DOSAGE-CONTROLLED STUDIES OF EELGRASS WASTING DISEASE:

PHENOLOGY OF INFECTION AND FACTORS MODERATING VIRULENCE

A Thesis
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Master of Science

by

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ABSTRACT

Coinciding with increased anthropogenic stressors and climate change, many marine diseases are on the rise. Understanding the epidemiology of marine disease that affect ecologically, economically, and culturally important species is a priority, but also a challenge given the nature of marine systems. In the first chapter of this thesis, we conduct a dosage-controlled inoculation method to enable quantitative investigations of aspects of phenology of eelgrass wasting disease infection, namely timescales and descriptors of characteristic infection, timescales of immunodynamics of newly established EGWD lesions and growth consequences of infection to the host over time. Observations of developing lesions showed that lesions first became visible 2 days post pathogenic exposure and leaf tissue can become totally necrotic in as soon as 12 days and furthermore suggested that the outcome of infection is largely contingent on the genotype of the host. Growth of plants that have been exposed to the pathogen are significantly lower than control plants by 12 days. Immuno-response as measured by induction of phenols is significantly higher in treatment plants by 12 days into the time series, and phenolic production is positively correlated with higher disease severity by the end of the experiment at 20 days.

The second chapter investigates factors affecting severity of infection, namely pathogenic isolates, pathogen dosage, temperature, and light. Severity of lesions on eelgrass varied among the 3 different isolates inoculated in laboratory trials. Disease severity increased with pathogen dosage from 10^4 to 10^6 cells ml^{-1}. In a dosage-controlled light and temperature 2-way factorial experiment consisting of 2 light regimes (diel light cycle and complete darkness) and 2 temperatures (11 and 18°C), *L. zosterae cell* growth rate in vitro was higher at the warmer temperature. In a companion experiment that tested the effects of light and temperature in in vivo inoculations, disease severity was higher in dark treatments and temperature was marginally significant.

Although the details of these studies are specific to infection of the eelgrass *Zostera marina* by the protist *Labyrinthula zosterae*, it provides a general modeling framework for studying the role of within-host disease dynamics in other increasing marine diseases. Our work with controlled inoculation methods evaluate EGWD host-pathogen interactions narrowing the knowledge gap of phenology of infection and factors affecting virulence of a widespread and historically devastating disease.

BIOGRAPHICAL SKETCH

Phoebe received her Bachelors in Environmental Science from Cornell University in 2016 with a concentration in Ocean Science. As an undergraduate, she joined the Harvell Lab as an undergraduate researcher, interning with PhD candidate Morgan Eisenlord carrying out field surveys assessing temporal and spatial variation of eelgrass wasting disease. It was beside Morgan that Phoebe began her investigations of eelgrass wasting disease dynamics.

Following her undergraduate career, Phoebe worked as the Harvell Lab technician and coordinator of an intensive undergraduate research program. This position was a promising opportunity to be a contributing member of the research community, empowering undergraduates to pursue futures in STEM and providing training opportunities to students to enable high level research. After two years of coordinating this program, Phoebe continued on to pursue a Master's degree under the tutelage of Dr. Drew Harvell and her former undergraduate advisor, Dr. Charles Greene.

ACKNOWLEDGMENTS

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Experimental Phenology of Dosage-controlled Eelgrass Wasting Disease Infection

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ABSTRACT: Coinciding with increased anthropogenic stressors and climate change, many marine diseases are on the rise. Understanding the epidemiology of marine diseases that affect ecologically, economically, and culturally important species is a priority, but also a challenge given the nature of marine systems. We conducted a dosage controlled experimental study with the tractable eelgrass wasting disease (EGWD) plant pathosystem over a time series of 20 days to elucidate the phenology of infection. This experiment revealed: (1) timescales and descriptors of characteristic infection, (2) growth and survival consequences of infection to the host over time and (3) timescales of immunodynamics of newly established EGWD lesions. Observations of developing lesions showed that lesions first became visible 2 days post pathogenic exposure, leaf tissue can become totally necrotic in as soon as 12 days, and furthermore suggested that the outcome of infection is largely contingent on the genotype of the host. Growth of plants that have been exposed to the pathogen are significantly lower than control plants by 20 days. Immuno-response as measured by induction of phenols is significantly higher in treatment plants by 2 days in the time series. Although the details of these studies are specific to infection of the eelgrass *Zostera marina* by the protist *Labyrinthula zosterae*, it provides a general modeling framework for studying the role of within-host disease dynamics in other increasing marine diseases. These studies are the first that evaluate EGWD host-pathogen criterion over the full timeframe of necrotic lesion development, investigating phenology of infection over time of a widespread disease.

KEY WORDS: *Labyrinthula zosterae* · Eelgrass wasting disease · *Zostera marina* · Seagrass

INTRODUCTION:

Marine diseases are capable of disturbing the structure and function of marine ecosystems (Ward & Lafferty 2004). The mass mortality of sea urchins in the Caribbean in 1983-1984 resulted in more

than 93% reduction of existing *Diadema* at surveyed locations, leading to the increase in algae abundance and consequent decrease in coral populations (Lessios 1988). Withering syndrome of abalone first observed in 1992 changed the course of an entire species complex (Crosson et al. 2014); once a thriving farmed fishery, now two species of abalone are endangered with their recoveries limited by disease (Harvell 2019 (book)). The sea star wasting disease first detected in 2013 has devastated over 20 species of asteroids (Hewson et al. 2014), including near extirpation of a subtidal star (Montecino-Latorre et al. 2016; Harvell et al. 2019). As emphasized by these examples of infectious diseases that devastate important herbivores, keystone predators, and foundation species, we have learned through experience that marine diseases can have profound impacts that cascade ecosystems and alter community structure. Despite their large ecological role, infectious marine diseases are elusive, and studying them is challenging; due to the slurry of microbes that are present in the oceans and an inability to culture many potential pathogens in the lab, successfully fulfilling Koch's postulate and confirming etiological agents is a formidable challenge, hindering investigative experiments in marine pathosystems (Ritchie et al. 2001).

Eelgrass wasting disease (EGWD) is another example of a marine infectious disease that targets a key ecosystem player. Eelgrass, *Zostera marina,* is one of the world's most widespread marine plants, forming extensive coastal meadows throughout temperate and tropical waters. It provides critical nursery habitat and sustenance for diverse marine fauna, and is an ecosystem engineer that removes and fixes excess carbon dioxide into its meadows and sediments, and reduces the presence of bacterial pathogens (Lamb et al. 2017). The EGWD outbreak of the 1930s emerged suddenly, leading to the documented loss of >90% along Atlantic coasts of Europe and the US (Short et al.1987). These ecosystem impacts were drastic: disruption of coastal food chains and fisheries, losses of major populations of migratory waterfowl (Short et al. 1986), and the disappearance of the

eelgrass limpet *Lottia alveus*—the first historical extinction of a marine invertebrate in an ocean basin (Carlton et al. 1991). Again, in the late 1980s, seagrasses along the Eastern coast suffered declines also attributed to EGWD (Short & Wyllie-Echeverria 1996). These epidemics occurred in two stages: the initial infection of eelgrass leaves followed by the subsequent mass mortality of eelgrass (Short et al. 1987). These initial infections were described as blackened or dark brown spots, streaks, and patches, and could wipe out lush meadows in as little as 2 years (Short, photo documentation).

In the burgeoning frontier of disease ecology, there still remain vital knowledge gaps regarding phenology of infection—timescales of within host establishment, subsequent host-pathogen dynamics, and symptomatic progression. Disease signs in natural systems are highly variable through time and space, and the inherent complexity of biotic interactions presents challenges and opportunities to understand and manage the dynamics of disease spread. Controlled inoculation experiments are an important approach in closing this knowledge gap, and yet few marine host-pathogen systems are amenable to manipulation. The EGWD plant-pathosystem is unusually tractable—the robust plants fare well in the lab culture with flowing seawater, the pathogen *Labyrinthula zosterae* is well described, easily isolated, cultured, and maintained from the field (Porter 1990), and there are honed quantitative methods developed for inoculations (Dawkins et al. 2018, Groner et al. 2014). Despite the tractability of the system and abundant scientific literature on myriad aspects of its epidemiology there is little information on the phenology of infection.

The goal of this research was to understand how a suite of characteristic features of host and pathogen respond to each other after exposure to a controlled inoculation and determine timescales and descriptors of characteristic infection. We conducted two experiments: In Experiment I, we qualitatively observe outward signs of lesion development over time. In Experiment II, we

quantitatively assess a host-pathogen profile at seven time points after inoculation: 4 h, 24 h, 2 d, 3 d, 6 d, 12 d, and 20 d. With such early and late time points throughout the course of infection, we intended to identify the point at which the host responds to the pathogen and observe its progressive response. Through these experimental trials, we address the following research questions:

I. By qualitatively investigating rates and patterns of lesion progression, within what timeframe do we see lesions causing tissue to become necrotic?
II. Do the rates and progression of phenolic acid production compare with times of disease severity?
III. Are Z. marina phenolics an immune-response induced by exposure to *L. zosterae*, and if so, within what time frame?
IV. Is there a host cost associated with infection?

MATERIALS AND METHODS:

Experimental Trials

Our experiments were conducted in the Ocean Acidification Experimental Lab at Friday Harbor Laboratories, University of Washington, on San Juan Island, Washington state (48.550° N, 123.008° W).

Zostera marina collection and preparation

Vegetative *Zostera marina* plants were collected from the intertidal eelgrass bed in Beach Haven, Orcas Island, WA (48.691° N, 122.952° W) on 15 May 2017. Specimens were collected from this site because it has a historically stable eelgrass population (Wyllie-Echeverria et al. 2010, Groner et al. 2014, 2016). For each individual plant collected, we collected at least one clonal ramet attached to

the same rhizome network to ensure equal genetic representation across control and experimental treatments. Immediately after collection, the plants were kept in cool seawater until they were placed in a 10 ppt salinity water bath at ambient temperature of 10°C at Friday Harbor Laboratories in an effort to reduce environmental baseline pathogen levels. After 12 h in this low salinity treatment, each shoot and its attached ramet were clipped into separate shoots with several cm of rhizome. Outer leaves were carefully removed, leaving just 1st and 2nd rank leaves. Second rank leaves were trimmed to standardize tissue area across individual shoots and remove any minor disease signs. Plants were then acclimated in continuously flowing seawater for 48 h (~30 ppt, ~12°C) before placement into their experimental mesocosms on 18 May 2017.

Pathogen inoculum preparation

Labyrinthula zosterae used for this experiment was isolated from an EGWD lesion on an eelgrass blade from Beach Haven and maintained on serum seawater agar (Muehlstein et al. 1991) through several passages to create sufficient total pathogenic cells for inoculations. *L. zosterae* cultured from Beach Haven have been shown to be virulent in previous experiments (Eisenlord, unpublished data). Though this pathogenic culture was not sequenced, we assume that this maintained isolate is comparable to any isolate that collected *Zostera marina* individuals would be exposed to in the field. *L. zosterae* cells were harvested, de-clumped using silicon glass beads and vortexing (Dawkins et al. 2018), and adjusted to a final concentration of 66.6 (SD±18.15) x 10^4 cells/mL.

Experimental set-up

Eelgrass shoots were held in 3.5 L chambers housed within 95 L coolers, and each chamber received its own 1 um filtered, aerated, flow-through seawater (~28-30 ppt, 12°C, 70 min seawater residence time). There were 3 individual ramets per chamber and 8 chambers in each of the 7 coolers. An

experimental chamber for each time point was paired with a control chamber of the same plant genotype (Fig 1). These pairs of chambers were assigned randomly to coolers and positions within coolers.

On 20 May 2017, individual *L. zosterae* shoots were placed in Whirlpak bags within their assigned chamber with 100 mL of sterile filtered seawater and inoculated with 1mL of prepared inoculum, for a final pathogenic exposure of 1.33 x 10^4 cells/mL. Shoots were totally submerged in their Whirlpaks and agitated after 12 h to ensure aerated conditions. Shoots were left exposed to this pathogenic dosage for 24 h before being removed from Whirlpak bags and attached with rubber bands at the base of their rhizomes to weighted mesh at the bottom of the chamber to ensure vertical orientation. Full-spectrum LED lights for aquatic plants (MarineLand, Blacksburg, VA) were placed above each cooler to provide the tanks with a 14:10 light:dark schedule.

Sampling procedure

Experiment I: Individual Lesion Progression

Zosterae marina shoots were sampled at three time points after exposure: 5 d, 12 d, and 20 d. For each time point, all experimental plants (*n=12)* and all treatment plants (*n=12*) were removed from their chambers, laid onto sterile transparencies, and scanned for lesion imaging. After scanning, plants were returned to their assigned chambers. The plants in this cooler were not destructively sampled, enabling observation of individual lesion development over time.

Experiment II: Time Series of Host-Pathogen Criteria

Zosterae marina shoots were destructively sampled at seven time points after exposure: 4 h, 24 h, 2 d, 3 d, 6 d, 12 d, and 20 d. For the first six time points, three experimental units of treatment plants (*n*

= 9) and three experimental units of control plants (n = 9) were destructively sampled. For the last time point seven, six experimental units of treatment plants (n = 18) and six experimental units of control plants (n = 18) were destructively sampled. At the end of each time point, the plants were removed from their chambers, laid onto sterile transparencies, and scanned to determine total tissue growth from start of the experiment, as well as for disease severity analysis. Disease severity (total lesion area/leaf section area x 100%) was quantified for each leaf for each plant using ImageJ. The second oldest leaf of each plant was sampled for phenolic acid content. We used the Folin-Denis method used to determine the phenolic concentrations in our samples (Harrison 1977; Arnold et al. 2012; Sánchez-Rangel et al. 2013), reporting phenolic concentrations as percentages of phenolic compounds per mass of plant.

STATISTICAL ANALYSES:

We used linear mixed effect models (package 'lme' in Rv. 3.3.1) to test response variables of disease severity, phenolic acid content, and total growth with time and treatment set as fixed effects and a random effect of tank. Two-way Type III ANOVAs were used to compare the control and experimental treatment over time. Tests were performed on the log scale to meet the assumptions of normality and homogeneity of variance. Reported p-values are Tukey adjusted for comparing a family of 7 estimates. All values of disease severity data were increased by .005 and logarithimically transformed.

RESULTS:

Experiment I: Individual Lesion Progression

Photo-analysis of our negative controls and treatments confirmed that our inoculation methods were effective. All control plants were free of disease signs. Of the 12 plants used in our treatments,

only 4 of them developed lesions with pale centers. The lesions in Figure 2 exhibit development of characteristic late-infection necrotic lesions. As described in previous studies, these lesions start off as dark brown to black speckled tissue that gradually expand longitudinally and converge into larger lesions with defined borders. Lesions begin to exhibit pale tissue at their centers where older infected tissue contains lower pathogenic cell abundance (Eisenlord, unpublished data).

Experiment II: Timeseries of Host-Pathogen Criteria

Control plants showed extremely low levels of disease—no higher than 3.5% disease severity at any given time point. We begin to see disease signs on our treatment blades day 2 after pathogenic exposure, with a significant increase in disease severity at 12 d ($p=0.0005$) and continued through day 20 ($p<0.0001$) (Table 1).

As for phenolic acid induction and the timeframe of this biological process, we determined that there is indeed a significant increase in plants that were exposed to the pathogen. While there is a general trend that the treatments have higher phenols than the control starting at the 24 h mark and onwards (Fig 3), we see a significant divergence in phenolic levels between the treatments and our controls at 2 days ($p=.008$), 6 days ($p=.043$), 12 days ($p=.001$) and 20 days ($p<.001$) (Table 3).

In investigation of consequence of infection to the host, we looked at differences in growth between control and treatment plants. Again, there is a general trend in our data that the control plants have consistently higher growth than those exposed to the pathogen, and we see a significant divergence by day 20 of our experiment ($p=0.0037$) (Table 5; Fig 5).

DISCUSSION:

Disease dynamics involve components and processes at multiple scales of biology, time, and space (Gutierrez et al. 2015). In the frontier of disease ecology, studies of infection phenology are lacking. Although it has been studied in a few economically important crop and human systems with culturable pathogens, case studies of phenology of infection in natural ecological systems are few. Increases in disease outbreaks in several marine groups lends urgency to understanding disease dynamics, particularly since few viable options currently exist to mitigate disease in the oceans (Ward & Lafferty 2004; Harvell 2019 (book)). Experiments with tractable systems with culturable pathogens like EGWD are crucial in understanding the spread of infectious agents and predicting the outcome of plant-pathogen interactions. Despite the tractability, historical relevance of the EGWD system, and depth of scientific literature, so much remains regarding its spatio-temporal epidemiological dynamics – timeframes for lesion development within which tissues become necrotic, immune response, and host costs for defense against infection. By controlling the pathogen dosage to ensure equal exposure in each shoot, we are able to qualitatively observe signs of lesion development over time and examine the relationships between a number of relevant pathogen and host criteria over a 20 d time period.

Experiment I provided photo documentation to image of individual plants and their respective lesions over time. Our observations show that lesions begin to appear as soon as 2 d after pathogenic exposure, and can develop pale, necrotic tissue as soon as 12 d. Of the 12 plants that were collected from spatially distinct rhizome networks used in our treatments, only 4 of them developed lesions with these pale centers characteristic of severe, late infection (Eisenlord, unpublished data). This provides insight into the variable disease levels found in the nature (Eisenlord, unpublished data)—some individual plant genotypes may be more susceptible to infection than others. Examples of other variable plant genotype susceptibility to infection can be

seen in the bean common mosaic virus (Anderson et al. 2004), wheat stripe rust (Finckh & Mundt 1992), and rust fungus in skeleton weed (Burdon et al. 1984). In these plant-pathosystems, genetic differences are important factors in determining differential disease response. The gene-for-gene hypothesis states that, for every gene for virulence in a pathogen, there is a corresponding susceptibility gene in the plant species (Flor 1971), suggesting that disease emergence occurs when the pathogenic strain has matching pathogenicity genes or host genotypes lack resistance to the pathogen (Anderson et al. 2004). In our experiments, only 1/3 of our plants collected from distinct rhizome networks developed late-infection, pale-centered lesions, and from those lesions, 50% of the lesions came from the same plant. Our data suggests that the individual genotype of the plant plays a role in the outcome of infection, but to confirm the effects of plant genetic diversity, plants should be further investigated with genotype sequencing.

In Experiment II, control plants were free of visual disease signs at all time points, confirming a successful selection of disease naïve plants and minimal effect of natural background disease level throughout our experiment in our treatment plants. Visual signs of disease began to appear on the treatment plants 2 d after exposure to the pathogen. Across all time points there is a large spread of lesion areas possibly due to the effect of genotype susceptibility/resistance, with a significant increase in disease severity at 12 d and 20 d.

When we look at levels of phenolic acids over time between control and treatment plants, there is a general trend that the treatments have higher phenols than the controls, and 2 d, 6 d, 12 d and 20 d. The divergence in phenols by 20 d in conjunction with the appearance and steady levels of disease severity suggests that the increased levels in phenolic acids are a gradual induction to infection, and have a lag in response. Pathogen defense in *Z. marina* specifically is understudied, but in general,

defense reactions in flowering plants are evolutionarily conserved (Brakel et al. 2014); there are purely physical barriers (e.g. wax cuticles or cell walls) and biochemical barriers such as secondary metabolites that inhibit pathogen growth (da Cunha et al. 2006). One important group of secondary metabolites are phenolic acids and their derivatives, which have various functions such as antioxidant capacity (Rice-Evans et al. 1996) and antimicrobial function (Nicholson & Hammerschmidt 1992). Phenolic compounds probably also play a role in the interaction between *Z. marina* and *L. zosterae*, since higher concentrations of phenolic acids were detected in infected as compared to healthy plants (Vergeer et al. 1995) and the expression of CYP73A—a gene which is involved in phenol synthesis—increased almost 80-fold upon infection (Brakel et al. 2014). The phenolics may affect the susceptibility of plants to EGWD. For example, the accumulation of phenolic acids in *Zostera marina* has been correlated with reduced microbial growth and herbivory (Harrison 1982), and with resistance to the wasting disease in mesocosm experiments (Buchsbaum et al. 1990) and in the field (Vergeer & Den Hartog 1991, 1994, Vergeer & Develi 1997). While phenolics are one of the most well studied secondary metabolites—phenolics are found in all higher terrestrial plant leaves—the regulation of their production in eelgrass has not been well studied. This inoculation experiment has pinpointed phenolic acids as a potent inducible defense response in *L. zosterae* to *Z. marina.*

Decreased growth of infected eelgrass by 20 d may indicate an allocation of *Z. marina* resources to the production of secondary metabolites, suggesting that there is indeed an incurred host cost associated with *L. zosterae* infection. Production of secondary metabolites in plants is often associated with reduced fitness in terms of lower growth and reproduction (Sagers & Coley 1995, Gershenzon 1994, Smedegaard-Petersen & Stolen 1981, Levin 1976); this trade-off between investment in plant defense and growth is known as an allocation cost. Future work will be needed

to disentangle the contribution of induced defense and simply metabolic consequences of lost tissue to the significant reduction in growth of infection plants.

Current disease levels of EGWD in the San Juan Islands are at outbreak levels at several sites and have increased in the last 7 years (Eisenlord et al., in prep). The scientific knowledge gaps that surrounded to the epidemics of the 1930s and 1980s poses interesting questions about the biotic interactions that modulate disease success and the phenology of infection. *L. zosterae* is an omnipresent pathogen and have been isolated from seagrass populations that do not show disease signs (Muehlstein et al. 1988). Some have suggested the pathogen may simply act as a secondary decomposer on senescent leaves until environmental conditions cause it to become virulent (Short et al. 1988, Vergeer & Den Hartog 1994, Den Hartog 1996). Within-host dynamics will shape the health of individual hosts, the transmission of pathogens across host populations, and our ability to predict future outcomes—determining critical values for key factors and their interactions is key. This study is a first step in characterizing such complex dynamics quantitatively. We look forward to the continuation and expansion of this work, adding other important criterion to fill out the host-pathogen disease profile over time.

FIGURES:

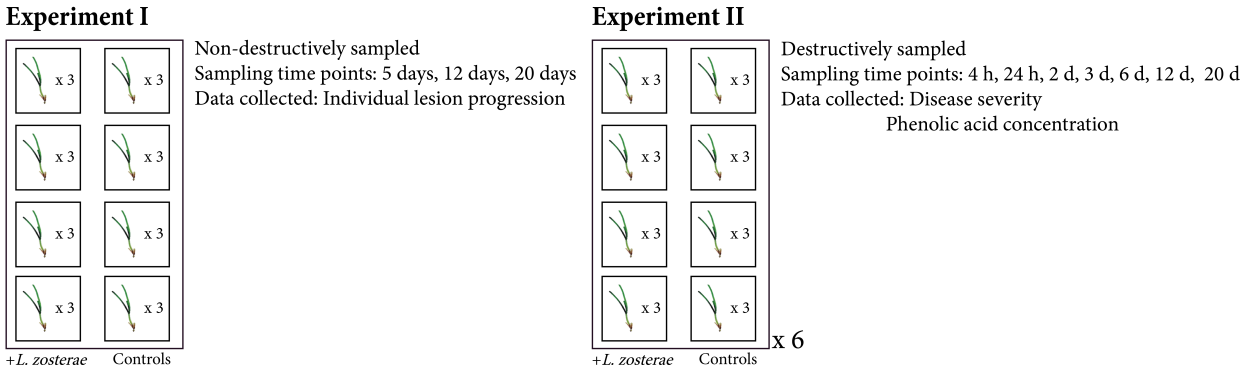

Figure 1 Simplified diagram of experimental designs for Experiment I and Experiment II. Larger rectangles represent 3.5 L cooler that housed the chambers containing three eelgrass shoots each, represented by smaller squares. Each cooler housed four chambers exposed to the pathogen and four control chambers. Pairs of chambers were assigned randomly to coolers and positions within coolers. For all analyses, a random effect for the tank was included in order to account for the non-independence of replicates within the tanks.

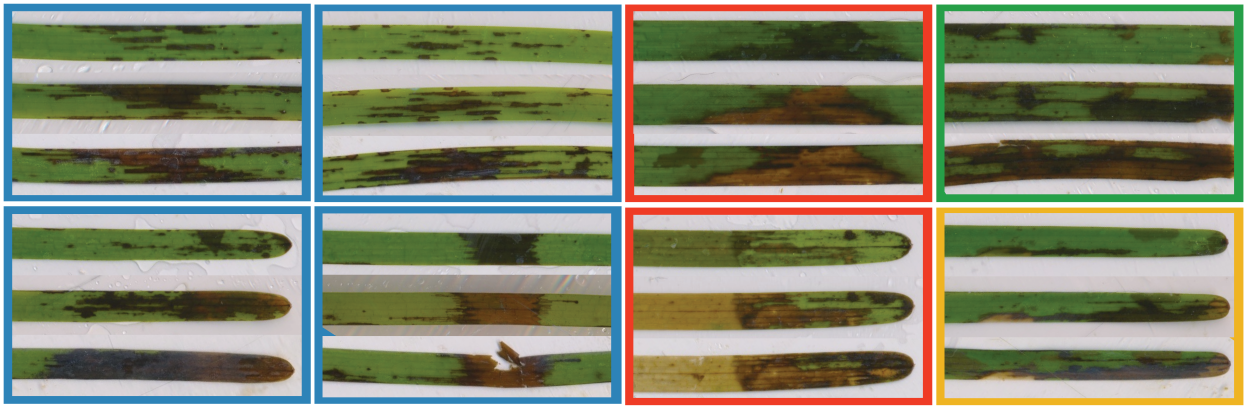

Figure 2 Images are sections of the eelgrass tissue that developed characteristic late-stage infection with pale tissue from 5 d, 12 d, and 20 d after pathogenic exposure in Experiment I. Lesion progressions with similarly colored borders were imaged from the same plant.

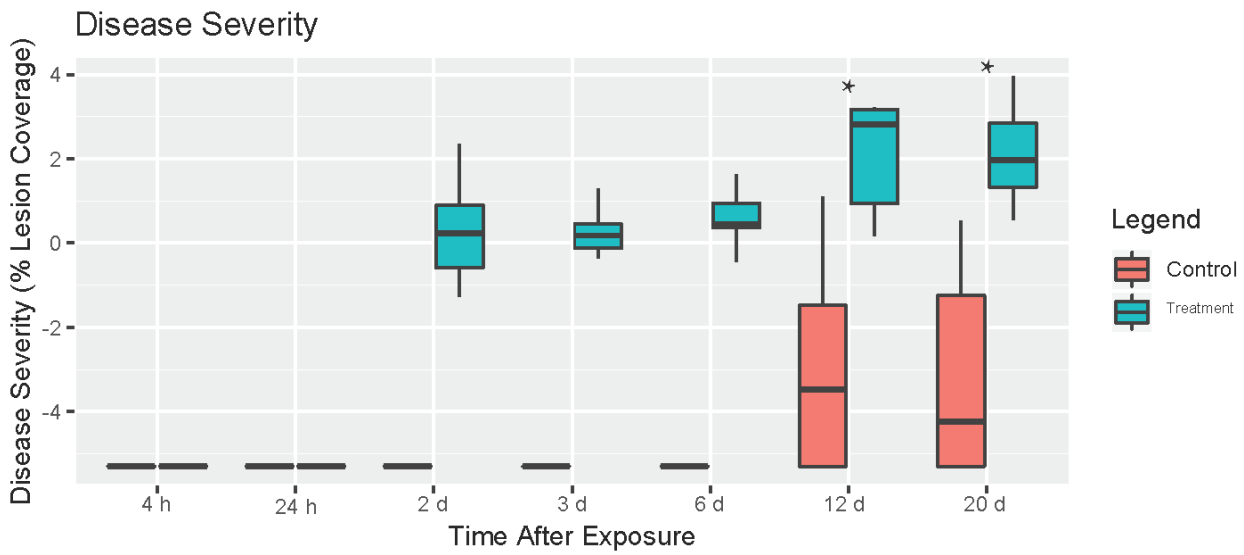

Figure 3: Box and whisker plot showing disease severity data. Asterisks denote divergence of control and treatment data as tested by type III ANOVA.

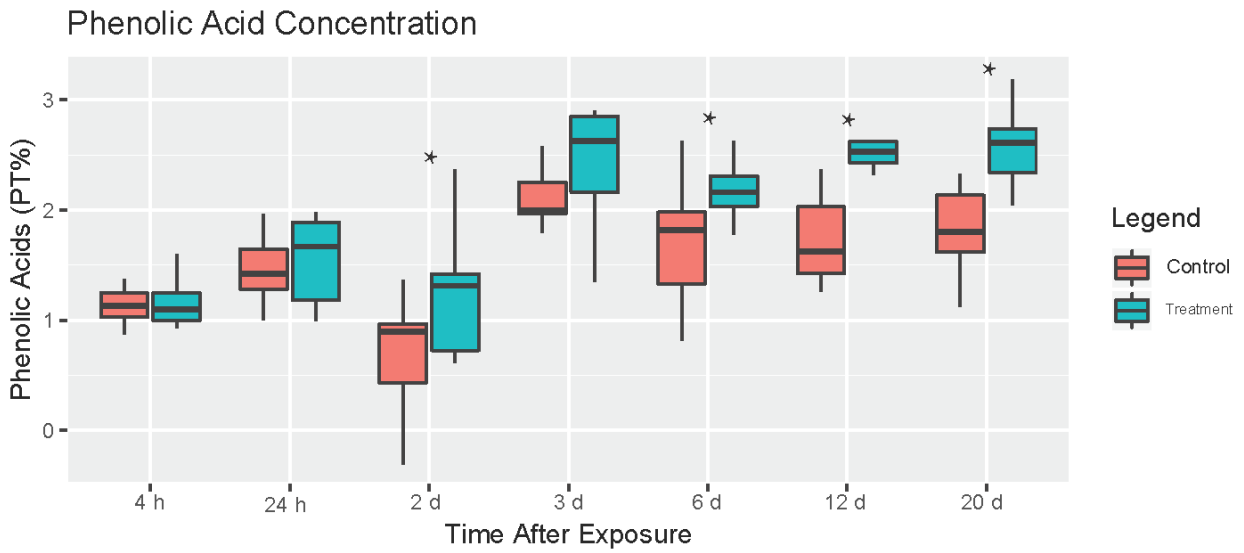

Figure 4: Box and whisker plot showing phenolic acid concentration data. Asterisks denote divergence of control and treatment data as tested by type III ANOVA.

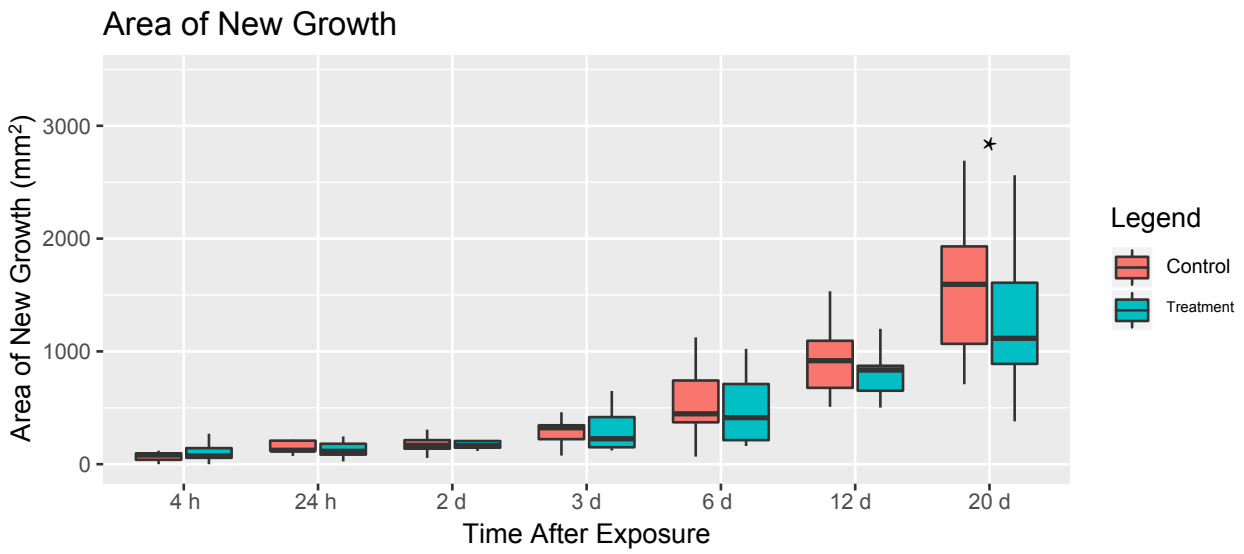

Figure 5: Box and whisker plot showing area of new growth data. Asterisks denote divergence of control and treatment data as tested by type III ANOVA.

TABLES:

Time Point	Control Estimate ± SE	Treatment Estimate ± SE	p
4 h	0.00 ± 1.73	0.00 ± 1.73	1.0000
24 h	0.00 ± 1.73	0.0322 ± 1.73	0.9895
2 d	0.00 ± 1.73	1.1002 ± 1.73	0.6550
3 d	0.00 ± 1.73	0.9355 ± 1.83	0.7123
6 d	0.00 ± 1.73	1.7270 ± 1.73	0.5914
12 d	0.3372 ± 1.73	9.7729 ± 1.73	**0.0005**
20 d	0.1732 ± 1.26	9.5707 ± 1.22	**< .0001**

Table 1: Two-way ANOVA comparisons for disease severity between control and experimental treatments. Tests are performed on the log scale. Reported p-values are Tukey adjusted for comparing a family of 7 estimates. Significant p-values ($p < 0.05$) are shown in bold.

Time Point Comparison (for Treatment)	Estimate	SE	p
4 h / 24 h	-0.0322	2.44	1.0000
4 h / 2 d	0.865	0.1750	0.9993
4 h / 3 d	0.256	0.0519	0.9998
4 h / 6 d	0.339	0.0734	0.9912
4 h / 12 d	0.248	0.0518	**0.0055**

4 h / 20 d	0.231	0.0408	**0.0013**
24 h / 3	1.318	0.2666	0.9994
24 h / 4	0.391	0.0790	0.9998
24 h / 5	0.516	0.1118	0.9920
24 h / 6	0.378	0.0788	**0.0057**
24 h / 7	0.352	0.0622	**0.0014**
2 d / 4	0.296	0.0600	1.0000
2 d / 6 d	0.392	0.0848	1.0000
2 d / 12 d	0.287	0.0598	**0.0180**
2 d / 20 d	0.267	0.0472	**0.0055**
3 d / 6 d	1.321	0.2861	0.9999
3 d / 12 d	0.967	0.2018	**0.0189**
3 d / 20 d	0.900	0.1592	**0.0061**
6 d / 12 d	0.732	0.1629	**0.0341**
6 d / 20 d	0.681	0.1315	**0.0120**
12 d / 20 d	0.931	0.1715	1.000

Table 2: Two-way ANOVA comparisons for disease severity within treated plants. Tests are performed on the log scale. Reported p-values are Tukey adjusted for comparing a family of 7 estimates. Significant p-values ($p < 0.05$) are shown in bold.

Time Point	Control Estimate ± SE	Treatment Estimate ± SE	p
4 h	3.21 ± 0.488	3.00 ± .428	0.7420
24 h	4.29 ± .614	4.56 ± .653	0.7664
2 d	1.93 ± .293	3.46 ± .495	**0.0083**
3 d	8.94 ± 1.279	11.68 ± 1.670	0.1960
6 d	5.55 ± 0.844	8.84 ± 1.437	**.0430**
12 d	4.81 ± 0.687	12.08 ± 1.836	**0.0001**
20 d	6.25 ± 0.651	12.98 ± 1.836	**< .0001**

Table 3: Two-way ANOVA comparisons for phenolic acid concentrations between control and experimental treatments. Tests are performed on the log scale. Reported p-values are Tukey adjusted for comparing a family of 7 estimates. Significant p-values ($p < 0.05$) are shown in bold.

Time Point Comparison (for Treatment)	Estimate	SE	p
4 h / 24 h	0.656	0.1328	0.3887
4 h / 2 d	0.865	0.1750	0.9906
4 h / 3 d	0.256	0.0519	**<.0001**
4 h / 6 d	0.339	0.0734	**0.0003**
4 h / 12 d	0.248	0.0518	**<.0001**
4 h / 20 d	0.231	0.0408	**<.0001**
24 h / 3	1.318	0.2666	0.8154

24 h / 4	0.391	0.0790	**0.0011**
24 h / 5	0.516	0.1118	0.0573
24 h / 6	0.378	0.0788	**0.0009**
24 h / 7	0.352	0.0622	**<.0001**
2 d / 4	0.296	0.0600	**<.0001**
2 d / 6 d	0.392	0.0848	**0.0019**
2 d / 12 d	0.287	0.0598	**<.0001**
2 d / 20 d	0.267	0.0472	**<.0001**
3 d / 6 d	1.321	0.2861	0.8535
3 d / 12 d	0.967	0.2018	1.0000
3 d / 20 d	0.900	0.1592	0.9965
6 d / 12 d	0.732	0.1629	0.7971
6 d / 20 d	0.681	0.1315	0.4361
12 d / 20 d	0.931	0.1715	0.9997

Table 4: Two-way ANOVA comparisons for phenolic acid concentrations within treated plants. Tests are performed on the log scale. Reported p-values are Tukey adjusted for comparing a family of 7 estimates. Significant p-values ($p < 0.05$) are shown in bold.

Time Point	Control Estimate ± SE	Treatment Estimate ± SE	p
4 h	70.4 ± 122.2	102.6 ± 122.2	0.8533
24 h	191.6 ± 122.2	128.9 ± . 122.2	0.7188
2 d	173.1 ± 122.2	183.3 ± 122.2	0.9534
3 d	289.7 ± 122.2	299.9 ± 129.8	0.9546
6 d	543.3 ± 122.2	479.5 ± 122.2	0.7143
12 d	946.8 ± 122.2	816.6 ± 122.2	0.4562
20 d	1633.3 ± 89.0	1247.1 ± 86.4	**0.0037**

Table 5: Two-way ANOVA comparisons for new growth between control and experimental treatments. Tests are performed on the log scale. Reported p-values are Tukey adjusted for comparing a family of 7 estimates. Significant p-values ($p < 0.05$) are shown in bold.

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Environment, dosage, and pathogen isolate moderate virulence in eelgrass wasting disease

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ABSTRACT: Eelgrass wasting disease, caused by the marine pathogen *Labyrinthula zosterae*, has the potential to devastate important eelgrass habitats worldwide. Although this host-pathogen interaction may increase under certain environmental conditions, little is known about how disease severity is impacted by multiple components of a changing environment. In this study, we investigated the effects of variation in 3 different *L. zosterae* isolates, pathogen dosage, temperature, and light on severity of infections. Severity of lesions on eelgrass varied among the 3 different isolates inoculated in laboratory trials. Our methods to control dosage of inoculum showed that disease severity increased with pathogen dosage from 10^4 to 10^6 cells ml^{-1}. In a dosage-controlled light and temperature 2-way factorial experiment consisting of 2 light regimes (diel light cycle and complete darkness) and 2 temperatures (11 and 18°C), *L. zosterae* cell growth rate *in vitro* was higher at the warmer temperature. In a companion experiment that tested the effects of light and temperature in *in vivo* inoculations, disease severity was higher in dark treatments and temperature was marginally significant. We suggest that the much greater impact of light in the *in vivo* inoculation experiment indicates an important role for plant physiology and the need for photosynthesis in slowing severity of infections. Our work with controlled inoculation of distinct *L. zosterae* isolates shows that pathogen isolate, increasing dosage of inoculum, increasing temperature, and diminishing light increase disease severity, suggesting *L. zosterae* will cause increased damage to eelgrass beds with changing environmental conditions.

KEY WORDS: Virulence · Environmental stress · *Labyrinthula zosterae* · Eelgrass wasting disease ·EGWD · *Zostera marina* · Seagrass

INTRODUCTION:

Eelgrass beds are among the most productive marine ecosystems in our oceans, providing a multitude of ecosystem services that include critical nursery habitats for economically important species, stabilization of sediments, carbon sequestration, and water filtration and purification (Lamb

et al. 2017, Nordlund et al. 2017), as well as valuable cultural benefits (Wyllie- Echeverria & Cox 2000, de la Torre-Castro & Rönn -bäck 2004). However, this valuable ecosystem is currently experiencing serious declines worldwide (Orth et al. 2006, Waycott et al. 2009). Although declines have most recently been associated with anthropogenic disturbances such as eutrophication, overharvesting, and sediment runoff (Barbier et al. 2011), the most remarkable historic declines in eelgrass beds have been attributed to outbreaks of eelgrass wasting disease (EGWD) (Renn 1934, 1935, Short et al. 1987, Muehlstein et al. 1991, Godet et al. 2008).

The disease symptoms associated with EGWD are blackened streaks and lesions with defined borders, often with a pale necrotic center (Muehlstein et al. 1991). During the 1930s, seagrass populations along the Atlantic coasts of Europe and North America suffered catastrophic declines, reaching 90% mortality (Renn 1934, 1935, Short et al. 1987, Godet et al. 2008). In the late 1980s, seagrasses in Cape Ann, Massachusetts, Great Bay, New Hampshire, and Niantic River, Connecticut (USA), suffered declines also attributed to EGWD (Short & Wyllie-Echeverria 1996). EGWD outbreaks have had detrimental environmental and ecological consequences, including alteration of sediment distribution, disruption of coastal food chains and fisheries, and losses of major populations of migratory waterfowl (Short et al. 1986). It was not until the acute declines along the western Atlantic coast in the 1980s that a *Labyrinthula* sp. was conclusively identified as the causative agent of EGWD (Short et al. 1987). In 1991, *L. zosterae* was named and confirmed to cause EGWD in the eelgrass species *Zostera marina* (Muehlstein et al. 1991).

L. zosterae has been considered an opportunistic pathogen, meaning it is present in the environment but only becomes pathogenic under specific conditions of host susceptibility and/or change in the environment (Burge et al. 2013, 2014, Groner et al. 2014). Examples of this type of pathogen

include the herpes viruses associated with high mortality of larval and juvenile stages of a number of molluscan species in commercial hatcheries and nurseries where pathogenesis is promoted by rearing conditions such as higher temperature and high density (Farley et al. 1972, Le Deuff et al. 1996, Arzul et al. 2001). Another pathogen considered an opportunist is Quahog Parasite Unknown, a thraustochytrid closely related to related to *L. zosterae*, which causes molluscan disease and mortalities regulated by temperature and salinity (Dahl 2015). Other examples include the temperature-dependent disease in corals caused by *Vibrio shiloi* AK1 and *V. coralliilyticus* (Frydenborg et al. 2014). Further study is needed to establish *L. zosterae* as opportunistic and understand the factors affecting its virulence in eelgrass.

In this study, we used controlled experiments to investigate the impact of light and temperature, 2 major environmental stressors to eelgrass in the Salish Sea, on the virulence of *L. zosterae*. We hypothesized that the interaction between these 2 stressors may lead to a new physiological regime for the *Z. marina* host and its corresponding *L. zosterae* pathogen isolate that facilitates increased EGWD. Under this new regime, the compromised immune response of the host and the increased virulence and/or increased growth rate of the pathogen may increase risk of a disease outbreak (Burge et al. 2014).

Recent work investigating environmental factors affecting EGWD has shown that multiple different strains of *L. zosterae* are present in the environment (M. E. Eisenlord unpubl. data). The characterization of a pathogen as opportunistic, when this pathogen may exist as a consortium of diverse strains, must consider that virulence may vary among strains. Virulence, used here as a metric of disease damage, is not an independent characteristic of the microbe; rather, it is contingent upon characteristics of the pathogen and the susceptibility of the host, as well as the environmental

context (Casadevall & Pirofski 2001). Thus, an increase in virulence driven by changes in the environment could be caused by either a community-level change of non-pathogenic strains switching to pathogenic strains, or by a change in respective strain pathogenicity. Recent work by Martin et al. (2016) has shown that both pathogenic and non-pathogenic strains of *L. zosterae* are widespread globally, but further studies are needed to elucidate the environmental factors that affect the virulence of these pathogenic strains and identify where they occur in nature.

Research investigating environmental factors that affect marine infectious disease is a priority in our changing oceans (Burge et al. 2013, 2016, Groner et al. 2016, Lafferty & Hofmann 2016). While direct impacts of a changing climate have been documented on diseases of corals, shellfish, and finfish (Harvell et al. 2002, Burge et al. 2013), these drivers, as well as the other environmental factors, have yet to be clearly linked to EGWD. In this study we tested how pathogen dosage, isolate, light, and temperature affect EGWD virulence. We investigated the combined influence of light and temperature on virulence and *L. zosterae* growth *in vitro*. We hypothesized that (1) *L. zosterae* isolates vary in virulence, (2*) L. zosterae* virulence increases with increased dosage, (3) *L. zosterae* virulence increases with warmer temperatures, and (4) *L. zosterae* virulence increases with reduced light. The goal of this study was to elucidate effects of environmental factors on the EGWD system.

MATERIALS AND METHODS:

Culturing and identification of the etiologic agent *Labyrinthula zosterae* cells were isolated from *Zostera marina* leaves with characteristic sharp-edged black or dark brown lesions symptomatic of EGWD (Muehlstein et al. 1991). Small sections of lesioned tissue were surface-rinsed with sterilized seawater, blotted dry, and placed onto serum seawater agar (SSA) plates, subsequently wrapped in

Parafilm and grown at 20-24°C. Cultures were then re-plated to create axenic cultures and transferred monthly. SSA was modified from Porter (1990) as described by Groner et al. (2014).

Identification of *L. zosterae* cells includes observation of characteristic cell growth on SSA media coupled with observation of characteristic cell morphology via light microscopy. *L. zosterae* has an identifiable growth pattern on SSA; irregular masses of aggregated cells form on the agar surface as well as within the agar matrix, exhibiting intricate patterns of branching along the culture's spreading margin (Muehlstein et al. 1991). The most obvious feature of the fusiform cells is their prominent central nuclei as well as gliding movement within their ectoplasmic networks (Muehlstein et al. 1991). *L. zosterae* identification is further confirmed by observation of fusiform or spindle-shaped vegetative cells linked with mucous strands via light microscopy.

Expt 1: Impact of dosage and temperature on L. zosterae *virulence*

We ran a full-factorial experiment comparing virulence of 3 *L. zosterae* dosage treatments (10^4, 10^5, and 10^6 cells ml^{-1}) at 2 temperatures (14 and 19°C) in *Z. marina* leaf tissue (*in vivo*).

Forty *Z. marina* plants were collected on 26 April 2015 from Collin's Cove, Friday Harbor, Washington (WA) (48° 32' 60" N, 123° 0' 36"W), and kept at ambient temperature in sterile seawater for 2 h to acclimate. We selected the third longest blade and recorded its length, using only blades with no visible signs of disease or physical damage. The third longest is less likely to have been infected than older blades (Groner et al. 2014), while still providing the length needed for this experiment. Each blade was scraped clean of epiphytes and fouling with a glass cover slide, gently to avoid mechanical damage to the leaf surface, and subsequently cut into 5 cm sections. The base and tip of each blade were discarded. The blades were then quickly rinsed in reverse osmosis water to

reduce potential *L. zosterae* contamination from seawater or the leaf exterior, as fresh water inhibits *Labyrinthula* (Muehlstein et al. 1988). Blades were then placed into a sterile seawater bath for 60 s and blotted dry.

The *L. zosterae* isolate (8.16.D) used for this experiment was isolated from non-flowering adult *Z. marina* shoots in 2011 that were collected from Picnic Cove, Shaw Island, WA (48° 34' 12" N, 122° 55' 12"W), in 2006 and subsequently grown in a continuous flow mesocosm at Friday Harbor Laboratories (Groner et al. 2014). Previous experiments have confirmed that this is a virulent isolate (Groner et al. 2014). *L. zosterae* cells were gently scraped from their SSA plates and suspended in filtered seawater, and a stock inoculum of 8.3 x 10^6 cells ml^{-1} was prepared using a hemocytometer (Groner et al. 2014). Inoculations of 3 cell concentrations were prepared by diluting the stock inoculum: 1:8 for a concentration of 10^6 cells ml^{-1} (high dosage), and then serially diluted 1:10 for concentration of 10^5 cells ml^{-1} (medium dosage) and 1:100 for a concentration of 10^4 cells ml^{-1} (low dosage).

Each dosage treatment consisted of 10 replicate 5 cm leaf sections. A sterile razor was used to score an 'X' through the center of each leaf section to serve as a controlled point of entry for the pathogen to reduce variability of infectivity. Ten ul of the dosage-controlled *L. zosterae* inoculum was pipetted directly onto the scored area of each eelgrass leaf section. While a number of transmission modes have proven to be successful, including infected leaf-drift, infected close-neighbor plant, and attached-infected leaf piece, we developed this dosage-controlled inoculation protocol specifically to test virulence (Martin et al. 2016). Experimental leaf sections were laid onto petri dishes containing a thin layer of sterile seawater and agar. This moist surface prevents the clips from desiccating during the inoculation.

In addition to the 10 replicates for each treatment, 5 replicates of negative controls, consisting of a 10 ml sham inoculum (0 cells ml^{-1} in sterile seawater) were inoculated. Five positive controls (a dosage of 10^4 L. zosterae cells ml^{-1}) placed directly onto the seawater serum agar were added to each temperature-controlled chamber to confirm inoculum viability. All plates were sealed with Parafilm and placed into climate-controlled chambers set to 12 consecutive hours of light per day. After 24 h, 2 ml of sterile seawater were added to all plates (except positive controls) to prevent blade desiccation. Pendant loggers (HOBO) recorded temperature in each of the climate-controlled chambers every 30 min throughout the experiment. The average ± SD temperatures of the incubators were 19.1 ± 0.09°C and 14.38 ± 2.31°C.

The experiment was terminated after 10 d. Each leaf section was photographed on the final day of the experiment. Lesion area was measured using ImageJ (Schneider et al. 2012). When examining leaf sections for the presence of *L. zosterae*, only distinct, dark brown or black-bordered lesions were measured (Muehlstein et al. 1991). We calculated disease prevalence (number of diseased leaf sections/total number of leaf sections x 100%) and severity (total lesion area/leaf section area) as per Groner et al. (2014).

Expt 2: Virulence of L. zosterae *isolates*

We compared the virulence of 3 *L. zosterae* isolates in *Z. marina* leaf tissue (*in vivo*) under controlled conditions (12:12 h light:dark cycle at 20°C). Twenty *Z. marina* plants were collected on 19 June 2016 from Indian Cove, Shaw Island, WA (48° 33' 36" N, 122° 56' 24"W), and kept at ambient temperature in filtered seawater for 3 h to acclimate. Only second-rank leaves with no signs of *L. zosterae* infection were used. Epiphytes and fouling were gently scraped off blade surfaces, and 4 cm

leaf sections (n = 8) were taken from each blade. The base and tip of each blade were discarded. Four leaf sections from each plant were distributed evenly across our 3 isolate treatments to spread potential genetic variation. The remaining leaf sections from each plant were used as negative controls.

The 3 isolates used for inoculations were cultured from the San Juan Islands (WA) on 8 June 2015: 1 isolate (Isolate A) from North Cove, San Juan Island (48° 43' 12" N, 123° 1' 48"W), and 2 isolates (Isolates B and C), from Shoal Bay, Lopez Island (48° 32' 60"N, 122° 52' 48"W). These isolates were chosen based on their variable growth rates in culture. Mean ± SD inoculums of (0.87 ± 0.22) x 10^5 cells ml^{-1} (Isolate A), (1.33 ± 0.39) x 10^5 cells ml^{-1} (Isolate B), and (0.77 ± 0.04) x 10^5 cells ml^{-1} (Isolate C) were prepared using a hemocytometer. Prior to cell quantification, each inoculum was centrifuged with 1 um zirconia/silica beads for 25 s to break up aggregations of clumped cells to improve confidence in our cell concentrations. Unlike in Expt 1, leaf tissue was not scored prior to inoculation since we were testing both infectivity and virulence of the isolates in this experiment. Leaf sections were laid onto petri dishes containing a thin layer of sterile seawater with agar and placed in temperature-controlled chambers set to a 12:12 h light:dark cycle at 20°C.

Pendant loggers that recorded light and temperature were placed on the center of each of the 3 shelves in 2 climate-controlled chambers, set to record every 30 min. Each treatment consisted of 20 experimental samples, 20 negative controls (sterile seawater sham inoculum), and 10 positive controls (inoculum on sterile seawater agar media). Twenty ul of the prepared inoculum were carefully pipetted onto the center of each experimental section. Petri dishes were wrapped with Parafilm to keep samples sealed. After 4 h, the samples were unwrapped and flooded with 3 ml of sterile seawater to keep the leaf sections hydrated. The experiment was terminated after 12 d. Photo

analysis using ImageJ was used as in Expt 1. Recording only characteristic lesions, we calculated disease prevalence and severity.

Expt 3: Impact of light and temperature on L. zosterae *virulence*

We ran a full-factorial experiment comparing the impact of 2 light conditions (12:12 h light:dark cycle and full dark) and 2 temperatures (12 and 18°C) on L. zosterae virulence in *Z. marina* leaf tissue (*in vivo*) and on the cell growth rates of the *L. zosterae* in liquid culture (*in vitro*). *Z. marina* shoots were collected on 31 March 2016 from False Bay, Friday Harbor, WA (48° 28' 48"N, 123° 4' 12"W), and kept at ambient temperature in sterile seawater for 2 h. The second-rank leaf was chosen and its length recorded. The blade was then gently scraped clean of epiphytes and fouling with a glass coverslip, and its central section was cut into 4 cm sections. The base and the tip of the blade were discarded. The 4 cm sections were placed in 0.37 um filtered seawater with an aerator under LED grow lights set on a 12 h diel light cycle to acclimate.

The *L. zosterae* isolate used for our experiments was cultured on 21 March 2016 from the eelgrass tank on the Friday Harbor Laboratories premises where the culture used in Expt 1 was isolated (see Expt 1 methods). An inoculum of (1.20 ± 0.27) x 10^5 cells ml^{-1} (mean ± SD) was centrifuged with 1 um zirconia/silica beads for 25 s to break up cell clumps and prepared using a hemocytometer (see Expt 2 methods).

Pendant loggers that recorded light and temperature were placed on the center of each of the 3 shelves in 2 climate-controlled chambers, set to record every 30 min. The average temperatures of the climate-controlled chambers were 12.02 ± 1.02°C and 18.74 ± 0.58°C, and respective average light intensities were 1100.39 ± 243.16 and 1066.27 ± 322.70 lux. The temperatures were chosen to

be consistent with temperature ranges observed in intertidal eelgrass beds in the San Juan Islands during summer months.

In vivo *experiment*

A single 2 mm cut was made across the center of each leaf section using a sterile razor. Each treatment consisted of 25 experimental samples, 15 negative controls (sterile seawater sham inoculum), and 10 positive controls (inoculum on sterile seawater agar media). Twenty ul of the prepared inoculum were carefully pipetted onto the center of the cut of each experimental section. Petri dishes were wrapped with Parafilm to keep samples sealed. After 4 h, the samples were unwrapped and flooded with 3 ml of sterile seawater to keep the leaf sections hydrated. The dark treatments were wrapped in 2 layers of tinfoil to ensure no light penetration after flooding was completed. The experiment was terminated 7 d after inoculations. Photo analysis using ImageJ was done as in Expts 1 and 2.

In vitro *experiment*

A cell growth experiment with the same full factorial treatments was run in tandem to the in vivo experiment within the same climate-controlled chambers. L. zosterae cells were diluted to 10^4 cells ml^{-1} in serum seawater broth within a 1.5 ml microcentrifuge tube (500 ul of 10^5 cells ml^{-1} L. zosterae inoculum combined with 1000 ul of serum seawater broth). The ingredients of serum seawater broth are identical to previously described SSA (Groner et al. 2014), omitting agar. Fifteen tubes were prepared for each treatment. Daily over 5 d, 3 replicates for each treatment were removed and counted 3 times each. Prior to cell quantification using a hemocytometer, each microcentrifuge tube was centrifuged with 1 um zirconia/silica beads for 25 s to break up aggregations of clumped cells and improve precision of counts.

STATISTICAL ANALYSES:

All analyses were done in R (v. 3.0.1) and were performed at a significance level of $\alpha = 0.05$, except in post hoc tests where Bonferroni-corrected p-values are given. Disease prevalence was analyzed using generalized linear models (GLMs) with binomial error distributions and logit links (function glm in the 'lme4' package, Bates et al. 2015).

Disease severities were analyzed using GLMs with beta error distributions and log links (function 'betareg' in the 'betareg' package, Cribari-Neto & Zeileis 2010). Beta models account for the proportion of clearly lesioned area for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Cell growth was analyzed using GLMs with negative binomial error distribution and log link (glmer.nb, MASS, Venables & Ripley 2002). Where appropriate, post hoc tests were performed using least square means analysis with Bonferroni correction of p-values ('lsmeans,' Lenth 2016). For each analysis, full models and all reduced models were compared using Akaike's information criterion (AIC). R code and datasets can be found in the supplemental.

Expt 1: Impact of dosage and temperature on L. zosterae *virulence*

Differences in levels of disease prevalence were analyzed using a GLM with a binomial error distribution and logit link (function 'glm' of the 'lme4' package). Dose and temperature were treated as continuous fixed factors, with dose characterized by the order of magnitude of the pathogen concentration. Model selection was based on AIC (Table 1). Differences in levels of disease severity were analyzed using a generalized linear model with a beta error distribution and log link (function betareg of the betareg package). Dose and temperature were treated as continuous fixed factors with dose characterized by the order of magnitude of the pathogen concentration. The models accounted

for the proportion of clearly lesioned area for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Model selection was based on AIC (Table 2).

Expt 2: Virulence of L. zosterae isolates

Differences in levels of disease prevalence across different *L. zosterae* isolates were analyzed using a GLM with a binomial error distribution and logistic link (function 'glm' of the 'lme4' [v1.1-13] package). The different isolates were treated as categorical fixed factors. Model selection was based on a likelihood ratio test between the model including isolate and the null model (function 'anova' with 'test=lrt' of the package 'stats' [v3.4.1]). To test for differences between the different isolates, a least-squares mean analysis was performed on the full model (function 'lsmeans' with Bonferroni p-value adjustment of the package 'lsmeans' [v2.26-3]) (Table 3).

Differences in levels of disease severity across different *L. zosterae* isolates were analyzed using a GLM with a beta error distribution and log link. The different isolates were treated as fixed factors and modeled against the proportion of lesioned tissue for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Model selection was based on AIC. To compare the differences between the different isolates, a least-squares mean analysis was performed on the full model (function 'lsmeans' with Bonferroni p-value adjustment of the package 'lsmeans' [v2.26-3]) (Table 5).

Expt 3: Impact of light and temperature on L. zosterae *virulence*

In vivo *experiment.*

Differences in levels of EGWD severity were analyzed using a GLM with a beta error distribution and log link. The full model included the interaction of both temperature and light treatments, and

all subsequent models were reduced from this model. Both light and temperature were treated as fixed factors, with light treatment as categorical and temperature treated as continuous. Severity was modeled as the proportion of diseased area for all clips transformed by the weighted average (Smithson & Verkuilen 2006). Model selection was based on AIC (Table 5).

In vitro *experiment.*

Differences in cell counts of *L. zosterae* were analyzed using a generalized linear mixed-effects model with a negative binomial error distribution and log link function 'glmer.nb' of the MASS package. The full model included the interaction of temperature, light, and time, with all subsequent models being reduced forms of this model. Light, temperature, and time were treated as fixed factors, with light treatment as categorical and temperature and time treated as continuous factors. The vial in which each sample was held was treated as a random factor to maintain consistency with the study design. Model selection was based on AIC (Table 6).

RESULTS:

Expt 1: Impact of dosage and temperature on Labyrinthula zosterae *virulence*

From comparison of models using AIC, we found that the model best supported by the data only includes dosage and not temperature when analyzing prevalence. From this model and the averaged model, we found that disease prevalence increases with the log of pathogen dose ($Z = 2.360$, $p = 0.0183$, relative importance [RI] = 1.0) and is only weakly influenced by temperature ($Z = 0.091$, $p = 0.9276$, RI = 0.36; Fig. 1). The analysis of severity found that the best model was the full model including the interaction of dosage and temperature. From this model and the averaged model, we found that that dosage ($Z = 2.251$, $p = 0.02$, RI = 0.99) was significant, while both temperature ($Z = 0.127$, $p = 0.89$, RI = 0.30) and the interaction of temperature and dosage were not ($Z = 0.259$, p

= 0.79, RI = 0.07; Fig. 1). L. zosterae cell growth in the positive controls showed that the inoculum was viable.

Expt 2: Virulence of L. zosterae *isolates*

Analysis of the 3 potentially unique isolates of *L. zosterae* and controls showed that the models allowing for differences in severity and prevalence between the different isolates fit the data better than the models that did not allow for the variation (prevalence likelihood ratio test: chi-squared = 22.456, $p < 0.001$; Fig. 2). From subsequent post hoc least squares means analysis with a Bonferroni p-value correction, we found 3 distinct groups for prevalence, with Isolate A from North Cove and Isolate B from Shoal Bay. For severity, Isolate C from Shoal Bay was significantly different from the rest. *L. zosterae* cell growth in the positive controls showed that the inoculum was viable.

Expt 3: Impact of light and temperature on L. zosterae *virulence*

In vivo *experiment*

Photo-analysis of our negative controls and treatments confirmed that our inoculation methods were effective. A total of 3 out of 60 of our negative controls showed disease, with lesion area confined to small specks of blackened tissue. The ubiquitous nature of the pathogen makes picking completely uninfected plants difficult, with the possibility of *L. zosterae* existing commensally in the plants before the experiment and shifting to pathogenic once the plants became stressed. Conversely, 99 out of the 100 inoculated leaf sections developed lesions, further supporting the effectiveness of our inoculation methods.

Comparison of the models using AIC showed that the best model includes only the light regime. The averaged model supports this and we can conclude that reductions in light increase severity of

the disease ($Z = 2.207$, $p = 0.02$), while increasing temperature does not impact severity ($Z = 0.177$, $p = 0.86$), and there are no interactive effects ($Z = 0.255$, $p = 0.82$) (Fig. 3). *L. zosterae* cell growth in the positive controls showed that the inoculum was viable.

In vitro *experiment*

The cell growth experiment was run for a total of 5 d. However, due to a lack of resources—either space or nutrients—cell growth in the 18°C treatment plateaued by Day 3. For this reason, Days 4 and 5 of the cell growth experiment were not included in our analysis.

From the comparison of the models using AIC, the best model includes a changing impact of temperature over time with a constant light effect. From this model, growing L. zosterae in complete darkness does not alter the growth rate, although there are consistently more cells in the culture at all time points ($Z = 3.828$, $p < 0.001$). The 2 different temperature treatments showed no difference at the start of the trial ($Z = 0.715$, $p = 0.475$), but the cells grown at the higher temperature showed an increased growth rate ($Z = 2.145$, $p = 0.0320$) (Fig. 4).

DISCUSSION:

Opportunistic pathogens are those that coexist commensally with their hosts, leading to patho - genesis only under specific conditions such as host immunosuppression or environmental change (Burge et al. 2013, 2014). Understanding the changeable biology of opportunistic pathogens is a large knowledge gap, gaining increased attention within disease ecology (Burge et al. 2013) as opportunistic outbreaks increase with ocean change (Burge et al. 2014). Since at least some strains of *Labyrinthula zosterae* are ubiquitous in the marine environment, the factors that facilitate an outbreak

of EGWD are likely to rely on either a change in environment or emergence of new strains. L. zosterae strains are readily culturable and so the EGWD pathosystem is unusually tractable to experimentally investigate key drivers of variation in virulence of infections.

We developed an improved method to control dosage in experimental inoculations of eelgrass to quantitatively test differences in *L. zosterae* performance across environments and for different isolates. Our dosing method provides increased control of cell concentrations to study the host-pathogen interaction between *Zostera marina* and *L. zosterae*. *L. zosterae* cells secrete an ectoplasmic filamentous net through bothrosome organelles by which their spindle-shaped cells can move and absorb nutrients (Porter 1972). This ectoplasmic net results in irregular, sticky aggregations of *L. zosterae* cells in culture which are difficult to count and impossible to quantitatively dose. In previous studies, inoculations of *L. zosterae* on healthy seagrass plants were performed by several methods: direct contact with active lesions (Vergeer et al. 1995), direct contact with gauze bandages containing the pathogenic *L. zosterae* cells (Brakel et al. 2014), or by inoculation in a liquid culture homogenized with a vortex and estimated with a hemocytometer (Groner et al. 2014). To ensure consistency in pathogenic cell concentrations across inoculations in this study, *L. zosterae* cells were vortexed for 25 s with 1 um zirconia/silica beads and diluted to appropriate concentrations with sterile seawater before being pipetted directly onto plant tissue. Vortexing the cells with glass beads broke up irregular aggregates of *L. zosterae* cells and allowed consistent cell counts between treatments.

Results from the *L. zosterae* dosage experiment show that higher concentration of pathogenic cells leads to higher disease severity at both temperatures. Inoculation of 10^6 pathogenic cells ml^{-1} caused 17.8% (1.67 times) higher severity compared to 10^5 cells ml^{-1}, and 30.4% (3.11 times) higher severity compared to 104 cells ml^{-1} (when temperature was held at 16.5°C). Further studies are needed to

determine environmental levels of *L. zosterae*, and subsequently test if patterns of higher disease severity at higher pathogenic cell concentrations hold up under naturally occurring conditions.

In the isolate inoculation experiment, we tested for differences in virulence of 3 pathogen isolates cultured from the San Juan Islands. The activity of our isolates fell into 2 significantly different categories of severity—1 isolate stood out as being much more virulent, with lesions developing approximately 25% faster, while the other 2 isolates grouped together at a lower severity with the control treatment. Over the course of our 12 d trial, our most virulent isolate fell into a higher prevalence category than the control treatment and the other 2 isolates, with at least 25% more leaf sections developing lesions. The high virulence isolate and 1 of the low-virulence isolates came from the same field site (Shoal Bay), showing that mixes of different virulence isolates exist in nature. This study has implications for future conservation and management of critical marine habitats. To offset the effects of eelgrass bed decline, conservation efforts have turned to transplanting eelgrass to accelerate recolonization and expansion at sites with suitable ecological conditions (for example Leschen et al. 2010, Goehring et al. 2015). Given our results that different isolates of *L. zosterae* can vary from relatively non-pathogenic to highly virulent, it is important to use care in moving transplants, as they might harbor unusually virulent isolates of *L. zosterae*. The introduction of novel isolates to different populations is risky, and is well documented as an epidemiological phenomenon causing spread of infectious disease. Our study suggests that more work needs to be done on strain diversity of this pathogen, since virulent strains coupled with transplantation stress will undermine restoration efforts. Use of transplants to accelerate growth and bed coalescence is a proactive way to restore declining eelgrass beds, but these transplants need to be informed by knowledge of local disease metrics to avoid increasing the range of more virulent isolates of *L. zosterae*.

The ability to culture *L. zosterae* allowed us to distinguish between the separate response of the pathogen in isolation (in vitro) and inside its eelgrass host (in vivo). Our in vitro study with 2 light levels and 2 temperatures at a constant dosage showed that *L. zosterae* cell growth is marginally affected by light levels, with slightly higher cell counts in dark treatments compared to light treatments. However, isolated pathogen cells grew much faster at the warmer temperature; cells of *L. zosterae* grew 2.13 times faster at 18°C than at 11°C. Thus the small effect of light was overwhelmed by the huge effect of temperature on in vitro growth of *L. zosterae* cells. The *in vivo* light/temperature experiment showed the opposite result. Disease severity was much higher in the dark, but only marginally affected by temperature. Since light level had an almost negligible affect on *in vitro* growth, we conclude that this significant role of light in the in vivo experiment is driven by the capability of *Z. marina* to mount a defense response to the infection. Therefore, plants grown in the dark were unable to mount an effective defense and consequently infections spread more rapidly.

Light, directly associated with photosynthetic capability, controls a plant's resources that could be allocated to produce secondary metabolites used as defense mechanisms. Secondary metabolites play an important role in plant disease resistance, but the biochemical basis of seagrass defense responses is understudied (Ross et al. 2008). Seagrasses produce a range of secondary metabolites, including phenolic-based compounds (Trevathan-Tackett et al. 2015). Phenolic-based compounds have diverse ecological roles in marine angiosperms and are capable of mediating biotic interactions on both broad and localized scales (Sieg & Kubanek 2013). One proposed mechanism for increased disease resistance in eelgrasses is the production of these phenols. Vergeer & Develi (1997) found that eelgrass plants kept under higher light intensities produced a higher concentration of phenolic compounds in response to infection than plants kept under low light intensities. They also found

lower infection rates at higher light intensities. Induction of phenols is a significant energy investment (Vergeer & Develi 1997), so it is no surprise that our data suggest that decreased light and thus photosynthetic capability is directly correlated with the ability of *Z. marina* to mount an effective response to infection. This finding may help explain variable levels of EGWD in nature between beds and across seasons that have variable canopy light conditions. Shifts in the environmental context of EGWD caused by climate change and physical intervention by overwater structures are imminent, and will have impacts on plant-pathosystem dynamics. Our results and previous studies (Kaldy 2014, Sullivan et al. 2017) suggest that increased temperature associated with recent trends of climate change may increase the risk of disease of *L. zosterae* in the marine environment. These results are consistent with earlier studies investigating the temperature range of *Labyrinthula* spp. (Young 1943, Pokorny 1967), but our study is the first to pair in vitro with *in vivo* experiments at a controlled dosage to quantify how increased temperatures directly increase reproductive rates of the pathogen. In the coming century, average annual temperatures in Washington are projected to rise at a rate of 0.1 to 0.6°C (0.2 to 1.0°F) decade^{-1} (Lawler & Mathias 2007). Global average temperatures have increased by 0.7°C (1.3°F) over the last century and are projected to rise between 1.1 and 6.4°C (2.0-11.5°F) by 2100 (Alley et al. 2007). Light attenuation events that may be caused by algal blooms or turbid plumes occur with increased frequency in coastal waters and during high water temperature periods in the summer (Kim et al. 2015). Furthermore, light quality and quantity are often altered by human actions in coastal areas (Zimmerman 2006), such as construction and expansion of structures such as piers, docks, and bridges (Short & Wyllie-Echeverria 1996, Shafer 1999), channel dredging (Moore et al. 1997, Longstaff & Dennison 1999), and runoff from watersheds (Longstaff & Dennison 1999, Cabello-Pasini et al. 2002). While the effects of light and temperature on eelgrass growth and survival as well as on EGWD dynamics have been investigated previously (Backman & Barilotti 1976, Vergeer et al.

1995, Hauxwell et al. 2001, Thom et al. 2008, Kim et al. 2015), our studies test hypotheses about the role of these environmental factors to facilitate EGWD with quantitative methods. These studies further elucidate the affects of shifting environmental parameters on both global and localized scales, and contribute to the body of literature that informs the building of coastal structures over seagrass beds that may exacerbate EGWD.

Understanding emergence of EGWD requires knowledge of its host-pathogen biology, which will involve the use of models parameterized for many environmental, ecological, and biological factors. Characterization of the status of *L. zosterae* as an opportunistic pathogen will help define the mechanisms of EGWD and may lead to further research on defining potential approaches for managing and projecting seagrass diseases. To improve our ability to predict and respond to eelgrass epidemics in wild populations, it will be necessary to take into consideration the interplay of abiotic factors in plant-pathosystem dynamics (Sullivan et al. 2013).

FIGURES:

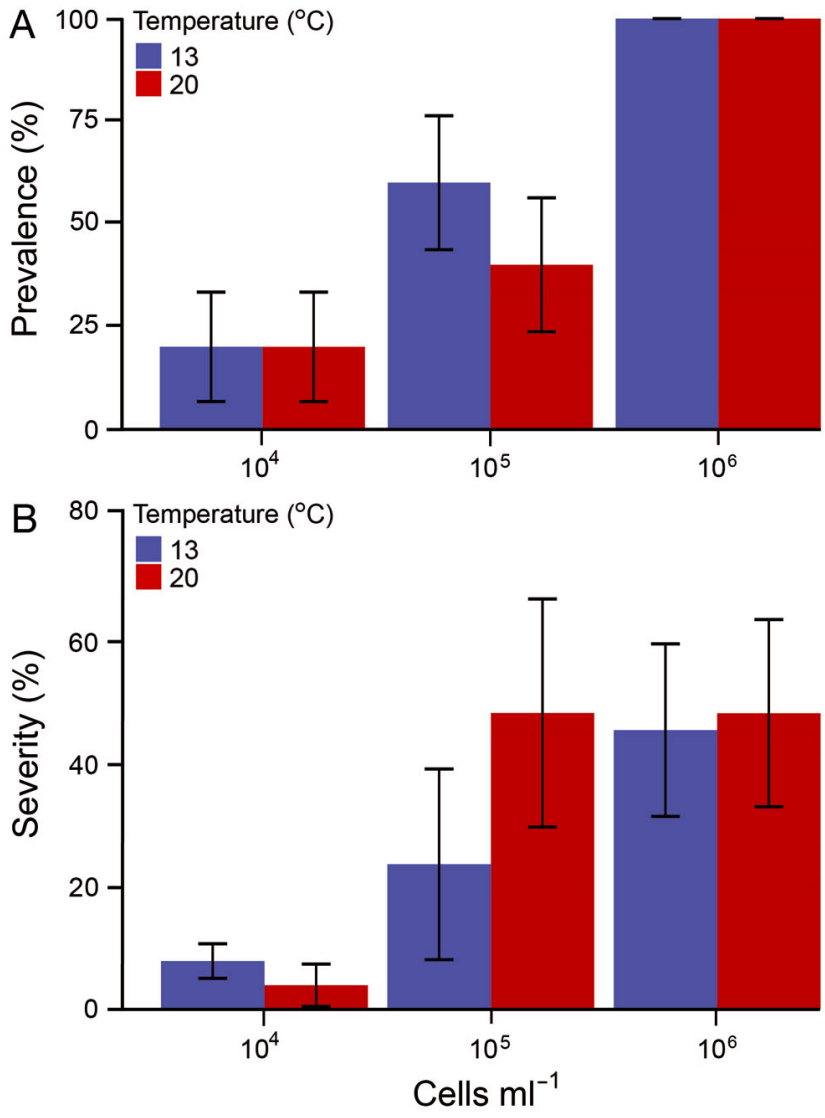

Fig. 1. Increasing pathogen dosage increases both prevalence and severity of eelgrass wasting disease. Bars represent the mean ±1 SE for (A) disease prevalence and (B) disease severity across 3 dosage treatments and 2 temperatures. Significant effects of temperature were only found in panel (B)

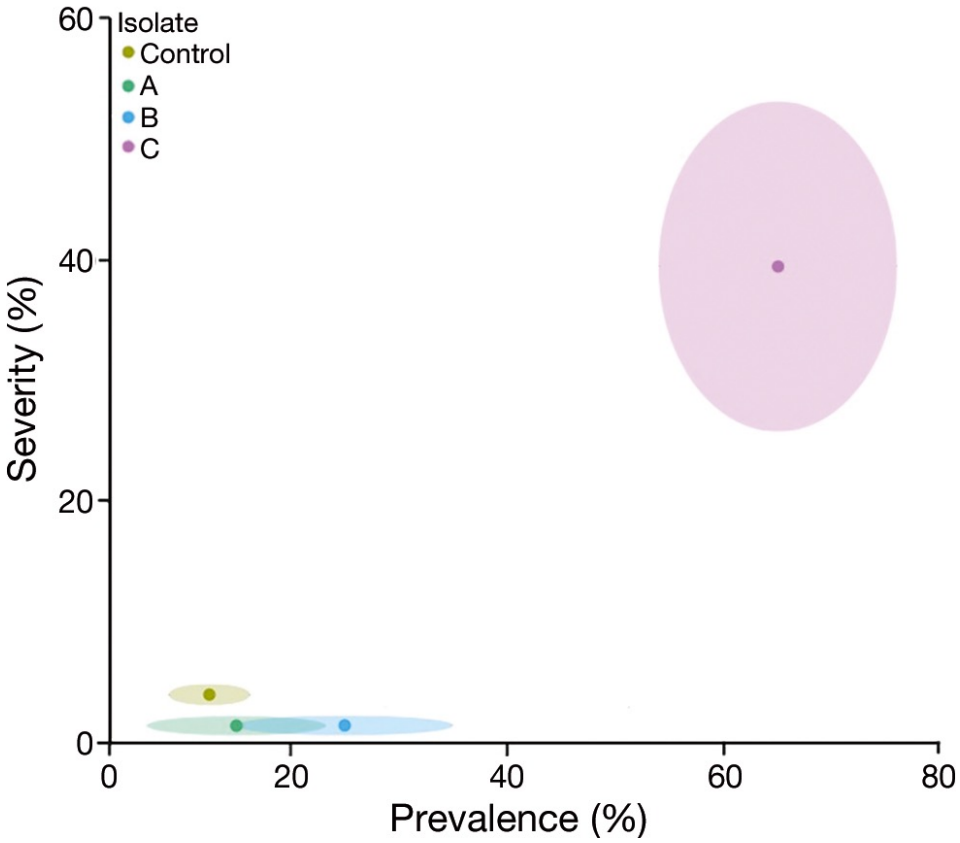

Fig. 2. Different isolates of *Labyrinthula zosterae* vary in disease prevalence and severity. Points represent mean levels of prevalence and severity for the different isolates with the ellipse boundaries representing ±1 SE. Differences in prevalence and severity between isolates are significant ($p < 0.05$)

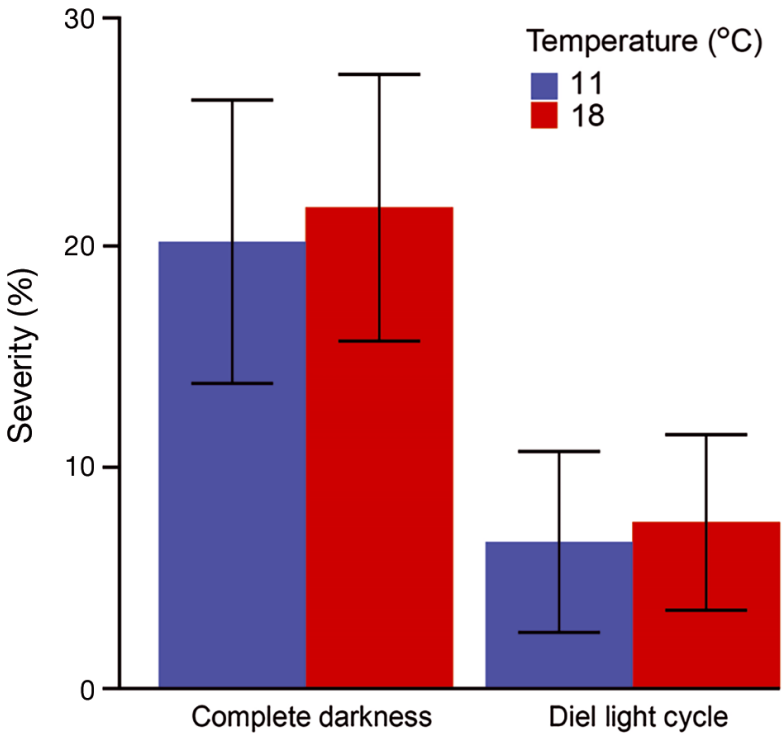

Fig. 3. *In vivo* experiment. Light and temperature influence eelgrass wasting disease severity. Mean ± SE disease severity showing that reductions in light and increasing temperatures both increase severity

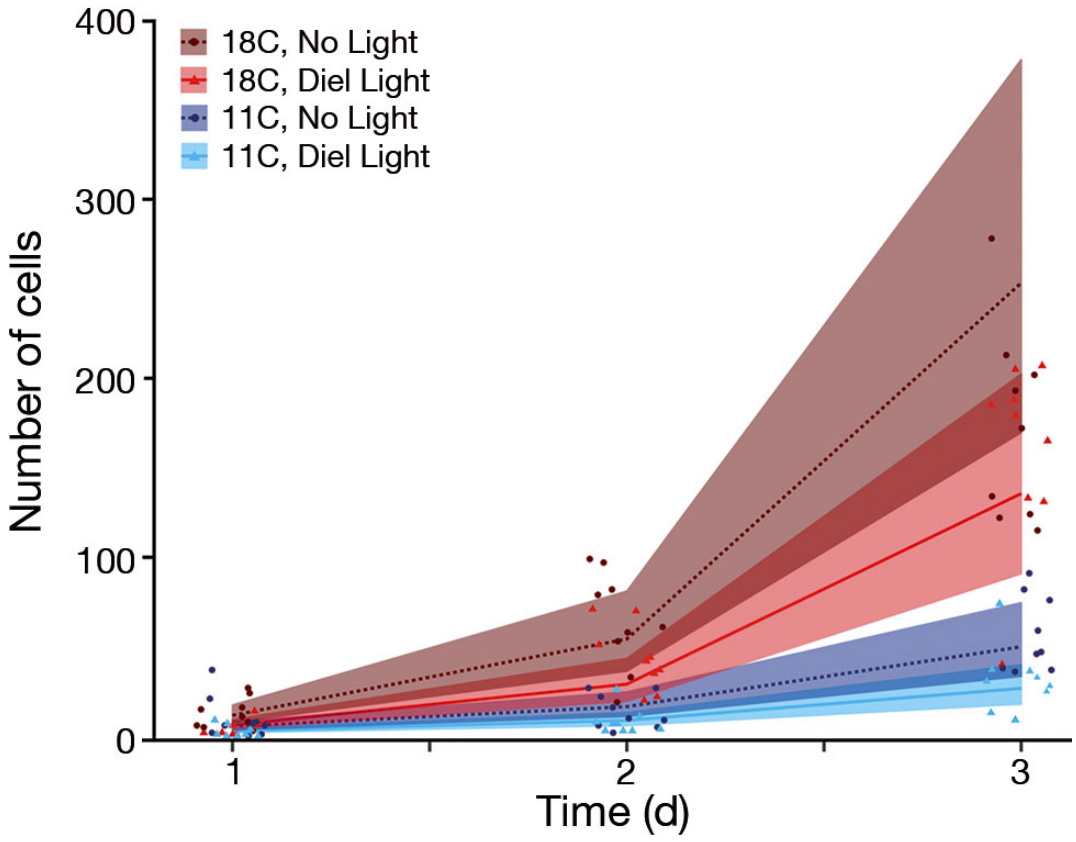

Fig. 4. *In vitro* experiment. Light and temperature influence the population growth rates of *Labyrinthula zosterae*. Predicted growth curves ±1 SE from the best model that has increasing temperature increasing the growth rate and a constant difference between the 2 light treatments. The points represent individual counts at various time points for each of the treatments

TABLES:

	[1] Full	[2] Additive	**[3] Dose**	[4] Temperature	[5] Null
Intercept estimate	−12.183	−9.926***	**−10.960*** **	0.910	0.268
Intercept SE	13.181	2.994	**2.678**	1.263	0.261
Dose estimate	2.781	2.313***	**2.2882*** **	NA	NA
Dose SE	2.723	0.555	**0.5485**	NA	NA
Temperature estimate	0.0661	−0.0699	**NA**	−0.0389	NA
Temperature SE	0.774	0.101	**NA**	0.0746	NA
Interaction estimate	−0.0281	NA	**NA**	NA	NA
Interaction SE	0.159	NA	**NA**	NA	NA
df	4	3	**2**	2	1
logLik	−25.661	−25.676	**−25.920**	−40.918	−41.053
AICc	60.049	57.782	**56.051**	86.046	84.176
Delta	3.998	1.731	**0**	29.995	28.125

Weight	0.0870	0.270	**0.642**	1.97×10^{-7}	5.02×10^{-7}

Model equations are:

[1] logit(Disease Presence) ~ $b_1 + b_2$Dose + b_3Temperature + b_4Dose×Temperature
[2] logit(Disease Presence) ~ $b_1 + b_2$Dose + b_3Temperature
[3] logit(Disease Presence) ~ $b_1 + b_2$Dose
[4] logit(Disease Presence) ~ $b_1 + b_2$Temperature
[5] logit(Disease Presence) ~ b_1

Table 1. Expt 1. Generalized linear model selection used to assess the effects of pathogen dose and temperature on eelgrass wasting disease prevalence. Bolded column represents the best model. LogLik: log-likelihood; AICc: Akaike's information criterian corrected for small sample size; NA: not applicable. ***p < 0.001

	[1] Full	[2] Additive	[3] Dose	[4] Temperature	[5] Null
Intercept estimate	**11.202*** **	−5.500***	−4.669***	−1.328***	−0.563***
Intercept SE	**0.753**	0.163	0.143	0.0748	0.0153
Dose estimate	**1.763*** **	0.746***	0.743***	NA	NA
Dose SE	**0.133**	0.0254	0.0254	NA	NA
Temperature estimate	**0.381*** **	0.0492***	NA	0.0462***	NA
Temperature SE	**0.0424**	0.00450	NA	0.00439	NA
Interaction estimate	**0.0592*** **	NA	NA	NA	NA
Interaction SE	**0.00751**	NA	NA	NA	NA
df	**4**	3	2	2	1
logLik	**7324.208**	−7355.825	−7415.761	−7841.884	−7897.363
AICc	**14657.796**	14718.450	14835.910	15688.156	15796.851
Delta	**0**	60.653	178.113	1030.359	1139.055
Weight	**1**	6.75×10^{-14}	2.10×10^{-39}	1.82×10^{-224}	4.54×10^{-248}

Model equations are:

[1] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Dose + β_3Temperature + β_4Dose×Temperature
[2] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Dose + β_3Temperature
[3] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Dose
[4] logit(Lesioned Area, Healthy Area) ~ $\beta_1 + \beta_2$Temperature
[5] logit(Lesioned Area, Healthy Area) ~ β_1

Table 2. Expt 1. Generalized linear model selection used to assess the effects of pathogen dose and temperature on eelgrass wasting disease severity. Bolded column represents the best model. Abbreviations as in Table 1. *** p < 0.001

Comparison	**Estimate**	**SE**	***Z*-ratio**	**p**
Control−A	**−0.211**	**0.712**	**−0.297**	**1.000**
Control−B	**−0.847**	**0.617**	**−1.373**	**1.000**
Control−C	**−2.565**	**0.578**	**−4.438**	**0.0001**
A−B	**−0.636**	**0.812**	**−0.784**	**1.000**
A−C	**−2.354**	**0.782**	**−3.009**	**0.0157**
B−C	**−1.718**	**0.697**	**−2.463**	**0.0827**

Table 3. Expt 2. Least-squares means table comparing eelgrass wasting disease prevalence across different pathogen isolates (A: North Cove; B: Shoal 1; C: Shoal 2). Significant p-values (p < 0.05) are shown in bold

Comparison	Estimate	SE	*Z*-ratio	p
Control−A	−0.032	0.024	−1.324	0.548
Control−B	−0.034	0.025	−1.392	0.505
Control−C	−0.152	0.040	−3.787	**0.001**
A−B	−0.002	0.033	−0.066	0.999
A−C	−0.120	0.045	−2.636	**0.041**
B−C	−0.117	0.045	−2.580	**0.049**

Table 4. Expt 2. Least-squares means table comparing eelgrass wasting disease severity across different pathogen isolates (A: North Cove; B: Shoal 1; C: Shoal 2). Significant p-values ($p < 0.05$) are shown in bold

Comparison	*W*	r	p
L12−L18	218.5	0.672307692	0.06965
D12−D18	288	0.886153846	3.8484
L12−D12*	485.5	1.493846154	0.0048876
L12−D18*	124	0.381538462	0.0009294
L18−D12	428.5	1.318461538	0.14994
L18−D18*	457	1.406153846	0.02718

Table 5. Expt 3. Statistical summary for differences in eelgrass wasting disease severity across treatments. Pairwise comparisons were completed using Wilcoxon rank sum. Reported p-values are Bonferroni-corrected. L: light; D: dark; number indicates temperature (°C). *significant at $p < 0.05$

Comparison	p
L12−L18*	0.005756
D12−D18*	0.0102862
L12−D12	0.7747686
L12−D18*	0.0031316
L18−D12*	0.0200634
L18−D18	0.9560524

Table 6. Expt 3. Statistical summary for differences in cell counts across treatments. *significant at $p < 0.05$. Treatments are defined in Table 5

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