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Host demography influences the prevalence and severity of eelgrass wasting disease

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ABSTRACT: Many marine pathogens are opportunists, present in the environment, but causing disease only under certain conditions such as immunosuppression due to environmental stress or host factors such as age. In the temperate eelgrass *Zostera marina*, the opportunistic labyrinthulomycete pathogen *Labyrinthula zosterae* is present in many populations and occasionally causes severe epidemics of wasting disease; however, risk factors associated with these epidemics are unknown. We conducted both field surveys and experimental manipulations to examine the effect of leaf age (inferred from leaf size) on wasting disease prevalence and severity in *Z. marina* across sites in the San Juan Archipelago, Washington, USA. We confirmed that lesions observed in the field were caused by active *Labyrinthula* infections both by identifying the etiologic agent through histology and by performing inoculations with cultures of *Labyrinthula* spp. isolated from observed lesions. We found that disease prevalence increased at shallower depths and with greater leaf size at all sites, and this effect was more pronounced at declining sites. Experimental inoculations with 2 strains of *L. zosterae* confirmed an increased susceptibility of older leaves to infection. Overall, this pattern suggests that mature beds and shallow beds of eelgrass may be especially susceptible to outbreaks of wasting disease. The study highlights the importance of considering host and environmental factors when evaluating risk of disease from opportunistic pathogens.

KEY WORDS: Labyrinthulomycetes · Opportunistic pathogens · Seagrass declines · *Zostera marina* · Seagrass · Marine diseases · *Labyrinthula zosterae*

INTRODUCTION

While disease is a natural component of all ecosystems, increases in global emerging diseases present a serious risk to multiple species and ecosystems (Jones et al. 2008). In the oceans, increases in the prevalence and severity of infectious diseases have been associated with changes to trophic interactions, biotic structure, age distributions, and hydrodynamics (reviewed by Burge et al. 2014). Marine diseases that affect ecosystem engineers such as corals, oysters, and seagrasses may be of particular concern, because population declines in these organisms can result in dramatic shifts in community structure

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(Harvell et al. 2004, Ward & Lafferty 2004, Burge et al. 2014). Accumulating evidence suggests that many of these diseases are caused by opportunistic pathogens, which are omnipresent in the environment but have increased in pathogenicity as a result of environmental changes that reduce host defenses against infection (reviewed by Burge et al. 2013). Understanding what can cause this increase in an opportunist's pathogenicity is important for predicting and mitigating disease.

Identification of environmental factors that can cause opportunists to switch from a commensal to a pathogenic state has recently become a research priority (Burge et al. 2013); however, population demographics of the host may also be important in determining the prevalence and severity of disease. In particular, the density, age-, and size-structure of a population may influence the severity, extent, and timing of disease as a result of changes to density-dependent transmission (e.g. Anderson & May 1979, Arneberg et al. 1998), and age- and size-dependent susceptibility to infection (e.g. Raffel et al. 2011). While these ideas have been explored in primary pathogens (i.e. pathogens that are not opportunists), less is known about the role that demography may play in epidemics of opportunistic pathogens.

Wasting disease in the temperate seagrass *Zostera marina* is a tractable system for investigating the role of demographic factors in driving patterns of opportunistic diseases. *Z. marina* inhabits coastal marine and estuarine ecosystems across the northern hemisphere and forms highly productive ecosystems that provide a variety of services, such as habitat for economically valuable invertebrates, fish, and marine birds, stabilization of coastal sediments, and biological filtration of terrestrial-derived nutrients (Wilson & Atkinson 1995, Moore & Short 2006, Orth et al. 2006, Fourqurean et al. 2012, Plummer et al. 2012). These marine angiosperms are highly susceptible to natural and human-caused environmental stress and disease (Short & Wyllie-Echeverria 1996, Orth et al. 2006). Eelgrass wasting disease in particular has caused dramatic, rapid population declines. In the early 1930s, an outbreak along the Atlantic coasts of North America and Europe resulted in up to 90% mortality of *Z. marina* populations (reviewed by Muehlstein et al. 1991). Less severe epidemics have been documented along the Atlantic coasts since the 1980s (Short et al. 1987). Since the early 1990s, a number of *Z. marina* beds across the Salish Sea in the Pacific Northwest of North America have experienced periodic mortalities from unknown causes (Berry et al. 2003, Wyllie-Echeverria et al. 2003, Dowty et al. 2010). It has been hypothesized that these mortality events may be partially due to wasting disease, which has been observed in the Salish Sea during the past few decades (Muehlstein et al. 1988, Muehlstein 1992).

The causative agent of wasting disease in *Zostera marina* is *Labyrinthula zosterae,* a marine protist (Muehlstein et al. 1988, 1991). Like many Labyrinthulomycetes, *L. zosterae* is an opportunistic pathogen that is ubiquitous in the marine environment. It is characterized by spindle-shaped or fusiform zoospores that secrete a mucus network, which it uses for cell adhesion and signaling, transport of nutrients, and motility (Porter 1972, Muehlstein et al. 1991). Analyses of *L. zosterae* in the San Juan Archipelago show a widespread population with little genetic variation, although strains vary considerably in virulence (Muehlstein et al. 1991).

Factors that contribute to an increased prevalence of eelgrass wasting disease, as well as the role of wasting disease in declines of *Zostera marina,* are poorly understood (Muehlstein et al. 1988). At a population level, *Z. marina* beds show within- and among-population variation in density and age structure (Bull et al. 2012), but the link between eelgrass age structure and risk of disease has not been addressed. The morphology of *Z. marina* makes it ideal for testing the effect of host age in opportunistic infections. In a typical plant, several leaves extend from a meristem along a single rhizome (Short & Duarte 2003), and shoots toward the distal end of the rhizome are progressively younger. Leaves within the same shoot differ in age, such that smaller leaves are younger (Tomlinson 1974). Therefore, the effect of age and size on disease can be examined both within and among shoots in a single ramet.

We used a field study and inoculation trials to (1) identify and quantify signs of wasting disease on *Zostera marina* at selected sites in the San Juan Archipelago; (2) confirm the etiologic agent of these signs to be *Labryinthula* spp.; and (3) examine the relationship between shoot size (a proxy for age structure) and susceptibility to wasting disease.

MATERIALS AND METHODS

Field study

Field sampling was in the San Juan Archipelago region of the Salish Sea in Washington, USA (Fig. 1). We chose this region due to ongoing declines in the distribution and density of *Zostera marina* since the early 2000s (Wyllie-Echeverria et al. 2003, 2010, Fer-

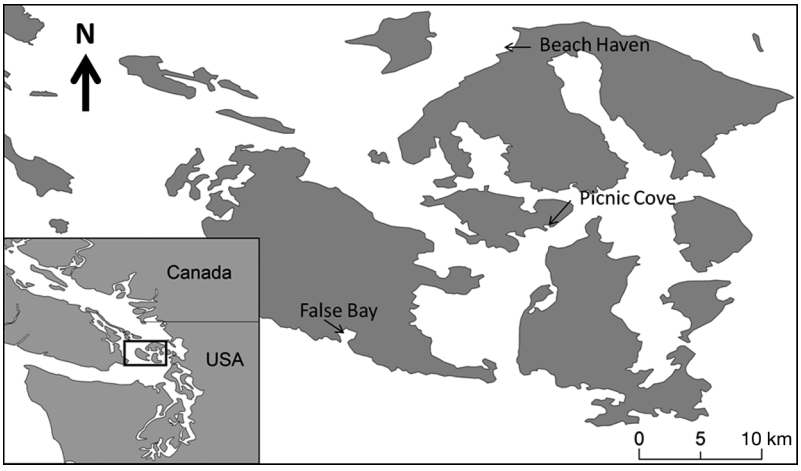

Fig. 1. Study sites in the San Juan Islands, Washington, USA

rier & Berry 2010). We chose 2 sites, Picnic Cove (48.566° N, 122.924° W) and False Bay (48.483° N, 123.074° W), with a history of decline, and 1 site (Beach Haven, 48.691° N, 122.952° W) with a relatively stable population (Wyllie-Echeverria et al. 2010). Picnic Cove is an embayment on the southern coast of Shaw Island. Beach Haven is on the northern coast of Orcas Island and represents a fringing coastline; it was also chosen for its exposure to oceanic currents and its proximity to the Fraser River effluent. False Bay is an embayment on the southwestern coast of San Juan Island and has experienced highly variable shoot densities since 2005 (S. Wyllie-Echeverria et al. unpubl. data). All surveys were conducted between 30 July and 3 August 2012.

Data were collected along 2 (Picnic Cove) or 3 (False Bay and Beach Haven) 10 m transects laid 1 m apart in both shallow and deep sites of the intertidal region. Transects were laid out parallel to the shore, with a distance of 6 m between shallow and deep sites and the midpoint between shallow and deep transects at −1 m Mean Lower Low Water (MLLW). All adult non-flowering shoots within 15 cm of each transect were counted, and the lengths of the longest leaf on each shoot and of all visible lesions due to wasting disease were measured. At Beach Haven, due to the rising tide and the high density of shoots, leaf and lesion measurements were only made directly under the transect tape. Lesions associated with wasting disease were identified by an irregular dark necrotic center surrounded by a black border (e.g. Burdick et al. 1993; Fig. 2). While other lesions on these leaves might have also been caused by *Labyrinthula zosterae*, we conservatively limited our identification to lesions with a black border.

Using these data, we generated measurements of disease prevalence (number of diseased leaves / total number of leaves × 100) and severity (total lesion length / leaf). We also counted the number of shoots in 3 quadrats (0.25 m^2 each) placed at 0, 5, and 10 m in each transect in order to determine site-level differences in population density.

Histology

From select transects, we sampled leaves for histological analysis to confirm our field diagnosis. At Picnic Cove, we sampled eelgrass along 2 transects: 14 healthy leaves (no visible lesions) and 13 diseased leaves (visible lesions) from transect 1, and 15 diseased leaves from transect 2. At Beach Haven and False Bay, we sampled 3 to 4 diseased leaves from each site. Leaves were fixed for 24 h in 4% seawater-buffered formalin, followed by storage in 70% ethanol. For the diseased leaves, areas including the leading edge of the lesion and adjacent healthy tissue were sampled. At Cornell University's Diagnostic Laboratory, leaves were embedded in paraffin, sectioned (5 µm), and stained with hematoxylin and eosin. Stained sections were randomized and viewed

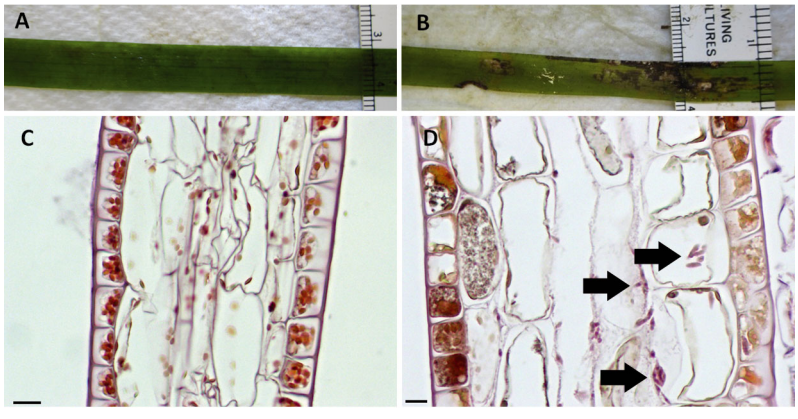

Fig. 2. *Zostera marina*. Samples collected from Picnic Cove, San Juan Islands, Washington, USA, in 2012: (A) outwardly healthy, (B) outwardly diseased, (C) histological section of (A), and (D) histological section of (B) showing *Labyrinthula* cells (arrows). Scale bar in (C,D) = 10 µm

under a bright-field Olympus BH-2 microscope with the Olympus DP-20 camera system. Sections were examined blindly (without demarcation of site or disease status) by a single viewer for the presence/absence of *Labyrinthula* spp. cells or other parasites within the tissues. The number of *Labyrinthula* spp. cells was counted in 3 fields of view for each slide.

Culturing and identification of etiologic agents in field studies

Leaves of *Zostera marina* with distinctive lesions were collected and brought back to the lab to culture *Labyrinthula* spp. Leaf sections with identified lesions were cut into pieces of ~0.5 cm, surface-rinsed with 70% ethanol, and then allowed to dry. These leaf sections were then put onto serum seawater agar (SSA) plates, wrapped in parafilm, and allowed to grow at room temperature. Samples were re-plated as the *Labyrinthula* grew or if fungal contamination became apparent on the plate. We made liquid cultures by inoculating serum seawater (SS) broth with a streak from the desired plate culture. Liquid cultures grew at room temperature for at least 7 d. In total, 4 isolates were cultured: 3 from False Bay and 1 from Beach Haven. The isolate with the fastest growth, FBSH1TD, which originated from False Bay, was verified as *Labyrinthula* spp. under the microscope and used for inoculation experiments (described below).

SSA was modified from Porter (1990): to 1 l of filter-sterilized seawater (salinity = 25 ppt) we added 12 g of USB Noble Agar, 1.5 mg of germanium dioxide, 0.1 g of yeast extract, 0.1 g of peptone, and 1.0 g of glucose. The mixture was autoclaved and tempered to 50 to 55°C before adding 10 ml of horse serum and 25 ml of 100× penicillin/streptomycin. SS broth followed the same recipe, but did not contain agar.

Inoculation trial

To determine the effect of leaf age on susceptibility to disease, we conducted a full-factorial experiment, consisting of 2 age treatments (younger and older leaves from a single shoot) and 3 infection treatments (2 *Labyrinthula* spp. strains and a sham inoculate). Each treatment was replicated on 10 unique ramets for a total of 60 experimental units.

On 22 October 2012, 10 healthy plants (free of lesions on visual inspection) with intact older (terminal) and younger (daughter) shoots on the same rhizome were collected at False Bay. The collected shoots were placed in a sea table at the Friday Harbor Laboratories (FHL), Washington, USA. In order to expose individuals from each shoot to all treatments, the second leaf of each terminal and daughter shoot was divided into 3 segments (5 cm each). Both the second and third leaves from the same shoot were used when a leaf was not long enough to obtain all individual segments. Segments were placed in individual sterile petri dishes with 25 ml of filtered seawater (FSW) and allowed to acclimate overnight in a light- and temperature-controlled incubator (12 h light: 12 h dark, 20°C). Leaf segments remained in the incubator for the entirety of the experiment except during inoculation and photo-documentation.

The experiment began on 23 October 2012. Two strains of *Labyrinthula* spp. were used in the inoculations: 8.16.D and FBSH1TD. FBSH1TD was isolated from False Bay (described above), and 8.16.D was isolated in 2011 from non-flowering adult *Zostera marina* shoots that were collected at Picnic Cove in 2006 and subsequently grown in a continuous flow mesocosm at FHL. Both strains were cultured in SS broth in 15 ml centrifuge tubes after initial isolation onto SSA. Immediately prior to inoculation, the liquid cultures were homogenized with a vortex, centrifuged, and resuspended in FSW. Resuspended samples were vortexed again and quantified using a hemocytometer. Approximately 1.37×10^4 cells were used in each inoculation. The inoculation method was modified from Muehlstein et al. (1988): 70 µl of inoculum (experimental treatments) or sterile seawater (negative controls) were pipetted onto autoclaved leaf segments of 2 to 3 cm and allowed to sit for 1 h. The inoculated autoclaved segments were then attached to the field-collected eelgrass leaves, with the inoculated side of the autoclaved segment in contact with the prepared leaf (Muehlstein et al. 1988). Thin plastic tubing cut into 1 cm sections and slit through on one side was used to 'clip' the autoclaved, infected piece to the uninfected leaf segment. The clipped leaves were placed in sterile petri dishes with 25 ml FSW and returned to the incubator. After 12 h, the plastic clips and autoclaved leaves were removed.

The experiment was terminated after 2.5 d, and all leaves were photographed. Lesions in the photographed leaf segments were scored blindly by 4 independent scorers. Scores were based on the percentage (to the nearest 10%) of tissue that had not deteriorated (i.e. a score of 0.1 corresponded to 10% health, a score of 0.2 corresponded to 20% health).

Statistical analyses

Field data

We used a multivariable 2-part random effects model to determine the effect of site, depth, and shoot length on the presence and severity of wasting disease (e.g. Kristoffersen et al. 2013). The first part of the model analyzed the odds that a shoot was infected using logistic regression and a binary error term:

$$\begin{aligned}\text{logit}(P_{(\text{infected})}) = {} & A_0 + A_1 \times \text{site1} + A_2 \times \text{site2} + \\ & A_3 \times \text{depth} + A_4 \times (\text{shoot length}) + \\ & A_5 \times (\text{shoot length}) \times \text{site1} + \\ & A_6 \times (\text{shoot length}) \times \text{site2} + u_1 \end{aligned} \quad (1)$$

where site 1 is False Bay, site 2 is Picnic Cove, and the reference site is Beach Haven (the healthiest of the 3 sites). A_0 is the model intercept, and u_1 is the error term.

The second part of the model used regression with a log-normal distribution to model the lesion length (cm):

$$\begin{aligned}\text{Lesion length} = {} & B_0 + B_1 \times \text{site1} + B_2 \times \text{site2} + \\ & B_3 \times \text{depth} + B_4 \times (\text{shoot length}) + \\ & B_5 \times (\text{shoot length}) \times \text{site1} + \\ & B_6 \times (\text{shoot length}) \times \text{site2} + u_2 \end{aligned} \quad (2)$$

where B_0 is the model intercept, and u_2 is an error term. For both parts of the model, we included a random effect of the transect location and the fixed effects of site, depth, and shoot length.

As a diagnostic tool, we compared the observed number of shoots with no lesions to the expected number of disease-free shoots as predicted by part 1 of the model. High agreement between these values (757.98 predicted disease free to 758 measured disease free) indicated a good model fit. For part 2 of the model, we evaluated the fit of the model by plotting the Anscombe residuals against the fitted values.

The model was implemented in SAS PROC NLMIXED (SAS® 9.1.3 for Windows, SAS Institute) and estimated by adaptive Gaussian quadrature using 12 quadrature points (Kristoffersen et al. 2013). The data were left-skewed, and a 0.2 power transformation was required to meet the assumption of normality for the second part of the model; however, the results for the 0.2 power transformed data and the log-normal data were similar. Therefore, we present the latter for part 2 of the model as the interpretation is more straightforward. The data met the assumption of homoscedasticity.

Histology

We used a generalized linear mixed model with a Poisson distribution to compare counts of *Labyrinthula* spp. cells in healthy leaves with healthy and diseased sections of diseased leaves collected on transect 1 at Picnic Cove. Since cells were counted in 3 spots for each histological sample, these 3 counts were nested as a random effect within each sample. These data were analyzed using the 'glmer' function in the 'lme4' package in R (v.2.15.2; R Development Core Team 2012).

Inoculation trial

Data on inoculation trials were analyzed using a linear mixed effects model (package 'lme4') in R (v.2.15.2). We included genotype as a random effect and evaluated the fixed effects of strain (control, 8.16.D or FBSH1TD), shoot (terminal or daughter), and the strain×shoot interaction. Post hoc analyses were conducted on Bonferroni-corrected p-values that were calculated using Markov chain Monte Carlo estimation with the 'mcposthoc.fnc' function in the 'lme4' package. In order to meet the assumption of normality, we analyzed the arcsine square-root transformations of the average rankings of health. The data were homoscedastic.

RESULTS

Field study

In total, 1542 shoots of *Zostera marina* were examined at 3 field sites in the San Juan Islands, of which 784 showed visual evidence of wasting disease. Disease prevalence was 63% at Picnic Cove, 53% at Beach Haven, and 33% at False Bay. The shoot lengths varied between 3 and 234 cm, and the size structure of shoots varied by site (Fig. 3). At all sites, less than 5% of the shoots surveyed were under 10 cm in length. Representation of size classes varied across sites for shoots greater than 40 cm: over 75% of shoots surveyed at Beach Haven and Picnic Cove, but only 50% of the shoots at False Bay, were over 40 cm in length. Beach Haven had the greatest mean (±SE) density of shoots, at 182 ± 20 shoots m^{-2}, while False Bay and Picnic Cove had 61 ± 16 and 55 ± 13 shoots m^{-2}, respectively.

Disease prevalence varied significantly as a function of depth, shoot length, and the interaction of site

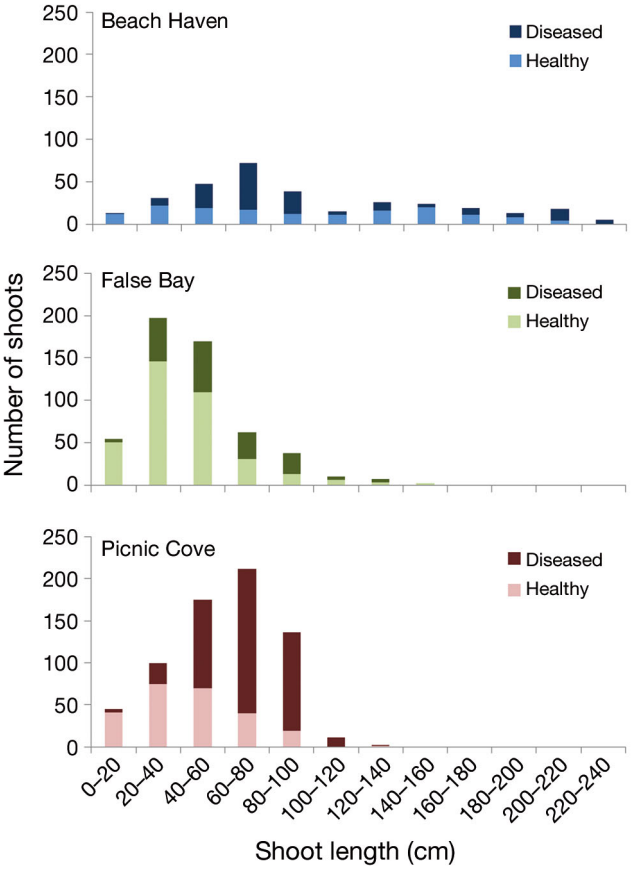

Fig. 3. *Zostera marina*. Counts of diseased and healthy leaves by size class for each site

and shoot length (Fig. 3, Table 1). Across all sites, controlling for shoot size, transects that were taken above –1 m MLLW had 73% greater disease prevalence than transects that were deeper than –1 m MLLW (t_{14} = 2.4, p = 0.031). Shoot length explained the most variation in disease prevalence (t_{14} = 14.45, p < 0.0001). At False Bay, the odds of having wasting disease increased by 58% for every 10 cm increase in shoot length (t_{14} = 1.97, p = 0.068), while at Picnic Cove the odds of having wasting disease increased by 84% for every 10 cm increase in shoot length (t_{14} = 5.21, p = 0.0001). In contrast, at the least impacted site, Beach Haven, the odds of having wasting disease only increased by 26% for every 10 cm increase in shoot length (t_{14} = –3.3, p = 0.005)

Disease severity (the total lesion length / leaf) was influenced by site, shoot length, and the interaction of these terms (Table 1). At False Bay and Picnic Cove, the total lesion length increased by 0.16 and 0.29 cm for every 10 cm increase in shoot length (t_{14} = –3.3, p = 0.069; and t_{14} = 5.21, p < 0.0001, respectively), while at the reference site, Beach Haven, the total lesion length increased by only 0.08 cm for every 10 cm increase in shoot length (Fig. 4). After controlling for shoot length, disease severity was lower at Picnic Cove than Beach Haven (t_{14} = –3.23, p = 0.006), and there was no difference in disease severity between False Bay and Beach Haven (t_{14} = –0.33, p = 0.75).

Table 1. *Zostera marina*. Coefficient estimates (see Eqs. 1 & 2), standard errors, *t*- and p-values, and odds ratios from a 2-part model. The model evaluates the influence of site, depth, shoot length, and the interaction of shoot length and site on the probability of being diseased (part 1) and the severity of disease (defined as the lesion length) for diseased plants (part 2). In both parts of the model, the reference site is Beach Haven, the reference depth is shallow, and the estimates for shoot length are based on 1 m intervals

Term	Coefficient estimate	SE	*t* (df = 14)	p	Odds ratio (95% CI)
Part 1 Logit model					
Intercept	–1.68	0.70	–2.4	0.031	
False Bay	–0.87	0.85	–1.03	0.32	0.417 (0.068, 2.58)
Picnic Cove	–0.69	0.86	–0.81	0.43	0.499 (0.08, 3.14)
Depth	–1.30	0.54	–2.4	0.031	0.272 (0.085, 0.87)
Shoot Length (m)	2.34	0.42	5.58	<0.0001	10.33 (4.21, 25.3)
False Bay × Shoot Length	2.27	0.68	3.32	0.005	9.67 (2.23, 41.9)
Picnic Cove × Shoot Length	3.78	0.66	5.73	<0.0001	43.7 (10.63, 179.8)
Part 2 Continuous model					
Intercept	0.90	0.27	3.35	<0.005	
False Bay	–0.11	0.34	–0.33	0.75	0.90 (0.44, 1.84)
Picnic Cove	–1.15	0.35	–3.23	0.006	0.32 (0.32, 0.15)
Depth	–0.28	0.17	–1.69	0.11	0.75 (0.53, 1.08)
Shoot Length (m)	0.80	0.21	3.8	0.002	2.23 (1.42, 3.51)
False Bay × Shoot Length	0.73	0.37	1.97	0.069	2.07 (0.94, 4.58)
Picnic Cove × Shoot Length	1.76	0.34	5.21	0.0001	5.81 (2.82, 11.98)

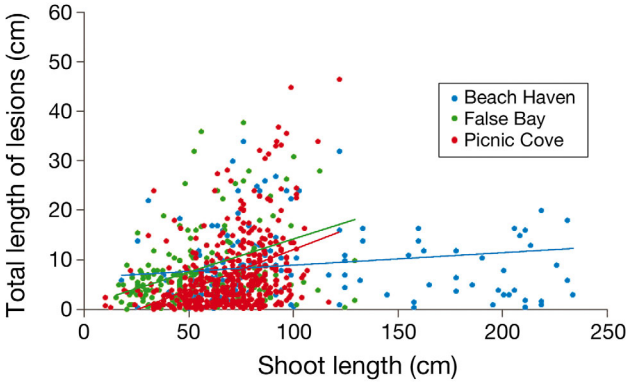

Fig. 4. *Zostera marina*. Linear regression of the relationship between shoot length and total lesion length at each site

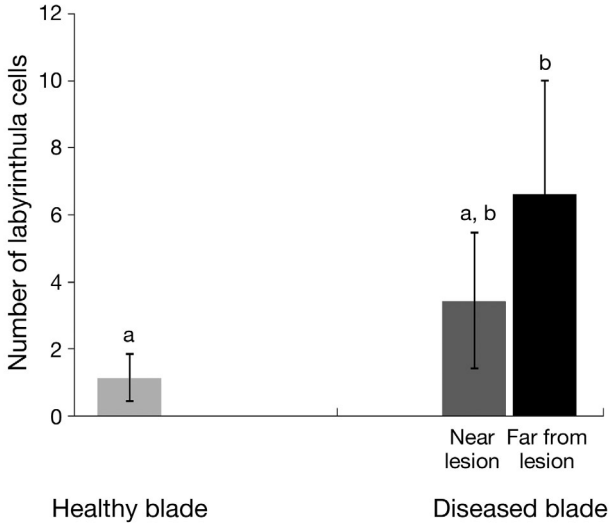

Fig. 5. *Labyrinthula* spp. Mean ± SE cell abundance using histology within healthy and diseased *Zostera marina* leaves. Letters indicate significant differences ($p < 0.05$)

Histology

We used histology of field samples to verify that observed lesions were caused by *Labyrinthula* pathogens. Because *Labyrinthula* spp. are ubiquitous and cells can appear in healthy tissue, we quantified the number of *Labyrinthula* spp. cells in healthy leaves, lesions, and healthy portions of diseased leaves.

Histological examination confirmed *Labyrinthula* spp. infection in all selected diseased leaves from all sites. The mean (±SE) numbers of cells per leaf varied among sites: Picnic Cove (transect 1: 3.45 ± 2.02 *Labyrinthula* spp. cells outside lesion, 6.64 ± 3.38 cells inside lesion area; transect 2: 2.50 ± 0.95 and 3.05 ± 0.88, respectively), Beach Haven (9.33 ± 5.86; 0.33 ± 0.33), and False Bay (4.67 ± 3.83; 6.3 ± 4.21).

At transect 1 at Picnic Cove, *Labyrinthula* spp. cell abundance in diseased lesions (6.64 ± 3.38) was significantly higher than in healthy leaves (1.15 ± 0.72; $z = -2.943$, $p = 0.0091$; Fig. 5). Cell abundance did not differ significantly between healthy leaves and healthy sections of diseased leaves (3.45 ± 2.02, $z = 1.131$, $p = 0.495$), nor between the inner and leading-edge portions of lesions on diseased leaves ($z = -1.812$, $p = 0.165$).

Inoculation trials

To determine how age structure might influence a population's susceptibility to wasting disease, we inoculated old and young leaves from the same plant with 1 of 2 different *Labyrinthula* spp. strains. Photographs of inoculated leaves were then scored according to the amount of degradation due to wasting disease (see 'Materials and methods' for details).

The health score of leaf sections in the inoculation trials was influenced by strain ($F_{2,45} = 9.022$, $p = 0.0005$) and the interaction of strain and shoot age ($F_{2,45} = 5.64$, $p = 0.006$; Fig. 6). Compared to FSW control inoculates, the health score of leaf sections was 8% lower for sections exposed to *Labyrinthula* spp. strain 8.16.D and 3% lower for sections exposed to *Labyrinthula* spp. strain FBSH1TD ($t_{45} = -4.21$, $p < 0.0001$; and $t_{45} = -2.55$, $p = 0.0142$, respectively). Older shoots had lower health scores than younger shoots; however, the difference depended on the strain to which they were exposed. Older shoots exposed to strain 8.16.D had a 10% lower health score than younger shoots ($t_{45} = 3.34$, $p < 0.002$), and older shoots exposed to strain FBSH1TD had a 2%

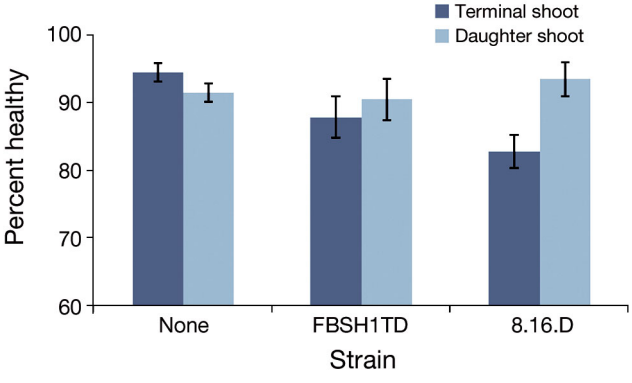

Fig. 6. *Labyrinthula zosterae*. Effect of *Zostera marina* shoot age and strain of *L. zosterae* on health. Mean % healthy shoots ± SE across *Z. marina* genets with older terminal shoots and younger daughter shoots (n = 10 genets)

lower health score, although the latter trend was not significant (t_{45} = 1.40, p = 0.17). There was no effect of age on the health score in the control treatment. By itself, age was not a significant predictor of shoot health (t_{45} = −1.25, p = 0.2).

DISCUSSION

Identification of factors that increase host susceptibility to opportunistic pathogens is crucial for understanding patterns of disease in infected populations. Previous studies of opportunistic pathogens show that environmental factors such as rising temperatures may correlate with disease outbreaks by increasing host susceptibility (reviewed by Burge et al. 2013). Here, we present evidence that population age and size structure also influence the prevalence and severity of a disease caused by an opportunistic pathogen. The field prevalence of wasting disease in *Zostera marina* is greater on larger, older leaves, and experimental exposure to *Labyrinthula zosterae* demonstrates increased susceptibility of older shoots to eelgrass wasting disease. While other environmental and genetic factors most likely contribute to patterns of disease in declining populations of this marine plant, the results of our study allow for the identification of susceptible populations based on measurable demographic properties.

Several isolates of *Labyrinthula* spp. (with differences in host specificity) and at least 2 species of *Phytophthora*, viz. *P. gemini* and *P. inundata*, can infect *Zostera marina* (Garcias-Bonet et al. 2011, Man in 't Veld et al. 2011). Proper identification of pathogens and verification of the etiologic agents of disease has been a challenge for *Z. marina*. Lesions similar to those caused by wasting disease can result from other stressors, including desiccation (Boese et al. 2003), invertebrate grazing (Hily et al. 2004, Boese et al. 2008), and mechanical damage (Boese et al. 2008). Moreover, because opportunistic pathogens are ubiquitous in many seagrass populations and do not always cause disease (Burge et al. 2013), identification by molecular or microbiological methods alone will not confirm an association with disease (e.g. Bockelmann et al. 2012, 2013). Indeed, in a subset of samples, histology indicated that *Labyrinthula* spp. cells are found in healthy *Z. marina* shoots as well as diseased ones, although the number of cells was consistently higher in tissue with lesions than in healthy tissue. We used both histology and isolation and re-infection with local strains to confirm that the lesions we identified were associated with a *Labyrinthula* pathogen. We recommend this paired approach for confident verification of both disease and the etiologic agent. Future work developing a qPCR diagnostic for Pacific strains of *L. zosterae* and immunohistochemistry for identifying cryptic *L. zosterae* cells would greatly enhance diagnostic capabilities.

The effect of shoot length on disease prevalence depended upon site such that a positive correlation was most apparent at the 2 disturbed sites, False Bay and Picnic Cove. The density of shoots was nearly 4 times greater at Beach Haven, suggesting that density-dependent transmission is not driving infection prevalence. In addition, both of the disturbed sites had smaller shoots than Beach Haven. This may be because larger shoots had decayed as a result of disease or simply because environmental conditions caused a different phenotypic expression of shoots (see Backman 1991). While Beach Haven experiences high wave action, has a steep slope, and faces to the north, Picnic Cove and False Bay are in south-facing shallow embayments and are characterized by warm water during low tides (S. Wyllie-Echeverria et al. unpubl. data). Collectively, these factors may contribute to increased disease after controlling for the effect of shoot size. Warmer temperatures promote *in vitro* growth of *Labyrinthula zosterae* (S. Wyllie-Echeverria et al. unpubl. data) and may be a source of stress to *Zostera marina* shoots growing in this region (e.g. Vergeer et al. 1995). At Picnic Cove in particular, *Z. marina* beds have been in decline since 2000 (Ferrier & Berry 2010, Wyllie-Echeverria et al. 2010). The site has fewer herbivores and higher densities of diatomaceous epiphytes in comparison to our other study sites (S. Wyllie-Echeverria pers. obs.), as well as high levels of hydrogen sulfide, which is toxic to adult plants and seedlings at high concentrations (Krause-Jensen et al. 2011, Dooley et al. 2013). Further work investigating how these ecological factors influence disease susceptibility may help to explain declines at Picnic Cove.

Across all sites, disease severity and prevalence increased with leaf length. An increase in disease prevalence with size or age is a common pattern, for example in invertebrates such as corals (Dube et al. 2002) and terrestrial plants such as the Port Orford cedar *Chamaecyparis lawsoniana* (Kauffman & Jules 2006). Several mechanisms may explain this pattern. Larger individuals have longer cumulative exposure times and greater surface area available for infection (e.g. *Daphnia* in Hall et al. 2007). In addition, exposure to stressors over time may decrease plant allocation to defenses, allowing infected plants to become

diseased (Zangerl & Bazzaz 1992). Larger plants may also experience more mechanical stress and herbivory, damaging leaf tissue and creating more infection opportunities for the pathogen (reviewed by Lafferty & Harvell 2014). An association between wasting disease lesions and *Zostera marina* leaf age has previously been noted (Burdick et al. 1993); however, those authors assumed that it was due to increased contact with infected plants and the trend was not statistically tested. Interestingly, the highest abundance of *Labyrinthula zosterae* cells using qPCR has been noted in the third longest leaf (Bockelmann et al. 2013). The histological results of our and other studies show that the highest densities of *L. zosterae* can be found at the leading edge of the lesion, or before darkening has occurred (Muehlstein 1992). Thus the most diseased leaf as assessed by the presence of lesions may not be the site of the greatest infection.

Our experimental inoculations suggest that the higher levels of disease in older shoots may be caused by an increase in susceptibility. Increased resistance with age and size has been observed in many plant species due to a change in nutrient status and activation of defense pathways as a result of development (reviewed by Develey-Rivière & Galiana 2007). On the other hand, decreased resistance has also been observed with age due to increased allocation of defenses to young tissue (Dube et al. 2002) and a trade-off between growth and defense (Bazzaz et al. 1987, Fine et al. 2006). In *Zostera marina,* experimental evidence suggests that phenols, particularly caffeic acid, may be associated with resistance to *Labyrinthula zosterae* (e.g. Buchsbaum et al. 1990, Vergeer et al. 1995, Vergeer & Develi 1997). Total phenol concentrations are generally decreased in older leaves (Ravn et al. 1994). More research is needed to understand the role of phenols in resistance to wasting disease; however, if phenols are an important mechanism for resistance, it would be consistent with our results. Other mechanisms not tested in this study, such as increased contact with diseased shoots or mechanical damage, may also contribute to increased disease in older shoots.

CONCLUSION

This study is the first demographic assessment of wasting disease in an area of recent seagrass declines. Our finding of nearly 50% prevalence across all sites suggests that the role of wasting disease as a factor in seagrass population decline warrants further investigation. Moreover, patterns of infection from both the field and laboratory inoculation trials suggest that older *Zostera marina* leaves at shallower depths may be more vulnerable to wasting disease. While demographic factors are well studied in primary (non-opportunistic) pathogens, they are often overlooked in opportunistic pathogens. The class Labyrinthulomycetes includes a variety of opportunistic pathogens associated with recent disease outbreaks, such as those infecting gorgonian corals (Burge et al. 2012) and hard clams (Ragan et al. 2000). The association between demography and disease, such as we found here, allows for identification of eelgrass populations that are at higher risk of disease based on demographic measurements. More broadly, these results highlight host population demographics as an important research area in the understanding of the epidemiology of these and other opportunistic pathogens.

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