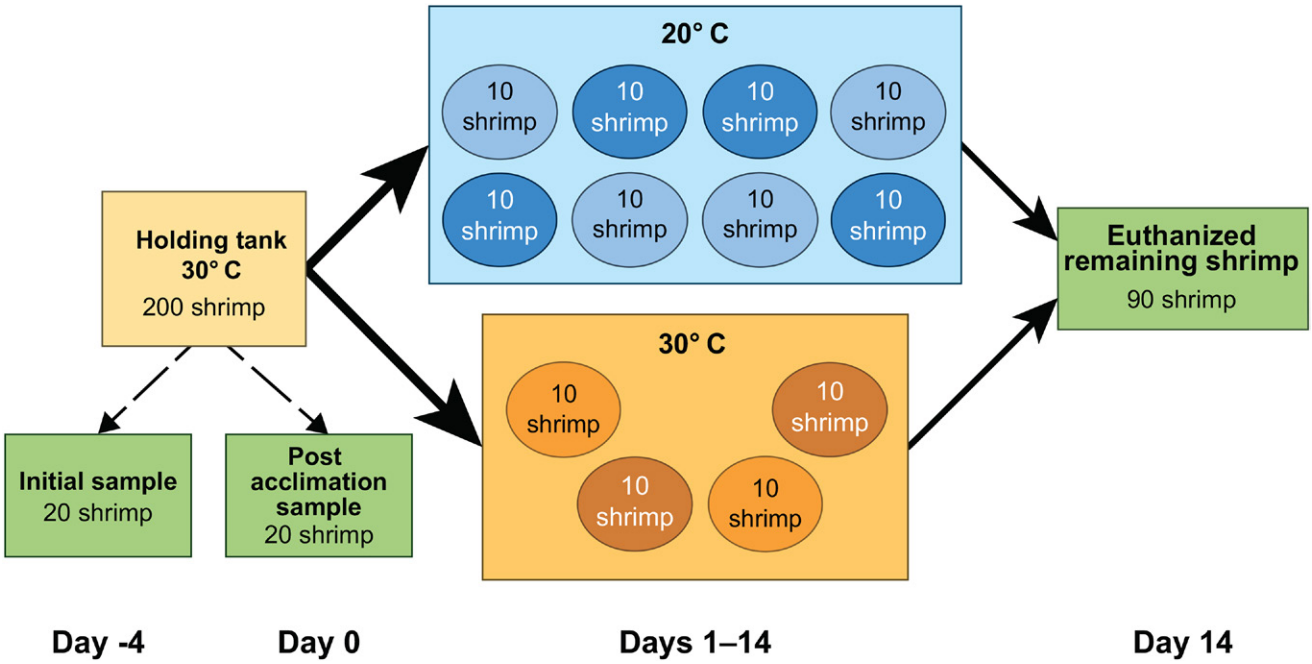

Figure 1. Diagram of experimental setup and sampling timeline. Approximately 200 white shrimp were purchased from a local bait shop in July 2019 and held in a holding tank with flowing estuary water (approximately 30°C). Twenty shrimp were euthanized to measure the prevalence of *Hyalophysa lynni* in the initial sample. Four days later, an additional 20 shrimp were sampled as the postacclimation sample and are representative of the animals distributed into the experimental treatments. Eighty shrimp were distributed into eight tanks and held at 20°C (blue circles and box) and medications were administered to four of these tanks (dark blue). Forty shrimp were distributed into four tanks surrounded by flowing estuary water (orange circles and box), which maintained the tanks at approximately 30°C, and medications were administered to two of these tanks (dark orange). After 14 days, the remaining 90 shrimp (64 from the 20°C treatments and 26 from the 30°C treatments) were euthanized and processed for *H. lynni* infection.

Probability of Infection and Survival

Logistic regression was used to detect differences in *Hyalophysa lynni* infection among the initial, postacclimation, and experimental treatments because logistic regression uses a binomial error distribution that corresponds to the binary nature of infection presence [i.e., a shrimp is infected (1) or uninfected (0) with *H. lynni*]. The probability that a shrimp was infected in the initial sample was compared with the probability that a shrimp was infected in the postacclimation sample to determine whether the holding period altered infection prevalence. The probability of infection in the postacclimation sample was then compared with the experimental treatments to determine whether these treatments reduced the probability of infection from this baseline measure. The experimental treatments were 30°C and 20°C with and without medications. To understand whether medication affected shrimp differently depending on the temperature, we performed an additional logistic regression comparing the probability of infection between the 20°C with medications and the 30°C with medications treatments.

Logistic regression was also used to understand how the probability of survival differed between experimental treatments. Specifically, we compared the probability of survival in the 20°C without medications treatment to the rest of the experimental treatments. To understand whether temperature-medication combinations affected shrimp survival, we compared the probability of survival in the 20°C treatment with medications to the 30°C with medications treatment and the probability of survival between the 30°C with and without medications.

Finally, the influence of tank was investigated as an additional random effect in the two 20°C treatments. Because of facility constraints, there was not sufficient replication of the 30°C treatments to statistically control for the random effect of tank. Instead, the two 20°C treatments (which had more replication) were analyzed with both a fixed effect and mixed effect logistic regression model (i.e., that included tank as a random effect). This additional analysis determined (1) whether the probability of infection between the two 20°C treatments was statistically different and (2) allowed insight into the effect of tank in our experimental treatments. Although the effect of tank was not analyzable statistically in the 30°C treatments, understanding the influence of tank in the 20°C treatments helps to contextualize the results of the experiments and provides confidence that the trends detected are not solely an artifact of the experimental design. All statistical analysis were performed using R (R Version 4.1.3 2022).

Minimum and Maximum Recovery Rate

The minimum and maximum recovery rate for each experimental treatment was calculated based on the estimated change in *Hyalophysa lynni* prevalence over the course of the experiment. Because shrimp must be euthanized to determine infection status, it was not possible to determine the infection status of each shrimp at the start of the experiment. Additionally, it was not possible to detect *H. lynni* infections on shrimp that suffered

mortality throughout the experiment due to rapid gill degradation postmortem. Thus, the *H. lynni* prevalence in the postacclimation sample (which was the pool from which experimental shrimp were randomly drawn) was used to estimate the number of infected and uninfected shrimp per treatment on day 0. The minimum recovery rate was deduced by assuming that all of the shrimp that died during the experiment were infected; therefore, any change in the number of uninfected shrimp in the treatment was due to infected shrimp recovering. This difference was then divided by the estimated initial number of infected individuals to calculate the percentage of shrimp that recovered from the infection. This is described by Eq. 1, where R_{min} is the minimum recovery rate for the treatment, I_0 is the number of infected shrimp in the treatment on day 0, U_0 is the number of uninfected shrimp in the treatment on day 0, and U_{14} is the number of uninfected shrimp in the treatment on day 14 (the end of the experiment):

$$R_{\min} = \frac{U_{14} - U_0}{I_0} \tag{1}$$

The maximum recovery rate was calculated by assuming the opposite extreme, that is, all the shrimp that died during the experiment were uninfected on day 0. Therefore, any additional change in uninfected shrimp on day 14, after mortalities are accounted for, were the result of shrimp that were infected on day 0 recovering from infection. This is described by Eqs. 2 and 3, where R_{max} is the maximum recovery rate of the treatment and D is the number of dead shrimp in the treatment:

if $D \leq U_0$

$$R_{\max} = \frac{U_{14} - (U_0 - D)}{I_0} \tag{2}$$

If the number of dead shrimp exceeded the number of uninfected shrimp on day 0, the excess deaths had to come from infected shrimp.

if $D > U_0$

$$R_{\max} = \frac{U_{14}}{I_0} \tag{3}$$

RESULTS

Overall, 75% of shrimp survived until the end of the study; however, the amount of mortality each treatment experienced varied greatly (Table 1). In the 20°C treatments, 64 out of the 80 shrimp survived, and 26 out of 40 shrimp survived in the 30°C treatments. Of the shrimp that survived until the end of the experiment, 40 tested positive for *Hyalophysa lynni* by PCR

TABLE 1.

Prevalence of sBG and shrimp survival.

	Sample group	Group level prevalence	Survival	sBG visual score		
					Count	Percentage
Baseline	Initial	85%	N/A	Clean	12	60%
				Light brown	3	15%
				Brown	4	20%
				Black	1	5%
	Postacclimation	80%	N/A	Clean	1	5%
				Light brown	12	60%
				Brown	4	20%
				Black	3	15%
Experimental treatments	20°C	78%	93%	Clean	36	97%
				Light brown	1	3%
				Brown	0	0%
				Black	0	0%
	20°C + medications	29%	66%	Clean	25	92%
				Light brown	1	4%
				Brown	1	4%
				Black	0	0%
	30°C	25%	80%	Clean	16	100%
				Light brown	0	0%
				Brown	0	0%
				Black	0	0%
	30°C + medications	0%	50%	Clean	10	100%
				Light brown	0	0%
				Brown	0	0%
				Black	0	0%

Treatment level prevalence (the number of shrimp testing positive for *Hyalophysa lynni* in the treatment divided by the total number of shrimp in that treatment), treatment level survival percentage (the proportion of shrimp that survived until the end of the experiment from that treatment), and sBG visual score count of the initial, postacclimation, and experimental treatments. The initial and postacclimation samples comprise a baseline estimate of sBG prevalence prior to the experiment, and do not have a survival metric (N/A) because they were not held for the same 14-day duration as the shrimp used in the experimental treatments.

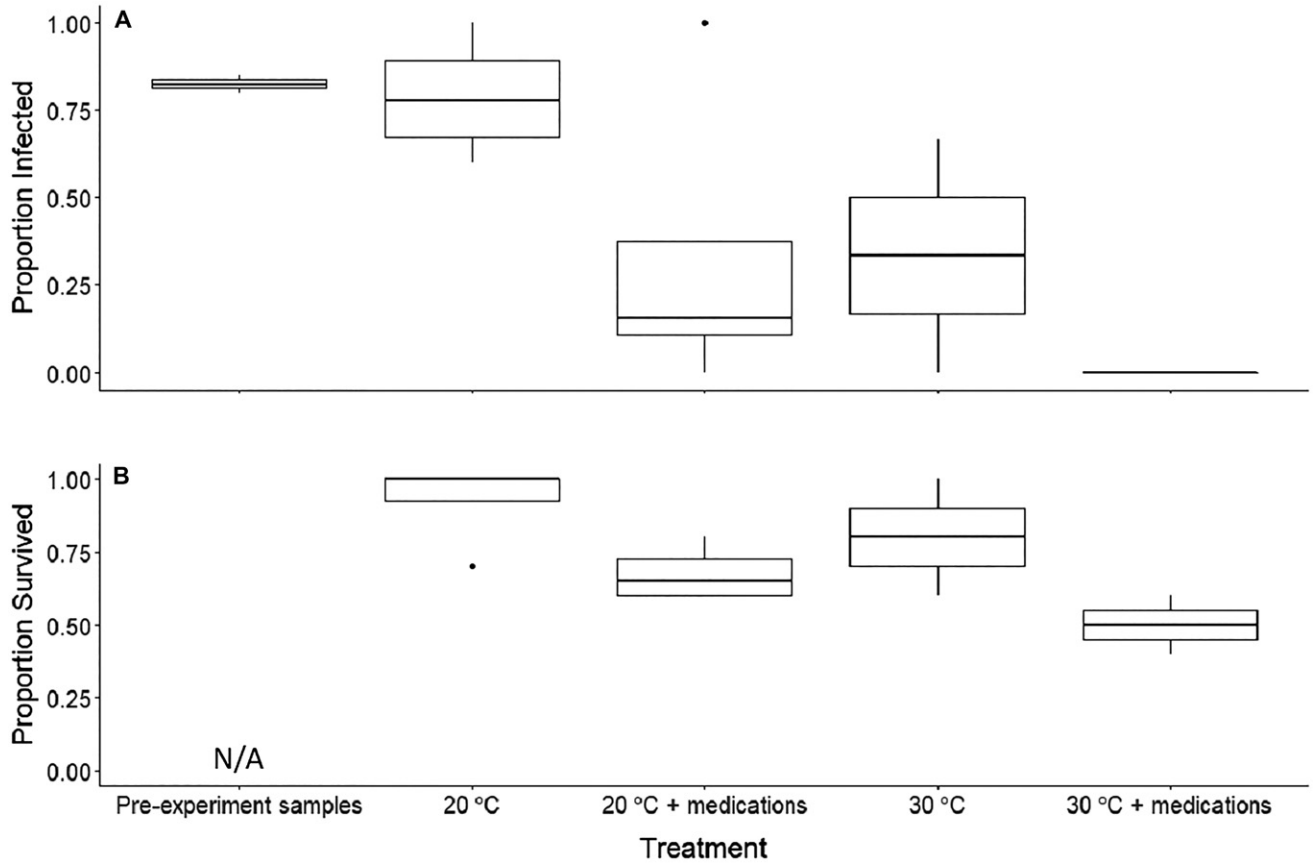

Figure 2. Tank-level prevalence and survival across experiment treatments. (A) The proportion of shrimp that tested positive for *Hyalophysa lynni* infection per tank and (B) the proportion that survived the duration across the pre-experiment samples and experimental treatments. The initial and postacclimation samples are combined in this figure for simplicity and labeled "Pre-experiment samples." Note that the preexperiment samples did not have a survival proportion recorded in (B) because they were not held for the same experimental duration. The solid horizontal line is the median tank-level prevalence, and the lower and upper hinges depict the first and third quartiles with the solid point displaying an outlier. The whiskers extend from the hinge to the largest value no further than 1.5× the interquartile range.

(44%), although only three shrimp (3%) exhibited any degree of visible melanization on their gill tissue. The treatment level prevalence (i.e., the number of shrimp testing positive for *H. lynni* in the treatment divided by the total number of shrimp in that treatment) was highest for the 20°C without medications (78%) and lowest in the 30°C with medications treatment (0%). The treatment level prevalence was similar for the 30°C without medications (25%) and the 20°C with medications treatment (29%). Except for the 20°C without medications treatment, the prevalence in the other treatments was reduced compared with the baseline samples, where 80% (initial sample) to 85% (postacclimation sample) of shrimp tested positive for infection. Interestingly, only 40% of shrimp in the initial sample had any notable gill melanization, whereas in the postacclimation sample taken 4 days later gill coloration had increased to occur in 95% of shrimp.

Probability of Infection and Survival

The probability of *Hyalophysa lynni* infection differed substantially between experimental treatments, but not between the initial and postacclimation samples (Table 1, Fig. 2A). The probability of infection was equal between shrimp from the initial sample and the postacclimation sample (Estimate = 0.3483, $Z = 0.415$, $P = 0.68$). Shrimp in the 20°C without medications treatment were also equally likely to test positive for *H. lynni* as shrimp from the postacclimation sample (Estimate = −0.098, $Z = -0.143$, $P = 0.89$). The shrimp in the 20°C with medications and the 30°C without medications treatment had a significantly lower probability of infection than shrimp from the postacclimation sample (Estimate = −2.251, $Z = -3.216$, $P = 0.001$ and Estimate = −2.485, $Z = -3.092$, $P = 0.002$, respectively). Since none of the shrimp in the 30°C with medications treatment tested positive for *H. lynni,* one positive entry was added to the 30°C with medications treatment to allow for statistical evaluation of this treatment. This analysis showed a significant difference between probability of infection in the 30°C with medications and the postacclimation sample (Estimate = −3.584, $Z = -3.004$, $P = 0.003$). Comparing the probability of infection between the 30°C with medications and the 20°C with medications treatments indicated that these treatments had an equal probability of infection (Estimate = −1.3322, $Z = -1.174$, $P = 0.241$), suggesting that medicine did not operate differently with temperature to influence infection probability.

The 30°C with medications treatment experienced the lowest survival, with only 50% of the shrimp surviving the 2-wk experimental period (Table 1, Fig. 2B). In contrast, 93% of the shrimp in the 20°C treatment survived. Logistic regression

found that the probability of a shrimp surviving until the end of the study was equivalent in the 20°C without medications and in the 30°C without medications treatments (Estimate = −1.126, Z = −1.373, P = 0.17). Both medicated treatments had a significantly lower probability of survival than the 20°C treatment without medications (20°C with medications: Estimate = −1.7814, Z = −2.587, P = 0.01, 30°C with medications: Estimate = −2.5123, Z = −3.356, P = 0.001). The two treatments with medications had an equal probability of survival (Estimate = −0.7309, Z = −1.304, P = 0.192), and the 30°C with medications treatment had a marginally lower probability of survival than the 30°C without medications (Estimate = −1.3863, Z = −1.936, P = 0.053).

When the effect of tank was controlled for in the logistic mixed effect model between the 20°C treatments, the 20°C with medications treatment resulted in a marginally significant lower probability of infection compared with the 20°C without medications treatment (Intercept = 1.789, Estimate = −2.939, Z = −1.902, P = 0.057). The logistic mixed effects regression model estimated the variance caused by tank in both of the 20°C treatments as 3.129. A fixed effects model resulted in a similar estimate of the reduction in probability of infection as the mixed effects model, but the estimate was highly significant (Estimate = −2.1529, Z = −3.708, P < 0.01). Thus, although the random effect of tank is likely important in all experimental treatments, the trends in the data reflect similar effects of medications when the tank variance is accounted for as when it is not.

Minimum and Maximum Recovery Rate

The treatments without medications provide an estimate of "natural" host recovery at the two experimental temperatures. In the 20°C without medications treatment, few shrimp were able to recover from the parasite (R_{min} =3%, R_{max} = 12.5%) and the prevalence remained equivalent to the baseline prevalence measured in the postacclimation sample (Tables 1 and 2). At 30°C without medications, more hosts were able to shed the parasite leading to an estimated recovery rate between (R_{min} = 50%, R_{max} = 75%). The estimated recovery rate in the 20°C with medications treatment ranged between (R_{min} = 34%, R_{max} = 59%). The estimated recovery rate in the 30°C with medications treatment was between (R_{min} = 38%, R_{max} = 63%), lower than that of the unmedicated treatment at that temperature, but similar to the recovery range of the 20°C with medications treatment.

DISCUSSION

Shrimp black gill that is caused by *Hyalophysa lynni* infection is an important disease present in the shrimp fishery of the Gulf of Mexico and South Atlantic Bight, and its seasonal patterns of prevalence in Georgia and South Carolina are well-documented (Frischer et al. 2022). The causal mechanisms behind this seasonal rise and fall of parasite prevalence, however, are poorly understood, in part, due to a lack of local SPF animals available for empirical research. This study provides a methodology for reducing the prevalence of *H. lynni* infection in captive shrimp and demonstrates that temperature and medication play an important role in the process. Although all treatments except for the 20°C without medications treatment reduced the prevalence of infection, when shrimp were subjected to the 30°C with medications treatment, the treatment level prevalence fell from approximately 80% to 0%. Additionally, because the recovery rate of the 30°C without medication treatment (50%–75%) was much greater than those held at 20°C without medications (3%–12.5%), this indicates that temperature is likely important for shrimp recovery. The ability to produce SPF shrimp is a critical advance that will facilitate future experimentation to understand the ecology of this parasite and help to inform management actions that work to preserve the fishery. Although this methodology was developed for the sBG system, similar protocols can likely be implemented to produce SPF crustaceans for use in other systems.

The reduced prevalence observed in the medicated treatments could have arisen from two mechanisms: the medications could be curing *Hyalophysa lynni* infections, or the medications are differentially causing mortality in infected shrimp. The 20°C with medications treatment had a higher range of recovery (34%–59%) than the 20°C without medications treatment (3%–12.5%), which suggests that the medications are reducing the level of *H. lynni* infection, at least at that temperature. In contrast, the estimated recovery rate range in the 30°C with medications treatment (38%–63%) is well within the "natural" recovery rate range estimated by the 30°C without medications treatment (50%–75%). Thus, the reduction in prevalence in the 30°C with medications treatment (0% prevalence) compared with the 30°C without medications (25% prevalence) could be entirely due to an increased infected mortality rate instead of the medications increasing the recovery rate.

Host survival was low in the treatments with medications (50% in the 30°C with medications treatment and 66% in the

TABLE 2.

Estimated minimum and maximum recovery rates of shrimp from *Hyalophysa lynni* infection in experimental treatments.

Treatment	Minimum recovery rate (%) R_{min}	Maximum recovery rate (%) R_{max}	Day 0		Day 14		Number dead (D)
			Uninfected U_0	Infected I_0	Uninfected U_{14}	Infected I_{14}	
20°C	3	12.5	8	32	9	28	3
20°C + medications	34	59	8	32	19	8	13
30°C	50	75	4	16	12	4	4
30°C + medications	38	63	4	16	10	0	10

The minimum recovery rate (R_{min}) was estimated by assuming all dead shrimp (D) were infected (Eq. 1) and the maximum recovery rate (R_{max}) was estimated by doing the reverse, where all dead shrimp were assumed to be uninfected (Eqs. 2 and 3). The numbers of uninfected (U_0) and infected (I_0) shrimp at day 0 were estimated by multiplying the prevalence of the postacclimation sample by the number of shrimp entering into each treatment. Values of uninfected (U_{14}) and infected (I_{14}) shrimp at day 14 were measured from euthanized shrimp at the end of the experiment.

20°C with medications treatment), and the probability of survival was significantly lower in the 30°C with medications treatment than the 30°C without medications treatment (80%) (Table 1, Fig. 2B). Cupramine and Metroplex are known to be toxic to invertebrates, and while the experiment attempted to mitigate this by reducing the dosage and shrimp densities, these compounds still contributed to the high mortality rates in medicated treatments. Several studies suggest that sBG increases shrimp mortality rates, particularly under stressful conditions (Frischer et al. 2022). Additionally, mortality may have contributed to this low prevalence of infection by removing heavily infected shrimp early on during the experiment by culling these shrimp. Culling results in both lower prevalence and lower transmission, allowing the uninfected hosts to remain uninfected and the lightly infected hosts to recover with reduced risk of reinfection (Murray et al. 2001). Therefore, it is possible that both mechanisms are playing a role in the observed reduction in prevalence in the treatments with parasiticides.

The similar range of recovery rates and the lack of statistical significance when comparing the infection probability between the two medicated treatments suggests that increased temperature does not improve the ability of the medications to reduce the probability of *Hyalophysa lynni* infection. Furthermore, it suggests that the sizable difference in net infection prevalence that exists between these treatments is driven not by temperature-dependent differences in drug efficacy on recovery, but rather by differential effects of temperature on mortality (Table 1, Fig. 2B). It is a common practice to increase the water temperature of aquaria when treating ornamental animals for a ciliate infection to induce the parasite into the free-swimming life cycle stage more rapidly. According to the manufacturer, it is at this free-swimming, infectious stage that the medications are most efficacious in treating. This increase in medicated host recovery at higher temperatures, however, was not evident in this study. Even without medications, there is a high range of recovery in the 30°C treatment, indicating the importance of temperature on the "natural" recovery rate. Temperature affects both host and parasite physiology in numerous complicated ways, and in this case, it appears that the shrimp host innate immune system was better able to perform at the elevated temperature than the infection mechanisms of the parasite (Le Moullac & Haffner 2000, Shields 2019).

The finding that the 20°C treatment produced shrimp with macroscopically clean gills but a high prevalence of infection (78%) points to both the importance of developing this medication protocol and lends insight into the interpretation of previous data. These results demonstrate that the visual presence of melanized gill tissue is not an accurate diagnostic tool to identify the presence of *Hyalophysa lynni* infection. Clean gills with a positive molecular diagnosis may indicate early or latent stages of infection where the shrimp host may not be suffering from gill tissue damage and may be unable to transmit the parasite to other animals. If this early/latent infected animal is exposed to conditions where *H. lynni* can advance through its life cycle, however, it will likely develop melanized gill tissue and can spread the infection to other shrimp. Research efforts that only use visual identification of melanized gill tissue as a diagnostic may underestimate the prevalence of *H. lynni* infections.

Caveats

Although the differences in prevalence between the experimental treatments are quite large, there are some important caveats when interpreting these findings and expanding upon them to produce SPF animals for use in experimental studies. Because of the low number of replicate tanks, the effect of tank was statistically unaccountable in the full experimental analysis. These effects are often quite important in studies housing multiple animals in aquaria (Hurlbert 1984). Treating every shrimp as an independent experimental unit without including tank as a random factor artificially increases the power in the experiment and elevates the risk of a Type 1 error. When the mixed effect logistic regression was performed on the treatments that had sufficient replication to control for the effect of tank (the 20°C and the 20°C with medications treatments), the effect of the medications remained marginally significant. This provides some confidence that the trends in these data are real, that is, that treatment effects persist even when accounting for the nonindependent nature of shrimp housed in the same tank.

Another consideration about the results of this study is that the shrimp that survive the 30°C with medications procedure may not represent the average health and disease resistance of wild white shrimp. The experimental animals have been subjected to numerous rounds of selection that would likely kill unhealthy individuals, including their initial collection from the wild and the stressful nature of the experiments. If the animals that were heavily infected were more likely to die in the 30°C with medications treatment, the shrimp that remain may be naturally more resistant to *Hyalophysa lynni* because there is individual variation in susceptibility to infection among crustaceans (Little & Ebert 2000). It is possible that the 30°C with medications protocol selected for animals that are naturally better defended against the parasite, which would artificially decrease the transmission rate and/or infection intensity if these animals are used in transmission experiments. These are important considerations when extrapolating the results from experiments utilizing shrimp that survived the 2-wk curing procedure to wild populations, as wild populations may be more vulnerable to *H. lynni* and have a different mortality rate than the cured shrimp.

Applications of SPF Hosts

The ability to cure crustaceans of gill parasites, including shrimp of *Hyalophysa lynni* infections, provides several exciting opportunities for future experimentation to understand the ecology of *H. lynni*, of aquatic infectious diseases in general, and for informing mechanistic, mathematical models of disease dynamics. Although there has been extensive monitoring of sBG prevalence, this metric is the result of at least three distinct processes, including transmission, mortality, and recovery, that cannot be disentangled based only on prevalence estimates. By utilizing SPF shrimp, the contributions of these processes can be isolated and uniquely quantified. Additionally, deploying SPF shrimp across the different habitats that white shrimp occupy over the course of their life history would determine where the shrimp are acquiring *H. lynni* infections. Future research could also develop methodologies to transmit *H. lynni* in laboratory conditions. This would allow researchers to experimentally

determine if *H. lynni* is transmitted in a density-dependent or -independent manner (Ryder et al. 2005).

In-laboratory transmission could also be used to measure how the transmission rate varies with temperature, therefore, attributing any observed seasonal changes in transmission to either temperature or the seasonal changes in local shrimp density. The results of such studies would inform predictive, mechanistic models that can simulate different management strategies to control or mitigate sBG. If sBG has density-dependent transmission, models can explore how an increase in fishing pressure could reduce the number of infected shrimp enough to significantly reduce transmission and "fish-out" the parasite (Ben-Horin et al. 2016). If transmission is dictated by seasonality, the fishing season could be adjusted to alter the prevalence of sBG in the catch. Management could also target fishing effort in or around the habitat where *Hyalophysa lynni* is most often spread with similar fishery consequences. Finally, if *H. lynni* transmission is influenced by temperature, a model can be built to explore how warming sea surface temperatures may affect sBG prevalence.

The ability to produce SPF crustaceans that are sourced from the local environment is of enormous benefit to the growing field of marine infectious disease ecology. There is increasing recognition of the impact of infectious diseases on both human and animal populations (Lafferty et al. 2015, Chakraborty & Maity 2020). Although there is advanced modeling theory that describes infectious disease dynamics in terrestrial systems, some of the assumptions made by these equations may not apply to aquatic systems, particularly those related to transmission (McCallum et al. 2004). Aquatic parasites and pathogens almost universally have free-living life stages and are able to infect hosts on very different spatial scales compared with most terrestrial systems (Lafferty 2017), and interact with the environment over these spatial scales. Utilizing SPF animals to measure rates of transmission across these environments will help target management actions and parameterize infectious disease modeling efforts (Ben-Horin et al. 2016). Further, utilizing SPF animals that were generated in the local environment allows for more direct application of experimental transmission results to the system of interest by accounting for local host adaptation to the environment and endemic parasites (Schade et al. 2014).

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