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Facilitation of a tropical seagrass by a chemosymbiotic bivalve increases with environmental stress

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Abstract

1. Facilitation of foundation species is critical to the structure, function and persistence of ecosystems. Understanding the dependence of the strength of this facilitation on environmental conditions is important for informed ecosystem management and for predicting the impacts of global change. In coastal seagrass habitats, chemosymbiotic lucinid bivalves can facilitate seagrasses by decreasing potentially toxic levels of sulphide in sediment porewater. However, variation in the strength of lucinid–seagrass facilitation with environmental context has not been experimentally investigated.
2. We tested the hypothesis that the presence of the tiger lucine *Codakia orbicularis* becomes more important to the growth and survival of the seagrass *Thalassia testudinum* under decreased light availability and increased sulphide stress. In a mesocosm experiment, we reduced average ambient-light to *T. testudinum* by 64% and/or increased sediment porewater sulphide concentrations by ~200% and compared growth and tissue chemistry of *T. testudinum* with and without *C. orbicularis*.
3. We found that *T. testudinum* was better able to maintain growth under shading and sulphide stress when *C. orbicularis* was present. *C. orbicularis* strongly decreased sediment porewater sulphide, an effect that minimized sulphur build-up in seagrass tissue and was likely achieved through bioirrigation as well as chemoautotrophy. The relative effects of *C. orbicularis* on *T. testudinum* growth were strongest in the presence of environmental stressors.
4. *Synthesis*. The strength of lucinid–seagrass facilitation increases under environmental conditions that hinder the ability of seagrass to detoxify sulphide. Our results provide evidence of a potential mechanism by which the spatiotemporal association between lucinids and seagrasses is maintained and support the

incorporation of interspecific facilitation into conservation and restoration strategies for foundation species in the face of increasing anthropogenic impact and global change.

KEYWORDS

coastal ecosystems, context dependence, facilitation, lucinid bivalves, positive species interactions, seagrass, sediment sulphide, *Thalassia testudinum*

1 | INTRODUCTION

Positive species interactions are increasingly acknowledged as important to the structure and function of ecosystems (Brooker et al., 2008; Bulleri, 2009; Stachowicz, 2001). The most critical of these facilitative interactions involve habitat-forming foundation species (Dayton, 1972), such as reef-building animals and forest trees, which modulate environmental conditions, resource availability and biotic stress across entire communities (Angelini, Altieri, Silliman, & Bertness, 2011; Ellison et al., 2005; Thomsen et al., 2010). Foundation species often form facultative mutualisms with other species (Hay et al., 2004; Stachowicz, 2001), in which both partners benefit but neither partner obligatorily depends on the interaction. For example, stony corals create substrate for sponges that retain carbon and nutrients within the reef system by recycling dissolved organic matter as detritus (de Goeij et al., 2013), and large-seeded tropical trees create forest habitat for large frugivores that disperse their seeds (Peres, Emilio, Schietti, Desmoulière, & Levi, 2016). A number of studies show that facultative mutualisms are vital to the persistence of foundation species, conferring benefits such as greater tolerance to environmental stress (e.g. thermal or drought; Afkhami, McIntyre, & Strauss, 2014; Angelini et al., 2016; Derksen-Hooijberg et al., 2019; Redman, Sheehan, Stout, Rodriguez, & Henson, 2002) and resistance to long-term loss of biomass (Peres et al., 2016).

Facilitation of a foundation species as part of a facultative mutualism is expected to be context-dependent, that is, the benefit of the partner to the foundation species will change in sign or strength across biotic or abiotic gradients. Changes to the outcome of the interaction are particularly likely during episodes of extreme stress that would otherwise result in mortality of the foundation species. For example, the cost to acacia trees of hosting some ant species may outweigh fitness gains from ant defence against typical herbivore damage; however, net benefits may be yielded over long periods when ants defend trees against elephant damage, which can lead to mortality of unprotected trees (Goheen & Palmer, 2010; Stanton & Palmer, 2011). Likewise, under nominally typical environmental conditions, the effect of ribbed mussels *Geukensia demissa* on the success of intertidal cordgrass *Spartina alterniflora* may range from positive (Bertness, 1984) to neutral (Altieri, Silliman, & Bertness, 2007). However, because mussels can facilitate cordgrass by alleviating sediment biogeochemical stressors, mussels may become especially important for cordgrass persistence and recovery following

drought-induced die-offs (Angelini et al., 2016). Global change is expected to increase the frequency and intensity of droughts, heat waves and other extreme climate events that stress foundation species and the ecosystems they support (Doney et al., 2012). Efforts to characterize environmental context dependence in positive species interactions are therefore important to our capacity to predict global change impacts and manage for the long-term persistence of key ecosystems (Maron, Baer, & Angert, 2014).

Recent research in seagrass ecosystems has shown that members of the bivalve family Lucinidae can facilitate seagrasses as part of a facultative mutualism by ameliorating phytotoxic levels of sulphides in sediment porewater (Reynolds, Berg, & Ziemann, 2007; van der Heide et al., 2012). Lucinids are a diverse group of infaunal clams that host sulphur-oxidizing Gammaproteobacteria in their gill tissue (Dubilier, Bergin, & Lott, 2008; Taylor, Glover, Smith, Dyal, & Williams, 2011). Lucinids are abundant in subtropical and tropical seagrass beds world-wide (van der Heide et al., 2012) and are closely associated with seagrasses in the geological record (Stanley, 2014). By virtue of their chemosymbionts, lucinids are typically the only conspicuous seagrass macrofauna with the ability to consume toxic sulphides produced from sulphate reduction, the dominant remineralization process in coastal sediments (Howarth, 1984). Seagrasses have evolved a variety of detoxification mechanisms to deal with consistent potential sediment sulphide toxicity, including radial oxygen release from roots, S^0 precipitation and incorporation of sulphur into plant tissues (Hasler-Sheetal & Holmer, 2015; Lamers et al., 2013). However, a number of environmental stressors can alter the ability of seagrasses to sustain detoxification and can increase the risk of seagrass mortality from sulphide exposure. These stressors include high temperatures (Koch, Schopmeyer, Kyhn-Hansen, & Madden, 2007), hypersalinity (Koch, Schopmeyer, Holmer, Madden, & Kyhn-Hansen, 2007), water column hypoxia (Holmer & Bondgaard, 2001), low light (Goodman, Moore, & Dennison, 1995; Holmer, Frederiksen, & Møllegaard, 2005) and excess sulphide from decomposition of adjacent seagrass (Carlson, Yarbro, & Barber, 1994). Thus, the ability of lucinids to decrease sediment porewater sulphide levels represents a clear mechanism by which lucinids can facilitate seagrass growth and survival (Heck & Orth, 2006; Reynolds et al., 2007; van der Heide et al., 2012).

Despite recent interest in lucinid-seagrass facilitation, information regarding how the facilitation may vary with environmental context is lacking. van der Heide et al. (2012) were the first to experimentally demonstrate lucinid facilitation of a seagrass. In their

experiment, seagrass biomass significantly increased with the addition of lucinids to mesocosms, whether or not porewater sulphide levels were also enhanced. The biomass increases were not readily attributable to other changes that could occur with lucinid addition and could alleviate resource limitation and/or promote seagrass growth, such as increased nutrient availability. In fact, porewater concentrations of ammonium and phosphate in mesocosms with lucinids were significantly decreased relative to mesocosms without lucinids (van der Heide et al., 2012). Thus, lucinid facilitation of seagrass was demonstrated when seagrass growth was not clearly inhibited or enhanced by environmental factors. Subsequent evaluation of a drought-triggered seagrass die-off event in the eastern Atlantic showed that lucinid–seagrass facilitation could be important under extreme environmental stress and suggested that the strength of the facilitation could depend on the timing and magnitude of that stress (de Fouw et al., 2016, 2018). However, environmental context dependence of lucinid–seagrass facilitation has not been confirmed or assessed in controlled experiments. Moreover, all of the above work was based in intertidal systems. Ecological theory and data predict that facilitation will increase in importance with increasing stress (the stress gradient hypothesis; Bertness & Callaway, 1994), particularly if that stress is abiotic (Chamberlain, Bronstein, & Rudgers, 2014). Understanding how the strength of lucinid–seagrass facilitation changes with environmental context may help to explain how and why seagrasses associate with lucinids through geological time not only in physically stressful intertidal areas, but also in deeper subtidal habitats where physical gradients have not typically been considered drivers of facilitation (Bulleri, 2009; but see Bennett et al., 2015). Finally, an experimental understanding of environmental context dependency in lucinid–seagrass facilitation may aid seagrass conservation and restoration efforts. Large-scale seagrass die-offs may be triggered by complex cascades of environmental stressors involving, for example, interactions among salinity, temperature, light availability and organic matter decomposition that lead to increased sulphide stress and widespread hypoxia (e.g. Koch, Schopmeyer, Nielsen, Kyhn-Hansen, & Madden, 2007). Deeper knowledge of where, when and to what extent lucinids may increase seagrass resistance to environmental stressors by ameliorating sediment sulphide levels adds an important component to our understanding of observed patterns of seagrass productivity and decline.

We designed a short-term (6.5-week) mesocosm experiment in south Florida to test whether the strength of lucinid–seagrass facilitation increases with increasing environmental stress. Our focal species were turtle grass *Thalassia testudinum*, a dominant primary producer in coastal seagrass communities in the Gulf of Mexico and Caribbean Sea (van Tussenbroek et al., 2006), and the tiger lucine *Codakia orbicularis*, a large lucinid commonly found in *T. testudinum* beds (Allen, 1958; Stanley, 1970). We evaluated the response of *T. testudinum* to low light and high sediment porewater sulphide in the presence or absence of *C. orbicularis*. Light availability is one of the primary determinants of seagrass growth and survival and may be affected in coastal environments by storms, runoff,

phytoplankton blooms and other natural and anthropogenic events. Among other effects, reduced light availability hampers photosynthesis-driven radial oxygen loss from seagrass roots (Frederiksen & Glud, 2006; Jovanovic, Pedersen, Larsen, Kristensen, & Glud, 2015) and thus hinders a major pathway by which seagrass can avoid excess sulphide intrusion (Hasler-Sheetal & Holmer, 2015; Lamers et al., 2013). Excessive and prolonged sulphide exposure is thought to be a proximate, though not ultimate, cause of several observed *T. testudinum* die-off events in Florida (Borum et al., 2005; Carlson et al., 1994; Johnson, Koch, Pedersen, & Madden, 2018; Koch, Schopmeyer, Nielsen, et al., 2007). We hypothesized that the presence of *C. orbicularis* would increase *T. testudinum* growth and survival even without added environmental stress, as in van der Heide et al. (2012). We further hypothesized that the importance of *C. orbicularis* presence to *T. testudinum* would increase as we added environmental stressors.

2 | MATERIALS AND METHODS

2.1 | Field collections

In fall 2017, we collected sediment, seagrass and lucinids from the east side of Key Biscayne, Florida, USA (25.708822°N, 80.150449°W). Sediment at the collection site is relatively carbonate-rich (53%–60% by acidified weight loss, $n = 4$) sand with low organic matter content (1.0%–1.7% by loss on ignition, $n = 5$). Segments of *T. testudinum* were collected from 1 to 2 m water depth by hand as apical horizontal rhizomes with two to four attached short shoots. Live *C. orbicularis* were collected from <30 cm sediment depth and separated from sediment in a 4-mm sieve. Sediment was collected adjacent to the seagrass collection area with shovels and sieved over a 2-mm mesh to remove larger material. Organisms and sediment were transported to the Smithsonian Marine Station (SMS) in Fort Pierce and held in aerated tanks with 20 μm -filtered ocean water. *C. orbicularis* individuals were allowed to burrow in sediment from the collection site for several days until needed for the experiment.

2.2 | Experimental design, set up and data collection

We planted *T. testudinum* segments in 48 custom 25-cm diameter PVC mesocosms consisting of an upper chamber of 14 cm depth and a lower chamber of 3 cm depth, separated by a perforated shelf and porous 100- μm polyester–cotton membrane (Figure S1). The upper chamber was filled with approximately 7 L of homogenized sediment, and the lower chamber was filled with seawater and sealed with a rubber septum. Each mesocosm received a total of nine short shoots, allocated among a 2-shoot, a 3-shoot and a 4-shoot rhizome segment (shoot density 183 m^{-2}). A coloured zip tie tag was placed just in front of the second short shoot on each segment, and the length from the apical end to the tag on each segment was measured prior to planting. Each mesocosm received two 5-cm Rhizon SMS

porewater samplers (Rhizosphere Research Products), placed on opposing sides of the mesocosm at approximately 0–5 and 5–10 cm depth.

Mesocosms were housed outdoors in six 416-L aquaculture tanks. Individual tanks were filled with 20 μm -filtered ocean water, aerated with two airlifts and covered loosely with a transparent polycarbonate roofing panel to prevent rainwater input and reduce evaporation. Mesocosm position was re-randomized within tanks during weekly 100% water changes. Epiphytic and microphyto-benthic loading were limited. Salinity was maintained at ~34–38 and adjusted downward when necessary with reverse osmosis (RO) water. Surface water pH was ~8.0–8.2, and dissolved oxygen was ~6.3–6.9 mg/L. One submersible aquarium heater (250W; Eheim) was added to each tank at the end of the 5-week acclimation period to stabilize night-time water temperatures.

The experiment was fully factorial, with three factors, eight treatments and six replicates per treatment. There were two levels of each factor: light exposure (ambient-light or shaded), sediment porewater sulphide exposure (ambient or enhanced-sulphide) and *C. orbicularis* presence (presence or absence of clams). Treatments were arranged in randomized complete blocks, with eight mesocosms in each tank.

Following the 5-week acclimation period, each shaded mesocosm received a single layer of pet-grade window screen held above the canopy by a plastic-coated wire frame. Target average light levels under shading were ~30–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, in the range of whole-plant compensation irradiances observed for *T. testudinum* (Lee, Park, & Kim, 2007). Ambient-light mesocosms received a frame only. Enhanced-sulphide mesocosms were injected with 30 ml of 100 mM sulphide solution, which was prepared with sodium sulphide trihydrate, adjusted to pH 7.1 and injected into the lower chamber of each mesocosm by syringe under anoxic conditions (see also van der Heide et al., 2012). Injections into enhanced-sulphide mesocosms were performed twice at the beginning of the experimental period and weekly thereafter. During the fourth and fifth weeks, the injection regime was adjusted to stabilize the highest sediment porewater sulphide concentrations around 5–6 mM. This target level was chosen in order to reduce the possibility of complete shoot loss in one or more treatments while maintaining a challenge to seagrass sulphide tolerance. Sulphide concentrations of 5–6 mM may induce above-ground die-off in *T. testudinum* (Ruiz-Halpern, Macko, & Fourqurean, 2008) or may be tolerated if other stressors, such as high temperature, are absent (Koch, Schopmeyer, Kyhn-Hansen, Madden, 2007). Clam-present mesocosms received three randomly selected *C. orbicularis*, one individual from each available group of 13–17, 19–24 and 25–36 mm shell height. The resulting clam density, 61 m^{-2} , was comparable to the highest field estimates (0–64 m^{-2} , $n = 3$) at the collection site. Individuals were tagged with bee tags and observed after placement in clam-present mesocosms to ensure burial. Survival throughout the experimental period was 90% (65 of 72 individuals), and all mesocosms retained at least one live *C. orbicularis*. Neither light nor sulphide exposure significantly affected the number of surviving *C. orbicularis* in each treatment (two-way Poisson GLM, $p > 0.60$; Table S1). The condition of

live *C. orbicularis* at the end of the experiment, which was assessed by determining the flesh:shell dry weight ratio (Lucas & Beninger, 1985) after drying for 1 week at 60°C, also did not differ significantly among treatments (two-way ANOVA, $p > 0.60$; Table S2).

Following acclimation, the experiment was conducted for 6.5 weeks. Temperature and light intensity at canopy level were monitored continuously at 10-min intervals in each tank by two HOBO Pendant loggers (Onset) under ambient-light and shaded mesocosms. Light intensities measured by loggers were converted to estimates of photosynthetically active radiation (PAR) by calibrating loggers to a 4 π PAR sensor (LI-193; LI-COR) over 24 hr (Long, Rheuban, Berg, & Zieman, 2012). The average of mean daily PAR over the experimental period across six tanks was 138 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in ambient-light and 49.6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under shades. The average of mean daily temperatures over the experimental period across six tanks was 25.7°C. All calculations excluded data from days involving water changes, major sampling events or other experimental activities with the potential to temporarily but substantially bias recorded light and temperature levels.

Sediment porewater for analysis of total dissolved sulphide ($\text{H}_2\text{S} + \text{HS}^- + \text{S}^{2-}$) and pH was collected by syringe from both Rhizon samplers in all mesocosms weekly. Porewater was processed the day of collection, prior to sulphide injections in enhanced-sulphide mesocosms. Porewater subsamples (5 ml) were fixed temporarily in an equal volume of sulphide antioxidant buffer (SAOB) to convert all sulphide to S^{2-} , which was measured with an ion-selective silver/sulphide electrode (HI4115; Hanna Instruments). The practical lower reporting limit was considered to be 10 μM . The unfixated remainder of each porewater sample (5–10 ml) was evaluated using a double-junction pH electrode with temperature compensation (S2900C/SAM; Senorex). Results from the two Rhizon samplers in each mesocosm were averaged to determine mean sulphide concentration and mean pH over 0–10 cm depth.

Samples of sediment porewater for dissolved nutrient analysis (~15 ml) were collected from the 5–10 cm sampler in each mesocosm at initiation and termination of the experiment. Samples of water from each tank were also collected at termination of the experiment, 1 week after the final complete water change. All samples were frozen until analysis. Concentrations of ammonium, ortho-phosphate and nitrate in porewater were determined colorimetrically on autoanalysers (Skalar Analytical; SEAL Analytical) by reaction with salicylate (Kempers & Zweers, 1986), ammonium molybdate (Henriksen, 1965) and sulphanilamide following cadmium reduction to nitrite (Wood, Armstrong, & Richards, 1967) respectively. Water column dissolved nutrient concentrations ranged from 1.8 to 11 μM nitrate, 0 to 6.5 μM ammonium and 0 to 0.004 μM phosphate.

Live shoots in each mesocosm were counted weekly. The percentage net shoot change was calculated as the ratio of the difference of initial and final live shoot counts to the initial live shoot count. One week prior to harvest, all visible shoots were marked with a syringe needle just above the leaf sheath (method modified from Zieman, 1974). New leaf growth was defined as the portion of leaves from the top of the sheath to holes displaced above the sheath.

Rhizome elongation rate was estimated as the difference in length, from planting to harvest, between the apical end and the coloured tag on each rhizome. To account for potential increases in growth with increased above-ground tissue, rhizome elongation rate was standardized to the initial number of short shoots on the segment (i.e. 2, 3 or 4 short shoots) and averaged across the live segments in a mesocosm to estimate a mean rhizome elongation rate for that mesocosm ($\text{mm day}^{-1} \text{shoot}^{-1}$). We also estimated rhizome elongation rates during the acclimation period for several sets of 'surrogate' rhizome segments, because our estimates of rhizome elongation in experimental mesocosms included growth that occurred during the acclimation as well as the experimental period. Three groups of three segments (comprising nine short shoots: one 2-shoot, one 3-shoot and one 4-shoot segment) were planted in 7.6-L buckets and maintained in a separate 242-L tank in the same way as the experimental mesocosms during acclimation. Rhizome elongation rates for these surrogate segments were used as an estimate of the range of rhizome growth that may have occurred during the acclimation period in the absence of any experimental treatment. At the end of the experimental period, rhizomes with no attached live shoots were often soft with a strong sulphidic odour. While defoliation and later recovery in response to environmental stress such as high temperatures or hypersalinity are possible (van Tussenbroek et al., 2006), the loss of structural integrity in these rhizomes was considered indicative of mortality and early decomposition. Dead rhizomes were not included in calculations of rhizome growth, regardless of whether they appeared to have elongated between planting and the end of the experiment.

After harvest, seagrass tissues were dried for 1 week at 60°C and weighed to determine dry biomass. Biomass of new leaf growth was converted to a growth rate over the period of leaf marking, standardized to the number of short shoots on a segment at the end of the experiment, and averaged across the live segments in a mesocosm to estimate a mean leaf growth rate for that mesocosm ($\text{mg d}^{-1} \text{shoot}^{-1}$). Subsamples of dried leaf and rhizome tissue from each segment were ground to fine powder in a ball mill and analysed for sulphur content (% dry weight) on an elemental analyser (Thermo Scientific). Dead segments were eliminated from statistical analyses to avoid confounding live tissue chemistry with potential tissue decomposition. Exploratory data analysis suggested that rhizome %S might be related to the number of short shoots on a segment at planting. Therefore, we considered %S of live rhizomes individually but calculated means across live segments in each mesocosm for all other parameters.

2.3 | Statistical analysis

Statistical analyses were performed in R v.3.5.3 (R Core Team, 2019). Seagrass growth (rhizome elongation rate, leaf biomass growth rate, percentage net shoot change), seagrass tissue chemistry (rhizome and leaf %S) and porewater concentrations of sulphide, ammonium and phosphate were analysed by linear mixed models (LMM)

in the R packages LME4 (Bates, Maechler, Bolker, & Walker, 2019) and LMERTEST (Kuznetsova, Brockhoff, & Christensen, 2019). Models used light exposure, sulphide exposure and *C. orbicularis* presence as crossed fixed factors and tank as a random blocking factor. For rhizome %S only, the model was extended to include the number of shoots on a segment at planting as an additive fixed factor and mesocosm as a random factor nested within tank. Statistical significance of fixed factors and their interactions was assessed by Type III ANOVA using the Satterthwaite approximation for degrees of freedom, and random effects were assessed using likelihood ratio tests. Data transformations were applied where necessary to meet the assumptions of linear modelling. Normality and homogeneity of variances were evaluated with graphical methods, including boxplots of observations; histograms and quantile-quantile plots of residuals from generated models; and plots of model residuals versus fitted values (Quinn & Keough, 2002). Pairwise differences between treatments were examined with Tukey-adjusted post hoc comparisons in the package EMMEANS (Lenth, 2019). Effects were considered significant at $\alpha = 0.05$. Average responses are given as $M \pm SE$.

3 | RESULTS

3.1 | Seagrass growth and survival

Rhizome elongation rates over the combined acclimation and experimental period were similar to the control treatment (ambient-light, ambient-sulphide, clams absent; $0.16 \pm 0.04 \text{ mm d}^{-1} \text{shoot}^{-1}$) in the presence of *C. orbicularis* but were lower when mesocosms experienced shading, enhanced-sulphide or both in the absence of *C. orbicularis* (Figure 1A). Thus, rhizome elongation was influenced by a significant three-way interaction among light exposure, sulphide exposure and *C. orbicularis* presence (LMM, $p = 0.035$; Table 1; Figure 1A).

Based on growth of the 'surrogate' segments during acclimation, we inferred that most experimental rhizome mortality occurred during the acclimation period and was related to the experimental treatments in only a few cases. The overall rhizome segment survival rate from planting to termination of the experiment was 83% (119 of 144 segments). Of the 25 dead segments, only seven had a calculated growth rate higher than the lowest growth rate observed among the nine surrogate segments during acclimation ($0.02 \text{ mm d}^{-1} \text{shoot}^{-1}$). Of these seven segments, which likely survived acclimation and later died, six were located in mesocosms without *C. orbicularis*. Including these seven segments in a supplemental analysis did not change our conclusions regarding the impact of the experimental treatments on rhizome elongation rates (Table S3; Figure S2).

Leaf growth at the end of the experiment was significantly higher overall when *C. orbicularis* was present (LMM, $p = 0.024$), and shading significantly decreased leaf growth rates only in combination with enhanced-sulphide (LMM, $p = 0.052$; Table 1;

FIGURE 1 Growth rates of (A) rhizomes and (B) leaves in *Thalassia testudinum* mesocosms with and without shading, enhanced sediment porewater sulphide and the lucinid clam *Codakia orbicularis*. Rhizome growth rates in each mesocosm were the mean elongation of one to three live rhizomes, standardized to the initial number of short shoots on each rhizome (two, three or four shoots), over an 11.5-week acclimation + experimental period. Leaf growth rates in each mesocosm were the mean dry biomass of one to three sets of new leaf tissue, standardized to the final number of short shoots comprising each leaf set, generated during 8–10 days at the end of the experiment. Displayed response values are $M \pm SE$, $n = 6$ mesocosms. Different letters indicate significant Tukey-adjusted pairwise comparisons ($p < 0.05$) following analysis by linear mixed models. n.s., not significant ($p > 0.05$)

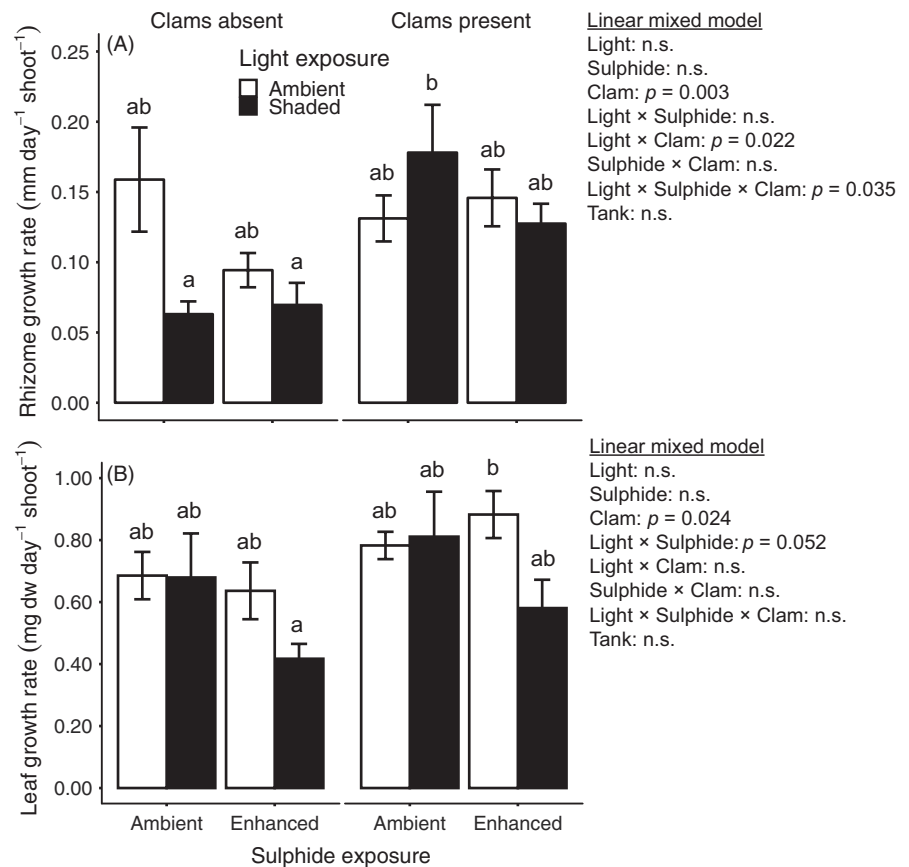


Figure 1B). Leaf growth was lowest relative to the controls ($0.69 \pm 0.08 \text{ mg d}^{-1} \text{ shoot}^{-1}$) in clam-absent mesocosms experiencing both shading and enhanced-sulphide ($0.42 \pm 0.05 \text{ mg d}^{-1} \text{ shoot}^{-1}$; Figure 1B).

Short shoot growth and survival over the experimental period were not significantly affected by light exposure, sulphide exposure or the presence of *C. orbicularis* (LMM, $p > 0.05$; Table 1; Figure S3). Net shoot losses and gains varied substantially across treatments (Figure S3). No mesocosm experienced complete loss of short shoots.

3.2 | Sediment porewater chemistry

Dissolved sulphide in sediment porewater was significantly increased by sulphide injections in the absence of *C. orbicularis* but significantly decreased in the presence of *C. orbicularis* regardless of whether sulphide was injected (LMM, sulphide \times clam interaction, $p < 0.001$; Table 1; Figure 2A). Mesocosms differentiated by porewater sulphide into three groups by the second week of the experiment: mesocosms with *C. orbicularis*; ambient-sulphide mesocosms without *C. orbicularis*; and enhanced-sulphide mesocosms without *C. orbicularis* (Figure S4). These groupings were maintained to the end of the experiment, with depth-averaged porewater sulphide concentrations $< 50 \mu\text{M}$ in most clam-present mesocosms but close to $\sim 2 \text{ mM}$ and $\sim 4 \text{ mM}$ in clam-absent mesocosms with ambient-sulphide

or enhanced-sulphide levels respectively (Figure S4; Figure 2A). Mesocosms experiencing the highest porewater sulphide levels were often visually distinguishable by dark grey colouration and/or white bacterial mats (possibly *Beggiatoa* sp.) on the sediment surface, indicating surface anoxia. Porewater pH exhibited a similar pattern to sulphide levels. Mesocosms differentiated into two groups by the first week of the experiment based on the presence (pH ~ 7.4) or absence (pH ~ 7.1) of *C. orbicularis* (Figure S5).

Porewater dissolved nutrient concentrations (ammonium, phosphate) were significantly lower in the presence of *C. orbicularis* (LMM, $p < 0.001$; Table 1; Figure 2B,C). Nitrate was non-detectable in all but one mesocosm, where the porewater nitrate concentration ($0.72 \mu\text{M}$) was less than the lowest measured concentration in surface water ($1.8 \mu\text{M}$).

3.3 | Seagrass tissue chemistry

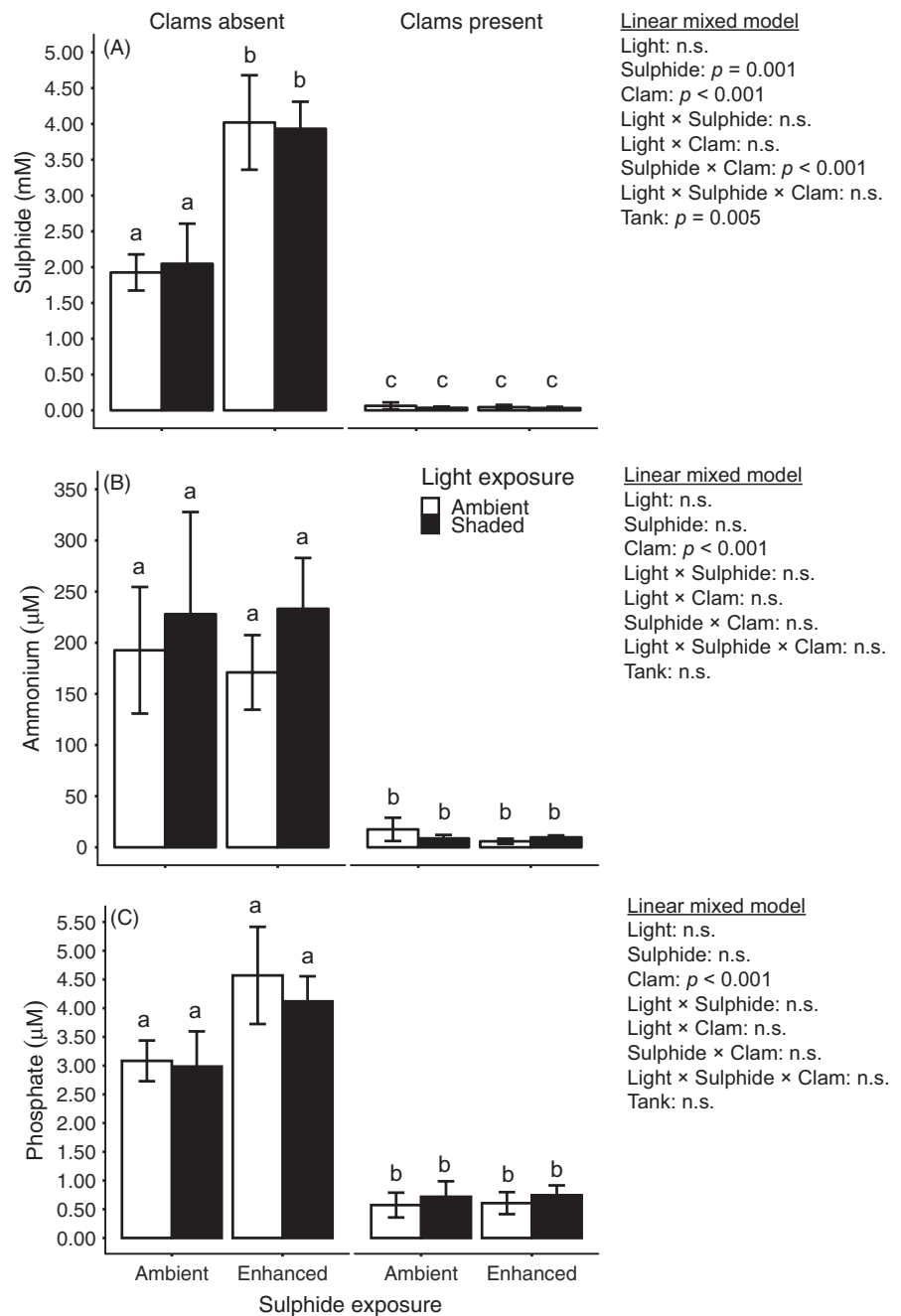
Sulphur content (% dry weight) of rhizomes was significantly affected by all treatments and by the initial number of shoots on a rhizome segment at planting (Table 1; Figure 3A). Rhizomes contained higher %S overall when they had fewer shoots at planting (LMM, $p = 0.002$), when *C. orbicularis* was absent (LMM, $p < 0.001$), and when mesocosms were shaded (LMM, $p = 0.038$) or sulphide enhanced (LMM, $p = 0.030$; Table 1; Figure 3A). Leaf %S was significantly higher overall, though also more variable, when *C. orbicularis*

TABLE 1 Results of ANOVA *F*-tests on linear mixed models of seagrass growth, sediment porewater chemistry and seagrass tissue chemistry according to light exposure (ambient-light or shaded), sediment porewater sulphide exposure (ambient or enhanced-sulphide) and *Codakia orbicularis* presence (presence or absence of clams). Tank was considered a random blocking factor and assessed using likelihood ratio tests (LRT). For rhizome %S only, the number of shoots on a rhizome segment at time of planting was an additive fixed factor and mesocosm was a random factor nested within tank. Data transformations are indicated. Significant ($\alpha = 0.05$) *p*-values are highlighted in bold

Rhizome elongation rate				Leaf growth rate			
	<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>		<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>
Light	1, 40	2.179	0.148	Light	1, 40	3.367	0.074
Sulphide	1, 40	2.266	0.140	Sulphide	1, 40	2.646	0.112
Clam	1, 40	9.954	0.003	Clam	1, 40	5.519	0.024
Light × Sulphide	1, 40	0.009	0.926	Light × Sulphide	1, 40	3.995	0.052
Light × Clam	1, 40	5.719	0.022	Light × Clam	1, 40	0.032	0.860
Sulphide × Clam	1, 40	0.125	0.726	Sulphide × Clam	1, 40	0.441	0.510
Light × Sulphide × Clam	1, 40	4.772	0.035	Light × Sulphide × Clam	1, 40	0.186	0.669
Tank	1	0.000	1.000	Tank	1	0.000	1.000
Percentage net shoot change				Porewater sulphide (final), $\sqrt{\sqrt{x}}$			
	<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>		<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>
Light	1, 35	0.193	0.663	Light	1, 35	0.049	0.827
Sulphide	1, 35	1.937	0.173	Sulphide	1, 35	11.885	0.001
Clam	1, 35	1.277	0.266	Clam	1, 35	773.849	<0.001
Light × Sulphide	1, 35	0.002	0.961	Light × Sulphide	1, 35	0.057	0.812
Light × Clam	1, 35	1.568	0.219	Light × Clam	1, 35	0.024	0.879
Sulphide × Clam	1, 35	0.505	0.482	Sulphide × Clam	1, 35	15.745	<0.001
Light × Sulphide × Clam	1, 35	0.026	0.874	Light × Sulphide × Clam	1, 35	0.0001	0.994
Tank	1	1.058	0.304	Tank	1	7.917	0.005
Porewater ammonium (final), $\ln(x + 1)$				Porewater phosphate (final), $\ln(x + 1)$			
	<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>		<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>
Light	1, 40	0.935	0.339	Light	1, 40	0.044	0.834
Sulphide	1, 40	0.045	0.834	Sulphide	1, 40	3.109	0.086
Clam	1, 40	195.721	<0.001	Clam	1, 40	142.686	<0.001
Light × Sulphide	1, 40	1.573	0.217	Light × Sulphide	1, 40	0.008	0.929
Light × Clam	1, 40	0.001	0.975	Light × Clam	1, 40	0.542	0.466
Sulphide × Clam	1, 40	0.388	0.537	Sulphide × Clam	1, 40	1.913	0.174
Light × Sulphide × Clam	1, 40	0.607	0.440	Light × Sulphide × Clam	1, 40	0.0001	0.993
Tank	1	0.000	1.000	Tank	1	0.000	1.000
Rhizome %S, $\ln(x/(1-x))$				Leaf %S, $\ln(x/(1-x))$			
	<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>		<i>df</i> (n,d)	<i>F</i> or LRT	<i>p</i>
Light	1, 40	4.590	0.038	Light	1, 40	0.306	0.583
Sulphide	1, 40	5.032	0.030	Sulphide	1, 40	1.626	0.210
Clam	1, 40	33.278	<0.001	Clam	1, 40	17.308	<0.001
No. shoots at planting	2, 78	6.868	0.002	Light × Sulphide	1, 40	0.127	0.724
Light × Sulphide	1, 40	1.420	0.240	Light × Clam	1, 40	0.001	0.981
Light × Clam	1, 40	0.164	0.687	Sulphide × Clam	1, 40	0.741	0.394
Sulphide × Clam	1, 40	2.562	0.117	Light × Sulphide × Clam	1, 40	0.022	0.883
Light × Sulphide × Clam	1, 40	0.102	0.751	Tank	1	0.000	1.000
Tank	1	0.000	1.000				
Tank/Mesocosm	1	4.082	0.043				

Abbreviation: *df* (n,d), numerator and denominator degrees of freedom.

FIGURE 2 Final sediment porewater concentrations of (A) total dissolved sulphide, (B) ammonium and (C) ortho-phosphate in *Thalassia testudinum* mesocosms with and without shading, enhanced sediment porewater sulphide and the lucinid clam *Codakia orbicularis*. Sulphide concentrations were averaged between two samples at 0–5 and 5–10 cm depth; porewater samples for ammonium and phosphate were collected at 5–10 cm depth. Displayed response values are $M \pm SE$, $n = 6$ mesocosms. Different letters indicate significant Tukey-adjusted pairwise comparisons ($p < 0.05$) following analysis by linear mixed models. n.s., not significant ($p > 0.05$)



was absent (LMM, $p < 0.001$; Table 1; Figure 3B). Both rhizome and leaf growth rates showed a variable but distinctly inverse relationship to tissue %S (Spearman correlation, rhizomes: $\rho = -0.29$, $p = 0.001$, leaves: $\rho = -0.37$, $p < 0.001$; Figures S6 and S7).

4 | DISCUSSION

Our study provides the first experimental evidence that the strength of facilitation of a seagrass by a chemosymbiotic clam, mediated by the impact of the clam on sediment porewater sulphide levels, increases with environmental stress. More specifically, we show that facilitation did not manifest in both rhizomes and leaves until

shading or sulphide stress was applied. This result is consistent with the stress gradient hypothesis, which predicts that facilitation will be more important where environmental stress is high (Bertness & Callaway, 1994; He, Bertness, & Altieri, 2013). Two other hypotheses were not fully supported. First, we did not observe a significant increase in seagrass rhizome growth solely with the addition of *C. orbicularis* to mesocosms, which contrasts with the observations of van der Heide et al. (2012). In that study, both above-ground and below-ground biomass increased with lucinid addition, whether or not sulphide was also added. Here, *T. testudinum* maintained rhizome growth and slightly increased leaf growth in the presence of *C. orbicularis*, but growth was decreased under shading and sulphide stress in the absence of *C. orbicularis*. Second, while we observed an

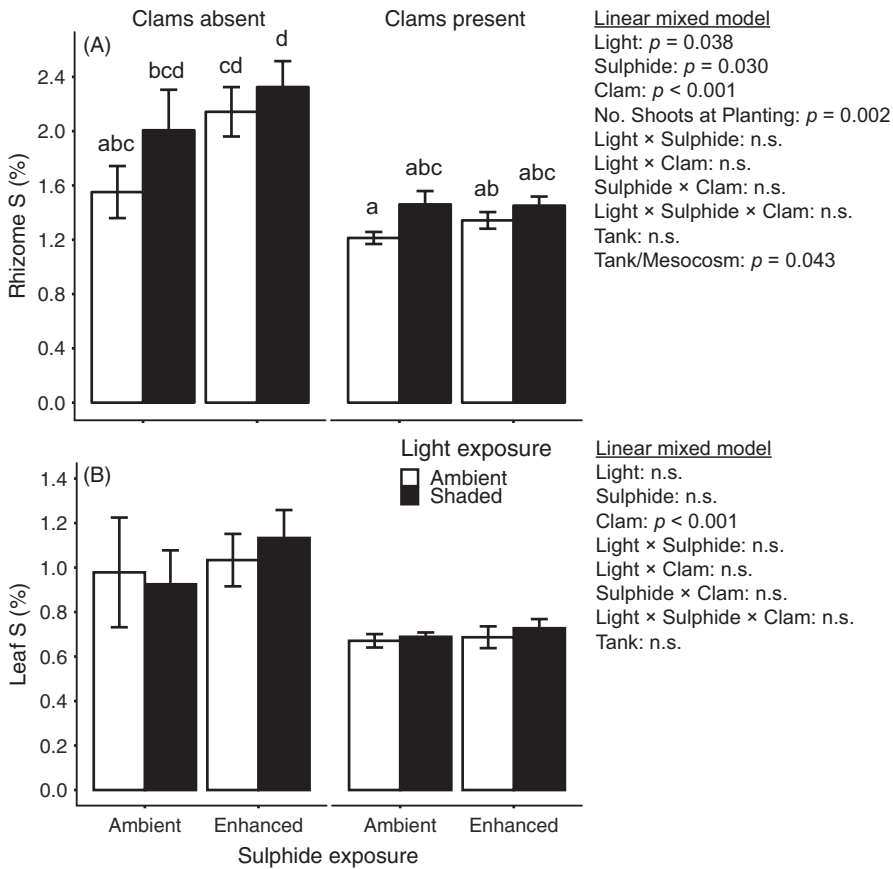


FIGURE 3 Sulphur content (% dry weight) of (A) rhizomes and (B) leaves in *Thalassia testudinum* mesocosms with and without shading, enhanced sediment porewater sulphide and the lucinid clam *Codakia orbicularis*. Displayed response values are $M \pm SE$, $n = 6$ mesocosms, where %S in each mesocosm is the mean from dried tissue of one to three live segments. Different letters indicate significant Tukey-adjusted pairwise comparisons ($p < 0.05$) following analysis by linear mixed models. n.s., not significant ($p > 0.05$)

increase in the importance of *C. orbicularis* to *T. testudinum* growth under environmental stress, the effects of shading and sulphide stress were not additive. Shading alone, rather than combined shading and sulphide enhancement, produced the greatest decrease in rhizome elongation rates in the absence of *C. orbicularis* relative to its presence.

Our conceptual model of increased lucinid-seagrass facilitation with increased environmental stress is summarized in Figure 4. When exposed to ambient-light and moderate sediment sulphide levels, *T. testudinum* rhizome growth is similar whether lucinids are present or absent. Leaf growth is slightly increased when lucinids are present. Under these conditions, light is not limiting to seagrass growth, and most sulphide stress can be managed by passive plant detoxification mechanisms, especially when dissolved sulphide in sediment porewater is strongly decreased by lucinid bioirrigation and symbiont chemoautotrophy. Under abnormally high sulphate reduction rates (mimicked in our experiment with sulphide injections), porewater sulphide accumulates. Shading reduces photosynthesis in *T. testudinum*, which reduces radial oxygen release from below-ground tissues (Frederiksen & Glud, 2006; Jovanovic et al., 2015), increases sulphide intrusion into those tissues (usually positively correlated with tissue sulphur content; Cambridge, Fraser, Holmer, Kuo, & Kendrick, 2012; Holmer & Hasler-Sheetal, 2014; Holmer et al., 2009) and decreases growth rates. However, *T. testudinum* maintains rhizome growth under low light and elevated sulphate reduction rates if lucinids are present because lucinids are

able to maintain porewater sulphide at low concentrations, minimizing sulphide intrusion and its negative effects on plant growth. Therefore, lucinid facilitation of *T. testudinum* becomes stronger when environmental stress increases.

In this study, rhizome sulphur content and growth rate were more dependent on environmental context than leaf sulphur content and growth rate. This pattern is likely a consequence of typical physiological responses of seagrasses to light and sulphide conditions and the relatively short duration of the experiment. Seagrass that is light limited may mobilize non-structural carbohydrate reserves from rhizomes, the primary storage tissue, to support leaf growth at the expense of below-ground growth (Alcoverro, Zimmerman, Kohrs, & Alberte, 1999). Below-ground seagrass tissues are most directly exposed to sulphides and are responsible for the majority of sulphide detoxification, some of which may involve active metabolism (Hasler-Sheetal & Holmer, 2015). Larger seagrass species, which include *T. testudinum*, are also more likely to limit sulphide intrusion into leaves, even if below-ground tissues are affected (Holmer & Kendrick, 2013). Therefore, a greater response of rhizomes than leaves to light and sulphide stress over the experimental period, in terms of sulphur content and growth, is not unexpected. Additionally, it is possible for sulphide to intrude into seagrass tissues under low apparent light or sulphide stress (Holmer & Kendrick, 2013; Holmer et al., 2009). If a low level of sulphide intrusion was occurring in the experiment under baseline conditions, then the decrease of porewater sulphide to very low levels upon addition of *C. orbicularis* may have

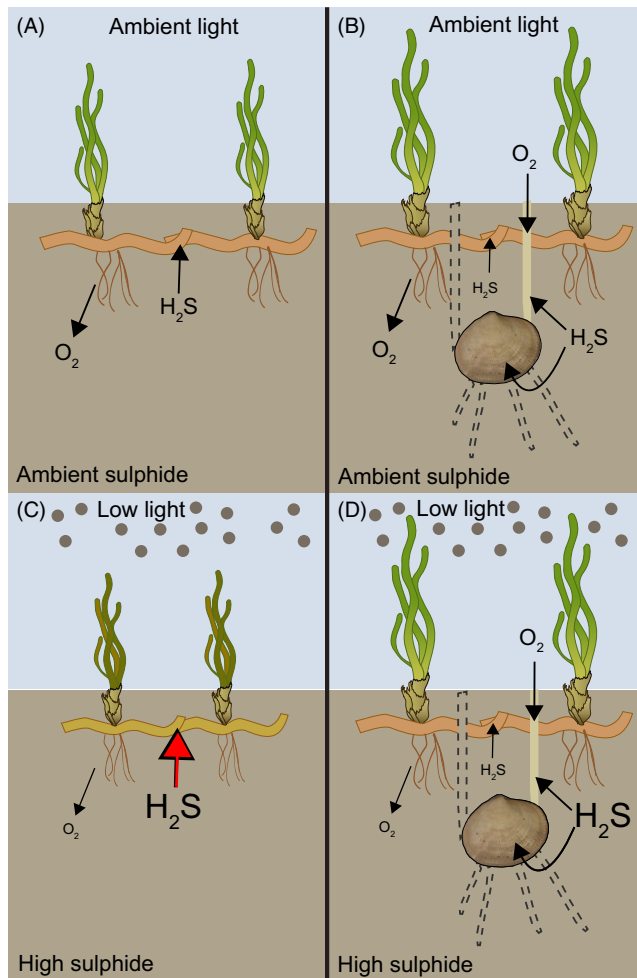


FIGURE 4 Conceptual model of facilitation of the seagrass *Thalassia testudinum* by the lucinid clam *Codakia orbicularis*. *T. testudinum* is depicted (A) under typical environmental conditions (ambient-light availability, ambient sediment sulphide production) without lucinids; (B) under typical conditions with lucinids; (C) under high environmental stress (low light, high sulphide) without lucinids; and (D) under high stress with lucinids. Lucinids decrease bulk sulphide levels in sediment, likely through direct consumption by their sulphur-oxidizing gill endosymbionts as well as through bioirrigation. Effects on *T. testudinum* growth are mediated by the balance between release of photosynthetically generated oxygen from seagrass roots and intrusion of phytotoxic sulphide into seagrass tissues. Facilitation of *T. testudinum* by lucinids is stronger when environmental stressors are present compared to when they are absent

released plants from this low-level stress and resulted in slightly increased leaf growth rates wherever *C. orbicularis* was present, regardless of shading or sulphide addition.

While *T. testudinum* tolerates a relatively wide range of environmental conditions (e.g. Koch, Schopmeyer, Holmer, Madden, et al., 2007; Koch, Schopmeyer, Kyhn-Hansen, Madden, 2007; Koch, Schopmeyer, Kyhn-Hansen, Madden, & Peters, 2007; Lee et al., 2007), we show that facilitation by *C. orbicularis* is relevant to *T. testudinum* growth under light stress, especially in combination with enhanced sediment sulphide stress. This suggests several

settings in which the presence of *C. orbicularis*, or other lucinids common in *T. testudinum* beds, might extend the range of *T. testudinum* in space and time. Lucinids may promote *T. testudinum* growth at the deep edges of beds, where light is limiting and where seagrass baseline light requirements can be further raised by chronically poor water or sediment quality (Kenworthy, Gallegos, Costello, Field, & di Carlo, 2014). Lucinids could also increase *T. testudinum* survival and recovery from phytoplankton blooms, storms or other events that not only reduce light availability, but may also stimulate sediment sulphide production by increasing organic matter inputs (e.g. Graco, Fariás, Molina, Gutiérrez, & Nielsen, 2001; Moeslund, Thamdrup, & Jørgensen, 1994). Such extensions to the range of *T. testudinum* as a result of the amelioration of environmental stress would be consistent with the stress gradient hypothesis and with the related idea that facilitation may expand a species' realized niche beyond the boundaries predicted by the fundamental niche (Bruno, Stachowicz, & Bertness, 2003; He & Bertness, 2014).

Based on our observations of increased porewater pH and strongly decreased concentrations of sulphide, ammonium and phosphate where *C. orbicularis* was present, we speculate that both bioirrigation and chemosymbiont metabolism contributed to the effects of *C. orbicularis* on porewater, and therefore on *T. testudinum*, in our study. A relatively strong role for bioirrigation would contrast with the conclusions of Reynolds et al. (2007). Based on an experiment with Rhodamine WT dye, Reynolds et al. (2007) suggested that oxygen introduction by the dwarf tiger lucine *Ctena orbiculata* accounted for only a small portion of sediment sulphide removal in *T. testudinum* sediments. We note that lucinid mixotrophy could also have influenced *C. orbicularis* behaviour and metabolism in our experimental system. Lucinids do not rely exclusively on their chemosymbionts for nutrition under natural conditions and may obtain an estimated 21%–48% of their carbon from suspension feeding depending on species and season (Cary, Vetter, & Felbeck, 1989; Johnson, Diouris, & Le Pennec, 1994; Kharlamenko, Kiyashko, Imbs, & Vyshkvartzev, 2001; van der Geest et al., 2014). Although *C. orbicularis* must be well-adapted to oligotrophic environments, the filtered seawater used in this study provided little to no suspended food. Therefore, the magnitude of the effects of *C. orbicularis* on sediment porewater characteristics in our study could have been influenced by alterations to bioirrigation activity or chemoautotrophy from typical rates. It is currently unclear whether lucinids would increase, decrease or maintain bioirrigation activity as they shift allocation of energy between nutritional modes. As lucinid facilitation of seagrasses is driven by their effects on porewater, this is an area for future research that would help identify and refine the contexts in which the facilitation is strongest.

Several modifications to the design of future studies are likely to enhance their utility with respect to applied seagrass management as well as improve basic knowledge of lucinid-seagrass interactions. This study considered the effect of lucinids on seagrass growth when seagrass was subjected to two simultaneous stressors, one stressor at a time or no added stressors. Examining seagrass responses over multiple levels of a single environmental

stressor, such as light availability across a range of water depths, would allow more detailed delineation of the shape of the relationship between those stressors and the strength of lucinid–seagrass facilitation (i.e. the severity–interaction relationship, *sensu* Brooker, Scott, Palmer, & Swaine, 2006). Further, no study has yet investigated how the strength of lucinid–seagrass facilitation may depend on lucinid density. For example, we do not know at what densities (and in which species) the facilitative effects of lucinids on seagrasses may plateau as competition among lucinids for sulphide resources increases. Additionally, we focused on lucinid alleviation of sulphide stress and did not specifically test other possible mechanisms by which bivalves may facilitate seagrass growth, such as the alleviation of nutrient limitation via biodeposition (Carroll, Gobler, & Peterson, 2008; Peterson & Heck Jr., 2001a, 2001b; Reusch, Chapman, & Gröger, 1994). Given the strong decreases in sediment porewater ammonium and phosphorus in the presence of lucinids in this study and in van der Heide et al. (2012), increased nutrient availability appears an unlikely mechanism of facilitation in this system. However, closer examination of the linkages among porewater nutrient concentrations, leaf C:N and C:P ratios, and lucinid presence or density in future studies should help to clarify the role of nutrients in lucinid–seagrass facilitation.

By showing experimentally that the strength of lucinid facilitation of seagrass increases with environmental stress, we set the stage for several lines of research that may continue to advance our understanding of lucinid–seagrass relationships and how context-dependent positive species interactions may be applied towards management goals. First, our results imply that where environmental conditions are stressful enough to promote sulphide intrusion into seagrass tissues, strong decreases in sediment sulphide due to lucinid bioirrigation and chemoautotrophy could confer a fitness advantage to seagrasses. We suggest that future studies address the hypothesis that facilitation of seagrasses by lucinids during periods of environmental stress may be a key mechanism by which the long-term association between these taxa is maintained. Second, while not designed to be a test of the stress gradient hypothesis *per se*, this study provides support from a subtidal marine system and from a facilitation with interacting partners that have very different resource requirements (Bulleri, 2009). Future research examining the stress gradient hypothesis in this system will join a relatively small group of studies testing predictions of the hypothesis outside of the plant–plant interaction studies that dominate current empirical support (e.g. Bulleri, Cristaudo, Alestra, & Benedetti-Cecchi, 2011; Gastaldi, Firstater, Navarte, & Daleo, 2017). Finally, consideration of environmental context dependence has the potential to enhance and refine the use of positive species interactions in the restoration of foundation species and their ecosystems (Brooker et al., 2008; Halpern, Silliman, Olden, Bruno, & Bertness, 2007; Padilla & Pugnaire, 2006; Silliman et al., 2015). For example, across a large set of experimental reforestations in a Mediterranean climate, the facilitative effect of nurse shrubs on growth and survival of woody seedlings was found to be strongest at low altitudes and on sunny,

drier slopes (Gómez-Aparicio et al., 2004). Recent work in US salt marshes shows that ribbed mussels increase cordgrass resilience to drought but are unlikely to mitigate all negative effects when cordgrass is subjected to repeated desiccation events (Angelini et al., 2016; Derksen-Hooijberg et al., 2019). In seagrass ecosystems, knowledge of lucinid abundances and distributions, in combination with environmental monitoring (e.g. turbidity, dissolved oxygen, temperature, salinity), may enhance predictions regarding which seagrass areas will be more or less vulnerable to die-off precipitated by environmental stress and which areas may recover more quickly following die-off events. Co-transplantation of lucinids may also enhance the success of seagrass transplanted for restoration, as has been experimentally demonstrated for ribbed mussels and cordgrass (Derksen-Hooijberg et al., 2018). Thus, consideration of context dependency in facilitation may assist managers seeking to refine relatively low-investment, high-reward conservation and restoration strategies in the face of increasing anthropogenic impact and global change.

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AUTHORS' CONTRIBUTIONS

D.W.C. designed the experiments with input from J.d.F., T.v.d.H., V.J.P., J.E.C. and B.J.P., analysed the data and wrote the manuscript; D.W.C., J.d.F., B.V.C., K.K. and J.E.C. conducted fieldwork and preparatory laboratory work; D.W.C., B.V.C. and K.K. performed the experiments; D.W.C., J.d.F. and T.v.d.H. generated nutrient and elemental analyses; J.d.F., T.v.d.H., V.J.P., J.E.C. and B.J.P. assisted with data interpretation. All the authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data used in this study are available in the Figshare Data Repository: <https://doi.org/10.6084/m9.figshare.12574010.v1> (Chin et al., 2020).

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SUPPORTING INFORMATION

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