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Pacific oysters are a sink and a potential source of the eelgrass pathogen, *Labyrinthula zosterae*

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ABSTRACT: Oyster aquaculture and seagrasses often co-occur and are each vital to the ecological and economic value of coastal ecosystems. Global declines in seagrasses, including *Zostera marina*, have recently been observed in association with multiple factors, including infection with diseases such as seagrass wasting disease (SWD), caused by the protist *Labyrinthula zosterae*. Protection of seagrasses has led to restrictions on oyster aquaculture due to perceived negative impacts on seagrass beds; however, positive impacts may also occur. An important aquaculture species, the Pacific oyster *Crassostrea gigas*, can filter *L. zosterae* from the water, potentially reducing pathogen transmission, although oysters may vector infection if they accumulate and release live *L. zosterae* into the water. We investigated whether oyster presence decreases lesion severity and infection intensity in eelgrass, or acts as a vector of *L. zosterae*, via laboratory and field experiments. In the laboratory, oysters and eelgrass were exposed to *L. zosterae* for 24 h and kept at 11°C or 18°C for 13 d. In the field, eelgrass ramets were deployed with and without oysters for 28 d adjacent to eelgrass known to have SWD. In the laboratory experiment, the presence of oysters significantly decreased lesion severity and infection intensity, but oysters previously exposed to *L. zosterae* did transmit the pathogen to naïve eelgrass. Temperature did not affect oyster ability to mitigate SWD; however, increased temperature significantly increased lesion severity. Oysters had no effect on SWD in the field. Further research is needed regarding the potential for oysters to vector *L. zosterae* and to quantify when oysters reduce SWD in the field.

KEY WORDS: Eelgrass · *Labyrinthula zosterae* · Oyster · Temperature · Transmission · Wasting disease

1. INTRODUCTION

Oysters and seagrasses are important for both the ecological function and economic value of coastal systems, with oyster aquaculture often co-occurring near seagrass beds. Oysters are a key aquaculture species: in 2018, oysters were the highest volume shellfish produced in the USA, resulting in $219 million in revenue (National Marine Fisheries Service 2021). Additionally, oysters provide valuable habitat (Gra-

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bowski & Peterson 2007), support benthic–pelagic coupling of nutrients (Newell et al. 2005), and improve water quality through filter feeding (Porter et al. 2004). Seagrasses, like oysters, are ecosystem engineers that provide habitat and nursery grounds, contribute to sediment stabilization (Potouroglou et al. 2017), efficiently sequester carbon (Duarte et al. 2005), and reduce bacterial pathogens detrimental to both humans and marine organisms (Lamb et al. 2017, Reusch et al. 2021). Seagrass coverage is decreasing globally due to a variety of factors including eutrophication, sedimentation, increases in sea surface temperature, and disease (Short & Wyllie-Echeverria 1996, Orth et al. 2006, Waycott et al. 2009, Sullivan et al. 2013). Resource managers are tasked with implementing restoration for seagrasses, including in the Salish Sea, located in the northeast Pacific, where seagrasses are considered a critical habitat (Christiaen et al. 2019). This type of protection can restrict oyster farming due to the perceived negative effects of bivalve aquaculture on seagrasses (Dumbauld et al. 2009). Oyster aquaculture can cause pulse and press ecological disturbances to the environment by affecting material processes, creating physical structure, and by increasing physical disruptions (Dumbauld et al. 2009). The effects of oyster aquaculture on seagrass beds vary with location and aquaculture method (Everett et al. 1995, Wisehart et al. 2007, Tallis et al. 2009), indicating that this relationship can be complicated and likely depends on a variety of factors.

Although oyster aquaculture may cause initial and periodic disturbances to eelgrass beds, filter-feeding oysters may mitigate one potential contributor to seagrass loss, seagrass wasting disease (SWD), by filtering the pathogen *Labyrinthula zosterae* out of the water. Infection by this pathogen leads to black necrotic lesions along seagrass blades and can ultimately lead to death of the plant (Muehlstein et al. 1988, 1991). In the 1930s, SWD contributed to a >90% loss of eelgrass *Zostera marina* beds along both coasts of the northern Atlantic (Cotton 1933). This loss in habitat had a devastating effect and contributed to major declines in migratory waterfowl populations (Cottam & Addy 1947) and lasting devastation to the bay scallop fishery (Oreska et al. 2017). Although monitoring for SWD has been inconsistent, it was detected in the 1980s along the northwest Atlantic seaboard from Nova Scotia, Canada, to North Carolina, USA, and the northeast Pacific in the Salish Sea, and has been associated with eelgrass declines at some locations (Short et al. 1987). Recently, in the San Juan Islands, WA, USA, located in the Salish Sea, increased SWD prevalence and severity occurred during and after unusually high summer temperatures (Groner et al. 2021). The dramatic repercussions of the 1930s epidemic demonstrate the cascading effects of seagrass habitat loss and highlight the need for continued efforts to understand the role of *L. zosterae* in seagrass population health (Short et al. 1987).

Bivalves have been used to reduce densities of pathogens and parasites, including larval salmon lice and *Himasthla elongatain* in cockles (reviewed by Burge et al. 2016). In laboratory trials, Pacific oysters reduced SWD severity in naturally infected *Z. marina* (Groner et al. 2018). In addition to filtering waterborne pathogens and reducing transmission, oysters could act as a source of infection if they maintain pathogen populations and transmit the pathogen to naïve hosts (reviewed by Burge et al. 2016). Understanding the role of oysters as a sink or source of *L. zosterae* is crucial when restoring eelgrass beds, implementing oyster aquaculture within or adjacent to eelgrass beds, and transporting oysters between aquaculture sites.

Temperature affects numerous marine host–pathogen interactions (Burge & Hershberger 2020), and the impacts of temperature on SWD in mixed oyster aquaculture–eelgrass beds are not well documented. Temperature alters bivalve filtration, with rates maximized at a thermal optimum and decreasing above and below that optimum (Gray & Langdon 2018). Climate change impacts may also affect pathogenicity; *Labyrinthula* spp. are widespread within marine environments, and they are not always pathogenic (Raghukumar 2002, Martin et al. 2016, Trevathan-Tackett et al. 2018). It is hypothesized that disease outbreaks associated with pathogenic *Labyrinthula* spp., including the 1930s SWD outbreak, correspond to unfavorable conditions for the host such as changes in salinity, decreased light availability (Young 1943, Giesen et al. 1990, McKone & Tanner 2009), and increased temperatures (Bull et al. 2012, Kaldy 2014, Groner et al. 2021). A better understanding of the effects of temperature on both the eelgrass–*L. zosterae*–oyster interactions and oyster filtration of *L. zosterae* is critical for implementing oyster aquaculture to mitigate SWD.

The goal of this study was to investigate the ability of Pacific oysters *Crassostrea gigas* to mitigate SWD in eelgrass *Z. marina* and to serve as vectors of this pathogen at both ambient and increased temperatures. Using laboratory trials, we tested the hypotheses that the presence of oysters can decrease lesion prevalence and severity, as well as *L. zosterae* infection intensity in *Z. marina*, and that infection of *Z. marina* with *L. zosterae* leads to decreased growth of infected eelgrass. We also hypothesized that oysters exposed to *L. zosterae* could act as a vector and

transmit the pathogen to naïve eelgrass. All results were hypothesized to be affected by temperature, with severity of lesions and *L. zosterae* infection intensity hypothesized to increase at 18°C compared to 11°C. Using a field experiment, we investigated the hypothesis that oyster presence would decrease both the prevalence and severity of SWD.

2. MATERIALS AND METHODS

We conducted these experiments in the San Juan Islands, an area where SWD is present (Groner et al. 2014), and where eelgrass die-offs have been recorded since 2002 (Christiaen et al. 2019).

2.1. Laboratory experiment

We conducted a laboratory experiment in the Ocean Acidification Experimental Laboratory (OAEL) at Friday Harbor Laboratories (FHL) in Friday Harbor, WA, USA (48.550°N, 120.008°W). Oysters were co-cultured with eelgrass under 5 unique treatments to investigate the ability of Pacific oysters to either mitigate or vector SWD.

2.1.1. Plant collection and processing

Eelgrass ramets were collected from Fourth of July Beach, Friday Harbor, WA, USA (48.463713° N, 122.991055° W) on 29 July 2019 at low tide (approx. –3 to 0 m Mean Low Water). Plants were haphazardly sampled every ~2 m across ~200 m. Collected ramets were immediately transported to FHL in seawater. Upon return, the oldest leaves of all plants were removed along with any signs of SWD (identified by black/brown lesions), leaving only the youngest 3 leaves. The remaining leaves were rinsed in 1 µm filtered seawater (FSW) to remove epiphytes and sand from the collection site. Plants were rinsed in low salinity seawater (1 µm FSW mixed with freshwater to a final salinity of 10 ppt) at ambient temperature for 12 h to minimize potential background infection of *Labyrinthula zosterae* (Muehlstein et al. 1988). The youngest 2 remaining blades with no visual signs of SWD were then cut to 15 cm length, and all other blades were removed. All roots were trimmed to one node and a pin prick was made through the sheath to track future changes in growth. During trimming, all plants were checked again to ensure those used were free from visual signs of disease. Ramets were tied to glass weights at the root to keep them oriented upright and placed into tanks at their corresponding temperatures (11°C or 18°C) and allowed to acclimate overnight. Due to a power outage, a temperature spike of 2–4°C was experienced overnight in all replicates (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/q014p295_supp.pdf). Once all treatments were back to the correct temperature, the eelgrass acclimated to treatment temperatures for ~24 h prior to inoculation.

2.1.2. Oyster collection

Pacific oysters (mean ± SE length: 36.00 ± 0.16 mm; Table S1) were collected from an oyster farm in Shelton, WA, USA, and transported on ice to FHL with a transfer permit from the state of Washington. Oysters were kept in ambient flow-through seawater with aeration until onset of the experiment and fed ~5 ml (~10 billion cells) of Shellfish Diet 1800 (Reed Mariculture) per 500 oysters daily. The flow-through seawater was turned off to allow feeding. Prior to the experiment, oysters were placed into 1 µm FSW for 24 h, followed by freshwater for 1 h, to remove any potential *L. zosterae*.

2.1.3. Inoculum

The *L. zosterae* isolate used for the laboratory experiment was cultured from a SWD lesion on eelgrass collected in July 2018 from Fourth of July Beach. Multiple replicates of the isolate were plated on serum seawater agar with antibiotics as per Porter (1990) and modified by Groner et al. (2014), wrapped in parafilm, and grown at 17°C for 7 d. *L. zosterae* cells were scraped from each plate with ~2 ml of 1 µm FSW into a 250 ml Eppendorf tube. To disrupt the mucus net formed by *L. zosterae* cells, the cells were briefly vortexed with 1 µm zirconia/silica beads (Dawkins et al. 2018). The cells were diluted and recounted on a hemocytometer until a final concentration of ~ 7×10^6 cells ml^{-1} was obtained.

2.1.4. Experimental set-up

The experiment was conducted using a nested design. The system used is that described in O'Donnell et al. (2013). Individual 4 l tanks (replicates) containing 3.5 l seawater and aeration were nested within larger coolers that dispersed FSW into each tank

(Fig. 1). Incoming seawater to FHL went through a series of filtration: sand filtration, 25 µm, 10 µm, 5 µm, and finally 1 µm. Filtered water was either heated or cooled to the appropriate temperature (18°C or 11°C) using a Honeywell UDA2182v controller before being dispersed into each tank. The outflow from each tank was UV sterilized to ensure *L. zosterae* from the experiment was not introduced into the environment. Each cooler was placed under full spectrum LED lights (MarineLand) on a 16 h light:8 h dark cycle with ~117 ± 6 µmol photons m^{-2} s^{-1}.

Each replicate contained 4 eelgrass ramets (with no signs of SWD) that were subjected to one of 5 treatments: (1) inoculated with *L. zosterae* (positive control), (2) alone (negative control; background infection assessment), (3) co-cultured with oysters (negative control with oysters), (4) inoculated with *L. zosterae* and co-cultured with oysters (*L. zosterae* with oysters), or (5) co-cultured with oysters previously exposed to *L. zosterae* (inoculated oysters; Fig. 1).

Each treatment was replicated 3 times at 18°C and twice at 11°C (Fig. 1). Replicates with oysters included 15 oysters placed in a mesh bag and suspended in the middle of the tank. One replicate per treatment was placed in 1 of 5 coolers. Temperature manipulation was done to unique coolers, 2 were set to 11°C and 3 at 18°C.

Inoculated treatments were exposed to a final concentration of ~7 × 10^3 *L. zosterae* cells ml^{-1}; negative controls were inoculated with 1 µm FSW. The *L. zosterae* inoculum and control inoculum were added simultaneously with 500 µl (~1 billion cells) of Shellfish Diet 1800 (Reed Mariculture) to promote oyster filtration. During inoculation, flow-through seawater was turned off for 24 h (aeration remained). After 24 h, the oysters inoculated with *L. zosterae* for the inoculated oyster treatment were added to a tank with naïve eelgrass shoots, and the flow-through seawater was turned on for all treatments. Every 2 d, the flow-through seawater was turned off for 1 h and all tanks received 500 µl of Shellfish Diet 1800 to allow for feeding. Growth of oysters with this feed has been accomplished in past laboratory settings (C. Burge, pers. comm), and active feeding by oysters in the present experiment was inferred by the presence of clearer water after 1 h. To reduce diatom growth and prevent plumbing clogs, 0.67 ppm (final concentra-

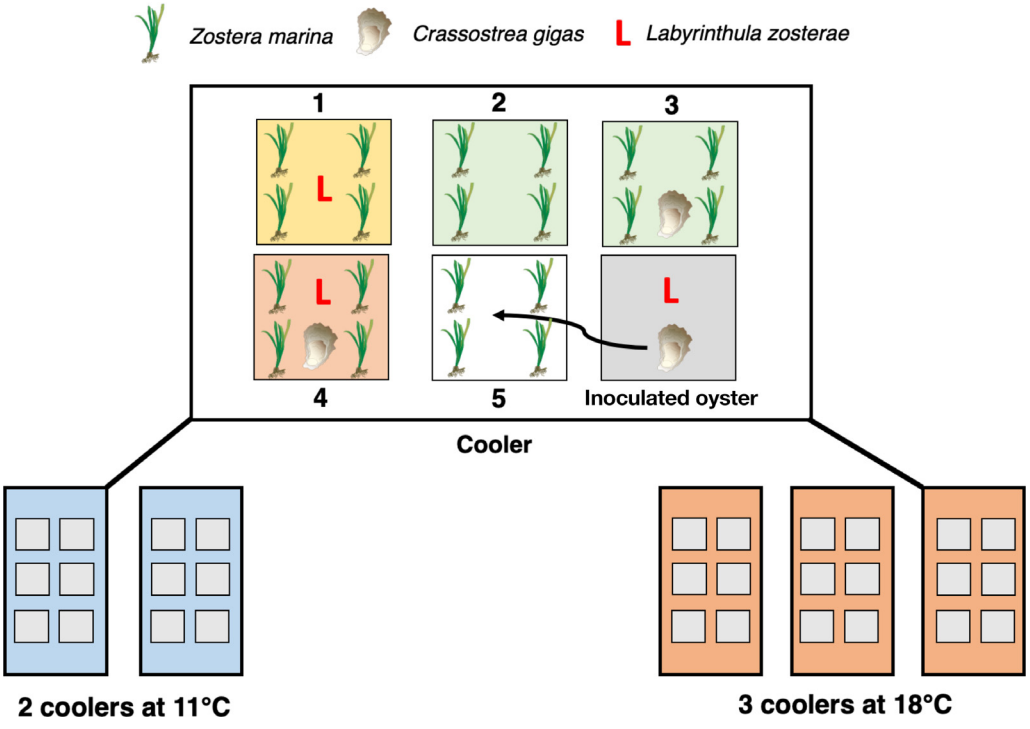

Fig. 1. The laboratory experiment set-up depicting treatments 1–5, with eelgrass *Zostera marina* either (1) inoculated with *Labyrinthula zosterae* (positive control), (2) alone (negative control; background infection assessment), (3) co-cultured with Pacific oysters *Crassostrea gigas* (negative control with oysters), (4) inoculated with *L. zosterae* and co-cultured with oysters (oyster and *L. zosterae*), or (5) co-cultured with oysters exposed to *L. zosterae* added (inoculated oysters). Each tank (replicate) contained 4 eelgrass plants. The 5 treatments were replicated twice at 11°C and 3 times at 18°C. Temperature treatments were maintained by placing a full set of biological replicates (1–5) in a cooler held at the target temperature

tion) of germanium dioxide (GeO_2) was added to the head tank every 24 h (Groner et al. 2018). In all coolers, A HOBO pendant wireless temperature logger (Onset) was placed into a separate tank that received flow-through seawater from each experimental cooler to monitor temperature throughout the experiment. The experiment ran for 13 d, after which eelgrass ramets were collected and sampled immediately.

2.1.5. Eelgrass sampling

Eelgrass ramets were removed from their tanks and cleaned of epiphytes and debris by gently scraping with a plastic ruler and wiping with a kimwipe. Once cleaned, each ramet was placed between 2 sterile, clear plastic sheets and scanned using a Canon CanoScan 9000F Mark II scanner at 600 dpi for later analysis of disease signs. Once scanned, the top 15 cm of the 2 pin-pricked blades from each plant were removed using a sterile technique and rinsed in freshwater to remove any *L. zosterae* on the outside of the leaf. The blades were placed into Eppendorf tubes and kept on ice until frozen at –80°C for qPCR analysis. See Text S1 for methods on image analysis for lesion severity and quantification of *L. zosterae* DNA.

2.2. Field experiment

2.2.1. Plant collection and processing

Eelgrass ramets were collected from Indian Cove, Shaw Island, WA, USA (48° 33.773′ N, 122° 56.078′ W), an eelgrass bed directly next to our deployment site, on 20 June 2019. Sampling was conducted as described in the laboratory experiment (see Section 2.1.1). After receiving a low salinity rinse, eelgrass ramets were potted in plastic containers filled with sterile sand. These containers were kept outside in UV-treated, ambient flow-through seawater with an additional full-spectrum LED light on a 16 h light:8 h dark cycle for 10 d prior to the experiment to reduce background infection. On Day 1 of the experiment, ramets were trimmed as they were prior to the laboratory experiment (see Section 2.1.1) before being used in the experiment.

2.2.2. Oyster sourcing

Pacific oysters (mean ± SE length: 86.30 ± 1.46 mm) were collected on an oyster farm in Thorndyke Bay, WA, USA, and transported on ice to FHL with a transfer permit from the state of Washington. The oysters were placed in ambient flow-through seawater for 3 d before deployment into the field.

2.2.3. Experimental design

The field experiment consisted of 2 subtidal treatments, one with and one without oysters, each of which was replicated 4 times for a total of 8 units (Fig. S2). Each unit consisted of a metal crate with 4 mesh bags that floated upright to surround the eelgrass ramets. Mesh bags were obtained from an oyster farm that routinely uses them to grow their oysters. Units with oysters included a total of 360 oysters allocated to the 4 mesh bags (n = 90 oysters per bag), and control units had empty bags. The eelgrass ramets were attached at the rhizome to nylon rope (n = 5 ramets per rope) and zip-tied to the bottom of the unit, with the rhizome facing down, surrounded by the 4 mesh bags. Each of the 8 units had 5 ropes for a total of 25 plants per unit and 100 plants per treatment.

The experimental units were deployed by boat in Picnic Cove, Shaw Island (48° 33.942′ N, 122° 55.448′ W), on 2 July 2019. Each unit was deployed in-between patches of eelgrass beds, approximately 2–3 m from the nearest eelgrass bed (not touching any eelgrass in the bed) and at least 5 m away from other units (Fig. S3). Control and experimental units were staggered so that 2 of the same treatment were not next to each other. Three HOBO TidbiT water temperature data loggers (Onset) were attached to random cages to monitor temperature during deployment. The cages were left at Picnic Cove for a total of 4 wk before being collected and sampled.

2.2.4. Sampling

After 4 wk, the units were retrieved, and ropes with attached eelgrass ramets were collected and sorted by rope into plastic bags. The bags were transported immediately back to FHL on ice. Ramets were processed as described in the laboratory experiment (see Section 2.1.5), except only the top 15cm of the youngest pin-pricked leaf was sampled for one eelgrass shoot per rope (n = 5 blades per crate, 20 blades per treatment). These samples were specifically chosen for the purpose of confirming the presence of *L. zosterae* DNA. The sampled blades were frozen at –80°C for DNA extraction and qPCR analysis. See Text S1 for methods on using ImageJ to quantify lesion severity and qPCR to quantify infection intensity.

2.3. Hypothesis testing

All statistical analyses were performed using R v.3.5.2 (R Core Team 2018). Generalized linear mixed models (GLMMs) were run using the package glmmTMB (Brooks et al. 2017). Due to the small sample size of our experiment, which limited statistical power, we fit all combinations of models (package MuMIn, Bartoń 2019) and selected the best-fit model based on minimizing Akaike's information criterion with a correction for small sample size (AICc). All additional models with a ΔAICc of <3 compared to the best model were also evaluated (see Tables S2–S7 for all models with a ΔAICc < 3 from the best fit model).

2.3.1. Laboratory experiments

To investigate the hypothesis that the presence of oysters decreases lesion severity and prevalence, data from 4 of the 5 experimental treatments were used: the positive control, the negative control, the negative control with oysters, and the *L. zosterae* with oysters treatment. Zeros within the data due to lack of disease or disease below the limit of visual detection were possible in this scenario. To include zeros in our analysis, severity of lesions was analyzed with a GLMM with a beta distribution and a logit link with zero inflation. Both the severity of lesions (the proportion of the top 15 cm of the oldest 2 leaves that had lesions; the conditional model), and the presence of lesions (the zero-inflated model) were modeled as a function of *L. zosterae* exposure, oyster presence, temperature, and all possible interactions. The zero-inflation portion of the model was analyzed for prevalence, while the conditional portion of the model was analyzed for severity. Random effects for tank (replicate) and the coolers that the tanks were placed in were included for each part of this additive model.

The same 4 experimental treatments were used to investigate the hypotheses that the presence of oysters decreases *L. zosterae* infection intensity, and that exposure with *L. zosterae* leads to decreased growth of *Zostera marina*. Infection intensity was analyzed with a GLMM with a gamma distribution and a log link; only samples with amplifiable *L. zosterae* DNA were used in this analysis. A GLMM with a Gaussian distribution and an identity link was used to investigate how infection with *L. zosterae* affected *Z. marina* growth. The total copies of *L. zosterae* DNA per mg of eelgrass tissue or the total new growth of each plant was modeled as a function of *L. zosterae* exposure, oyster presence, temperature, and all possible interactions. Random effects for tank and cooler were included in both models.

To investigate the hypothesis that oysters previously exposed to *L. zosterae* could vector the pathogen (based on DNA presence) and cause SWD lesions, data from the negative control with oysters and the vectored oyster treatment were used. Severity of lesions was analyzed with a GLMM with a beta distribution and a logit link. Infection intensity was analyzed with a GLMM with a gamma distribution and a log link; only qPCR-positive samples were used in the analysis. The severity of lesions or the total copies of *L. zosterae* DNA per mg of eelgrass was modeled as a function of inoculated oyster presence, temperature, and all possible interactions. Random effects for tank and cooler were included for both models.

2.3.2. Field experiments

Data from the field experiment were used to investigate the hypothesis that oyster presence would decrease SWD prevalence and severity in the field. Prevalence of SWD (the number of plants showing visible lesions on either blade) was analyzed with a GLMM with a binomial distribution and a logit link. Severity of lesions (the proportion of the top 15 cm of the oldest 2 leaves that had lesions) was analyzed with a GLMM with a beta distribution and a logit link; only samples with visual signs of lesions were used in this analysis. Prevalence or severity was modeled as a function of treatment (oysters versus no oysters), and a random effect for experimental unit was included in both models.

3. RESULTS

3.1. Laboratory experiments

The severity of lesions was significantly greater in the absence of oysters, with exposure to *Labyrinthula zosterae*, and at the warmer temperature (Fig. 2a, Table 1). A zero-inflation model was run to analyze both prevalence and severity of lesions. The best-fit conditional part of the model (explaining lesion severity) included fixed effects for exposure to *L. zosterae*, oyster presence and temperature, but not their interactions (Table S2). Lesion severity was 1.9 times higher in eelgrass cultured without oysters than in eelgrass cultured in the presence of oysters ($p = 0.02$). Exposure to *L. zosterae* caused lesion severity to be 8.0 times higher than in non-exposed

ramets ($p < 0.01$). Overall, lesion severity was 2.9 times higher in ramets kept at 18°C than in those kept at 11°C ($p < 0.01$).

The prevalence of lesions varied with oyster presence and *L. zosterae* exposure. Lesion prevalence was 100% in eelgrass shoots inoculated directly with *L. zosterae* (with and without oysters) at both temperatures (Fig. 2b, Table 1). The best-fit zero-inflation part of the model (explaining lesion presence/absence) included fixed effects for *L. zosterae* exposure and oyster presence, but not their interaction (Table S2). The zero-inflation model indicated that oyster presence significantly increased the log odds of lesion prevalence ($p = 0.03$). Prevalence of lesions in the negative control without oysters was 38% and 17% at 11°C and 18°C, respectively. Lesion prevalence in plants from the negative control with oysters was 50% and 67% at 11°C and 18°C, respectively. *Labyrinthula zosterae* exposure did not influence prevalence ($p > 0.05$).

Amplifiable *L. zosterae* DNA was detected in 100% of the eelgrass ramets inoculated directly with *L. zosterae* (with and without oysters) at both temperatures. No *L. zosterae* DNA was amplified from the negative controls (with and without oysters) at either temperature, so they were excluded from analyses of *L. zosterae* DNA. Oyster presence significantly decreased the quantity of *L. zosterae* DNA copies mg^{-1} of dry eelgrass tissue, and the effect of oyster presence was not influenced by temperature (Fig. 2c, Table 1). The best-fit model for *L. zosterae* infection intensity included a fixed effect for only oyster presence (Table S3). Eelgrass ramets exposed to *L. zosterae* without oysters present had 1.65 times higher *L. zosterae* DNA copies mg^{-1} of dry tissue than ramets exposed with oysters present ($p = 0.01$).

Growth of *Zostera marina* was significantly lower in *L. zosterae*-exposed plants compared to controls, significantly lower at 11°C compared to 18°C, and trended lower in the absence of oysters (Fig. 2d, Table 1). The best-fit model for growth included fixed effects for temperature and exposure to *L. zosterae*, but not their interaction (Table S4). Eelgrass ramets that were not exposed to *L. zosterae* had 1.34 times more total growth than plants exposed to *L. zosterae* ($p < 0.01$). Eelgrass at 18°C had 1.29 times more total growth than plants at 11°C ($p < 0.01$). A non-significant trend was observed of increased growth in the presence of oysters when exposed to *L. zosterae* ($p = 0.09$).

Co-culture of eelgrass ramets with inoculated oysters resulted in amplifiable *L. zosterae* DNA detected in all but 2 ramets with a mean ± SE of $1.15 \times 10^5 \pm 3.89 \times 10^4$ DNA copies mg^{-1} dry eelgrass tissue,

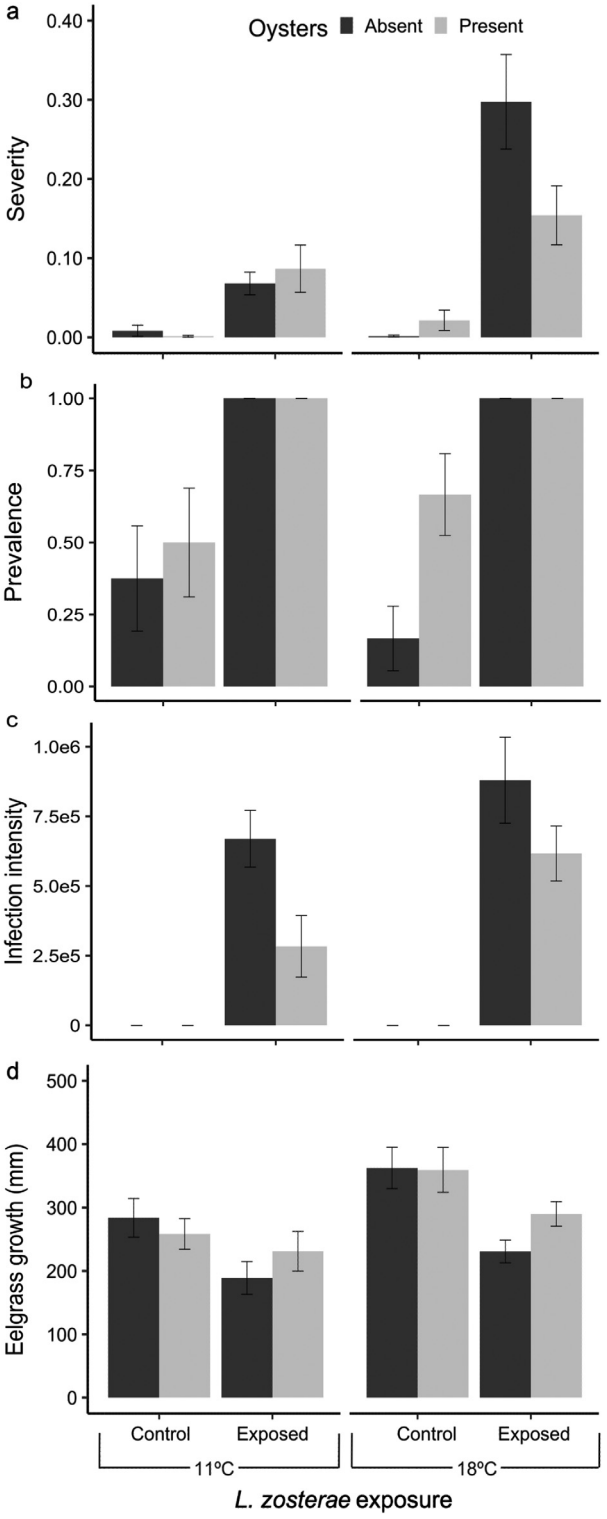

Fig. 2. Mean (a) seagrass wasting disease (SWD) severity, (b) prevalence of lesions, (c) infection intensity (copies of *L. zosterae* DNA per mg of eelgrass tissue) and (d) eelgrass growth (total new growth) of eelgrass ramets either exposed or not exposed (control) to *L. zosterae* in the presence or absence of oysters at 11°C or 18°C. Error bars represent ±1 SE

Table 1. Laboratory experiment results from the best-fit models (lowest AICc value). (a) Severity of lesions was analyzed with a generalized linear mixed model (GLMM) with a beta distribution and a logit link with zero inflation. Both the severity of lesions (the proportion of the top 15 cm of the oldest 2 leaves that had lesions; the conditional model) and the presence of lesions (the zero-inflated model) were modeled as a function of *Labyrinthula zosterae* exposure, oyster presence and temperature, and all possible interactions. (b) Infection intensity was analyzed with a GLMM with a gamma distribution and a log link; only samples with amplifiable *L. zosterae* DNA were used in this analysis. The total copies of *L. zosterae* DNA per mg of eelgrass tissue was modeled as a function of *L. zosterae* exposure, oyster presence, temperature, and all possible interactions. (c) Eelgrass growth was analyzed with a GLMM with a Gaussian distribution and an identity link was used to investigate how infection with *L. zosterae* affected *Zostera marina* growth. The total new growth of each plant was modeled as a function of *L. zosterae* exposure, oyster presence, temperature, and all possible interactions. Random effects for tank and cooler were included in all models

	Estimate	SE	*z*	p
(a) Severity and prevalence				
Conditional model				
Intercept	−3.61	0.35	−10.23	<0.01
Labyrinthula zosterae (exposed)	1.68	0.28	5.91	<0.01
Oysters (present)	−0.49	0.22	−2.26	0.02
Temperature (18°C)	0.87	0.23	3.78	<0.01
Zero-inflation model				
Intercept	1.01	0.52	2.13	0.03
Labyrinthula zosterae (exposed)	−29.93	3.68×10^5	0.00	0.99
Oysters (present)	−1.5	0.69	−2.18	0.03
(b) Infection intensity				
Intercept	13.66	0.21	65.34	<0.01
Oysters (present)	−0.62	0.25	−2.46	0.01
(c) Eelgrass growth				
Intercept	282.59	18.13	15.59	<0.01
Labyrinthula zosterae (exposed)	−83.38	19.61	−4.25	<0.01
Temperature (18°C)	70.46	20.0	3.53	<0.01

which was ~6 times lower than eelgrass directly inoculated with *L. zosterae*. No *L. zosterae* DNA was amplified in the negative control with oysters. The best-fit model for lesion severity in the presence of inoculated oysters was the null model, indicating no effect of inoculated oyster presence or temperature on lesion severity (Table S5). The severity of lesions in the presence of inoculated oysters (mean ± SE: 5.63 ± 2.07%) was not significantly different from the negative control with oysters (mean ± SE: 2.24 ± 1.27%), and was not affected by temperature.

3.2. Field experiment

The average water temperature during the field experiment deployment was 11.76°C (see Fig. S4). Due to blades and shoots breaking off during the experiment or collection and the inability to collect data from the top 15 cm of the 2 oldest leaves, 59 of 100 eelgrass ramets from the units with oysters and 67 of 100 from the units without oysters were removed from the analysis. Prevalence of SWD in eelgrass co-cultured with oysters was 90.24 ± 4.69% (mean ± SE; n = 41), and prevalence of SWD in eelgrass without oysters was 90.91 ± 5.08% (n = 33). The best-fit model for prevalence was the null model, indicating that the presence of oysters had no effect on the prevalence of SWD in outplanted eelgrass (Table S6).

The severity of SWD in eelgrass outplanted with oysters was 2.58 ± 1.00% (mean ± SE), and the severity of SWD in eelgrass outplanted without oysters was 2.97 ± 0.71%. The best-fit model was the null model, indicating the presence of oysters in the field did not affect SWD severity (Table S7). The presence of *L. zosterae* DNA was confirmed through qPCR analysis. Of the 20 blades sampled per treatment, 20% of blades contained amplifiable *L. zosterae* DNA in the presence of oysters, and 35% contained *L. zosterae* DNA without oysters. Sampling for DNA analysis was not randomized, and therefore no statistics on infection intensity were run.

4. DISCUSSION

The laboratory study demonstrates that the negative impacts of *Labyrinthula zosterae* on *Zostera marina* health and growth can be partially mitigated by Pacific oysters. In the laboratory, *Crassostrea gigas* decreased both the severity of lesions and the infection intensity of eelgrass ramets exposed to *L. zosterae* compared to shoots exposed without oysters present. Exposure to *L. zosterae* caused a significant increase in lesions and copies of *L. zosterae* DNA, indicating that exposure to *L. zosterae* successfully caused infection in the eelgrass ramets.

Oysters were able to significantly decrease lesion severity and *L. zosterae* infection intensity, likely by filtering waterborne *L. zosterae* cells before they were able to infect the eelgrass ramets. *Labyrinthula* spp. are typically 11–18 µm in length and 3–5 µm in width (Muehlstein et al. 1988), which falls within known particle filtration sizes (3–20 µm) of *C. gigas* (Ward et al. 1998). *C. gigas* can filter and retain pro-

tists that range in size from 4 to 72 µm in length and may use protists as a food source especially when phytoplankton abundance is low (Dupuy et al. 1999). *L. zosterae* spreads via waterborne transmission in both the laboratory and the field (M. E. Eisenlord & M. V. Agnew unpubl. data). Thus, oysters have to filter the pathogenic cells prior to reaching the eelgrass ramets, decreasing the amount of cells that plants are exposed to, which can directly decrease lesion severity (Dawkins et al. 2018). Oysters can reduce SWD severity in previously infected ramets (Groner et al. 2018), which, combined with the results from this study, suggests that oysters may be beneficial at early and middle stages of an infection.

Lesion severity was significantly higher in the present study in eelgrass ramets held at 18°C compared to 11°C, consistent with other laboratory and field studies. Kaldy (2014) reported naturally infected *Z. marina* from the Pacific Northwest had significantly increased SWD lesion severity at 25°C compared to 10°C (although increased NO_3 concentrations may also have had an effect), and an analysis of 9 eelgrass beds over 5 yr in the Pacific Northwest correlated higher summer temperatures with summer SWD prevalence (Groner et al. 2021). In contrast, other laboratory experiments found no changes in SWD severity or prevalence in *Z. marina* kept at 11°C or 18°C (Dawkins et al. 2018), or 22°C to 27°C (Brakel et al. 2019); however, these studies were run for half as much time as the present study and that of Kaldy (2014). Combined, these observations indicate that in the Pacific Northwest, *L. zosterae* may have increased negative effects on *Z. marina* at the temperatures expected over the next century.

Oyster presence decreased lesion severity and *L. zosterae* infection intensity, and this was not significantly affected by temperature. The maximum filtration rate of *C. gigas* occurs at 19–20°C when tested from 10°C to 25°C (Gray & Langdon 2018) and 5°C to 32°C (Bougrier et al. 1995), suggesting that the oyster's ability to filter *L. zosterae* may increase to this point, but beyond which may not be beneficial. The average temperatures and maximum filtration rates of bivalves should be considered according to location for the purpose of mitigating SWD. Local filter feeders may have varying ability to filter *L. zosterae* out of the water based on particle selection, filtering volume, and temperature and salinity preferences. For example, the native oyster species in the Pacific Northwest, *Ostrea lurida*, has a lower clearance rate and a higher optimum filtration temperature than *C. gigas* (Gray & Langdon 2018). Furthermore, modeling indicates that the filtration services of *C. gigas* are significantly greater than those of *O. lurida* (Gray et al. 2019). These laboratory trials suggest that *C. gigas* may be a good mitigation tool for SWD in the Pacific Northwest; however, further experiments are needed to validate this result in the field.

Oyster presence significantly increased lesion prevalence, likely due to increased prevalence in the negative controls with oysters present. Small visible lesions were present in the control ramets not exposed to *L. zosterae*, but these plants did not contain amplifiable *L. zosterae* DNA. These small lesions (mean ± SE: 17.57 ± 8.51 mm^2) could represent background infections from the field that were eliminated prior to or during the experiment or contained *L. zosterae* below the limit of detection for our assay. Alternatively, these lesions could result from other stressors that cause lesions similar to those observed in SWD, such as invertebrate grazing and heat stress (Groner et al. 2014). A correlation between lesion size and qPCR detection would be helpful to set a cut-off when calculating prevalence and severity of lesions, since inclusion of small lesions increases the visual prevalence despite lack of *L. zosterae* DNA. There was 100% lesion prevalence in the *L. zosterae* exposed eelgrass and 43% lesion prevalence in the negative controls. We could not test this pattern statistically due to the lack of variation in the exposed treatment, which violates the assumption of homoscedasticity.

Eelgrass exposed to *L. zosterae* grew more slowly than unexposed plants, and those reared at 18°C grew significantly more than those held at 11°C. Other studies have also seen increased *Z. marina* growth with temperatures between 15°C and 20°C compared to lower (5–10°C) or higher (20–30°C) temperatures (Nejrup & Pedersen 2008), and a significant linear relationship of increased growth with increasing temperatures from 10°C to 24°C (Kaldy 2014). *L. zosterae* likely infects seagrass by degrading the plant cell walls and destroying the contents of the cell (Muehlstein 1992), which leads to necrotic lesions and significant impacts on the photosynthetic capacity of the lesion area and surrounding green tissue (Ralph & Short 2002). Having a growth rate that exceeds SWD lesion expansion rate is key for eelgrass survival if infected with *L. zosterae*, since additional eelgrass growth allows for continued photosynthesis despite lesion presence (Brakel et al. 2019). Although not significant, total growth of eelgrass co-cultured with oysters and exposed to *L. zosterae* was higher as compared to eelgrass not co-cultured with oysters (see Fig. 2d). Filtration of *L. zosterae* by oysters can possibly mitigate infection and consequential lesions.

Seagrasses typically spread rhizomes through hypoxic, sulfide-rich sediments, and their leaves have high light requirements to provide oxygen to non-photosynthetic tissue (reviewed in Ralph et al. 2007). Necrotic lesions such as those caused by *L. zosterae* lead to a decrease in photosynthesis and, consequently, decreased energy production. Filtration of *L. zosterae* by oysters may mitigate infection and consequential lesions, allowing the eelgrass to have increased photosynthetic capacity and more energy for growth or pathogen defense.

Oysters were able to transmit *L. zosterae* in our laboratory study, confirmed by the presence of *L. zosterae* DNA in eelgrass ramets co-cultured with oysters previously exposed to *L. zosterae*. However, the severity of lesions did not differ between the inoculated oyster treatment and the controls, which may be due to a low number of *L. zosterae* cells transmitted by the oysters, or the presence of non-viable cells. The detection of *L. zosterae* DNA via qPCR in this study does not distinguish between living and dead cells; therefore, further research on the viability of the *L. zosterae* cells transmitted by *C. gigas* is needed.

The potential transmission of *L. zosterae* still has important implications when considering transportation of oysters from one area to another. In Washington state, bivalves including oysters and manila clams are often transported from one bay to another for various reasons, including storage prior to shipment and movement between farms, which occasionally results in transportation from near one eelgrass bed to another. Shellfish transfer permits are required for all transportation of shellfish in Washington state, although additional unregulated transfers may occur. Transported bivalves that originate from a restricted shellfish area (known to have oyster drills or European green crabs) go through a washing process or dilute chlorine dip treatment to prevent the spread of harmful organisms, but SWD is not considered in transfers (B. Blake pers. comm.).

Bivalves have been known to introduce fish bacterial pathogens (e.g. that infect Arctic char *Salvelinus alpinus*; Starliper 2001) as well as exotic species such as macroalgae and toxic phytoplankton when moved between locations (reviewed by Mckindsey et al. 2007). Oysters have also been known to accumulate large amounts of human bacterial pathogens in their tissue, including various *Vibrio* species and *Escherichia coli* (Murphree & Tamplin 1995). Depuration of bivalves has been an effective strategy used to remove bacterial pathogens (Starliper 2001), and it is possible that depuration of the oysters prior to outplant, and/or a freshwater rinse (as was done for our control treatments), could reduce the risk of spreading *L. zosterae* as well. Further experiments to determine the exact mechanism for this transmission (via filtering, attached to shell, etc.) and the viability of the cells are needed to decide the risk of spread, and effective ways to prevent spreading of *L. zosterae* via oyster movement if necessary.

Unlike our laboratory experiment, the field experiment did not demonstrate significant effects of oysters on SWD. We lost a large amount of eelgrass tissue during collection. This resulted in the use of only 41% of the eelgrass outplanted with oysters and 33% of the eelgrass outplanted without oysters, all of which had minimal SWD severity (average <3%). Thus, it is possible that *L. zosterae* infections were present in the blades that were lost during the experiment, which may have skewed our results. Some blades with lesions did not contain amplifiable *L. zosterae* DNA, which may be the result of old lesions lacking *L. zosterae* cells or due to other stressors that cause lesions similar to those observed in SWD. This observation emphasizes the importance of combining both visual and molecular techniques when assessing *L. zosterae* infection (Groner et al. 2014). Despite a lack of measurable effects of oyster presence on SWD in the field, this experiment lays the groundwork for future field experiments with oysters and eelgrass and could be utilized in other locations.

Our experiment did not look at other common stressors or compounding environmental impacts that may affect oyster filtration or the eelgrass–*L. zosterae* host–pathogen interaction. Different results may arise when considering a combination of stressors to the eelgrass, *L. zosterae*, or oysters. For example, multiple studies have only found negative effects of *L. zosterae* on seagrass under high temperatures when combined with another stressor, including high salinity and low light conditions (Brakel et al. 2019, Jakobsson-Thor et al. 2020). Decreased light availability alone has shown to increase SWD severity on sections of blade (Dawkins et al. 2018) and to increase lesion coverage in whole plants by 35% compared to high light conditions (Jakobsson-Thor et al. 2020). Low light conditions do not appear to have any positive effects on *L. zosterae* in culture, and therefore increased infection is likely due to the negative effects on the host (Dawkins et al. 2018). If low light conditions affect the susceptibility of seagrass to SWD, then shading due to physical structure from oyster aquaculture may negatively impact seagrass beds. Conversely, oyster aquaculture and consequential oyster filtration could provide refuge to a seagrass bed and an overall net increase in light

availability through top-down control of phytoplankton (reviewed by Herbert et al. 2016), especially during algal blooms that otherwise could be detrimental to seagrass beds (Bologna et al. 2007). Implementation of specific oyster culturing and harvesting methods can be optimized to minimize initial and periodic disturbance (Ferriss et al. 2019), which would allow for a net positive effect of oyster aquaculture regarding filtration for both light attenuation and reduction in SWD. Additionally, placement of oyster aquaculture adjacent to or in front of seagrass meadows may be beneficial to seagrass habitat. Heavy wave action caused a decline in transplantation success of *Z. marina* in the Wadden Sea, and the depth of *Z. marina* beds increases when shelter is present to decrease water turbidity (van Katwijk & Hermus 2000). Modeling also indicates that *Z. marina* patch size and percent cover decrease with increasing wave energy (Uhrin & Turner 2018). Optimal placement of physical structures such as oyster aquaculture or living shorelines in the form of oyster reefs may decrease wave attenuation and consequently increase seagrass coverage.

Overall, our study demonstrates the potential for oyster aquaculture to improve eelgrass health. The ability for *C. gigas* to mitigate SWD by decreasing both lesion severity and *L. zosterae* infection intensity in *Z. marina* in the laboratory makes them an effective sink for this pathogen. Oysters also act as a source of *L. zosterae* in this laboratory study. Examination of the potential for oysters to vector *L. zosterae* in the field warrants further study, including controlled laboratory experiments to determine how oysters spread *L. zosterae* (on their shell, in their pseudofeces, etc.), the viability of transmitted cells, and measuring *L. zosterae* concentration in oysters moved between field sites. Should oysters spread *L. zosterae* in the field, preventative measures should be taken to minimize the chance of introducing this pathogen into naïve eelgrass beds. *L. zosterae* can cause infection at concentrations as low as 6 cells ml^{-1} (Eisenlord & Agnew unpubl. data). Future research to quantify waterborne concentrations of *L. zosterae* and to measure oyster filtration and transmission at these concentrations would be valuable. Mitigation of SWD via oyster filtration can be beneficial for affected eelgrass populations especially in temperate regions such as the Pacific Northwest, which are experiencing rapid warming. Although temperature increases have the potential to exacerbate the effects of SWD, *C. gigas* may continue to filter the pathogen successfully at higher temperatures. Aside from filtering *L. zosterae*, the improvement of water quality should also have a net positive impact on eelgrass populations, although further field studies should be conducted to ensure that these benefits translate on a larger scale.

Data availability. All data and statistical code can be found on figshare: https://figshare.com/s/1de022c6578f15180478.

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